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Constitutive Activity of the Androgen Receptor

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Abstract

Prostate cancer (PCa) is the most frequently diagnosed cancer in the United States. The androgen receptor (AR) signaling axis is central to all stages of PCa pathophysiology and serves as the main target for endocrine-based therapy. The most advanced stage of the disease, castration resistant prostate cancer (CRPC), is presently incurable and accounts for most PCa mortality. In this review, we highlight the mechanisms by which the AR signaling axis can bypass endocrine-targeted therapies and drive progression of CRPC. These mechanisms include alterations in growth factor, cytokine, and inflammatory signaling pathways, altered expression or activity of transcriptional co-regulators, AR point mutations, and AR gene amplification leading to AR protein overexpression. Additionally, we will discuss the mechanisms underlying the synthesis of constitutively active AR splice variants (AR-Vs) lacking the COOH-terminal ligand binding domain, as well as the role and regulation of AR-Vs in supporting therapeutic resistance in CRPC. Finally, we summarize the ongoing development of inhibitors targeting discrete AR functional domains as well as the status of new biomarkers for monitoring the AR signaling axis in patients.

Keywords

androgen receptor; splice variant; prostate cancer; castration resistant prostate cancer; CRPC; constitutive activity; antiandrogen; NTD; LBD

1. INTRODUCTION

Androgens and the androgen receptor (AR) have pleiotropic roles in regulating male development and normal function of the male urogenital system. The best-defined role for AR is in the development and biological regulation of the prostate gland, due to decades of research focused on improving survival and quality of life for men with prostate cancer (PCa). In the United States, PCa is the most frequently diagnosed male cancer and the second leading cause of male cancer-related death (Siegel, Naishadham, & Jemal, 2013). PCa that is confined to the prostate capsule can be treated by prostatectomy or radiotherapy. However, for more advanced or metastatic prostate cancer, androgen deprivation therapy (ADT) is the standard systematic treatment. At all stages of disease progression, most

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CONFLICT OF INTEREST

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prostate cancer cells are dependent on activity of the AR transcription factor. Even when the disease has progressed to a lethal castration resistant prostate cancer (CRPC) phenotype resistant to ADT, the AR remains a viable target for prostate cancer therapy.

This review will focus on the basic constitutive functions of the AR in androgen signaling in PCa, and the role of constitutively active AR splice variants in driving therapeutic resistance in CRPC. We will discuss mechanistic aspects of the AR transcriptional regulatory network, and potential therapeutic approaches to target constitutive AR signaling that emerges during progression to CRPC.

2. BASIC ANDROGEN RECEPTOR FUNCTIONS

2.1. Ligands for AR activation

The AR is a member of the nuclear receptor superfamily of ligand-regulated transcription factors (Mangelsdorf et al., 1995). In the un-liganded state, AR is localized to the cytosol in a complex with molecular chaperones, Hsp40, 70 and 90. Androgen binding induces conformational changes in the AR protein, which exposes a nuclear localization signal that can be bound by nuclear import proteins such as importin- α . This nuclear import complex then facilitates AR translocation to the nucleus. Nuclear AR binds as a dimer to androgen responsive elements (ARE) in promoters and enhancers of target genes that are regulated by androgen/AR activity (Dehm, & Tindall, 2007).

Testosterone is the major androgen in the circulation, synthesized by Leydig cells in the testes. Testosterone synthesis in the testes is regulated by luteinizing hormone release from the pituitary gland, which is under upstream control by gonadotropin-releasing hormone release from the hypothalamus. Testosterone can enter prostate cells through passive diffusion, where it is converted to the more potent ligand, 5 α -dihydroxytestosterone (DHT), by 5 α reductase enzyme activity. Additional androgens in the bloodstream include dihydroepiandrosterone (DHEA) androstenediol, and androstenedione. These androgens are weak agonists for the AR, but can be converted to testosterone and/or DHT in peripheral tissues.

2.2. AR regulation and function in normal prostate gland

The earliest cell determination step for development of the male reproductive tract occurs in the embryo, but terminal cell differentiation takes place during sexual maturation (Matusik et al., 2008). A critical role for androgen/AR signaling in this process is evidenced by loss of function mutations in the AR underlying androgen insensitivity syndrome (AIS), wherein impaired AR function is unable to regulate the male development program and female phenotypes develop in XY genetic males (Dehm, & Tindall, 2011). The prostate gland is the component of the male reproductive system that produces the majority of seminal fluid. The glandular acini are organized as luminal and basal epithelial cell layers within a supportive stroma. Prostate tissue develops from the urogenital sinus (Cunha, & Rieke, 2011), which is dependent on activity of AR proteins expressed in the urogenital sinus mesenchyme, the outer layer of embryonic connective tissue surrounding the urogenital sinus epithelium. The intricate signaling relationship between these tissue compartments was demonstrated by seminal work showing that AR expressed in urogenital sinus mesenchyme is sufficient to

transform adult bladder epithelium or bladder stem cells into prostatic glands (Cunha et al., 2011). A similar tissue signaling relationship exists in normal adult prostate, wherein stromal AR is a critical regulator of epithelial cell proliferation, survival and differentiation. Conversely, AR expressed in normal prostate epithelial cells is mainly responsible for regulating genes required for synthesis of proteins that are secreted into the prostate lumen (Cunha, Cooke, & Kurita, 2004). Interestingly, it appears that PCa cell of origin can be either basal or luminal epithelial cells, with luminal-derived disease possibly representing a more aggressive subset (Wang et al., 2013).

2.3. AR regulation and function in primary prostate cancer and castration resistant prostate cancer

The pioneering work of Huggins and Hodges (Huggins, & Hodges, 1972) provided the first demonstration that prostate cancer is an androgen-dependent disease. Subsequent molecular cloning of AR cDNA from human and mouse made possible a large number of studies demonstrating that AR transcriptional activity is critical for all stages of prostate cancer development and progression. Indeed, various unbiased next-generation sequencing platforms have been used to perform genomic characterization of PCa at different stages, and results have consistently confirmed a critical role for activity of the androgen/AR axis (Baca et al., 2013; Berger et al., 2011; Taylor et al., 2010). Additionally, whole genome sequencing analysis of 11 early onset prostate cancers indicated that androgens, through the AR, likely participate in shaping the somatic alternation landscape (Weischenfeldt et al., 2013). This is further supported by studies demonstrating that androgen treatment can induce AR-mediated proximity of TMPRSS2 and ERG or ETV1 loci, ultimately leading to gene fusion and ensuing PCa initiation via androgen-driven overexpression of the gene fusion products (Lin et al., 2009; Tomlins et al., 2005).

These critical roles for AR in PCa development and progression provide the underlying rationale for the initial efficacy of ADT as the standard treatment for advanced, relapsed and metastatic prostate cancer. However, resistance and progression to CRPC invariably develops as prostate cancer cells acquire the ability to grow under conditions of reduced androgen availability. However, as will be discussed in subsequent sections, re-activation of the androgen/AR signaling axis is a central component of this resistance. Identifying the mechanisms underlying AR re-activation in CRPC has provided opportunities for successful development of new therapies targeting the AR signaling axis. Despite these advances, resistance remains a major barrier to substantial improvements in survival for CRPC patients.

2.4. Biochemical properties of different functional domains of AR

The AR shares a similar domain organization with other members of the nuclear receptor (NR) superfamily (Evans, 1988), particularly closely-related steroid receptors including progesterone, glucocorticoid and estrogen receptors. The AR gene is located on the X-chromosome at position Xq11-12 and is 183 kilobases in length. It consists of eight exons which are separated by relatively long introns, some of which harbor cryptic exons as illustrated in Figure 1A. Based on NCBI AR reference sequence NM_00044.2, the AR protein is 919 amino acids in length and has a molecular weight of 110 kDa. The full-length

AR protein is polymorphic as a result of variable-length stretches of polyglutamines and polyglycines within the NH₂-terminal domain (NTD). AR exon 1 encodes the entire AR NTD, which comprises about 60% of the AR protein, and exons 2 and 3 each encode one zinc finger of the AR DNA binding domain (DBD). The AR hinge region is encoded by the 5' portion of exon 4, and the remaining exons 4-8 encode the AR ligand binding domain (LBD) and transcriptional activation function-2 (AF-2) domain (Fig. 1B).

The NTD of nuclear receptors are the most divergent domains in terms of their amino acid sequence and overall length. Additionally, the 3-dimensional structure of all steroid receptor NTDs is unknown, which is due to the inherent structural flexibility of these domains. This structural flexibility is likely important for nuclear receptors to process and transmit a diversity of cellular signals. The large central part of the AR NTD has been termed activation function-1 (AF-1) to reflect the importance of this domain for AR transactivation activity and interaction with co-regulators (Heemers, & Tindall, 2007). This AF-1 domain can be separated into two discrete activation domains termed transactivation unit 1&5, TAU1 (aa 101-360) and TAU5 (aa361-490) (Jenster, van der Korput, van Vroonhoven, van der Kwast, Trapman, & Brinkmann, 1991) (Fig. 1B). Domain mapping experiments have shown that two separate deletions within the TAU1 domain each lead to abolished androgen-dependent transcriptional activity in LNCaP and C4-2 cells. Therefore, the TAU1 domain has been further sub-divided into discrete AF1a and AF1b units (Dehm, & Tindall, 2006). In more precise mapping experiments, AF1a activity has been narrowed to a Leu-Lys-Asp-Ile-Leu (LKDIL) motif, which is similar in sequence composition to the nuclear receptor (NR) box consensus sequence LXXLL found in many steroid receptor-associated co-regulatory proteins (Chamberlain, Whitacre, & Miesfeld, 1996). In these studies, deletion of the LKDIL motif inhibited the transcriptional activity of the AR to a similar extent as removal of the complete AF1a fragment. Whereas the TAU1 domain of the AR appears to be an absolute requirement for transcriptional activation, the TAU5 domain has been shown to play a more context-dependent role. For example, deletion of the TAU5 domain from the full-length AR protein results in enhanced ligand-dependent transcriptional activity in androgen-dependent LNCaP prostate cancer cells, but reduced ligand-independent transcriptional activity in castration-resistant C4-2 prostate cancer cells (Christiaens et al., 2002; Dehm et al., 2006). Subsequent detailed mapping analysis of the TAU5 domain identified a Trp-His-Thr-Leu-Phe (WHTLF) motif which was confirmed to be important for ligand-independent, but not ligand-dependent AR transcriptional activity (Dehm, Regan, Schmidt, & Tindall, 2007). Interestingly, in addition to this transcriptional function, the AR WHTLF motif in TAU5 has also been shown to mediate an intra-molecular NH₂/COOH terminal domain interaction with the AR AF-2 domain (He, Minges, Lee, & Wilson, 2002). There is also a Phe-Glu-Asn-Leu-Phe (FQNLF) motif in the AR NTD that can mediate the NH₂/COOH terminal domain interaction via the same AF-2 region. While the precise mechanism by which the AR NH₂/COOH terminal domain interaction regulates AR function is not entirely clear, it appears to be important for dynamic interaction with co-activator proteins (Askew, Bai, Blackwelder, & Wilson, 2010; He, Kempainen, & Wilson, 2000).

The DNA binding domain (DBD) is the most highly conserved region among steroid receptors, consisting of about 80 amino acids. The AR DBD has two zinc fingers, each of

which is characterized by four cysteine residues coordinating a single zinc atom (Fig. 1C). The first zinc finger contains the P-box residues, which make direct DNA contact with an ARE half-site, thus conferring base-pair specificity. The second zinc finger mediates AR:AR dimerization via its D-Box residues with an AR molecule bound to an adjacent ARE half-site (Shaffer, Jivan, Dollins, Claessens, & Gewirth, 2004). This allows AR to bind as a dimer to an inverted repeat element (IR3) ARE in a head-to-head fashion, with a higher affinity for this sequence than other steroid receptors such as GR, PR and MR.

The hinge region of the AR was originally thought to be a flexible linker connecting the DBD and LBD regions of the AR. Later studies identified that the hinge region contains the nuclear localization signal (NLS) required for binding to importin- α and subsequent nuclear localization (Jenster, Trapman, & Brinkmann, 1993; Simental, Sar, Lane, French, & Wilson, 1991). Detailed biochemical analyses by different groups have also suggested that AR NLS residues may play additional roles in DNA binding affinity, transactivation, and intranuclear mobility, although these studies have not ruled out effects on nuclear localization as the basis for these observations (Chan, Li, & Dehm, 2012; Cutress, Whitaker, Mills, Stewart, & Neal, 2008; Tanner et al., 2010).

The ligand binding domain (LBD) of AR consists of 11 helices and 1 β -sheet organized as an antiparallel sandwich motif with a hydrophobic pocket to accommodate ligand binding. Ligand binding induces conformational changes resulting in the relocation of helix 12. This leads to the formation of a hydrophobic groove by helices 3, 4, 5, and 12, which mediates binding to LXXLL motifs present in AR co-regulators as well as the FQNLF and WHTLF motifs in the AR NTD (He et al., 2004). Various structures of the AR LBD in complex with diverse ligands and co-regulator peptide fragments have been solved by x-ray crystallography (He et al., 2004). These studies have shown that different steroid ligands possess varied affinities for the ligand binding pocket as a result of forming different hydrophobic contacts and electrostatic interactions at both extremities of the steroid nucleus (Pereira de Jesus-Tran, Cote, Cantin, Blanchet, Labrie, & Breton, 2006). More recently, a COOH-terminal protein:protein interaction surface termed BF3 was shown to influence AF-2 function allosterically (Estebanez-Perpina et al., 2007). Mechanistically, the BF3 domain has been shown to mediate AR binding to the AR co-regulator FKBP52 (De Leon et al., 2011). The importance of BF3 for AR function is supported by the finding that clinically-relevant mutations in BF3 regions are associated with both PCa and AIS (Gottlieb, Beitel, Nadarajah, Paliouras, & Trifiro, 2012).

3. MODES OF CONSTITUTIVE AND HYPERSENSITIVE ACTIVITY OF FULL-LENGTH ANDROGEN RECEPTOR

3.1. AR Activation by growth factor signaling pathways

In order for AR to direct transcriptional programs in the prostate, an extensive array of posttranslational modifications is required. Those protein post-translational modifications of AR include phosphorylation, acetylation, methylation, sumoylation and ubiquitination, regulated by the extracellular signals induced by growth factor and other signaling pathways. The precise amino acid locations on the AR protein to which these different

protein posttranslational modifications are conjugated have been studied extensively and have been reviewed previously (van der Steen, Tindall, & Huang, 2013). In this section, we will highlight three major signaling pathways which play a significant role in regulating constitutive or ligand-hypersensitive activity of full length AR.

The insulin-like growth factor-1 (IGF-1) pathway has been shown to play a key role in regulating cell growth, and elevation of serum IGF-1 levels has been associated with risk of prostate cancer development (Chan et al., 1998). IGF-1 can stimulate PSA secretion in LNCaP prostate cancer cells by 5-fold in the absence of androgen *in vitro* (Culig et al., 1994) and stable transfection of an antisense IGF-IR expression construct inhibits prostate tumor growth in mice (Burfeind, Chernicky, Rininsland, & Ilan, 1996). Notably, androgen independent growth of human tumor xenografts was associated with a dramatic increase of the IGF type I receptor (IGF-IR) mRNA levels (Nickerson, Chang, Lorimer, Smeekens, Sawyers, & Pollak, 2001). Moreover, androgen treatment can up-regulate IGF-IR expression in LNCaP cells, which is associated with activation of the Src-extracellular signal-regulated kinase pathway (Pandini et al., 2005). This positive feed-forward loop between IGF-1 and AR is likely to have a major functional impact on constitutive functions of the AR in PCa progression. Additionally, treatment of IGF2 in LNCaP and 22Rv1 cells resulted in the increased expression of steroidogenic enzymes including cytochrome p450 family member (CYP17A1), aldo-keto reductase family member (AKR1C3) and hydroxysteroid dehydrogenase (HSD17B3) (Lubik et al., 2013). Targeting the IGF axis in PCa with fitgitumumab, a human monoclonal antibody against IGF-1R has recently been tested in clinical trials. Fitgitumumab was able to achieve PSA declines in localized prostate cancer patients (Chi et al., 2012). In another study, ganitumab (formerly AMG 479), a human antibody that inhibits binding of IGF-1 and IGF-II to IGF-IR, was shown to inhibit growth of VCaP prostate cancer xenografts, as well as enhance the growth inhibitory effects of ADT (Fahrenholtz, Beltran, & Burnstein, 2013).

The role of epidermal growth factor (EGF) and its tyrosine kinase receptor, EGFR, have also been strongly implicated in prostate cancer progression via activation of the MAPK pathway (Lamont, & Tindall, 2011). HER-2/neu is a member of the EGFR family of receptor kinases which is overexpressed in breast and ovarian cancers. HER2/neu is expressed at high levels in androgen-independent sublines of the LAPC-4 xenograft, which has been shown to activate AR function under castrate conditions through mechanisms that cannot be blocked by casodex. This indicates that activated EGFR signaling supports a constitutive mode of AR activation (Craft, Shostak, Carey, & Sawyers, 1999). Mechanistically, the EGF-induced activation AR transcriptional activity can be executed by MAPK-dependent phosphorylation of AR S515 and by protein kinase C (PKC)-dependent phosphorylation of AR S578 (Ponguta, Gregory, French, & Wilson, 2008). In line with this, inhibitors of the MAPK pathway have been shown to inhibit HER2/neu mediated AR constitutive activity in prostate cancer cells (Yeh, Lin, Kang, Thin, Lin, & Chang, 1999). In addition, dasatinib, a Src inhibitor, has been shown to block EGF-induced phosphorylation of AR Tyr-534 (Liu, Karaca, Zhang, Gioeli, Earp, & Whang, 2010). However, a phase II clinical trial with the second generation anti-HER-2 antibody, pertuzumab, was not an effective treatment for patients with CRPC (de Bono, Bellmunt, Attard, Droz, Miller, & Flechon, 2007).

Nevertheless, the co-administration of EGFR inhibitors erlotinib and AG1478, or HER-2 inhibitors trastuzumab and AG879 induced apoptosis in androgen-sensitive prostate cancer cells (Chen et al., 2011a).

The involvement of the PI3K/PTEN/Akt pathway in prostate cancer development has been reviewed previously (Grossmann, Huang, & Tindall, 2001). However, three recent studies have provided new insights into the role of this pathway in cross-talk with AR signaling in prostate cancer progression (Carver et al., 2011; Chen et al., 2011b; Mulholland et al., 2011). In human and murine prostate tumors, AR transcriptional output is decreased under conditions of Pten inhibition. Additionally, inhibition of PI3K leads to activation of AR signaling by releasing negative feedback from HER2 kinases (Carver et al., 2011). Another study using tissue specific and conditional knockout of AR in mouse prostate epithelium demonstrated that AR loss promoted the growth of Pten null cancer cells. Mechanistically, this was shown to be due to blockade of AR-mediated activation of the Hsp90 co-chaperone, FKBP5, which promotes Akt inhibition via the Akt phosphatase PHLPP (Mulholland et al., 2011). Indeed, PHLPP-mediated Akt inhibition required FKBP5 activity, and PHLPP knock-out caused mouse prostate neoplasia. When combined with partial PTEN loss, PHLPP knock-out caused mouse prostate carcinoma (Chen et al., 2011b). Overall, elucidating the mechanisms of cross-talk between the PI3K and AR pathways has led to the concept that combinational targeting of these two pathways may be effective in advanced CRPC. Indeed, a recent study showed that the AKT inhibitor AZD5365 in combination with bicalutamide delayed the progression of CRPC in a preclinical model (Thomas et al., 2013).

3.2. AR activation by cytokines and inflammatory signaling

Currently, IL-6 is the best-characterized cytokine with regard to AR regulation in prostate cancer (Nguyen, Li, & Tewari, 2013). The binding of IL-6 to the IL-6 receptor leads to downstream activation of JAK tyrosine kinases and subsequent phosphorylation and activation of STAT proteins. Elevated serum levels of IL-6 are frequent in CRPC patients and IL-6 can stimulate the androgen-independent growth of prostate cancer cells in vitro and in vivo (Malinowska et al., 2009). IL-6 treatment increased testosterone levels in LNCaP cells by upregulating the expression level of steroidogenic enzymes such as HSD3B2 and AKR1C3 (Chun et al., 2009). The mechanism of the IL-6 function in androgen hypersensitivity may also be mediated through increased expression of SRC2, as demonstrated by IL-6 treatment in higher passage LNCaP cells (Feng, Tang, Sun, Chun, Evans, & Gao, 2009). Interestingly, STAT3 activation can be inhibited by suppressor of cytokine signaling (SOCS) family proteins. Importantly, SOCS2 has been identified as a direct AR transcriptional target by microarray analysis in prostate cancer cells (Hoefler et al., 2013; Iglesias-Gato et al., 2013). However, the function of this negative feedback loop may be impaired in CRPC, perhaps through reduced levels of SOCS2 expression.

An additional role for inflammatory signaling in the regulation of constitutive AR transcriptional activity has recently been reported to occur through NF-kappaB. In this study secretory proteins from neuroendocrine cells were shown to lead to activation of the NF-kappaB pathway in LNCaP cells (Jin et al., 2008). Sustained activation of NF-kappaB activity was shown to maintain a high level of AR in the nucleus, thereby facilitating AR-

mediated target gene activation under castrate condition. Mechanistically, this may be due to a direct interaction of p52 with the AR NTD. Indeed, this interaction has been shown to enhance AR nuclear localization and recruitment of the AR co-regulator p300 (Nadiminty et al., 2010). Since p52 can interact with the AR NTD, this may have further importance for enzalutamide resistance mediated by truncated AR-V proteins (Nadiminty et al., 2013).

3.3. AR activation by co-regulators

Co-regulators activate or repress AR-dependent transcription via direct protein-protein interactions with the AR or components of the AR transcriptional complex. The list of known co-regulators for AR is growing and was reviewed recently (Heemers et al., 2007). The mechanisms by which AR co-regulators influence AR transcriptional output are diverse, including biochemical functions such as recruitment of basic transcriptional machinery components, chromatin remodeling complexes, modulating activity of histone modifying enzymes in the nucleus, as well as modulating activity of AR-bound chaperones and co-chaperones in the cytosol.

FKBP51 (encoded by the FKBP5 gene) is a well-characterized AR target gene (Makkonen, Kauhanen, Paakinaho, Jaaskelainen, & Palvimo, 2009) sharing 70% protein homology with FKBP52, which binds the AR BF3 domain (De Leon et al., 2011). FKBP51 expression has been shown to be upregulated in relapsed LAPC-4 tumors grown in castrated mice (Ni, Yang, Gioeli, Frierson, Toft, & Paschal, 2010). FKBP51 regulates the recruitment of the cochaperone p23 to ATP-bound Hsp90, promoting formation of a FKBP51-Hsp90-p23 superchaperone complex, which maintains AR in a high-affinity conformation competent for ligand binding. Therefore, FKBP51 enhances androgen-stimulated AR transcriptional activity and androgen-dependent cell growth. Although expression of FKBP52 is not regulated by the AR, FKBP52 functions as a positive regulator of AR signaling by promoting the formation a stable complex with Hsp90-FKBP52-AR which is critical for receptor translocation (Storer, Dickey, Galigniana, Rein, & Cox, 2011). Interestingly, although Hsp90 inhibitors can inhibit nuclear localization and transcriptional activity of full-length AR, Hsp90 inhibitors are ineffective at inhibiting nuclear localization or transcriptional activity of AR-Vs (Chan et al., 2012; Gillis et al., 2013; Shafi, Cox, & Weigel, 2013).

P300/CBP and p160/steroid receptor co-activator (SRC) are two major classes of AR co-activators possessing acetyltransferase activity. Both of these classes enhance AR transactivation by promoting formation of activation complexes between the AR-associated enhancer and/or promoter and the transcription start site of AR target genes (Wang, Carroll, & Brown, 2005). Increased expression of p160 coactivators SRC-1, SRC-2 (also referred to as TIF2 or, GRIP-1, or NCoA2) and SRC-3 (also referred to as AIB) are associated with prostate cancer progression (Xu, Wu, & O'Malley, 2009). SRC proteins can interact with the AR through both the AF-1 and AF-2 domains (Bevan, Hoare, Claessens, Heery, & Parker, 1999). Increased expression of SRC2 has been shown to support AR transcriptional activity in prostate cancer cells under castrate conditions (AgoulNIK, & Weigel, 2006). More recently, prostate cancer-associated mutations in the SPOP E3 ligase were found to affect regulation of SRC-3 (Barbieri et al., 2012; Grasso et al., 2012; Li et al., 2011). Multiple

SPOP mutants discovered in prostate cancer were found to be defective in binding SRC-3, thereby preventing ubiquitin-mediated proteasomal degradation of SRC-3 (Geng et al., 2013).

p300 and CBP have also been shown to be important for IL-6 mediated activation of AR in the absence of androgens, and this effect has further been shown to require STAT3 (Debes, Schmidt, Huang, & Tindall, 2002). Interestingly, another function of p300/CBP is to acetylate lysine residues in the AR NLS located in the hinge region (Fu et al., 2000). These acetylation events have been shown to be critical for AR transcriptional activity, although it is not clear whether this is due to impaired nuclear localization or impaired transcriptional activation function of nuclear AR. Interestingly, despite these important roles demonstrated for these co-activators in prostate cancer, siRNA-mediated knock-down of p300, SRC-1, -2, or -3 individually had modest effects on AR transcriptional activation of PSA expression in the LNCaP prostate cancer cell line, indicating there may be a certain degree of functional redundancy (Wanget al., 2005).

LSD1 and JMJD2C are two lysine demethylases that interact with AR and catalyze removal of methyl groups from histone H3 proteins that are methylated at lysine 4 or lysine 9 (H3K4, H3K9) (Wissmann et al., 2007). Whereas LSD1 can only remove methyl groups from mono- or di-methylated H3K4 or H3K9, JMJD2C can catalyze removal of tri-methyl marks. The role of these demethylases in modulating AR activity appears to be dichotomous, with one report indicating that LSD1 and JMJD2C act in a cooperative manner to stimulate AR-dependent gene transcription in normal prostate or prostate carcinoma (Wissmann et al., 2007). Conversely, a separate study has indicated that LSD1 functions as an AR co-repressor that supports AR-mediated transcriptional repression of its own expression (Cai et al., 2011). In this case, physiological levels of androgens led to AR-mediated recruitment of LSD1 to an ARE-driven enhancer in AR intron 2, thereby inhibiting expression of the AR gene. Importantly, castrate levels of androgens relieved this LSD1-mediated repression, leading to increased levels of AR expression. Under castrate conditions, this increase in AR protein levels led to enhanced transcriptional activation of a subset of proliferation-associated AR target genes (Cai et al., 2011).

In addition to regulation of transcriptional output, H3K4 methylation has also been shown to influence AR binding dynamics at genomic AREs. For example, H3K4 mono- and di-methylation are epigenetic marks important for AR binding to ARE-containing enhancers (Lupien et al., 2008). The putative importance of these marks for AR function in PCa has been further supported by whole exome sequence analysis of lethal CRPC, which has indicated that many components of the H3K4 methyltransferase complex (such as MLL2 & 3, UTX and ASXL1) are mutated in tumor tissues (Barbieri et al., 2012; Grasso et al., 2012). Although the precise mechanisms by which these alterations may affect constitutive or hypersensitive functions of the AR during AR-targeted therapy are not completely clear, modulating these epigenetic signals may represent an alternative way to inhibit AR function in CRPC.

3.4. AR gene amplification and overexpression of AR protein

Genomic amplification of the AR locus at Xq11-q12 was first reported nearly two decades ago, with 7 out of 23 cases of CRPC studied displaying AR gene amplification as determined by comparative genomic hybridization and/or fluorescence in situ hybridization (Visakorpi et al., 1995). This early observation has been validated extensively, most recently with next-generation sequencing and copy number microarrays (Grasso et al., 2012). Mechanistically, AR gene amplification has been shown to result in increased AR mRNA and protein expression. In untreated prostatectomy specimens, IHC analysis demonstrated that increased AR tumor nuclei staining correlated with a shorter survival time in advanced PCa samples, indicating that AR protein levels are important determinants of disease progression (Donovan et al., 2010). Indeed, increased AR mRNA and protein levels were observed in prostate cancer xenografts as they progressed to CRPC, and a 2-fold increase in AR expression in LNCaP cells was sufficient for androgen hypersensitization and induction of a CRPC growth phenotype (Chen et al., 2004). In this study, increased AR expression was also sufficient to convert the antiandrogen bicalutamide into an AR agonist.

3.5. AR point mutations

The first AR gene mutations were discovered as an underlying basis for AR loss-of-function in androgen sensitivity syndrome (AIS). Conversely, somatic AR mutations in prostate cancer are almost always associated with diverse gains-of function (Gottlieb et al., 2012). For example, mutations in the AR coding region have been shown to promote AR transcriptional activity by hypersensitizing the receptor to castrate levels of androgens, altering ligand specificity such that AR can be activated by alternative steroids and even antiandrogens, altering the topology of protein:protein interaction surfaces and thereby influencing interactions with co-regulatory proteins, altering affinity of the DBD for AREs, and affecting dynamics of nuclear translocation. A recent whole exome sequencing study demonstrated that AR somatic mutations did not occur in untreated and localized cancer, but were present in 10% of CRPC (Grasso et al., 2012). The nature and role of AR point mutations in CRPC progression has been reviewed in detail elsewhere (Brooke, & Bevan, 2009). Here, we will focus on discrete AR point mutations that alter AR responses to antagonists.

LNCaP was the first cell line discovered to have a missense T877A mutation in the ligand binding domain (Veldscholte, Berrevoets, Brinkmann, Grootegoed, & Mulder, 1992). This T877A mutation has served as a prototype for many additional mutations in the AR LBD, which appear to be more frequent in patients treated with antiandrogens (O'Mahony, Steinkamp, Albertelli, Brogley, Rehman, & Robins, 2008). Notably, four recurrent mutations, H874Y, T877A, T877S, and F876L, occur in the ligand binding domain resulting in adoption of an agonist conformation and co-activator recruitment when bound to antiandrogens (Bohl, Gao, Miller, Bell, & Dalton, 2005; Bohl, Wu, Miller, Bell, & Dalton, 2007). Notably, F876L is a hotspot mutation in CRPC arising in cell lines and tissues from patients treated with the second generation antiandrogens, enzalutamide and ARN-509 (Joseph et al., 2013). This F876L mutation is located in helix 11 of the LBD, and molecular dynamics simulations have indicated that F876L AR bound to enzalutamide leads to positioning of LBD helix 12 in an agonist-like conformation and formation of an AF-2

interaction surface that is competent for co-activator binding (Joseph et al., 2013). In addition to these well-defined LBD mutations, additional somatic mutations from CRPC patients have been observed in the AR NTD, and shown to be constitutively active in AR reporter assays (Hay, & McEwan, 2012). Identifying the detailed mechanisms by which these diverse AR point mutations can drive therapeutic resistance is anticipated to reveal opportunities to overcome these resistance mechanisms. For example, addition of bulkier substituents to the enzalutamide/ARN-509 chemical scaffold led to the development of a new class of AR antagonists that could inhibit aberrant transcriptional activity of the AR F876L mutant (Balbas et al., 2013).

4. DISCOVERY OF CONSTITUTIVELY ACTIVE AR SPLICE VARIANTS

4.1. Identity and origin of AR-Vs

Androgen receptor splice variants (AR-V) are truncated AR species lacking the AR LBD. Because these species retain the transcriptionally active NTD/DBD core, they display constitutive transcriptional activity and are insensitive to agonists or antagonists (Dehmet al., 2011). This is in contrast to AR gene amplification and LBD point mutations, which appear to retain at least some degree of ligand-dependence for supporting growth of CRPC cells. The first clues to AR-Vs in CRPC came from studies with relapsed CRPC tumors from the CWR22 xenograft model of prostate cancer progression. These studies revealed the presence of a lower molecular weight AR species in Western blot experiments that was recognized by antibodies targeted to the AR NTD, but not the AR LBD (Tepper et al., 2002). This low molecular weight protein appeared to be associated with disease progression and therapeutic resistance, as it was not detected from the original, hormone-dependent CWR22 xenograft. Initially, this truncated AR protein species was reported to be a calpain-mediated protein cleavage product of full length AR (Libertini, Tepper, Rodriguez, Asmuth, Kung, & Mudryj, 2007). However, subsequent studies with small interference RNAs (siRNAs) demonstrated that knock-down of full-length AR with a siRNA targeting AR exon 7 had no effect on expression of the truncated AR protein species in 22Rv1 cells (Dehm, Schmidt, Heemers, Vessella, & Tindall, 2008). This indicated that the truncated AR protein species expressed in 22Rv1 cells resulted from alternative splicing, which was further supported by the observation that this species could be eliminated with siRNA targeted to AR exon 1 (Dehm et al., 2008). Subsequent studies using 3' RACE and various additional PCR-based approaches demonstrated the existence of multiple AR-Vs in 22Rv1 cells, as well as additional prostate cancer cell lines, xenograft tumors, mouse models of prostate cancer, and patient specimens (Brand, & Dehm, 2013; Dehm et al., 2011; Nyquist & Dehm, 2013a).

The first AR-Vs to be reported were found to result from splicing of a novel cryptic exon 2b (also referred to as CE4) into AR mRNA. Two AR mRNAs, consisting of contiguously-spliced AR exons 1/2/2b or 1/2/3/2b were identified (Dehm et al., 2008), but a subsequent study with an antibody specific for the amino acids encoded by exon 2b demonstrated that only the AR 1/2/3/2b mRNA was translated into an AR-V protein in 22Rv1 cells (Chan et al., 2012). AR 1/2/3/2b displayed constitutive transcriptional activity in reporter assays with ARE-driven reporters, mediated constitutive activation of endogenous AR target genes, and

supported androgen independent growth (Chan et al., 2012). These data indicated that expression and activity of AR-Vs could support continued androgen signaling during AR-targeted therapy (Dehm et al., 2008). Further studies identified additional AR-Vs in 22Rv1 as well as a related cell line, CWR-R1. Each of these AR-Vs was similar to 1/2/3/2b, but contained alternate 3' terminal exons in place of exon 2b. These exons, termed CE1, CE2, CE3 and CE5, are located in AR intron 3 (Guo et al., 2009; Hu et al., 2009) (Fig. 1A). Importantly, two separate studies demonstrated that one of these AR-Vs, named AR-V7 (also referred to as AR3) encoded by contiguously-spliced AR exons 1/2/3/CE3, was expressed widely at the mRNA and protein level in prostate cancer cell lines, xenografts, and patient tissues (Guo et al., 2009; Hu et al., 2009). Since these initial reports, various additional AR-Vs have been discovered. For example, an AR-V arising from contiguously-spliced AR exons 1/2/3/4/8 (referred to as ARv567es) was identified by RT-PCR in several human prostate cancer xenografts as well as clinical specimens (Sun et al., 2010). Through the use of tiling genomic microarrays, a cryptic exon 9 downstream of the AR locus was identified that could splice into various AR mRNAs (Hu, Isaacs, & Luo, 2011). Another strategy based on modified 3' RACE followed by next-generation sequencing with RNA isolated from VCaP cells identified various additional cryptic exons in AR intron 3 (Watson et al., 2010). This modified 3'RACE/next generation sequencing workflow was also used with RNA isolated from the murine Myc-CaP model of PCa, leading to the identification of additional cryptic exons located hundreds of kilobases distal and proximal to the murine AR locus (Watson et al., 2010). Clearly, these studies have demonstrated that there is great diversity in the AR-Vs that can be expressed in prostate cancer cells. However, this diversity only affects the extreme COOH-terminal portion of the AR-V protein, indicating that the NTD/DBD core may be the most important determinant of constitutive transcriptional activity.

4.2. Constitutive transcriptional activity of AR-Vs

Experiments with androgen-responsive promoter reporter constructs have established that diverse AR-Vs can function as constitutively active transcription factors in various prostate cancer cell lines (Chan et al., 2012; Dehm et al., 2008; Guo et al., 2009; Watson et al., 2010). In most cases, the magnitude of AR-V-mediated transactivation in these promoter reporter assays is similar to the magnitude of transcriptional activation that can be induced by full-length AR following stimulation with DHT or synthetic androgens such as R1881 or mibolerone (Chan et al., 2012; Dehm et al., 2008). This observation provides further support for the idea that the AR LBD is a regulatory domain that blocks strong activity of the NH₂-terminal TAU1 and TAU5 transcriptional activation modules in the absence of androgens (Simental et al., 1991). In addition to androgen-responsive promoter-reporter constructs, AR-Vs have been shown to support constitutive activation of classical androgen responsive transcriptional targets such PSA, hK2, and FKBP5 (Chan et al., 2012; Guo et al., 2009). For example, expression of these endogenous AR target genes was downregulated in 22Rv1 cells following siRNA-mediated knock-down of AR-Vs (Dehm et al., 2008). However, despite this consistent observation with promoter-reporter constructs and endogenous genes, the basis for constitutive AR-V transcriptional activity has been the subject of debate. Interrogation of the mechanisms leading to nuclear import of full-length AR has demonstrated that the NLS located in the hinge region is a bipartite motif that binds to

importin- α and is absolutely required for full-length AR nuclear localization following ligand stimulation (Cutress et al., 2008; Jenster et al., 1993; Simental et al., 1991). For most AR-Vs, this NLS is disrupted by the presence of the 3' terminal cryptic exon, which would suggest that there would be impairment in nuclear import. However, deletion and mutagenesis experiments revealed that the AR NTD/DBD core is able to support a relatively high level of constitutive nuclear localization through mechanisms that are not yet clear (Chan et al., 2012). This basal level of AR-V nuclear localization appears to be augmented or enhanced in the ARv567es and AR-V7 variants, because ARv567es retains the wild-type AR hinge region and cryptic exon CE3 encodes functional NLS-like lysine and arginine residues in the COOH-terminus of AR-V7 (Chan et al., 2012).

4.3. Genomic alteration in the generation of AR-Vs

Given these important roles for AR-Vs in supporting constitutive transcriptional activity, identifying the mechanisms leading to functional AR-V synthesis in CRPC has been an area of interest. Studies with cell line and xenograft models where AR-Vs were first discovered has indicated that somatic rearrangements in the AR locus are an important mechanism underlying functional AR-V expression. For instance, the 22Rv1 cell line was found to harbor an intragenic tandem duplication of a 35kb genomic segment harboring AR exon 3 as well as cryptic exons 2b, CE1, CE2, and CE3 (Li, Alsagabi, Fan, Bova, Tewfik, & Dehm, 2011). Gain-of-function AR gene rearrangement contrasts with loss-of-function AR gene rearrangements and ensuing splicing alterations that had been reported in androgen insensitivity syndromes (Dehm et al., 2011). In line with a rearrangement-based mechanism, a 48kb intragenic deletion was discovered in AR intron 1 in a sub-population of the heterogeneous CWR-R1 cell line that displayed high levels of AR-V7 expression (Li et al., 2012). Additionally, LuCaP 86.2 xenograft tissue, which was established from CRPC bladder metastasis and expresses high levels of ARv567es (Sun et al., 2010) was found to harbor an intragenic deletion of AR exons 5, 6, and 7 (Li et al., 2012). LuCaP 136 tissue, which was established from CRPC abdominal ascites and also expresses high levels of AR v567es, was found to harbor an intragenic inversion of this same segment encompassing AR exons 5-7 (Nyquist et al., 2013b). A clear cause-effect relationship between AR gene rearrangements and expression of AR v567es was demonstrated by generating these tissue-associated rearrangements *de novo* in a prostate cancer cell line using transcription activator-like effector nuclease (TALEN) technology (Nyquist et al., 2013b). Targeted deletion or inversion of the genomic segment encompassing AR exons 5-7 prevented expression of full-length AR but induced expression of AR v567es. This also provided a clear demonstration that AR-Vs could support constitutive AR transcriptional activity autonomously, independent of full-length AR (Nyquist et al., 2013b).

In addition to a rearrangement-based mechanism, de-regulated AR splicing dynamics may also play a role in AR-V synthesis in CRPC cells. Several studies have demonstrated that castration or treatment with antiandrogens leads to rapid induction of AR-V7 expression in VCaP cells as well as LNCaP sublines derived from long-term passage (Hu et al., 2012; Liu, Xie, Sun, Plymate, Mostaghel, & Dong, 2013b). The mechanism for this acute regulation of the AR-V7 in prostate cancer cells was found to be related to AR gene transcription initiation and elongation rates. This agrees with the observation that increased AR-V7

expression in these models occurred coordinately with increased full-length AR mRNA expression. Using an AR minigene harboring AR exons 3, CE3, and 4, two RNA splicing enhancers were identified within and near exon CE3, and the RNA splicing factors U2AF65 and ASF/SF2 were shown to bind these enhancers and regulate splicing of exon CE3 downstream of AR exon 3 (Liu et al., 2013b).

4.4. Clinical relevance of AR-Vs

The demonstration that genomic rearrangements are responsible for AR-V synthesis in CRPC cell lines, xenografts, and clinical tissue have identified at least one mechanistic scenario where AR-Vs can function as drivers of therapeutic resistance. In addition to this clonal selection basis for AR-V expression, AR-Vs also display acute increases in expression upon castration or treatment with antiandrogens. These key observations strongly implicate the clinical importance of AR-Vs in CRPC (Dehm et al., 2008; Guo et al., 2009; Hu et al., 2009; Hu et al., 2012; Watson et al., 2010). Indeed, a recent study investigating the levels of AR-Vs in hormone-naïve and CRPC metastases found that expression of AR-Vs was increased in CRPC compared to hormone naïve bone metastases (Hornberg et al., 2011). The transcripts for AR-V7 and AR v567es were detected in 100% and 23% of the 30 samples of CRPC bone metastases, respectively. Most significantly, the tissues studied in this cohort that demonstrated a higher immunostaining score were associated with a shorter duration of patient survival. A subsequent study with this cohort of CRPC bone metastases identified a possible reciprocal relationship between AR-V mRNA levels and mRNA expression levels of the steroidogenic enzyme, AKR1C3 (Jernberg et al., 2013). While this study points to a possible inverse relationship between resistance associated with steroidogenesis vs. AR-Vs, a contrasting study of abiraterone resistance in mice bearing CRPC xenografts demonstrated that relapsed tumors displayed increased expression of the CYP17A1 in addition to increased levels of full-length AR and AR-Vs (Mostaghel et al., 2011). While these mRNA-based studies have been informative, more definitive protein measurements have provided a clearer picture of the role of AR-Vs in CRPC progression. To date, AR-V7 is the only AR-V for which a COOH-terminal specific antibody suitable for tissue staining has been developed. Immunohistochemistry with this antibody has demonstrated that AR-V7 protein is frequently expressed in CRPC (Guo et al., 2009; Hu et al., 2012; McGrath et al., 2013). An additional strategy has been to co-stain tissues with two antibodies targeted to the AR protein at either the NH₂- or COOH-termini. This surrogate approach to assessing AR-V expression demonstrated that CRPC was associated with a decrease in staining intensity for the antibody recognizing the AR COOH-terminus relative to the antibody recognizing the AR NH₂-terminus (Zhang et al., 2011).

To model the functions of AR-Vs *in vivo*, two transgenic mouse models have been developed wherein AR-V7 or v567es cDNA were expressed from the prostate epithelium-specific probasin promoter (Liu et al., 2013a; Sun et al., 2013). Prostate-specific expression of v567es in the prostate promoted the formation of prostatic intraepithelial neoplasia (PIN), beginning at week 16, which progressed to invasive adenocarcinoma at one year of age. Microarray-based analysis of gene expression in prostate tissues from Pb-AR^{v567es} suggested a reprogrammed transcriptome enriched in cell cycle related genes and genes involved in tumor initiation and progression (Liu et al., 2013a). However, transgenic AR3

(V7) also induced PIN at one year of age in 43% of 22 mice studied, which was concurrent with expression of the autocrine/paracrine growth factors TGF β 2 and IGF1 (Sun et al., 2013). Overall, these studies demonstrate that AR-V proteins can induce tumorigenesis *de novo*. These two AR-V transgenic models will be valuable for understanding the role of AR-V's *in vivo* during PCa progression.

5. MODE OF ACTION OF CONSTITUTIVE ACTIVE AR AND AR-V IN GENE REGULATION

5. 1. Pioneer factors in AR gene regulation

Pioneer factors are a group of proteins that were first discovered as being important for developmental control of transcription (Zaret, & Carroll, 2011). These proteins are able to bind within closed and condensed chromatin, thereby leading to increased local chromatin accessibility. FoxA1 and GATA-4 were the first two pioneer factors shown to convert compacted chromatin to open chromatin by binding to histones 3 and 4 (Cirillo, Lin, Cuesta, Friedman, Jarnik, & Zaret, 2002). A role for FoxA1 in AR regulation was first revealed by studies demonstrating that FoxA1 functions as a regulator of endoderm development required for AR-mediated transcriptional activation of the rat probasin gene promoter and the human PSA gene. This was due to FoxA1 binding directly to FoxA1 recognition sites adjacent to AREs within core enhancer elements (Gao et al., 2003). Biochemical studies also demonstrated physical interactions between the AR DBD and hinge domains with the forkhead domain of FoxA1. Subsequent genome-wide studies have demonstrated that other transcription factors such as GATA2 and Oct1 may also function as collaborating factors in the AR-FoxA1 regulatory complex (Wang et al., 2007).

Multiple genome-wide studies, using complementary techniques such as chromatin immunoprecipitation-sequencing (ChIP-seq), global run-on-sequencing (GRO-seq) and RNA sequencing (RNA-seq) have further reinforced an important role for FoxA1 in regulating AR function in prostate cancer cells. For example, three separate ChIP-seq studies have demonstrated that the majority of genomic AR binding sites are adjacent to a forkhead motif (Sahu et al., 2011; Tan, Chang, Chng, Wansa, Sung, & Cheung, 2012; Wang et al., 2011). Knock-down of FoxA1 expression using siRNA resulted in re-distribution of these AR binding sites across the genome, indicating that FoxA1 defines sites of AR function (Sahu et al., 2011; Tan et al., 2012; Wang et al., 2011). Whole-exome sequencing of 50 lethal CRPC obtained by rapid autopsy and 11 high-grade localized prostate cancers identified FoxA1 somatic mutations in 3.4% of the samples studied (Grasso et al., 2012). This finding of FoxA1 mutations in prostate cancer is consistent with another whole-exome sequencing study that investigated 112 prostate tumor and normal tissue pairs, although the locations of FoxA1 mutations were different between these two studies (Barbieri et al., 2012). Exogenous expression of wild-type FoxA1 as well as several of these FoxA1 mutants increased proliferation of the LNCaP cell line under castrate conditions *in vivo* and increased xenograft growth *in vivo* (Grasso et al., 2012). However, the mechanism by which these FoxA1 mutants may affect the function of wild-type FoxA1 is not clear. Mechanistically, FoxA1 overexpression appears to enhance LNCaP cell proliferation by forcing genome-wide re-distribution of AR binding sites, resulting in an overall increase in the number of

AR genomic binding regions (Robinson et al., 2013). There are also likely to be important AR-independent functions of FoxA1 in prostate cancer, as shRNA knockdown of FoxA1 increased SLUG expression in several different prostate cell lines, and expression of FoxA1 mutants in AR-null DU145 cells showed reduced capacity for inhibiting cell motility compared with wild-type FoxA1 (Jin, Zhao, Ogden, Bergan, & Yu, 2013). Overall, the precise role of FoxA1 in regulating androgen/AR axis during PCa progression is still not fully established.

5. 2. Co-regulators interaction with AR-Vs

Binding of co-regulators to the AR NTD have pleiotropic roles in AR (and likely AR-V) regulation, including nuclear targeting, ARE binding, and spatial and temporal control of transcriptional output. The mechanism by which established AR co-regulators may bind to and regulate AR-Vs is just beginning to be elucidated, yet several important studies have provided new insights. Here, we highlight these recent studies that have investigated interactions between AR-Vs and co-regulatory proteins.

FHL2 has been shown to function as a co-activator of full-length AR (Muller et al., 2000). Additionally, FHL2 can act as a co-regulator for AR-Vs, and this function may be important for CRPC progression (McGrath et al., 2013). Interestingly, nuclear localization and co-activator function of FHL2 requires calpain-mediated cleavage and liberation of an active 90kDa COOH-terminal fragment of filamin. This 90kDa C-terminal fragment of filamin can form a ternary complex with FHL2 and AR-V7 as assessed by co-immunoprecipitation experiments. Noteworthy, this C-terminal filamin-FHL2-AR-V transcriptional complex exists in CRPC but not in benign prostate tissue or localized prostate cancer. Importantly, this complex is also able to enhance the constitutive transcriptional activity of AR-V7 under castrate conditions.

Vav3 is a Rho GTPase and a co-activator for full-length AR. Vav3 has also been shown to interact with AR-V7 (Peacock, Fahrenholtz, & Burnstein, 2012). Functionally, Vav3 enhanced constitutive transcriptional activity of AR-V7 by directly binding to AR-V7 and promoting nuclear localization. In line with this finding, Vav3 knockdown with shRNA led to reduced levels of AR-V7 in the nucleus.

FOXO1 has been shown to function as a co-repressor of full-length AR, inhibiting transcriptional activity of AR induced by androgen binding as well as IL-6 signaling (Liu, Li, Gan, Kao, & Huang, 2008). FOXO1 binds the TAU5 domain of full-length AR, as well as AR-V7. FOXO1 binding to TAU5 inhibits the association of this domain with p160 SRC co-activators (Bohrer et al., 2013). Ectopic expression of FOXO1 in 22Rv1 cells decreased expression of the endogenous genes that are transcriptional targets of AR-Vs, indicating that FOXO1 and upstream signaling pathways regulating FOXO1, such as PTEN/AKT, could serve as therapeutic targets to inhibit constitutive AR-V transcriptional activity in CRPC cells. This result is in agreement with another study, which found that AKT-mediated phosphorylation of FOXO1 caused retention of FOXO1 protein in the cytoplasm, thereby promoting transcriptional activity of AR-Vs (Mediwala et al., 2013).

5. 3. Gene targets of AR-Vs

Since the initial identification of AR-Vs, a central question has been whether AR-Vs regulate the same sets of transcriptional targets as full-length AR, or whether there may exist distinct sets of transcriptional targets that could distinguish AR-V vs. full-length AR activity. Several microarray-based studies have been conducted to address this question (Guo et al., 2009; Hu et al., 2009; Hu et al., 2012; Sun et al., 2010). Ectopic expression of AR-V7 and AR v567es cDNAs in LNCaP cells demonstrated that AR-Vs are able to regulate the expression of canonical targets of full-length AR such as PSA, hK2, FKBP5, TMPRSS2, ORM1, ORM2, and Grb10 under castrate conditions. Another study employing an inducible AR-V7 transgene in LNCaP cells demonstrated that AR-V7 and full-length AR regulated a similar set of transcriptional targets (Tsai, Boucher, Martinez, Tepper, & Kung, 2012). In cells harboring AR gene rearrangements, direct comparison of the transcriptomes regulated by full-length AR and AR-Vs demonstrated on a genome-wide scale that the target gene repertoires are largely similar (Li, Chan, Brand, Hwang, Silverstein, & Dehm, 2013; Nyquist et al., 2013b). Overall, these studies provide strong support for the idea that AR-Vs mediate therapeutic resistance by recapitulating the basic transcriptional activity of full length AR under castrate conditions. However, interesting differences in the sets of genes activated by AR-Vs vs. full-length AR have also been noted (Guo et al., 2009; Li et al., 2013). For instance, rapid induction of AR-Vs in VCaP and late-passage LNCaP cells is associated with induction of a set of genes associated with cell-cycle progression (Hu et al., 2012). These genes did not appear to be full-length AR targets, as treatment of these same cells with androgens did not result in their induction (Hu et al., 2012). However, a separate study demonstrated that many of these cell cycle-associated genes were responsive to activity of full-length AR as well as AR-Vs, but in a biphasic manner (Li et al., 2013). Similarly, following shRNA-mediated knock-down of AR-V7 in 22Rv1 cells, AKT expression was repressed, indicating that AKT is a selective AR-V7 target in this cell line. Indeed, ChIP studies demonstrated that AR-V7, but not full-length AR, could engage with an ARE in the AKT promoter region (Guo et al., 2009). However, cellular context may be important for this, because siRNA-mediated knock-down of AR-V7 in CWR-R1 cells had no effect on expression of AKT mRNA. Overall, while knowledge in this area has advanced considerably since AR-Vs were first identified, the precise sets of genes regulated by AR-Vs that are critical regulators of CRPC cell growth during AR targeted therapies has yet to be fully established.

6. TARGETING CONSTITUTIVE ACTIVITY OF ANDROGEN RECEPTOR IN PROSTATE CANCER

6.1. Current status of drugs targeting endocrine regulation of AR

The majority of men with primary localized PCa will be treated by radical prostatectomy or radiotherapy, as these treatment modalities are associated with a high rate of cure. However, in cases of advanced, relapsed or metastatic disease, ADT achieved by single-agent or combined treatment with AR antagonists such as bicalutamide or flutamide or gonadotropin releasing hormone agonists such as leuprolide is very effective (Knudsen, & Scher, 2009).

The identification of resistance mechanisms to these first-generation modes of ADT led to the development of abiraterone acetate as a second generation AR axis inhibitor. Abiraterone binds and inhibits the CYP17A1 enzyme required for androgen biosynthesis in testes, adrenals, and tumor tissue (Ferraldeschi, Pezaro, Karavasilis, & de Bono, 2013). Clinical trials have demonstrated that abiraterone acetate prolongs overall survival in CRPC patients that have received prior taxane chemotherapy (de Bono et al., 2011), as well as chemotherapy-naïve patients (Ryan et al., 2013). However, despite this clinical benefit, resistance to abiraterone is a major clinical challenge. Importantly, constitutive activity of AR-Vs may be one mechanism of abiraterone resistance, as evidenced by induced expression of several AR-Vs in xenograft-based models following development of resistance to abiraterone (Mostaghel et al., 2011).

Enzalutamide and ARN-509 are additional next-generation AR axis inhibitors developed to address clinical resistance to first-generation ADT. Enzalutamide and ARN-509 are AR antagonists that bind directly to the AR LBD with a higher affinity than bicalutamide or flutamide. ARN-509 is related structurally to enzalutamide with greater *in vivo* activity in CRPC xenograft models (Clegg et al., 2012). Similar to abiraterone, enzalutamide prolongs overall survival of CRPC patients (Scher et al., 2012). However, another similarity with abiraterone therapy is that resistance to enzalutamide limits clinical efficacy (Scher et al., 2010). One mechanism of clinical resistance is selection for cells harboring an AR F876L mutation, which converts enzalutamide to an agonist (Balbas et al., 2013; Joseph et al., 2013; Korpai et al., 2013). Additionally, prostate cancer cell lines that harboring AR gene rearrangements and expressing high levels of AR-Vs are resistant to enzalutamide due to constitutive AR-V driven activity of the broad androgen transcriptional program (Li et al., 2013).

Apart from the conventional strategy of re-designing drugs that bind to the androgen binding pocket of the AR LBD, targeting alternative functional domains on the AR protein may also have therapeutic efficacy. For example, a structure-based screen for drugs that could bind to the AR BF3 domain and inhibit the AR:SRC-2 interaction identified three non-steroidal anti-inflammatory drugs (flufenamic acid, tolfenamic acid, and meclofenamic acid) as well as the low abundance thyroid hormone 3, 3', 5-triiodothyroacetic acid (Estebanez-Perpina et al., 2007). Similarly, several BF3 inhibitors were identified in a large-scale *in silico* screen that inhibited AR transcriptional activity with no apparent toxicity (Lack et al., 2011). Interestingly, flufenamic acid is very similar to MJC13, which represent another class of inhibitors that prevent hormone-dependent dissociation of the Hsp90-FKBP52-AR complex (De Leon et al., 2011). Indeed, MJC13 had been shown to inhibit transcriptional activity of full-length AR. However, because the BF3 domain is not present in AR-Vs, MJC13 is ineffective at suppressing constitutive AR-V transcriptional activity in prostate cancer cells (Shafi et al., 2013). Nevertheless, these compounds may be exploited as a new class of antiandrogens that could overcome full-length AR-dependent resistance mechanisms.

Blocking the AR DBD: DNA interaction represents another therapeutic opportunity to inhibit AR transcriptional activity. However, targeting the AR DBD is notoriously difficult due to the structural similarities among steroid receptor DBDs. One opportunity may be to exploit unique structural details of the AR DBD: DNA interaction, such as increased

reliance on the AR: AR dimerization interface for stability on AREs as compared with other nuclear receptors (Shaffer et al., 2004). Indeed, even for AR-Vs, there appear to be amino acid residues in the second zinc finger (which makes up part of the dimerization interface) that are important for constitutive AR-V transcriptional activity. For instance, mutational analysis of amino acids located COOH-terminal from the AR DBD in AR-Vs indicated that the first two basic residues in the AR NLS (RK), are important for AR-V transcriptional activity (Chan et al., 2012). However, mutation of these residues also partially inhibited nuclear localization, indicating that further investigation is needed to discern the precise role(s) of these residues in regulating DNA binding vs. nuclear localization (Chan et al., 2012). Despite the clear functional importance of the AR DBD for function of full-length AR and AR-Vs, there are few compounds that have been identified that exert their activity through this domain. One strategy employed cell-permeable Pyrrole (Py)-Imidazole (Im) polyamides designed to bind to the consensus ARE. An ARE-targeted Py-Im polyamide was shown to inhibit the expression of classical AR targets in LNCaP cells by occupying AR binding sites in the PSA and FKBP5 enhancers (Nickols, & Dervan, 2007). This polyamide strategy has also been developed for additional steroid receptors, and X-ray crystallography of a Py-Im polyamide bound to DNA has indicated that these polyamides act as allosteric modulators that alter the DNA helical structure such that steroid receptors are no longer compatible for binding (Chenoweth, & Dervan, 2010). This direct antagonism of the AR:DNA interaction with small molecules may provide an alternative for targeting AR action in prostate cancer cells.

6.2. Therapeutic approaches to inhibit activity of the AR NH₂-terminal domain

The AR NTD is the engine of transcriptional activation mediated by full-length AR and AR-Vs. Therefore targeting AR transactivation domains such as TAU1 and TAU5 would be postulated to be an efficacious strategy. However, the intrinsic structural disorder of the AR NTD has represented a barrier to drug development (McEwan, 2012). Despite these barriers, EPI-001 has been identified through high-throughput screening as a lead compound that can inhibit transcriptional activity of the AR NTD (Andersen et al., 2010). EPI-001 is a chlorinated bisphenol A diglycidic ether (BADGE) that has been shown to interact directly with the AR NTD and block protein:protein interactions between AR and CBP. EPI-001 has two chiral centers, and synthesis of each of the 4 possible stereoisomers (EPI-002 through -005) revealed that EPI-002 had the most potent anti-AR effects as well as lowest toxicity in mouse xenograft studies (Myung et al., 2013). Additionally, each of the EPI compounds were shown to block AR and AR-V mediated transcriptional activity. Additional data demonstrating the precise location on the AR NTD to which these EPI compounds engage is likely to reveal opportunities to further optimize potency and specificity for inhibition of full-length AR as well as AR-Vs.

6.3. Biomarkers for monitoring persistent AR signaling

The finding that resistance to second-generation AR axis inhibitors such as abiraterone and enzalutamide frequently occur through AR-dependent mechanisms has highlighted the importance of the development of biomarkers for monitoring activity of the AR axis during therapy. This is expected to enable to the identification of resistance mechanisms that are active in individual patients, thereby revealing opportunities for therapeutic re-targeting.

However, the major challenges to achieving this goal are the lack of clinically-validated biomarkers available for monitoring AR axis activity as well as the issues with directly sampling tumor cells or tumor cell-derived material from patients.

Resistance to abiraterone acetate is associated with increased expression of steroidogenic enzymes such as FASN, CYP17A1, HSD3B1, HSD3B3, and SD5A1 and UGT2B17 (Mitsiades et al., 2012; Montgomery et al., 2008; Mostaghel et al., 2011), which can facilitate intratumoral de novo steroidogenesis (Locke et al., 2008). Additionally, recurrent mutations in HSD3B1 are associated with abiraterone resistance in prostate cancer cell lines as well as clinical tissues, and can drive abiraterone resistance when expressed ectopically (Chang et al., 2013). Overall these alterations in the sequence and/or expression levels of steroidogenic enzymes have been reported in diverse sources, including prostate cancer cell lines, human xenografts, circulating tumor cells, and metastatic tissue from patients (Mitsiades et al., 2012; Montgomery et al., 2008; Mostaghel et al., 2011). The development of reliable assays for these steroidogenic enzymes, or key steroid metabolites that are synthesized by these enzymes, may serve as biomarkers for monitoring the efficacy of abiraterone acetate in CRPC patients.

Serum-based assays for PSA expression in prostate cancer patients is currently the gold standard for monitoring treatment efficacy and disease progression. CRPC is a complex and heterogeneous disease, and simple monitoring of serum PSA level is not adequate for delineating the precise status of the AR signaling axis in patients. Therefore, a more diverse range of AR targets that have been identified by genome-wide methods may provide a more robust readout of AR axis activity in CRPC patients. For example, a core set of 16 direct AR transcriptional target genes have recently been identified by ChIP-seq with CRPC tissues that may be useful for evaluating continued AR signaling (Sharma et al., 2013).

Recent studies have also identified a key role for AR in regulating central metabolism and metabolic flux in prostate cancer cells (Decker, Zheng, He, Bowman, Edwards, & Jia, 2012; Massie et al., 2011; Sharma et al., 2013; Tan et al., 2012). Therefore, metabolic profiling may provide another avenue for evaluating the status of the AR axis in patient specimens. However, advances in the use of metabolic signatures for monitoring prostate cancer patients is still complicated by several factors such as sample collection and the large variability in metabolite levels assessed through mass spectrometry-based methods. As an alternative to direct monitoring of metabolites, CAMKK2 is a central regulator of metabolism and a direct AR transcriptional target in prostate cancer cells that may serve as a biomarker of AR-regulated metabolic function (Frigo et al., 2011; Massie et al., 2011).

Blood and urine are two convenient specimens for biomarker development, as sampling of these compartments avoids the logistical challenges of directly sampling tumor tissue. Isolation of circulating tumor cells (CTCs) from blood of CRPC patients has allowed for relatively non-invasive evaluation of AR gene amplification (Shaffer et al., 2007). Recent implementation of advanced microfluidic CTC capture platforms has been used to monitor cellular expression of two AR-associated biomarkers, PSA and PSMA. These studies revealed heterogeneity in the presence of CTCs within patients that displayed signatures of AR axis activity (PSA+/PSMA-), AR axis inactivity (PSA-/PSMA+) as well as mixed PSA

+PSMA+ signatures (Miyamoto et al., 2012). Circulating plasma DNA has also been used successfully to assess resistance-associated AR alterations. For example, a beads, emulsion, amplification, magnetic (BEAMing) assay was recently used to assess the presence of the AR F876L mutation associated with enzalutamide and ARN-509 resistance in patient samples (Joseph et al., 2013).

In summary, development of biomarkers that can provide robust evaluation of AR axis alterations is expected to enable the identification of resistance mechanisms operating in individual patients. Harnessing these readouts will likely lead to more effective use of next-generation AR-targeted agents.

7. FUTURE DIRECTIONS

Over the past decade, there have been tremendous advances in our understanding of the biology of AR in driving CRPC progression and alterations that drive continued AR-axis signaling in CRPC cells. As summarized in Fig. 2, constitutive or ligand-hypersensitive activity of the full length AR as well as AR-Vs can be augmented by cross-talk with other hormonal, growth factor, and cytokine/inflammatory signaling pathways. An immediate opportunity to enhance treatment of patients could be the optimization of combinational therapies with AR axis inhibitors and clinically-approved inhibitors of these signaling pathways.

It will also be important to develop therapies targeted to sites on the AR protein independent of the LBD. Targeting the AR NTD remains an attractive concept, as this is the region of the protein responsible for transcriptional activity. However, success with AR NTD targeting may not be possible without breakthroughs in elucidation of the three dimensional structure of this highly disordered protein domain. The challenges to AR NTD structural determination could be overcome by molecular modeling, NMR, or X-ray crystallization following segmentation of the full NTD into smaller discrete modules. Besides the AR NTD, the AR DBD and BF3 site provide additional target sites for development of small molecule inhibitors. Alternatively, a complete understanding of the biochemical basis for constitutive transcriptional activity of the AR NTD/DBD core, such as interactions with chaperone proteins, nuclear import proteins, co-regulators, chromatin modifying enzymes, and components of the basal transcriptional machinery is expected to reveal opportunities for therapeutic intervention with the goal of inhibiting constitutive transcriptional activity of the AR.

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ABBREVIATIONS

ADT Androgen deprivation therapy

AF	Transcriptional activation function
AR	Androgen receptor
ARE	Androgen response element
AR-V	Androgen receptor splice variant
BF	Binding function
CRPC	Castration resistant prostate cancer
CTC	Circulating tumor cell
CTD	COOH-terminal domain
DBD	DNA binding domain
LBD	Ligand binding domain
PCa	Prostate cancer

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Figure 1.

Modular organization of the AR gene and relationship with functional protein domains. (A) Schematic representation of the AR gene locus. Canonical exons are blue, cryptic exons are orange. (B) Functional protein domains of full-length AR and AR-Vs, v567es, 123/2b, 123/CE1, 123/CE2, and 123/CE3 (AR-V7 or AR3) showing amino acid sequences of their unique COOH-termini. (C) Structure of the two zinc fingers in the AR DBD. P-box residues are light blue; D-box residues are light brown. Details are provided in the text.

Figure 2. Pathways regulating constitutive or ligand-hypersensitive activity of full length AR and AR-Vs. Altered AR expression caused by AR gene amplification, point mutations, gene rearrangements, and splicing alterations can be augmented by cross-talk with various hormonal, growth factor, and cytokine/inflammatory signaling pathways. Details are provided in the text.