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## Androgen receptor functions in castration-resistant prostate cancer and mechanisms of resistance to new agents targeting the androgen axis

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### Abstract

The metabolic functions of androgen receptor (AR) in normal prostate are circumvented in prostate cancer (PCa) to drive tumor growth, and the AR also can acquire new growth-promoting functions during PCa development and progression through genetic and epigenetic mechanisms. Androgen deprivation therapy (ADT, surgical or medical castration) is the standard treatment for metastatic PCa, but patients invariably relapse despite castrate androgen levels (castration-resistant PCa, CRPC). Early studies from many groups had shown that AR was highly expressed and transcriptionally active in CRPC, and indicated that steroids from the adrenal glands were contributing to this AR activity. More recent studies showed that CRPC cells had increased expression of enzymes mediating androgen synthesis from adrenal steroids, and could synthesize androgens *de novo* from cholesterol. Phase III clinical trials showing a survival advantage in CRPC for treatment with abiraterone (inhibitor of the enzyme CYP17A1 required for androgen synthesis that markedly reduces androgens and precursor steroids) and for enzalutamide (new AR antagonist) have now confirmed that AR activity driven by residual androgens makes a major contribution to CRPC, and led to the recent Food and Drug Administration approval of both agents. Unfortunately, patients treated with these agents for advanced CRPC generally relapse within a year and AR appears to be active in the relapsed tumors, but the molecular mechanisms mediating intrinsic or acquired resistance to these AR-targeted therapies remain to be defined. This review outlines AR functions that contribute to PCa development and progression, the roles of intratumoral androgen synthesis and AR structural alterations in driving AR activity in CRPC, mechanisms of action for abiraterone and enzalutamide, and possible mechanisms of resistance to these agents.

### Keywords

androgen; androgen receptor; prostate cancer; castration-resistant prostate cancer

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#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

## INTRODUCTION

The standard systemic treatment for prostate cancer (PCa) since the 1940s has been androgen deprivation therapy (ADT, surgical or medical castration) to suppress androgen receptor (AR) transcriptional activity, but most patients relapse within several years with disease that is generally more aggressive and is currently referred to as castration-resistant PCa (CRPC). The past several years have seen a paradigm shift in therapy for CRPC, as it is now widely accepted that the AR is active and stimulating the growth of these cancers that relapse despite castrate levels of androgens (testosterone and dihydrotestosterone). Historically, studies from many groups had indicated that AR was reactivated in CRPC, and that it was driven at least in part by residual steroid hormones from the adrenal glands. Indeed, during the late 1940s and 1950s adrenalectomy or hypophysectomy were shown to be effective secondary hormonal therapies for men who relapsed after orchiectomy.<sup>1</sup> These surgical approaches were subsequently replaced by medical therapies such as prednisone and ketoconazole that suppressed adrenal androgen synthesis, which were similarly effective in a subset of patients based on clinical and biochemical criteria (decrease in serum prostate-specific antigen (PSA)).<sup>2-4</sup> Based on the hypothesis that residual androgens were still driving AR after castration, clinical trials were conducted in the 1980s and 1990s to determine whether the efficacy of castration (orchiectomy or GnRH agonist) could be improved by addition of a direct AR antagonist (flutamide, nilutamide or bicalutamide) to block the effects of residual androgens.<sup>5,6</sup> While meta-analyses of these studies showed a survival benefit for the combined therapies, the magnitude of the benefit was very small and enthusiasm for further therapies beyond castration to suppress AR waned.

Despite these disappointing clinical results, immunohistochemical and molecular studies on patient samples in the 1990s showed that AR was highly expressed and transcriptionally active (based on high-level expression of AR-regulated genes such as PSA) in CRPC.<sup>7-10</sup> Moreover, *AR* gene amplification in CRPC,<sup>11</sup> and the identification of ARs with gain of function mutations in AR antagonist-treated patients that could be strongly activated by the antagonists,<sup>8,12</sup> showed that these tumors were under strong selective pressure to maintain AR activity. Subsequent studies in xenograft models similarly showed increased AR and restoration of AR activity in tumors that relapsed after castration,<sup>13-15</sup> and RNA interference and related approaches established that AR was still required for growth in these CRPC models.<sup>16,17</sup> Studies showing relatively high levels of androgens in CRPC samples from patients,<sup>18,20</sup> in conjunction with studies showing that these tumors had increased expression of androgen synthetic enzymes,<sup>10,20</sup> established androgen synthesis by tumor cells as a mechanism for AR reactivation in CRPC.<sup>21</sup>

Most recently, phase III clinical trials of abiraterone (inhibitor of the enzyme CYP17A1 required for androgen synthesis) and enzalutamide (more effective direct AR antagonist) in CRPC established that further AR suppression can extend patient survival, and led to Food and Drug Administration approval of these agents.<sup>22-24</sup> Unfortunately, while the majority of patients who have relapsed after castration respond initially to these agents, the overall survival advantage in advanced disease (post chemotherapy) is still modest (4–6 months), and most responding patients relapse within 1–2 years with evidence of renewed AR activity. In order to build on these recent advances in AR-targeted therapies for PCa, it is

clearly critical to better understand the critical functions of AR and mechanisms mediating its reactivation, and to develop strategies that can overcome these mechanisms. This review focuses on AR functions in PCa and mechanisms of action and resistance to agents targeting AR in CRPC.

## AR STRUCTURE AND NORMAL FUNCTION AS A TRANSCRIPTIONAL ACTIVATOR

The AR is a transcription factor with a large N-terminal transactivation domain (NTD) (exon 1), a C-terminal ligand-binding domain (LBD) (exons 4–8), a central DNA-binding domain (DBD) (exons 2–3), and a hinge region between the DNA-binding domain and LBD that contributes to nuclear localization and degradation (Figure 1). The unliganded AR associates with an HSP90 chaperone complex in the cytoplasm and undergoes proteasome-mediated degradation in the absence of ligand. Similarly to other nuclear receptors, binding of agonist ligands (testosterone or dihydrotestosterone) causes a shift in the position of helix 12 in the AR LBD towards helices 3–5, which stabilizes ligand binding and generates a hydrophobic cleft for binding of leucine-x-x-leucine-leucine (LxxLL) motifs found in many transcriptional coactivator proteins.<sup>25,26</sup> A unique feature of AR is that an LxxLL-like motif in the AR N terminus (amino acids 23–27, FQNLF) binds to this hydrophobic cleft, which further stabilizes helix 12 and ligand binding (AR–N–C terminal interaction).<sup>27,28</sup> Fluorescence resonance energy transfer studies show that this N–C interaction is initially intramolecular in the cytoplasm, but shifts towards intermolecular in the nucleus and may have some role in nuclear localization, although its precise function is not clear.<sup>29,32</sup> Interestingly, fluorescence resonance energy transfer data also suggest that the N–C interaction may be disrupted when AR binds chromatin, possibly in order to allow for coactivator binding.<sup>30</sup>

The agonist-liganded AR then translocates to the nucleus, dimerizes and binds to specific sequences (androgen-responsive elements (AREs)) found in the regulatory regions of AR-target genes. AR binding to these sites displaces a weakly associated central nucleosome and is generally dependent upon prior binding of the transcription factor FOXA1, which has been termed a ‘pioneer transcription factor,’ as it is needed to initially unwind the chromatin around the ARE so it becomes accessible for AR binding.<sup>33,34</sup> The AR also undergoes multiple post-translational modifications in response to agonist binding, including phosphorylation, methylation, acetylation, ubiquitylation and sumoylation.<sup>35</sup> These modifications have roles in regulating AR stability, cellular localization and transcriptional activity, although the precise mechanisms by which many of these modifications modulate AR structure and interactions with other proteins or DNA remain to be determined (see below). A series of transcriptional coactivator/chromatin-modifying proteins are then recruited through interactions with the AR NTD and LBD. This complex of proteins recruited by AR then interacts by chromatin looping with the promoter regions to stimulate the transcription of a large number of AR-regulated genes.<sup>36,37</sup>

Consistent with the normal function of androgens in the prostate being to drive the differentiated functions of luminal epithelial cells, these AR-stimulated genes include

seminal fluid proteins (such as prostate-specific antigen) and multiple genes in metabolic pathways required to support high levels of protein and lipid synthesis.<sup>38,39</sup> Significantly, while androgens can clearly stimulate PCa growth, and androgen deprivation causes a G0/G1 cell cycle arrest,<sup>40,41</sup> most genes driving cell cycle progression in response to androgen do not appear to be directly regulated by AR. One mechanism mediating the cell cycle progression in response to androgens is an increase in TORC1 activity and subsequent TORC1-mediated increase in the translation of D-cyclins.<sup>39</sup> The decrease in TORC1 activity after androgen deprivation and the increase in TORC1 activity in response to androgen likely reflect the ability of AR to stimulate cellular metabolism by increasing the expression of multiple membrane transporters and other genes driving lipid and protein synthesis (see above).

Androgen deprivation also increases levels of the cyclin-dependent kinase inhibitor p27, while androgen stimulation promotes the rapid degradation of p27.<sup>40,42</sup> Recent data indicate that the rapid p27 degradation reflects androgen stimulation of TORC2, the subsequent phosphorylation and activation of AKT, and phosphorylation of p27 by AKT at a site that enhances p27 degradation (threonine 157).<sup>43</sup> The androgen-mediated stimulation of TORC2 appears to be independent of transcription, but its mechanism remains to be determined. Interestingly, this AKT site on human p27 is not conserved in the mouse, and a recent study using a tetracycline-inducible myristoylated-AKT indicated that AKT-driven proliferation in mouse prostate epithelium is independent of p27 degradation.<sup>44</sup> These observations suggest that studies in mouse models may underestimate the oncogenic activity of PI3 kinase/AKT-pathway activation.

In contrast to p27, the agonist-liganded AR binds to a site on the cyclin-dependent kinase inhibitor *p21* gene and directly increases p21 transcription and protein expression.<sup>45</sup> As p21 can, in some contexts, stimulate cell cycle progression by increasing assembly of cyclin D/CDK4 complexes, this may be a mechanism that contributes to androgen-stimulated PCa growth. Finally, it should be noted that AR is also weakly expressed by subsets of cells in the prostate stroma, and that AR in these cells can stimulate the expression of growth factors such as KGF/FGF7.<sup>46</sup> Through this mechanism, AR in stromal cells may indirectly regulate growth of the epithelium, and loss of these stromal factors likely contributes to prostate involution after castration.

## AR FUNCTION AS A TRANSCRIPTIONAL REPRESSOR

In addition to its well established function as a transcriptional activator, gene expression studies from many groups have shown that expression of multiple genes is decreased in response to androgen. For many of these androgen-repressed genes, this is likely secondary to transcriptional effects on other genes. However, AR can repress the expression of some genes more directly through interactions with other transcriptional activators or by functioning as a transcriptional repressor. The most studied example of the former mechanism is AR interaction with SP1, which can interfere with SP1-mediated transactivation of genes including the luteinizing hormone  $\beta$ -subunit and c-MET.<sup>47,48</sup> Other transcription factors that may be similarly antagonized through interaction with the agonist-liganded AR include RUNX2, JUN and SMAD3.<sup>49</sup> Moreover, AR binding of  $\beta$ -catenin,

which is a critical coactivator for the T-cell factor transcription factors that mediate canonical Wnt/ $\beta$ -catenin signaling, may suppress this pathway.<sup>50,55</sup>

The agonist-liganded AR also may function more directly as a transcriptional repressor by recruiting corepressors including ALIEN, DAX1, HEY, AES, PHB and SHP.<sup>56-61</sup> However, more studies are needed to address whether these corepressors are targeted to AR on a specific set of genes, and whether any such targeting is determined by features of the ARE or by other mechanisms. The corepressors, NCoR and SMRT, which associate strongly with the LBD of unliganded nonsteroid nuclear receptors and mediate their transcriptional repression functions, can also associate weakly with the agonist-liganded AR (probably through the AR NTD) (Figure 1).<sup>62-65</sup> In the absence of ligand, nonsteroid nuclear receptors still bind chromatin and the coactivator-binding site in their LBD is enlarged (due to displacement of helix 12), allowing them to accommodate extended LxxLL-like motifs (CoRNR boxes) in NCoR and SMRT. While the effects of NCoR and SMRT on the agonist-liganded AR are modest, their enhanced recruitment may contribute to the activity of some AR antagonists (see below). Finally, androgen-mediated transcriptional repression has been linked to AR recruitment of EZH2, a histone methyltransferase and component of the polycomb-repressive complex 2, which increases the repressive H3K27me3 mark.<sup>66,67</sup>

Recent studies have shown that AR also associates with a histone demethylase, lysine-specific demethylase 1 (LSD1, KDM1A), and that LSD1 can function both as a coactivator and corepressor for AR.<sup>68-70</sup> LSD1 was identified initially in corepressor complexes and shown to function as a transcriptional corepressor by demethylating mono- and dimethylated lysine 4 on histone 3 (H3K4me1,2) (due to its catalytic mechanism, LSD1 cannot demethylate trimethylated lysines).<sup>71</sup> However, it was subsequently shown that LSD1, when associated with AR and possibly other transcription factors including ER $\alpha$ , can function as a transcriptional coactivator and mediate demethylation of repressive mono- and dimethylated H3K9.<sup>68,72-75</sup> Indeed, CHIP studies have shown that LSD1 is localized to androgen-responsive elements in multiple androgen-stimulated genes, and that LSD1 inhibition markedly attenuates androgen-stimulated expression of these genes.<sup>68,69</sup>

In contrast to LSD1 function as a coactivator for androgen-stimulated genes, it functions as an AR corepressor on androgen-repressed genes including the *AR* gene. A recent study showed that the agonist-liganded AR functions directly as a transcriptional repressor on the *AR* gene by binding to a site in the second intron of the *AR* gene, where it recruits LSD1.<sup>70</sup> Significantly, androgen-mediated AR and LSD1 binding to this site was associated with demethylation of H3K4me1 and H3K4me2, and LSD1 knockdown or inhibitors prevented this demethylation and downregulation of *AR* gene expression. Together these findings suggest that a switch in LSD1 substrate specificity from H3K4 to H3K9 may mediate its functions as an AR corepressor versus a coactivator, respectively. Recent studies indicate that phosphorylation of histone 3 on threonine 11 (H3T11ph) by an AR-associated serine/threonine kinase, protein kinase C-related kinase 1 (PRK1, PKN1) enhances the ability of LSD1 to demethylate repressive H3K9me1,2.<sup>76</sup> Moreover, phosphorylation of histone 3 on threonine 6 (H3T6ph) by a distinct kinase (protein kinase C  $\beta$ 1, PKC $\beta$ 1) was found to suppress the LSD1-mediated demethylation of H3K4me1,2.<sup>77</sup> These results suggest that the dual recruitment of PKN1 and PKC $\beta$ 1 by AR at androgen-stimulated genes may switch

LSD1 substrate specificity from H3K4 to H3K9 (Figure 1). However, it should be emphasized that LSD1 mediates some degree of both H3K4 and H3K9 demethylation at androgen-repressed and -activated genes,<sup>70</sup> and that alterations in H3K4 and H3K9 methylation may be the result of alterations in transcription rather than being causal. Therefore, further mechanisms are likely contributing to the activities of AR and LSD1 on androgen-stimulated versus -repressed genes.

In addition to the *AR* gene, androgens similarly repress the expression of at least two enzymes mediating androgen synthesis in prostate cells (AKR1C3 and HSD17B6) by this LSD1-dependent mechanism.<sup>70,78</sup> This is consistent with a physiological negative feedback loop that can adjust AR levels and intracellular androgen levels to maintain AR signaling in response to fluctuations in serum androgen levels. By rapidly increasing *AR* gene transcription and androgen synthesis, this feedback loop may be a mechanism that helps PCa cells initially to adapt to ADT. Significantly, androgen-repressed genes are also highly enriched for genes that mediate DNA synthesis and cell cycle progression, with at least a subset being repressed by an LSD1-dependent mechanism.<sup>70</sup> This result is consistent with a normal physiological role of AR being to drive differentiation rather than proliferation, and with previous studies showing that androgens, particularly at higher concentrations, can suppress rather than stimulate growth of PCa cells.<sup>70</sup> The androgen repression of these genes mediating DNA synthesis and cell cycle progression is presumably overridden in PCa by other oncogenic signal-transduction pathways that strongly activate these genes.

These dual functions of AR as a transcriptional activator and repressor have important implications for ADT (Figure 2). PCa cells may initially respond to androgen deprivation due to the central role of AR in regulating metabolic functions, so that androgen deprivation essentially starves the cells and represses pathways including TORC1. However, androgen deprivation may also allow cells initially to maintain a low level of AR activity by increasing *AR* gene transcription and androgen synthesis. Tumor cells then further adapt over time and partially restore AR signaling, based on mRNA levels for AR-regulated genes in CRPC clinical samples.<sup>10,79</sup> These levels are presumably adequate to support critical metabolic functions, but not adequate to impair the expression of AR-repressed genes mediating androgen synthesis, DNA synthesis and cell cycle progression. Significantly, high levels of androgen can repress these genes and arrest tumor growth in some CRPC cell line and xenograft models,<sup>70</sup> but the efficacy of this approach in patients is unclear and would likely be limited by tumor heterogeneity. Nonetheless, these findings support the development of novel AR antagonists or other agents that can selectively impair AR transcriptional activator functions, and/or enhance its repressor functions, as these may be efficacious in CRPC.

## NOVEL AR FUNCTIONS IN PCA DEVELOPMENT AND PROGRESSION TO CRPC

The identification of frequent fusions between the strongly AR-regulated *TMPRSS2* gene and the Ets family transcription factor *ERG* gene, as well as additional fusions involving *TMPRSS2* or other AR-regulated genes, established that AR acquires new functions in PCa.<sup>80</sup> Reported downstream functions of *ERG* in PCa include increased invasion, increased



expression of EZH2 and down-regulation of AR expression and transcriptional activity.<sup>81,82</sup> However, while it appears clear that ERG contributes to PCa development, the relevant downstream effectors and functions of ERG in PCa have remained to be established. A recent study found that the SOX9 transcription factor, which regulates ductal morphogenesis in fetal prostate and maintenance of stem/progenitor cells in adult tissues,<sup>83-86</sup> is a downstream effector of ERG in *TMPRSS2:ERG* fusion-positive PCa.<sup>87</sup> Specifically, ERG in PCa cells binds to a site 3' of the SOX9 gene and opens a cryptic AR-binding site, so that androgens strongly stimulate SOX9 in *TMPRSS2:ERG* fusion-positive PCa cells both by driving ERG expression and directly through this ERG-dependent AR-regulated enhancer in the SOX9 gene (Figure 3). Further studies showed that SOX9 mediates the increased invasion downstream of ERG *in vitro*, and that SOX9 knockdown could markedly impair the *in vivo* growth of PCa cells expressing the *TMPRSS2:ERG* fusion gene.<sup>87</sup> In genetically engineered mouse models, SOX9 knockdown impairs PCa development driven by MYC and SV40 T antigen,<sup>83</sup> while SOX9 overexpression in prostate on a *Pten*<sup>-/+</sup> background results in high-grade dysplastic lesions that progress to invasive PCa in a subset of mice.<sup>87,88</sup>

SOX9 is expressed at higher levels in *TMPRSS2:ERG* fusion-positive versus -negative PCa,<sup>87</sup> but is also expressed in fusion-negative tumors where it may be regulated by pathways including MAP kinase and Wnt/ $\beta$ -catenin.<sup>89,90</sup> SOX9 expression appears to be further increased in CRPC,<sup>90</sup> and higher levels of SOX9 in residual tumor correlated with early relapse in a neoadjuvant clinical trial of androgen deprivation combined with docetaxel and estramustine.<sup>91</sup> Significantly, SOX9 expression is repressed by androgens in *TMPRSS2:ERG* negative PCa cells and in mouse prostate after castration, so that androgen deprivation may increase SOX9 in fusion-negative tumors.<sup>87,88</sup> This may have therapeutic implications, as androgen deprivation combined with radiation or cytotoxic chemotherapy may be more effective in fusion-positive tumors expressing high levels of SOX9, that are downregulated by castration, than in fusion-negative tumors. However, further studies are needed to determine whether this increase in SOX9 after androgen deprivation is biologically significant, and whether it affects responses to particular therapies.

Further studies using ChIP-seq methods coupled with gene expression microarrays to comprehensively identify genes that are directly regulated by AR indicate that AR acquires further new functions in CRPC. In particular, AR in CRPC cell lines was found to execute a distinct transcriptional program that included the direct activation of a set of M-phase cell cycle genes such as *CDK1* and *UBE2C* that are overexpressed in CRPC.<sup>37</sup> This reprogramming could in part reflect novel intracellular AR ligands or distinct activities of an unliganded AR, but increased H3K4 methylation at AR-binding sites in these genes suggest a role for epigenetic alterations. Significantly, previous studies have indicated that AR signaling, based on expression of genes that are normally stimulated by androgen, decreases in higher Gleason grade tumors,<sup>92</sup> and in CRPC.<sup>10,79,92</sup> This is consistent with lower androgen levels in CRPC, but may in addition reflect epigenetic reprogramming and narrowing of the spectrum of genes that are AR regulated with tumor progression.

Most recently, EZH2 has been identified as an AR coactivator that may contribute to altering AR function in CRPC.<sup>93</sup> EZH2 is a histone methyltransferase associated with the polycomb-repressive complex 2 that is upregulated in CRPC, and had been presumed to be functioning

by progressive silencing of tumor suppressor genes through H3K27 trimethylation. However, this recent study found that EZH2 forms a complex with AR in CRPC cells that is recruited to the cis-regulatory elements of AR-target genes including CDK1 and UBE2C, and that it functions as an AR coactivator on these genes by a mechanism that is dependent on its methyltransferase activity, but independent of its ability to methylate H3K27.<sup>93</sup> This AR interaction and coactivator function of EZH2 is mediated by AKT-dependent phosphorylation of EZH2 on serine 21, which also impairs its ability to methylate H3K27 and presumably directs it to one or more alternative substrates. Finally, recent data indicate that AR splice variants lacking the LBD, which are increased in CRPC (see below), may regulate a distinct set of genes that include genes driving cell cycle progression.<sup>94</sup> These findings could reflect novel interactions between AR splice variants and EZH2, but further studies are needed to determine their molecular basis.<sup>95,96</sup>

## AR REACTIVATION IN CRPC AND RESISTANCE TO CYP17A1 INHIBITION MEDIATED BY INTRATUMORAL ANDROGEN SYNTHESIS

The normal prostate has the enzymatic machinery to synthesize testosterone from serum precursors supplied by the adrenal glands, and this testosterone is normally converted to the higher affinity ligand dihydrotestosterone by 5 $\alpha$ -reductases (SRD5A1 and SRD5A2), primarily the type 2 5 $\alpha$ -reductase (SRD5A2) in normal prostate (Figure 4). Intraprostatic synthesis may not be a major source of androgen in men with intact testes and normal circulating testosterone, but can become a substantial source after ADT.<sup>21</sup> Indeed, several studies have shown that intraprostatic androgen levels do not decline as markedly as serum levels after ADT.<sup>97-101</sup> Moreover, an analysis of AR-regulated gene expression in prostatectomy specimens in patients receiving neoadjuvant (treatment prior to prostatectomy) ADT found that while several androgen-regulated genes (*NDRG1*, *FKBP5*, and *TMPRSS2*) were reduced, many others were not suppressed.<sup>102</sup> Taken together, these data demonstrated that intraprostatic androgen synthesis can be a significant source of androgen and may buffer primary PCa against the acute effects of ADT.

Early studies examining intraprostatic androgen levels in men with CRPC and intact prostates suggested that androgen synthesis may be further increased,<sup>103,104</sup> and recent studies using more reliable mass spectrometry methods have now clearly established that intraprostatic androgen levels in men with CRPC are not substantially reduced relative to levels in eugonadal men.<sup>18,19</sup> Moreover, a study in metastatic CRPC samples showed that androgen levels in many of the CRPC samples were actually increased relative to control tissues, indicating that the progression to CRPC was associated with increased intratumoral accumulation or synthesis of androgen.<sup>20</sup> In parallel with these assessments of androgen levels, gene expression studies in CRPC bone marrow metastases identified increased expression of enzymes mediating androgen synthesis from weak adrenal androgens as a molecular mechanism mediating the restoration of androgen levels in CRPC, with the largest increases being in the levels of *AKR1C3* mRNA, which reduce androstenedione to testosterone (approximately five-fold median increase, with marked increases in approximately one-third of cases).<sup>10</sup> This study also found significant increases in type 1 *SRD5A* and in *HSD3B2*, which converts DHEA to androstenedione.<sup>10</sup> Subsequent studies in



CRPC clinical samples have similarly found increased expression of enzymes mediating androgen synthesis, including AKR1C3 and SRD5A1.<sup>20,105,106</sup>

CRPC cells also may have increased expression of enzymes mediating cholesterol and fatty acid synthesis, which may be driven by increased SREBP activity and is consistent with their enhanced lipid synthesis.<sup>9,107</sup> However, while it now appears clear that PCa cells adapt to ADT by enhancing their ability to metabolize weak androgens produced by the adrenal glands, whether they can synthesize physiologically significant amounts of androgen *de novo* from cholesterol has been less clear. Studies conducted primarily in LNCaP cells and xenografts showed that enzymes required for *de novo* steroid synthesis (including CYP17A1, the enzyme that catalyzes 2 steps in androgen synthesis to generate DHEA, see Figure 4), were increased in castration-resistant sublines and could generate detectable levels of androgens.<sup>108,110</sup> However, studies in CRPC clinical samples have not consistently found increases in these enzymes.<sup>10,20,105</sup> More recent studies in CRPC cell line and xenograft models have now confirmed that PCa cells can generate androgens *de novo* from cholesterol at levels that are adequate to drive AR transcriptional activity and tumor growth.<sup>78,111</sup> Significantly, AR activity and growth of these CRPC xenografts could be suppressed by the CYP17A1 inhibitor abiraterone, and CYP17A1 mRNA was increased in xenografts that progressed after initial responses. Moreover, CYP17A1 mRNA also appeared to be increased in a small set of CRPC clinical samples from patients treated with a CYP17A1 inhibitor.<sup>78</sup>

Taken together, these findings suggest that CYP17A1-dependent *de novo* androgen synthesis may not be a major mechanism driving AR in tumors that relapse after standard ADT, where there are very high serum levels of precursor steroids that can be taken up by the tumor cells and converted to testosterone and dihydrotestosterone. However, this mechanism may become more important in patients treated with abiraterone or other CYP17A1 inhibitors that markedly decrease adrenal gland synthesis of precursor steroids. While abiraterone would presumably also be targeting CYP17A1 and androgen synthesis in tumor cells, relative resistance could emerge through mechanisms including CYP17A1 overexpression or mutations, increased synthesis of upstream substrates (possibly including novel substrates that may have higher affinity for CYP17A1 and be metabolized through alternative pathways), or increased drug metabolism or efflux (Figure 4). Alternatively, as abiraterone does not completely ablate serum precursor steroids (particularly DHEA-S, which is still present at substantial levels and can be transported and metabolized to DHEA by steroid sulfatase in PCa cells), tumor cells may adapt by further enhancing their ability to take up and metabolize steroid precursors downstream of CYP17A1. Further studies of clinical samples from CRPC patients with intrinsic and/or acquired resistance to CYP17A1 inhibitors will be critical to determine the role of these and other (see below) resistance mechanisms and to assess therapies targeting these mechanisms.

## STRUCTURAL ALTERATIONS IN AR-MEDIATING RESISTANCE TO CASTRATION AND CYP17A1 INHIBITION

Increased AR expression as a result of *AR* gene amplification,<sup>11</sup> or other mechanisms that increase *AR* gene transcription in CRPC,<sup>70,112</sup> can clearly contribute to increasing AR responses to low levels of androgen. Mutations in the AR LBD can alter its structure so that it is activated by alternative ligands including progesterone, hydrocortisone, estradiol and certain AR antagonists.<sup>8,12,113-115</sup> These latter mutations are found at increased frequency in patients treated with the AR antagonists flutamide (codons 874 and 877) and bicalutamide (codon 741), but are uncommon in patients treated with medical or surgical castration.<sup>12,116</sup> However, as serum levels of progesterone and other upstream steroids are increased in patients treated with CYP17A1 inhibitors, mutant ARs that are activated by these upstream steroids may emerge as an important resistance mechanism.<sup>78</sup>

AR splice variants that have lost the LBD, and are therefore constitutively active in the absence of ligand, also have been identified in CRPC.<sup>117-123</sup> In the majority of these variants, exon 3 (encoding the 3' end of the DNA-binding domain) is spliced to a cryptic exon in the intron between exons 3 and 4, while exon 4 is spliced out of frame to exon 8 in another major variant. Based on mRNA levels, these variants generally appear to be expressed at low levels relative to the full length AR, and the extent to which they contribute to AR activity in CRPC (either as homodimers or as heterodimers with full length AR) is not yet clear. However, they can be expressed at high levels and drive androgen-independent growth in cells with deletions in the AR locus that impair expression of the full length AR.<sup>96,124</sup> Therefore, it seems likely that genetic or epigenetic mechanisms mediating expression of AR variants at levels that can contribute substantially to AR-pathway reactivation will emerge as a resistance mechanism in response to agents that markedly deplete androgens such as CYP17A1 inhibitors or to novel AR antagonists targeting the LBD (see below).<sup>96,111</sup> AR antagonists that target the NTD are currently being developed and may be active against these variants.<sup>125</sup>

Studies from many groups have identified post-translational modifications (serine/threonine phosphorylation, tyrosine phosphorylation, acetylation, methylation, ubiquitylation and sumoylation) of AR that can enhance AR activity in response to low levels of androgens, and may thereby contribute to AR reactivation in CRPC and to intrinsic and/or acquired resistance to CYP17A1 inhibitors.<sup>35</sup> Proteins mediating these modifications that may have increased activity in CRPC include CDK1, AKT, SRC, ACK1, p38, JNK, RNF6 and SET9. SRC and ACK1 have been reported to tyrosine phosphorylate AR at distinct sites on the NTD and enhance AR transcriptional activity at low-androgen levels.<sup>126-128</sup> CDK1 activated during M-phase phosphorylates serine 81 in the AR NTD, a site that is phosphorylated by CDK9 during interphase and enhances AR transcriptional activity.<sup>129-131</sup> AKT can phosphorylate serine 213, but the major kinase targeting this site may be PIM1.<sup>132-135</sup> Phosphorylation of this site can mediate MDM2 binding and AR degradation, but this appears to be context dependent and this site may also increase AR levels and modulate AR nuclear localization.<sup>132,134,136-138</sup> Recent reports in PTEN-deficient mice indicate that PI3 kinase-pathway activation decreases AR expression, but the role of AR phosphorylation by

AKT in this model remains to be defined.<sup>139,140</sup> Phosphorylation of serine 650 in the AR hinge region by p38 and JNK stimulates AR nuclear export, and dephosphorylation of this site by PP1 can enhance AR nuclear retention.<sup>141,142</sup> The ubiquitin ligase RNF6 is increased in CRPC and can enhance AR activity through non-lysine 48-linked polyubiquitylation, which presumably modulates its interaction with other chromatin-associated proteins.<sup>143</sup> Finally, methylation of a site in the AR hinge region by SET9 can enhance the N–C terminal interaction and may increase AR activity in CRPC.<sup>144,145</sup>

Increased expression or activity of transcriptional coactivator proteins (including SRC2/TIF2, which is amplified in a subset of PCa), or decreases in corepressor proteins, also may enhance AR activity.<sup>146–150</sup> Further proteins that associate with AR and may modulate its activity in CRPC include EZH2 and LSD1 (see above), and additional histone methyltransferases and demethylases.<sup>69,151</sup> Interestingly, exome sequencing indicates that multiple chromatin-modifying enzymes are mutated in advanced CRPC,<sup>152,153</sup> suggesting that AR activity in general, or on subsets of genes (such as M-phase genes, see above), may be enhanced through changes in chromatin structure.

## MECHANISMS OF ACTION AND RESISTANCE TO AR ANTAGONISTS

Nonsteroidal AR antagonists including bicalutamide and hydroxyflutamide (active metabolite of flutamide) bind to the steroid-binding pocket in the AR LBD and presumably mediate an alternative conformational change that interferes with formation of the coactivator-binding site (crystal structures of the wild-type AR LBD bound to antagonists have not yet been obtained). Mutations in codons 874 or 877 alter the steroid-binding pocket and allow hydroxyflutamide to generate an agonist conformation and function as a strong agonist, while a mutation in codon 741 similarly allows bicalutamide to function as a potent AR agonist.<sup>26,154,155</sup> Importantly, CHIP and FRAP studies have shown that bicalutamide can still stimulate nuclear accumulation and binding of the wild-type AR to chromatin (although more weak and transient binding compared to androgen), and that its antagonist activity reflects ineffective recruitment of coactivator proteins.<sup>32,156,157</sup> In contrast, enzalutamide stimulates less nuclear accumulation of AR, and reporter gene and related studies show that the enzalutamide liganded AR does not bind to chromatin (Figure 1).<sup>158</sup> A sensitive fluorescence resonance energy transfer assay indicates that this may reflect more effective impairment of the AR–N–C terminal interaction, and possibly AR interaction with other proteins. However, the precise basis for this loss of chromatin binding, which likely contributes to the improved efficacy of enzalutamide versus bicalutamide in CRPC, remains to be determined.<sup>158</sup> Another AR antagonist related to enzalutamide that is currently in clinical trials (ARN-509),<sup>159</sup> as well as a series of recently reported unrelated AR antagonists, similarly inhibit AR chromatin binding.<sup>160</sup>

The agonist-liganded AR can also interact weakly with transcriptional corepressor proteins including NCoR and SMRT, and RNA interference targeting NCoR and SMRT can enhance androgen-stimulated AR transcriptional activity (see above). This AR interaction with NCoR and SMRT appears to be mediated primarily by the AR NTD, as the agonist-liganded coactivator-binding site in the AR LBD cannot accommodate the extended CoRNR boxes in NCoR and SMRT that mediate binding to other unliganded nuclear receptors. CHIP studies

indicate that AR recruitment of NCoR and SMRT are increased by the AR antagonist bicalutamide, which may reflect an alternative non-agonist conformation of the AR LBD that can better accommodate CoRNR box binding.<sup>36,161,162</sup> The interaction between AR and NCoR/SMRT also can be further enhanced by mifepristone.<sup>64,65</sup> However, the extent to which NCoR/SMRT recruitment contributes to the antagonist activity of bicalutamide is not clear, as bicalutamide still functions as an AR antagonist in cells treated with NCoR and SMRT small interfering RNA.<sup>163</sup>

One study found that bicalutamide could function as an AR agonist in the setting of inflammation due to NCoR/SMRT translocation out of the nucleus, but the extent of this agonist activity and whether factors in addition to decreased nuclear NCoR/SMRT mediate this activity are not clear.<sup>164</sup> Bicalutamide also has detectable partial agonist activity in cells that are transiently or stably overexpressing AR.<sup>14</sup> However, this activity is very weak compared to the potent activity mediated by true agonists or by bicalutamide-stimulated Y741-mutant ARs, and bicalutamide still functions as an antagonist of androgen-stimulated AR activity in cells that overexpress AR. Nonetheless, it is reasonable to suggest that a number of factors, including altered expression or post-translational modification of AR, coactivators or corepressors, may enhance the very weak partial agonist activity of bicalutamide adequately to achieve a threshold level of AR activity required to support tumor growth. However, it remains to be determined whether bicalutamide resistance in CRPC reflects bicalutamide function as an agonist for wild-type AR that can drive the substantial AR reactivation in CRPC, versus lack of potency in competing for increased intratumoral androgens, or other mechanisms. Mechanisms mediating intrinsic or acquired AR-pathway resistance to enzalutamide remain to be determined, but similarly to bicalutamide may include AR mutations, constitutively active AR splice variants lacking the LBD (see above), or increased intratumoral androgens. Moreover, further novel mechanisms may emerge that can drive chromatin binding by the enzalutamide liganded wild-type AR.

## CONCLUSIONS

It is now apparent that increased intratumoral androgen synthesis and subsequent partial restoration of AR transcriptional activity make a significant contribution to CRPC. To build on the clinical success of abiraterone and enzalutamide, it will clearly be necessary to identify and target mechanisms of resistance to these and related agents (and/or to employ these agents earlier in the course of the disease). Multiple mechanisms may contribute to restoration of AR activity after treatment with CYP17A1 inhibitors, including further increases in intratumoral androgen synthesis (de novo or from serum DHEA-S) and alterations in AR or associated proteins that can enhance AR responses to very low levels of ligand. The former mechanism may be addressed by targeting additional steps in androgen synthesis or possibly by addition of an AR antagonist, while the latter may be addressed by combination therapies with agents blocking kinase or other pathways that enhance AR activity. Resistance to enzalutamide or other AR antagonists may be mediated by similar mechanisms, or by mechanisms that stimulate partial agonist activity, and there is clear interest in the development of additional antagonists that may circumvent these mechanisms by enhancing AR degradation. As treatment with flutamide or bicalutamide selects mutant ARs that are activated by these agents, it also is reasonable to assume that mutant ARs will

emerge that have decreased affinity or are stimulated by enzalutamide. The increased levels of pregnanes in patients treated with CYP17A1 inhibitors may similarly select for AR mutations (including those in codons 874 and 877) that enhance AR activation by progesterone or other steroids upstream of CYP17A1. Increased expression of AR variants lacking the LBD also may emerge as a significant mechanism of resistance to inhibitors of steroid synthesis and to antagonists targeting the LBD, which should spur the further development of agents targeting the AR NTD or DNA-binding domain. Finally, one can anticipate that more effective targeting of AR will lead to the increased emergence of tumors that are no longer dependent on AR, and that will require alternative therapeutic strategies.

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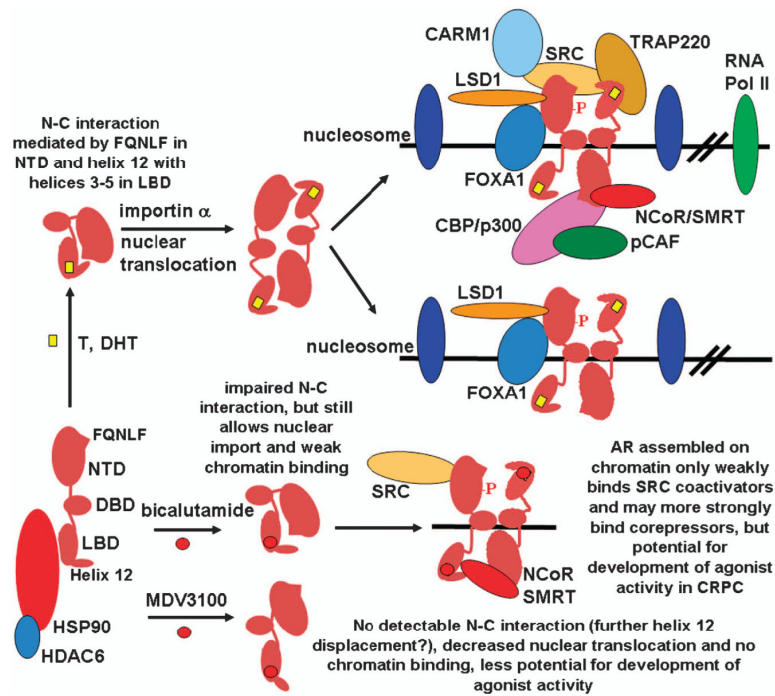


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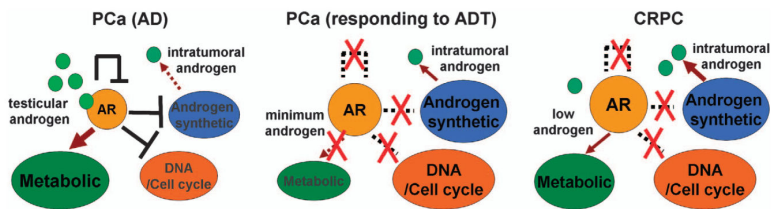
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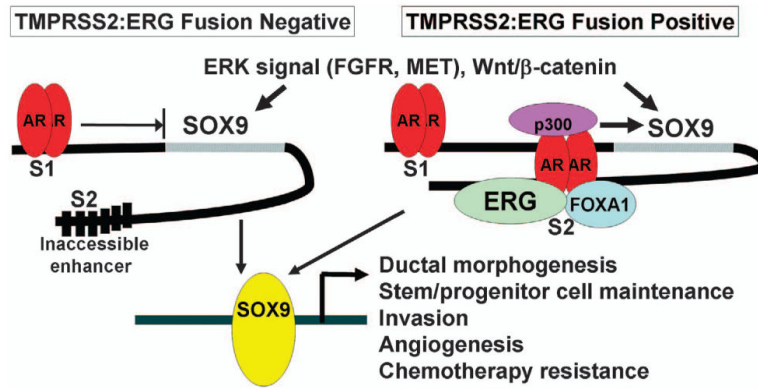


**Figure 1.**

AR structure and responses to binding agonist and antagonist ligands. Androgen binding mediates a conformational change in the position of helix 12 in the LBD. Binding to an FQNLF peptide in the NTD mediates an initial intramolecular N–C interaction, and a subsequent intermolecular interaction may contribute to nuclear localization. AR then binds to androgen-responsive elements at sites that are generally bound initially by the FOXA1 transcription factor, which has been termed a ‘pioneer transcription factor,’ as it opens chromatin locally so AR can access the ARE. These sites also are generally marked by H3K4me2 containing nucleosomes. AR binding displaces a weakly associated central nucleosome, and initiates the assembly of multiple coactivator and chromatin-modifying proteins that loop to the promoter to initiate transcription. LSD1 functions as a critical coactivator for androgen-stimulated genes that is associated with H3K9me2 demethylation, and corepressor for androgen-repressed genes that is associated with H3K4me2 demethylation, but the precise LSD1 mechanisms of action on androgen-stimulated versus -repressed genes remain to be established. The bicalutamide-liganded AR associates more transiently with chromatin, does not effectively mediate coactivator recruitment, and has increased corepressor recruitment. The MDV3100 (enzalutamide) liganded AR localizes in both the nucleus and cytoplasm, but does not detectably associate with chromatin, which may reflect further displacement of helix 12 and abrogation of the N–C interaction.

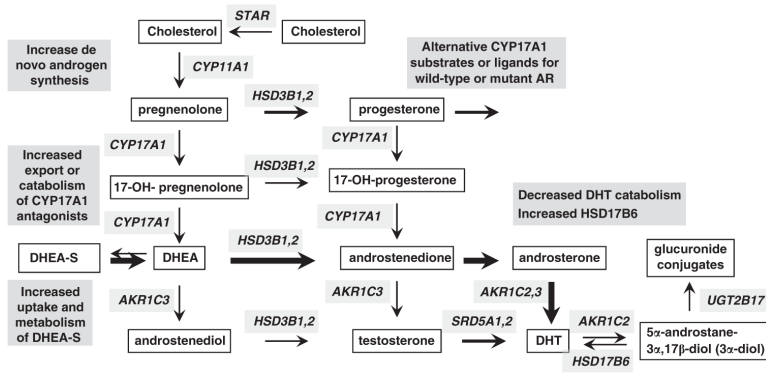


**Figure 2.** Increased expression of androgen-repressed genes may contribute to PCa progression after ADT. In the presence of testicular androgen, AR stimulates PCa growth through its positive effects on metabolic genes while its repression of genes mediating DNA synthesis/cell cycle progression is circumvented by activated oncogenic pathways. The initial response to ADT reflects downregulation of metabolic pathways mediating lipid and protein synthesis, but an undesirable effect is to relieve repression of the *AR* gene, and of genes regulating androgen synthesis and DNA synthesis/cell cycle progression. In CRPC, mechanisms including increased intratumoral androgen synthesis partially restore AR activity and its metabolic functions, but this AR activity is not adequate to decrease expression of the androgen-repressed genes controlling functions that include DNA synthesis/cell cycle progression.



**Figure 3.**

SOX9 is downstream effector of ERG and AR in TMPRSS2:ERG fusion-positive PCa. SOX9 expression can be stimulated by extracellular signals including FGFs and HGF (mediated by their receptors and downstream ERK), and by WNTs through the canonical WNT/β-catenin pathway. AR in fusion-negative cells binds weakly to a site 5' of the *SOX9* gene (S1 site) and can weakly repress basal SOX9 expression (possibly by displacing positive transcription factors). In fusion-positive cells, ERG binds to a cryptic ARE 3' of the *SOX9* gene, with subsequent binding of FOXA1 and strong androgen-stimulated SOX9 expression.



**Figure 4.** Increased intratumoral androgen synthesis in CRPC and potential mechanisms of resistance to CYP17A1 inhibitors.

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