Published in final edited form as:

Int J Biochem Cell Biol. 2014 September ; 54: 49–59. doi:10.1016/j.biocel.2014.06.013.

Androgen Receptor and its Splice Variant, AR-V7, Differentially Regulate FOXA1 Sensitive Genes in LNCaP Prostate Cancer Cells

William C. Krause, Ayesha A. Shafi, Manjula Nakka, and Nancy L. Weigel

Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030

Abstract

Prostate cancer (PCa) is an androgen-dependent disease, and tumors that are resistant to androgen ablation therapy often remain androgen receptor (AR) dependent. Among the contributors to castration-resistant PCa are AR splice variants that lack the ligand-binding domain (LBD). Instead, they have small amounts of unique sequence derived from cryptic exons or from out of frame translation. The AR-V7 (or AR3) variant is constitutively active and is expressed under conditions consistent with CRPC. AR-V7 is reported to regulate a transcriptional program that is similar but not identical to that of AR. However, it is unknown whether these differences are due to the unique sequence in AR-V7, or simply to loss of the LBD. To examine transcriptional regulation by AR-V7, we have used lentiviruses encoding AR-V7 (amino acids 1-627 of AR with the 16 amino acids unique to the variant) to prepare a derivative of the androgen-dependent LNCaP cells with inducible expression of AR-V7. An additional cell line was generated with regulated expression of AR-NTD (amino acids 1-660 of AR); this mutant lacks the LBD but does not have the AR-V7 specific sequence. We find that AR and AR-V7 have distinct activities on target genes that are co-regulated by FOXA1. Transcripts regulated by AR-V7 were similarly regulated by AR-NTD, indicating that loss of the LBD is sufficient for the observed differences. Differential regulation of target genes correlates with preferential recruitment of AR or AR-V7 to specific cis-regulatory DNA sequences providing an explanation for some of the observed differences in target gene regulation.

Keywords

Androgen receptor; Nuclear receptors; Prostate cancer; Transcription; and Cell biology

© 2014 Elsevier Ltd. All rights reserved.

To whom correspondence should be addressed: Nancy L. Weigel, Department of Molecular & Cellular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX, 77030, USA, Tel.: (713)798-6234; FAX: (713)790-1275; nweigel@bcm.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. INTRODUCTION

In PCa, regulation of AR by hormone is disrupted. While androgen deprivation therapy (ADT) to inhibit AR activation is initially effective, tumors develop resistance to ADT, a condition called castration-resistant (or castration-recurrent) prostate cancer (CRPC). Most tumors remain AR-dependent despite reduced levels of circulating androgens. Understanding the mechanisms of AR reactivation and the functional consequences are critical for the development of new AR-targeted treatment strategies for CRPC.

The AR is a member of the nuclear receptor superfamily, and has the characteristic organization consisting of an amino-terminal domain (NTD), DNA-binding domain (DBD), hinge region, and ligand-binding domain (LBD) (Fig. 1A). Binding of androgen (testosterone or DHT) initiates a stepwise activation process leading to regulation of gene expression. Unlike most nuclear receptors, AR transcriptional activity is predominantly conferred by the NTD (Rundlett et al. 1990; Jenster et al. 1995), and the NTD is the primary binding site for the p160 coactivators (Bevan et al. 1999; Ma et al. 1999).

Alternative splicing of the AR to generate constitutively active isoforms has been identified as a potential mechanism for restoring AR transcriptional activity in the context of CRPC. Loss of the LBD is a common feature of these splice variants (Dehm et al. 2008; Guo et al. 2009; Hu et al. 2009, 2011; Sun et al. 2010). The AR-V7 (AR3) variant has received substantial attention for its potential role in CRPC. At the transcript level, this isoform is composed of exons 1 through 3, which encode the NTD and DBD and a unique 16 amino acid sequence at the carboxyl terminus encoded by a cryptic exon (CE3) in the third intron of AR (Guo et al. 2009; Hu et al. 2009). Prostate specific antigen (PSA), transmembrane protease serine 2 (TMPRSS2), and protocadherin 11 (PCDH11) are well known AR target genes. However, reports of AR-V7-dependent gene regulation are inconsistent, with early reports suggesting that it does not regulate common AR targets such as PSA (Guo et al. 2009). Others have reported that target gene regulation is overlapping but not identical to that of AR-FL (Guo et al. 2009; Hu et al. 2012). Additionally, there is debate regarding whether some of the apparently unique targets are a result of the hormone concentrations used (Li et al. 2013). Moreover, a molecular basis for differences in target gene selection is lacking.

Target gene expression by full-length AR (AR-FL) requires binding to specific chromatin sites surrounding regulated genes. Pioneering transcription factors, such as forkhead box protein A1 (FOXA1 or HNF3A), recognize these regions and make them accessible to AR-FL (Lupien et al. 2008). FOXA1 can also be repressive, as genes that are regulated by AR-FL only in the absence of FOXA1 have also been identified (Sahu et al. 2011; Wang et al. 2011). Although the AR amino acids that interface with FOXA1 have not been identified, this interaction has been localized to regions downstream of the DBD (Gao et al. 2003, 2005). AR deletion mutants that are truncated after the DBD have diminished functional interaction with FOXA1 (Wang et al. 2011). Thus, AR target genes that are sensitive to FOXA1 levels for regulation might be differentially responsive to AR-V7.

Although some PCa cell lines express AR-V7, functional analyses of AR-V7 in these systems are challenging. The constitutive expression of AR-V7 in CWR22Rv1 and VCaP cell lines (Guo et al. 2009; Hu et al. 2009) makes it difficult to dissociate the activities of AR-FL and the variant; furthermore, in the VCaP line, variant expression is a small fraction of total AR. Although AR-V7 is more abundant in 22Rv-1 cells, the AR carries a point mutation in the LBD (Tan et al. 1997) and a duplication of a portion of the DBD (Chlenski et al. 2001; Tepper et al. 2002), which could influence target gene selection by AR-FL and its variants. Therefore, we generated several cell lines with inducible expression of AR-V7 and a line with inducible expression of an artificially truncated version of AR, called AR-NTD. The artificial AR-NTD isoform lacks the LBD and is constitutively active (Shafi et al. 2013), but it does not contain the unique sequence found in AR-V7. By comparing the activity of AR-V7 and AR-NTD, we can determine what, if any, of the unique transcriptional activity of AR-V7 is attributable to this short, unique sequence. Comparing receptor activity across these modified cell lines and those with and without endogenous AR-V7 we find that AR-V7 regulates a number of well characterized AR target genes including PSA. However, several genes, which have previously been shown to be co-regulated by FOXA1, are differentially regulated by AR variants. Consistent with this, we find differences in the binding of AR-FL and AR-V7 to distinct sites in the regions surrounding these target genes.

2. EXPERIMENTAL PROCEDURES

2.1 Cell Culture

Parental LNCaP, C4-2, and parental VCaP, were cultured as previously described (Agoulunik et al. 2006; Nakka et al. 2013). 22Rv-1 cells were cultured under the same conditions as LNCaP cells. Unless indicated, all cells were transferred to steroid depleted (charcoal stripped serum) medium at the beginning of experiments.

2.2 Generation of stable cell lines

The LNCaP^{AR-V7/pLenti} cell line was prepared using the ViraPower T-REx Lentiviral Gateway Expression System (Invitrogen). Total mRNA was isolated from VCaP cells, and cDNA was generated with SuperScript II reverse transcriptase (Invitrogen). AR-V7 (Guo et al. 2009) and AR-NTD (amino acids 1-660) were PCR amplified with the iProof GC Master Mix (Bio-Rad) in 5% DMSO using a common forward primer with variant specific reverse primers (Table 1). Primers incorporated the CACC sequence necessary for TOPO cloning on the 5' end of the forward primer. Products were cloned into the pENTR/D-TOPO entry vector (Invitrogen). 2 μ L of the cloning reaction was transformed into a vial of One Shot Chemically Competent E. coli. DNA was extracted from transformed colonies and sequenced. Lentiviruses were prepared using the pLenti6.2/V5-DEST Gateway Vector and the manufacturer's protocol and transduced into an early passage LNCaP cell line previously infected with lentivirus to constitutively express the TET repressor. Transduced LNCaP cells were selected with 4.5 μ g/mL Blasticidin (Invitrogen) and 350 μ g/mL Geneticin (Cellgro).

The LNCaP^{AR-V7/pHage}, LNCaP^{AR-NTD/pHage}, and VCaP^{AR-V7/pHage} cell lines were generated by cloning the appropriate AR variant cDNA into the pHage vector (originally provided by Dr. Jianping Jin, University of Texas Health Science Center, Houston, TX). Subsequently, the pENTR/D-TOPO entry vector was inserted into pHage using the Gateway Cloning System (Invitrogen). Parental LNCaP or VCaP cells were transduced with the vector under 350 µg/mL Geneticin selection.

2.3 Western Blot

Protein lysates were prepared and analyzed as described (AgoulNIK et al. 2005) using 5–10 µg of protein. Blots were incubated with the following primary antibodies at indicated dilutions: mouse anti-AR441 (Nazareth et al. 1999) 1:5000 (BCM Proteomics Core facilities), mouse anti-AR-V7 1:5000 (Precision Antibody), rabbit anti-PSA 1:2000 (Dako), rabbit anti-FOXA1 1:1000 (Abcam) mouse anti-tubulin 1:10000 (Upstate), and mouse anti-Actin 1:5000 (Thermo Scientific). Proteins were detected using horseradish peroxidase conjugated secondary anti-mouse (GenDEPOT) or anti-rabbit (GE Healthcare) antibodies both at 1:10000 and Pierce ECL Plus chemiluminescent reaction (Thermo Scientific).

2.4 Transfection of siRNA

Cells were transiently transfected with 8.3 nM siRNA against AR-FL exon 7 (Ambion #1539), pan-AR exon 1 (Li et al. 2012), FOXA1 (Thermo Scientific #M-010319), or control (Ambion #4638). LNCaP^{AR-V7/pLenti} cells were transfected using the Lipofectamine RNAiMAX reagent (Invitrogen), and the LNCaP^{AR-V7/pHage} and 22Rv-1 cells were transfected using Lipofectamine 2000 (Invitrogen) all according to the manufacturer's instructions.

2.5 Luciferase Assay

Expression constructs for AR-regulated luciferase (GRE₂E1bluc) as well as β-galactosidase have been described (AgoulNIK et al. 2003). LNCaP cells in 6-well plates were transfected with 250 ng of luciferase and 30 ng of β-galactosidase plasmids using Lipofectin (Invitrogen) according to the manufacturer's protocol. Luciferase activity was measured using the Luciferase Assay System (Promega). β-galactosidase activity was measured as previously described (Bai and Weigel 1996), and used to normalize luciferase activity levels.

2.6 mRNA Quantification

Cells were lysed in TRIzol (Life Technologies) and RNA was purified according to the manufacturer's protocol. 1 µg of total RNA was converted into cDNA using M-MuLV reverse transcriptase (New England Biolabs). Transcript expression was measured via the incorporation of SYBR Green (Life Technologies) using the Applied Biosystem 7500 Real-Time PCR System (Life Technologies). Primers for PSA, TMPRSS2, PCDH11, and 18S have been described (AgoulNIK et al. 2008). Additional primers were designed using Primer3 (Rozen and Skaletsky 2000) (see Table 1 for sequences). qPCR data were analyzed by the comparative C_T or the relative standard curve method, using 18S rRNA as the reference transcript.

2.7 Chromatin Immunoprecipitation

ChIP was performed as described (Narayanan et al. 2005) with the following modifications. Cells were treated with 0.75% formaldehyde for 10 minutes at room temperature; excess formaldehyde was quenched with 125 mM glycine. Cell pellets were resuspended and sonicated in FA lysis buffer (50 mM HEPES-KOH pH7.5, 140 mM NaCl, 1 mM EDTA pH8, 1% Triton X-100, 0.1% Sodium Deoxycholate, 0.1% SDS, and protease inhibitors). Samples were processed simultaneously via bath sonication (Sonicator 4000, Misonix). Power was adjusted to deliver approximately 100 watts per sonication cycle and the samples were sonicated with 5 cycles of 30 seconds each. 8.3% of the diluted lysate was reserved as the input and the remainder was pre-cleared with 25 μ L of DynaBeads (Invitrogen Life Technologies). Immunoprecipitations were performed with 50 μ L of Dynabeads in combination with 2 μ g of rabbit anti-mouse IgG (Invitrogen), 1 μ g of AR-N20 antibody (Santa Cruz Biotechnology), or with 2 μ g AR-V7 antibody (Precision Antibody). Immunoprecipitated DNA fragments were measured by qPCR using the primer sequences in Agoulnik et al. (2006) and Table 1. Enrichment of DNA fragments after ChIP was quantified as a percentage of the input amount.

2.8 Data Analysis

Experiments were performed on at least three separate occasions. For Western blot and luciferase data, representative examples are presented. For all qPCR and ChIP-qPCR assays, data from each of the experimental replicates were pooled for analysis. Statistical tests were performed using Prism 5.0 software (GraphPad).

3. RESULTS

3.1 Generation of an LNCaP cell line with inducible expression of AR-V7

Most PCa and cell lines that express the AR-V7 variant also express AR-FL (Guo et al. 2009; Hu et al. 2009; Watson et al. 2010; Hörnberg et al. 2011). To model this scenario in cell culture, we modified LNCaP cells to inducibly express AR-V7 (LNCaP^{AR-V7/pLenti}). Addition of doxycycline (Dox) relieved repression by the TET repressor permitting AR-V7 induction. We also created an LNCaP line with inducible expression of AR-NTD (LNCaP^{AR-NTD/pHage}) (Shafi et al. 2013). The schematic in figure 1A highlights the differences in the composition of the protein domains among the AR forms. Using an isoform-specific antibody, Western blotting of lysates from the LNCaP^{AR-V7/pLenti} line revealed that the inducible AR variant was not detectable in the absence of Dox, but there was a dose-dependent increase in AR-V7 protein expression following Dox addition (Fig. 1B). Since AR-V7 was undetectable in the absence of Dox we could compare the activities of AR-FL and AR-V7 by treating the cells with the synthetic ligand R1881 or Dox, respectively. To confirm that the AR-V7 was active, we transfected the cells with an AR responsive luciferase reporter under the control of two AR response elements and assayed reporter activity after 24 hours of treatment with Dox or R1881. There was a dose-dependent increase in luciferase activity, and the maximum activity was significantly greater than that induced by AR-FL using a saturating concentration (10 nM) of R1881 (Fig. 1C). Subsequent analysis (see Fig. 2, below) indicated that the expression of AR-V7 in response to 0.25 ng/mL Dox was similar to the expression level of hormone-stabilized AR-FL with 10 nM

R1881. Although no epitope exists in the AR-NTD that allowed for antigenic discrimination relative to AR-FL, Western blotting of protein lysates from the inducible LNCaP^{AR-NTD/pHage} cell line using a pan-AR antibody revealed a band at approximately 80 kDa in response to induction with Dox. This molecular weight is consistent with the anticipated size of AR-NTD. As expected, Dox induced transcription of the luciferase reporter in the LNCaP^{AR-NTD/pHage} cell line (data not shown).

3.2 AR-V7 regulates canonical AR target genes

Next, we compared the regulation of endogenous AR target genes by AR-V7 with regulation by AR-FL. Relative protein levels of AR-FL and AR-V7 were determined by Western blot using a pan-AR antibody that detects both full-length and variant receptors (Fig. 2A). Treatment with R1881 increased the level of AR-FL protein (110 kDa) relative to the vehicle control, a known effect resulting from stabilization of the receptor by ligand binding (Kemppainen et al. 1992). Treatment with three concentrations of Dox yielded a dose-dependent increase in the band at approximately 80 kDa, corresponding to AR-V7. Expression of the variant at the three Dox conditions was lower, similar, or much greater than that of hormone-stabilized AR-FL. Induction of AR-V7 by the highest Dox concentration occurred at the expense of AR-FL expression. Activation of AR-FL by R1881 or induction of AR-V7 increased PSA protein expression (Fig. 2A).

Using the same range of Dox concentrations as in 2A, we found the transcripts up-regulated (PSA and TMPRSS2) or down-regulated (PCDH11 and AR-FL) by AR-FL were also regulated by AR-V7 (Fig. 2B). An autoinhibitory feedback loop for AR mRNA expression has been documented previously (Quarmby et al. 1990; Yeap et al. 1999; Cai et al. 2011). Notably, the highest levels of Dox-induced AR-V7 were less effective in inducing PSA and TMPRSS2 than the moderate level that corresponded to the level of endogenous AR-FL. The enhanced sensitivity of qPCR allowed us to detect significant increases in the up-regulated target genes by AR-V7 even at the lowest level of variant induction. Significant down-regulation of PCDH11 and AR-FL was only achieved by the moderate and higher levels of AR-V7 induction.

To determine if the decreased sensitivity of down-regulated genes to low levels of AR-V7 was a property of the variant receptor or of the gene itself, we compared the activity of AR-FL on the regulation of TMPRSS2 and PCDH11 following activation over a range of R1881 concentrations (Fig. 2C). Statistically significant induction of TMPRSS2 was achieved at somewhat lower concentrations of R1881 than those required to significantly down-regulate PCDH11 probably because of the wider range of regulation of TMPRSS2. However, at 0.1 nM R1881, PCDH11 was closer to optimal repression than was TMPRSS2 to optimal induction. Overall, AR-V7 is somewhat less effective than AR-FL in inducing TMPRSS2 and in repressing PCDH11.

Whether AR-V7 requires AR-FL to regulate endogenous target genes had been controversial (Watson et al. 2010; Hu et al. 2011). Thus, we depleted AR-FL using an siRNA that specifically targeted exon 7 of AR-FL. 48 hours after transfection, AR-FL protein was undetectable by Western blot, and transcript levels were reduced by more than 90 percent

relative to transfection with a control siRNA (Fig. 2D). Despite the lack of AR-FL, Dox-induced AR-V7 still regulated PSA and PCDH11 (Fig. 2E).

3.3 Identification of target genes differentially regulated by AR and its variants

FOXA1 both positively and negatively regulates AR activity (Sahu et al. 2011; Wang et al. 2011). We asked whether AR-V7 and AR-NTD can induce target genes that require FOXA1 for AR-FL mediated induction. As expected, R1881 induced RASSF3 and EXTL2 in both cell lines (Fig. 3A). However, neither was induced by AR-V7 or by AR-NTD.

We then tested whether transcripts whose induction by AR-FL is inhibited by FOXA1 are also differentially regulated by the AR variants. As reported previously (Sahu et al. 2011), R1881 did not induce expression of EDN2 or ETS2 (Fig. 3B). Remarkably, both of these transcripts were significantly up-regulated by AR-V7. One exception to these patterns of regulation was LRIG1, which has been reported to require FOXA1 for its induction. Although we confirmed LRIG1 induction by R1881, it also was induced by AR-V7, but higher levels of AR-V7 were needed for optimal induction (Fig. 3C). The differential activity of AR-V7 at these genes was mirrored by AR-NTD. Higher concentrations of Dox were required to induce AR-NTD, likely because of the lentivirus (pHage) used to construct the line. Nonetheless, the level of protein expression of AR-NTD relative to AR-FL under these conditions was similar to that of AR-V7 relative to AR-FL (Fig. 3D).

Some AR-FL target genes, particularly those associated with cell cycle regulation, are differentially regulated by low and high doses of hormone (Li et al. 2013). To ensure that a similar phenomenon was not contributing to the apparent AR-V7-specific regulation of EDN2 and ETS2, we compared induction of these genes by AR-V7 to that by AR-FL at several concentrations of R1881. PSA mRNA was increased at all doses of R1881 and by AR-V7 induction; however, even 0.1 nM, R1881 failed to significantly increase EDN2 or ETS2 mRNA expression (Fig. 3E) suggesting intrinsic difference in the capacity of AR variants to regulate these genes.

3.4 AR-V7-specific target genes are regulated independently of FOXA1 expression

To confirm the reported role of FOXA1 in specifying the activities of AR-FL and to test more directly whether it altered the activity of the variants, we reduced FOXA1 expression using siRNA (Fig. 4A and B). In FOXA1 depleted cells, PSA mRNA expression was regulated by R1881 and by AR-V7 induction as expected (Sahu et al. 2011; Wang et al. 2011). Moreover, the R1881-induced increase in the FOXA1-dependent genes RASSF3 and EXTL2 was lost as previously reported, but this had no effect on the inability of AR-V7 to regulate these genes (Fig. 4B). The regulation of EDN2 and ETS2 by AR-V7 was unaltered (Fig. 4B). Depletion of FOXA1 eliminated the R1881-mediated repression of EDN2 and raised basal levels, but was not sufficient to cause substantial induction by R1881. ETS2 basal levels were also increased by the reduction of FOXA1, but we did not detect additional R1881-mediated induction. This differs from a previous report, which described induction of these genes in FOXA1 depleted cells (Sahu et al. 2011). Although our reduction in FOXA1 was sufficient to inhibit induction of RASSF3 and EXTL2 by AR-FL, it is formally possible

that a better depletion would have increased responsiveness of EDN2 and ETS2 to R1881-mediated activation of AR-FL.

3.5 AR-V7-specific activity is consistent across multiple cell lines

To confirm that the unique activities of the AR variants were not unique to the LNCaP cell lineage, we generated the VCaP^{AR-V7/pHage} cell line. By Western blot, using the pan-AR antibody, we confirmed induction of AR-V7 in response to Dox treatment (Fig. 5A). Treatment of the VCaP^{AR-V7/pHage} cells with either R1881 (10 nM) or Dox (300 ng/mL) increased expression of PSA (Fig. 5B). As in the LNCaP^{AR-V7/pLenti} line, expression of very high levels of AR-V7 prevented induction of PSA (data not shown). EDN2 and ETS were upregulated following AR-V7 induction with 300 ng/mL Dox but not by R1881 (Fig. 5B).

Unlike the C4-2 and LNCaP cell lines, the 22Rv-1 cell line endogenously expresses abundant AR-V7 (Guo et al., 2009 and Fig. 5C). In agreement with the data from our inducible cell lines, we found that basal expression of the AR-V7 specific target genes, EDN2 and ETS2, was significantly higher in 22Rv-1 cells than in either C4-2 or parental LNCaP cells when grown under charcoal-stripped serum conditions (Fig. 5D). To determine whether the higher basal levels were dependent on AR, we used siRNA against exon 1 of AR to deplete all isoforms of AR (these cells express AR-V7 and other variants) (Fig. 5E and F). There was a significant decrease in PSA as expected (Dehm et al. 2008) and a reduction in EDN2 and ETS2 (Fig. 5F), which was consistent with AR activity contributing to the elevated expression.

3.6 AR-FL and AR-V7 preferentially bind to specific regulatory elements

FOXA1 controls the accessibility of cis-regulatory elements to the AR. Our dose response studies of gene regulation showed that reducing levels of R1881 did not change gene specificity for AR-FL. Thus, we used optimal R1881 (10 nM) and levels of Dox that induced similar levels of AR-V7 for our ChIP assays. Using ChIP-qPCR with a pan-AR antibody, we first compared the enrichment of a known AR binding site from the PSA enhancer region (Agoulnik et al. 2006) following activation of AR-FL or AR-V7. PSA is a FOXA1-independent AR target gene, and activation of either AR-FL or AR-V7 caused significant enrichment of the enhancer in immunoprecipitated receptor-DNA complexes when compared to control treatment (Fig. 6A). No enrichment was observed with a non-specific IgG.

Next we used the available ChIP-Seq data set from Wang et al (2011) to identify AR binding sites in the vicinity of the differentially regulated genes. One site upstream of RASSF3, site 12-89, was only bound by AR-FL in the presence of FOXA1 (Wang et al. 2011) (Fig. 6B, upper). Using the pan-AR antibody, we observed much stronger enrichment of this site in response to R1881 than to induction of AR-V7 by Dox (Fig. 6B, middle left); however, some AR-V7 recruitment could be detected using an AR-V7-specific antibody (Fig. 6B, lower left). A second binding site, 12-529, was located further upstream of site 12-89, and binding of AR-FL to this site was inhibited by the presence of FOXA1 (Wang et al. 2011). Remarkably, our ChIP-qPCR data indicated that this site was bound by AR-V7, but binding by AR-FL was not statistically significant. (Fig. 6B, middle right). Recruitment

of AR-V7 to this site was also detected using the AR-V7-specific antibody (Fig. 6B, lower right).

Similar analyses of the ChIP-Seq data for AR binding sites near EDN2 identified several sites at which FOXA1 inhibited binding by AR-FL (Wang et al. 2011) (Fig. 6C, upper). Analysis of one of these sites, site 1-223, by ChIP-qPCR using the pan-AR antibody demonstrated that AR-V7 binding was greater than R1881-induced binding of AR-FL (Fig. 6C, lower) which was consistent with AR-V7-specific induction. Because AR binding to all of the cis-regulatory sites within a 100 kilobase region surrounding EDN2 was shown by ChIP-Seq to be similarly inhibited by FOXA1, we did not analyze these sites further. Locations for FOXA1 and AR binding sites for EXTL2 and ETS2 are shown in Fig. 6D.

3.7 Simultaneous activation of AR-FL and AR-V7 competitively influence gene expression

To assess the consequence of simultaneous activation of AR-FL and AR-V7, we compared gene expression in LNCaP^{AR-V7/pHage} cells treated with R1881, DOX, or a combination of the two. AR isoform expression in response to the various treatments was confirmed by Western blot (Fig. 7A). Parallel qPCR analysis of TMPRSS2 demonstrated that, as expected, activation of either AR isoform alone increased mRNA expression, and this induction was not significantly attenuated by simultaneous activation of AR-FL and AR-V7 (Fig. 7B, left). Treatment with R1881 but not Dox significantly increased expression of the AR-FL-specific transcript, RASSF3. However, when both AR-FL and AR-V7 were activated, the mRNA expression level returned to baseline (Fig. 7B, middle). In contrast, the expression of EDN2 was up-regulated specifically by AR-V7, and this induction was not attenuated by simultaneous activation of both AR-V7 and AR-FL (Fig. 7B, right).

4. DISCUSSION

Identifying differences in transcriptional activity between AR-FL and its variants and defining the mechanistic basis for such differences are essential to understanding the role that these variants play in CRPC. To examine transcriptional regulation by AR-V7, we developed a panel of cell lines with inducible expression of this alternatively spliced variant of the AR. Using these models, we find a number of similarities, but also striking differences between the activities of AR-FL and AR-V7.

Although AR-V7 regulates each of the canonical AR target genes that we tested (Fig. 2), the extent of AR-V7 regulation was lower than that obtained with 10 nM R1881, and this was true regardless of time or level of Dox induction (data not shown). Several features of AR-V7 may contribute to this reduced activity. AR-V7 lacks the LBD and is thus unable to recruit factors via this domain. Similarly, absence of the LBD eliminates the amino terminal/carboxyl terminal interactions in AR that have been reported to be required for optimal induction of a PSA promoter construct (He et al. 2002). Although it is formally possible that the unique 16 amino acid sequence in AR-V7 alters its activity, the AR-NTD exhibited similar relative levels of induction. Differences in the extent of transcriptional activity of AR-FL and AR-V7 at specific target genes could also be secondary to differential induction of facilitating proteins such as PDEF. Androgen-induced PDEF (now renamed SPDEF) cooperates with AR to induce PSA (Oettgen et al. 2000). AR-V7 does not induce SPDEF

expression (data not shown), so the reduced induction of PSA may be due to lower levels of SPDEF.

Induction of a transcriptional reporter increased with increasing level of AR-V7 (Fig. 1), but induction of PSA and TMPRSS2 by AR-V7 was biphasic (Fig. 2B). This may explain why reports of the capacity of AR-V7 to induce PSA have been inconsistent; high levels of AR-V7 do not induce PSA. Unlike most nuclear receptors in which the transcriptional activity is concentrated within the LBD or requires both the amino-terminus and the LBD, most of the AR's ability to induce gene transcription is conferred by the NTD. AR is also unique in that this region forms the strongest interactions with members of the p160 family of coactivators (Bevan et al. 1999; Ma et al. 1999). Thus, constitutively active AR-V7 is likely to compete for coregulators that facilitate the activation of some AR target genes, leading to transcriptional interference at high levels of induction. However, we find that the inhibitory effect of high levels of AR-V7 expression is not universal, suggesting that one or more factors required for expression of PSA and TMPRSS2 may be limiting.

We examined FOXA1 sensitive genes as candidates for differential regulation by AR isoforms because the AR region that interacts with FOXA1 is missing in AR-V7. In general, we observe an inverse relationship between a gene's requirement for FOXA1 and the ability of AR-V7 to induce its transcription. The genes that we identified as AR-V7 specific were consistently regulated in multiple cell lines, were regulated independently of AR-FL and FOXA1 expression, and could not be induced by AR-FL at any concentration of R1881.

The failure of AR-V7 to induce RASSF3 and EXTL2 might have been a result of its inability to bind to any AR cis-regulatory sites. Although it is formally possible that the epitope is differentially accessible, the cells expressed similar levels of AR-FL and AR-V7, and an antibody that recognizes an epitope common to both isoforms was used. Additionally, this antibody recognized AR-V7 in other expression as well as ChIP assays (see below). Our analysis of binding to sites near RASSF3 showed R1881-dependent recruitment to the site bound by AR-FL in FOXA1 positive cells, but no statistically significant binding by AR-V7 (Fig. 6B). An AR-V7 specific antibody which would not have detected basal binding of unliganded AR-FL did detect some binding to this site. Surprisingly, AR-V7 did bind to another site that is ordinarily accessible to AR only when FOXA1 is depleted and RASSF3 is not induced by R1881. This recruitment was detected with both antibodies. Intriguingly, simultaneous activation/expression of AR-FL and AR-V7 inhibited the induction of RASSF3 (Fig. 7B), which suggests that the binding by AR-V7 to one or both of these sites upstream of RASSF3 prevents productive transcription of the gene by AR-FL. In the case of EDN2, assuming equal reaction with the pan-AR antibody, AR-V7 preferentially interacted with a cis-regulatory site upstream of this locus when compared to AR-FL (Fig. 6C). Accordingly, simultaneous activation of AR-FL was not able to block the induction of EDN2 by AR-V7 (Fig. 7B). In general, the two AR-FL specific genes contain multiple FOXA1 binding sites and each contains a site that appears to overlap with a site bound by AR in FOXA1 containing cells (Fig. 6B and 6D). In contrast AR binding sites near EDN2 and ETS2, the AR-V7 regulated genes, do not overlap with FOXA1 sites (Fig. 6C and 6D). Thus, AR-V7 may not be able to interact with FOXA1 at the composite sites to induce transcription of RASSF3 and EXTL2 whereas it appears to be able to overcome the

distal repression phenomena previously reported for FOXA1 at genes such as EDN2 (Sahu et al. 2011; Wang et al. 2011).

In addition to loss of the LBD, AR-V7 is distinguished from AR-FL by the inclusion of a unique 16 amino acid sequence encoded by nucleotides that are intronic in the full-length transcript (Guo et al. 2009; Hu et al. 2009). In our studies, AR-NTD mimicked the activity of AR-V7 suggesting that loss of the LBD/hinge causes most, if not all, of the unique variant activities. This further suggests that other reported active variants will have similar activities. Therefore, the biology of the tumor may depend upon the levels of splice variants, but there may be little difference in the activities of the individual variants. In the case of AR-V7, the function of the unique sequence may be to substitute for the endogenous nuclear localization signal (Chan et al. 2012), which is retained in AR-NTD.

Alternatively spliced AR variants, such as AR-V7, that lack the regulatory LBD are well suited to contribute to PCa. Our data support the hypothesis that switching from an AR-FL to an AR-V7 transcriptional program may promote tumor growth and proliferation both by substituting for AR-FL and via the expression of a unique set of target genes. In particular, ETS2 and EDN2 have been implicated in cellular transformation, growth, and migration (Sementchenko et al. 1998; Nelson et al. 2003). In the prostate, loss of FOXA1 has been associated with dedifferentiation of epithelial tissue (DeGraff et al. 2014). The apparent gain-of-function exhibited by AR-V7 relative to AR-FL could therefore increase the proliferative or oncogenic potential of tumors and highlights the need to find therapies that inhibit activity of these variants.

Acknowledgments

We thank W.E. Bingman III for technical assistance. This work was supported by Cancer Prevention and Research Institute of Texas (CPRIT) RP100320 (NLW), the Prostate Cancer Foundation, and T32 HD07165 (WCK and AAS). We thank the Department of Molecular and Cellular Biology Tissue Culture Core for technical assistance and the Proteomics Shared Resource of the NCI supported Dan L. Duncan Cancer Center (P30CA125123) for production of the AR441 monoclonal antibody.

References

- Agoulnik IU, Bingman WE, Nakka M, Li W, Wang Q, Liu XS, et al. Target gene-specific regulation of androgen receptor activity by p42/p44 mitogen-activated protein kinase. *Mol Endocrinol*. 2008 Nov; 22(11):2420–32. [PubMed: 18787043]
- Agoulnik IU, Krause WC, Bingman WE, Rahman HT, Amrikachi M, Ayala GE, et al. Repressors of androgen and progesterone receptor action. *J Biol Chem*. 2003 Aug 15; 278(33):31136–48. [PubMed: 12771131]
- Agoulnik IU, Vaid A, Bingman WE, Erdeme H, Frolov A, Smith CL, et al. Role of SRC-1 in the promotion of prostate cancer cell growth and tumor progression. *Cancer Res*. 2005 Sep 1; 65(17):7959–67. [PubMed: 16140968]
- Agoulnik IU, Vaid A, Nakka M, Alvarado M, Bingman WE, Erdem H, et al. Androgens modulate expression of transcription intermediary factor 2, an androgen receptor coactivator whose expression level correlates with early biochemical recurrence in prostate cancer. *Cancer Res*. 2006 Nov 1; 66(21):10594–602. [PubMed: 17079484]
- Bai W, Weigel NL. Phosphorylation of Ser211 in the chicken progesterone receptor modulates its transcriptional activity. *J Biol Chem*. 1996 May 31; 271(22):12801–6. [PubMed: 8662804]
- Bevan CL, Hoare S, Claessens F, Heery DM, Parker MG. The AF1 and AF2 Domains of the Androgen Receptor Interact with Distinct Regions of The AF1 and AF2 Domains of the Androgen Receptor

- Interact with Distinct Regions of SRC1. *Mol Cell Biol.* 1999; 19(12):8383–92. [PubMed: 10567563]
- Cai, C.; He, HH.; Chen, S.; Coleman, I.; Wang, H.; Fang, Z., et al. *Cancer Cell.* Vol. 20. Elsevier Inc; 2011 Oct 18. Androgen receptor gene expression in prostate cancer is directly suppressed by the androgen receptor through recruitment of lysine-specific demethylase 1; p. 457-71.
- Chan SC, Li Y, Dehm SM. Androgen receptor splice variants activate AR target genes and support aberrant prostate cancer cell growth independent of the canonical AR nuclear localization signal. *J Biol Chem.* 2012 Apr 24; 287(23):19736–49. [PubMed: 22532567]
- Chlenski A, Nakashiro K, Ketels K, Korovaitseva G, Oyasu R. Androgen receptor expression in androgen-independent prostate cancer cell lines. *Prostate.* 2001 Apr; 47(1):66–75. [PubMed: 11304731]
- DeGraff, DJ.; Grabowska, MM.; Case, TC.; Yu, X.; Herrick, MK.; Hayward, WJ., et al. *Lab Invest.* Nature Publishing Group; 2014 May 19. FOXA1 Deletion in Luminal Epithelium Causes Prostatic Hyperplasia and Alteration of Differentiated Phenotype; p. 1-14.00 Advanced Online Publication
- Dehm SM, Schmidt LJ, Heemers HV, Vessella RL, Tindall DJ. Splicing of a novel androgen receptor exon generates a constitutively active androgen receptor that mediates prostate cancer therapy resistance. *Cancer Res.* 2008 Jul 1; 68(13):5469–77. [PubMed: 18593950]
- Gao N, Ishii K, Mirosevich J, Kuwajima S, Oppenheimer SR, Roberts RL, et al. Forkhead box A1 regulates prostate ductal morphogenesis and promotes epithelial cell maturation. *Development.* 2005 Aug; 132(15):3431–43. [PubMed: 15987773]
- Gao N, Zhang J, Rao Ma, Case TC, Mirosevich J, Wang Y, et al. The role of hepatocyte nuclear factor-3 alpha (Forkhead Box A1) and androgen receptor in transcriptional regulation of prostatic genes. *Mol Endocrinol.* 2003 Aug; 17(8):1484–507. [PubMed: 12750453]
- Guo Z, Yang X, Sun F, Jiang R, Linn DE, Chen H, et al. A novel androgen receptor splice variant is up-regulated during prostate cancer progression and promotes androgen depletion-resistant growth. *Cancer Res.* 2009 Mar 15; 69(6):2305–13. [PubMed: 19244107]
- He B, Lee LW, Minges JT, Wilson EM. Dependence of Selective Gene Activation on the Androgen Receptor NH2- and COOH-terminal Interaction. *J Biol Chem.* 2002 May 8; 277(28):25631–9. [PubMed: 12000757]
- Hörnberg E, Ylitalo EB, Crnalic S, Antti H, Stattin P, Widmark A, et al. Expression of androgen receptor splice variants in prostate cancer bone metastases is associated with castration-resistance and short survival. *PLoS One.* 2011 Jan; 6(4):e19059. [PubMed: 21552559]
- Hu R, Dunn TA, Wei S, Isharwal S, Veltri RW, Humphreys E, et al. Ligand-Independent Androgen Receptor Variants Derived from Splicing of Cryptic Exons Signify Hormone-Refractory Prostate Cancer. *Cancer Res.* 2009 Jan 1; 69(1):16–22. [PubMed: 19117982]
- Hu R, Isaacs WB, Luo J. A Snapshot of the Expression Signature of Androgen Receptor Splicing Variants and Their Distinctive Transcriptional Activities. *Prostate.* 2011 Nov; 71(15):1656–67. [PubMed: 21446008]
- Hu R, Lu C, Mostaghel EA, Yegnasubramanian S, Gurel M, Tannahill C, et al. Distinct Transcriptional Programs Mediated by the Ligand-Dependent Full-Length Androgen Receptor and Its Splice Variants in Castration-Resistant Prostate Cancer. *Cancer Res.* 2012 Jul 6; 72:3457–62. [PubMed: 22710436]
- Jenster G, van der Korput HAGM, Trapman J, Brinkmann AO. Identification of Two Transcription Activation Units in the N-terminal Domain of the Human Androgen Receptor. *J Biol Chem.* 1995 Mar 31; 270(13):7341–6. [PubMed: 7706276]
- Kemppainen JA, Lane MV, Sar M, Wilson EM. Androgen Receptor Phosphorylation, Turnover, Nuclear Transport, and Transcriptional Activation. *J Biol Chem.* 1992; 267(2):968–74. [PubMed: 1730684]
- Li Y, Chan SC, Brand LJ, Hwang TH, Silverstein KAT, Dehm SM. Androgen Receptor Splice Variants Mediate Enzalutamide Resistance in Castration-Resistant Prostate Cancer Cell Lines. *Cancer Res.* 2013 Jan 15; 73(2):483–9. [PubMed: 23117885]
- Li Y, Hwang TH, Oseth LA, Hauge A, Vessella RL, Schmechel SC, et al. AR Intragenic Deletions Linked to Androgen Receptor Splice Variant Expression and Activity in Models of Prostate Cancer Progression. *Oncogene.* 2012 Jan 23; 31(45):1–9. [PubMed: 21643006]

- Lupien M, Eeckhoutte J, Meyer Ca, Wang Q, Zhang Y, Li W, et al. FoxA1 translates epigenetic signatures into enhancer-driven lineage-specific transcription. *Cell*. 2008 Mar 21; 132(6):958–70. [PubMed: 18358809]
- Ma H, Hong H, Huang SM, Irvine RA, Webb P, Kushner PJ, et al. Multiple signal input and output domains of the 160-kilodalton nuclear receptor coactivator proteins. *Mol Cell Biol*. 1999 Sep; 19(9):6164–73. [PubMed: 10454563]
- Nakka, M.; Agoulnik, IU.; Weigel, NL. *Int J Biochem Cell Biol*. Vol. 45. Elsevier Ltd; 2013 Apr. Targeted disruption of the p160 coactivator interface of androgen receptor (AR) selectively inhibits AR activity in both androgen-dependent and castration-resistant AR-expressing prostate cancer cells; p. 763-72.
- Narayanan R, Adigun AA, Edwards DP, Weigel NL. Cyclin-Dependent Kinase Activity Is Required for Progesterone Receptor Function : Novel Role for Cyclin A / Cdk2 as a Progesterone Receptor Coactivator Cyclin-Dependent Kinase Activity Is Required for Progesterone Receptor Function : Novel Role for Cyclin. *Mol Cell Biol*. 2005; 25(1):264–77. [PubMed: 15601848]
- Nazareth LV, Stenoi DL, Bingman WE, Jamesa J, Wu C, Zhang Y, et al. A C619Y mutation in the human androgen receptor causes inactivation and mislocalization of the receptor with concomitant sequestration of SRC-1 (steroid receptor coactivator 1). *Mol Endocrinol*. 1999 Dec; 13(12):2065–75. [PubMed: 10598582]
- Nelson J, Bagnato A, Battistini B, Nisen P. The endothelin axis: emerging role in cancer. *Nat Rev Cancer*. 2003 Feb; 3(2):110–6. [PubMed: 12563310]
- Oettgen P, Finger E, Sun Z, Akbarali Y, Thamrongsak U, Boltax J, et al. PDEF, a Novel Prostate Epithelium-specific Ets Transcription Factor, Interacts with the Androgen Receptor and Activates Prostate-specific Antigen Gene Expression. *J Biol Chem*. 2000 Jan 14; 275(2):1216–25. [PubMed: 10625666]
- Quarmany VE, Yarbrough WG, Lubahn DB, French FS, Wilson EM. Autologous Down-Regulation of Androgen Receptor Messenger Ribonucleic Acid. *Mol Endocrinol*. 1990 Jan 1; 4(1):22–8. [PubMed: 2325667]
- Rozen, S.; Skaletsky, H. Primer 3 on the WWW for general users and for biologist programmers. In: Krawetz, S.; Misener, S., editors. *Methods Mol Biol*. Totowa, NJ: Humana Press; 2000. p. 365-86.
- Rundlett SE, Wu X-P, Miesfeld RL. Functional Characterizations of the Androgen Receptor Confirm that the Molecular Basis of Androgen Action Is Transcriptional Regulation. *Mol Endocrinol*. 1990 May 1; 4(5):708–14. [PubMed: 2274054]
- Sahu B, Laakso M, Ovaska K, Mirtti T, Lundin J, Rannikko A, et al. Dual role of FoxA1 in androgen receptor binding to chromatin, androgen signalling and prostate cancer. *EMBO J*. 2011 Oct 5; 30(19):3962–76. [PubMed: 21915096]
- Sementchenko VI, Schweinfest CW, Papas TS, Watson DK. ETS2 function is required to maintain the transformed state of human prostate cancer cells. *Oncogene*. 1998 Dec 3; 17(22):2883–8. [PubMed: 9879994]
- Shafi, AA.; Cox, MB.; Weigel, NL. *Steroids*. Vol. 78. Elsevier Inc; 2013 Jun. Androgen receptor splice variants are resistant to inhibitors of Hsp90 and FKBP52, which alter androgen receptor activity and expression; p. 548-54.
- Sun, S.; Sprenger, CCT.; Vessella, RL.; Haugk, K.; Soriano, K.; Mostaghel, EA., et al. *J Clin Invest*. Vol. 120. American Society for Clinical Investigation; 2010. Castration resistance in human prostate cancer is conferred by a frequently occurring androgen receptor splice variant; p. 2715
- Tan J, Sharief Y, Hamil KG, Gregory CW, Zang D, Sar M, et al. Dehydroepiandrosterone activates mutant androgen receptors expressed in the androgen-dependent human prostate cancer xenograft CWR22 and LNCaP cells. *Mol Endocrinol*. 1997; 11(4):450–9. [PubMed: 9092797]
- Tepper CG, Boucher DL, Ryan PE, Ma A, Xia L, Lee L-F, et al. Characterization of a novel androgen receptor mutation in a relapsed CWR22 prostate cancer xenograft and cell line. *Cancer Cell*. 2002; 62(22):6606–14.
- Wang, D.; Garcia-Bassets, I.; Benner, C.; Li, W.; Su, X.; Zhou, Y., et al. *Nature*. Vol. 474. Nature Publishing Group; 2011 Jun 16. Reprogramming transcription by distinct classes of enhancers functionally defined by eRNA; p. 390-4.

- Watson PA, Chen YF, Balbas MD, Wongvipat J, Socci ND, Viale A, et al. Constitutively active androgen receptor splice variants expressed in castration-resistant prostate cancer require full-length androgen receptor. *Proc Natl Acad Sci U S A*. 2010 Sep 28; 107(39):16759–65. [PubMed: 20823238]
- Yeap BB, Krueger RG, Leedman PJ. Differential Posttranscriptional Regulation of Androgen Receptor Gene Expression by Androgen in Prostate and Breast Cancer Cells. *Endocrinology*. 1999 Jul 1; 140(7):3282–91. [PubMed: 10385425]

Highlights

- AR-V7 is a hormone-independent variant of the androgen receptor (AR).
- We generated cells with inducible AR-V7 expression to compare its activity to AR.
- We observe gene-specific differences in DNA binding and induction by AR and AR-V7.
- Genes co-regulated by FOXA1 are misregulated by AR-V7.

FIGURE 1. Inducible expression of constitutively active AR variants

A, schematic illustration of the differences in functional domains among AR-FL, AR-V7, and AR-NTD. N-terminal domain (NTD), DNA binding domain (DBD), hinge (H), variant sequence (V), and ligand binding domain (LBD). B, LNCaP^{AR-V7/pLenti} cells were treated in duplicate with indicated concentrations of Dox for 24 hours. Western blots were probed with antibodies against AR-V7 (upper) or Actin (lower). C, cells were treated as indicated for 24 hours. Transcriptional activities of AR-FL and AR-V7 were measured using a transiently transfected AR responsive Luciferase reporter and normalized to β-gal activity. Error bars indicate SEM. n = 3. Statistical differences between groups were determined by ANOVA with Bonferroni post-hoc test. *, $p < 0.01$.

FIGURE 2. Analysis of AR-V7 regulation of endogenous targets

A, inducible LNCaP^{AR-V7/pLenti} cells were treated for 24 hours with the indicated amounts of vehicle control, Dox, or R1881. Changes in protein level were analyzed by Western blot using antibodies that recognize: total AR (upper), Actin (middle), or PSA (lower). B, cells were treated in parallel with those in A and changes in expression of the indicated genes were quantified by qPCR. C, parental LNCaP cells were treated with increasing concentrations of R1881 and changes in expression of the indicated target genes were quantified by qPCR. D–E, LNCaP^{AR-V7/pLenti} cells in complete medium were transiently transfected with siRNA targeting AR-FL. Medium was replaced with charcoal-stripped serum and cells were treated as indicated. Depletion of AR-FL protein (D left) and transcript (D right) was monitored by Western blot using a pan-AR antibody and by qPCR, respectively. In parallel, changes in target gene expression following induction of AR-V7 were quantified by qPCR (E). For all panels, error bars indicate the upper and lower bounds of the 95% confidence interval. n = 3 experiments performed in triplicate. Statistical differences between groups were determined by ANOVA with Bonferroni post-hoc test (B and E), or between treatment and control groups by Dunnett's multiple comparison test (C). *, $p < 0.01$.

FIGURE 3. Differential regulation by AR and its variants of target genes that are co-regulated by FOXA1

A–C, LNCaP^{AR-V7/pLenti} or LNCaP^{AR-NTD/pHage} cells were treated, as indicated, for 24 hours and transcripts measured by qPCR. The activities of AR-FL, AR-V7, and AR-NTD were compared across a subset of target genes that have been previously demonstrated to require (A and C) or be inhibited (B) by the expression of FOXA1. Error bars indicate the upper and lower bounds of the 95% confidence interval. n = 3 experiments performed in triplicate. D, expression levels of AR-NTD in the LNCaP^{AR-NTD/pHage} line were determined by Western blot using the pan-AR antibody. E, LNCaP^{AR-V7/pHage} cells were treated for 24 hours, and expression of transcripts measured by qPCR. Error bars indicate standard error of the mean. n = 3 experiments performed in triplicate. For all qPCR data, statistical differences between groups were determined by ANOVA with Bonferroni post-hoc test. *, $p < 0.01$.

FIGURE 4. Selective Regulation of target genes by AR-FL, but not AR-V7, depends on FOXA1
 48 hours after transfection with siRNA targeting FOXA1 or a nonspecific control sequence LNCaP^{AR-V7/pHage} cells were placed in 10% charcoal-stripped serum, and treated for an additional 24 hours with 10 nM R1881 or 20 ng/mL Dox, as indicated. **A**, Protein levels of AR-FL, AR-V7, and FOXA1 were assessed by Western blot using the pan-AR and FOXA1 antibodies. **B**, expression of the indicated target genes was measured by qPCR. Error bars indicate the upper and lower bounds of the 95% confidence interval. $n = 3$ experiments performed in triplicate. Statistical differences within groups transfected with the same siRNA were determined by one-way ANOVA with Bonferroni post-hoc test. *, $p < 0.01$.

FIGURE 5. Variant-specific target genes are regulated in multiple cell lines with inducible or endogenous expression of AR-V7
A–B, VCaP^{AR-V7/pHage} cells in 10% charcoal-stripped serum were treated for 24 hours with the indicated amounts of Dox or R1881. AR-FL and AR-V7 protein levels were assessed by Western blot using the AR-441 antibody (*A*), and in parallel the relative expression levels of the given target genes were determined by qPCR (*B*). Error bars indicate the upper and lower bounds of the 95% confidence interval. *C*, 22Rv-1, C4-2, and LNCaP PCa cell lines were maintained in charcoal stripped-serum for 48 hours and then treated with vehicle or 10 nM R1881 for 24 hours. Endogenous expression levels of AR-FL and AR-V7 were compared by Western blot using the AR-441 antibody. *D*, LNCaP, C4-2, and 22Rv-1 cells were maintained in charcoal-stripped serum for 48 hours and expression of the indicated target genes was measured by qPCR. Error bars indicate the upper and lower bounds of the 95% confidence interval. *n* = 3 experiments performed in triplicate. Statistical differences between groups were determined by ANOVA with Bonferroni post-hoc test. *, $p < 0.01$. *E–F*, 22Rv-1 cells in complete medium were transiently transfected with pan-AR or control siRNA for 48 hours. AR protein levels were measured by Western blot using the pan-AR antibody (*E*) and changes in expression of the indicated target genes were analyzed by qPCR (*F*). Error bars indicate standard error of the mean. *n* = 3 experiments performed in triplicate. Statistical differences between groups were determined by one-way Student's T-test. *, $p < 0.01$.

FIGURE 6. AR-FL and AR-V7 preferentially recognize distinct cis-regulatory binding sites
 LNCaP^{AR-V7/pLenti} were treated for 24 hours with 0.25 ng/mL Dox or 10 nM R1881. AR recruitment to the indicated sites was measured by ChIP-qPCR using the AR-N20 antibody, which recognizes all AR forms (grey bars), the AR-V7 antibody (black bars) or a control IgG antibody (open bars). Enrichment of the binding site sequence in response to either treatment was quantified as a percentage of the input amount. *A*, ChIP-qPCR analysis of AR-FL and AR-V7 binding to the PSA enhancer. *B–C*, upper panels show UCSC Genome browser view of the genomic regions surrounding *RASSF3* (*B*) and *EDN2* (*C*). Vertical bars in overlaid tracks indicate peaks of binding events by FOXA1 and AR, both in the presence or absence of FOXA1, as captured by Wang et al. (2011) (GSE27682). Labeled binding sites (12-529, 12-89, and 1-223) were analyzed for AR recruitment in response to isoform-specific treatments by ChIP-qPCR (lower panels). Error bars indicate the SEM. *n* = 3 experiments. Statistical differences between control and activating treatments when pulled-down with the AR-N20 antibody were determined by ANOVA with Bonferroni post hoc test. *, *p* < 0.05. The effect of treatment in the AR-V7 ChIP was determined by two-way Student's T-test. *, *p* < 0.05. *D*, Comparison of the overlap between AR and FOXA1 cis-regulatory elements on target genes that are specifically activated by AR-V7 (*ETS2*) or by AR-FL (*EXTL2*).

FIGURE 7. AR-V7 antagonizes the activity of AR-FL on a subset of genes with distinct receptor binding sites

LNCaP^{AR-V7/pHage} cells in 10% charcoal-stripped serum were treated for 24 hours with Dox or R1881. In the combined treatment groups Dox was added 6 hours prior to R1881, to allow time for expression of AR-V7. *A*, total AR protein levels in response to treatment were assessed by Western blot using a pan-AR antibody; blots were also probed for Actin expression to ensure equal loading. *B*, mRNA expression of the indicated target genes was quantified by qPCR. Error bars indicate the SEM. *n* = 3 experiments performed in triplicate. Statistical differences between groups were determined by ANOVA with Bonferroni post-hoc test. *, *p* < 0.01.

Table 1

Primer sequences for qPCR and ChIP-qPCR assays.

Primer Type	Target	Forward	Reverse
PCR	AR-V7	CACCATGGAAGTGCAGTTAGGGCTGGGAAGGGTCTACCCT	TCAGGGTCTGGTCATTTGAGATGCTTGCAATTGCC
PCR	AR-NTD	CACCATGGAAGTGCAGTTAGGGCTGGGAAGGGTCTACCCT	TCACAGCTTCTGGGTTGTCTCCTCAGTGGGGCTGGT
qPCR	EXTL2	CCTGAACTGGAAACCAATGC	TCAGGAAATTGCTGCCAAA
qPCR	RASSF3	GACGCCGAGGACTTCTTCTT	TGCTGAGGTAAGTGTGGGTTT
qPCR	ETS2	TGGCTTCAAAAAGGAACAGC	CCTCTGCAGATTCACGTTCA
qPCR	EDN2	TGGGTGAACACTCCTGAACA	AGGCAGAAGGTGGCACAG
qPCR	LRIG1	CCGAACCTACAGGAAGTGTACC	TGCGAATCTTGTGTGCTG
qPCR	FOXA1	GTGGCTCCAGGATGTTAGGA	GAGTAGGCCTCCTGCGTGT
ChIP-qPCR	12-529	CATAAACCTGGACCTGGAA	CCTCCCATTCCTTGAGTAG
ChIP-qPCR	12-89	GCTGGGGATATGAGGACACA	TCAGCTTTGCTACAGGATTTC
ChIP-qPCR	1-223	AGTCATTTCCCATCGACAC	CTTCTACTGGGGCTGCAAAC