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**14. ABSTRACT**

Androgen depletion therapy for advanced prostate cancer (PCa) invariably fails, and PCa recurs with an aggressive and lethal castration-resistant (CR) phenotype. Although androgen depletion inhibits activity of the androgen receptor (AR), continued AR function is important for resistance to androgen depletion. Therefore, even though this stage of the disease is often referred to as androgen-independent (AI), CRPCa remains an AR-dependent disease. Recently, alternatively-spliced, COOH-terminally truncated AR isoform variants have been identified in CRPCa cells and clinical tissues. These isoform variants function as constitutively active AR transcription factors that can support the CRPCa phenotype in model systems. The purpose of this project is to understand the mechanisms underlying increased expression and activity of these truncated AR isoform variants in CRPCa. Our work provides the first evidence that structural alterations in the AR gene may underlie disrupted AR splicing patterns at this stage of the disease. This knowledge could lead to better treatments or management of patients with CRPCa.

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## INTRODUCTION

Androgen depletion therapy for advanced prostate cancer (PCa) invariably fails, and PCa recurs with an aggressive and lethal androgen-refractory or castration-resistant (CR) phenotype (also referred to as androgen-depletion-independent, abbreviated ADI). Although androgen depletion inhibits activity of the androgen receptor (AR), continued AR function is important for resistance to androgen depletion. Therefore, even though this stage of the disease is often referred to as androgen-independent (AI), CRPCa remains an AR-dependent disease. Recently, alternatively-spliced, COOH-terminally truncated AR isoform variants have been identified in CRPCa cells and clinical tissues [1-5]. These isoform variants function as constitutively active AR transcription factors that can support the CRPCa phenotype in model systems. The purpose of this project is to understand the mechanisms underlying increased expression and activity of these truncated AR isoform variants in CRPCa. The scope of the research is: 1. To study the structure of the AR gene and investigate whether intragenic rearrangements, including deletions and duplications, may underlie disrupted AR splicing patterns; and 2: to elucidate the biochemical properties of individual truncated AR isoform variants and determine whether different variants have differential capacities to support specific aspects of the CRPCa phenotype.

## BODY

### Findings resulting from TASK 1: Determine the locations of *AR* genomic break-points in 22Rv1 cells and AI LuCaP 35 xenografts

In our YR1 report, we outlined the following research findings that resulted from completing project milestones:

1) The altered AR splicing patterns observed in the 22Rv1 cell line are linked to a 35kb tandem duplication encompassing AR exon 3 and flanking cryptic exons. We defined the nucleotide sequence of the break fusion junctions and demonstrated that this rearrangement explained the synthesis of the AR 1/2/3/2b mRNA. These findings are outlined in the attached publication (Li et al., *Cancer Research*, 2011) [6].
2) The CWR-R1 cell line, which also expresses high levels of truncated AR variants, harbors decreased copy number within the AR exon 1 region. This discovery was facilitated by our development of multiplex ligation-dependent probe assay (MLPA) as a new method to query AR copy number at distributed loci along the length of the gene.

In our YR2 report, we outlined the following research findings that resulted from completing project milestones:

1) The preliminary data in Fig. 6A of our application indicated that the LuCaP 35 model of prostate cancer progression displayed unbalanced AR gene copy number when tumors progress to a CRPCa stage. These data were based on a quantitative PCR-based assay that we had developed, which has since been replaced by MLPA. As outlined in Fig. 1C of our recent publication (Li et al., *Oncogene*, 2012) [7], MLPA failed to detect overt AR gene copy number imbalances in CRPCa LuCaP 35 xenografts.
2) As a result of this negative finding in LuCaP 35, we shifted our focus to characterizing the intron 1 copy number loss in CWR-R1 in more detail. As outlined in our recent

publication, (Li et al., *Oncogene*, 2012) [7], a subset of cells in the CWR-R1 cell line harbors a 48kb deletion within AR intron 1. We were successful in mapping the break fusion junction of this intragenic deletion which revealed a signature of non-homologous end joining (Fig. 5 of Li et al., *Oncogene*, 2012) [7]. Further, we demonstrated that this intragenic deletion marked the CRPCa sub-population in this heterogeneous cell line that expressed high levels of the truncated AR 1/2/3/CE3 variant and grew under androgen independent conditions (Fig. 7 of Li et al., *Oncogene*, 2012) [7].

3) During the funding period, the LuCaP 86.2 xenograft was identified as a model of CRPCa that expressed high levels of the truncated AR v567es variant arising from the splicing machinery skipping AR exons 5-7 [4]. Using MLPA, we found that the LuCaP 86.2 xenograft displayed reduced copy number of AR exons 5-7 (Fig. 1c of Li et al., *Oncogene*, 2012) [7]. We were successful in mapping the break fusion junction of this novel 8.5kb intragenic deletion of AR exons 5-7, which revealed a signature of non-homologous end joining (Fig. 2 of Li et al., *Oncogene*, 2012) [7].

All Task 1 studies were completed during the 3-year funding period.

**Findings resulting from TASK 2: Determine whether unbalanced AR amplification occurs in ADI vs AD PCa**

In our YR2 report, we reported that we had made good progress as a result of completing the following project milestones:

1) We used MLPA to assess AR gene copy number in androgen-dependent PCa cell lines, androgen-dependent PCa xenografts, and PCa prostatectomy specimens. Overall, these androgen-dependent PCa cells displayed one intact AR gene copy, with the exception of the LuCaP 35 xenograft, which displayed four balanced copies of the AR gene (Fig. 1 of Li et al., Oncogene, 2012) [7]. These data support the concept that AR gene copy number imbalance does not occur in hormone naïve PCa.
2) We also used MLPA to assess AR gene copy number in CRPCa cell lines, xenografts, and rapid autopsy specimens. Overall, we found frequent AR gene amplification and/or complex patterns of AR gene copy number imbalance. These data support the concept that AR gene copy number imbalances may be an important component of CRPCa progression.

In the final funding period (YR3), we have made the following research findings as a result of completing project milestones:

1) Rather than perform RT-PCR analysis of AR target genes, we elected to carry out a more comprehensive genome-wide microarray expression analysis to identify the sets of genes activated by truncated AR variants vs. full-length AR. Overall, we found that truncated AR variants were able to support a gene expression program very similar to the transcriptional program activated by full-length AR in the presence of androgens[8] (Fig. 4 of Li et al., Cancer Research, 2013).

All Task 2 studies were completed during the 3-year funding period.

**Findings resulting from TASK 3: Determine the biochemical properties of individual truncated AR isoforms**

In our YR2 report, we outlined the following research findings that resulted from completing project milestones:

1) The AR NH2-terminal domain (NTD) and the DNA binding domain (DBD) represent the core requirement for truncated AR variants to support androgen-independent growth of the 22Rv1 cell line (Figs. 9D and E of Chan et al., *JBC*, 2012) [9]. This is also the core domain required for truncated AR variants to induce an androgen-independent growth phenotype in androgen-dependent LNCaP cells (Figs. 9A and B of Chan et al., *JBC*, 2012) [9].
2) The AR NTD/DBD core is sufficient for truncated AR variants to activate endogenous AR target genes (Fig. 8 of Chan et al., *JBC*, 2012) [9].

In the final funding period (YR3), we have made the following research findings as a result of completing project milestones:

1) We elected to switch our xenograft-based experiments to the CWR-R1 cell line in order to understand the significance of a 50kb deletion that we discovered in intron 1 of the AR gene[7]. We demonstrated that a 50kb intragenic deletion served as a sensitive marker of individual cells in the CWR-R1 cell line that expressed high levels of truncated AR variant proteins and displayed resistance to the next-generation antiandrogens enzalutamide[7, 8]. Using nude mouse xenograft assays as outlined in Task 3c, we demonstrated that CWR-R1 cells negative for this 50kb deletion formed tumors in intact mice and were driven by full-length AR expression (CWR-R1 early, see Fig. 1). Conversely, CWR-R1 cells positive for this 50kb deletion formed tumors in castrated mice and were driven by truncated AR variant expression (CWR-R1 late, see Fig. 1).

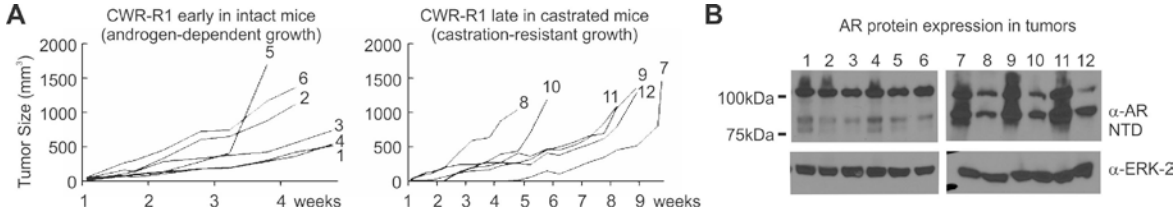


**Fig. 1 (A)** Two populations of CWR-R1 cells were grown as androgen-dependent or CRPC xenografts. **(B)** AR protein expression in xenografts.

All Task 3 studies were completed during the 3-year funding period.

**Findings resulting from TASK 4: Test transcriptional activities of individual and paired truncated AR isoforms to identify the molecular basis for differential transcriptional activity**

In our YR2 report, we outlined the following research findings that resulted from completing project milestones:

1) The AR NTD/DBD core is sufficient for truncated AR variants to access the nucleus in prostate cancer cells. Certain truncated AR variants such as AR 1/2/3/CE3 display more efficient nuclear localization due to reconstitution of the canonical bipartite AR nuclear localization signal (NLS) by cryptic exon splicing, but this reconstituted NLS is not an important biochemical determinant of truncated AR variant activity (Fig. 1-4 and 7 of Chan et al., *JBC*, 2012) [9].

In the final funding period (YR3), we have made the following research findings as a result of completing project milestones:

1) We have performed ChIP in LNCaP cells transfected with lentivirus encoding the AR 1/2/3/CE3 (also referred to as AR-V7) variant (Fig. 2). These data demonstrated that AR 1/2/3/CE3 was able to bind to androgen response elements (AREs) that are bound by full-length AR. These data support the concept that truncated AR variants bind to the same genomic sites as full-length AR.

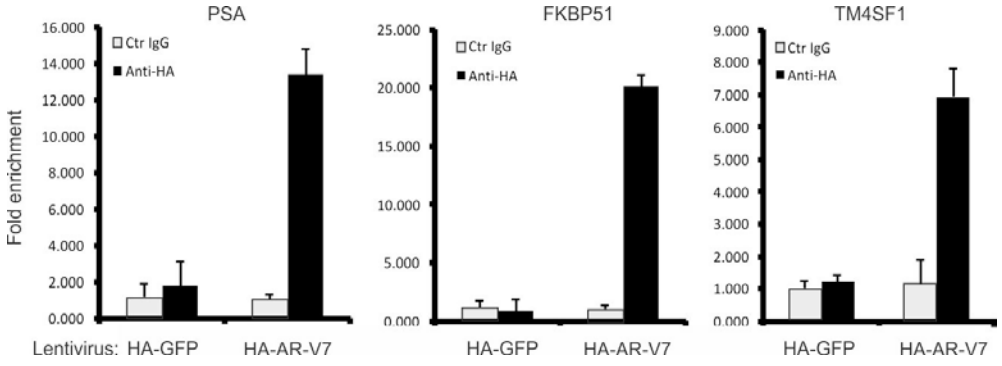


**Fig. 2** ChIP-qPCR of lentivirus-infected LNCaP cell lysates to test binding of HA-tagged AR-V7 (HA-AR-V7) to defined AREs in the enhancers of the PSA, FKBP51 and TM4SF1 genes.

All Task 4 studies were completed during the 3-year funding period.

## KEY RESEARCH ACCOMPLISHMENTS

- The first reports of structural alterations involving the AR gene in PCa as a mechanism for synthesis for truncated AR variants supporting the CRPCa phenotype (Li et al., *Cancer Research*, 2011, Li et al., *Oncogene*, 2012).

- Development of a novel MLPA assay for assessing AR gene structure in prostate cancer cell lines, xenografts, and tissue specimens (Figs. 1 and 2).

- The first report of unbalanced AR gene architectures in CRPCa.

- Identification that the canonical AR nuclear localization signal (NLS) is not required for truncated AR variants to access the nucleus, bind chromatin, and activate AR target genes in PCa cells (Chan et a., *JBC*, 2012).

- Identification that AR gene rearrangements mark individual cells in heterogeneous tumor populations that are resistant do enzalutamide as a result of truncated AR variants driving constitutive activity of the AR transcriptional program (Li et al., *Cancer Research*, 2013).

## REPORTABLE OUTCOMES

**Manuscripts**

1. Bohrer, LR, Liu P, Zhong J, Pan Y, Angstman J, Brand LJ, **Dehm SM***, Huang H*. FOXO1 binds to the TAU5 motif and inhibits constitutively active androgen receptor splice variants. *The Prostate*, 73:1017-27, 2013. *****Corresponding authors

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8. **Dehm SM,** Tindall DJ. Alternatively spliced androgen receptor variants. *Endocrine Related Cancer*, 18:R183-196, 2011.

9. Li Y, Alsagabi M, Fan D, Bova GS, Tewfik AH, **Dehm SM**. Intragenic rearrangement and altered RNA splicing of the androgen receptor in a cell-based model of prostate cancer progression. *Cancer Res.*, 71:2108-17, 2011.
   *****Comment in *Pigment Cell & Melanoma Research*. 25: 293, 2012.

**Presentations**

Molecular Genetics and Medicine Symposium, Duke University, June 24, 2013, Durham, NC.

Genitourinary Research/Koch Center Seminar Series, M.D. Anderson Cancer Center, December 11, 2012, Houston, TX.

Terry Fox Seminar Series, Prostate Centre at Vancouver General Hospital. December 7, 2012. Vancouver, Canada.

Department of Biology, Hamline University, November 30, 2012, Minneapolis, MN.

Androgens 2012. November 8-10, 2012. Helsinki, Finland.

Prostate Cancer Foundation Scientific Retreat. October 25-27, 2012. Carlsbad, CA.

Prostate Cancer Research Program Seminar Series, Fred Hutchinson Cancer Research Center. October 4, 2012, Seattle, WA.

American Urological Association (AUA)/Society of Basic Urologic Research (SBUR) Summer Conference: Role of Hormones in Urologic Health and Development. August 11-12, 2012 AUA Headquarters, Linthicum, MD

Modulating Androgen Receptor Splice Variants in Castration-Resistant Prostate Cancer (MARS-CRPC). June 27, 2012. Dallas, TX.

Endocrine Society Annual Meeting (ENDO). June 23-26, 2012 Houston, TX.

Joint Research Seminar Series, Thomas Jefferson University. March 29, 2012. Philadelphia, PA.

American Association for Cancer Research (AACR) Special Conference: Advances in Prostate Cancer Research. February 6-9, 2012, Orlando. FL.

Society of Basic Urologic Research (SBUR) Spring Program at the American Urological Association (AUA) Annual Meeting, May 14, 2011. Washington, DC.

Department of Defense Prostate Cancer Research Program Innovative Minds in Prostate Cancer Today (IMPaCT) Meeting, "Game-Changing Research" Plenary Session, March 9-11, 2011. Orlando, FL.

Androgen Receptor Signaling in Prostate Cancer: Translating Biology into Clinical Practice. December 6-7, 2010. Arlington, VA.

**Databases**
Androgen receptor splice variants mediate enzalutamide resistance in castration-resistant prostate cancer cell lines
NCBI GEO Accession #GSE41784

**Grant Applications**
RSG-12-031-01 Dehm (PI) 01/01/2012-12/31/2015
American Cancer Society
"AR Gene Structure Alterations and Prostate Cancer Progression"
Role: Principal Investigator
Status: FUNDED

R01CA174777 Dehm (PI) 04/01/2013-03/31/2018
"AR Gene Rearrangements and AR Signaling in Prostate Cancer
Role: Principal Investigator
Status: FUNDED

SPORE in Prostate Cancer Tindall (PI) 09/01/2012-08/31/2017
"Role of the AR Amino Terminal Domain in Prostate Cancer Progression"
Role: Project 1 Leader
Status: PENDING

Transformative Impact Award Plymate (PI) 09/01/2013-08/31/2016
Department of Defense Prostate Cancer Research Program
"Targeting the aberrant androgen receptor in advanced, treatment resistant prostate cancer"
Role: Sub-award Co-I (Prime Institution PI: Steve Plymate, U of Washington)
Status: RECOMMENDED FOR FUNDING

## CONCLUSION

Rearrangements in the AR gene represent a new mechanism of CRPCa development and progression. Our work demonstrates that AR gene rearrangements drive splicing alterations favoring synthesis of truncated AR variants that can function as constitutive, ligand-independent AR transcription factors. These truncated AR variants are impervious to PCa therapies targeted to the AR ligand binding domain.

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7. Li, Y., T.H. Hwang, L. Oseth, A. Hauge, R.L. Vessella, S.C. Schmechel, B. Hirsch, K.B. Beckman, K.A. Silverstein, and S.M. Dehm, *AR intragenic deletions linked to androgen receptor splice variant expression and activity in models of prostate cancer progression.* Oncogene, 2012. **31**(45): p. 4759-67.
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## APPENDICES

APPENDIX 1:
Li Y, Alsagabi M, Fan D, Bova GS, Tewfik AH, Dehm SM. Intragenic rearrangement and altered RNA splicing of the androgen receptor in a cell-based model of prostate cancer progression. *Cancer Res.,* 20:2108-17, 2011

APPENDIX 2:
Li, Y., T.H. Hwang, L. Oseth, A. Hauge, R.L. Vessella, S.C. Schmechel, B. Hirsch, K.B. Beckman, K.A. Silverstein, and S.M. Dehm, AR intragenic deletions linked to androgen receptor splice variant expression and activity in models of prostate cancer progression. *Oncogene,* 2012. In Press.

APPENDIX 3:
Chan, S.C., Y. Li, and S.M. Dehm, 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. 287(23): p. 19736-49.

APPENDIX 4:
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Cancer Research

# Intragenic Rearrangement and Altered RNA Splicing of the Androgen Receptor in a Cell-Based Model of Prostate Cancer Progression

Yingming Li, Majid Alsagabi, Danhua Fan, et al.

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Cancer Research

# Intragenic Rearrangement and Altered RNA Splicing of the Androgen Receptor in a Cell-Based Model of Prostate Cancer Progression

Yingming Li[1], Majid Alsagabi[2], Danhua Fan[3], G. Steven Bova[4], Ahmed H. Tewfik[2], and Scott M. Dehm[1,5]

## Abstract

Androgen depletion for advanced prostate cancer (PCa) targets activity of the androgen receptor (AR), a steroid receptor transcription factor required for PCa growth. The emergence of lethal castration-resistant PCa (CRPCa) is marked by aberrant reactivation of the AR despite ongoing androgen depletion. Recently, alternative splicing has been described as a mechanism giving rise to COOH-terminally truncated, constitutively active AR isoforms that can support the CRPCa phenotype. However, the pathologic origin of these truncated AR isoforms is unknown. The goal of this study was to investigate alterations in AR expression arising in a cell-based model of PCa progression driven by truncated AR isoform activity. We show that stable, high-level expression of truncated AR isoforms in 22Rv1 CRPCa cells is associated with intragenic rearrangement of an approximately 35-kb *AR* genomic segment harboring a cluster of previously described alternative AR exons. Analysis of genomic data from clinical specimens indicated that related *AR* intragenic copy number alterations occurred in CRPCa in the context of *AR* amplification. Cloning of the break fusion junction in 22Rv1 cells revealed long interspersed nuclear elements (LINE-1) flanking the rearranged segment and a DNA repair signature consistent with microhomology-mediated, break-induced replication. This rearrangement served as a marker for the emergence of a rare subpopulation of CRPCa cells expressing high levels of truncated AR isoforms during PCa progression *in vitro*. Together, these data provide the first report of *AR* intragenic rearrangements in CRPCa and an association with pathologic expression of truncated AR isoforms in a cell-based model of PCa progression. *Cancer Res; 71(6); 2108–17. ©2011 AACR.*

## Introduction

Prostate cancer (PCa) is the most frequently diagnosed male cancer in the United States and the second leading cause of male cancer deaths (1). Normal prostate tissue requires androgens for healthy function and cellular homeostasis. Androgens exert their cellular action by binding to the androgen receptor (AR), a 110-kDa transcription factor and member of the steroid nuclear receptor family (2). Initially, PCa depends on normal androgenic activation of the AR for ongoing growth and survival and presents as an androgen- and AR-dependent disease. Therefore, androgen depletion is the standard systemic therapy for locally advanced or metastatic PCa (3). The limitation of androgen depletion is that PCa eventually recurs with a lethal, castration-resistant phenotype. Although this stage of the disease seems to be independent of normal androgenic signaling, it is well established that castration-resistant PCa (CRPCa) remains AR-dependent through various mechanisms of aberrant AR activation and the AR remains an important therapeutic target for CRPCa (4).

*AR* mutations, which can broaden AR ligand specificity, and *AR* amplification, which can lead to AR protein overexpression, are 2 genomic mechanisms that can support the CRPCa phenotype (5–14). Ligand-independent AR activation has also been described and can occur through enhanced dependence on mitogenic signaling cascades that converge on the AR and associated transcriptional coregulators (15). More recently, alternative splicing was described as a mechanism of aberrant AR activation in CRPCa (16–20). Wild-type AR is a modular protein with an $NH_2$-terminal (NTD) transcriptional activation function-1 (AF-1) domain, a central DNA-binding domain (DBD), and a dual-function COOH-terminal ligand-binding domain/AF-2 domain. Splicing of cryptic exons or exon skipping can yield truncated AR isoforms consisting of the NTD, DBD, and short, variable-length C-terminal extensions

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**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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(16–20). These truncated AR isoforms are constitutively active and can support various features of the CRPCa phenotype such as the androgen-independent activation of *AR* target genes and androgen-independent growth. Importantly, truncated AR isoforms have been observed in various PCa cell lines, xenografts, and clinical samples, which supports an important role in disease progression (16–20).

Alternative splicing is a widespread mechanism for increasing diversity from a single gene (21), and normal regulation of this process is disrupted in pathologic conditions such as cancer (22). The discovery of alternatively spliced AR isoforms has underscored the importance of understanding how AR splicing may be disrupted in CRPCa. This could provide clues to how truncated AR isoforms play a pathologic role at later stages of the disease. Therefore, the purpose of this study was to investigate the mechanisms underlying changes in AR isoform expression in a cell-based model of PCa progression.

## Materials and Methods

### Cell culture

Benign prostate BPH-1 cells were generously provided by Dr. Haojie Huang (University of Minnesota) and cultured in RPMI 1640 (Invitrogen) with 10% FBS (Invitrogen). The CRPCa 22Rv1 cell line was obtained from American Type Culture Collection and cultured in RPMI 1640 medium with 10% FBS. Androgen-dependent PCa CWR22Pc cells were generously provided by Dr. Marja Nevalainen (Thomas Jefferson University; ref. 23) and cultured in RPMI 1640 supplemented with 10% FBS, 2.5 mmol/L L-glutamine, and 0.8 nmol/L dihydrotestosterone (DHT; Sigma). Cell growth in RPMI 1640 medium containing 10% charcoal-stripped serum (CSS) ± 1 nmol/L DHT was monitored by crystal violet staining. For androgen response experiments, cells were cultured in RPMI 1640 + 10% CSS for 48 hours, treated at $t = 0$ with 1 nmol/L DHT (Sigma) or vehicle (EtOH), and then harvested at indicated time points. For long-term androgen deprivation, 22Pc cells were cultured in RPMI 1640 + 10% CSS for 7 days and then split to fresh plates in RPMI 1640 + 10% CSS. Cells were trypsinized and reseeded in RPMI 1640 + 10% CSS after an additional 10 days to disperse emerging foci of growth. Samples were harvested following 7, 12, 17, 22, 27, and 32 days of culture in RPMI 1640 + 10% CSS.

### Western blot

Western blotting of CWR22Pc and 22Rv1 lysates with AR (Santa Cruz N-20), ERK-2 (Santa Cruz D-2), and ARV-7 (Precision Antibody no. AG10008) antibodies was conducted exactly as described (16).

### Quantitative real-time RT-PCR

Total cellular RNA was isolated from CWR22Pc and 22Rv1 cells as described (24). RNA was reverse transcribed using a RT kit and an oligo(dT) primer (Roche). Absolute quantitation of AR mRNA species was carried out using forward and reverse primers listed in Supplementary Table S1. Concurrently, quantitative PCR with serial dilutions of plasmids harboring wild-type AR, AR 1/2/2b, AR 1/2/3/2b, AR 1/2/3/CE1, AR 1/2/3/CE2, and AR 1/2/3/CE3 cDNAs was carried out using a SYBRGreen FastMix reaction cocktail (PerfeCTa; VWR Life Sciences) and an iCycler instrument (BioRad) exactly as described (16). Threshold cycle ($C_t$) of amplification values obtained from cDNA standards were used to construct $C_t$ versus cDNA standard copy number standard curves. The values of $C_t$ obtained from real-time reverse transcriptase PCR (RT-PCR) were plotted on these standard curves to derive copy number values for individual AR mRNA isoforms. For relative quantitation, fold expression change relative to glyceraldehyde 3-phosphate dehydrogenase was determined by the comparative $C_t$ method ($2^{-\Delta\Delta C_t}$).

### Genomic PCR

Genomic DNA was isolated from BPH-1, CWR22Pc, and 22Rv1 cells, using a NucleoSpin kit (Clontech). Genomic DNA from clinical CRPCa tissues was isolated as described previously (25). PCR primers were designed using the Primer3 program of the MacVector software package and are listed in Supplementary Table S1. For copy number determination, quantitative PCR with serial dilutions of BPH-1 genomic DNA was carried out for each primer pair, using SYBRGreen fastmix and an iCycler instrument. The values of $C_t$ obtained from BPH-1 genomic DNA dilutions were used to construct $C_t$ versus genomic copy number standard curves, with the inference that one BPH-1 genome contains one copy of the X chromosome and therefore one copy of the target region. The values of $C_t$ obtained from test genomic DNA in real-time PCR reactions were plotted on these standard curves to derive genomic copy numbers for each of the PCR target regions. For conventional PCR, genomic DNA was amplified using a Taq Polymerase PCR kit (Qiagen), according to the manufacturer's protocol. For long-range PCR, genomic DNA was amplified using outward facing primers (Supplementary Table S1) and a LongRange PCR kit (Qiagen). Cloned PCR products originating from the *AR* locus were completely sequenced to identify the 22Rv1 *AR* locus break fusion junction.

### Affymetrix Genome-Wide Human SNP Array 6.0 analysis

Affymetrix SNP6.0 profiling of primary PCa (26) and metastatic CRPCa (25) was done in previous studies. Raw data in .CEL format was obtained from the Gene Expression Omnnibus web site (accession numbers GSE18333 and GSE14996). Copy numbers were calculated for each probe set, using Partek Genomics Suite 6.4 analysis software with default settings. Briefly, for each probe set, raw intensity was corrected for fragment length and sequence and the geometric means of allele intensity values were scaled to 1 (0 in $\log_2$ space). Copy number was calculated from these summarized intensities by normalizing intensity of each individual tumor samples to the mean intensity of the pooled noncancerous samples. Probe-level copy number data were used as input in an algorithm designed to determine the collection of breakpoints that satisfy the maximum



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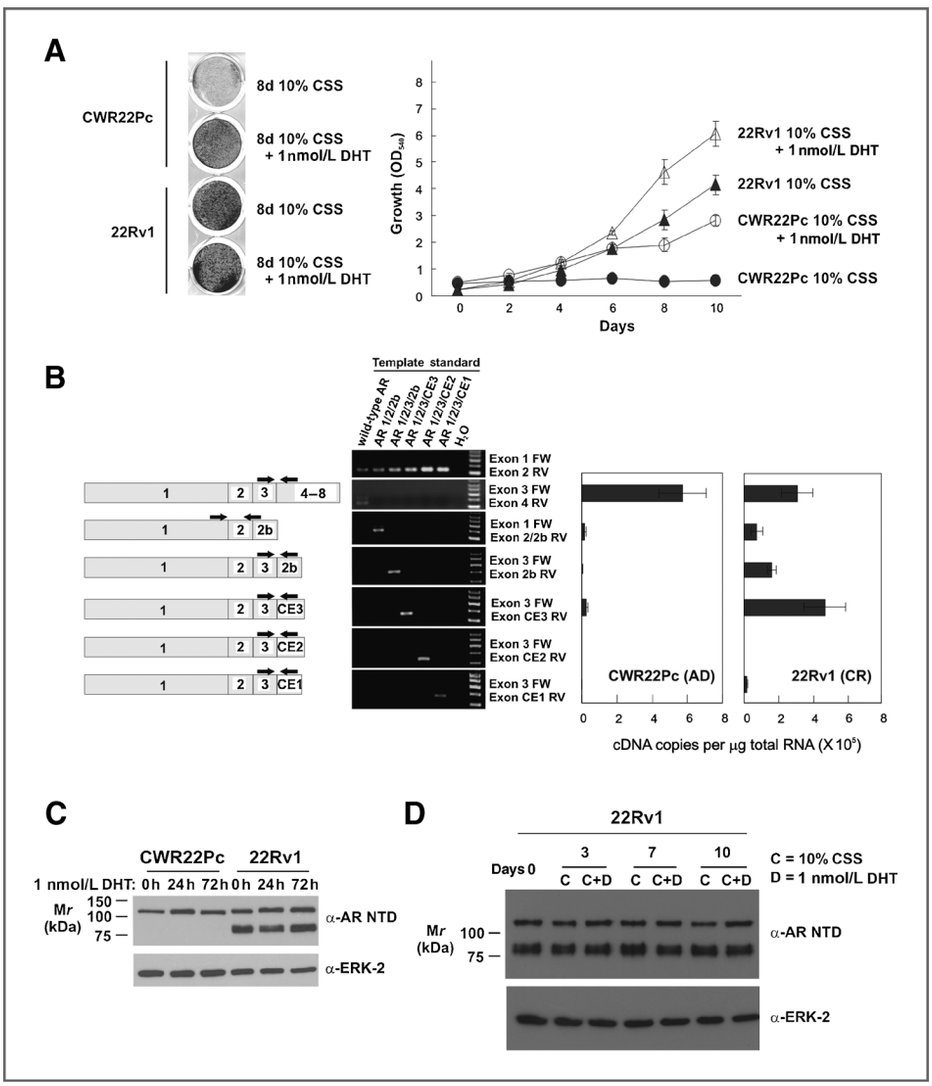


Figure 1. Efficient and stable synthesis of alternatively spliced *AR* mRNA isoforms in CRPCa cells. A, growth of CWR22Pc and 22Rv1 cells in the presence or absence of androgens. B, plasmid templates harboring depicted cDNAs were subjected to PCR with indicated primer pairs. Right, mRNAs from CWR22Pc and 22Rv1 cells were subjected to quantitative RT-PCR, using indicated primer sets. $C_t$ values obtained from quantitative RT-PCR reactions were converted to copy number by plotting sample $C_t$ values on $C_t$ versus copy number standard curves constructed from concurrent quantitative PCR analysis of serial dilutions of plasmid templates. Data represent mean ± standard error from 2 independent experiments, each conducted in triplicate ($n = 6$). C, AR Western blots of CWR22Pc and 22Rv1 cells following 3-day treatment with 1 nmol/L DHT. ERK-2, loading control. D, AR Western blot of 22Rv1 cells following 10-day culture in steroid-depleted medium containing 1 nmol/L DHT or vehicle control. ERK-2, loading control.

likelihood between the input data and the noise-free version. The detailed algorithm is described in the Supplementary Methods section and is available in MATLAB (The MathWorks) upon request.

## Results

### *AR* intragenic rearrangement and aberrant *AR* mRNA splicing

The CWR22Pc cell line was recently established from the CWR22 human PCa xenograft (23). This cell line is androgen dependent for growth, which is in contrast to the CWR22-derived CRPCa 22Rv1 cell line (Fig. 1A; ref. 27). Recent reports have shown that alternatively spliced, truncated AR isoforms support constitutive AR-mediated transcription and androgen-independent proliferation of 22Rv1 cells (16–18). We therefore examined whether these isoforms were also synthesized in CWR22Pc cells. Using different PCR primer sets with different amplification efficiencies to identify the various *AR* mRNA isoforms precludes the use of the differential $C_t$ of amplification ($2^{-\Delta\Delta C_t}$) method for determining relative expression by real-time PCR. We therefore pursued RT-PCR–based absolute quantification (Fig. 1B). As previously reported, full-length *AR* expression and high-level expression of the *AR* 1/2/2b, *AR* 1/2/3/2b, and *AR* 1/2/3/CE3 isoforms were observed at the mRNA and protein level in 22Rv1 cells (Fig. 1B and Supplementary Fig. S1). In androgen-dependent CWR22Pc cells, full-length *AR* expression was predominant but expression of AR 1/2/3/CE3 mRNA and protein was also detectable (Fig. 1B and Supplementary Fig. S2). No substantial change in these AR expression patterns was observed following 24 or 72 hours of treatment with androgens (Fig. 1C and



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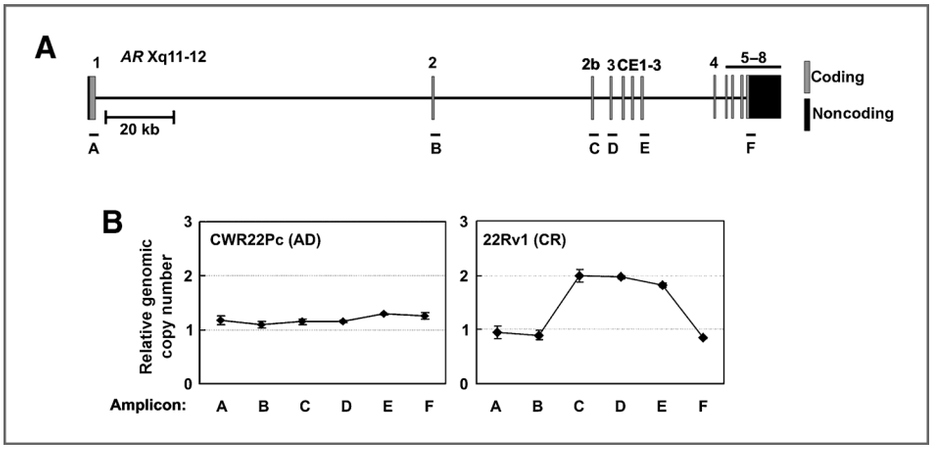


**Figure 2.** Alternatively spliced *AR* exons are contained on a rearranged genomic segment in 22Rv1 cells. A, schematic of the approximately 180-kb *AR* locus at Xq11-12. PCR amplicons used for copy number determination are labeled A to F. B, genomic DNA from CWR22Pc and 22Rv1 cells was subjected to quantitative PCR, using amplicon primer pairs indicated in A. $C_t$ values were converted to copy number by plotting sample $C_t$ values on $C_t$ versus copy number standard curves constructed from serial dilutions of BPH-1 genomic DNA. Data represent mean ± standard error from 2 independent experiments, each conducted in triplicate ($n = 6$).

Supplementary Fig. S2). Similarly, AR isoform expression was stable during 10 days of 22Rv1 cell culture in the presence or absence of androgens (Fig. 1D). Together, these data show that both androgen-dependent CWR22Pc and CRPCa 22Rv1 cells can synthesize truncated AR isoforms but 22Rv1 cells can sustain stable, high-level expression.

Because the full-length *AR* mRNA in 22Rv1 cells has 2 copies of exon 3, resulting in an AR DBD with 3 zinc fingers (28), and because 22Rv1 cells can efficiently synthesize mRNAs with contiguously spliced exons 1, 2, 3, and 2b (16), we hypothesized that a genomic aberration in the 180-kb *AR* locus at Xp11-12 may underlie the stable splicing alterations observed in these cells. We therefore interrogated copy number at distributed loci along the length of the *AR* gene. Strikingly, in castration-resistant 22Rv1 cells, we observed increased copy number of AR exons 2b, 3, and CE3, suggesting a rearrangement involving this genomic segment (Fig. 2). This aberration was not observed in the androgen-dependent CWR22Pc cell line (Fig. 2). These data therefore suggest that alternative AR isoforms, which support the castration-resistant phenotype of 22Rv1 cells, may arise via enhanced splicing of alternative exons harbored on a rearranged genomic segment in the *AR* locus.

### *AR* intragenic copy number alterations in metastatic CRPCa tissues

To determine whether *AR* intragenic copy number alterations occurred in human CRPCa, we analyzed high-resolution Affymetrix Genome-Wide Human SNP Array 6.0 (SNP6.0) data derived from clinical primary PCa ($n = 44$ tissues) from 44 patients and metastatic CRPCa ($n = 58$ tissues) from 14 patients (25, 26). To localize the boundaries of putative breakpoints, we used a dynamic program that estimates the number and locations of segments adaptively on the basis of probe-level data. This analysis revealed a high incidence of rearrangement in conjunction with *AR* amplification, only in CRPCa, which to our knowledge is a novel phenomenon that has not been described (Supplementary Fig. S3). Because the outcome of the 22Rv1 *AR* rearrangement seemed to be a focal copy number increase of a segment between *AR* exons 2 and 4, resulting in higher dosage of exon 3 and alternative exons relative to *AR* exon 4 (Fig. 2), we asked whether these phenomena occurred in clinical PCa. Indeed, focal copy number increases were observed between *AR* exons 2/3 and 3/4 in 12 of 58 (20.7%) metastases from 6 of 14 (42.9%) subjects, which presented as rearrangement of a segment harboring some or all alternative *AR* exons 2b or CE1-3 (Fig. 3). For most of these CRPCa samples, the outcome was the higher gene content of a segment containing *AR* exon 3 and alternative exons compared with *AR* exon 4 (Supplementary Fig. S4). These alterations were not observed in genomic DNA samples from these subjects' normal tissue (Supplementary Fig. S5). Focal copy number increase of this segment in a CRPCa subject was confirmed using targeted quantitative PCR (Supplementary Fig. S6). SNP6.0 analysis revealed no changes in overall *AR* copy number or focal alterations in this region in 44 primary PCa samples (Supplementary Fig. S4), indicating that CRPCa patients are more likely to harbor this rearrangement in at least one of their tumors than patients with localized, androgen-dependent PCa (6/14 vs. 0/44; $P = 0.000074$, Fisher's exact test). Overall, these data suggest that the region encompassing *AR* exon 3 may represent a "hotspot" for intragenic rearrangement in CRPCa.

### *AR* breakpoint junction boundaries lie within LINE-1 elements in 22Rv1 cells

To establish with more precision the breakpoint junctions between *AR* exons 2 and 2b and *AR* exons CE3 and 4 in 22Rv1



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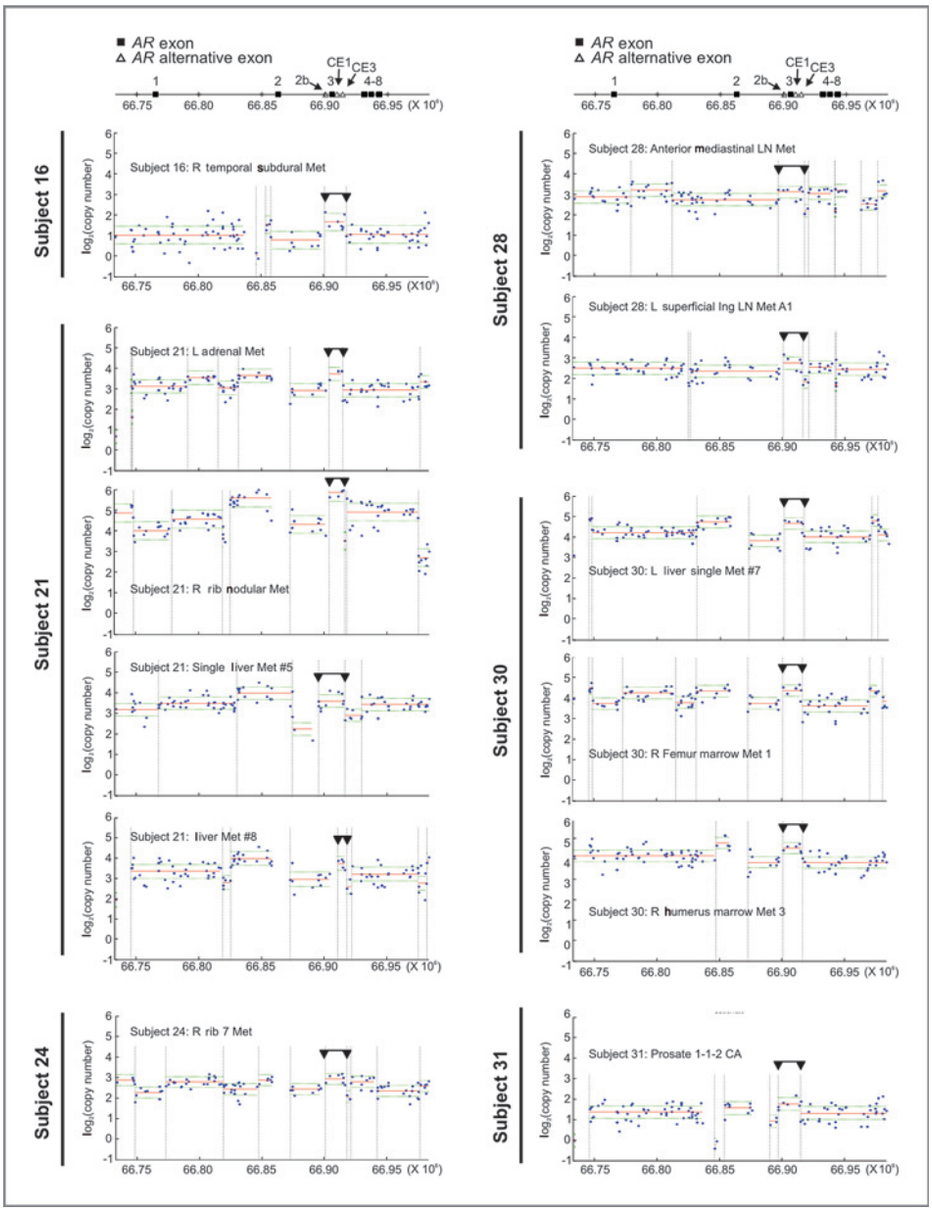


**Figure 3.** AR intragenic rearrangements in CRPCa detected by Affymetrix Genome Wide SNP 6.0 Array analysis of metastatic tissues. Top, exon organization of the *AR* locus on Xq11-12 and chromosome position (human genome build 19, hg19) is indicated at the top of each panel. All panels shown are individual tissue samples from CRPCa metastases. Blue dots, probe-level copy number; horizontal red lines, mean segment copy number; horizontal green dashed lines, SD; and dashed vertical lines, segment boundaries defined by the segmentation algorithm. Black horizontal lines with downward-facing arrowheads, a region of focal copy number alteration similar to 22Rv1 cells.

cells, we carried out higher-resolution copy number interrogation (Fig. 4A). Using this approach, we mapped the 5′ breakpoint between *AR* exons 2 and 2b to a resolution of 4 kb (Fig. 4B). Concurrently, we mapped the 3′ breakpoint between *AR* exons CE3 and 4 to a resolution of 8 kb (Fig. 4B). Attempts to map the 5′ or 3′ breakpoints with higher resolution yielded real-time PCR products associated with very low $C_t$ values in both reference and test DNA samples, indicating repetitive sequence. Indeed, analysis of public reference genome sequence revealed long interspersed nuclear elements (LINE-1) and low complexity (TA)*n* repeats and AT-rich sequence in both of these regions (Fig. 4C).

It is common for the endpoints of genomic deletions or insertions to map to repetitive elements such as LINE-1, although the underlying mechanisms are not fully established (29, 30). One possibility is that extensive homology between LINE-1 elements at breakpoint junctions could lead to deletion on one sister chromatid and duplication on the other via nonallelic homologous recombination (NAHR; ref. 31). Pairwise alignments between the 5′ LINE-1 fragments and the full-length 3′ LINE-1 element identified a longer than 1-kb stretch of 87% sequence identity with one particular 5′ LINE-1 fragment, implicating NAHR as the basis for this rearrangement (Supplementary Fig. S7). Therefore, we conducted long-range PCR by using 2 pairs of outward facing



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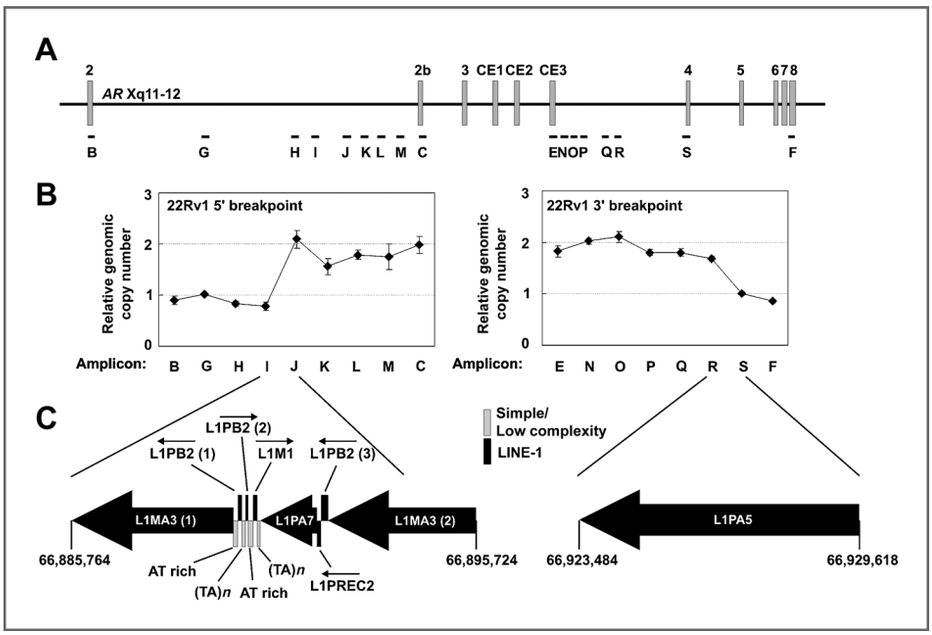


**Figure 4.** Fine mapping of *AR* intragenic rearrangement segment boundaries in 22Rv1 cells. A, schematic of the *AR* locus at Xq11-12. PCR amplicons used for copy number determination are labeled B, C, E, F, and G to S. B, genomic DNA from 22Rv1 cells was subjected to quantitative PCR, using amplicon primer pairs indicated in panel A. $C_t$ values were converted to copy number by plotting sample $C_t$ values on $C_t$ versus copy number standard curves constructed from serial dilutions of BPH-1 genomic DNA. C, schematic of repetitive element organization at the 5′ and 3′ boundaries of the 22Rv1 duplicated AR segment in the reference human genome. Elements were defined by RepeatMasker 3.0 (41). Black arrows indicate the directional orientation of L1 elements, which are named on the basis of their evolutionary origin and sequence divergence, with relative ages (oldest to youngest) L1M1 > L1MA3 > L1PB2 > L1PREC2 > L1PA7 > L1PA5 (41, 42).

primers to isolate the breakpoint junction in 22Rv1 cells (Fig. 5A and Supplementary Fig. S8). This resulted in long PCR products of 6,723 and 4,762 bp (Supplementary Fig. S8). Sequencing of cloned PCR products revealed that they were identical over the common 4,762 bp and localized the 22Rv1-specific 5′ and 3′ breakpoints to genomic positions 66,889,976 and 66,924,525, respectively (Fig. 5B and C). Analysis of the break fusion junction revealed 27 bp of

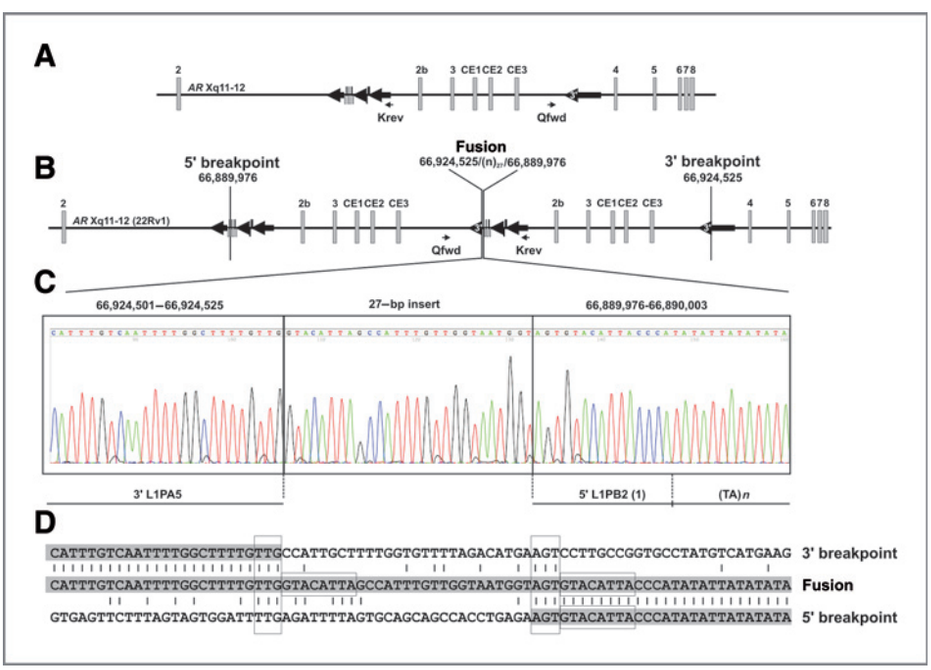


**Figure 5.** Outward-facing PCR to isolate the 22Rv1 *AR* tandem duplication. A, schematic of the *AR* locus at Xq11-12 with locations of primers used for outward-facing long-range PCR. B, schematic of the *AR* locus in 22Rv1 cells as revealed by sequencing of cloned long-range PCR products. C, electropherogram sequence of the *AR* break fusion junction in 22Rv1 cells, including a novel 27-bp insert. D, sequence alignments of the 3′ breakpoint, the 22Rv1 break fusion junction, and the 5′ breakpoint. Sequence contained in the break fusion junction is shaded in gray. Regions of microhomology are boxed.



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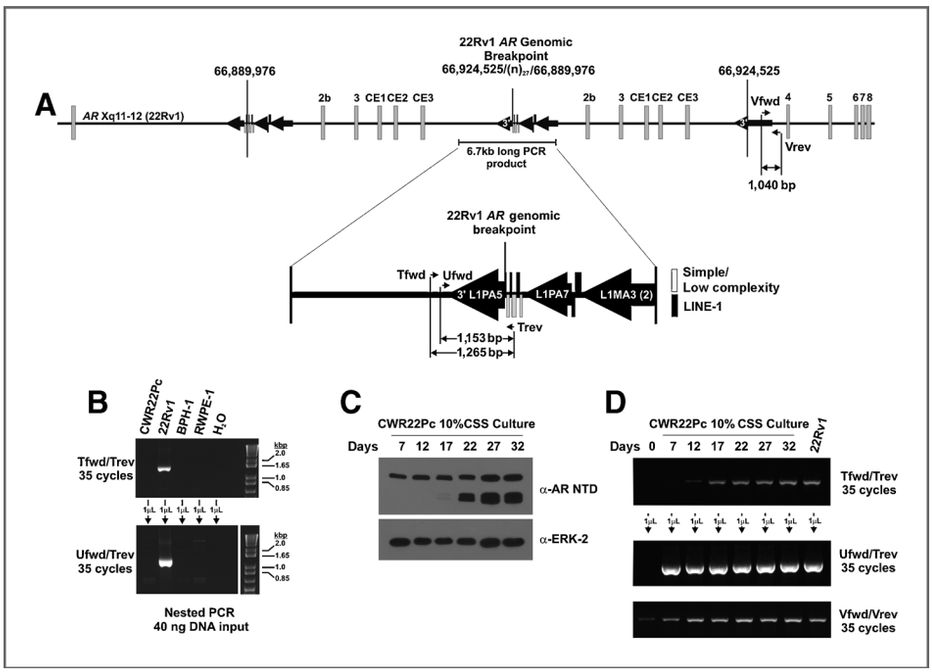


**Figure 6.** Concurrent emergence of *AR* intragenic rearrangement, androgen-independent growth, and high-level truncated AR isoform expression during CWR22Pc castration. A, schematic of the 22Rv1 *AR* locus and locations of primers used for nested PCR. B, conventional PCR was carried out using Tfwd/Trev primers and 40 ng of input DNA from the indicated cell lines. An aliquot of this reaction was used in a second nested PCR reaction using Ufwd/Trev primers. C, AR Western blot of CWR22Pc castration time course. CWR22Pc cells were cultured in androgen-depleted medium for the indicated time points. ERK-2, loading control. D, nested PCR of CWR22Pc castration time course. Reactions were carried out exactly as described in panel B.

inserted sequence (Fig. 5C). The origin of this sequence was not apparent by BLASTN and BLAT searches; however, the first 8 bp of this sequence perfectly matched an 8-bp motif at the 5′ breakpoint. Sequence alignments of the cloned break fusion junction and the 5′ and 3′ breakpoints showed virtually no extended homology through this region (Fig. 5D). However, regions of 3-bp microhomology were found at the breakpoints (Fig. 5D). Microhomology at the breakpoints and inserted sequence at the fusion site argues against NAHR and supports a microhomology-mediated break-induced replication (MMBIR; ref. 32) mechanism of segmental duplication in 22Rv1 cells.

### Emergence of CRPCa cells during long-term CWR22Pc castration

Using conventional and nested PCR strategies, we confirmed that the *AR* breakpoint observed in 22Rv1 cells was indeed restricted to this cell line (Supplementary Fig. S8C and Fig. 6B). Previous studies have shown that androgen-dependent CWR22Pc xenograft tumors initially regress during castration but eventually recur with a CRPCa phenotype (23). To probe the link between *AR* intragenic rearrangement and CRPCa, we cultured CWR22Pc cells over a 1-month period in androgen-depleted medium. During the first 12 days of culture, no changes in AR protein expression patterns were observed (Fig. 6C). Interestingly, the 22Rv1 breakpoint was detected in CWR22Pc cells by nested PCR after 7 days of castration (Fig. 6D). The sensitivity of this nested PCR approach was determined to be as low as 1 to 2 genomes in limiting dilution assays (Supplementary Fig. S8D), indicating that the subpopulation of cells harboring this rearrangement was very rare. By day 17, discrete proliferative foci were apparent, which coincided with faint expression of truncated AR isoforms (Fig. 6C) and detection of the 22Rv1 breakpoint via conventional PCR (Fig. 6D). On day 17, cells were trypsinized and reseeded to disperse these proliferative foci. By day 22 and onward, androgen-independent cell growth was apparent, as was the expression of truncated AR isoforms. Together, these findings show that *AR* intragenic rearrangement is linked to high-level truncated AR isoform expression and CRPCa growth in a cell-based model of PCa progression.

## Discussion

Recent reports describing the synthesis and function of truncated, constitutively active AR isoforms have provided a novel and conceptually simple mechanism for the resistance of CRPCa cells to androgen depletion (16–19). However, the mere presence of truncated AR isoforms does not correlate perfectly with androgen responsiveness, which highlights the importance of quantitative understanding in this area. This is especially apparent from a recent study showing that AR 1/2/3/CE3 [also termed AR-V7 (18) or AR-3 (17)] increases during progression to CRPCa but is also expressed in benign prostate tissue and hormone naive PCa (17). Because truncated AR isoforms were originally identified in CRPCa cells derived from the CWR22 model, the recent establishment of an androgen-dependent cell line from CWR22 xenografts has permitted an evaluation of the



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changes in *AR* mRNA splicing regulation that may occur during PCa progression in a lineage-related context. One striking difference between androgen-dependent CWR22Pc cells and 22Rv1 CRPCa cells was the expression profile of full-length and alternatively spliced *AR* mRNAs. Although alternatively spliced *AR* mRNAs and protein were detectable in both cell lines, we found that 22Rv1 cells had an enhanced capacity to efficiently synthesize *AR* 1/2/2b, *AR* 1/2/3/2b, and *AR* 1/2/3/CE3 mRNAs. We further showed tandem duplication of an approximately 35-kb segment harboring these alternative exons as a likely basis for the deregulation of AR mRNA splicing observed in 22Rv1 cells. Interestingly, a recent study identified 2 additional alternative exons expressed in VCaP cells that are clustered on this segment between AR exons CE1 and CE3 (20). Mechanistically, such a rearrangement could impair normal splicing by lengthening of the already vast distance between the *AR* transcription start site and *AR* exon 4, increasing the likelihood of incorporating 1 of the 2 sets of alternative exons preceding exon 4, disrupting the normal genomic organization of cis-acting intronic and exonic splicing elements or any combination of these possibilities. It will be important to elucidate a clear cause–effect mechanism, but technical limitations such as the size of the *AR* locus (~180 kb) and even larger aberrant locus in 22Rv1 cells (~215 kb) will have to be addressed.

AR overexpression is common in CRPCa, and *AR* gene amplification is thought to be a main driver of increased AR protein expression (33, 34). Most prior assessments of *AR* amplification in PCa tissues employed FISH, which lacks resolution and does not permit accurate copy number assessment along the length of the *AR* gene (7, 13, 34–36). Our findings indicate that a subset of amplified *AR* loci in CRPCa harbor intragenic rearrangements similar to 22Rv1, in addition to other alterations, which would clearly lead to a reconfigured *AR* exon organization for many of these alleles. It will therefore be important to conduct a comprehensive study of the relationship between *AR* intragenic rearrangements and levels of alternatively spliced AR isoforms in CRPCa to determine whether there is selection for intragenic rearrangement or whether intragenic rearrangement is simply arising as a by-product of *AR* gene amplification. Long-term castration of CWR22Pc cells suggests that *AR* intragenic rearrangement and the CRPCa growth phenotype are linked and that enrichment for cells with this genomic alteration occurs because of a selective advantage under castrate conditions. It is also possible that larger-scale genomic rearrangements may play a role in disrupted AR splicing, as evidenced by the recent identification of the truncated mAR-V4 isoform in the Myc-CaP mouse model, which results from alternative splicing of a cryptic exon nearly 1 Mb upstream of the mouse *AR* locus (20). Together, these findings indicate that tissues displaying *AR* intragenic rearrangements should be prioritized for further studies of AR splice variants and their importance to PCa prognosis and therapeutic response.

It will also be important to map and sequence the break fusion junctions within the *AR* locus in individual CRPCa metastases to obtain a more complete understanding of the mechanisms and significance of *AR* intragenic rearrangements. Our work revealed LINE-1 elements at the 5′ and 3′ ends of the 22Rv1 AR rearrangement. Transposable elements (TE) are implicated in the genesis of rearrangements underlying TE-related genetic diseases, including cancer (29), and often arise through NAHR. However, sequencing the 22Rv1 *AR* break fusion junction revealed a 27-bp insertion of unknown origin, which opposes a NAHR-based model. Indeed, stressed cancer cells are deficient in NAHR (37) and cancer-specific rearrangements frequently contain insertions ranging from 1 to 154 bp of so-called nontemplate sequence at the break fusion junction (38–40). Therefore, a new model, MMBIR has recently been proposed to account for this class of break fusion junctions in cancer cells (32).

In summary, our work describes a novel AR intragenic rearrangement in the 22Rv1 model of PCa progression, which is linked to enhanced synthesis of truncated AR isoforms and androgen-independent growth. We further show that similar genomic rearrangements occur in metastatic CRPCa specimens. It will be important in future studies to define whether intragenic *AR* rearrangements directly cause disrupted AR splicing, because a scenario of *AR* intralocus breaks leading to enhanced synthesis of truncated AR isoforms indicates that there may be little plasticity in the repertoire of AR isoforms synthesized. This would potentially limit the effectiveness of manipulating "alternative" splicing as a therapy for CRPCa. Nevertheless, a genomic basis for pathologic AR isoform expression may serve as a stable mechanism-based marker for resistance to androgen depletion therapies.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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ORIGINAL ARTICLE

# AR intragenic deletions linked to androgen receptor splice variant expression and activity in models of prostate cancer progression

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Reactivation of the androgen receptor (AR) during androgen depletion therapy (ADT) underlies castration-resistant prostate cancer (CRPCa). Alternative splicing of the *AR* gene and synthesis of constitutively active COOH-terminally truncated AR variants lacking the AR ligand-binding domain has emerged as an important mechanism of ADT resistance in CRPCa. In a previous study, we demonstrated that altered AR splicing in CRPCa 22Rv1 cells was linked to a 35-kb intragenic tandem duplication of AR exon 3 and flanking sequences. In this study, we demonstrate that complex patterns of *AR* gene copy number imbalances occur in PCa cell lines, xenografts and clinical specimens. To investigate whether these copy number imbalances reflect *AR* gene rearrangements that could be linked to splicing disruptions, we carried out a detailed analysis of *AR* gene structure in the LuCaP 86.2 and CWR-R1 models of CRPCa. By deletion-spanning PCR, we discovered a 8579-bp deletion of AR exons 5, 6 and 7 in the LuCaP 86.2 xenograft, which provides a rational explanation for synthesis of the truncated AR v567es AR variant in this model. Similarly, targeted resequencing of the *AR* gene in CWR-R1 cells led to the discovery of a 48-kb deletion in AR intron 1. This intragenic deletion marked a specific CWR-R1 cell population with enhanced expression of the truncated AR-V7/AR3 variant, a high level of androgen-independent AR transcriptional activity and rapid androgen independent growth. Together, these data demonstrate that structural alterations in the *AR* gene are linked to stable gain-of-function splicing alterations in CRPCa.

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**Keywords:** prostate cancer; androgen receptor variants; castration-resistant; intragenic rearrangement, AR alternative splicing

## INTRODUCTION

PCa initially presents as an androgen- and androgen receptor (AR)-dependent disease. Therefore, suppressing the production or action of androgens, which inhibits AR transcriptional activity, leads to stabilization or regression of advanced PCa. The duration of response to androgen depletion therapy (ADT) is variable with the end point typically marked by rising serum levels of prostate specific antigen, an AR transcriptional target gene and rapid growth of PCa metastases. This transition from androgen-dependent to castration-resistant prostate cancer (CRPCa) is frequently due to aberrant AR re-activation despite ongoing treatment with AR-targeted therapies.[1–4] Various mechanisms have been advanced to explain AR activation in CRPCa cells. These include *AR* gene amplification and/or AR protein over-expression,[5–12] point mutations that permit promiscuous AR transcriptional responses[13–20] and intra-tumor steroid synthesis or sequestration.[21–24] This knowledge has driven the clinical development of new inhibitors of androgen production and AR signaling,[25] including the CYP17 inhibitor abiraterone acetate, which has been recently shown to increase overall survival in patients with metastatic CRPCa.[26]

Synthesis of truncated AR variant proteins via AR alternative splicing has recently emerged as an additional mechanism of ADT resistance in PCa.[27–31] These proteins lack the AR ligand-binding domain, display constitutive, ligand-independent transcriptional activity, and mediate androgen-independent growth of PCa cells in various model systems.[27–31] Increased expression of the AR3 variant protein (also termed AR-V7[29]) in PCa prostatectomy specimens is associated with biochemical recurrence following surgery.[28] In addition, increased mRNA expression of alternatively spliced AR variants in PCa bone metastases is associated with shorter survival.[32] Therefore, understanding the mechanisms leading to increased synthesis of these species could provide important prognostic information, or perhaps guide more effective use of therapies that inhibit ligand-dependent AR activity.

Truncated AR variants proteins were originally discovered and functionally characterized in the CRPCa 22Rv1 and CWR-R1 cell lines,[27–29] and the LuCaP 86.2 PCa xenograft.[30] In 22Rv1 cells, a 35-kb AR intragenic tandem duplication is linked to altered splicing of full-length AR, as well as synthesis of truncated AR variants.[33] However, the mechanisms driving AR splicing

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alterations outside of the 22Rv1 model have remained elusive. Therefore, the purpose of this study was to investigate the link between *AR* gene structure alterations and enhanced synthesis of truncated AR variants in CRPCa.

## RESULTS

### *AR* gene structure complexity in CRPCa

In a previous study, we analyzed high-resolution whole-genome copy number data from CRPCa metastasis, which implicated frequent AR copy number imbalance concurrent with AR amplification at this stage of the disease.[33] To investigate this phenomenon directly, we employed a multiplex ligation-dependent probe assay (MLPA) with probe sets targeted to coding exons in the *AR* gene (Figure 1a). This MLPA approach detected the 22Rv1 duplication involving exon 3, as well as 20-fold amplification of the *AR* gene in VCaP cells (Figure 1b). Androgen-dependent PCa tissue obtained from xenografts (Figure 1c) or clinical specimens (Figure 1d) displayed one intact *AR* gene copy, with the exception of the LuCaP 35 xenograft, which displayed four copies of the *AR* gene. However, CRPCa tissue obtained from xenografts (Figure 1c) or autopsy specimens (Figure 1d) displayed frequent *AR* gene amplification and/or complex patterns of *AR* gene copy number imbalance. These data suggest that imbalances in *AR* gene copy number may be important for CRPCa progression.

To investigate this phenomenon in more detail, we focused on the LuCaP 86.2 xenograft, which expresses high levels of the truncated AR v567es variant arising from the mRNA splicing machinery skipping exons 5, 6 and 7.[30] LuCaP 86.2 displayed reduced genome copy number of these exons, indicating a mixed cell population with ~50% of cells harboring an intragenic deletion (Figure 1c). Deletion-spanning PCR yielded products consistent with an intragenic deletion encompassing AR exons 5, 6 and 7 (Figures 2a and b). Sequence analysis verified a 8579-bp deletion (Figure 2c) with microhomology at the 5′ and 3′ break fusion junctions, which signifies a non-homologous end joining mechanism of origin (Figure 2d). Together, these data implicate focal intragenic deletion as a novel mechanism underlying synthesis of the truncated AR v567es variant in the LuCaP 86.2 xenograft.

### Stable AR mRNA splicing alterations in CRPCa CWR-R1 cells

MLPA analysis indicated that the CRPCa CWR-R1 cell line does not harbor any *AR* gene copy number alterations, despite previous studies demonstrating that these cells express truncated AR variants.[28] To confirm altered splicing in these cells, we assessed AR mRNA isoform levels using an absolute quantification reverse transcription (RT)–PCR assay. To correct for variability in *AR* gene dosage and rates of AR transcription, we scaled copy number for each AR mRNA isoform relative to full-length AR. This approach revealed changes in the ratios of full-length AR mRNA and alternatively spliced AR isoforms in CRPCa CWR-R1 and 22Rv1 cells (Figure 3b). Conversely, androgen-dependent CWR22Pc, LNCaP and VCaP cells expressed predominantly full-length AR mRNA (Figure 3b). To test for plasticity in the expression of truncated AR variant expression in CWR-R1 cells, we knocked down full-length AR mRNA using an AR exon 7-targeted siRNA. No changes in truncated AR protein expression were observed following 48 h or 72 h of knock down, whereas an AR exon 1-targeted siRNA completely abolished all AR protein expression in these cells (Figure 3c). Similarly, no changes in truncated AR variant protein expression were observed in CWR-R1 cells following 24 h or 72 h of androgen stimulation (Figure 3d). These data indicate that the altered AR mRNA splicing pattern in CWR-R1 cells is stable and is unlikely an acute cellular response to manipulations of androgen or AR levels.

### A novel intragenic deletion in CWR-R1 cells identified by paired-end *AR* gene resequencing

As MLPA only interrogates AR copy number at coding exons, which represent less than 1.5% of the 180-kb *AR* gene, we

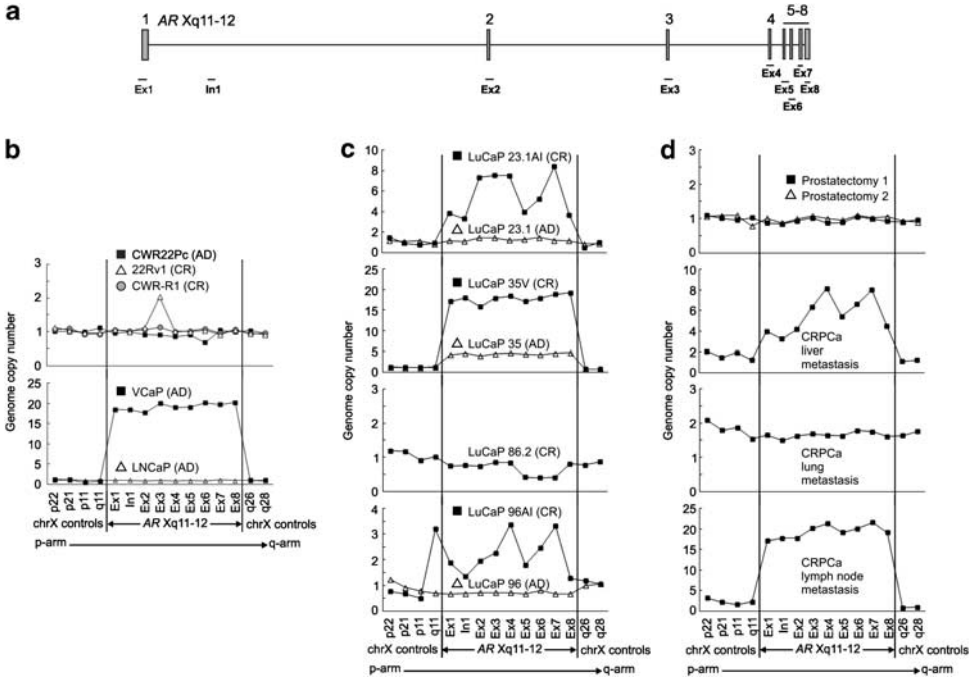


**Figure 1.** Diverse and complex patterns of *AR* gene copy number imbalance in CRPCa. (**a**) Schematic of the *AR* gene with relative locations of MLPA probes used for targeted copy number analysis. Genomic DNA from (**b**) androgen-dependent (AD) and castration-resistant (CR) PCa cell lines, (**c**) PCa xenografts, including AD/CR pairs propagated in intact/castrated male mice, and (**d**) clinical PCa was subjected to MLPA to evaluate genomic copy number across the AR locus and X chromosome.


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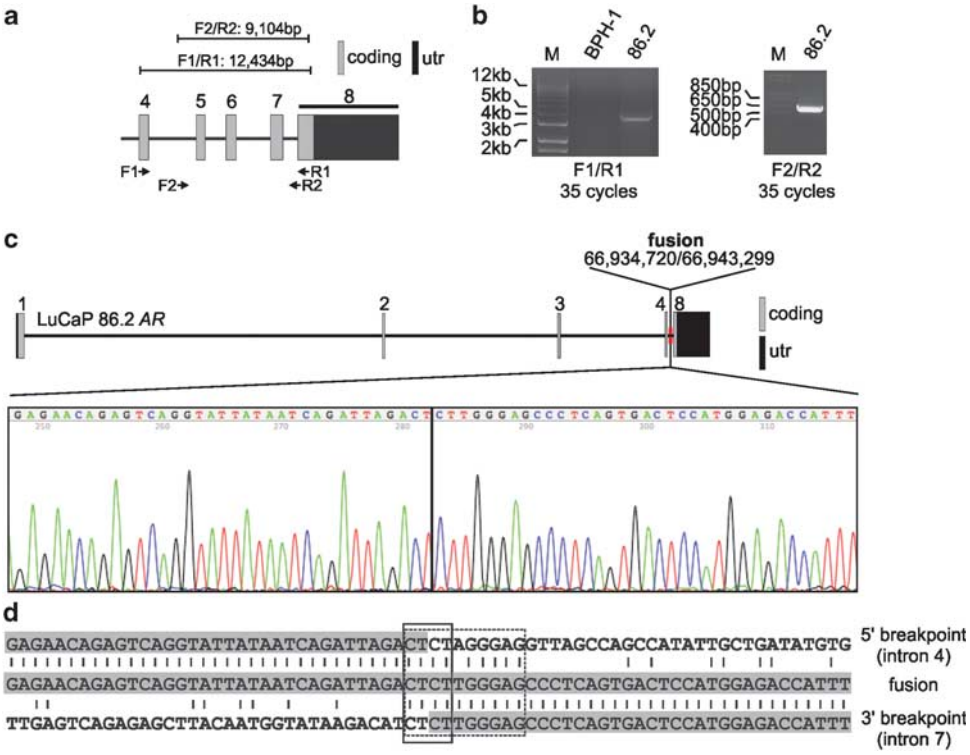


**Figure 2.** Intragenic deletion encompassing AR exons 5, 6 and 7 in the LuCaP 86.2 xenograft model. (**a**) Relative positions of deletion-spanning PCR primers and expected PCR fragment sizes based on the hg19 build of the human genome. (**b**) CWR-R1 and BPH-1 genomic DNA was subjected to nested PCR using primer sets indicated in (**a**). (**c**) PCR products from (**b**) were cloned and sequenced using the Sanger method. The electropherogram peak trace and *AR* gene structure resulting from the 8579-bp intragenic deletion are shown. (**d**) Alignment of the 5′-deletion breakpoint, the 3′-deletion breakpoint, and the deletion fusion revealed 4 bp of perfect microhomology (solid box) and 10 bp of extended microhomology with 1 bp mismatch (dashed box). Sequence retained in the break fusion junction is shaded in gray.

analyzed the nucleotide sequence and structure of the entire AR locus in CWR-R1 cells using a combination of liquid-phase sequence capture and Illumina paired-end massively parallel sequencing (Supplementary Figure 1 and Supplementary Table 1). Androgen-dependent CWR22Pc and CRPCa 22Rv1 cells were sequenced concurrently. As 49.9% of the AR locus is composed of repetitive DNA,[34] which precludes the design of capture baits to these regions, we were unable to obtain sequence for nearly a quarter of the *AR* gene using this strategy (Supplementary Table 1). In particular, no sequence was obtained within three 'gaps' of contiguous repetitive elements spanning >6 kb each (Figure 4 and Supplementary Figures 1 and 2). Gaps 2 and 3 harbor the 5′ and 3′ breakpoints of the previously identified 22Rv1 tandem duplication[33] and no paired-end reads representing this structural alteration were detected (Supplementary Table 2). However, sequence coverage plots revealed increased copy number within this region, with transition points located within gaps 2 and 3 (Supplementary Figures 2 and 3). No copy number imbalances in this region were observed in CWR22Pc or CWR-R1 cells (Supplementary Figure 2). Point mutations and indels were observed at various frequencies in all three cell lines, including the previously-identified H874Y coding mutation at position chrX:66 943 543 (Supplementary Tables 3–5). However, no prevalent sequence alterations were observed in splice donor/acceptor sequences in CWR-R1 and 22Rv1 cells (Supplementary Tables 4 and 5). Strikingly, structural variant analysis of paired-end reads using the Hydra workflow[35] identified a ~48-kb intragenic deletion within AR exon 1 in a sub-population of the CWR-R1 cell line, which was also apparent from a relative decrease in sequence coverage peak height within this region (Figure 4, Supplementary Table 2 and Supplementary Figure 4). To confirm this structural alteration in CWR-R1 cells, we performed nested PCR using primers spanning the deletion (Figures 5a and b). Sanger sequencing of cloned PCR products revealed deletion of 48 476 bp from AR intron 1 (Figures 5c and d). Alignments of the 5′ and 3′ break fusion junctions demonstrated 3 bp of microhomology, implicating non-homologous end joining as the mechanism underlying this deletion (Figure 5e).

To quantify the prevalence of this ~48-kb deletion, we performed MLPA with probe pairs custom-designed to query copy number at regular intervals along the length of the *AR* gene (Figure 6a). MLPA probe pairs targeted within this 48-kb region displayed a ~20–30% decrease in copy number (Figure 6b). There were no copy number alterations in this region detected by MLPA in CWR22Pc or 22Rv1 cells (Figure 6b), and nested PCR with deletion-spanning primers did not generate products in CWR22Pc or 22Rv1 cells (Figure 6c). Similarly, only CWR-R1 cells yielded 76 bp Illumina sequencing reads that could be aligned to a 130-bp template harboring this specific breakpoint sequence (Supplementary Figure 5). Together, these data demonstrate that a 48 476-bp deletion within intron 1 of the *AR* gene is restricted to 20–40% of the cells in the CWR-R1 cell line.

## Enrichment for cells harboring AR intron 1 deletion during castration

The levels of truncated AR isoforms expressed in CWR-R1 cells are markedly higher when these cells are grown as xenografts in castrated mice versus intact mice.[28] All the analyses in our study had been performed with CWR-R1 cells that had been cultured in complete medium (contains androgens) for 10–20 passages, which would allow the growth of both androgen-dependent and

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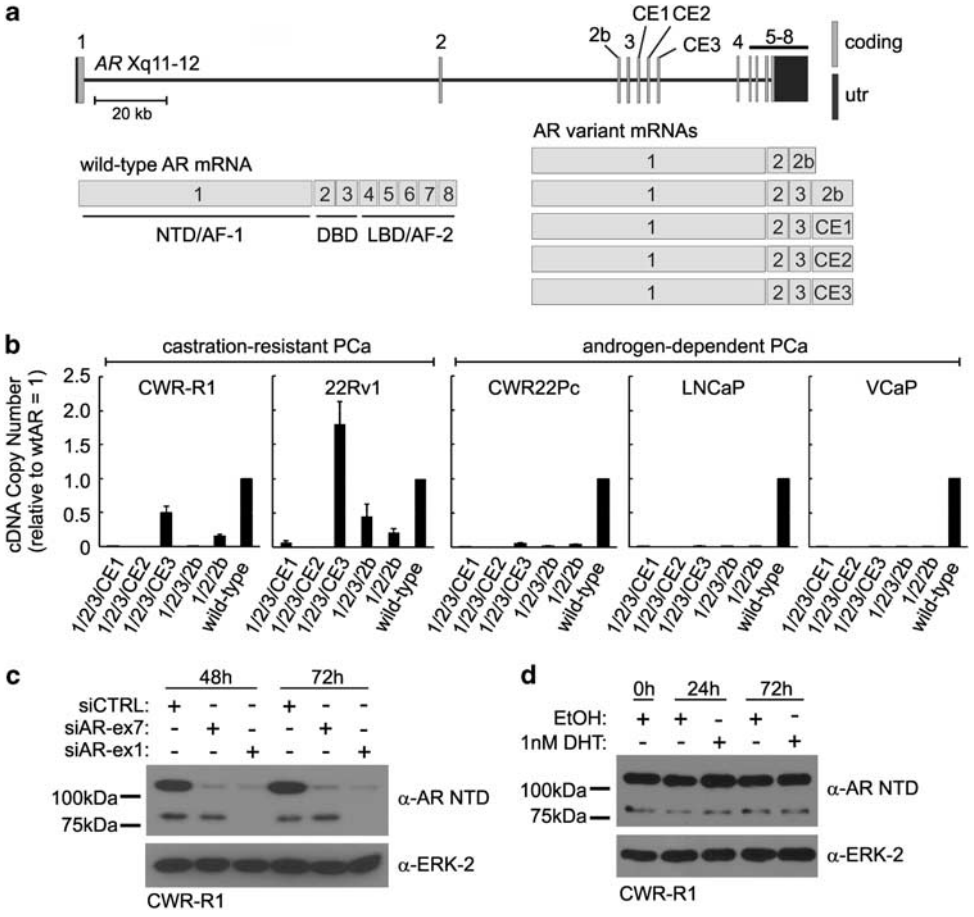


**Figure 3.** Stable, high-level expression of truncated AR variants in CWR-R1 cells. (**a**) AR genomic organization and exon composition of alternatively spliced AR mRNA isoforms reported in cell lines derived from the CWR22 xenograft. (**b**) RNA from indicated PCa cells lines was subjected to quantitative RT–PCR with isoform-specific primer sets, and Ct values were converted to copy number by plotting on standard curves. (**c**) CWR-R1 cells were electroporated with a control siRNA or siRNAs targeted to AR exon 1 or exon 7 and analyzed by western blot with indicated antibodies 48 h and 72 h post-transfection. (**d**) CWR-R1 cells were treated with 1 nM dihydrotestosterone (DHT) or vehicle (EtOH) for indicated times and analyzed by western blot with indicated antibodies.

CRPCa cell populations. Therefore, we compared *AR* gene structure in early passage CWR-R1 cells (referred to as CWR-R1 early) to CWR-R1 cells that were cultured in the absence of androgens for 20 passages (referred to as CWR-R1-late). Remarkably, no copy number decrease within AR intron 1 was apparent following MLPA analysis of CWR-R1 early cells (Figure 7a), despite a positive nested PCR signal for this deletion (Supplementary Figure 6). Conversely, MLPA probe signal in this region was nearly completely lost in CWR-R1 late cells (Figure 7a), indicating that this deletion was a marker of the CRPCa cell sub-population. Consistent with this deletion underlying the AR splicing patterns in CWR-R1 cells, expression of the truncated AR 1/2/3/CE3 variant (also referred to as AR-V7[29] or AR3[28]) was low in CWR-R1 early cells, regardless of whether they were cultured in whole serum or steroid-depleted serum (Figure 7b). However, CWR-R1 late cells displayed high-level expression of AR 1/2/3/CE3 protein (Figure 7b). These changes at the protein level corresponded with a stable shift in splicing favoring the AR 1/2/3/CE3 AR mRNA isoform in CWR-R1 late versus CWR-R1 early cells (Supplementary Figure 7). CWR-R1 cells cultured in the presence of androgens for 20 passages displayed an intermediate AR 1/2/3/CE3 protein expression pattern (Figure 7b). Immunostaining of cells grown under castrate conditions demonstrated increased nuclear expression of the AR $NH_2$-terminal domain versus the AR COOH-terminal domain in CWR-R1 late cells, but not CWR-R1 early cells (Supplementary Figure 8). Together, these data demonstrate that cells harboring this ~48-kb deletion within AR exon 1 display a splicing switch that favors stable, high-level expression of the truncated AR 1/2/3/CE3 variant.

To investigate whether there may be functional consequences to these differences in truncated AR variant expression, we examined AR transcriptional activity in CWR-R1 early and CWR-R1 late cells. AR transactivation in response to the synthetic androgen mibolerone was higher in CWR-R1 early cells than CWR-R1 late cells and knock down of full-length AR inhibited this androgen response in both cell lines (Figure 7c). Interestingly, knock down of AR expression with siRNAs targeted to either AR exon 1 or exon 7 inhibited androgen-independent transcriptional activity in CWR-R1 early cells, but only siRNA targeted to AR exon 1 had this effect in CWR-R1 late cells (Figure 7c). These data indicate that androgen independent AR activity in CWR-R1 early cells is dependent on full-length AR expression, whereas androgen-independent AR activity in CWR-R1 late cells is mediated by truncated AR variants through a mechanism that is independent of full-length AR. To investigate differential siRNA sensitivity and a differential role for full-length AR in more detail, we compared the androgen-independent growth of CWR-R1 early and CWR-R1 late cells transfected with AR-targeted siRNAs. Consistent with their selection under castrate conditions, CWR-R1 late cells displayed a rapid androgen-independent growth rate, which was inhibited by


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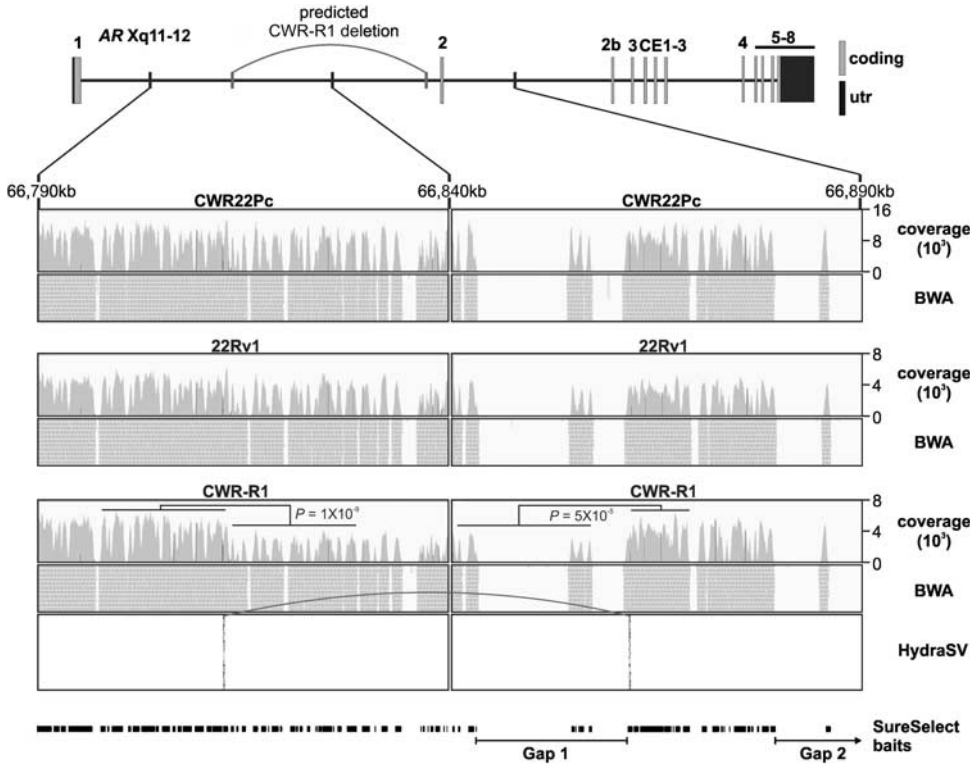


**Figure 4.** CWR-R1 cells harbor an intragenic deletion in AR intron 1. A schematic of the AR locus is illustrated at the top. Paired-end sequence reads were mapped to the hg19 build of the human genome using Burrows–Wheeler Alignment (BWA) and visualized using Integrated Genomics Viewer (IGV). Two 50-kb windows-spanning genomic positions 66 790 000 and 66 890 000 are shown for each cell line, indicating positions and depth of coverage of BWA-mapped reads. Discordantly mapped paired-end reads identified by the Hydra workflow are shown for CWR-R1 and the relative location of this predicted deletion is indicated on the AR locus schematic. Differences in coverage peak maxima at sites flanking the predicted deletion breakpoints were compared using *t*-tests. Positions of individual SureSelect baits used for AR sequence capture are tiled across the bottom. Two large gaps of extended repetitive sequence precluding design of capture baits are indicated.

siRNA targeted to AR exon 1, but not AR exon 7 (Figure 7d). Conversely, CWR-R1 early cells grew slowly under androgen-independent conditions during this short time-course and there was limited response to AR-targeted siRNA (Figure 6d).

## DISCUSSION

The prevalence of *AR* gene mutations in CRPCa reported by different groups has been variable, but appears to be low at approximately 10%.[13–20] These data suggest that the majority of CRPCa harbors a wild-type *AR* gene. However, mutations have been evaluated historically by sequence analysis of AR cDNA and/or AR coding exons, which represents only ~1.5% of the 180 245-bp *AR* gene.[36] The identification and clinical validation of truncated AR variant synthesis as an important mechanism of PCa therapy resistance has raised the possibility that splicing disruptions may be due to non-coding *AR* gene alterations. Indeed, our previous work defined a ~35-kb AR intragenic tandem duplication in 22Rv1 cells, which is a rational explanation for the altered splicing pattern in this cell line.[33] In this study, we have defined two additional simple *AR* gene structural alterations that are linked to the pathological AR splicing patterns in the LuCaP 86.2 and CWR-R1 models of PCa progression. These data, combined with MLPA analysis of additional CRPCa specimens, indicate that the prevalence of *AR* gene alterations in tumors resistant to ADT may be higher than previously anticipated. Although targeted methods such as MLPA are useful for identifying deletions or duplications that involve probe-binding sites, this study has illustrated that unbiased evaluation of the entire *AR* gene sequence and structure is a preferable approach. However, our work has also demonstrated that high-throughput approaches are challenged by substantial repeat in the AR locus. Indeed, paired-end reads diagnostic of the 35-kb 22Rv1 AR intragenic tandem duplication were not obtained in our study, despite 6000-fold maximal on-target sequence coverage in these cells. This indicates that many *AR* gene structural alterations would also go undetected using larger-scale sequence capture or whole-genome sequencing approaches.[37,38] Therefore, to define the prevalence and spectrum of the *AR* gene structure alterations that may exist in clinical CRPCa, methodology optimization must be a prerequisite.

Previous analysis of genome-wide copy number data from clinical CRPCa specimens suggested that complex patterns of copy number gain and copy number loss occurred along the length of the *AR* gene,[33] which is supported by MLPA analysis in this study. Here, we demonstrated that large deletions involving intron 1 are associated with enhanced synthesis of the truncated AR 1/2/3/CE3 variant (also referred to as AR-V7 or AR3) and a growth advantage under castrate conditions. In the CWR-R1 model, castration-mediated enrichment for cells harboring intron 1 deletion resulted in an overall population that exhibited levels of AR 1/2/3/CE3 that were equivalent to or greater than the levels of full-length AR. This is important because a recent study of surgical specimens of CRPCa bone metastases with an antibody specific for the AR NTD demonstrated that protein expression of truncated AR variants can reach similar high levels relative to full-length AR.[32] Moreover, patients with CRPCa bone metastases that displayed the highest levels of alternatively spliced, truncated AR mRNA variants had shorter cancer-specific survival after metastasis

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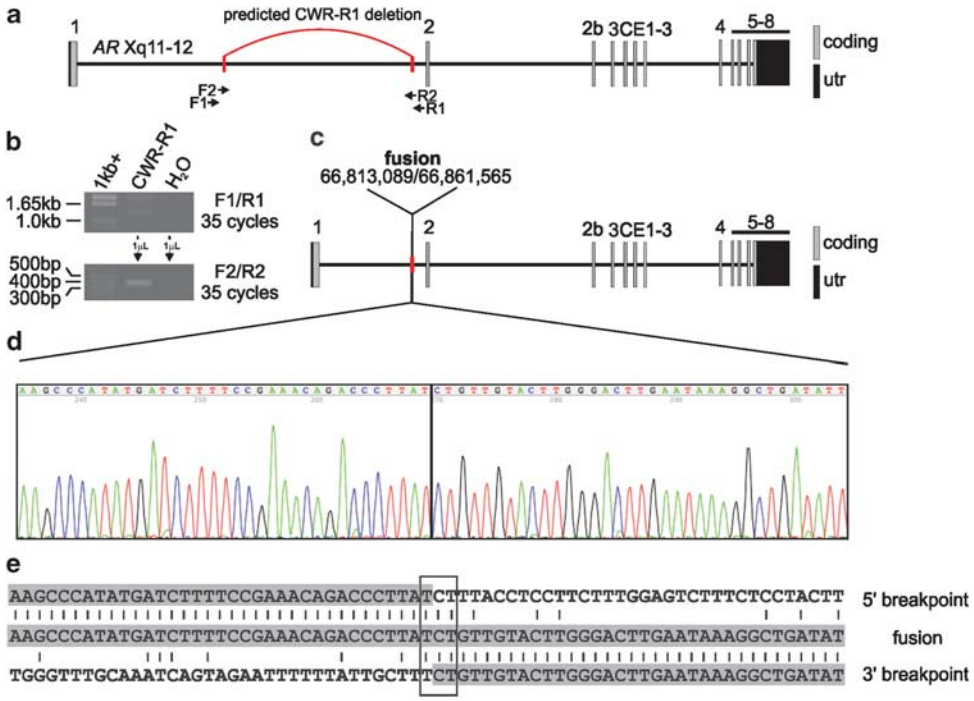


**Figure 5.** Intragenic deletion involving 48 476 bp of AR intron 1 in CWR-R1 cells. (**a**) Schematic of the AR locus with relative positions of deletion-spanning PCR primers. (**b**) CWR-R1 genomic DNA was subjected to nested PCR using indicated primer sets indicated in (**a**). (**c**) Schematic of the CWR-R1 AR locus harboring a 48 476-bp intron 1 deletion. (**d**) PCR products from (b) were cloned and sequenced using the Sanger method. The electropherogram peak trace is shown. (**e**) Alignment of the 5′-deletion breakpoint, the 3′-deletion breakpoint, and the deletion fusion revealed 3 bp of microhomology (boxed). Sequence retained in the break fusion junction is shaded in gray.

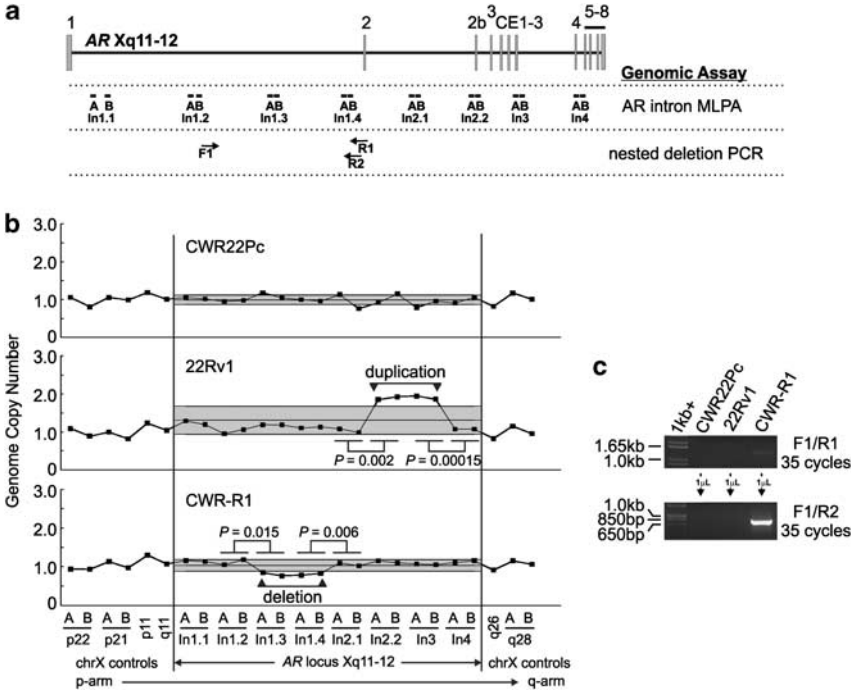


**Figure 6.** Intragenic deletion is restricted to a CWR-R1 subpopulation. (**a**) Schematic of the *AR* gene with relative locations of primers and probes used for targeted genomic assays. (**b**) Genomic DNA was subjected to MLPA to evaluate genomic copy number across the AR locus and X chromosome. Relative positions of AR-specific MLPA probes are indicated in (**a**). Gray boxes represent the mean ± s.d. of all AR locus probes from two independent experiments. Probe pairs displaying copy number greater than one s.d. away from the mean AR copy number are diagnostic of duplication or deletion. Differences in copy number measured by probe pairs flanking predicted duplication or deletion breakpoints were compared with *t*-tests. (**c**) Genomic DNA was subjected to nested PCR using primer sets depicted in (**a**).


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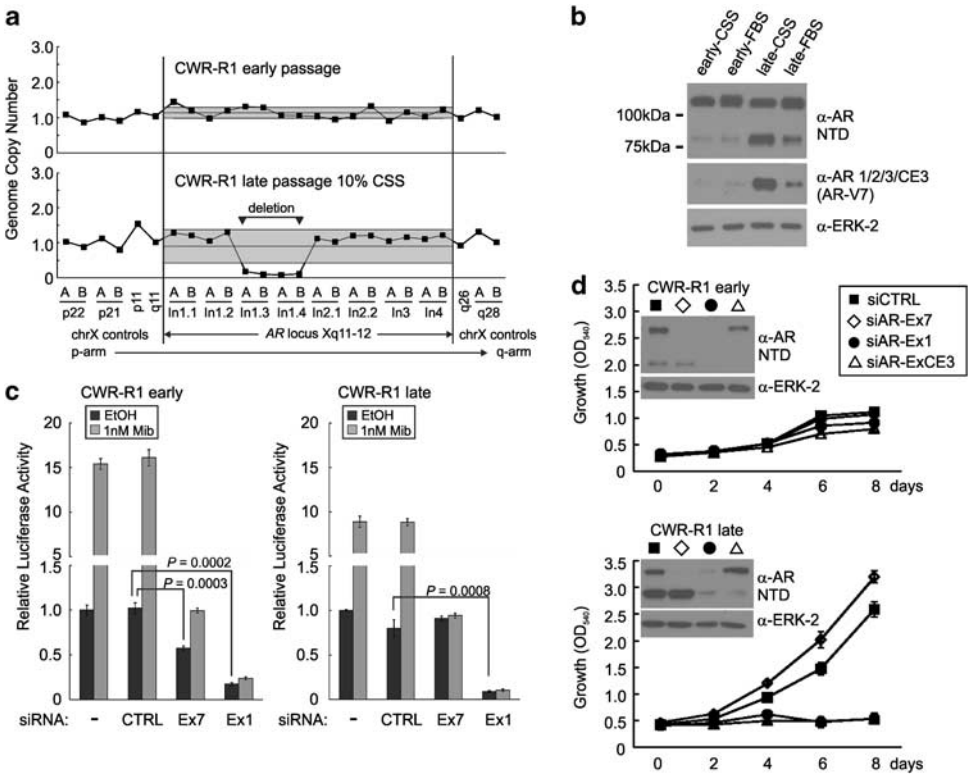


**Figure 7.** CWR-R1 cells with intron 1 deletion are castration-resistant and dependent on truncated AR variant function. (**a**) Genomic DNA from early-passage CWR-R1 cells and CWR-R1 cells cultured for 20 passages under castrate conditions was subjected to MLPA to evaluate genomic copy number across the AR locus and X chromosome. Gray boxes represent the mean ± s.d. of all AR locus probes from two independent experiments. Probe pairs diagnostic of deletion are indicated. (**b**) Lysates from early-passage CWR-R1 cells cultured in the presence (fetal bovine serum (FBS)) or absence (charcoal-stripped serum (CSS)) of androgens for 24 h and CWR-R1 cells cultured in the presence (FBS) or absence (CSS) of androgens for 20 passages (late) were analyzed by western blot with indicated antibodies. (**c**) Early-passage CWR-R1 cells and CWR-R1 cells cultured for 20 passages under castrate conditions (late) were transfected with MMTV-Luc, non-targeted control (CTRL) siRNA, or siRNAs targeted to AR exon 1 or 7. Cells were grown 24 h in serum-free medium and treated with 1 nM mibolerone (synthetic androgen) or EtOH (vehicle control) for 24 h. Luciferase activity was determined. Data represent the mean ± s.e. from two independent experiments, each performed in duplicate. MMTV promoter activity without androgens and siRNAs was arbitrarily set to 1. (**d**) Early-passage CWR-R1 cells and CWR-R1 cells cultured for 20 passages under castrate conditions (late) were transfected with non-targeted CTRL siRNA, or siRNAs targeted to AR exon 1 or 7. Growth of transfected cells under castrate conditions was monitored every 2 days.

surgery than other CRPCa patients.[32] Therefore, increased expression of truncated AR variants is an important component of clinical PCa progression. The data in this study strongly suggests that alterations in the architecture of the *AR* gene may underlie these disruptions in normal splicing patterns. With this in mind, it is important to point out that a true 'alternative splicing' mechanism has not yet been elucidated for the *AR* gene. Rather, models that exhibit levels of truncated AR variant expression sufficient to drive the CRPCa phentoype (such as 22Rv1, CWR-R1 and LuCaP 86.2) have dramatic changes in the *AR* gene template. These AR intragenic duplications and deletions that we have defined all appear to result from homologous recombination-independent mechanisms such as microhomology-mediated break-induced replication in 22Rv1[33] and non-homologous end joining in LuCaP 86.2 and CWR-R1. Therefore, the exact locations of breakpoints in the AR locus are unlikely to be recurrent between specimens. Intriguingly, these three models each display a unique splicing signature and repertoire of truncated AR variant protein expression. Therefore, it is tempting to speculate that different patterns of *AR* gene alteration may give rise to different AR splicing patterns in clinical CRPCa. This would argue that a complete understanding of the role of truncated AR variants in CRPCa progression will require that individual tumors be evaluated for splicing alterations using unbiased detection methods rather than targeted approaches focused on known AR variants.

Overexpression of the AR 1/2/3/CE3 variant (also referred to as AR-V7 or AR3), the AR v567es variant, or a truncated AR variant of mouse origin (mAR-V4) in LNCaP cells can induce androgen-independent expression of AR target genes and growth under castrate conditions *in vitro* and *in vivo*.[28,30,31] Interestingly, treatment of these engineered LNCaP cells with the next-generation anti-androgen MDV3100 or knock down of full-length AR resulted in reversal of these CRPCa features.[31] These data indicate that truncated AR variants require full-length AR to support a CRPCa phenotype. However, this is in opposition to our studies with CRPCa models that endogenously express high levels of truncated AR variants and harbor apparent gain-of-function structural alterations in the *AR* gene.[27,33] For example, in this study, knock down of full-length AR had no effect on androgen-independent AR activity or androgen-independent growth in late-passage CWR-R1 cells. However, knock down of AR 1/2/3/CE3 inhibited these parameters. We have also demonstrated this differential response to isoform-targeted siRNAs in the 22Rv1 cell line.[27] Conversely, early-passage CWR-R1 cells displayed modest androgen-independent growth and measurable androgen-independent AR activity, which was inhibited following knock down of full-length AR. These data demonstrate that the CWR-R1 cell line is heterogeneous and that growth conditions can have dramatic effects on the relative proportions of androgen-dependent cells and CRPCa cells, which may explain a previous report where

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CWR-R1 cells displayed decreased proliferation and increased apoptosis in response to full-length AR knockdown.[28] With this in mind, it is also important to note that the LuCaP 86.2 xenograft tissue evaluated in this study was propagated in an intact male mouse, and MLPA data reflected an approximate 50/50 mixture of cells with either one intact *AR* gene copy or one *AR* gene copy with a 8579-bp deletion of exons 5, 6 and 7. If the cell population harboring the 8549-bp intragenic deletion is indeed the cell population which synthesizes the AR v567es variant, these cells would not be able to synthesize full-length AR and would be truly independent of full-length AR activity.[30] Therefore, a more thorough investigation of the requirement for full-length AR is warranted, as this will provide important insights to resistance mechanisms that may circumvent clinical responses to current and next-generation therapies targeting the AR ligand-binding domain.[25]

In summary, this study represents the first report of intragenic deletions involving coding and non-coding sequences in the *AR* gene in CRPCa, which we have linked to expression of truncated AR variants that support the CRPCa phenotype. Therefore, structural alterations in the *AR* gene may represent a widespread, yet previously unanticipated, mechanism of therapy resistance in PCa. Our findings provide justification for large-scale investigation of *AR* gene structure and splicing patterns in clinical specimens.

## MATERIALS AND METHODS

### PCa tissues

Genomic DNA samples from the LuCaP series of PCa xenografts and de-identified clinical CRPCa tissue were obtained from the University of Washington Prostate Cancer Biorepository, which was developed and managed by one of the co-authors (RLV) and has been described in previous publications.[30,39,40] De-identified prostatectomy tissue samples were obtained under the direction of the University of Minnesota BioNet tissue resource, which was developed and managed by one of the co-authors (SCS). Cores of PCa tissue (1 mm) were obtained from archival formalin-fixed, paraffin-embedded prostatectomy blocks using a tissue microarrayer (Beecher Instruments, Sun Prairie, WI, USA) and genomic DNA was isolated using a RecoverAll kit (Applied Biosystems/Ambion, Austin, TX, USA).

### Cell culture

The 22Rv1 (number CRL-2505), LNCaP (number CRL-1740) and VCaP (number CRL-2876) cell lines were obtained from ATCC (Manassas, VA, USA) and cultured according to ATCC protocol. CWR22Pc cells[41] were generously provided by Dr Marja Nevalainen (Thomas Jefferson University) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2.5 mM L-glutamine and 0.8 nM dihydrotestosterone (Sigma, St Louis, MO, USA). CWR-R1 cells[42] were a kind gift from Dr Elizabeth Wilson (UNC Chapel Hill) and cultured in RPMI 1640 + 10% fetal bovine serum. For androgen response experiments, cells were cultured in RPMI 1640 + 10% steroid-depleted, charcoal-stripped serum for 48 h, treated at $t = 0$ with 1 nM dihydrotestosterone (Sigma) or vehicle (EtOH), and then harvested at indicated time points. For long-term culture experiments, CWR-R1 cells were cultured in RPMI 1640 + 10% charcoal-stripped serum. Cells were trypsinized and re-seeded in the appropriate medium when flasks attained 80% confluence.

### Transient transfections

The CWR-R1 cell line was electroporated with siRNAs targeted to AR exon 7 (target sequence: 5′-GGAACUCGAUCGUAUCAUU-3′) or AR exon 1 (5′-CAAG GGAGGUUACACCAAA-3′) and/or an MMTV-LUC reporter as described.[27] Growth of electroporated cells was monitored by crystal violet staining as described.[33] Luciferase activity was measured as described.[27]

*Quantitative real-time RT–PCR.* RNA isolation and absolute quantification RT–PCR analysis of alternatively-spliced AR mRNA isoforms was performed as described.[33] To correct for different levels of wild-type AR mRNA expression among the PCa cell lines, copy numbers of AR mRNA isoforms were scaled relative to wild-type AR mRNA copy number in each cell line (set to 1). For relative quantification RT–PCR, fold change in expression levels were determined by the comparative Ct method using the equation $2^{-\Delta\Delta Ct}$.

*Genomic PCR.* Genomic PCR was performed as described,[33] using primer pairs listed in Supplementary Table 6.

### Western blot

Western blotting with AR NTD (N-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA), AR CTD (Santa Cruz C-19), ERK-2 (Santa Cruz D-2) and ARV-7 (number AG10008, Precision Antibody, Columbia, MD, USA) antibodies was performed as described.[33]

### Multiplex ligation-dependent probe assay

MLPA for AR coding sequence was performed using a commercially available kit (P074, MRC Holland, Amsterdam, The Netherlands) as per the manufacturer's protocol. Briefly, 100 ng of genomic DNA was hybridized at 60 °C for 18 h with MLPA probemix. Hybridized probes were ligated and amplified by PCR with labeled universal primers provided with the MLPA kit. PCR reactions were diluted 1:10 in formamide containing ROX-500 size standards (Applied Biosystems, Carlsbad, CA, USA), denatured and resolved by capillary electrophoresis using a Genetic Analyzer 3130XL (Applied Biosystems). Electropherogram peak areas were obtained using Peak Scanner software (Applied Biosystems). Peak areas for samples and calibration control (HPV-7 prostate epithelial cell genomic DNA) were block-normalized using X-chromosome p-arm controls and then normalized to HPV-7 copy number with the inference that the HPV-7 genome contains one copy of the *AR* gene. MLPA for AR intron sequences was performed using the exact same protocol with a commercially available reagent kit (EK1, MRC Holland) and custom-designed oligonucleotide probes (Supplementary Table 7). Probe pairs that each displayed copy number values outside of one s.d. from the mean copy number of all AR locus probes from two independent experiments were determined to have increased or decreased copy number at that location.

### Paired-end library creation, sequence capture and next-generation sequencing

Genomic DNA from CWR22Pc, 22Rv1 and CWR-R1 cells was fragmented using an S220 ultra-sonicator (Covaris, Woburn, MA, USA) with Agilent SureSelect parameters. A Bioanalyzer DNA 1000 chip (Agilent, Santa Clara, CA, USA) was used to verify DNA samples sheared with fragment peaks between 150–200 bp. Paired-end sequencing libraries were generated from sheared DNA samples using a SureSelect Library Preparation Kit (Agilent) and amplified for sequence capture as per the manufacturer's protocol. The amplified DNA libraries were hybridized and captured using overlapping, tiled SureSelect baits (Agilent) custom-designed to provide two times coverage of non-repetitive regions of the AR locus (Supplementary Table 8). Target-enriched libraries were amplified for 16-cycles to add index tags and generate sufficient template for flowcell clustering. Final libraries were quantified via quantitative PCR (Kapa Biosystems, Woburn, MA, USA), normalized and pooled before clustering on a single lane of a flowcell. The flowcell was loaded on a Genome Analyzer IIx (GAIIx, Illumina, San Diego, CA, USA) for paired-end sequencing at 76 cycles ($2 \times 76$ bp). Data analysis methodology is provided as Supplemental Information.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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# Androgen Receptor Splice Variants Activate Androgen Receptor Target Genes and Support Aberrant Prostate Cancer Cell Growth Independent of Canonical Androgen Receptor Nuclear Localization Signal*[S]

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**Background:** Truncated AR splice variants support castration-resistant prostate cancer.
**Results:** The AR NTD/DBD core is sufficient for AR variants to access the nucleus, activate AR target genes, and support androgen-independent prostate cancer cell growth.
**Conclusion:** Diverse truncated AR variants are constitutively active transcription factors.
**Significance:** These novel biochemical properties could lead to the development of new prostate cancer therapies.

**Synthesis of truncated androgen receptor (AR) splice variants has emerged as an important mechanism of prostate cancer (PCa) resistance to AR-targeted therapy and progression to a lethal castration-resistant phenotype. However, the precise role of these factors at this stage of the disease is not clear due to loss of multiple COOH-terminal AR protein domains, including the canonical nuclear localization signal (NLS) in the AR hinge region. Despite loss of this NLS, we show that diverse truncated AR variant species have a basal level of nuclear localization sufficient for ligand-independent transcriptional activity. Whereas full-length AR requires Hsp90 and importin-$\beta$ for active nuclear translocation, basal nuclear localization of truncated AR variants is independent of these classical signals. For a subset of truncated AR variants, this basal level of nuclear import can be augmented by unique COOH-terminal sequences that reconstitute classical AR NLS activity. However, this property is separable from ligand-independent transcriptional activity. Therefore, the AR splice variant core consisting of the AR $NH_2$-terminal domain and DNA binding domain is sufficient for nuclear localization and androgen-independent transcriptional activation of endogenous AR target genes. Indeed, we show that truncated AR variants with nuclear as well as nuclear/cytoplasmic localization patterns can drive androgen-independent growth of PCa cells. Together, our data demonstrate that diverse truncated AR species with varying efficiencies of nuclear localization can contribute to castration-resistant PCa pathology by driving persistent ligand-independent AR transcriptional activity.**

The androgen receptor (AR)[2] is a modular steroid receptor transcription factor, composed of a large, disordered $NH_2$-terminal domain (NTD), a central DNA binding domain (DBD), and a COOH-terminal ligand binding domain (LBD) and transcriptional activation function-2 coactivator binding interface (1). Androgen depletion therapy (ADT) for locally advanced or metastatic prostate cancer (PCa) relies on blocking androgen production and/or androgen binding to the AR LBD. However, most PCa mortality is due to the development of castration-resistant PCa (CRPCa), which is driven by pathologic reactivation of the AR during ADT (2). An ongoing AR dependence of CRPCa supports the concept that AR is a lineage survival factor in prostate cells (3), and this knowledge has led to the development of additional strategies for AR inhibition, including the CYP17 inhibitor abiraterone, which extends lifespan in a subset of CRPCa patients by further suppressing androgen production in the adrenal cortex as well as tumor tissue (4).

One possible mechanism of aberrant AR reactivation during ADT is the synthesis of COOH-terminal truncated AR variants lacking the AR LBD and activation function-2 domain (5). These AR variants arise through alterations in splicing and encode protein products composed of the AR NTD/DBD core and novel COOH-terminal extensions of variable length and sequence (6–10). Antibodies have been developed for the COOH-terminal extension of the truncated AR-V7 variant (also referred to as AR3), demonstrating that expression levels increase concomitant with stage of hormonal progression (7). Moreover, increased expression of AR-V7 in hormone-naïve prostatectomy specimens is associated with disease relapse

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[S] This article contains supplemental Experimental Procedures, Table 1, and Figs. 1–8.

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[2] The abbreviations used are: AR, androgen receptor; NTD, $NH_2$-terminal domain; DBD, DNA binding domain; LBD, ligand binding domain; ADT, androgen depletion therapy; PCa, prostate cancer; CRPCa, castration-resistant PCa; NLS, nuclear localization signal; ARE, androgen response element; LUC, luciferase.

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after surgery (7). In surgical specimens of skeletal CRPCa metastases, levels of AR-V7 and AR-V1 mRNA are inversely correlated with survival (11). These studies indicate that changes in AR splicing are important for clinical CRPCa progression and may drive resistance to therapies that require an intact AR LBD, including next-generation inhibitors such as abiraterone (12).

Currently, it is unclear whether diverse truncated AR variants can activate the AR transcriptional program because many of these species are predicted to have lost the canonical AR nuclear localization signal (NLS) in the exon 4-encoded hinge region. Similar to other steroid receptors, AR transcriptional activity is tightly regulated by ligand binding. In the unbound, inactive state, the AR is a predominantly cytoplasmic protein bound by heat shock family chaperone proteins and immunophilins (13). AR nuclear import proceeds via a classical pathway wherein nuclear translocation is initiated by ligand binding, which exposes the bipartite AR NLS and allows binding to the importin-$\alpha$ adapter protein and importin-$\beta$ carrier protein, translocation through the nuclear core complex, and Ran-dependent release into the nucleus (14, 15). Because domains required for interaction with these pathways appear to be lost, it has been suggested that many truncated AR variants may have exclusively cytoplasmic functions or even function as dominant negative species (7, 9, 10). Therefore, how these diverse species may be involved in CRPCa progression is not clear. To address this, we investigated the biochemical properties of individual truncated AR variants with the goal of identifying general molecular features that could be exploited for developing new CRPCa therapies.

## EXPERIMENTAL PROCEDURES

*Cell Lines and Culture Conditions*—LNCaP, 22Rv1, DU145, 293T, and Cos-7 cells were obtained from American Type Culture Collection (ATCC). LNCaP, 22Rv1, and DU145 cells were maintained in RMPI 1640 (Invitrogen) with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 $\mu$g/ml streptomycin in a 5% $CO_2$ incubator at 37 °C. The 293T and Cos-7 cell lines were cultured in DMEM with 10% FBS. For androgen response experiments, cells were seeded in 10% charcoal-stripped serum for 48 h. Cells were then stimulated for 24 h by replacement of growth medium with medium containing 1 nM mibolerone (Biomol) or dihydrotestosterone (Sigma).

*Antibodies and siRNA Reagents*—Small interfering RNAs (targeted to AR exons 1, 3, and 7) were purchased from Dharmacon. Primary antibodies specific for the AR NTD (N-20, catalogue #sc-816; A-441, catalogue #sc-7305), ERK-2 (D-2, catalogue #sc-1647), HA tag (F-7, catalogue #sc-7392), and $\alpha$-tubulin (catalogue #sc-23948) were purchased from Santa Cruz Biotechnology. An antibody specific for lamin A/C (4C11, catalogue #4777) was purchased from Cell Signaling.

*Plasmids*—Plasmid constructs harboring full-length AR (p5HBhAR-A), AR 1/2/3/2b, MMTV-LUC, −5746 PSA-LUC, and 4XARE-E4-LUC have been described (6, 16). Construction details for plasmid and lentiviral reagents used in this study are provided in the supplemental Experimental Procedures.

*Luciferase Reporter Gene Assays*—LNCaP cells were transfected by electroporation exactly as described (6, 17). DU145 cells were transfected using Superfect reagent (Qiagen) exactly as described (6). For all reporter-based experiments, 24–48 h post-transfection cells were re-fed with serum-free medium containing 1 nM mibolerone or 0.1% ethanol as vehicle control for 24 h. Cells were harvested in 1× passive lysis buffer provided in a Dual Luciferase Assay kit (Promega). Activities of the firefly and *Renilla* luciferase reporters were assayed using a Dual Luciferase Assay kit as per the manufacturer's recommendations. Transfection efficiency was normalized by dividing firefly luciferase activity by *Renilla* luciferase activity. Data presented represent the mean ± S.E. from at least three independent experiments, each performed in triplicate.

*Subcellular Fractionation*—LNCaP cells were transfected under androgen-free conditions with expression vectors encoding truncated AR variants using PolyExpress (Excellgen) exactly as per the manufacturer's protocol. 22Rv1 cells were electroporated under androgen-free conditions with siRNA targeted to AR exon 7 (Dharmacon) as described (6). Transfected cells were cultured 24 h post-transfection and then treated for 24 h with medium containing 1 nM mibolerone or 0.1% ethanol. After treatment, transfected cells were washed in 1× phosphate-buffered saline (PBS), harvested in hypotonic buffer (250 mM sucrose, 20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and a 1× final concentration of Roche Applied Science Mini complete protease inhibitor), and incubated for 20 min on ice. Cells were lysed by 10 passages through 25-gauge needles. The cytosolic fraction (supernatant) was collected by centrifugation at 720 × *g*, 5 min, 4 °C. The nuclear pellet was washed twice by resuspending in 500 $\mu$l of hypotonic buffer followed by 10 passages through 25-gauge needles. Nuclei were pelleted by centrifugation at 3000 × *g* for 10 min at 4 °C. Isolated cytosolic and nuclear fractions were resuspended in 1× Laemmli buffer (18), boiled for 5 min, and then loaded equally by volume for Western blot analysis.

*Western Blot*—Cell lysates in 1× Laemmli buffer were subjected to Western blot analysis as described (6). Blots were incubated with primary antibodies overnight at 4 °C and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies at room temperature for 2 h. Blots were developed by incubation with Super Signal chemiluminescence reagent (Pierce) and exposed to film.

*Immunofluorescence*—Cos-7, LNCaP, and DU145 cells were seeded in complete medium on coverslips the day before transfection. Cells were transfected under androgen-free conditions with 1 $\mu$g of expression vector encoding full-length AR or truncated AR variants using Lipofectamine 2000 (Invitrogen) for Cos-7 cells, electroporation for LNCaP cells, or Superfect (Qiagen) for DU145 cells. The next day culture medium was replaced with serum-free medium containing 1 nM mibolerone or 0.1% ethanol. Cells were maintained an additional 24 h and then fixed with ice-cold methanol for 10 min, permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature, and blocked with 1× PBS containing 10% FBS for 1 h at room temperature. Cells were incubated overnight at 4 °C with AR-N20 diluted 1:1000 or HA-F7 diluted 1:500 in PBS. Unbound primary antibody was removed by three washes with PBS, and then cells were incubated with fluorescein isothiocya-

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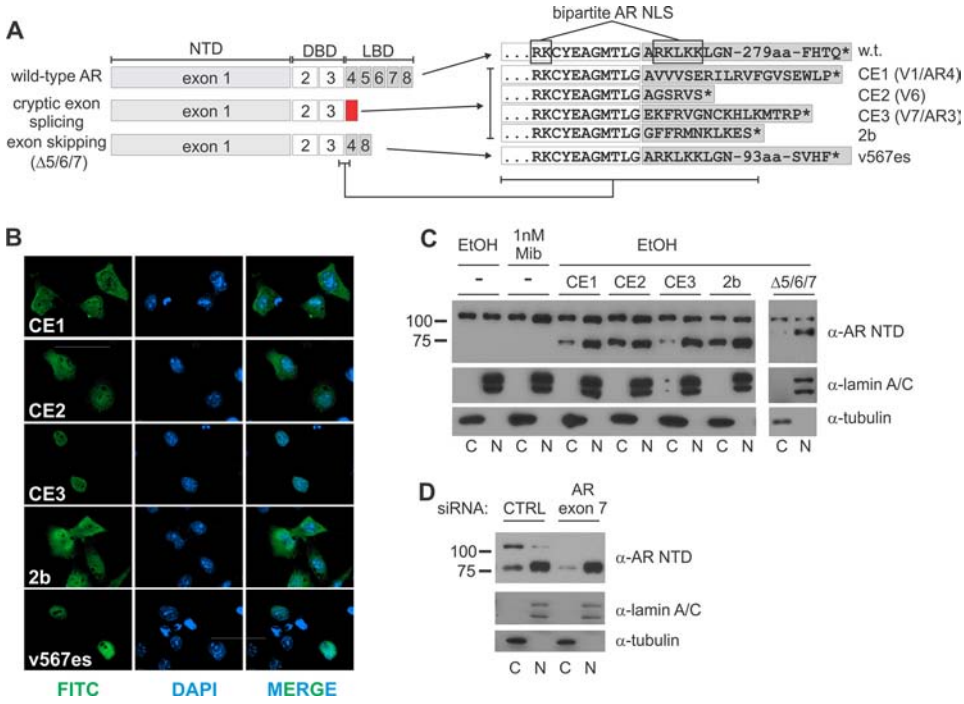


FIGURE 1. **Constitutive nuclear localization of truncated AR variants with diverse COOH-terminal extensions.** *A*, shown is a schematic of COOH-terminal tails of truncated AR variants aligned with the AR hinge region. The multiple names that have been assigned to several of these variants are indicated (7, 8). *B*, Cos-7 cells expressing truncated AR variants 1/2/3/CE1, 1/2/3/CE2, 1/2/3/CE3, 1/2/3/2b, or v567es were stained with an antibody specific for the AR NTD (FITC signal), and nuclei were stained with DAPI. Stained cells were visualized by confocal microscopy. Representative images are shown for three color channels (FITC, DAPI, and merged FITC/DAPI). *C*, LNCaP cells expressing truncated AR variants were cultured in serum-free medium containing 1 nM mibolerone (*Mib*, synthetic androgen) or ethanol (*EtOH*, vehicle control). Cell lysates were separated into nuclear (*N*) and cytoplasmic (*C*) fractions and analyzed by Western blot with antibodies specific for the AR NTD, lamin A/C (nuclear marker), and tubulin (cytoplasmic marker). *D*, 22Rv1 cells were electroporated with non-targeted siRNA (*CTRL*) or siRNA targeted to AR Exon 7. Cells were cultured, fractionated, and analyzed by Western blot exactly as described in *C*.

nate (FITC)-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) at a 1:500 dilution in PBS containing 0.2 $\mu$g/ml 4′,6-diamidino-2-phenylindole (DAPI) for 1 h at room temperature. Slides were washed with PBS and mounted with Mowoil (Calbiochem), and images were captured using a confocal laser scanning microscope (Olympus Fluoview FV500) equipped with a 40× objective. To optimize microscopy data for print media, hue adjustments were made to all immunofluorescence images (*red*, +90; *green*, +90; *blue*, −90) using CorelDraw software.

*Lentivirus Packaging and Transduction*—Lentivirus expressing GFP, AR 1/2/3/CE3, 1/2/3/CE3-R617A/K618A, 1/2/3/CE3-K629A/R631A, AR 1–627aa, AR 1/2/3/2b, and AR v567es was prepared using a standard third generation packaging system in 293T cells. Briefly, 293T cells were cotransfected with lentivirus vectors and packaging vectors pCMVΔR8.91 (19) and pMD.G (20) at a ratio of 4:3:1 using Lipofectamine 2000. Medium containing lentivirus was collected from 12 to 96 h post-transfection and concentrated using the Lenti-X Concentrator (Clontech). Titers for LNCaP transductions were tested and normalized to achieve protein expression levels equivalent to endogenous AR.

*Total RNA Extraction and Quantitative Real Time RT-PCR*—Total RNA was isolated from LNCaP cells using TRIzol (Invitrogen). RNA was reverse-transcribed using a Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science) following the manufacturer's instructions. cDNA was subjected to quantitative PCR using PerfeCTa SYBR Green FastMix (Quanta Biosciences) with primer sets specific for PSA, hK2, TMPRSS2, FKBP51, and GAPDH (see supplemental Table 1). -Fold change in mRNA expression levels was calculated by the comparative Ct method using the formula $2^{-(\Delta\Delta Ct)}$ and GAPDH as calibrator as described (6).

*Analysis of Cell Growth by Crystal Violet Mitogenic Assay*—Lentivirus-transduced LNCaP cells were seeded at equal density on 24-well plates. Lentivirus-transduced 22Rv1 cells were electroporated with small interference RNA (siRNA) targeted to AR exon 1 as described (6, 17) and seeded at equal density on 24-well plates. At the indicated time points, cells were fixed and stained with crystal violet as described (21).

## RESULTS

*Truncated AR Variants with Diverse COOH-terminal Extensions Are Constitutively Nuclear Transcription Factors*—The majority of AR transcriptional activity is mediated by the AR NTD and deletion of the AR LBD results in constitutive, ligand-independent activity (22). However, early biochemical studies that established these fundamental principles employed AR fragments that retained the bipartite NLS in the AR hinge region. Most truncated AR variants expressed in CRPCa have a disrupted bipartite NLS due to altered splicing (Fig. 1*A*). Therefore, it has been proposed that many of these species would not be able to access the nucleus, which is a prerequisite for transcriptional activation. However, this theory has not been rigorously tested. Therefore, we transfected cells with diverse truncated AR variants and performed immunofluorescence

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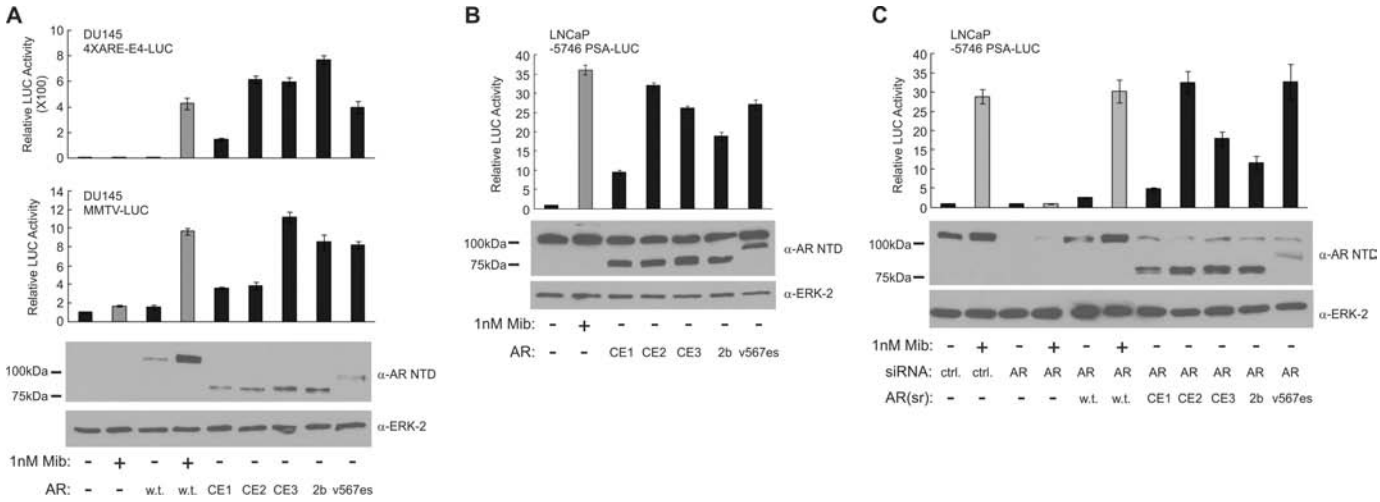


FIGURE 2. **Constitutive transcriptional activity of truncated AR variants with diverse COOH-terminal extensions.** *A*, DU145 cells were transiently transfected with 4XARE-E4-LUC or MMTV-LUC reporters and expression plasmids encoding full-length AR or truncated variants 1/2/3/CE1, 1/2/3/CE2, 1/2/3/CE3, 1/2/3/2b, and v567es. Cells were cultured in serum-free medium containing 1 nM mibolerone (*Mib*) or vehicle control (ethanol). Cell lysates were analyzed by luciferase assay and Western blot with antibodies specific for the AR NTD and ERK-2 (loading control). *Bars* represent mean ± S.E. from at least three independent experiments, each performed in duplicate. Reporter activity in the absence of transactivator or ligand was arbitrarily set to 1. *B*, LNCaP cells were transiently transfected with a −5746 PSA-LUC reporter and truncated AR variants and analyzed by luciferase assay and Western blot as in *A*. *C*, LNCaP cells were transiently transfected and analyzed exactly as described in *B* with the exception of the use of AR exon 7-targeted siRNA and siRNA-resistant AR expression constructs (denoted *sr* (17)).

microscopy (Fig. 1*B* and see supplemental Fig. 1) and biochemical fractionation (Fig. 1*C*). Truncated AR variants displayed significant differences in predominance of nuclear localization, but all were able to access the nucleus. Nuclear localization patterns were the same in AR-null Cos-7 and DU145 cells and AR-dependent LNCaP cells, indicating that nuclear localization is not affected by cell type or presence of full-length AR (Fig. 1, *B* and *C*, and see supplemental Fig. 1). To test this directly, we selectively knocked-down full-length AR expression in 22Rv1 cells using siRNA targeted to AR exon 7 and observed no effect on the nuclear localization of endogenous truncated AR variants (Fig. 1*D*). Therefore, truncated AR variants with the AR NTD/DBD core and diverse COOH-terminal extensions are nuclear proteins.

To test whether the observed differences in nuclear localization between truncated AR variants underlie differences in transcriptional activity, we performed transient transfection experiments in AR-null DU145 PCa cells and AR-dependent LNCaP cells (Fig. 2, *A* and *B*, and see supplemental Fig. 2*A*). Different truncated AR variants displayed varying strengths of transcriptional activity in a promoter-dependent but cell line-independent manner. To test a requirement for full-length AR, we performed knockdown/re-expression experiments in LNCaP cells. Knockdown of full-length AR completely inhibited ligand-dependent promoter activity, and re-expression of siRNA-resistant full-length AR restored this ligand-dependent activity. Conversely, re-expression of individual siRNA-resistant truncated AR variants was able to restore promoter activity in the absence of ligand (Fig. 2*C* and see supplemental Fig. 2*B*). The levels of transcriptional activity in these assays were nearly identical to the levels observed in the absence of knock-down (Fig. 2, *B* and *C*, and see supplemental Fig. 2), confirming that full-length AR is not required for this activity.

*Reconstitution of Bipartite AR NLS Enhances Truncated AR Variant Nuclear Localization but Not Transcriptional Activity*—Despite the observation that individual truncated AR variants exhibited varying degrees of transcriptional activity on AR-responsive promoters, there did not appear to be a perfect relationship between efficiency of nuclear localization and transcriptional activity. This suggests that nuclear localization is not the primary determinant of AR variant transcriptional activity. To test this concept, we focused on the AR-V7/AR3 variant generated by splicing of AR exons 1/2/3/CE3, which displayed strong nuclear localization and high transcriptional activity. This variant contains two clusters of basic amino acids in the COOH-terminal tail encoded by AR exon CE3, one of which aligns with the second basic amino acid cluster of the wild-type AR bipartite NLS (Fig. 3*A*). Consistent with previous reports (15, 23), a K630A/K632A/K633A compound mutation blocked ligand-induced nuclear localization of full-length AR (Fig. 3*B*). Alanine mutation of Lys-629 and Arg-631 in the truncated AR 1/2/3/CE3 variant, which both align to the second basic amino acid cluster in the full-length AR NLS, shifted 1/2/3/CE3 expression from predominantly nuclear to a mixed nuclear/cytoplasmic pattern (Fig. 3*B* and see supplemental Fig. 3). Conversely, alanine mutation of Lys-636 and Lys-639 in the truncated AR 1/2/3/CE3 variant had no effect on nuclear localization (Fig. 3*B*). We next asked whether dominant negative Ran Q69L, which prevents the carrier importin-$\beta$ from releasing cargo into the nucleus (24), could alter subcellular distribution of the truncated AR1/2/3/CE3 variant. Indeed, dominant negative Ran Q69L caused a shift in AR 1/2/3/CE3 expression to a mixed nuclear/cytoplasmic pattern (Fig. 3*C*). A similar effect of dominant negative Ran Q69L was observed on the nuclear K636A/K639A mutant version of AR 1/2/3/CE3, but no effect was observed for the nuclear/cytoplasmic K629A/R631A mutant version, showing that the dominant negative effects of

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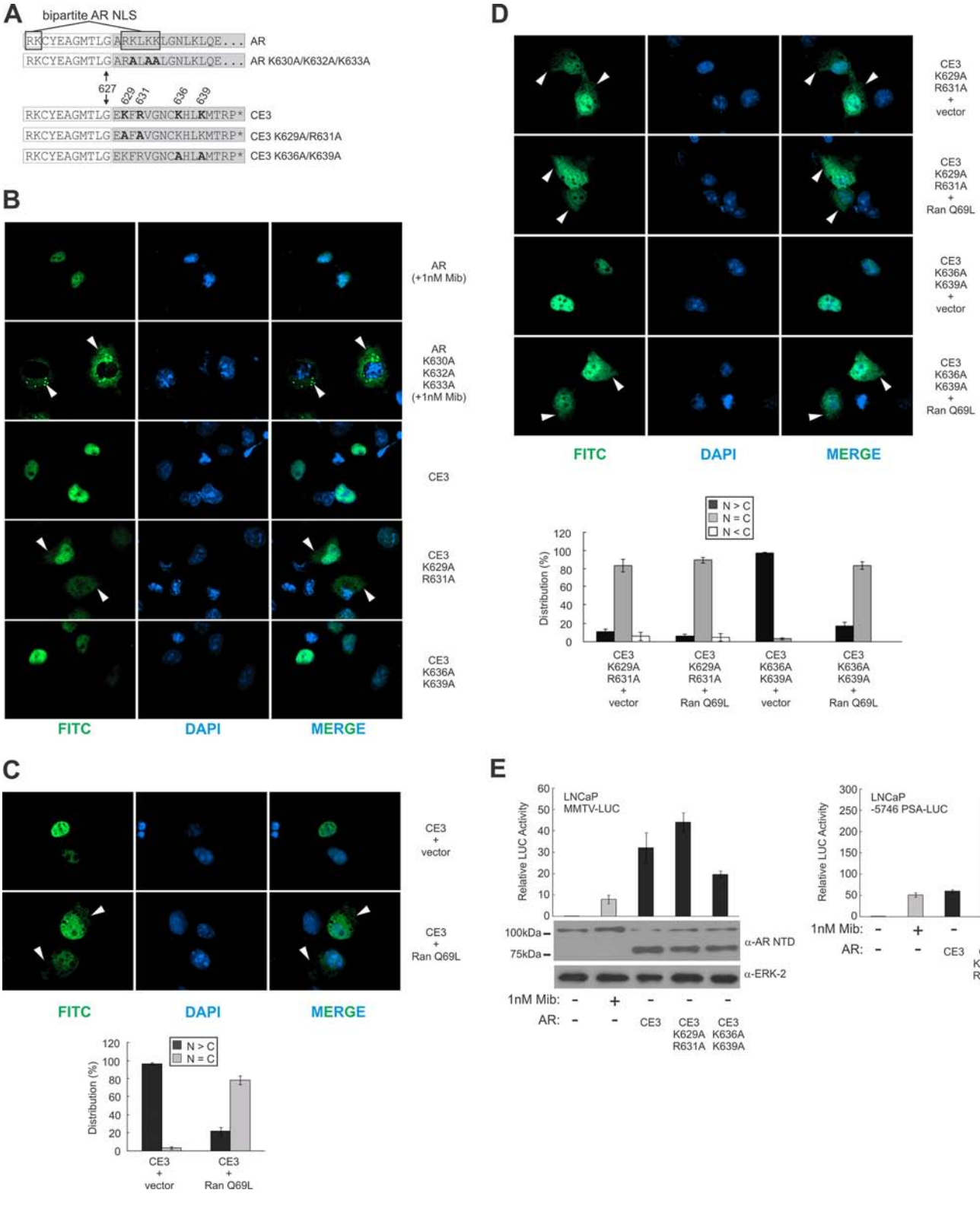
Ran Q69L were mediated through the Lys-629/Arg-631 motif (Fig. 3*D*). These data indicate that AR 1/2/3/CE3 displays enhanced nuclear localization because amino acid residues Lys-629 and Arg-631 reconstitute the second half of the bipartite AR NLS. Notably, alanine mutations in these residues did not have the same magnitude of effect as for full-length AR, indicating alternate modes of nuclear import existed for truncated AR variants. Indeed, the K629A/R631A mutant version of AR 1/2/3/CE3 displayed a paradoxical higher level of transcriptional activity on various AR-responsive promoters in AR-dependent LNCaP cells and AR-null DU145 cells, further confirming that the classical mode of AR nuclear import is not the

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main determinant of truncated AR variant activity (Fig. 3*E* and see supplemental Fig. 4).

To test this concept more rigorously, we asked whether increasing the basic amino acid content in the short COOH-terminal tail of the truncated AR 1/2/3/CE2 variant would enhance nuclear localization by reconstituting bipartite AR NLS function. Alignment of the COOH-terminal tails of AR 1/2/3/CE2 and AR 1/2/3/CE3 revealed that they both harbor arginine at position 631, but AR 1/2/3/CE2 harbors glycine at position 629 (Fig. 4*A*). As predicted, generation of a G629K mutant, which mimics the basic amino acid cluster in the AR 1/2/3/CE3 COOH-terminal tail, led to enhanced nuclear localization (Fig. 4*B*) that was reversed by dominant negative Ran Q69L (Fig. 4*C*). However, despite this enhanced nuclear localization, transcriptional activity on an AR-responsive promoter was unchanged (Fig. 4*D*).

These data indicate that truncated AR variants possess an intrinsic, basal level of nuclear localization that may be supported by a pathway(s) different from that required by full-length AR. This may be due to unmasking of additional regulatory signals or loss of the strong COOH-terminal AR nuclear export sequence by truncation of the AR LBD (25). Indeed, even nuclear localization of the AR v567es variant, which retains the AR exon 3/4 splice junction and wild-type AR NLS sequence, was only modestly affected by dominant negative Ran Q69L (see supplemental Fig. 5). We, therefore, queried whether activity of the Hsp90 chaperone complex, which is required upstream of importin-$\alpha/\beta$ for AR ligand binding and translocation to the nucleus (13), was required for nuclear access of truncated AR variants. A complete block in androgen-induced nuclear import of full-length AR was observed after treatment of cells with the Hsp90 inhibitor, 17-*N*-allylamino-17-demethoxygeldanamycin (Fig. 5*A*), but there was no effect of this inhibitor on constitutive nuclear localization of the truncated AR 1/2/3/CE3 and v567es variants (Fig. 5, *B* and *C*). These data thus confirm that the canonical pathways required for nuclear import of full-length AR are not required for nuclear import of truncated AR variants.

*AR NTD Encoded by Exon 1 and AR DBD Encoded by Exons 2 and 3 Are Sufficient for Nuclear Localization and Transcriptional Activity of Truncated AR Variants*—We next turned our attention to the role of the first basic amino acid cluster in the bipartite AR NLS, which is encoded by AR exon 3. Even though the AR NLS/importin-$\alpha$ crystal structure did not reveal a direct binding role for this part of the NLS (15), we postulated that this region may be able to compensate in the absence of the second, main cluster of basic amino acids. Therefore, we generated R617A/K618A compound mutations in full-length AR and truncated AR variants (Fig. 6*A*). Interestingly, this compound mutation did not inhibit ligand-dependent transcriptional activity or nuclear localization of full-length AR (Fig. 6, *B* and *C*), but this mutation impaired transcriptional activity of truncated AR variants (Fig. 6*D*). The R617A/K618A compound mutant version of the truncated AR 1/2/3/CE3 variant, but not the 1/2/3/CE2 variant, displayed impairment in nuclear access (Fig. 6*E* and see supplemental Fig. 6), indicating that this motif plays a minor role in truncated AR variant nuclear localization. More significantly, these data suggested that the intrinsic, basal level of nuclear localization displayed by truncated AR variants proceeds in a manner completely independent of either basic amino acid cluster in the bipartite AR NLS. To test this idea directly, we generated a R617A/K618A/K629A/R631A compound mutant of the truncated AR 1/2/3/CE3 variant to neutralize both basic amino acid clusters (Fig. 7*A*). This compound mutant version of the truncated AR 1/2/3/CE3 variant displayed roughly equal distribution in the nucleus and cytoplasm, demonstrating that basal nuclear localization of truncated AR variants could proceed independently from the canonical AR NLS (Fig. 7*B*). However, this compound mutant was transcriptionally inactive (Fig. 7, *C* and *D*), illustrating that these residues in the second $\alpha$-helix of the AR DBD are important for transcriptional function.

To probe the requirement for this first cluster of basic amino acids in more detail, we generated a synthetic truncated AR variant composed of only the exon 1-encoded NTD and exon 2/3-encoded DBD (AR amino acids 1–627) as well as a R617A/K618A compound mutant version of this synthetic AR variant (Fig. 7*A*). The synthetic AR 1–627 variant displayed approximately equal expression in the nucleus and cytoplasm (Fig. 7*B*) as well as strong ligand-independent transcriptional activity on AR-responsive promoters (Fig. 7, *C* and *D*). This finding confirms that COOH-terminal tail sequences encoded by exons spliced after exon 3 are not required for transcriptional function. However, the R617A/K618A compound mutant version of the synthetic AR 1–627 variant was transcriptionally inactive (Fig. 7, *C* and *D*). This compound mutant was still able to access the nucleus, but a pattern consistent with protein aggregation was also noted (Fig. 7*B*). These data further implicate a unique and important role for exon 3-encoded Arg-617 and Lys-618 in structural integrity of the AR DBD in the context of truncated AR variants but not full-length AR.

FIGURE 3. **The COOH-terminal tail encoded by AR exon CE3 reconstitutes the canonical AR NLS.** *A*, shown is a schematic of alanine substitutions in full-length AR and the truncated AR 1/2/3/CE3 variant. *B*, Cos-7 cells expressing parental and mutant plasmids illustrated in *A* were maintained in serum-free medium containing 1 nM mibolerone (*Mib*, synthetic androgen) or ethanol (vehicle control) before staining with an antibody specific for the AR NTD (FITC signal). Nuclei were stained with DAPI. Stained cells were visualized by confocal microscopy, and representative images are shown for three color channels (FITC, DAPI, and merged FITC/DAPI). Increased cytoplasmic localization is denoted by *white arrows*. *C*, Cos-7 cells were transfected with an AR 1/2/3/CE3 expression plasmid and dominant negative Ran Q69L. Cells were stained and subjected to confocal microscopy as in *A*. Increased cytoplasmic localization is denoted by *white arrows*. Quantification of predominantly nuclear ($N > C$) *versus* equal nuclear and cytoplasmic ($n = C$) expression is shown at the *bottom*. Bars represent the mean ± S.D. of 100 cells scored in three independent experiments. *D*, Cos-7 cells were transfected with mutant plasmids indicated in *A* along with dominant-negative RanQ69L (or vector control). Cells were stained and subjected to confocal microscopy as in *A*. Increased cytoplasmic localization is denoted by *white arrows*. Quantification of predominantly nuclear ($N > C$), equal nuclear and cytoplasmic ($n = C$) or predominantly cytoplasmic ($N < C$) expression is shown at the *bottom*. *Bars* represent mean ± S.D. of 100 cells scored in three independent experiments. *E*, LNCaP cells were transiently transfected with MMTV-LUC or −5746-LUC reporters and parental and mutant plasmid constructs depicted in *A*. Cell lysates were analyzed by luciferase assay and Western blot with antibodies specific for the AR NTD and ERK-2 (loading control). *Bars* represent the mean ± S.E. from at least three independent experiments, each performed in triplicate. Reporter activity in the absence of transactivator or ligand was arbitrarily set to 1.

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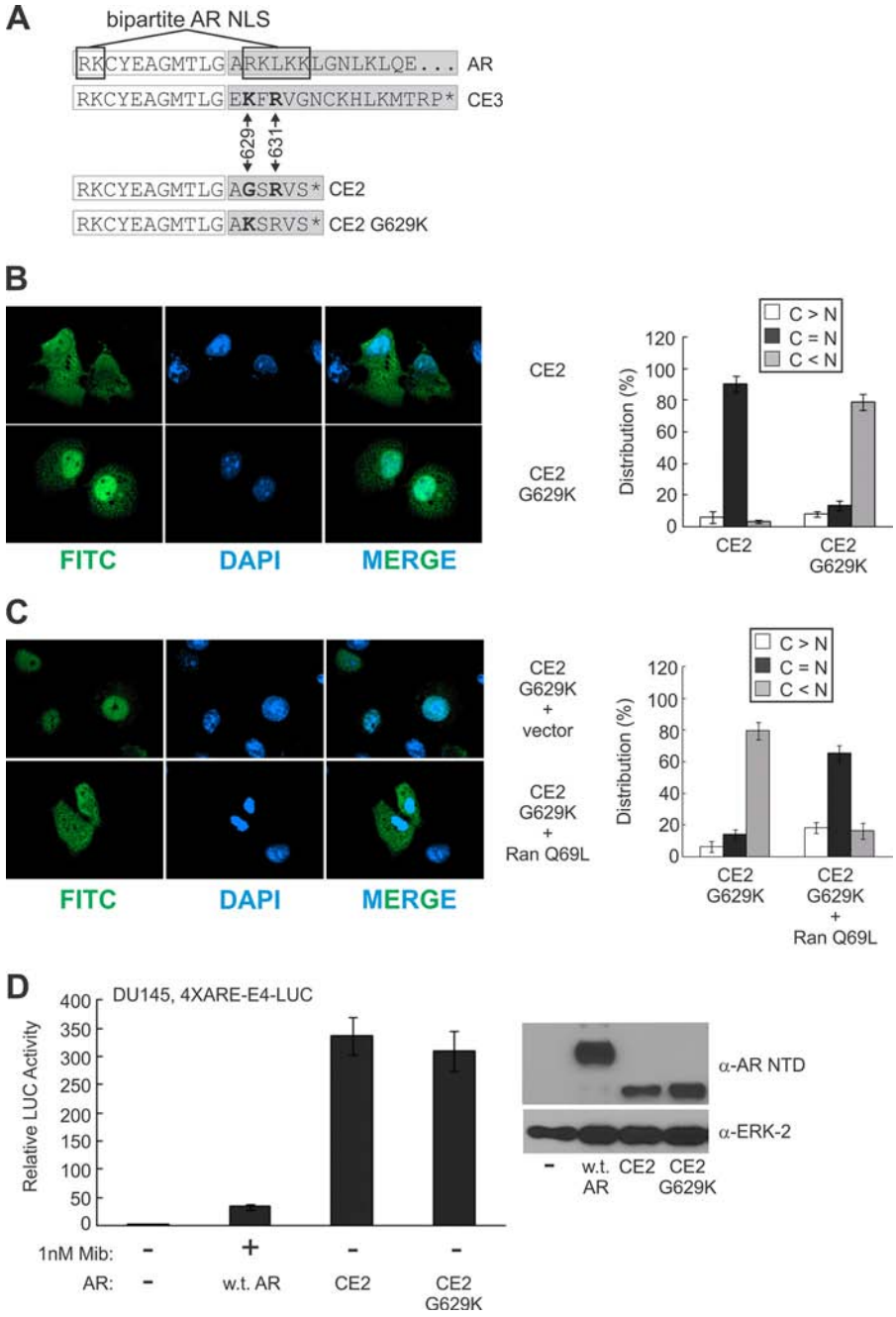


FIGURE 4. **NLS reconstitution does not enhance transcriptional activity of the truncated AR 1/2/3/CE2 variant.** *A*, shown is alignment of full-length AR, AR 1/2/3/CE3, and AR 1/2/3/CE2 and location of the G629K mutation. *B*, *left*, Cos-7 cells expressing 1/2/3/CE2 and the G629K mutant were maintained in serum-free medium and stained with an antibody specific for the AR NTD (FITC signal). Nuclei were stained with DAPI. Stained cells were visualized by confocal microscopy, and representative images are shown for three color channels (FITC, DAPI, and merged FITC/DAPI). *Right*, shown is quantification of predominantly nuclear ($N > C$), equally distributed nuclear and cytoplasmic ($n = C$), or predominantly cytoplasmic ($N < C$) expression. Bars represent mean ± S.D. of 100 cells scored in 3 independent experiments. *C*, *left*, Cos-7 cells were transfected as in *B* with the addition of dominant-negative RanQ69L (or vector control). *Right*, shown is quantification of predominantly nuclear ($N > C$), equally distributed nuclear and cytoplasmic ($n = C$), or predominantly cytoplasmic ($N < C$) expression. *Bars* represent mean ± S.D. of 100 cells scored in 3 independent experiments. *D*, *left*, DU145 cells were transfected with a 4XARE-E4-LUC reporter and parental/mutant plasmid constructs depicted in *A*. *Bars* represent mean ± S.E. from at least three independent experiments, each performed in triplicate. Reporter activity in the absence of transactivator or ligand was arbitrarily set to 1. *Right*, cell lysates were analyzed by luciferase assay and Western blot with antibodies specific for the AR NTD and ERK-2 (loading control).

*AR NTD/DBD Fragment Is Sufficient for Constitutive, Ligand-independent Transcriptional Activation of AR Target Genes*—To determine whether these biochemical principles applied to endogenous AR target genes, we generated lentivirus encoding GFP, AR 1/2/3/CE3, AR 1/2/3/CE3 R617A/K618A, AR 1/2/3/CE3 K629A/R631A, or the synthetic AR 1–627 variant (Fig. 8*A*). We chose to study transcriptional responses of PSA, hK2, TMPRSS2, and FKBP51 because promoters and enhancers of these genes are regulated by a variety of classical and selective androgen response elements (AREs) that may reflect differential requirements for AR COOH-terminal extensions (26). Infection of LNCaP cells with lentivirus encod-

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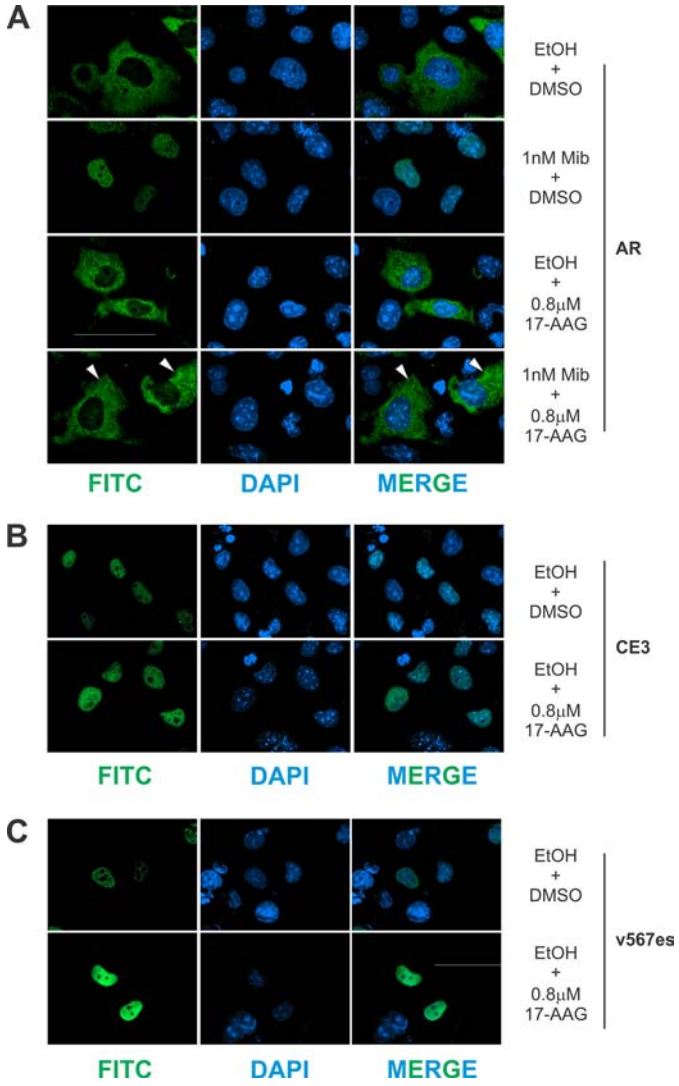


FIGURE 5. **Truncated AR variants do not require Hsp90 activity for nuclear localization.** Cos-7 cells expressing full-length AR (*A*), AR 1/2/3/CE3 (*B*), or AR v567es (*C*) were maintained in serum-free medium containing the indicated combinations of 1 nM mibolerone (*Mib*, synthetic androgen), ethanol (*EtOH*, vehicle control), 0.8 μM 17-*N*-allylamino-17-demethoxygeldanamycin (*17-AAG*, Hsp90 inhibitor), and dimetylsulfoxide (*DMSO*, vehicle control) before staining with an antibody specific for the AR NTD (FITC signal). Nuclei were stained with DAPI. Stained cells were visualized by confocal microscopy, and representative images are shown for three color channels (FITC, DAPI, and merged FITC/DAPI). *White arrowheads* are shown to highlight cytoplasmic expression caused by 17-*N*-allylamino-17-demethoxygeldanamycin.

ing the truncated AR 1/2/3/CE3 variant elicited androgen-independent transcriptional activation of these AR target genes (Fig. 8*B*). Inactivation of the reconstituted AR 1/2/3/CE3 NLS by K629A/R631A mutations or truncation after position 627 still resulted in robust, ligand-independent transcriptional activation of the same AR target genes (Fig. 8*B*). However, inactivation of the Arg-617/Lys-618 motif in the second α-helix of the AR DBD of AR 1/2/3/CE3 impaired transcriptional activation of PSA, hK2, and FKBP51 target genes. Noteworthy, the TMPRSS2 gene did not display impairment in androgen-independent expression levels in response to any of these mutations in the AR DBD or COOH-terminal tail (Fig. 8*B*). Together, these data illustrate a new paradigm wherein the AR NTD/DBD core encoded by exons 1, 2, and 3 are sufficient for a basal level of access to the nucleus, engagement with endogenous AR target genes, and efficient transcriptional activation of these target genes in an androgen-free environment. Because CRPCa is characterized by persistent AR transcriptional activity under conditions of ADT, these findings indicate that AR variants consisting of the AR NTD and DBD may contribute to disease progression regardless of the identity of their COOH-terminal extensions.

*AR 1/2/3/2b Protein Is Expressed in 22Rv1 Cells and Can Support Androgen-independent Growth of Prostate Cancer Cells*—The CRPCa 22Rv1 cell line displays altered AR splicing patterns as a result of a 35-kb intragenic AR tandem duplication encompassing AR exon 3 (27, 28). We and others have demonstrated that 22Rv1 cells express two separate mRNAs harboring cryptic exon 2b composed of contiguously spliced AR exons 1/2/2b or AR exons 1/2/3/2b. The AR 1/2/2b variant is different from other truncated AR variants that have been reported because it encodes the NTD and first zinc finger of the AR DBD (see supplemental Fig. 7*A*). Because there is concern that many of the variant AR mRNAs that have been identified in 22Rv1 and other models may simply be spurious splicing byproducts that are degraded by nonsense-mediated RNA decay (29), we asked whether these protein species are expressed in 22Rv1 cells. To test for AR 1/2/2b expression, we first employed siRNA specific for AR exon 3, which would not target the AR 1/2/2b isoform. Transfection of 22Rv1 cells with this siRNA led to complete ablation of all truncated AR variant protein expression, indicating that AR 1/2/2b is not a protein constituent in these cells (see supplemental Fig. 7*B*). Next, we developed polyclonal antibodies specific for the COOH-terminal extension encoded by AR exon 2b. Crude antisera as well as antibodies purified on an immobilized peptide affinity column were able to immunoprecipitate an ~80-kDa AR polypeptide from 22Rv1 lysates (see supplemental Fig. 7*C*). Moreover, affinity-purified exon 2b polyclonal antibodies recognized a truncated AR variant species in Western blot experiments that was the same size as ectopic AR 1/2/3/2b protein (see supplemental Fig. 7*D*). Therefore, we conclude that AR 1/2/3/2b is expressed as a mature protein species in 22Rv1 cells.

The predominantly nuclear AR 1/2/3/CE3 variant (referred to as AR-V7 or AR3) is also expressed as a mature protein in 22Rv1 cells and can induce androgen-independent growth when expressed in androgen-dependent LNCaP cells (7, 8). In subcellular localization assays, AR 1/2/3/2b protein displayed a mixed cytoplasmic/nuclear localization pattern (Fig. 1, *B* and *C*). Therefore, we chose to employ the AR 1/2/3/2b variant to determine whether truncated AR species with partial nuclear localization could also support androgen-independent growth of PCa cells. We infected LNCaP cells with lentivirus encoding AR 1/2/3/2b, AR 1/2/3/CE3, or AR v567es and found that each of these truncated AR variants could induce an androgen-independent growth phenotype (Fig. 9*A*). These data confirm that classical NLS activity is independent from truncated AR variant function in PCa cells. Intriguingly, during the course of these studies we noted variability in the magnitude of androgen-independent growth that each AR variant induced in LNCaP cells. Paradoxically, we noted that higher levels of truncated AR variant expression consistently resulted in lower levels of

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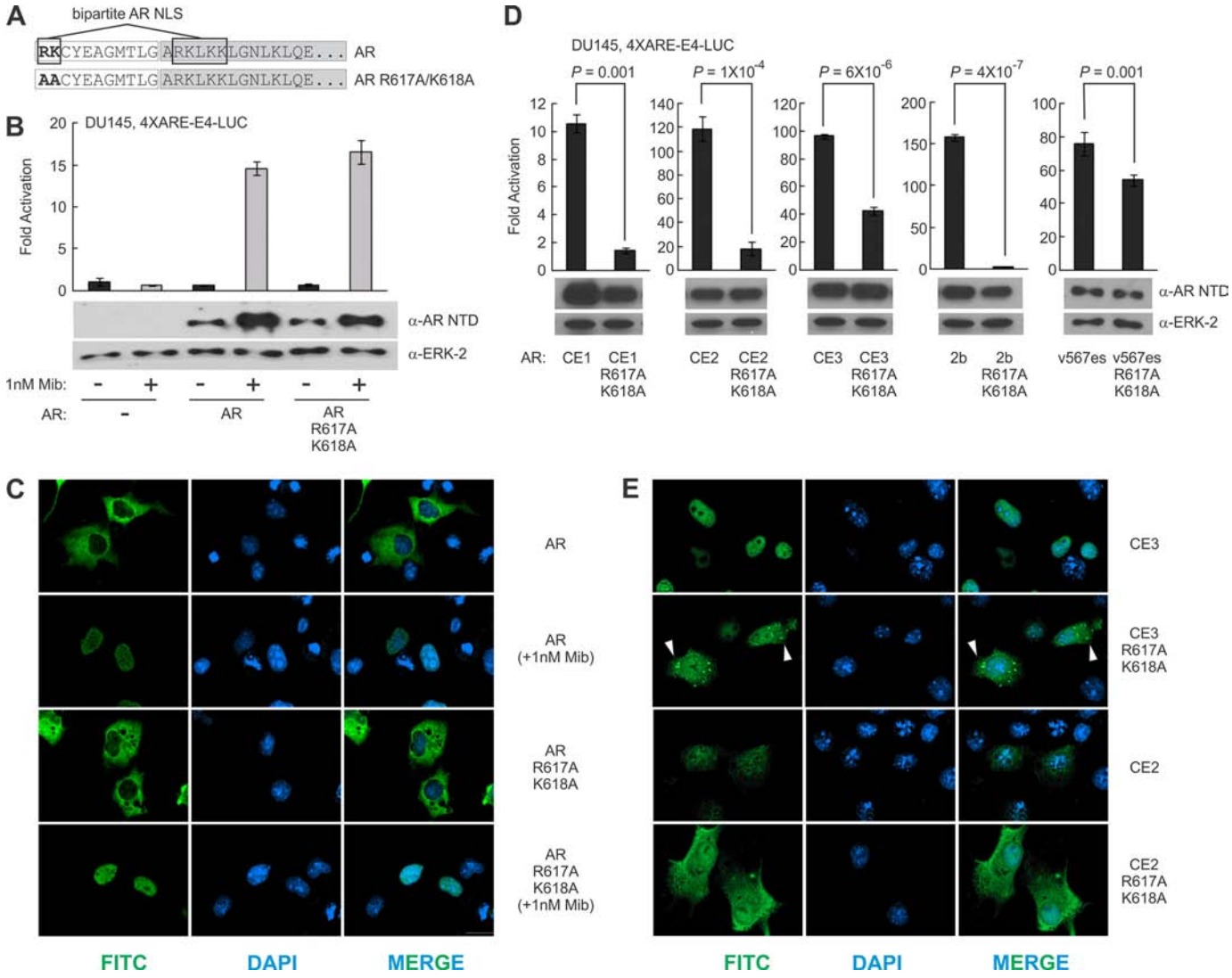


FIGURE 6. **A unique requirement for the Arg-617/Lys-618 motif in the AR DBD terminal helix.** *A*, shown is a schematic of the AR hinge region and location of the Arg-617/Lys-618 motif. *B*, DU145 cells were transiently transfected with 4XARE-E4-LUC and full-length AR or a R617A/K618A mutant. Cells were cultured in serum-free medium containing 1 nM mibolerone (*Mib*) or vehicle control (ethanol). Cell lysates were analyzed by luciferase assay and Western blot with antibodies specific for the AR NTD and ERK-2 (loading control). *Bars* represent the mean ± S.E. from at least three independent experiments, each performed in triplicate. Reporter activity in the absence of transactivator or ligand was arbitrarily set to 1. *p* values were derived using *t* tests. *C*, Cos-7 cells expressing parental or mutant plasmids illustrated in *A* were maintained in serum-free medium containing 1 nM mibolerone (*Mib*, synthetic androgen) or ethanol (vehicle control) before staining with an antibody specific for the AR NTD (FITC signal). Nuclei were stained with DAPI. Stained cells were visualized by confocal microscopy, and representative images are shown for three color channels (FITC, DAPI, and merged FITC/DAPI). Cytoplasmic localization resulting from the R617A/K618A mutation is denoted by *white arrows*. *D*, DU145 cells were transiently transfected with 4XARE-E4-LUC and parental/mutant truncated AR variants exactly as in *B*. *E*, Cos-7 cells were transfected with parental/mutant truncated AR variants, stained, and imaged exactly as in *C*. Increased cytoplasmic localization resulting from the CE3 R617A/K618A mutation is denoted by *white arrows*.

androgen-independent growth. This observation is reminiscent of the biphasic growth response of LNCaP cells wherein androgen concentrations in the 0.1–1 nM range induce proliferation, but higher doses are growth-suppressive despite robust induction of AR target genes (30–32). Indeed, LNCaP cells transduced with lentivirus encoding AR 1/2/3/CE3 displayed a dose-dependent increase in expression of PSA and TMPRSS2 but were growth-suppressed at a high expression level (Fig. 9, *B* and *C*). This growth suppression was also observed when lentivirus-infected LNCaP cells were assayed the presence of androgens (see supplemental Fig. 8*A*). Moreover, under these conditions the AR 1/2/3/CE3 variant appeared to inhibit dihydrotestosterone-induced AR transcriptional activity perhaps via competition for ARE binding or feedback inhibition of full-length AR expression (33) (see supplemental Fig. 8*B*). In similar dose-response experiments with AR 1/2/3/2b or AR v567es, androgen-independent growth of LNCaP cells was apparent at lower expression levels, but higher levels of expression blocked this effect (see supplemental Fig. 8, *C* and *D*). Together, these data indicate that truncated AR variants can support androgen-independent PCa cell growth regardless of nuclear localization efficiency. To test this we performed AR knockdown/re-expression experiments in the 22Rv1 cell line (Fig. 9, *D* and *E*). Knocking-down AR expression using an exon 1-targeted siRNA inhibited androgen-independent growth, which was fully restored by lentiviral re-expression of siRNA-resistant AR 1/2/3/CE3 (Fig. 9, *D* and *E*). The nuclear/cytoplasmic AR 1/2/3/CE3 K629A/R631A mutant was also able to effectively

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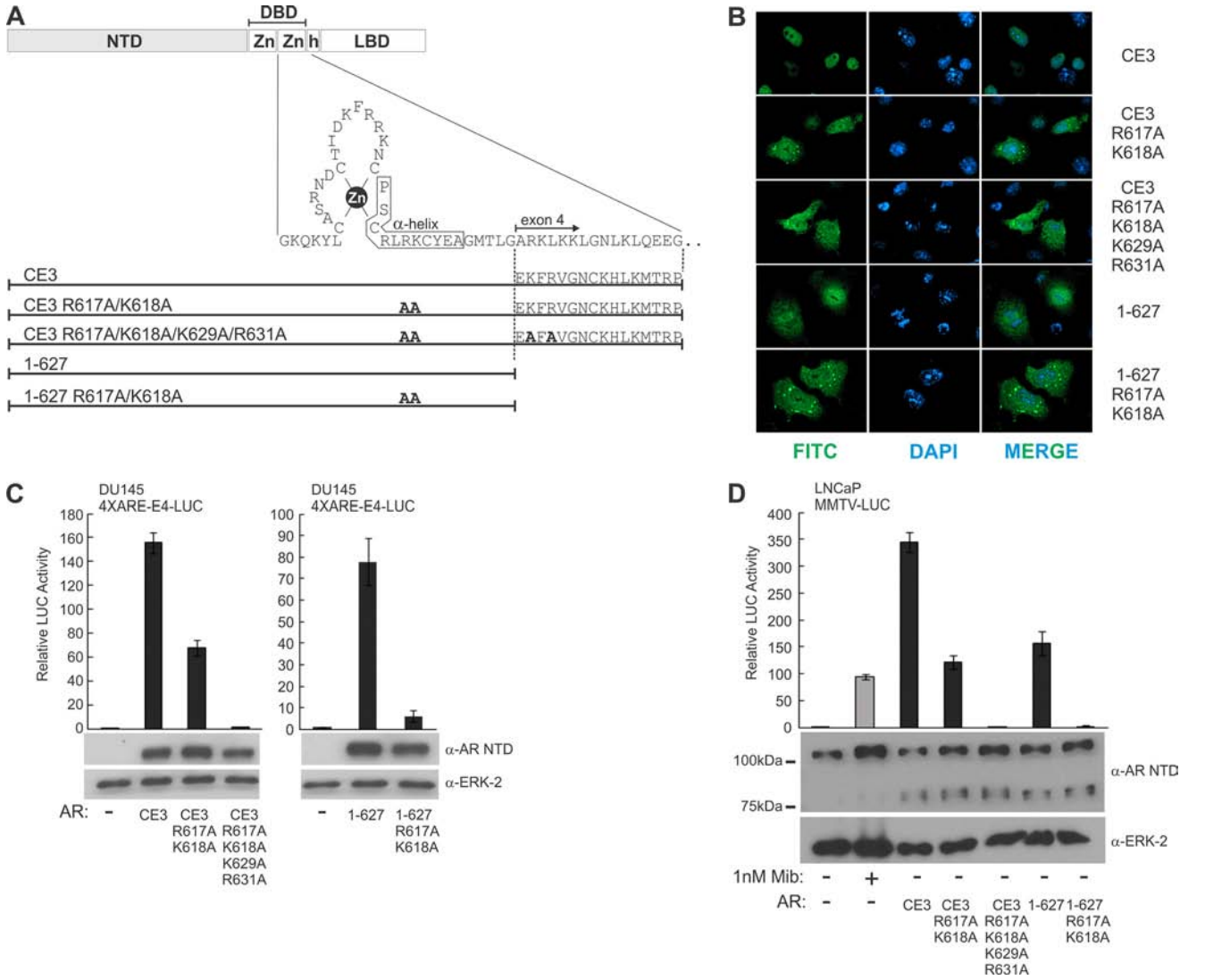


FIGURE 7. **The AR NTD/DBD core is sufficient for nuclear localization and transcriptional activity of truncated AR variants.** *A*, shown is a schematic of COOH-terminal deletions and alanine substitution mutations relative to key structural motifs in the second zinc finger of the AR DBD and hinge region. Zinc finger and α-helix locations were adapted from a prior depiction of the AR DBD crystal structure (46). *B*, Cos-7 cells expressing constructs depicted in *A* were maintained in serum-free medium and stained with an antibody specific for the AR NTD (FITC signal). Nuclei were stained with DAPI. Stained cells were visualized by confocal microscopy, and representative images are shown for three color channels (FITC, DAPI, and merged FITC/DAPI). *C*, DU145 cells were transiently transfected with 4XARE-E4-LUC and constructs depicted in *A*. Cell lysates were analyzed by luciferase assay and Western blot with antibodies specific for the AR NTD and ERK-2 (loading control). *Bars* represent mean ± S.E. from at least three independent experiments, each performed in triplicate. *D*, LNCaP cells were transiently transfected with MMTV-LUC and constructs depicted in *A*. Luciferase activity and transgene expression were assessed exactly as described in *C*. *Mib*, mibolerone.

restore androgen-independent growth, but the AR 1/2/3/CE3 R617A/K618A mutant displayed impairment (Fig. 9, *D* and *E*), which is consistent with the effects of these mutations on truncated AR variant transcriptional activity (Fig. 8, *A* and *B*).

In summary, our data demonstrate that a subset of truncated AR variants display enhanced nuclear localization due to reconstitution of classical NLS activity. However, a high, basal level of nuclear localization is mediated by the AR NTD/DBD core encoded by AR exons 1, 2, and 3. This basal level of nuclear localization is sufficient for strong, androgen-independent transcriptional activation of AR target genes and induction of androgen-independent growth. Therefore, truncated AR variants with diverse COOH-terminal extensions can contribute to CRPCa pathology by driving persistent AR activity during ADT.

## DISCUSSION

Steroid receptors have extensive similarities in DBD and LBD structure, and a wealth of mechanistic information exists for steps leading to transcriptional activation. A key requirement for transcriptional activity of steroid receptors is interaction of unliganded receptors with the Hsp90 chaperone machinery. This interaction occurs exclusively through the LBD via direct binding to Hsp40 and Hsp70 (13, 34, 35). The Hsp90 chaperone complex association maintains AR in a conformation that inhibits DNA binding (36) and has high affinity for androgens (37). Chaperone release from steroid receptors is ligand-dependent, which results in a conformational change that exposes the bipartite AR NLS (RKCYEAGMTLGARKLKK) in the flexible hinge region between the AR DBD and

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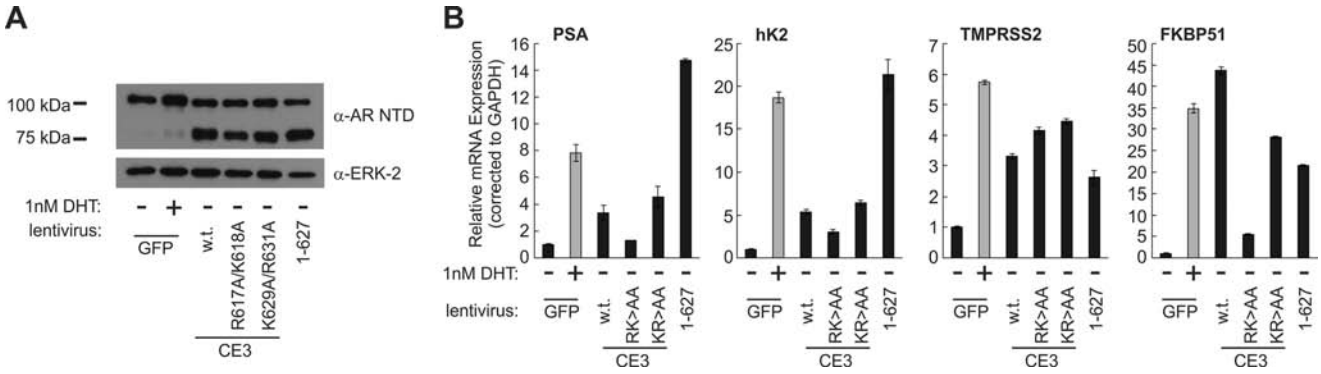


FIGURE 8. **The AR NTD/DBD core is sufficient for transcriptional activation of endogenous AR target genes.** *A*, LNCaP cells were transduced with lentivirus encoding GFP, parental and mutant versions of AR 1/2/3/CE3, or AR 1–627. Transduced cells were maintained under serum-free conditions and stimulated with 1 nM dihydrotestosterone (*DHT*) or vehicle control (ethanol) for 24 h before Western blot analysis with antibodies specific for the AR NTD or ERK-2 (loading control). *B*, LNCaP cells were transduced exactly as in *A*, and RNA was subjected to quantitative RT-PCR using primer sets specific for GAPDH, PSA, hK2, TMPRSS2, and FKBP51. Expression is shown relative to GAPDH as determined using the formula $2^{-\Delta\Delta Ct}$. *Bars* represent the mean ± S.D. from a triplicate experiment representative of three biological replicates.

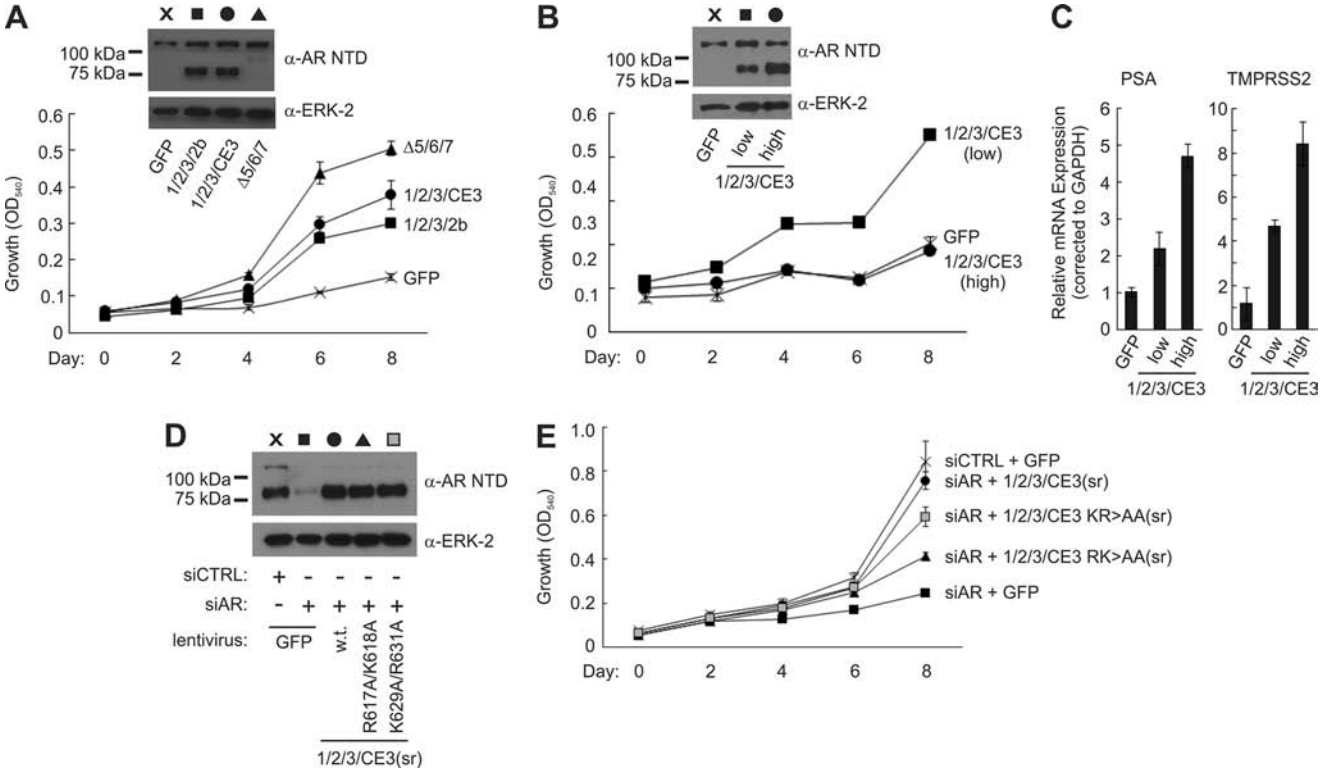


FIGURE 9. **Truncated AR variants support biphasic, androgen-independent growth of PCa cells.** *A*, LNCaP cells transduced with lentivirus encoding AR 1/2/3/2b, AR 1/2/3/CE3, or AR v567es were maintained in medium containing 10% charcoal-stripped (steroid-depleted) serum (10% charcoal-stripped serum). *Inset*, transduced cell lysates were analyzed by Western blot with antibodies specific for the AR NTD or ERK-2 (loading control). *Graph*, transduced cells were seeded on 24-well plates in medium containing 10% charcoal-stripped serum. At the indicated time points, cells were fixed and stained with crystal violet. Intensity of crystal violet staining ($A_{540}$) was used as a surrogate of cell number. Data represent the mean ± S.D. from a quadruplicate experiment representative of three biological replicates. *B*, LNCaP cells were transduced with increasing doses of lentivirus encoding AR 1/2/3/CE3 and subjected to Western blot (*inset*) and growth assay (*graph*) exactly as in *A*. *C*, LNCaP cells transduced as in *B* were subjected to quantitative RT-PCR using primer sets specific for GAPDH, PSA, and TMPRSS2. Expression is shown relative to GAPDH as determined using the formula $2^{-\Delta\Delta Ct}$. *Bars* represent the mean ± S.D. from a triplicate experiment representative of three biological replicates. *D*, 22Rv1 cells were transduced with lentivirus encoding GFP or siRNA-resistant forms (denoted *sr* (17)) of AR 1/2/3/CE3, AR 1/2/3/CE3 R617A/K618A, or AR 1/2/3/CE3 K629A/R631A and electroporated with siRNA targeted to AR exon 1 as indicated. Transduced/electroporated cells were subjected to Western blot with antibodies specific for the AR NTD or ERK-2 (loading control). *E*, 22Rv1 cells transduced/electroporated as in *D* were seeded on 24-well plates in medium containing 10% charcoal-stripped serum. At the indicated time points, cells were fixed and stained with crystal violet. Intensity of crystal violet staining ($A_{540}$) was used as a surrogate of cell number. Data represent the mean ± S.D. from a quadruplicate experiment representative of two biological replicates.

LBD. The crystal structure of the AR NLS peptide in complex with importin-α has revealed that the second cluster of basic amino acids (RKLKK), which is encoded by AR exon 4, engages with the major NLS binding site in importin-α (15). Interestingly, data presented here demonstrate that truncated AR variants display a constitutive, basal level of nuclear localization sufficient for ligand-independent transcriptional activity regardless of whether they harbor the exon 4-encoded NLS or NLS-like COOH-terminal extensions. Moreover, we have demonstrated that truncated AR variants access the nucleus inde-

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pendently of the Hsp90 chaperone complex. Therefore, the signals that are critical for the regulatory cycle of the full-length AR appear to be dispensable for truncated AR variants.

It is well established that removal of the AR LBD results in ligand-independent AR transcriptional activity mediated by the AR NTD, but these early studies involved the use of constructs with deletions after the hinge region to retain the complete AR NLS (22, 38). The straightforward idea that NLS-mediated nuclear localization and transcriptional activity are intertwined has also led to controversy over the significance of many truncated AR variants that have been identified to date. For example, it has been postulated that truncated AR variants retaining the exon 4-encoded AR NLS would be functional transcription factors, whereas others would be transcriptionally incompetent (9, 10). This concept was supported by the demonstration that individual truncated AR variants display varying degrees of transcriptional activity (7) and an apparent correlation between the extent of nuclear localization and transcriptional activation (10). However, the current study demonstrates that transcriptional strength of individual truncated AR variants is a promoter-dependent phenomenon and independent of the magnitude of subcellular localization. Indeed, point mutations that inhibited the constitutive nuclear localization of the AR 1/2/3/CE3 isoform did not inhibit transcriptional activity. Similarly, point mutations that enhanced the nuclear localization of the AR 1/2/3/CE2 variant did not increase transcriptional activity. Even a synthetic truncated AR variant (1–627) that completely lacked the importin-$\alpha$ binding site (15) was transcriptionally active in a ligand-independent manner. Because these mechanistic principles were independent of promoter or cell line and were also extended to clinically relevant endogenous AR target genes, we conclude that constitutive transcriptional activity is likely an inherent property of truncated AR variants retaining the NTD/DBD core encoded by AR exons 1, 2, and 3. Our data also demonstrate that truncated AR variants with this NTD/DBD core can support androgen-independent growth when expressed in PCa cells. However, we have also shown that individual truncated AR variants need to be evaluated for growth-promoting effects very cautiously, especially in LNCaP cells, because the constitutive, androgen-independent transcriptional activity appears to induce a biphasic growth response in a fashion similar to high-dose androgen treatment (30–32). The molecular basis for the biphasic androgen effect is largely unknown but may arise from AR-mediated activation of differentiation-promoting genes or AR-mediated transcriptional inhibition of the AR promoter at higher androgen concentrations (33).

If the canonical nuclear localization signal is dispensable for truncated AR variant function, then what are the mechanisms that promote their entry into the nucleus? A previous live cell imaging study with fluorescence-labeled AR fragments demonstrated that the AR NTD has strong nuclear import activity in isolation (39). Moreover, deletion of the RKLKK motif in full-length AR has been shown to impair nuclear localization but induces a paradoxical superactivity in response to androgens (40), which may be due to enhanced intranuclear mobility (41). However, studies where the AR NLS has been manipulated by mutation and/or deletion have historically been difficult to interpret because lysine residues in the AR NLS are also acetylation targets (42, 43). The fact that the AR 1–627 fragment employed in this study could access the nucleus and activate endogenous AR target genes with high efficiency clearly confirms that functional nuclear targeting signals exist outside of the AR hinge/LBD region. Based on these findings, we conclude that the canonical NLS in the AR hinge region is not the only determinant of AR nuclear access and transcriptional activity in PCa cells.

Intriguingly, a previous study demonstrated that androgen-independent growth induced by truncated AR variants required full-length AR (10). One explanation for this phenomenon could be a physical interaction between full-length AR and truncated AR variants, which is supported by *in vitro* experiments with overexpressed full-length AR and AR v567es (9). However, we have recently demonstrated that the LuCaP 86.2 xenograft, where the AR v567es variant was discovered, harbors a 8.5-kb deletion of AR exons 5, 6, and 7 in the sole AR gene copy at Xq11–12 (44). This would preclude a scenario where full-length AR and AR v567es could co-exist in the same cell in this model. Similarly, in this and other studies we have demonstrated that knockdown of full-length AR in the 22Rv1 and CWR-R1 models of CRPCa has no effect on androgen-independent expression of AR target genes or androgen-independent growth. Conversely, knock-down of endogenously expressed truncated AR variants inhibits both of these parameters (28, 44). These data strongly support the concept that truncated AR variants possess the biochemical properties required to independently support ongoing PCa cell growth during ADT. Indeed, PCa cells with AR intragenic rearrangements and altered splicing patterns have a competitive growth advantage under castrate conditions (28, 44). The finding that intragenic copy number imbalances are frequent in CRPCa (44) portends complex and diverse AR splicing patterns in human tumors, which is also supported by unbiased tiling array studies (27). Antibody development efforts in this and previous studies show that rearrangement-dependent splicing alterations do indeed give rise to translated, functional proteins such as AR 1/2/3/2b and AR 1/2/3/CE3 (7, 8). Our data establish new biochemical principles that will be critical for studying the role of known as well as yet-to-be-discovered truncated AR variant species in PCa progression.

Our data also highlight the potential application of targeting truncated AR variants for therapy of CRPCa. For example, inhibitors such as EPI-001, a bisphenol A-derivative that appears to interfere with the function of transcriptional activation domains in the AR NTD (45), would be anticipated to block transcriptional activity and growth-supporting functions of truncated AR variants. Our data also show that truncated AR variants have a unique requirement for structural integrity of the second $\alpha$-helix in the AR DBD, which might be an additional focal point for inhibitory strategies. We have further shown that higher levels of overexpression of the truncated AR 1/2/3/CE3 variant in LNCaP cells leads to paradoxical lower levels of cell growth, especially under conditions where androgens are present. Androgen-induced expression of AR target genes was also reduced after expression of the truncated AR 1/2/3/CE3 variant. This indicates that targeted inhibition of

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truncated AR variants may be most effective when activity of full-length AR is durably suppressed. However, it is also possible that these results may reflect competition between ligand-bound AR and truncated AR variants for ARE binding and/or an acute sensitivity of LNCaP cells to excessive levels of AR signaling. Further investigation in additional models is required to fully understand the potential application and optimal conditions for targeting truncated AR variants in CRPCa.

In summary, our data demonstrate that truncated AR variants with the NTD/DBD core are constitutively active, ligand-independent transcription factors that can support androgen-independent growth of PCa cells. Expression of truncated AR variants is frequent in CRPCa metastases and associated with poor prognosis (11). Therefore, AR variant species with the NTD/DBD core should be viewed as key therapeutic targets at this stage of the disease.

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Cancer Research

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*Priority Report*

Cancer Research

# Androgen Receptor Splice Variants Mediate Enzalutamide Resistance in Castration-Resistant Prostate Cancer Cell Lines

Yingming Li[3], Siu Chiu Chan[3], Lucas J. Brand[1,3], Tae Hyun Hwang[2,3], Kevin A.T. Silverstein[4], and Scott M. Dehm[3,5]

## Abstract

Persistent androgen receptor (AR) transcriptional activity underlies resistance to AR-targeted therapy and progression to lethal castration-resistant prostate cancer (CRPC). Recent success in retargeting persistent AR activity with next generation androgen/AR axis inhibitors such as enzalutamide (MDV3100) has validated AR as a master regulator during all stages of disease progression. However, resistance to next generation AR inhibitors limits therapeutic efficacy for many patients. One emerging mechanism of CRPC progression is AR gene rearrangement, promoting synthesis of constitutively active truncated AR splice variants (AR-V) that lack the AR ligand-binding domain. In this study, we show that cells with AR gene rearrangements expressing both full-length and AR-Vs are androgen independent and enzalutamide resistant. However, selective knock-down of AR-V expression inhibited androgen-independent growth and restored responsiveness to androgens and antiandrogens. In heterogeneous cell populations, AR gene rearrangements marked individual AR-V–dependent cells that were resistant to enzalutamide. Gene expression profiling following knock-down of full-length AR or AR-Vs showed that AR-Vs drive resistance to AR-targeted therapy by functioning as constitutive and independent effectors of the androgen/AR transcriptional program. Further, mitotic genes deemed previously to be unique AR-V targets were found to be biphasic targets associated with a proliferative level of signaling output from either AR-Vs or androgen-stimulated AR. Overall, these studies highlight AR-Vs as key mediators of persistent AR signaling and resistance to the current arsenal of conventional and next generation AR-directed therapies, advancing the concept of AR-Vs as therapeutic targets in advanced disease. *Cancer Res; 73(2); 1–7. ©2012 AACR.*

## Introduction

Androgen depletion therapy (ADT) achieves clinical regression or disease stabilization for men with advanced prostate cancer (PCa). However, castration-resistant or -recurrent prostate cancer (CRPC) invariably develops due to aberrant reactivation of the androgen/androgen receptor (AR) signaling axis (1). This has been confirmed clinically through the recent retargeting of persistent androgen/AR activity in CRPC patients with next generation AR-axis inhibitors such as the potent antiandrogen enzalutamide (2, 3). Despite an increase in overall survival, *de novo* resistance to enzalutamide precludes responses for many patients, and resistance can develop rapidly in initial responders (4). However, mechanisms that could mediate resistance to enzalutamide are poorly understood.

Altered AR mRNA splicing and synthesis of COOH-terminally truncated AR variant (AR-V) proteins lacking the AR ligand-binding domain (LBD) is one mechanism that has been postulated to drive an overall resistance to conventional and next generation ADT (5). Indeed, the transcriptionally active $NH_2$-terminal domain (NTD) and central DNA binding domain are sufficient for AR-Vs to function as ligand-independent transcription factors (6). Moreover, AR-Vs are frequently expressed in CRPC metastases (7, 8), and high mRNA and/or protein expression levels in PCa tissues predict disease progression and shorter survival (7, 9, 10). However, despite being able to induce a CRPC growth phenotype, ectopically expressed AR-Vs were shown to remain dependent on activity of endogenous full-length AR (11). Therefore, it has been concluded that constitutive AR-V activity in CRPC tissues could be overcome by targeting full-length AR with antiandrogens.

Recent studies showing AR gene rearrangements in CRPC that underlie functional AR-V expression and activity have revealed scenarios where full-length AR activity may not be required in CRPC cells (12, 13). Foremost, the LuCaP 86.2

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xenograft derived from CRPC bladder metastasis harbors an 8.5-kb intragenic deletion of AR exons 5 to 7, which prevents full-length AR synthesis but favors expression of a truncated AR-V species encoded by mRNA lacking exons 5 to 7 (12). The 22Rv1 and CWR-R1 models of CRPC also harbor underlying AR gene rearrangements, leading to coexpression of full-length AR and AR-Vs (12, 13). The effects of targeting full-length AR on AR-V function have not been evaluated in these rearrangement-driven models. Therefore, the purpose of this study was to test the roles of full-length and AR-V species in supporting the CRPC phenotype and mediating responsiveness to enzalutamide in the context of rearrangement-driven changes in AR splicing.

## Materials and Methods

### Cell culture

The 22Rv1 (#CRL-2505) and LNCaP (#CRL-1740) cell lines were obtained from the American Type Culture Collection (ATCC) and cultured according to the ATCC protocol. The ATCC ensures authenticity of these human cell lines using short tandem repeat (STR) analyses. All experiments with these cells were conducted within 4 months of resuscitation of frozen cell stocks prepared within 3 passages of receipt from the ATCC. CWR-R1 cells (14) were a kind gift from Dr. Elizabeth Wilson (University of North Carolina at Chapel Hill, Chapel Hill, NC) and cultured in RPMI-1640 with 10% FBS. Authentication of the CWR-R1 cell line was conducted by sequence-based validation of 2 signature AR gene alterations: an AR H874Y point mutation and a 50-kb intragenic deletion within AR intron 1 (13). Sequence-based authentication of CWR-R1 was carried out every 5 to 10 passages, and cells were kept in culture no longer than 3 months after authentication unless otherwise indicated. Details of cell treatment with bicalutamide (AstraZeneca) or enzalutamide (a kind gift from Dr. Michael Jung, University of California, Los Angeles, Los Angeles, CA) are provided in the Supplementary Material.

### Transient transfections

Cells were electroporated with siRNAs targeted to AR Exon 7 (15), AR Exon 1 (15), AR Exon 2b (15), AR Exon CE3 (10), or a mouse mammary tumor virus-luciferase (MMTV-LUC) reporter as described previously (15). Growth of electroporated cells was monitored by crystal violet staining as described earlier (12). Luciferase activity was measured as previously described (15).

### Lentiviral infections

LNCaP cells were infected with increasing titers of lentivirus encoding AR 1/2/3/CE3 and AR Δ5/6/7 as described previously (6). Infected cells were maintained in RPMI1640 with 10% charcoal-stripped, steroid-depleted serum (CSS) for 48 hours and then switched to serum-free medium for 24 hours before lysis.

### Western blot

Western blotting with AR NTD (N-20; Santa Cruz Biotechnology), AR CTD (C-19; Santa Cruz Biotechnology), and ERK-2 (D-2; Santa Cruz Biotechnology) antibodies was carried out as described previously (12).

### Quantitative reverse transcription-PCR

Total RNA was extracted from 22Rv1, CWR-R1, and LNCaP cells as described previously (16). Primers and quantitative reverse transcription PCR (qRT-PCR) conditions for assessment of prostate specific antigen (PSA), hK2, and TMPRSS2 mRNA expression have been described previously (15). Androgen- and AR variant-responses of M–phase-specific genes were assessed using specific primers with sequences provided in Supplementary Material. Fold change in mRNA expression levels was calculated by the comparative $C_t$ method, using the formula $2^{-(\Delta\Delta Ct)}$ and GAPDH as calibrator, as described previously (15).

### Genomic PCR

Primers and PCR conditions for deletion-spanning PCR of the AR intron 1 deletion in CWR-R1 cells have been described previously (13).

### Gene expression analysis with Illumina Beadchips

CWR-R1 cells that had been maintained in long-term culture in RPMI1640 with 10% CSS were used for global gene expression profiling. These CWR-R1 cells were electroporated with siRNAs targeting AR exon 1, 7, or CE3, and seeded in RPMI with 10% CSS. Following 48-hour recovery, cells were switched to serum-free RPMI1640 and treated for 24 hours with 1 nmol/L dihydrotestosterone (DHT) or 0.01% (v/v) vehicle control (ethanol). Details of RNA isolation, Illumina Beadchip hybridization, and data analysis are provided as Supplementary Material. Data are available through NCBI's Gene Expression Omnibus (GSE41784).

## Results and Discussion

### AR-Vs are sufficient for resistance to enzalutamide in 22Rv1 cells

The CRPC 22Rv1 cell line is characterized by a 35-kb tandem duplication encompassing AR exon 3 (12). This rearrangement is associated with enhanced mRNA and protein expression of truncated AR-Vs AR 1/2/3/2b and AR 1/2/3/CE3 (also referred to as AR-V7/AR3; refs. 6, 9, 10, 12). The 22Rv1 cells display robust growth under castrated conditions, which was unaffected by the antiandrogens bicalutamide or enzalutamide (Fig. 1A). However, both bicalutamide and enzalutamide were able to antagonize androgen-mediated activation of the AR target genes PSA and hK2 (Supplementary Fig. S1A) as well as an AR-responsive MMTV-LUC reporter (Fig. 1B), showing that these drugs can achieve on-target inhibition of full-length AR in these cells. Similarly, androgen-induced MMTV activity was blocked following selective knockdown of full-length AR (Fig. 1B). However, knockdown of AR-Vs resulted in robust inhibition of constitutive, androgen-independent MMTV-LUC activity. Similarly, constitutive, androgen-independent expression of PSA and hK2 was blocked by AR-V knockdown, but not by manipulations that block full-length AR (bicalutamide, enzalutamide, full-length AR knockdown, or AR knockdown combined with antiandrogens, Fig. 1C). Interestingly, constitutive AR-V activity appeared to maintain TMPRSS2 expression at a maximal AR-inducible level, as there was no response to



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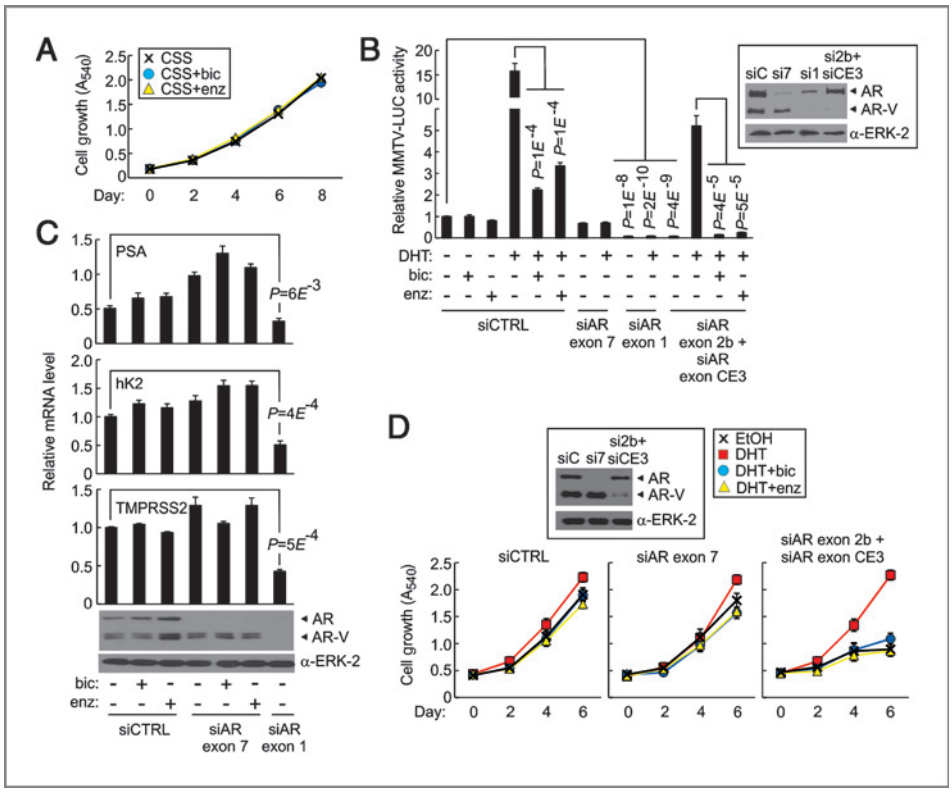


Figure 1. AR-Vs support resistance to full-length AR targeting in 22Rv1 cells. A, 22Rv1 cells were cultured under castrate (CSS) conditions with 10 μmol/L bicalutamide (bic) or 1 μmol/L enzalutamide (enz). B, MMTV promoter activities in siRNA-transfected cells treated under castrate conditions with 1 nmol/L DHT, 10 μmol/L bicalutamide, or 1 μmol/L enzalutamide. Data represent mean ± SE from at least 3 independent experiments, each conducted in duplicate. Inset, Western blot with antibodies targeted to the AR NTD or an internal control (ERK-2). Locations of full-length AR and truncated AR-Vs are indicated. C, 22Rv1 cells were transfected with siRNAs under castrate conditions. Gene expression was assessed by quantitative RT-PCR. Bars represent mean ± SD from 2 biological replicates, each carried out in duplicate. Western blots were carried out as in B. D, 22Rv1 cells were transfected and treated as in B. Growth was assessed at indicated time-points. Data represent mean ± SD from a quadruplicate experiment representative of 2 biological replicates. Inset, Western blots were carried out as in B.

androgens (Supplementary Fig. S1B), but expression was inhibited by AR-V knockdown (Fig. 1C). Together, these data show that AR-Vs are independent effectors of constitutive AR transcriptional activity in these cells. To test the biological implications of this, we assessed the effects of androgens and antiandrogens on the growth of 22Rv1 cells under conditions of full-length versus AR-V knockdown. Remarkably, knockdown of AR-Vs, but not full-length AR, reduced the androgen-independent growth rate of these cells and restored robust growth-responsiveness to androgens (Fig. 1D). Perhaps more importantly, AR-V knockdown restored the ability of antiandrogens to inhibit this newly acquired androgen-dependent growth phenotype (Fig. 1D). On the basis of this finding, we conclude that AR-Vs are sufficient for resistance of 22Rv1 cells to therapies targeting full-length AR, including enzalutamide.

### AR gene rearrangements mark AR-V-driven, enzalutamide-resistant cells in heterogenous PCa cell populations

Recently, we discovered a 48-kb AR intron 1 deletion in a subset of cells in the heterogeneous CWR-R1 cell line (ref. 13; Fig. 2A). Single-cell cloning revealed that cells positive for the 48-kb deletion displayed high-level expression of the AR 1/2/3/CE3 variant (Fig. 2B). Conversely, cells that were negative for the 48-kb deletion expressed predominantly full-length AR (Fig. 2B). Sub-clones negative for the 48-kb AR intragenic deletion displayed a basal level of androgen-independent growth, which was enhanced by DHT (Fig. 2C). This basal level of androgen-independent growth was reduced by treatment with bicalutamide or enzalutamide, indicating that full-length AR was required (Fig. 2C). Conversely, sub-clones positive for the 48-kb AR intragenic deletion displayed rapid androgen-independent growth, which was unaffected by androgens (Supplementary Fig. S2) or antiandrogens (Fig. 2D). However, selective knockdown of AR 1/2/3/CE3 inhibited androgen-independent growth of these rearrangement-positive cells (Fig. 2D).

To verify that this property of enzalutamide resistance was not restricted to a few rare cells, we tested the effects of antiandrogens on a version of the CWR-R1 cell line that had been propagated for the long term under castrated conditions. We have previously shown that long-term castration enriches for the AR intron 1 deletion-positive population (13), which AR gene copy number analysis showed was approximately 97% of



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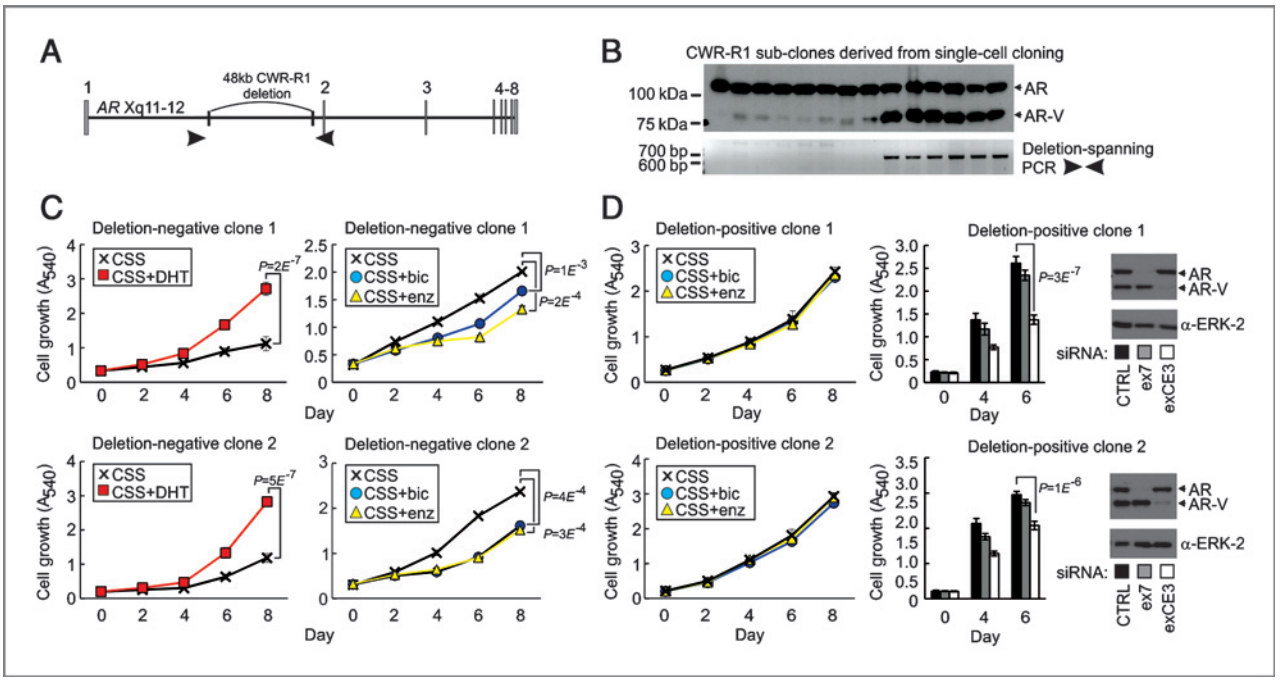


Figure 2. Rearrangement-positive CWR-R1 cells are resistant to full-length AR targeting. A, schematic of the AR locus with location of PCR primers for deletion analysis. B, CWR-R1 single-cell clones were assessed for AR-V expression by Western blot analysis using an antibody specific for the AR NTD. Concurrently, genomic DNA was isolated and subjected to deletion-specific PCR. C, deletion-negative clones were cultured under castrate conditions with 1 nmol/L DHT, 10 μmol/L bicalutamide, or 1 μmol/L enzalutamide. Growth was assessed at indicated time-points. Data represent mean ± SD from a quadruplicate experiment representative of 2 biological replicates. D, deletion-positive clones were cultured, treated, and subjected to growth assays as in C. Deletion-positive clones were further transfected with siRNAs and subjected to growth assays at days 0, 4, and 6. Data represent mean ± SD from a quadruplicate experiment representative of 2 biological replicates.

the population (Supplementary Fig. S3A). Similar to 22Rv1 cells, androgen-independent growth of deletion-enriched CWR-R1 cells was insensitive to bicalutamide and enzalutamide (Supplementary Fig. S3B). Nevertheless, antiandrogens were able to achieve on-target activity and inhibit androgen-induced MMTV-LUC activation (Supplementary Fig. S3C). However, only AR-V knockdown was able to inhibit constitutive, androgen-independent MMTV activity (Supplementary Fig. S3C). This is in line with our previous work showing that androgen-independent growth of deletion-enriched CWR-R1 cells is not affected by knockdown of full-length AR, but can be blocked by knockdown of AR 1/2/3/CE3 (13). Therefore, we conclude that the 48-kb AR intron 1 deletion can discriminate between individual cells in the heterogeneous CWR-R1 cell line that are enzalutamide-responsive and cells that are driven by AR-V activity and resistant to inhibition of full-length AR.

### AR-Vs are independent effectors of the androgen/AR transcriptional program

AR-Vs have been reported to induce unique transcriptional targets such as AKT1 (9), which may play a role in enzalutamide resistance (17). However, in AR intron 1 deletion-enriched CWR-R1 cells, we did not observe any changes in AKT1 expression following AR 1/2/3/CE3 knockdown (Supplementary Fig. S4). Therefore, to understand the mechanistic basis for AR-V–mediated resistance to enzalutamide, we carried out gene expression profiling of deletion-enriched CWR-R1 cells. Because constitutive activity of AR-Vs can mask androgen/AR induction targets (and vice versa) we assessed the androgen/AR transcriptional program following AR 1/2/3/CE3 knockdown and assessed the AR-V transcriptional program following full-length AR knockdown (Fig. 3A and Supplementary Fig. S5). Many, but not all, of the genes responsive to androgen/AR activity were similarly activated/repressed in a constitutive manner by AR 1/2/3/CE3 in these cells (Fig. 3B). This suggested that the AR-V transcriptional program represented a subset of the broader androgen/AR transcriptional program. Indeed, when we focused on AR-V responsive genes, nearly all were regulated in the exact same manner by androgen/AR activity (Fig. 3C). These data confirm that AR-Vs are constitutive and independent effectors of the AR transcriptional program, which explains why androgens and AR-Vs can support maximal growth of the same cell line in an interchangeable fashion (Fig. 1D). In line with this idea, the only knowledge-based multi-gene signaling networks identified as being associated with androgen/AR and AR-V gene signatures had AR as the prominent central hub (Supplementary Fig. S6).

These findings are in opposition to a recent study showing that AR-Vs have gene signatures distinct from full-length AR, including a set of genes involved in M-phase cell-cycle progression (18). To understand the basis for this discrepancy, we used gene set enrichment analysis (GSEA; ref. 19) to test the response of this AR-V-specific set of M-phase genes (18) in CWR-R1 cells. This AR-V-responsive M-phase gene set was



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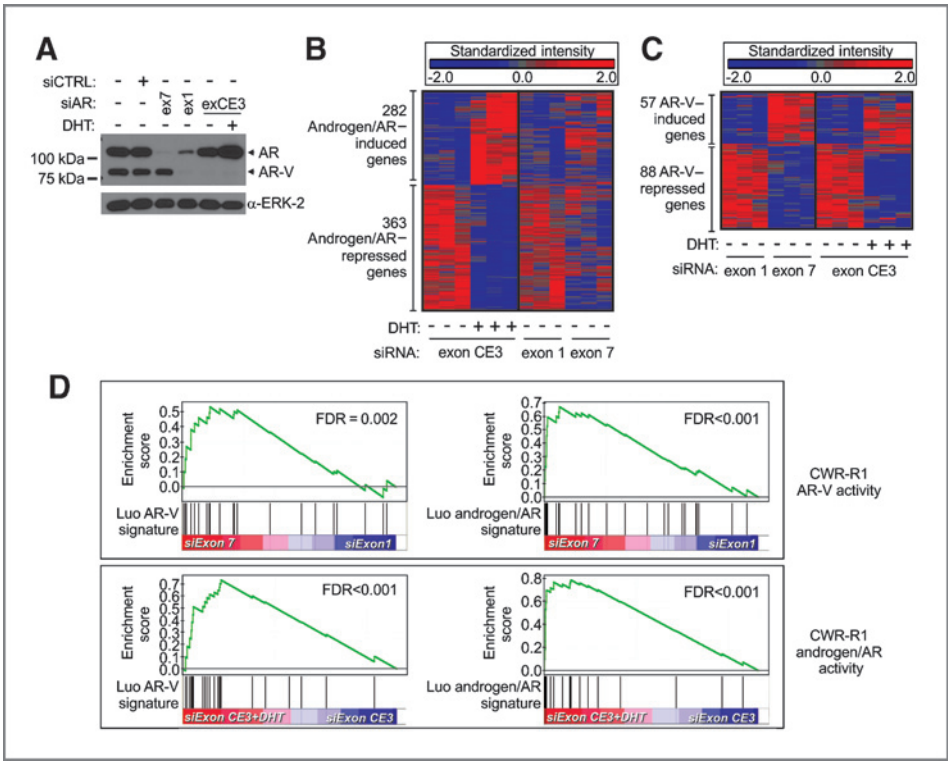


Figure 3. AR-Vs support the androgen/AR transcriptional program. A, CWR-R1 cells transfected with siRNAs specific for full-length and/or truncated AR-Vs were treated with 1 nmol/L DHT under castrate conditions. Western blots were carried out with antibodies specific for the AR NTD or a loading control (ERK-2). Two additional biological replicates are provided in Supplementary Fig. S5. B, heat-map of the androgen/AR gene expression program (left 2 columns) with comparison of the responses of these genes to AR-V activity (right 2 columns). Androgen/AR targets are defined as those genes showing differential expression in variant knockdown cells (siAR exon CE3) treated with DHT versus vehicle control. C, heat-map of the AR-V gene expression program (left 2 columns) with comparison of the responses of these genes to androgen/AR activity (right 2 columns). AR-V targets are defined as those genes showing differential expression in cells transfected with siRNA targeting AR exon 7 versus AR exon 1. D, gene set enrichment analysis of AR-V-specific (18) or full-length AR-specific (18) gene signatures in gene expression datasets supported by AR-Vs (top) or androgen/AR (bottom).

positively enriched in both androgen/AR and AR-V gene expression datasets derived from CWR-R1 cells (Fig. 3D). Similarly, a gene set deemed to be full-length AR-specific (18) was positively enriched in both of these CWR-R1–derived gene expression datasets (Fig. 3D). Therefore, these signatures could not discriminate between AR-V and androgen/AR activity in CWR-R1 cells.

AR-Vs have been shown to drive biphasic AR signaling in a manner similar to androgens (6). Therefore, we hypothesized that differences previously noted between AR-V and full-length AR transcriptional programs could have risen from comparing different strengths of AR transcriptional output from AR-Vs compared with androgens. To test this, we carried out GSEA with gene expression datasets derived from LNCaP cells treated with 1 nmol/L DHT (a pro-proliferative dose) or 100 nmol/L DHT (an antiproliferative dose). As expected, the full-length AR signature displayed positive enrichment in both the 1 nmol/L DHT and 100 nmol/L DHT gene expression datasets (Supplementary Fig. S7). Conversely, the AR-V-specific signature displayed positive enrichment in the 1 nmol/L DHT dataset, but strong negative enrichment in the 100 nmol/L DHT dataset (Fig. 4A). Therefore, these data indicate that the AR-V-specific signature does not discriminate between AR-V and full-length AR signaling, but rather reflects proliferative versus growth-suppressive levels of AR signaling output. To test this further, we treated LNCaP cells with androgens at concentrations that cover the range of proliferative and growth suppressive doses (0.1–100 nmol/L DHT) and assessed expression of M-phase genes *UBE2C*, *CDCA5*, *ZWINT*, and *CCNA2*. Whereas PSA expression increased concomitant with increasing androgen concentration, all of the M–phase-specific genes displayed a biphasic response: induction at low androgen concentrations and/or repression at higher doses (Fig. 4B). Similarly, when increasing titers of lentivirus encoding the AR 1/2/3/CE3 (AR-V7/AR3) variant (Fig. 4C) or the AR Δ5/6/7 ($ARv567^{es}$) variant (Fig. 4D) were used for infection, similar biphasic responses were observed for M–phase-specific genes, but not PSA. Therefore, these data challenge the notion that AR-Vs have acquired unique transcriptional targets and provide strong support for the concept that AR-Vs are independent effectors of the androgen/AR transcriptional program.

In summary, these data provide the first demonstration that AR-V expression driven by AR gene rearrangements can mediate resistance to therapies targeting full-length AR, including the next generation antiandrogen enzalutamide/MDV3100.



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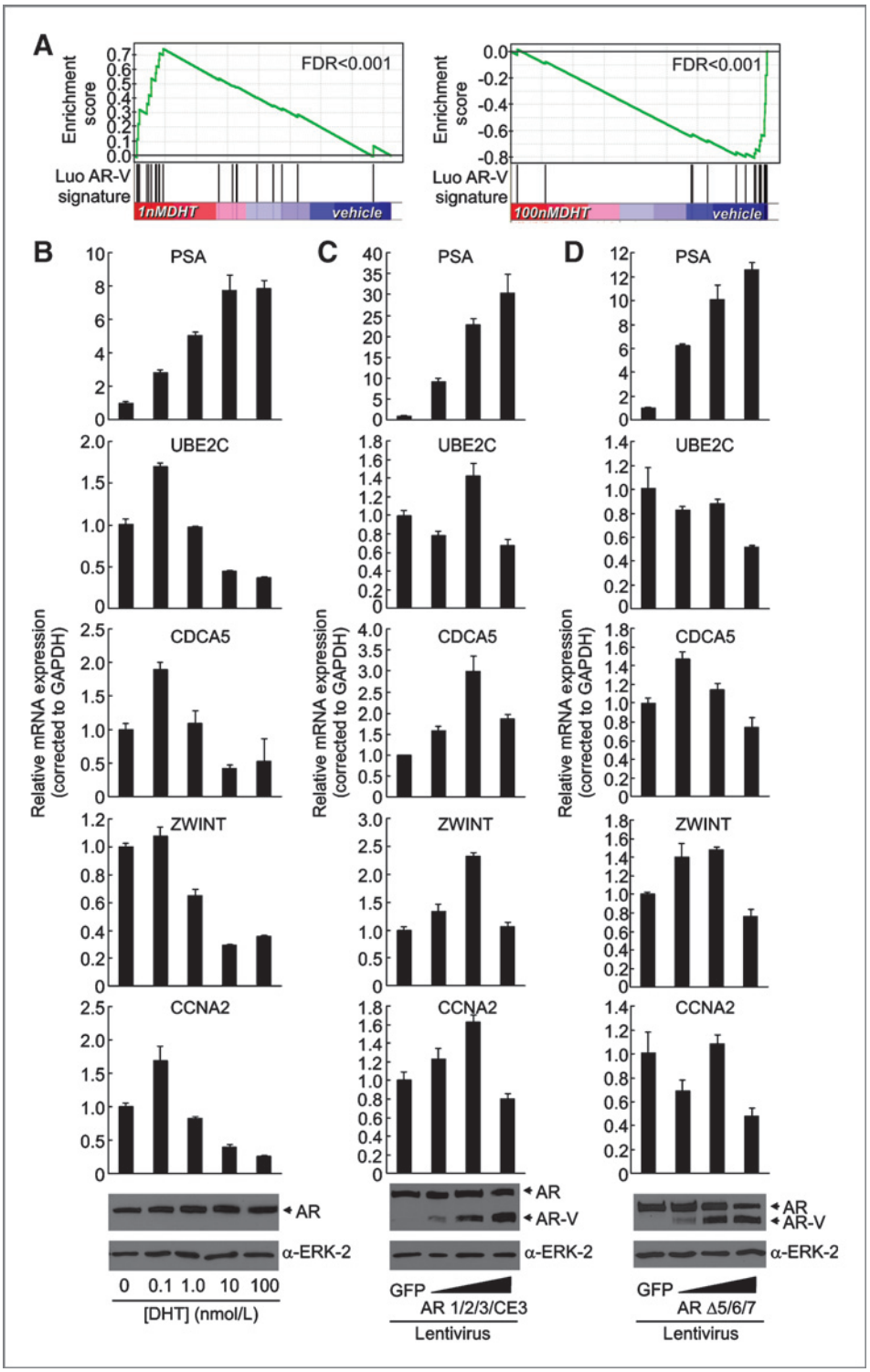


**Figure 4.** M-phase cell-cycle genes display a biphasic response to both androgen/AR signaling and AR-V signaling. A, gene set enrichment analysis of AR-V–specific gene signatures in gene expression datasets derived from LNCaP cells treated with 1 nmol/L DHT (GSE26483, left) versus 100 nmol/L DHT (GSE7868, right). B, LNCaP cells were treated with increasing concentrations of androgens and subjected to quantitative RT-PCR for indicated genes. Bars represent mean ± SD from a triplicate experiment representative of 2 biological replicates. Western blots were carried out using antibodies specific for the AR NTD or loading control (ERK-2). C, LNCaP cells were infected with increasing titers of lentivirus encoding AR 1/2/3/CE3. RNA and protein analyses were carried out as in B. D, LNCaP cells were infected with increasing titers of lentivirus encoding AR Δ5/6/7. RNA and protein analyses were carried out as in B.

These studies are significant because resistance, either *de novo* or acquired during therapy, is a major clinical limitation for new AR-axis inhibitors (4, 20). Importantly, the majority of patients that display disease progression on enzalutamide also display rising PSA, indicating that enzalutamide-resistant tumors remain driven by persistent AR activity (3). AR-Vs are overexpressed in a subset of CRPC metastases and correlate with poor survival (7). Mechanistically, our data show that AR-Vs mediate enzalutamide resistance in CRPC through their activities as independent effectors of the AR transcriptional program, driving persistent activation of a large subset of AR target genes at a level of output sufficient to support cell



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proliferation. Overall, these studies with cell line models of CRPC provide proof-of-concept for reversing enzalutamide resistance through inhibition of AR-V expression and/or activity.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

**Conception and design:** Y. Li, S.M. Dehm
**Development of methodology:** Y. Li, S. Chiu Chan, S.M. Dehm
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** Y. Li, S. Chiu Chan, S.M. Dehm
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** Y. Li, S. Chiu Chan, T. Hyun Hwang, K.A. Silverstein, S. M. Dehm
**Writing, review, and/or revision of the manuscript:** S. Chiu Chan, K.A. Silverstein, S.M. Dehm
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** S. Chiu Chan, L.J. Brand, S.M. Dehm
**Study supervision:** S.M. Dehm

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