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Androgen receptor splice variants mediate enzalutamide resistance in castration-resistant prostate cancer cell lines

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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 re-targeting 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-Vs) that lack the AR ligand binding domain. In this study, we demonstrate 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 demonstrated 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.

Keywords

androgen receptor; prostate cancer; enzalutamide resistance; truncated AR splice variants; castration-resistant

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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 re-activation of the androgen/androgen receptor (AR) signaling axis (1). This has been confirmed clinically through the recent re-targeting 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 demonstrating 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 xenograft derived from CRPC bladder metastasis harbors an 8.5 kb intragenic deletion of AR exons 5–7 which prevents full-length AR synthesis but favors expression of a truncated AR-V species encoded by mRNA lacking exons 5–7 (12). The 22Rv1 and CWR-R1 models of CRPC also harbor underlying AR gene rearrangements, leading to co-expression 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 ATCC and cultured according to ATCC protocol. ATCC ensures authenticity of these human cell lines using short tandem repeat (STR) analyses. All experiments with these cells were performed within 4 months of resuscitation of frozen cell stocks prepared within 3 passages of receipt from ATCC. CWR-R1 cells (14) were a kind gift from Dr. Elizabeth Wilson (UNC Chapel Hill) and cultured in RPMI 1640 + 10% FBS. Authentication of the CWR-R1 cell line was performed by sequence-based validation of two signature AR gene alterations: AR H874Y point mutation and 50kb intragenic deletion within AR intron 1 (13). Sequence-based authentication of CWR-R1 was performed every 5–10 passages, and cells were kept in culture no longer than 3 months after authentication unless otherwise indicated. Details of cell treatment with bicalutamide (Astra Zeneca) or enzalutamide (a kind gift from Dr. Michael Jung, UCLA) are provided as Supplementary material.

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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 an MMTV-LUC reporter as described (15). Growth of electroporated cells was monitored by crystal violet staining as described (12). Luciferase activity was measured as 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 (6). Infected cells were maintained in RPMI 1640 + 10% CSS for 48h and then switched to serum free medium for 24h prior to lysis.

Western Blot

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

Quantitative RT-PCR

Total RNA was extracted from 22Rv1, CWR-R1, and LNCaP cells as described (16). Primers and quantitative reverse transcription PCR (qRT-PCR) conditions for assessment of PSA, hK2, and TMPRSS2 mRNA expression have been described (15). Androgen- and AR variant-responses of M-phase specific genes were assessed using specific primers with sequences provided as Supplementary material. 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 (15).

Genomic PCR

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

Gene Expression Analysis with Illumina Beadchips

CWR-R1 cells that had been maintained in long term culture in RPMI 1640 + 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 + 10% CSS. Following 48h recovery, cells were switched to serum-free RPMI 1640 and treated for 24h with 1nM 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 35kb 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) (6, 9, 10, 12). 22Rv1 cells display robust growth under castrate conditions, which was unaffected by 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. 1A) as well as an AR-responsive MMTV-luciferase 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 knock-down of full-length AR (Fig. 1B). However, knock-down of AR-Vs resulted in robust inhibition of constitutive, androgen-independent MMTV-LUC activity. Similarly,

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constitutive, androgen-independent expression of PSA and hK2 was blocked by AR-V knock-down, but not by manipulations that block full-length AR (bicalutamide, enzalutamide, full-length AR knockdown, or AR knock-down 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 androgens (Supplementary Figure 1B), but expression was inhibited by AR-V knock-down (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 vs. AR-V knock-down. Remarkably, knock-down 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 knock-down restored the ability of antiandrogens to inhibit this newly-acquired androgen-dependent growth phenotype (Fig. 1D). Based on 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 48kb AR intron 1 deletion in a subset of cells in the heterogeneous CWR-R1 cell line (13) (Fig. 2A). Single cell cloning revealed that cells positive for the 48kb deletion displayed high-level expression of the AR 1/2/3/CE3 variant (Fig. 2B). Conversely, cells that were negative for the 48kb deletion expressed predominantly full-length AR (Fig. 2B). Sub-clones negative for the 48kb 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 48kb AR intragenic deletion displayed rapid androgen-independent growth which was unaffected by androgens (Supplementary Fig. 2) 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 long-term under castrate 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 ~97% of the population (Supplementary Fig. 3A). Similar to 22Rv1 cells, androgen-independent growth of deletion-enriched CWR-R1 cells was insensitive to bicalutamide and enzalutamide (Supplementary Fig. 3B). Nevertheless, antiandrogens were able to achieve on-target activity and inhibit androgen-induced MMTV-LUC activation (Supplementary Fig. 3C). However, only AR-V knock-down was able to inhibit constitutive, androgen-independent MMTV activity (Supplementary Fig. 3C). This is in line with our previous work demonstrating that androgen-independent growth of deletion-enriched CWR-R1 cells is not affected by knock-down of full-length AR, but can be blocked by knock-down of AR 1/2/3/CE3 (13). Therefore, we conclude that the 48kb 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

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CWR-R1 cells, we did not observe any changes in AKT1 expression following AR 1/2/3/CE3 knock-down (Supplementary Fig. 4). Therefore, to understand the mechanistic basis for AR-V-mediated resistance to enzalutamide, we performed 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 knock-down and assessed the AR-V transcriptional program following full-length AR knock-down (Fig. 3A and Supplementary Fig. 5). 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. 6).

These findings are in opposition to a recent study demonstrating 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) (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 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 vs. 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 arisen from comparing different strengths of AR transcriptional output from AR-Vs vs. androgens. To test this, we performed GSEA with gene expression datasets derived from LNCaP cells treated with 1nM DHT (a pro-proliferative dose) or 100nM DHT (an anti-proliferative dose). As expected, the “full-length AR” signature displayed positive enrichment in both the 1nM DHT and 100nM DHT gene expression datasets (Supplementary Fig. 7). Conversely, the “AR-V-specific” signature displayed positive enrichment in the 1nM DHT dataset, but strong negative enrichment in the 100nM DHT dataset (Fig. 4A). Therefore, these data indicate that the “AR-V-specific” signature does not discriminate between AR-V vs. full-length AR signaling, but rather reflects proliferative vs. 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 to 100nM 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.

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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. 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 demonstrate 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 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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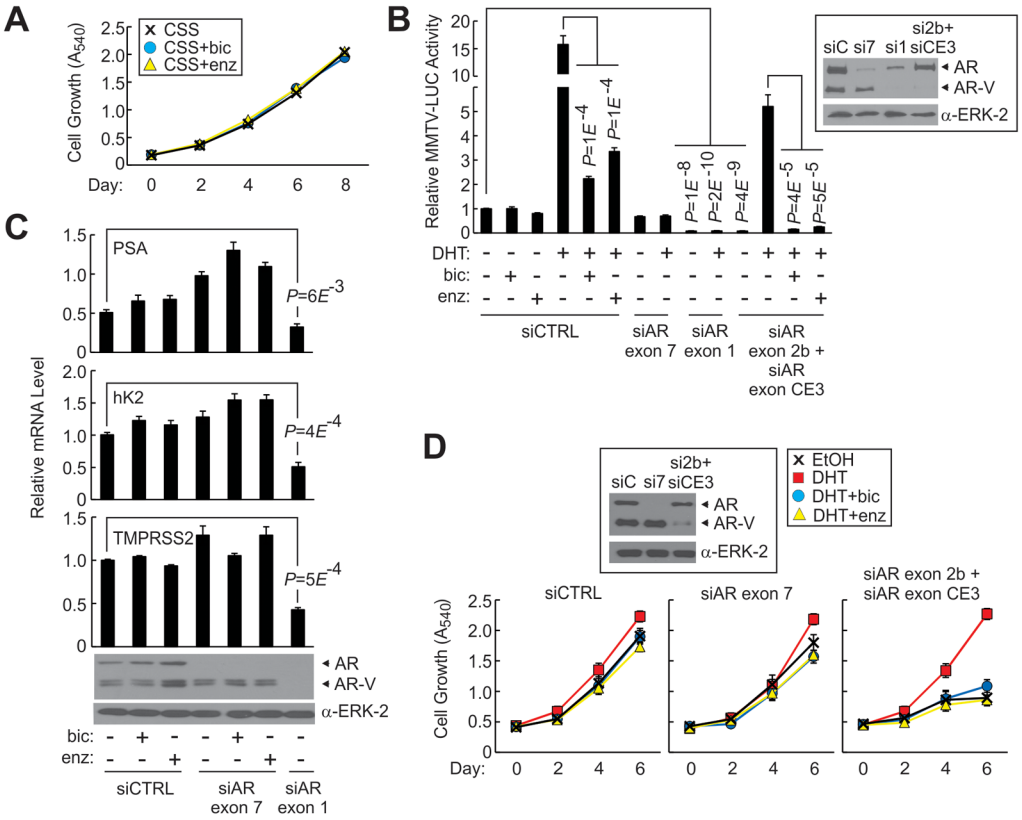

Figure 1.

AR-Vs support resistance to full-length AR targeting in 22Rv1 cells. (A) 22Rv1 cells were cultured under castrate (CSS) conditions with 10μM bicalutamide (bic) or 1μM enzalutamide (enz). (B) MMTV promoter activities in siRNA-transfected cells treated under castrate conditions with 1nM DHT, 10μM bicalutamide, or 1μM enzalutamide. Data represent mean +/− S.E. from at least three independent experiments, each performed 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 +/− S.D. from two biological replicates, each performed in duplicate. Western blots were performed as in (C). (D) 22Rv1 cells were transfected and treated as in (B). Growth was assessed at indicated time-points. Data represent mean +/− S.D. from a quadruplicate experiment representative of two biological replicates. Inset: Western blots were performed as in (B).

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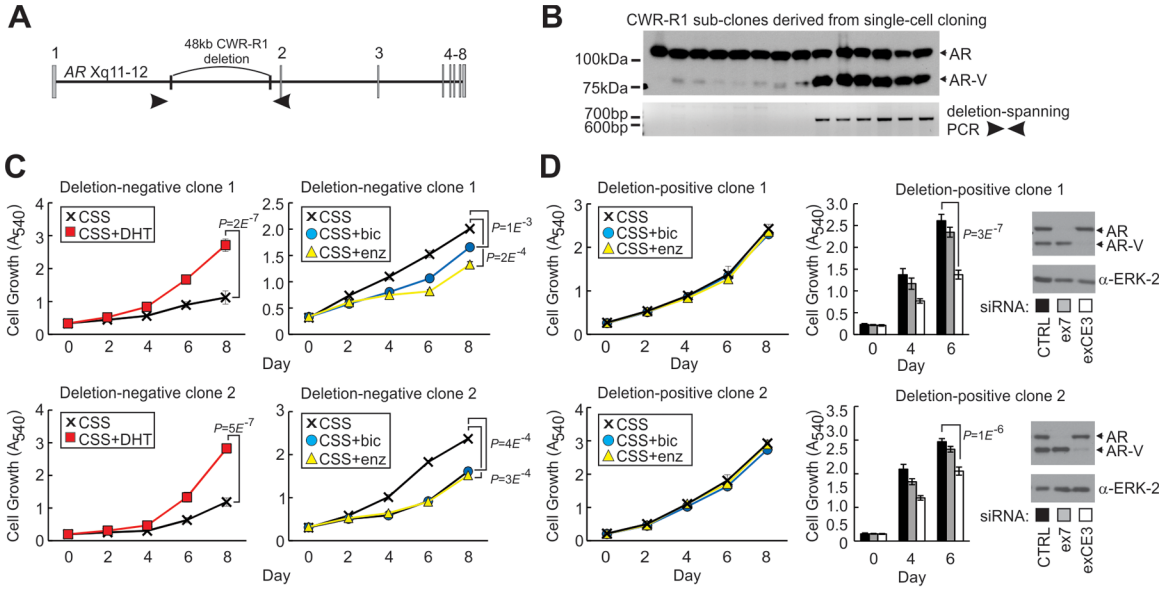

Figure 2.

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 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 1nM DHT, 10μM bicalutamide, or 1μM enzalutamide. Growth was assessed at indicated time-points. Data represent mean +/− S.D. from a quadruplicate experiment representative of two 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 +/− S.D. from a quadruplicate experiment representative of two biological replicates.

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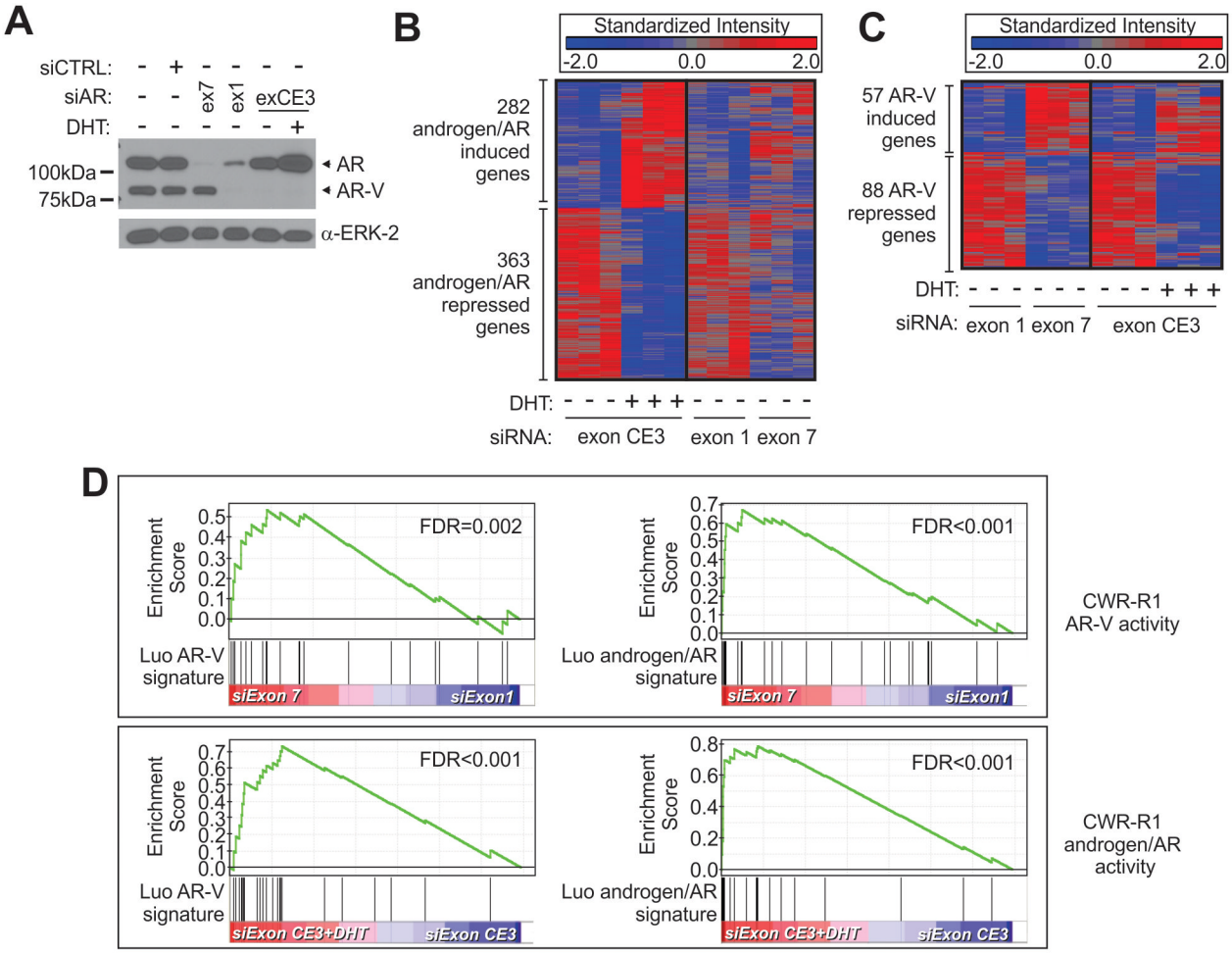

Figure 3.

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 1nM DHT under castrate conditions. Western blots were performed with antibodies specific for the AR NTD or a loading control (ERK-2). Two additional biological replicates are provided in Supplementary Fig. 5. (B) Heat-map of the androgen/AR gene expression program (left two columns) with comparison of the responses of these genes to AR-V activity (right two columns). Androgen/AR targets are defined as those genes demonstrating differential expression in variant knock-down cells (siAR exon CE3) treated with DHT vs. vehicle control. (C) Heat-map of the AR-V gene expression program (left two columns) with comparison of the responses of these genes to androgen/AR activity (right two columns). AR-V targets are defined as those genes demonstrating differential expression in cells transfected with siRNA targeting AR exon 7 vs. AR exon 1. (D) Gene set enrichment analysis (GSEA) 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).

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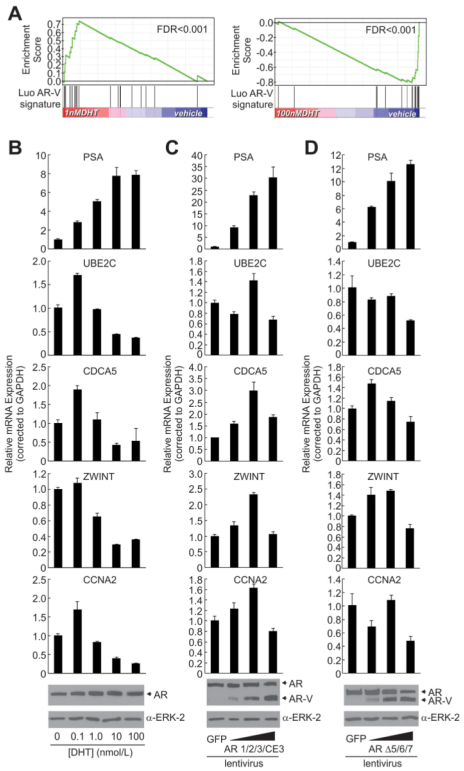

Figure 4.

M-phase cell cycle genes display a biphasic response to both androgen/AR signaling and AR-V signaling. (A) Gene set enrichment analysis (GSEA) of an AR-V-specific gene signatures in gene expression datasets derived from LNCaP cells treated with 1nM DHT (GSE26483, left) vs. 100nM 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 +/− S.D. from a triplicate experiment representative of two biological replicates. Western blots were performed 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 analysis was performed as in (B). LNCaP cells were infected with increasing titers of lentivirus encoding AR Δ5/6/7. RNA and protein analysis was performed as in (B).

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