Distinct transcriptional programs mediated by the ligand-dependent full-length androgen receptor and its splice variants in castration-resistant prostate cancer

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Abstract

Continued androgen receptor (AR) signaling is an established mechanism underlying castration-resistant prostate cancer (CRPC), and suppression of AR signaling remains a therapeutic goal of CRPC therapy. Constitutively active androgen receptor splice variants (AR-Vs) lack the AR ligand-binding domain (AR-LBD), the intended target of androgen deprivation therapies (ADT) including CRPC therapies such as abiraterone and MDV3100. While the canonical full-length AR (AR-FL) and AR-Vs are both increased in CRPC, their expression regulation, associated transcriptional programs, and functional relationships have not been dissected. In this study, we show that suppression of ligand-mediated AR-FL signaling by targeting AR-LBD leads to increased AR-V expression in two cell line models of CRPC. Importantly, treatment-induced AR-Vs activated a distinct expression signature enriched for cell cycle genes without requiring the presence of AR-FL. Conversely, activation of AR-FL signaling suppressed the AR-V signature and activated expression programs mainly associated with macromolecular synthesis, metabolism, and differentiation. In prostate cancer cells and CRPC xenografts treated with MDV3100 or abiraterone, increased expression of two constitutively active AR-Vs, AR-V7 and ARV567ES, but not AR-FL, paralleled increased expression of the AR-driven cell cycle gene UBE2C. Expression of AR-V7, but not AR-FL, was positively correlated with UBE2C in clinical CRPC specimens. Together, our findings support an adaptive shift toward AR-V-mediated signaling in a subset of CRPC tumors as the AR-LBD is rendered inactive, suggesting an important mechanism contributing to drug resistance to CRPC therapy.

Introduction

Androgen deprivation therapy (ADT) for advanced prostate cancer is designed to disrupt the androgen receptor (AR) pathway (1). The intended therapeutic target is the full-length androgen receptor (AR-FL), complete with an intact ligand-binding domain (LBD). Prostate tumors that progress despite first-line ADT (e.g., LHRH analogs), generally termed castration-resistant prostate cancer (CRPC), frequently demonstrate continued AR signaling driven by intratumoral androgens as well as elevated levels of AR-FL (2, 3). In support of the importance of ligand-driven AR-FL signaling in CRPC, a number of clinically effective endocrine therapies targeting AR-LBD were recently developed to treat CRPC patients (e.g., abiraterone, MDV3100) (4, 5). Nevertheless, the majority of patients progress shortly after treatment, again with reactivated AR signaling (4, 5).

Androgen receptor splice variants that lack the functional LBD (AR-Vs) were recently decoded and characterized (6-11), with some (e.g., AR-V7, ARV567ES) (7, 9, 10) but not all (8) facilitating ligand-independent AR signaling in CRPC. AR-Vs originate from AR transcripts with insertions of cryptic exons downstream of the coding sequences for AR DNA-binding domain (DBD) (6, 9-11), or with deletions of exons coding for AR-LBD (7, 8). These alterations in AR transcripts disrupt the AR open reading frame, leading to truncated AR proteins with the intact N-terminal domain (NTD), AR-DBD, and a short variant-specific peptide replacing the functional AR-LBD. AR-Vs are capable of activating canonical AR-FL regulated genes (e.g., KLK3, TMPRSS2, NKX3.1) in the absence of AR-FL signaling (6-11), raising the possibility that AR-Vs and AR-FL may direct a similar transcriptional program, and that elevated AR-Vs in CRPC may compensate for AR-FL signaling. However, previous studies also suggest that AR-Vs are not as potent as AR-FL in inducing the expression of AR-FL genes (9). Since AR-FL and AR-Vs are both overexpressed in CRPC, and AR-Vs are less abundant than AR-FL (6, 9), the role of ligand-independent AR signaling in the context of suppressed yet still active AR-

FL signaling is not clear. In addition, regulation of expression of endogenous AR-Vs and their functional relationships with AR-FL signaling have not been discerned in the context of ADT. In this study, we employed a set of detection and targeting tools that differentiate the AR-FL and AR-Vs to investigate the functional interplay and distinctions between AR-FL and AR-V. The cumulative *in vitro* and *in vivo* evidence supports the importance of AR-V-mediated signaling in mediating responses to CRPC therapies targeting AR-LBD.

Materials and Methods

Cell line models and treatments. LNCaP, CWR22Rv1, and VCaP human prostate cancer cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Early passage cells were used in all experiments. LNCaP95 is an androgen-independent cell line derived from the parental LNCaP cells (provided by Dr. Alan K. Meeker, Johns Hopkins University, Baltimore, MD). LNCaP95 cells and the parental LNCaP cells demonstrate qualitatively similar AR transcriptional responses (Supplemental Figure 1). Previous studies have evaluated and estabished the expression status for both AR-FL and AR-V in these 4 cell lines (9). Stable clones of AR-FL positive and AR-FL negative LNCaP cells were developed by EB (Supplemental Methods). In all comparisons involving the treatment of cells with or without R1881 (NEN, Boston, MA), cells were cultured for indicated time periods in culture media without phenol red (Invitrogen, Carlsbad, CA) supplemented with 10% charcoal-stripped FBS (CSS) (Invitrogen) with or without 1 nM R1881 (NEN, Boston, MA). For siRNA treatment, cells reaching ~70% confluence were treated with siRNA for 24 hours and then cultured for 48 hours with or without 1 nM R1881. Target sequences for specific knockdown of different AR molecules were described previously (9). MDV3100 was obtained from Medivation (San Francisco, CA). VCaP and LNCaP 95 cells were treated with MDV3100 dissolved in DMSO at the indicated concentrations for 24 hours with or without 1 nM R1881 (NEN). Transient transfection with AR-Vs was performed as described previously (7-9).

Western blot analysis. Antibodies utilized in this study include anti-AR N20 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-AR-V7, anti-PSA (Santa Cruz Biotechnology), anti-ERG (C-17, Santa Cruz Biotechnology), anti-UBE2C (Boston Biochem, Cambridge, MA), and anti-β-actin (Sigma-Aldrich, St. Louis, MO). The anti-AR-V7 antibody is a mouse monoclonal antibody developed using peptide sequences specific to AR-V7 (CKHLKMTRP) (Supplemental Figure 2).

Immunofluorescence staining. Cells were grown on chamber slides to ~80% confluence in culture media supplemented with 10% FBS. At 24 hours after the indicated treatments, cells were fixed for 10 minutes using fresh made 4% paraformaldehyde and then with 0.2% Triton X-100 in 1x PBS for 5 min at room temperature (RT). Fixed cells were incubated with the primary anti-AR-V7 antibody for 2 hrs at RT. Secondary antibody was an Alexa Fluor 488-conjugated goat anti-mouse antibody (1:200 dilution) (Invitrogen).

Gene set enrichment analysis. Expression data was generated using the Agilent Whole Genome Expression Arrays (Agilent Technologies, Santa Clara, CA) at Johns Hopkins University (by JL) and the University of Washington (by PSN), analyzed using GeneSpring GX 11.5 (Agilent Technologies), and subjected to gene set enrichment analysis (12). Other details are described in Supplemental Methods. Raw expression microarray data have been submitted to GEO (GSE36549).

CRPC Xenografts. Abiraterone-treated xenograft tumor samples derived from the LuCaP35CR xenograft line was obtained from EAM. Detailed methods for treatment, tumor collection, and mRNA analysis were fully described previously (13).

Tissue microarray analysis. CRPC tissues on the TMAs were procured as previously described (14). Immunohistochemical staining for AR-V7, AR-FL, and UBE2C was optimized and

performed by JE. De-paraffinized TMA slides were placed in 10 mM Tris, 1 mM EDTA (pH 9.0) and steamed for 40 min for antigen retrieval. The primary antibodies were incubated with each slide for 1 hour at room temperature. The EnVision reagent (DAKO Corp., Carpenteria, CA) was used for color development. Protein expression levels were scored by a semi-automated method as previously described (15). Scoring data were analyzed using SPSS (SPSS Inc., Chicago, IL) and GraphPad (GraphPad Software, Inc., La Jolla, CA).

Results and Discussion

Regulation of AR-V expression by AR-FL. AR-FL and AR-V are both overexpressed in clinical CRPC specimens (9), and induced in castrate conditions in CRPC xenografts (6), suggesting that increased AR-V levels may be coupled with enhanced transcription of the AR gene, and that AR-V function may require the presence of AR-FL (6). Because individual AR-V levels are typically lower than AR-FL (6, 8, 9), we utilized two cell lines, LNCaP95 and VCaP, that recapitulate the relative expression levels of AR-FL and AR-Vs in clinical CRPC specimens, i.e., detectable but lower levels of AR-Vs than AR-FL (9). AR-FL signaling in the presence of R1881 was suppressed (Figure 1A) by siRNA targeting the AR-LBD (AR-LBD siRNA), by ligand depletion (R1881-), or by MDV3100, a potent anti-androgen that targets the AR-LBD (4). We demonstrate that suppression of ligand-mediated AR-FL signaling by all three of these AR-LBD targeting strategies leads to an increase of the aggregate AR-V signal (AR-Vs) (Figure 1A). Increased AR-V protein expression following suppression of AR-LBD was confirmed by an increase in AR-V7, as shown by Western blot (Figure 1A) and immunofluorescence staining (Figure 1B) using a variant-specific monoclonal antibody (Supplemental Figure 2). We conclude that AR-V protein expression may not parallel that of AR-FL following suppression of AR-FL signaling (Figure 1A). Instead, AR-V protein levels are negatively regulated by ligand-mediated AR-FL signaling irrespective of the AR-FL protein levels, suggesting an adaptive shift toward AR-V-mediated signaling after therapy targeting AR-LBD.

Distinctive transcriptional programs mediated by AR-FL and AR-Vs. To dissect the transcriptional programs induced by AR-V-mediated signaling, we took two approaches: first we examined transcriptional changes driven by forced expression of AR-Vs in the presence or absence of AR-FL signaling by gene set enrichment analysis (GSEA) (12). Transient expression of exogenous AR-V7 in parental LNCaP cells (Supplemental Figure 3) induced expression of cell cycle genes, under both androgen-depleted or androgen-stimulated conditions (Supplemental Figures 4). On the other hand, top gene sets increased by ligand-dependent AR-FL are dominated by those related to biosynthesis, metabolism, and secretion (Supplemental Figure 5). For illustration, we generated two gene sets, "AR-V7 UP" and "AR-FL UP", each containing 25 probes that contributed to the core enrichment of top ranked gene sets driven by AR-V7 and AR-FL. The two representative gene sets demonstrate clearly independent expression patterns (Figure 2A). Other canonical AR-FL regulated genes (KLK3, TMPRSS2, NKX3.1) follow the same pattern as those in the "AR-FL UP" gene set (not shown). To further determine whether expression of the AR-V genes requires the presence of endogenous AR-FL, we generated stable LNCaP clones with or without endogenous AR-FL protein (Supplemental Methods), and transiently transfected these clones with AR-V7 or ARV567ES in androgen-deprived conditions (Supplemental Figures 6-7). In this independent series of expression profiles (Figure 2B), the "AR-V7 UP" gene set remains as the top ranked gene set induced by ARV567ES or AR-V7 (not shown), and the absence of endogenous AR-FL did not attenuate induction of the "AR-V7 UP" signature (Figure 2B, Supplemental Figure 8). Thus, the presence of AR-FL is not required for induction of cell cycle genes by constitutively active AR-V7 and AR-V567ES.

As a second approach to further corroborate the functional distinctions between AR-FL and AR-Vs, we analyzed gene expression correlates of endogenous AR-Vs following suppression of canonical AR-FL signaling only or suppression of both AR-FL and AR-Vs (Figure 2C, Supplemental Figures 9-12). In LNCaP95 cells, the "AR-V7 UP" signature is again the top gene set enriched for up-

regulation following an increase of endogenous AR-V7 induced by AR-FL suppression (Supplemental Figure 11). Knockdown of both AR-FL and AR-Vs abrogated expression of the "AR-V7 UP" gene set (Figure 2C), confirming the essential role of AR-Vs. In contrast, the canonical AR-FL target genes demonstrated a change of direction opposite to those driven by AR-V, with the "AR-FL UP" gene set as the top ranked gene set enriched for down-regulation following suppression of AR-FL (Supplemental Figure 11). Increased expression of the "AR-V7 UP" gene set was further confirmed in VCaP cells following suppression of AR-FL signaling by either AR-LBD siRNA or MDV3100 (Supplemental Figure 12).

Correlation between AR-V and UBE2C after treatment with MDV3100 and abiraterone. The "AR-V7 UP" gene set included UBE2C (Figure 2). Previous studies established a direct regulation of UBE2C expression by AR in LNCaP-abl cells under androgen-deprived conditions (16). Our findings (Figure 2) suggest that UBE2C expression in our CRPC models may be driven by AR-Vs but not by AR-FL. To validate the relationship between UBE2C and AR-V expression following suppression of AR-FL signaling, we first show that in LNCaP95 cells treated with MDV3100, the expression of UBE2C parallels that of AR-Vs but not AR-FL (Figure 3A). Both AR-FL and constitutively active AR-Vs are elevated in the LuCaP35CR xenografts following treatment with abiraterone (13). We further show that in those abiraterone-treated xenografts, AR-Vs, but not AR-FL, are significantly correlated with UBE2C and other cell cycle genes at mRNA levels (Figure 3B and 3C, Supplemental Table I). From these findings (Figures 1-3), we reason that an adaptive shift toward AR-V mediated-signaling may contribute to resistance to MDV3100 and abiraterone.

***In Vivo* correlation between AR-V and UBE2C in CRPC.** Suppression of AR-FL does not induce AR-V expression in the parental LNCaP and CWR22RV1 cells (Supplemental Figure 13), in which high levels of AR-V7 may be caused by genomic alterations not frequently seen in clinical

specimens (17). Consistent with the role of AR-V, we did not observe increased cell cycle gene expression after suppression of AR-FL signaling in these two cell lines (not shown). Thus, cell type-specific regulation of endogenous AR-V expression may explain why suppression of AR-FL inhibits tumor growth in some models of CRPC but not the others (6). Our results nevertheless suggest that AR-Vs, rather than AR-FL, may play a key role in supporting castration-resistant growth in at least a subset of CRPC. We investigated the relative importance of AR-FL and AR-V *in vivo* by immunohistochemistry analyzing the correlation between AR-FL and UBE2C, and between AR-V7 and UBE2C, in transurethral resection of the prostate (TURP) specimens derived from patients who developed obstructive urinary symptoms after hormone therapy (Figure 4A, Supplemental Figure 14, Supplemental Table II). AR-V7, but not AR-FL, is significantly correlated with UBE2C (Figure 4B, 4C), further supporting that AR-Vs, rather than AR-FL, mediates cell cycle gene expression in at least a subset of CRPC specimens. This protein expression data is consistent with findings from an mRNA based study demonstrating elevated cell cycle gene expression detected in bone metastasis expressing higher levels of AR-Vs (18).

In summary, this study reveals the functional interplay between AR-FL and AR-Vs when AR-LBD is rendered inactive by ADT. Importantly, the combined *in vitro* and *in vivo* data predict an adaptive shift toward AR-V- mediated signaling with effective CRPC therapies targeting AR-LBD. These studies indicate that therapeutic efficacy of agents targeting AR-LBD may be compromised as a consequence of this adaptive shift, and that early detection of this shift may be utilized to guide treatment decisions. In addition, novel agents for CRPC (19) may be designed to suppress the activation of transcriptional programs directed by the AR-Vs. Overall, given the recently expanded therapeutic options for metastatic CRPC (20), the present findings may stimulate efforts to target adaptive AR-V signaling for treatment selection and to overcome resistance to CRPC therapy.

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Figure legend

Figure 1. Regulation of AR-V expression by AR-FL signaling in LNCaP95 and VCaP cells. A: Increased AR-Vs following suppression of AR-FL by ligand depletion (R1881-), siRNA targeting AR-LBD (AR-LBD siRNA), or MDV3100 (10μM). Protein levels of AR (by the N20 antibody that detects both AR-FL and AR-Vs), AR-V7, PSA, ERG, and beta-actin were assessed by Western blot. B: Immunofluorescent images showing decreased or loss of AR-V7 nuclear staining in the presence of 1nM R1881.

Figure 2. Distinctive expression patterns of gene sets representing the core transcriptional output of AR-V7 and AR-FL. A. Expression of the "AR-V7 UP" and "AR-FL UP" gene sets in parental LNCaP cells transiently transfetced with AR-V7 in the presence (R1881) or absence (CSS) of AR-FL signaling. B. Expression of the "AR-V7 UP" gene set in stable clones of LNCaP cells with ($AR\text{-}FL^{+)}$ or without ($AR\text{-}FL^{-}$) endogenous AR-FL following transient transfection with either AR-V7 or ARV567ES. Each experiment was repeated 3 times. C. Expression profiles of the "AR-FL UP" and "AR-V7 UP" gene sets in LNCaP95 cells following suppression of AR-FL only (AR-LBD siRNA), or both AR-FL and AR-Vs (AR-DBD siRNA), in the presence or absence of 1nM R1881.

Figure 3. Increased expression of UBE2C parallels that of AR-Vs following treatment with MDV3100 and abiraterone. A. Increased AR-V7 and UBE2C expression following MDV3100 treatment in the presence of 1nM R1881 in LNCaP95 cells. B. Correlation of UBE2C and AR (AR-V7, ARV567ES, and AR-FL) expression in LuCaP35CR xenografts treated with control vehicles. C. Correlation of UBE2C and AR (AR-V7, ARV567ES, and AR-FL) expression in LuCaP35CR xenografts treated with abiraterone.

Figure 4. Tissue microarray analysis of correlation between AR (AR-FL and AR-7) and UBE2C in castration-resistant prostate specimens (n=67). A. Representative high power TMA images showing nuclear staining for AR-FL, AR-V7, and UBE2C. B. No correlation between AR-FL and UBE2C (r=0.01, p>0.05) in 67 CRPC specimens. C. Significant correlation between AR-V7 and UBE2C (r=0.41, p<0.05) in 67 CRPC specimens.

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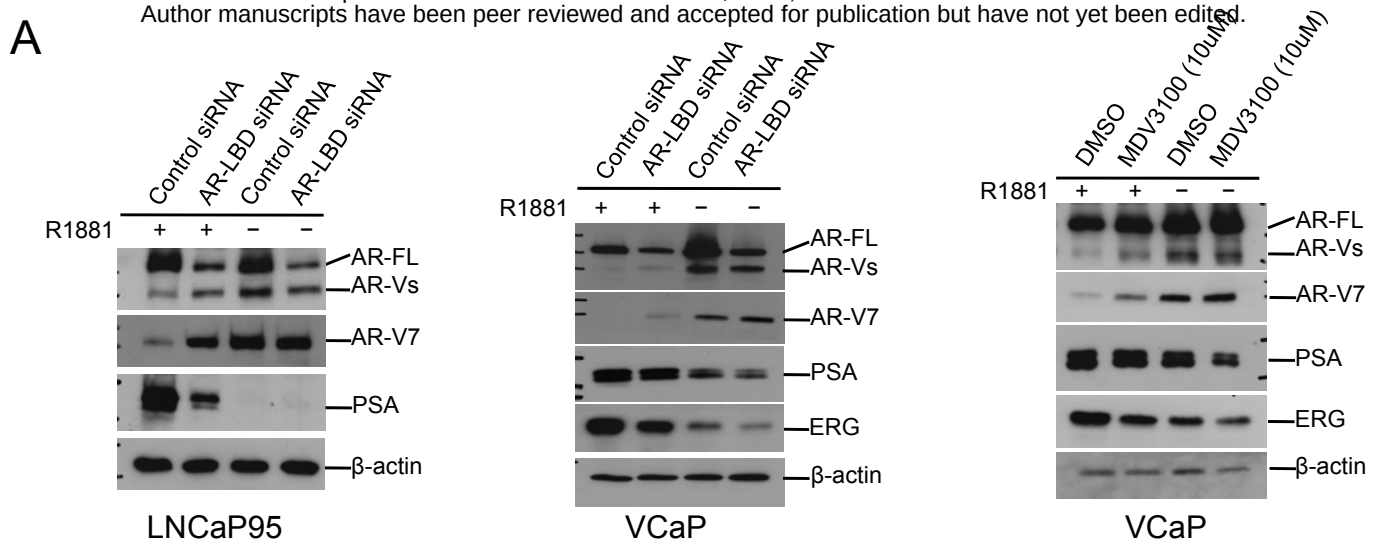

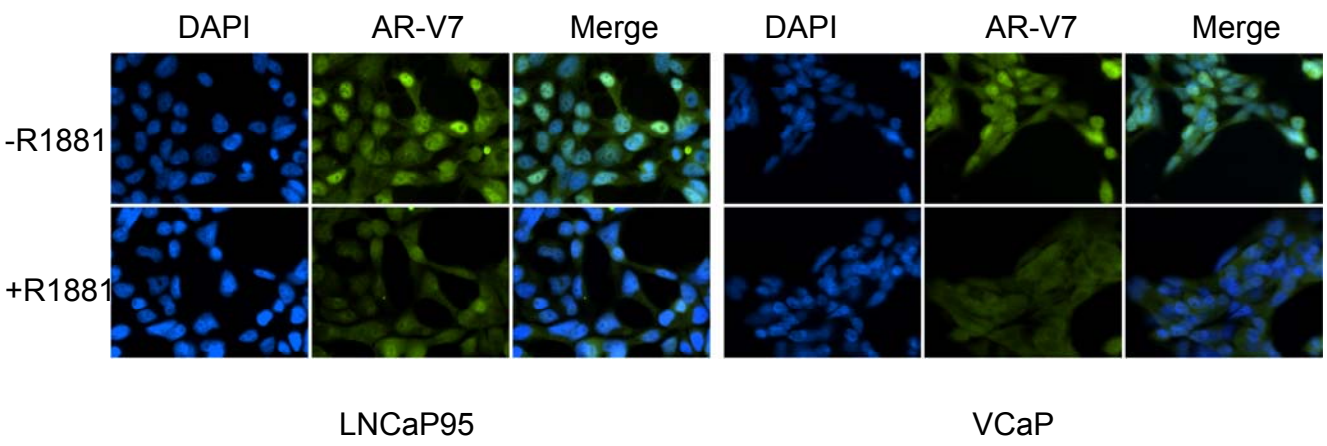

Figure 1

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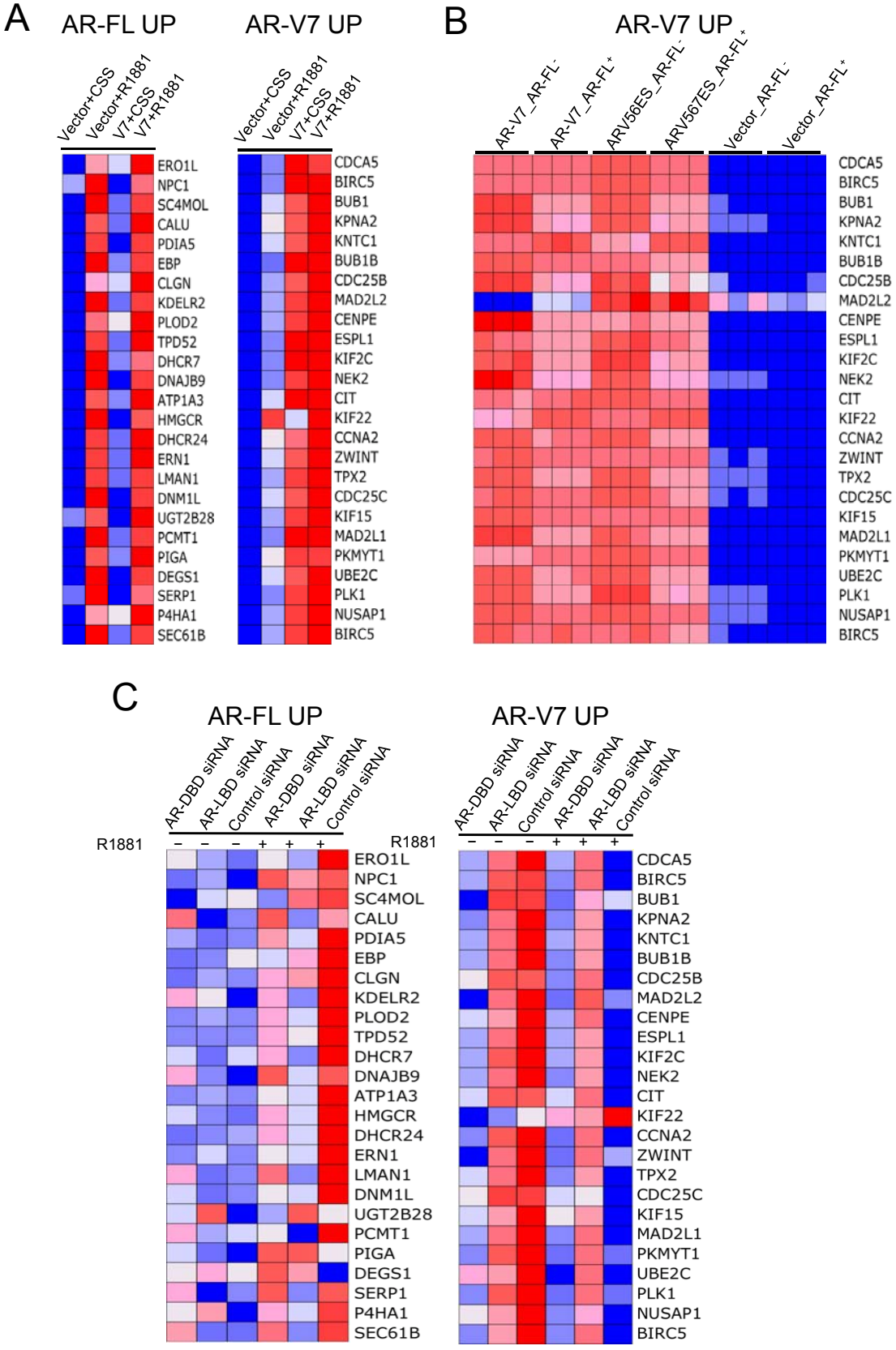

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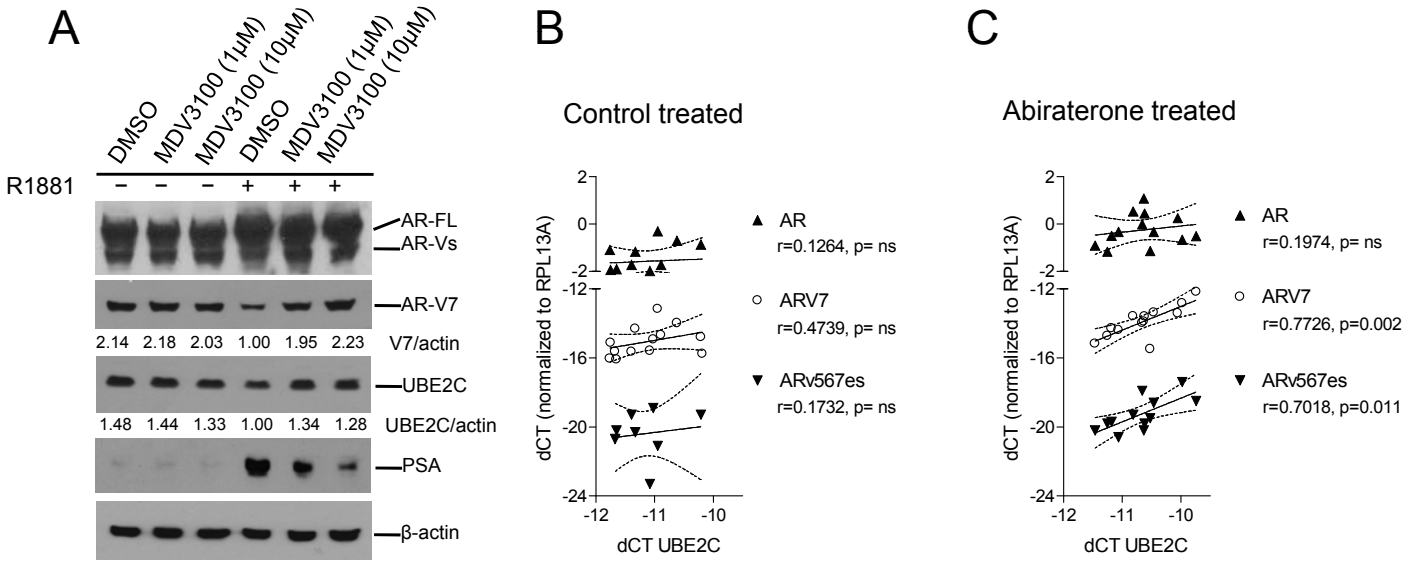
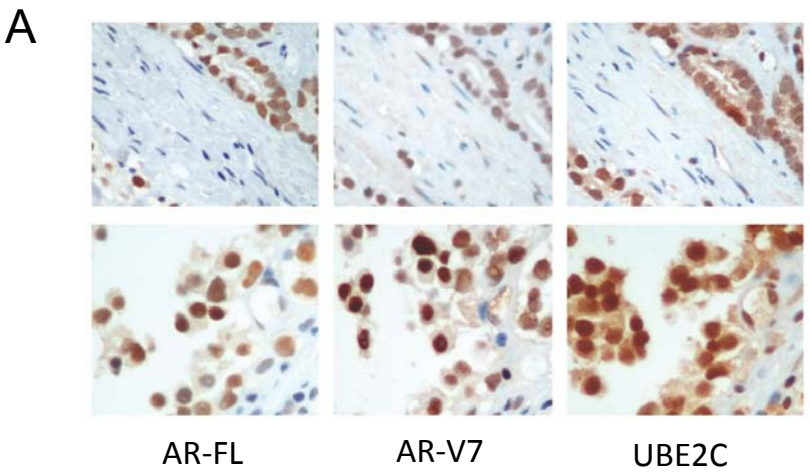
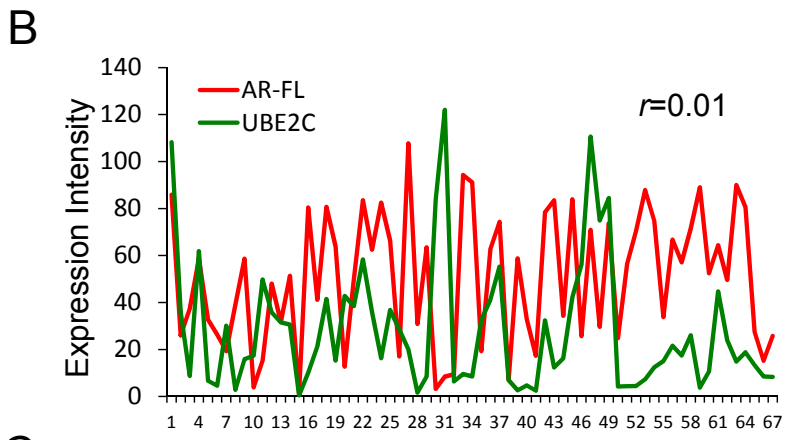


Figure 3





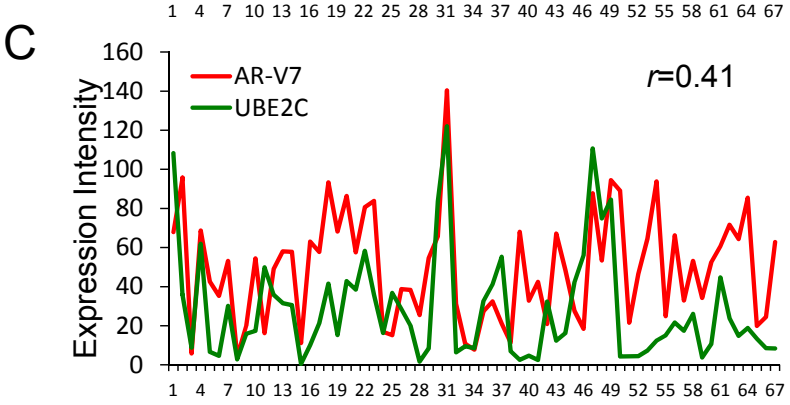


Figure 4

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