Role of androgen receptor splice variant-7 (AR-V7) in prostate cancer resistance to 2nd-generation androgen receptor signaling inhibitors

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Abstract
The role of truncated androgen receptor splice variant-7 (AR-V7) in prostate cancer biology is an unresolved question. Is it simply a marker of resistance to 2nd-generation androgen receptor signaling inhibitors (ARSi) like abiraterone acetate (Abi) and enzalutamide (Enza) or a functional driver of lethal resistance via its ligand-independent transcriptional activity? To resolve this question, the correlation between resistance to ARSi and genetic chances and expression of full length AR (AR-FL) vs. AR-V7 were evaluated in a series of independent patient-derived xenografts (PDXs). While all PDXs lack PTEN expression, there is no consistent requirement for mutation in TP53, RB1, BRCA2, PIK3CA, or MSH2, or expression of SOX2 or ERG and ARSi resistance. Elevated expression of AR-FL alone is sufficient for Abi but not Enza resistance, even if AR-FL is gain-of-function (GOF) mutated. Enza resistance is consistently correlated with enhanced AR-V7 expression. In vitro and in vivo growth responses of Abi-/Enza-resistant LNCaP-95 cells in which CRISPR-Cas9 was used to knockout AR-FL or AR-V7 alone or in combination were evaluated. Combining these growth responses with RNAseq analysis demonstrates that both AR-FL- and AR-V7-dependent transcriptional complementation are needed for Abi/Enza resistance.

Introduction
Androgen stimulates androgen receptor (AR)-dependent transcriptional regulation within prostate stromal cells activating secretion of a combination of paracrine growth and survival factors (e.g., IGF, EGF, FGFs) while simultaneously repressing secretion of paracrine death inducing factors (e.g., TGF-β ligands) [1, 2]. In the presence of physiologic androgen, prostate stromal cells secrete sufficient paracrine growth and survival factors to maintain homeostatic epithelial cell turnover, preventing gland regression without inducing neoplastic overgrowth [2]. Under these conditions, epithelial homeostasis is maintained and androgen-induced cell autonomous AR-dependent signaling within prostate epithelial cells induces their terminal differentiation [i.e., expression of prostate-specific differentiation marker genes such as prostate-specific antigen (PSA) and human kallikrein-2] [3]. This differentiation suppresses their proliferation, despite the chronic presence of high levels of stromal cell-derived paracrine growth factors [3].

During prostatic carcinogenesis, there is conversion from AR-regulated stromal paracrine dependency by normal
prostate epithelium to cancer cells acquiring autonomous stromal cell-independent AR-stimulated malignant growth [2, 4]. Such cell autonomous growth involves losing normal AR function as a growth suppressor and instead acquiring ability to act as an oncogenic GOF stimulator of malignant growth [2, 3]. These oncogenic acquisitions “addict” prostate cancer cells to cell autonomous AR signaling. This addiction can involve cancer cells acquiring cell autonomous ligand-dependent AR transcription preventing their apoptotic cell death while also inducing proliferation, making these cancers AR-dependent for their lethal growth [5]. Alternatively, prostate cancer cells can lose their dependence on AR survival signaling while retaining a sensitivity to AR signaling to enhance their rate of malignant proliferation [2]. This makes them AR-sensitive, but not absolutely dependent upon such continuous signaling. Regardless of whether AR signaling addiction results in dependency vs. sensitivity, it provides rationale for the use of androgen deprivation therapy (ADT) for metastatic prostate cancer [2].

Eventual resistance to first-line (i.e., primary) ADT utilizing luteinizing hormone-releasing hormone (LHRH) analogs to suppress circulating testosterone (T) to a castrate level alone or in combination with Casodex (a 1st generation antiandrogen) is essentially universal. Such castration-resistant prostate cancer (CRPC) patients are subsequently given 2nd-generation AR signaling inhibitors (ARSi) to suppress AR signaling using steroid synthesis inhibitors [e.g., abiraterone acetate, (Abi)] to eliminate non-testicular androgen ligands and/or next-generation ligand binding domain (LBD) antagonists [e.g., enzalutamide (Enza)] that target the full length AR (AR-FL) protein [6]. Subsequent resistance to these 2nd-generation ARSi is also essentially universal and often associated with significantly elevated expression of both AR-FL and truncated AR splice variant-7 (AR-V7) [7]. AR-V7 originates from contiguous splicing of AR exons 1, 2, and 3 with the cryptic exon 3 (CE3) present within the canonical intron 3 of the AR gene. This generates a transcript, which encodes for a truncated protein lacking C-terminal LBD, thus acquiring ligand-independent transcriptional activity [8–10].

While expression of AR-V7 protein is rare in primary PC, nuclear AR-V7 expression is detectable in response to primary ADT alone in most patients, and further increases during Abi or Enza therapy [6, 7]. This raises the question of whether AR-V7 protein expression is simply associated with enhanced AR-FL expression as a marker of resistance to ARSi, or whether a critical level of AR-V7 is required for such ARSi-refractory lethal cancer growth. Consistent with this latter possibility is the observation that AR-V7 has cistromes and thus transcriptional outputs that are distinct from those directed by AR-FL and which are consistent with genomic features of disease progression in a low-androgen environment [11]. Thus, a series of PDXs in which the genetic and phenotypic changes were followed before and after the development of ARSi resistance was used to determine the role of AR-V7 in this progression. In addition, in vitro and in vivo growth and transcriptional response of Abi- and Enza-resistant LNCaP-95 (LN-95) cells to CRISPR-Cas9 knockout (KO) of AR-FL vs. AR-V7 alone or in combination were evaluated.

Results

Response of prostate cancer PDX lacking AR-V7 to Abi and Enza

CWR22 PDX is derived from a primary prostate cancer with an AR H875Y mutation from a hormone treatment-naive European-American patient [12]. Its xenograft growth in adult male NSG mice is highly androgen sensitive as documented by its regression following castration with a subset (i.e., 40%) eventually relapsing [13]. Serial passage in castrated hosts of one of the relapses produced the CWR22-RH PDX so named because it is castration-resistant and was produced at Hopkins [13]. CWR22-RH grows equally well in an intact or castrated NSG mouse with a doubling time (DT) of 10 ± 2 days. Histologically, like the parental CWR22, it is a poorly differentiated adenocarcinoma (Fig. 1a), which expresses c-Myc and Ki67 in >80% of cells (Table 1). Like the parental CWR22, CWR22-RH cells express prostate-specific HOXB13, and express luminal cell-specific, but not basal cell or NE-specific, markers (Table 1 and Fig. 2). CWR22-RH secretes PSA (serum PSA of 249 ± 41-ng/ml/g tumor). Genetically, it retains the heterogeneous LOF mutation in BRCA2 (E984fs) and loss of homozygosity (LOH) and LOF TP53 [G154F] mutation from the parental CWR22 (Table 1).

There are several unique genetic changes associated with castration resistance of the CWR22-RH. These include a LOH and a LOF truncating PTEN [T321fs] mutation (Table 1) resulting in these cells being null for PTEN protein expression (Fig. 1b). In addition, during relapse to castration, CWR22-RH acquired an additional AR T878A mutation and is thus hemizygous for H875Y/T878A double mutation (Table 1). This double-mutated AR is highly expressed in nuclei of CWR22-RH cells (Fig. 1c) at a 25-fold higher level of AR-FL protein compared to normal prostate luminal cells. This is consistent with such elevation in AR-FL protein being the most common molecular determinant of resistance to first-line ADT in CRPC patients [14, 15]. This elevation in AR-FL, however, is not accompanied by detectable AR-V7 protein expression (Fig. 1d). Double AR mutations in codons 875 and 878
result in a GOF, because such ARs are strongly stimulated by progesterone binding, which is only a very weak agonist for wild-type AR [15]. This is consistent with CWR22-RH growing equally well in intact vs. castrated hosts (Table 1), since castration does not lower serum progesterone in mice (1–2 ng/ml) [16]. Daily oral treatment of castrated adult male NSG mice bearing CWR22-RH tumors with a therapeutically effective dose of Abi (i.e. 0.5 mmol/kg [17]) no growth inhibitory effect (Fig. 1e). This resistance is predictable because Abi inhibits steroid metabolism downstream from progesterone and castration does not lower serum progesterone levels in mice [15, 16].

Despite its Abi resistance, growth of the CWR22-RH PDX in castrated adult male NSG mice is profoundly inhibited by daily oral treatment with a therapeutically effective dose of Enza (10 mg/kg [18]), even though it has high expression of double-mutated (i.e., H875Y/T878A) AR-FL protein (Fig. 1f). This is not unexpected since Enz blocks progesterone binding to AR and thus can inhibit progesterone-induced growth of PCA cells [19].

AR-FL vs. AR-V7 expression in prostate cancer PDXs resistant to Abi and Enza

LvCaP-2 PDX is derived from a liver metastasis obtained at rapid autopsy from a 75-year-old European-American who following a prostate biopsy (Gleason Sum 9) was treated over a 3-year period with ADT, then Abi, docetaxel plus Carboplatin, and finally Enza (Supplementary Fig. 1a). Histologically, CRPC PDX is a poorly differentiated adenocarcinoma (Fig. 3a). LvCaP-2 has wild-type AR, which it expresses at a 52-fold higher mRNA level (Table 1) and an 11-fold higher AR-FL protein level compared to normal prostate luminal cells with a low level of AR-V7 protein that it loses with serial passaging in intact hosts (Fig. 3b, inset). Essentially all LvCaP-2 cells exhibit nuclear
|                      | CWR22          | CWR22-RH       | LvCaP-2        | LvCaP-2R       | SkCaP-1        | SkCaP-1R       | LNCaP          | LNCaP-95       |
|----------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| RNAseq classification| AR+ PCa        | AR+ PCa        | AR+/NE+ PCa    | AR+/NE+ PCa    | AR+ PCa        | AR+ PCa        | AR+ PCa        | AR+ PCa        |
| Tissue of origin     | Primary        | Liver Met      | Skin Met       | SkCaP-1        | LNCaP          | LNCaP-95       |                |                |
| Patient treatment    | None           | ADT, Abi,      | ADT, Taxane,   |                |                |                |                |                |
|                      |                | Carboplatin,   | Abi, Carboplatin, and Enza |                |                |                |                |                |
|                      |                |                |                |                |                |                |                |                |
| Histology            | Poorly         | Poorly         | Poorly         | Poorly         | Poorly         | Poorly         | Poorly         | Poorly         |
|                      | differentiated | differentiated | differentiated | differentiated | differentiated | differentiated | differentiated | differentiated |
|                      | adenocarcinoma | adenocarcinoma | adenocarcinoma | adenocarcinoma | adenocarcinoma | adenocarcinoma | adenocarcinoma | adenocarcinoma |
| In vivo growth       | Yes            | No             | Yes            | No             | Yes            | No             | Yes            | No             |
| response to ADT      |                |                |                |                |                |                |                |                |
| Xenograft            |                |                |                |                |                |                |                |                |
| doubling time        |                |                |                |                |                |                |                |                |
|                      | 11 ± 3 days    | 10 ± 2 days    | 10 ± 3 days    | 9 ± 2 days     | 14 ± 5 days    | 18 ± 4 days    | 12 ± 5 days    | 6 ± 3 days     |
|                      | (intact host)  | (intact or     | (intact host)  | (intact or     | (intact host)  | (intact or     | (intact or     | (intact or     |
|                      |                | castrate host) |                | castrate host) |                | castrate host) | castrate host) | castrate host) |
| AR                   | Homozygous GOF | Homozygous GOF | Wild type +    | Wild type +    | Wild type      | Wild type      | Homozygous     | Homozygous     |
|                      | H878A mutation | Double GOF     | low to no V7   | V7             | Wild type + V7 | Wild type + V7 | T878A GOF      | T878A GOF      |
|                      |                | H875Y and T878A mutation |                |                |                |                | mutation       | mutation       |
| Normalized AR mRNA   | 4              | 11             | 52             | 256            | 4              | 388            | 17             | 30             |
| Normalized AR protein| 6              | 25             | 11             | 50             | 7              | 80             | 33             | 59             |
| AR-FL/AR-V7 protein ratio | >100:1       | >100:1         | >100:1         | 6:1            | >100:1         | 12:1           | >100:1         | 8:1            |
| TP53                 | Heterozygous LOF G154F mutation | Heterozygous LOF G154F mutation | LOF T211fs mutation | LOF T211fs mutation | Wild type | Wild type | Wild type | Wild type |
| PTEN                 | Wild type      | Heterozygous LOF T321fs mutation | LOH and hemizygous deleterious R130Q mutation | LOH and hemizygous deleterious R130Q mutation | Wild type | Wild type | Wild type | Wild type |
| ERG                  | No             | No             | No             | No             | Yes            | Yes            | No             | No             |
| c-Myc                | >80%           | >80%           | >80%           | >80%           | >50%           | >50%           | Yes            | Yes            |
| Nkx3.1               | Yes            | Yes            | Yes            | Yes            | Yes            | Yes            | Yes            | Yes            |
| Ki67                 | 72 ± 27%       | 83 ± 6%        | 80 ± 6         | 75 ± 9%        | 45 ± 3%        | 39 ± 4%        | 47 ± 12%       | 82 ± 12%       |
| PSA                  | Yes            | Yes            | Yes            | Yes            | Yes            | Yes            | Yes            | Yes            |
| Serum PSA (ng/mL/g)  | 462 ± 67       | 249 ± 41       | 59 ± 11        | 25 ± 6         | 284 ± 51       | 44 ± 12        | 185 ± 34       | 50 ± 10        |
|                      | (intact host)  | (castrate host)| (intact host)  | (castrate host)| (intact host) | (castrate host)| (castrate host)| (castrate host)|
| PSMA                 | Yes            | Yes            | Focal          | Focal          | >50%           | >50%           | Yes            | Yes            |
localization of AR protein (Fig. 3b). At the transcriptome and protein level (Table 1), a high proportion (>80%) of the parental LvCaP-2 cells express c-Myc (Fig. 3c) and Ki67 (Fig. 3d). Besides expressing prostate-specific HOXB13 (Fig. 3e), it expresses luminal cell-specific (Fig. 3f–h), but not basal cell, markers (Table 1 and Fig. 2). It does express NE markers, however and is thus an “amphicrine” carcinoma [20]. Genetically, it has a hemizygous LOF truncating mutation in TP53 (T211fs) and hemizygous deleterious mutation (R130Q) in PTEN [21] with loss of PTEN protein expression. While LvCaP-2 has wild-type RB1, there is only limited focal expression of RB1 protein. It secrets PSA [serum PSA of 59 ± 11-ng/ml/g tumor (Table 1)].

When adult male hosts bearing the LvCaP-2 PDX are castrated, the cancer stops growing for ~1 month before relapsing (Fig. 3i). Passage of a relapsing tumor in castrated hosts results in a variant, termed LvCaP-2R, that grows equally well in intact vs. castrated hosts [DT of 10 ± 3 days vs. 9 ± 2 days, respectively (Fig. 3j)]. Growth of LvCaP-2R in a castrated mouse is resistant to daily oral treatment with 0.5 mmol/kg of Abi (Fig. 3k).

Histologically (Fig. 3l) and phenotypically (Table 1 and Fig. 2), this Abi-resistant LvCaP-2R remains a poorly differentiated amphicrine adenocarcinoma with retained expression of NKX3.1 (Fig. 3m), PSA, HOXB13, and PSMA (Table 1). LvCaP-2R has a 50% decrease in RB1 mRNA with undetectable expression of RB1 protein, and an additional fivefold increase in AR mRNA compared to the
parental LvCaP-2, raising the level to 256-fold higher than in normal prostate luminal cells (Table 1 and Fig. 2). This results in a 4.7-fold increase in total AR protein in LvCaP-2R in castrated hosts vs. parental LvCaP-2 in intact mice (Fig. 3n), which is 50-fold higher total AR protein than in normal prostatic luminal cells (Table 1).

Importantly, progression of LvCaP-2 to the Abi-resistant LvCaP-2R variant is associated with the gain of AR-V7 protein expression at a ratio of 6 to 1 [AR-FL: AR-V7] (Fig. 3o). This translates to an eightfold higher level of AR-V7 protein in LvCaP-2R than the level of AR-FL expression in normal prostate luminal cells. AR is located not only in the cytoplasm, but also strongly present in nuclei of essentially all LvCaP-2R cells despite being in castrated hosts (Fig. 3n). Growth of the Abi-resistant LvCaP-2R PDX in castrated NSG adult male mice is not inhibited by daily oral treatment with 10 mg/kg of Enza (Fig. 3p). Likewise, Enza treatment had no effect on serum PSA expressed as ng/ml/g of tumor (i.e., 25 ± 4 vs. 22 ± 6 for controls vs. Enza-treated, respectively).

To address the generalizability of coordinated AR-FL and AR-V7 expression in the development of Abi and Enza resistance, an additional PDX, termed SkCaP-1, was evaluated. The SkCaP-1 PDX is derived from a biopsy of a CRPC skin metastasis obtained from a 52-year-old European-American who underwent a radical prostatectomy (Gleason Sum 7), and subsequently progressed over a 12-year period to sequential treatment with salvage XRT/ADT/Taxane/Abi/Carboplatin/Enza treatment before rapid autopsy (Supplementary Fig. 1b). Histologically, it is a poorly differentiated adenocarcinoma (Fig. 4a). In addition to expressing prostate-specific HOXB13, it expresses...
luminal cell-specific including AR (Fig. 4b), Nkx3.1, and PSMA. e Growth rate of SkCaP-1 in intact (i.e., ADT equivalent) mice with subsequent regression and relapse in castrate (i.e., ARSi equivalent) male NSG mice (n = 5 each). AR-FL and AR-V7 immunoblots of SkCaP-1 vs. SkCaP-1R (inset). f IHC (200×) of SkCaP-1 for Ki67. g Abi and Enza resistance of SkCaP-1R in vivo (n = 3 each). h H & E histology (200×) of SkCaP-1R. IHC (200×) of i AR, j PSA, k c-Myc, and l Ki67 in SkCaP-1R PDX.

Fig. 4 Characterization of SkCaP-1 and SkCaP-1R. a H & E histology (200×) of SkCaP-1. IHC (200×) of SKCaP-1 for b AR, c Nkx3.1, and d PSMA. e Growth rate of SkCaP-1 in intact (i.e., ADT equivalent) mice with subsequent regression and relapse in castrate (i.e., ARSi equivalent) male NSG mice (n = 5 each). AR-FL and AR-V7 immunoblots of SkCaP-1 vs. SkCaP-1R (inset). f IHC (200×) of SkCaP-1 for Ki67. g Abi and Enza resistance of SkCaP-1R in vivo (n = 3 each). h H & E histology (200×) of SkCaP-1R. IHC (200×) of i AR, j PSA, k c-Myc, and l Ki67 in SkCaP-1R PDX.

When adult male mice bearing established SkCaP PDXs are castrated, cancers regress over a 40-day period to a non-palpable size before relapsing (Fig. 4e). Passage of such a relapsing cancer results in a variant, SkCaP-1R, that grows equally well in intact vs. castrated hosts [DT of 18 ± 4 days] (Table 1). Growth of the SkCaP-1R in castrated adult male NSG mice is not inhibited by daily oral treatment with Abi or Enza over a 3-week period (Fig. 4g). Neither Abi nor Enza treatment has an effect on serum PSA expressed as ng/ml/g of tumor (i.e., 50 ± 8 for controls vs. 54 ± 12 for Abi vs. 44 ± 12 for Enza).

Histologically, Abi/Enza-resistant SkCaP-1R remains a poorly differentiated adenocarcinoma (Fig. 4h). It retains expression of prostate-specific HOXB13 and luminal cell-specific including AR (Fig. 4i) and PSA (Fig. 4j); but not basal cell or NE-specific, markers (Table 1 and Fig. 2). A major transcriptional difference between SkCaP-1R growing in castrated hosts is an additional 12-fold increase in AR mRNA compared to the SkCaP-1 growing in intact hosts (Fig. 2), raising the level to 388-fold higher than in normal prostate luminal cells (Table 1). This results in an 11-fold increase in total AR protein in SkCaP-1R in castrated hosts vs. parental SkCaP-1 in intact mice, which is 80-fold higher total AR protein than in normal prostatic luminal cells. Progression to the Abi/Enza-resistant SkCaP-1R variant is associated with an enhanced expression of AR-V7 protein at a ratio of AR-FL to AR-V7 of 12:1 (Fig. 4e, inset). This translates to a sixfold higher level of AR-V7 protein in SkCaP-1R than the level of AR-FL in normal prostate luminal cells (Table 1). AR is located in nuclei of essentially all SkCaP-1R cells despite being in a castrated host (Fig. 4i). This is consistent with their retained expression of c-Myc (Fig. 4k) and Ki67 (Fig. 4l).
LN-95 variant as a prototypic model of ARSi resistance

These results document that elevated expression of AR-FL alone is sufficient for Abi, but not Enza resistance, even if AR-FL has a GOF mutation and that Enza resistance is correlated with a critical level of AR-V7 expression. To test the role of AR-FL vs. AR-V7 in CRPC resistance to Enza directly requires an Enza-resistant cell line amenable to CRISPR-Cas9 gene KO that expresses both AR-FL and AR-V7. These conditions are met by a variant of the LNCaP cell line known as the LNCaP-95 (a.k.a. LN-95). LNCaP is derived from a supraclavicular lymph node metastasis from a CRPC patient [22]. It expresses wild-type RB1, but has a 2-bp deletion in codon 6 in PTEN leading to a LOF frame shift mutation [23], and has a GOF AR T878A mutation [24], and methylation of the GSTP1 and TGFβR2 promoters resulting in a loss of expression of these latter 2 proteins [25–27]. Thus, LN-95 is not sensitive to ADT-induced cell death [25, 27–30]. This is significant because LNCaP cells are passaged in phenol red-containing RPMI-1640 media supplemented with 10% fetal bovine serum (i.e., FBS media) [22]. FBS media contains a castrate serum level of T [i.e., 22.0 ± 6.1 pg/ml (55.1–97.5 pM)] [31]. LNCaP cells have microsatellite instability due to homozygous deletion of exons 9 to 16 in the mismatch repair gene hMSH2, resulting in truncation and LOF of the protein [32]. Thus, LNCaP is genetically unstable and accumulates mutations during serial in vitro passaging. This provides a mechanism for why LNCaP acquires a faster growth rate coupled with a decrease in PSA expression and acquisition of resistance during serial in vitro culture [33].

In low-androgen FBS media, LNCaP cells express a high level of mutated AR-FL (T878A) protein [i.e., 33-fold higher than normal prostate luminal epithelial cells (Fig. 1d)] [18], but no detectable level of AR-V7 (Fig. 5a). In this low-androgen media, AR signaling is functional as documented by its secretion of 70 ng of PSA/ml of media/10⁶ cells per day. Functional AR-signaling is also confirmed by the fact that addition of Enza (10 μM) to the FBS media inhibits LNCaP growth by ~75% (Fig. 5b) due to the inhibition of AR-dependent cell autonomous autocrine signaling [4]. Similarly, in vitro growth of LNCaP cells is inhibited by ~90% when cultured in phenol red-free RPMI-1640 media supplemented with 10% charcoal-stripped FBS [C/S media (Fig. 5b)], containing and even more depleted

Fig. 5 Characterization of LNCaP variant under long-term ARSi-equivalent conditions (i.e., LN-95 cells). a AR-FL and AR-V7 immunoblot of LNCaP vs. LN-95 variant and quantification via densitometry. b Cell number after 5 days of in vitro growth of LN-95 in 10% FBS media, 10% FBS media containing 10-μM enzalutamide, or 10% CS-FBS media vs. LNCaP growth under the same conditions with asterisks denoting significant difference at p < 0.05. e Growth rate of LN-95 in castrated (i.e. ARSi equivalent) vs. LNCaP in intact (i.e., ADT equivalent) mice. d Abi resistance of LN-95 xenografts in vivo (n = 3 each). black = vehicle control; gray = Abiraterone treated. e In vivo growth response of LN-95 growing in castrated (i.e., ARSi equivalent) male NSG mice given daily oral dosing with 25 mg of enzalutamide/kg/d vs. vehicle controls (n = 5 each).
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Role of AR-FL vs. AR-V7 in resistance of LN-95 cells to Enza

To address the role of AR-FL vs. AR-V7 in Enza resistance, the growth response to Enza was compared between LN-95 cells in which CRISPR-Cas9 was used to KO either AR-FL or AR-V7 alone or in combination (Fig. 6a). Clones of LN-95 cells were obtained in androgen-depleted C/S media in which AR-FL or AR-V7 alone or in combination were knocked out as validated by sequence analysis (Supplementary Fig. 2), Western blotting (Fig. 6b), and IHC (Fig. 6c). IHC documents that in single KOs, the remaining AR-FL or AR-V7 is localized in the nuclei despite being in androgen-depleted C/S media (Fig. 6c). This is significant for the AR-FL KO cells that only express AR-v7, because this documents that AR-V7, which contains the classical nuclear localization domain (i.e., AA608–628) [10, 38] translocates to the nuclei even though it lacks the LBD. This ability of the AR-V7 protein to nuclear translocate without co-expression of AR-FL is confirmed by cell fractionation and Western blot analysis (Fig. 6d). In these AR-FL KO cells, nuclear AR-V7 is transcriptionally active even without AR-FL as demonstrated by its ability to increase transcription of a subset of AR target genes (Fig. 6e). These target genes are defined by their transcriptional down-regulation in total AR (i.e., AR-FL/AR-V7) double KO cells and transcriptional upregulation by the addition of synthetic androgen (i.e., R1881) to parental LN-95 cells and AR-v7 KO cells that only express AR-FL (Fig. 6f). Significantly, when R1881 is added to the media, the LN-95 cells decrease their AR RNA expression by 40% (p < 0.05) and stop expressing a detectable level of AR-V7 protein, and this loss of AR-V7 protein does not occur in AR-FL KO cells (Fig. 6b). This supports that there is an autoregulatory negative feedback loop between level of ligand-dependent AR-FL signaling and AR-V7 expression as described previously [35, 39].

The in vitro growth of LN-95 cells is not dependent on, but is augmented by AR signaling as documented by the >75% reduction (p < 0.05) in growth of the total AR-KO vs. parental LN-95 cells in the androgen-depleted C/S media (Fig. 6f). As expected, Enza treatment did not decrease further the depressed growth of the total AR-KO cells (Fig. 6f). Similar growth depression (p < 0.05) also occurs in both AR-FL and AR-V7 single KO cells and as expected Enza has no effect upon the depressed growth of AR-FL single KO cells expressing only AR-V7 (Fig. 6f). Significantly, Enza treatment had no effect upon the depressed growth of the LN-95 AR-V7 KO cells expressing only AR-FL (Fig. 6f). This is consistent with their growth already being maximally depressed by the loss of AR-V7. These results document that signaling from both AR-FL and AR-v7 is required for maximal growth of LN-95 cells in the androgen-depleted C/S media.

This conclusion is supported by RNAseq analysis. There are a series of 32 signature genes whose expression is significantly (i.e., >1.4-fold) AR stimulated vs. 19 genes AR repressed (Table 2) in parental LN-95 cells growing optimally in androgen-depleted C/S media vs. AR-null (i.e., total AR-KO) cells whose growth is maximally depressed.
Thirteen out of the 32 (41%) AR-stimulated signature genes and 8 of 19 (42%) AR-repressed signature genes cannot be attributed specifically to either AR-FL or AR-V7 (i.e., they were not affected by KO of either AR-FL or AR-V7 alone). There are 10 of the 32 (31%) stimulated and 9 of 19 (47%) repressed genes, however, whose expression is regulated only by AR-FL (i.e., they were affected by KO of AR-FL but not AR-V7), consistent with the need for signaling by both receptors for maximal growth. Conversely, there is only one of the signature-stimulated genes (i.e., PRKACB) whose expression is repressed only in AR-V7 expressing cells. There are several AR-stimulated and AR-repressed genes (e.g., IGFBP3 and PSD4) whose expression is repressed by AR-FL, but stimulated by AR-V7 (Table 2). The data are consistent with overlapping and also distinct functional roles characterized previously and suggest the need for signaling by both receptors for maximal growth.

These results document that combined AR-FL plus AR-V7-dependent transcriptional regulation is needed for both growth stimulation under Abi-equivalent conditions and resistance to Enza. These results are not limited to the in vitro growth response. In xenograft studies, total AR-KO...
Table 2 Genes whose transcription is stimulated or repressed in parental LN-95, AR-FL KO, or AR-V7 KO cells vs. AR-null (i.e., total AR-KO) cells growing in androgen-depleted C/S media.

| Genes whose transcription is stimulated or repressed | AR+/AR− | AR-FL only/AR− | AR-V7 only/AR− | Function |
|-----------------------------------------------------|---------|----------------|---------------|----------|
| **Upregulated genes**                               |         |                |               |          |
| Both                                                |         |                |               |          |
| KLK3                                                | 8.5     | 11.2           | 20.9          | Prostate-specific serine-type endopeptidase (chymotrypsin) activity |
| NKX3-1                                              | 6.2     | 1.5            | 8.5           | Prostate-specific DNA-binding transcription factor |
| PPP3CA                                              | 4.8     | 2.6            | 4.6           | Calcineurin A protein phosphatase |
| PPAP2A                                               | 4.2     | 2.2            | 6.2           | Phospholipid phosphatase |
| GPC6                                                | 3.8     | 1.5            | 3.9           | Glycosylphosphatidylinositol-anchored heparan sulfate proteoglycan |
| BEX2                                                | 3.5     | 4.0            | 1.9           | Increases proliferation via the JNK/c-Jun pathway |
| NEDD4L                                               | 3.4     | 2.8            | 4.1           | E3 ubiquitin-ligase for TGFBR1 and Smad2 |
| BTG1                                                | 2.7     | 2.0            | 3.4           | Enzyme binding and transcription co-repressor activity |
| KLK4                                                | 2.5     | 1.8            | 2.0           | Serine-type endopeptidase (trypsin) activity and serine-type peptidase activity |
| SLC25A36                                             | 2.4     | 2.2            | 1.6           | Mitochondrial function through transporting pyrimidine nucleotides for mtDNA/RNA synthesis |
| TRPV6                                                | 1.9     | 1.6            | 1.9           | Calmodulin binding and calcium channel activity |
| FKBPs                                               | 1.7     | 1.6            | 1.7           | Peptidyl-prolyl cis-trans isomerase activity and FK506 binding |
| STEAP2                                               | 1.6     | 1.8            | 1.8           | Fe/Cu transporter activity and ferric-chelate reductase activity |
| UGT2B11                                              | 26.6    | 43.9           | 1.4           | Carbohydrate binding and glucuronosyltransferase activity |
| CD55                                                | 3.7     | 5.0            | 1.0           | Lipid binding and virus receptor activity |
| UGT2B15                                              | 2.7     | 4.3            | 1.1           | Carbohydrate binding and glucuronosyltransferase activity |
| CTAGE5                                               | 2.4     | 2.6            | 1.4           | Receptor in the endoplasmic reticulum required for collagen VII (COL7A1) secretion |
| GTPBP2                                               | 1.8     | 2.8            | 1.1           | GTP binding and GTPase activity |
| NEAT1                                                | 1.8     | 1.9            | 1.0           | Long non-coding RNA (IncRNA) |
| SLC38A1                                              | 1.7     | 1.7            | 1.2           | Neutral amino acid transmembrane transporter activity and amino acid: sodium symporter |
| BEST1                                                | 1.5     | 2.4            | 1.3           | Chloride channel activity |
| SLC43A1                                              | 1.5     | 1.8            | −1.3          | Sodium-independent, high affinity transport of large neutral amino acids |
| NCOA1                                                | 1.4     | 2.9            | −1.1          | Transcriptional co-activator for steroid and nuclear hormone receptors |
| **AR-FL only**                                       |         |                |               |          |
| UGT2B11                                              | 26.6    | 43.9           | 1.4           | Carbohydrate binding and glucuronosyltransferase activity |
| CD55                                                | 3.7     | 5.0            | 1.0           | Lipid binding and virus receptor activity |
| UGT2B15                                              | 2.7     | 4.3            | 1.1           | Carbohydrate binding and glucuronosyltransferase activity |
| CTAGE5                                               | 2.4     | 2.6            | 1.4           | Receptor in the endoplasmic reticulum required for collagen VII (COL7A1) secretion |
| GTPBP2                                               | 1.8     | 2.8            | 1.1           | GTP binding and GTPase activity |
| NEAT1                                                | 1.8     | 1.9            | 1.0           | Long non-coding RNA (IncRNA) |
| SLC38A1                                              | 1.7     | 1.7            | 1.2           | Neutral amino acid transmembrane transporter activity and amino acid: sodium symporter |
| BEST1                                                | 1.5     | 2.4            | 1.3           | Chloride channel activity |
| SLC43A1                                              | 1.5     | 1.8            | −1.3          | Sodium-independent, high affinity transport of large neutral amino acids |
| NCOA1                                                | 1.4     | 2.9            | −1.1          | Transcriptional co-activator for steroid and nuclear hormone receptors |
| **AR-V7 only**                                       |         |                |               |          |
| KLK2                                                | 3.7     | 1.0            | 14.0          | Serine-type endopeptidase (trypsin) activity |
| CALD1                                               | 2.5     | 1.4            | 2.6           | Actin- and myosin-binding protein |
| PRKD1                                               | 2.1     | 1.4            | 3.0           | Serine/threonine-protein kinase involved in the regulation of MAPK8/JNK1 |
| CD276                                                | 2.1     | 1.0            | 1.9           | Signaling receptor binding |
| SSF2A                                                | 1.8     | 1.0            | 2.3           | Structural integrity and/or signal transduction |
| GMNN                                                | 1.8     | 1.0            | 2.2           | Geminin DNA replication inhibitor |
| PRKCD                                                | 1.5     | 1.3            | 2.8           | Calcium-independent, phospholipid- and diacylglycerol (DAG)-dependent serine/threonine-protein kinase |
| IGFBP5                                              | 1.5     | −1.5           | 5.0           | Fibronectin binding and insulin-like growth factor I binding |
| GULP1                                                | 2.0     | 1.1            | 1.9           | Modulates cellular glycosphingolipid and cholesterol transport |
| **Downregulated genes**                             |         |                |               |          |
| Both                                                |         |                |               |          |
| NR3C1                                                | −7.1    | −9.4           | 5.4           | Glucocorticoid receptor |
| LDOC1                                                | −4.1    | −3.7           | −2.9          | Regulates the transcriptional response mediated by the nuclear factor kappa B |
| NR4A2                                                | −3.7    | −4.5           | −2.2          | DNA-binding transcription factor activity and protein heterodimerization activity |
| PLA2G2A                                              | −2.8    | −1.5           | −1.4          | Calcium ion binding and phospholipase A2 activity |
cells in Abi-equivalent castrated mice have no AR protein expression (Fig. 7a), and their growth is much slower than parental LN-95 cells expressing both AR-FL and AR-V7, but still faster than LNCaP (Fig. 7b). In contrast, growth of AR-FL KO cells retaining AR-V7 nuclear expression (Fig. 7a) is only minimally decreased in Abi-equivalent castrated mice (Fig. 7c). Growth of AR-V7 KO cells only expressing AR-FL is slower than in parental LN-95 and only slightly faster than total AR-KO cells (Fig. 7d).

### Discussion

The central question regarding the clinical significance of AR-V7 splice variant expression in CRPC is whether it is simply a marker of enhanced AR transcription characteristic of resistance to 2nd-generation ARSi like Abi and Enza or whether it has a functional role in driving such resistance. To address this issue, the present study utilized independently derived PDXs in which the genetic and phenotypic changes could be followed before and after the development of ARSi resistance. While all of the PDXs lacked PTEN expression, there is not a consistent requirement for mutation in TP53, RB1, BRCA2, PIK3CA, or MSH2, or expression of SOX2 or ERG and ARSi resistance. In contrast, the combined results document that elevated expression of AR-FL alone is sufficient for Abi but not Enza resistance. This is true even if AR-FL has a gain-of-function (GOF) mutation.

Enza resistance requires both high AR-FL expression plus a critical level of AR-V7 expression. This conclusion is supported by several previous publications. For example, when Enza-sensitive LNCaP cells are engineered to express a threefold higher level of AR-FL protein, but without AR-V7 expression raising their total normalized AR protein to 99-fold greater than normal, the in vitro and in vivo growth of these cells remained Enza-sensitive [18]. In fact, this is the basis for the clinical development of Enza as a 2nd-generation ARSi. An interesting corollary to these findings is that Abi resistance of the CWR22-RH PDX involves a 25-fold elevated expression of GOF double-mutated AR-FL compared to normal with no detectable expression of AR-V7 does not produce Enza resistance. In contrast, in another CWR22 variant (i.e., CWR22Rv1), there is a genomic alteration (i.e., exon 3 duplication) accompanying the gain of AR-V7 expression resulting in resistance to Enza [40]. In the current study, Enza resistance requires both a >50-fold increase in AR-FL and AR-V7 protein expression at a level that is approximately seven- to eightfold higher than AR-FL protein expression in normal prostate epithelium.

These results raise the question of the mechanism for enhanced AR-V7 expression in the lethal progression of CRPC. Along these lines, copy-number gains in the AR

### Table 2 (continued)

| Gene     | AR+/AR− | AR-FL only/AR− | AR-V7 only/AR− | Function                                                                 |
|----------|---------|----------------|----------------|--------------------------------------------------------------------------|
| GLI3     | −2.7    | −3.2           | −2.0           | Transcriptional activator and a repressor of the sonic hedgehog (Shh) pathway |
| ZKSCAN3  | −2.6    | −1.8           | −2.7           | Transcriptional repressor of autophagy                                    |
| GRB10    | −2.5    | −2.8           | −2.5           | SH3/SH2 adaptor suppress signals from insulin and insulin-like growth factor receptors. |
| GPC1     | −2.1    | −1.9           | −1.6           | Cell surface proteoglycan that inhibits FGF-mediated signaling            |
| FAM198B  | −2.3    | −4.7           | 1.4            | Golgi-associated kinase 1B                                                |
| SEMA6A   | −2.0    | −2.4           | 1.1            | Cell surface receptor for PLXNA2                                           |
| CAMK2N1  | −2.3    | −2.1           | −1.2           | Calcium/calmodulin-dependent protein kinase II inhibitor                   |
| HOXB13   | −1.8    | −1.6           | −1.3           | Homeobox B13, which regulates AR activity                                  |
| QSOX1    | −1.8    | −2.2           | 1.2            | Protein disulfide isomerase activity and flavin-linked sulfhydryl oxidase activity |
| CDK1     | −1.6    | −1.7           | −1.2           | Ser/Thr protein kinase                                                    |
| JAG1     | −1.4    | −3.2           | 1.4            | Ligand for notch 1 receptor                                               |
| SESN1    | −1.3    | −2.3           | 1.2            | Intracellular leucine sensor that negatively regulates the TORC1 signaling pathway |
| CAPNS1   | −1.2    | −1.6           | 1.4            | Calcium ion binding and calcium-dependent cysteine-type endopeptidase activity |
| PSD4     | −1.3    | −1.4           | 1.5            | Phospholipid binding and ARF guanyl-nucleotide exchange factor activity    |
| AR-V7 only | PRKACB | 1.4           | 1.1           | −10.9                      | Protein kinase cAMP-activated catalytic subunit beta                     |

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locus were reported more than 25 years ago as a major mechanism for resistance of metastatic prostate cancer to first-line ADT [41]. Recent studies determined that while rare in primary prostate cancer in hormonally naïve patients, AR amplification occurs in the majority of mCRPCs, which is coupled with an amplified tandem duplication of a non-coding AR enhancer element located 624 kb upstream of AR [42–44]. This makes such co-amplification the most common molecular change in mCRPC and provides a mechanistic basis for the significant elevation in AR mRNA expression in most, but not all, mCRPCs. This elevation in overall AR transcription may be sufficient, even without additional changes in efficiency of AR mRNA splicing, to produce adequate expression of AR-V7 mRNA, and thus protein to drive ARSi resistance. This is particularly possible since AR-V7 RNA is not a substrate of nonsense-mediated decay [45].

There are however, additional genomic alterations and mRNA splicing changes that have been suggested to effect regulatory mechanisms for AR-V7 expression in mCRPC [46]. Resolving how these alterations affect AR-V7 expression is critical for identifying therapies for preventing and/or inhibiting enhanced AR-V7 expression from driving lethal progression of CRPC. Along these
lines, the present LN-95 KO studies confirm earlier documentation [35, 39] that there is autoregulatory negative feedback between the level of ligand-dependent AR-FL transcription and AR-V7 expression. Such an autoregulatory negative feedback may explain paradoxical therapeutic response of prostate cancer patients resistant to 2nd-generation ARSi to bipolar androgen therapy (BAT) in which patients are rapidly cycled between a castrate to supraphysiologic level of T (SPT) [13]. In metastatic CRPC patients progressing on Enza, BAT results in resensitization when rechallenged with Enza [47]. This regaining of response to Enza is consistent with such SPT suppressing expression of AR-V7 thus preventing its transcriptional complementation with AR-FL transcriptional regulation needed for Enza resistance. Presently, this is being tested.

Materials and methods

Detailed procedures describing cell culture, proliferation assays, cytogenetic, genetic and epigenetic characterization, plasmid construction, and transfection of CRISPR-Cas9 vectors, isolation of clonal cell lines by FACS, RNaseq, Western blot analysis, IHC, animal studies, and statistical analyses are included in the Supplemental Materials and Methods document, including Supplementary Figs. 1–3 and Supplementary Tables 1 and 2.

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Compliance with ethical standards

Conflict of interest

ESA is a paid consultant/advisor to Janssen, Astellas, Sanofi, Dendreon, Pfizer, Amgen, AstraZeneca, Bristol Myers Squibb, Bayer, Clovis, and Merck; has received research funding (to his institution) from Janssen, Johnson & Johnson, Sanofi, Dendreon, Genentech, Novartis, Bristol Myers Squibb, AstraZeneca, Clovis, and Merck. ESA and JL are co-inventors of an AR-V7 biomarker technology that has been licensed to Qiagen.

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