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14. ABSTRACT

There has been a major focus on the androgen receptor (AR) pathway as the principal therapeutic target for CRPC including recently approved therapies such as next-generation antiandrogen enzalutamide and abiraterone. Despite these advances that provide temporary respite, almost all patients will go on to die from progressive and resistant prostate cancer. Therefore, there is an urgent need to identify resistant pathways that perpetuate disease progression. We provided preliminary data demonstrating that p52 increases AR variant V7 (AR-V7) expression and enhances prostate cancer cell resistance to next-generation antiandrogen enzalutamide treatment. We hypothesize that overexpression of p52 signaling activates resistance pathways to enzalutamide and co-targeting p52 will overcome treatment resistance. In this project, we will examine the potential mechanisms underlying p52-mediated treatment resistance (Aim 1). Aim 2 will validate the efficacy of co-targeting p52 to overcome treatment resistance to enzalutamide. We hope to identify the mechanisms of adaptive/resistant pathways that are responsible for enzalutamide resistance, and provide a rationale for therapeutic co-targeting to overcome enzalutamide resistance.

15. SUBJECT TERMS
P52, androgen receptor, androgen receptor variants, enzalutamide

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Introduction

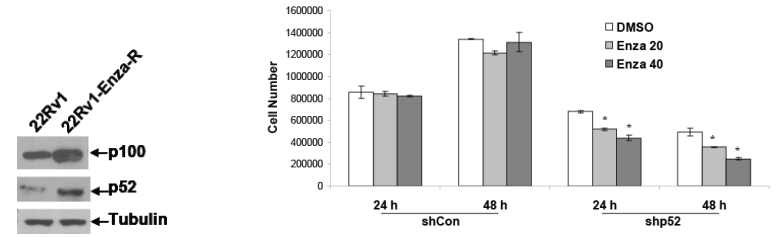
Background: Accumulating evidence suggests that abnormal activation of androgen receptor (AR) including AR variants such as AR-V7 contributes to castration-resistant prostate cancer (CRPC) growth. There has been a major focus on the androgen receptor (AR) pathway as the principal therapeutic target for CRPC including recently approved therapies such as next-generation antiandrogen enzalutamide and abiraterone. Despite these advances that provide temporary respite, almost all patients will go on to die from progressive and resistant prostate cancer. Therefore, there is an urgent need to identify resistant pathways that perpetuate disease progression during an effective AR blockade. NF-κB functions as a master transcription factor in regulating the expression of genes implicated in cell survival and chemo resistance. Numerous studies demonstrate that non-canonical NF-κB2/p52 (p52) is overexpressed in prostate cancer and overexpression of p52 facilitates CRPC progression through activating AR signaling and protecting cells from apoptotic death. We provided preliminary data demonstrating that p52 increases AR variant V7 (AR-V7) expression and enhances prostate cancer cell resistance to next-generation antiandrogen enzalutamide treatment.
Hypothesis: We hypothesize that overexpression of p52 signaling activates resistance pathways to enzalutamide and co-targeting p52 will overcome treatment resistance.
Specific aims: 1. Determine the potential mechanisms of p52-mediated treatment resistance in prostate cancer cells. 2. Co-targeting p52 to overcome treatment resistance to enzalutamide.

Keywords
NF-κB2/p52, Androgen receptor, Variants, enzalutamide, resistance

Accomplishments

We have made significant progress in Major Task 1: *Determining the potential mechanisms of p52-mediated treatment resistance* To understand the molecular mechanisms that may be responsible for enzalutamide resistance, we demonstrate that NF-κB2/p52 (p52) may play a crucial role in the development of resistance to enzalutamide and that the interplay between p52 and the AR signaling axis may be one of the underlying mechanisms. We showed that C4-2B and CWR22Rv1 cells chronically treated with enzalutamide were found to express higher levels of NF-κB2/p52. Downregulation of NF-κB2/p52 in CWR22Rv1 cells chronically treated with enzalutamide rendered them more sensitive to cell growth inhibition by enzalutamide. Analysis of the expression levels of AR splice variants by qRT-PCR and Western blotting revealed that LNCaP cells expressing p52 exhibit higher expression of AR splice variants. Downregulation of expression of NF-κB2/p52 in VCaP and CWR22Rv1 cells by shRNA abolished expression of splice variants. Downregulation of expression of either full length AR or the splice variant AR-V7 led to an increase in sensitivity of CaP cells to enzalutamide. These results collectively demonstrate that resistance to enzalutamide may be mediated by NF-κB2/p52 via activation of AR and its splice variants.

As illustrated in **Fig 1**, we examined the expression levels of NF-κB2/p52 in resistant cells by qRT-PCR and by Western blotting. CWR22Rv1 cells treated chronically with enzalutamide exhibited higher levels of both precursor p100 as well as p52, indicating that CaP cells resistant to enzalutamide may upregulate the endogenous levels of NF-κB2/p52. To test whether downregulation of p52 resensitizes these cells to enzalutamide, we transfected shRNAs specific to p52 into CWR22Rv1 cells treated chronically with enzalutamide (expressing higher levels of p52) and examined cell growth after 24 and 48 h. Downregulation of p52 after transfection was confirmed by qRT-PCR. As shown in **Fig. 1B**, cells transfected with p52 shRNA were increasingly sensitive to enzalutamide compared to control CWR22Rv1-Enza-R cells, indicating that expression of p52 may be necessary for the survival of cells treated chronically with enzalutamide. These results collectively demonstrate that NF-κB2/p52 may regulate the induction of resistance to enzalutamide in CaP cells.



A B

Fig 1. CaP cells treated chronically with Enzalutamide upregulate the expression of NF-kB2/p52. **A**) CWR22Rv1 cells treated chronically with Enzalutamide exhibit higher endogenous levels of both p100 and p52. **B**) CWR22Rv1 cells treated chronically with Enzalutamide were transfected with either control shRNA or shRNA against NF-kB2/p52 and were treated with 0, 20 or 40 μM Enzalutamide. Cell numbers were counted after 24 and 48 h. Results are presented as means±SD of 2 experiments performed in triplicate. * denotes $P \leq 0.05$. Cells transfected with shRNA against p52 exhibited lower cell survival when treated with Enzalutamide.

We have made significant progress in Major Task 2 and 3: Determining the potential mechanisms underlying p52-mediated AR-V7 production and whether hnRNP A1 and hnRNP A2 are responsible for AR-V7 generation. We demonstrated that NF-kappaB2/p52 regulates AR-V7 expression via hnRNPA1 and c-Myc

We reported earlier that activation of NF-kappaB2/p52 promotes progression to CRPC and enzalutamide resistance via the generation of AR variants, specifically AR-V7. Our previous findings also indicated that NF-kappaB2/p52 may regulate c-Myc expression. Hence, we examined whether NF-kappaB2/p52 plays a role in the elevated expression of hnRNPA1 and c-Myc in PCa using lysates from LNCaP cells stably expressing p52 (LN-p52). Protein levels of AR-V7, hnRNPA1 and c-Myc were elevated in LN-p52 cells, while no appreciable differences were found in the expression of hnRNPA2 (Fig. 5A, left panel). These results were confirmed using LNCaP cells expressing p52 under the control of a Tet-inducible promoter (LN/TR/p52). Induction of p52 expression led to increases in expression levels of AR-V7, hnRNPA1 and c-Myc (Fig. 2A, right panel), indicating that upregulation of AR-V7 by p52 may be mediated by hnRNPA1 and c-Myc. To examine the relationship between hnRNPA1, c-Myc and AR-V7 in LN-p52 cells, we analyzed levels of AR-V7 by Western blotting in LN-p52 cells transfected with hnRNPA1 or hnRNPA2 siRNAs. Downregulation of hnRNPA1 abrogated the expression of AR-V7 in LN-p52 cells, while downregulation of hnRNPA2 did not have an appreciable effect on AR-V7 protein levels (Fig. 2B, left panel). Protein

levels of c-Myc were also downregulated. In addition, expression of hnRNPA1 and AR-V7 were abolished as a result of downregulation of c-Myc expression in LN-p52 cells

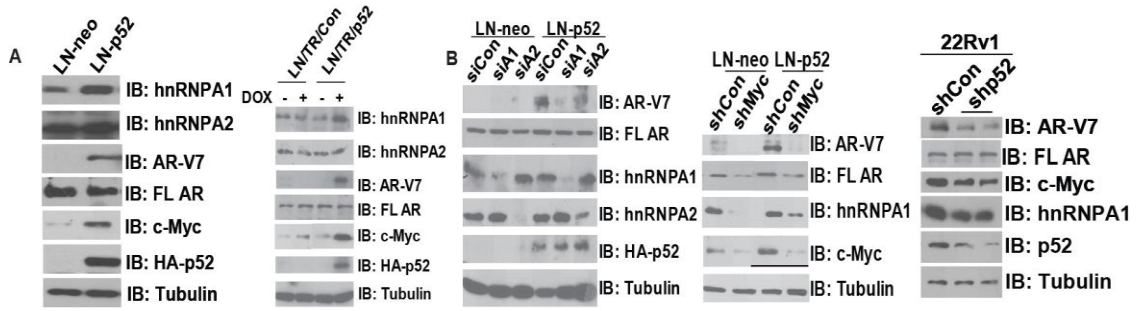


Fig 2. NF-kappaB2/p52 regulates expression of c-Myc and hnRNPA1. A) *Left panel*, Immunoblotting for hnRNPA1, c-Myc and AR-V7 in LN-p52 cells. *Right panel*, Immunoblotting for hnRNPA1, c-Myc and AR-V7 in LN/TR/p52 cells. B) *Left panel*, Western blotting for AR-V7 in LN-p52 cells transfected with hnRNPA1 siRNA. *Middle panel*, Immunoblotting for AR-V7 and hnRNPA1 in LN-p52 cells transfected with c-Myc shRNA. *Right panel*, Immunoblotting for AR-V7, hnRNPA1 and c-Myc in 22Rv1 cells transfected with p52 shRNA.

transfected with c-Myc shRNA (Fig. 2B, middle panel).

To confirm these findings in a cell line with constitutive expression of both p52 and AR-V7, we analyzed levels of AR-V7, hnRNPA1 and c-Myc by immunoblotting in 22Rv1 cells transfected with p52 shRNA. Downregulation of p52 in 22Rv1 cells abrogated expression of AR-V7, hnRNPA1 and c-Myc (Fig. 2B, right panel). These results demonstrate that NF-kappaB2/p52 may modulate generation of AR-Vs by regulation of hnRNPA1 and c-Myc.

We also demonstrated that PCa cells resistant to enzalutamide exhibit higher levels of splicing factors

As our results demonstrate that expression of hnRNPA1 and AR variants may be positively correlated with each other in PCa cells, and AR-V7 expression has been shown to be involved in the acquisition of resistance to enzalutamide, we tested the correlation between levels of AR variants and hnRNPA1 in PCa cells that have acquired resistance to enzalutamide. 22Rv1-Enza-R and C4-2B-Enza-R cell lines exhibited higher levels of AR-V7 and hnRNPA1 (Fig. 3A), indicating that expression of hnRNPA1 may be positively correlated with expression of AR-Vs. Furthermore, expression levels of c-Myc and NF-kappaB2/p52 were also elevated in enzalutamide-resistant cells, confirming the importance of the NF-kappaB2/p52:c-Myc:hnRNPA1:AR-V7 axis in enzalutamide resistance. No significant differences were observed in the expression of hnRNPA2 in 22Rv1-Enza-R and C4-2B-Enza-R cells compared to their parental cells (Fig. 3A right & middle panels). To confirm these results *in vivo*, we analyzed extracts from xenograft tumors derived from C4-2B and C4-2B-Enza-R cells using antibodies against AR-V7 and hnRNPA1. Higher levels of AR-V7 were observed in xenografts derived from C4-2B-Enza-R cells, which was correlated well with higher levels of hnRNPA1 and c-Myc (Fig. 3A right panel), confirming our observations that expression of AR-Vs is positively correlated with expression of hnRNPA1 in PCa cell lines resistant to enzalutamide.

Next, we tested whether downregulation of hnRNPA1 affects endogenous levels of AR-Vs in 22Rv1-Enza-R cells. Transfection of hnRNPA1 siRNA abrogated levels of

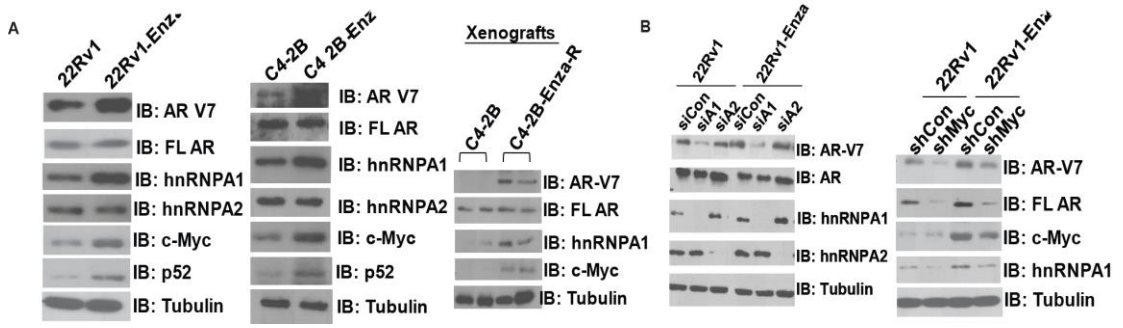


Fig 3. A) *Left panel*, 22Rv1 cells resistant to enzalutamide (22Rv1-Enza-R) express higher levels of AR-V7, hnRNPA1, c-Myc and NF-kappaB2/p52. *Middle panel*, C4-2B cells resistant to enzalutamide (C4-2B-Enza-R) express higher levels of hnRNPA1, AR-V7, c-Myc and NF-kappaB2/p52. *Right panel*, Xenografts from C4-2B-Enza-R cells exhibit higher levels of AR-V7, hnRNPA1 and c-Myc. B) *Left panel*, Western analysis of AR-V7 in 22Rv1-Enza-R cells transfected with hnRNPA1 siRNA. *Right panel*, Expression of AR-V7 and hnRNPA1 in 22Rv1-Enza-R cells transfected with c-Myc shRNA. All results are shown as representative images from 2 experiments performed in duplicate.

AR-V7 in 22Rv1-Enza-R cells (Fig. 3B left panel). Similarly, downregulation of c-Myc by specific shRNA reduced expression levels of AR-V7 and hnRNPA1 in 22Rv1 and 22Rv1-Enza-R cells (Fig. 3B right panel), confirming the c-Myc: hnRNPA1: AR-V7 axis in PCa cells.

Key outcomes:

- We demonstrated that p52 enhances enzalutamide resistance.
- We demonstrated that p52 induces ARv7 expression.
- We demonstrated that p52 induced enzalutamide resistance is mediated by ARv7.
- We demonstrated that NF-kappaB2/p52 regulates AR-V7 expression via hnRNPA1 and c-Myc
- We demonstrated that enzalutamide resistant cells express higher levels of ARv7, hnRNPA1, and c-Myc.

Impact

This proposes studies will not only uncover a novel pathway involved in resistant CRPC development, but may also provide proof-of-concept experiments for future development of therapies targeting resistant pathways that are responsible for acquired treatment resistance, and to increase the magnitude and duration of the benefits of second-generation antiandrogen.

Changes/problems
N/A

Products

Publications:

1. Nadiminty N, Tummala R, Liu C, Yang J, Lou W, Evans CP, **Gao AC**. NF-kappaB2/p52 induces resistance to Enzalutamide in Prostate Cancer: Role of androgen receptor and its variants. Mol Cancer Ther, 2013. 12: p. 1629-1637. PMCID:PMC3941973
2. Nadiminty N., Tummala R, Liu C, Lou W, Evans CP, **Gao AC**. NF-kB/p52:c-myc:hnRNPA1 pathway regulates expression of androgen receptor splice variants and enzalutamide sensitivity in prostate cancer. Mol Cancer Ther, 2015 Aug;14 (8):1884-95. PMID:26056150

Participants & other collaborating organizations

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Special reporting requirements

Appendices

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Molecular Cancer Therapeutics

NF-κB2/p52 Induces Resistance to Enzalutamide in Prostate Cancer: Role of Androgen Receptor and Its Variants

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Molecular Cancer Therapeutics

NF-κB2/p52 Induces Resistance to Enzalutamide in Prostate Cancer: Role of Androgen Receptor and Its Variants

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Abstract

Resistance of prostate cancer cells to the next-generation antiandrogen enzalutamide may be mediated by a multitude of survival signaling pathways. In this study, we tested whether increased expression of NF-κB2/p52 induces prostate cancer cell resistance to enzalutamide and whether this response is mediated by aberrant androgen receptor (AR) activation and AR splice variant production. LNCaP cells stably expressing NF-κB2/p52 exhibited higher survival rates than controls when treated with enzalutamide. C4-2B and CWR22Rv1 cells chronically treated with enzalutamide were found to express higher levels of NF-κB2/p52. Downregulation of NF-κB2/p52 in CWR22Rv1 cells chronically treated with enzalutamide rendered them more sensitive to cell growth inhibition by enzalutamide. Analysis of the expression levels of AR splice variants by quantitative reverse transcription PCR and Western blotting revealed that LNCaP cells expressing p52 exhibit higher expression of AR splice variants. Downregulation of expression of NF-κB2/p52 in VCaP and CWR22Rv1 cells by short hairpin RNA abolished expression of splice variants. Downregulation of expression of either full-length AR or the splice variant AR-V7 led to an increase in sensitivity of prostate cancer cells to enzalutamide. These results collectively demonstrate that resistance to enzalutamide may be mediated by NF-κB2/p52 via activation of AR and its splice variants. *Mol Cancer Ther; 12(8); 1–9. ©2013 AACR.*

Introduction

Localized prostate cancer is dependent on androgens, and the majority of patients respond to androgen ablation. However, virtually every patient will develop castration-resistant prostate cancer (CRPC) and no longer respond to androgen deprivation therapy (ADT). Persistent androgen receptor (AR) activation remains an important player in CRPC progression. CRPC cells often continue to express AR and AR axis genes (1, 2), implying that the AR is active in AR-positive CRPC cells. Such observations form the basis for continued attempts to target the AR axis and for the development of next-generation antiandrogens such as enzalutamide (formerly MDV3100). Enzalutamide binds to the AR with greater affinity than bicalutamide and inhibits its nuclear translocation and expression of its target genes (3). Despite initial success, development of resistance is a contraindication for its use in many patients, and as demographics change, an increasing number of patients are likely to develop resistance to enzalutamide. The mechanisms leading to resistance have been poorly understood, even though a recent report showed that AR splice variants play a major role in development of resistance (4). AR splice variants lack the ligand-binding domain targeted by enzalutamide and variants such as AR-V7 are postulated to be constitutively active. The mechanistic aspects of regulation of variant expression leading to resistance against enzalutamide are unknown. Therefore, an urgent need exists to fully understand the mechanisms of resistance and to devise ways to overcome them.

The classical NF-κB pathway involving the p65/p50 heterodimer has been shown to be constitutively activated in several cancers including prostate cancer (5). The non-canonical NF-κB pathway involves the processing of p100 to NF-κB2/p52 via the recruitment of NF-κB–inducing kinase (NIK) and subsequent activation of IκB kinase α (IKKα). The processing of p100 to p52 is a tightly controlled event in many cells and tissues (6–9). The functional significance of p100 processing has been confirmed by genetic evidence from humans and mice (10). Overproduction of p52 has been observed in several solid tumors including breast and prostate cancers (11, 12). Our previous studies showed that NF-κB2/p52 induces castration-resistant growth in LNCaP cells (13), that several genes involved in processes such as cell growth,

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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proliferation, cell movement are potential targets of NF-κB2/p52 (14), and that NF-κB2/p52 induces aberrant activation of the AR in a ligand-independent manner and thus promotes castration resistance (15).

In this study, we report that NF-κB2/p52 promotes resistance of prostate cancer cells to enzalutamide. We show that increased resistance of prostate cancer cells expressing p52 to enzalutamide may be mediated by induction of AR splice variants (such as AR-V7) and by activation of the AR axis by p52.

Materials and Methods

Cell lines and reagents

LNCaP, CWR22Rv1, and VCaP cells were obtained from the American Type Culture Collection (ATCC). All experiments with cell lines were performed within 6 months of receipt from ATCC or resuscitation after cryopreservation. ATCC uses short tandem repeat (STR) profiling for testing and authentication of cell lines. C4-2B cells were kindly provided and authenticated by Dr. Leland Chung, Cedars-Sinai Medical Center, Los Angeles, CA. Cells were cultured in RPMI containing either 10% complete FBS or 10% charcoal/dextran-stripped FBS (CS-FBS) and penicillin/streptomycin. LNCaP passage numbers less than 20 were used throughout the study. VCaP cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. NF-κB2/p52 (K-27), AR (441; mouse monoclonal), hemagglutinin (HA), and tubulin antibodies were purchased from Santa Cruz Biotechnologies. Antibodies against AR-V7 splice variant were kindly provided by Dr. Jun Luo (Department of Urology, Johns Hopkins University, Baltimore, MD). All other reagents were of analytical grade and obtained from local suppliers. Sso Fast Eva Green qPCR Supermix was from Bio-Rad.

Generation of stable cell lines

Stable cell lines of LNCaP expressing NF-κB2/p52 (LN-p52) were generated by transfection of plasmids containing the cDNA and selection of clones after application of selective pressure with appropriate antibiotics. LNCaP cells expressing p52 under the control of a tetracycline-inducible cassette (LN/TR/p52) were generated using the ViraPower lentiviral transduction system (Invitrogen).

Cell growth assays

Cells were transfected with plasmids or treated with the indicated reagents, and viable cell numbers were determined at various time points using a Coulter cell counter.

Western blot analysis

Cells were lysed in high-salt buffer containing 50 mmol/L HEPES, pH 7.9, 250 mmol/L NaCl, 1 mmol/L EDTA, 1% NP-40, 1 mmol/L phenylmethylsulfonylfluoride (PMSF), 1 mmol/L Na vanadate, 1 mmol/L NaF, and protease inhibitor cocktail (Roche) as described earlier (16). Total protein was estimated using the Coomassie Protein Assay Reagent (Pierce). Equal amounts of protein were loaded on 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in PBST (1× PBS + 0.1% Tween-20) and probed with primary antibodies in 1% bovine serum albumin (BSA). The signal was detected by ECL (GE Healthcare) after incubation with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies.

Real-time quantitative reverse transcription PCR

Total RNAs were extracted using TRIzol reagent (Invitrogen). cDNAs were prepared after digestion with RNase-free RQ1 DNase (Promega). The cDNAs were subjected to real-time reverse transcription PCR (RT-PCR) using Sso Fast Eva Green Supermix (Bio-Rad) according to the manufacturer's instructions and as described previously (15). Each reaction was normalized by coamplification of actin. Triplicates of samples were run on default settings of Bio-Rad CFX-96 real-time cycler.

Clonogenic assays

Anchorage-dependent clonogenic ability assays were performed as described previously (13). Briefly, cells were seeded at low densities (400 cells/dish) in 10-cm culture plates. The plates were incubated at 37°C in media containing either 10% FBS or 10% charcoal-stripped FBS (CS-FBS) and were left undisturbed for 14 days. At the end of the experiment, cells were fixed with methanol, stained with crystal violet, and the numbers of colonies were counted.

Luciferase assays

Cells were transfected with reporters along with plasmids and AR and AR-V7 siRNAs as indicated in the figures. Cell lysates were subjected to luciferase assays with the Luciferase Assay System (Promega).

Statistical analyses

Data are shown as means ± SD. Multiple group comparison was performed by one-way ANOVA followed by the Scheffe procedure for comparison of means. $P \leq 0.05$ was considered significant.

Results

Prostate cancer cells expressing NF-κB2/p52 are resistant to enzalutamide and bicalutamide

LN-neo (LNCaP cells expressing the empty vector) and LN-p52 cells (LNCaP cells stably expressing p52) were treated with 0 and 20 μmol/L enzalutamide or bicalutamide in media containing either complete FBS or charcoal-stripped FBS, and cell growth was examined after 48 hours. Dimethyl sulfoxide (DMSO) was used as the vehicle control. As shown in Fig. 1A, cells stably expressing p52 exhibited better cell survival ability when exposed to enzalutamide or bicalutamide compared to control LN-neo cells. To confirm these experiments, we treated LN-neo or LN-p52 cells with 0, 20, and 40 μ mol/L enzalutamide or bicalutamide and performed clonogenic assays. As shown

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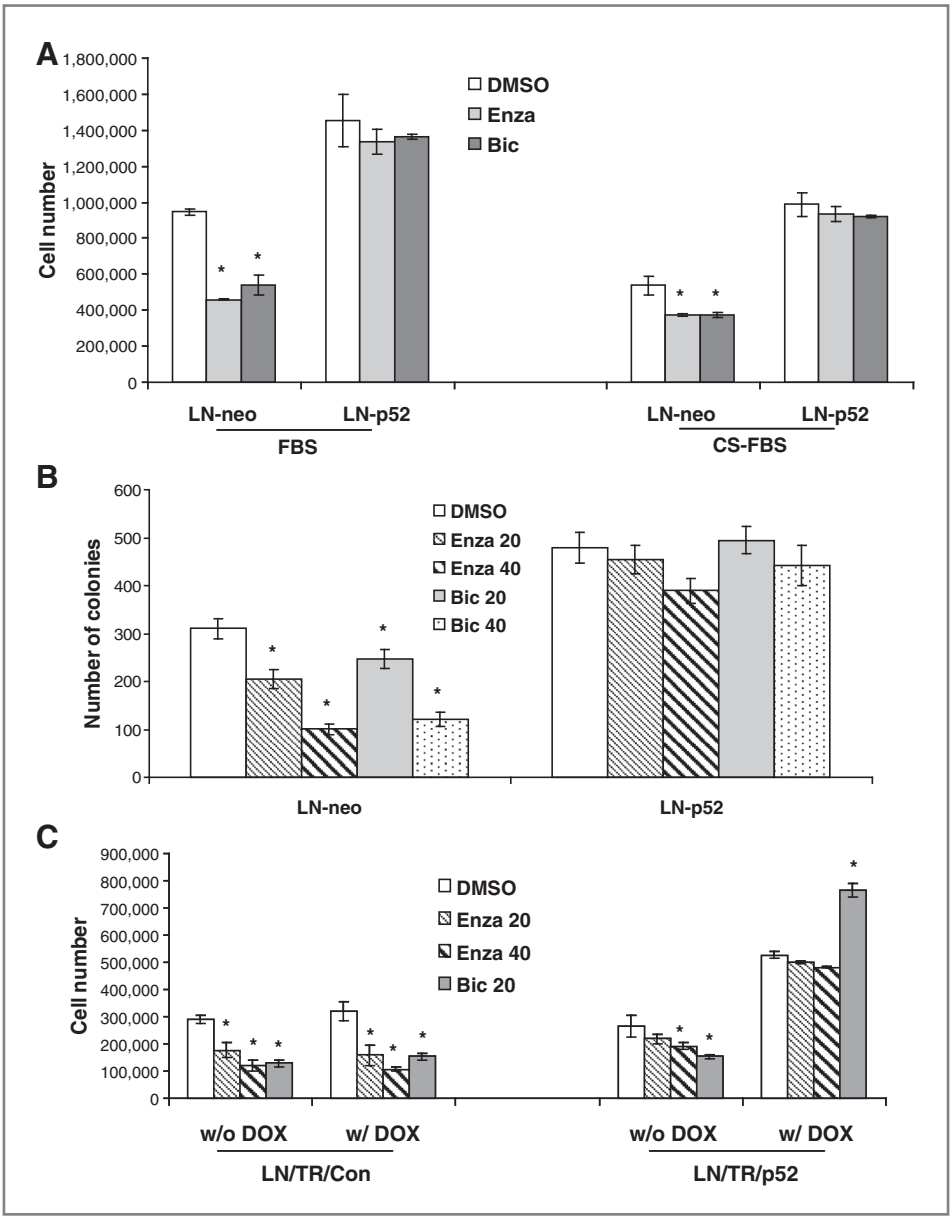

Figure 1. NF-κB2/p52-expressing prostate cancer cells are resistant to enzalutamide. A, LNCaP cells stably expressing p52 (LN-p52) and control LNCaP cells (LN-neo) were treated with 0 and 20 μmol/L enzalutamide or bicalutamide in media containing either FBS or CS-FBS, and cell numbers were counted after 48 hours. Results are presented as mean ± SD of 3 experiments performed in triplicate. LN-p52 cells exhibited higher survival rates when treated with enzalutamide or bicalutamide than LN-neo cells. B, LN-neo and LN-p52 cells were treated with 0, 20, or 40 μmol/L enzalutamide or bicalutamide, and clonogenic assays were performed. Results are presented as mean ± SD of 2 experiments performed in triplicate. LN-p52 cells formed higher numbers of colonies than LN-neo cells when treated with enzalutamide or bicalutamide. C, LN/TR/p52 cells [expressing p52 under the control of a tetracycline (tet)-inducible promoter] and control LN/TR/Con cells were treated with 0, 20, or 40 μmol/L enzalutamide or 20 μmol/L bicalutamide in the presence or absence of 0.5 μmol/L doxycycline (DOX), and cell numbers were counted after 48 hours. Results are presented as mean ± SD of 3 experiments performed in triplicate. *, $P \leq 0.05$. LN/TR/p52 cells displayed higher survival rates when p52 expression was induced with DOX, compared to uninduced LN/TR/p52 cells as well as LN/TR/Con cells.

in Fig. 1B, LN-neo cells were highly sensitive to both enzalutamide and bicalutamide and formed fewer colonies, whereas the number of colonies formed by cells expressing p52 was significantly higher, indicating that NF-κB2/p52 may induce resistance to enzalutamide and bicalutamide in prostate cancer cells. To further confirm these results, we used the tetracycline-inducible system to induce p52 expression in LNCaP cells and tested enzalutamide and bicalutamide sensitivity. We treated LN/TR/Con and LN/TR/p52 cells with 0, 20, and 40 μ mol/L enzalutamide or bicalutamide and performed growth assays. As shown in Fig. 1C, induction of expression of p52 by doxycycline significantly enhanced the ability of LN/TR/p52 cells to survive in the presence of enzalutamide or bicalutamide compared to control LN/TR/Con cells. These results collectively demonstrate that prostate cancer cells expressing higher levels of NF-κB2/p52 are more resistant to enzalutamide and bicalutamide compared to cells which do not express p52.

Prostate cancer cells chronically treated with enzalutamide exhibit higher levels of NF-κB2/p52

Our previous studies showed that most androgen-dependent prostate cancer cell lines do not express detectable levels of endogenous NF-κB2/p52 (13). Hence, to test whether prostate cancer cells resistant to enzalutamide exhibit higher levels of p52, we treated CWR22Rv1 cells with 5 to 10 μ mol/L enzalutamide chronically for more than 10 months. The resultant cells showed higher cell survival rates when treated with enzalutamide. We examined the expression levels of NF-κB2/p52 in these cells by quantitative RT-PCR (qRT-PCR) and Western

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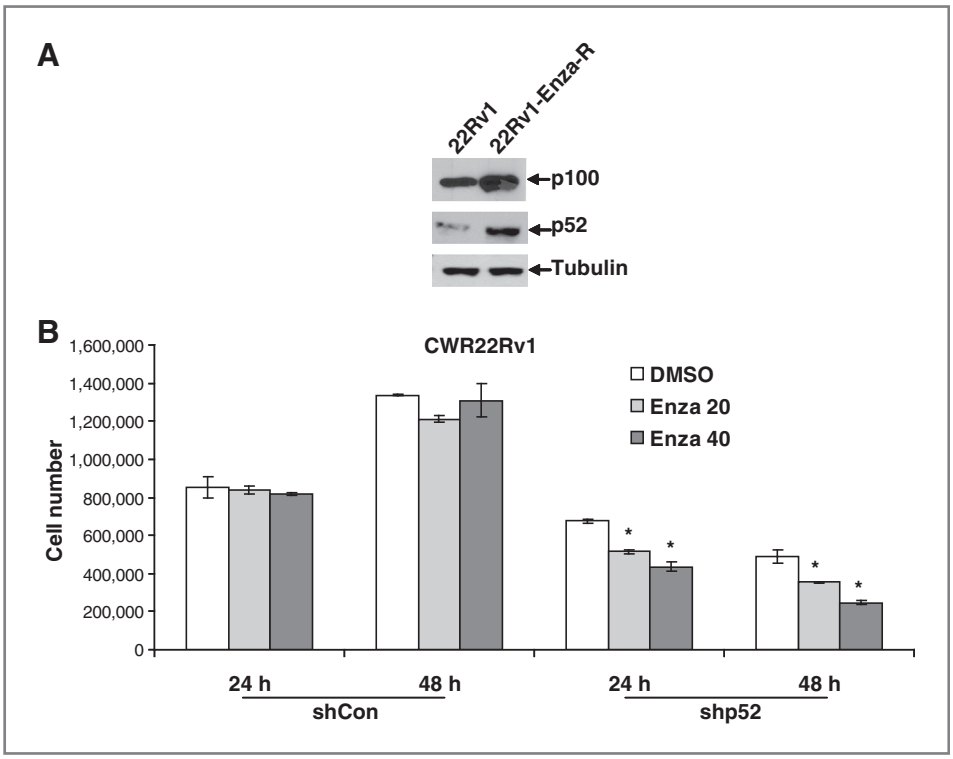

Figure 2. Prostate cancer cells treated chronically with enzalutamide upregulate the expression of NF-κB2/p52. A, CWR22Rv1 cells treated chronically with enzalutamide exhibit higher endogenous levels of both p100 and p52. B, CWR22Rv1 cells treated chronically with enzalutamide were transfected with either control shRNAs or shRNAs against NF-κB2/p52 and were treated with 0, 20, or 40 μmol/L enzalutamide. Cell numbers were counted after 24 and 48 hours. Results are presented as mean ± SD of 2 experiments performed in triplicate. *, $P \leq 0.05$. Cells transfected with shRNAs against p52 exhibited lower cell survival when treated with enzalutamide.

blotting. As shown in Fig. 2A, CWR22Rv1 cells treated chronically with enzalutamide exhibited higher levels of both precursor p100 as well as p52, indicating that prostate cancer cells resistant to enzalutamide may upregulate the endogenous levels of NF-κB2/p52. To test whether downregulation of p52 resensitizes these cells to enzalutamide, we transfected short hairpin RNAs (shRNA) specific to p52 into CWR22Rv1 cells treated chronically with enzalutamide (expressing higher levels of p52) and examined cell growth after 24 and 48 hours. Downregulation of p52 after transfection was confirmed by qRT-PCR. As shown in Fig. 2B, cells transfected with p52 shRNA were increasingly sensitive to enzalutamide compared to control CWR22Rv1-Enza-R cells, indicating that expression of p52 may be necessary for the survival of cells treated chronically with enzalutamide. These results collectively demonstrate that NF-κB2/p52 may regulate the induction of resistance to enzalutamide in prostate cancer cells.

NF-κB2/p52 enhances expression of AR splice variants

It has been shown that higher levels of AR splice variants may be responsible for the resistance to enzalutamide in prostate cancer (4), hence, we tested whether NF-κB2/p52 regulates the expression of AR splice variants. Total RNAs from LNCaP and C4-2B cells transfected with either empty vector or p52 in media containing either complete or charcoal-stripped FBS (CS-FBS) were analyzed by qRT-PCR for the expression levels of full-length (FL) AR as well as the major splice variant AR-V7. As shown in Fig. 3A, expression of p52 enhanced the expression levels of the splice variant AR-V7 in both FBS and CS-FBS, whereas expression of FL AR remained unchanged in LNCaP cells (left). These results were confirmed by Western blotting using antibodies specific for FL AR and AR-V7 (right). Similar results were observed in C4-2B cells, in which expression of p52 enhanced the expression levels of AR-V7, whereas expression levels of FL AR were unaffected (Fig. 3B). To substantiate these results, we examined expression levels of FL AR and AR-V7 in LN-neo and LN-p52 cells by qRT-PCR and Western blotting and found that expression levels of AR-V7 were elevated in LN-p52 cells compared to LN-neo cells (Fig. 3C). We also analyzed expression levels of AR-V7 in xenografts of LNCaP cells expressing p52 and found that xenografts expressing p52 exhibited significantly higher levels of AR-V7 mRNA compared to control LNCaP cell xenografts (Fig. 3D). These findings show that NF-κB2/p52 may induce upregulation of the expression of AR-V7.

Downregulation of NF-κB2/p52 abrogates expression of AR splice variants

Next, we tested whether NF-κB2/p52 was necessary for the enhanced expression of AR splice variants. VCaP and CWR22Rv1 prostate cancer cells express endogenous levels of AR splice variants AR-V1, AR-V5, AR-V7, AR-1/2/2b, and AR-1/2/3/2b. We transfected shRNA specific to p52 into VCaP and CWR22Rv1 cells and examined the expression levels of these splice variants by qRT-PCR using specific primers. As shown in Fig. 4A and B (left), downregulation of p52 reduced the expression levels of most of the splice variants significantly, whereas levels of

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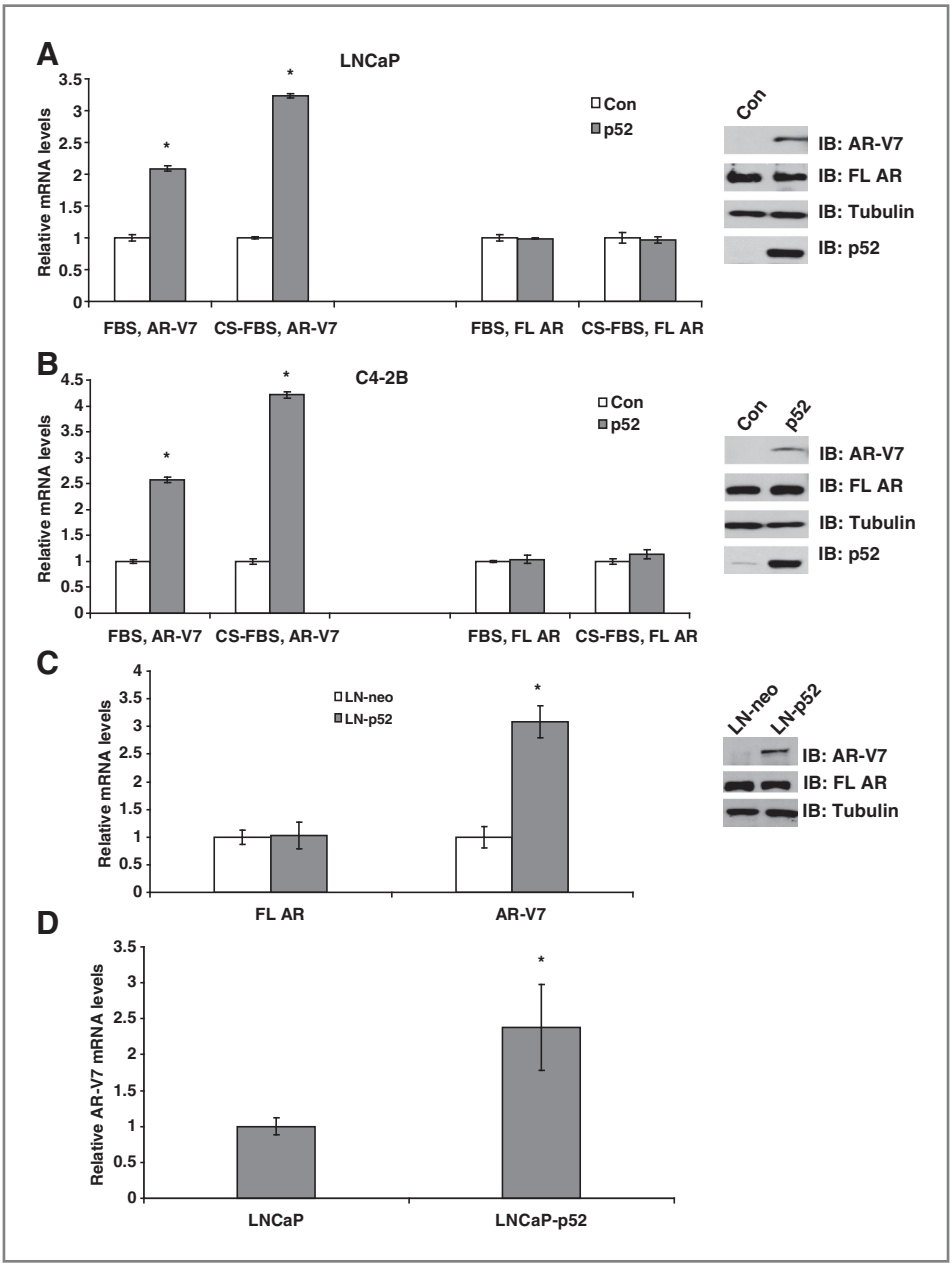

Figure 3. NF-κB2/p52 induces higher expression of AR splice variants. Total RNAs from LNCaP (A) and C4-2B (B) cells transfected with empty vector or p52 were analyzed by qRT-PCR for the expression of FL AR and AR-V7 in media containing either FBS or CS-FBS. Expression of p52 enhanced the levels of AR-V7, whereas levels of FL AR remained unchanged. Right, immunoblotting of above lysates with antibodies specific against either FL AR or AR-V7. C, total RNAs from LN-p52 and LN-neo cells were analyzed by qRT-PCR for the expression levels of FL AR or AR-V7. LN-p52 cells showed higher levels of expression of AR-V7 compared to LN-neo cells, whereas FL AR levels were unaffected. Right, immunoblotting of above lysates with antibodies against FL AR or AR-V7. D, expression levels of AR-V7 were enhanced in xenografts from LNCaP cells expressing p52 compared to xenografts from parental LNCaP cells. Results are presented as mean ± SD of 2 experiments performed in triplicate. *, $P \leq 0.05$.

FL AR remained unaffected. These results were confirmed for AR-V7 expression by Western blotting using antibodies specific against AR-V7 and FL AR in VCaP and CWR22Rv1 cells (Fig. 4A and B, right), indicating that expression of p52 may be necessary for the synthesis of AR splice variants.

Downregulation of FL AR and AR-V7 increases sensitivity of p52-expressing prostate cancer cells to enzalutamide

LNCaP cells stably expressing p52 (LN-p52) exhibit higher levels of AR-V7. We also assessed expression levels of other members of the NF-κB family by Western blotting and found that their levels were not altered (Supplementary Fig. S1), indicating that the effect on AR-V7 expression was mainly due to the expression of NF-κB2/p52. Next, we analyzed expression levels of antiapoptotic proteins such as Bcl-xL, survivin, and cyclin D1 in LN-p52 cells compared to LN-neo cells and found that LN-p52 cells express higher levels of Bcl-xL, survivin, and cyclin D1 (Supplementary Fig. S2), indicating that activation of antiapoptotic genes may play an important role in resistance against enzalutamide. As reported previously, the cells also exhibit aberrant activation of AR in the absence of androgen and exhibit castration-resistant growth (15). In the current study, we also showed that LN-p52 cells are resistant to enzalutamide-induced growth inhibition compared to control LN-neo cells. Hence, to test whether

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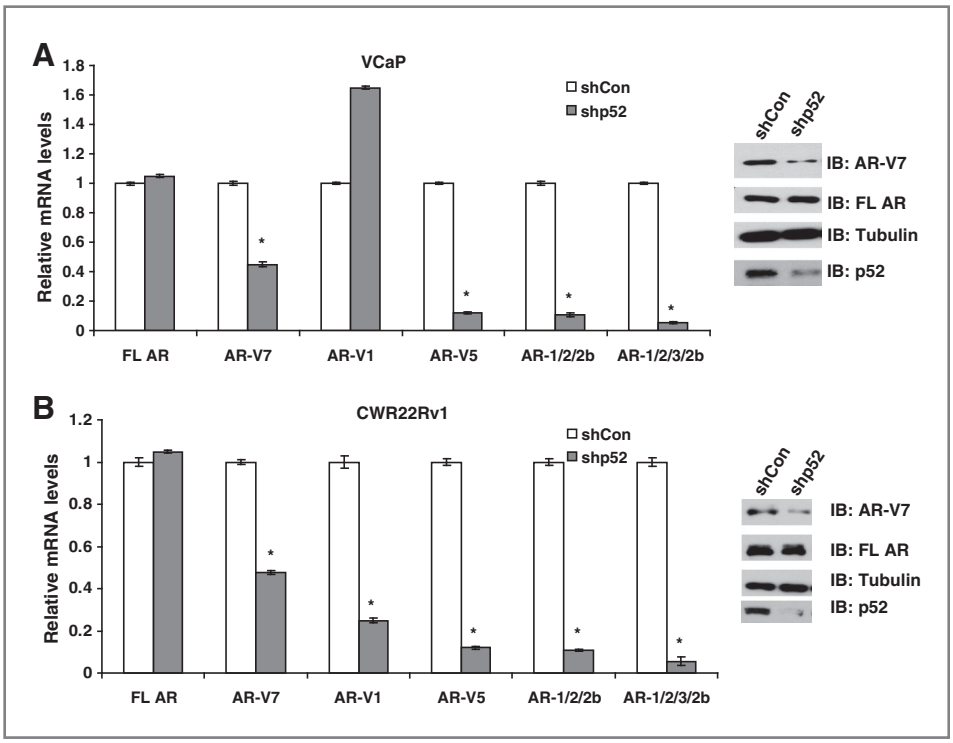

Figure 4. Downregulation of NF-κB2/p52 in prostate cancer (CaP) cells reduces expression of AR splice variants. VCaP (A) and CWR22Rv1 (B) cells were transfected with either control shRNAs or shRNAs against p52 and expression levels of the indicated AR splice variants were analyzed by qRT-PCR. Downregulation of p52 led to a decrease in synthesis of AR splice variants, whereas expression levels of FL AR remained unchanged. Right panels show immunoblots of above lysates with antibodies against FL AR or AR-V7. Results are presented as mean ± SD of 3 experiments performed in triplicate. *, $P \leq 0.05$.

FL AR or AR-V7 plays a role in the p52-induced resistance to enzalutamide, we transfected siRNAs specific against either FL AR or AR-V7 into LN-neo and LN-p52 cells and monitored cell growth in response to enzalutamide. As shown in Fig. 5A, downregulation of either FL AR or AR-V7 reduced growth of control LN-neo cells by ∼20%, and enzalutamide itself reduced growth of LN-neo cells by ∼50%. No additional reduction of growth was observed in LN-neo cells when FL AR or AR-V7 was downregulated in the presence of enzalutamide, showing that inhibition of either FL AR or AR-V7 had no effect on the sensitivity of LN-neo cells to enzalutamide. In LN-p52 cells which express higher levels of AR-V7, downregulation of either FL AR or AR-V7 reduced growth by ∼50% in the presence of enzalutamide, thus resensitizing LN-p52 cells to enzalutamide. In other words, LN-p52 cells are more sensitive to enzalutamide when expression of either FL AR or AR-V7 was inhibited. These results suggest that resistance of LN-p52 cells to enzalutamide is mediated by alterations in the AR signaling pathway and demonstrate that activation of the AR axis by p52 plays an important role in the p52-induced resistance to enzalutamide. To confirm these results and test whether downregulation of FL AR or AR-V7 modulates p52-induced AR activation, we co-transfected a luciferase reporter containing the enhancer and promoter regions of PSA (PSA-E/P-Luc) along with p52 and siRNAs against FL AR or AR-V7 into VCaP and CWR22Rv1 cells. The cells were treated with either vehicle or 20 μmol/L enzalutamide and luciferase assays performed. VCaP and CWR22Rv1 cells express higher endogenous levels of p52, and our previous studies show that p52 induces ligand-independent activation of AR (13, 15). As shown in Fig. 5B and as shown in our previous studies (15), p52 induces activation of AR-mediated target gene transcription, which was abolished by downregulation of either FL AR or AR-V7. p52-induced activation of AR was unaffected by enzalutamide treatment. Treatment with enzalutamide further enhanced the suppressive effect of siRNAs against FL AR or AR-V7 on p52-induced AR-mediated target gene transcription. These results demonstrate that activation of AR signaling is necessary for the p52-induced resistance against enzalutamide. Similar results were obtained in CWR22Rv1 cells (Fig. 5C), showing that the interplay between FL AR, AR-V7 and NF-κB2/p52 may be critical in the development of resistance to enzalutamide in prostate cancer cells. These results implicate the activation of the AR signaling axis by p52 via FL AR and its splice variants as being responsible for the induction of resistance against enzalutamide.

Discussion

Next-generation antiandrogens such as enzalutamide and inhibitors of androgen synthesis such as abiraterone have revolutionized the standard of care for patients with both early- and late-stage prostate cancer. Despite their successes and continuing widespread use, threat of development of resistance looms large (17, 18). The understanding of mechanisms by which resistance against these agents may develop in prostate cancer cells may be critical for early intervention strategies in the event of development of resistance. Enzalutamide binds to the ligand-binding domain of AR and inhibits its nuclear translocation, DNA binding, and transactivation of target

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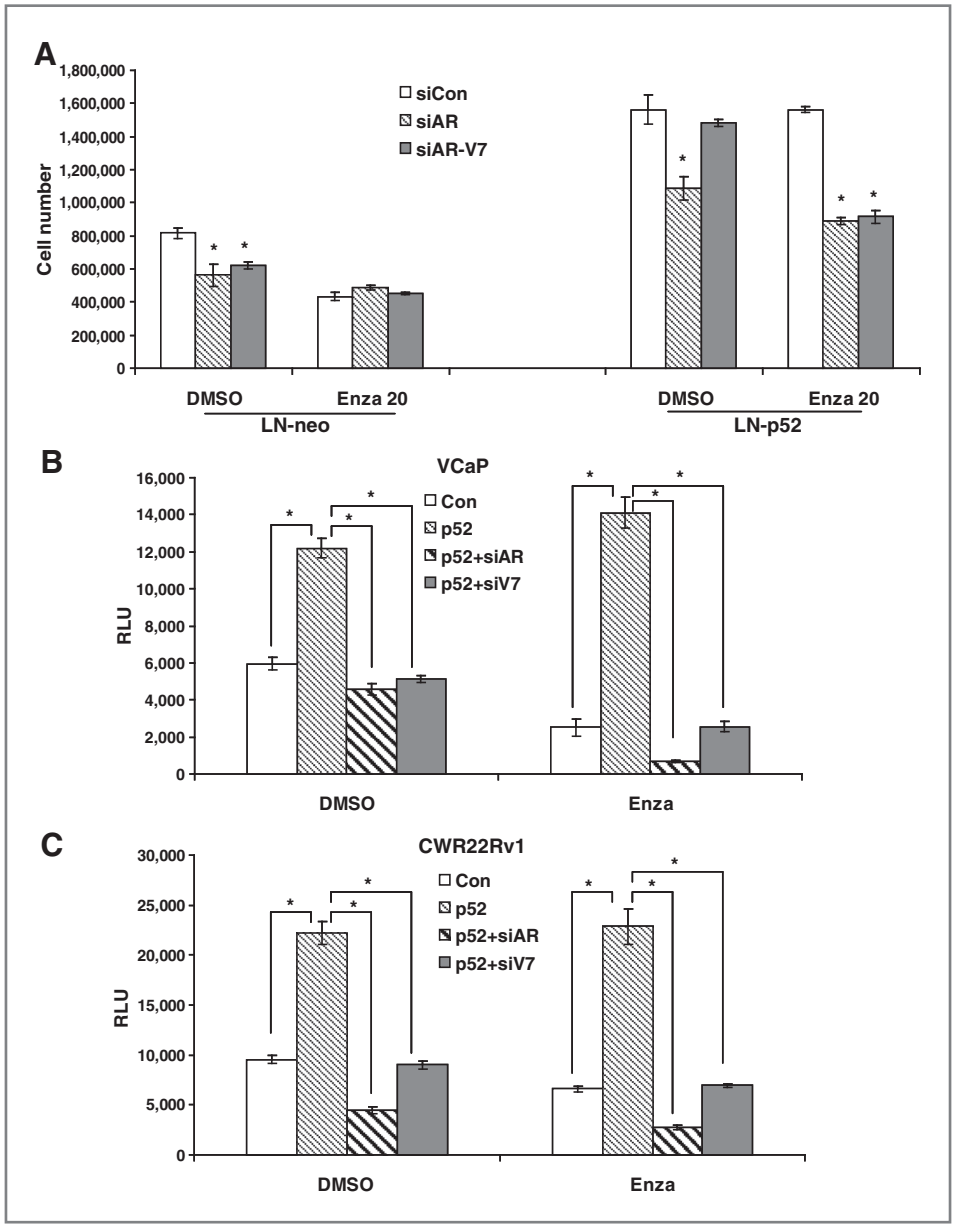

Figure 5. Downregulation of FL AR and AR-V7 increases sensitivity of p52-expressing prostate cancer (CaP) cells to enzalutamide. A, LN-p52 and LN-neo cells were transfected with siRNAs specific to either FL AR or AR-V7 and were treated with 0 or 20 μmol/L enzalutamide. Cell numbers were counted after 48 hours. Results are presented as mean ± SD of 3 experiments performed in triplicate. Downregulation of either FL AR or AR-V7 increased sensitivity of LN-p52 cells to enzalutamide. VCaP (B) and CWR22Rv1 (C) cells were transfected with PSA-E/P-Luc reporter, empty vector, or p52 together with siRNAs against FL AR or AR-V7. Cells were treated with 0 or 40 μmol/L enzalutamide, and luciferase assays were performed after 48 hours. Results are presented as mean ± SD of 2 experiments performed in triplicate. *, $P \leq 0.05$. Downregulation of either FL AR or AR-V7 suppressed p52-induced activation of AR in both VCaP and CWR22Rv1 cells.

genes (3). In the current study, we show that NF-κB2/p52 may play a crucial role in the development of resistance to enzalutamide and that the interplay between p52 and the AR signaling axis may be one of the underlying mechanisms. Even though enzalutamide and bicalutamide have similar mechanisms of action, clinically patients who progress on bicalutamide may respond to enzalutamide, indicating the existence of different mechanisms of resistance and that NF-κB2/p52 may be one of the many mediators of resistance. Our studies also show that prostate cancer cells treated chronically with enzalutamide may develop resistance against the agent via upregulation of expression of p52. These findings have important implications for therapeutic regimen in which patients are treated for long periods of time with enzalutamide. Further studies are warranted to test whether blocking of cellular signaling pathways in combination with antiandrogens may prove beneficial.

Our current data demonstrate that signaling networks such as p52 and AR interactions can mediate resistance to therapies targeting FL AR, including the next-generation antiandrogen, enzalutamide. The significance of these studies lies in the fact that resistance, either *de novo* or acquired, is one of the major clinical limitations for new AR inhibitors. The majority of patients who display disease progression on enzalutamide also display increasing prostate-specific antigen (PSA), indicating that enzalutamide-resistant tumors remain driven by persistent AR activity. AR variants are overexpressed in a subset of CRPC metastases and correlate with poor survival

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(19, 20). As AR splice variants lack the ligand-binding domain, they may be insensitive to inhibition by both bicalutamide and enzalutamide. One of the pioneering studies about enzalutamide showed that it may be effective against cells expressing higher levels of AR splice variants, although this fact remains to be substantiated (21). Even though AR variants have been hypothesized to be independent mediators of castration resistance (4), FL AR may still be necessary and may augment the castration-resistant response (21). Conflicting results have been obtained about the distinct transcriptional programs activated by AR variants and FL AR in prostate cancer cells (4, 22). It is probable that such perceived differences are due to experimental platforms used and do not reflect physiologic deviations. It is also possible that AR variants execute a "tumor-specific" program, which is a part of the broader transcriptional program of the FL AR, and hence are enriched in tumors. This may not necessarily mean that the FL AR is no longer a player in the progression of prostate cancer. It would be more likely that co-operation between FL AR and its splice variants is the driver behind CRPC progression, rather than a distinct and dominant transcriptional program driven by the splice variants alone.

Our earlier studies showed that NF-κB2/p52 promotes castration-resistant progression of prostate cancer by activating the AR in conditions of androgen deprivation (13, 15). In this study, we showed that p52 also induces expression of AR splice variants. Since the mechanism of action of enzalutamide is the inhibition of AR activation, we hypothesized that prostate cancer cells expressing higher levels of p52 may be resistant to enzalutamide. Our current results confirm the hypothesis and point to the role of the interaction between AR and p52 as being one of the critical turns during the progression to castration resistance.

In summary, our study demonstrates a link between persistent activation of the AR by NF-κB2/p52 and development of resistance to enzalutamide in prostate cancer. Future points of interest would be whether overcoming these networks and improving the efficacy of currently available clinical agents represents a viable area of research.

Disclosure of Potential Conflicts of Interest

C.P. Evans has other commercial research support from, honoraria from speakers bureau from, and is a consultant/advisory board member for Medivation/Astellas. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: N. Nadiminty, J. Yang, C.P. Evans, A.C. Gao
Development of methodology: N. Nadiminty, A.C. Gao
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Nadiminty, R. Tummala, W. Lou, C.P. Evans, A.C. Gao
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N. Nadiminty, R. Tummala, C.P. Evans, A.C. Gao
Writing, review, and/or revision of the manuscript: N. Nadiminty, R. Tummala, C.P. Evans, A.C. Gao
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Liu, J. Yang, W. Lou, A.C. Gao
Study supervision: N. Nadiminty, C.P. Evans, A.C. Gao

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Molecular Cancer Therapeutics

NF-κB2/p52:c-Myc:hnRNPA1 Pathway Regulates Expression of Androgen Receptor Splice Variants and Enzalutamide Sensitivity in Prostate Cancer

Nagalakshmi Nadiminty[1], Ramakumar Tummala[1], Chengfei Liu[1], Wei Lou[1], Christopher P. Evans[1,2], and Allen C. Gao[1,2]

Abstract

Castration-resistant prostate cancer (CRPC) remains dependent on androgen receptor (AR) signaling. Alternative splicing of the AR to generate constitutively active, ligand-independent variants is one of the principal mechanisms that promote the development of resistance to next-generation antiandrogens such as enzalutamide. Here, we demonstrate that the splicing factor heterogeneous nuclear RNA-binding protein A1 (hnRNPA1) plays a pivotal role in the generation of AR splice variants such as AR-V7. hnRNPA1 is overexpressed in prostate tumors compared with benign prostates, and its expression is regulated by NF-κB2/p52 and c-Myc. CRPC cells resistant to enzalutamide exhibit higher levels of NF-κB2/p52, c-Myc, hnRNPA1, and AR-V7. Levels of hnRNPA1 and AR-V7 are positively correlated with each other in prostate cancer. The regulatory circuit involving NF-κB2/p52, c-Myc, and hnRNPA1 plays a central role in the generation of AR splice variants. Downregulation of hnRNPA1 and consequently of AR-V7 resensitizes enzalutamide-resistant cells to enzalutamide, indicating that enhanced expression of hnRNPA1 may confer resistance to AR-targeted therapies by promoting the generation of splice variants. These findings may provide a rationale for cotargeting these pathways to achieve better efficacy through AR blockade. *Mol Cancer Ther; 14(8); 1884–95. ©2015 AACR.*

Introduction

Prostate cancer remains the second most lethal disease for males in western countries. The development of abiraterone and enzalutamide marked the continuing success of androgen deprivation therapy (ADT) practiced for more than 70 years, reinforcing the concept that androgen receptor (AR) is the key factor for metastatic castration-resistant prostate cancer (CRPC) progression and lethality. However, like earlier ADT, these new therapies have a short efficacy due to primary or acquired resistance. A major form of ADT-resistance in prostate cancer is the generation of AR splicing variants that lack the ligand-binding domain, thus evading binding of antiandrogens such as bicalutamide and enzalutamide. Several reports have documented the expression of alternatively spliced AR-Vs lacking the C-terminal ligand binding domain in prostate cancer cells, which are constitutively nuclear and active even in the absence of androgens, thus indicating their potential role in the acquisition of the CRPC phenotype. Expression of these variants arises from the inclusion of cryptic exons located in intron 2 and 3 of the *AR* gene, which inserts premature stop codons and termination sites, yielding shorter AR proteins of 75 to 80 kDa lacking the androgen-binding domain (1, 2). Truncated AR-Vs such as AR-V7 (AR3) and AR^{v567es} can function independently of full-length AR and their selective knockdown can suppress androgen-independent growth of CRPC cells. Alternatively, AR-Vs may play important roles in activating the full length AR in a ligand-independent manner (3). AR-Vs confer resistance to not only AR-targeted therapies (4, 5) but to conventional chemotherapeutics such as taxanes used as first line therapies against CRPC (6). These splice variants are rapidly induced after androgen deprivation and are suppressed after restoration of androgen supply (7). The mechanisms mediating increased expression of aberrant AR-Vs in prostate cancer are still largely unknown. One possible cause of defective splicing is the genomic rearrangement and/or intragenic deletions of the *AR* locus in CRPC (8). Alternatively, aberrant expression of specific splicing factors in prostate cancer cells may also contribute to unbalanced splicing and aberrant recognition of cryptic exons in the *AR* gene. Understanding the molecular mechanism of AR-Vs production will facilitate the design of mechanism-based inhibitors, extending the efficacy of current ADT, and possibly treating progression of CRPC and prolonging patient survival.

The importance of alternative messenger RNA splicing in regulatory circuits is underscored by the fact that >90% of human genes encode transcripts that undergo at least one alternative splicing event with a frequency higher than 10% (9, 10). Alternative splicing plays important roles in development, physiology, and disease and is often disturbed in inflammatory disorders and cancers (11, 12). Alternative splicing modulates the generation of protein isoforms with distinct structural and functional properties

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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or affects mRNA stability, by the insertion of premature stop codons, and translatability, by altering microRNA target sites (13). Two nuclear RNA-binding protein families, heterogeneous nuclear ribonucleoproteins (hnRNP) and serine/arginine-rich proteins (SR), play pivotal roles in regulation of alternative splicing. The hnRNP family consists of ~20 members which bind to splicing silencers located in exons or introns to promote exon exclusion and act as splicing repressors (13). The best characterized proteins of this group are hnRNPA1 and hnRNPA2, which share a high degree of sequence and functional homology (14). HnRNPA1 and hnRNPA2 are overexpressed in various kinds of tumors and serve as early tumor biomarkers (15–17). The SR family includes >20 members, which bind to splicing enhancers and predominantly function to counterbalance the activity of hnRNP proteins (18). Splicing factor 2/alternative splicing factor (SF2/ASF), the best-characterized member of the SR family, is upregulated in multiple human cancers, including lung and cervical cancers, and plays important roles in the establishment and maintenance of cellular transformation (19). During tumor progression, stimuli from the tumor microenvironment may affect the expression and/or activity of splicing regulatory factors, thus perturbing the physiological splicing program of genes involved in cellular processes. An increasing body of evidence indicates that splicing variants of many cancer-related genes can directly contribute to the oncogenic phenotype and to the acquisition of resistance to therapeutic treatments (11, 12, 20). Hence, understanding the functional role(s) of cancer-associated alternative splicing variants and the mechanisms underlying their production offers the potential to develop novel diagnostic, prognostic, and more specific anticancer therapies.

In this study, we investigated the mechanisms involved in aberrant splicing of AR transcripts in a constitutively occurring setting as well as in response to chronic treatment with enzalutamide. Our results show that the splicing factor hnRNPA1 plays a major role in generation of AR-Vs. We also demonstrate that enhanced expression of hnRNPA1 may be mediated by c-Myc and NF-κB2/p52, thus paving the way for increase in transcript numbers of constitutively active splice variants and contributing to CRPC therapy resistance.

Materials and Methods

Cell lines and reagents

LNCaP, CWR22Rv1, and VCaP cells were obtained in 2001 from the American Type Culture Collection (ATCC) and were cultured in RPMI containing 10% complete FBS and penicillin/streptomycin. ATCC uses short tandem repeat (STR) profiling for testing and authentication of cell lines. C4-2B cells were kindly provided and authenticated by Dr. Leland Chung, Cedars-Sinai Medical Center, Los Angeles, CA, USA, in 2006. All experiments with these cell lines were performed within 6 months of resuscitation after cryopreservation. LNCaP cells stably expressing NF-κB2/p52 were generated by stable transfection of LNCaP cells with plasmids expressing NF-κB2/p52 as described previously (21) and were not authenticated further. 22Rv1 and C4-2B cells resistant to enzalutamide (22Rv1-Enza-R and C4-2B-Enza-R) were generated by chronic culture of 22Rv1 and C4-2B cells in enzalutamide as described previously (22, 23) and were not authenticated further. Antibodies against NF-κB2/p52 (K-27), AR (441; mouse monoclonal), HA, tubulin, U2AF65, and ASF/SF2 were from Santa Cruz Biotechnologies. Antibodies against splicing factors hnRNPA1 (9H10) and hnRNPA2B1 (DP3B3) were from Sigma-Aldrich and AbCam, respectively. Sso Fast Eva Green qPCR Supermix was from Bio-Rad. All other reagents were of analytical grade and obtained from local suppliers.

Cell growth assays

Plasmid transfections were performed using Attractene transfection reagent (Qiagen). Oligonucleotide siRNA transfections were performed using Lipofectamine 2000 transfection reagent (Invitrogen). Viable cell numbers were determined using a Coulter cell counter (Beckman Coulter).

Western blot analysis

Cells were lysed in high salt buffer containing 50 mmol/L Hepes pH 7.9, 250 mmol/L NaCl, 1 mmol/L EDTA, 1% NP-40, 1 mmol/L PMSF, 1 mmol/L Na Vanadate, 1 mmol/L NaF, and protease inhibitor cocktail (Roche). Total protein was estimated using the Coomassie Protein Assay Reagent (Pierce). Equal amounts of protein were loaded on 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in PBST (1× PBS + 0.1% Tween-20) and probed with indicated primary antibodies in 1% BSA. The signal was detected by ECL (Millipore) after incubation with the appropriate horseradish peroxidase–conjugated secondary antibodies.

Real-time quantitative RT-PCR

Total RNAs were extracted using TRIzol reagent (Invitrogen). One microgram of total RNAs were subjected to digestion with RNase-free RQ1 DNase (Promega) and cDNAs were prepared using ImProm^{II} Reverse Transcriptase (Promega) according to manufacturer's instructions. The cDNAs were analyzed by real-time reverse transcription-PCR (RT-PCR) using Sso Fast Eva Green Supermix (Bio-Rad) as described previously (24). Each reaction was normalized by coamplification of actin. Triplicates of samples were run on a Bio-Rad CFX-96 real-time cycler.

RNA immunoprecipitation assay (RIP)

RIP assays were performed as described earlier (25). RNA-protein complexes were cross-linked using 1% formaldehyde. Nuclear extracts were immunoprecipitated with antibodies against indicated RNA-binding splicing factors. Isotype-matched IgG was used as control. Bound RNAs were purified, reverse transcribed, and the levels of indicated transcripts were analyzed by qPCR. Splice sites in the full length AR pre-mRNA were detected using ESRsearch and ESEFinder programs (Supplementary Fig. S1).

Human clinical specimens

Paired benign and tumor prostate tissue extracts were described previously (26). Total RNAs from human clinical specimens used for measurement of splicing factor transcript levels were described previously (27).

Gene expression omnibus analysis

Two separate data sets from NCBI GEO were screened independently for expression levels of hnRNPA1, hnRNPA2, U2AF65, and SF2/ASF. GDS1439 (28) compared specimens of benign prostatic hyperplasia with clinically localized primary prostate cancer and metastatic prostate cancer. GDS2545 (29) compared normal prostate specimens without any pathology, normal prostate adjacent to tumor, primary prostate tumor, and metastatic

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prostate cancer. Significant differences between groups were determined using Microsoft Excel Tools.

Oncomine analysis

Data sets generated from four comparisons of normal prostate tissue with prostate carcinoma: Lapointe_prostate (30), Wallace_prostate (31), Singh_prostate (32), and Yu_prostate (33) were analyzed using the differential expression function of Oncomine.

Statistical analyses

Data are shown as means ± SD. Multiple group comparison was performed by one-way ANOVA followed by the Scheffe procedure for comparison of means. $P \leq 0.05$ was considered significant.

Results

HnRNPA1 regulates the expression of AR variants

To test whether generation of AR variants by alternative splicing is dependent upon expression of hnRNPs, we analyzed expression levels of full-length AR and of variants such as AR-V7, AR-V1, AR-V5, AR-1/2/2b, and AR-1/2/3/2b using qRT-PCR in 22Rv1 and VCaP prostate cancer cells transfected with siRNAs against hnRNPA1 and hnRNPA2. Downregulation of hnRNPA1 and hnRNPA2 decreased the expression levels of AR variants in 22Rv1 and VCaP cells (Fig. 1A and B; Supplementary Fig. S2A and S2B). Insets in Fig. 1A and B confirm the downregulation of hnRNPA1 and hnRNPA2 by specific siRNAs. The downregulation of hnRNPA1 and the resultant suppression of AR-V7 protein levels were confirmed by Western blot analysis (Fig. 1C). The protein levels of AR-V7 variant were decreased in both 22Rv1 and VCaP cells transfected with hnRNPA1 siRNA. These results indicate that hnRNPA1 may regulate the generation of AR-Vs in prostate cancer cells.

Next, we tested whether overexpression of hnRNPA1 affects the expression levels of AR-Vs. LNCaP cells were transfected with full-length hnRNPA1 cDNA and levels of AR-Vs were analyzed by Western blotting and qRT-PCR. Overexpression of hnRNPA1 enhanced AR-V7 protein levels in LNCaP cells, which possess undetectable endogenous levels of AR-V7 protein (Fig. 1D). qRT-PCR confirmed that overexpression of hnRNPA1 significantly enhanced the mRNA levels of AR-V7, AR-V5, AR-1/2/2b, and AR-1/2/3/2b variants in LNCaP cells (Fig. 1E; Supplementary Fig. S2C). Inset in Fig. 1E confirms the overexpression of hnRNPA1 after transfection in LNCaP cells. These results using downregulation as well as overexpression of hnRNPA1 suggest that hnRNPA1 plays an important role in the generation of AR splice variants.

Recruitment of hnRNPA1 to splice sites in AR pre-mRNA is increased in enzalutamide-resistant cells

We found hnRNP binding sites (UAGGGA) in the full-length AR mRNA using sequence analysis and ESRSearch program (Supplementary Fig. S1). To determine whether hnRNPA1 is recruited to splice sites in the AR pre-mRNA, we performed RNA Immunoprecipitation (RIP) assays using specific antibodies against hnRNPA1 and hnRNPA2 in 22Rv1 versus 22Rv1-Enza-R and C4-2B versus C4-2B-Enza-R cell lines. The 22Rv1-Enza-R and C4-2B-Enza-R cell lines were generated by chronic exposure to enzalutamide and display resistance to enzalutamide (22, 23). The degree of recruitment of hnRNPA1 to AR-V7 splice sites was significantly higher in 22Rv1-Enza-R cells compared with parental 22Rv1 cells (Fig. 2A), indicating that hnRNPA1 may promote

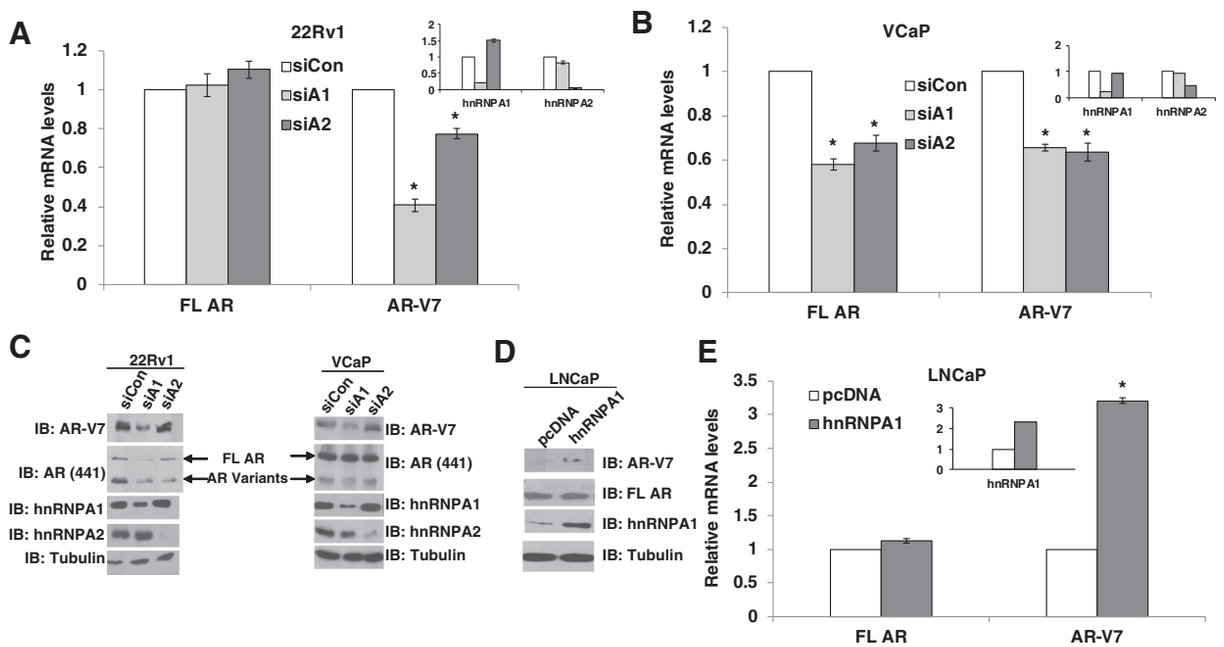

Figure 1.
HnRNPA1 promotes generation of AR splice variants. qPCR to determine the expression levels of AR-V7 and FL AR in 22Rv1 (A) and VCaP (B) cells transfected with hnRNPA1 or hnRNPA2 siRNAs. C, Western blot analysis in 22Rv1 and VCaP cells transfected with hnRNPA1 or hnRNPA2 siRNAs. D, Western blotting for AR-V7 in LNCaP cells transfected with hnRNPA1 cDNA. E, qPCR analysis in LNCaP cells transfected with hnRNPA1 cDNA. Results are presented as means ± SD of three experiments performed in triplicate. *, $P \leq 0.05$.

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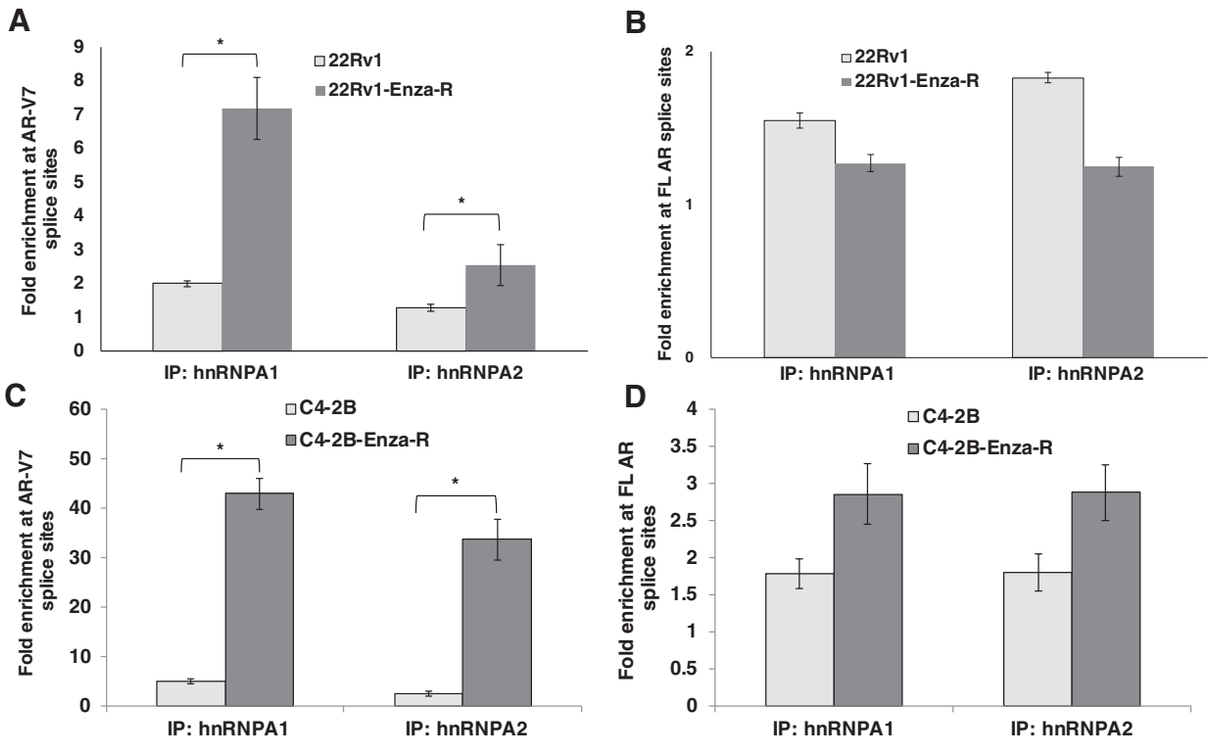

Figure 2.
Recruitment of hnRNPA1 and hnRNPA2 to splice sites in AR pre-mRNA is enhanced in 22Rv1-Enza-R and C4-2B-Enza-R enzalutamide-resistant prostate cancer cells compared with 22Rv1 and C4-2B parental cells respectively. RIP assays for splice sites of AR-V7 (A) and AR (B) in 22Rv1-Enza-R compared with 22Rv1 and AR-V7 (C) and AR (D) in C4-2B-Enza-R compared with C4-2B. Results are presented as means ± SD of two experiments performed in duplicate. *, $P \leq 0.05$.

generation of AR-V7 in prostate cancer cells resistant to enzalutamide. Even though the recruitment of hnRNPA2 to AR-V7 splice sites was also enhanced in 22Rv1-Enza-R cells compared with 22Rv1 cells, the recruitment of hnRNPA1 was several fold higher than that of hnRNPA2 (Fig. 2A). No significant differences were observed in the recruitment of either hnRNPA1 or hnRNPA2 to FL AR splice sites between 22Rv1 parental and 22Rv1-Enza-R cells (Fig. 2B). We also analyzed recruitment of hnRNPA1 and hnRNPA2 to splice sites for other AR-Vs such as AR-V1, AR-V5, AR-1/2/2b, and AR-1/2/3/2b (Supplementary Fig. S3A–S3D). Recruitment of hnRNPA1 to AR-1/2/2b splice sites was significantly higher in 22Rv1-Enza-R cells (Supplementary Fig. S3C), indicating that hnRNPA1 may play a selective role in generation of AR splice variants. In addition, recruitment of hnRNPA2 to AR-1/2/3/2b splice sites was significantly enhanced in 22Rv1-Enza-R cells (Supplementary Fig. S3D). These results imply that different splicing factors may function cooperatively to promote generation of AR-Vs in enzalutamide-resistant prostate cancer cells. These results also confirm that hnRNP proteins are physically recruited to splice sites in the AR pre-mRNA with the degree of recruitment increasing in prostate cancer cells resistant to enzalutamide, indicating that hnRNP proteins may drive the generation of AR splice variants leading to enzalutamide resistance.

To confirm the above results in another enzalutamide-resistant cell line, we analyzed the C4-2B versus C4-2B-Enza-R cell line pair. Our results showed that recruitment of hnRNPA1 and hnRNPA2 to AR-V7 splice sites was significantly enhanced in C4-2B-Enza-R cells compared with parental C4-2B cells (Fig. 2C), whereas, similar to 22Rv1-Enza-R cells, recruitment of either hnRNPA1 or hnRNPA2 to FL AR splice sites was not altered significantly in C4-2B-Enza-R cells compared with parental C4-2B cells (Fig. 2D). In all cases, the fold enrichment of hnRNPA1 at splice sites on AR pre-mRNA was much higher compared with hnRNPA2, indicating that hnRNPA1 may play a more central role in promoting the expression of AR-Vs (Supplementary Fig. S3E–S3H). These results collectively demonstrate that different splicing factors may play context- and cell type-dependent roles in prostate cancer cells in alternative splicing of the AR.

Expression levels of hnRNPA1 are elevated in prostate cancer tissues

To determine whether increased expression of splicing factors and AR-Vs is associated with prostate cancer, we examined the expression levels of hnRNPA1 and hnRNPA2 by immunoblotting in lysates from 27 archived paired benign and tumor prostate clinical samples. Levels of hnRNPA1 and hnRNPA2 were elevated in ∼44% of tumor tissues compared with matched benign tissues (Fig. 3A; Supplementary Fig. S4A). These results were correlated positively with the protein expression levels of AR-V7, which were enhanced in ∼48% of tumor tissues compared with their benign counterparts (Fig. 3A and Supplementary Fig. S4A). In addition, expression levels of hnRNPA1 and hnRNPA2 were low or undetectable in 9 of 12 and 6 of 12 donor prostates, respectively. These observations

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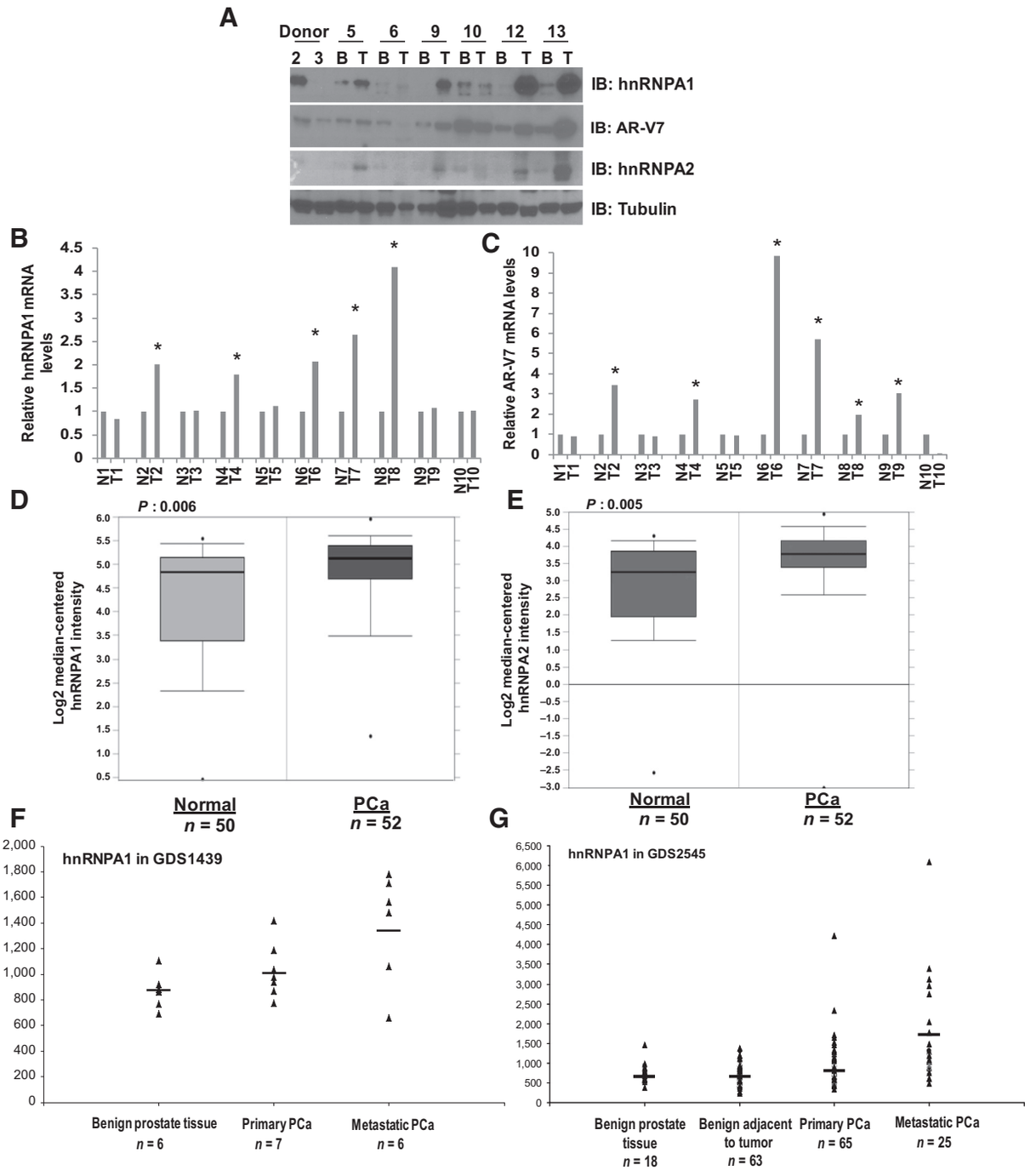

Figure 3.
Expression levels of hnRNPA1 and AR-V7 are positively correlated with each other. A, representative immunoblot for hnRNPA1, hnRNPA2, and AR-V7 in lysates from 27 paired benign and tumor patient samples. qRT-PCR of mRNA levels of hnRNPA1 (B) and AR-V7 (C) in 10 paired normal and tumor clinical prostate samples. Results are presented as means ± SD of two experiments performed in triplicate. *, $P \leq 0.05$. Relative expression levels of hnRNPA1 (D) and hnRNPA2 (E) in the representative Singh_prostate ($n = 102$) data set from Oncomine. Relative expression levels of hnRNPA1 in GDS1439 data set (F) and in GDS2545 data set (G) from GEO.

were also correlated with expression levels of AR-V7, which were low or undetectable in 8 of 12 donor tissues (Table 1).

We also analyzed the mRNA levels of hnRNPA1, hnRNPA2, and AR-V7 in archival total RNAs extracted from 10 pairs of matched benign and tumor clinical prostate specimens (27, 34). Transcript levels of hnRNPA1 were elevated in 5 of 10 tumor tissues compared with matched benign tissues with no appreciable differences between tumor and benign being observed in the other 5 of 10

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Table 1. Summary of the Western blot analysis of levels of hnRNPA1, hnRNPA2, and AR-V7 in 27 paired benign and tumor prostate clinical samples

Gene	T > B	T < B	T = B	High in donor	Low in donor
hnRNPA1	12 (44%)	8 (29%)	7 (26%)	3/12	9/12
AR-V7	13 (48%)	7 (25%)	7 (26%)	4/12	8/12
hnRNPA2	12 (44%)	8 (29%)	7 (26%)	6/12	6/12

samples (Fig. 3B). Transcript levels of AR-V7 were elevated in 6 of 10 tumor tissues compared with their matched benign counterparts (Fig. 3C), demonstrating that expression of hnRNPA1 and AR-V7 may be positively correlated with each other in human prostate cancer. No significant differences were observed in mRNA levels of hnRNPA2 between matched tumor and benign prostate tissues (Supplementary Fig. S4B).

To further confirm our findings, we analyzed expression levels of hnRNPA1 and hnRNPA2 in clinical prostate cancer tissues using publicly available data sets from Gene Expression Omnibus (GEO) and Oncomine. Results from an analysis of Oncomine data sets revealed that expression levels of hnRNPA1 and hnRNPA2 are significantly elevated in prostate tumor tissues compared with benign prostates in 17 of 21 and 15 of 21 data sets, respectively (Supplementary Fig. S4C). Results from a representative data set, Singh_prostate ($n = 102$) from Oncomine are shown (Fig. 3D and E). An analysis of GEO revealed that expression levels of hnRNPA1 and hnRNPA2 were elevated in primary as well as metastatic prostate cancer compared with benign prostates (Fig. 3F and G and Supplementary Fig. S4D). Data regarding expression levels of AR-Vs were not available in these data sets, but nonetheless, these results indicate that elevated levels of hnRNPA1 may contribute to prostate cancer development and progression. Our findings correlate well with studies showing that expression levels of AR-V7 are elevated in ∼40% of CRPC tissues (2, 35), indicating that elevated expression of hnRNPA1 in prostate tumors may contribute to generation of higher levels of AR-Vs.

Expression of hnRNPA1 is regulated by c-Myc

Previous studies indicated that hnRNPA1 and c-Myc exhibit positive reciprocal regulation (36). C-Myc enhances hnRNPA1 expression transcriptionally, whereas hnRNPA1 regulates c-Myc via alternative splicing. Previous studies also showed that c-Myc is one of the transcription factors which regulate transcription of the AR (37). Hence, we analyzed the status of c-Myc or hnRNPA1 when the expression of either was downregulated in prostate cancer cells. Lysates from 22Rv1 and VCaP cells transfected with shRNA against c-Myc were analyzed using specific antibodies against hnRNPA1. Downregulation of c-Myc reduced protein levels of hnRNPA1 significantly (Fig. 4A). Similarly, lysates from LNCaP, 22Rv1, and VCaP cells transfected with siRNA against hnRNPA1 were examined by immunoblotting using specific antibodies against c-Myc. Downregulation of hnRNPA1 reduced protein levels of c-Myc (Fig. 4B). These results confirm that hnRNPA1 and c-Myc exhibit reciprocal regulation in prostate cancer cells. We also analyzed whether reduction in hnRNPA1 levels by c-Myc shRNA affects expression of AR-Vs in 22Rv1 and VCaP cells. Western blotting and qRT-PCR analyses showed that levels of AR-Vs, including that of AR-V7, were abrogated due to depletion of hnRNPA1 caused by downregulation of c-Myc (Fig. 4A–D; Supplementary Fig. S5). These findings support an important role for c-Myc in the generation of AR splice variants.

To analyze whether overexpression of hnRNPA1 can overcome the effects of downregulation of Myc, we suppressed expression of c-Myc using shRNA in 22Rv1 cells followed by overexpression of hnRNPA1. The results showed that even though suppression of c-Myc reduced mRNA as well as protein levels of both FL AR and AR-V7, subsequent overexpression of hnRNPA1 restored the expression of AR-V7 fully while having minimal effect on FL AR (Fig. 4E and F). These results indicate that hnRNPA1 primarily regulates generation of alternative splice variants of the AR and not the generation of the FL AR.

NF-κB2/p52 regulates AR-V7 expression via hnRNPA1 and c-Myc

We reported earlier that activation of NF-κB2/p52 promotes progression to CRPC and enzalutamide resistance via the generation of AR variants, specifically AR-V7 (21, 23, 24). Our previous findings also indicated that NF-κB2/p52 may regulate c-Myc expression. Hence, we examined whether NF-κB2/p52 plays a role in the elevated expression of hnRNPA1 and c-Myc in prostate cancer using lysates from LNCaP cells stably expressing p52 (LN-p52). Protein levels of AR-V7, hnRNPA1, and c-Myc were elevated in LN-p52 cells, whereas no appreciable differences were found in the expression of hnRNPA2 (Fig. 5A, left). These results were confirmed using LNCaP cells expressing p52 under the control of a Tet-inducible promoter (LN/TR/p52). Induction of p52 expression led to increases in expression levels of AR-V7, hnRNPA1, and c-Myc (Fig. 5A, right), indicating that upregulation of AR-V7 by p52 may be mediated by hnRNPA1 and c-Myc. To examine the relationship between hnRNPA1, c-Myc and AR-V7 in LN-p52 cells, we analyzed levels of AR-V7 by Western blotting in LN-p52 cells transfected with hnRNPA1 or hnRNPA2 siRNAs. Downregulation of hnRNPA1 abrogated the expression of AR-V7 in LN-p52 cells, whereas downregulation of hnRNPA2 did not have an appreciable effect on AR-V7 protein levels (Fig. 5B, left). Protein levels of c-Myc were also downregulated, keeping in line with earlier findings that hnRNPA1 and c-Myc regulate each other (36). In addition, expression of hnRNPA1 and AR-V7 were abolished as a result of downregulation of c-Myc expression in LN-p52 cells transfected with c-Myc shRNA (Fig. 5B, middle).

To confirm these findings in a cell line with constitutive expression of both p52 and AR-V7, we analyzed levels of AR-V7, hnRNPA1, and c-Myc by immunoblotting in 22Rv1 cells transfected with p52 shRNA. Downregulation of p52 in 22Rv1 cells abrogated expression of AR-V7, hnRNPA1, and c-Myc (Fig. 5B, right). These results demonstrate that NF-κB2/p52 may modulate generation of AR-Vs by regulation of hnRNPA1 and c-Myc.

Prostate cancer cells resistant to enzalutamide exhibit higher levels of splicing factors

As our results demonstrate that expression of hnRNPA1 and AR variants may be positively correlated with each other in prostate cancer cells, and AR-V7 expression has been shown to be involved in the acquisition of resistance to enzalutamide (3, 23), we tested the correlation between levels of AR variants and hnRNPA1 in

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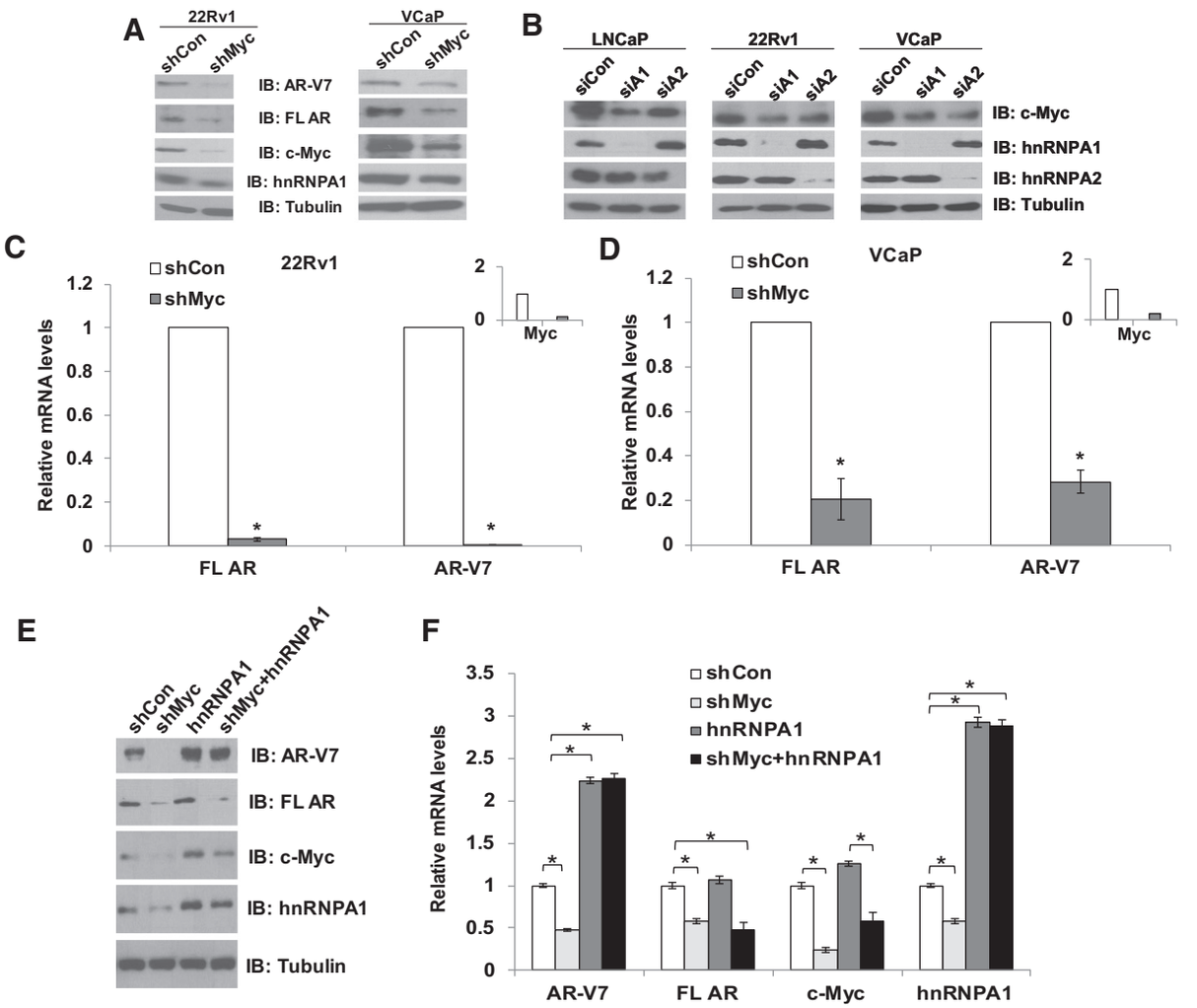

Figure 4.
Reciprocal regulation between c-Myc and hnRNPA1 is responsible for the generation of AR splice variants. A, Immunoblotting for hnRNPA1 and AR-V7 in 22Rv1 and VCaP cells transfected with c-Myc shRNA. B, immunoblotting for c-Myc in LNCaP, 22Rv1, and VCaP cells transfected with hnRNPA1 or hnRNPA2 siRNAs. qRT-PCR for mRNA levels of full-length AR and AR-V7 in 22Rv1 (C) and VCaP (D) cells transfected with c-Myc shRNA. Insets show the expression of c-Myc mRNA in cells transfected with c-Myc shRNA. Protein (E) and mRNA (F) levels of AR-V7, FL AR, c-Myc, and hnRNPA1 in 22Rv1 cells transfected with c-Myc shRNA with or without overexpression of hnRNPA1. Results are presented as means ± SD of two experiments performed in triplicate. *, $P \leq 0.05$.

prostate cancer cells that have acquired resistance to enzalutamide. 22Rv1-Enza-R and C4-2B-Enza-R cell lines exhibited higher levels of AR-V7 and hnRNPA1 (Fig. 5C), indicating that expression of hnRNPA1 may be positively correlated with expression of AR-Vs. Furthermore, expression levels of c-Myc and NF-κB2/p52 were also elevated in enzalutamide-resistant cells, confirming the importance of the NF-κB2/p52:c-Myc:hnRNPA1:AR-V7 axis in enzalutamide resistance. No significant differences were observed in the expression of hnRNPA2 in 22Rv1-Enza-R and C4-2B-Enza-R cells compared with their parental cells (Fig. 5C, right and middle). To confirm these results *in vivo*, we analyzed extracts from xenograft tumors derived from C4-2B and C4-2B-Enza-R cells using antibodies against AR-V7 and hnRNPA1. Higher levels of AR-V7 were observed in xenografts derived from C4-2B-Enza-R cells, which was correlated well with higher levels of hnRNPA1 and c-Myc (Fig. 5C, right), confirming our observations that expression of AR-Vs is positively correlated with expression of hnRNPA1 in prostate cancer cell lines resistant to enzalutamide.

Next, we tested whether downregulation of hnRNPA1 affects endogenous levels of AR-Vs in 22Rv1-Enza-R cells. Transfection of hnRNPA1 siRNA abrogated levels of AR-V7 in 22Rv1-Enza-R cells (Fig. 5D, left). Similarly, downregulation of c-Myc by specific shRNA reduced expression levels of AR-V7 and hnRNPA1 in 22Rv1 and 22Rv1-Enza-R cells (Fig. 5D, right), confirming the c-Myc:hnRNPA1:AR-V7 axis in prostate cancer cells.

To confirm the importance of the link between NF-κB2/p52, c-Myc, hnRNPA1, and AR-V7 in prostate cancer, we analyzed the correlation between their expression levels at mRNA and protein levels in paired benign and tumor prostate clinical samples from Fig. 3. Transcript (left) and protein (right) levels of NF-κB2/p52, c-Myc, hnRNPA1, and AR-V7 were positively correlated with each other (Fig. 5E), demonstrating that the NF-κB2/p52:c-

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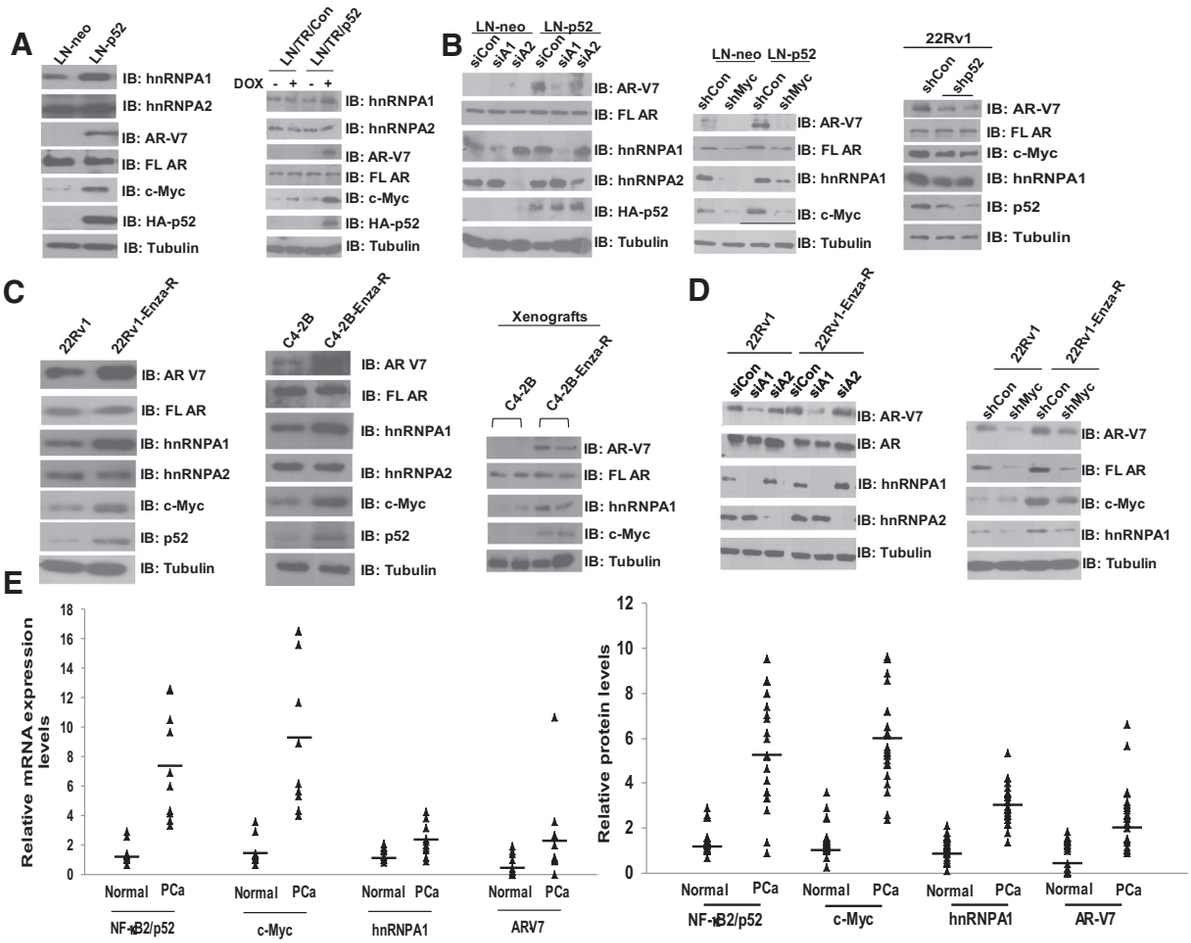

Figure 5.

NF-κB2/p52 regulates expression of c-Myc and hnRNPA1. A, left, immunoblotting for hnRNPA1, c-Myc, and AR-V7 in LN-p52 cells; right, immunoblotting for hnRNPA1, c-Myc, and AR-V7 in LN/TR/p52 cells. B, left, Western blotting for AR-V7 in LN-p52 cells transfected with hnRNPA1 siRNA; middle, immunoblotting for AR-V7 and hnRNPA1 in LN-p52 cells transfected with c-Myc shRNA; right, immunoblotting for AR-V7, hnRNPA1, and c-Myc in 22Rv1 cells transfected with p52 shRNA. C, left, 22Rv1 cells resistant to enzalutamide (22Rv1-Enza-R) express higher levels of AR-V7, hnRNPA1, c-Myc, and NF-κB2/p52; (middle) C4-2B cells resistant to enzalutamide (C4-2B-Enza-R) express higher levels of hnRNPA1, AR-V7, c-Myc, and NF-κB2/p52; right, xenografts from C4-2B-Enza-R cells exhibit higher levels of AR-V7, hnRNPA1, and c-Myc. D, left, Western blot analysis of AR-V7 in 22Rv1-Enza-R cells transfected with hnRNPA1 siRNA; right, expression of AR-V7 and hnRNPA1 in 22Rv1-Enza-R cells transfected with c-Myc shRNA. All results are shown as representative images from two experiments performed in duplicate. E, left, chart depicting the positive correlation between mRNA levels of NF-κB2/p52, c-Myc, hnRNPA1, and AR-V7 in 10 paired benign and tumor prostate clinical samples; right, chart depicting the correlation between relative protein levels of NF-κB2/p52, c-Myc, hnRNPA1, and AR-V7 in 27 paired benign and tumor prostate clinical samples. Band intensities in immunoblots were quantified using ImageJ software and plotted as arbitrary units. The horizontal lines represent the median of each series.

Myc:hnRNPA1:AR-V7 axis plays a vital role in prostate cancer and in the development of castration and therapy resistance.

Suppression of hnRNPA1 resensitizes enzalutamide-resistant prostate cancer cells to enzalutamide

To examine the functional relevance of regulation of AR alternative splicing by hnRNPA1, we tested whether down-regulation of hnRNPA1 resulting in decreased levels of AR-V7 resensitizes enzalutamide-resistant cells to enzalutamide. We examined cell survival in 22Rv1 and 22Rv1-Enza-R cells transfected with hnRNPA1 or hnRNPA2 siRNAs and treated with 0 and 20 μmol/L enzalutamide for 48 hours. Reduced expression of hnRNPA1 enhanced the sensitivity of enzalutamide-resistant 22Rv1-Enza-R cells to enzalutamide (Fig. 6A), indicating that upregulation of AR-V7 expression by hnRNPA1 may be required to sustain the acquired resistance of 22Rv1-Enza-R cells to enzalutamide. Downregulation of hnRNPA1 and hnRNPA2 also enhanced the sensitivity of LN-neo and LN-p52 cells transfected with hnRNPA1 or hnRNPA2 siRNAs and treated with 20 μmol/L enzalutamide to enzalutamide (Fig. 6B). Suppression of hnRNPA1 expression reduced cell survival by ∼40% to 50% when enzalutamide-resistant LN-p52 cells were treated with enzalutamide. Suppression of hnRNPA1 expression also reduced survival of VCaP cells when treated with enzalutamide (Fig. 6C), confirming the essential nature of AR variants in these cells.

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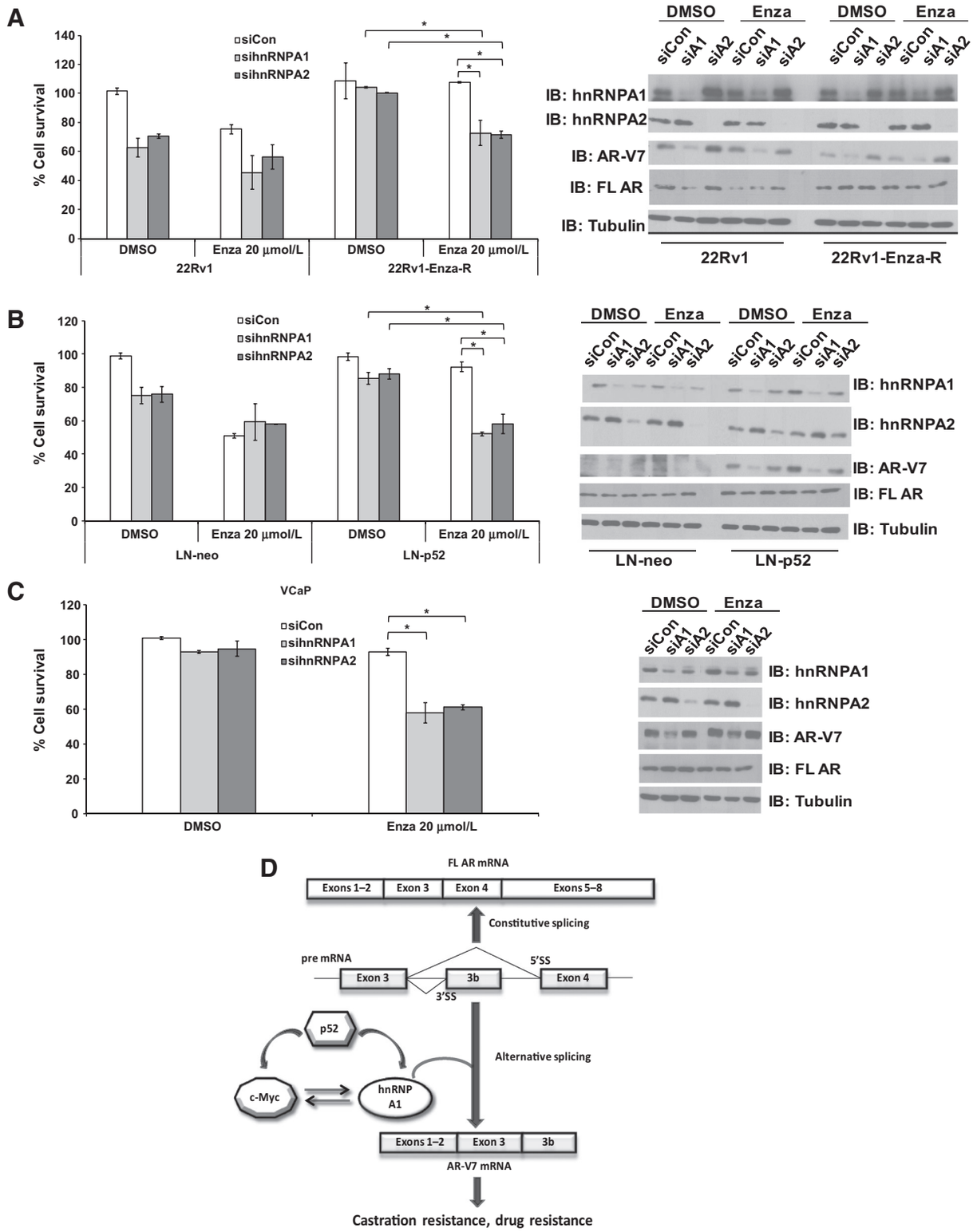

Figure 6.
Suppression of hnRNPA1 restores enzalutamide sensitivity of enzalutamide-resistant prostate cells. A, left, cell survival in 22Rv1-Enza-R cells transfected with hnRNPA1 or hnRNPA2 siRNAs and treated with vehicle or 20 μmol/L enzalutamide. Cell numbers were counted after 48 hours; right, immunoblots confirm the downregulation of hnRNPA1 or hnRNPA2 and of AR-V7. B, left, cell survival in LN-p52 cells transfected with hnRNPA1 or hnRNPA2 siRNAs and treated with vehicle or 20 μmol/L enzalutamide. (*Continued on the following page.*)

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Our findings collectively demonstrate that hnRNPA1 plays a major role in the generation of AR splice variants. Expression of hnRNPA1 is modulated by NF-κB2/p52 via c-Myc (Fig. 6D). Our results point to the enhanced expression of hnRNPA1 in prostate tumors being instrumental in inducing alternative splicing of the precursor AR mRNA.

Discussion

A large number of previous studies have shown that C-terminally truncated AR-Vs are expressed in prostate cancer cells and even in normal prostate epithelial cells (35, 38) and promote CRPC progression under androgen deprivation (39, 40). In addition, enhanced expression of AR-V7 confers resistance to next-generation therapeutics such as enzalutamide and abiraterone (4, 5). These studies attest to the importance of AR-Vs in CRPC and cotargeting the mechanisms which contribute to their generation may increase the efficacy of currently used AR-targeted therapies and prolong time to development of resistance. Our findings in this study demonstrate that the splicing factor hnRNPA1 plays a major role in the alternative splicing of AR mRNA.

HnRNPA1 is a multifunctional RNA-binding protein involved in the regulation of RNA biogenesis. HnRNPA1 is under the transcriptional control of the c-Myc proto-oncogene and modulates the splicing of PKM2, activating the metabolic switch to aerobic glycolysis that is a hallmark of cancer cells (11, 36). HnRNPA1 also regulates alternative splicing of genes involved in invasion and metastasis such as *Rac1* and *Ron* (11). Increased expression of hnRNPA1 has been documented in proliferating and transformed cells (20) and in lung, breast, colon, renal cell carcinomas, and gliomas (41–46). HnRNPs cooperate with other splicing factors to generate pro-oncogenic and proinflammatory molecules in cancers (41, 47). Silencing of hnRNPA1 and A2 promotes apoptosis in human and mouse cancer cell lines, while having no effect on normal epithelial and fibroblastic cell lines (48).

In this study, hnRNPA1 binding sites (UAGGGA) were identified in AR mRNA using sequence analysis and ESRSearch program. Downregulation of hnRNPA1 significantly reduced the expression of AR-Vs such as AR-V7, while not affecting full-length AR. HnRNPA1 binding sites were not detected at full length AR splice sites in the AR pre-mRNA. Moreover, the slight decrease seen in either full length AR mRNA or protein levels in VCaP or 22Rv1 cells respectively (Fig. 1) was not observed consistently in all experiments. In consideration of these observations, we concluded that hnRNPA1 does not play a significant role in splicing of full length AR from AR pre-mRNA. RNA-binding assays revealed that hnRNPA1 is recruited to splice sites for AR splice variants. Enhanced expression of hnRNPA1 was observed in prostate cancer tissues compared with their benign counterparts, which was correlated positively with expression of AR-V7. Increased expression of hnRNPA1 was also correlated with higher levels of AR-V7 in prostate cancer cells with acquired resistance to enzalutamide. Exploration of the mechanisms revealed that c-Myc and NF-κB2/p52 contribute to the development of therapy resistance in prostate cancer cells by inducing hnRNPA1 expression and thereby ligand-independent AR-Vs. Downregulation of hnRNPA1 resensitized enzalutamide-resistant cells to enzalutamide, indicating that suppression of hnRNPA1 resulting in suppression of AR-Vs reversed the acquired resistance to enzalutamide. These data led us to conclude that hnRNPA1 is the central player in a splicing regulatory circuit involving c-Myc, NF-κB2/p52, and AR.

Our study demonstrates that elevated levels of splicing factors such as hnRNPA1 promote expression of alternative splice forms of AR. Relative amounts of splicing factors have been proposed to determine alternative splicing (45). A recent study by Liu and colleagues (49) showed that recruitment, and not expression, of splicing factors SF2/ASF and U2AF65 determines the generation of AR splice variants in enzalutamide resistance. We found higher levels of hnRNPA1 in prostate cancer clinical samples compared with SF2/ASF (data not shown), indicating that higher expression of a splicing factor, and not simply its recruitment to splice sites under certain conditions, may determine the levels of alternative splice forms. These results are supported by previous studies which showed that the relative levels of hnRNPA1 expression increased to a greater extent than those of SF2/ASF in lung tumors (44). Of note, analysis of expression levels of U2AF65 and SF2/ASF in prostate cancer tissues using GEO and Oncomine data sets revealed that expression levels of both splicing factors are elevated in prostate cancer tissues compared with benign counterparts (Supplementary Fig. S6), lending credence to our observations that enhanced expression of splicing factors may be one of the principal mechanisms driving generation of AR-Vs. Elevated levels of hnRNPA1 may conceivably change the splicing milieu of a broad spectrum of proteins in addition to that of the AR, with splicing of CD44 being an example. But splice variants of AR have been demonstrated to play major roles in resistance to enzalutamide, underlining the importance of our results in splicing regulation of the AR. Furthermore, earlier studies indicated that the splice variant AR-1/2/3/2b is also generated by intragenic genomic rearrangement of the AR gene due to duplication of exon 3 (1). Our studies consistently found transcripts corresponding to this splice variant in LNCaP, C4-2B, and VCaP cells, albeit at extremely low levels. These observations warrant further exploration of the mechanisms involved in generation of this splice variant and further validation.

In summary, we demonstrated that hnRNPA1, in concert with NF-κB2/p52 and c-Myc, regulates the generation of AR-Vs in prostate cancer cells and that the NF-κB2/p52:c-Myc:hnRNPA1:AR-V7 axis (Fig. 6D) plays a pivotal role in the development and maintenance of resistance to androgen blockade. These findings may have important implications in targeting AR-Vs and the splicing factors responsible to overcome acquired enzalutamide resistance in prostate cancer.

(*Continued.*) Cell numbers were counted after 48 hours; right, immunoblots confirm the downregulation of hnRNPA1 or hnRNPA2 and AR-V7. C, left, cell survival in VCaP cells transfected with hnRNPA1 or hnRNPA2 siRNAs and treated with vehicle or 20 μmol/L enzalutamide. Cell numbers were counted after 48 hours; right, immunoblots confirm the downregulation of hnRNPA1 or hnRNPA2 and of AR-V7. Results are presented as means ± SD of three experiments performed in triplicate. *, $P \leq 0.05$. D, schematic representation of the alternative splicing of AR mRNA regulated by the NF-κB2:c-Myc:hnRNPA1 axis.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Nadiminty, R. Tummala, A.C. Gao
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N. Nadiminty, C.P. Evans, A.C. Gao
Writing, review, and/or revision of the manuscript: N. Nadiminty, C.P. Evans, A.C. Gao
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Nadiminty, R. Tummala, C. Liu, W. Lou, A.C. Gao
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