Androgen receptor splice variants dimerize to transactivate target genes

Duo Xu1,2,11*, Yang Zhan2*, Yanfeng Qi2, Bo Cao1,2, Shanshan Bai1,2, Wei Xu3, Sanjiv S. Gambhir4, Peng Lee5, Oliver Sartor6,7, Erik K. Flemington8, Haitao Zhang8, Chang-Deng Hu9, Yan Dong1,2,10

1College of Life Sciences, 10National Engineering Laboratory for AIDS Vaccine, 11School of Nursing, Jilin University, Changchun, China; 2Departments of Structural and Cellular Biology, Urology, 7Medicine, and 8Pathology and Laboratory Medicine, Tulane University School of Medicine, Tulane Cancer Center, New Orleans, LA; 3Mc Ardle Laboratory for Cancer Research, University of Wisconsin, Madison, WI; 4Bio-X Program and Department of Radiology, Stanford University School of Medicine, Stanford, CA; 5Department of Pathology, New York University School of Medicine, New York, NY; 9Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN.

*, contributed equally

Correspondence: Yan Dong, 1430 Tulane Avenue SL-49, New Orleans, LA 70112; Phone: 504-988-4761; Email: ydong@tulane.edu

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ABSTRACT

Constitutively-active androgen receptor splice variants (AR-V) lacking the ligand-binding domain have been implicated in the pathogenesis of castration-resistant prostate cancer and in mediating resistance to newer drugs that target the androgen axis. AR-V regulate expression of both canonical AR targets and a unique set of cancer-specific targets that are enriched for cell cycle functions. However, little is known about how AR-V control gene expression. Here we report that two major AR-V, termed AR-V7 and AR-V567es, not only homodimerize and heterodimerize with each other but also heterodimerize with full-length androgen receptor (AR-FL) in an androgen-independent manner. We found that heterodimerization of AR-V and AR-FL was mediated by N- and C-terminal interactions and by the DNA-binding domain of each molecule, whereas AR-V homodimerization was mediated only by DNA-binding domain interactions. Notably, AR-V dimerization was required to transactivate target genes and to confer castration-resistant cell growth. Our results clarify the mechanism by which AR-V mediate gene regulation and provide a pivotal pathway for rational drug design to disrupt AR-V signaling, as a rational strategy for effective treatment of advanced prostate cancer.

Precis

Results clarify how variant splice forms of the androgen receptor function to drive the malignant character of advanced prostate cancer, providing key mechanistic insights that will promote rational drug design for more effective treatment of this deadly disease.
INTRODUCTION

Recurrence with lethal castration-resistant prostate cancer (CRPC) after androgen deprivation therapy remains the major challenge in treatment of advanced prostate cancer (1,2). Significant advances in our understanding of continued androgen receptor (AR) signaling in CRPC have led to the development and FDA approval of two next-generation androgen-directed therapies, the androgen biosynthesis inhibitor abiraterone and the potent AR antagonist enzalutamide (3,4). These drugs heralded a new era of prostate cancer therapy. However, some patients present with therapy-resistant disease, and most initial responders develop acquired resistance within months of therapy initiation (3,4). The resistance is typically accompanied by increased prostate-specific antigen (PSA), indicating reactivated AR signaling (3,4). Accumulating evidences indicate that prostate tumors can adapt to these androgen-directed therapies, including abiraterone and enzalutamide, by signaling through constitutively-active alternative splicing variants of AR (AR-Vs) (5-17).

To date, 15 AR-Vs have been identified (18). Structurally, AR-Vs have insertions of cryptic exons downstream of the exons encoding the DNA-binding domain (DBD) or deletions of the exons encoding the ligand-binding domain (LBD), resulting in a disrupted AR open reading frame and expression of LBD-truncated AR (6,7,9,15,19,20). Since the N-terminal domain, which contains the most critical transactivation domain of the receptor (AF1), and the DBD remain intact in the majority of the AR-Vs, many AR-Vs display ligand-independent transactivation. AR-V7 (aka AR3) and ARv567es (aka AR-V12) are two major AR-Vs expressed in clinical specimens (7-10,15,17). They localize primarily to the nucleus, activate target-gene expression in a ligand-independent manner, and promote castration-resistant growth of prostate
cancer cells both \textit{in vitro} and \textit{in vivo} (7,9,15,19-21). Strikingly, patients with high levels of expression of AR-V7 or detectable expression of AR$^{v567es}$ in prostate tumors have a shorter survival than other CRPC patients (8). Moreover, AR-V7 expression in circulating tumor cells of CRPC patients is associated with resistance to both abiraterone and enzalutamide (17). These findings indicate an association between AR-V expression and a more lethal form of prostate cancer, and also highlight the importance of AR-Vs in limiting the efficacy of androgen-directed therapies.

AR-V7 and AR$^{v567es}$ can regulate the expression of both canonical AR targets and a unique set of targets enriched for cell-cycle function independent of the full-length AR (AR-FL) (7,10,15). AR-V7 and AR$^{v567es}$ can also activate AR-FL in the absence of androgen by facilitating AR-FL nuclear localization and co-regulate the expression of canonical AR targets (5). It has long been appreciated that dimerization is required for AR-FL to regulate target-gene expression (22), but little is known about AR-V dimerization. Coimmunoprecipitation of endogenous AR$^{v567es}$ and AR-FL (15) and co-occupancy of the PSA promoter by AR-V7 and AR-FL (5) suggest that AR-Vs may form heterodimers with AR-FL. However, whether AR-Vs homodimerize or heterodimerize with each other and whether the dimerization is required for AR-Vs to regulate target genes and to confer castration-resistant cell growth are currently unknown.

Dimerization of AR-FL is mediated mainly through N/C-terminal interactions, via the FxxLF motif in the N-terminal domain and the coactivator groove in the LBD, and DBD/DBD interactions, via the dimerization box (D-box) (22). Since the FxxLF motif and the D-box (Fig.
1A) are maintained in the majority of the AR-Vs identified, we hypothesize that these AR-Vs can form heterodimers with each other as well as homodimers via DBD/DBD interactions and that they can also form heterodimers with AR-FL via DBD/DBD and N/C interactions. In the present study, we tested this hypothesis by using the bimolecular fluorescence complementation (BiFC) and bioluminescence resonance energy transfer (BRET) assays, which have complementary capabilities for characterizing protein-protein interactions in live cells. BiFC allows direct visualization of subcellular locations of the interactions (23), while BRET allows real-time detection of complex formation (24,25).
MATERIALS AND METHODS

Cell Lines and Reagents

LNCaP, PC-3, DU145, VCaP, and HEK-293T cells were obtained from the American Type Culture Collection, and cultured as described (26). C4-2 was provided by Dr. Shahriar Koochekpour. All the cell lines were authenticated on April 1, 2015 by the method of short tandem repeat profiling at the Genetica DNA Laboratories. Enzalutamide was purchased from Selleck Chemicals.

Plasmid Construction

To generate different BiFC-fusion constructs of AR-FL, AR-V7, and ARv567es, we PCR amplified the AR-FL, AR-V7, and ARv567es cDNAs from their respective expression construct, and cloned the PCR amplicons separately into a TA-cloning vector (Promega). Fusion constructs of AR-FL, ARv567es, and AR-V7 with either VN or VC were generated by subcloning the cDNAs from the TA-plasmids into the Sall and XhoI sites of the pBiFC-VN155 and pBiFC-VC155 vectors. The mutant BiFC-AR-V and BiFC-AR-FL constructs with mutations at the FxxLF motif (F23,27A/L26A) and/or D-box (A596T/S597T) were generated by site-directed mutagenesis by using the Q5 site-Directed Mutagenesis Kit (New England BioLabs). BRET-fusion constructs of AR-FL, AR-V7, and ARv567es were generated by subcloning the AR-FL, AR-V7, and ARv567es cDNA from the respective TA-plasmids into the BamHI and XbaI sites of the pcDNA3.1-RLuc8.6 and TurboFP635 vectors (24). The doxycycline-inducible ARv567es lentiviral construct was generated by subcloning the ARv567es cDNA from its TA-plasmid first into the pDONR221 vector (Invitrogen) and subsequently into the doxycycline-inducible pHAGE-Ind-EF1a-DEST-
GH lentiviral construct by using the Gateway Cloning System (Invitrogen). All plasmids were sequence verified.

**DNA Transfection and Reporter Gene Assay**

PC-3 and HEK-293T cells were transfected by using the TransIT-2020 (Mirus Bio LLC) and TurboFect reagents (Thermo Scientific), respectively, per instruction of the manufacturer. DU145, C4-2, and LNCaP cells were transfected by using the Lipofectamine 2000 and Plus reagent (Invitrogen) as described (27). Reporter gene assay was performed as previously described (28) with either an androgen-responsive element-luciferase plasmid (ARE-luc) containing three ARE regions ligated in tandem to the luciferase reporter or a luciferase construct driven by three repeats of an AR-V-specific promoter element of the ubiquitin-conjugating enzyme E2C (UBE2C) gene (UBE2C-luc). To ensure an even transfection efficiency, we conducted the transfection in bulk, and then split the transfected cells for luciferase assay.

**Immunofluorescence Staining**

Cells were transfected with indicated plasmids on Poly-D-Lysine-coated coverslips (neuVitro) and cultured in phenol red-free medium supplemented with 10% charcoal-stripped fetal bovine serum. For the dihydrotestosterone (DHT) groups, 1 nM DHT was added at 24 hr after transfection. At 48 hr after transfection, cells were fixed with 70% ethanol, and incubated with a pan-AR antibody (PG-21, Millipore; 1:200) overnight at 4°C and subsequently with Alexa Fluor 488-conjugated secondary antibody (Invitrogen; 1:1000) for 1 hr at room temperature in the dark. Nuclei were then stained with 4',6-diamidino-2-phenylindole (DAPI). Confocal images
were obtained by using a Leica TCS SP2 system with a 40X oil-immersion objective on a Z-stage.

**BiFC Analysis**

Cells were co-transfected with different BiFC fusion constructs. At 48 hr after transfection, cells were incubated with Hoechst33342 (Invitrogen) and observed by fluorescence microscopy (Olympus, Japan). For flow cytometry quantitation of BiFC signals, the pDsRed2-C1 construct (Clontech) was co-transfected with the BiFC fusion constructs. At 48 hr after transfection, cells were trypsinized, and the Venus and DsRed fluorescence were analyzed by flow cytometry.

**Western Analysis**

The procedure was described previously (29). The anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Millipore), anti-AR (N-20, Santa Cruz), anti-HSP70 (Abcam), anti-Turbo-red-fluorescent-protein (Abcam), and anti-Renilla-luciferase (Thermo Scientific) antibodies were used.

**Quantitative Reverse Transcription-PCR (qRT-PCR) and Cell Growth Assay**

qRT-PCR was performed as described (30), and the qPCR primer-probe sets were from IDT. Cell growth was determined by the Sulforhodamine (SRB) assay as described (31). To ensure an even transduction efficiency, we conducted the transduction of the cells with packaged lentivirus in bulk, and then split the transduced cells for qRT-PCR and SRB assays.
BRET Assay

Cells were either transfected with an RLuc BRET fusion plasmid or co-transfected with an RLuc and a TFP BRET fusion plasmid. At 72 hr post transfection, cells were detached with 5 mM EDTA in PBS and resuspended in PBS with 1% sucrose. Cells were counted and seeded in triplicate into a 96-well white-wall microplate at $10^5$ cells per well. Freshly prepared coelenterazine (Nanolight Technology) in water was added to the cells at a final concentration of 25 μM. BRET readings at 528 nm and 635 nm were obtained immediately with a Synergy 2 microplate reader (BioTek). The BRET ratio was calculated by subtracting the ratio of 635-nm emission and 528-nm emission obtained from cells coexpressing the RLuc and TFP fusion proteins from the background BRET ratio resulting from cells expressing the RLuc fusion protein alone in the same experiment: BRET ratio = (emission at 635 nm)/(emission at 528 nm) – (emission at 635 nm RLuc only)/(emission at 528 nm RLuc only).

Statistical Analysis

The Student's two-tailed t test was used to determine the mean differences between two groups. $P < 0.05$ is considered significant. Data are presented as mean ± SEM.
RESULTS

Characterization of AR-FL and AR-Vs in BiFC fusion proteins

For BiFC analysis of interaction between proteins A and B, the two proteins are fused separately to either the N- or C-terminal fragment of the Venus fluorescent protein (VN or VC, Fig. 1B). If the two proteins dimerize, the interaction allows re-generation of the Venus fluorescent protein to emit fluorescent signal (23). Since BiFC depends on the relative orientation of the fusion proteins (23), we generated all possible combinations of N- and C-terminal fusions by cloning the AR-FL, ARV567es, or AR-V7 cDNA either in front of or after VN or VC. Different pairs of fusion protein constructs were transfected into the AR-null PC-3 cells (to avoid confounding effect of endogenous AR), and the fusion protein constructs exhibiting the highest BiFC signals (Fig. 1C) were chosen for further analysis. The trans-activating abilities of the fusion proteins were tested by the reporter gene assay. Although the protein fusion affected the relative activities of the fusion proteins (Figs. 1D and S1), all the fusion proteins can trans-activate target genes. Immunofluorescence assay further showed that the AR-FL and AR-Vs in the fusion proteins have the same subcellular localizations as the respective non-fusion AR isoform (Fig. 1E). Collectively, the data indicated that AR-FL and AR-Vs are functional in the fusion proteins.

BiFC detection of AR-V/AR-FL heterodimerization

To assess the ability of AR-V7 and ARV567es to heterodimerize with AR-FL, we cotransfected the AR-V- and AR-FL BiFC fusion constructs into PC-3 cells and quantitated the Venus fluorescence signal by flow cytometry. Both AR-V7 and ARV567es dimerized with AR-FL, and the dimerization did not require androgen (Fig. 2A & 2B). To delineate the dimerization
interface, we generated mutant BiFC-AR-V constructs with mutations at the FxxLF motif (F23,27A/L26A) and/or D-box (A596T/S597T). FxxLF motif and D-box mediate AR-FL homodimerization through N/C and DBD/DBD interactions, respectively (22). Only mutating both motifs abolished AR-V/AR-FL dimerization (Fig. 2A & 2B), indicating that both N/C and DBD/DBD interactions mediate the dimerization. Mutating one motif did not lead to significant change of BiFC signal (Fig. 2A & 2B), likely due to compensation of the loss of one mode of interaction by the other. Similar results were obtained in DU145 and HEK-293T cells (Figs. S2 and S3). Intriguingly, although ARv567es/AR-FL dimerization was observed in both the cytoplasm and the nucleus, AR-V7/AR-FL dimerization was detected primarily in the nucleus in the vast majority of the cells (Figs. 2A, 2B, S2A, S2B, S3A, and S3B).

Pretreatment of cells with DHT attenuated AR-V7/AR-FL dimerization, and this effect was blocked by the antiandrogen enzalutamide (Fig. 2C). Conversely, DHT pretreatment produced minimal effect on the dimerization of AR-V7 and the FxxLF-motif-mutated AR-FL (Fig. 2D), which lost the ability to homodimerize upon androgen treatment (Ref. (32) and Fig. S4B). These data indicate that AR-V7 may compete with AR-FL for dimerizing with AR-FL. Notably, the expression of each of the wild-type and mutant fusion proteins was confirmed by Western blotting (Fig. 2E). Collectively, our data demonstrated androgen-independent dimerization between AR-V and AR-FL, and indicated that AR-V/AR-FL dimerization may attenuate androgen induction of AR-FL homodimerization.

BiFC detection of AR-V/AR-V dimerization
We further showed that, like liganded AR-FL (Figs. 3A and S4), both AR-Vs can form a homodimer when expressed alone (Figs. 3B, 3C, S2C, S2D, S3C, and S3D). The homodimerization can also occur when AR-V is co-expressed with AR-FL and even when it is expressed at a much lower level than AR-FL (Fig. S5). Moreover, AR-V7 and ARv567es can heterodimerize (Fig. 3D). Mutating D-box, but not the FxxLF motif, abolished AR-V/AR-V interactions, indicating that AR-Vs homodimerize and heterodimerize with each other through DBD/DBD interactions. Interestingly, similar to AR-V7/AR-FL dimerization, AR-V7/AR-V7 dimerization was detected primarily in the nucleus (Figs. 3B, S2C, and S3C). However, ARv567es/ARv567es and AR-V7/ARv567es dimerization were observed in both the nucleus and the cytoplasm (Figs. 3C, 3D, S2D, and S3D).

Characterization of AR-FL and AR-Vs in BRET fusion proteins

We then used the newest BRET system, BRET6 (24), to confirm the BiFC results. BRET6 is based on energy transfer between the Rluc8.6 Renilla luciferase (Rluc) energy donor and the turbo red fluorescent protein (TFP) energy acceptor when the donor and acceptor are brought into close proximity by their fused proteins (Fig. 4A). Similar to BiFC, BRET also depends on the relative orientation of the fusion proteins. We therefore generated all possible combinations of N- and C-terminal fusions by cloning the AR-FL, ARv567es, or AR-V7 cDNA either in front of or after Rluc or TFP. Different pairs of the fusion protein constructs were transfected into the AR-null HEK-293T cells (to avoid confounding effect of endogenous AR), and the fusion protein constructs exhibiting the highest BRET signals (Fig. 4B) were chosen for further analysis. The expression of these fusion proteins was confirmed by Western blotting (Fig. 4C). Furthermore, their abilities to trans-activate were validated by luciferase assay with the co-
transfection of the ARE-luc plasmid (Fig. 4D), indicating that AR-FL and AR-Vs are functional in the BRET fusion proteins.

**BRET confirmation of AR-V/AR-FL and AR-V/AR-V dimerization**

Figure 5 shows the BRET saturation curves for different combinations of the BRET fusion proteins in HEK-293T cells. The BRET ratios increased hyperbolically and rapidly saturated with the increase in the ratio of energy acceptor to energy donor, indicating specific protein-protein interaction (33). Similar to the BiFC data, mutating the FxxLF-motif and/or the D-box inhibited AR-V/AR-FL and AR-V/AR-V dimerization (Fig. S6). Thus, the BRET data confirmed the BiFC results, showing the ability of AR-Vs to heterodimerize with AR-FL and to homodimerize. AR<sup>v567es</sup>/AR<sup>v567es</sup> interaction was further demonstrated by co-immunoprecipitation assay (Fig. S7).

**Dimerization is required for AR-V action**

To assess the requirement of dimerization for AR-V action, we first performed reporter gene assay with the wild-type or the dimerization mutants of AR-V. As shown in Figure 6A, the dimerization mutants completely lost the ability to trans-activate, indicating a requirement of dimerization for AR-V transactivation. We then analyzed the ability of the wild-type and dimerization mutants of AR-Vs to regulate target-gene expression and castration-resistant growth of prostate cancer cells. To this end, we infected the AR-FL-expressing LNCaP cells with lentivirus encoding AR-V7 or doxycycline-inducible AR<sup>v567es</sup>. Mutation of the FxxLF motif alone or both the FxxLF motif and D-box attenuated AR-V induction of androgen-independent expression of the canonical AR target PSA and the AR-V-specific target UBE2C (Figs. 6B) as
well as castration-resistant cell growth (Fig. 6C). The data indicated the requirement of
dimerization for AR-Vs to regulate target genes and to confer castration-resistant cell growth.
DISCUSSION

The present study represents the first to show the dimeric nature of AR-Vs in live cells. Using BiFC and BRET assays, we showed that AR-V7 and AR\(^{\text{v567es}}\) not only homodimerize and heterodimerize with each other but also heterodimerize with AR-FL. The dimerization does not require androgen. By mutating the FxxLF motif in the N-terminal domain and/or D-box in DBD of AR-Vs, we further showed that AR-V/AR-FL dimerization is mediated by both N/C and DBD/DBD interactions, whereas AR-V/AR-V dimerization is through DBD/DBD interactions. Since AR-Vs lack the C-terminal domain, the N/C interactions between AR-V and AR-FL is mediated presumably via the FxxLF motif of AR-V and the C-terminal domain of AR-FL. Significantly, dimerization mutants of AR-Vs lose the ability to trans-activate target genes and to confer castration-resistant cell growth, indicating the requirement of dimerization for important functions of AR-Vs.

Our finding on AR-V/AR-FL interaction is in accordance with the previous reports on AR\(^{\text{v567es}}\) and AR-FL coimmunoprecipitation (15) as well as on AR-V7 and AR-FL co-occupancy of the PSA promoter (5), providing a direct evidence for their dimerization. Interestingly, we found that the androgen-independent dimerization between AR-V and AR-FL may mitigate androgen induction of AR-FL homodimerization. This could constitute a mechanistic basis for the ability of AR-Vs to attenuate androgen induction of AR-FL activity (5). To date, functional studies of AR-Vs have been focused mostly on their ability to regulate gene expression independent of AR-FL. Since AR-Vs are often co-expressed with AR-FL in biological contexts, it is conceivable that the ability of AR-Vs to heterodimerize with and activate AR-FL in an
androgen-independent manner could be equally important as their AR-FL-independent activity to castration resistance.

We and others showed previously that AR-V7 and ARV567es localize constitutively to the nucleus and can facilitate AR-FL nuclear entry (5,15), indicating that the initial interaction between AR-V and AR-FL is likely to be in the cytoplasm. This is supported by our data showing both cytoplasmic and nuclear localization of ARV567es/AR-FL dimerization. Intriguingly, AR-V7/AR-FL dimerization is detected primarily in the nucleus in the vast majority of the cells. This may be due to the re-generation of the Venus fluorescent protein from the VN and VC fragments being slower than AR-V7/AR-FL nuclear translocation. Interestingly, AR-V7/AR-V7 dimerization was also detected primarily in the nucleus, whereas ARV567es/ARV567es and AR-V7/ARV567es dimerization were observed in both the nucleus and the cytoplasm. Whether this is also due to slower re-generation of the Venus fluorescent protein than AR-V7/AR-V7 nuclear translocation or AR-V7 entering the nucleus as a monomer requires further investigation. In addition, the majority of the post-translational modification sites of AR-FL are retained in AR-Vs (34). These post-translational modifications regulate AR-FL trans-activating activity, possibly via the interaction of AR-FL with other proteins or with itself (34). It is very likely that these post-translational modifications may impact AR-V dimerization and transactivation and therefore deserve further investigation.

We reported previously that AR-V binds to the promoter of its specific target UBE2C without AR-FL, but co-occupies the promoter of the canonical AR target PSA with AR-FL in a mutually-dependent manner (5). Furthermore, knockdown of AR-FL and AR-V both result in
reduced androgen-independent PSA expression, but only AR-V knockdown downregulates UBE2C expression (5). The data, together with the findings from the present study, indicate that AR-Vs regulate their specific targets as an AR-V/AR-V dimer but control the expression of canonical AR targets as an AR-V/AR-FL dimer. Interestingly, while mutating D-box alone does not significantly mitigate AR-V/AR-FL dimerization, the mutation abolishes the ability of AR-V to induce the expression of PSA and UBE2C as well as to promote castration-resistant cell growth. A plausible explanation is that, although D-box-mutated AR-V can dimerize with AR-FL, the dimer cannot bind to DNA to regulate the expression of target genes. This, together with the finding that D-box-D-box interactions are required for the formation of androgen-induced AR-FL intermolecular N/C interactions (32), indicates that disrupting D-box-D-box interactions could lead to inhibition of not only AR-V/AR-V dimerization and transactivation but also AR-FL activation induced by either AR-Vs or androgens. Thus, disrupting D-box-D-box interactions may represent a more effective means to suppress AR signaling than targeting the LBD of AR.

In summary, we demonstrated the dimeric nature of AR-Vs in live cells and identified the dimerization interface. Significantly, we showed that proper dimerization is required for AR-V functions. The research therefore represents a key step in delineating the mechanism by which AR-Vs mediate gene regulation. This is vital for developing effective therapeutic strategies to disrupt AR-V signaling and provide more effective treatments for prostate cancer.
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References


FIGURE LEGENDS

Figure 1. AR-FL and AR-Vs in BiFC fusion proteins are functional. (A) Schematic representation of AR-FL, AR-V7, and AR<sup>v567es</sup> protein structure. The DBD is composed of two zinc fingers. NTD, N-terminal domain; H, hinge region; U, unique C-terminal sequence. D-box and FxxLF motif mediate AR-FL dimerization. (B) A schematic of the principle of the BiFC assay. VFP, Venus fluorescent protein. (C) Schematic diagram of the constructs used in the BiFC assay. (D) Luciferase assay showing AR trans-activating activity in PC-3 cells co-transfected with the indicated BiFC construct and the ARE-luc plasmid. *, P < 0.05 from mock control. (E) Immunofluorescent (IF) staining showing protein fusion does not change subcellular localization of AR-FL, AR-V7, or AR<sup>v567es</sup>. The indicated expression construct or BiFC fusion construct was transfected into PC-3 cells, and IF staining was conducted at 48 hr after transfection. DAPI, nuclear stain. Scale bars, 10 µm. Cells were cultured under androgen-deprived condition unless specified. DHT, 1 nM for 24 hr.

Figure 2. AR-V7 and AR<sup>v567es</sup> heterodimerize with AR-FL through both N/C and DBD/DBD interactions. wt, wild-type; F-mut, FxxLF-motif mutant; D-mut, D-box mutant; FD-mut, FxxLF-motif and D-Box double mutant. Hoechst, nuclear stain. Scale bars, 10 µm. *, P < 0.05. (A & B) Dimerization was detected by the BiFC assay in PC-3 cells under androgen-deprived condition. Right panels, quantitation of BiFC signals by flow cytometry. (C & D) Pretreatment with androgen attenuates the dimerization between AR-V7 and wt AR-FL (C) but not the dimerization between AR-V7 and F-mut AR-FL (D). PC-3 cells were treated with 1 nM DHT with or without 10 µM enzalutamide (Enz) right after transfection with the indicated BiFC constructs, and BiFC signal was assessed at 48 hr after transfection. Right panel, quantitation of
BiFC signals by flow cytometry. (E) Western blotting with a pan-AR antibody showing expression of the BiFC-fusion proteins. Individual fusion construct was transfected into PC-3 cells cultured under androgen-deprived condition unless specified. DHT, 1 nM for 24 hr.

**Figure 3. AR-V and AR-V dimerize through DBD/DBD interactions.** AR-FL homodimerization (A), AR-V7 homodimerization (B), ARv567es homodimerization (C), and AR-V7/ARv567es heterodimerization (D) were detected by BiFC assay in PC-3 cells under androgen-deprived condition unless specified. DHT, 1 nM for 24 hr. Right panels, quantitation of BiFC signals by flow cytometry. wt, wild-type; F-mut, FxxLF-motif mutant; D-mut, D-box mutant. Hoechst, nuclear stain. Scale bars, 10 µm. *, P < 0.05. In contrast to AR-FL/AR-FL and AR-V7/AR-V7 dimerization, which were detected mainly in the nucleus (>90%), ARv567es/ARv567es and AR-V7/ARv567es dimerization were observed in both the nucleus (37% and 57%, respectively) and the cytoplasm (63% and 43%, respectively).

**Figure 4. AR-FL and AR-Vs in BRET fusion proteins are functional.** (A) A schematic of the principle of the BRET assay. (B) Schematic diagram of the constructs used in the BRET assay. RLuc, RLuc8.6 luciferase; TFP, TurboFP635 fluorescent protein. (C) Western blotting with a pan-AR antibody showing expression of the BRET-fusion proteins. Individual fusion construct was transfected into HEK-293T cells cultured under androgen-deprived condition. (D) Luciferase assay showing AR trans-activating activity in HEK-293T cells co-transfected with the indicated BRET construct and the ARE-luc plasmid. Cells were cultured under androgen-deprived condition unless specified. DHT, 1 nM for 24 hr. *, P < 0.05 from mock control.
Figure 5. BRET assay confirmation of AR-V/AR-FL and AR-V/AR-V dimerization.
Indicated BRET fusion constructs were co-transfected into HEK-293T cells at different ratios, and BRET signal was measured after the addition of the coelenterazine substrate. Lower panels, Western blotting with an antibody against TFP, RLuc, or HSP70 showing the levels of the fusion proteins expressed. Cells were cultured under androgen-deprived condition.

Figure 6. Dimerization mutants of AR-Vs lose ability to trans-activate and to promote castration-resistant cell growth. A. Wild-type or dimerization mutant of AR-V was co-transfected with the ARE-luc plasmid, and cells were cultured under androgen-deprived condition. B & C. LNCaP cells were infected in bulk with lentivirus encoding wild-type or dimerization mutant of AR-V7 (left panel) or doxycycline-inducible wild-type or dimerization mutant of ARv567es (right panel). At 24 hr after infection, cells were reseeded and treated with or without 200 ng/ml doxycycline and incubated for an additional 48 hr for qRT-PCR analysis of target genes (B) or for the indicated time for SRB assay of cell growth (C). Western blotting confirmed AR-V expression. *, P < 0.05 from control cells.
**Figure 1**

A. Schematic representation of D-box motifs in AR-FL-VN and AR-FL-VC constructs. D-box motifs are indicated by red boxes. 

B. Diagram showing VN: N-terminal fragment of VFP and VC: C-terminal fragment of VFP.

C. Diagrams illustrating the constructs used: AR-FL-VN, AR-FL-VC, AR-V7-VN, AR-V7-VC, AR\(^{v567}es\)-VN, AR\(^{v567}es\)-VC, AR-V7, AR\(^{v567}es\), and their respective linkers.

D. Bar graph showing relative luciferase activity for different constructs with mock and DHT treatments. Asterisks indicate statistically significant differences.

E. Immunofluorescence images showing AR expression with DAPI merged for different constructs under Ctrl and DHT conditions.
Figure 2
Figure 3
Figure 4

A) No BRET

B) BRET

C) Relative Luciferase Activity

D) Relative Luciferase Activity
Figure 6

(A) Relative Luciferase Activity

(B) Relative mRNA Levels

(C) Cell Growth Relative to Day 0

Legend:
- mock Ctrl
- wt AR-V7
- D-mut AR-V7
- FD-mut AR-V7
Androgen receptor splice variants dimerize to transactivate target genes

Duo Xu, Yang Zhan, Yanfeng Qi, et al.

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