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Identification of ELK1 interacting peptide segments in the androgen receptor

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Abstract

Prostate cancer (PCa) growth requires tethering of the androgen receptor (AR) to chromatin by the ETS domain transcription factor ELK1 to coactivate critical cell proliferation genes. Disruption of the ELK1-AR complex is a validated potential means of therapeutic intervention in PCa. AR associates with ELK1 by co-opting its two ERK docking sites, through the amino-terminal domain (A/B domain) of AR. Using a mammalian two-hybrid assay, we have now functionally mapped amino acids within the peptide segments 358-457 and 514-557 in the A/B domain as required for association with ELK1. The mapping data was validated by GST (glutathione S-transferase)-pulldown and BRET (bioluminescence resonance energy transfer) assays. Comparison of the relative contributions of the interacting motifs/segments in ELK1 and AR to coactivation of ELK1 by AR suggested a parallel mode of binding of AR and ELK1 polypeptides. Growth of PCa cells was partially inhibited by deletion of the upstream segment in AR and nearly fully inhibited by deletion of the downstream segment. Our studies have identified two peptide segments in AR that mediate functional association of AR with its two docking sites in ELK1. Identification of the ELK1 recognition sites in AR should enable further structural studies of the ELK1-AR interaction and rational design of small molecule drugs to disrupt this interaction.

Keywords

Prostate cancer; Androgen receptor; ELK1; ERK; D-box; FXFP motif

Introduction

Prostate cancer (PCa) growth and progression typically depend on transcriptional activity of the androgen receptor (AR) [1-5]. Therefore, the mainstay long-term treatment of residual or recurrent PCa is inhibition of androgen signaling, achieved by testosterone suppression. This is typically accomplished by blocking testosterone synthesis in the testes, most commonly

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Conflict of interest

The authors declare that they have no conflicts of interest.

by suppressing the hypothalamus-pituitary-gonad axis (chemical castration) [6]. Resistance to castration is often overcome by the additional use of androgen antagonists (enzalutamide, apalutamide, and darolutamide) or an inhibitor of testosterone synthesis within the tumor (abiraterone acetate) [7]. Patients eventually develop resistance to the treatments, even though the resistant tumors continue to depend on AR for growth. In the resistant tumors, growth signaling may be sustained through overexpression of AR or splice variants of AR in which the hormone binding domain is absent [8, 9]. Resistance could also occur due to hormone-independent phosphorylation of AR, changes in the co-regulator complement of AR or mutations in AR [10-13]. In addition to the problem of treatment resistance, testosterone suppression is burdensome because of its adverse effects, both acute and long term, on a variety of normal differentiated tissues [14-16]. To address these limitations of testosterone suppression, we have recently established the possibility of selectively disrupting a complex of AR with the transcription factor ELK1 that constitutes a growth signaling axis of AR that is critical for supporting PCa growth at all stages, including tumors resistant to current AR-targeted treatments [17-19].

ELK1 is an ETS (erythroblast transformation specific) family transcription factor that occurs as a downstream effector in the MAPK (mitogen-activated protein kinase) pathway and is a member of the ternary complex factor (TCF) subfamily. ELK1 binds to a core GGA sequence that is purine-rich [20]. ELK1 associates with many cell proliferation genes in a repressive or passive manner. When ELK1 is phosphorylated by ERK (extracellular signal-regulated kinase) it is hyper-stimulated, resulting in transient activation of its target genes and also association of ELK1 with serum response factor (SRF) for activation of immediate early genes [21, 22]. Notably, chromatin sites at which AR binds are highly enriched for DNA elements that bind ELK1 [23]. We have reported that ELK1 is required for complete or partial activation of a substantial proportion (~ 27 percent) of all genes activated by androgen in PCa cells [17]. Anchoring of AR to a set of chromatin sites by ELK1 in PCa cells enabled constitutive activation of critical cell proliferation genes by AR. ELK1-dependent gene activation by AR was not associated with ELK1 phosphorylation or MAPK signaling and was unaffected by inactivation of the transactivation domain of ELK1 [17, 18]. Using a variety of complimentary approaches, we have shown that AR binds to ELK1 ($Kd = 2x10^{-8}$ M) by coopting the two ERK docking motifs of ELK1, through the amino-terminal A/B domain of AR which lacks the hormone binding site. That binding of AR to ELK1 was essential for growth, was proven by the dominant-negative effect of an AR binding site mutant of ELK1 on growth of PCa cells that were insensitive to inhibition of MEK [18]. The A/B domain of AR supported ELK1-dependent gene activation, and so did splice variants of AR [17, 18]. Only AR-dependent tumor cells (including castration-resistant and enzalutamide-resistant tumors expressing AR splice variants) were dependent on ELK1 for cell, colony and tumor growth [17, 18]. The interaction of AR with ELK1 selectively supported activation of a critical set of AR target genes that were exclusively enriched for genes supporting cell cycle progression and mitosis [17, 18]. ELK1 was generally not required for regulation of AR target genes that were unrelated to cell growth [17, 18]. ELK1 was also reported by us to be an independent and strong prognosticator of PCa recurrence [24]. It follows therefore that disruption of the ELK1-AR complex could selectively suppress growth of PCa/castration-resistant PCa (CRPC) tumors, including

tumors dependent on AR splice variants, without significant effects on other functions of testosterone/AR and without affecting the normal functions of ELK1. Accordingly, a prototype small molecule inhibitor, KCI807, bound to AR, blocked ELK1 binding to AR, selectively inhibited ELK1-dependent transcriptional activity of AR and inhibited growth of cell line-derived and patient-derived prostate tumor xenografts that were resistant to castration and enzalutamide [19].

To facilitate further rational drug design to target the AR-ELK1 complex, it is desirable to identify the recognition segments within AR for the two AR docking sites in ELK1 that were previously identified by us. Toward this end, we first used a two-hybrid assay as well as an appropriate control assay and systematic deletion mapping of the AR A/B domain. We validated the mapped ELK1 binding segments by a GST (glutathione S-transferase)-pulldown assay and an *in situ* bioluminescence resonance energy transfer (BRET) assay. We then functionally validated the mapping data in the full-length AR, followed by confirming the role of the mapped segments in supporting AR-dependent growth of PCa cells.

Results

Deletion mapping to identify ELK1 recognition segments in the A/B domain of AR

The AR polypeptide is organized into several domains that include an amino-terminal A/B domain, a DNA binding domain, a hinge region, and a carboxyl-terminal ligand binding domain (Fig. 1A). The A/B domain includes a transcriptional activation function (AF1), which is ligand-independent and includes two units, TAU1 and TAU5. The ligand binding domain contains a ligand-dependent transcriptional activation function, AF2. Androgen binding within the ligand binding domain is typically needed for AR to translocate to the nucleus; however, splice variants lacking this domain have been shown to induce androgen response genes and support growth in PCa cells. We have previously reported that the A/B domain is necessary and sufficient for binding to ELK1 [17, 18]. Therefore, we used the A/B domain to map the ELK1 recognition segments of AR.

We used a mammalian two-hybrid assay and deletion mutagenesis to map the regions within AR(A/B) required for interaction with ELK1. Although the A/B domain has transcriptional activity, our rationale for using the AR(A/B)-VP16 fusion protein (rather than the A/B domain alone) was to ensure that if a deletion in the A/B domain abrogated its transcriptional activity, binding to ELK1 could still be monitored via the transcriptional activity of VP16, as long as we included a control assay, as described in the following paragraph. We used recombinant AR-negative HeLa cells harboring a minimal TATA box-dependent promoter-luciferase reporter construct containing five Gal4 elements located upstream of a TATA box (*GAL4-TATA-Luc*) and constitutively expressing a Gal4-ELK1 fusion protein in which the DNA binding domain of ELK1 was replaced with the Gal4 DNA binding domain. These cells were transiently transfected with expression plasmids for AR(A/B)-VP16 fusion constructs. The luciferase reporter activity was used to quantify binding of the AR(A/B)-VP16 constructs to ELK1.

AR(A/B)-VP16 fusion constructs containing consecutive, overlapping deletions within the AR(A/B) region were tested in the two-hybrid assay and compared with a wild

type AR(A/B)-VP16 fusion protein containing no deletions (Fig. 1B). Expression of the AR(A/B)-VP16 fusion proteins was monitored for data normalization by western blotting using one of two antibodies to AR with different epitopes to enable probing of all the deletion constructs (Fig. 1C). Five constructs exhibited decreased promoter activity relative to the wild-type construct; they were deletion 307-407, deletion 357-470, deletion 489-539, deletion 535-557, and deletion 552-557 (Fig. 1B). The deletion 457-514 construct did not display decreased promoter activity (Fig. 1B). Based on these results, there are apparently two segments within the A/B domain of AR that are required for functional association with ELK1; the two segments span amino acids 357-457 and 514-557. In addition, deletion 1-105 and 107-207 showed increased promoter activity, possibly because they include sites of corepressor binding to the A/B domain.

As deletions made within AR(A/B) could influence the inherent transcriptional activity of the AR(A/B)-VP16 fusion constructs independently of their ability to interact with ELK1, it was necessary to directly test the effect of the deletions in control studies conducted in parallel with the mapping studies above. We accomplished this using a control assay in which recombinant HeLa cells harboring the *GAL4-TATA-Luc* promoter-reporter were transiently transfected with expression plasmids for Gal4-AR(A/B)-VP16 double fusion constructs containing either no deletion or the deletions within AR(A/B) that were tested in Fig. 1B (Fig. 1C). Expression of the fusion proteins was monitored for data normalization by western blotting using antibodies to AR (Fig. 1E). None of the fusion constructs containing deletions showed a decrease in promoter activity, indicating that the inherent transcriptional activity of AR(A/B)-VP16 was not decreased by the deletions (Fig. 1D). As expected, deletion of residues 1-105 and 107-207 showed increased promoter activity in the control assay in Fig. 1D, consistent with the observation in Fig. 1B. Additionally in Fig. 1D, deletions 307-407, 357-470 and 489-539 also showed increased transcriptional activity, attributable to loss of repressor binding. However, these deleted segments were obviously also in the regions needed for the A/B domain to bind to ELK1 in the two-hybrid assay resulting in reduced transcriptional activity for these deletions in Fig. 1B. Therefore, the decrease in promoter activity in some of the constructs seen in the initial mapping experiment in Fig. 1B was due to decreased interaction between AR and ELK1, rather than a decrease in the intrinsic transcriptional activity of the AR(A/B)-VP16 fusion protein.

To further confirm that the polypeptide segment spanning amino acids 457 to 514, which apparently separated two distinct ELK1-interacting segments in the AR(A/B) domain (Fig. 1B), was not required for interaction with ELK1, it was necessary to exclude the possibility that deletion of this relatively large segment (57 amino acids) did not actually influence the promoter activity by significantly altering the distance between the two putative ELK1-interacting segments. To address this question, we made smaller sequential internal deletions of five amino acids within this segment and tested the effects of the deletions in the above two-hybrid assay (Fig. 2A). Western blots were used for monitoring fusion protein expression for data normalization (Fig. 2B). None of the smaller deletions significantly affected promoter activity, confirming that the peptide segment spanning amino acids 457 to 514 separates two distinct ELK1 interaction segments.

Collectively, the above data identify two distinct segments within the A/B domain of AR that are required for transcriptional coactivation of ELK1 by AR. The two segments span amino acids 357-457 and 514-557.

Validation of mapped ELK1 recognition segments in full-length AR

To confirm that the interaction of full-length AR with ELK1 required two ELK1 recognition segments (amino acids 357-457 and 514-557) mapped above within the AR(A/B) domain, we next tested the effect of deleting the upstream or the downstream segment in wtAR on the ability of the receptor to synergize with ELK1. The constructs were transfected into recombinant HeLa cells harboring the *GAL4-TATA-Luc* promoter-reporter and constitutively expressing the Gal4-ELK1 fusion protein. The cells were treated with either testosterone (to enable nuclear translocation of AR) or vehicle control (Fig. 3A). Expression of the AR constructs was confirmed by western blotting (Fig. 3B). Compared with the reporter luciferase activity induced by wtAR in a testosterone-dependent manner, the testosterone-dependent induction of luciferase was significantly decreased by deletion of amino acids 358-459 and abolished by deletion of amino acids 514-562 (Fig. 3A). The data confirm that the two segments required for interaction of the AR(A/B) domain with ELK1 also represent the ELK1-interacting segments in the full-length AR.

Interacting partners among the two ELK1 recognition segments in AR and the two AR docking sites in ELK1

The two segments in AR required for functional association with ELK1 presumably interact with the two sites within ELK1 previously identified as the docking sites for AR [18], in either a parallel or anti-parallel orientation of the two proteins. To examine the possibilities, we co-transfected HeLa cells with the *GAL4-TATA-Luc* promoter-reporter and the expression plasmid for wtAR. The cells were additionally co-transfected with an expression plasmid for Gal4-ELK1 or Gal4-ELK1 containing either a deletion of the upstream AR docking site (D-box) or a deleterious mutation in the downstream AR docking site (FxFP motif). Interaction between AR and ELK1 was monitored in terms of the testosterone-induced luciferase activity (Fig. 3C). Expression of the AR and ELK1 constructs was confirmed by western blot using antibodies to AR and to Gal4 (Fig. 3D). We observed that disruption of the upstream AR docking site in ELK1 partially inhibited the ELK1-AR synergy, whereas disruption of the downstream AR docking site abolished the synergy (Fig. 3C). Taken together with the data in Fig. 3A, which shows that loss of the upstream ELK1-interacting segment in AR results in only partial loss of its synergy with ELK1, whereas loss of the downstream segment in AR results in a greater loss of the synergy, it is possible that AR and ELK1 interact in a parallel binding mode (as shown in Fig. 3E), wherein the upstream and downstream sites in AR associate respectively with the upstream and downstream sites in ELK1.

Validation of ELK1 binding segments in AR using orthogonal methods

To further validate the mapped ELK1 binding segments within AR, we performed orthogonal assays, one using purified proteins and another in the *in situ* context.

First, we performed a GST-pulldown assay using purified His-tagged ELK1 and a purified GST-tagged AR fragment comprising amino acids 334-566 (Fig. 4A**, left panel)**. Protein input was monitored by Coomassie blue staining (Fig. 4A**, right panel).** As seen in Fig. 4A, the AR peptide 334-566 specifically pulled down ELK1, consistent with physical association of the ELK1-interacting AR segments mapped above with the ELK1 protein.

Next, bioluminescence resonance energy transfer (BRET) was used to further validate the ELK1 interacting segments in AR *in situ* using HEK293T cells (Fig. 4B). The cells were transfected with expression plasmids for Turbo-ELK1 and an RLuc construct fused with an AR-V7 polypeptide in which most of the A/B domain (amino acids 50-357) was deleted, leaving intact the region of the A/B domain that encompasses the two ELK1-interacting segments (amino acids 358-557). BRET coupling between the ELK1 and AR fusions increased hyperbolically with increasing acceptor/donor ratios, indicative of specific binding between ELK1 and the AR polypeptide (Fig. 4B), further validating the region within the A/B domain mapped as the ELK1 binding region. Data presented in Supplemental Fig. 1 shows an additional (negative) control for the BRET assay using an AR construct with a double mutation that resulted in loss of the BRET signal.

Physiological validation of the ELK1 interacting segments in AR

As association with ELK1 is critical for the AR-dependent growth of established PCa model cell lines [17], it was of interest to examine the role of the two ELK1 interacting segments in AR in supporting the growth of PCa cells. For this purpose, we chose to examine 22Rv1 cells, which are aggressively growing PCa cells that express both full length AR and the AR-V7 splice variant. 22Rv1 cells are entirely dependent on the full-length AR for growth and colony formation [25]. In these cells, we first ectopically stably overexpressed AR with a deletion of either ELK1 interacting segment mapped above. We then used shRNA to knock down the endogenous full-length AR in parental 22Rv1 cells or in either one of the recombinant cell lines. The endogenous AR in 22Rv1 cells could be distinguished from the exogenous AR deletion constructs by western blot, based on its higher apparent molecular weight (Fig. 5A). Although the shRNA targeted both the endogenous AR and the exogenous AR deletion mutants (but not AR-V7), it virtually completely knocked down the endogenous AR in both the parental and the recombinant cell lines (Fig. 5A). However, in the recombinant cells treated with AR shRNA, there was residual expression of ectopic AR deletion constructs at levels that either matched or exceeded the expression of endogenous AR in the parental 22Rv1 cells treated with control (non-targeted) shRNA (Fig. 5A). Loss of endogenous AR resulted in suppression (~90 percent) of colony growth in parental 22Rv1 cells (Fig 5B). In the cells expressing AR (deletion 358-462) and AR (deletion 514-562), depletion of endogenous AR resulted in inhibition of colony formation by ~30 percent and ~75 percent, respectively. We did not include cells expressing ectopic full-length AR because this was not essential and also because we were unable to achieve substantial overexpression compared with endogenous AR. The results indicate that AR with deletion of either one of the two ELK1 interacting segments is unable to substitute for the wtAR in supporting growth of PCa cells. Further studies will be needed to determine why deletion of the downstream ELK1 binding segment appears to impact more severely on the ability of AR to support cell growth compared with deletion of the upstream segment.

Discussion

The two-hybrid assay used in this study of AR enabled systematic deletion analysis of the A/B domain to map the ELK1 interacting segments of the A/B domain while controlling for any unrelated effects of the deletions using an appropriate one-hybrid assay. In this manner, two peptide segments in the A/B domain were determined to be necessary and sufficient for the association of AR with ELK1, an upstream segment comprising amino acids 358 to 457 and a downstream segment, comprising amino acids 514 to 557. Other regions of the A/B domain were not required for the association of AR with ELK1. The mapping data was validated by deletion analysis of the full-length AR protein, which showed that both the downstream and upstream segments were required for optimal functional association. The mapping data was further supported by orthogonal assays including GST-pulldown and *in situ* BRET assays. Finally, the mapped segments were physiologically validated by demonstrating their contribution to the growth of PCa cells. Additionally, the studies suggest that two discrete regions in AR associate with the two previously mapped AR recognition motifs within ELK1 in a parallel orientation of the AR and ELK1 polypeptides.

Members of the nuclear receptor superfamily of transcription factors, including the steroid receptor subfamily, share a common structural organization comprising five major polypeptide domains [26]. Two of these domains, the DNA binding domain (DBD) and the ligand binding domain (LBD), are significantly conserved [26], and numerous publications have reported their crystal structures [27, 28]. The amino-terminal domain shows little conservation even among steroid receptors [26] and has an intrinsically disordered structure that has confounded attempts to crystallize it [29]. However, this domain plays a critical functional role through interactions with a variety of transcriptional coregulators [30]. The amino terminal A/B domain of AR is functionally dynamic, as it not only binds both general coactivators and also coactivators that are specific to AR function [31] but also interacts with and modulates the functions of the LBD [32] and the DBD [33]. Indeed, although the A/B domain of AR does not directly contact DNA, it does influence the DNA sequence selectivity of the DBD [33]. Therefore, it is unsurprising that the A/B domain of AR confers on the receptor its unique ability to associate with ELK1 as a coactivator and that it contains within it the structural segments needed for this association.

Approximately 70 percent of the A/B domain of AR comprises the ligand-independent transcriptional activation function 1 (AF-1) encompassing the functional subdomains Tau1 and Tau5. The downstream ELK1 interaction segment mapped in this study lies outside AF-1, at the transition to the DBD. The upstream segment partially overlaps Tau1 and Tau5. Therefore, while tethering of AR to chromatin by ELK1 through the downstream segment may not significantly interfere with the ability of AR to activate the associated target genes, it is possible that occlusion of a part of AF-1 in a complex with ELK1 due to its association with the upstream AR segment may confer selectivity in coactivator binding to AR in Pca cells.

The amino terminal domains of steroid receptors have different lengths and show little amino acid sequence homology. However, they characteristically share certain structural features, including an amino acid compositional bias towards small residues (G, A),

uncharged hydrophilic residues (N, Q, S, T) and proline (P) residues that collectively contribute to a generally disordered structure, interspersed with small segments of charged and hydrophobic residues that are more structured, serving as likely sites of binding of other proteins [34]. It has been suggested that these structural features of the NTDs allow for the flexibility and receptor specificity required for the characteristic protein-protein interactions of each steroid receptor [34]. Inspection of the amino acid sequences of the two ELK1 binding segments in AR (Fig. 6) shows that while the longer (~100 amino residues) upstream segment displays four clusters of mostly charged and large hydrophobic residues in between apparently unstructured (flexible) stretches, the shorter (~ 50 amino residues) downstream segment predominantly comprises charged and large hydrophobic residues, characteristic of a more rigid structure. Comparison of either ELK1 interacting segment in AR with other nuclear receptors (estrogen receptor isoform 1, progesterone receptor isoform A, glucocorticoid receptor isoform alpha, and mineralocorticoid receptor isoform 1) using the basic local alignment search tool (BLAST) did not show significant sequence similarity, consistent with our observation that AR is the only steroid receptor that has the ability to avidly bind to ELK1.

Structural modeling of the binding of AR to ELK1 through the mapped segments could be challenging at this time in the absence of physical data on the structure of the A/B domain of AR. However, the mapping data reported here should now enable the use of the appropriate synthetic peptides for detailed structural studies. There is currently a great deal of interest in developing small molecule drugs for Pca that would target the A/B domain of AR, as they would overcome limitations of conventional AR antagonists that target the LBD [35]. Significantly, the findings reported here should facilitate investigation of the mode of target interaction of the platform antagonist KCI807, which binds to AR and disrupts its association with ELK1. It is possible that KCI807 binds to one of the two ELK1 interacting segments in AR, or it could bind elsewhere in AR to induce AR domain interactions that disrupt the AR-ELK1 complex. Future studies should resolve these questions to aid in the development of novel KCI807-based drug molecules.

Methods

Reagents and cell lines

22Rv1 (CRL-2505) and HeLa (CCL-2) cells were purchased from the American Type Culture Collection (Manassas, VA). Recombinant HeLa cells which harbor a minimal TATA-dependent promoter-luciferase reporter with five upstream Gal4 elements (*GAL4-TATA-LUC*) were made by stably transducing a vector containing *GAL4-TATA-LUC*. HeLa cells with stably integrated *GAL4-TATA-LUC* that express the Gal4-ELK1 fusion protein, with the Gal4 DNA binding domain substituting the ETS DNA binding domain of ELK1, were a kind gift from Dr. Johann Hofman (Innsbruck Medical University). 22Rv1 cells stably expressing ectopic mutant AR were made by stably transducing a vector containing each type or mutant AR as described below. Culture media for recombinant and parental HeLa cells was DMEM supplemented with 10% FBS plus a mixture of 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine (Invitrogen). Culture media for the recombinant HeLa cells also included one or more of the following antibiotics for

selection: 100 μg/mL hygromycin (10687010, Invitrogen) (for Gal4-ELK1) and 100 or 400 μg/mL geneticin (10131027, Invitrogen) for *GAL4-TATA-LUC*. Culture media for parental 22Rv1 cells was RPMI 1640 supplemented with 10% FBS and 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine mixture (Invitrogen) for collection of conditioned media. Culture medium for 22Rv1 cells expressing ectopic mutant AR was RPMI 1640 supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine mixture (Invitrogen), and 0.4 μg/mL puromycin (P8833, Sigma). Rabbit monoclonal antibody to AR (ab133273, 1:500) and mouse monoclonal antibody to AR (ab9474, 1:250) were purchased from Abcam. Antibody to GAPDH (sc-47724, 1:3000) was purchased from Santa Cruz Biotechnology. Rabbit monoclonal antibody to ELK1 (ab32106,1:1000) was purchased from Abcam. The source of rabbit polyclonal to AR (GTX100056, 1:1000) was GeneTex. GST-HRP conjugate (RPN1236, 1:3000) was purchased from Amersham. Mouse monoclonal antibody (AC-15, 1:25,000) was purchased from Sigma-Aldrich. Testosterone (T-1500) was purchased from Sigma. The source of Lipofectamine™-2000 (11668027) was Invitrogen.

Purified proteins

His-tagged ELK1 was expressed in baculovirus-infected Sf9 cells and was purified by using nickel-agarose affinity chromatography. 200 mM imidazole was used to elute the protein and the protein was then dialyzed against 20 mM HEPES, pH 7.9, containing 10% glycerol, 20 mM KCl, 2 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM benzamidine, and 0.5 mM DTT. SDS-PAGE was used to confirm that the purity of the protein was at least 85%. GST-tagged AR334-566 expressed from Rosetta(DE3) *E. coli* cells was purified by glutathione-sepharose affinity chromatography. Protein was eluted with 10 mM reduced glutathione, followed by dialysis against HBS-N (10 mM HEPES pH 7.4, 150 mM NaCl) plus 0.2 mM EDTA and 1mM DTT.

Plasmids

The pSG5-VP16-AR(A/B) construct was previously generated in our laboratory. The AR expression plasmid pSG5-hAR was a kind gift from Lirim Shemshedini (University of Toledo, Toledo, OH). The *GAL4-TATA-LUC* pG5*Luc* plasmid (E249A), VP16 pACT plasmid (E246A), and pBIND Gal4 plasmid (E245A) were purchased from Promega in the CheckMate Mammalian Two-hybrid System (E2440). The pRL plasmid encoding *Renilla* luciferase (E2271) was purchased from Promega. The pCDH-CMV-MCS-EF1-puro cDNA Cloning and Expression Lentivector plasmid (CD510B-1) used for all lentiviral constructs was purchased from System BioSciences. shRNA targeting AR (TRCN0000003718) and non-targeting control shRNA (SHC016-1EA) in the lentivirus expression plasmid pLKO.1-puro were obtained from Sigma-Aldrich. BRET constructs pcDNA3.1-Rluc8.6 and pcDNA3.1-Turbo635 were kindly gifted by Dr. Yan Dong (Tulane University, New Orleans, LA), and they were used to construct pcDNA3.1-Rluc-AR-V7Δ50-357 and pcDNA3.1-Turbo-ELK1. pGEX4T1-AR334-566 was obtained from Addgene (104198) and pFastbac1-ELK1-his was previously generated [36].

Production of AR constructs with mutations

The QuikChange II-XL Site-Directed Mutagenesis kit (200522) from Agilent or the Q5 Site-Directed Mutagenesis kit (E0554S) from New England Biolabs were used to make

deletion constructs for AR(A/B) and full-length AR, unless otherwise noted. For the VP16-AR(A/B) fusion proteins, a pSG5-AR(A/B)-VP16 construct previously generated in our lab, containing wild type A/B, was used as the template. For the full-length AR constructs, a pSG5 expression vector containing wild type AR was used as the template. All AR constructs correspond to NCBI reference number NM_000044. AR(A/B) domain constructs include residues 1-557. Primer sequences used for site directed mutagenesis are as follows: AR(A/B) Δ107-207 forward 5'- CGAGCGCAGCACCTTCAGCTGCCCCA – 3', reverse 5' – GGCCCCACAGGCAGCGGGAGAGCG – 3';
AR(A/B) Δ207-307 forward 5' – CAGTATCCGAAGGCAGCGAGTATTCCC – 3', reverse 5' – TTGAAAGGGGAATACTCGCTGCCTTCG – 3';
AR(A/B) Δ257-357 forward 5' – GTGGAGGCGTTGGAGCATGCGTACCAG – 3', reverse 5' – CTCTGGTACGCATGCTCCAACGCCTCC – 3';
AR(A/B) Δ307-407 forward 5' – GAGCACTGAAGATACTTATGGGGACCT – 3', reverse 5' – TCGCCAGGTCCCCATAAGTATCTTCAG – 3',
AR(A/B) Δ357-470 forward 5' – GCACTGGACGAGGCAGGCGGCGG – 3', reverse 5' – CCGCCGCCTGCCTCGTCCAGTGC – 3'; AR(A/B) Δ457-514 forward 5' – GTGGTGGGGGTGGTCCCAGTCCCA – 3', reverse 5' – GTGGGACTGGGACCACCCCCACCAC – 3'; AR(A/B) 489-539 forward 5' – CTCGGCCCCCTCAGTTGGAGACTGCCAG – 3', reverse 5' – CTGGCAGTCTCCAACTGAGGGGGCCGAG – 3'; AR(A/B) 485-491 forward 5' – CGGCTACACTCGGGCGGGCCAGGAAA – 3', reverse 5' – TTTCCTGGCCCGCCCGAGTGTAGCCG – 3'; AR(A/B) Δ496-500 forward 5' – CGGGCCAGGAAAGCGATGTGTGGTACCC – 3', reverse 5' – GGGTACCACACATCGCTTTCCTGGCCCG – 3'; AR(A/B) Δ506-510 forward 5' – GCACCTGATGTGTGGTACCCTAGAGTGCCCTAT – 3', reverse 5' – ATAGGGCACTCTAGGGTACCACACATCAGGTGC – 3';
AR Δ358-459 forward 5' – CTGGACGAGGCAGCTGGCGGCGG – 3', reverse 5' – CCGCCGCCAGCTGCCTCGTCCAG – 3'; and AR Δ514-557 forward 5'- GAGCAGAGTGCCCCTCGATCGAGGCG – 3', reverse 5' – CGCCTCGATCGAGGGGCACTCTGCTC – 3'

The AR(A/B)-VP16 deletion 1-105 construct was made by cloning the VP16-AR(A/B) fusion protein consisting of amino acids 106-557 from a pACT-AR(A/B) expression construct that was previously generated in our laboratory into the pSG5 vector, using the BamHI and AfIII restriction sites.

The AR(A/B)-VP16 constructs containing deletions 535-557 and 552-557 were made from AR(A/B)-VP16 mutagenesis constructs previously generated in our laboratory in the pACT expression vector which contained each of the two deletions. The regions containing each of the deletions within the A/B domain were cloned into the pSG5-AR(A/B)-VP16 construct using the KpnI restriction site. The digested vector and insert were dephosphorylated prior to ligation using calf intestinal alkaline phosphatase (CIP) obtained from New England Biolabs (M02905) to ensure proper ligation orientation.

The VP16-AR(A/B)-Gal4 fusion constructs were made by cloning the yeast Gal4 DBD from the pBIND vector downstream of A/B into each of the existing AR(A/B)-VP16

deletion constructs using the NotI and XbaI restriction sites, using custom primers designed to add the downstream XbaI restriction site and stop codon at the C-terminal (forward: 5' – TCCTGAAAGGCGGCCGCATGAAGCTACTGTCTTC – 3', reverse: 5' – CGGGAATTCCGTCTAGAGTTACGATACAGTCAACTG – 3').

The ELK1 mutant constructs are described in [18].

Bioluminescence resonance energy transfer (BRET)

Transfection of HEK293T cells with pcDNA3.1-Rluc-AR-V7Δ50-357 and pcDNA3.1-Turbo-ELK1 was performed at different ratios using TransIT-LT1 transfection reagent (MIR 2300) from Mirus Bio. Two days after transfection, 1×10^5 cells per well were seeded again in white-walled, 96-well plates. Two hours later, 25μM coelenterazine was added and BRET emissions were measured at 535 nm (Rluc) and 635 nm (Turbo) using a CLARIOstar (BMG Labtech) dual plate reader.

GST pull down

Glutathione sepharose beads (5 uL per sample) were first blocked with 0.1% BSA in PBS at room temperature for 1 hour and then washed sequentially twice with 500 uL PBS and once with HBS-N supplemented with 0.1% Triton. Then, HBS-N (200 uL) was added to each bead sample, and protein as required (1.5μg GST/GST-AR334-566/ELK1-His). The samples were then incubated for 2 hours at room temperature. Then they were washed three times with 500 uL HBS-N with a 5 minute incubation at room temperature and resuspended in SDS-PAGE buffer for western blot.

Lentivirus-mediated transduction

Mutant AR plasmids and vector control were packaged in 293FT cells as previously described [17, 37]. 24 hours prior to infection, 22Rv1 cells were plated in phenol red-free medium supplemented with 10% heat-inactivated charcoal-stripped FBS and 2 mm l-glutamine at a confluency of 30%. The following day, the cells were infected with the vector lentivirus or mutant AR lentivirus containing a deletion of either the upstream or downstream ELK1 interaction segment, with Polybrene (8 μg/mL) and incubated for five hours. After incubation, the virus was replaced with RPMI-1640, 10% FBS, plus 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine. After a two-week recovery period, the cells were put under selection in 0.4 μg/mL puromycin. As needed, cells were then re-infected with control shRNA or AR shRNA lentivirus to knock down AR.

Colony formation assay

Following lentivirus-mediated knockdown of AR 72h post-transfection, empty vector or mutant AR-overexpressing 22Rv1 cells were plated in triplicate in 6-well plates at a density of 4000 cells per well using phenol red-free conditioned media that had been collected from parental 22Rv1 cells grown for 48 or 72 hours in monolayer culture. Colony forming cells were grown at 37 °C in 5% CO2 with a media change every 3 to 4 days for 7-8 days until colonies had formed. Colonies were fixed in methanol and stained with crystal violet. All treatments were conducted in triplicate. Colony counting was performed using a Gelcount colony counter (Oxford Optronix).

Other experimental methods

The methods for transient transfections, luciferase reporter assays, Checkmate mammalian two-hybrid assay, and western blotting analysis were as previously described [17-19]. In the luciferase assays, we used protein expression on western blots to compare transfection and expression of various constructs rather than normalization to Renilla luciferase to avoid variability in co-transfection and squelching effects known to occur with the Renilla luciferase expression plasmid.

Statistical Analysis

The expression levels were depicted with mean and standard deviation. For comparisons between two groups, the expression levels were checked for their distribution and log-transformed to meet the normality assumptions, followed by either paired or unpaired t-tests as appropriate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

All of the data described in this study are included in the manuscript.

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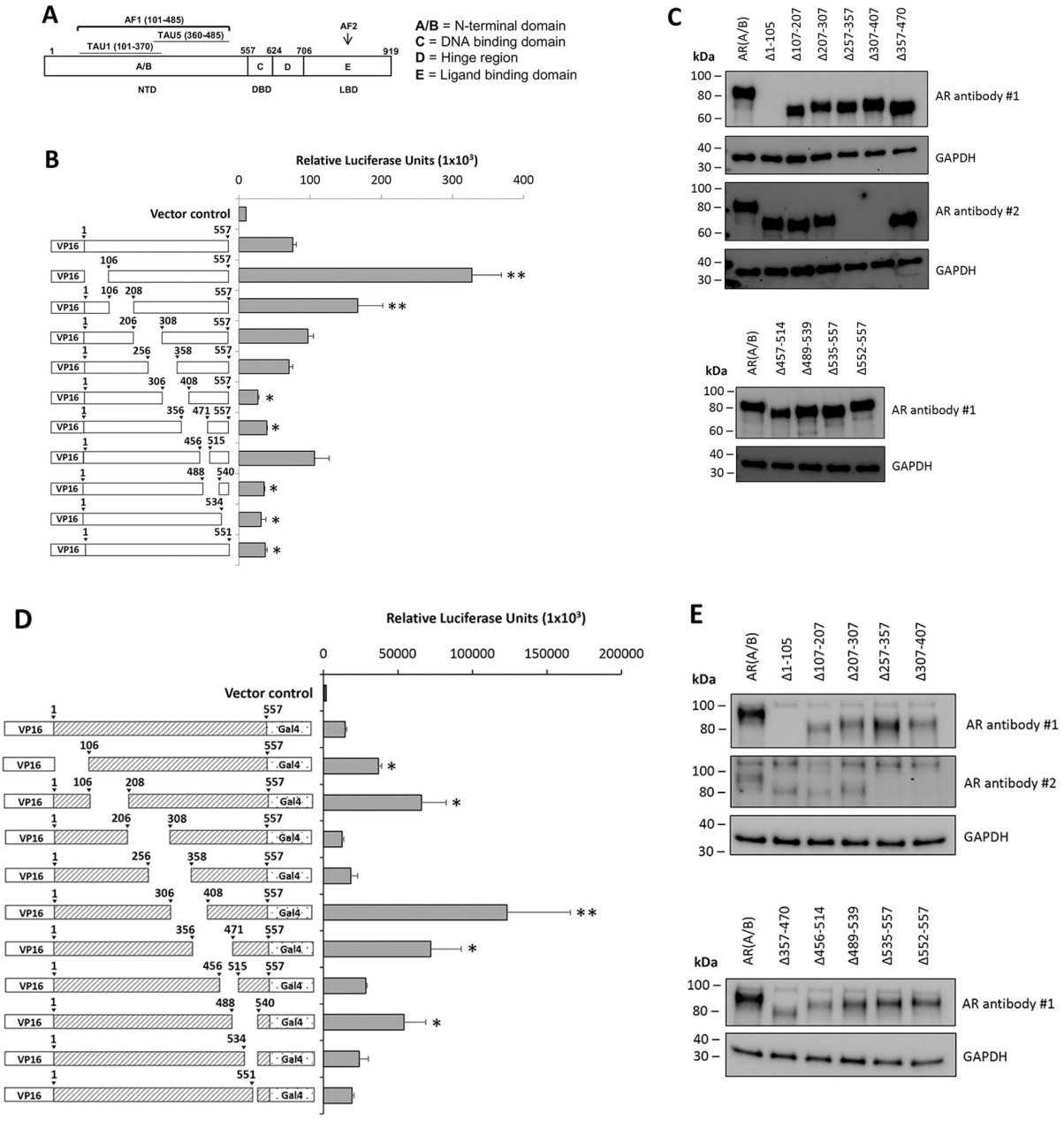

Figure 1: Mapping regions of the A/B domain of AR required for interaction with ELK1 by deletion analysis.

A. Schematic of the structural organization of AR including functional domains and sub-domains. **B.** HeLa cells harboring the GAL4-TATA-LUC promoter-reporter and expressing a Gal4-ELK1 fusion protein, were transfected with expression plasmids for AR(A/B)-VP16 fusion constructs containing no deletion or consecutive, overlapping deletions within the A/B domain. Cells were harvested 48h post-transfection for preparation of cell lysates to measure luciferase activities that were normalized to protein expression from western blots and plotted. $*P < 0.03$, $**P < 0.02$. **C.** From a parallel set of wells transfected as described for Panel B, cell lysates were probed by western blotting with antibodies against AR or GAPDH (loading control). To ensure detection of all the deletion mutants by western blot,

two separate AR antibodies with different epitopes were used. Antibody #1 corresponds to Abcam catalog number ab133273, and antibody #2 corresponds to Abcam catalog number ab9474. **D.** HeLa cells harboring GAL4-TATA-LUC (but not expressing Gal4-ELK1) were transfected with double fusion constructs of the A/B domain of AR (Gal4-AR[A/B]-VP16) containing no deletion or the same consecutive deletions within the A/B domain used in Panel B. Cells were harvested 48h after transfection and cell lysates prepared to measure luciferase activities that were normalized to protein expression from western blots and plotted. $*P < 0.02$, $**P = 0.006$. **E.** From a parallel set of wells transfected as described for Panel D, cell lysates were probed by western blotting with antibodies against AR or GAPDH (loading control). To ensure detection of all the deletion mutants by western blot, two separate AR antibodies with different epitopes were used. Antibody #1 corresponds to Abcam catalog number ab133273, and antibody #2 corresponds to Abcam catalog number ab9474.

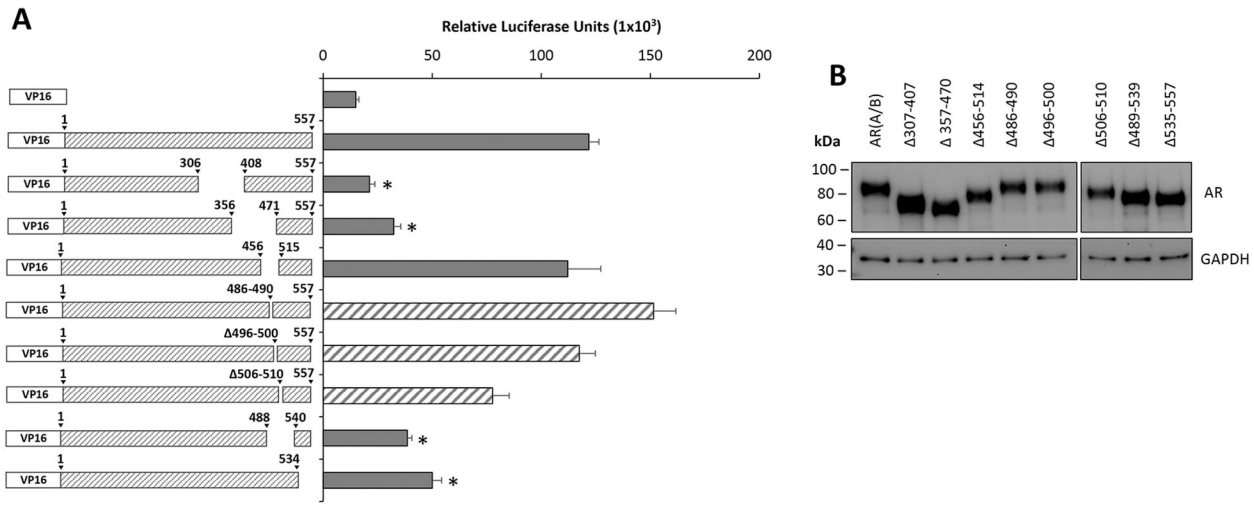

Figure 2: Further deletion analysis of the region spanning amino acids 457 to 514 within AR[A/B].

A. HeLa cells harboring the GAL4-TATA-LUC promoter-reporter and stably expressing a Gal4-ELK1 fusion protein, were transfected with expression plasmids for AR(A/B)-VP16 fusion constructs containing no deletion or deletions within the region aa457 to aa514 which lies between the two putative ELK1 interaction segments mapped in Fig. 1. Cells were harvested 48h post-transfection and cell lysates prepared for measurement of luciferase activities that were normalized to protein expression from western blots and plotted. $*P < 0.003$. **B.** From a parallel set of wells transfected as described for Panel A, cell lysates were probed by western blotting with antibodies against AR or GAPDH (loading control).

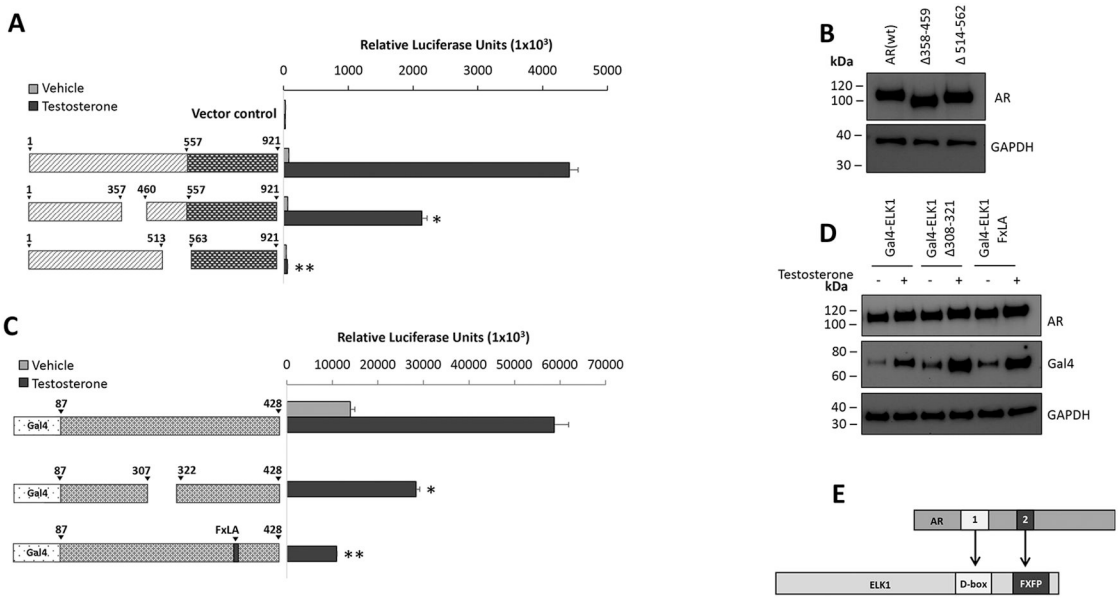

Figure 3: Validation of putative ELK1 interacting segments using full-length AR and testing the orientation of AR and ELK1 in their binary complex.

A. HeLa cells harboring the GAL4-TATA-LUC promoter-reporter and constitutively expressing a Gal4-ELK1 fusion protein, were transfected with expression plasmids for either wild type or mutant AR containing deletions of the putative ELK1 interacting segments, identified in Figure 1. At the time of transfection, cells were treated with testosterone (10 nM) to allow nuclear translocation of AR or vehicle control. 48h post- transfection, cells were harvested for preparation of lysates that were used for measurement of luciferase activity. **P* = 0.001, ***P* = 0.00002. **B.** From a parallel set of wells transfected as described for Panel A, cell lysates were probed by western blotting with antibodies against AR or GAPDH (loading control). **C.** HeLa cells co-transfected with a plasmid containing GAL4-TATA-LUC and expression plasmids for wild type AR. Expression plasmids for either wild type or mutant Gal4-ELK1 fusion proteins containing either a deletion of the D-box segment (Δ308-321) or a deleterious mutation in the FxFP motif in which FQFP was mutated to FQLA were also co-transfected. At the time of transfection, cells were treated with testosterone (10 nM) to allow nuclear translocation of AR or vehicle control. Forty-eight hours after transfection, cell lysates were prepared for measurement of luciferase activity. **P* = 0.0005, ***P* = 0.0003. **D.** From a parallel set of wells transfected as described for Panel A, cell lysates were probed by western blotting with antibodies against AR or GAPDH (loading control). **E.** Schematic illustrating parallel mode of binding of AR and ELK1.

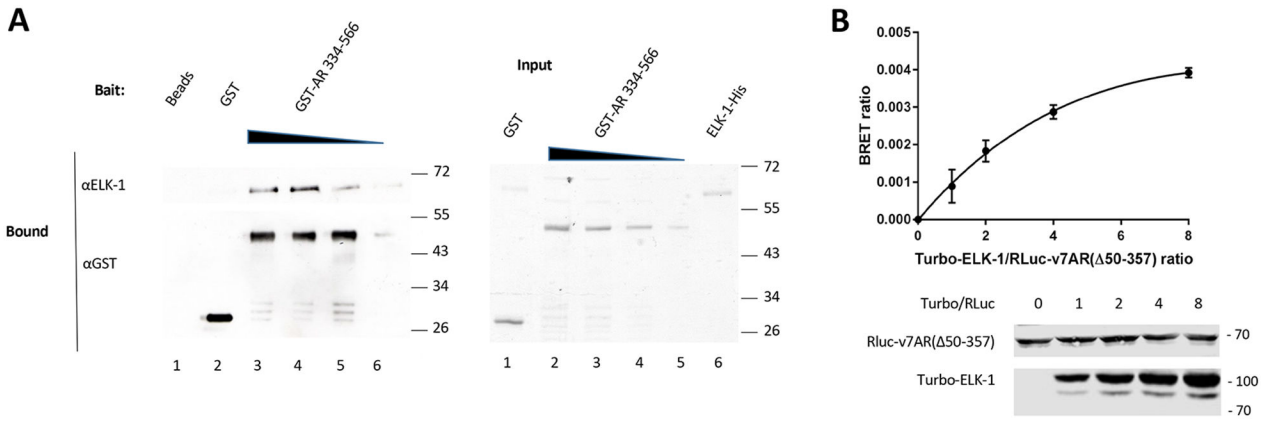

Figure 4: Validation of putative ELK1 interacting segments using orthogonal and *in situ* assays.

A. GST pulldown assay for binding of an AR peptide fragment containing the ELK1-interacting segments with wild type ELK1. Purified His-tagged ELK1 and a purified GST-tagged AR fragment consisting of amino acids 334-566 were incubated with GST beads for two hours. Following washing, the proteins were probed by western blotting with antibodies against ELK1, and GST. Coomassie staining was used to monitor protein input. **B.** BRET assay to confirm binding between wild type ELK1 and a mutant AR protein with regions not critical for interaction with ELK1 deleted. HEK293T cells were transfected with different ratios of TurboELK1 fusion and Rluc-AR-V7 truncated variant protein with amino acids 50-357 additionally deleted. Two days post-transfection, cells were replated in 96 well plates. Two hours later, coelenterazine was added and BRET emissions were measured. The 635 nm/535 nm ratios are normalized to the ratios obtained from cells transfected with RLuc construct alone, with representative western blot showing AR and ELK1 protein expression.

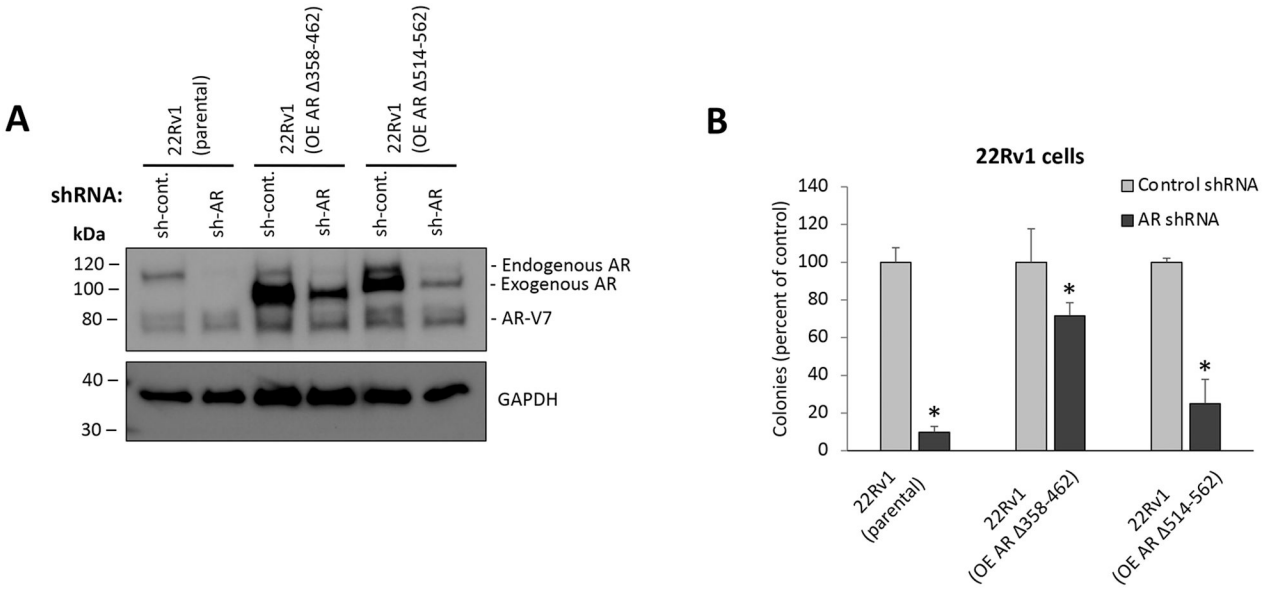

Figure 5: Effect of deleting ELK1 interaction segments on the ability of AR to support colony growth in PCa cells.

22Rv1 cells stably expressing ectopic AR mutants with deletion of either one of the two ELK1 interaction segments or vector control transduced cells were used. Each line was infected with lentivirus carrying either a control (nontargeting) shRNA plasmid or an AR-targeted shRNA plasmid. In **Panel A**, 72 hours after infection, the cell lysates were probed by western blotting with an antibody to detect both the endogenous AR and the shorter ectopic mutant ARs, using GAPDH as the loading control. The blot western blot shows virtually complete knockdown of the endogenous AR by the AR shRNA with residual ectopic mutant ARs whose levels are comparable to or greater than the original level of endogenous AR seen in the control cells. As the shRNA did not target the endogenous AR-V7 variant in the 22Rv1 cells, both AR-V7 and GAPDH serve as loading controls for each recombinant cell line. In **Panel B**, the cells treated in parallel as described in Panel A, and 72 hours after infection they were plated for colony formation in conditioned media. After 7-8 days, the colonies were stained and counted. * $P < 0.05$.

Peptide segment 1 (A358-G459):
AYQSRDYYNFPLALAGPPPPPPPPHPHARIKLENPLDYGSAWAAAAAQCRYGDLASLHGAGAAGPGSGSPSAAASSS
WHTLFTAEEGQLYGPCGGGGGGGGG

Peptide segment 2 (Y514-I562):
YPSPTCVKSEMGPWMDSYSGPYGDMRLETARDHVLPIDYYFPPQKTCLI

Figure 6: Amino acid sequences of the two ELK1-interacting peptide segments in AR.
The red font denotes hydrophobic and charged amino acid residues that are known to contribute to a more ordered structure. Stretches of amino acid residues contributing disordered (flexible) structural elements (amino acids G, A, S, T, N, Q and P) are underlined.