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Coregulator Control of Androgen Receptor Action by a Novel Nuclear Receptor-Binding Motif\*

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\*Running title: A novel AR coregulator motif

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**Background:** The interaction surface of coactivators and the androgen receptor (AR) is an important target for prostate cancer therapeutics.

**Results:** A new interface formed by binding of the sequence (GARRPR) and the allosteric pocket (BF-3) of the AR has been identified.

**Conclusion:** GARRPR binding modulates AR activity.

**Significance:** The GARRPR/BF-3 interaction is a novel regulatory hub for AR activity.

#### ABSTRACT

The androgen receptor (AR) is a ligand-activated transcription factor that is essential for prostate cancer development. It is activated by androgens through its ligand-binding domain (LBD) that consists predominantly of 11 alpha helices. Upon ligand binding, the last helix is reorganized to an agonist conformation termed activator function-2 (AF-2) for coactivator binding. Several coactivators bind to the AF-2 pocket through conserved LXXLL or FXXLF sequences to enhance the activity of the receptor. Recently, a small compound-binding surface adjacent to AF-2 has been identified as an allosteric modulator of the AF-2 activity and is termed binding function-3 (BF-3). However, the role of BF-3 *in vivo* is currently unknown and little is understood about what proteins can bind to it. Here we demonstrate that a duplicated GARRPR motif at the N-terminus of the co-chaperone Bag-1L functions through the BF-3 pocket. These findings are supported by the fact that a

selective BF-3 inhibitor or mutations within the BF-3 pocket abolish the interaction between the GARRPR motif(s) and the BF-3. Conversely, amino acid exchanges in the two GARRPR motifs of Bag-1L can impair the interaction between Bag-1L and AR without altering the ability of Bag-1L to bind to chromatin. Furthermore, the mutant Bag-1L increases androgen-dependent activation of a subset of AR-targets in a genome-wide transcriptome analysis, demonstrating a repressive function of the GARRPR/BF-3 interaction. We have therefore identified GARRPR as a novel BF-3 regulatory sequence important for fine-tuning the activity of the AR.

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Androgens regulate both normal and malignant prostate cell growth and proliferation by binding to the androgen receptor (AR), a ligand-inducible factor that belongs to the family of nuclear receptors (1–3). Like other members in this family, the AR has a modular structure. It consists of an N-terminal domain for transactivation (subdivided into transactivation units 1 and 5;  $\tau$ 1: amino acids 100-360 and  $\tau$ 5: amino acids 360-528), a highly conserved central DNA-binding domain (DBD) and a carboxy-terminal ligand-binding domain (LBD) (4).

The LBD of most nuclear receptors, including the AR, consists of 12 alpha helices (numbered 1-12; although helix 2 is missing in the AR compared to other nuclear receptors). Upon hormone binding, the 12th helix is reorganized to an agonist conformation generating a hydrophobic surface termed activation function-

2 (AF-2) for coactivator binding. Several coactivators bind to this hormone-induced conformation via a conserved “LXXLL” sequence to enhance the transcriptional activity of the receptor (5–7). In addition, the AF-2 binds strongly to a <sup>23</sup>FQNLF<sup>27</sup> motif at its N-terminus (8–11) to generate an N/C-terminal intramolecular interaction for alternative coactivator binding (4, 12). More recently a topologically distinct surface adjacent to, but different from AF-2, has been identified in a screen of chemical libraries for small coactivator inhibitors. This surface has been proposed as an additional protein binding site for AR regulatory proteins and has been termed binding function-3 (BF-3) (13). However, not much is known about its *in vivo* function except that it may be a site for protein-protein interaction.

One of the factors known to modulate the activity of the AR is the cochaperone Bag-1L. This cochaperone belongs to a family of polypeptides translated from the same mRNA by a leaky scanning mechanism generating four isoforms in humans (Bag-1L, Bag-1M, Bag-1 and Bag-1S) (14, 15). These isoforms differ in their N-terminal sequences but have a conserved C-terminal domain (otherwise known as the BAG domain) with which they bind the molecular chaperone Hsp70/Hsc70 as nucleotide exchange factors (16, 17). Besides Hsp70/Hsc70 binding, the Bag-1 proteins interact with and regulate the activities of several cellular proteins ranging from Bcl-2 and steroid receptors to growth factor receptors (18). Bag-1L, the largest member of this family possesses a N-terminal nuclear localization sequence (NLS) and is

therefore localized to the nucleus where it functions as a transcriptional regulator (15, 19, 20).

Immunohistological studies have shown that Bag-1L is expressed in the basal cells of benign prostate tissue but the site of its expression is changed in prostate carcinoma to the secretory epithelium where the AR is expressed (21). The AR and Bag-1L are therefore expressed in the same cell-type in prostate carcinoma suggesting a mechanistic link between the two proteins in this disease. Furthermore, overexpression of Bag-1L potentiates the transactivation action of the AR and domain mapping experiments have shown that the C-terminal region of Bag-1L contributes to the enhancement of the receptor activity (22, 23). While deletion or single amino acid substitutions within the C-terminus abolish the ability of Bag-1L to enhance AR activity and to bind Hsp70/Hsc70 (24), our earlier studies suggest that sequences other than those in the C-terminal region may contribute to the regulation of AR activity (21).

In this report we have identified a novel hexapeptide repeat sequence GARRPR in the N-terminal region of Bag-1L as an additional interaction site for AR and have shown that it functions through the BF-3 pocket in the AR-LBD. We have further demonstrated that mutations in the hexapeptide motifs impair binding of Bag-1L to the AR and differentially alter the androgen response of the receptor. The GARRPR sequence therefore defines a novel regulatory motif for modulating the activity of the AR through the BF-3 domain.

## EXPERIMENTAL PROCEDURES

*Plasmids* - pcDNA3-Bag-1L and pGex4T.1-Bag-1L were provided by J. Reed. pcDNA3.1-HA-BagN128 was generated by PCR amplification of the first 128 amino acids of Bag-1L and cloned into the pcDNA3.1-HA vector. pGex3X-Bag-1L $\Delta$ C and pGex4T.1-Bag-1L(1-127) were previously described (21). pGex-Bag-1L(1-80), (1-60), (1-40), (1-20), (21-80), (41-80) and (61-80) were generated by PCR amplification of Bag-1L and cloned into the pGex4T.1 vector. Similarly, pGex4T.1-HAP1, -NcoA4, PAK6, -RP9, -RRP36, -HAP1(G/P), -PAK6(G/P), -NcoA4(G/P), -RP9(G/P), -RRP36(G/P) were cloning into pGex4T.1. AR domains AF1,  $\tau$ 5, the hinge region and the DBD were cloned into pET28-GB1 vector. pET28-AR-LBD and Gal4DBD-AR-LBD mutants were provided by Eva Estébanez-Perpiñá. For the mammalian two-hybrid assay, BagN128 was cloned in frame with the Gal4VP16 transactivation domain in the pVP16 vector (Clontech). Gal4DBD-AR-LBD and pVP16-AR TAD were previously described (25). pGL3-MMTV and Ubi-Renilla luciferase constructs have already been described (26, 27). pG5 $\Delta$ E4-38Luc has also been described (28). pSG5-AR was provided by Jorma J. Palvimo. Plasmids poZC, pCG-gagpol and PCG-VSV-G for retroviral production were a kind gift from Guillaume Adelmant, while poZC-Bag-1L wild-type or N-terminal mutant were cloned in house.

*Cell Culture and stable transfection* - LNCaP cells were cultured in RPMI 1640 medium

(GIBCO) and MCF7, T47D and HeLa cells in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS) at 37 °C with an atmosphere of 5% CO<sub>2</sub>. Stable transfections were carried out using FuGene 6 transfection reagent (Roche Diagnostics) according to the manufacturer's protocol and stable transfectants were selected with geneticine (G418, Sigma-Aldrich). For stable retroviral transfection, 293T cells (cultured in DMEM, 10% FBS) were transfected with the retroviral vectors pCG-gagpol, pCG-VSV-G and the poZC constructs (poZC alone, poZC-Bag1L or poZC-Bag1LNmut), using Superfect transfection agent (Qiagen) according to the manufacturer's protocol. Subsequently, LNCaP cells were infected with the virus and selected using magnetic beads (M-450 Dynabeads, Invitrogen) coupled with IL-2-antibody (Millipore).

*Proximity Ligation Assay (PLA)* - LNCaP cells were starved for 72 h and then treated with vehicle or 10 nM DHT for 4 h. Cells were fixed and permeabilized using 4% paraformaldehyde and 0.2% Triton X-100. LNCaP cells were then blocked with 10% FBS and incubated with anti-AR (N-20, Santa Cruz), -Bag-1 (CC9E8, Santa Cruz) or -IgG (mouse IgG, BD Biosciences; rabbit serum, Sigma-Aldrich) antibodies. Protein-protein interactions were analyzed using Duolink-based *in situ* PLA (Sigma-Aldrich), according to the manufacturer's instructions. Signals were quantified using a confocal Leica SPE microscope and the Duolink ImageTool. 300 cells were analyzed per treatment.

*Bag-1 knockdown and quantitative real-time PCR analysis* -  $1 \times 10^5$  LNCaP cells were transfected with control siRNA (sense 5'-GGCUACGUCCAGGAGCGCACC-3', antisense 5'-GGUGCGCUCCUGGACGUAGCC-3') or Bag-1 siRNA (sense 5'-AGAACAGUCCACAGGAA-3', antisense 5'-UCUCCUGUGGACUGUUCU-3') using HiPerFect transfection reagent (Qiagen) according to the manufacturer's protocol. Cells were then starved and treated with 100 nM DHT for 16 h. Thereafter, cells were harvested and lysed in NP-40 buffer (20 mM Tris-HCl pH 8.0, 400 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS and protease inhibitors) for SDS-PAGE and Western blotting. Alternatively, cells were taken up in PeqGold RNApure (PiqLab Biotechnologie) or Trizol (Invitrogen) for RNA preparation using the RNeasy Mini purification kit (Qiagen). cDNA was synthesized for use in real-time quantitative PCR (qRT-PCR) analysis with either QuantiTect Reverse Transcription kit (Qiagen) or using enzymes and buffers for cDNA synthesis from Promega.

*Gene expression and microarray analysis* - LNCaP cells stably expressing poZC vector, or poZC-Bag-1L wild-type or mutant were starved for 72 h and then treated with vehicle or 10 nM DHT for 4 h. Subsequently, total RNA was isolated as described above. Biological triplicate RNAs were hybridized to human U133 plus 2.0 expression array (Affymetrix) at the Dana-Farber Cancer Institute (DFCI) Microarray Core

Facility. Gene expression data was normalized and log-scaled using the RMA algorithm (29) and the RefSeq probe definition (30). For genes with more than one probeset, the probeset with the highest average expression was used for further analysis. Differentially expressed genes were determined by Linear Models for Microarray Data (LIMMA) algorithm (31). The microarray data have been submitted to the Gene Expression Omnibus (GEO) repository under the accession number GSE51524.

*GST pull-down experiments* - Expression of GST-tagged proteins and GST pull-down experiments were performed as previously described (32, 33).

*Chromatin-Immunoprecipitation (ChIP), ChIP-re-ChIP and co-IP* - LNCaP cells were starved for 72 hours and then treated with DHT or vehicle for 4 h. Thereafter ChIP was performed as previously described (34, 35) using following antibodies: anti-AR (N20, Santa Cruz), Bag-1L (36) and HA-tag (ab9110, Abcam). As a control, rabbit IgG (Santa Cruz) was employed. ChIP DNA was purified using the PCR purification kit (Qiagen) and quantified using the Quant-iT dsDNA HS assay kit (Invitrogen).

ChIP-re-ChIP was carried out as described for ChIP, with following alterations: After the initial round of overnight IP (with AR, Bag-1L or IgG antibody), the magnetic beads were incubation in 10 mM DTT for 30 min at 37 °C. Subsequently, the DTT was quenched with RIPA buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 0.1% SDS, 1% Triton X-100, 0.1% NaDOC and

protease inhibitors) with 0.3 M NaCl. Free chromatin complexes were then subjected to a second IP using different antibodies of choice.

Endogenous co-IP was carried out as previously described (37) with following alterations: After nuclear lysis and overnight incubation with anti-Bag-1L or HA-tag antibody, protein complexes were precipitate with magnetic beads. Beads were then washed five times, 10 min each, and complexes eluted by boiling in Laemmli sample buffer (Bio-Rad).

*Fluorescence Polarization* - For the fluorescence polarization experiments, 5 nM fluorescently-labeled Bag-1L (Bag-1L(1-20): FITC-MAQRGGARRPRGDRERLGSR, Bag-1L(61-80): FITC-RGAAAGARRPRMKKKTRRRS) or SRC-2 peptide (FITC-HDSKGQTKLLQLLTTKSDQM) were incubated with serially diluted AR-LBD (4 to 0.4  $\mu$ M) in binding buffer (50 mM NaPO<sub>4</sub> pH 6.5, 50 mM KCl, 1 mM DTT) and 10  $\mu$ M DHT. The samples were then equilibrated for 45 min at ambient temperature. For the competition experiments, 400 to 0.4 nM of Bag-1L core (GARRPR) or SRC-2 peptides (HDSKGQTKLLQLLTTKSDQM) were incubated with 4  $\mu$ M AR-LBD and 5 nM fluorescent Bag-1 or SRC-2 peptide. Binding was measured using polarization (excitation  $\lambda$ = 485 nm, emission  $\lambda$ = 530 nm) on a Synergy H1 Hybrid Reader (Biotek).

*Synthesis of 2-((2-(2,6-dimethylphenoxy)ethyl)thio)-1H-benzo[d]imidazole* - The BF-3

inhibitor 2-((2-(2,6-dimethylphenoxy)ethyl)thio)-1H-benzo[d]imidazole was synthesized in house essentially as described (38), with minor modifications in the addition of 1H-benzimidazole-2-thiol to 2-(2-bromoethoxy)-1,3-dimethylbenzene in the presence of potassium iodide and carbonate. The latter reactant was synthesized by allowing 1,2-dibromoethane to react with a commercially available 2,6-dimethylphenol in the presence of potassium carbonate as a base at 60 °C, as previously described (39).

*Molecular modeling* - The GARRPR peptide and CPD49 were docked into the BF-3 pocket of AR using DOCK 3.5.54 (40, 41). Six AR complex structures (PDB IDs, 2PIT, 2PIU, 2PIV, 2PIW, 2PIX, and 2PKL) (42) were used individually in order to capture receptor conformational flexibility. For each docking experiment, the protein in one complex structure was used as the rigid receptor, with all of the waters removed. The original ligand in the corresponding complex crystal structure was used to generate the matching spheres. Multiple conformations of the peptide and CPD49 were pre-generated and placed in the binding pocket guided by the matching spheres. Ligand poses were scored through a grid-based method calculating van der Waals forces, electrostatic interactions and the ligand desolvation energy. The highest-ranking poses from each of the six docking experiments were combined and visually examined in the context of their respective receptor environment. The best pose was chosen based on its polar and non-polar interactions between the ligands and

the receptor, as well as the shape complementarity with the binding pocket.

## RESULTS

*Bag-1L is important for AR action* - To study the significance of the regulation of AR action by Bag-1L, we first determined whether these two proteins interact in LNCaP prostate cancer cells using co-immunoprecipitation. We were able to detect AR by Western blotting when Bag-1L was immunoprecipitated, both in the absence and presence of the androgen dihydrotestosterone (DHT) (Fig. 1A). We could additionally show that Bag-1 and AR interact in LNCaP cells by an *in situ* proximity ligation assay (43). This assay uses oligonucleotide-generated high intensity fluorescent signals to assess *in situ* whether two proteins are located in close proximity to one another. The ligation assay performed with Bag-1 (that recognizes all Bag-1 isoforms) and AR antibodies showed positive punctuated perinuclear signals in the LNCaP cells in the absence and presence of DHT. However, the number of fluorescence signals per nucleus was slightly enhanced in the presence of the hormone (Fig. 1B). Little or no fluorescent signals were observed when AR or Bag-1 antibodies were used together with an isotype IgG control antibody.

To determine the contribution of Bag-1L to AR response, we next downregulated the expression of Bag-1 in LNCaP cells using RNA interference and determined the expression of two well-studied, androgen-regulated genes, PSA and FKBP5 (44). siRNA transfection significantly reduced the level of expression of the Bag-1

isoforms (Fig. 2A). Concomitant with this reduction was a downregulation of the androgen-mediated transcription of the two genes, which was highly significant for FKBP5 (Fig. 2B). This shows that Bag-1 proteins contribute to an efficient, androgen-mediated transcription.

Next we investigated whether Bag-1L is recruited to AR enhancer regions of the PSA and FKBP5 genes using a Bag-1L-specific antibody (36). We were able to identify solo occupancy of either AR (Fig. 2C) or Bag-1L (Fig. 2D) using chromatin immunoprecipitation (ChIP). Furthermore, we could demonstrate a partial co-occupancy of Bag-1L and AR at these enhancer elements, shown by a comparable or increased fold enrichment in the ChIP-re-ChIP relative to the solo ChIP (compare Fig. 2C with 2E and 2D with 2F) (45). No co-occupancy of the two proteins was observed in the re-ChIP experiments when the antibody for either of the proteins was substituted with IgG (data not shown).

*Bag-1L interacts with the AR* - To determine which Bag-1L domains are important for AR binding we carried out pull-down experiments with GST-fused truncations of Bag-1L and lysate from androgen-treated LNCaP cells (Fig. 3A). We could confirm the results of Knee *et al.*, 2001, which showed that the C-terminal domain of Bag-1L binds the AR (Bag-1L $\Delta$ 214, construct 5; Figs. 3B and 3C). Similarly, all other Bag-1L constructs containing this part of the protein also bound the AR (Bag-1L $\Delta$ 115 and  $\Delta$ 122, constructs 3 and 4; Fig. 3B). Intriguingly, a construct lacking the C-terminus altogether also

interacted with the AR (Bag-1L $\Delta$ C, construct 2; Fig. 3B). This binding could be traced to the first 128 N-terminal amino acids of Bag-1L (BagN128, construct 6) since amino acids 141 to 298 failed to bind the receptor (BagULD, construct 7; Fig. 3C). We have therefore identified an additional interaction site for AR on the N-terminus of Bag-1L.

To show that the BagN128/AR interaction can also occur in cells (*in situ*), we performed a mammalian one-hybrid assay in HeLa cells where we transfected a construct coding for a Gal4DBD-BagN128 fusion or just Gal4DBD alone together with a Gal4 binding site-luciferase reporter gene and an AR expression vector. The transfected cells were then treated with or without DHT and the reporter gene activity was measured. A basal reporter gene activity was observed for the Gal4DBD alone or in combination with AR, independent of androgen treatment. However, when Gal4DBD-BagN128 and AR were transfected together, an increase in the reporter gene activity was observed in the presence of DHT (Fig. 3D). This demonstrates a cellular interaction of the N-terminal sequence of Bag-1L with AR in the presence of hormone.

*A duplicated hexapeptide motif for AR binding*

The N-terminal sequence of Bag-1L (BagN128) consists of an unidentified sequence, the NLS and a TR/QSEEX repeat region (construct 1; Fig. 4A). To determine precisely which region is responsible for AR binding, we carried out GST pull-down experiments utilizing deletion mutants of BagN128 (Fig. 4A) and

lysate of androgen-treated LNCaP cells. Our results show that AR binding is localized to the 1-80 but not to the 81-128 amino acids of Bag-1L (constructs 2 and 3; Fig. 4B). Further C- and N-terminal truncations of the 1-80 amino acid sequence all interacted with the AR. The smallest peptides bound by the AR were identified as amino acids 1-20 and 61-80 (constructs 6 and 9; Fig. 4B).

The only identical feature that could explain why these two peptides bind the AR is a motif “GARRPR” that is present in both sequences (Fig. 4C). We therefore postulated that the GARRPR motif is the interaction site of the AR. To validate this, we substituted the hexapeptide sequence with alanine residues and repeated the above-described GST pull-down assay (Figs. 4D to 4F). These alanine substitutions completely abolished the interaction between the Bag-1L peptides and the AR, confirming the importance of the GARRPR motif for AR binding (Figs. 4E and F: compare constructs 3 with 4 and 5 with 6). To determine the selectivity of this hexapeptide sequence for the AR compared to other nuclear receptors, we repeated the GST pull-down assays substituting LNCaP cell lysate for those from MCF7, HeLa or T47D cells (Fig. 4G). These lysates contain high levels of estrogen (ER $\alpha$ ), glucocorticoid (GR) or progesterone receptors (PR), respectively. ER $\alpha$  proved similar to AR in recognizing the GARRPR motif (Fig. 4G, top row). However, although GR and PR bound both the 1-128 and the 1-80 N-terminal peptides of Bag-1L, they failed to bind the two shorter peptides (constructs 3 and? 5; Figs. 4D and 4G),

suggesting that their binding sites are localized outside these two sequences.

*The GARRPR motif binds the AR-LBD* - To determine the region of the AR that binds the GARRPR motif, fluorescence polarization experiments were carried out with the two Bag-1L peptides (amino acids 1-20 and 61-80) labeled with fluorescein isothiocyanate (FITC), and purified domains of the AR covering the hinge-LBD (amino acids 646-919), the  $\tau 5$  region (amino acids 360-548), the DBD (amino acids 552-636), the hinge region (amino acids 610-685) and, as a control, GST. Both Bag-1L peptides bound the hinge-LBD (Fig. 5A) but not the other AR domains or the GST control (data not shown). The binding affinities of the Bag-1L peptides for the AR-LBD were estimated by Sigma plot to be  $K_D = 2.0 \pm 0.6 \mu\text{M}$  for Bag-1L(1-20) and  $3.3 \pm 1.3 \mu\text{M}$  for Bag-1L(61-80). A positive control peptide, the high affinity nuclear receptor box of SCR-2 that contains the LXXLL core motif (46), also bound the AR-LBD with comparable affinity ( $K_D = 2.8 \pm 1.6 \mu\text{M}$ ; Fig. 5B). Given the similar affinities of the Bag-1L and SRC-2 peptides for AR-LBD, we questioned if these peptides could directly compete for binding to the AR-LBD. In line with our hypothesis, binding of the GARRPR-containing Bag-1L peptides to the AR-LBD could be outcompeted by SRC-2 (Fig. 5C) and conversely, SRC-2 binding was outcompeted by an unlabeled GARRPR core sequence (Fig. 5D). These results suggest that the binding sites of the Bag-1L and SRC-2 peptides must be similar or in close proximity to one another. Alternatively,

binding of one of the peptides may allosterically hinder binding of the other.

*The Bag-1L peptides interact with the BF-3 pocket of the AR-LBD* - Detailed studies of the AR AF-2 have shown that it forms an interaction surface for LXXLL-binding proteins or proteins with LXXLL variants with bulky aromatic hydrophobic groups such as FXXLF (11, 47). Given the highly charged nature of the GARRPR motif, it is therefore unlikely that it interacts with the AF-2 domain. It is more likely that it interacts with the recently described BF-3 domain, which is found next to the AF-2 and which allosterically modulates the activity of this domain.

Interaction of peptides to BF-3 has been difficult to demonstrate, but more recently a group of ligand derivatives of 2-((2-phenoxyethyl)thio)-1H-benzimidazole was shown to bind specifically to the BF-3 pocket of the AR (38, 48). One of these ligands is 2-((2,6-dimethylphenoxy)ethyl)thio)-1H-benzo[d]imidazole (compound 49 (CPD49); Fig. 6A) that exerts significant anti-androgen potency in LNCaP and Enzalutamide-resistant prostate cancer cell lines (38, 48). We therefore used this ligand to determine whether it would compete with the GARRPR peptides for binding to the BF-3 pocket.

In a mammalian two-hybrid assay in which we determined binding of the GARRPR-containing Bag-1L peptides to the AR-LBD, CPD49 had no significant inhibitory effect on the activity of the LBD alone (Fig. 6B; black line) but potently inhibited the interaction of the Bag-1L peptides

with the LBD with  $IC_{50}$  values of 4 and 10  $\mu M$  for Bag-1L(1-20) and Bag-1L(61-80), respectively (Fig. 6B; blue and red lines). This compares favorably to the  $IC_{50}$  values of 3.3 and 6.8  $\mu M$  for the inhibition of PSA secretion in LNCaP and Enzalutamide-resistant LNCaP cell lines, respectively (13, 38)(13, 38)(13, 38)(13, 38).

Our molecular modeling studies show that CPD49 binds to the BF-3 domain by making extensive non-polar contacts with amino acids F673, P723, and F826 that form the BF-3 pocket, in addition to favorable electrostatic interactions between its nitrogen atom and E837 of the BF-3 (Fig. 6C). CPD49 binding would therefore likely interfere with the binding of the peptides to the BF-3 domain since the proline residue of the peptide core (GARRPR) prefers to sit in the hydrophobic cavity of the BF-3 domain (Fig. 6D). In addition, favorable electrostatic interactions appear to occur between the G(1), A(2), R(3), R(4) and R(6) of the peptide core with amino acids E837, N727, G724, E829 and E837 that form the BF-3 pocket (Fig. 6D).

To further confirm the interaction of the Bag-1L peptides with the BF-3 domain, we performed a mammalian two-hybrid assay with Gal4DBD fused to the wild-type AR-LBD or LBD domains harboring mutations in the BF-3 pocket (Fig. 6E). These mutations either line the BF-3 pocket itself (F826, L830, N833, R840) or are at the boundary of the BF-3 and AF-2 domains (R726) (13, 49, 50). In addition, we included mutant V757, which is located at the end of helix 5 of the AR-LBD outside the BF-3 pocket (49). Although the Bag-1L(1-20) and

Bag-1L(61-80) peptides interacted with the wild-type AR-LBD, their interaction with most of the BF-3 mutants was strongly compromised (Fig. 6E). The only exception was the interaction with F826, where a substitution of an arginine (R) suppressed but did not abolish binding of the Bag-1L peptides to the AR-LBD. This F826R mutation has previously been reported to have an anomalous behavior in interaction studies with other coactivators (49). The control mutation at V757 did not affect binding of the Bag-1L peptides (Fig. 6E). Together these results strongly suggest that the BF-3 pocket is an essential domain for the interaction of the Bag-1L peptides with the AR-LBD.

*Identification of a signature motif* - A systematic analysis of the contribution of the individual residues in the hexapeptide motif of Bag-1L was carried out to determine the exact binding signature for Bag-1L to the AR. Each amino acid in the GARRPR cores of Bag-1L(1-20) and Bag-1L(61-80) was consecutively substituted with alanine residues. These mutants were then fused to GST and used in a pull-down assay with lysate from LNCaP cells previously treated with or without androgen. Androgen-mediated binding was destroyed when any single amino acid of the core residues GARRPR was exchanged for alanine moieties in the Bag-1L(1-20) peptide (Fig. 7A). This was however not the case for the Bag-1L(61-80) peptide where only the exchange of the penultimate two amino acids xxxRPx to alanine decreased binding (Fig. 7B).

In a subsequent attempt to define the contribution of the individual residues in the

hexapeptide motif, protein blast search was carried out to determine whether homologies of the Bag-1L GARRPR sequence exist in other proteins. Over 70 proteins with near complete or partial homologies were identified and peptides encompassing the homologous regions were synthesized. These peptides were then spotted onto membranes using an automated SPOT synthesizer (51) and hybridized to (bacterially-purified) AR-LBD. A wide variety of peptides containing complete to partial homology to the hexapeptide core were found to interact with the AR-LBD (Fig. 7C and Supplementary Table 1). A sequence logo derived from the validated peptide sequences identified G(1) and P(5) as the crucial residues for AR binding (Fig. 7D).

To further confirm the contribution of G(1) and P(5) within the GARRPR motif for binding to the AR-LBD, some positively identified peptides from the SPOT assay were further validated in a GST pull-down experiment. These included the ribosomal RNA processing protein 36 (RRP36), retinitis pigmentosa 9 (RP), huntington associated protein 1 (HAP1), nuclear receptor coactivator 4 (NcoA4) and p21 protein (Cdc42/Rac9-activated kinase 6 (PAK6)), the latter three of which are known to bind the AR (52–54). These peptides mostly show complete sequence identity with the core GARRPR, with the exception of NcoA4 and PAK6, which only share 2-3 homologous residues (but including G(1) and P(5)) with the GARRPR motif. We could confirm a hormone-dependent binding of all the peptides to the AR with the exception of PAK6, where binding was weaker in the presence than in the absence of hormone (Fig.

7E). Since PAK6 and NcoA4 contain only limited homologies to the GARRPR core, it is likely that only the conserved amino acids at positions 1 and 5 (GxxxPx) contribute to the interaction with the AR-LBD. In GST pull-down experiments we could confirm this by exchanging the conserved amino acids G(1) and P(5) to alanine, which indeed reduced binding of all the peptides to the liganded AR-LBD (Fig. 7F).

*The N-terminal mutant of Bag-1L is defective in AR binding* - Having established that residues G(1) and P(5) in the GARRPR motif are essential for the interaction with the AR, we next investigated the effect mutations of these residues in the two Bag-1L peptides would have on their interaction with the AR. Using a GST pull-down assay we could show that an exchange of residues G(1) and P(5) with alanine residues destroys the androgen-dependent binding of both Bag-1L(1-20) and Bag-1L(61-80) to the receptor (Fig. 8A). Furthermore, fluorescence polarization experiments confirmed that binding of the Bag-1L peptides to the AR-LBD is reduced upon the mutation (Fig. 8B).

We next introduced the G6A/P10A/G66A/P70A substitutions that decreased the BagN128-AR binding, into the full-length Bag-1L; the resulting Bag-1L protein has a mutated N-terminal end but an intact C-terminal BAG-domain. We created LNCaP cells stably overexpressing HA-, Flag-tagged versions of the wild-type or mutant Bag-1L proteins, and as a control, LNCaP cells expressing the empty vector construct. Since the GARRPR mutations

are close to the NLS (position 70-76), as well as another region (amino acids 17-50) that has been reported to contribute to the nuclear localization of Bag-1L (23, 55), we carried out immunofluorescence microscopy to determine if the cellular localization of Bag-1L (or AR) is altered by the mutations. The immunofluorescence experiments showed no change in localization of the mutant Bag-1L compared with the wild-type protein (Fig. 9A), demonstrating that the mutations do not affect the nuclear localization of Bag-1L (or the AR). We next tested the expression levels of Bag-1L and AR in the stable cell lines. Bag-1L expression levels were comparable between the wild-type and mutant Bag-1L expressing cell lines, and Bag-1L overexpression (whether wild-type or mutant) did not change the endogenous expression levels of the AR (Fig. 9B). Lastly, we tested the ability of the full-length, GARRPR-mutant Bag-1L protein to interact with the AR by co-immunoprecipitation (Fig. 9C). We could not detect any significant interaction (both hormone-dependent or -independent) between the full-length Bag-1L and AR when the GARRPR motifs were mutated, which is in agreement with the findings for the mutated Bag-1L peptides (Fig. 8A).

*The N-terminal Bag-1L mutations cause alterations in AR transactivation* - Having established that the GARRPR mutations impair the interaction of Bag-1L with AR (Fig. 9C), we postulated that Bag-1L-mediated regulation of AR action might also be affected by these mutations. To prove this, we performed genome-

wide gene expression profiling in the LNCaP Bag-1L wild-type and mutant overexpressing cell lines, as well as the LNCaP vector control cells. We focused entirely on AR-regulated genes by culturing the cells in the absence and presence of DHT and only took into consideration differentially expressed genes with a fold change of 1.75 or higher upon hormone treatment (and a p-value of 0.05 or less). Comparison of the DHT-regulated transcriptome of the vector, wild-type and Bag-1L mutant-transfected LNCaP cells revealed a significant number of differentially up-regulated genes that could be clustered in five different categories (Fig. 10A). The total number of down-regulated genes was modest (n=7) and therefore these genes were excluded from the analysis.

The biggest gene cluster we identified, defined as “all common genes”, consists of a number of genes that were all highly expressed (>1.75 fold) in all three datasets. This is in contrast to the next cluster (“WT+NMut only”), which only contains genes that were upregulated in the Bag-1L wild-type and mutant overexpressing cells but showed low (< 1.75 fold) or no differential expression in the vector expressing cells. This finding is in agreement with previous studies that showed that Bag-1L overexpression potentiates the transactivation action of the AR (21, 22, 24). Correspondingly, gene annotation analysis revealed that the genes uniquely upregulated in the “WT+NMut” cell lines are associated with changes in cell signaling and proliferation.

We also identified a significant number of genes that were differentially transcribed across

all three cell lines and were therefore categorized as being uniquely expressed; the exception is the vector (Vec) category which includes two genes that are also expressed in the wild-type cells. The largest and most significant of the unique categories belongs to the N-terminal mutant overexpressing cells (NMut), which points towards an exclusive function of the GARRPR mutations in the modulation of the AR-mediated transcriptome. The majority (53.6%) of the genes identified within this group are associated with metabolic processes.

We validated the expression of a select number of genes by qRT-PCR, which we had defined as being unique to the Bag-1L GARRPR mutant cell line (based on the microarray data). As expected, the expression of all tested genes was significantly higher in the Bag-1L mutant cell line compared with their respective expression levels in the vector and Bag-1L overexpressing cells (Fig. 10B). Concomitantly, AR binding at regulatory regions of these genes, as determined by AR ChIP, was higher in the Bag-1L mutant than in the vector or Bag-1L wild-type cells (Fig. 10C). In comparison, Bag-1L binding remained unaltered at the same AR regulatory sites across the three different cell lines (data not shown). Thus, the mutation of the GARRPR motif of Bag-1L, which causes the inhibition of the Bag-1L/AR interaction, triggers an increase in binding and activity of the AR at selected target genes, without altering the direct binding of Bag-1L to chromatin. Together, our findings indicate that binding of Bag-1L to the AR through the (un-mutated) GARRPR motif contributes to the suppression of a subset of AR-regulated genes.

## **DISCUSSION**

In this work we have analyzed the regulation of AR by the cochaperone Bag-1L. We could show that N-terminal amino acid sequences of Bag-1L bind to the AR and contribute to the transactivation function of the receptor. Furthermore, we identified a repeat hexapeptide motif “GARRPR” in Bag-1L as an essential sequence for binding to the AR-LBD. This motif functions through sequences that constitute the newly identified allosteric regulator BF-3 in the AR-LBD and mutations in this motif cause the loss of Bag-1L/AR interaction and alter the transactivation function of the AR.

Previous studies to identify sequences on coactivators that bind the LBD of nuclear receptors using phage display libraries have only identified variants of the LXXLL motif (11, 47). For example, peptides bound to the AR coactivator binding domain include a combination of aromatic residues and the canonical leucine-rich sequence of LXXLL such as FXXLF, FXXLW, WXXLF, WXXVW, FXXFF or FXXYF (11, 47). Similarly, peptides that interact with the coactivator binding domain of another steroid receptor, the estrogen receptor  $\alpha$  (ER $\alpha$ ) can be categorized into three classes of LXXLL motifs that differ in their flanking sequences, but not in the canonical motifs themselves (56).

Well-characterized AR coactivators include the p160/SRC family of proteins that bind to the  $\tau 5$  of the AR through a glutamine-rich sequence independent of their intrinsic LXXLL motifs (57–59). A coactivator MED1 has also been

shown to bind to the  $\tau 1$  region at the N-terminus through two noncanonical  $\alpha$ -helical motifs but independent of its LXXLL motifs (60). The GARRPR sequence we have identified in the present study presents yet another mode of AR regulation and differs both structurally and functionally from the LXXLL motif. First, it is highly charged compared to the LXXLL motif, which is highly hydrophobic. Second, it exhibits target specificity, since it binds the AR and the ER $\alpha$ , but not the GR or the PR. This is therefore unlike the LXXLL motif, which interacts with the coactivator pocket, activation function-2 (AF-2) of several nuclear receptors, including the AR. However like the LXXLL motif, the GARRPR motif binds to the AR-LBD. Compellingly, our studies show that the interaction of the AR-LBD with the GARRPR-containing Bag-1L peptides can be outcompeted with a LXXLL-containing SRC-2 peptide. Similarly, the AR-LBD/SRC-2 interaction can be competed off by a GARRPR core peptide. This might suggest that the GARRPR and LXXLL motifs bind to closely related sequences on the AR. Alternatively, binding by one peptide may produce allosteric changes in the flanking region of AR that may affect binding by the other. However, it is more plausible to us that the GARRPR sequence binds to the newly identified allosteric region of the AR-LBD termed binding function-3 (BF-3) (42), rather than the AF-2 pocket. We present three pieces of evidence that support this possibility. First, the use of a small, specific, molecule inhibitor of the BF-3 destroys binding of the Bag-1L peptides to the AR-LBD. Second, molecular modeling

shows that the GARRPR fits into the BF-3 pocket with the proline residue localized in the hydrophobic cavity of this pocket. Third, single amino acid exchanges in the BF-3 pocket, but not an outside sequence, destroy binding of the Bag-1L peptides to the LBD in a mammalian two-hybrid assay. Together these results strongly suggest that the Bag-1L peptides bind to the BF-3 pocket of the AR.

Although the BF-3 pocket has been postulated to bind regulatory proteins, it has thus far been difficult to demonstrate factor-specific binding to this site because of its weak affinity for proteins (13). So far only FKBP52, a cochaperone of Hsp90 that binds the AR/Hsp90 complex and regulates ligand binding to the AR, has been shown to function through the BF-3 pocket. However, a direct demonstration of binding is lacking (61). Studies on a possible interaction sequence of FKBP52 with the BF-3 pocket suggested the involvement of alanine and proline residues in the sequence "GSAGSPP" at the N-terminus of FKBP52 (61, 62). This sequence is strikingly similar to the GARRPR motif we identified but it is completely different from the LXXLL coactivator motif that binds the AF-2 domain of the AR. Nonetheless there are differences in the functional consequences of the binding of FKBP52 and Bag-1L to the AR-LBD. The former binds through its N-terminal FK506 binding domain and is required for hormone binding, while the latter interaction is through a so far uncharacterized N-terminal Bag-1L sequence that seems to control the transactivation function of the AR. Future structural experiments will be needed to

distinguish between the interactions of these two proteins with the BF-3 of the AR. For now, our results with Bag-1L agree with the notion that agents that bind to the BF-3 pocket allosterically regulate the transactivation function of the AR (13).

Studies on how BF-3-lining residues affect AR activity show that mutations in the BF-3 pocket alter the ability of AF2-binding coactivators to modulate the transactivation function of the AR (49). Residues in the BF-3 pocket are also targets for prostate cancer and androgen-insensitivity syndrome (AIS) mutations (63–67). Our finding that the GARRPR motifs of Bag-1L bind to the BF-3 would therefore strongly implicate Bag-1L in the development or progression of AIS and/or prostate cancer. In our study we could show that mutations in the GARRPR motif that destroy binding to the BF-3 pocket did not inhibit AR-mediated gene expression, but rather increased the expression of a subset of androgen-regulated genes mediating metabolic processes. Previous studies on AR-mediated upregulation of genes involved in metabolism have pointed to a role of the AR in the normal prostate epithelium in driving the synthesis of seminal fluid (68, 69). Therefore our finding that the mutant Bag-1L increases the expression of AR-regulated genes involved in metabolism indicates a shift from the involvement of Bag-1L in promoting AR action in prostate cancer towards a normal prostate function.

The N-terminus is not the only part of Bag-1L that binds the AR. The C-terminus of Bag-1L has been shown to bind the AR *in vitro* as identified by GST pull-down studies (21, 23). However our *in vivo* studies show a complete impairment of binding of Bag-1L to the AR after mutating the GARRPR sequence at the N-terminus of Bag-1L. Thus, we can conclude that in the cellular context, the N-terminal sequence of Bag-1L is very important for AR binding. In addition, the N-terminus of Bag-1L also binds DNA through lysine- and arginine-rich sequences between amino acids 68 and 81 at its N-terminus (70–72). We therefore postulate that the association of Bag-1L with the AR, as demonstrated in this work, allows the (intact) AR/Bag-1L complexes to bind to enhancer elements of AR target genes (with both proteins binding to chromatin). However, when the Bag-1L/AR interaction is disrupted by mutations in the GARRPR motifs, although the interaction of Bag-1L and chromatin still persists, the AR is now free to bind more avidly to distinct enhancer sequences. This would explain the increased AR binding and androgen response of a subset of genes following the overexpression of the mutated Bag-1L in LNCaP cells, as shown in our experiments.

Our findings therefore offer an opportunity for studying the mechanism of repression and de-repression in the control of AR transactivation in prostate cancer cells.

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## FOOTNOTES

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## FIGURE LEGENDS

### FIGURE 1. AR and Bag-1L interact *in situ*

A. Co-immunoprecipitation of endogenous Bag-1L and AR in LNCaP cells using a Bag-1L-specific antibody for the IP and an antibody against AR, to evaluate binding. An IgG IP was carried out simultaneously, as a negative control. 1/10<sup>th</sup> of the input samples are shown and confirm equal protein loading. B. Proximity ligation assay (PLA) was performed in LNCaP cells treated with vehicle or 10 nM DHT for 4 h. Antibodies against AR, Bag-1 or IgG control were used. The quantification of the PLA dots (scored as dots per nucleus) is shown on the right. P-values were calculated using Mann-Whitney-U-test; \* p < 0.05.

**FIGURE 2. Bag-1 is necessary for AR-dependent gene expression and co-localizes with AR at androgen-regulated enhancers**

A, B. LNCaP cells were transfected with control or Bag-1 siRNA and treated with vehicle or 100 nM DHT for 16 h. A. Western blot of the Bag-1 knockdown using anti-Bag-1 and, as a control,  $\alpha$ -Tubulin antibodies. B. qRT-PCR of PSA and FKBP5 expression in control (Ctrl.si) or Bag-1 knockdown cells (Bag-1si). Data represent the mean of three independent experiments, each in duplicate  $\pm$  SEM. P-values were calculated using standard t-test; \*\*  $p < 0.01$ . C, D. Chromatin immunoprecipitation (ChIP) of AR (C) or Bag-1 (D) was performed in LNCaP cells treated with vehicle (open bars) or 10 nM DHT (closed bars) for 4 h. The results are represented as fold enrichment over input and are the averages of three replicates  $\pm$  SD. E, F. Complex formation between AR and Bag-1L was analyzed by ChIP-re-ChIP, immunoprecipitating first with AR followed by Bag-1L ChIP (E) or by Bag-1L ChIP followed by IP with an AR antibody (F).

**FIGURE 3. The N- and C-termini of Bag-1L bind the AR**

A. Schematic diagrams of wild-type Bag-1L and its deletion mutants. B, C. GST/Bag-1L fusion proteins, as indicated in (A), were used in a pull-down assays with lysate from LNCaP cells treated with 10 nM R1881 for 20 h. AR binding was determined by Western blotting using an anti-AR antibody. GST-proteins were visualized using Ponceau staining. D. Luciferase assay of HeLa cells transfected with the indicated vector constructs and treated with vehicle (open bars) or 100 nM DHT (filled bars) for 16 h. Data points represent the mean of three independent experiments  $\pm$  SEM; \*\*\*  $p \leq 0.005$ .

**FIGURE 4. Identification of the N-terminal AR binding site of Bag-1L**

A. Schematic diagrams of different truncations of the 128 N-terminal amino acids of Bag-1L. B. GST pull-down experiments with the GST-fusion proteins shown in (A) and LNCaP cell lysate. Protein binding was visualized by Western blotting using an anti-AR antibody. Equal protein loading was confirmed by Ponceau staining. C. The amino acid sequence of Bag-1L(1-20) and Bag-1L(61-80) with the GARRPR motif highlighted in blue. D. Schematic diagrams of different truncations of the 128 amino acids of Bag-1L, with the GARRPR motifs either intact (constructs 1-3 and 5) or all the GARRPR amino acids converted to alanine residues (constructs 4 and 6). E. GST pull-down experiments with the GST-fused proteins shown in (D) and lysate from LNCaP cells. Western blotting was carried out using an antibody against AR. Equal protein loading was confirmed by Ponceau staining. F. Densitometry analysis of the AR interaction with the BagN128

truncations (as shown in (E)). The graph represents AR band intensities with reference to the AR/BagN128 interaction signal. Data points represent the mean of four independent experiments  $\pm$  SEM. G. GST pull-down experiments with the GST-fusion proteins shown in (D) and lysates from MCF7, HeLa or T47D cells. Binding was visualized by Western blotting using antibodies against ER $\alpha$ , GR or PR. Equal protein loading was confirmed with an anti-GST antibody.

**Figure 5. The GARRPR motif binds the AR-LBD**

A, B. Fluorescence polarization experiments using serially-diluted AR-LBD and FITC-labeled Bag-1L(1-20) (blue line) and Bag-1L(61-80) peptide (red line; A) or FITC-labeled SRC-2 peptide (green line; B). C, D. Peptide competition experiments were performed using conditions for maximum polarization of FITC-labeled peptide and AR-LBD. The competition was carried out with unlabeled SRC-2 peptide (C) or unlabeled core GARRPR hexapeptide (D).

**FIGURE 6. The integrity of the BF-3 domain is essential for binding of the GARRPR motif**

A. Structure of the BF-3 inhibitor 2-((2-(2,6-dimethylphenoxy)ethyl)thio)-1H-benzo[d]imidazole (CPD49). B. Luciferase assay of HeLa cells transfected with Bag-1L(1-20), Bag-1L(61-80) or a control vector construct and treated with different concentrations of CPD49 and 100 nM DHT for 16 h. Data points are shown as DHT-mediated fold induction and the lines demonstrate the non-linear regression between data points. The results are the mean of three independent experiments  $\pm$  SD. C, D. Modeling experiment between the BF-3 pocket of the AR-LBD and the BF-3 inhibitor, CPD49 (C), or the GARRPR peptide (D). Residues of the BF-3 pocket involved in contact formation are indicated. E. Luciferase assay of HeLa cells transfected with indicated vector constructs and either wild-type AR-LBD (wt) or different AR-LBD mutants. The experiments were carried out in the presence of vehicle (open bars) or 16 h 100 nM DHT treatment (filled bars). Data points represent the mean of at least three independent experiments  $\pm$  SEM.

**FIGURE 7. Identification of essential amino acids in the GARRPR motif required for AR binding**

A, B. GST pull-down experiments between the lysate from LNCaP cells treated with 100 nM DHT for 1 h and GST-fused Bag-1L(1-20) (A) or Bag-1L(61-80) peptides (B). Each amino acid of the GARRPR motif was consecutively substituted by an alanine residue (shown in red). Results are represented as the observed mean binding compared with Bag-1L wild-type peptide

binding in three independent experiments  $\pm$  SEM. Open bars represent vehicle-treated and closed bars DHT-treated conditions. C. The Bag-1L peptide sequences and sequences of other AR-binding proteins with the GARRPR sequence (or variants thereof) shown in red. D. Sequence logo of the binding motif of all peptides tested in (C) as determined by <http://weblogo.berkeley.edu/>. E. The peptides shown in (C) were GST-tagged and incubated with LNCaP cell lysate treated with vehicle or 100 nM DHT for 1 h. Protein binding was confirmed by Western blotting using an anti-AR antibody. GST-proteins were visualized using Ponceau staining. F. GST-tagged peptide versions of the sequences described in (C), either in their intact form (wt) or with the G and P of their GxxxPx motives exchanged to alanine residues (mut), were incubated with LNCaP cell lysate treated with 100 nM DHT for 1 h. Protein binding was identified by Western blotting using an anti-AR antibody. GST-proteins were visualized using Ponceau staining.

**FIGURE 8. Mutation of the GARRPR peptides decreases their binding to the AR-LBD**

A. GST pull-down experiments with GST-fused wild-type Bag-1L(1-20) or Bag-1L(61-80) peptides, or the same peptides with alanine exchanges at the G and P positions of the GARRPR motif. The experiment was carried out using LNCaP cell lysate treated with vehicle or 100 nM DHT for 1 h. Binding was analyzed by Western blotting using an anti-AR antibody. GST-proteins were visualized by Ponceau staining. B. Fluorescence Polarization experiments were performed using serially diluted AR-LBD and FITC-labeled Bag-1L(1-20) (blue line; A) or Bag-1L(61-80) (red line; B) and their corresponding mutant peptides as shown by black or grey lines for Bag-1L(1-20) or Bag-1L(61-80), respectively.

**FIGURE 9. The integrity of the Bag-1L GARRPR motif is essential for AR binding**

A. HeLa cells were transfected with constructs for AR and HA-tagged wild-type or mutant Bag-1L, and then treated with vehicle or 100 nM DHT for 16 h. Immunostaining was carried out with anti-AR or HA-antibodies. Cell nuclei were stained with Draq5. B. The Western blot of Bag-1L (Bag-1L, HA-tag and Flag-tag) and AR expression was performed using nuclear cell lysates of vector-transfected control cells (Vector) or LNCaP cells overexpressing HA-, FLAG-tagged wild-type (WT) or N-terminal mutant Bag-1L (NMut). Cells were either treated with vehicle (-) or DHT (+) for 4 h prior to cell lysis. Actin levels are shown as a control of equal protein loading. C. Co-immunoprecipitation of Bag-1L and AR in LNCaP cells stably overexpressing HA-, FLAG-tagged wild-type (Bag-1L WT) or N-terminal mutant Bag-1L (Bag-1L NMut). The IP was performed using anti-HA-tag or IgG control antibodies and an antibody against AR, to evaluate

binding. Equal protein loading ( $1/10^{\text{th}}$  of the input samples) was confirmed by probing for Bag-1L, AR and Actin expression.

**FIGURE 10. The N-terminal mutant of Bag-1L has an elevated AR-mediated transcription profile**

A. Heatmap of the expression profiles, shown as DHT-induced log<sub>2</sub> fold changes, of vector-control cells (Vector) or LNCaP cells stably overexpressing wild-type (WT) and N-terminal mutant Bag-1L (NMut). B. qRT-PCR validation of select DHT-upregulated genes unique to the N-terminal Bag-1L mutant (as shown in (A)). DHT-induced fold changes for the control cells (Vector; open bars), Bag-1L wild-type (WT; grey bars) and Bag-1L N-terminal mutant cells (NMut; full bars) are shown. Results are the averages of three independent experiments, three replicates each  $\pm$  SD. C. ChIP-qPCR of AR at select enhancer sites of genes upregulated in the LNCaP cells overexpressing the Bag-1L GARRPR mutant. DHT-induced fold enrichments over inputs are shown for the control cells (Vector; open bars), the Bag-1L wild-type (WT; brown bars) and Bag-1L N-terminal mutant cells (NMut; pink bars). Results are the averages of three replicates  $\pm$  SD.

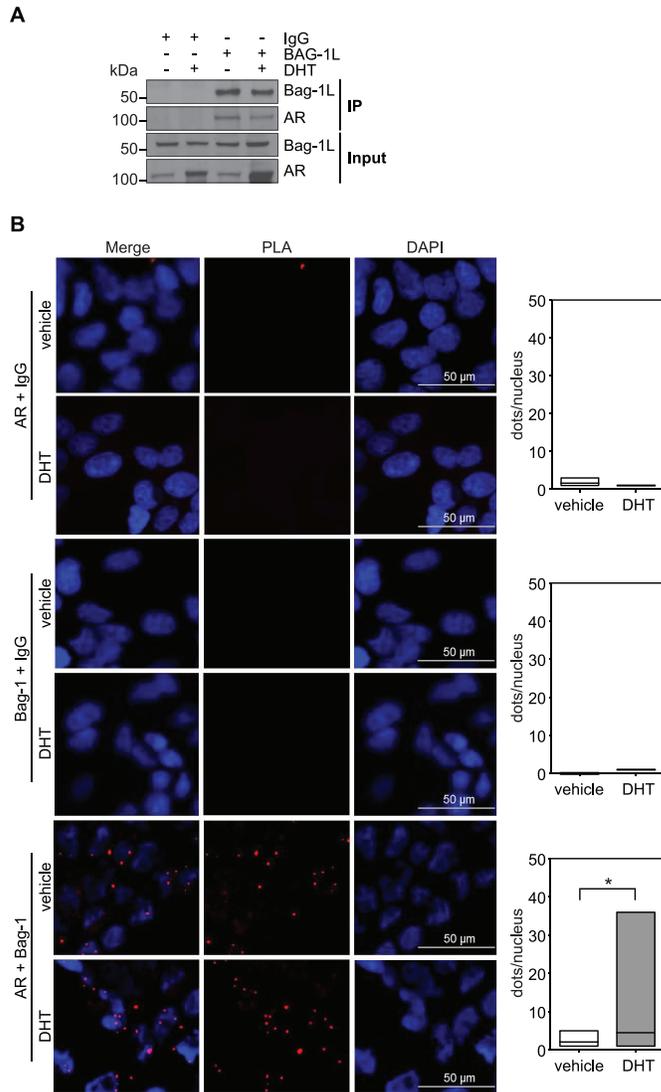


Figure 1

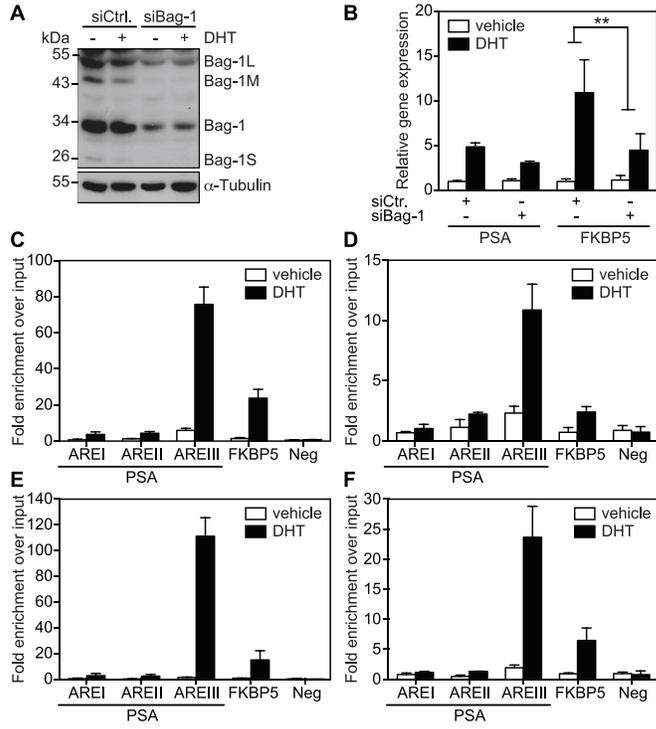


Figure 2

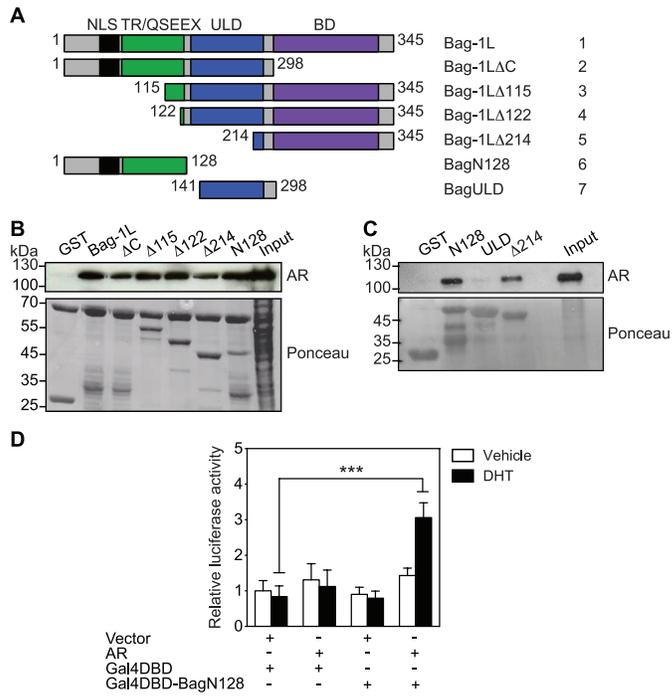


Figure 3

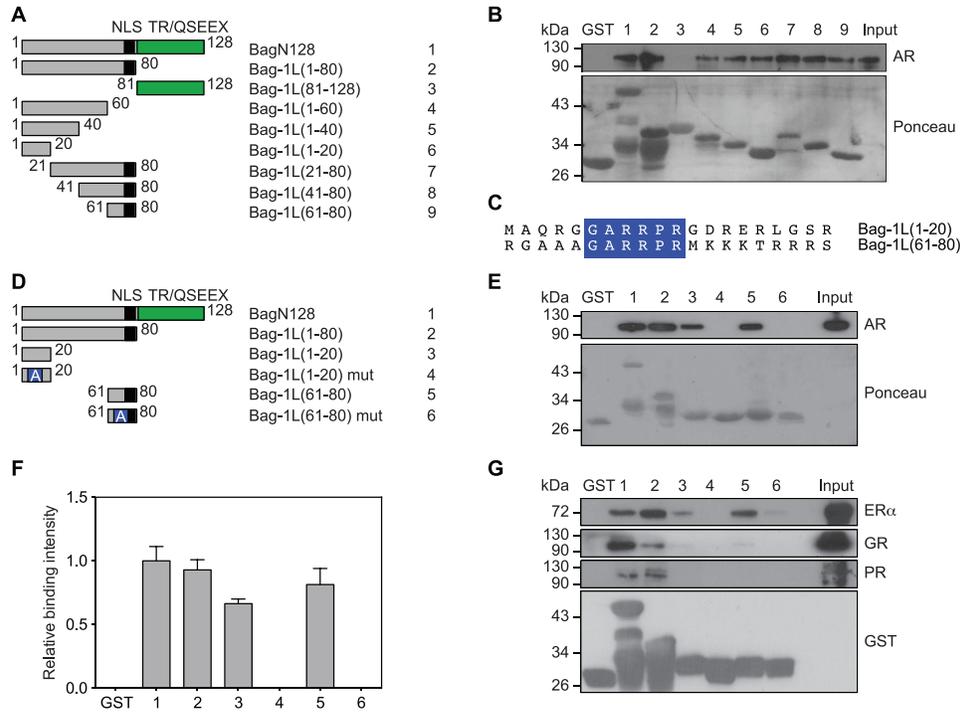


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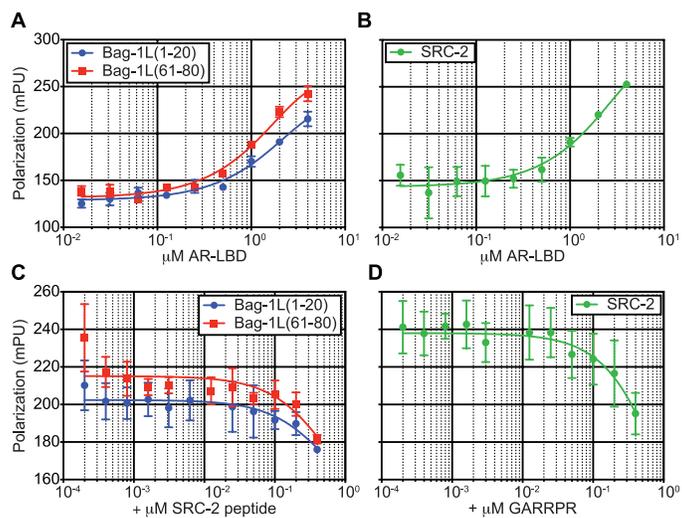


Figure 5

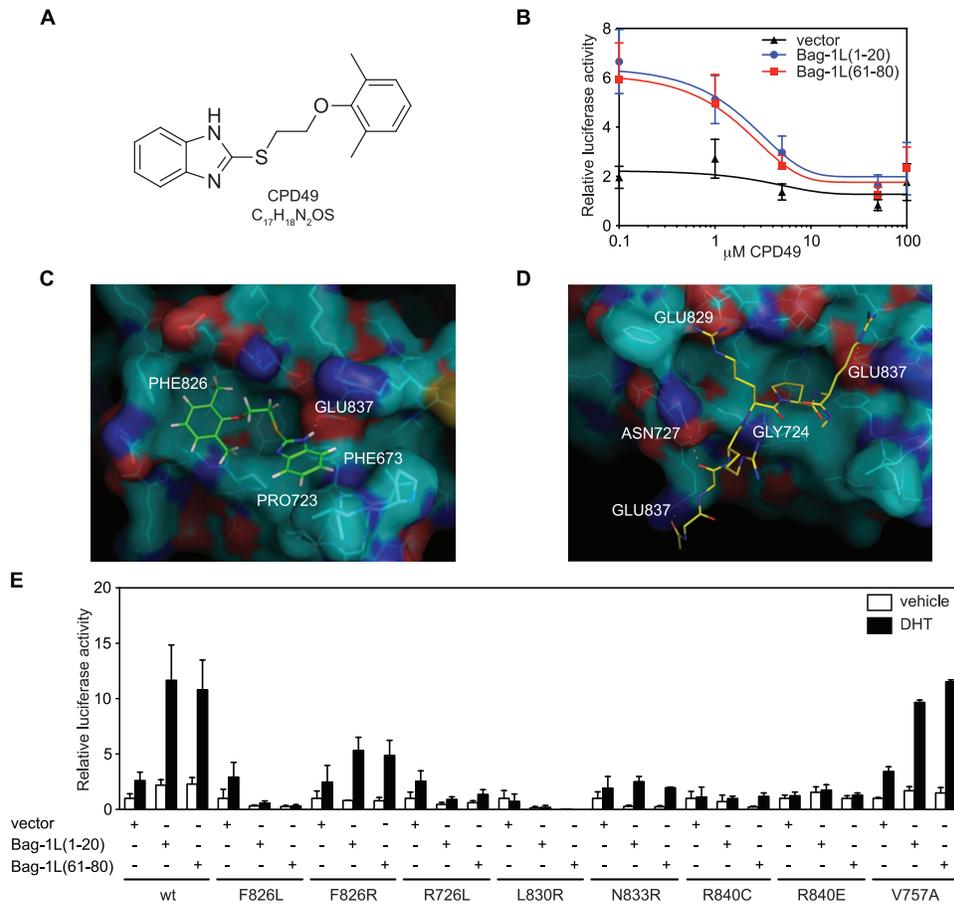


Figure 6

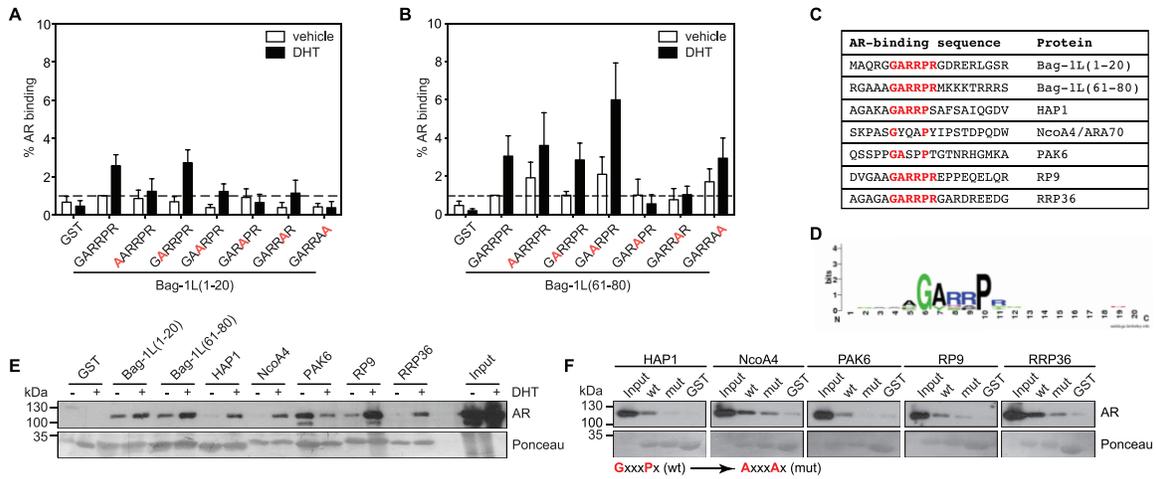


Figure 7

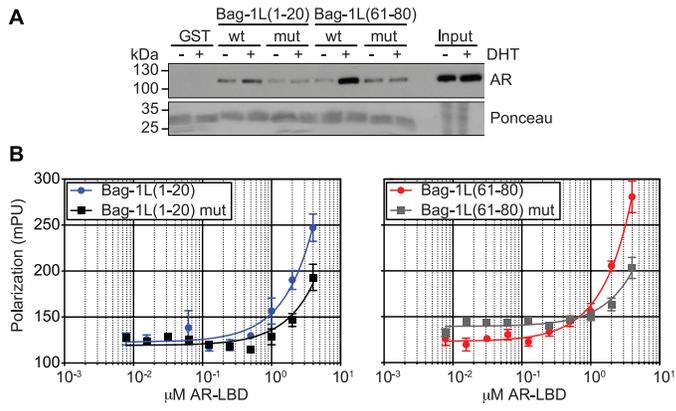


Figure 8

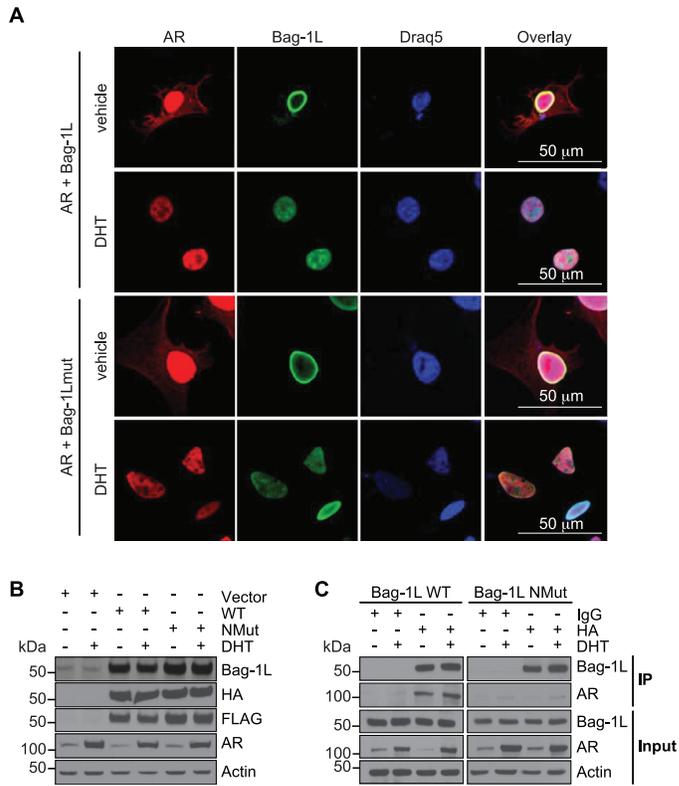


Figure 9

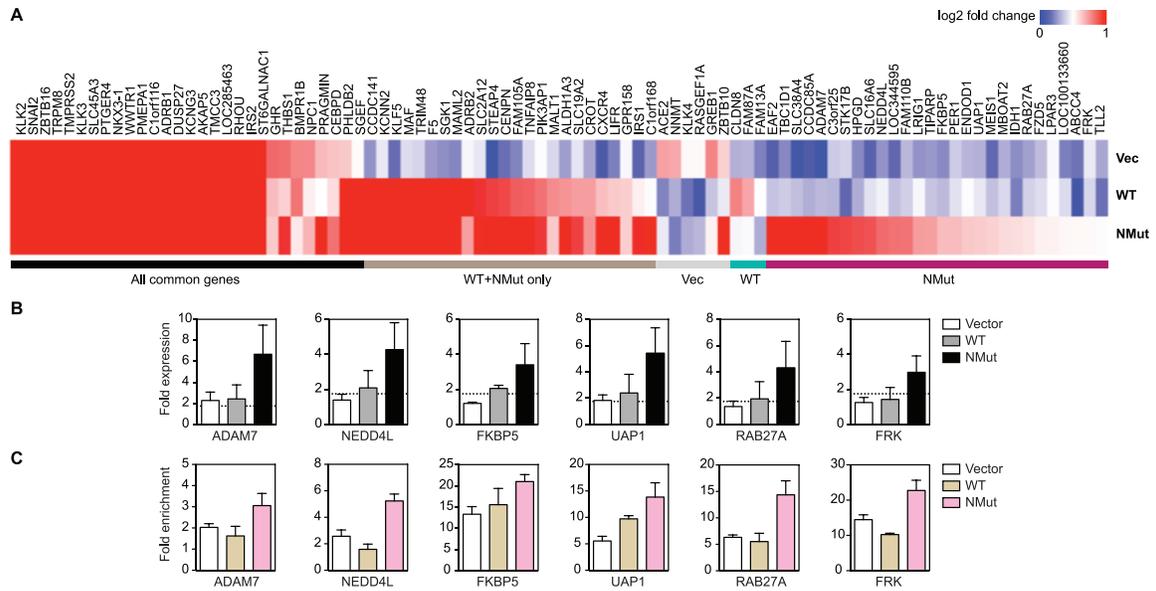


Figure 10