UNIVERSITAT DE BARCELONA

# Characterization of alternative therapeutic sites on the androgen receptor and novel protein-protein associations

Laia Rodríguez Carbó

Tesi doctoral

# CHARACTERIZATION OF ALTERNATIVE THERAPEUTIC SITES ON THE ANDROGEN RECEPTOR AND NOVEL PROTEIN-PROTEIN ASSOCIATIONS

Laia Rodríguez Carbó

Departament de Bioquímica i Biologia Molecular

Facultat de Biologia

Novembre de 2015

Universitat de Barcelona

Programa de Doctorat de Biomedicina

Facultat de Biologia

Departament de Bioquímica i Biologia Molecular

# CHARACTERIZATION OF ALTERNATIVE THERAPEUTIC SITES ON THE ANDROGEN RECEPTOR AND NOVEL PROTEIN-PROTEIN ASSOCIATIONS

*Caracterització de Superfícies Terapèutiques alternatives en el Receptor d'Andròg ens i Noves Associacions Proteïna-Proteïna*

Memòria presentada per Laia Rodríguez Carbó per optar al grau de Doctora per la Universitat de Barcelona

La interessada,

Laia Rodríguez Carbó

Vistiplau de la directora,

Dra. Eva Estébanez Perpiñá

# TABLE OF CONTENTS

# FIGURES

## INTRODUCTION

## RESULTS AND DISCUSSION

# TABLES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ADT | Androgen-deprivation therapy |
| AF-1 | Activation function-1 |
| AF-2 | Activation function-2 |
| AIS | Androgen insensitivity syndromes |
| AR | Androgen receptor |
| ARA | Androgen receptor associated protein |
| ARE | Androgen response element |
| ARMC9 | Armadillo repeat containing 9 |
| BF-3 | Binding function-3 |
| CAIS | Complete androgen insensitivity syndrome |
| CPA | Cyproterone acetate |
| CRPC | Castration resistant prostate cancer |
| DBD | DNA-binding domain |
| DHT | 5α-dihydrotestosterone |
| DMSO | Dimethyl sulfoxide |
| ER | Estrogen receptor |
| FBS | Fetal bovine serum |
| FKBP52 | 52 kDa FK506 binding protein |
| FUS | Fused in Ewing's Sarcoma |
| FXR | Farnesoid X receptor |
| GR | Glucocorticoid receptor |
| GRIP1 | Glucocorticoid receptor interacting protein 1 |
| H | Helix |
| HAT | Histone acetyltransferase |
| LBD | Ligand-binding domain |
| LBP | Ligand-binding pocket |
| M2H | Mammalian two-hybrid |
| MAIS | Mild androgen insensitivity syndrome |
| MAPK8IP1 | Mitogen-activated protein kinase 8 interacting protein 1 |
| MD | Molecular Dynamics |

| | |
|---|---|
| MR | Mineralocorticoid receptor |
| NAE | NEDD8-specific E1 activating enzyme |
| NSAA | Non-steroidal anti-androgen |
| N/C interaction | Amino-terminal/carboxy-terminal interaction |
| NCoR | Nuclear receptor corepressor |
| NEDD8 | Neuronal precursor cell-expressed developmentally down-regulated 8 |
| NR | Nuclear receptor |
| NTD | amino-terminal domain |
| Nurr1 | Nur-related protein 1 |
| PAIS | Partial androgen insensitivity syndrome |
| PCa | Prostate cancer |
| PDB | Protein Data Bank |
| PPAR | Peroxisome proliferator-activated receptor |
| PR | Progesterone receptor |
| PTM | Posttranslational modification |
| Rab11FIP3 | Rab11 family-interacting protein 3 |
| RAR | Retinoic acid receptor |
| SARM | Selective androgen receptor modulator |
| SBMA | Spinal and bulbar muscular atrophy |
| SFBS | Charcoal stripped fetal bovine serum |
| SMRT | Silencing mediator of retinoid and thyroid receptors |
| SR | Steroid receptor |
| SRC | Steroid receptor coactivator |
| TR | Thyroid receptor |
| Uba3 | Ubiquitin-like modifier-activating enzyme 3 |
| Ubc9 | Ubiquitin conjugating enzyme 9 |
| UBL | Ubiquitin-like proteins |
| VDR | Vitamin D receptor |
| WT | Wild-type |
| Y2H | Yeast two-hybrid |

# I. INTRODUCTION

# 1. INTRODUCTION

## 1.1. THE ANDROGEN RECEPTOR (AR): AN OVERVIEW

The androgen receptor (AR) (NR3C4, nuclear receptor subfamily 3, group C, gene 4) (Centenera et al., 2008; Lubahn et al., 1988), together with the estrogen receptor isoforms (ER), glucocorticoid receptor (GR), progesterone receptor (PR) and mineralocorticoid receptor (MR), belongs to the steroid receptor (SR) group of nuclear receptors (NRs) (Evans and Mangelsdorf, 2014; Mangelsdorf et al., 1995). The NR superfamily, which is composed of a total of 48 transcription factors in humans, is comprised by the endocrine receptors (steroid and non-steroid hormone receptors), the adopted orphan receptors, and the so-called orphan receptors, for which the ligand remains unknown, not agreed upon or in some examples there appears to be no endogenous ligand (Benoit et al., 2006; Gallastegui et al., 2015) (**Figure 1-1**).

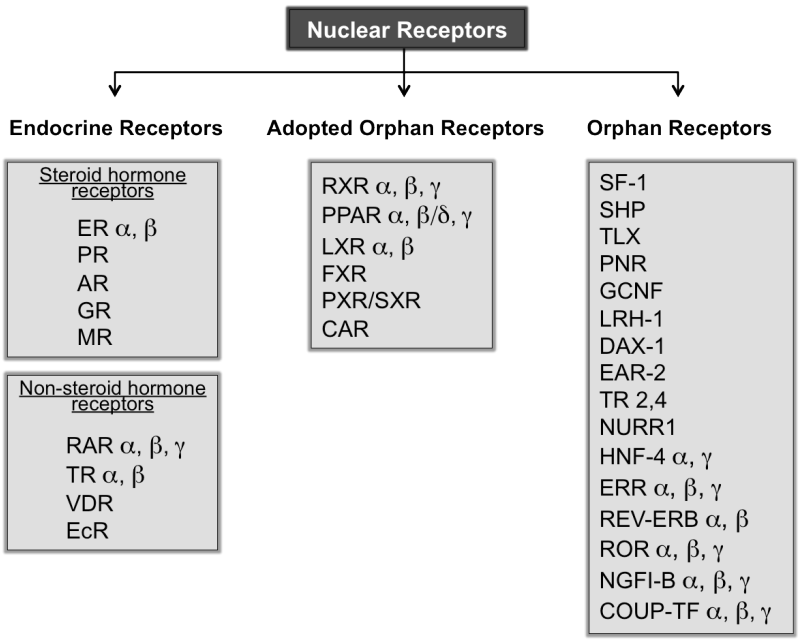


**Figure 1-1.** A classification of the NR superfamily.

NRs can be divided in endocrine receptors (steroid and non-steroid hormone receptors) that bind with high affinity ligands that act as hormones; the so-called adopted orphan receptors that recognize with low affinity several ligands; and the orphan receptors that lack identifiable ligands or the ones found are still controversial in the NR field.

NRs represent the largest group of eukaryotic transcription factors and regulate a wide variety of physiological functions, including cell development, reproduction, homeostasis and metabolism (Evans and Mangelsdorf, 2014; Gallastegui et al., 2015).

Importantly, this superfamily is involved in major human pathologies and carcinogenesis, representing one of the most important pharmacological targets in the clinic (Burris et al., 2013; Evans and Mangelsdorf, 2014).

AR is a ligand-dependent transcription factor that by binding to testosterone and its metabolite 5α-dihydrotestosterone (DHT), the two most potent natural androgens, regulates the expression of specific genes. Appropriate androgen signaling is necessary for a wide range of developmental and physiological responses, such as male sexual differentiation and pubertal sexual maturation, as well as the maintenance of spermatogenesis and male reproductive organs (Claessens et al., 2008).

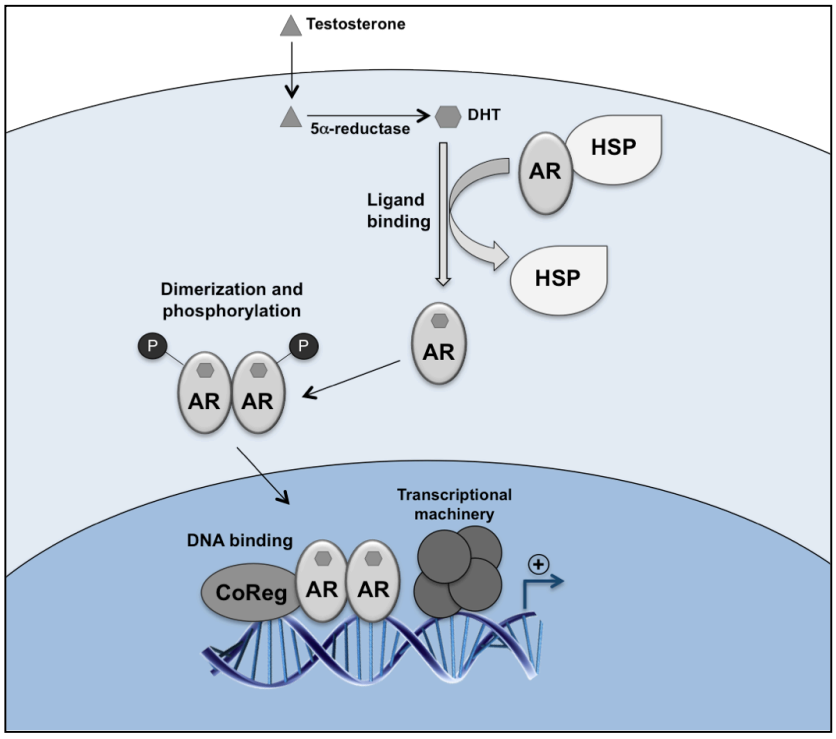


**Figure 1-2.** Androgen actions.

Testosterone enters prostate cells, where it is converted to DHT by the enzyme 5α-reductase. Binding of DHT to AR induces dissociation from chaperones and receptor phosphorylation (P). AR then dimerizes and translocates to the nucleus, where it binds to the promoter region of target genes. Coregulator proteins (CoReg) and members of the basal transcriptional machinery are further recruited, activating (or repressing) target genes.

Androgen action takes place in a mechanism that involves multiple steps (**Figure 1-2**). In the absence of hormone, AR is thought to be inactive or maintained in a resting conformation in the cytoplasm complexed to heat-shock proteins/chaperones. After testicular synthesis, testosterone is transported to target tissues, such as the prostate, where it is converted into DHT. The binding of DHT to AR promotes the dissociation of the chaperone complex, dimerization, phosphorylation and translocation of AR into the nucleus, where it binds to the androgen response elements in the promoter region of target genes (Centenera et al., 2008). At the promoter, members of the basal transcriptional machinery and other coregulators are further recruited, resulting in the activation or repression of AR-responsive genes (Claessens et al., 2008; Palvimo, 2012).

## 1.2. AR STRUCTURE AND DOMAIN ORGANIZATION

Encoded by a single copy gene located on the X chromosome long arm and organized in eight exons, the AR is a 919-amino acid protein (although it can vary depending on the length of the polyglutamine and polyglycine stretches) with an apparent molecular weight of 110 kDa (Chang et al., 1995; Lubahn et al., 1988).

Like other members of the NR superfamily, AR is composed of three major functional domains: the amino-terminal domain (NTD), a central DNA-binding domain (DBD), and a carboxyl-terminal ligand-binding domain (LBD), which is linked to the former by a flexible hinge region (**Figure 1-3**). Within the NTD resides the constitutively active activation function-1 (AF-1), whereas the ligand-dependent AF-2 is located in the LBD (Centenera et al., 2008; Claessens et al., 2008; Jenster et al., 1995).

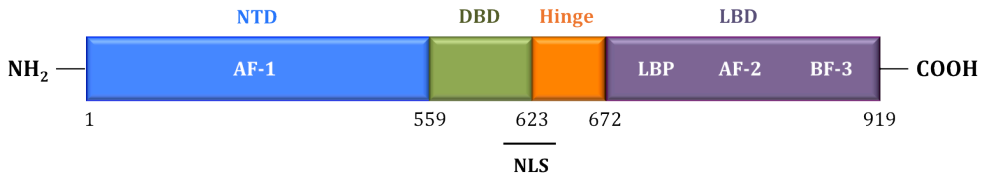


**Figure 1-3.** Schematic representation of the AR functional domains.
Domain organization of AR with indications of the N-terminal domain (NTD) in blue, the DNA-binding domain (DBD) in green, the hinge region in orange, and the ligand-binding domain (LBD) in purple. Numbers indicate the boundaries of the different domains. AF-1, activation function-1; NLS, nuclear localization signal; LBP, ligand-binding pocket; AF-2, activation function-2; BF-3, binding function-3 are indicated.

### 1.2.1. The AR N-terminal domain

Encoded by the first exon of the *AR* gene, the NTD comprises the largest part of AR, accounting for more than half of the size of the receptor (**Figure 1-3**). This domain is characterized by the presence of a polymorphic polyglutamine stretch (starting at position 57) and a polyglycine repeat (starting at residue 449) (Palazzolo et al., 2008) (**Figure 1-4**), the length of which are highly variable among the human population. The expansion of both stretches has been correlated with the incidence of diseases (La Spada et al., 1991) and has been shown to affect the folding and structure of the NTD (McEwan et al., 2007).

The NTD, considered as the major activation domain of AR, contains the ligand-independent AF-1, which harbors the transcription activation units Tau-1 and Tau-5. The latter covers residues from positions 360 to 528 (the amino acid numbering system in this thesis is based on the Genbank mRNA sequence M20132.1) and is considered an autonomous activation domain, as this fragment retains the activation potential in the absence of the LBD (Jenster et al., 1995). On the other hand, Tau-1, residing between amino acids 173 and 203, is more dependent on the presence of the LBD.

Located at positions 23-27 of the NTD, the $^{23}$FQNLF$^{27}$ motif, highly conserved among different species (Claessens et al., 2008), is responsible for the N/C interaction between the NTD and the LBD of AR, which is key in AR functions in the cell (Berrevoets et al., 1998; He et al., 1999; 2000).

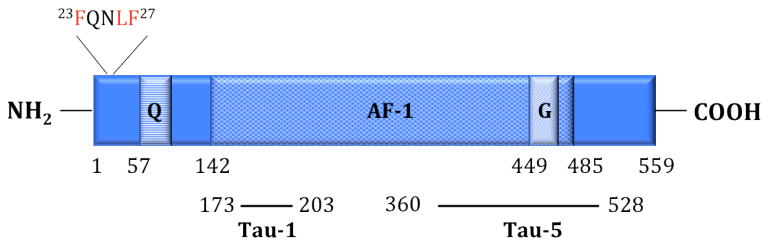


**Figure 1-4.** Schematic representation of the NTD of AR.
AF-1 spans from residue 142 to 485. The polyglutamine (Q) and polyglicine (G) repeats start at positions 57 and 449, respectively. Tau-1 expands from aminoacid 173 to 203, whereas Tau-5 spans from residue 360 to 528. The localization of the FxxLF motif responsible for AR N/C interaction is also shown.

The NTD is the least conserved domain between all NRs and due to its intrinsically disordered nature (McEwan, 2011) no structure has been elucidated thus far.

### 1.2.2. The AR DNA-binding domain

The AR DBD is formed by approximately 70 amino acids encoded by exons 2 and 3, and is the most structurally conserved domain among all members of the NR superfamily (Shaffer et al., 2004) (**Figure 1-3**). It consists of two coordination complexes, each one composed of four cysteines and a zinc atom. The first zinc-coordinated module makes the base-specific contact with the major groove of DNA, whereas the second one is involved in the DNA-dependent dimerization (Centenera et al., 2008; Claessens et al., 2008).

### 1.2.3. The AR hinge region

The hinge region, located between amino acids 623 and 671 of the human AR and encoded partially by exons 3 and 4, can be defined as a flexible linker that connects the last α-helix of the DBD with the first α-helix of the LBD (Claessens et al., 2008; Clinckemalie et al., 2012; Deeb et al., 2008) (**Figure 1-3**). Although its sequence is poorly conserved, the hinge region of all SRs contains the bipartite nuclear localization signal (NLS), which is responsible for the translocation of the protein to the nucleus. Additionally, the hinge region of AR contains several sites for post-translational modifications, which seem to be responsible for modulating AR activity in different cellular settings (Deeb et al., 2008).

### 1.2.4. The AR ligand-binding domain

The AR LBD is a highly structured domain encoded by exons 4-8 organized as a twelve α-helical sandwich-like structure (Estébanez-Perpiñá et al., 2005; Hur et al., 2004; Matias et al., 2000; Sack et al., 2001) (**Figure 1-5**). Within this domain resides the ligand-binding pocket (LBP), which is formed by helices 3, 5, 7, 11 and 12, together with the β-sheet preceding H6.

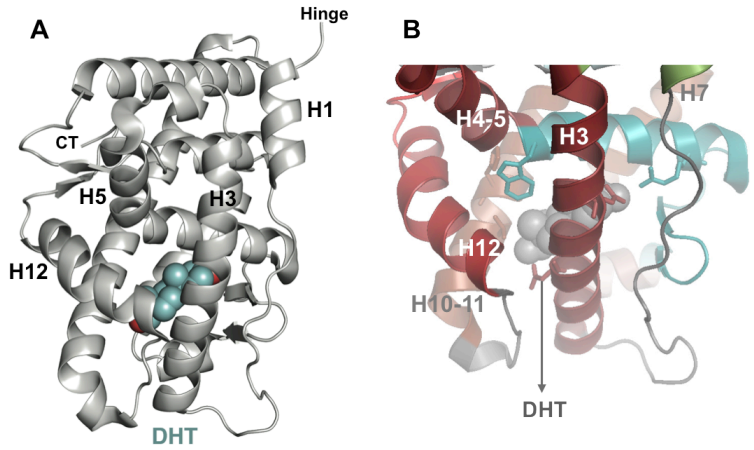


**Figure 1-5.** AR LBD featuring the encapsulated LBP that contains the hormone.
**(A)** Schematic representation of the AR LBD showing the hidden DHT (blue), key AF-2 helices (H) 3, 5, and 12; and H1. **(B)** Schematic representation of the LBP, formed by helices 3, 5, 7, 11 and 12, where the hydrophobic DHT (grey) binds to and it is protected from the solvent.

Unlike the DBD of AR, with only one single elucidated crystal structure (Shaffer et al., 2004), the LBD of the wild type as well as different mutated forms of AR complexed with a variety of ligands has been solved (Estébanez-Perpiñá et al., 2005; 2007; Hur et al., 2004; Matias et al., 2000; Sack et al., 2001). However, there is no structure of the AR LBD in either the apo-form (protein in the absence of hormone), the wild type AR in the antagonist-bound conformation, or as a part of the multidomain/full-length AR. All the crystal structures of AR LBD complexed to antiandrogens contain mutations in the LBP rendering the receptor active (Bohl et al., 2005; 2007).

Besides the cocooned LBP, the LBD of AR harbors on its surface the coactivator binding pocket also known as the AF-2 pocket, which is an important site for protein-protein interactions with coregulatory proteins (van de Wijngaart et al., 2012), and the so-called binding function-3 (BF-3) (Buzón et al., 2012; Estébanez-Perpiñá et al., 2007; Grosdidier et al., 2012), which has been associated with allosteric modulation of AR function, and where several protein-protein recognition events have been mapped (De Leon et al., 2011; Grosdidier et al., 2012; Jehle et al., 2014).

1.2.4.1. Activation Function-2 Pocket (AF-2)

By analogy with other NRs, whose apo- and holo-structures have already been solved, agonist binding to the LBP of AR is thought to induce the folding and repositioning of H12, sealing the LBP like a mouse trap and completing the AF-2 pocket (Darimont et al., 1998; Estébanez-Perpiñá et al., 2005) (**Figure 1-6**).

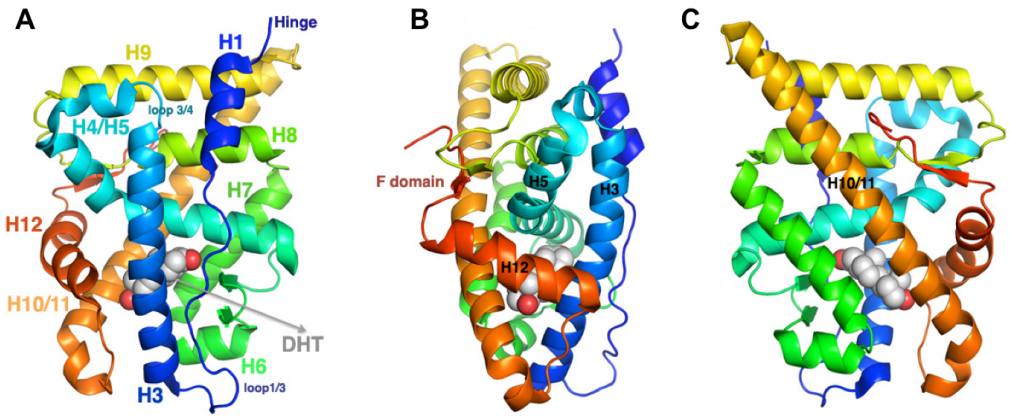


**Figure 1-6.** Secondary- structure representation of the AR ligand-binding domain.
Schematic representation of the AR LBD helices and connecting loops. **(A)** Front view in standard orientation to display fully the AF-2 pocket, formed by helices 3, 4, 5 and 12. **(B)** 90º rotation of AR LBD depicted in (A) to show H12 (red) in the agonist/active conformation **(C)** An additional 90º rotation of AR LBD depicted in (B) to show the backside of the AR LBD. DHT is depicted in grey.

Several residues (V713, V716, K717, K720, R726, V730, Q733, M734, I737, Q738, M895, E897 and I899) from H3, H4, H5 and H12 shape the AF-2 pocket (**Figure 1-6**), a solvent-exposed hydrophobic cleft that serves as a docking site for short hydrophobic peptide motifs and NR boxes present in AR coactivator proteins (Estébanez-Perpiñá et al., 2005; Hur et al., 2004)

The AR AF-2 differs from other NRs in that it binds very weakly to LxxLL (where L is a leucine and x is any amino acid) signature sequences (Gallastegui et al., 2015; Heery et al., 1997). Instead, it accommodates preferentially the bulkier aromatic-rich motifs present both in AR specific-coactivators and the AR NTD (Estébanez-Perpiñá et al., 2007; Hur et al., 2004). In contrast to many other SRs, a unique feature of the AR is that a strong interaction between the NTD and the LBD (N/C interaction) is required for optimal receptor function, as the H12 of hormone-bound AR is stabilized by this interaction, which is also facilitated by several coactivators (Berrevoets et al., 1998; He et al., 2000). Numerous studies have

shown that this N/C interaction is initially intramolecular in the cytoplasm before becoming intermolecular once in the nucleus (van Royen et al., 2012). It has also been suggested that the N/C interaction may be disrupted when AR binds to chromatin, possibly to facilitate the recruitment of coactivators (Lonard and O'Malley, 2007; van Royen et al., 2012; 2007)

Two regions of the AR NTD, $^{23}$FQNLF$^{27}$ (FxxLF) and $^{433}$WHTLF$^{437}$ (WxxLF), can mediate binding of the N- and C-terminal regions of AR. These motifs interact with the AF-2 pocket and compete favorably with the LxxLL-containing coactivator proteins for ligand-dependent binding to the LBD (Estébanez-Perpiñá et al., 2007; 2005; He et al., 2000; 2002; Hur et al., 2004)

The AF-2 of AR displays low intrinsic transactivation properties, a feature that correlates with the low affinity of this domain for the canonical LxxLL-bearing coactivators. In addition, there are two clusters of oppositely charged residues on the AR LBD surface, at the opposite ends of the AF-2, that assist in orienting the bound NR motifs in the AF-2 groove (Estébanez-Perpiñá et al., 2007; Hur et al., 2004). Particularly interesting are lysine 720 (K720) and glutamate 897 (E897), which form the so-called electrostatic charge clamp (**Figure 1-7**) functioning as capping residues that stabilize the interaction between the AF-2 and the coactivator by making backbone contact with FxxLF motifs (Estébanez-Perpiñá et al., 2005; He et al., 2002; He and Wilson, 2003).

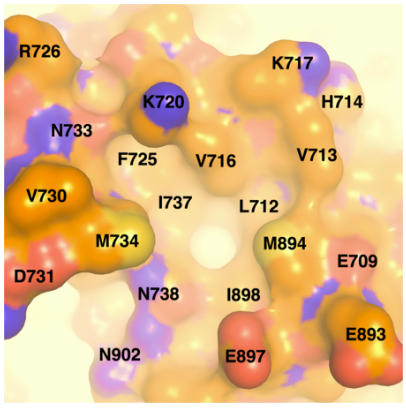


**Figure 1-7.** Residues lining the AF-2 pocket, which has three subpockets.

The charge clamp residues K720 and E897 are located at opposite poles of the coactivator binding groove and help positioning the interacting coregulator on AR surface.

### 1.2.4.2. Binding function-3 pocket (BF-3)

Several lines of evidence show that the AF-2 groove may not be the sole protein-protein interface governing macromolecular assembly upon AR LBD surface (De Leon et al., 2011; Estébanez-Perpiñá et al., 2007; Grosdidier et al., 2012; Jehle et al., 2014). It has been observed that the AF-2 pocket is reshaped not only by the ligand binding to the LBP, but also by the interaction of small molecules elsewhere on the surface of the LBD (Estébanez-Perpiñá et al., 2007). Structural and mutational studies unexpectedly identified a novel surface-exposed pocket termed BF-3, which is able to remodel and allosterically modulate the AF-2 site, affecting the recruitment of coactivators (Buzón et al., 2012; Estébanez-Perpiñá et al., 2007; Grosdidier et al., 2012).

BF-3 is a concave pocket that lies at a distinct but topographically adjacent surface to the AF-2 coactivator-binding groove. The BF-3 site, comparable in size and depth to the AF-2 pocket, is solvent exposed and has a hydrophobic nature, features that are in line with its postulated role as a protein-protein interaction surface. AR BF-3 is outlined by several LBD-forming helices, and roughly resembles a rectangle with rounded corners.

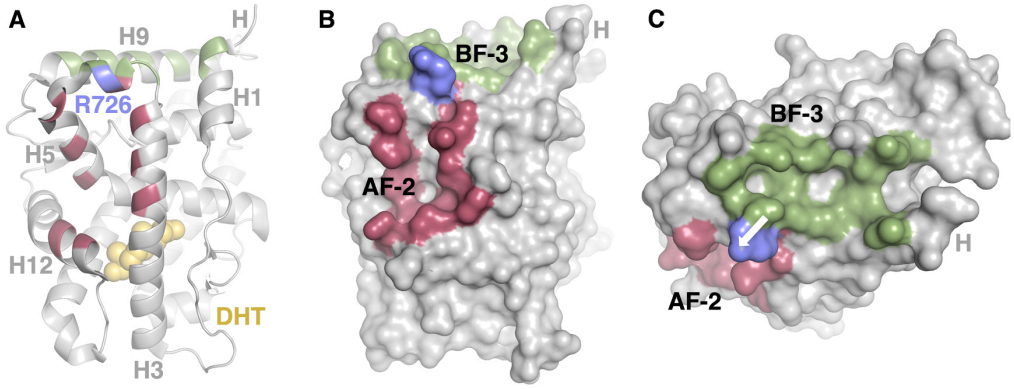


**Figure 1-8.** AR LBD features several interaction surfaces that modulate its pathophysiological actions.

**(A)** Schematic secondary structure representation of AR LBD showing the location of the hormone (DHT, space-filling model in yellow) in the LBP, and helices (H)1, H3, H5, H9 and H12 (grey). Some residues lining the AF-2 pocket are highlighted in raspberry and some key BF-3 residues are colored green. The residue R726 belonging to the boundary between AF-2 and BF-3 is shown in blue. **(B)** Space-filling model showing AR LBD surface (gray) and relative locations of AF-2 and BF-3 pockets. **(C)** 90º rotation of AR LBD depicted in (B) to display BF-3 fully. A white arrow indicates that conformational changes are transmitted by R726 (blue) from BF-3 towards AF-2. AR hinge (H) further delimits the perimeter of BF-3. (Extracted from Buzón V*, Carbó LR* et al. (Buzón V et al. 2012)).

By representing the LBD domain as in **Figure 1-8**, the BF-3 pocket is delimited by AR residues from the $NH_2$-terminal part of H1 (Q670, P671, I672 and F673), some residues of H3/loop 3-4 (P723, G724, R726 and N727) and numerous residues that span almost the whole length of H9 (F826, E829, L830, N833, E837, and R840).

Accessibility to the perimeter of BF-3 is further delimited in a canyon-like manner by the most C-terminal part of the AR hinge domain together with the loop between helices 9-10/11 and the upper limit of the AF-2 pocket. Hence, there are two residues (R726 and N727) that can be defined as the boundary between those two interfaces. Additionally, residue L830, present at the floor of the pocket, divides AR BF-3 in two sub-compartments (**Figure 1-9**). In contrast to the trifurcated AR AF-2 pocket, there seems to be no opposite charge-clusters delimiting the BF-3 groove.

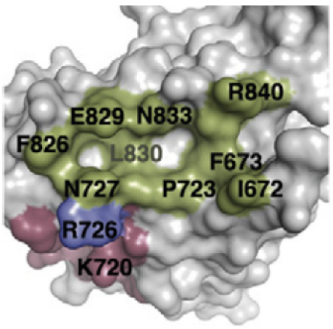


**Figure 1-9.** Residues lining the BF-3 pocket.
The AR LBD surface is shown in grey. The BF-3 lining residues are shown in green, whereas the AF-2 groove is visualized in raspberry, and residues R726, in the boundary of AF-2 and BF-3, is depicted in blue. (Modified from Grosdidier et al., 2012)).

Physiological relevant partners of AR BF-3 have started to emerge although further studies are needed to corroborate whether BF-3 is only a docking surface for NR co-chaperones such as FKBP52 and BAG-1L (De Leon et al., 2011; Grosdidier et al., 2012; Jehle et al., 2014).

## 1.3. ALLOSTERY IN THE ANDROGEN RECEPTOR

Allostery in NRs has been attracting increasing attention in the last few years (Mackinnon et al., 2014). This is not surprising as long-range communication between sites of a protein that are located wide apart has been shown to be essential for its function in many cellular processes (Süel et al., 2003). Protein allostery seems to be a universal phenomenon: a perturbation at a site (e.g.

substrate binding, covalent modification, a pathogenic or disease-linked mutation) causes a functional change at a distant site of the protein by altering its conformation and/or dynamics. It is a cooperative event, positive or negative, up- or down-regulating the function of proteins, respectively (Mackinnon et al., 2014).

NRs are flexible transcription factors that regulate networks of target genes by acting in a dynamic way. The conformational perturbations induced by the ligand binding in NRs play an important role, as they control transactivation and coactivator recruitment. The best-described allosteric rearrangement in NRs upon ligand accommodation is the conformational change that takes place in H12, whose position determines the transcriptional readout of the receptor, acting as a molecular switch (Watanabe et al., 2010). In the agonist-bound conformation, the H12 forms one side of the AF-2 pocket, allowing the recruitment of coactivator proteins. In contrast, the bulky side chains of antagonists block the H12 from assuming an agonist position, preventing the formation of a productive AF-2 and the interaction with coactivators (Edwards, 2000). Therefore, through its dynamic localization, the H12 provides allosteric control of transcription. However, the conformational changes described in NRs are not exclusively limited to the LBD. Despite having traditionally been considered simple docking sites, the role of DNA response elements as allosteric modulators of receptor function is now well documented. DNA binding sites are known to influence the activity of NRs by imparting conformational changes onto the DBD and neighboring domains (i.e. AF-2), as well as by altering the affinity of the receptor for other ligands (Meijsing et al., 2009). Additionally, interdomain and inter-receptor communication has been reported for the thyroid and vitamin D receptors (Putcha BD, 2009; Zhang et al., 2011)

Interestingly, an allosteric relationship between the AF-2 and BF-3 pockets of the AR LBD has been suggested and we have showed it as part of this thesis (Buzón et al., 2012; Estébanez-Perpiñá et al., 2007; Grosdidier et al., 2012; Jehle et al., 2014). On the one hand, it has been shown that compound binding to the BF-3 pocket induces the remodeling of AF-2 surface, impairing the binding of coactivator peptides and, consequently, evidencing that allosteric communication between the AF-2 and BF-3 grooves exists (Estébanez-Perpiñá et al., 2007). On the other hand,

BF-3 mutants have been shown to act as allosteric elicitors of conformational changes that are transmitted towards AF-2, affecting the function of the AR LBD. Furthermore, several residues belonging to BF-3 and AF-2 surface pockets have been revealed as key players of an allosteric network that may influence multiple aspects of AR LBD function (Grosdidier et al., 2012).

Furthermore, it has been reported that an interdomain communication between the DBD and the LBD of AR exists (Helsen et al., 2012), as well as it has been demonstrated that coactivators induce sequence-specific perturbations distant from the binding pocket, even in the presence of the same ligand (Zakharov et al., 2011). The resulting distinct conformational states of the protein complexes may dictate tissue specificity by altering the interaction/affinity between the downstream components of the transcriptional machinery and the response elements in the DNA.

## 1.4. AR COREGULATORS

Transcriptional regulation by NRs involves ordered and dynamic protein-protein interactions between the receptor, associated coregulators, and the RNA polymerase II transcriptional machinery at the chromatin of target genes. Coregulators are broadly defined as proteins that interact with NRs to enhance (coactivators) or reduce (corepressors) transactivation of target genes without significantly altering the basal transcription rate or typically binding to DNA (Heemers and Tindall, 2007). More than 200 coregulators have been identified to interact with AR, making this receptor one of the most social of the NR superfamily (van de Wijngaart et al., 2012).

Coactivators generally possess intrinsic histone acetyl transferase (HAT) activity and are characterized by their ability to potentiate NR transactivation through the interaction with the NTD and/or LBD of agonist-bound receptors. Conversely, corepressors mediate the repressive effect of unliganded or antagonist-bound NRs and, in some cases, even of agonist-liganded NRs by recruiting histone deacetylase complexe (McKenna et al., 1999).

Only AR coregulators relevant for this thesis will be shortly described.

### 1.4.1. AR coactivators

Numerous coactivators have been identified to interact with and enhance the ligand-dependent transcriptional activity of AR (Culig, 2016; Heinlein and Chang, 2002; van de Wijngaart et al., 2012). They may either serve as bridging or adaptor molecules, binding to NRs themselves, recruiting additional proteins (e.g. HATs) or interacting with the basal transcriptional machinery; or may function to facilitate ligand binding, promote nuclear translocation, or mediate signal transduction. Most of the AR coactivators identified to date also stimulate the activity of other NRs and transcription factors (Culig, 2016; Heinlein et al., 1999; Lanzino et al., 2005; van de Wijngaart et al., 2012). Therefore, very few AR-interacting proteins seem to be exclusive for AR, and even most of these AR specific-coregulators show promiscuity with other NRs (van de Wijngaart et al., 2012).

#### 1.4.1.1. The steroid receptor coactivator (SRC) family

The p160 steroid receptor coactivator (SRC) family is comprised by the three homologous members SRC1 (NCOA1), SRC2 (NCOA2/GRIP1/TIF2) and SRC3 (NCOA3/RAC3/ACTR/AIB1/TRAM1). SRCs play an important role in promoting cancer initiation, progression and metastasis. All SRCs have been found to be overexpressed in many types of human cancers and, several studies have demonstrated that both SRC1 and SRC3 are overexpressed in prostate tumors (Agoulnik et al., 2005; Dasgupta and O'Malley, 2014).

SRCs serve as a platform for the assembly of coactivator complexes on the regulatory regions of genes that are targeted by agonist-bound NRs. Although possessing intrinsic HAT activity, p160 proteins primarily serve as bridging factors for recruiting HAT-containing coactivator complexes in a promoter- and NR-specific context (Leo and J. D. Chen, 2000). Because SRC genes are subject to amplification and overexpression in various human cancers (Lonard et al., 2007; Xu et al., 2009), this family is the most extensively characterized of all the transcriptional coactivators.

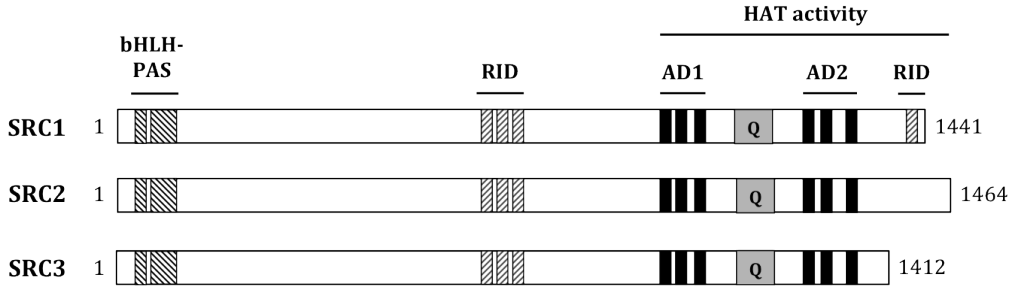


**Figure 1-10.** Schematic representation of the functional domains of the SRC family members. The location of conserved structural and functional domains are indicated by filled or textured boxes. bHLH, basic helix-loop-helix; PAS, Per/ARNT/Sim domain; RID, receptor interaction domain; AD, transcriptional activation domains; Q, glutamine-rich region; HAT, histone acetyltransferase domain (only in SRC1 and SRC2)

SRCs encompass three functional domains (**Figure 1-10**). The N-terminal region is the most conserved part and is required for protein-protein interactions; the central receptor interaction domain (RID) contains three conserved LxxLL motifs responsible for direct association with NRs; and the carboxyl-terminus harbors two transcriptional activation domains (AD1 and AD2), which allows the recruitment of HATs and methyltransferases to the chromatin, essential for SRC-mediated transcriptional activation (Leo and J. D. Chen, 2000). Moreover, the C-terminus of SRC1 and SRC3 have been shown to contain HAT activity domains (Dasgupta and O'Malley, 2014; Heinlein and Chang, 2002).

Despite the three central LxxLL motifs of SRCs mediate the binding to most NRs, this does not seem to be the case for AR. As its AF-2 is transcriptionally weak, the coactivator recruitment to AR differs from that of most NRs. Whereas the major part of SRs bind to coactivators via contact points in the LBD, different parts within the AR NTD seem to be also involved in protein-protein interactions with AR (van de Wijngaart et al., 2012). While SRC1 and SRC2 bind to the AR AF-2, this interaction is not essential for coactivation (Brooke et al., 2008). Instead, both coactivators bind to the NTD and possibly the DBD (Alen et al., 1999b), interaction that does not require the coactivators to contain intact LxxLL motifs (Bevan et al., 1999; Mäki et al., 2006).

### 1.4.1.2. Androgen receptor associated (ARA) proteins

The androgen receptor-associated protein 70 (ARA70/NCOA4) was the first ligand-dependent AR coactivator to be identified (T. Gao et al., 1999; Heinlein et al., 1999; Z. X. Zhou et al., 2002). However, despite it was initially thought to be AR-specific, subsequent studies indicated that it also interacts with several other NRs (Alen et al., 1999a; Heinlein et al., 1999; Kollara and Brown, 2012). In addition to ARA70, the non-related ARA55, ARA54, ARA24, ARA160 and ARA267 are also known to interact with AR (Sampson et al., 2001).

ARA70 is a 614 amino acid-protein that contains two NR interaction motifs: an LxxLL motif located in the N-terminal region (amino acids 92-96) and an FxxLF motif at residues 328-332 (Heinlein et al., 1999) (**Figure 1-11**). While the LxxLL motif is essential for the binding of ARA70 to most NRs, the region responsible for the interaction with the LBD of AR resides within amino acids 321-441, where the FxxLF motif is located, although residues flanking this motif also seem to play an important role in the functional binding to AR (He et al., 2002; He and Wilson, 2003). Furthermore, in addition to the LBD, it has been shown that ARA70 is also recruited at the NTD of AR (Z. X. Zhou et al., 2002).

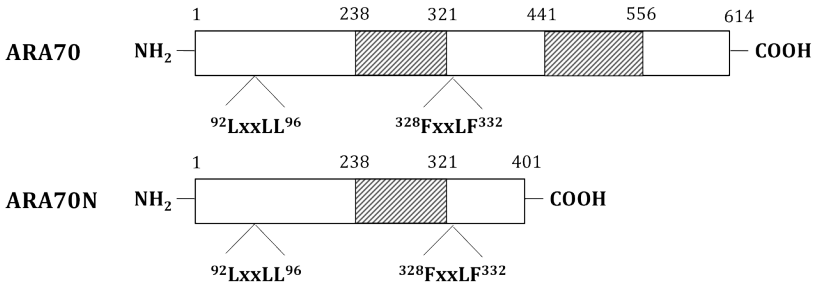


**Figure 1-11.** Schematic representation of human ARA70 and ARA70N variant.
The FxxLF motif located at amino acids 328-332 is involved in AR and PR interaction, whereas the LxxLL motif located at amino acids 92-96 is implicated in the binding to VDR, TR and PPARγ. Amino acids 238-321 and 441-556, indicated by textured boxes, are key for optimal AR coactivation. ARA70N consists of the first 401 amino acids of ARA70, containing thus both the LxxLL and FxxLF motifs.

ARA70 has been associated to many carcinomas, although its altered expression in prostate cancer (PCa) is still controversial. Cell-based reporter assays with the N-terminal part of ARA70 (ARA70N, residues 1-401) (**Figure 1-11**) have demonstrated that overexpression of ARA70N enhances the activity of AR by

ligands other than androgens, diminishing the success of hormonal therapies followed in the treatment of PCa (Kollara and Brown, 2012). In addition to ligand specificity, ARA70 overexpression seems to enhance the agonist activity of antiandrogens in human prostate cancer cells by directly interacting with AR (Saad and Fizazi, 2015).

## 1.4.2. AR corepressors

By the classical definition, corepressors function as negative regulators in order to repress or silence basal transcriptional activity by recruiting chromatin-modifying enzymes. However, a large number of proteins have been identified to regulate negatively AR activity through alternate mechanisms, including chromatin modification, regulation of AR N/C interaction, abrogation of AR-chromatin association or nuclear translocation, and inhibition of coactivator recruitment (Perissi et al., 2010; M. M. Wong et al., 2014)

The best-studied AR corepressors so far are the nuclear receptor corepressor (NCoR/NCOR1) and the silencing mediator of retinoic and thyroid hormone receptor (SMRT/NCOR2), which dock to and/or around the AF-2 pocket (Nagy et al., 1999).

### 1.4.2.1. NCoR and SMRT

NCoR and SMRT, two large (ca. 2500 aa) homologous proteins with an overall sequence identity of 40%, mediate repression by recruiting histone deacetylases, which compact nucleosomes into tight and inaccessible structures.

In its unliganded state, AR seems not to interact strongly with NCoR or SMRT; however, both corepressors associate intensely with the antagonist-bound AR LBD. Interestingly, also in its agonist-activated state, AR can be inhibited through direct NCoR and SMRT recruitment (van de Wijngaart et al., 2012). While the effects of both corepressors on the agonist-liganded AR are modest, their enhanced recruitment may contribute to the activity of some AR antagonists.

NCoR and SMRT contain multiple repression domains (**Figure 1-12**) that serve as docking platforms for the recruitment of additional components, including histone deacetylases, in the corepressor transcriptional complexes (Nagy et al., 1999).

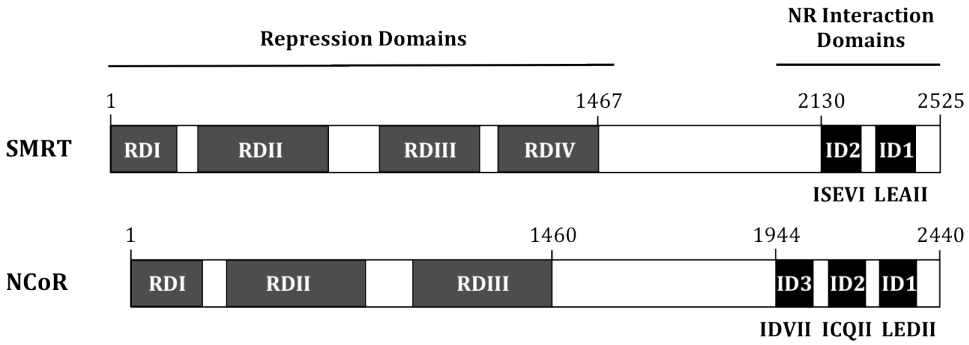


**Figure 1-12.** Schematic representation of the functional domains of SMRT and NCoR.
The transcriptional repression domains (RD) and NR interaction domains (ID) are indicated by grey and black boxes, respectively.

Short conserved autonomous motifs located in the C-terminus of both NCoR and SMRT, termed interaction domains, mediate binding to NRs. These corepressor nuclear receptor (CoRNR) boxes, which contain the internal signature motif (I/L)xx(I/V)I, are likely to adopt an amphipathic $\alpha$-helical conformation (Nagy et al., 1999; Perissi et al., 1999). Similarly to the NR box present in coactivator proteins, the CoRNR box docks to residues found in H3, H5, and H6 of the AF-2 hydrophobic groove, suggesting that there may be a competitive relationship between corepressor dissociation and coactivator recruitment, possibly by recognition of a common or overlapping binding site (Perissi et al., 1999). Under normal conditions, the ligand-dependent positioning of H12 relative to the LBD dictates whether coactivator or corepressor binding to AF-2 is favored. In the unliganded receptor, the extended conformation of H12 away from the LBD enables the recruitment of the bulky CoRNR boxes. By contrast, the agonist-induced repositioning of H12 close to the LBD results in the release of corepressors, switching the steric accessibility of the binding surface to prevent the CoRNR boxes from binding. Indeed, crystal structures of agonist bound-LBDs with a peptide from ID2 of SMRT confirmed that the liganded-corepressor peptide binds in the same groove as the coactivator, rendering coactivator and corepressor recruitment mutually exclusive (Heldring et al., 2007; Nagy et al., 1999).

## 1.5. DEREGULATED ROLE OF AR IN DISEASES

Medical geneticists have identified more spontaneous somatic mutations in human *AR* than in any other gene (http://androgendb.mcgill.ca/). While abnormalities resulting in an attenuation of the AR response to androgens produce male infertility and feminization, excessive stimulation of the AR results in other pathologies, being PCa the most common one. Disruptions of the molecular structure, protein-protein interactions or mechanisms of AR regulation can cause PCa, lead to clinical phenotypes that span from mild (MAIS) and partial (PAIS) to complete androgen insensitivity syndromes (CAIS), or even be responsible for the rare spinal and bulbar muscular atrophy (SBMA) neurodegenerative disease, also known as Kennedy's disease (Buchanan et al., 2001a; Katzenwadel and Wolf, 2015; D. Robinson et al., 2015) (**Figure 1-13**).

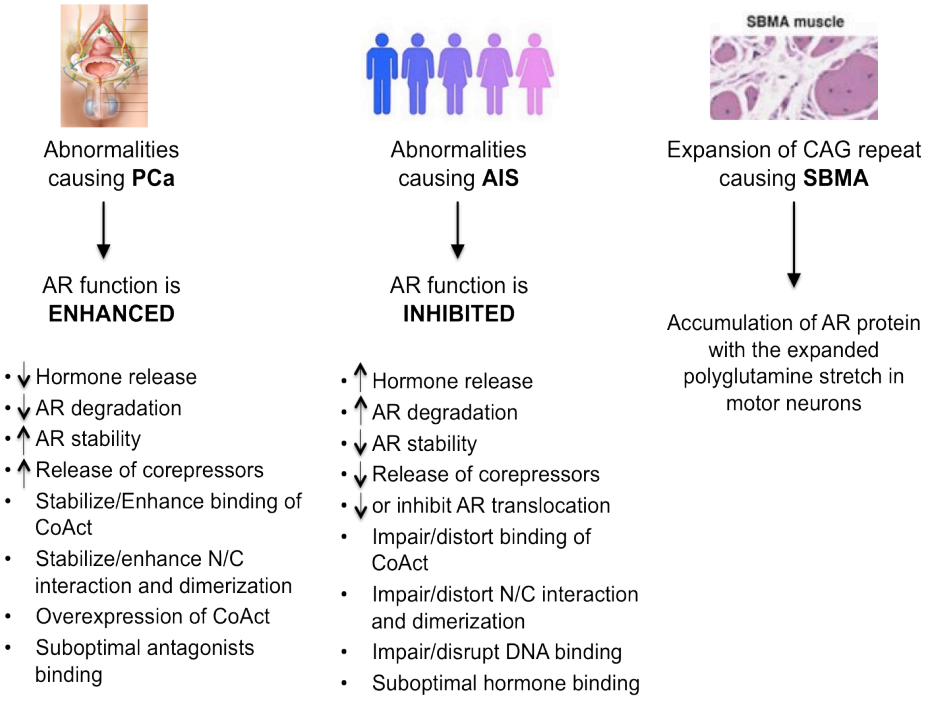


**Figure 1-13.** Deregulation of the AR axis can result in prostate cancer, androgen insensitivity syndromes and spinal and bulbar muscular atrophy.

PCa is generally associated with an enhanced function of the AR (Gain of function), whereas androgen insensitivity syndromes (AIS) are related to a reduced action of the AR (Loss of function). Spinal and bulbar muscular atrophy (SBMA) is caused by an extension of the polyglutamine stretch present at AR NTD. However, a reduction in the number of polyglutamine has been associated to PCa and the molecular mechanisms underlying this are not understood.

Additionally, although the expression of AR has mainly been related to the progression of PCa, other types of carcinomas, such as ovarian tumor and a subset of breast cancers termed molecular apocrines, have also been shown to express AR (Chadha et al., 1993; Hickey et al., 2015; J. L. L. Robinson et al., 2011).

### 1.5.1. Prostate Cancer

It is well known that AR plays a key role in promoting the development of PCa, currently the second most common cancer in men and the fifth leading cause of cancer deaths in males (Lorente et al., 2015). This is the reason why AR represents the most important therapeutic target for PCa treatment to date (Attard et al., 2015; Helsen et al., 2014; Y. N. S. Wong et al., 2014).

Androgen-deprivation therapy (ADT) to suppress AR transcriptional activity represents the standard treatment for metastatic PCa (Saad and Fizazi, 2015). These regimens are frequently used in combination with antiandrogens, AR antagonists that bind and block the AR LBD, in order to inhibit both the production of androgens and the action of AR. Unfortunately, after the initially effective response, it has been known for years that most tumors progress to castration resistant PCa (CRPC) after prolonged antiandrogen treatment, for which no curative therapy is still available (Attard et al., 2015; Katzenwadel and Wolf, 2015; Saad and Fizazi, 2015; Tian et al., 2015).

Despite many suggestions for a possible mechanism for the development of the androgen-responsiveness state of prostate tumors, the exact process underlying the transition to androgen independency is still not clear. However, since AR is required at all stages of the disease and remains the critical factor for the survival of the majority of the castration resistant tumor cells, it is widely accepted that AR is the crucial driver of PCa progression (D. Robinson et al., 2015).

### 1.5.1.1. Mechanisms conferring castration resistance

Mechanisms by which AR can be reactivated in CRPC include overexpression of AR, gain-of-function mutations, aberrant post-translational modifications, alternative splicing events, and cofactor deregulation (D. Robinson et al., 2015; Yuan et al., 2013) (**Figure 1-14**).

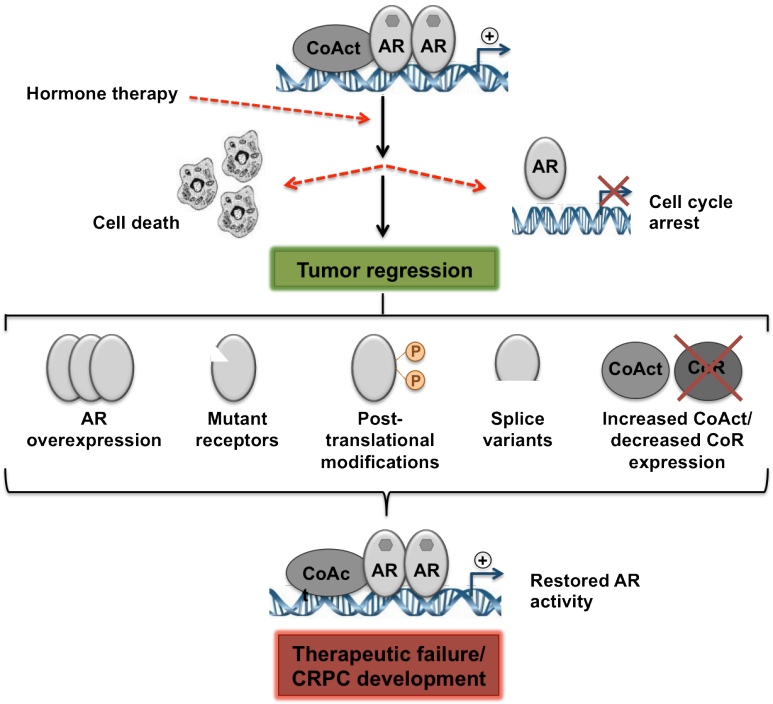


**Figure 1-14.** AR reactivation in castration resistant PCa development.
Successful targeting of AR activity results in tumor cell death or cell cycle arrest. However, adaptation events, including gene overexpression, genetic variations, post-translational modifications, alternative splicing, and cofactor deregulation, restore AR signaling, leading to CRPC, which currently has no cure. AR, Androgen Receptor; CoAct, coactivator; CoR, corepressor.

Increased AR expression as a result of either *AR* gene amplification or other mechanisms that increase *AR* gene transcription has been proposed as one of the mechanisms for progression of PCa after hormone therapy, resulting, in most cases, in AR overexpression and hypersensitivity to androgens (Linja and Visakorpi, 2004). In order to adapt to castrate levels of circulating androgens, PCa cells amplify the *AR* gene copy number, so that even under extremely low hormone levels, AR can still be activated.

Genetic variations in the *AR* affecting its activity have also been shown to influence PCa risk. The polyglutamine repeat length in the AR NTD has been inversely correlated with AR activity: the shorter the CAG repeat, the more aggressive the PCa is, as well as the earlier age of onset and likelihood of recurrence (Heinlein and Chang, 2004). Moreover, more than 1000 different mutations located predominantly in the DBD- and LBD-coding regions of AR, have been identified in PCa tissue (Tan et al., 2015). Most of the PCa mutations identified in the LBD of AR cluster in 3 different regions: codons 701-730, codons 874-910, and codons 670-678 (Buchanan et al., 2001a). Mutations in the 30-amino acid region spanning from position 701 to 730, which contains most of the conserved residues involved in ligand recognition and specificity, result in an increased sensitivity to low levels of circulating androgens and in receptor variants that exhibit an altered response to a wider range of steroid hormones and pharmaceutical antiandrogens (Mohler et al., 2012). A second cluster is located in the region flanking the AF-2 pocket (amino acids 874 to 910), receptor mutants that have also demonstrated to response to a broader spectrum of androgenic and non-androgenic ligands (Matias et al., 2000). Finally, PCa AR variants arising from mutations in the boundary of the hinge and LBD (residues 670-678) have demonstrated a greater transactivation activity in response to several ligands (Buchanan et al., 2001b). All reported AR mutations found in PCa are catalogued in the Androgen Receptor Gene Mutations Database World Wide Web Server at the Lady Davis Institute for Medical Research, available at http://www.mcgill.ca/androgendb/.

Studies from many groups have identified post-translational modifications (phosphorylation, acetylation, methylation, ubiquitylation and sumoylation) of AR that enhance AR activity in response to low levels of androgens, which may contribute to AR reactivation in CRPC (Koryakina et al., 2014; van der Steen et al., 2013).

Some CRPC tumors express AR splice variants that have lost their LBD, resulting in constitutively active receptors in the absence of ligand (Claessens et al., 2014).

Finally, as already mentioned in Section 1.5, alterations in the balance of AR-specific coregulators important for AR signaling, including increased expression levels and enhanced transcriptional activity of AR coactivators, as well as

decreased levels of corepressor proteins also result in an enhanced AR activity and, consequently, in tumor growth (Linja et al., 2004).

### 1.5.2. Androgen Insensitivity Syndromes

AIS is an X-linked recessive genetic disease defined as a condition resulting from complete (CAIS) or partial (PAIS) resistance to the biological actions of androgens in an XY male with normal testis and production of androgen levels (Mongan et al., 2015).

More than 1000 mutations in the *AR* gene have been reported in AIS patients (Gottlieb et al., 2012). The most severe mutations are generally associated with a CAIS phenotype, which result in a complete loss of AR function, manifested as phenotypic, though sterile, females (Wisniewski et al., 2000). PAIS is less frequent than CAIS and is characterized by varying degrees of masculinization of the external genitalia (ranging from almost complete feminization to almost normal masculinization) due to partial androgen responsiveness and missense mutations that attenuate AR activity. The mildest form of AIS (MAIS) is caused by an AR gene mutation found in a small number of infertile males who have no genital abnormalities (Zuccarello et al., 2008) (**Figure 1-15**).

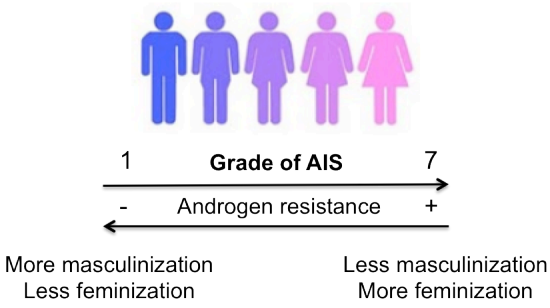


**Figure 1-15.** Grades of AIS.
There are seven grades of AIS, spanning from mild to complete AIS, including partial AIS.

Approximately 80% of *AR* gene mutations identified in its LBD leading to CAIS are localized in amino acid regions 688-710, 749-780, and 831-866. Since AR mutations leading to AIS decrease AR activity, whereas AR mutations in PCa are selected to enhance AR transactivation capacity, it is not surprising that PCa AR mutations do not overlap (except for residues 701-710) with mutations that cause AIS (Buchanan et al., 2001a).

### 1.5.3. Spinal and Bulbar Muscular Atrophy

Also known as Kennedy's disease, SBMA is an inherited neuromuscular degenerative condition caused by extension of the polyglutamine repeat length at the NTD of AR, ranging from 40 to 62 repeats, and resulting in an androgen-dependent gain of function in the mutant protein (Kumar et al., 2011; La Spada et al., 1991).

In SBMA, as in the other polyglutamine diseases, there is an inverse correlation between the number of glutamines and the age of onset, and a direct correlation with the severity of the illness, adjusted by the age of examination (Beilin et al., 2000). Furthermore, SBMA is unique among all polyglutamine diseases in that AR has a specific ligand that favors the nuclear translocation of the protein, and this ligand-dependent intracellular trafficking of the receptor appears to play an important role in the pathogenesis of SBMA (Katsuno et al., 2012). Despite several putative models have been proposed, it is yet not clear how the polyglutamine amplifications contribute to pathogenesis (Grunseich et al., 2013).

## 1.6. TARGETING THE ANDROGEN RECEPTOR IN THE TREATMENT OF PROSTATE CANCER

AR antagonists are compounds that interfere with the biological effects of androgens and are frequently used in the treatment of androgen-related pathologies. So far, all drugs approved by the U.S. Food and Drug Administration (FDA) target the LBP of the AR, competing with the natural hormones (Mohler et al., 2012). The prevalent model postulates that by dislodging the H12 from its position against the body of the LBD, AR antagonists prevent the formation of a productive AF-2 pocket, blocking the recruitment of the coactivator proteins required to activate transcription of target genes. This type of inhibition can, therefore, be considered as an allosteric modulation of AR activity, since an inhibitor bound to the LBP impedes the recruitment of proteins at a distant site.

Based on the distinct conformational changes induced in the AR LBD by androgens and antiandrogens, it can be postulated that the different transcriptional activities displayed by either full agonists, partial agonists or full antagonists, are the result

of the recruitment of different coregulators (Iain J McEwan and Raj Kumar, 2015). This differential recruitment can be considered as a special form of ligand-selective modulation of the AR LBD and could be applied in a broader sense to the tissue-selective modulation of androgen action, where the balance of coactivators and corepressors levels may ultimately determine the final activity of AR (Berrevoets et al., 2004).

### 1.6.1. Antiandrogens

PCa cells depend normally on testosterone and DHT to stimulate cell function, growth and division. Antiandrogens are oral compounds that compete with endogenous androgens for AR binding (Mohler et al., 2012). When antagonists interact with the LBP of the receptor, an inactive LBD is formed, disabling the binding of coactivators and/or enabling the recruitment of corepressors, and ultimately preventing the receptor from being activated. By inducing conformational changes in the receptor, these molecules block androgen-regulated genes transcription, resulting in cell apoptosis and inhibition of tumor growth (Golshayan and Antonarakis, 2013). According to their structure, antiandrogens can be classified as steroidal and non-steroidal agents. The formers were first developed in the late 1960s and can be distinguished by their physiologic progestational effects, whereas the non-steroidal drugs act only at the AR and are generally better tolerated by patients (Mohler et al., 2012).

The steroidal antiandrogen cyproterone acetate (CPA) (Cyprostat®, Androcur®) and the non-steroidal bicalutamide (Casodex®), flutamide (Eulexin®, Cytomid®), nilutamide (Nilandron®) and enzalutamide (Xtandi®) are the current commercialized antiandrogenic drugs used as first-line for PCa hormonal treatment (Mohler et al., 2012) (**Figure 1-16**).

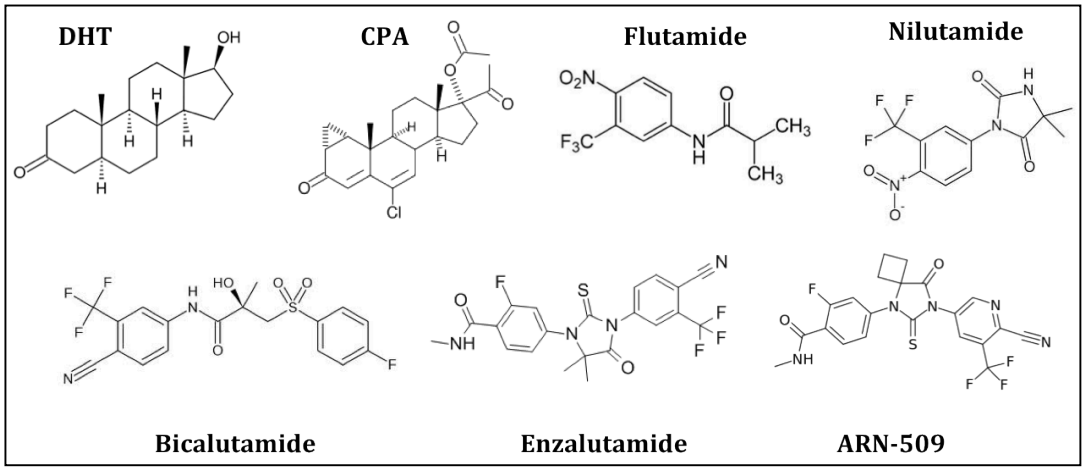


**Figure 1-16.** Chemical structure of the AR natural ligand dihydrotestosterone (DHT), the steroidal antiandrogen cyproterone acetate (CPA), the first-generation non-steroidal antiandrogens flutamide, nilutamide, and bicalutamide; and the second-generation enzalutamide and ARN-509.

#### 1.6.1.1. Steroidal antiandrogens

The first antiandrogen approved by the FDA back in 1989 was CPA, a synthetic derivative of hydroxyprogesterone that blocks the AR and inhibits the release of the luteinizing hormone, decreasing serum testosterone levels (Mohler et al., 2012). However, the severe drawbacks (e.g. hepatotoxicity, cardiovascular side effects, suppression of libido, and loss of erectile potency) related to the effects of CPA on other steroid receptors and its low efficacy have limited their clinical use.

#### 1.6.1.2. Non-steroidal antiandrogens

Non-steroidal antiandrogens (NSAAs), which avoid the constrains of the steroidal ones, were first used for advanced and metastatic PCa in 1989. Available drugs are the first-generation NSAAs flutamide, bicalutamide, and nilutamide; and the second-generation NSAA enzalutamide.

Bicalutamide and nilutamide originated from flutamide and thus share a similar chemical structure, avoiding the adverse effects related to the steroid scaffold. Other advantages over CPA are that they do not cause hepatotoxicity in long-term treatments neither compromise the sexual potency of the patient. The efficacy of bicalutamide in clinical trials has been reported to be equivalent to flutamide but

its stability and tolerability in terms of adverse effects are improved. Unlike bicalutamide, nilutamide does not seem to have any benefit over flutamide and manifests the least favorable safety profile (Haendler and Cleve, 2012).

In *in vitro* cell-based assays, bicalutamide has demonstrated to disrupt the N/C interaction and reduce the recruitment of AR coactivators, inhibiting gene expression and cell growth (Klotz and Schellhammer, 2005).

In the search of novel AR inhibitors with improved selectivity and potency, in August 2012, the FDA approved the commercialization of the second-generation NSAA termed enzalutamide, formerly known as MDV3100, for the treatment of metastatic CRPC (Ning et al., 2013). Enzalutamide displays approximately an eight-fold higher binding affinity for the LBD of AR compared to bicalutamide and, in contrast to other antiandrogens, blocks both the translocation of the receptor to the nucleus and DNA binding (Lorente et al., 2015). Moreover, enzalutamide is likely to be active in all patients with metastatic CRPC resistant to other antiandrogens (Vogelzang, 2012) and, most importantly, has already been proven to remain efficient in CRPC models overexpressing AR (Brasso et al., 2015).

Currently under clinical development, ARN-509, which differs from enzalutamide by only one single atom, is being developed as a novel antiandrogen for PCa treatment (Rathkopf et al., 2013). Similarly to enzalutamide, ARN-509 prevents AR nuclear translocation and DNA binding, is remarkably more potent than bicalutamide, and inhibits growth and androgen-mediated gene transcription in PCa cells overexpressing AR (Clegg et al., 2012). Importantly, ARN-509 has demonstrated an improved efficacy over enzalutamide in a CRPC xenograft model, suggesting that it may have a higher therapeutic index (Clegg et al., 2012).

#### 1.6.1.3. Resistance to antiandrogens

Unfortunately, as already mentioned in Section 1.5.1, despite an initial good response, resistance to AR antiandrogens emerges invariably over time, limiting the therapeutic efficacy for many PCa patients.

It has been widely accepted that the acquired mutations in AR represent an important cause for drug resistance in PCa. Antiandrogens lead to the selection of

mutations (i.e. W741C, W741L, T877A) that turns these drugs from AR antagonists to perfect activators of AR, resulting in an uncontrolled growth of the tumor, and highlighting the urgent need for next-generation antiandrogens able to overcome these compensatory mechanisms developed by cancer cells. An altered pharmacological activity was first reported with AR mutation T877A, frequently found in androgen-independent prostate tumors, which is able to recognize CPA and flutamide as an agonist (Bohl et al., 2007; Haendler and Cleve, 2012). Mutants W741L and W741C have also been demonstrated to be activated by bicalutamide (Haendler and Cleve, 2012) and, more recently, a novel mutant F826L has been shown to turn enzalutamide and ARN-509 into AR agonists (Joseph et al., 2013).

Synthesis of constitutively active truncated AR splice variants lacking the LBD, *AR* gene rearrangements and the activation of GR signaling have also been suggested to have an important role in enzalutamide resistance (Claessens et al., 2014; Tian et al., 2015).

#### 1.6.1.4. Next-generation antiandrogens

Despite the fact that big improvements have been made in the development of new antiandrogens that circumvent mutation-based resistance, it still remains a big challenge in the field. The identification of mutations that broaden ligand selectivity and confer resistance to AR antagonists has been suggested as a basis for the development of new drugs (Balbas et al., 2013). Therefore, similar structural molecular-modeling techniques could be used to simulate receptor-drug complexes and design compounds that retain activity in the presence of mutations. In addition, several structural-based design strategies have been used to develop novel AR inhibitors able to combat drug resistance (Tian et al., 2015). Up to date, 89 AR LBD structures are deposited in Protein Database Bank (PDB, www.pdb.org); however, there is still no crystal structure of the AR LBD in the antagonist-bound conformation available, which would provide a better basis for the structure-based design of antiandrogens.

Besides the AR LBP, there are other regulatory sites that can be therapeutically exploited. Several compounds have already demonstrated to effectively target the AR NTD in CRPC treatment (Tan et al., 2015). Targeting the intrinsically

unstructured NTD, although a big challenge, it is a promising approach to inhibit constitutively active AR splice variants that lack the LBD. Fewer strategies to target the DBD have been explored so far, probably because of the high sequence homology among the DBD of all NR members (Tan et al., 2015). Finally, alternative druggable surfaces on the LBD of AR, including the AF-2 and BF-3 pockets, have been suggested as putative target sites for the development of novel therapeutic strategies, which could help overcoming the gain-of-function mutations selected and the currently available antiandrogens (Tian et al., 2015). Indeed, some FDA-approved drugs have been shown to bind the BF-3 pocket (Estébanez-Perpiñá et al., 2007; Munuganti et al., 2013), and a compound with excellent antiandrogen potency has recently been demonstrated to target the BF-3 pocket (Munuganti et al., 2014), evidencing its pharmacological potential.

### 1.6.2. Selective androgen receptor modulators

One of the major challenges in the rational design of new AR-targeted drugs is to generate novel chemical compounds that regulate just one or some of the multiple activities carried out by AR in order to achieve the pharmacological desired effect. Recent developments in our understanding of AR structure and mode of action have contributed to the development of selective AR modulators (SARMs), tissue-selective AR ligands. The major goal of SARMs is to eliminate the undesirable side-effects of treatment by increasing the specificity for AR and improving the tissue selectivity of pharmacological activities (W. Gao and Dalton, 2007). Differential recruitment of coregulators due to distinct conformational changes imparted onto SARM-liganded ARs is thought to be a major mechanism contributing to their tissue specificity, ultimately leading to transcriptional control of distinct subsets of genes in a cell- and tissue-selective manner (Mohler et al., 2009).

Due to the drawbacks that steroidal antiandrogens have shown, great efforts to identify non-steroidal SARMs with an improved, differentiated profile are currently being made. The major challenge in the rational design of SARMs lies in their ability to clearly differentiate between anabolic and androgenic activities (Mohler et al., 2009). Several SARMs for different clinical indications are currently under preclinical and clinical development (Narayanan et al., 2008).

# II. OBJECTIVES

# 2. OBJECTIVES

The androgen receptor (AR) is a ligand-activated transcription factor that plays a crucial role in the correct development, differentiation, and function of male reproductive organs. Alterations in the AR protein or in the AR signaling pathway result in pathology such as prostate cancer (PCa), which is the fifth leading cause of cancer-related death in men in most western industrialized countries. Moreover, according to the American Cancer Society, about one man in six will be diagnosed with PCa during his lifetime.

The AR represents the major clinical target in the treatment of PCa and, to date, all the FDA-approved drugs against the AR target the ligand-binding pocket (LBP) of the receptor, competing with the natural hormones. Unfortunately, prolonged treatments with antiandrogens invariably fail, rendering them ineffective and resulting in the development of castration-resistant PCa (CRPC). Thus, there is pressing need for more potent and selective novel AR antagonists capable of blocking the action of the AR in tumors that are resistant to conventional antiandrogens. The binding function-3 (BF-3) pocket on the LBD of AR has been shown to be a hot spot for the recruitment of small molecules, highlighting the possibility that this surface could be considered as a novel potential therapeutic target site to modulate the action of the receptor in AR-related diseases.

Our initial hypothesis was that BF-3 may be a protein-protein interaction site, which may play a physiological role in AR action. Therefore, the major goals of this PhD project has been to further determine the biological role of the newly described BF-3 regulatory surface, as well as to identify and better characterize BF-3-interacting proteins that may modulate AR pathophysiological actions so as to use this pocket to design allosteric modulators with enhanced selectivity.

The main specific goals covered in this PhD project are the following:

(1) **Study of the conservation of the BF-3 pocket among NRs**. The conservation of the BF-3 pocket in terms of structural identity, as well as shape, size and depth of the groove, was analyzed in several NRs by superimposing their available crystal structures. Their structure-based sequence conservation was

also analyzed. We propose the BF-3 pocket as a druggable target to obtain NR selectivity.

(2) **Effect of BF-3 pocket single-point mutations on key AR functions**. Importantly, several residues involved in the formation of the BF-3 pocket have been found to be mutated in patients suffering from PCa and AIS (Gottlieb et al., 2012). Besides, it was demonstrated that small compounds binding to the BF-3 site induced conformational changes in BF-3, which were transmitted to the AF-2 groove, affecting AR activity. Thus, several residues lining the AF-2 and BF-3 pockets were mutated in order to determine the allosteric responses they induce in the receptor function to further elucidate the molecular mechanisms by which point mutations are linked to disease. Specifically, the effect that BF-3 mutations exert on AR activity and interaction with AR coregulators and its N-terminal domain, as well as the impact on AR nuclear translocation have been evaluated.

(3) **Identification of BF-3-Interacting Proteins.** One of the main goals of this thesis work has been to identify the role of the BF-3 pocket on the macromolecular associations of AR with known or newly characterized coregulatory proteins. Discovering which proteins interact with and regulate AR by binding to the BF-3 opens a new line of research in the treatment of PCa, as such proteins themselves could represent new targets to modulate AR function under pathological conditions.

(4) **Effect of antiandrogens on the binding of WT AR LBD and BF-3 mutants to AR coregulators**. It is well known that some AR mutations escape from the antagonistic activity of antiandrogens, which act as AR agonists instead of blocking AR activity. With the objective of assessing if BF-3 mutants potentiate or repress coregulators recruitment, a high-throughput yeast two-hybrid assay was set up in the laboratory, which facilitated the evaluation of BF-3 mutants interaction with different AR coregulators in the presence of several antiandrogens. The set-up developed in the host laboratory during this thesis allowed as a surrogate method to evaluate the role of AR H12 in macromolecular associations.

# III. MATERIALS AND METHODS

# 3. MATERIALS AND METHODS

## 3.1. MATERIALS

All restriction and modifying enzymes were purchased from New England BioLabs. Oligonucleotides were synthesized by Sigma-Aldrich. The expand high fidelity$^{PLUS}$ PCR system (Roche) was used for all PCR reactions, which were performed in a GeneAmp PCR System 2400 (Applied Biosystems).

For cloning and growing plasmids, competent *E. coli* DH5α cells (Invitrogen) were transformed following standard heat shock protocols. Sequences of all constructs were verified by Macrogen Inc. Desired plasmid DNA was purified with NucleoSplin Gel and PCR clean-up, NucleoSpin Plasmid and NucleoBond Xtra Midi kits (Macherey-Nagel).

Dymethyl sulfoxide (DMSO), dihydrotestosterone (DHT), hydroxyflutamide, bicalutamide and mifepristone were obtained from Sigma-Aldrich. The synthetic androgen mibolerone was purchased from PerkinElmer Life Sciences. Methyltrienolone (R1881) and enzalutamide (MDV3100) were a kind gift from Bayer and Medivation, respectively.

The 3-amino-1,2,4-triazole (3AT) used in the yeast two-hybrid screens was purchased from Sigma.

## 3.2. PLASMID CONSTRUCTS AND PRIMERS

### 3.2.1. Mammalian expressions constructs

pM-AR LBD (646-919) WT and BF-3 mutants I672R, R726L and N833R; pCMV AR full-length WT and BF-3 mutants I672R, G724R, F826A, F826R and R840E; and 5xGAL4-Luciferase (LUC) reporter plasmid (containing five GAL4 response elements upstream of a minimal promoter) were previously described (Estébanez-Perpiñá et al., 2007; 2005). All other mutations (N727K, V757A, F826L, F826R, L830A, L830R, R840A, R840C, R840E and R840H) were made using the

QuickChange Site-Directed Mutagenesis Kit (Stratagene) and the following pair of primers:

| | |
|---|---|
| N727K-Forward: | 5'-CTTGCCTGGCTTCCGCAAATTACACGTGGACGACC-3' |
| N727K-Reverse: | 5'-GGTCGTCCACGTGTAATTTGCGGAAGCCAGGCAAG-3' |
| V757A-Forward: | 5'-CGATCCTTCACCAATGCCAACTCCAGGATGCTC-3' |
| V757A-Reverse: | 5'-GAGCATCCTGGAGTTGGCATTGGTGAAGGATCG-3' |
| F826L-Forward: | 5'-CTGAAAAATCAAAAATTATTTGATGAACTTCG-3' |
| F826L -Reverse: | 5'-CGAAGTTCATCAAATAATTTTTGATTTTTCAG-3' |
| F826R-Forward: | 5'-CTGAAAAATCAAAAACGTTTTGATGAACTTCG-3' |
| F826R -Reverse: | 5'-CGAAGTTCATCAAAACGTTTTTGATTTTTCAG-3' |
| L830A-Forward: | 5'-CAAAAATTCTTTGATGAAGCTCGAATGAACTACATC-3' |
| L830A -Reverse: | 5'-GATGTAGTTCATTCGAGCTTCATCAAAGAATTTTTG-3' |
| L830R-Forward: | 5'-CAAAAATTCTTTGATGAACGTCGAATGAACTACATC-3' |
| L830R -Reverse: | 5'-GATGTAGTTCATTCGACGTTCATCAAAGAATTTTTG-3' |
| R840A-Forward: | 5'-CAAGGAACTCGATGCTATCATTGCATGC-3' |
| R840A -Reverse: | 5'-GCATGCAATGATAGCATCGAGTTCCTTG-3' |
| R840C-Forward: | 5'-TACATCAAGGAACTCGATTGTATCATTGCATGCAAAAGA-3' |
| R840C -Reverse: | 5'-TCTTTTGCATGCAATGATACAATCGAGTTCCTTGATGTA-3' |
| R840E-Forward: | 5'-CAAGGAACTCGATGAAATCATTGCATGC-3' |
| R840E -Reverse: | 5'-GCATGCAATGATTTCATCGAGTTCCTTG-3' |
| R840H-Forward: | 5'-TACATCAAGGAACTCGATCATATCATTGCATGCAAAAGA-3' |
| R840H -Reverse: | 5'-TCTTTTGCATGCAATGATATGATCGAGTTCCTTGATGTA-3' |

The Renilla Luciferase Control Vector (pRL) and the pSG5-GRIP1, VP16-AR NTD (1-504), VP16-NCoR (1925-End), and VP16-SMRT (2025-End) plasmids were kind gifts from Paul Webb (Houston, USA). pcDNA3-Uba3 was obtained from Kenneth P Nephew (Indiana, USA), whereas pcDNA3-HAN-Rab11FIP3 was received from Kazuhisa Nakayama (Kyoto, Japan). The TAT-GRE-EIB-LUC reporter plasmid and the PDM-LACZ-β-GAL control vector were available at Charlote Bevan's lab (London, UK).

The sequence of all constructs was checked using the corresponding primer pairs:

AR-Reverse: 5'-CTGGGTGTGGAAATAGATGGG-3'
pCMV-Forward: T3 promoter (5'-ATTAACCCTCACTAAAG-3')
pCMV-Reverse: T7 promoter (5'-TAATACGACTCACTATAGGG-3')
pM-Forward: 5'-TCATCGGAAGAGAGTAGT-3'
pM-Reverse: 5'-GTATGGCTGATTATGATC-3'
VP16-Forward: 5'-GCCGACTTCGAGTTTGAG-3'
VP16-Reverse: 5'-GTATGGCTGATTATGATC-3'
pcDNA3-Forward: T7 promoter
pcDNA3-Reverse: SP6 (5'-ATTTAGGTGACACTATAG-3')
pSG5-Forward: T7 promoter

### 3.2.2. Yeast two-hybrid constructs

The bait is the protein for which interacting proteins in a cDNA library is trying to be found, whereas the prey is a protein or protein fragment isolated from a cDNA library as a bait potential interactor. The former is always fused with the DNA binding domain of the GAL4 transcription factor, while the latter is a fusion with its activation domain. The Gateway system (Invitrogen) was used to clone the baits (AR LBD (646-919) WT and BF-3 mutants) into the pDEST32 vector and the preys (the NR coregulators SRC1, SRC3, β-catenin, caveolin-1, FUS, FKBP52 (1-262), and SMRT (2025-2525)) into the pDEST22 vector. cDNAs encoding baits and preys were generated by a first standard PCR using a specific pair of oligonucleotides for each protein:

AR-FWD: 5'-AACCTGTACTTCCAGTCCTCCAGCACCACCAGCCCCACTGAG-3'
AR-REV: 5'-GTACAAGAAAGCTGGGTCTCACTGGGTGTGGAAATAGATGGG-3'
SRC1-FWD: 5'-AACCTGTACTTCCAGTCCATGAGTGGCCTCGGGGACAGTTCA-3'
SRC1-REV: 5'-GTACAAGAAAGCTGGGTCTCATTCAGTCAGTAGCTGCTGAAGGAG-3'
SRC3-FWD: 5'-AACCTGTACTTCCAGTCCATGAGTGGATTAGGAGAAAACTTG-3'
SRC3-REV: 5'-GTACAAGAAAGCTGGGTCTCAGCAGTATTTCTGATCAGGACC-3'
β-cat-FWD: 5'-AACCTGTACTTCCAGTCCATGGCTACTCAAGCTGATTTGATG-3'
β-cat-REV: 5'-GTACAAGAAAGCTGGGTCTCACAGGTCAGTATCAAACCAGGC-3'
Cav1-FWD: 5'-AACCTGTACTTCCAGTCCATGTCTGGGGGCAAATACGTAGAC-3'

Cav1-REV: 5'-GTACAAGAAAGCTGGGTCTCATATTTCTTTCTGCAAGTTGATGCG-3'
FUS-FWD: 5'-TTGTACAAAAAAGCAGGCTTCGCCTCAAACGATTATACCC-3'
FUS-REV: 5'-GTACAAGAAAGCTGGGTCTTAATACGGCCTCTCCCTG-3'
FKBP52-FWD: 5'-TTGTACAAAAAAGCAGGCTTCATGACAGCCGAGGAGATG-3'
FKBP52-REV: 5'-GTACAAGAAAGCTGGGTGTTAATTCATCTCCCAAGACTCC-3'
SMRT-FWD: 5'-TTGTACAAAAAAGCAGGCTTCCCGGACCCGCCGG-3'
SMRT-REV: 5'-GTACAAGAAAGCTGGGTGTTACTCGCTGTCGGAGAGTGTCTCGTA-3'

The above primers added the attB1 and attB2 sites (needed to recombine with the entry pDONR221/ZEO vector) at 5' and 3' end, respectively. The PCR products containing the attB sites were then amplified by a second PCR round with the oligonucleotides attB-FWD 5'-GGGGACAAGTTTGTACAAAAAAGCA-GGCT-3' and attB-REV 5'-GGGGACCACTTTGTACAAGAAAGCTGGGT-3', followed by a BP recombination reaction with pDONR221/ZEO vector (Invitrogen). Finally, an LR reaction (Invitrogen) was performed in order to transfer the AR cDNAs into the pDEST32 vector and the AR coregulator cDNAs into the pDEST22 vector, generating the bait and prey plasmids, respectively. The sequence of all obtained constructs was verified using the corresponding pair of primers:

pDONR-FWD M13F (5'-GTAAAACGACGGCCAGT-3').
pDONR-REV M13R-pUC (5'-CAGGAAAC-AGCTATGAC-3').
pDEST22-FWD 5'-TATAACGCGTTTGGAATCACT-3'.
pDEST22-REV 5'-AGCCGACAACCTTGA-TTGGAGAC-3'
pDEST32-FWD 5'-AACCGAAGTGCG-CCAAGTGTCTG-3'.
pDEST32-REV 5'-AGC-CGACAACCTTGATTGGAGAC-3'.

pDONR201 ARA70 was purchased from Plasmid (http://plasmid.med.harvard.edu/PLASMID/). It was recloned into the pDEST22 vector performing the Gateway LR reaction.

Uba3, ARMC9, Rab11FIP3 and MAPK8IP1 human clones isolated from the large-scale Y2H screens, which came in the pEXP-AD502 plasmid, were identified by sequencing them with the oligonucleotide pair pEXP-AD502-FWD 5'-TATAACG-CGTTTGGAATCACT-3' and pEXP-AD502-REV 5'-TAAATTTCTGGCAAGGTAGAC-3'. They were transferred into the pDEST22 vector by means of the LR reaction,

generating the prey plasmid needed to validate the interaction by the forward one-to-one Y2H.

Mutants of Uba3 NR boxes, $^{63}$LQFAA$^{67}$, $^{179}$LISAA$^{183}$, and the double mutant $^{63}$LQFAA$^{67}$/$^{179}$LISAA$^{183}$, were generated using the pair of primers Uba3 $^{63}$LQFAA$^{67}$-FWD 5'CCGAGCACTGAATCTCTCCAGTTCGCGGCAGATACATGTAAAGTTC-3' and UBA3 $^{63}$LQFAA$^{67}$-REV 5'-GAACTTTACATGTATCTGCCGCGAACTGGAGAGATTCAGT-GCTCGG-3', and Uba3 $^{179}$LISAA$^{183}$-FWD 5'-GGCATGCTGATATCTGCTGCAAATTATG-AAGATGGTGTCTTAGATC-3' and Uba3 $^{179}$LISAA$^{183}$-REV 5'-GATCTAAGACACCATC-TTCATAATTTGCAGCAGATATCAGCATGCC-3'.

## 3.3. METHODS

### 3.3.1. Cell culture

Human cervix adenocarcinoma epithelial (HeLa) cells were a kind gift from Dr. Jens Lüders, whereas COS-1 (CV-1 in Origin carrying the SV40 genetic material) cells were available at Dr. Charlotte Bevan's lab. Both cell lines were maintained at 37ºC and 5% $CO_2$ in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) containing 4.5 g/liter D-glucose, 0.58 g/liter L-glutamine, 0.11 g/liter sodium pyruvate, and supplemented with GlutaMAX (Gibco), 10% fetal bovine serum (FBS; Gibco) or 10% fetal calf serum (FCS; First Link (UK)), 100 u/ml penicillin and 100 μg/ml streptomycin.

### 3.3.2. Transient transfection assays

#### 3.3.2.1. AR LBD transient transfection assays

For AR LBD transfection assays, HeLa cells were collected in fresh medium containing 10% charcoal-stripped FBS and seeded into 24-well culture plates at a density of 1,5x10$^5$ cells per well. The following day, cells were transfected using FuGENE HD transfection reagent (Promega) as described by the manufacturer. The DNA mixture was composed of 300 ng/well of LUC reporter construct; 2.5 ng/well of pRL; 100 ng/well of pM-AR LBD (646-919) WT, pM-mutant AR LBD or empty control vector; and 100 ng/well of pSG5-GRIP1, VP16-AR NTD, VP16-NCoR, VP16-

SMRT, pcDNA3-Uba3, pcDNA3-HAN-Rab11FIP3, or empty control plasmid. Six hours after transfection, the cells were incubated with vehicle (1%, v/v, DMSO), or hormone (0,1, 1, 10 or 100 nM, as indicated) (DHT, dissolved in DMSO) for 16 hours. Finally, cells were washed twice with cold phosphate-buffered saline and lysed in 100μl passive lysis buffer (Promega). LUC and pRL activities were measured on 25 μl of the extracts in a GloMax 96 Microplate Luminometer (Promega) using the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's instructions. pRL counts were used to assess transfection efficiency and normalize LUC readings. The values shown are the averages of at least three independent experiments performed in triplicate. Error bars indicate Standard Error of the Mean (SEM) values.

#### 3.3.2.2. AR full-length transient transfection assays

For transfection with full-length AR, COS-1 cells were plated out in 24-well dishes in starvation medium (phenol red free-DMEM (Gibco) supplemented with 5% FCS). Twenty-four hours later, cells were transfected using calcium phosphate with 50 ng pCMV AR WT or BF-3 mutant, 100 ng PDM-LACZ-β-GAL and 1 μg TAT-GRE-E1B-LUC. The following day, cells were washed twice with starvation medium and then incubated with increasing concentrations of hormone (0-0,01-0,1-1-10-100 nM mibolerone, dissolved in ethanol) before being lysed in passive lysis buffer. Luciferase and β-galactosidase activities were measured using the LucLite Plus (PerkinElmer Life Sciences) and Galacto-Light Plus System (Applied Biosystems) kits, respectively, as recommended by the manufacturers. β-galactosidase activity was used to assess transfection efficiency and normalize luciferase readings. The values shown are the averages of at least three independent experiments performed in triplicate. Error bars indicate SEM values.

## 3.3.3. Immunoblotting

### 3.3.3.1. AR LBD immunoblotting

For AR LBD western blot analysis, HeLa cells were transfected with 1 μg AR expression constructs (as described in section 2.3.2.1). Twenty-four hours after hormone treatment, cells were washed twice with cold PBS, lysed with cold RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate and 50 mM Tris pH 7.5) supplemented with complete protease inhibitors (Roche Diagnostics) and centrifuged for 10 min at 14,000 rpm. Equal protein concentrations were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on NuPAGE 4-12% Bis-Tris gels (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membranes. Western immunoblotting was performed using a polyclonal antibody against the C-terminus of AR (C-19, Santa Cruz Biotechnology), followed by incubation with a horseradish peroxidase-conjugated mouse anti-rabbit antibody (Abcam). AR proteins were visualized by a reaction with Luminata Forte Western HRP substrate (Millipore).

### 3.3.3.2. AR FL immunoblotting

For AR FL immunoblotting, COS-1 cells were transfected with 50 ng pCMV-AR WT or BF-3 mutant using calcium phosphate (as described in section 2.3.2.2), incubated with 10 nM mibolerone for 16 hours and lyse with reporter lysis buffer. 20 μg total protein was loaded onto a 12% SDS-PAGE and transferred to a PVDF membrane using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). AR proteins were detected using mouse anti-AR-441 (1:1000, Dako) primary antibody, followed by incubation with goat anti-mouse HRP secondary antibody (1:2000, Dako). Mouse anti-β-actin primary antibody (1:5000, Abcam) was used as loading control.

## 3.3.4. Immunofluorescence

COS-1 cells were grown in DMEM medium on sterile glass coverslips in 24-well dishes to 50% confluence for 24 hours. On the next day, cells were transfected with

1 μg pCMV AR WT or BF-3 mutant using FuGENE HD and incubated in starvation medium for a subsequent 24 hours in the presence (10 nM) or absence of mibolerone. After a washing step with ice-cold PBS, cells were fixed in 1% formaldehyde in PBS for 10 min. They were then permeabilized in 0,1% Trition X-100 in PBS for 10 min and blocked with 10% goat serum in PBS for 30 min. Cells were then incubated for 60 min with mouse anti-AR-441 primary antibody (1:200 dilution in 10% goat serum-PBS) and washed with blocking buffer. After 15 minutes in 10% goat serum-PBS, cells were incubated with Alexa Fluor 488 goat anti-mouse secondary antibody (Invitrogen) for 1 h and washed extensively in PBS. Finally, coverslips were mounted on slides with VECTASHIELD containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories), sealed, air-dried and analyzed on a Zeiss Meta 512 confocal microscope.

### 3.3.5. Yeast transformation

Yeast strain MaV203 (Invitrogen) transformations were performed using the LiAcO/salmon sperm carrier DNA/PEG method as described in ProQuest two-hybrid system user manual (Invitrogen). MaV203 cells were first made competent and then transformed with bait and prey plasmids together with an excess of carrier DNA in a LiAcO and PEG solution. After an incubation of 30 minutes at 30 ºC, DMSO was added and cells were heat shocked at 42 ºC (for 50 minutes in the large-scale transformations and for 7 minutes in the one-to-one and high-throughput screens). Transformed MaV203 cells were then plated on SD-L-T (lacking leucine and tryptophan) control plates and grown at 30 ºC for 3 days to select transformants.

### 3.3.6. Large-scale yeast two-hybrid screens

Large-scale Y2H screens were performed using an adult human brain cDNA prey library (Invitrogen) against DHT-bound AR LBD (646-919) WT as described in the ProQuest two-hybrid system user manual (Invitrogen). AR LBD bait was transformed into MaV203 yeast strain in a first small-scale transformation step and grown in plates lacking leucine (SD-L) to select bait-transformed yeast cells. After a 3 day-incubation at 30ºC, a replica clean of the transformants was made

and plates were incubated for 3 additional days. Bait-containing yeast cells were then transformed against an adult human brain cDNA prey library and plated into selective plates: SD1 (SD lacking leucine, tryptophan and histidine and supplemented with 20 mM 3AT), SD2 (SD lacking leucine, tryptophan and histidine and supplemented with 50 mM 3AT) and SD4 (SD lacking leucine, tryptophan and uracil). After a 5 day-incubation at 30ºC, positive growing colonies were picked up and cultured in prey selective liquid medium (SD-T, lacking tryptophan). Prey plasmid DNAs were then extracted from cultures and shuttled in *E. coli* DH5α strain to enable DNA extraction and sequencing. Finally, gene identification by BLASTp (NCBI) was performed.

### 3.3.7. Yeast two-hybrid validation using forward one-to-one Y2H

1 μg of bait (pDEST32-AR LBD WT) and 1 μg of prey (pDEST22-Uba3, pDEST22-Rab11FIP3, pDEST22-ARMC9, or pDEST22-MAPK8IP1) plasmids were pair-wise cotransformed into MaV203 cells (as described in section 3.3.5) in a 96-well array format. Cotransformed cells were plated onto SD-L-T control plates, incubated for 3 days at 30ºC, replicated onto SD2 (SD lacking leucine, tryptophan and histidine, supplemented with 50 mM 3AT), SD3 (SD lacking leucine, tryptophan and histidine, supplemented with 75 mM 3AT) and SD4 (SD lacking leucine, tryptophan and uracil) selective plates containing 100 nM DHT, and incubated at 30ºC for 3 additional days to detect *HIS3* or *URA3* reporters induction.

### 3.3.8. High-throughput yeast two-hybrid screens of AR LBD BF-3 mutants

1 μg of bait (pDEST32 AR LBD WT or corresponding BF-3 mutant) and prey (pDEST22-ARA70, pDEST22-SRC3, pDEST22-SMRT, pDEST22-SRC1, pDEST22-β-catenin, pDEST22-caveolin-1, pDEST22-FUS, pDEST22-FKBP52, pDEST22-ARMC9, pDEST22-MAPK8IP3, pDEST22-Rab11FIP3, pDEST22-Uba3, pDEST22-$^{63}$LQFLL$^{67}$ or pDEST22-$^{179}$LISLL$^{183}$) plasmids were cotransformed into MaV203 cells (as described in section 3.3.5) in a 96-well plate. In order to analyze specific interactions in the presence of DHT or different antiandrogens, selected colonies

were resuspended in TE buffer and incubated for 3 days on SD2, SD3 and SD4 selective plates containing either vehicle (DMSO), 100 nM DHT or 10 μM of the corresponding antiandrogen (hydroxyflutamide, bicalutamide, mifepristone or enzalutamide).

# IV. RESULTS & DISCUSSION

# 4. RESULTS AND DISCUSSION

## 4.1. BINDING FUNCTION-3: A CONSERVED SURFACE ON THE LIGAND-BINDING DOMAIN OF NUCLEAR RECEPTORS FOR ALLOSTERIC CONTROL

### 4.1.1. Introduction

NRs form a large superfamily of transcription factors that participate in virtually every key biological process. Their enormous functional plasticity as transcription factors relates in part to NR-mediated interactions with hundreds of coregulatory proteins upon ligand binding to their LBD. Some coregulator association relates to the distinct residues that shape the AF-2 coactivator-binding pocket, a surface groove that primarily determines the preference and specificity of protein-protein interactions. However, the highly conserved AF-2 pocket in the NR superfamily appears to be insufficient to account for NR subtype specificity. Additional protein-protein interaction surfaces, most notably on their LBD, may contribute in the modulation of NR function. In the case of the AR LBD surface, structural and functional data highlighted the presence of another site named BF-3, which lies at a distinct but topographically adjacent surface to AF-2. Importantly, AR BF-3 is a hot spot for mutations involved in PCa and AIS, and some FDA-approved drugs bind at this site. Moreover, structural studies suggested an allosteric relationship between AF-2 and BF-3, as occupancy of the latter affected coactivator recruitment to the former. The fact that AR BF-3 pocket is a druggable site evidences its pharmacological potential. Compounds that may affect allosterically NR function by binding to BF-3 open promising avenues to develop type-specific NR modulators. This section will focus on the evidence found in several NRs suggesting a physiological role for the BF-3 surface pocket.

### 4.1.2. The BF-3 Pocket is Highly Conserved Among Steroid Receptors

To determine whether the BF-3 pocket is also present in other NRs, the atomic coordinates of several NR LBDs solved by X-ray crystallography were superimposed. In order to visualize the BF-3 concavity in ERα (PDB 1ERE), its hinge and H1 residues were manually removed, as the hinge is folded against the body of the LBD, docking in its corresponding BF-3 pocket as a lid and impeding its correct visualization.

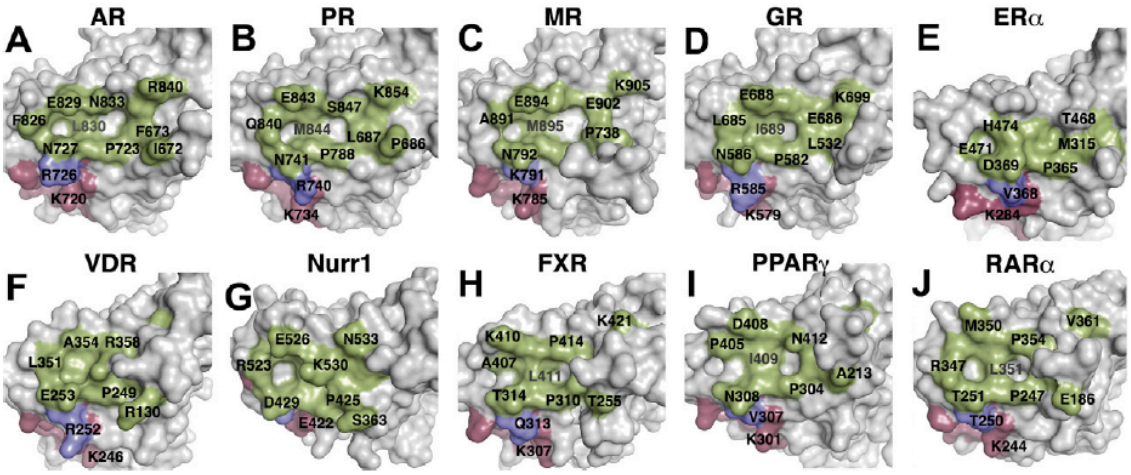


**Figure 4-1.** Comparison of NR BF-3 pockets by structural superimposition.

Close-ups of the BF-3 pockets from different NRs. The NR surfaces are shown in grey; the BF-3 lining residues, in green; the AF-2 pocket is visualized in raspberry, and residue R726, in blue. AF-2 and BF-3 key residues are labeled in black. The residue located at the bottom of the BF-3 pocket is indicated in dark grey **(A)** AR (PDB 1T5Z). **(B)** PR (PDB 1A28). **(C)** MR (PDB 2AA7). **(D)** GR (PDB 1P93). **(E)** ERα (PDB 1ERE). The first residues belonging to the hinge domain have been removed to expose the BF-3 pockets. **(F)** VDR (PDB 3A78). **(G)** Nurr1 (PDB 1OVL). **(H)** FXR (PDB 1OSH). **(I)** PPARγ (PDB 1PRG). **(J)** RARα (PDB 3KMR).

Comparing the BF-3 pockets from the structural superimpositions, it was revealed that the BF-3 site in AR is highly conserved among the steroid receptors PR (PDB 1A28), MR (PDB 2AA7), and GR (PDB 1P93), being the BF-3 pocket of PR and MR the most similar to that of AR (**Figure 4-1**). Contrarily, the level of conservation of AR BF-3 in the ER isoforms is more discrete.

**Table 1** lists the residues of various examined NRs that are located in the topologically equivalent position in their solved three-dimensional structures to AR BF-3 residues (template AR PDB code 1T5Z), whereas **Table 2** resumes the sequence identity among their LBDs, and their corresponding AF-2 and BF-3 pockets obtained by primary sequence alignment in comparison to AR. The alignments of AF-2 and BF-3 were manually corrected to take into account the structural information given in **Table 1**.

| NR | AR | PR | MR | GR | ERα | VDR | Nurr1 | FXR | PPARγ | TRα | TRβ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PDB code | 1T5Z | 1A28 | 2AA7 | 1P93 | 1ERE | 3A78 | 1OVL | 1OSH | 1PRG | 2HT9 | 1BSH |
| BF-3 residue | I672 | P686 | S737 | T531 | M315 | R130 | S363 | T255 | A213 | D166 | E220 |
| | F673 | L687 | P738 | L532 | V316 | I131 | L364 | L256 | L214 | L167 | L221 |
| | P723 | P737 | P788 | P582 | P365 | P249 | P425 | P310 | P304 | P237 | P291 |
| | G724 | G738 | G789 | G583 | G366 | G250 | G426 | G311 | G305 | M238 | M292 |
| | R726 | R740 | K791 | R585 | V368 | R252 | A428 | Q313 | V307 | S240 | F293 |
| | N727 | N741 | N792 | N586 | D369 | D253 | D429 | T314 | N308 | E241 | C294 |
| | F826 | Q840 | A891 | L685 | E471 | L351 | R523 | A407 | P405 | K337 | R391 |
| | E829 | E843 | E894 | E688 | H474 | A354 | E526 | K410 | D408 | K340 | K394 |
| | L830 | M844 | M895 | I689 | I475 | I355 | L527 | L411 | I409 | S341 | Y395 |
| | N833 | S847 | N898 | T692 | V478 | R358 | K529 | P414 | N412 | A344 | S398 |
| | E837 | E851 | E902 | E696 | I482 | T362 | N533 | V418 | A416 | A348 | A402 |
| | R840 | K854 | K905 | K699 | T485 | T365 | C354 | K421 | L419 | H351 | H405 |

**Table 1.** Residues of various NR LBD crystal structures located in the topologically equivalent position to the residues that form the BF-3 pocket in the AR.

The atomic coordinates deposited (PDB codes) are indicated. Identical residues found in most of the analyzed NRs are shaded in light grey. Identical or conserved residues featured in at least two of all the analyzed NRs for a given residue in topologically related positions are shaded in dark grey.

The residues that show the highest degree of conservation among the SRs corresponding BF-3 pockets (taking into consideration the sequence and structural identity, as well as the shape, size and depth of the cavity) are the tandem of arginine and asparagine found at the boundary between AF-2 and BF-3 pockets of AR (R726 and N727), which is conserved in all steroid receptors but ER (**Table 1**). These residues have been identified as the AF-2/BF-3 linker and have been shown to engage in contacts with surface inhibitors, playing an important role in transmitting information to AF-2 by undergoing structural changes

(Estébanez-Perpiñá et al., 2007).

| | AR pairwise identity (%) | | |
|---|---|---|---|
| | **LBD** | **AF-2** | **BF3** |
| **PR** | 54,8 | 52,6 | 46,2 |
| **MR** | 51,6 | 42,1 | 46,2 |
| **GR** | 49,6 | 57,9 | 46,2 |
| **RXRα** | 21,4 | 40,0 | 21,4 |
| **ERα** | 20,2 | 42,1 | 14,3 |
| **ERβ** | 18,6 | 36,8 | 23,1 |
| **Nurr1** | 16,3 | 15,8 | 30,8 |
| **RARα** | 15,1 | 31,6 | 21,4 |
| **TRβ** | 14,7 | 42,1 | 6,3 |
| **VDR** | 14,6 | 26,3 | 23,1 |
| **PPARγ** | 14,5 | 36,8 | 31,3 |
| **TRα** | 14,3 | 42,1 | 6,3 |
| **FXR** | 13,9 | 38,9 | 20,0 |

**Table 2.** Sequence identity among some NR LBD domains.

The LBD column indicates the primary sequence alignment (done with Genious alignment package) without including structural information. The AF-2 and BF-3 columns show the sequence identity of their corresponding AF-2 and BF-3 lining residues in comparison to AR. The alignments of AF-2 and BF-3 have been manually corrected to include the structural information given in Table 1. The total number of AF-2 lining residues included is 19, whereas the included BF-3 residues are 16.

The crystal structure of the GR LBD identified a second charge-clamp in the AF-2 pocket that was also implicated in making contacts with coactivator peptides (Bledsoe et al., 2004). Interestingly, one of the members of this second charge clamp is R585, which corresponds to the AR BF-3 R726, residue that has been demonstrated to adopt the C-terminal capping role in AR, stabilizing the bound coactivator peptide (Estébanez-Perpiñá et al., 2005; X. E. Zhou et al., 2010).

The homologous residues to AR P723 and G724 are almost invariably structurally conserved among all the NRs studied, fact that was already pointed out before (Wurtz et al., 1996). In the case of the TR isoforms, the residue topologically equivalent to G724 is a methionine. Thornton et al. studied the evolution and structure-function implications of the AR and identified a group of residues that was conserved in the AR clade, for which they hypothesized to be involved in AR-specific aspects of receptor function (Thornton and Kelley, 1998). Three of these residues resulted to be R840, Q670 and I672, which belong to the AR BF-3 pocket,

but are not conserved in the other SR members. Interestingly, the structural superimpositions revealed that the equivalent position to AR R840 is also preferentially occupied by a positively charged lysine in other NRs (**Figure 4-1** and **Table 1**).

Although the primary sequence conservation of the LBDs of orphan NRs Nurr1, FXR, RXRs, RARs, PPARs, VDR, and DAX1 show modest sequence identity to the AR LBD, their BF-3 pockets exhibit a striking conservation degree at the amino acid level, although minor than the one presented by SRs (**Table 2**). FXR, RARs, PPARs, VDR and Nurr1 possess BF-3 grooves that resemble in shape and depth the one described for AR (**Figure 4-1F-J**). The side chain of K530 in Nurr1 (AR N833) protrudes from H9 occupying part of the BF-3 groove. AR BF-3 pocket is divided by the presence of a leucine in position 830 (**Figure 4-1**). There seems to be a preference for a leucine, isoleucine or methionine in the homologous positions of the studied NRs, highlighting the hydrophobic nature of this groove. The conformation of the side chain of the residues in this position is responsible for further dissecting this pocket into two sockets (**Figure 4-1A-C** and **Figure 4-1H-J**). AR BF-3 presents a very low degree of conservation compared to both TR isoforms, and the cavity resemblance is low (not shown). However, additional surfaces on TRβ (named sites 1, 2 and 3) were identified and, although such surfaces are distinct from BF-3, some of the residues superimpose to certain key AR BF-3 residues (Marimuthu et al., 2002).

### 4.1.3. Discussion

Structure-based site-directed mutagenesis of several AR BF-3 lining residues have been shown to profoundly affect AR transcriptional activity *in vitro*. Moreover, the fact that some of the mutations greatly enhanced AR activity suggested that AR BF-3 site may be a corepressor site or may increase coactivator recruitment (Estébanez-Perpiñá et al., 2007). Most importantly, several naturally occurring mutations in patients either with PCa, AIS or infertility problems co-localize with AR BF-3 pocket (Estébanez-Perpiñá et al., 2007; Gottlieb et al., 2012). Therefore, we have further studied whether mutations on residues that belong to the putative

BF-3 sites of several NRs are associated with pathology or have been shown to affect NR function *in vitro*.

On TRβ LBD surface, Marimuthu et al. scanned more than hundred mutations and tested the effects of individual or combined mutations on corepressor binding (Marimuthu et al., 2002). Interestingly, some of the TR residues they identified superimpose with AR BF-3 forming-residues. Mutant W219K (AR BF-3 P671) affected the binding of different repressor interaction domains; variant Y406K (AR I841, adjacent to AR BF-3 R840) impaired TR binding to NCoR; whereas mutants Q396R (AR R831, which precedes AR BF-3 L830) and L401R (AR BF-3 K836) weakened NCoR corepressor binding. Three of the four mutations also showed partial defects in coactivator binding and only minor defects in ligand binding, concluding that these sites could modulate NCoR binding through allosteric mechanisms (Marimuthu et al., 2002).
In PPARγ, point mutation V307A (AR BF-3 R726) was shown to diminish, but not abolish, receptor transactivation function (S. Chen et al., 2000), whereas mutation V316I (AR L674) in ERα has been associated with recurrent breast cancer.

VDR mutant F251C (AR BF-3 F725), which has been identified in patients with an autosomal recessive disease called hereditary vitamin D-resistant rickets, shows ligand resistance, reduced transactivation and defective heterodimerization, although its activity can be partially rescued by addition of RXRa (Malloy PJ, 2001). Furthermore, mutant L254G (AR BF-3 L728) is capable of binding ligand normally but cannot form an heterodimeric complex with RXR (Whitfield et al., 1995).
Analysis of GR mutations resulting in relative glucocorticoid resistance lead to the identification of mutant E688K (AR BF-3 E829), which has been associated with normal steroid binding properties but with no dexamethasone-dependent transactivation function (Brönnegård M, 1995). Furthermore, the double mutant E684A/E688A (AR BF3 K825/E829) showed protein stabilization upon mifepristone and dexamethasone binding, yielding GR crystals in the presence of mifepristone and NCoR peptide (Schoch et al., 2010). In their crystals, the mutated GR BF-3 residues E684A and E688A are involved in crystal packing interactions.

The crystal structure of ERα PDB 1ERE (Brzozowski et al., 1997) and the full-length PPARγ-RXR heterodimer structure reported by Chandra et al. (Chandra et al., 2008) (PDB code 3E00, 3DZY and 3DZU) indicate that BF-3 may be a docking site for the hinge domain in some contexts. Whether this is representative of other NRs cannot be extrapolated from the crystal structures available. One can speculate that the hinge could be covering the BF-3 pocket as a lid in some NR conformations and exposing it under different macromolecular complexes. Mutations at the hinge domain or beginning of H1 of several NRs have been shown to affect their ligand-dependent transactivation function or recruitment of cognate protein partners suggesting that the zone along H1 may be a docking area for corepressors and chaperones or a site that would trigger conformational changes affecting protein recruitment to AF-2 or ligand binding (Buchanan et al., 2001a; Gelmann, 2002; Gottlieb et al., 2012; Nascimento et al., 2006; Safer et al., 1998; Tetel et al., 1997). Whether the BF-3 pocket engages in crucial contacts with chaperones, coactivators or corepressors requires additional experimentation. The hinge region in AR has been shown to inhibit ligand and coactivator-mediated transactivation (Q. Wang et al., 2001), whereas in RAR and TRs, it has been demonstrated to be a binding site for corepressors (J. D. Chen and Evans, 1995; Hörlein et al., 1995). In addition, the association site for ER interaction with the TATA-binding protein-associated factor TAFII30 has also been mapped to the hinge region as well (Jacq et al., 1994), and in PR, the hinge seems to be required for positive cooperative binding of progesterone to the PR LBD and for PR homodimerization (Tetel et al., 1997).

Whether these effects are only related to the hinge *per se* or by the BF-3 pocket needs experimental proof. It can only be speculated that this highly conserved site on the surface of many NRs may have a role in some physiologically relevant protein-protein interactions of the LBD as having solvent-exposed hydrophobic cavities in the cell is not energetically favored. Importantly, several naturally occurring mutations co-localizing with the BF-3 site of different NRs have been associated with pathology.

Molecular and Cellular Endocrinology 348 (2012) 394–402

Contents lists available at SciVerse ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Review

# A conserved surface on the ligand binding domain of nuclear receptors for allosteric control

Víctor Buzón [a,1], Laia R. Carbó [a,1], Sara B. Estruch [a], Robert J. Fletterick [b], Eva Estébanez-Perpiñá [a,*]

[a] Institut de Biomedicina (IBUB), Universitat de Barcelona (UB), Baldiri Reixac 15-21, Parc Científic de Barcelona (PCB), 08028 Barcelona, Spain
[b] Department of Biochemistry and Biophysics, University of California, San Francisco (UCSF), 600 16th Street, San Francisco, CA, USA

ARTICLE INFO

*Article history:*
Available online 22 August 2011

*Keywords:*
Androgen receptor
Ligand binding domain
Crystal structures
AF-2 pocket
BF-3 pocket
Nuclear receptors

ABSTRACT

Nuclear receptors (NRs) form a large superfamily of transcription factors that participate in virtually every key biological process. They control development, fertility, gametogenesis and are misregulated in many cancers. Their enormous functional plasticity as transcription factors relates in part to NR-mediated interactions with hundreds of coregulatory proteins upon ligand (e.g., hormone) binding to their ligand binding domains (LBD), or following covalent modification. Some coregulator association relates to the distinct residues that shape a coactivator binding pocket termed AF-2, a surface groove that primarily determines the preference and specificity of protein–protein interactions. However, the highly conserved AF-2 pocket in the NR superfamily appears to be insufficient to account for NR subtype specificity leading to fine transcriptional modulation in certain settings. Additional protein–protein interaction surfaces, most notably on their LBD, may contribute to modulating NR function. NR coregulators and chaperones, normally much larger than the NR itself, may also bind to such interfaces. In the case of the androgen receptor (AR) LBD surface, structural and functional data highlighted the presence of another site named BF-3, which lies at a distinct but topographically adjacent surface to AF-2. AR BF-3 is a hot spot for mutations involved in prostate cancer and androgen insensitivity syndromes, and some FDA-approved drugs bind at this site. Structural studies suggested an allosteric relationship between AF-2 and BF-3, as occupancy of the latter affected coactivator recruitment to AF-2. Physiological relevant partners of AR BF-3 have not been described as yet. The newly discovered site is highly conserved among the steroid receptors subclass, but is also present in other NRs. Several missense mutations in the BF-3 regions of these human NRs are implicated in pathology and affect their function *in vitro*. The fact that AR BF-3 pocket is a druggable site evidences its pharmacological potential. Compounds that may affect allosterically NR function by binding to BF-3 open promising avenues to develop type-specific NR modulators.



**Contents**



*Abbreviations:* AF-2, activation function-2; AIS, androgen insensitivity syndrome; AR, androgen receptor; BF-3, binding function-3; ER, estrogen receptor; DAX1, protein dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1; DBD, DNA binding domain; DHT, dihydrotestosterone; FDA, food and drug administration; FXR, farnesoid X receptor; GR, glucocorticoid receptor; HVDRR, hereditary vitamin D-resistant rickets; LBD, ligand binding domain; LBP, ligand binding pocket; MR, mineralocorticoid receptor; N-CoR, nuclear receptor corepressor; NLS, nuclear-localization signal; NRs, nuclear receptors; NTD, N-terminal domain; Nurr1, Nur-related protein 1; PCa, prostate cancer; PDB, protein data bank; PPAR, peroxisome proliferator activated receptor; PR, progesterone receptor; RAR, retinoid acid receptor; RXR, retinoid X receptor; SR, steroid receptor; SRC, steroid receptor coactivator; TR, thyroid receptor; TIF, transcriptional intermediary factor-2; VDR, vitamin D receptor.

* Corresponding author. Tel.: +34 93403 1119.
*E-mail address:* evaestebanez@ub.edu (E. Estébanez-Perpiñá).

[1] These authors contributed equally to this work.

0303-7207/$ - see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mce.2011.08.012

## 1. Introduction

The implication of nuclear receptors (NRs) in a wide variety of complex biological processes and pathologies make them major pharmacological targets (Mangelsdorf et al., 1995; Resche-Rigon and Gronemeyer, 1998; Altucci and Gronemeyer, 2001; Escriva et al., 2004; Gronemeyer et al., 2004; Szanto et al., 2004; Evans, 2005; Chen, 2008; Feldman et al., 2008; Hansen and C.T., 2008; Narayanan et al., 2008; Ellmann et al., 2009; McEwan, 2009; Huang et al., 2010; Huang and G.C., 2010; Mukherjee and Mani, 2010; Oosterveer et al., 2010; Sadar, 2011; Shapiro et al., 2011; Gao, 2010). NRs are highly social proteins and multiple protein partnerships modulate their context- and time-dependent activities (McKenna et al., 1998; Hermanson et al., 2002; Amoutzias et al., 2007; Lonard and O'Malley, 2006; Lonard et al., 2010; Jung et al., 2005; McInerney et al., 1998). NR modular architecture is behind this plethora of functions, and reflects their common evolutionary origin (Evans, 1988; Thornton, 2001; Laudet, 2003; Ortlund et al., 2007). NRs are composed of an amino-terminal domain (NTD), a central DNA-binding domain (DBD), a hinge region, and a carboxyl-terminal ligand binding domain (LBD) (Carson-Jurica et al., 1990; Jenster et al., 1991; Wurtz et al., 1996; Moras and Gronemeyer, 1998; Steinmetz et al., 2001). Each domain performs distinctive functions through the recruitment of DNA and/or cognate proteins, and exhibit distinct hormone-dependency (Jenster et al., 1995; Berrevoets et al., 1998; Schoenmakers et al., 1999; Christiaens et al., 2002; Callewaert et al., 2006; Bain et al., 2007; Centenera et al., 2008; Bevan et al., 1999; Claessens et al., 2008).

The vast majority of structural and functional studies in this field have focused in structural characterization on isolated LBDs from different receptors. The hormone-responsive LBD features a common canonical structure with the hormone nesting in its interior (Wurtz et al., 1996, 1998; Moras and Gronemeyer, 1998; Renaud et al., 1995; Wagner et al., 1995; Bourguet et al., 2000; Renaud and Moras, 2000; Greschik and Moras, 2003; Gao et al., 2005; Ingraham and Redinbo, 2005). The highly conserved protein–protein interaction site on the LBD surface named AF-2 pocket, which recruits coactivators, has also been extensively analyzed throughout this superfamily (Feng et al., 1998; Shiau et al., 1998; Collingwood et al., 1998; Darimont et al., 1998; Warnmark et al., 2003; Nagy and Schwabe, 2004; He et al., 2004; Hur et al., 2004; Estebanez-Perpina et al., 2005). The intramolecular conformational changes triggered upon ligand-binding on the most C-terminal part of NR LBDs has been studied in many NRs, whose apo- as well as holo-structures are available with different agonists or antagonists (Shiau et al., 1998; Darimont et al., 1998). The so-called "mouse-trap model" for helix 12 repositioning was then proposed based upon these structures and the dynamics of this intramolecular switch are crucial for NR function. On one hand, the large number of LBD crystal structures available, of members of almost all NR subgroups, evidences the great functional versatility of this fold, but on the other hand raises the question of how these proteins achieve specificity or fine-tuning as numerous partner proteins are shared among NR subtypes (Lonard and O'Malley, 2006; Lonard et al., 2010; McInerney et al., 1998; Smith and O'Malley, 2004). The balance between promiscuity towards specificity in determining productive and specific macromolecular assemblies leading to transcriptional regulation may also be influenced by either more subtle LBD conformational states, which may have been overseen by the more static view offered by X-ray crystallography, or by the presence of additional, non-identified protein–protein docking surfaces on the LBD (Lonard and O'Malley, 2006; Lonard et al., 2010; Glass and Rosenfeld, 2000; Rosenfeld and Glass, 2001; Rosenfeld et al., 2006; Marimuthu et al., 2002; Estébanez-Perpiñá et al., 2007).

Several lines of evidence show that the AF-2 groove may not be the sole protein–protein interface dictating macromolecular assembly upon LBD engagement in various NRs. Using a combination of X-ray crystallography and functional assays, we identified a novel site on the human androgen receptor (AR) LBD surface, and named it binding function-3 (BF-3) (Estébanez-Perpiñá et al., 2007). Previous studies with other NRs have also highlighted protein–protein interacting surfaces distinct from AF-2, some of which overlap with AR BF-3. Structure-based sequence alignments of multiple NR LBDs show that the BF-3 pocket is conserved among steroid receptors (SRs), as well as being present in other major NRs. A number of missense mutations that map to the AR BF-3 pocket have been linked to prostate cancer, infertility, and/or androgen insensitivity syndromes. Several mutations in the BF-3

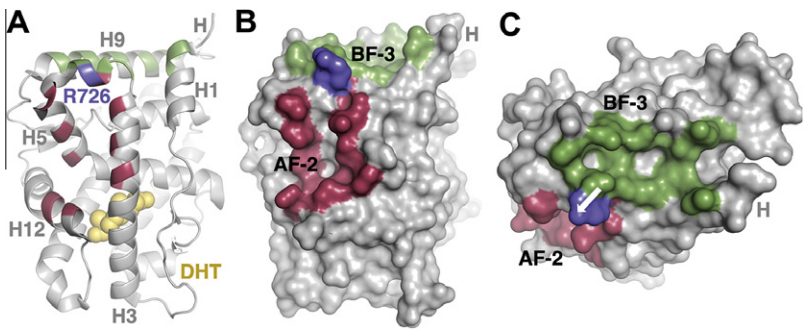


**Fig. 1.** Androgen receptor LBD representation. (A) Schematic of AR LBD showing the location of the buried hormone in the ligand binding pocket (DHT, space filling model in yellow), and helices 1 (H1), 3 (H3), 5 (H5), 9 (H9) and 12 (H12) (grey). Some residues lining the AF-2 pocket are highlighted in raspberry. Some key BF-3 residues are colored green. The residue R726 belonging to the boundary between AF-2/BF-3 is shown in blue. (B) Space-filling model showing AR LBD surface (grey) and relative location of AF-2 and BF-3 pockets. (C) 90° rotation of AR LBD depicted in (B) to display BF-3 fully. A white arrow indicates that conformational changes are transmitted by R726 (blue) from BF-3 towards AF-2. AR hinge (H) further delimits the perimeter of BF-3.

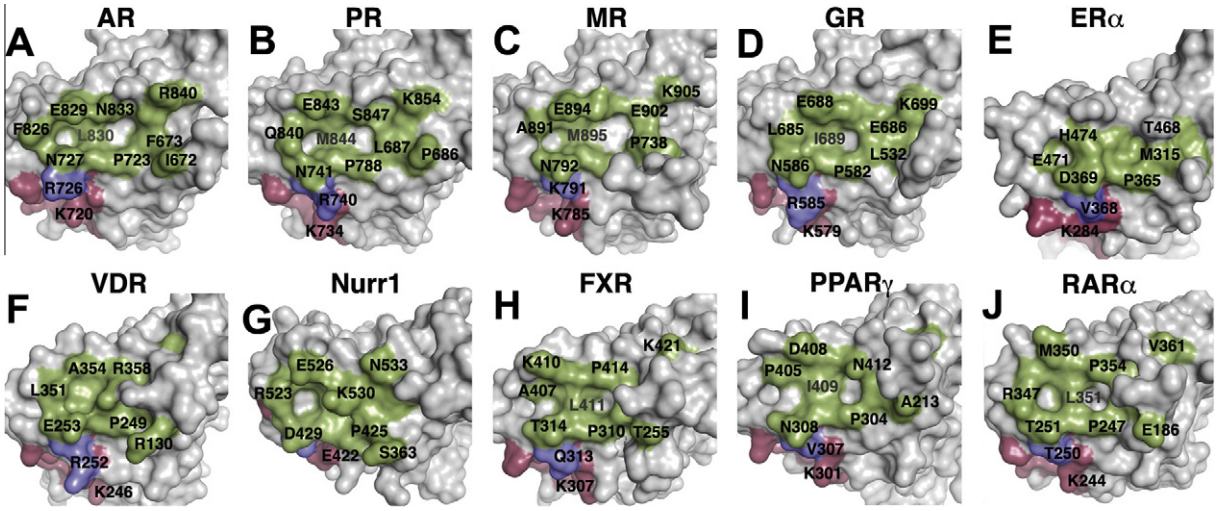


**Fig. 2.** Comparisons of NR BF-3 pockets by structural superimposition. Close-ups of the BF-3 pockets from different NR analyzed. The NR surfaces are shown in grey whereas the BF-3 lining residues are shown in green and the AF-2 pocket is visualized in raspberry. Residue R726 is also depicted in blue. (A) AR (1T5Z) (Estebanez-Perpina et al., 2005), (B) PR (1A28) (Williams and Sigler, 1998), (C) MR (2AA7) (Bledsoe et al., 2005), (D) GR (1P93) (Bledsoe et al., 2004), (E) ERα (1ERE) (Bourguet et al., 2000), (F) VDR (3A78) (Sato et al., 2009), (G) Nurr1 (1OVL) (Wang et al., 2003), (H) FXR (1OSH) (Downes et al., 2003), (I) PPARγ (1PRG) (Nolte et al., 1998), (J) RARα (3KMR) (le Maire et al., 2010). Key residues from BF-3 and AF-2 are labeled in black. The residue located at the bottom of the BF-3 pocket is indicated in dark grey. The first residues in ERα (1ERE) belonging to its hinge domain have been removed to expose the BF-3 pocket.

site of other related NRs have also been associated with pathology or abnormal NR function *in vitro*. This review will focus on the evidence found in several NRs suggesting a physiological role for the BF-3 surface pocket.

## 2. Androgen receptor binding function-3 (AR BF-3)

The BF-3 pocket in the AR was unexpectedly found by X-ray crystallography, *in vitro* transcriptional assays, and site-directed mutagenesis (Estébanez-Perpiñá et al., 2007). BF-3 is concave and topographically adjacent to but distinct from the AF-2 coactivator-binding groove on the LBD (Fig. 1). The BF-3 pocket, comparable in size and depth to the AF-2 pocket, is solvent exposed and has a hydrophobic nature, features that are in line with its postulated role as a protein–protein interaction site (Figs. 1 and 2). AR BF-3 is outlined by several LBD-forming helices, and roughly resembles a rectangle with rounded corners (Fig. 2A). By representing the LBD domain as in Fig. 1, the BF-3 is delimited by AR residues from the $NH_2$-terminal part of H1 (Q670, P671, I672 and F673), some

**Table 1**
BF-3 residues of various NR LBD crystal structures located in the topologically equivalent positions. The atomic coordinates deposited (PDB code) are indicated.

| NR | AR | PR | MR | GR | ERα | VDR | Nurr1 | FXR | PPARγ | TRα | TRβ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PDB code | 1T5Z | 1A28 | 2AA7 | 1P93 | 1ERE | 3A78 | 1OVL | 1OSH | 1PRG | 2HT9 | 1BSH |
| BF-3 residues | I672 | P686 | S737 | T531 | M315 | R130 | S363 | T255 | A213 | D166 | E220 |
| | F673 | L687 | P738 | L532 | V316 | I131 | L364 | L256 | L214 | L167 | L221 |
| | P723 | P737 | P788 | P582 | P365 | P249 | P425 | P310 | P304 | P237 | P291 |
| | G724 | G738 | G789 | G583 | G366 | G250 | G426 | G311 | G305 | M238 | M292 |
| | R726 | R740 | K791 | R585 | V368 | R252 | A428 | Q313 | V307 | S240 | F293 |
| | N727 | N741 | N792 | N586 | D369 | D253 | D429 | T314 | N308 | E241 | C294 |
| | F826 | Q840 | A891 | L685 | E471 | L351 | R523 | A407 | P405 | K337 | R391 |
| | E829 | E843 | E894 | E688 | H474 | A354 | E526 | K410 | D408 | K340 | K394 |
| | L830 | M844 | M895 | I689 | I475 | I355 | L527 | L411 | I409 | S341 | Y395 |
| | N833 | S847 | N898 | T692 | V478 | R358 | K529 | P414 | N412 | A344 | S398 |
| | E837 | E851 | E902 | E696 | I482 | T362 | N533 | V418 | A416 | A348 | A402 |
| | R840 | K854 | K905 | K699 | T485 | T365 | C354 | K421 | L419 | H351 | H405 |

Identical residues found in most of the NR analyzed hereby are shaded in light grey. In dark grey are also shaded identical or conserved residues (i.e. R and K; E and D) featured in at least two NR analyzed for a given residue in topologically-related positions. PPAR residue D408 (AR BF-3 E829) has been highlighted in light grey, as it is also a negatively-charged residue as highlighted in the same colour.

**Table 2**
Sequence identity among some NR LBD domains. The LBD column indicates the primary sequence alignment (Geneious alignment package) without including structural information. The AF-2 and BF-3 columns show the sequence identity of their corresponding AF-2 and BF-3 lining residues in comparison to AR. The alignments of AF-2 and BF-3 have been manually corrected to take into account the structural information given in Table 1. The total number of AF-2 lining residues included is 19. The total number of BF-3 lining residues included is 16. The percentage of structural identity is indicated.

| | AR pairwise identity (%) | | |
|---|---|---|---|
| | LBD | AF-2 | BF-3 |
| PR | 54,8 | 52,6 | 46,2 |
| MR | 51,6 | 42,1 | 46,2 |
| GR | 49,6 | 57,9 | 46,2 |
| RXRα | 21,4 | 40,0 | 21,4 |
| ERα | 20,2 | 42,1 | 14,3 |
| ERβ | 18,6 | 36,8 | 23,1 |
| SF1 | 17,9 | 40,0 | 9,1 |
| Nurr1 | 16,3 | 15,8 | 30,8 |
| RARα | 15,1 | 31,6 | 21,4 |
| LRH1 | 15,0 | 47,4 | 14,3 |
| RARγ | 14,7 | 31,6 | 21,4 |
| TRβ | 14,7 | 42,1 | 6,3 |
| VDR | 14,6 | 26,3 | 23,1 |
| PPARγ | 14,5 | 36,8 | 31,3 |
| TRα | 14,3 | 42,1 | 6,3 |
| FXR | 13,9 | 38,9 | 20,0 |
| DAX1 | 13,6 | 20,0 | 18,2 |

residues of H3/loop 3–4 (P723, G724, R726 and N727) and numerous residues that span almost the whole length of H9 (F826, E829, L830, N833, E837, and R840) (Fig. 2 and Table 1). Accessibility to the perimeter of BF-3 is further delimited in a canyon-like manner by the most C-terminal part of the AR hinge domain together with the loop between H9–H10/11 and the upper limit of the AF-2 pocket (Fig. 1C). Hence, there are some residues (R726 and N727) that can be defined as a boundary between those two interfaces (Figs. 1 and 2 and Table 1). Additionally, residue L830, present at the floor of the pocket, divides AR BF-3 in two sub-compartments (Fig. 2A). In contrast to the trifurcated AR AF-2 pocket (Estebanez-Perpina et al., 2005; Estébanez-Perpiñá et al., 2007) (Fig. 1B), there seems to be no opposite charge-clusters delimiting the BF-3 groove. Several structures of AR LBD in the presence of DHT were solved in complex with compounds previously identified in a fluorescent polarization assay (Estébanez-Perpiñá et al., 2007; Moore et al., 2010). Such compounds were visualized binding to either AF-2 or BF-3, or both AF-2 + BF-3 by X-ray crystallography. Crystal structure comparison of those structures with or without the presence of coactivator peptides bound to the AF-2 pocket, revealed noticeable structural differences (Estébanez-Perpiñá et al., 2007). Such structural rearrangements originated in several BF-3 forming residues far away from the AF-2 pocket (for instance R840 or F826) and seemed to be transmitted by the residues located at the boundary between them (Fig. 2A). The question whether these conformational changes are a crystallographic artifact could be raised. How can we address the possibility that an organic compound at the high concentrations used in the crystal soaks did not cause the changes in structure? Several compounds of different chemical character and altered binding interactions showed similar structural changes. Hence, subtle changes originated in BF-3 allosterically affected AF-2 causing some remodeling that impaired coactivator peptide binding to AF-2. Detailed analysis of such residues led to the formulation that some residues seemed to be linking both pockets (R726 and N727) (Fig. 2A). The conclusion is that agents at the AR BF-3 pocket may remodel the neighboring AF-2 surface affecting its capability to engage in contacts with the coactivator peptides, and possibly proteins (Estébanez-Perpiñá et al., 2007).

## 3. The BF-3 pocket is highly conserved among steroid receptors

To determine whether the BF-3 pocket is also present in other NRs, we have superimposed the atomic coordinates of several NR LBDs solved by X-ray crystallography (Fig. 2). Table 1 lists the BF-3 residues of the various NRs examined that are located in the topologically equivalent position in the solved three-dimensional structure of AR BF-3 residues (template AR PDB code 1T5Z (Estebanez-Perpina et al., 2005), whereas Table 2 resumes the sequence identity among their LBDs by primary sequence alignment and the sequence identity of their corresponding AF-2 and BF-3 pockets in comparison to AR. The alignments of AF-2 and BF-3 have been manually corrected to take into account the structural information given in Table 1.

Structural superimpositions reveal that the AR BF-3 pocket is highly conserved among the steroid receptor members progesterone receptor (PR) (Williams and Sigler, 1998), mineralocorticoid receptor (MR) (Bledsoe et al., 2005), and glucocorticoid receptor (GR) (Bledsoe et al., 2004). However, the level of conservation of AR BF-3 with the estrogen receptor (ER) isoforms is more discrete (Brzozowski et al., 1997) (Fig. 2A–E, Table 2). This conservation refers to sequence and structural identity as well as the shape, size and depth of the cavity. Among them, the PR and MR BF-3 pockets are the most similar to the one found in AR (Fig. 2B and C). In a provocative pose, the ERα hinge in PDB 1ERE is folded against the body of the LBD and docks in its corresponding BF-3 pocket acting as a lid (Brzozowski et al., 1997). To visualize ERα BF-3 concavity, we have manually removed the hinge and H1 residues of 1ERE as represented in Fig. 2E. The residues with higher degree of conservation among the steroid receptors corresponding BF-3 pockets are the ones engaged in contacts with the surface inhibitors, and also the ones that underwent the structural changes transmitting information to AF-2 (Estébanez-Perpiñá et al., 2007). The tandem of arginine (R) and asparagine (N) residues at the boundary between BF-3 and AF-2 is conserved in all steroid receptors but ER (Table 1). These residues were identified as the AR BF-3/AF-2 linkers (R726 and N727) as seen by X-ray crystallography and functional assays (Estébanez-Perpiñá et al., 2007). The crystal structure of GR LBD identified a second charge-clamp in AF-2 implicated in contacting with coactivator peptides. One of the members of this second charge clamp is R585 (AR BF-3 R726) Bledsoe et al., 2004. The AR-LBD crystal structure in complex with the SRC3–2 peptide also revealed that R726 adopts the C-terminal capping role and stabilization of the bound coactivator peptide (Estebanez-Perpina et al., 2005; Zhou et al., 2010).

The homologous residues to AR P723 and G724 are almost invariably structurally conserved among all the NRs studied, fact that was already pointed out before (Wurtz et al., 1996). In the case of the TR isoforms, the residue topologically equivalent to G724 is a methionine (Table 1). Thornton et al. studied the evolution and structure–function implications of the AR and identified a group of residues conserved in the AR clade (Thornton and Kelley, 1998). They hypothesized that these residues were involved in AR-specific aspects of receptor function. Among these residues they highlighted three that are not conserved in the other SR members, and which belong to the AR BF-3 pocket, R840, Q670 and I672 (Estébanez-Perpiñá et al., 2007). However, structural superimpositions reveal that the position equivalent to R840 in AR LBD is preferentially occupied also by a positively charged lysine in other NRs (e.g., GR, PR, and FXR) (Williams and Sigler, 1998; Bledsoe et al., 2004; Downes et al., 2003) (Fig. 2 and Table 1).

Although the primary sequence conservation of the LBDs of the orphan Nurr1, FXR, RXRs, RARs, PPARs, VDR, and DAX1 have modest sequence identity to AR LBD, their BF-3 pockets exhibit a striking conservation level at the amino acid level, although lesser to the one present among steroid receptors (Table 2). FXR, RARs,

PPARs, VDR and Nurr1 have BF-3 grooves that resemble in shape and depth the one described for AR (Fig. 2F–J) (Downes et al., 2003; le Maire et al., 2010; Nolte et al., 1998; Sato et al., 2009; Wang et al., 2003). The side chain of K530 in Nurr1 (AR BF-3 N833) protrudes from H9 occupying part of the groove (Fig. 2G). AR BF-3 pocket is divided by the presence of a leucine in position 830 (Fig. 2A). There seems to be a preference for a leucine, isoleucine or methionine in the homologous positions of the NRs studied, hence the hydrophobic nature of this groove. The conformation of the side chain of the residue in this position is responsible for further dissecting this pocket into two sockets (Fig. 2A–C and H–J). AR BF-3 presents a very low degree of conservation compared to both TR isoforms, and the cavity resemblance is low (not shown). However, additional surfaces on TRβ (named sites 1, 2 and 3) were identified. Such surfaces are distinct than BF-3 but some of the residues that they identified superimpose to certain key AR BF-3 residues as mentioned later on (Marimuthu et al., 2002).

## 4. Mutations in BF-3 are associated with pathology or altered NR function

We have performed a manual literature search using Pubmed and browsed several web-based NR databases to extract mutation data on residues that belong to the putative BF-3 sites of the several NRs we have analyzed hereby to study whether mutations co-localizing with this site are associated with pathology or have been shown to affect NR function *in vitro* (Gottlieb et al., 1998, 2004; Van Durme et al., 2003; Horn et al., 2001; Horn and LA, 2004; Folkertsma et al., 2004). Structure-based site-directed mutagenesis of several AR BF-3 lining residues was shown to profoundly affect AR transcriptional activity *in vitro*. Furthermore, the fact that some of the mutations greatly boosted AR activity suggested that AR BF-3 site may be a corepressor site or increase coactivator recruitment (Estébanez-Perpiñá et al., 2007). Most importantly, several naturally occurring mutations co-localize with AR BF-3 pocket in patients either with prostate cancer, infertility problems or androgen insensitivity syndromes (AIS), the latter being disorders of sex-development (Estébanez-Perpiñá et al., 2007; Gottlieb et al., 2004; Tilley et al., 1996; Brinkmann, 2001). The mutation data found for several NR putative BF-3 sites have been classified hereby according to whether they are localized in the hinge or H1, the loop connecting H3–H4/5 (loop 3–4), or H9. The NR mutant variants we mention hereby in the review have indicated into brackets the corresponding AR BF-3 residues that occupy the topologically equivalent positions in space.

### 4.1. Mutations at the Hinge domain or $NH_2$-terminal part of H1

It has been observed that NR hinge domain seems to exert an inhibitory role in ligand and coactivator-mediated transactivation, to modulate DNA binding and translocation to the nucleus and serve as the binding domain for many proteins (Gottlieb et al., 2004; Zhou et al., 1994; Tetel et al., 1997; Safer et al., 1998; Poukka et al., 1999; Buchanan et al., 2001; Wang et al., 2001; Gelmann, 2002; Nascimento et al., 2006; Haelens et al., 2007). AR deletion mutants of the hinge residues 628–646 created super-active AR variants (Wang et al., 2001). Because mutations in AR hinge are commonly associated with prostate cancer, it is crucial to characterize the molecular mechanisms by which this region exerts its repressor effect on ligand-activated and coactivator-mediated AF2 activity. In AR, missense mutations in residues located at the boundary between the hinge and H1 have been described (Buchanan et al., 2001; Estébanez-Perpiñá et al., 2007, #64)}. Such residues (Q668R, I670T, F671I), which border on one site the ridge of BF-3, were identified in human prostate cancer and the autochthonous transgenic adenocarcinoma of the mouse prostate model and resulted in AR that exhibited several-fold increased activity compared with wild-type AR in response to DHT, estradiol, progesterone, adrenal androgens, and the AR antagonist, hydroxyflutamide, without an apparent effect on receptor levels, ligand binding kinetics, or DNA binding (Buchanan et al., 2001; Han et al., 2001). Buchanan et al. suggested that some of these mutations could be responsible for the onset of hormone refractory disease in some patients (Buchanan et al., 2001). Estebanez et al. described mutants that turned AR into a super-receptor (Q670R, I672T, and I672R) while F673R decreased AR transcriptional activity (Estébanez-Perpiñá et al., 2007). Another mutation more upstream on AR hinge (R629W) was identified in a patient with severe undermasculinization (AIS) and poor response to exogenous androgens (Deeb et al., 2008). Both mutants Q670R and I672T have been associated to prostate cancer and AIS patients (Estébanez-Perpiñá et al., 2007; Gottlieb et al., 2004; Shi et al., 2002).

In the ERα, several mutations have been described in metastatic or recurrent breast cancer, metastatic lymph nodes or recurrent breast cancer tissue from patients with ER-positive primary tumors (Osborne, 1998; Mueller-Fahrnow and Egner, 1999). Some of these residues line ER BF-3-site and localize in its hinge and H1. Mutation V316I (AR L674) in ERα BF-3 pocket has been associated with recurrent breast cancer, whereas mutant T311A has been described in endometrial cancer and this threonine is phosphorylated by p38 mitogen-activated protein kinase (Lee and Bai, 2002). Mutation T311A did not affect estrogen binding but compromised its interaction with coactivators *in vitro* and *in vivo* (Lee and Bai, 2002). It has been also shown that phosphorylation of residue S305 seems to alter the orientation and activity of bound coactivator to AF-2 without affecting the affinity of coactivator binding (Umekita et al., 1998). ERα K303R is a hypersensitive mutant associated with human breast cancer. This mutant exhibited increased interactions with members of the SRC family of coactivators but not increased estrogen binding affinity and seemed to have lost negative regulation by the F domain (Herynk et al., 2010).

A point mutation in the hinge domain of TRβ in several members of a family with generalized thyroid hormone resistance has been described. This mutant bound with high affinity to various thyroid hormone response elements but binding to the hormone was threefold reduced, suggesting that TRβ hinge domain is important for full ligand-binding activity (Behr and Loos, 1992). On TRβ LBD surface, Marimuthu et al. scanned more than hundred mutations and tested the effects of individual or combined mutations on corepressor binding (Marimuthu et al., 2002). One of this identified surfaces overlaps with the TR AF-2 pocket but extends further underneath it. A second site is a concave surface running alongside H1, and a third one is located above H11, an area nearby but distinct from the dimerization area. Some of the TR residues they identified superimpose with AR BF-3 forming-residues. Site 2 mutant W219K (AR BF-3 P671) affected the binding of different repressor interaction domains. Mutant Y406K was also identified (AR I841, which is adjacent to AR BF-3 R840) and impaired TR binding to corepressor N-CoR. Site 3 mutants Q396R (AR R831, which precedes AR BF-3 L830) and L401R (AR BF-3 K836) weakened N-CoR corepressor binding. Mutations from TR sites 2 and 3 also showed partial defects in coactivator binding and only minor defects in ligand binding similar to some TR mutants found in patients with the syndrome of resistance to thyroid hormone concluding that these sites could modulate N-CoR binding through allosteric mechanisms (Marimuthu et al., 2002; Ribeiro et al., 2001).

### 4.2. Mutations at the boundary between BF-3 and AF-2

The residues that are shared between the AF-2 and BF-3 pockets function as a structural relay in the AR (Estébanez-Perpiñá et al.,

2007). The BF-3/AF-2 boundary is a target for prostate cancer and AIS mutations which have variously been shown to influence androgen binding and dissociation, coregulator recruitment, N/C interaction and transcriptional activity (Estébanez-Perpiñá et al., 2007; Gottlieb et al., 2004; Horn et al., 2001; Gelmann, 2002; Mononen et al., 2000; Thompson et al., 2001; Gruber et al., 2003; Koivisto et al., 2004). Mutants AR R726L (associated with prostate cancer, alcoholism and phobia) (Mononen et al., 2000; Thompson et al., 2001; Gruber et al., 2003; Koivisto et al., 2004; Yan et al., 2004) and mutant AR N727K has been identified in mild androgen insensitivity syndrome patients (MAIS) (Wang et al., 2001; Lim et al., 2000). Targeted mutagenesis of N727 (N727A) eliminate AR LBD activity (Fig. 1C), similar to inhibition obtained with mutations in AF-2 (Estébanez-Perpiñá et al., 2007). Likewise, mutations at P723 or G724 reduced activity *in vitro* (Estébanez-Perpiñá et al., 2007). Mutation and R726L using an AR LBD construct seemed though to diminish the receptor activity in HeLa cells (Estébanez-Perpiñá et al., 2007).

In the PPARγ, the point mutation V307A (AR BF-3 R726) was shown to diminish but not abolish receptor transactivation function in COS1 cells using a luciferase assay (Chen et al., 2000).

VDR mutant F251C (AR BF-3 F725) has been identified in patients with and autosomal recessive disease called hereditary vitamin D-resistant rickets (HVDRR). This mutant VDR shows ligand resistance, reduced transactivation and defective heterodimerization. Activity was partially rescued by addition of RXRα (Malloy PJ et al., 2001). Furthermore, VDR mutant L254G (AR BF-3 L728) binds ligand normally but is defective in its ability to form a heterodimeric complex with RXR on a vitamin D responsive element (Whitfield et al., 1995).

ERα mutant V364E (AR L722, which precedes the highly conserved proline in loop 3–4 in most of the NRs) induced a superactive response to estrogen when the mutant was expressed alone, and showed a dominant-negative when expressed with wild-type ER (Wurtz et al., 1998; Wrenn and Katzenellenbogen, 1993; McInerney et al., 1996; Herynk and Katzenellenbogen, 2004).

### 4.3. Mutations in Helix 9

AR BF-3-lining residues belonging to H9 are a target for several mutations identified in patients: L830P (prostate cancer) (Gottlieb et al., 2004), F826L (AIS) (Wong et al., 2008) and multiple R840 variants (AIS) (Lim et al., 2000; Beitel et al., 1994; Melo et al., 1999, 2003; Mazen et al., 2004). On the other hand, R840E in both AR LBD and full-length receptor (AR-FL) and L830R abolished its activity *in vitro*, whereas mutant F826R slightly diminished AR-FL function (Estébanez-Perpiñá et al., 2007; Lim et al., 2000). Mutations in these residues diminished coactivator binding *in vitro* although none of them make direct contact with the coregulator peptides visualized by X-ray crystallography (Estebanez-Perpina et al., 2005; Estébanez-Perpiñá et al., 2007). However, several mutation in AR BF-3 pocket located in H9 induce activating receptors in a luciferase assay with AR LBD: F826A, E829R, E829A and N833R. Residue R840 was identified as undergoing major rearrangements upon compound binding to AR BF-3 pocket visualized by X-ray crystallography (Estébanez-Perpiñá et al., 2007). Several mutants implicated in patients with varying degrees of AIS have been described for residue R840: R840C, R840G, R840H and R840S (Gottlieb et al., 2004; Beitel et al., 1994; Giwercman et al., 1998).

Analysis of GR mutations resulting in relative glucocorticoid resistance, both familial glucocorticoid resistance and directed mutagenesis, identified two regions of clustered mutations (Hollenberg and E.R., 1988; Pierrat et al., 1994; Brönnegård and Carlstedt-Duke, 1995). In most of the cases, the mutation affected steroid binding and transactivation, but this was not always the case. Brönnegad et al. resumed a number of identified mutants with reduced glucocorticoid-dependent transactivation in both yeast cells and CV-1 cells. One of such GR mutants is E688K (AR BF-3 E829), which has been associated with normal steroid binding properties but with no dexamethasone (Dex)-dependent transactivation function (Brönnegård and Carlstedt-Duke, 1995). Schoch et al. selected clusters of lysine or glutamic on the GR LBD surface and distant from the steroid and the AF-2 binding sites for mutagenesis studies replacing them by alanine and serine mutations in an attempt to decrease surface entropy to search for conditions that may yield GR LBD crystals with improved diffraction quality (Schoch et al., 2010). The double GR mutant E684A (AR BF3 K825) and E688A (AR BF-3 E829) showed, in a fluorescence thermal shift assay, protein stabilization upon mifepristone and dex-binding and yielded GR crystals in the presence of mifepristone and NCoR peptide. In their crystals, the mutated GR BF-3 residues A684 and A688 are involved in crystal packing interactions.

In the mouse GR, several point mutations were identified in a region named τ2 (residues 533–562), which reduced or eliminated hormone binding although none of these residues interacts directly with hormone. These mutants affect hormone binding indirectly by disrupting hydrophobic contacts between H1 and the other helices or a salt bridge of a conserved residue K673 in H9. The mutants were thought to expel H1 from its usual position affecting as a result the hormone-binding pocket (Milhon et al., 1997, 1994).

## 5. Unanswered questions

The shape and characteristics of the BF-3 pocket in the NRs examined suggest a possible role for protein–protein interactions. So far, only small compounds are described to bind this surface pocket by the AR LBD. Several mutations associated with pathology and affecting receptor function *in vitro* have been described in many NRs. Whether the BF-3 pocket is an authentic protein–protein interface needs to be further explored. No specific BF-3 protein binder has been described as yet. Also, the current NR crystal structures with coactivator motifs do not suggest whether this pocket may be an additional docking site (auxiliary site or exosite) for known coregulatory proteins. The role of the hinge region of NRs is not as well understood as other parts of the receptor except for PPARγ in its complex with RXR (Chandra et al., 2008). The crystal structure of ERα PDB 1ERE (Brzozowski et al., 1997) and the full-length PPARγ-RXR heterodimer structure reported by Chandra et al. (Chandra et al., 2008) (PDB code 3E00, 3DZY and 3DZU) indicate that BF-3 may be a docking site for the hinge domain in some contexts. Whether this is representative of other NRs cannot be extrapolated from the crystal structures available. One can speculate that the hinge could be covering the BF-3 pocket as a lid in some NR conformations and exposing it under different macromolecular complexes. Mutations at the hinge domain or beginning of H1 of several NRs have been shown to affect their ligand-dependent transactivation function or recruitment of cognate protein partners suggesting that the zone along H1 may be a docking area for corepressors and chaperones or a site that would trigger conformational changes affecting protein recruitment to AF-2 or ligand binding (Gottlieb et al., 2004; Zhou et al., 1994; Tetel et al., 1997; Safer et al., 1998; Poukka et al., 1999; Buchanan et al., 2001; Wang et al., 2001; Gelmann, 2002; Nascimento et al., 2006). Whether the BF-3 pocket engages in crucial contacts with chaperones, coactivators or corepressors requires additional experimentation. AR hinge region contains a phosphorylation site and several acetylation sites as well as being identified as the putative recruiting domain for several proteins (Gottlieb et al., 2004; Faus and Haendler, 2006). In addition, it contains a bipartite nuclear-localization signal (NLS) and has been shown to play a role in

proteasome-mediated transcription (Tanner et al., 2004). It has been shown that this domain inhibits ligand and coactivated-mediated transactivation (Wang et al., 2001). In RAR and TRs, the hinge has been shown to be a binding site for corepressors (Hörlein et al., 1995; Chen and Evans, 1995). In addition, the binding site for ER interaction with the TATA-binding protein-associated factor TAFII30 has also been mapped to the hinge region as well (Jacq et al., 1994). In the PR, Tetel et al. showed that the hinge is required for positive cooperative binding of progesterone to the PR LBD and PR homodimerization (Tetel et al., 1997). Whether these effects are only related to the hinge *per se* or by the BF-3 pocket needs experimental proof. We can only speculate that this highly conserved site on the surface of many NRs may have a role in some physiologically relevant protein–protein interactions of the LBD in the cell as having hydrophobic cavities exposed to the solvent are not energetically favored. Furthermore, several naturally occurring mutations colocalizing with the BF-3 site of different NRs are associated with pathology.

It is evident that NR LBD crystal structures with coactivator or corepressor motifs are only a reductionist snapshot of what may be happening in the cell when the full-size coregulator contacts with the full-length NR. What the hereby-mentioned structures evidenced is the fact that intra-domain subtle rearrangements transmitted along AR LBD surfaces may be a mechanism also modulating macromolecular complex formation upon this domain and that BF-3 pocket may be a legitimate protein–protein interaction and activation site in the context of the full size receptor.

## 6. Concluding remarks

NRs are important pharmaceutical targets. Most of the current research focuses on finding ligands to modulate receptor function in several pathological conditions, such as the metabolic syndrome, prostate and breast cancers, to name a few. Most of the compounds that target NR ligand binding pocket have serious secondary effects due to cross-reactivity among different NR subfamilies. Developing NR modulators that would target LBD surface pockets may be a novel way to find class-specific drugs. In particular, targeting the BF-3 surface may open new promising alternatives to current therapeutics.

## Acknowledgements

E.E.P. is supported by a RyC-2007 fellowship and a SAF-2008-03562 Project, both from the Ministerio de Ciencia e Innovación (MICINN, Gobierno de España), a Marie-Curie International Reintegration Grant (IRG-code 224812) and the Marató de TV3 Grant-Malalties Rares (2010) that funds V.B. L.R.C. is recipient of a FPI Grant from the MICINN. SBE is recipient of a PPL Grant from the Asociación Española Contra el Cáncer (AECC). We thank Dr. Pablo Fuentes-Prior and Dr. Jens Lüders for useful comments on the manuscript.

## References

Altucci, L., Gronemeyer, H., 2001. Nuclear receptors in cell life and death. Trends Endocrinol. Metab. 12, 460–468.
Amoutzias, G.D., Pichler, E.E., Mian, N., De Graaf, D., Imsiridou, A., Robinson-Rechavi, M., Bornberg-Bauer, E., Robertson, D.L., Oliver, S.G., 2007. A protein interaction atlas for the nuclear receptors: properties and quality of a hub-based dimerisation network. BMC Syst. Biol. 31, 1–34.
Bain, D.L., Heneghan, A.F., Connaghan-Jones, K.D., Miura, M.T., 2007. Nuclear receptor structure: implications for function. Annu. Rev. Physiol. 69, 201–220.
Behr, M., Loos, U., 1992. A point mutation (Ala229 to Thr) in the hinge domain of the c-erbA beta thyroid hormone receptor gene in a family with generalized thyroid hormone resistance. Mol. Endocrinol. 6, 1119–1126.
Beitel, L.K., K.-E.P., Kaufman, M., Lumbroso, R., DiGeorge, A.M., Killinger, D.W., Trifiro, M.A., Pinsky, L., 1994. Substitution of arginine-839 by cysteine or histidine in the androgen receptor causes different receptor phenotypes in cultured cells and coordinate degrees of clinical androgen resistance. J. Clin. Invest. 94, 546–554.
Berrevoets, C.A., Doesburg, P., Steketee, K., Trapman, J., Brinkmann, A.O., 1998. Functional interactions of the AF-2 activation domain core region of the human androgen receptor with the amino-terminal domain and with the transcriptional coactivator TIF2 (transcriptional intermediary factor2). Mol. Endocrinol. 12, 1172–1183.
Bevan, C.L., Hoare, S., Claessens, F., Heery, D.M., Parker, M.G., 1999. The AF1 and AF2 domains of the androgen receptor interact with distinct regions of SRC1. Mol. Cell. Biol. 19, 8383–8392.
Bledsoe, R.K., Stewart, E.L., Pearce, K.H., 2004. Structure and function of the glucocorticoid receptor ligand binding domain. Vitam. Horm. 68, 49–91.
Bledsoe, R.K., Madauss, K.P., Holt, J.A., Apolito, C.J., Lambert, M.H., Pearce, K.H., Stanley, T.B., Stewart, E.L., Trump, R.P., Willson, T.M., Williams, S.P., 2005. A ligand-mediated hydrogen bond network required for the activation of the mineralocorticoid receptor. J. Biol. Chem. 280, 31283–31293.
Bourguet, W., Vivat, V., Wurtz, J.M., Chambon, P., Gronemeyer, H., Moras, D., 2000. Crystal structure of a heterodimeric complex of RAR and RXR ligand-binding domains. Mol. Cell 5, 289–298.
Brinkmann, A.O., 2001. Molecular basis of androgen insensitivity. Mol. Cell. Endocrinol. 179, 105–109.
Brönnegård, M., Carlstedt-Duke, J., 1995. The genetic basis of glucocorticoid resistance. Trends Endocrinol. Metab. 6, 160–164.
Brzozowski, A.M., P.A., Dauter, Z., Hubbard, R.E., Bonn, T., Engstrom, O., Ohman, L., Greene, G.L., Gustafsson, J.A., Carlquist, M., 1997. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389, 753–758.
Buchanan, G., Yang, M., Harris, J.M., Nahm, H.S., Han, G., Moore, N., Bentel, J.M., Matusik, R.J., Horsfall, D.J., Marshall, V.R., Greenberg, N.M., Tilley, W.D., 2001. Mutations at the boundary of the hinge and ligand binding domain of the androgen receptor confer increased transactivation function. Mol. Endocrinol. 15, 46–56.
Callewaert, L., Van Tilborgh, N., Claessens, F., 2006. Interplay between two hormone-independent activation domains in the androgen receptor. Cancer Res. 66, 543–553.
Carson-Jurica, M.A., Schrader, W.T., O'Malley, B.W., 1990. Steroid receptor family: structure and functions. Endocr. Rev. 11, 201–220.
Centenera, M.M., Harris, J.M., Tilley, W.D., Butler, L.M., 2008. The contribution of different androgen receptor domains to receptor dimerization and signaling. Mol. Endocrinol. 22, 2373–2382.
Chandra, V., Huang, P., Hamuro, Y., Raghuram, S., Wang, Y., Burris, T.P., Rastinejad, F., 2008. Structure of the intact PPAR-gamma-RXR- nuclear receptor complex on DNA. Nature 456, 350–356.
Chen, T., 2008. Nuclear receptor drug discovery. Curr. Opin. Chem. Biol. 12, 418–426.
Chen, J.D., Evans, R., 1995. A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature 377, 397–403.
Chen, S., J.B., Li, Y., Aster, S., McKeever, B., Mosley, R., Moller, D.E., Zhou, G., 2000. Both coactivator LXXLL motif-dependent and -independent interactions are required for peroxisome proliferator-activated receptor gamma (PPARgamma) function. J. Biol. Chem. 275, 3733–3736.
Christiaens, V., Bevan, C.L., Callewaert, L., Haelens, A., Verrijdt, G., Rombauts, W., Claessens, F., 2002. Characterization of the two coactivator-interacting surfaces of the androgen receptor and their relative role in transcriptional control. J. Biol. Chem. 277, 49230–49237.
Claessens, F., Denayer, S., Van Tilborgh, N., Kerkhofs, S., Helsen, C., Haelens, A., 2008. Diverse roles of androgen receptor (AR) domains in AR-mediated signaling. Nucl Recept Signal 6, e008.
Collingwood, T.N., Wagner, R., Matthews, C.H., Clifton-Bligh, R.J., Gurnell, M., Rajanayagam, O., Agostini, M., Fletterick, R.J., Beck-Peccoz, P., Reinhardt, W., Binder, G., Ranke, M.B., Hermus, A., Hesch, R.D., Lazarus, J., Newrick, P., Parfitt, V., Raggatt, P., de Zegher, F., Chatterjee, V.K., 1998. A role for helix 3 of the TRbeta ligand-binding domain in coactivator recruitment identified by characterization of a third cluster of mutations in resistance to thyroid hormone. EMBO J. 17, 4760–4770.
Darimont, B., Wagner, R., Apriletti, J., Stallcup, M., Kushner, P., Baxter, J., Fletterick, R., Yamamoto, K., 1998. Structure and specificity of nuclear receptor-coactivator interactions. Genes Dev. 12, 3343–3356.
Deeb, A., Jääskeläinen, J., Dattani, M., Whitaker, H.C., Costigan, C., Hughes, I.A., 2008. A novel mutation in the human androgen receptor suggests a regulatory role for the hinge region in amino-terminal and carboxy-terminal interactions. J. Clin. Endocrinol. Metab. 93, 3691–3696.
Downes, M., Verdecia, M.A., Roecker, A.J., Hughes, R., Hogenesch, J.B., Kast-Woelbern, H.R., Bowman, M.E., Ferrer, J.-L., Anisfeld, A.M., Edwards, P.A., Rosenfeld, J.M., Alvarez, J.G.A., Noel, J.P., Nicolaou, K.C., Evans, R.M., 2003. A chemical, genetic, and structural analysis of the nuclear bile acid receptor FXR. Mol. Cell 11, 1079–1092.
Ellmann, S., S.H., Thiel, F., Beckmann, M.W., Strick, R., Strissel, P.L., 2009. Estrogen and progesterone receptors: from molecular structures to clinical targets. Cell. Mol. Life Sci. 66, 2405–2426.
Escriva, H., Bertrand, S., Laudet, V., 2004. The evolution of the nuclear receptor superfamily. Essays Biochem. 40, 11–26.
Estebanez-Perpina, E., Moore, J.M., Mar, E., Delgado-Rodrigues, E., Nguyen, P., Baxter, J.D., Buehrer, B.M., Webb, P., Fletterick, R.J., Guy, R.K., 2005. The

molecular mechanisms of coactivator utilization in ligand-dependent transactivation by the androgen receptor. J. Biol. Chem. 280, 8060–8068.
Estébanez-Perpiñá, E., Arnold, L.A., Arnold, A.A., Nguyen, P., Rodrigues, E.D., Mar, E., Bateman, R., Pallai, P., Shokat, K.M., Baxter, J.D., Guy, R.K., Webb, P., Fletterick, R.J., 2007. A surface on the androgen receptor that allosterically regulates coactivator binding. Proc. Natl. Acad. Sci. USA 104, 16074–16079.
Evans, R., 1988. The steroid and thyroid hormone receptor superfamily. Science 13, 889–895.
Evans, R., 2005. The nuclear receptor superfamily: a rosetta stone for physiology. Mol. Endocrinol. 19, 1429–1438.
Faus, H., Haendler, B., 2006. Post-translational modifications of steroid receptors. Biomed. Pharmacother. 60, 520–528.
Feldman, P.L., L.M., Henke, B.R., 2008. PPAR modulators and PPAR pan agonists for metabolic diseases: the next generation of drugs targeting peroxisome proliferator-activate. Curr. Top. Med. Chem. 8, 728–749.
Feng, W., Ribeiro, R.C., Wagner, R.L., Nguyen, H., Apriletti, J.W., Fletterick, R.J., Baxter, J.D., Kushner, P.J., West, B.L., 1998. Hormone-dependent coactivator binding to a hydrophobic cleft on nuclear receptors. Science 280, 1747–1749.
Folkertsma, S., van Noort, P., Van Durme, J., Joosten, H.J., Bettler, E., Fleuren, W., Oliveira, L., Horn, F., de Vlieg, J., Vriend, G., 2004. A family-based approach reveals the function of residues in the nuclear receptor ligand-binding domain. J. Mol. Biol. 341, 321–335.
Gao, W., 2010. Androgen receptor as a therapeutic target. Adv. Drug Deliv. Rev. (Epub ahead of print).
Gao, W., Bohl, C.E., Dalton, J.T., 2005. Chemistry and structural biology of androgen receptor. Chem. Rev. 105, 3352–3370.
Gelmann, E.P., 2002. Molecular Biology of the Androgen Receptor. J. Clin. Oncol. 20, 3001–3015.
Giwercman, Y.L., Nikoshkov, A., Lindsten, K., Byström, B., Pousette, A., Chibalin, A.V., Arvidsson, S., Tiupakov, A., Semitcheva, T.V., Peterkova, V., Hagenfeldt, K., Ritzén, E.M., Wedell, A., 1998. Functional characterisation of mutation in the ligand-binding domain of the androgen receptor gene in patients with androgen insensitivity syndrome. Hum. Genet. 10.
Glass, C.K., Rosenfeld, M.G., 2000. The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev. 14, 121–141.
Gottlieb, B., Lehvaslaiho, H., Beitel, L.K., Lumbroso, R., Pinsky, L., Trifiro, M., 1998. The androgen receptor gene mutations database. Nucleic Acids Res. 26, 234–238.
Gottlieb, B., Beitel, L.K., Wu, J.H., Trifiro, M., 2004. The androgen receptor gene mutations database (ARDB). update 23, 6.
Greschik, H., Moras, D., 2003. Structure-activity relationship of nuclear receptor-ligand interactions. Curr. Top. Med. Chem. 3, 1573–1599.
Gronemeyer, H., Gustafsson, J.A., Laudet, V., 2004. Principles for modulation of the nuclear receptor superfamily. Nat. Rev. Drug Discov. 3, 950–964.
Gruber, S.B., Chen, H., Tomsho, L.P., Lee, N., Perrone, E.E., Cooney, K.A., 2003. R726L androgen receptor mutation is uncommon in prostate cancer families in the united states. Prostate 54, 306–309.
Haelens, A., Tanner, T., Denayer, S., Callewaert, L., Claessens, F., 2007. The hinge region regulates DNA binding, nuclear translocation, and transactivation of the androgen receptor. Cancer Res. 67, 4514–4523.
Han, G., Foster, B.A., Mistry, S., Buchanan, G., Harris, J.M., Tilley, W.D., Greenberg, N.M., 2001. Hormone status selects for spontaneous somatic androgen receptor variants that demonstrate specific ligand and cofactor dependent activities in autochthonous prostate cancer. J. Biol. Chem. 276, 11204–11213.
Hansen, M.K., C.T., 2008. Nuclear receptors as drug targets in obesity, dyslipidemia and atherosclerosis. Curr. Opin. Investig. Drugs 9, 247–255.
He, B., Gampe Jr., R.T., Kole, A.J., Hnat, A.T., Stanley, T.B., An, G., Stewart, E.L., Kalman, R.I., Minges, J.T., Wilson, E.M., 2004. Structural basis for androgen receptor interdomain and coactivator interactions suggests a transition in nuclear receptor activation function dominance. Mol. Cell 16, 425–438.
Hermanson, O., Glass, C.K., Rosenfeld, M.G., 2002. Nuclear receptor coregulators: multiple modes of modification. Trends Endocrinol. Metab. 13, 55–60.
Herynk, M.H., F.S., 2004. Estrogen receptor mutations in human disease. Endocr. Rev. 25, 869–898.
Herynk, M.H., H.T., Cui, Y., Niu, A., Corona-Rodriguez, A., Fuqua, S.A., 2010. A hypersensitive estrogen receptor alpha mutation that alters dynamic protein interactions. Breast Cancer Res. Treat. 2.
Hollenberg, S.M., E.R., 1988. Multiple and cooperative transactivation domains of the human glucocorticoid receptor. Cell 55, 899–906.
Hörlein, A.J., N.A., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Söderström, M., Glass, C.K., et al., 1995. Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature 377, 397–404.
Horn, F., L.A., LA, F.E., 2004. Automated extraction of mutation data from the literature: application of MuteXt to G protein-coupled receptors and nuclear hormone receptors. Bioinformatics 20, 557–568.
Horn, F., V.G., Cohen, F.E., 2001. Collecting and harvesting biological data: the GPCRDB and NucleaRDB information systems. Nucleic Acids Res. 29, 346–349.
Huang, W., G.C., 2010. Nuclear receptors and inflammation control: molecular mechanisms and pathophysiological relevance. Arterioscler. Thromb. Vasc. Biol. 30, 1542–1549.
Huang, P., C.V., Rastinejad, F., 2010. Structural overview of the nuclear receptor superfamily: insights into physiology and therapeutics. Annu. Rev. Physiol. 17, 247–272.
Hur, E., Pfaff, S.J., Sturgis, P.E., Hanne, G., Buehrer, B.M., Fletterick, R.J., 2004. Recognition and accommodation at the androgen receptor coactivator binding interface. PLoS 2, 363.
Ingraham, H.A., Redinbo, M.R., 2005. Orphan nuclear receptors adopted by crystallography. Curr. Opin. Struct. Biol. 15, 708–715.
Jacq, X., B.C., Lutz, Y., Davidson, I., Chambon, P., Tora, L., 1994. Human TAFII30 is present in a distinct TFIID com- plex and is required for transcriptional activation by the estrogen receptor. Cell 79, 107–117.
Jenster, G., van der Korput, H.A., van Vroonhoven, C., van der Kwast, T.H., Trapman, J., Brinkmann, A.O., 1991. Domains of the human androgen receptor involved in steroid binding, transcriptional activation, and subcellular localization. Mol. Endocrinol. 5, 1396–1404.
Jenster, G., van der Korput, H.A., Trapman, J., Brinkmann, A.O., 1995. Identification of two transcription activation units in the N-terminal domain of the human androgen receptor. J. Biol. Chem. 270, 7341–7346.
Jung, S.Y., Malovannaya, A., Wei, J., O'Malley, B.W., Qin, J., 2005. Proteomic analysis of steady-state nuclear hormone receptor coactivator complexes. Mol. Endocrinol. 19, 2451–2465.
Koivisto, P.A., Hyytinen, E.R., Matikainen, M., Tammela, T.L., Ikonen, T., Schleutker, J., 2004. Germline mutation analysis of the androgen receptor gene in Finnish patients with prostate cancer. J. Urol. 171, 431–433.
Laudet, V., 2003. The nuclear receptor superfamily. J. Cell Sci. 116, 585–586.
le Maire, A., Teyssier, C., Erb, C., Grimaldi, M., Alvarez, S., de Lera, A.R., Balaguer, P., Gronemeyer, H., Royer, C.A., Germain, P., Bourguet, W., 2010. A unique secondary-structure switch controls constitutive gene repression by retinoic acid receptor. Nat. Struct. Mol. Biol. 17, 801–807.
Lee, H., Bai, W., 2002. Regulation of estrogen receptor nuclear export by ligand-induced and p38-mediated receptor phosphorylation. Mol. Cell. Biol. 22, 5835–5845.
Lim, J., Ghadessy, F.J., Abdullah, A.A., Pinsky, L., Trifiro, M., Yong, E.L., 2000. Human androgen receptor mutation disrupts ternary interactions between ligand, receptor domains, and the coactivator TIF2 (transcription intermediary factor 2). Mol. Endocrinol. 14, 1187–1197.
Lonard, D.M., O'Malley, B.W., 2006. The expanding cosmos of nuclear receptor coactivators. Cell 125, 411–414.
Lonard, D.M., Kumar, R., O'Malley, B.W., 2010. Minireview: the SRC family of coactivators: an entrée to understanding a subset of polygenic diseases? Mol. Endocrinol. 24, 279–285.
Malloy PJ, Z.W., Zhao, X.Y., Pehling, G.B., Feldman, D., 2001. A novel inborn error in the ligand-binding domain of the vitamin D receptor causes hereditary vitamin D-resistant rickets. Mol. Genet. Metab. 73, 138–148.
Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., Evans, R.M., 1995. The nuclear receptor superfamily: the second decade. Cell 83, 835–839.
Marimuthu, A., Feng, W., Tagami, T., Nguyen, H., Jameson, J.L., Fletterick, R.J., Baxter, J.D., West, B.L., 2002. TR surfaces and conformations required to bind nuclear receptor corepressor. Mol. Endocrinol. 16, 271–286.
Mazen, I., Lumbroso, S., Abdel, Ghaffar S., Salah, N., Sultan, C., 2004. Mutation of the androgen receptor (R840S) in an Egyptian patient with partial androgen insensitivity syndrome: review of the literature on the clinical expression of different R840 substitutions. J. Endocrinol. Invest. 27, 57–60.
McEwan, I.J., 2009. Nuclear receptors: one big family. Methods Mol. Biol. 505, 3–18.
McInerney, E.M., I.B., Shapiro, D.J., Katzenellenbogen, B.S., 1996. A transcriptionally active estrogen receptor mutant is a novel type of dominant negative inhibitor of estrogen action. Mol. Endocrinol. 10, 1519–1526.
McInerney, E.M., Rose, D.W., Flynn, S.E., Westin, S., Mullen, T.M., Krones, A., Inostroza, J., Torchia, J., Nolte, R.T., Assa-Munt, N., Milburn, M.V., Glass, C.K., Rosenfeld, M.G., 1998. Determinants of coactivator LXXLL motif specificity in nuclear receptor transcriptional activation. Genes Dev. 12, 3357–3368.
McKenna, N.J., Nawaz, Z., Tsai, S.Y., Tsai, M.J., O'Malley, B.W., 1998. Distinct steady-state nuclear receptor coregulator complexes exist in vivo. Proc. Natl. Acad. Sci. USA 95, 11697–11702.
Melo, K.F., Latronico, A.C., Costa, E.M., Billerbeck, A.E., Mendonca, B.B., Arnhold, I.J., 1999. A novel point mutation (R840S) in the androgen receptor in a Brazilian family with partial androgen insensitivity syndrome. Hum. Mutat. 14, 353.
Melo, K.F., Mendonca, B.B., Billerbeck, A.E., Costa, E.M., Inacio, M., Silva, F.A., Leal, A.M., Latronico, A.C., Arnhold, I.J., 2003. Clinical, hormonal, behavioral, and genetic characteristics of androgen insensitivity syndrome in a Brazilian cohort: five novel mutations in the androgen receptor gene. J. Clin. Endocrinol. Metab. 88, 3241–3250.
Milhon, J., Kohli, K., Stallcup, M.R., 1994. Genetic analysis of the N-terminal end of the glucocorticoid receptor hormone binding domain. J. Steroid Biochem. Mol. Biol. 51, 11–19.
Milhon, J., Lee, S., Kohli, K., Chen, D., Hong, H., Stallcup, M.R., 1997. Identification of amino acids in the tau 2-region of the mouse glucocorticoid receptor that contribute to hormone binding and transcriptional activation. Mol. Endocrinol. 11, 1795–1805.
Mononen, N., Syrjäkoski, K., Matikainen, M., Tammela, T.L., Schleutker, J., Kallioniemi, O.P., Trapman, J., Koivisto, P.A., 2000. Two percent of Finnish prostate cancer patients have a germ-line mutation in the hormone-binding domain of the androgen receptor gene. Cancer Res. 60, 6479–6481.
Moore, T.W., Mayne, C.G., Katzenellenbogen, J.A., 2010. Minireview: not picking pockets: nuclear receptor alternate-site modulators (NRAMs). Mol. Endocrinol. 24, 683–695.
Moras, D., Gronemeyer, H., 1998. The nuclear receptor ligand-binding domain: structure and function. Curr. Opin. Cell Biol. 10, 384–391.

Mueller-Fahrnow, A., Egner, U., 1999. Ligand-binding domain of estrogen receptors. Curr. Opin. Biotechnol. 10, 550–556.
Mukherjee, S., Mani, S., 2010. Orphan nuclear receptors as targets for drug development. Pharm. Res. 27, 1439–1468.
Nagy, L., Schwabe, J.W., 2004. Mechanism of the nuclear receptor molecular switch. Trends Biochem. Sci. 29, 317–324.
Narayanan, R., M.M., Bohl, C.E., Miller, D.D., Dalton, J.T., 2008. Selective androgen receptor modulators in preclinical and clinic. Nucl. Recept. Signal 6.
Nascimento, A., Dias, S.M., Nunes, F.M., Aparício, R., Ambrosio, A.L., Bleicher, L., Figueira, A.C., Santos, M.A., de Oliveira Neto, M., Fischer, H., Togashi, M., Craievich, A.F., Garratt, R.C., Baxter, J.D., Webb, P., Polikarpov, I., 2006. Structural rearrangements in the thyroid hormone receptor hinge domain and their putative role in the receptor function. J. Mol. Biol. 360, 586–598.
Nolte, R., Wisely, G., Westin, S., Cobb, J., Lambert, M., Kurokawa, R., Rosenfeld, M., Willson, T., Glass, C., Milburn, M., 1998. Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-gamma. Nature 395, 137–143.
Oosterveer, M.H., G.A., Groen, A.K., Kuipers, F., 2010. The liver X receptor: control of cellular lipid homeostasis and beyond Implications for drug design. Prog. Lipid Res. 49, 343–352.
Ortlund, E.A., Bridgham, J.T., Redinbo, M.R., Thornton, J.W., 2007. Crystal structure of an ancient protein: evolution by conformational epistasis. Science 317, 1544–1548.
Osborne, C., 1998. Steroid hormone receptors in breast cancer management. Breast Cancer Res. Treat. 51, 227–238.
Pierrat, B., H.D., Chambon, P., Losson, R., 1994. A highly conserved region in the hormone-binding domain of the human estrogen receptor functions as an efficient transactivation domain in yeast. Gene 143, 193–200.
Poukka, H., Aarnisalo, P., Karvonen, U., Palvimo, J.J., Janne, O.A., 1999. Ubc9 interacts with the androgen receptor and activates receptor-dependent transcription. J. Biol. Chem. 274, 19441–19446.
Renaud, J.P., Moras, D., 2000. Structural studies on nuclear receptors. Cell. Mol. Life Sci. 57, 1748–1769.
Renaud, J.-P., Rochel, N., Ruff, M., Vivat, V., Chambon, P., Gronemeyer, H., aDM, 1995. Crystal structure of the RAR-7 ligand-binding domain bound to all-trans retinoic acid. Nature 378, 681–689.
Resche-Rigon, M., Gronemeyer, H., 1998. Therapeutic potential of selective modulators of nuclear receptor action. Curr. Opin. Chem. Biol. 2, 501–507.
Ribeiro, R.C., Feng, W., Wagner, R.L., Costa, C.H., Pereira, A.C., Apriletti, J.W., Fletterick, R.J., Baxter, J.D., 2001. Definition of the surface in the thyroid hormone receptor ligand binding domain for association as homodimers and heterodimers with retinoid X receptor. J. Biol. Chem. 276, 14987–14995.
Rosenfeld, M.G., Glass, C.K., 2001. Coregulator codes of transcriptional regulation by nuclear receptors. J. Biol. Chem. 276, 36865–36868.
Rosenfeld, M.G., Lunyak, V.V., Glass, C.K., 2006. Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. Genes Dev. 20, 1405–1428.
Sadar, M., 2011. Small molecule inhibitors targeting the ''achilles' heel'' of androgen receptor activity. Cancer Res. 71, 1208–1213.
Safer, J.D., C.R., Hollenberg, A.N., Wondisford, F.E., 1998. Defective release of corepressor by hinge mutants of the thyroid hormone receptor found in patients with resistance to thyroid hormone. J. Biol. Chem. 273, 30175–30182.
Sato, Y., S.R., Antony, P., Rochel, N., Moras, D., 2009. Crystal structure of the human VDR ligand binding domain bound to the natural metabolite 1alpha,25-dihydroxy-3-epi-vitamin D3.
Schoch, G.A., D.A.B., Stihle, M., Burger, D., Bär, D., Benz, J., Thoma, R., Ruf, A., 2010. Molecular switch in the glucocorticoid receptor: active and passive antagonist conformations. J. Mol. Biol. 395, 568–577.
Schoenmakers, E., Alen, P., Verrijdt, G., Peeters, B., Verhoeven, G., Rombauts, W., Claessens, F., 1999. Differential DNA binding by the androgen and glucocorticoid receptors involves the second Zn-finger and a C-terminal extension of the DNA-binding domains. Biochem. J. 341 (Pt 3), 515–521.
Shapiro, D.J., M.C., Cherian, M.T., 2011. Small molecule inhibitors as probes for estrogen and androgen receptor action. J. Biol. Chem. 286, 4043–4048.
Shi, X.B., Ma, A.H., Xia, L., Kung, H.J., de Vere White, R.W., 2002. Functional analysis of 44 mutant androgen receptors from human prostate cancer. Cancer Res. 62, 1496–1502.
Shiau, A.K., Barstad, D., Loria, P.M., Cheng, L., Kushner, P.J., Agard, D.A., Greene, G.L., 1998. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95, 927–937.
Smith, C.L., O'Malley, B., 2004. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. Endocr. Rev. 25, 45–71.
Steinmetz, A.C., Renaud, J.P., Moras, D., 2001. Binding of ligands and activation of transcription by nuclear receptors. Annu. Rev. Biophys. Biomol. Struct. 30, 329–359.
Szanto, A., Narkar, V., Shen, Q., Uray, I.P., Davies, P.J., Nagy, L., 2004. Retinoid X receptors: X-ploring their (patho)physiological functions. Cell Death Differ. 11, S126–143.
Tanner, T., C.F., Haelens, A., 2004. The hinge region of the androgen receptor plays a role in proteasome-mediated transcriptional activation. Ann. N. Y. Acad. Sci. 1030.
Tetel, M.J., J.S., Carbajo, P., Ladtkow, T., Skafar, D.F., Edwards, D.P., 1997. Hinge and amino-terminal sequences contribute to solution dimerization of human progesterone receptor. Mol. Endocrinol. 11, 1114–1128.
Thompson, J., Saatcioglu, F., Janne, O.A., Palvimo, J.J., 2001. Disrupted amino- and carboxyl-terminal interactions of the androgen receptor are linked to androgen insensitivity. Mol. Endocrinol. 15, 923–935.
Thornton, J.W., 2001. Evolution of vertebrate steroid receptors from an ancestral estrogen receptor by ligand exploitation and serial genome expansions. Proc. Natl. Acad. Sci. USA 98, 5671–5676.
Thornton, J.W., Kelley, D.B., 1998. Evolution of the androgen receptor: structure–function implications. BioEssays 20, 860–869.
Tilley, W.D., Buchanan, G., Hickey, T.E., Bentel, J.M., 1996. Mutations in the androgen receptor gene are associated with progression of human prostate cancer to androgen independence. Clin. Cancer Res. 2, 277–285.
Umekita, Y., S.Y., Yoshida, H., 1998. Estrogen receptor mutations and changes in estrogen receptor and progesterone receptor protein expression in metastatic or recurrent breast cancer. Jpn. J. Cancer Res. 89, 27–32.
Van Durme, J.J., B.E., Folkertsma, S., Horn, F., Vriend, G., 2003. NRMD: nuclear receptor mutation database. Nucleic Acids Res. 31, 331–333.
Wagner, R.L., Apriletti, J.W., McGrath, M.E., West, B.L., Baxter, J.D., Fletterick, R.J., 1995. A structural role for hormone in the thyroid hormone receptor. Nature 378, 690–697.
Wang, Q., Lu, J., Yong, E.L., 2001. Ligand- and coactivator-mediated transactivation function (AF2) of the androgen receptor ligand-binding domain is inhibited by the cognate hinge region. J. Biol. Chem. 276, 7493–7499.
Wang, Z., Benoit, G., Liu, J., Prasad, S., Aarnisalo, P., Liu, X., Xu, H., Walker, N.P., Perlmann, T., 2003. Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors. Nature 423, 555–560.
Warnmark, A., Treuter, E., Wright, A.P., Gustafsson, J.A., 2003. Activation functions 1 and 2 of nuclear receptors: molecular strategies for transcriptional activation. Mol. Endocrinol. 17, 1901–1909.
Whitfield, G.K., H.J., Nakajima, S., MacDonald, P.N., Thompson, P.D., Jurutka, P.W., Haussler, C.A., Haussler, M.R., 1995. A highly conserved region in the hormone-binding domain of the human vitamin D receptor contains residues vital for heterodimerization with retinoid X receptor and for transcriptional activation. Mol. Endocrinol. 9, 1166–1179.
Williams, S., Sigler, P., 1998. Atomic structure of progesterone complexed with its receptor. Nature 393, 392–396.
Wong, H.Y., Hoogerbrugge, J.W., Pang, K.L., van Leeuwen, M., van Royen, M.E., Molier, M., Berrevoets, C.A., Dooijes, D., Dubbink, H.J., van de Wijngaart, D.J., Wolffenbuttel, K.P., Trapman, J., Kleijer, W.J., Drop, S.L., Grootegoed, J.A., Brinkmann, A.O., 2008. A novel mutation F826L in the human androgen receptor in partial androgen insensitivity syndrome; increased $NH_2^-$/COOH-terminal domain interaction and TIF2 co-activation. Mol. Cell. Endocrinol. 292, 69–78.
Wrenn, C.K.K., Katzenellenbogen, B.S., 1993. Structure-function analysis of the hormone binding domain of the human estrogen receptor by region-specific mutagenesis and phenotypic screening in yeast. J. Biol. Chem. 268, 24089–24098.
Wurtz, J.M., Bourguet, W., Renaud, J.P., Vivat, V., Chambon, P., Moras, D., Gronemeyer, H., 1996. A canonical structure for the ligand-binding domain of nuclear receptors. Nat. Struct. Biol. 3, 206.
Wurtz, J.M., E.U., Heinrich, N., Moras, D., Mueller-Fahrnow, A., 1998. Three dimensional models of estrogen receptor ligand binding domain complexes, based on related crystal structures and mutational and structure-activity relationship data. J. Med. Chem. 41, 1803–1814.
Yan, J., Feng, J., Goldman, D., Cook Jr., E.H., Craddock, N., Jones, I.R., Heston, L.L., Sommer, S.S., 2004. Mutation scanning of the androgen receptor gene in patients with psychiatric disorders reveals highly conserved variants in alcoholic and phobia patients. Psychiatr. Genet. 14, 57–60.
Zhou, Z.X., S.M., Simental, J.A., Lane, M.V., Wilson, E.M., 1994. A ligand-dependent bipartite nuclear targeting signal in the human androgen receptor. Requirement for the DNA-binding domain and modulation by $NH_2$-terminal and carboxyl-terminal sequences. J. Biol. Chem. 269, 13115–13123.
Zhou, X.E., Suino-Powell, K.M., Li, J., He, Y., Mackeigan, J.P., Melcher, K., Yong, E.L., Xu, H.E., 2010. Identification of SRC3/AIB1 as a preferred coactivator for hormone-activated androgen receptor. J. Biol. Chem. 285, 9161–9171.

## 4.2. MUTATIONS IN THE BINDING FUNCTION-3 POCKET OF THE ANDROGEN RECEPTOR ALTER MULTIPLE FUNCTIONS OF THE RECEPTOR

### 4.2.1. Introduction

Because of its pivotal role in PCa and AIS, AR represents a major therapeutic target. It has been proposed that small molecules recruited to surfaces on the LBD could regulate AR activity in CRPC and discovered several modulators of AR function. Surprisingly, the most effective compounds bound preferentially to the BF-3 pocket instead of the AF-2 groove. Whether BF-3 is a protein-protein interaction site remains to be proved but different BF-3 mutations identified both in PCa and AIS patients have been demonstrated to affect strongly AR activity. Further, comparison of AR X-ray structures with and without bound molecules at BF-3 and AF-2 showed structural coupling between both pockets.

In order to try to envision a possible role for the BF-3 pocket, a mutagenesis study to address the *in vitro* functional effects that BF-3 mutations may exert on AR was performed. The transactivation and coactivation capacities of the agonist-bound AR LBD wild-type (WT) and several BF-3 mutants, as well as their N/C interaction and binding to NCoR and SMRT was tested. Moreover, the transactivation activity and nuclear translocation of selected BF-3 variants were also assessed using full-length constructs. The mutated residues are located either lining the BF-3 pocket (I672, F826, N833, R840) or at the boundary between AF-2 and BF-3 (R726, N727) (**Figure 4-2**). Additionally, for control purposes, a mutation (V757A) located at the end of H5 (distant from both studied surfaces) was also included. The selected dataset of mutations studied herein includes variant forms that have been associated with PCa (R726L and V757A), mutants that have been reported in AIS patients (N727K and F826L), as well as mutations that have not been described in any pathology yet (I672R, F826R, N833, R840A, and R840E).

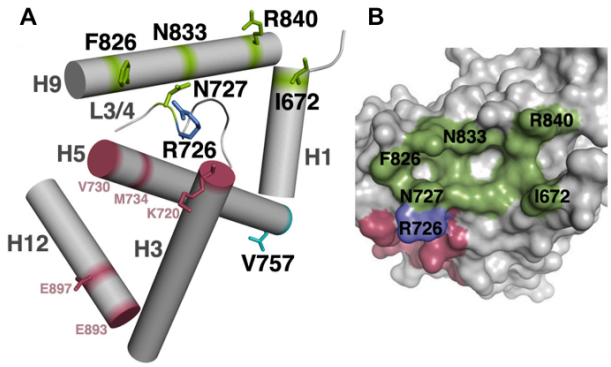


**Figure 4-2.** Structure-based localization of chosen studied mutations in the AR LBD.
**(A)** Simplified model representation of AR LBD structure. Helices H1, H3, H4-H5, H9 and H12 are depicted as grey cylinders. The AF-2 coactivator-binding pocket is limited by H3, H5 and H12, while the BF-3 pocket is formed by H1, H9 and the loop linking H3 with H4-5 (L3/4), which is shown as a thin grey wire. The studied BF-3 residues are shown as sticks and colored green. Key AF-2 residues are highlighted in raspberry and the charge clamp residues K720 and E897 are shown as raspberry sticks. The residues R726 and N727 belonging to the boundary between AF-2 and BF-3 pockets are shown as dark blue and green sticks, respectively. Residue V757, located at the end of H5, is depicted as a blue stick. **(B)** Solid surface representation of AR LBD, in grey; showing the residues lining BF-3, in green; AF-2, in raspberry; and residues R726, in blue.

### 4.2.2. BF-3 Mutations Affects AR AF-2 Activity

Evaluating the transactivation ability of all mutants at different hormone concentrations, it was found that I672R, N833R, and R840A behaved as super-AR variants at the highest amount of both DHT (**Figure 4-3**). While the activity of N833R and R840A was similar to that of WT at all lower DHT concentrations, I672R already displayed a greater transactivation capacity at 10 nM. Interestingly, N833R and R840A were the only mutations, together with R840E, that were inactive at a concentration of 1 nM DHT. Mutant F826L manifested a moderately enhanced AR AF-2 activity at 10 nM and 100 nM DHT, despite not showing a super-AR behavior. The activation level of mutations R726L, N727K, V757A and F826R resembled to that of WT at all hormone concentrations, although F826R significantly attenuated the receptor activity at 100 nM DHT. Finally, replacing the arginine at position 840 by a glutamic acid (R840E) completely abolished AR LBD activity, being inactive at all hormone concentrations.

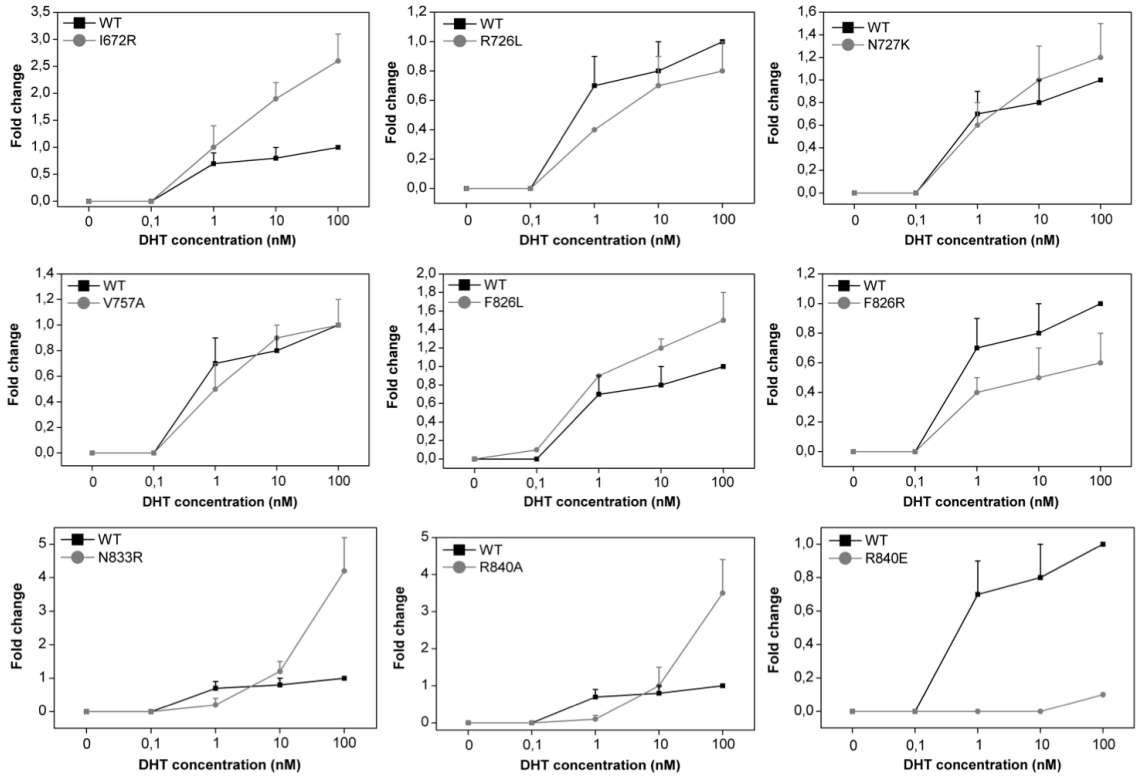


**Figure 4-3.** AF-2 transactivation activity of the AR LBD BF-3 mutants.

HeLa cells were transfected with 100 ng cDNA of the AR LBD WT or the corresponding BF-3 mutant and treated with increasing concentrations of DHT (0-0,1-1-10-100 nM). AR LBD WT at 10 nM = 1. Results are the mean of three independent experiments performed in triplicate.

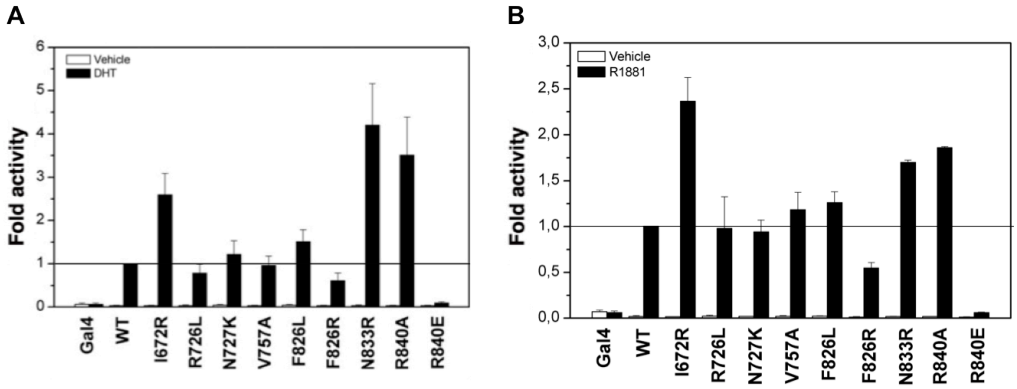


**Figure 4-4.** AF-2 transcriptional activity of AR LBD WT and BF-3 mutants.

AR AF-2 activity in the absence (vehicle) and presence of 100 nM DHT **(A)** or 100 nM R1881 **(B)**. WT AR LBD AF-2 activity in the presence of hormone = 1. Results are the mean of five independent experiments performed in triplicate.

The results obtained at the highest concentration of the synthetic DHT analogue R1881 (metribolone, methyltrienolone) were very similar to those found with DHT, although the three AR super-mutant variants did not show such an elevated activity (**Figure 4-4**).

The WT and all mutant AR LBDs investigated exhibited comparable levels of expressed protein as assessed by western blot (**Figure 4-5**).

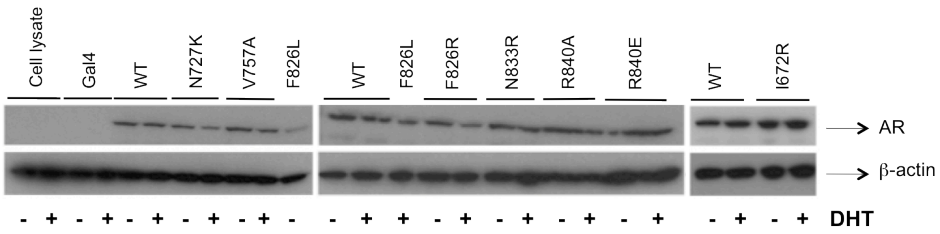


**Figure 4-5.** Protein expression level of AR LBD WT and BF-3 mutants.
Western blot showing the protein expression level of AR LBD WT and BF-3 mutants in the absence (-) and presence (+) of hormone (100 nM).

### 4.2.3. BF-3 Mutations Affect AR LBD Coactivation by GRIP1 and Disturb the N/C Interdomain Interaction

The functionality of the studied AR LBDs was further investigated by measuring the AR AF-2 transactivation capacity in the presence of an AR coactivator, as well as by addressing the interaction of the AR LBD with its NTD.

The effect of the coactivator glucocorticoid receptor interacting protein 1 (GRIP1), also termed transcription intermediary factor 2 and SRC2, was first determined on the activity of the WT and BF-3 forms of the AR LBD. GRIP1, which is known to associate with the AR AF-2 pocket in a hormone-dependent manner, enhanced the activity of all mutants but with variations in the extent of its potentiation (**Figure 4-6A**). The coactivation fold increase of mutants R726L and V757A by GRIP1 was slightly more elevated than the one exhibited by the WT, while F826R clearly manifested a higher increase in activity in the presence of GRIP1 when compared to the WT. Interestingly, on the one hand, super-mutant N833R showed the smallest fold change in coactivation, without reaching WT-levels; whereas, on the other hand, the coactivation that GRIP1 exerted on the dead mutant R840E was very similar to that of the WT form. Finally, variants I672R, N727K, F826L and

R840A displayed a coactivation fold increase comparable to that of WT.

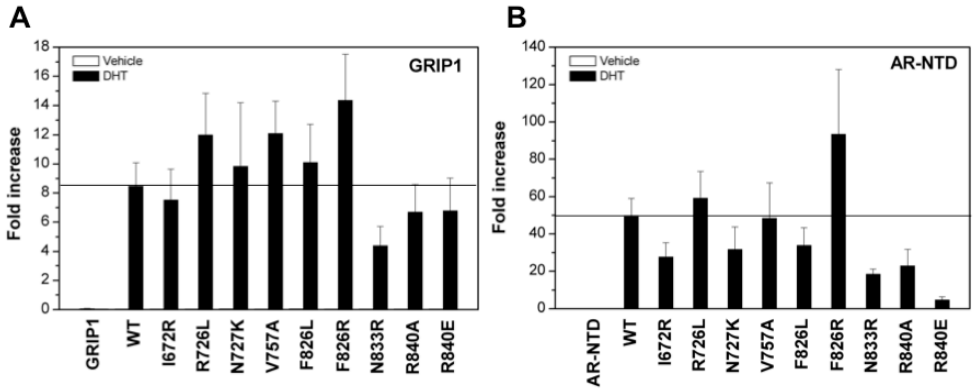


**Figure 4-6.** AR AF-2 coactivation by GRIP1 and N/C interaction of the WT and BF-3 mutant LBDs.

**(A)** AF-2 coactivation of AR LBD WT and BF-3 mutants by GRIP1, in the absence (vehicle) and presence of 100 nM hormone (DHT). **(B)** Mammalian two-hybrid showing the interaction of the LBD of AR LBD WT and the different BF-3 mutants with the AR NTD in the absence (vehicle) and presence (DHT) of 100 nM hormone. AR LBD WT in the absence of GRIP1/AR-NTD = 1. Results are the mean of three independent experiments performed in triplicate.

To assess whether mutations in the BF-3 pocket affect the interaction between the N- and C-terminal domains of AR, a mammalian two-hybrid was performed. Again, most of the BF-3 mutants demonstrated an altered AR N/C interaction although the profiles were different from that observed with GRIP1. Interestingly, the three super-mutant AR LBDs, I672R, N833R and R840A, exhibited the weakest association with the NTD (**Figure 4-6B**). Moreover, the replacement of the phenylalanine at position 826 by an arginine, which exposed the lowest receptor transactivation capacity (Figure 4-3) and the highest fold increase in the presence of GRIP1 (Figure 4-6A), revealed the strongest binding to the NTD. Mutants R726L and V757A, associated with PCa, manifested an interaction similar to the one showed by the WT; whereas variants N727K and F826L, related to AIS, displayed a mild impairment in the recruitment of the NTD. Finally, substituting the arginine at position 840 for a glutamic acid seemed to completely prevent the receptor from associating with its NTD.

### 4.2.4. BF-3 Alters the Association of the AR LBD with the NR Corepressors NCoR and SMRT

Since the AF-2 groove of DHT-liganded AR is known to interact weakly with NR corepressors, a mammalian two-hybrid assay was used to evaluate how BF-3 mutants affect the association with corepressors NCoR and SMRT. As seen with the AR NTD, BF-3 mutants broadly influenced the capacity of AR to bind to corepressors and, interestingly, most of them showed a weaker interaction with both NCoR (**Figure 4-7A**) and SMRT (**Figure 4-7B**) when compared to the WT, suggesting a possible corepressor role for the BF-3 pocket. Super-mutant R840A displayed the weakest association with the receptor interacting domains of both corepressors, although I672R, V757A, F826L and the dead mutant R840E also reduced the binding to the two corepressors. Super-mutant N833R and variant F826R demonstrated an impaired interaction with SMRT, but maintained a WT-like association with NCoR, whereas N727K exhibited a decreased binding to NCoR but left the interaction with SMRT unaffected. Finally, mutation R726L had little effect on corepressor binding.

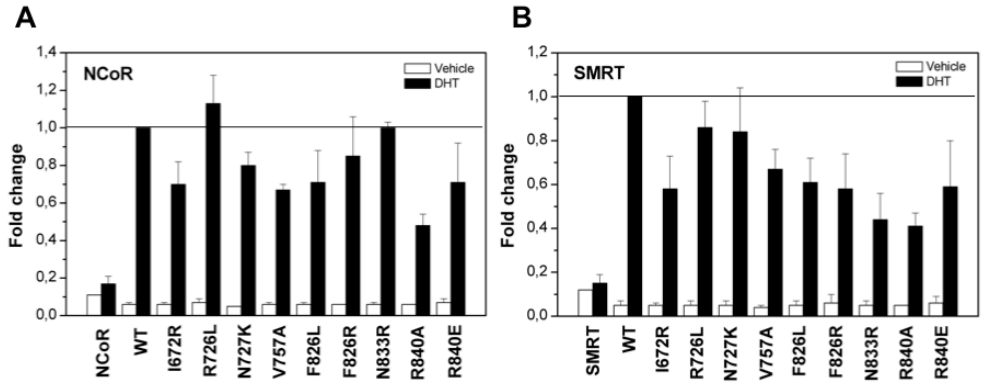


**Figure 4-7.** AR LBD WT and BF-3 mutants interaction with NCoR and SMRT.

Mammalian two-hybrid assays showing the interaction between all studied AR LBDs and NCoR **(A)** or SMRT **(B)** in the absence (vehicle) or presence (DHT) of 10 nM hormone. Interaction of the AR LBD WT with NCoR/SMRT = 1. Results are the mean of three independent experiments performed in triplicate.

### 4.2.5. Effect of the BF-3 mutations on the Activity of the Full-Length Androgen Receptor

To further examine the effects that mutations in the BF-3 pocket may exert on the AR, their influence in the nuclear import of the receptor was also explored. As the nuclear translocation signal of the AR resides at the end of the DBD and beginning of the hinge region, for this study the full-length receptor was explored. Because the consequences that BF-3 mutations may exercise on the activity of the full-length receptor might differ from those observed in the context of the isolated LBD, the transactivation capacity of the full-length mutants at different concentrations of mibolerone, an anabolic steroid with a chemical structure similar tot that of the DHT, was first tested.

Interestingly, mutants I672R and R840A, the isolated LBD of whom exhibited an abnormally enhanced transactivation capability at the highest hormone concentration, manifested a slightly lower activity than the WT in the context of the full-length receptor (**Figure 4-8**). Additionally, they both proved a WT-like activity at 10 nM mibolerone, although the LBD of I672R had evidenced an improved transactivation capacity when the cells were stimulated with 10 nM DHT. Furthermore, as already observed for the LBD of variant R840A, it needed a higher concentration of mibolerone than the WT in order to be activated. Mutants N727A and V757A displayed an activation profile almost identical to that of the WT and to the one obtained with their isolated LBDs (with a lysine substitution at position 727 instead of the asparagine). R840E again demonstrated a defective transactivation capacity at all hormone concentrations, despite at 100 nM mibolerone exhibited a more elevated activity than its isolated LBD. Finally, mutant G724R, which was not tested before, needed higher amounts of hormone to be activated, being inactive at the lowest concentrations of mibolerone, showing an attenuated activity at 10 nM, and reaching a WT-like activity at the highest concentration.

No significant differences were seen at protein expression levels (**Figure 4-9**).

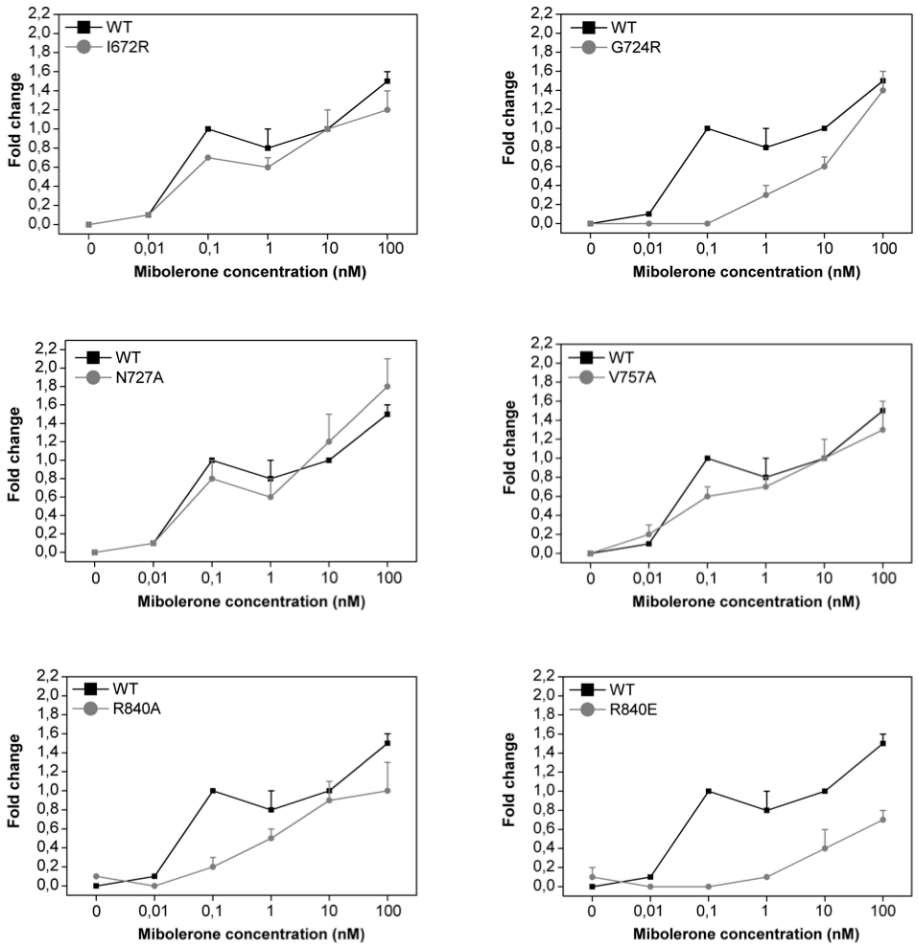


**Figure 4-8.** Activity of the AR full-length BF-3 mutants.

COS-1 cells were transfected with 50 ng DNA of the AR WT or the corresponding BF-3 mutants and treated with increasing concentrations of mibolerone (0-0,01-0,1-1-10-100 nM). AR WT at 10 nM mibolerone = 1. Results are the mean of three independent experiments performed in triplicate.

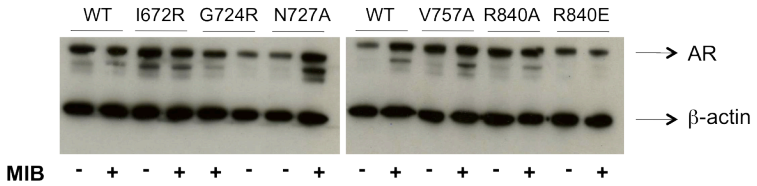


**Figure 4-9.** All BF-3 mutants are expressed at the same level than the WT receptor.

Western blot showing the protein expression level of full-length AR WT and BF-3 mutants in the absence (-) and presence (+) of 10 nM mibolerone (MIB).

### 4.2.6. The Androgen Receptor Mutant R840E Exhibits a Decreased Nuclear Import

Finally, after having assessed the consequences of the BF-3 mutations on the transactivation capacity of the AR AF-2 in the context of the full-length receptor, and in order to investigate whether these mutations disturb the nuclear translocation of the receptor, the cytoplasmic-nuclear trafficking of the full-length AR was studied by immunofluorescence. Cells were transfected with the corresponding expression construct and left untreated or stimulated with hormone for 15 minutes, 2 hours and 16 hours, when samples were collected and the immunofluorescence was performed.

In the absence of hormone, all proteins were located in the cytoplasm (**Figure 4-10**). Under the stimulation of mibolerone, the AR WT entered rapidly into the nucleus, completing the import process in 15 minutes (**Figure 4-10A**). However, the cytoplasmic-nuclear trafficking of all BF-3 variants was significantly decreased, since only a very low proportion of the proteins was nuclear in the presence of mibolerone, indicating that an incubation of 15 minutes was not enough time for the mutant receptors to translocate to the nucleus. The 2-hour treatment revealed interesting observations. Stimulating the cells with hormone for 2 hours induced the nuclear import of all BF-3 mutants, but part of R840E still persisted in the cytoplasm (**Figure 4-10B**). No differences were appreciated between the 2-hour and the 16-hour incubation, as the WT receptor and all BF-3 mutants (with the exception of R840E) were exclusively nuclear (**Figure 4-10C**). Not even treating the cells with mibolerone for 16 hours, did R840E manage to complete the import process, denoting it may need more time or a higher concentration of hormone to translocate completely to the nucleus.

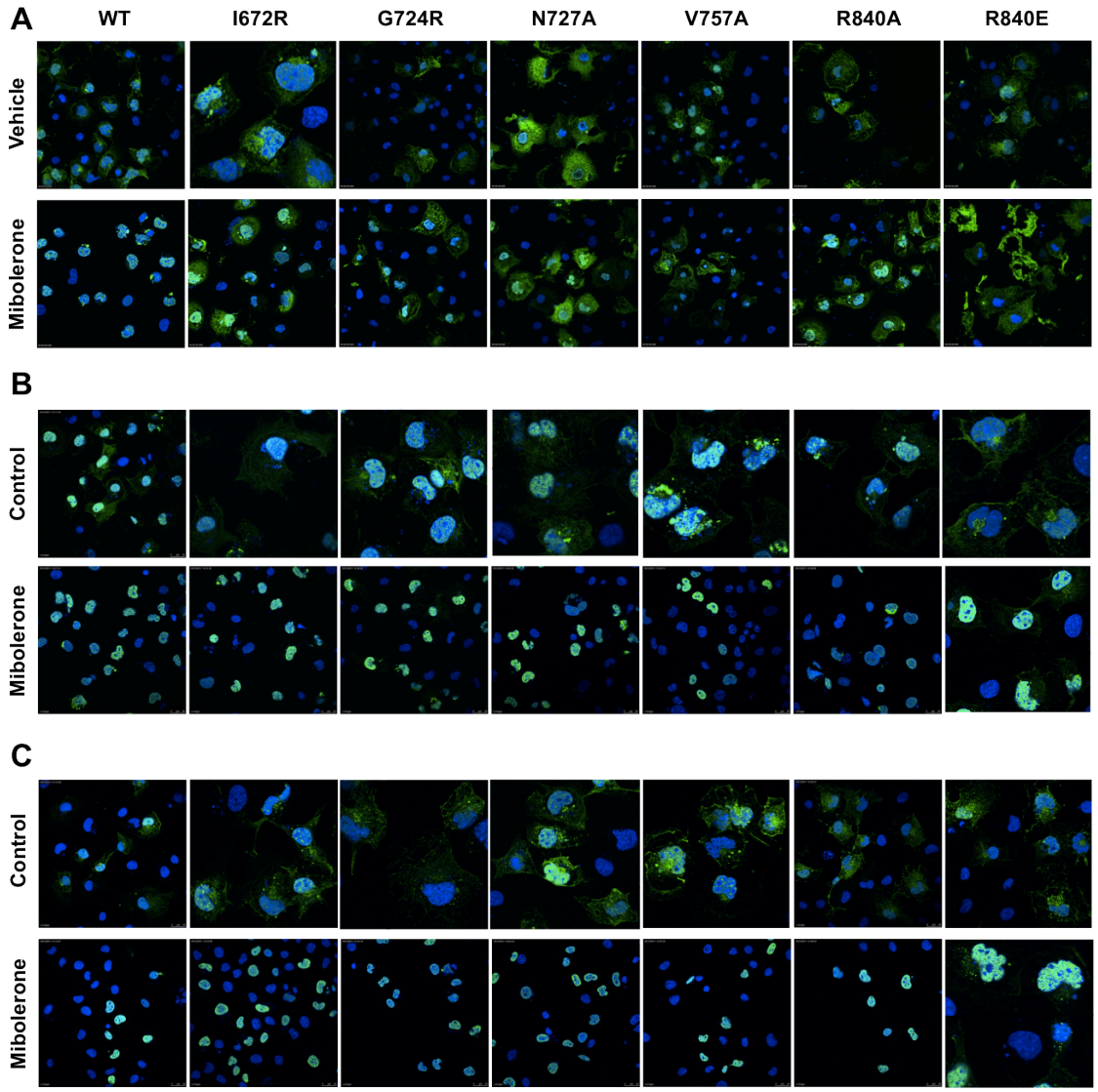


**Figure 4-10.** Nuclear translocation studies of the AR WT and BF-3 mutants.

COS-1 cells were transfected with 1 μg AR WT or the corresponding BF-3 mutant and left untreated (control) or stimulated with mibolerone (10 nM) for 15 minutes **(A),** 2 hours **(B)**, and 16 hours **(C)**. Confocal immunofluorescence was performed using anti-AR antibody (green) and cells were counterstained with DAPI (blue). Cellular localization of the expressed AR proteins was analyzed by a confocal microscopy at 60x magnification.

## 4.2.7. Discussion

Hundreds of mutations responsible for PCa and AIS have been documented in the *AR* gene, altering androgen binding and dissociation, transactivation, and coregulator recruitment, as well as its N/C-terminal domains interaction (Gottlieb et al., 2012). This study confirms and complements previous mutational analysis, which already revealed that BF-3 variants alter overall AR activity in different

ways (Ahmed et al., 2000; Estébanez-Perpiñá et al., 2007; MacLean et al., 2004; Petroli et al., 2011; Thompson et al., 2001; Tilley et al., 1996; Yong et al., 1994), demonstrating that the BF-3 pocket is highly important for AR function. Here, it is shown that mutants either in BF-3 or lying between AF-2 and BF-3 produce a wide variety of effects, ranging from super-activators (I672R, N833R, R840A) and moderate enhancers (F826L), to weak inhibitors (F826R) and dead mutants (R840E), including variants that maintain a WT-like activity (R726L, N727K). Different consequences on GRIP1 coactivation, as well as diverse outcomes in NTD and corepressor interactions have also been presented (summarized in **Table 3**).

| | | | AR LBD ACTIVITY | | | | AR INTERACTION | | |
|---|---|---|---|---|---|---|---|---|---|
| **AR LBD** | **Site** | **Associated with** | **1 nM** | **10 nM** | **100 nM** | **GRIP1** | **NTD** | **NCoR** | **SMRT** |
| WT | | | | WT | WT | WT | WT | WT | WT |
| I672R | BF-3 | | WT | ++ | ++ | WT | - | - | - |
| R726L | AF-2 BF-3 | PCa | - | WT | WT | + | WT | WT | WT |
| N727K | AF-2 BF-3 | AIS | WT | WT | WT | WT | - | - | WT |
| V757A | H5 | PCa | WT | WT | WT | + | WT | - | - |
| F826L | BF-3 | AIS | WT | + | + | WT | - | - | - |
| F826R | BF-3 | | - | - | - | + | + | WT | - |
| N833R | BF-3 | | - | WT | +++ | -- | -- | WT | -- |
| R840A | BF-3 | | - | WT | +++ | WT | -- | -- | -- |
| R840E | BF-3 | | -- | -- | -- | WT | --- | - | - |

**Table 3.** Summary of the experimental results obtained with the AR LBD BF-3 mutants.
Table showing the location of the mutations, the pathology they have been related to, the AF-2 activity, the coactivation by GRIP1, and the interaction with the N-terminal domain of AR, as well as with NCoR and SMRT.

Although the AR mutation I672R has never been found in any AR-related disease in patients, a threonine substitution has been identified in a human PCa tumor (Tilley et al., 1996). When I672 is substituted by an arginine in the isolated LBD construct, the receptor maintained a WT-like activity at low hormone levels. However, as earlier reported (Estébanez-Perpiñá et al., 2007), it was greatly enhanced at supraphysiological concentrations of hormone, although the coactivation by GRIP1 was quite similar to that of WT, and its interaction with the NTD, weaker. Importantly, the binding to both corepressors was markedly

affected, being considerably feebler than the WT, maybe explaining the I672R super-activating effect. Contrarily, the full-length construct maintained a WT-like activity. The replacement of the hydrophobic isoleucine by the positively charged arginine seemed to have a profound effect on the transactivation capacity of the LBD but remained impassive in the full-length construct.

Variants G724S, G724D, G724A, and G724V have been documented in CAIS patients (Ahmed et al., 2000; Hannema et al., 2004; Jääskeläinen et al., 2006; MacLean et al., 2004), mutants G724S, G724D and G724V resulting in no hormone binding (Ahmed et al., 2000; Jaaskelainen, 2006), whereas G724A maintained normal levels of androgen association (MacLean et al., 2004). AR activity of substitutions G724D and G724V has been demonstrated to be impaired in the presence of 1 nM hormone, reaching AR WT transactivational capacity at a concentration of 10 nM and 100 nM, respectively (Hannema et al., 2004; Jaaskelainen, 2006). In line with these results, we illustrate that the full-length receptor mutant G724R possessed a critically reduced activity in the presence of low hormone levels but reached a WT-like transactivation at the highest tested concentration, as it has previously been shown (Estébanez-Perpiñá et al., 2007). Interestingly, all SRs have a glycine at position 724, in a conserved block between helices 2 and 3 of the LBD (Matias et al., 2000), suggesting it is essential for normal receptor function. Moreover, in the case of AR, G724 is located in a region known to be involved not only in ligand binding but also in the AR N/C-terminal interaction (Jaaskelainen, 2006).

Mutation R726L has been identified in several men suffering from PCa (Elo et al., 1995; Hyytinen et al., 2002; Koivisto et al., 2004; Koivisto et al., 1999; Mononen et al., 2000), and different and contradictory effects on AR transactivation, coactivation by SRCs, N/C interaction, and binding affinities for p160 proteins have been reported (Estébanez-Perpiñá et al., 2007; Hay and McEwan, 2012; Thompson et al., 2001). Similarly to what Thompson et al. demonstrated, we show that R726L transactivation activity and N/C interaction were WT-like, whereas AF-2 was activated by GRIP1 a little better than the WT receptor. The binding either to NCoR or SMRT was unaffected. Arginine 726 can be defined as a boundary between AF-2 and BF-3 grooves and it seems to play an important role in transmitting the

conformational changes from the former pocket to the latter, linking both surfaces (Estébanez-Perpiñá et al., 2007; 2005; Grosdidier et al., 2012). In addition, the crystal structures of the AR LBD in complex with peptides derived from different SRCs have revealed that R726 adopts the C-terminal capping role, stabilizing the bound coactivator peptide (Estébanez-Perpiñá et al., 2005). Thus, although according to our results, this mutant cannot be related to PCa by an alteration in any of the molecular mechanism we tested, its implication in the disease could be ascribed to an enhanced interaction with AR coactivators.

AR variant N727K was detected in a 51-year-old man suffering from MAIS (Yong et al., 1994), and has been shown to display a disrupted ligand-dependent transactivation function, as well as defective protein-protein interactions between receptor domains and coactivator proteins, without altering any ligand-binding characteristic of the receptor (Lim, 2000; Yong, 2003). Here all activities were WT-like, except from the binding to the NTD and NCoR, which were slightly weaker than the exhibited by the WT. When this asparagine is replaced by an alanine in the full-length construct, the mutant form also behaved as the WT in terms of the transactivation capacity. Similarly to R726, the asparagine at position 727 lies in the boundary between the AF-2 and BF-3 pockets, linking both surfaces, and transmitting information from the former to the latter (Estébanez-Perpiñá et al., 2007), maybe playing a role in the recruitment of coactivators at AF-2.

F826L mutant was reported in a 3-year-old boy with PAIS, exposing an activity identical to the WT form with respect to ligand binding, transactivation and repression but, unexpectedly, the N/C-terminal interaction and GRIP1 coactivation were found to be enhanced in comparison to the WT (H. Y. Wong et al., 2008). In contrast to their study, our results illustrate that the receptor activity was marginally higher than the WT, and the GRIP1 coactivation, WT-like. The interaction between domains was faintly impaired, whereas with corepressors was substantially compromised. An amino acid substitution at AR position 826, changing the phenylalanine for an arginine has never been described yet. Despite the vaguely diminished transactivation activity, mutant F826R showed a better coactivation by GRIP1 and a stronger binding to the NTD than the WT. The

recruitment of NCoR was very similar to that of the WT, but the interaction with SMRT was severely weakened.

Mutations at residue N833 in the *AR* gene associated to pathology have never been identified yet, although its deletion has been reported in a CAIS patient (Audi et al., 2010). We illustrate that when this residue was replaced by an arginine, the receptor behaved as a super-AR at the highest hormone concentration. However, at 10 nM DHT, the transactivation capacity was identical to that of the WT, whereas at 1 nM, was completely inactive, suggesting that the low transactivation capacity can be overcome in the presence of high concentrations of androgen. Surprisingly, in spite of being the mutant that exposed the most elevated activity at 100 nM hormone, was the one that presented the weakest coactivation by GRIP1 and the feeblest N/C-interaction. Furthermore, the binding to SMRT was also substantially reduced, although the recruitment of NCoR remained unaffected.

Finally, multiple mutations at residue R840 (R840C, R840G, R840H, R840S) have been described in patients, all of them leading to variable degrees of AIS (Beitel et al., 1994; Bevan et al., 1996; Bouvattier et al., 2002; Georget et al., 1998; Lundberg Giwercman et al., 1998; McPhaul and Marcelli, 1992; Szafran et al., 2009), although neither a replacement by an alanine nor a glutamic have ever been reported. Detailed molecular studies have established that the substitution of this arginine affect multiple functions of the receptor (Beitel et al., 1994; Bevan et al., 1996; Marcelli et al., 1994; Szafran et al., 2009), emphasizing a decreased transactivation function in a wide range of different hormones concentration (Bevan et al., 1996; Marcelli et al., 1994). Here it was shown that similarly to N833R, variant R840A conferred a greatly boosted activity to the receptor at the highest hormone concentration, showed a WT-activity at 10 nM DHT, and was completely inactive at 1 nM, demonstrating its ability to increase activity in response to an increase in hormone concentration. Coactivation by GRIP1 was WT-like, and interaction with both the NTD and the corepressors was significantly compromised, being the weakest of all mutants. Surprisingly, the full-length construct manifested a defective transactivation at all hormone concentrations, results that are more in line with the published data for mutant R840C (Bevan et al., 1996). On the other hand, completely the opposite was observed for the glutamic mutant R840E, which

was not capable of being activated by the hormone, nor interacting with the NTD. However, the fold increase in activity obtained in the presence of GRIP1 was comparable to that of WT. The binding to both corepressors was faintly impaired. Despite the full-length construct displayed an improved activity when compared to the isolated LBD, it never reached WT levels. Probably, reversing the arginine positive charge with the negatively charged glutamic kills the receptor.

PCa-related I672R, and AIS-associated G724R, N727A, R840A, and R840E full-length BF-3 variants were further characterized by assessing whether these mutations disturbed the nuclear translocation of the receptor. The positively charged NLS, which is highly conserved among all SRs (Cutress et al., 2008) plays an important role in nuclear trafficking. AR possesses a bipartite NLS, in which the first (minor) cluster ($^{617}$RKCY$^{620}$) is located within the terminal $\alpha$-helix of the second zinc finger of the AR DBD, whereas the major cluster ($^{629}$RKLKK$^{633}$) resides in the hinge domain (Z. X. Zhou et al., 1994). The mutation of several residues within the AR NLS have been associated with both PCa and AIS (Gottlieb et al., 2012), despite it is not clear whether the nuclear import or transcriptional activity of these AR mutants is affected. However, mutating the lysines in the acetylation motif ($^{630}$KLKK$^{633}$) within the AR NLS has been shown to influence the intracellular location of AR, DNA binding, and receptor folding and aggregation, as well as increase or prevent the binding of specific coregulators (Tanner T, 2004; Tanner et al., 2010).

Here we show that the BF-3 mutants did not enter into the nucleus as rapidly as the WT, since the latter was almost exclusively nuclear after treating the cells with mibolerone for only 15 minutes, whereas the mutants still persisted in the cytoplasm. Stimulating the cells with hormone for 2 hours revealed interesting observations. While the nuclear translocation process was completed for mutants I672R, G724R, N727A, and R840A, only a low proportion of R840E was nuclear. No differences were visible between the 2-hour treatment and the 16-hour treatment, as the longest stimulation with mibolerone also failed to complete the nuclear translocation of variant R840E. This finding was especially exciting because this variant needed higher concentrations of mibolerone to be activated and proved a diminished transactivation capacity compared to the WT receptor, opening the

possibility that partially decreased nuclear import might be responsible for its defective activity.

Interestingly, it has been described that when cells are incubated in the presence of a high concentration of hormone (1 μM), the kinetics of the AIS mutant R840C was indistinguishable from the kinetics of normal receptor. However, the nuclear import at 10 nM (the same concentration that has been employed in our assays) decreased partially, and at 1 nM DHT, just a low proportion of AR R840C was nuclear (Georget et al., 1998). Thus, the trafficking rate of the mutant receptor seems to decrease as a function of hormone concentration, which maintains constant for the WT, indicating that only supraphysiological concentrations of hormone are capable of inducing the nuclear import of the mutant receptor. Therefore, it might be possible that by incubating the cells with higher hormone concentrations or for longer periods of times, the mutant R840E could maybe manage to complete the nuclear import process.

It is evident from the many published studies that no simple correlation exists between the severity of the receptor dysfunction observed in *in vitro* cell assays and the clinical phenotype, in part because same mutations can apparently cause different phenotypes. The arginine to cysteine substitution in residue 840, for example, has been identified in two brothers with very different degrees of virilization (Bevan et al., 1996). This observation reveals that in most cases knowledge of the AR mutation does not provide enough information to predict the individual phenotype, as factors outside the AR coding region can profoundly influence the expression of AR itself or influence the available levels of circulating androgens. However, the *in vitro* functional consequences of AR mutations may indicate how a patient carrying that mutation may respond to androgen therapy. Currently, PAIS patients raised as males may be given high doses of androgens in early infancy or puberty in an attempt to improve virilization (Bevan et al., 1996). For example, mutants N833R and R840A seem to be inactive at 1 nM DHT but reach WT-like activity at 10 nM DHT, indicating that this defect may be overcome by increasing hormone levels. If the *in vitro* performance of the receptor mimics the performance of the receptor *in vivo*, the virilization of patients carrying N833R

and R804A mutations may occur in response to increased circulating concentrations of hormone.

# Allosteric Conversation in the Androgen Receptor Ligand-Binding Domain Surfaces

Solène Grosdidier,* Laia R. Carbó,* Víctor Buzón, Greg Brooke, Phuong Nguyen, John D. Baxter,‡ Charlotte Bevan, Paul Webb, Eva Estébanez-Perpiñá,† and Juan Fernández-Recio†

Joint Barcelona Supercomputing Center (BSC)-Institute for Research in Biomedicine (IRB) Research Programme in Computational Biology (S.G., J.F.-R.), Life Sciences Department, Barcelona Supercomputing Center, 08034 Barcelona, Spain; Institute of Biomedicine (IB) (L.R.C., V.B., E.E.-P.), University of Barcelona (UB), Barcelona Science Park, 08028 Barcelona, Spain; Genomic Medicine (P.N., J.D.B., P.W.), The Methodist Hospital Research Institute, Houston Texas; and Androgen Signalling Laboratory (G.B.,C.B.), Department of Surgery and Cancer, Imperial College London, London W12 0NN, United Kingdom

Androgen receptor (AR) is a major therapeutic target that plays pivotal roles in prostate cancer (PCa) and androgen insensitivity syndromes. We previously proposed that compounds recruited to ligand-binding domain (LBD) surfaces could regulate AR activity in hormone-refractory PCa and discovered several surface modulators of AR function. Surprisingly, the most effective compounds bound preferentially to a surface of unknown function [binding function (BF-3)] instead of the coactivator-binding site [activation function-2 (AF-2)]. Different BF-3 mutations have been identified in PCa or androgen insensitivity syndrome patients, and they can strongly affect AR activity. Further, comparison of AR x-ray structures with and without bound ligands at BF-3 and AF-2 showed structural coupling between both pockets. Here, we combine experimental evidence and molecular dynamic simulations to investigate whether BF-3 mutations affect AR LBD function and dynamics possibly via allosteric conversation between surface sites. Our data indicate that AF-2 conformation is indeed closely coupled to BF-3 and provide mechanistic proof of their structural interconnection. BF-3 mutations may function as allosteric elicitors, probably shifting the AR LBD conformational ensemble toward conformations that alter AF-2 propensity to reorganize into subpockets that accommodate N-terminal domain and coactivator peptides. The induced conformation may result in either increased or decreased AR activity. Activating BF-3 mutations also favor the formation of another pocket (BF-4) in the vicinity of AF-2 and BF-3, which we also previously identified as a hot spot for a small compound. We discuss the possibility that BF-3 may be a protein-docking site that binds to the N-terminal domain and corepressors. AR surface sites are attractive pharmacological targets to develop allosteric modulators that might be alternative lead compounds for drug design. **(*Molecular Endocrinology* 26: 0000–0000, 2012)**

Androgen receptor (AR, NR3C4) is a ligand-activated transcription factor (1) that belongs to the nuclear receptor (NR) superfamily (2). AR plays specific roles in male development, prostate cancer (PCa), androgen insensitivity syndromes (AIS), and the rare neurodegenerative spinal and bulbar muscular atrophy (3–13). Like other NR, AR displays a modular architecture, composed of an N-terminal domain (NTD), a DNA-binding domain (DBD), a hinge region, and a C-terminal ligand-binding domain (LBD) (14). AR LBD adopts the canonical NR LBD fold (15): a three-layered $\alpha$-helical sandwich with the ligand buried inside the hydrophobic ligand-binding pocket (LBP) [Fig. 1, supporting information (SI); and

ISSN Print 0888-8809 ISSN Online 1944-9917
Printed in U.S.A.

doi: 10.1210/me.2011-1281 Received October 12, 2011. Accepted April 23, 2012.

* S.G. and L.R.C. contributed equally to this work.
† E.E-P and J.F-R. are co-corresponding authors.
‡ Deceased, October 5, 2011.

Abbreviations: AF-2, Activation function-2; AIS, androgen insensitivity syndromes; AR, androgen receptor; BF-3 and -4, binding functions 3 and 4; DBD, DNA-binding domain; DHT, dihydrotestosterone; FxxLF, phenylalanine-rich motif; GRIP1, GR-interacting protein1; H12, helix 12; LBD, ligand-binding domain; LBP, ligand-binding pocket; LxxLL, Leucine-rich motif; MD, molecular dynamics; N-CoR, nuclear receptor corepressor; $NH_2$/COOH interaction, amino-terminal/carboxy-terminal interaction; NTD, N-terminal domain; NR, nuclear receptor; PCa, prostate cancer; SMRT, silencing mediator of retinoid and thyroid receptors; SI, supporting information; WT, wild-type.

**TABLE 1.** Summary of the experimental results obtained *in vitro* using AF-2 transcriptional activation and mammalian 2-hybrid assays with AR LBD WT and mutant variants, and computational parameters from their corresponding MD simulations.

| AR LBD | Site | Luciferase Activity | Fold Increase Activity with GRIP1 | Fold Increase NTD Interaction | Fold Increase NCoR Interaction | Fold Increase SMRT Interaction | rel mean all b-factor | rel mean BF-3 b-factor | rel mean AF-2 b-factor | BF-3-AF-2 corr ($r^2$) | BF-3-LBP corr ($r^2$) | avgeASA DHT |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WT | | WT | WT | WT | WT | WT | 1.00 | 1.00 | 1.00 | 0.34 | 0.13 | 0.33 |
| **Super-Activating** | | | | | | | | | | | | |
| N833R | BF-3 | VERY HIGH | LOWER | VERY LOW | WT | LOWEST | 0.90 | 1.36 | 1.07 | 0.12 | 0.05 | 1.90 |
| R840A | BF-3 | VERY HIGH | WT | VERY LOW | LOWEST | LOWEST | 0.87 | 0.94 | 1.26 | 0.50 | 0.17 | 1.22 |
| I672R | H1-BF-3 | VERY HIGH | WT | VERY LOW | LOWER | LOWER | 1.14 | 1.53 | 0.92 | 0.87 | 0.03 | 0.19 |
| **PCa** | | | | | | | | | | | | |
| V757A | H5 | WT | HIGHER | HIGHER-MILD | LOWER | LOWER | 1.38 | 1.64 | 1.14 | 0.89 | 0.10 | 0.14 |
| R726 L | BF-3 AF-2 | WT | HIGHER | HIGHER-MILD | WT | WT | 1.13 | 1.34 | 1.14 | 0.72 | 0.36 | 2.48 |
| **AIS** | | | | | | | | | | | | |
| N727K (MAIS) | BF-3 AF-2 | HIGHER-MILD | HIGHER-MILD | LOWER-MILD | LOWER | WT | 0.83 | 1.14 | 1.33 | 0.76 | 0.16 | 3.73 |
| F826 L (PAIS) | BF-3 | HIGHER-MILD | HIGHER-MILD | LOWER-MILD | LOWER | LOWER | 0.83 | 1.04 | 0.91 | 0.61 | 0.01 | 1.70 |
| **Inhibiting** | | | | | | | | | | | | |
| F826R | BF-3 | LOWER | HIGHEST | HIGHEST | WT | LOWER | 0.92 | 0.81 | 1.12 | 0.49 | 0.16 | 0.33 |
| R840E | H1-BF-3 | DEAD | WT | LOWEST | LOWER | LOWER | 0.86 | 0.87 | 1.03 | 0.50 | 0.01 | 0.25 |

Rel, Relative.

Table 1]. The LBD harbors a major coactivator binding surface [activation function-2 (AF-2)], which acts as a docking site for short hydrophobic peptide motifs (NR boxes) featured in AR coactivators and in the AR NTD and mediates AR functional amino/carboxy (N/C)-terminal interaction (16–20).

Androgen binding is known to trigger widespread structural and dynamic alterations within the AR LBD, although detailed structural data are missing. By analogy with other NR LBDs, unliganded (apo-) AR LBD may feature a dislodged helix 12 (H12) adopting an unstructured molten globule organization. Upon admission of the hormone into the core of the LBD, the overall stability of the domain is increased, achieving a more defined structure (21). The best-described allosteric rearrangement in NRs upon ligand binding takes place with the conformational change of H12 that completes AF-2 (22).

AR LBD is subject to mutations in advanced PCa and AIS (Androgen Receptor Gene Mutations Database: http://androgendb.mcgill.ca). PCa mutations often result in increased transactivation or expanded ligand binding preference (5, 7, 8, 10, 23, 24). Conversely, AIS mutations usually reduce AR activity and cause varying degrees of fertility problems and undervirilization (4, 6, 9, 13, 25). Whereas AR mutations that arise in both diseases commonly affect known functional regions of the protein, including the ligand-binding pocket and AF-2 surface, many others affect regions of the AR surface with no assigned function, implying that they disrupt as yet undefined aspects of AR activity.

AR is the pharmacological target for antiandrogens used in PCa treatment. Current PCa clinical treatments involve combinations of androgen-deprivation therapy and antiandrogens (*e.g.* bicalutamide) that inhibit AR action by competing for androgen binding and displacing H12 to prevent formation of a productive AF-2 pocket. Unfortunately, prolonged antiandrogen treatment results in emergence of hormone-refractory PCa with poor prognosis; incompletely defined mechanisms result in reactivation of AR in the absence of androgens and presence of antiandrogens (12, 26–29).

We have proposed that the AR surface may harbor attractive sites for intervention with small molecules (30–32). AR AF-2 undergoes subtle induced fit rearrangements upon coactivator binding and several residue side chains (*e.g.*, K720, M734, and M894) move to create hydrophobic subpockets that bind apolar side chains of coactivator NR boxes and can deepen further to accommodate bulky hydrophobic side chains of W/FxxLF motifs that characterize AR LBD binding peptides within the NTD- and AR-specific coactivators (19, 20). These surface cavities are attractive targets for small molecules and, because AR is reactivated in recurrent PCa, such small molecules could inhibit growth of both early-stage PCa and late-stage hormone-refractory forms of the disease (30–33). In a previous study, we screened chemical libraries for small molecules that inhibit coactivator binding to AF-2 (30). Surprisingly, several compounds, including Triac and members of the fenamic acid series of antiinflammatories, inhibited AR/LxxLL peptide interactions but preferentially were localized by x-ray crystallography to a distinct surface, binding function-3 (BF-3) (30); BF-3 which is topologically adjacent to, but distinct from, AF-2, displays characteristics of a protein-binding site, and is target for PCa and AIS mutations (30–32). Site-

directed mutagenesis of BF-3-lining residues confirmed its modulating role in AR activity (30). Furthermore, comparisons of AR LBD crystal structures with and without Triac at BF-3 suggested that compound binding triggers allosteric alterations that propagate to AF-2 and inhibit coregulator binding (30). Thus, x-ray structures suggest that BF-3 and AF-2 pockets are structurally coupled and that allosteric communication between them exists, but the succession of conformational changes and function of these effects are not clear.

Here, we employed a combination of *in vitro* transactivation assays, mammalian two-hybrid assays with AR LBD, and computational molecular dynamics (MD) simulations to understand how mutations in residues in or near BF-3 may influence AR function and dynamics and how allosteric communication between BF-3 with AF-2 may take place. Our data show that mutations in BF-3 act as allosteric elicitors of conformational changes that are transmitted towards AF-2, and that this allosteric communication affects AR LBD function as experimentally shown *in vitro*. Furthermore, a series of residues from BF-3, the boundary of BF-3/AF-2, and AF-2 are structurally interconnected and allosterically coupled. Moreover, our data indicate that several residues belonging to BF-3 and AF-2 surface pockets are key players of an allosteric network that may influence multiple aspects of AR LBD function.

## Materials and Methods

### Cell culture and transfection assays with AR LBD

Transcriptional activities of wild-type (WT) and mutant human AR LBD GAL4-DBD constructs (GAL4-AR LBD) were determined in transient cotransfection assays using human cervix adenocarcinoma epithelial HeLa cells. Vectors and assay procedures were previously described (20, 30). The GK1-Luciferase (LUC) reporter plasmid used contained five GAL4 response elements upstream of a minimal promoter. GAL4-AR LBD WT and constructs of mutants I672R, R726L, F826R, N833R, and R840E have been previously described (30). Mutants N727K, V757A, F826L, and R840A were made by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). HeLa cells were maintained in Dulbecco's modified Eagle, 4.5 g/liter D-glucose medium (GIBCO) containing 10% fetal bovine serum, 0.58 g/liter L-glutamine, 0.11 g/liter sodium pyruvate, 100 u/ml penicillin, and 100 mg/ml streptomycin. Cells were collected 24 h before transfection in fresh medium containing 10% charcoal-stripped fetal bovine serum and seeded in 24-well culture plates (Corning, Inc., Corning, NY) at a density of $1.5 \times 10^5$ cells per well. They were transfected using FuGENE HD reagent (Promega Corp., Madison, WI) as described by the manufacturer. The DNA mixture was composed of 300 ng/well of GK1-LUC; 2.5 ng/well of *Renilla*-LUC; 100 ng/well of WT or mutant GAL4-AR LBD or empty control vector; and 100 ng/well of pSG5-GR-interacting protein (GRIP)1, VP16- AR NTD (1-504), and VP16-NCoR (1925–2440) or VP16-SMRT (2025–2525) plasmids. The cells were incubated 5 h after transfection with vehicle (1%, vol/vol, dimethylsulfoxide) or hormone [dihydrotestosterone (DHT), dissolved in dimethylsulfoxide], which was purchased from Sigma-Aldrich (St. Louis, MO). Finally, cells were washed twice with PBS and lysed in 100 $\mu$l of passive lysis buffer (Promega). LUC and *Renilla*-LUC activities were measured on 25 $\mu$l of the extracts in a GloMax 96 Microplate Luminometer (Promega) using the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's instructions.

### Western blot analysis

For AR Western blot analysis, HeLa cells were transfected with 1 $\mu$g of AR LBD expression constructs as described above. Cells were washed with cold PBS 24 h after hormone treatment, lysed with cold RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate and 50 mM Tris, pH 7.5) supplemented with complete protease inhibitors (Roche Diagnostics, Indianapolis, IN) and centrifuged for 10 min at 14,000 rpm. Lysates were boiled and loaded onto a NuPAGE 4–12% Bis-Tris gel (Invitrogen, Carlsbad, CA), after which proteins were transferred to a polyvinylidene difluoride membrane. Western immunoblotting was performed using a polyclonal antibody against the C-terminus of AR (C-19, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), followed by incubation with a horseradish peroxidase-conjugated mouse antirabbit antibody (Abcam, Cambridge, MA). AR LBDs were visualized by a reaction with Luminata Forte Western HRP substrate (Millipore).

### Molecular dynamic simulations

#### *Preparation of AR LBD input structures*

We used the x-ray structure of AR LBD in complex with the hormone dihydrotestosterone (DHT) (PDB code 1T7T; 1.7 Å resolution) (19) as starting template to model all the mutants described herein, using the AMBER module LEAP. The PDB file was converted into an AMBER structure file using LEAP. The preparation of the files for the hormone DHT was done with the AMBER modules ANTECHAMBER and LEAP.

As a first preparation step, the crystallographic water molecules were removed from the structure. Then a quick minimization step was performed to optimize the structure within the force field *in vacuum*. Later on, each AR LBD structure to study was placed in a periodic truncated octahedron box of TIP3P water molecules (the minimum distance between protein and walls of the box was set at 12 Å). $Na^+$ and $Cl^-$ counterions were then added to the solvent bulk of protein/water with LEAP to neutralize the system and achieve 150 mM NaCl concentration.

### System setup and simulation protocol

We performed the MD simulations using the force field AMBER parm99 of the AMBER10 package (34, 35). Our equilibration protocol for each MD simulation started by 1 psec minimization with a 50 kcal/(mol · Å$^2$) harmonic potential to restrain the protein atoms to minimize the solvent molecules. Then, we applied 40 psec of MD simulation at constant pressure (1 atm) with a 12 Å nonbonding cut-off distance during which

the temperature was raised from 50 to 300K using Langevin dynamics, applying restraints of 25 kcal/(mol · Å$^2$) to all the protein atoms. After this, we ran 40 psec MD simulation with restraints of 10 kcal/(mol · Å$^2$), and another 40 psec MD simulation with restraints of 5 kcal/(mol · Å$^2$) on all the protein atoms. Then, we applied 20 psec MD simulation with 5 kcal/(mol · Å$^2$) restraints only to the protein backbone atoms, and another 20 psec MD simulation with 1 kcal/(mol · Å$^2$) restraints on the protein backbone atoms as well. Finally, we ran 60 psec MD simulations in which all the atoms of the system were able to move freely. After the above-mentioned system equilibration step, 40 nsec of MD simulation were run for each mutant/complex at constant pressure (1 atm) and a temperature of 300 K. The particle mesh Ewald method was used, with a collision frequency of 0.2 psec$^{-1}$ excluding bonds involving hydrogen atoms.

### B-Factors derived from MD simulation

We calculated the temperature factors (B-factors) from the MD simulation, using the *ptraj* AMBER tool (35). B-Factors were computed as atomic positional fluctuations multiplied by $8/3\pi^2$ and then mass weighted and averaged for each residue. For the sake of comparison, the original B factors derived from x-ray diffraction data in the AR LBD PDB 1T7T were also mass weighted and averaged by residue.

### Analysis of cross-correlation matrices

Correlation matrices, representing all the residue-residue pair-wise root mean square deviation correlations along the complete MD trajectory, were computed using the *ptraj* AMBER tool (35). The standard convention was used with a positive value between 0 and 1 reflecting correlated motion and a negative coefficient between 0 and −1 reflecting anticorrelated motion. Atoms with correlated motions move in phase whereas atoms with perfect anticorrelated motion move in antiphase.

### Accessible surface area calculations

The hormone DHT accessible surface area (ASA) along the simulations was computed with the ICM-Browser program (www.molsoft.com), based on the center of a spherical probe of 1.4 Å radius rolling over the structure of AR bound to the hormone (36). This parameter gives a measure of the solvent accessibility of DHT, which is buried within the LBP.

### Analysis of surface cavities along MD simulations

To identify the evolution of surface cavities or ligand pockets during the MD simulations, we used the fpocket program and its module MDpocket developed to track the persistence of pockets within MD trajectories (37, 38). MDpocket ran fpocket iteratively on 100 trajectory snapshots (extracted every 400 psec) to compute $\alpha$-spheres (defined as those in contact with four atoms without containing any internal atom inside). Then the density of the conserved positions of the $\alpha$-spheres during the trajectory was calculated. High-density regions corresponded to stable and well-defined cavities whereas lower densities indicated transient pockets. To visualize all volumes detected by MDpocket for each trajectory, we used an isovalue of 2.19, which selects the top 1% of the detected volume for the WT density data.

### Contacting pairs and distance calculations

We calculated all contact pairs (residue-residue minimal interatomic distance <4 Å) formed by residues in the peptide-binding region (*i.e.* those at a distance <4 Å from the ARA70 peptide in the structure 1T5Z after superimposing AR coordinates) (20), in the snapshots generated every 400 psec from the MD trajectories. We compared the frequency of such contact pairs in the mutants with respect to the WT.

## Results

### BF-3 mutations alter AR AF-2 activity

To address functional effects of different BF-3 mutations, we tested *in vitro* the transactivation activity of agonist-bound WT AR LBD (WT AR LBD) and nine AR LBD mutants (Fig. 1) and performed in parallel an exhaustive comparison of dynamics of WT AR and mutant ARs using MD simulation (see Figs. 2–5 and SI). The chosen mutated residues are located either lining the BF-3 pocket (I672, F826, N833, R840) or at the boundary between AF-2 and BF-3, hence part of both pockets (R726, N727) (Fig. 1, A and B). Additionally, we chose for control purposes a mutation (V757A) located at the end of H5, hence distant from both studied pockets (Fig. 1A). The selected dataset of mutations studied herein includes: I672R (http://androgendb.mcgill.ca), V757A (39–40), and R726L (41–46), associated with PCa; F826L (47) and N727K (48), found in AIS patients, as well as mutations that have not been associated with pathology (F826R, R840A, R840E, and N833R) (Fig. 1, A and B, and Table 1). We have not studied mutations F826L, N727K, and R840A before (Fig. 1 and Table 1). WT and all mutant AR LBDs investigated exhibit comparable levels of expressed protein as assessed by Western blot (Fig. 1C).

We observe that mutants I672R and N833R behave as super-AR variants, as earlier reported (30), as well as the new mutant R840A. Mutants N727K and F826L also moderately enhance AR AF-2 activity although without exhibiting super-AR behavior. Also along previous observations, F826R and R726L marginally reduce, and R840E totally abolishes AR LBD activity *in vitro* (Fig. 1D and Table 1).

### BF-3 mutations affect AR LBD activation by GRIP1

We additionally investigated the functionality of the studied AR LBDs by measuring AR AF-2 transactivation activity *in vitro* in the presence of coactivator and by addressing AR LBD interaction either with its N-terminal domain or corepressors using a mammalian two-hybrid experiment.

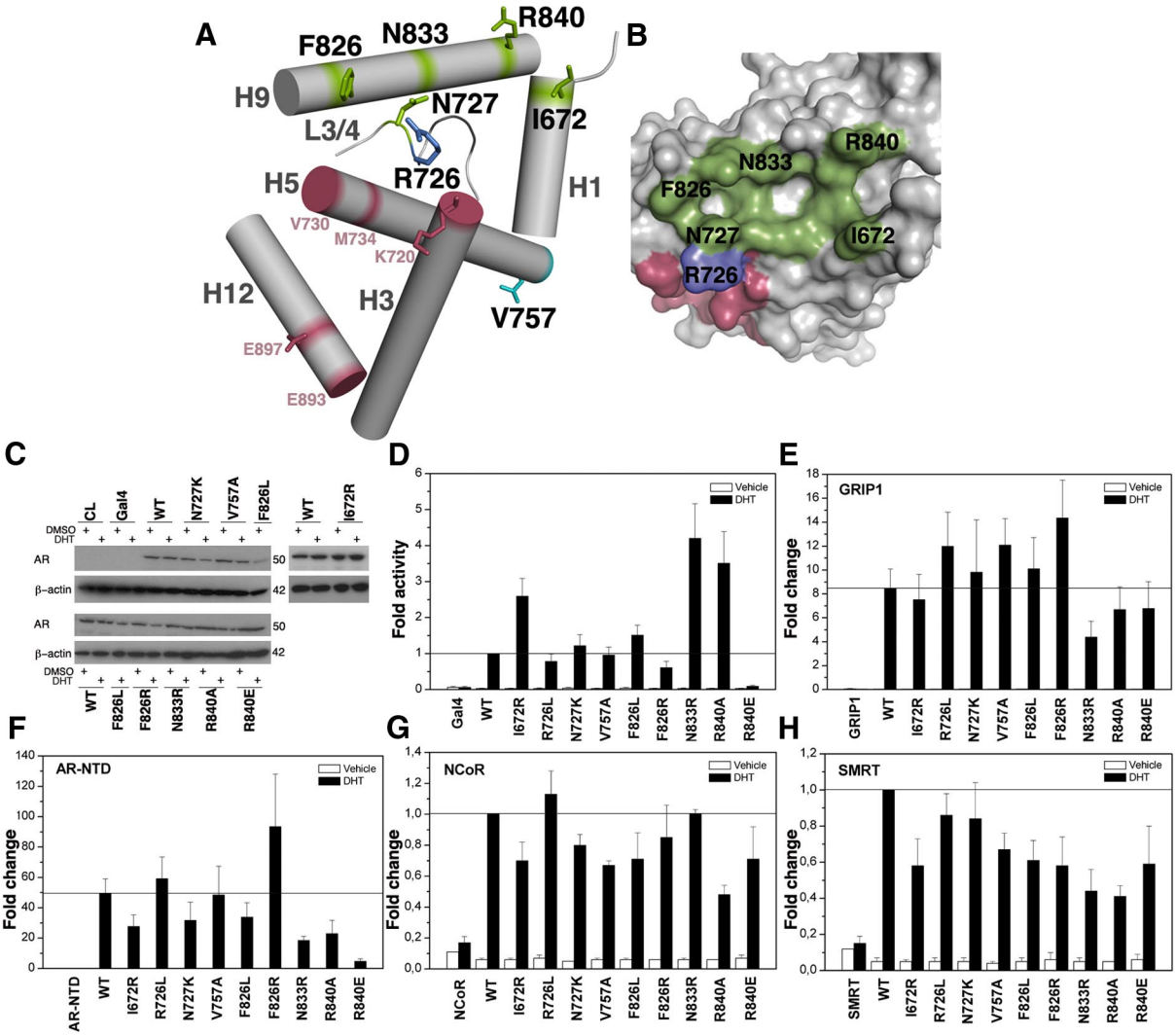


**FIG. 1.** Mutations in AR LBD, AF-2 transcriptional activation, and mammalian 2-hybrid assays. A, Simplified model representation of AR LBD structure. Helices H1, H3, H4–5, H9, and H12 are depicted as *gray cylinders*. The AF-2 coactivator binding pocket is lined by H3, H5, and H12, whereas the BF-3 pocket is formed by H1, H9, and the loop linking H3 with H4–5 (L3/4), which is shown as a *thin gray wire*. BF-3 residues studied herein are shown as *green sticks*. Key AF-2 residues implicated in engaging in important contacts with coactivator peptides as shown in previous crystal structures are highlighted in *raspberry,* and the charge clamp residues K720 and E897 are shown as *rasperry sticks*. The residues R726 and N727 belonging to the boundary between AF-2 and BF-3 pockets are shown as *dark blue* and *green sticks*, respectively. The residue V757 the mutation of which, V757A, has been associated with PCa is depicted as a *blue stick* located at the end of H5 and is surface exposed. B, Solid-surface representation of AR LBD in *gray* showing the residues lining BF-3 in *green*, the residues lining AF-2 in *raspberry* and residue R726 in *blue*. C, Western blot showing the protein expression level of all AR LBDs. D and E, AR AF-2 activity in the absence (D) or presence (E) of GRIP1 coactivator. WT AR LBD AF-2 activity is 100%. F–H, Mammalian two-hybrid assays with (F) AR NTD domain, and corepressors (G) N-CoR or (H) SMRT. HeLa cells were transfected with 100 ng DNA and treated with 100 nM (D–F) or 10 nM (G and H) DHT. Results are the mean of at least five independent experiments performed in triplicate.

We first determined effects of the coactivator GRIP1 on WT and mutant AR activity (Fig. 1E). GRIP1 is the mouse orthologue of the human protein transcription intermediary factor 2, which is known to interact with the AR AF-2 pocket in a hormone-dependent manner (49). GRIP1 enhances activity of all of the mutants, but there are variations in the extent of GRIP1 potentiation (Fig. 1E and Table 1). GRIP1 enhances activity of super-mutants I672R and R840A similarly to WT, but fold increase in activity of supermutant N833R by GRIP1 is lower. GRIP1 also enhances activity of PCa mutants V757A and R726L, AIS-associated mutations F826L and N727K, although to a lesser degree and rescues activity of the medium-inhibiting mutant F826R, resulting in highest fold increase among all the studied AR LBD mutants. Even the very weak activity of the R840E mutant is enhanced by GRIP1. Thus, some BF-3 mutations (notably N833R and F826R) alter the extent of GRIP1 coactivation, but none abolish GRIP1 interaction.

### BF-3 mutations affect the N/C interdomain interaction

To assess whether mutations at the BF-3 pocket affect AR LBD/NTD (N/C) interaction, a mammalian two-hybrid was performed (Fig. 1F and Table 1). Again, some of the BF-3 mutants altered AR N/C interaction, but the pattern of effects was different from that with GRIP1. All three supermutant AR LBDs, I672R, N833R, and R840A, show a decreased interaction with the NTD as compared with WT. Mutants R726L and V757A, associated with PCa, display a WT or moderately higher increase in their interaction with the NTD. Medium-inhibiting mutant F826R, which exhibited the highest increase in activity in the presence of coactivator, also exhibits the largest fold induction with the NTD. Mutants N727K and F826L, associated with AIS, show mild impairment in NTD interaction, superactivating mutant N833R exhibits the lowest capacity for N/C interaction, and activity of the severely impaired mutant R840E is not enhanced by VP16-NTD overexpression, unlike the case with GRIP1.

### BF-3 mutations alter AR interaction with N-CoR and SMRT

Because DHT-liganded AR also interacts weakly with NR corepressors (50), we used a mammalian two-hybrid assay to assess how BF-3 mutants affect this interaction. The chosen corepressors were the silencing mediator of retinoid and thyroid receptors (SMRT) and the nuclear receptor corepressor (N-CoR) (Fig. 1, G and H).

As seen with GRIP1 and the AR NTD, BF-3 mutants broadly affect the capacity of AR to bind corepressors (Fig. 1, G and H, and Table 1). Both supermutants I672R and R840A exhibit a significant decrease in their ability to interact with the receptor-interacting domains of corepressors SMRT and N-CoR, being R840 the one that disrupts such interactions the most among all the AR LBD herein studied. V757A, F826L, and the severe mutant R840E also reduced corepressor interaction. Supermutant N833R and mutant F826R show impaired interaction with SMRT, but maintain a WT-like interaction with N-CoR, whereas N727K decreases interaction with N-CoR but leaves interaction with SMRT unaffected. Finally, R726L has little effect on corepressor interaction in these assays.

### AR LBD MD simulations

To understand the structural and dynamic effects of the above-described mutations, we first analyzed WT AR LBD by running MD simulation for 40 nsec in explicit solvent, after an initial step to allow equilibration of AR LBD. The WT AR LBD structure appeared stable along the dynamics, and there was no evidence of large-scale reorganization or denaturation processes (Fig. 2, A and B).

The overall apparent mobility of WT AR LBD amino acids along the MD trajectory resembled that of the AR LBD x-ray structure PDB 1T7T (19). We estimated residue B-factor values by computing fluctuations of each residue along the MD simulation. Figure 2C shows these values against B-factor values derived from electron density uncertainties in the AR LBD structure (19). In general, B-factors derived from MD simulation were similar to the crystallographic ones, implying that our simulation produces a reasonably accurate representation of the molecular motions that are detected in the crystal although the most N-terminal helix of AR LBD (H1) and amino acids 687–695 (H1-H3 loop) appear more flexible in the simulation whereas two highly flexible regions [amino acids 844–850 (H9-H10) and 880–886 (H11 and H11/12 loop)] appear more rigid than equivalent regions in the x-ray structure.

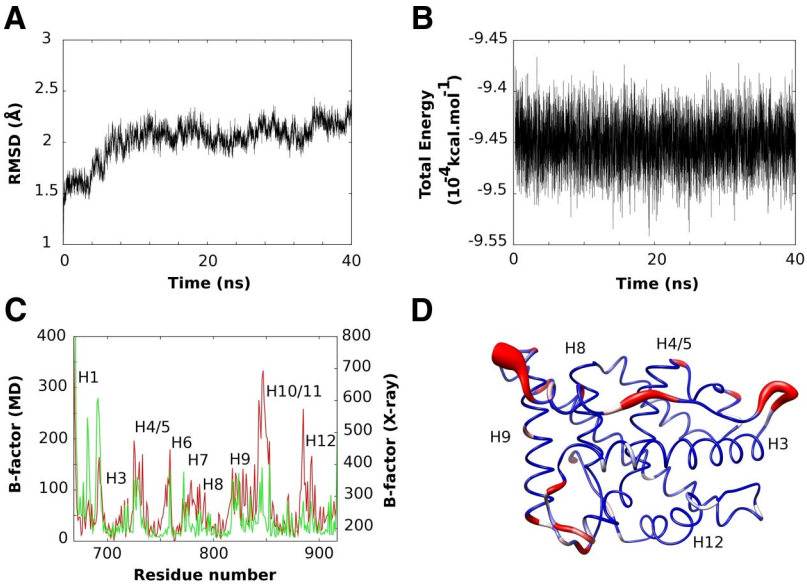


**FIG. 2.** Simulation of AR LBD WT and mutants. A, Evolution of root mean square deviation (RMSD) with respect to the initial structure along the MD trajectory. B, Evolution of global energy along MD trajectory. C, Comparison between experimental (in *red*) and AMBER-based simulated B factors (in *green*). For consistency, experimental B factors from PDB 1T7T were transformed to be comparable to simulated values (see *Materials and Methods* for details). AMBER B-factors per residue were computed from an ensemble of 200 frames along the MD trajectory selected every 200 psec. D, Simulated B-factors mapping on AR. The AR receptor B-factors are shown as *worms with variable thickness and color* according to their corresponding value (B-factors $<$ 46 in *blue*; 46 $<$ B-factors $<$ 77 in *white*; B-factors $>$ 119 in *red*). ns, Nanoseconds.

Interestingly, the B-factor measurements flag the H3-H4/5 loop and the S3-H9 loop as relatively flexible compared with other regions of the AR LBD; this was seen both in MD simulations and in B-factors derived from the x-ray structure (Fig. 2C and Supplemental Fig.1 for mutant B-factors fluctuations). Both regions are located in the boundary of AF-2 and BF-3 pockets, suggesting a possible dynamic link between these binding surfaces (Fig. 2D).

Next, we studied the effects of mutations on AR LBD dynamics, by obtaining 40 nsec MD trajectories for each of the mutants (Supplemental Fig. S2). Interestingly, consideration of mobility of the entire LBD reveals that mutations I672R, F826L, and N727K, which enhance AR LBD activity, exhibit larger average B-factors (Table 1) indicative of higher mobility. This is not true for supermutants N833R and R840A. On the contrary, inhibitory AR mutations generally display smaller average B-factors, indicative of reduced mobility (Table 1). Correlation of B-factors with activity is even more striking when mobility of only the BF-3 residues is considered (again, excluding N833R and R840A). There is no similar correlation between mobility of the AF-2 residues and activity (Table 1).

## AR mutations reveal allosteric coupling between BF-3 and AF-2

To analyze how specific AR mutations affect dynamics of BF-3 and other regions of the LBD and pinpoint possible allosteric effects, we computed the motion correlation of all AR residues against each other along the MD trajectories, shown as correlation matrices (see Supplemental Fig. 3 and *Materials and Methods*). The cross-correlation plots for WT activating (I672R, F826L) and inhibiting (R840E) mutations are shown in Supplemental Fig. 3. The values for WT AR LBD (Supplemental Fig. 3) show regions that are correlated (in *red*) or anticorrelated (in *blue*) along the MD trajectory. There is little obvious coupling between different regions of the WT AR LBD. Interestingly, the correlation matrix for AR I672R shows significant differences: there is stronger correlation between motion of residues 672–673 (H1, the mutation site), residues 710–740 (H3 and loop 3/4, mostly AF-2 and the boundary region of AF-2/BF-3) and residues 820–840 (the BF-3 lining loop S3/H9 and H9). In the case of F826L, we observe, in general, less motion correlation between regions than with I672R, but there is significant correlation between the region of the mutated residue (H9, close to BF-3) and the adjacent AF-2 pocket and this is stronger than WT. Increased correlation of mobility of BF-3 and AF-2 is not seen with the N833R and R840A mutants. In the case of R840E mutant, there is less motion correlation and no clearly correlated regions that can be highlighted. Reductions in correlation between mobility of the BF-3 and AF-2 pockets are also seen with other BF-3 mutants that reduce AR LBD activity.

Calculation of correlation coefficients ($r^2$) along the MD trajectories between the residues forming BF-3, AF-2, and LBP (where DHT is bound) sites underscores impressions from the mobility correlation matrices (Table 1). Activating mutants (with the exception of N833R and R840A) have BF-3/AF-2 correlation $r^2$ more than 0.7 (significantly higher than WT), whereas inhibiting mutants have $r^2$ around 0.5–0.6, smaller than that of the activating mutants, but still higher than WT. Thus, there is increased allosteric coupling between BF-3 and AF-2 when the transcriptional output is enhanced activation.

We do not observe any correlation between AF-2 or BF-3 and residues that line the LBP for WT AR or any of the analyzed AR mutants. Curiously, if we exclude the most activating mutants (N833R, R840A, I672R, and F826L), there is some correlation between the average DHT ASA relative to WT and activity (Supplemental Fig. 4). This raises the possibility that enhanced plasticity of LBP observed during MD simulations may favor AR activity.

## BF-3 mutations change dynamics of AF-2

Because the results obtained from our MD simulations suggest close structural communication between BF-3 and AF-2 surface pockets, we analyzed effects of BF-3 mutations on AF-2 conformation in more detail. To do this, we computed all possible contact pairs of AF-2 residues that exhibited at least a 15% reduction or increase in their pair-wise interaction frequency relative to WT AR during simulations (see *Materials and Methods* for details). Figure. 3 and Supplemental Figs. 5 and 6 show evolution of contacts between selected residue pairs along the simulations.

BF-3 mutations result in significant changes in pair-wise interactions within AF-2 residues over time. For activating mutants I672R and F826L, the proximity between the AF-2 key residues K720-M734 is greatly reduced relative to WT AR or R840E (Fig. 3). In contrast, K720 (one of the charge clamp AF-2 residues) forms more extensive contacts with H729, N727, L728, and V730. Among other AF-2 residues, M734 establishes frequent contacts with L712, M894, and I898, whereas V730 forms more frequent contacts with V716 in the context of both activating mutants. Effects of the AR R840E (inhibitor) mutation are opposite to that of the activating mutants. Specifically, pair-wise interactions between K720 and M734 are enhanced, whereas interactions between R726, K720, Q733, and V730 appear less frequently,

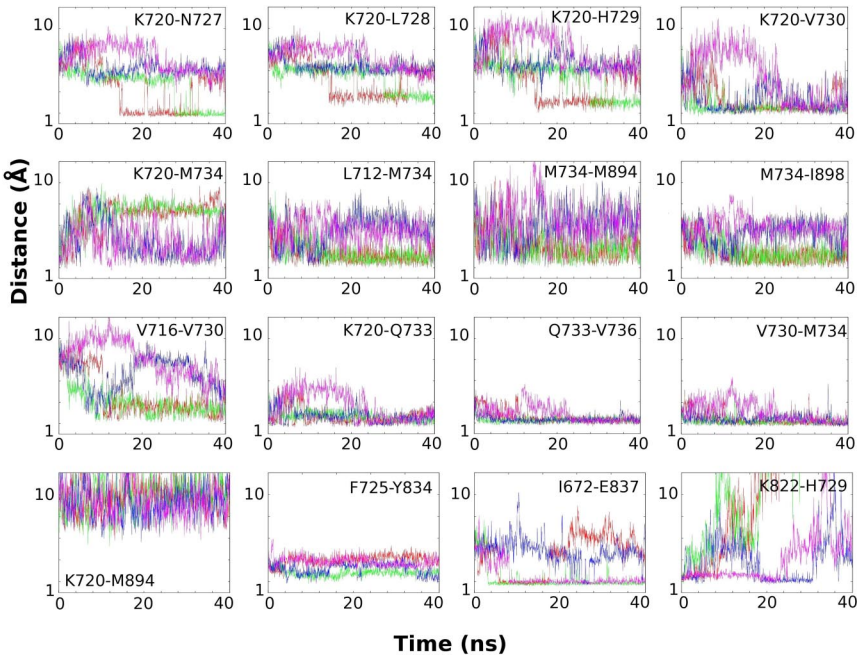


**FIG. 3.** Evolution of pair-wise minimal contact distance for selected residue pairs in WT AR (*blue*), I672R mutant (*green*), F826L mutant (*red*), and R840E mutant (*magenta*) along the MD trajectory for selected residue pairs. ns, Nanoseconds.

V730 remains further away from K720 and V716, and contacts between K720 and Q733 are less prevalent (whereas contacts between K720 and H729, N727, or L728 are never formed, as in WT).

The net effect of these changes in pair-wise interactions is that there are alterations in the frequency of formation of AF-2 subpockets that accommodate bulky side chains of $F_1xxLF_5$ peptides present in AR-specific coactivators (Supplemental Fig. 7). In the x-ray WT AR structure (PDB 1T7T) (19), K720 and M734 are in contact, closing the entrance for the second F side chain (at position 5) of the FxxLF motif (Fig. 4A). During the WT MD simulation, these residues open frequently to create an organization that resembles that of AR in complex with the ARA70 FxxLF motif (PDB 1T5Z) (20) (Fig. 4B). In the I672R mutant simulations, K720 and M734 separate even more widely and frequently, opening a larger hydrophobic pocket (Fig. 4E), an effect that can also be seen in the other activating mutant F826L (data not shown). By contrast, in the AR R840E (inhibitor) mutant, the arrangement of K720 and M734 along the simulation resembles that of the original AR x-ray structure PDB 1T7T (19), and the deep AF-2 subpocket fails to open (Fig. 4F), unlike the dynamic opening and closing of the subpocket seen in simulations with WT AR (Fig. 4D). Thus, MD simulations seem to suggest that BF-3 mutations alter the propensity of AF-2 to form subpockets that accommodate FxxLF peptides; activating mutations enhance subpocket formation, whereas the inhibitory mutation reduces subpocket formation.

## Allosteric paths on AF-2

We examined snapshots of the MD simulations to search for dynamic structural rearrangements that could be responsible for the allosteric transmission of information from BF-3 to AF-2 (Fig. 5). Analysis of the simulations of activating AR mutants I672R and F826L revealed a large conformational change in the H3-H4/5 loop (residues 723–734) toward the end of the simulation (see a conformational snapshot of I672R in Fig. 5B). Movements of this loop relative to the initial position (Supplemental Fig. 8) were more extensive than the ones seen in WT AR or any other mutant simulation [interestingly, the mutants with higher fold increase in activity in the presence of GRIP1 (V757A, R726L and, to a lower extent, F826R), have these loops more extensively moved than WT along the dynamics]. Within the H3–H4/5 loop, there are striking changes in the spatial location of residues H729 and V730 (Fig. 3). A possible mechanism for this effect is that the I672R side chain interacts with E837 (located in H9 and forming a salt bridge with R840 in WT AR). This effect would alter H9 position and free the H3–H4/5 loop so that K720 (H3) can interact with H729 (H3-H4/5 loop). H729 moves from being in contact with a lysine residue (K822, loop S3-H9) to contacting K720 backbone as a result of the new loop conformation.

Similar analysis for F826L (Fig. 5E) also reveals opening of the H3–H4/5 loop. Early in the MD simulation, L826-N823 interaction breaks leading to distortion of the S3-H9 loop and breakage of K822-H729 contact. This effect, which is not seen in WT AR, leaves H729 free to interact with K720 and results in a H729-K720 conformation that is exactly the same as the one seen in the I672R simulation and is not found in any other mutant. By contrast, the K822-H729 contact is more stable in the simulations with the R840E inhibitory mutant relative to WT-AR and certainly much more stable than in activating mutants (Fig. 3), underscoring the potential importance of this interaction in BF-3/AF-2 communication.

These new conformations of the H3-H4/5 and S3-H9 loops (especially in the activating mutants) open a small hydrophobic cavity around Y739 (H4) that is adjacent to

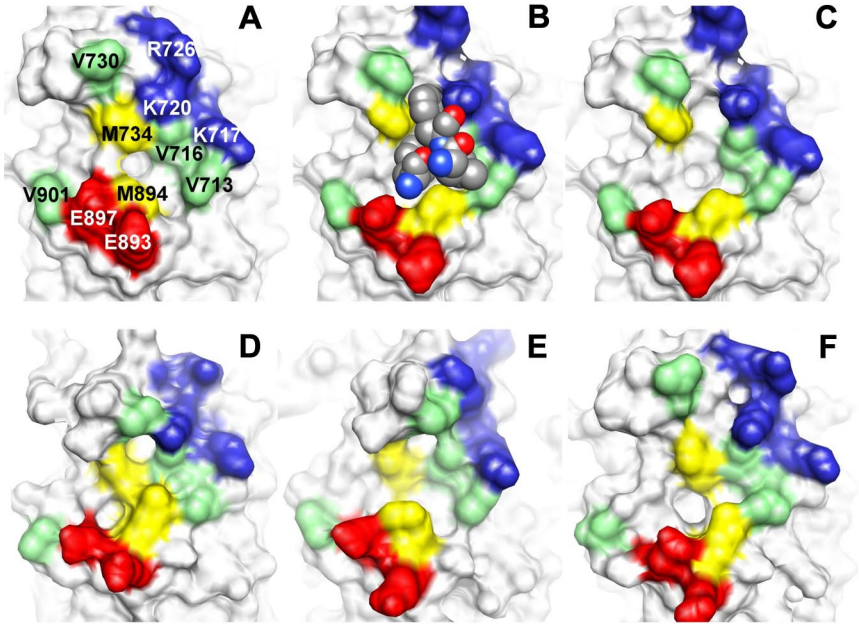


**FIG. 4.** Surface residues of the AF-2 pocket. A, WT AR x-ray structure, PDB 1T7T. B, AR bound to ARA70 peptide x-ray structure, PDB 1T5Z. C, AR bound to ARA70 peptide, with peptide removed for the sake of clarity. D, WT AR during MD simulation (snapshot taken at 10.4 nsec, representative of the first third of the trajectory). E, I672R mutant during MD simulation (snapshot taken at 29.6 nsec, representative of the last half of the trajectory). F, R840E mutant during MD simulation (snapshot taken at 4 nsec, representative of the first half of the trajectory).

AF-2 and close to BF-3. Comparison of the organization of this cavity reveals that it is closed throughout the simulations with the inhibitor mutation R840E.

## Discussion

We have previously discovered a small molecule-binding surface on the AR LBD (BF-3), and our mutational analysis has revealed that it is highly important for AR function (30). AR mutants that affect BF-3 and have been documented in PCa and AIS variously influence androgen binding and dissociation, coregulator recruitment, N/C-interaction, and transactivation (6, 7, 30, 42, 47, 51–69). We envision two possible physiological roles for BF-3, which are not mutually exclusive. First, our combined functional and computational assays support our previous hypothesis that BF-3 is an allosteric modulator of the adjacent AF-2 pocket, affecting its function. Second, BF-3 may be a protein-protein interaction site for coregulator proteins.

Our studies confirm and extend our previous data, which show that BF-3 mutants alter overall AR activity in different, and hard to predict, ways. We show that mutants in BF-3 and residues that lie between BF-3 and AF-2 produce a range of effects from superactivators (I672R, N833R, R840A), moderate enhancers (F826L, N727K), weak inhibitors (F826R, R726L), and very potent inhibitors (R840E). We have also shown a wide range of effects upon coregulator binding; BF-3 mutations do not completely inhibit functional interactions with GRIP1 or NTD but do affect coactivation differentially by both proteins. This stands in marked contrast to effects of mutations in the AF-2 surface that consistently block AR interactions with GRIP1, the AR NTD, and other coregulators. Finally, BF-3 mutations moderately inhibit corepressor interactions, but, here again, there are diverse effects with some mutations strongly inhibiting N-CoR and SMRT binding, others exhibiting milder effects or no effects, and some distinguishing between N-CoR and SMRT.

Our MD simulations do not systematically explain effects of all AR BF-3 mutations but do suggest a plausible general explanation for their diverse effects on AR activity; BF-3 is coupled to AF-2, and BF-3 mutations alter the propensity of the AF-2 surface to form deep subpockets that accommodate bulky side chains of coregulator motifs. MD simulation on AR LBD shows a dynamic link between BF-3 and AF-2, and two regions that form the boundary between AF-2 and BF-3 pockets play a key role in allosteric communication: the H3-H4/5 loop, where R726 and N727 are located, and the S3-H9 loop, where F826 resides. Interestingly, consideration of mobility of the entire LBD reveals that three mutations that enhance AR LBD *in vitro* activity (I672R, F826L, N727K), exhibit a greater mobility than WT. On the contrary, AR inhibitory mutations generally feature smaller mobility with respect to WT. Analysis of the flexibility per pocket indicates that BF-3 flexibility degree is inversely correlated to the AR LBD *in vitro* function, but there is no correlation with the AF-2 mobility.

BF-3 mutations induce conformational changes in several side chains of the adjacent AF-2 pocket. For activating mutants I672R and F826L, residues K720 and M734 stand out as already observed in the x-ray crystal structures of coactivator peptides bound to AF-2 (20). Pairwise residue contacts with several key AF-2 residues are manifested, specially implicating L712, H729, N727, L728, V730, and H12 M894 and I898. Effects of the AR R840E (inhibitor) mutation on the observed pair-wise contact formation are opposite to that of the activating mutants. The net effect of these changes in pair-wise in-

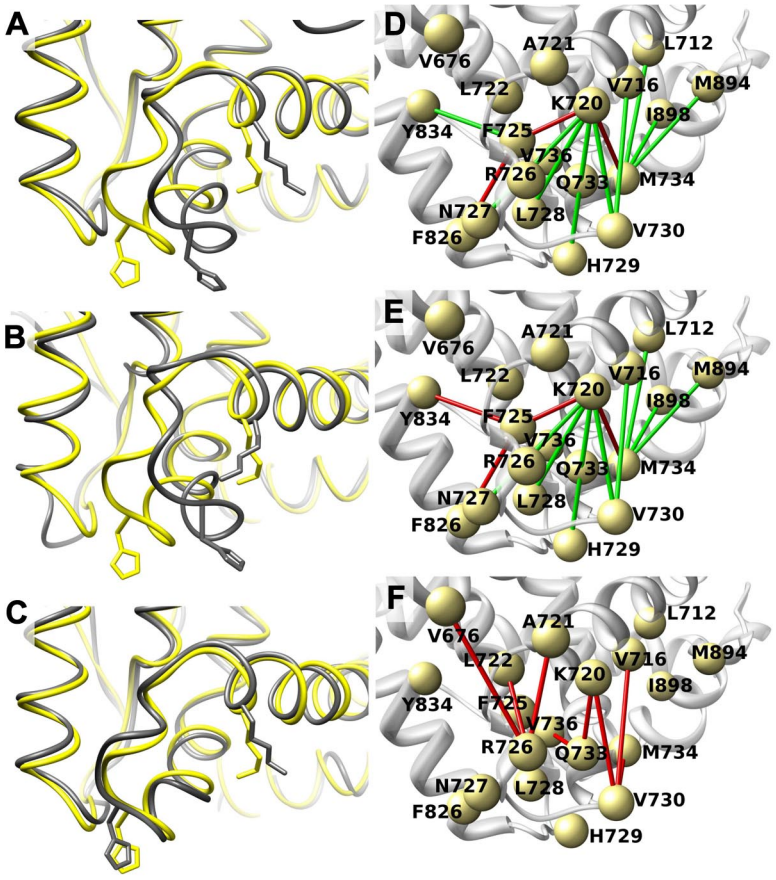


**FIG. 5.** Conformational changes in WT AR and mutants during MD simulation. A, Detail of the H3–H4/5 loop in WT AR during MD simulation (in *gray*, snapshot taken at 10.4 nsec, representative of the first third of the trajectory) with respect to the x-ray structure in *yellow*. B, I672R mutant after MD simulation (in *gray*, snapshot taken at 29.6 nsec, representative of the last half of the trajectory) compared with WT AR x-ray structure (in *yellow*). C, Same loop detail in R840E mutant after MD simulation (in *gray*, snapshot taken at 4 nsec, representative of the first half of the trajectory) compared with WT AR x-ray structure (in *yellow*). D, Residue pairs in which contact frequency during MD simulation in I672R mutant significantly change with respect to the AR WT simulation; residues are represented as spheres, and the link is colored in *red* or *green* depending whether the contact frequency in the corresponding AR mutant MD simulation is significantly smaller or larger, respectively, than in WT AR MD simulation. E and F, Similar analysis for F826L mutant (E), and for R840E mutant (F).

teractions is alterations in the frequency of formation of AF-2 subpockets that accommodate NR boxes present in coactivator peptides and the NTD domain. There is thus a shift in the conformational ensemble of the AF-2 groove. Residues K720 and M734, which are in a closed conformation in the x-ray WT AR structure without coactivator peptide (PDB 1T7T) (19), remain more frequently along the MD trajectory in the AF-2 open conformation, similar to the one observed in the crystal structure of AR bound to the ARA70 FxxLF motif (PDB 1T5Z) (20). In the I672R and F826L mutant MD simulation, K720 and M734 separate even more widely, frequently opening a larger hydrophobic pocket. However, K720 and M734 are found in the closed conformation along the MD trajectory in the inhibitory AR mutant R840E. Thus, MD simulation suggests that BF-3 mutations alter the propensity of AF-2 to reorganize its subpockets to accept the entering coactivator peptides. Whereas several activating mutations enhance the formation of subpockets, inhibitory ones reduce them.

Other paths may also be involved in the allosteric transmission of information from BF-3 to AF-2, and from/to other areas of the receptor. Our studies have evidenced possible roles for identified residues located in the H3-H4/5 and S3-H9 loops that are responsible for conformational changes and allosteric cross talk among AR LBD surfaces. These new conformations of the H3-H4/5 and S3-H9 loops (especially in the activating mutants) open a small hydrophobic cavity around Y739 (H4) that is adjacent to AF-2 and close to BF-3. Comparison of the organizations of this cavity reveals that it is closed throughout the simulations in the inhibitor mutation R840E. Interestingly, we observed weak binding of an apolar small molecule (salycylaldehyde) at this cavity adjacent to AF-2 in our initial screening of surface inhibitor compounds (PDB 2PIR) (30). Y739 is one of the residues that interacted with this surface binder drug, and, most importantly, K822 (loop S3-H9) was at a distance that allowed electrostatic stabilization of the drug (30). We call this groove "binding function 4" (BF-4). We did not observe correlation between AF-2 or BF-3 and the residues that line the LBP for WT AR or any of the AR analyzed mutants. However, it is interesting to point out that if the most activating mutants are excluded (N833R, R840A, I672R, and F826L), there seems to be a correlation between the average ASA of the LBP, where DHT is cocooned, indicating that an increase in LBP adaptability during the MD simulation may influence AR activity.

Our data suggest that two superactivating BF-3 mutations may work by a distinct mechanism. Our MD simulations suggest that N833R and R840A do not exhibit larger overall flexibility than WT AR, unlike I672R and other activating mutations, suggesting that they enhance AR activity via a mechanism that differs from allosteric communication. Because BF-3 exhibits characteristics of a protein interaction surface, it is attractive to speculate that both mutations could alter direct contacts with an unknown protein(s) that could either potentiate or silence AR function. Several lines of evidence suggest that BF-3

could be involved in protein contact. Equivalent regions of the thyroid hormone receptor (70) and the nuclear receptor LRH-1 bind to corepressor and the wnt-signaling dependent coactivator β-catenin (71). Furthermore, functional evidence links this region of AR to contacts with the chaperone FKBP52 (72). It is even possible that BF-3 might contact GRIP1 coactivator, the NTD, and corepressors and that these proteins have an interaction surface on AR LBD that extends beyond the AF-2 pocket toward BF-3 as we have previously suggested (30, 33).

## Acknowledgments

We dedicate this work to the memory of Professor John D. Baxter, who sadly passed away just a few days before the submission of this manuscript. Professor J. D. Baxter's passion for endocrinology and leadership has inspired many young scientists like us during these years.

We thank Drs. A. T. Hagler and M. Orozco for fruitful discussions. We thank Drs. P. Fuentes-Prior (Hospital de Sant Pau, Barcelona), J. Lüders Institute for Research in Biomedicine (IRB, Barcelona), and M. Vegetti for useful comments on the manuscript. We thank E. Hondares (IBUB-University of Barcelona) for useful technical advice.

Address all correspondence and requests for reprints to: Juan Fernández-Recio, Barcelona Supercomputing Center (BSC), Spain. e-mail: juanf@bsc.es; Eva Estébanez-Perpiñá, Institute of Biomedicine (IBUB), University of Barcelona (UB), Spain. e-mail: evaestebanez@ub.edu.

This work was supported by Plan Nacional I+D+i grants SAF-2008-03562, SAF-2011-29681 and BIO2010-22324 [MICINN (Ministerio de Ciencia e Innovación), Gobierno de España], and Marie-Curie International Reintegration Grant IRG-code 224812 (EU). E.E.P. is supported by a RyC (Programa Ramón y Cajal)-2007 fellowship. L.R.C. is recipient of a FPI (Formación de Personal Investigador) fellowship from the MICINN. Obtained funding: E.E.P., J.F.R.; Overall responsibility: E.E.P., J.F.R. G.B. is the recipient of the Martin Harris Research Fellowship.

Disclosure Summary: Conception and design: E.E.P., J.F.R.; Analysis and interpretation: S.G., L.R.C., V.B., G.B., C.B., P.W., E.E.P., J.F.R.; Data collection: S.G., L.R.C., V.B., P.N.; Writing the article: E.E.P., J.F.R., P.W. with contributions of C.B., S.G., L.R.C., V.B., J.D.B.; Critical revision of the article: E.E.P., J.F.R., C.B., P.W., J.D.B.; Final approval of the article: E.E.P., J.F.R., C.B., P.W.

## References

1. **Lubahn DB, Joseph DR, Sar M, Tan J, Higgs HN, Larson RE, French FS, Wilson EM** 1988 The human androgen receptor: complementary deoxyribonucleic acid cloning, sequence analysis and gene expression in prostate. Mol Endocrinol 2:1265–1275
2. **Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schütz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM** 1995 The nuclear receptor superfamily: the second decade. Cell 83:835–839
3. **La Spada AR, Wilson EM, Lubahn DB, Harding AE, Fischbeck KH** 1991 Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. Nature 352:77–79
4. **Quigley CA, French FS** 1994 Androgen insensitivity syndromes. Curr Ther Endocrinol Metab 5:342–351
5. **Brinkmann A, Trapman J** 2000 Genetic analysis of androgen receptors in development and disease. Adv Pharmacol:317–341.
6. **Brinkmann AO** 2001 Molecular basis of androgen insensitivity. Mol Cell Endocrinol 179:105–109
7. **Buchanan G, Greenberg NM, Scher HI, Harris JM, Marshall VR, Tilley WD** 2001 Collocation of androgen receptor gene mutations in prostate cancer. Clin Cancer Res 7:1273–1281
8. **Gottlieb B, Beitel LK, Wu J, Elhaji YA, Trifiro M** 2004 Nuclear receptors and disease: androgen receptor. Essays Biochem 40:121–136
9. **Hughes IA, Deeb A** 2006 Androgen resistance. Best Pract Res Clin Endocrinol Metab 20:577–598
10. **Brooke GN, Bevan CL** 2009 The role of androgen receptor mutations in prostate cancer progression. Curr Genomics 10:18–25
11. **Tindall DaM, Mohler J, eds.** 2009 Androgen action in prostate cancer. ISBN 978-0-387-69177-0
12. **Knudsen KE, Penning TM** 2010 Partners in crime: deregulation of AR activity and androgen synthesis in prostate cancer. Trends Endocrinol Metab 21:315–324
13. **Szafran AT, Sun H, Hartig S, Shen Y, Mediwala SN, Bell J, McPhaul MJ, Mancini MA, Marcelli M** 2011 Androgen receptor mutations associated with androgen insensitivity syndrome: a high content analysis approach leading to personalized medicine. Adv Exp Med Biol 707:63–65
14. **Jenster G, van der Korput HA, van Vroonhoven C, van der Kwast TH, Trapman J, Brinkmann AO** 1991 Domains of the human androgen receptor involved in steroid binding, transcriptional activation, and subcellular localization. Mol Endocrinol 5:1396–1404
15. **Matias PM, Donner P, Coelho R, Thomaz M, Peixoto C, Macedo S, Otto N, Joschko S, Scholz P, Wegg A, Bäsler S, Schäfer M, Egner U, Carrondo MA** 2000 Structural evidence for ligand specificity in the binding domain of the human androgen receptor. Implications for pathogenic gene mutations. J Biol Chem 275:26164–26171
16. **Heery DM, Kalkhoven E, Hoare S, Parker MG** 1997 A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature 387:733–736
17. **He B, Kemppainen JA, Wilson EM** 2000 FXXLF and WXXLF sequences mediate the $NH_2$-terminal interaction with the ligand binding domain of the androgen receptor. J Biol Chem 275:22986–22994
18. **Christiaens V, Bevan CL, Callewaert L, Haelens A, Verrijdt G, Rombauts W, Claessens F** 2002 Characterization of the two coactivator-interacting surfaces of the androgen receptor and their relative role in transcriptional control. J Biol Chem 277:49230–49237
19. **Hur E, Pfaff SJ, Sturgis PE, Hanne G, Buehrer BM, Fletterick RJ** 2004 Recognition and accommodation at the androgen receptor coactivator binding interface. PLoS Biol 2:E274
20. **Estébanez-Perpiñá E, Moore JM, Mar E, Delgado-Rodrigues E, Nguyen P, Baxter JD, Buehrer BM, Webb P, Fletterick RJ, Guy RK** 2005 The molecular mechanisms of coactivator utilization in ligand-dependent transactivation by the androgen receptor. J Biol Chem 280:8060–8068
21. **Jasuja R, Ulloor J, Yengo CM, Choong K, Istomin AY, Livesay DR, Jacobs DJ, Swerdloff RS, Miksovská J, Larsen RW, Bhasin S** 2009 Kinetic and thermodynamic characterization of dihydrotestosterone-induced conformational perturbations in androgen receptor ligand-binding domain. Mol Endocrinol 23:1231–1241
22. **Watanabe C, Watanabe H, Tanaka S** 2010 An interpretation of positional displacement of the helix12 in nuclear receptors: preex-

istent swing-up motion triggered by ligand binding. Biochim Biophys Acta 1804:1832–1840
23. **McPhaul MJ** 2008 Mechanisms of prostate cancer progression to androgen independence. Best Pract Res Clin Endocrinol Metab 22: 373–388
24. **Nacusi LP, Tindall DJ** 2009 Androgen receptor abnormalities in castration-recurrent prostate cancer. Expert Rev Endocrinol Metab 4:417–422
25. **Gottlieb B, Pinsky L, Beitel LK, Trifiro M** 1999 Androgen insensitivity. Am J Med Genet 89:210–217
26. **Huggins C** 1967 Endocrine-induced regression of cancers. Science 156:1050–1054
27. **Culig Z, Hoffmann J, Erdel M, Eder IE, Hobisch A, Hittmair A, Bartsch G, Utermann G, Schneider MR, Parczyk K, Klocker H** 1999 Switch from antagonist to agonist of the androgen receptor bicalutamide is associated with prostate tumour progression in a new model system. Br J Cancer 81:242–251
28. **Bohl CE, Gao W, Miller DD, Bell CE, Dalton JT** 2005 Structural basis for antagonism and resistance of bicalutamide in prostate cancer. Proc Natl Acad Sci USA 102:6201–6206
29. **Yuan X, Balk SP** 2009 Mechanisms mediating androgen receptor reactivation after castration. Urol Oncol 27:36–41
30. **Estébanez-Perpiñá E, Arnold LA, Arnold AA, Nguyen P, Rodrigues ED, Mar E, Bateman R, Pallai P, Shokat KM, Baxter JD, Guy RK, Webb P, Fletterick RJ** 2007 A surface on the androgen receptor that allosterically regulates coactivator binding. Proc Natl Acad Sci USA 104:16074–16079
31. **Axerio-Cilies P, Lack NA, Nayana RS, Chan KH, Yeung A, Leblanc E, Guns E, Rennie P, Cherkasov A** 2011 Inhibitors of androgen receptor activation function-2 (AF2) site indentified through virtual screening. J Med Chem 54:6197–6205
32. **Estébanez-Perpiñá E, Jouravel N, Fletterick RJ** 2007 Perspectives on designs of antiandrogens for prostate cancer. Expert Opin Drug Discov 2:1341–1355
33. **Buzón V, Carbó LR, Estruch SB, Fletterick RJ, Estébanez-Perpiñá E** 2012 A conserved surface on the ligand binding domain of nuclear receptors for allosteric control. Mol Cell Endocrinol 348:394–402
34. **Wang P, Cieplak P, Kollman PA** 2000 How well does a restrained electrographic potential (RESP) model perform in calculating conformational energies of organic and biological molecules? J Comput Chem 21:1049–1074
35. **Case DA, Cheatham III TE, Darden T, Gohlke H, Luo R, Merz Jr KM, Onufriev A, Simmerling C, Wang B, Woods RJ** 2005 The Amber biomolecular simulation programs. J Comput Chem 26: 1668–1688
36. **Lee B, Richards FM** 1971 The interpretation of protein structures: estimation of static accessibility. J Mol Biol 55:379–400
37. **Le Guilloux V, Schmidtke P, Tuffery P** 2009 Fpocket: an open source platform for ligand pocket detection. BMC Bioinformatics 10:168
38. **Schmidtke P, Souaille C, Estienne F, Baurin N, Kroemer RT** 2010 Large-scale comparison of four binding site detection algorithms. J Chem Inf Model 50:2191–2200
39. **Marcelli M, Ittmann M, Mariani S, Sutherland R, Nigam R, Murthy L, Zhao Y, DiConcini D, Puxeddu E, Esen A, Eastham J, Weigel NL, Lamb DJ** 2000 Androgen receptor mutations in prostate cancer. Cancer Res 60:944–949
40. **Duff J, Davies P, Watt K, McEwan IJ** 2006 Structural dynamics of the human androgen receptor: implications for prostate cancer and neurodegenerative disease. Biochem Soc Trans 34:1098–1102
41. **Elo JP, Kvist L, Leinonen K, Isomaa V, Henttu P, Lukkarinen O, Vihko P** 1995 Mutated human androgen receptor gene detected in a prostatic cancer patient is also activated by estradiol. J Clin Endocrinol Metab 80:3494–3500
42. **Koivisto PA, Schleutker J, Helin H, Ehren-van Eekelen C, Kallioniemi O-P, Trapman J** 1999 Androgen receptor gene alterations and chromosomal gains and losses in prostate carcinomas appearing during finasteride treatment for benign prostatic hyperplasia. Clin Cancer Res 5:3578–3582
43. **Mononen N, Syrjäkoski K, Matikainen M, Tammela TL, Schleutker J, Kallioniemi OP, Trapman J, Koivisto PA** 2000 Two percent of Finnish prostate cancer patients have a germ-line mutation in the hormone-binding domain of the androgen receptor gene. Cancer Res 60:6479–6481
44. **Hyytinen ER, Haapala K, Thompson J, Lappalainen I, Roiha M, Rantala I, Helin HJ, Jänne OA, Vihinen M, Palvimo JJ, Koivisto PA** 2002 Pattern of somatic androgen receptor gene mutations in patients with hormone-refractory prostate cancer. Lab Invest 82: 1591–1598
45. **Koivisto PA, Hyytinen ER, Matikainen M, Tammela TL, Ikonen T, Schleutker J** 2004 Germline mutation analysis of the androgen receptor gene in Finnish patients with prostate cancer. J Urol 171: 431–433
46. **Yan J, Feng J, Goldman D, Cook Jr EH, Craddock N, Jones IR, Heston LL, Sommer SS** 2004 Mutation scanning of the androgen receptor gene in patients with psychiatric disorders reveals highly conserved variants in alcoholic and phobia patients. Psychiatr Genet 14:57–60
47. **Wong HY, Hoogerbrugge JW, Pang KL, van Leeuwen M, van Royen ME, Molier M, Berrevoets CA, Dooijes D, Dubbink HJ, van de Wijngaart DJ, Wolffenbuttel KP, Trapman J, Kleijer WJ, Drop SL, Grootegoed JA, Brinkmann AO** 2008 A novel mutation F826L in the human androgen receptor in partial androgen insensitivity syndrome; increased NH2-/COOH-terminal domain interaction and TIF2 co-activation. Mol Cell Endocrinol 292:69–78
48. **Yong EL, Ng SC, Roy AC, Yun G, Ratnam SS** 1994 Pregnancy after hormonal correction of severe spermatogenic defect due to mutation in androgen receptor gene. Lancet 344:826–827
49. **Lim J, Ghadessy FJ, Abdullah AA, Pinsky L, Trifiro M, Yong EL** 2000 Human androgen receptor mutation disrupts ternary interactions between ligand, receptor domains, and the coactivator TIF2 (transcription intermediary factor 2). Mol Endocrinol 14:1187–1197
50. **Dotzlaw H, Moehren U, Mink S, Cato AC, Iñiguez Lluhí JA, Baniahmad A** 2002 The amino terminus of the human AR is target for corepressor action and antihormone agonism. Mol Endocrinol 16: 661–673
51. **Imasaki K, Hasegawa T, Okabe T, Sakai Y, Haji M, Takayanagi R, Nawata H** 1994 Single amino acid substitution (840Arg→His) in the hormone-binding domain of the androgen receptor leads to incomplete androgen insensitivity syndrome associated with a thermolabile androgen receptor. Eur J Endocrinol 1:569–574
52. **Beitel LK, Kazemi-Esfarjani P, Kaufman M, Lumbroso R, DiGeorge AM, Killinger DW, Trifiro MA, Pinsky L** 1994 Substitution of arginine-839 by cysteine or histidine in the androgen receptor causes different receptor phenotypes in cultured cells and coordinate degrees of clinical androgen resistance. J Clin Invest 94:546–554
53. **Melo KF, Latronico AC, Costa EM, Billerbeck AE, Mendonca BB, Arnhold IJ** 1999 A novel point mutation (R840S) in the androgen receptor in a Brazilian family with partial androgen insensitivity syndrome. Hum Mutat 14:353
54. **Nazareth LV, Stenoien DL, Bingman III WE, James AJ, Wu C, Zhang Y, Edwards DP, Mancini M, Marcelli M, Lamb DJ, Weigel NL** 1999 A C619Y mutation in the human androgen receptor causes inactivation and mislocalization of the receptor with concomitant sequestration of SRC-1 (steroid receptor coactivator 1). Mol Endocrinol 13:2065–2075
55. **Buchanan G, Yang M, Harris JM, Nahm HS, Han G, Moore N, Bentel JM, Matusik RJ, Horsfall DJ, Marshall VR, Greenberg NM, Tilley WD** 2001 Mutations at the boundary of the hinge and ligand binding domain of the androgen receptor confer increased transactivation function. Mol Endocrinol 15:46–56
56. **Wang Q, Lu J, Yong EL** 2001 Ligand- and coactivator-mediated

transactivation function (AF2) of the androgen receptor ligand-binding domain is inhibited by the cognate hinge region. J Biol Chem 276:7493–7499
57. **Tahiri B, Auzou G, Nicolas JC, Sultan C, Lupo B** 2001 Participation of critical residues from the extreme C-terminal end of the human androgen receptor in the ligand binding function. Biochemistry 40:8431–8437
58. **Gelmann EP** 2002 Molecular biology of the androgen receptor. J Clin Oncol 20:3001–3015
59. **Ong YC, Kolatkar PR, Yong EL** 2002 Androgen receptor mutations causing human androgen insensitivity syndromes show a key role of residue M807 in helix 8-helix 10 interactions and in receptor ligand-binding domain stability. Mol Hum Reprod 8:101–108
60. **McPhaul MJ** 2002 Molecular defects of the androgen receptor. Recent Prog Horm Res 57:181–194
61. **Shi XB, Ma AH, Xia L, Kung HJ, de Vere White RW** 2002 Functional analysis of 44 mutant androgen receptors from human prostate cancer. Cancer Res 62:1496–1502
62. **Gruber SB, Chen H, Tomsho LP, Lee N, Perrone EE, Cooney KA** 2003 R726L androgen receptor mutation is uncommon in prostate cancer families in the United States. Prostate 54:306–309
63. **Mazen I, Lumbroso S, Abdel Ghaffar S, Salah N, Sultan C** 2004 Mutation of the androgen receptor (R840S) in an Egyptian patient with partial androgen insensitivity syndrome: review of the literature on the clinical expression of different R840 substitutions. J Endocrinol Invest 27:57–60
64. **Quigley CA, Tan JA, He B, Zhou ZX, Mebarki F, Morel Y, Forest MG, Chatelain P, Ritzén EM, French FS, Wilson EM** 2004 Partial androgen insensitivity with phenotypic variation caused by androgen receptor mutations that disrupt activation function 2 and the NH(2)- and carboxyl-terminal interaction. Mech Ageing Dev 125:683–695
65. **Ledig S, Jakubiczka S, Neulen J, Aulepp U, Burck-Lehmann U, Mohnike K, Thiele H, Zierler H, Brewer C, Wieacker P** 2005 Novel and recurrent mutations in patients with androgen insensitivity syndromes. Horm Res 63:263–269
66. **Yen JL, Chang KH, Sheu JC, Lee YJ, Tsai LP** 2005 Partial androgen insensitivity syndrome with R840H mutation in androgen receptor: report of one case. Acta Paediatr Taiwan 46:101–105
67. **Haelens A, Tanner T, Denayer S, Callewaert L, Claessens F** 2007 The hinge region regulates DNA binding, nuclear translocation, and transactivation of the androgen receptor. Cancer Res 67:4514–4523
68. **Deeb A, Jääskeläinen J, Dattani M, Whitaker HC, Costigan C, Hughes IA** 2008 A novel mutation in the human androgen receptor suggests a regulatory role for the hinge region in amino-terminal and carboxy-terminal interactions. J Clin Endocrinol Metab 93:3691–3696
69. **Tanner TM, Denayer S, Geverts B, Van Tilborgh N, Kerkhofs S, Helsen C, Spans L, Dubois V, Houtsmuller AB, Claessens F, Haelens A** 2010 A 629RKLKK633 motif in the hinge region controls the androgen receptor at multiple levels. Cell Mol Life Sci 67:1919–1927
70. **Marimuthu A, Feng W, Tagami T, Nguyen H, Jameson JL, Fletterick RJ, Baxter JD, West BL** 2002 TR surfaces and conformations required to bind nuclear receptor corepressor. Mol Endocrinol 16:271–286
71. **Yumoto F, Nguyen P, Sablin EP, Baxter JD, Webb P, Fletterick RJ** 2012 2012 Structural basis of coactivation of liver receptor homolog-1 by β-catenin. Proc Natl Acad Sci USA 109:143–148
72. **De Leon JT, Iwai A, Feau C, Garcia Y, Balsiger HA, Storer CL, Suro RM, Garza KM, Lee S, Kim YS, Chen Y, Ning YM, Riggs DL, Fletterick RJ, Guy RK, Trepel JB, Neckers LM, Cox MB** 2011 Targeting the regulation of androgen receptor signaling by the heat shock protein 90 cochaperone FKBP52 in prostate cancer cells. Proc Natl Acad Sci USA 108:11878–11883

# 4.3. IDENTIFICATION OF NOVEL ANDROGEN RECEPTOR-INTERACTING PROTEINS

## 4.3.1. Introduction

Hundreds of proteins have been reported to interact with and/or regulate AR, making this receptor one of the most social NRs. A series of protein-protein recognition steps and concomitant conformational changes both in AR and the interacting proteins are believed to occur, although the detailed molecular mechanisms of AR regulation are still far from being understood. Current models of NR function favor the view that the tissue-specific actions of the AR depend both on the ligand as well as on the specific cellular context of coregulators. Thus, the physiological and pharmacological action of ligands (both androgens and antiandrogens used in the clinic) is interconnected with the binding of coregulators to AR.

In order to identify novel interactors and possible coregulators of AR, the hormone-bound AR LBD extended by a few residues of the hinge domain (646-919) was used as bait to perform yeast two-hybrid (Y2H) screens against both an adult human brain and prostate cDNA libraries. Putative novel AR interactors, together with AR coactivators ARA70 and SRC3, and the NR corepressor SMRT, were further validated in a one-to-one high-throughput (HT) Y2H assay. Finally, all interactions, including BF-3 mutants, were tested in the presence of several antiandrogens.

## 4.3.2. Identification of ARMC9, MAPK8IP1 and Uba3 as Novel AR Interactors

With the objective of assuring complete library coverage and find new AR interactors, seven Y2H screens were completed, in which repeatedly appeared several well-known and characterized AR coactivators such as gelsolin, β-catenin, and ARA70, evidencing the robustness of the Y2H setup. Furthermore, in the presence of DHT, four new putative AR interactors were isolated: one clone of

armadillo repeat containing 9 (ARMC9); three clones of mitogen-activated protein kinase 8-interacting protein 1 (MAPK8IP1); two clones of Rab11 family-interacting protein 3 (Rab11FIP3); and four clones of the ubiquitin-like modifier activating enzyme 3 (Uba3), the catalytic subunit of the NEDD8-specific E1 activating enzyme (NAE) involved in the neddylation pathway. From the 817 residues that form ARMC9, the region that was found to interact with AR spanned from the second amino acid to residue 307. All three MAPK8IP1 clones started at position 465, one of them stopping at amino acid 686, and the other two extending to residue 711, the last one. Both Rab11FIP3 clones covered from amino acid 601 to the end of the protein at position 756. Finally, all Uba3 clones comprised the first 300 N-terminal residues of the protein, representing the 65% of the full-length protein, which contains 463 amino acids.

To further verify the interaction between AR and the newly identified proteins, a 96 well-format one-to-one HT-Y2H was set up in the laboratory. This format of the Y2H screen enabled the exploration of the binding of these and other AR coregulators to the AR LBD BF-3 mutants, in the absence and presence of DHT or antiandrogens. Besides the new recently identified AR interacting proteins, the following AR coregulators were included in the screen: ARA70 (an FxxLF-containing AR coactivator), SRC1 and SRC3 (LxxLL-containing AR coactivators); the NR corepressor SMRT, β-catenin, caveolin-1, Fused in Ewing's Sarcoma (FUS), and the 52 kDa FK506 binding protein (FKBP52) cochaperone.

Excitingly, the interaction between the LBD of AR and three of the four putative AR binders fished in the Y2H screens of the adult human brain cDNA library, ARMC9, MAPK8IP1 and Uba3, was confirmed in the one-to-one HT-Y2H in the presence of hormone, although the binding of AR to MAPK8IP1 and Uba3 could not be validated on SD4 growing media. Contrarily, Rab11FIP3 could not be confirmed as an AR LBD-interacting protein (**Figure 4-11**). All clones grew in the master plates demonstrating that all colonies contained both the bait and the prey plasmids.

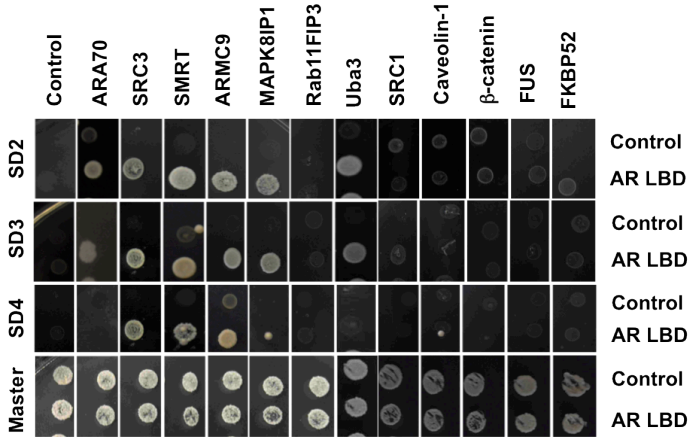


**Figure 4-11.** ARA70, SRC3, SMRT, ARMC9, MAPK8IP1, and Uba3 interact with the AR LBD in the presence of DHT.

Interaction validation between DHT-bound AR LBD WT and the identified ARMC9 (2-307), MAPK8IP1 (465-711), RAB11FIP3 (601-756) and Uba3 (1-300) clones by forward one-to-one Y2H. ARA70, SRC3, SMRT, SRC1, caveolin, β-catenin, FUS, and FKBP52 were also tested for AR LBD binding. Yeast transformants were plated on SD2 (lacking Leu, Trp, His, and supplemented with 50 mM 3AT) plates **(A)**, SD3 (lacking Leu, Trp, His, and supplemented with 75 mM 3AT) plates **(B)**, and SD4 (lacking Leu, Trp, Ura) selective plates **(C)**, all of them containing 100 nM DHT. **(D)** Master plates (lacking Leu and Trp) showing that all clones contained both bait and prey plasmids. The experiment was performed three times, although only the results of a single assay are shown.

SRC3 and SMRT grew in SD2, SD3 and SD4 selection media by activating both *HIS3* and *URA3* reporter genes in the presence of DHT, indicating they strongly interact with ligand-bound AR, whereas ARA70 was only able to activate the former reporter. Unexpectedly, the one-to-one HT-Y2H assay could not demonstrate the binding of AR to neither of the known AR coregulators SRC1, β-catenin, caveolin-1, FUS, or FKBP52.

### 4.3.3. Surface Mutations in the AF-2 and BF-3 Pockets Disrupt the Interaction of the AR LBD with known and novel Coregulators

Our previous results characterized a series of residues from BF-3, the boundary of AF-2/BF-3 and AF-2 pockets that are structurally interconnected and allosterically coupled indicating that several residues belonging to those surface grooves are key players of an allosteric network that influence multiple aspects of AR LBD function (Buzón et al., 2012; Estébanez-Perpiñá et al., 2007; Grosdidier et al., 2012). As

physiological relevant partners of AR BF-3 are still under investigation and with the objective to evaluate a possible role for the BF-3 pocket in the recruitment of proteins, the recently established HT-Y2H assay was used to study some of these AR mutants in the context of their association with both the previously mentioned AR coregulators and the putative newly identified interactors. In particular, the binding of AR LBD WT was analyzed and compared to a selected dataset of AR variants including K720A (one of the charge clamp residues, located at the AF-2 pocket), residues R726L and N727K (residing at the boundary of BF-3/AF-2) as well as the BF-3 pocket mutants F826A, F826L, F826R, N833R, R840A and R840E (location of all mutations are shown in Figure 4-2).

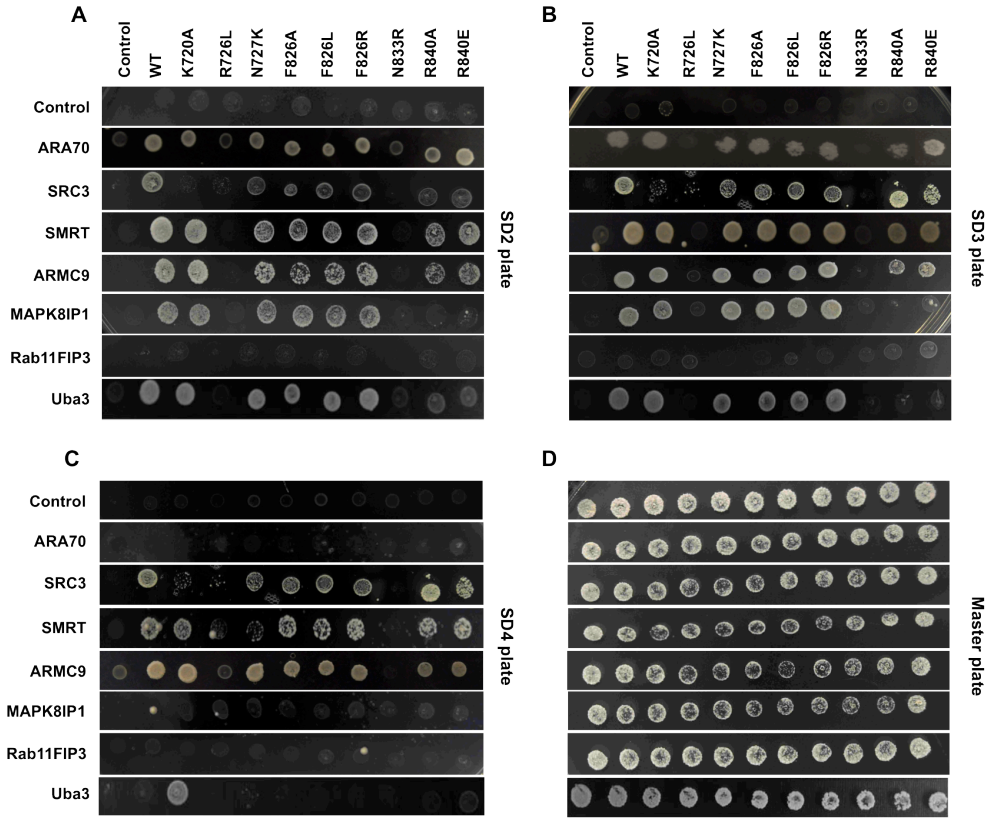


**Figure 4-12.** Interaction of the AR LBD WT and BF-3 mutants with AR coregulators and new putative AR interactors.

Interaction validation between DHT-bound AR LBD WT and the identified ARMC9 (2-307), MAPK8IP1 (465-711), and Uba3 (1-300) clones by forward one-to-one Y2H. AR LBD mutants were also tested for interaction with the newly identified AR coregulators, as well as ARA70, SRC3 and SMRT. Yeast transformants were plated on SD2 (lacking Leu, Trp, His, and supplemented with 50 mM 3AT) plates **(A)**, SD3 (lacking Leu, Trp, His, and supplemented with 75 mM 3AT) plates **(B)**, and SD4 (lacking Leu, Trp, Ura) selective plates **(C)**, all of them containing 100 nM DHT. **(D)** Master plate (lacking Leu and Trp) showing that all clones contained both bait and prey plasmids. The experiment was performed three times, although only the results of a single assay are shown.

Most of the substitutions had no effect on the binding of AR to the tested coregulators in the presence of DHT, although there are some exceptions that are worth mentioning (**Figure 4-12A-C**). Firstly, variants R726L and N833R were not able to recruit any protein in the presence of DHT. Secondly, mutating the charge-clamp residue K720 to an alanine prevented AR from associating with SRC3, while was the only one able to interact with Uba3 in SD4 selective medium, as neither variant N727K nor any of the substitutions at position 826 were capable of activating the *URA3* reporter gene in SD4 plates (the binding to all other coregulators remained unaffected). And lastly, replacing the arginine at position 840 by either an alanine or a glutamic acid disrupted the binding of AR to MAPK8IP1 and Uba3, but maintained the association with the rest of the tested proteins.

On the other hand, similar to what observed for the AR LBD WT, none of the mutants that were able to bind ARA70 in SD2 and SD3 plates could interact with the AR coregulator in SD4 plates. And as it was obtained with WT construct, none of the AR LBD variants was capable of associating with Rab11FIP3 in the presence of hormone.

In addition, none of the proteins was capable of interacting with neither the AR LBD WT nor any of the mutants in the absence of hormone (**Figure 4-13**).

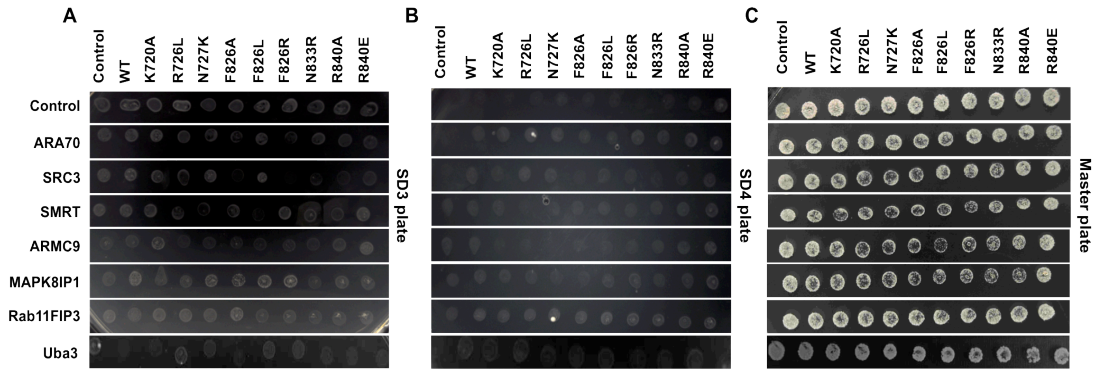


**Figure 4-13.** AR interactors do not bind to AR in the absence of hormone (Apo AR LBD).

In the absence of hormone, none of the proteins is able to interact with AR, neither on SD2 (lacking Leu, Trp, His, and supplemented with 50 mM 3AT) **(A)**, or SD4 (lacking Leu, Trp, Ura) **(B)** selective plates. **(C)** Master plate (lacking Leu and Trp) showing that all clones contained both bait and prey plasmids. The experiment was performed three times, although only the results of a single assay are shown.

As observed for the AR LBD WT, none of the mutant forms could interact with the known AR interacting proteins SRC1, β-catenin, caveolin-1, FUS, or FKBP52 in the established one-to-one HT-Y2H (**Figure 4-14**).

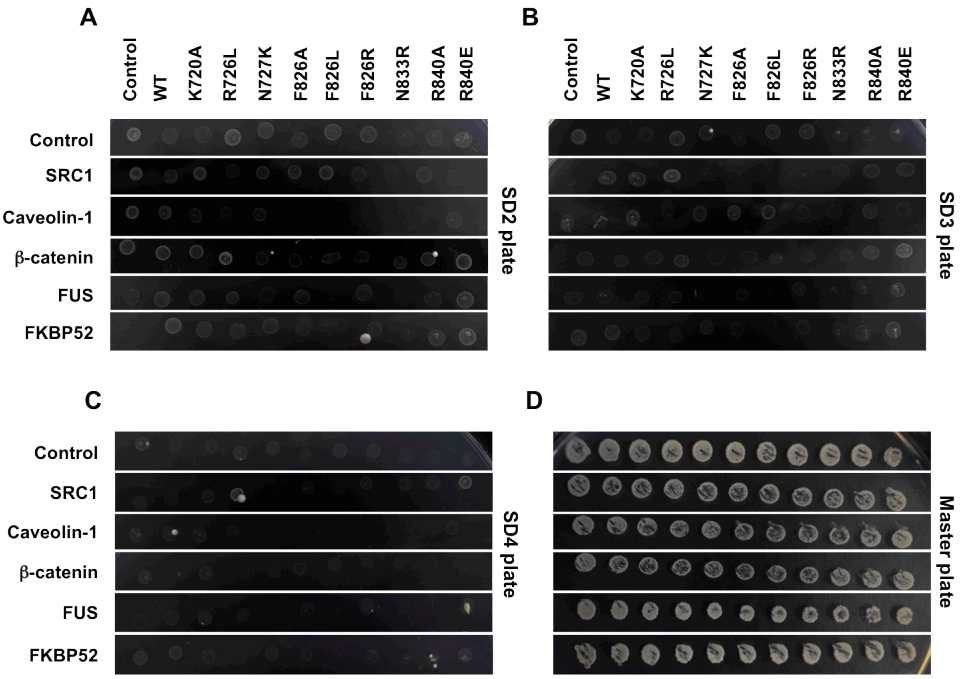


**Figure 4-14.** The interaction between AR LBD and SRC1, caveolin-1, β-catenin, FUS, or FKBP52 could not be demonstrated by the one-to-one high-throughput Y2H.

Yeast transformants were plated on SD2 (lacking Leu, Trp, His, and supplemented with 50 mM 3AT) plates **(A)**, SD3 (lacking Leu, Trp, His, and supplemented with 75 mM 3AT) plates **(B)**, and SD4 (lacking Leu, Trp, Ura) selective plates **(C)**, all of them containing 100 nM DHT. **(D)** Master plates (lacking Leu and Trp) showing that all clones contained both bait and prey plasmids. The experiment was performed three times, although only the results of a single assay are shown.

### 4.3.4. Uba3 Interacts with the LBD of AR Through NR boxes

All the Uba3 clones that were isolated from the adult human brain cDNA library contained two NR boxes, $^{63}$LQFLL$^{67}$ and $^{179}$LISLL$^{183}$ (**Figure 4-15**), which resemble to other LxxLL motifs found in known AR coactivators (e.g. the three members of the SRC family), suggesting the participation of these two sequences in the association between the AR LBD and Uba3.

| | | | | | |
|---|---|---|---|---|---|
| **1** | MADGEEPEKK | RRRIEELLAE | KMAVDGGCGD | TGDWEGRWNH | VKKFLERSGP |
| **51** | FTHPDFEPST | ES**LQFLL**DTC | KVLVIGAGGL | GCELLKNLAL | SGFRQIHVID |
| **101** | MDTIDVSNLN | RQFLFRPKDI | GRPKAEVAAE | FLNDRVPNCN | VVPHFNKIQD |
| **151** | FNDTFYRQFH | IIVCG***LDSII*** | ARRWINGM**LI** | **SLL**NYEDGVL | DPSS***IVPLI***D |
| **201** | GGTEGFKGNA | RVILPGMTAC | IECTLELYPP | QVNFPMCTIA | SMPRLPEHCI |
| **251** | EYVRMLQWPK | EQPFGEGVPL | DGDDPEHIQW | IFQKSLERAS | QYNIRGVTYR |
| **301** | LTQGVVKRII | PAVASTNAVI | AAVCATEVFK | IATSAYIPLN | NYLVFNDVDG |
| **361** | LYTYTFEAER | KENCPACSQL | PQNIQFSPSA | KLQEVLDYLT | NSASLQMKSP |
| **401** | AITATLEGKN | RTLYLQSVTS | IEERTRPNLS | KTLKELGLVD | GQELAVADVT |
| **451** | TPQTVLFKLH | FTS | | | |

**Figure 4-15.** Amino acid sequence of the human Uba3 (NCBI Reference Sequence NP_003959.3).
The two NR boxes, $^{63}$LQFLL$^{67}$ and $^{179}$LISLL$^{183}$, are shown in bold and are underlined; whereas the two NR corepressor-like motifs, $^{166}$LDSII$^{170}$ and $^{195}$IVPLI$^{199}$, are indicated in bold and italic.

In order to evaluate whether these NR boxes are implicated in the binding of Uba3 to AR, the last two leucines of each motif were replaced by alanines and the HT-Y2H with the AR LBD WT, K720A and BF-3 mutants was performed.

The disruption of these two single motifs either separately or together prevented Uba3 from interacting with the AR LBD WT (**Figure 4-16B-C**), indicating that both NR boxes are involved in the recruitment of AR. However, interestingly, mutating the first NR box did not impede Uba3 from associating with the charge clamp mutant K720A, which was still able to bind to Uba3 (**Figure 4-16B-C**). Nevertheless, the second NR box mutant of Uba3, as well as the double mutant, failed to interact with K720A, highlighting the importance of the second NR box in this association. None of the other BF-3 mutants was capable of recruiting any of the Uba3 variant forms.

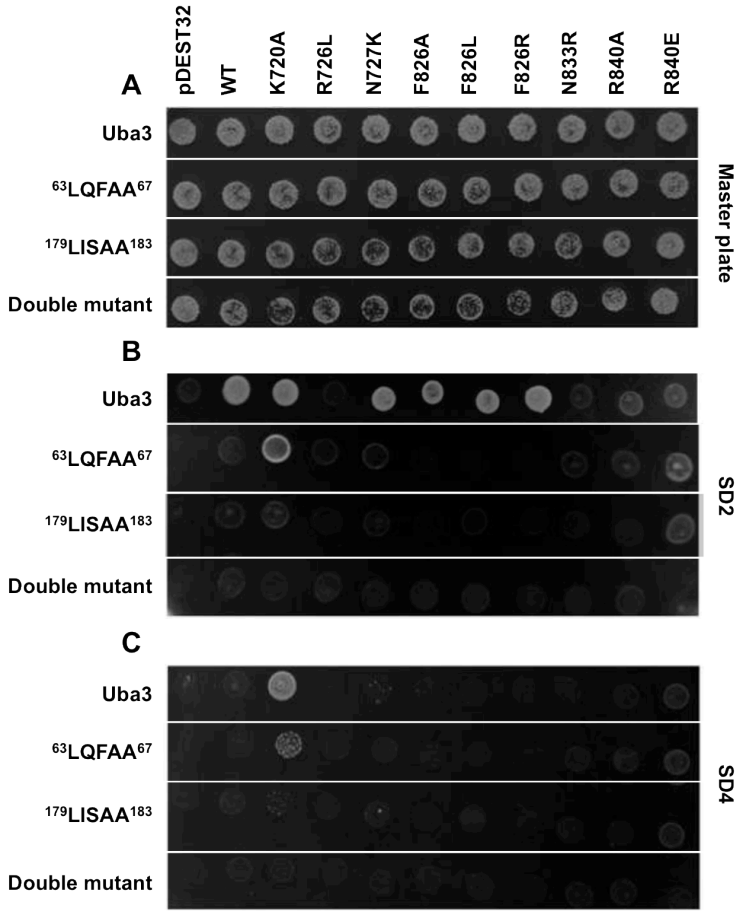


**Figure 4-16.** Uba3 binds to AR LBD through NR boxes.

Uba3 mutants were tested for interaction with AR LBD WT, K720A, and BF-3 mutants. Yeast transformants were plated on master plates (lacking Leu and Trp) **(A)**; and SD2 (lacking Leu, Trp, His, and supplemented with 50 mM 3AT) **(B)**, and SD4 (lacking Leu, Trp, and Ura) **(C)** selective plates in the presence of 100 nM DHT. The same experiment was performed in triplicate.

### 4.3.5. SRC3, ARMC9 and Uba3 Can Bind to the AR LBD in the Presence of Antiandrogens

Leaving aside the well-known AR coregulators, whose interaction with the DHT-bound AR LBD could not be demonstrated in our HT-Y2H set up, the binding of AR LBD to ARA70, SRC3, SMRT, ARMC9, MAPK8IP1, Rab11FIP3, and Uba3 was further explored in the presence of several AR antagonists (hydroxyflutamide, bicalutamide, mifepristone and enzalutamide) to evaluate their influence on AR LBD conformation and association with these proteins. Hydroxyflutamide, bicalutamide and the recently FDA-approved enzalutamide are three antiandrogens used in the treatment of PCa, whereas mifepristone is a potent

antagonist of the PR that also shows a weak antiandrogenic activity, and which has additionally been shown to increase the interaction of AR with corepressors (Song, 2003).

On the one hand, hydroxyflutamide, the first tested antiandrogen, did not promote the association of any of the examined proteins with neither the AR LBD WT nor any of the BF-3 mutants (**Figure 4-17**).

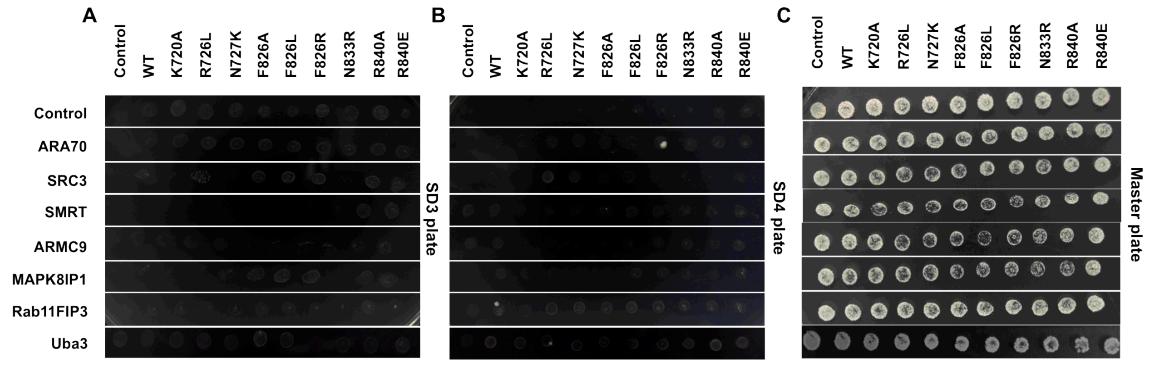


**Figure 4-17.** Hydroxyflutamide do not promote the interaction of AR LBD with AR coregulators.
None of the coregulators is able to bind to the AR LBD, neither on SD2 (lacking Leu, Trp, His, and supplemented with 50 mM 3AT) **(A)**, nor SD4 (lacking Leu, Trp, Ura) **(B)** selective plates. The experiment was performed three times, although only the results of a single assay are presented.

Surprisingly, however, SRC3, ARMC9 and Uba3 were able to associate with the AR LBD WT in the presence of bicalutamide (**Figure 4-18A-B**), mifepristone (**Figure 4-18C**) and enzalutamide (**Figure 4-18F**), although the binding of Uba3 to AR promoted by bicalutamide seemed to be weak. ARMC9 activated both *HIS3* and *URA3* reporter genes in SD3 and SD4 plates, respectively, in the presence of the three antiandrogens, whereas SRC3 only activated both reporters with the bicalutamide-bound AR. The interaction between the AR LBD WT and Uba3 could only take place in SD3 selective medium.

On the other hand, neither ARA70, SMRT, Rab11FIP3 or MAPK8IP1 were capable of binding to the AR LBD WT in the presence of any of the tested compounds. Not even the mifepristone-bound AR LBD WT was able to recruit SMRT.

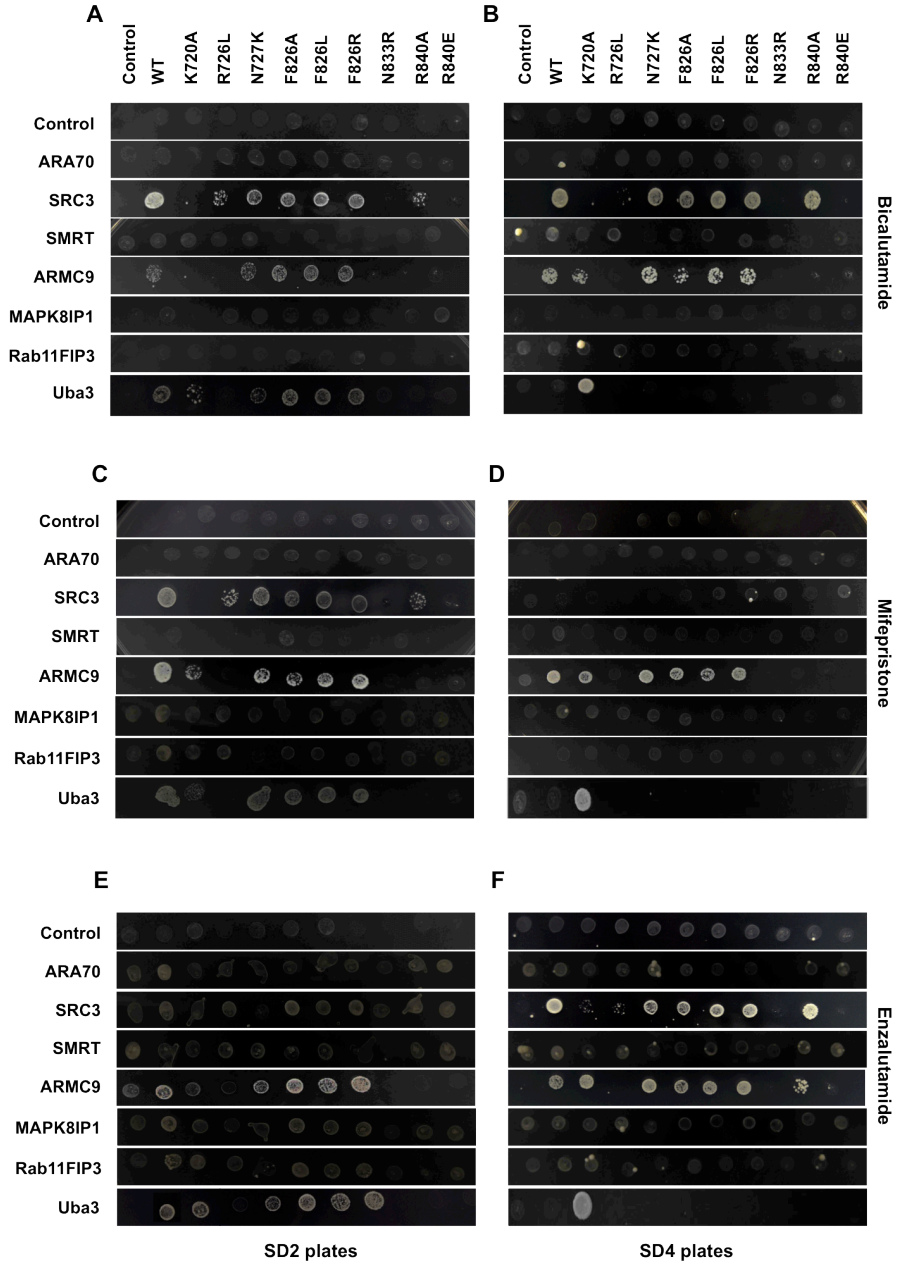


**Figure 4-18.** SRC3, ARMC9, and Uba3 interact with the AR LBD in the presence of antiandrogens.
Yeast transformants were plated on SD2 (lacking Leu, Trp, His, and supplemented with 50 mM 3AT) **(A,C,E)**, and SD4 selective plates (lacking Leu, Trp, and Ura) **(B,D,F)** containing 10 μg bicalutamide **(A,B)**, mifepristone **(C,D)** or enzalutamide **(E,F)**. The same experiment was performed in triplicate.

Regarding the AR LBD BF-3 mutants, the charge clamp mutant K720A was not capable of binding to SRC3 neither in the presence of DHT or any antiandrogen; on the contrary, the replacement of the lysine by an alanine conferred the AR LBD the capability of binding to ARMC9 and Uba3 on SD4 plates in the presence of bicalutamide, mifepristone, and enzalutamide. Excitingly, in the case of Uba3, this

association was not seen with the AR LBD WT with any of the three antiandrogens. In SD3 selective medium, only enzalutamide promoted the binding of AR K720A to Uba3 (this association in the presence of bicalutamide and mifepristone is controversial). Surprisingly, variant R726L, which did not recruit any of the tested AR coregulators in the presence of DHT, seemed to associate weakly with SRC3 on SD2 plates in the presence of bicalutamide and mifepristone. As seen with DHT (**Figure 4-12**A-C), the binding of the AR variants N727K, F826A, F826L and F826R to both SRC3 and ARMC9 was induced by bicalutamide, mifepristone and enzalutamide, although SRC3 could not activate neither the *URA3* reporter gene in the presence of mifepristone, nor the *HIS3* reporter in the presence of enzalutamide, as observed also with DHT. Mutant N833R was not able to interact with any of the two proteins in the presence of any of the tested antiandrogens. Finally, the substitution of the arginine at position 840 by a glutamic acid prevented AR from interacting with SRC3 and ARMC9 in the presence of bicalutamide, mifepristone and enzalutamide, whereas the replacement by an alanine retained the capacity of associating with SRC3.

**Table 4** summarizes the most relevant results found in the HT-Y2H assays with the AR LBD WT construct in the absence and presence of DHT or the antiandrogens hydroxyflutamide, bicalutamide, mifepristone or enzalutamide.

| Protein | No hormone | | DHT | | Hydroxy-flutamide | | Bicalutamide | | Mifepristone | | Enzalutamide | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *HIS3* | *URA3* | *HIS3* | *URA3* | *HIS3* | *URA3* | *HIS3* | *URA3* | *HIS3* | *URA3* | *HIS3* | *URA3* |
| ARA70 | | | | | | | | | | | | |
| SRC3 | | | | | | | | | | | | |
| SMRT | | | | | | | | | | | | |
| ARMC9 | | | | | | | | | | | | |
| MAPK8IP1 | | | | | | | | | | | | |
| Rab11FIP3 | | | | | | | | | | | | |
| Uba3 | | | | | | | | | | | | |

**Table 4.** Summary of the Y2H results obtained with the AR LBD WT.

Summary of the Y2H results obtained with the AR LBD WT in the absence of hormone, or in the presence of 100 nM DHT, or 10 mM hydroxyflutamide, bicalutamide, mifepristone, or enzalutamide. Green, interaction; red, no interaction.

### 4.3.6. Rab11FIP3 and Uba3 Reduce the Transactivation Capacity of the AR LBD, Repress its Coactivation by GRIP1, and Impair the N/C Interaction

Despite Rab11FIP3 could not be validated as an AR interactor in the HT-Y2H screens, luciferase assays in HeLa cells were performed in order to find out whether it was able to regulate AR somehow. Surprisingly, when cells were treated with DHT, Rab11FIP3 repressed AR LBD in a dose-dependent manner (**Figure 4-19A**) almost abolishing AR transactivation completely.

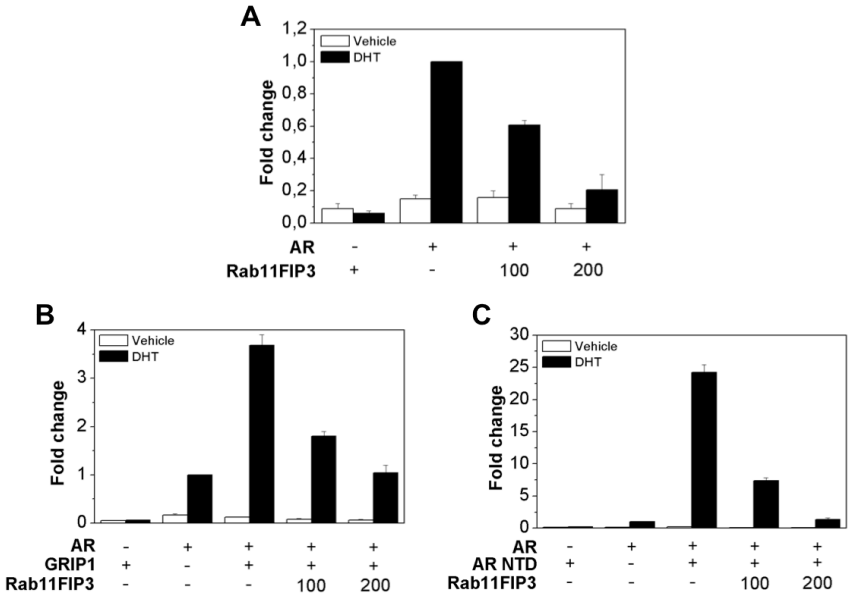


**Figure 4-19.** Rab11FIP3 represses the activity of AR LBD, attenuates its coactivation by GRIP1, and blocks the N/C interaction.

**(A)** AF-2 activity of the LBD of AR. **(B)** AF-2 coactivation by GRIP1. **(C)** N/C interaction between the LBD and the NTD of AR. Cells were transfected with 100 ng AR LBD alone or together with 100 ng GRIP1 or NTD, and increasing amounts of Rab11FIP3 (0-100-200 ng), and left untreated (vehicle) or stimulated with 10 nM hormone (DHT). Results are the mean of three independent experiments performed in triplicate. AR LBD = 1.

Additionally, Rab11FIP3 was also capable of attenuating the coactivation of AR by GRIP1, lowering this coactivation down to the baseline activity of the AR LBD alone (**Figure 4-19B**). Finally, an *in vitro* M2H assay in the absence and presence of Rab11FIP3 was performed in order to assess its effect on the interaction between the NTD and the AF-2 pocket of AR. As it has already been shown before, the NTD strongly binds to the LBD of AR in the presence of hormone. However, the addition of Rab11FIP3 severely compromised the interaction of the LBD with the N-

terminal domain of AR (**Figure 4-19C**).

On the other hand, as Uba3 is known to repress the function of some NRs (e.g. ER), the functional effects of Uba3 on AR LBD in *in vitro* reporter luciferase assays was also assessed (Fan et al., 2002).

In the presence of DHT, Uba3 inhibited AR in a dose-dependent manner, despite not abolishing the AR transactivation ability completely (**Figure 4-20A**). Under the stimulation of hormone, Uba3 was also able to attenuate the coactivation of AR by GRIP1, lowering this coactivation down to the baseline activity of the AR LBD alone at the highest amount of Uba3 tested (**Figure 4-20B**). Moreover, elevated concentrations of Uba3 seemed to block GRIP1 coactivation in the absence of hormone, although this repression was very modest.

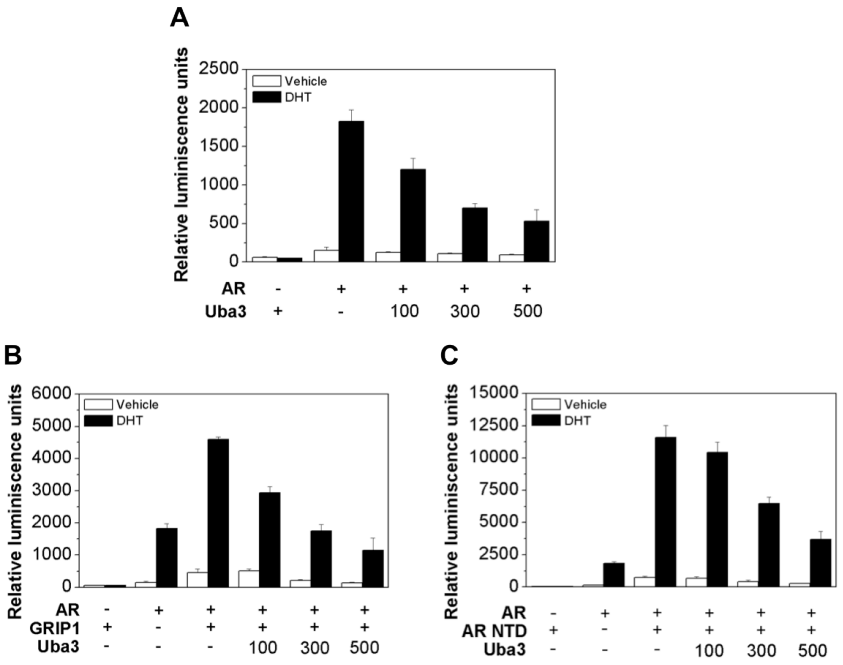


**Figure 4-20.** Uba3 represses the activity of AR LBD, attenuates its coactivation by GRIP1, and blocks the N/C interaction.

**(A)** AF-2 activity of the LBD of AR. **(B)** AR LBD coactivation by GRIP1. **(C)** N/C interaction between the LBD and the NTD of AR. Cells were transfected with 100 ng AR LBD alone or together with 100 ng GRIP1 or NTD, and increasing amounts of Uba3 (0-100-300-500 ng), and left untreated (vehicle) or stimulated (DHT) with hormone (10 nM). Experiments were performed three times in triplicate.

To test whether the binding of Uba3 to AR LBD has any effect on the interaction between the NTD and the AF-2 pocket of AR, an *in vitro* M2H assay in the absence and presence of Uba3 was performed, showing that high concentrations of Uba3

weakened the interaction between the N- and C-terminal domains of the AR (**Figure 4-20C)**.

### 4.3.7. Discussion

By means of a Y2H system, we have identified ARMC9, MAPK8IP1, Rab11FIP3 and Uba3 as new direct AR interactors. The Y2H analysis is a widely used technique for the discovery of protein-protein interactions. In the last 10 years, Y2H system has been developed as a tool that allows the systematic, large-scale analysis of protein-protein interactions and it is currently one of the most powerful methods to construct protein-protein interaction maps (Worseck et al., 2012). Therefore, with the objective of identifying novel AR protein interactors, which may bind to the BF-3 site, we performed several Y2H screens against a human adult brain and prostate cDNA libraries using the DHT-bound human AR LBD as bait. In other to further validate these proteins as new AR binders, a HT-Y2H array was set-up in our laboratory. Y2H analysis is a low sensitivity method, as data obtained are of high precision but low coverage, requiring repeated screens to capture most detectable interactions (Weimann et al., 2013). In contrast, the major advantage of this HT-Y2H matrix is that all protein pairs are tested with equal probabilities and the exact same interaction array can be repeated.

Despite the binding of ARMC9 to AR has never been reported before, this interaction was validated in the HT-Y2H matrix in the presence of DHT and, interestingly, in the presence of bicalutamide, mifepristone and enzalutamide (**Table 5**). ARMC9 is an armadillo-containing protein, a large family of evolutionary conserved proteins characterized by the presence of a tandem repeat copy of a 42-residue motif. Each armadillo-repeat unit consists of 3 α-helices, which allows these proteins to interact with numerous partners (Berthon and Stratakis, 2014). The most well-known member of this family is β-catenin, an AR coregulator that has been demonstrated to interact with AR AF-2 to significantly enhance AR ligand-dependent transactivation, increase sensitivity and alter its ligand specificity (Song et al., 2003; Truica et al., 2000). Interestingly, the binding of ARMC9 to AR could take place in the presence of antiandrogens, suggesting that

similarly to β-catenin, ARMC9 may broaden the range of agonists capable of activating AR. Consequently, a plausible implication of ARMC9 in PCa may not be ruled out, as mutated forms of β-catenin have been identified in human PCa (Voeller et al., 1998).

| Protein | No hormone | | DHT | | Hydroxy-flutamide | | Bicalutamide | | Mifepristone | | Enzalutamide | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *HIS3* | *URA3* | *HIS3* | *URA3* | *HIS3* | *URA3* | *HIS3* | *URA3* | *HIS3* | *URA3* | *HIS3* | *URA3* |
| WT | | | | | | | | | | | | |
| K720A | | | | | | | | | | | | |
| R726L | | | | | | | | | | | | |
| N727K | | | | | | | | | | | | |
| F826A | | | | | | | | | | | | |
| F826L | | | | | | | | | | | | |
| F826R | | | | | | | | | | | | |
| N833R | | | | | | | | | | | | |
| R840A | | | | | | | | | | | | |
| R840E | | | | | | | | | | | | |

**Table 5.** Summary of all Y2H interactions obtained between the different AR LBD constructs and ARMC9.

Summary of the Y2H results obtained in the absence of hormone, and in the presence of 100 nM DHT, or 10 mM hydroxyflutamide, bicalutamide, mifepristone, or enzalutamide. Green, interaction; red, no interaction.

Despite not much literature is available, MAPK8IP1, also known as C-Jun-amino-terminal kinase-interacting protein 3, has been identified as a scaffolding protein critical for autophagosome transport in neurons (Fu and Holzbaur, 2014), and it has been identified as an anti-apoptotic gene downregulated in breast cancers treated with photodynamic therapy (Silva et al., 2014). Here we show that MAPK8IP3 is a novel DHT-bound AR interactor, which, in contrast to ARMC9, could not associate to the receptor in the presence of antiandrogens, maybe because of its anti-apoptic function.

Rab11FIP3, also termed arfophilin-1 and eferin, is a Rab11-binding protein known to be involved in endosomal trafficking during cytokinesis. Rab proteins constitute the largest family of small GTPases, and Rab11 is a well-characterized regulator of endocytic and recycling pathways, known to associate with a wide range of post-Golgi organelles, including endosomes (Shiba et al., 2006). Interestingly, despite the interaction between the AR LBD and Rab11FIP3 could not be validated in the HT-Y2H matrix, transfection assays in HeLa cells demonstrated that Rab11FIP3 is

able to repress AR transactivation and coactivation by GRIP1, as well as completely block the N/C-terminal interaction. Whether these effects are mediated by direct contact with AR needs further experimental proof.

Additionally, we have shown that Uba3 directly binds to the LBD of AR and that this interaction takes place through two NR-interacting LxxLL motifs located at residues 63-37 and 179-183 of Uba3, as the mutation of any of these two NR boxes disrupted the binding to AR, suggesting that the AF-2 coactivator-binding pocket or overlapping areas on the AR LBD may be their site of contact. Surprisingly, bicalutamide, mifepristone and enzalutamide promoted the association between the AR LBD and Uba3 (**Table 6**).

| Protein | No hormone | | DHT | | Hydroxy-flutamide | | Bicalutamide | | Mifepristone | | Enzalutamide | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *HIS3* | *URA3* | *HIS3* | *URA3* | *HIS3* | *URA3* | *HIS3* | *URA3* | *HIS3* | *URA3* | *HIS3* | *URA3* |
| WT | | | | | | | | | | | | |
| K720A | | | | | | | | | | | | |
| R726L | | | | | | | | | | | | |
| N727K | | | | | | | | | | | | |
| F826A | | | | | | | | | | | | |
| F826L | | | | | | | | | | | | |
| F826R | | | | | | | | | | | | |
| N833R | | | | | | | | | | | | |
| R840A | | | | | | | | | | | | |
| R840E | | | | | | | | | | | | |

**Table 6.** Summary of all Y2H interactions obtained between the different AR LBD constructs and Uba3.

Summary of the Y2H results obtained in the absence of hormone, and in the presence of 100 nM DHT, or 10 mM hydroxyflutamide, bicalutamide, mifepristone, or enzalutamide. Green, interaction; red, no interaction.

Transfection studies in mammalian cells revealed that Uba3 inhibits AR transactivation, diminishes AR transcriptional coactivation by GRIP1, and interferes in the N/C interaction. Uba3 is the catalytic subunit of the NEDD8-specific E1 activating enzyme (NAE), which catalyzes the first reaction of protein neddylation, a newly characterized post-translational modification that adds a covalent conjugation of a NEDD8 moiety onto conserved lysine residues of substrate proteins, modulating their stability. Similar to ubiquitination, the neddylation pathway is a multistep process involving the action of NAE, a heterodimer consisting of the proteins AppBp1 and Uba3; as well as an E2

conjugating enzyme and an E3 ligase (**Figure 4-21**).

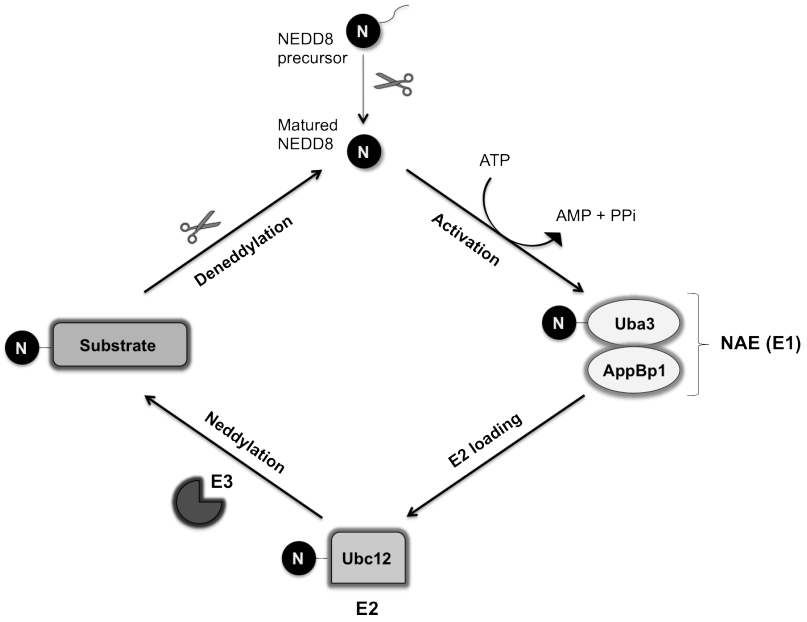


**Figure 4-21.** NEDD8 is a ubiquitin-like protein that becomes covalently conjugated to a limited number of cellular proteins.

NEDD8 activation and conjugation to substrates is catalyzed via an enzymatic cascade that is homologous to ubiquitination, involving NEDD8's E1 (NAE), E2 and E3.

Importantly, the neddylation pathway has been demonstrated to be overactivated in several cancer types, including PCa, and its inhibition by MLN4294, a compound that binds to NAE blocking the subsequent enzymatic cascades for protein neddylation, has proved to inhibit cell proliferation and survival in PCa cell lines (X. Wang et al., 2014).

Interestingly, the highly related ER is known to physically bind to Uba3 in the presence of hormone. Using the AF-2 and hinge region of ERα as bait in a Y2H screen, Uba3 was isolated and characterized, demonstrating its direct interaction with ligand-occupied ERα and ERβ (Fan et al., 2002). Moreover, sustaining our results, Fan et al. showed that Uba3 inhibits ER, AR and PR transactivation in mammalian cells and revealed that the neddylation activity is essential for mediating the suppression of ER transactivation function. Nevertheless, in flat opposition, Ubc9, a homologue of the E2 class of conjugating enzymes involved in the covalent linking of the ubiquitin-like protein SUMO-1, has been demonstrated to be able to enhance AR transcriptional activity in a fashion independent on its ability to catalyse SUMO-1 conjugation (Poukka, 1999). Thus, the mechanism by

which these ubiquitin-like proteins alter SRs can be dual: on the one hand, they function as coregulatory proteins governing the activity of SRs, while on the other hand, they modify them covalently, regulating their function. Whether the effects we observe on the impairment of AR functions are only due to its association with Uba3 or neddylation is also involved cannot be derived from our studies. If AR undergoes neddylation and whether its neddylation is required for Uba3-mediated inhibition of its activity still needs further investigation.

Despite the protein sequence of the human AR does not seem to contain the identified putative neddylation motif within the conserved IVRIMKMR sequence (Pan et al., 2004), it does comprise several lysine residues, most of them being surface-exposed, which could be responsible for accepting the NEDD8 moiety. Although so far, there are no publications on whether AR is a protein that undergoes neddylation, Prof. J. Don Chen (New Jersey, USA) has suggested that the lysine at position 630 in the AR hinge domain may be responsible for accepting the NEDD8 moiety (personal communication), despite this residue is already target for acetylation and methylation. Our AR LBD construct (646-919) contains part of the hinge region but does not include Lys630, what would suggest that, unlike what it has been demonstrated for ER, the inhibitory effect that Uba3 exerts on AR would be independent from the neddylation activity, in the hypothetical scenario that AR underwent neddylation.

What we additionally demonstrate hereby is that the disruption of one of the NR-like boxes contained in the primary structure of Uba3 weakened or prevented it from interacting with the AR LBD, pointing these NR boxes as the recruitment site for AR. Moreover, the presence of these two LxxLL motifs suggests that the protein-protein interface in AR involves the AF-2 coactivator-binding groove and/or an overlapping surface area that would also comprise the BF-3 pocket. Furthermore, the analysis of the crystal structure of the AppBp1-Uba3-NEDD8 complex (PDB 1R4M) shows that the $^{63}$LQFLL$^{67}$ motif forms an $\alpha$-helix (ESLQFLLDT) at the surface of Uba3 completely accessible to AR, which could be hypothesized as the association site for the AF-2 site on the AR LBD (**Figure 4-22**). In addition, our Y2H assays with several AR LBD mutants also indicate that the docking sites of both proteins may involve overlapping areas between the AF-2 and BF-3 pockets, without discarding the allosteric structural coupling between

both surface sites. Unexpectedly, we observed that AR was capable of interacting with UBA3 in the presence of the antiandrogens bicalutamide, mifepristone and enzalutamide.

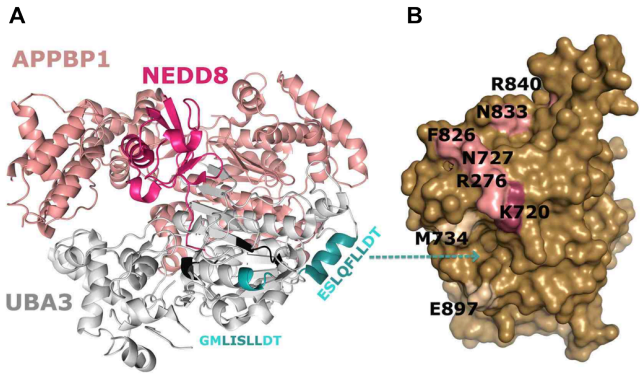


**Figure 4-22.** Computer analysis of protein-protein interaction sites on Uba3 surface.
**(A)** Analysis of the crystal structure of the AppBP1-Uba3-NEDD8 compex (PDB 1R4M). AppBp1 is shown in salmon; the catalytic subunit Uba3, in grey; and the NEDD8 moiety, in pink. The two LxxLL motifs ($^{63}$LQFLL$^{67}$ and $^{179}$LISLL$^{183}$) are depicted in cyan. **(B)** AR LBD surface representation highlighting the BF-3 surface residues that have been mutated (in pink), as well as the AF-2 charge clamp residue K720 (in purple). M734 and E897 are also shown.

Besides validating the four putative AR interacting proteins, the HT-Y2H array also served to assess the binding of a set of BF-3 mutants to several known AR coregulators in the absence and presence of hormone, as well as different antiandrogens. The interaction of AR with ARA70, SRC3, and the NR corepressor SMRT, was promoted in the presence of DHT. Contrarily, the binding of SCR1, caveolin-1, β-catenin, FUS and FKBP52 to DHT-bound AR could not be established in our Y2H matrix. Both caveolin-1 and β-catenin are well-known AR-interacting proteins responsible for enhancing the ligand-dependent AR transactivation (Lu et al., 2001; Song et al., 2003), whereas FUS has recently been demonstrated to interact with AR, promoting its transactivational activity (Haile et al., 2011). FKBP52 is known to regulate AR activity and is thought to bind to the BF-3 surface (De Leon et al., 2011).

Interestingly, SRC3 was able to bind to AR in the presence of bicalutamide, mifepristone and enzalutamide (**Table 7**). It is well-know that SRC3 interacts with AR, enhancing its transcriptional activity (Gnanapragasam et al., 2001). However, besides being a NR coactivator, SCR3 plays a key role in the tumorigenesis and

progression of PCa. Its implication in driving the development of CRPC has been demonstrated, and its level of expression has been correlated with the severity and prognosis of the clinical disease (Ma et al., 2011; Tien et al., 2013). Because it is known to be required for CRPC development, it may not be surprising that antiandrogens potentiate the association of AR to SRC3. Importantly, a small molecule binding to the receptor interacting domain of SRC3 has demonstrated to reduce its concentration in PCa cell lines (Y. Wang et al., 2011), suggesting SRC3 as a new therapeutic target to abrogate CRCP progression.

| Protein | No hormone | | DHT | | Hydroxy-flutamide | | Bicalutamide | | Mifepristone | | Enzalutamide | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *HIS3* | *URA3* | *HIS3* | *URA3* | *HIS3* | *URA3* | *HIS3* | *URA3* | *HIS3* | *URA3* | *HIS3* | *URA3* |
| WT | | | | | | | | | | | | |
| K720A | | | | | | | | | | | | |
| R726L | | | | | | | | | | | | |
| N727K | | | | | | | | | | | | |
| F826A | | | | | | | | | | | | |
| F826L | | | | | | | | | | | | |
| F826R | | | | | | | | | | | | |
| N833R | | | | | | | | | | | | |
| R840A | | | | | | | | | | | | |
| R840E | | | | | | | | | | | | |

**Table 7**. Summary of all Y2H interactions obtained between the different AR LBD constructs and SRC3.

Summary of the Y2H results obtained in the absence of hormone, and in the presence of 100 nM DHT, or 10 mM hydroxyflutamide, bicalutamide, mifepristone, or enzalutamide. Green, interaction; red, no interaction.

On the other hand, SMRT, which associates strongly with the LBD of unliganded non-steroid NRs, is known to interact weakly with the agonist-liganded AR. Despite it has been suggested that this interaction is primarily mediated through the AR NTD (Yuan et al., 2013), a clear association between the corepressor and the AR LBD was obtained in the Y2H matrix. Furthermore, it has been demonstrated that both bicalutamide and mifepristone induce the recruitment of SMRT by AR (Song, 2003; Yuan et al., 2013), although this data was not reproduced in our Y2H system.

Hydroxyflutamide did not promote the interaction of any of the AR LBD mutants with any of the examined AR coregulators in any of the tested conditions.

Finally, regarding the BF-3 mutant forms, it is worthy mentioning that variants R726L and N833R were not capable of binding to any of the proteins in the presence of any of the tested compounds, with the exception of R726L, which, surprisingly, was able of interacting with SRC3 in the presence of bicalutamide and mifepristone. As discussed in Section 4.2.7, residue R726 is known to stabilize the binding of coactivator peptides (Estébanez-Perpiñá et al., 2005), suggesting it may be essential for the interaction of AR with coregulators. On the other hand, adding a positive charge to the asparagine at position 833 disrupted the binding of AR to its coregulators, indicating the importance of an uncharged side chain at this position. Mutant K720A disrupted the association of AR with SRC3, consistent with published data demonstrating the interaction of an SRC2 peptide with the lysine at position 720 (Estébanez-Perpiñá et al., 2005), a highly conserved residue in NRs, located in the upper par of the so-called electrostatic charge clamp. Interestingly, the replacement of the arginine at position 840 by either an alanine or a glutamic acid prevented AR from recruiting Uba3, indicating that either Uba establishes direct contact with this residue or that the positive charge at this position is essential.

To conclude, whether ARMC9, MAPK8IP1, Rab11FIP3 and Uba3 are true novel AR interactors need further experimental proof. Nevertheless, these results strongly suggest that Uba3 binds to the AR LBD and that this association may involve overlapping areas between the AF-2 and BF-3 pockets, indicating a possible role of the BF-3 site in protein-protein interaction.

# V. GENERAL DISCUSSION

# 5. GENERAL DISCUSSION

The role of AR in the development and progression of PCa has raised an increasing interest in this NR, which has become the most important therapeutic target for the treatment of this disease (Tian et al., 2015). Current PCa clinical treatments involve combination of androgen deprivation therapy and antiandrogens in order to inhibit both the synthesis of testicular androgen and AR activation. Nevertheless, despite the initial good response, most tumors progress to the so-called CRPC, a lethal form of cancer for which no curative therapy exists nowadays. Several mechanisms for the development of CRPC have been described (Tan et al., 2015), including mutations in the LBP of AR that result in a decreased specificity and selectivity for ligands, rendering the receptor responsive to non-androgen molecules.

An altered pharmacological activity was first reported with AR mutation T877A, frequently found in androgen-independent prostate tumors, which is able to recognize CPA and flutamide as an agonist (Bohl et al., 2007; Haendler and Cleve, 2012). Bicalutamide has been demonstrated to stimulate AR activity in mutants W741L and W741C (Haendler and Cleve, 2012). And more recently, a novel variant F826L switching enzalutamide and ARN509 from AR antagonists to AR agonists has been identified (Joseph et al., 2013). The crystal structure of mutant T877A in complex with hydroxyflutamide (Bohl et al., 2005) reveals that the H12 adopts the agonistic conformation, and the structural superimposition of this structure, W741L AR mutant in complex with bicalutamide, and the DHT-bound AR LBD demonstrated perfect alignments of H12, confirming the mutant-driven switch of these antiandrogens from AR antagonist to AR agonist (Tian et al., 2015). Therefore, these gain-of-function mutations represent a great challenge in the design of new compounds capable of overcoming the mechanisms that lead to drug resistance. Fortunately, available structures of the WT and mutant AR forms in complex with different ligands provide a clear explanation on how changes in a single residue can dramatically change the specificity and affinity of the receptor. Additionally, this structural information is currently being used in rational drug design, an application of a structure-function relationship, which is becoming

widely utilized in modern medicinal chemistry for the development of selective ligands (Tan et al., 2015). The structure of the full length AR would provide a complete picture of the receptor, necessary to understand interdomain interactions. However, crystallization of full-length receptors remains a big challenge. Only two structures of NRs including all functional domains have been solved up to date (Tan et al., 2015): hepatocyte nuclear factor 4 alpha (NF-4$\alpha$) homodimers and PPAR/RXR heterodimers complexed with their DNA elements and coactivator peptides (Chandra et al., 2008; 2013). Moreover, no antagonistic or apo AR LBD structure is available yet.

Despite current clinically available antiandrogens mainly target the AR LBP (Tian et al., 2015), several other regulatory sites in the surface of AR, including the AF-2 and BF-3 pockets in the LBD, have attracted increasing attention in the development of novel drugs. On the one hand, the AF-2 groove represents an attractive target to modulate AR activity because its inhibition would presumably disrupt the N/C-terminal interaction and the recruitment of coregulators to the LBD. In fact, the viability of targeting the coactivator-binding pocket has already been demonstrated (Tan et al., 2015). On the other hand, several compounds have been reported to bind the BF-3 pocket (Estébanez-Perpiñá et al., 2007; Munuganti et al., 2014; 2013), a recently discovered surface adjacent to AF-2, which is target for PCa and AIS mutations (Gottlieb et al., 2012). Importantly, BF-3-interacting compounds have demonstrated to inhibit the proliferation of enzalutamide-resistant PCa cell lines and to reduce the expression of AR target genes (Munuganti et al., 2014; 2013), evidencing the pharmacological potential of this druggable site.

In this thesis we first demonstrate that the newly identified BF-3 site in the human AR is highly conserved among the SR subclass, and that naturally occurring mutations associated with pathology and affecting the function of several NRs *in vitro* colocalize with their putative BF-3 sites. Interestingly, as shown in Table 1, all SRs have a glycine at position 724, in a conserved block between helices 2 and 3 of the LBD (Matias et al., 2000), suggesting it is essential for normal receptor function. Moreover, in the case of AR, G724 is located in a region known to be involved not only in ligand binding but also in the AR N/C-terminal interaction (Jaaskelainen, 2006). Despite the N/C interaction has not bee assessed for this

mutant, our results show that the full-length construct is completely inactive at 0.1 nM DHT, possess a defective transactivation capacity at 1 and 10nM hormone and reaches WT levels at high DHT concentrations, showing the importance of this residue in AR normal function. The tandem of arginine and asparagine found at the boundary between AF-2 and BF-3 sites (R726 and N727) are also highly conserved in SRs. Importantly, both residues have been identified to play an important role in transmitting information from BF-3 to AF-2 by undergoing structural rearrangements (Estébanez-Perpiñá et al., 2007), as well as to play a key role in allosteric communication (Grosdidier et al., 2012; Mackinnon et al., 2014). In most of the functions assessed in *in vitro* cell assays, both mutants showed to be WT-like.

Secondly, the shape and characteristics of the BF-3 pocket in the examined NRs suggest a possible role for protein-protein interactions and physiological relevant partners of AR BF-3 (i.e. FKBP52 and BAG-1L) have started to emerge (De Leon et al., 2011; Grosdidier et al., 2012; Jehle et al., 2014). In order to identify possible BF-3 novel interactors, we performed Y2H screens of an adult human brain cDNA library. In the presence of DHT, four new putative AR binders were isolated: ARMC9, MAPK8IP1, Rab11FIP3, and Uba3. Three of them, ARMC9, MAPK8IP1, and Uba3 were further validated by the matrix Y2H. Despite the association between Rab11FIP3 and AR could not be confirmed in the one-to-one HT-Y2H assay, *in vitro* assays demonstrated that Rab11FIP3 is able to inhibit the AR LBD transactivation capacity, attenuate its coactivation by GRIP1 and disrupt the N/C interaction. Similar *in vitro* results were obtained with Uba3. Importantly, several BF-3 mutants disrupted the interaction between the AR LBD and AR coregulators, supporting the possible role of BF-3 in protein-protein interactions.

Moreover, we performed site-directed mutagenesis of BF-3 lining residues in order to envision a possible role for BF-3 in AR activity. Our studies confirm and complement previous data showing that BF-3 mutants alter overall AR activity in different ways (Ahmed et al., 2000; MacLean et al., 2004; Petroli et al., 2011; Thompson et al., 2001; Tilley et al., 1996; Yong et al., 1994). We demonstrate that mutations in BF-3 and residues lying between AF-2 and BF-3 are responsible for a wide range of effects on AR transactivation capacity, from superactivators to very

potent inhibitors, and that such outcomes are influenced by hormone concentration and the AR construct used (full-length versus the isolated LBD). Different consequences upon regulator coactivation and functional interaction with the NTD have also been shown. Finally, BF-3 mutations have exhibited diverse effects on corepressor binding in transfected cells: whereas most of the mutations impair NCoR and SMRT interaction, others exhibit milder or no effects, and a few distinguish between NCoR and SMRT.

Describing briefly the most exciting findings, it is interesting to highlight that the two BF-3 mutants that manifested the highest activity at 100 nM DHT, N833R and R840A, showed a WT-like activity at 10 nM and a low activity at 1 nM, showing that increased hormone concentrations can help overcome low transactivation capacities, specially important in patients diagnosed with AIS and carrying AR mutations. The other super-AR mutation, I672R, leaded to an abnormally raised activity at both 10 nM and 100 nM DHT, and a WT-like transactivation at 1 nM hormone, whereas contrarily manifested a defective interaction with the NTD and both corepressors. The results obtained with mutation F826L were analogous to that of I672R, although the AF-2 activity was not so high. On the other hand, replacing the phenylalanine at this position by an arginine resulted in a decreased receptor transactivation capacity but an enhanced coactivation by GRIP1 and a stronger interdomain binding. The association with SMRT was deficient, while the recruitment of NCoR was comparable to the one seen with the WT. Finally, substituting the arginine at position 840 for a glutamic acid (thus, reversing its charge) killed the activity of the receptor, although the coactivation exerted by GRIP1 reached the same level as the WT. Importantly, all BF-3 mutants with the exception of R726L and N727K, located in the boundary between AF-2 and BF-3 sites, exhibited a defective interaction with SMRT in the mammalian two-hybrid assays.

In the one-to-one Y2H, variants R726L and N833R disrupted the binding of AR LBD to all tested coregulators, including SMRT; and the replacement of the arginine at position 840 by either an alanine or a glutamic acid prevented the AR LBD from recruiting MAPK8IP1 and Uba3, suggesting this residue may be involved in contacting both proteins. Surprisingly, AR LBD was capable of associating to SCR3,

ARMC9 and Uba3 in the presence of antiandrogens; and both bicalutamide and mifepristone promoted the binding of AR LBD R726L to SRC3. The arginine at position 726 resides in a positive cluster in helix 3 and participate in the binding of the FxxLF motif found in the AR NTD as well as LxxLL motifs present in SRC coactivators (Dubbink, 2004; Estébanez-Perpiñá et al., 2007; 2005; He et al., 2004). Moreover, mutant R726L has shown to impair the binding of SRC coactivators and the N/C interaction (Hay and McEwan, 2012), possibly explaining why no interaction was obtained in the Y2H, although our mammalian two-hybrid demonstrated a WT-like interdomain interaction. Moreover, this mutation is implicated in a 6-fold increased risk of PCa (Mononen et al., 2000), which may be the reason why this variant is able to associate to SCR3 in the presence of antiandrogens.

Most of the times, it is difficult to correlate a specific missense mutation in a NR with its associated phenotype in patients, as the *in vitro* conditions, i.e. the construct tested (full-length vs isolated LBD), the hormone concentration, or the cell line used, can change dramatically the results obtained. The GRIP1 coactivation, N/C interaction and transactivation activity obtained cannot explain in all cases the corresponding phenotypes caused by the analyzed AR LBD mutants. Some mutations give *in vitro* the opposite results than one would expect from the phenotype in patients, where the whole receptor is present, sometimes even in an altered environment of coregulators or other factors crucial for its activity. However, such *in vitro* assays are widely used in the NR field, as indirect indications for an altered AR functioning can be derived (Bevan et al., 1996).

Taken all results together, two possible physiological roles for BF-3 are envisioned. BF-3 mutants have been shown to act as allosteric elicitors of conformational changes that are transmitted towards AF-2, affecting the function of the AR LBD (Estébanez-Perpiñá et al., 2007; Grosdidier et al., 2012). Furthermore, several residues belonging to BF-3 and AF-2 surface pockets have been revealed as key players of an allosteric network that may influence multiple aspects of AR LBD function (Estébanez-Perpiñá et al., 2007; Grosdidier et al., 2012; Mackinnon et al., 2014). On the other hand, it is also plausible to speculate that BF-3 mutations alter direct contacts with proteins such as Uba3, either potentiating or silencing AR

function. BF-3 surface might even contact GRIP1 coactivator, the NTD or NCoR/SMRT corepressors, and these proteins may interact with the AR LBD in a region that extends beyond the AF-2 pocket towards BF-3, as it has been previously suggested (Estébanez-Perpiñá et al., 2007). It is also plausible that the BF-3 may be a docking site for the hinge domain in some contexts, as the crystal structures of ERα (PDB 1ERE) and the full-length PPARγ-RXR heterodimer (PDB codes 3E00, 3DZY and 3DZU) indicate. Whether this is representative of other NRs cannot be extrapolated from the available crystal structures. One can only speculate that the hinge could be covering the BF-3 pocket as a lid in some NR conformations, exposing it under different macromolecular complexes.

To conclude, NRs are master regulators of reproduction, development, and metabolism and, despite all the difficulties associated with their pleiotropic action and their abundant side effects, they are still major targets in the development of new drugs. In fact, it is estimated that up to 10% of current available drugs are acting via NRs (Laudet, 2015). However, most of the compounds that target NR LBPs have serious secondary effects due to cross-reactivity among different NR subfamilies. Developing NR modulators targeting LBD surface pockets may be a novel way to find class-specific drugs. In particular, targeting the BF-3 surface may open new promising alternatives to current therapeutics. Compounds that may affect allosterically NR function by binding to BF-3 open promising avenues to develop type-specific NR modulators.

# VI. CONCLUSIONS

# 6. CONCLUSIONS

1. The BF-3 site in the human AR is highly conserved among the steroid receptor subclass, and despite the fact that it is also present in other NRs (with a lower level of conservation), it can represent a druggable site for controlling NR function and develop a new generation of modulators.

2. The AR BF-3 site is an allosteric modulator of the adjacent AF-2 pocket, hence compounds targeted to this site will modulate protein-protein associations of the AR involved in pathology.

3. Mutations in the BF-3 pocket and residues lying between the AF-2 and BF-3 sites affect AR transactivation and coactivation by GRIP1, as well as the AR LBD interaction with the NTD, and NR corepressors NCoR and SMRT.

4. BF-3 is a protein-protein interaction site for coregulatory proteins.

5. ARMC9, MAPK8IP1 and Uba3 are putative novel AR interactors.

6. Rab11FIP3 and Uba3 inhibit the AR LBD transactivation capacity, attenuate its coactivation by GRIP1 and disrupt the N/C interaction, pointing towards the fact that these proteins are AR corepressors not previously described.

7. SRC3, ARMC9 and Uba3 are capable of binding to the AR LBD in the presence of antiandrogens.

8. There are certain protein-protein interactions and complex formation of AR with known coregulators in the presence of antiandrogens. Hence, the model that all antiandrogens yield a non-productive or distorted AF-2 pocket need reevaluation.

9. The complex associations of AR with known or novel coregulators maintained in the presence of antiandrogens indicate that the AF-2 pocket and Helix 12 may exhibit more conformational functionality than previously described.

# VII. REFERENCES

# 7. REFERENCES


Agoulnik, I.U., Vaid, A., Bingman, W.E., Erdeme, H., Frolov, A., Smith, C.L., Ayala, G., Ittmann, M.M., Weigel, N.L., 2005. Role of SRC-1 in the promotion of prostate cancer cell growth and tumor progression. Cancer Res. 65, 7959–7967. doi:10.1158/0008-5472.CAN-04-3541

Ahmed, S.F., Cheng, A., Dovey, L., Hawkins, J.R., Martin, H., Rowland, J., Shimura, N., Tait, A.D., Hughes, I.A., 2000. Phenotypic features, androgen receptor binding, and mutational analysis in 278 clinical cases reported as androgen insensitivity syndrome. Journal of Clinical Endocrinology & Metabolism 85, 658–665.

Alen, P., Claessens, F., Schoenmakers, E., Swinnen, J.V., Verhoeven, G., Rombauts, W., Peeters, B., 1999a. Interaction of the putative androgen receptor-specific coactivator ARA70/ELE1alpha with multiple steroid receptors and identification of an internally deleted ELE1beta isoform. Mol. Endocrinol. 13, 117–128.

Alen, P., Claessens, F., Verhoeven, G., Rombauts, W., Peeters, B., 1999b. The androgen receptor amino-terminal domain plays a key role in p160 coactivator-stimulated gene transcription. Mol. Cell. Biol. 19, 6085–6097.

Attard, G., Parker, C., Eeles, R.A., Schröder, F., Tomlins, S.A., Tannock, I., Drake, C.G., de Bono, J.S., 2015. Prostate cancer. Lancet. doi:10.1016/S0140-6736(14)61947-4

Audi, L., Fernández-Cancio, M., Carrascosa, A., Andaluz, P., Torán, N., Piró, C., Vilaró, E., Vicens-Calvet, E., Gussinyé, M., Albisu, M.A., Yeste, D., Clemente, M., Hernández de la Calle, I., Del Campo, M., Vendrell, T., Blanco, A., Martínez-Mora, J., Granada, M.L., Salinas, I., Forn, J., Calaf, J., Angerri, O., Martínez-Sopena, M.J., Del Valle, J., García, E., Gracia-Bouthelier, R., Lapunzina, P., Mayayo, E., Labarta, J.I., Lledó, G., Sánchez Del Pozo, J., Arroyo, J., Pérez-Aytes, A., Beneyto, M., Segura, A., Borrás, V., Gabau, E., Caimarí, M., Rodríguez, A., Martínez-Aedo, M.J., Carrera, M., Castaño, L., Andrade, M., Bermúdez de la Vega, J.A., Grupo de Apoyo al Síndrome de Insensibilidad a los Andrógenos (GrApSIA), 2010. Novel (60%) and recurrent (40%) androgen receptor gene mutations in a series of 59 patients with a 46,XY disorder of sex development. J. Clin. Endocrinol. Metab. 95, 1876–1888. doi:10.1210/jc.2009-2146

Balbas, M.D., Evans, M.J., Hosfield, D.J., Wongvipat, J., Arora, V.K., Watson, P.A., Chen, Y., Greene, G.L., Shen, Y., Sawyers, C.L., 2013. Overcoming mutation-based resistance to antiandrogens with rational drug design. eLife 2, e00499. doi:10.7554/eLife.00499

Beilin, J., Ball, E.M., Favaloro, J.M., Zajac, J.D., 2000. Effect of the androgen receptor CAG repeat polymorphism on transcriptional activity: specificity in prostate and non-prostate cell lines. Journal of Molecular Endocrinology 25, 85–96.

Beitel, L.K., Kazemi-Esfarjani, P., Kaufman, M., Lumbroso, R., DiGeorge, A.M., Killinger, D.W., Trifiro, M.A., Pinsky, L., 1994. Substitution of arginine-839 by

cysteine or histidine in the androgen receptor causes different receptor phenotypes in cultured cells and coordinate degrees of clinical androgen resistance. J. Clin. Invest. 94, 546–554. doi:10.1172/JCI117368

Benoit, G., Cooney, A., Giguere, V., Ingraham, H., Lazar, M., Muscat, G., Perlmann, T., Renaud, J.-P., Schwabe, J., Sladek, F., Tsai, M.-J., Laudet, V., 2006. International Union of Pharmacology. LXVI. Orphan nuclear receptors. Pharmacol. Rev. 58, 798–836. doi:10.1124/pr.58.4.10

Berrevoets, C.A., Doesburg, P., Steketee, K., Trapman, J., Brinkmann, A.O., 1998. Functional interactions of the AF-2 activation domain core region of the human androgen receptor with the amino-terminal domain and with the transcriptional coactivator TIF2 (transcriptional intermediary factor2). Mol. Endocrinol. 12, 1172–1183.

Berthon, A., Stratakis, C.A., 2014. From β-catenin to ARM-repeat proteins in adrenocortical disorders. Horm. Metab. Res. 46, 889–896. doi:10.1055/s-0034-1389993

Bevan, C.L., Brown, B.B., Davies, H.R., Evans, B.A., Hughes, I.A., Patterson, M.N., 1996. Functional analysis of six androgen receptor mutations identified in patients with partial androgen insensitivity syndrome. Hum. Mol. Genet. 5, 265–273.

Bevan, C.L., Hoare, S., Claessens, F., Heery, D.M., Parker, M.G., 1999. The AF1 and AF2 domains of the androgen receptor interact with distinct regions of SRC1. Mol. Cell. Biol. 19, 8383–8392.

Bledsoe, R.K., Stewart, E.L., Pearce, K.H., 2004. Structure and function of the glucocorticoid receptor ligand binding domain. Vitam. Horm. 68, 49–91. doi:10.1016/S0083-6729(04)68002-2

Bohl, C.E., Miller, D.D., Chen, J., Bell, C.E., Dalton, J.T., 2005. Structural basis for accommodation of nonsteroidal ligands in the androgen receptor. J. Biol. Chem. 280, 37747–37754. doi:10.1074/jbc.M507464200

Bohl, C.E., Wu, Z., Miller, D.D., Bell, C.E., Dalton, J.T., 2007. Crystal structure of the T877A human androgen receptor ligand-binding domain complexed to cyproterone acetate provides insight for ligand-induced conformational changes and structure-based drug design. J. Biol. Chem. 282, 13648–13655. doi:10.1074/jbc.M611711200

Bouvattier, C., Carel, J.-C., Lecointre, C., David, A., Sultan, C., Bertrand, A.-M., Morel, Y., Chaussain, J.-L., 2002. Postnatal changes of T, LH, and FSH in 46,XY infants with mutations in the AR gene. Journal of Clinical Endocrinology & Metabolism 87, 29–32. doi:10.1210/jcem.87.1.7923

Brasso, K., Thomsen, F.B., Schrader, A.J., Schmid, S.C., Lorente, D., Retz, M., Merseburger, A.S., Klot, von, C.A., Boegemann, M., de Bono, J., 2015. Enzalutamide Antitumour Activity Against Metastatic Castration-resistant Prostate Cancer Previously Treated with Docetaxel and Abiraterone: A Multicentre Analysis. European Urology 68, 317–324. doi:10.1016/j.eururo.2014.07.028

Brooke, G.N., Parker, M.G., Bevan, C.L., 2008. Mechanisms of androgen receptor

activation in advanced prostate cancer: differential co-activator recruitment and gene expression. Oncogene 27, 2941–2950. doi:10.1038/sj.onc.1210955

Brönnegård M, C.-D.J., 1995. The genetic basis of glucocorticoid resistance. Trends Endocrinol. Metab. 6, 160–164.

Brzozowski, A.M., Pike, A.C., Dauter, Z., Hubbard, R.E., Bonn, T., Engström, O., Ohman, L., Greene, G.L., Gustafsson, J.A., Carlquist, M., 1997. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389, 753–758. doi:10.1038/39645

Buchanan, G., Greenberg, N.M., Scher, H.I., Harris, J.M., Marshall, V.R., Tilley, W.D., 2001a. Collocation of androgen receptor gene mutations in prostate cancer. Clin. Cancer Res. 7, 1273–1281.

Buchanan, G., Yang, M., Harris, J.M., Nahm, H.S., Han, G., Moore, N., Bentel, J.M., Matusik, R.J., Horsfall, D.J., Marshall, V.R., Greenberg, N.M., Tilley, W.D., 2001b. Mutations at the boundary of the hinge and ligand binding domain of the androgen receptor confer increased transactivation function. Mol. Endocrinol. 15, 46–56.

Burris, T.P., Solt, L.A., Wang, Y., Crumbley, C., Banerjee, S., Griffett, K., Lundåsen, T., Hughes, T., Kojetin, D.J., 2013. Nuclear receptors and their selective pharmacologic modulators. Pharmacol. Rev. 65, 710–778. doi:10.1124/pr.112.006833

Buzón, V., Carbó, L.R., Estruch, S.B., Fletterick, R.J., Estébanez-Perpiñá, E., 2012. A conserved surface on the ligand binding domain of nuclear receptors for allosteric control. Molecular and Cellular Endocrinology 348, 394–402. doi:10.1016/j.mce.2011.08.012

Centenera, M.M., Harris, J.M., Tilley, W.D., Butler, L.M., 2008. The contribution of different androgen receptor domains to receptor dimerization and signaling. Mol. Endocrinol. 22, 2373–2382. doi:10.1210/me.2008-0017

Chadha, S., Rao, B.R., Slotman, B.J., van Vroonhoven, C.C., van der Kwast, T.H., 1993. An immunohistochemical evaluation of androgen and progesterone receptors in ovarian tumors. Hum. Pathol. 24, 90–95.

Chandra, V., Huang, P., Hamuro, Y., Raghuram, S., Wang, Y., Burris, T.P., Rastinejad, F., 2008. Structure of the intact PPAR-gamma-RXR- nuclear receptor complex on DNA. Nature 456, 350–356. doi:10.1038/nature07413

Chandra, V., Huang, P., Potluri, N., Wu, D., Kim, Y., Rastinejad, F., 2013. Multidomain integration in the structure of the HNF-4α nuclear receptor complex. Nature 495, 394–398. doi:10.1038/nature11966

Chang, C., Saltzman, A., Yeh, S., Young, W., Keller, E., Lee, H.J., Wang, C., Mizokami, A., 1995. Androgen receptor: an overview. Crit. Rev. Eukaryot. Gene Expr. 5, 97–125.

Chen, J.D., Evans, R.M., 1995. A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature 377, 454–457. doi:10.1038/377454a0

Chen, S., Johnson, B.A., Li, Y., Aster, S., McKeever, B., Mosley, R., Moller, D.E., Zhou, G., 2000. Both coactivator LXXLL motif-dependent and -independent interactions are required for peroxisome proliferator-activated receptor

gamma (PPARgamma) function. J. Biol. Chem. 275, 3733–3736.

Claessens, F., Denayer, S., Van Tilborgh, N., Kerkhofs, S., Helsen, C., Haelens, A., 2008. Diverse roles of androgen receptor (AR) domains in AR-mediated signaling. Nucl Rec Sig 6, e008. doi:10.1621/nrs.06008

Claessens, F., Helsen, C., Prekovic, S., Van den Broeck, T., Spans, L., Van Poppel, H., Joniau, S., 2014. Emerging mechanisms of enzalutamide resistance in prostate cancer. Nat Rev Urol 11, 712–716. doi:10.1038/nrurol.2014.243

Clegg, N.J., Wongvipat, J., Joseph, J.D., Tran, C., Ouk, S., Dilhas, A., Chen, Y., Grillot, K., Bischoff, E.D., Cai, L., Aparicio, A., Dorow, S., Arora, V., Shao, G., Qian, J., Zhao, H., Yang, G., Cao, C., Sensintaffar, J., Wasielewska, T., Herbert, M.R., Bonnefous, C., Darimont, B., Scher, H.I., Smith-Jones, P., Klang, M., Smith, N.D., De Stanchina, E., Wu, N., Ouerfelli, O., Rix, P.J., Heyman, R.A., Jung, M.E., Sawyers, C.L., Hager, J.H., 2012. ARN-509: A Novel Antiandrogen for Prostate Cancer Treatment. Cancer Research 72, 1494–1503. doi:10.1158/0008-5472.CAN-11-3948

Clinckemalie, L., Vanderschueren, D., Boonen, S., Claessens, F., 2012. The hinge region in androgen receptor control. Molecular and Cellular Endocrinology 358, 1–8. doi:10.1016/j.mce.2012.02.019

Culig, Z., 2016. Androgen Receptor Coactivators in Regulation of Growth and Differentiation in Prostate Cancer. J. Cell. Physiol. 231, 270–274. doi:10.1002/jcp.25099

Cutress, M.L., Whitaker, H.C., Mills, I.G., Stewart, M., Neal, D.E., 2008. Structural basis for the nuclear import of the human androgen receptor. J. Cell. Sci. 121, 957–968. doi:10.1242/jcs.022103

Darimont, B.D., Wagner, R.L., Apriletti, J.W., Stallcup, M.R., Kushner, P.J., Baxter, J.D., Fletterick, R.J., Yamamoto, K.R., 1998. Structure and specificity of nuclear receptor-coactivator interactions. Genes Dev. 12, 3343–3356.

Dasgupta, S., O'Malley, B.W., 2014. Transcriptional coregulators: emerging roles of SRC family of coactivators in disease pathology. Journal of Molecular Endocrinology 53, R47–59. doi:10.1530/JME-14-0080

De Leon, J.T., Iwai, A., Feau, C., Garcia, Y., Balsiger, H.A., Storer, C.L., Suro, R.M., Garza, K.M., Lee, S., Kim, Y.S., Chen, Y., Ning, Y.-M., Riggs, D.L., Fletterick, R.J., Guy, R.K., Trepel, J.B., Neckers, L.M., Cox, M.B., 2011. Targeting the regulation of androgen receptor signaling by the heat shock protein 90 cochaperone FKBP52 in prostate cancer cells. Proc. Natl. Acad. Sci. U.S.A. 108, 11878–11883. doi:10.1073/pnas.1105160108

Deeb, A., Jaaskelainen, J., Dattani, M., Whitaker, H.C., Costigan, C., Hughes, I.A., 2008. A novel mutation in the human androgen receptor suggests a regulatory role for the hinge region in amino-terminal and carboxy-terminal interactions. Journal of Clinical Endocrinology & Metabolism 93, 3691–3696. doi:10.1210/jc.2008-0737

Dubbink, H.J., 2004. Distinct Recognition Modes of FXXLF and LXXLL Motifs by the Androgen Receptor. Mol. Endocrinol. 18, 2132–2150. doi:10.1210/me.2003-0375

Edwards, D.P., 2000. The role of coactivators and corepressors in the biology and

mechanism of action of steroid hormone receptors. J Mammary Gland Biol Neoplasia 5, 307–324.

Elo, J.P., Kvist, L., Leinonen, K., Isomaa, V., Henttu, P., Lukkarinen, O., Vihko, P., 1995. Mutated human androgen receptor gene detected in a prostatic cancer patient is also activated by estradiol. Journal of Clinical Endocrinology & Metabolism 80, 3494–3500.

Estébanez-Perpiñá, E., Arnold, L.A., Arnold, A.A., Nguyen, P., Rodrigues, E.D., Mar, E., Bateman, R., Pallai, P., Shokat, K.M., Baxter, J.D., Guy, R.K., Webb, P., Fletterick, R.J., 2007. A surface on the androgen receptor that allosterically regulates coactivator binding. Proc. Natl. Acad. Sci. U.S.A. 104, 16074–16079. doi:10.1073/pnas.0708036104

Estébanez-Perpiñá, E., Moore, J.M.R., Mar, E., Delgado-Rodrigues, E., Nguyen, P., Baxter, J.D., Buehrer, B.M., Webb, P., Fletterick, R.J., Guy, R.K., 2005. The molecular mechanisms of coactivator utilization in ligand-dependent transactivation by the androgen receptor. J. Biol. Chem. 280, 8060–8068. doi:10.1074/jbc.M407046200

Evans, R.M., Mangelsdorf, D.J., 2014. Nuclear Receptors, RXR, and the Big Bang. Cell 157, 255–266. doi:10.1016/j.cell.2014.03.012

Fan, M., Long, X., Bailey, J.A., Reed, C.A., Osborne, E., Gize, E.A., Kirk, E.A., Bigsby, R.M., Nephew, K.P., 2002. The activating enzyme of NEDD8 inhibits steroid receptor function. Mol. Endocrinol. 16, 315–330.

Fu, M.-M., Holzbaur, E.L.F., 2014. MAPK8IP1/JIP1 regulates the trafficking of autophagosomes in neurons. Autophagy 10, 2079–2081. doi:10.4161/auto.34451

Gallastegui, N., Mackinnon, J.A.G., Fletterick, R.J., Estébanez-Perpiñá, E., 2015. Advances in our structural understanding of orphan nuclear receptors. Trends Biochem. Sci. 40, 25–35. doi:10.1016/j.tibs.2014.11.002

Gao, T., Brantley, K., Bolu, E., McPhaul, M.J., 1999. RFG (ARA70, ELE1) interacts with the human androgen receptor in a ligand-dependent fashion, but functions only weakly as a coactivator in cotransfection assays. Mol. Endocrinol. 13, 1645–1656.

Gao, W., Dalton, J.T., 2007. Expanding the therapeutic use of androgens via selective androgen receptor modulators (SARMs). Drug Discovery Today 12, 241–248. doi:10.1016/j.drudis.2007.01.003

Gelmann, E.P., 2002. Molecular Biology of the Androgen Receptor. Journal of Clinical Oncology 20, 3001–3015. doi:10.1200/JCO.2002.10.018

Georget, V., Terouanne, B., Lumbroso, S., Nicolas, J.C., Sultan, C., 1998. Trafficking of androgen receptor mutants fused to green fluorescent protein: a new investigation of partial androgen insensitivity syndrome. Journal of Clinical Endocrinology & Metabolism 83, 3597–3603. doi:10.1210/jcem.83.10.5201

Gnanapragasam, V.J., Leung, H.Y., Pulimood, A.S., Neal, D.E., Robson, C.N., 2001. Expression of RAC 3, a steroid hormone receptor co-activator in prostate cancer. Br. J. Cancer 85, 1928–1936. doi:10.1054/bjoc.2001.2179

Golshayan, A.R., Antonarakis, E.S., 2013. Enzalutamide: an evidence-based review

of its use in the treatment of prostate cancer. CE 8, 27–35. doi:10.2147/CE.S34747

Gottlieb, B., Beitel, L.K., Nadarajah, A., Paliouras, M., Trifiro, M., 2012. The androgen receptor gene mutations database: 2012 update. Hum. Mutat. 33, 887–894. doi:10.1002/humu.22046

Grosdidier, S., Carbó, L.R., Buzón, V., Brooke, G., Nguyen, P., Baxter, J.D., Bevan, C., Webb, P., Estébanez-Perpiñá, E., Fernández-Recio, J., 2012. Allosteric conversation in the androgen receptor ligand-binding domain surfaces. Mol. Endocrinol. 26, 1078–1090. doi:10.1210/me.2011-1281

Grunseich, C., Rinaldi, C., Fischbeck, K., 2013. Spinal and bulbar muscular atrophy: pathogenesis and clinical management. Oral Dis. doi:10.1111/odi.12121

Haendler, B., Cleve, A., 2012. Recent developments in antiandrogens and selective androgen receptor modulators. Molecular and Cellular Endocrinology 352, 79–91. doi:10.1016/j.mce.2011.06.002

Haile, S., Lal, A., Myung, J.-K., Sadar, M.D., 2011. FUS/TLS is a co-activator of androgen receptor in prostate cancer cells. PLoS ONE 6, e24197. doi:10.1371/journal.pone.0024197

Hannema, S.E., Scott, I.S., Hodapp, J., Martin, H., Coleman, N., Schwabe, J.W., Hughes, I.A., 2004. Residual activity of mutant androgen receptors explains wolffian duct development in the complete androgen insensitivity syndrome. Journal of Clinical Endocrinology & Metabolism 89, 5815–5822. doi:10.1210/jc.2004-0709

Hay, C.W., McEwan, I.J., 2012. The Impact of Point Mutations in the Human Androgen Receptor: Classification of Mutations on the Basis of Transcriptional Activity. PLoS ONE 7, e32514. doi:10.1371/journal.pone.0032514.s001

He, B., Bai, S., Hnat, A.T., Kalman, R.I., Minges, J.T., Patterson, C., Wilson, E.M., 2004. An androgen receptor NH2-terminal conserved motif interacts with the COOH terminus of the Hsp70-interacting protein (CHIP). J. Biol. Chem. 279, 30643–30653. doi:10.1074/jbc.M403117200

He, B., Kemppainen, J.A., Voegel, J.J., Gronemeyer, H., Wilson, E.M., 1999. Activation function 2 in the human androgen receptor ligand binding domain mediates interdomain communication with the NH(2)-terminal domain. J. Biol. Chem. 274, 37219–37225.

He, B., Kemppainen, J.A., Wilson, E.M., 2000. FXXLF and WXXLF sequences mediate the NH2-terminal interaction with the ligand binding domain of the androgen receptor. J. Biol. Chem. 275, 22986–22994. doi:10.1074/jbc.M002807200

He, B., Minges, J.T., Lee, L.W., Wilson, E.M., 2002. The FXXLF motif mediates androgen receptor-specific interactions with coregulators. J. Biol. Chem. 277, 10226–10235. doi:10.1074/jbc.M111975200

He, B., Wilson, E.M., 2003. Electrostatic modulation in steroid receptor recruitment of LXXLL and FXXLF motifs. Mol. Cell. Biol. 23, 2135–2150.

Heemers, H.V., Tindall, D.J., 2007. Androgen receptor (AR) coregulators: a diversity of functions converging on and regulating the AR transcriptional complex. Endocr. Rev. 28, 778–808. doi:10.1210/er.2007-0019

Heery, D.M., Kalkhoven, E., Hoare, S., Parker, M.G., 1997. A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature 387, 733–736. doi:10.1038/42750

Heinlein, C.A., Chang, C., 2004. Androgen receptor in prostate cancer. Endocr. Rev. 25, 276–308.

Heinlein, C.A., Chang, C., 2002. Androgen receptor (AR) coregulators: an overview. Endocr. Rev. 23, 175–200.

Heinlein, C.A., Ting, H.J., Yeh, S., Chang, C., 1999. Identification of ARA70 as a ligand-enhanced coactivator for the peroxisome proliferator-activated receptor gamma. J. Biol. Chem. 274, 16147–16152.

Heldring, N., Pawson, T., McDonnell, D., Treuter, E., Gustafsson, J.-A., Pike, A.C.W., 2007. Structural insights into corepressor recognition by antagonist-bound estrogen receptors. J. Biol. Chem. 282, 10449–10455. doi:10.1074/jbc.M611424200

Helsen, C., Dubois, V., Verfaillie, A., Young, J., Trekels, M., Vancraenenbroeck, R., De Maeyer, M., Claessens, F., 2012. Evidence for DNA-Binding Domain-Ligand-Binding Domain Communications in the Androgen Receptor. Molecular and Cellular Biology 32, 3033–3043. doi:10.1128/MCB.00151-12

Helsen, C., Van den Broeck, T., Voet, A., Prekovic, S., Van Poppel, H., Joniau, S., Claessens, F., 2014. Androgen receptor antagonists for prostate cancer therapy. Endocrine Related Cancer 21, T105–18. doi:10.1530/ERC-13-0545

Hickey, T.E., Irvine, C.M., Dvinge, H., Tarulli, G.A., Hanson, A.R., Ryan, N.K., Pickering, M.A., Birrell, S.N., Hu, D.G., Mackenzie, P.I., Russell, R., Caldas, C., Raj, G.V., Dehm, S.M., Plymate, S.R., Bradley, R.K., Tilley, W.D., Selth, L.A., 2015. Expression of androgen receptor splice variants in clinical breast cancers. Oncotarget. doi:10.18632/oncotarget.6296

Hörlein, A.J., Näär, A.M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Söderström, M., Glass, C.K., 1995. Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature 377, 397–404. doi:10.1038/377397a0

Hur, E., Pfaff, S.J., Payne, E.S., Grøn, H., Buehrer, B.M., Fletterick, R.J., 2004. Recognition and accommodation at the androgen receptor coactivator binding interface. PLoS Biol. 2, E274. doi:10.1371/journal.pbio.0020274

Hyytinen, E.-R., Haapala, K., Thompson, J., Lappalainen, I., Roiha, M., Rantala, I., Helin, H.J., Jänne, O.A., Vihinen, M., Palvimo, J.J., KOIVISTO, P.A., 2002. Pattern of somatic androgen receptor gene mutations in patients with hormone-refractory prostate cancer. Lab. Invest. 82, 1591–1598.

Iain J McEwan, P., Raj Kumar, P., 2015. Nuclear Receptors: From Structure to the Clinic. Springer.

Jaaskelainen, J., 2006. Human androgen receptor gene ligand-binding-domain mutations leading to disrupted interaction between the N- and C-terminal domains. Journal of Molecular Endocrinology 36, 361–368. doi:10.1677/jme.1.01885

Jacq, X., Brou, C., Lutz, Y., Davidson, I., Chambon, P., Tora, L., 1994. Human TAFII30

is present in a distinct TFIID complex and is required for transcriptional activation by the estrogen receptor. Cell 79, 107–117.

Jääskeläinen, J., Mongan, N.P., Harland, S., Hughes, I.A., 2006. Five novel androgen receptor gene mutations associated with complete androgen insensitivity syndrome. Hum. Mutat. 27, 291. doi:10.1002/humu.9405

Jehle, K., Cato, L., Neeb, A., Muhle-Goll, C., Jung, N., Smith, E.W., Buzón, V., Carbó, L.R., Estébanez-Perpiñá, E., Schmitz, K., Fruk, L., Luy, B., Chen, Y., Cox, M.B., Bräse, S., Brown, M., Cato, A.C.B., 2014. Coregulator control of androgen receptor action by a novel nuclear receptor-binding motif. Journal of Biological Chemistry 289, 8839–8851. doi:10.1074/jbc.M113.534859

Jenster, G., van der Korput, H.A., Trapman, J., Brinkmann, A.O., 1995. Identification of two transcription activation units in the N-terminal domain of the human androgen receptor. J. Biol. Chem. 270, 7341–7346.

Joseph, J.D., Lu, N., Qian, J., Sensintaffar, J., Shao, G., Brigham, D., Moon, M., Chow Maneval, E., Chen, I., Darimont, B., Hager, J.H., 2013. A clinically relevant androgen receptor mutation confers resistance to 2nd generation anti-androgens enzalutamide and ARN-509. Cancer Discovery. doi:10.1158/2159-8290.CD-13-0226

Katsuno, M., Tanaka, F., Adachi, H., Banno, H., Suzuki, K., Watanabe, H., Sobue, G., 2012. Pathogenesis and therapy of spinal and bulbar muscular atrophy (SBMA). Prog. Neurobiol. 99, 246–256. doi:10.1016/j.pneurobio.2012.05.007

Katzenwadel, A., Wolf, P., 2015. Androgen deprivation of prostate cancer: Leading to a therapeutic dead end. Cancer Lett. 367, 12–17. doi:10.1016/j.canlet.2015.06.021

Klotz, L., Schellhammer, P., 2005. Combined androgen blockade: the case for bicalutamide. Clin Prostate Cancer 3, 215–219.

Koivisto, P.A., Hyytinen, E.-R., Matikainen, M., Tammela, T.L.J., Ikonen, T., Schleutker, J., 2004. Germline mutation analysis of the androgen receptor gene in Finnish patients with prostate cancer. The Journal of Urology 171, 431–433. doi:10.1097/01.ju.0000089774.99728.ef

Koivisto, P.A., Schleutker, J., Helin, H., Ehren-van Eekelen, C., Kallioniemi, O.P., Trapman, J., 1999. Androgen receptor gene alterations and chromosomal gains and losses in prostate carcinomas appearing during finasteride treatment for benign prostatic hyperplasia. Clin. Cancer Res. 5, 3578–3582.

Kollara, A., Brown, T.J., 2012. Expression and function of nuclear receptor co-activator 4: evidence of a potential role independent of co-activator activity. Cellular and Molecular Life Sciences (CMLS) 69, 3895–3909. doi:10.1007/s00018-012-1000-y

Koryakina, Y., Ta, H.Q., Gioeli, D., 2014. Androgen receptor phosphorylation: biological context and functional consequences. Endocrine Related Cancer 21, T131–45. doi:10.1530/ERC-13-0472

Kumar, R., Atamna, H., Zakharov, M.N., Bhasin, S., Khan, S.H., Jasuja, R., 2011. Role of the androgen receptor CAG repeat polymorphism in prostate cancer, and spinal and bulbar muscular atrophy. Life Sci. 88, 565–571.

doi:10.1016/j.lfs.2011.01.021

La Spada, A.R., Wilson, E.M., Lubahn, D.B., Harding, A.E., Fischbeck, K.H., 1991. Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. Nature 352, 77–79. doi:10.1038/352077a0

Lanzino, M., De Amicis, F., McPhaul, M.J., Marsico, S., Panno, M.L., Andò, S., 2005. Endogenous coactivator ARA70 interacts with estrogen receptor alpha (ERalpha) and modulates the functional ERalpha/androgen receptor interplay in MCF-7 cells. J. Biol. Chem. 280, 20421–20430. doi:10.1074/jbc.M413576200

Laudet, V., 2015. Nuclear Receptors and Development: From drugs to embryos and back again. Biochim. Biophys. Acta 1849, 71–72. doi:10.1016/j.bbagrm.2014.08.011

Leo, C., Chen, J.D., 2000. The SRC family of nuclear receptor coactivators. Gene 245, 1–11.

Lim, J., 2000. Human Androgen Receptor Mutation Disrupts Ternary Interactions between Ligand, Receptor Domains, and the Coactivator TIF2 (Transcription Intermediary Factor 2). Mol. Endocrinol. 14, 1187–1197. doi:10.1210/me.14.8.1187

Linja, M.J., Porkka, K.P., Kang, Z., Savinainen, K.J., Jänne, O.A., TAMMELA, T.L.J., Vessella, R.L., Palvimo, J.J., Visakorpi, T., 2004. Expression of androgen receptor coregulators in prostate cancer. Clin. Cancer Res. 10, 1032–1040.

Linja, M.J., Visakorpi, T., 2004. Alterations of androgen receptor in prostate cancer. J. Steroid Biochem. Mol. Biol. 92, 255–264. doi:10.1016/j.jsbmb.2004.10.012

Lonard, D.M., Lanz, R.B., O'Malley, B.W., 2007. Nuclear receptor coregulators and human disease. Endocr. Rev. 28, 575–587. doi:10.1210/er.2007-0012

Lonard, D.M., O'Malley, B.W., 2007. Nuclear receptor coregulators: judges, juries, and executioners of cellular regulation. Mol. Cell 27, 691–700. doi:10.1016/j.molcel.2007.08.012

Lorente, D., Mateo, J., Zafeiriou, Z., Smith, A.D., Sandhu, S., Ferraldeschi, R., de Bono, J.S., 2015. Switching and withdrawing hormonal agents for castration-resistant prostate cancer. Nat Rev Urol 12, 37–47. doi:10.1038/nrurol.2014.345

Lu, M.L., Schneider, M.C., Zheng, Y., Zhang, X., Richie, J.P., 2001. Caveolin-1 interacts with androgen receptor. A positive modulator of androgen receptor mediated transactivation. J. Biol. Chem. 276, 13442–13451. doi:10.1074/jbc.M006598200

Lubahn, D.B., Joseph, D.R., Sullivan, P.M., Willard, H.F., French, F.S., Wilson, E.M., 1988. Cloning of human androgen receptor complementary DNA and localization to the X chromosome. Science 240, 327–330.

Lundberg Giwercman, Y., Nikoshkov, A., Lindsten, K., Byström, B., Pousette, A., Chibalin, A.V., Arvidsson, S., Tiulpakov, A., Semitcheva, T.V., Peterkova, V., Hagenfeldt, K., Ritzén, E.M., Wedell, A., 1998. Functional characterisation of mutations in the ligand-binding domain of the androgen receptor gene in patients with androgen insensitivity syndrome. Hum. Genet. 103, 529–531.

Ma, G., Ren, Y., Wang, K., He, J., 2011. SRC-3 has a role in cancer other than as a

nuclear receptor coactivator. Int. J. Biol. Sci. 7, 664–672.

Mackinnon, J.A.G., Gallastegui, N., Osguthorpe, D.J., Hagler, A.T., Estébanez-Perpiñá, E., 2014. Allosteric mechanisms of nuclear receptors: insights from computational simulations. Molecular and Cellular Endocrinology 393, 75–82. doi:10.1016/j.mce.2014.05.017

MacLean, H.E., Ball, E.M.A., Rekaris, G., Warne, G.L., Zajac, J.D., 2004. Novel androgen receptor gene mutations in Australian patients with complete androgen insensitivity syndrome. Hum. Mutat. 23, 287. doi:10.1002/humu.9221

Malloy PJ, Z.W.Z.X.P.G.F.D., 2001. A novel inborn error in the ligand-binding domain of the vitamin D receptor causes hereditary vitamin D-resistant rickets. Mol. Genet. Metab. 73, 138–148.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., Evans, R.M., 1995. The nuclear receptor superfamily: the second decade. Cell 83, 835–839.

Marcelli, M., Zoppi, S., Wilson, C.M., Griffin, J.E., McPhaul, M.J., 1994. Amino acid substitutions in the hormone-binding domain of the human androgen receptor alter the stability of the hormone receptor complex. J. Clin. Invest. 94, 1642–1650. doi:10.1172/JCI117507

Marimuthu, A., Feng, W., Tagami, T., Nguyen, H., Jameson, J.L., Fletterick, R.J., Baxter, J.D., West, B.L., 2002. TR surfaces and conformations required to bind nuclear receptor corepressor. Mol. Endocrinol. 16, 271–286. doi:10.1210/mend.16.2.0777

Matias, P.M., Carrondo, M.A., Coelho, R., Thomaz, M., Zhao, X.-Y., Wegg, A., Crusius, K., Egner, U., Donner, P., 2002. Structural Basis for the Glucocorticoid Response in a Mutant Human Androgen Receptor (AR ccr) Derived from an Androgen-Independent Prostate Cancer. J. Med. Chem. 45, 1439–1446. doi:10.1021/jm011072j

Matias, P.M., Donner, P., Coelho, R., Thomaz, M., Peixoto, C., Macedo, S., Otto, N., Joschko, S., Scholz, P., Wegg, A., Basler, S., Schafer, M., Egner, U., Carrondo, M.A., 2000. Structural evidence for ligand specificity in the binding domain of the human androgen receptor. Implications for pathogenic gene mutations. J. Biol. Chem. 275, 26164–26171.

Mäki, H.E., Waltering, K.K., Wallén, M.J., Martikainen, P.M., TAMMELA, T.L.J., van Weerden, W.M., Vessella, R.L., Visakorpi, T., 2006. Screening of genetic and expression alterations of SRC1 gene in prostate cancer. Prostate 66, 1391–1398. doi:10.1002/pros.20427

McEwan, I.J., 2011. Intrinsic disorder in the androgen receptor: identification, characterisation and drugability. Mol. BioSyst. 8, 82. doi:10.1039/c1mb05249g

McEwan, I.J., Lavery, D., Fischer, K., Watt, K., 2007. Natural disordered sequences in the amino terminal domain of nuclear receptors: lessons from the androgen and glucocorticoid receptors. Nucl Rec Sig 5, e001. doi:10.1621/nrs.05001

McKenna, N.J., Lanz, R.B., O'Malley, B.W., 1999. Nuclear receptor coregulators: cellular and molecular biology. Endocr. Rev. 20, 321–344.

McPhaul, M.J., Marcelli, M., 1992. Molecular defects in the androgen receptor causing androgen resistance. J. Invest. Dermatol. 98, 97S–99S.

Meijsing, S.H., Pufall, M.A., So, A.Y., Bates, D.L., Chen, L., Yamamoto, K.R., 2009. DNA binding site sequence directs glucocorticoid receptor structure and activity. Science 324, 407–410. doi:10.1126/science.1164265

Mohler, M.L., Bohl, C.E., Jones, A., Coss, C.C., Narayanan, R., He, Y., Hwang, D.J., Dalton, J.T., Miller, D.D., 2009. Nonsteroidal selective androgen receptor modulators (SARMs): dissociating the anabolic and androgenic activities of the androgen receptor for therapeutic benefit. J. Med. Chem. 52, 3597–3617. doi:10.1021/jm900280m

Mohler, M.L., Coss, C.C., Duke, C.B., III, Patil, S.A., Miller, D.D., Dalton, J.T., 2012. Androgen receptor antagonists: a patent review (2008 – 2011). Expert Opin. Ther. Patents 22, 541–565. doi:10.1517/13543776.2012.682571

Mongan, N.P., Tadokoro-Cuccaro, R., Bunch, T., Hughes, I.A., 2015. Androgen insensitivity syndrome. Best Practice & Research Clinical Endocrinology & Metabolism 29, 569–580. doi:10.1016/j.beem.2015.04.005

Mononen, N., Syrjäkoski, K., Matikainen, M., Tammela, T.L., Schleutker, J., Kallioniemi, O.P., Trapman, J., Koivisto, P.A., 2000. Two percent of Finnish prostate cancer patients have a germ-line mutation in the hormone-binding domain of the androgen receptor gene. Cancer Res. 60, 6479–6481.

Munuganti, R.S.N., Hassona, M.D.H., Leblanc, E., Frewin, K., Singh, K., Ma, D., Ban, F., Hsing, M., Adomat, H., Lallous, N., Andre, C., Jonadass, J.P.S., Zoubeidi, A., Young, R.N., Guns, E.T., Rennie, P.S., Cherkasov, A., 2014. Identification of a potent antiandrogen that targets the BF3 site of the androgen receptor and inhibits enzalutamide-resistant prostate cancer. Chemistry & Biology 21, 1476–1485. doi:10.1016/j.chembiol.2014.09.012

Munuganti, R.S.N., Leblanc, E., Axerio-Cilies, P., Labriere, C., Frewin, K., Singh, K., Hassona, M.D.H., Lack, N.A., Li, H., Ban, F., Tomlinson Guns, E., Young, R., Rennie, P.S., Cherkasov, A., 2013. Targeting the Binding Function 3 (BF3) Site of the Androgen Receptor Through Virtual Screening. 2. Development of 2-((2-phenoxyethyl) thio)-1 H-benzimidazole Derivatives. J. Med. Chem. 56, 1136–1148. doi:10.1021/jm3015712

Nagy, L., Kao, H.Y., Love, J.D., Li, C., Banayo, E., Gooch, J.T., Krishna, V., Chatterjee, K., Evans, R.M., Schwabe, J.W., 1999. Mechanism of corepressor binding and release from nuclear hormone receptors. Genes Dev. 13, 3209–3216.

Narayanan, R., Mohler, M.L., Bohl, C.E., Miller, D.D., Dalton, J.T., 2008. Selective androgen receptor modulators in preclinical and clinical development. Nucl Rec Sig 6, e010. doi:10.1621/nrs.06010

Nascimento, A.S., Dias, S.M.G., Nunes, F.M., Aparicio, R., Ambrosio, A.L.B., Bleicher, L., Figueira, A.C.M., Santos, M.A.M., de Oliveira Neto, M., Fischer, H., Togashi, M., Craievich, A.F., Garratt, R.C., Baxter, J.D., Webb, P., Polikarpov, I., 2006. Structural rearrangements in the thyroid hormone receptor hinge domain and their putative role in the receptor function. Journal of Molecular Biology 360, 586–598. doi:10.1016/j.jmb.2006.05.008

Ning, Y.M., Pierce, W., Maher, V.E., Karuri, S., Tang, S.-H., Chiu, H.-J., Palmby, T., Zirkelbach, J.F., Marathe, D., Mehrotra, N., Liu, Q., Ghosh, D., Cottrell, C.L., Leighton, J., Sridhara, R., Ibrahim, A., Justice, R., Pazdur, R., 2013. Enzalutamide for treatment of patients with metastatic castration-resistant prostate cancer who have previously received docetaxel: U.S. Food and Drug Administration drug approval summary. Clin. Cancer Res. 19, 6067–6073. doi:10.1158/1078-0432.CCR-13-1763

Palazzolo, I., Gliozzi, A., Rusmini, P., Sau, D., Crippa, V., Simonini, F., Onesto, E., Bolzoni, E., Poletti, A., 2008. The role of the polyglutamine tract in androgen receptor. J. Steroid Biochem. Mol. Biol. 108, 245–253. doi:10.1016/j.jsbmb.2007.09.016

Palvimo, J.J., 2012. The androgen receptor. Molecular and Cellular Endocrinology 1–3. doi:10.1016/j.mce.2012.01.016

Pan, Z.-Q., Kentsis, A., Dias, D.C., Yamoah, K., Wu, K., 2004. Nedd8 on cullin: building an expressway to protein destruction. Oncogene 23, 1985–1997. doi:10.1038/sj.onc.1207414

Perissi, V., Jepsen, K., Glass, C.K., Rosenfeld, M.G., 2010. Deconstructing repression: evolving models of co-repressor action. Nat. Rev. Genet. 11, 109–123. doi:10.1038/nrg2736

Perissi, V., Staszewski, L.M., McInerney, E.M., Kurokawa, R., Krones, A., Rose, D.W., Lambert, M.H., Milburn, M.V., Glass, C.K., Rosenfeld, M.G., 1999. Molecular determinants of nuclear receptor-corepressor interaction. Genes Dev. 13, 3198–3208.

Petroli, R.J., Maciel-Guerra, A.T., Soardi, F.C., de Calais, F.L., Guerra-Junior, G., de Mello, M.P., 2011. 830F in Severe Forms of AIS_BMC2011_deMello. BMC Research Notes 4, 173. doi:10.1186/1756-0500-4-173

Poukka, H., 1999. Ubc9 Interacts with the Androgen Receptor and Activates Receptor-dependent Transcription. Journal of Biological Chemistry 274, 19441–19446. doi:10.1074/jbc.274.27.19441

Putcha BD, F.E., 2009. Direct interdomain interactions can mediate allosterism in the thyroid receptor. J. Biol. Chem. 284, 22517–24.

Quigley, C.A., Tan, J.-A., He, B., Zhou, Z.-X., Mebarki, F., Morel, Y., Forest, M.G., Chatelain, P., Ritzén, E.M., French, F.S., Wilson, E.M., 2004. Partial androgen insensitivity with phenotypic variation caused by androgen receptor mutations that disrupt activation function 2 and the NH2- and carboxyl-terminal interaction. Mechanisms of Ageing and Development 125, 683–695. doi:10.1016/j.mad.2004.08.007

Rathkopf, D.E., Morris, M.J., Fox, J.J., Danila, D.C., Slovin, S.F., Hager, J.H., Rix, P.J., Chow Maneval, E., Chen, I., Gönen, M., Fleisher, M., Larson, S.M., Sawyers, C.L., Scher, H.I., 2013. Phase I study of ARN-509, a novel antiandrogen, in the treatment of castration-resistant prostate cancer. J. Clin. Oncol. 31, 3525–3530. doi:10.1200/JCO.2013.50.1684

Robins, D.M., 2012. Androgen receptor gene polymorphisms and alterations in prostate cancer: Of humanized mice and men. Molecular and Cellular

Endocrinology 352, 26–33. doi:10.1016/j.mce.2011.06.003

Robinson, D., Van Allen, E.M., Wu, Y.-M., Schultz, N., Lonigro, R.J., Mosquera, J.-M., Montgomery, B., Taplin, M.-E., Pritchard, C.C., Attard, G., Beltran, H., Abida, W., Bradley, R.K., Vinson, J., Cao, X., Vats, P., Kunju, L.P., Hussain, M., Feng, F.Y., Tomlins, S.A., Cooney, K.A., Smith, D.C., Brennan, C., Siddiqui, J., Mehra, R., Chen, Y., Rathkopf, D.E., Morris, M.J., Solomon, S.B., Durack, J.C., Reuter, V.E., Gopalan, A., Gao, J., Loda, M., Lis, R.T., Bowden, M., Balk, S.P., Gaviola, G., Sougnez, C., Gupta, M., Yu, E.Y., Mostaghel, E.A., Cheng, H.H., Mulcahy, H., True, L.D., Plymate, S.R., Dvinge, H., Ferraldeschi, R., Flohr, P., Miranda, S., Zafeiriou, Z., Tunariu, N., Mateo, J., Perez-Lopez, R., Demichelis, F., Robinson, B.D., Schiffman, M., Nanus, D.M., Tagawa, S.T., Sigaras, A., Eng, K.W., Elemento, O., Sboner, A., Heath, E.I., Scher, H.I., Pienta, K.J., Kantoff, P., de Bono, J.S., Rubin, M.A., Nelson, P.S., Garraway, L.A., Sawyers, C.L., Chinnaiyan, A.M., 2015. Integrative clinical genomics of advanced prostate cancer. Cell 161, 1215–1228. doi:10.1016/j.cell.2015.05.001

Robinson, J.L.L., Macarthur, S., Ross-Innes, C.S., Tilley, W.D., Neal, D.E., Mills, I.G., Carroll, J.S., 2011. Androgen receptor driven transcription in molecular apocrine breast cancer is mediated by FoxA1. EMBO J. 30, 3019–3027. doi:10.1038/emboj.2011.216

Saad, F., Fizazi, K.S., 2015. Androgen Deprivation Therapy and Secondary Hormone Therapy in the Management of Hormone-Sensitive and Castration Resistant Prostate Cancer. Urology. doi:10.1016/j.urology.2015.07.034

Sack, J.S., Kish, K.F., Wang, C., Attar, R.M., Kiefer, S.E., An, Y., Wu, G.Y., Scheffler, J.E., Salvati, M.E., Krystek, S.R., Weinmann, R., Einspahr, H.M., 2001. Crystallographic structures of the ligand-binding domains of the androgen receptor and its T877A mutant complexed with the natural agonist dihydrotestosterone. Proc. Natl. Acad. Sci. U.S.A. 98, 4904–4909. doi:10.1073/pnas.081565498

Safer, J.D., Cohen, R.N., Hollenberg, A.N., Wondisford, F.E., 1998. Defective release of corepressor by hinge mutants of the thyroid hormone receptor found in patients with resistance to thyroid hormone. J. Biol. Chem. 273, 30175–30182.

Sampson, E.R., Yeh, S.Y., Chang, H.C., Tsai, M.Y., Wang, X., Ting, H.J., Chang, C., 2001. Identification and characterization of androgen receptor associated coregulators in prostate cancer cells. J. Biol. Regul. Homeost. Agents 15, 123–129.

Schoch, G.A., D'Arcy, B., Stihle, M., Burger, D., Bär, D., Benz, J., Thoma, R., Ruf, A., 2010. Molecular switch in the glucocorticoid receptor: active and passive antagonist conformations. Journal of Molecular Biology 395, 568–577. doi:10.1016/j.jmb.2009.11.011

Shaffer, P.L., Jivan, A., Dollins, D.E., Claessens, F., Gewirth, D.T., 2004. Structural basis of androgen receptor binding to selective androgen response elements. Proc. Natl. Acad. Sci. U.S.A. 101, 4758–4763. doi:10.1073/pnas.0401123101

Shiba, T., Koga, H., Shin, H.-W., Kawasaki, M., Kato, R., Nakayama, K., Wakatsuki, S., 2006. Structural basis for Rab11-dependent membrane recruitment of a family of Rab11-interacting protein 3 (FIP3)/Arfophilin-1. Proc. Natl. Acad. Sci. U.S.A.

103, 15416–15421. doi:10.1073/pnas.0605357103

Silva, J.C., Ferreira-Strixino, J., Fontana, L.C., Paula, L.M., Raniero, L., Martin, A.A., Canevari, R.A., 2014. Apoptosis-associated genes related to photodynamic therapy in breast carcinomas. Lasers Med Sci 29, 1429–1436. doi:10.1007/s10103-014-1547-y

Song, L.-N., Herrell, R., Byers, S., Shah, S., Wilson, E.M., Gelmann, E.P., 2003. Beta-catenin binds to the activation function 2 region of the androgen receptor and modulates the effects of the N-terminal domain and TIF2 on ligand-dependent transcription. Mol. Cell. Biol. 23, 1674–1687.

Song, L.N., 2003. Antiandrogen Effects of Mifepristone on Coactivator and Corepressor Interactions with the Androgen Receptor. Mol. Endocrinol. 18, 70–85. doi:10.1210/me.2003-0189

Süel, G.M., Lockless, S.W., Wall, M.A., Ranganathan, R., 2003. Evolutionarily conserved networks of residues mediate allosteric communication in proteins. Nat. Struct. Biol. 10, 59–69. doi:10.1038/nsb881

Szafran, A.T., Hartig, S., Sun, H., Uray, I.P., Szwarc, M., Shen, Y., Mediwala, S.N., Bell, J., McPhaul, M.J., Mancini, M.A., Marcelli, M., 2009. Androgen Receptor Mutations Associated with Androgen Insensitivity Syndrome: A High Content Analysis Approach Leading to Personalized Medicine. PLoS ONE 4, e8179. doi:10.1371/journal.pone.0008179.t003

Tan, M.H.E., Li, J., Xu, H.E., Melcher, K., Yong, E.-L., 2015. Androgen receptor: structure, role in prostate cancer and drug discovery. Acta Pharmacol. Sin. 36, 3–23. doi:10.1038/aps.2014.18

Tanner T, C.F.H.A., 2004. The hinge region of the androgen receptor plays a role in proteasome-mediated transcriptional activation. Ann. N. Y. Acad. Sci. 1030.

Tanner, T.M., Denayer, S., Geverts, B., Van Tilborgh, N., Kerkhofs, S., Helsen, C., Spans, L., Dubois, V., Houtsmuller, A.B., Claessens, F., Haelens, A., 2010. A 629RKLKK633 motif in the hinge region controls the androgen receptor at multiple levels. Cellular and Molecular Life Sciences (CMLS) 67, 1919–1927. doi:10.1007/s00018-010-0302-1

Tetel, M.J., Jung, S., Carbajo, P., Ladtkow, T., Skafar, D.F., Edwards, D.P., 1997. Hinge and amino-terminal sequences contribute to solution dimerization of human progesterone receptor. Mol. Endocrinol. 11, 1114–1128. doi:10.1210/mend.11.8.9963

Thompson, J., Saatcioglu, F., Janne, O.A., Palvimo, J.J., 2001. Disrupted amino- and carboxyl-terminal interactions of the androgen receptor are linked to androgen insensitivity. Mol. Endocrinol. 15, 923–935. doi:10.1210/mend.15.6.0647

Thornton, J.W., Kelley, D.B., 1998. Evolution of the androgen receptor: structure-function implications. Bioessays 20, 860–869. doi:10.1002/(SICI)1521-1878(199810)20:10<860::AID-BIES12>3.0.CO;2-S

Tian, X., He, Y., Zhou, J., 2015. Progress in antiandrogen design targeting hormone binding pocket to circumvent mutation based resistance. Front Pharmacol 6, 57. doi:10.3389/fphar.2015.00057

Tien, J.C.-Y., Liu, Z., Liao, L., Wang, F., Xu, Y., Wu, Y.-L., Zhou, N., Ittmann, M., Xu, J., 2013. The steroid receptor coactivator-3 is required for the development of castration-resistant prostate cancer. Cancer Research 73, 3997–4008. doi:10.1158/0008-5472.CAN-12-3929

Tilley, W.D., Buchanan, G., Hickey, T.E., Bentel, J.M., 1996. Mutations in the androgen receptor gene are associated with progression of human prostate cancer to androgen independence. Clin. Cancer Res. 2, 277–285.

Truica, C.I., Byers, S., Gelmann, E.P., 2000. Beta-catenin affects androgen receptor transcriptional activity and ligand specificity. Cancer Res. 60, 4709–4713.

van de Wijngaart, D.J., Dubbink, H.J., van Royen, M.E., Trapman, J., Jenster, G., 2012. Androgen receptor coregulators: Recruitment via the coactivator binding groove. Molecular and Cellular Endocrinology 352, 57–69. doi:10.1016/j.mce.2011.08.007

van der Steen, T., Tindall, D.J., Huang, H., 2013. Posttranslational modification of the androgen receptor in prostate cancer. IJMS 14, 14833–14859. doi:10.3390/ijms140714833

van Royen, M.E., Cunha, S.M., Brink, M.C., Mattern, K.A., Nigg, A.L., Dubbink, H.J., Verschure, P.J., Trapman, J., Houtsmuller, A.B., 2007. Compartmentalization of androgen receptor protein-protein interactions in living cells. J. Cell Biol. 177, 63–72. doi:10.1083/jcb.200609178

van Royen, M.E., van Cappellen, W.A., de Vos, C., Houtsmuller, A.B., Trapman, J., 2012. Stepwise androgen receptor dimerization. J. Cell. Sci. 125, 1970–1979. doi:10.1242/jcs.096792

Voeller, H.J., Truica, C.I., Gelmann, E.P., 1998. Beta-catenin mutations in human prostate cancer. Cancer Res. 58, 2520–2523.

Vogelzang, N.J., 2012. Enzalutamide — A Major Advance in the Treatment of Metastatic Prostate Cancer. N Engl J Med 367, 1256–1257. doi:10.1056/NEJMe1209041

Waltering, K.K., Urbanucci, A., Visakorpi, T., 2012. Androgen receptor (AR) aberrations in castration-resistant prostate cancer. Molecular and Cellular Endocrinology 1–6. doi:10.1016/j.mce.2011.12.019

Wang, Q., Lu, J., Yong, E.L., 2001. Ligand- and coactivator-mediated transactivation function (AF2) of the androgen receptor ligand-binding domain is inhibited by the cognate hinge region. J. Biol. Chem. 276, 7493–7499. doi:10.1074/jbc.M009916200

Wang, X., Li, L., Liang, Y., Li, C., Zhao, H., Ye, D., Sun, M., Jeong, L.S., Feng, Y., Fu, S., Jia, L., Guo, X., 2014. Targeting the neddylation pathway to suppress the growth of prostate cancer cells: therapeutic implication for the men's cancer. Biomed Res Int 2014, 974309. doi:10.1155/2014/974309

Wang, Y., Lonard, D.M., Yu, Y., Chow, D.-C., Palzkill, T.G., O'Malley, B.W., 2011. Small molecule inhibition of the steroid receptor coactivators, SRC-3 and SRC-1. Mol. Endocrinol. 25, 2041–2053. doi:10.1210/me.2011-1222

Watanabe, C., Watanabe, H., Tanaka, S., 2010. An interpretation of positional displacement of the helix12 in nuclear receptors: preexistent swing-up motion

triggered by ligand binding. Biochim. Biophys. Acta 1804, 1832–1840. doi:10.1016/j.bbapap.2010.06.009

Weimann, M., Grossmann, A., Woodsmith, J., Özkan, Z., Birth, P., Meierhofer, D., Benlasfer, N., Valovka, T., Timmermann, B., Wanker, E.E., Sauer, S., Stelzl, U., 2013. A Y2H-seq approach defines the human protein methyltransferase interactome. Nat. Methods 10, 339–342. doi:10.1038/nmeth.2397

Whitfield, G.K., Hsieh, J.C., Nakajima, S., MacDonald, P.N., Thompson, P.D., Jurutka, P.W., Haussler, C.A., Haussler, M.R., 1995. A highly conserved region in the hormone-binding domain of the human vitamin D receptor contains residues vital for heterodimerization with retinoid X receptor and for transcriptional activation. Mol. Endocrinol. 9, 1166–1179. doi:10.1210/mend.9.9.7491109

Wisniewski, A.B., Migeon, C.J., Meyer-Bahlburg, H.F., Gearhart, J.P., Berkovitz, G.D., Brown, T.R., Money, J., 2000. Complete androgen insensitivity syndrome: long-term medical, surgical, and psychosexual outcome. Journal of Clinical Endocrinology & Metabolism 85, 2664–2669.

Wong, H.Y., Hoogerbrugge, J.W., Pang, K.L., van Leeuwen, M., van Royen, M.E., Molier, M., Berrevoets, C.A., Dooijes, D., Dubbink, H.J., van de Wijngaart, D.J., Wolffenbuttel, K.P., Trapman, J., Kleijer, W.J., Drop, S.L.S., Grootegoed, J.A., Brinkmann, A.O., 2008. A novel mutation F826L in the human androgen receptor in partial androgen insensitivity syndrome; increased NH2-/COOH-terminal domain interaction and TIF2 co-activation. Molecular and Cellular Endocrinology 292, 69–78. doi:10.1016/j.mce.2008.06.016

Wong, M.M., Guo, C., Zhang, J., 2014. Nuclear receptor corepressor complexes in cancer: mechanism, function and regulation. Am J Clin Exp Urol 2, 169–187.

Wong, Y.N.S., Ferraldeschi, R., Attard, G., de Bono, J., 2014. Evolution of androgen receptor targeted therapy for advanced prostate cancer. Nat Rev Clin Oncol 11, 365–376. doi:10.1038/nrclinonc.2014.72

Worseck, J.M., Grossmann, A., Weimann, M., Hegele, A., Stelzl, U., 2012. A stringent yeast two-hybrid matrix screening approach for protein-protein interaction discovery. Methods Mol. Biol. 812, 63–87. doi:10.1007/978-1-61779-455-1_4

Wurtz, J.M., Bourguet, W., Renaud, J.P., Vivat, V., Chambon, P., Moras, D., Gronemeyer, H., 1996. A canonical structure for the ligand-binding domain of nuclear receptors. Nat. Struct. Biol. 3, 206.

Xu, J., Wu, R.-C., O'Malley, B.W., 2009. Normal and cancer-related functions of the p160 steroid receptor co-activator (SRC) family. Nat. Rev. Cancer 9, 615–630. doi:10.1038/nrc2695

Yong, E.L., 2003. Androgen receptor gene and male infertility. Human Reproduction Update 9, 1–7. doi:10.1093/humupd/dmg003

Yong, E.L., Ng, S.C., Roy, A.C., Yun, G., Ratnam, S.S., 1994. Pregnancy after hormonal correction of severe spermatogenic defect due to mutation in androgen receptor gene. The Lancet 344, 826–827.

Yuan, X., Cai, C., Chen, S., Chen, S., Yu, Z., Balk, S.P., 2013. AR functions in CRPC and mechanisms of resistance to new agents targeting the androgen axis_Oncogene_2013. Oncogene 1–11. doi:10.1038/onc.2013.235

Zakharov, M.N., Pillai, B.K., Bhasin, S., Ulloor, J., Istomin, A.Y., Guo, C., Godzik, A., Kumar, R., Jasuja, R., 2011. Dynamics of coregulator-induced conformational perturbations in androgen receptor ligand binding domain. Molecular and Cellular Endocrinology 1–8. doi:10.1016/j.mce.2011.03.003

Zhang, J., Chalmers, M.J., Stayrook, K.R., Burris, L.L., Wang, Y., Busby, S.A., Pascal, B.D., Garcia-Ordonez, R.D., Bruning, J.B., Istrate, M.A., Kojetin, D.J., Dodge, J.A., Burris, T.P., Griffin, P.R., 2011. DNA binding alters coactivator interaction surfaces of the intact VDR-RXR complex. Nat. Struct. Biol. 18, 556–563. doi:10.1038/nsmb.2046

Zhou, H.-J., Yan, J., Luo, W., Ayala, G., Lin, S.-H., Erdem, H., Ittmann, M., Tsai, S.Y., Tsai, M.-J., 2005. SRC-3 is required for prostate cancer cell proliferation and survival. Cancer Res. 65, 7976–7983. doi:10.1158/0008-5472.CAN-04-4076

Zhou, X.E., Suino-Powell, K., Ludidi, P.L., McDonnell, D.P., Xu, H.E., 2010. Expression, purification and primary crystallographic study of human androgen receptor in complex with DNA and coactivator motifs. Protein Expr. Purif. 71, 21–27. doi:10.1016/j.pep.2009.12.002

Zhou, Z.X., He, B., Hall, S.H., Wilson, E.M., French, F.S., 2002. Domain interactions between coregulator ARA(70) and the androgen receptor (AR). Mol. Endocrinol. 16, 287–300.

Zhou, Z.X., Sar, M., Simental, J.A., Lane, M.V., Wilson, E.M., 1994. A ligand-dependent bipartite nuclear targeting signal in the human androgen receptor. Requirement for the DNA-binding domain and modulation by NH2-terminal and carboxyl-terminal sequences. J. Biol. Chem. 269, 13115–13123.

Zuccarello, D., Ferlin, A., Vinanzi, C., Prana, E., Garolla, A., Callewaert, L., Claessens, F., Brinkmann, A.O., Foresta, C., 2008. Detailed functional studies on androgen receptor mild mutations demonstrate their association with male infertility. Clinical Endocrinology 68, 580–588. doi:10.1111/j.1365-2265.2007.03069.x

# VIII. RESUM DE LA TESI DOCTORAL

# 8. RESUM DE LA TESI DOCTORAL

## 8.1. INTRODUCCIÓ

### 8.1.1. El receptor d'andròGens (AR): introducció

El receptor d'andròGens (AR) (NR3, receptor nuclear subfamília 3, grup C, gen 4) (Lubahn et al., 1988), juntament amb el receptor d'estròGens (ER), el receptor de glucocorticoide (GR), el receptor de progesterona (PR) i el receptor de mineralcorticoids (MR), conformen el grup dels receptors d'esteroides (SR) dins de la superfamília de receptors nuclears (NRs) (Evans and Mangelsdorf, 2014; Mangelsdorf et al., 1995). En humans, aquesta superfamília està formada per un total de 48 factors de transcripció, i està composada pels receptors endocrins (esteroides i no esteroides), els receptors orfes adoptats i els anomenats receptors orfes, el lligand dels quals no es coneix encara o semblen no tenir lligand endogen (Benoit et al., 2006; Gallastegui et al., 2015).

Els NRs representen el grup de factors de transcripció eucariòtics més nombrós i regulen una gran varietat de funcions fisiològiques, incloent el desenvolupament cel·lular, la reproducció, la homeòstasi i el metabolisme (Evans and Mangelsdorf, 2014; Gallastegui et al., 2015). A més, aquesta superfamília està involucrada en la major part de patologies i tumors humans, motiu pel qual són una de les dianes farmacològiques més importants en la clínica (Burris et al., 2013).

En l'absència d'hormona, l'AR sembla estar inactiu en el citoplasma, acomplexat amb xaperones. La unió de la 5α-dihidrotestosterona (DHT), però, provoca la dissociació del complex de xaperones, dimerització, fosforilació i translocació del receptor al nucli, on activarà o reprimirà els gens corresponents (Claessens et al., 2008; Palvimo, 2012).

### 8.1.2. Estructura del receptor d'andròGens

L'AR és una proteïna de 919 aminoàcids (tot i que la seva llargada por variar en funció del nombre de repeticions en els trams de poliglutamina i poliglicina),

format per 3 dominis funcionals: el domini N-terminal (NTD), el domini central d'unió a l'ADN (DBD), i el domini d'unió al lligand (LBD), aquest últim unit a l'anterior a través d'una regió frontissa (Centenera et al., 2008; Lubahn et al., 1988).

L'NTD comprèn la part més gran del receptor i és el domini menys conservat d'entre tots els NRs. Se'l considera el major domini d'activació de l'AR, ja que conté la funció d'activació-1 (AF-1), la qual està constitutivament activa. En les posicions 23-27, hi ha localitzat el motiu $^{23}$FQNLF$^{27}$, altament conservat entre les diferents espècies i responsable de la interacció entre els dominis NTD i LBD del receptor, crucial pel bon funcionament d'AR en la cèl·lula (Claessens et al., 2008; He et al., 2000).

El DBD està format per uns 70 residus i estructuralment és el domini més conservat en tots els membres de la superfamília dels NRs (Gelmann, 2002). Està constituït per dos complexes de coordinació, cadascun format per quatre cisteïnes i un àtom de zinc, involucrats en la unió amb l'ADN i la dimerització del receptor (Centenera et al., 2008; Claessens et al., 2008).

La regió frontissa, localitzada entre els residus 623 i 671 del receptor, és un enllaç flexible que connecta el DBD i l'LBD (Claessens et al., 2008). Tot i que la seva seqüència està poc conservada, aquesta regió conté la senyal de localització nuclear (NLS) en tots els SRs, responsable de la translocació de la proteïna al nucli (Deeb et al., 2008).

Per últim, l'LBD és un domini altament estructurat que conté la butxaca d'unió al lligand (LBP), la butxaca d'unió a coactivadors, també coneguda com funció d'activació-2 (AF-2), i l'anomenada funció d'unió-3 (BF-3) (Estébanez-Perpiñá et al., 2007; 2005). L'AF-2 de l'AR difereix d'altres NRs en què uneix molt feblement els motius LxxLL (on L és una leucina i x és un aminoàcid qualsevol) (Heery et al., 1997), mentre que interacciona preferiblement amb els motius rics en aminoàcids aromàtics presents tant en coactivadors específics d'AR com en e l'NTD (Estébanez-Perpiñá et al., 2007; Hur et al., 2004). L'AF-2 de l'LBD presenta una baixa transactivació intrínseca i conté dos grups de residus amb càrregues contràries situats en extrems oposats que participen en la correcta orientació dels interactors d'AR en la butxaca AF-2 (Estébanez-Perpiñá et al., 2007; Hur et al.,

2004). Especialment interessants són la lisina de la posició 720 (K720) i el glutàmic del residu 897 (E897), els quals ajuden a estabilitzar la unió del coactivador (Estébanez-Perpiñá et al., 2005; He et al., 2002). D'altra banda, la butxaca BF-3, de mida i profunditat similars a l'AF-2, és una superfície hidrofòbica i exposada al solvent, característiques que suporten la seva participació en la interacció amb d'altres proteïnes (Estébanez-Perpiñá et al., 2007). La concavitat BF-3 està delimitada per residus localitzats a la part N-terminal de l'hèlix (H) 1 (Q670, P671, I672 i F673), alguns residus de l'H3/bucle 3-4 (P723, G724, R726 i N727), i nombrosos aminoàcids situats al llarg de l'H9 (F826, E829, L830, N833, E837, i R840). Els residus R726 i N727 es troben situats a la frontera de l'AF-2 i el BF-3. Fins a dia d'avui cap proteïna fisiològicament rellevant per la funció d'AR, a part de les coxaperones FKBP52 i BAG-1L (De Leon et al., 2011; Jehle et al., 2014), ha demostrat unir-se al BF-3.

S'ha suggerit que entre les butxaques AF-2 i BF-3 existeix una relació al·lostèrica (Buzón et al., 2012; Estébanez-Perpiñá et al., 2007; Grosdidier et al., 2012). D'una banda, s'ha demostrat que compostos que s'uneixen al BF-3 indueixen la remodelació de la superfície AF-2, impedint la interacció de pèptids coactivadors; i, d'altra banda, mutants de BF-3 han demostrat actuar com a inductors al·lostèrics de canvis conformacionals transmesos a l'AF-2, afectant la funció de l'LBD de l'AR.

### 8.1.3. Coreguladors de del receptor d'andròGens

Més de 200 coreguladors han estat identificats com a interactors de l'AR (van de Wijngaart et al., 2012). De manera genèrica, els coreguladors es classifiquen en proteïnes que interaccionen amb els NRs per potenciar (coactivadors) o reduir (corepressors) la transcripció de gens diana (Heemers and Tindall, 2007).

Molt pocs coactivadors identificats fins avui dia interaccionen exclusivament amb l'AR, ja que la majoria també estimulen l'activitat d'altres NRs i factors de transcripció (Culig, 2016; Heinlein et al., 1999; Lanzino et al., 2005; Waltering et al., 2012). Inclús els interactos específics de l'AR, també mostren promiscuïtat amb altres NRs.

En primer lloc, tenim els coactivadors dels receptors d'esteroides (SRC), una família formada per l'SRC1 (NCOA1), l'SRC2 (NCOA2/GRIP1/TIF2) i l'SRC3 (NCOA3/RAC3/ACTR/AIB1/TRAM1), implicats en el desenvolupament de diversos tipus de càncer (Agoulnik et al., 2005; Dasgupta and O'Malley, 2014; H.-J. Zhou et al., 2005). La regió N-terminal dels SRCs és la més conservada i la necessària per la interacció entre proteïnes, el domini central d'interacció amb receptors conté tres motius LxxLL conservats responsables de l'associació directa amb els NRs i e l'extrem més C-terminal conté dos dominis d'activació transcripcional essencials per la funció dels SRCs (Leo and J. D. Chen, 2000). Malgrat els tres motius LxxLL centrals són els responsable de la unió a la major part de NRs, aquest no és el cas de l'AR. L'SRC1 i SRC2 interacciones amb l'AF-2 de l'AR, però aquesta associació no és essencial per la coactivació del receptor (Brooke et al., 2008). En canvi, tots dos coactivadors han demostrat unir-se a l'NTD i, possiblement, també al DBD de l'AR, interacció en la qual els motius LxxLL no són imprescindibles (Bevan et al., 1999).

Un altre coactivador de l'AR molt conegut és la proteïna 70 associada a l'AR (ARA 70/NCOA4), la qual va ser el primer coactivador d'AR identificat dependent de lligand (Heinlein et al., 1999). No obstant, tot i que inicialment es va pensar que era específic d'AR, més endavant es va veure que també interacciona amb altres NRs (Heinlein et al., 1999; Kollara and Brown, 2012). L'ARA70 és una proteïna de 614 residus que conté dos motius d'interacció a NRs: un motiu LxxLL i un motiu FxxLF. Mentre que el primer motiu és essencial per la unió d'ARA70 a la majoria dels NRs, la regió responsable de la interacció amb l'LBD de l'AR resideix entre els residues 321-441, on es localitza el motiu FxxLF (He et al., 2002). L'ARA70 s'ha associat a diversos carcinomes, tot i que l'alteració de la seva expressió en el càncer de pròstata (PCa) encara no està clara (Kollara and Brown, 2012).

D'altra banda, tenim els corepressors de l'AR, entre els quals destaquen el corepressor dels NRs (NCoR/NCOR1) i el silenciador dels receptors retinoic i tiroide (SMRT/NCOR2), els quals s'uneixen a l'AF-2 i/o voltants de l'AR (Nagy et al., 1999). En absència de lligand, l'AR no sembla unir-se massa fortament amb l'NCoR i l'SMRT. Aquesta interacció, en cavi, és molt intensa quan l'AR està unit a un antagonista i, inclús, a algun agonista (van de Wijngaart et al., 2012).

L'associació amb els NRs està mediada a través d'uns motius curts i conservats en la regió C-terminal anomenats motius de corepressió dels NRs (CoRNR) (Nagy et al., 1999; Perissi et al., 1999).

## 8.1.4. Disfunció del receptor d'andròGens en patologies

Genetistes mèdics han identificat més mutacions somàtiques espontànies en l'AR que en qualsevol altre gen (http://androgendb.mcgill.ca/). Mentre que les anomalies que provoquen una atenuació de l'AR en resposta als andròGens provoca infertilitat i feminització, una excessiva estimulació de l'AR resulta en altres patologies, sent el PCa la més comuna.

### 8.1.4.1. Càncer de pròstata (PCa)

L'AR juga un paper crucial en el desenvolupament del PCa, actualment el segon tipus de càncer més comú en homes i la cinquena causa de mort per càncer més freqüent en homes (Lorente et al., 2015). Per aquest motiu, actualment l'AR representa la diana terapèutica més important en el tractament del PCa (Attard et al., 2015; Y. N. S. Wong et al., 2014). El tractament més comú consisteix en la combinació de la teràpia de privació d'andròGens i l'ús d'antiandròGens (antagonistes d'AR que bloquegen l'LBD) per tal d'inhibir tant la síntesi d'andròGens com l'acció de l'AR. Malauradament, tot i la resposta inicialment eficaç, la majoria de tumors progressen a PCa resistents a la castració (CRPC), pel qual avui dia no existeix cap teràpia curativa (Katzenwadel and Wolf, 2015; Saad and Fizazi, 2015). Els mecanismes a través dels quals l'AR pot reactivar el CRPC inclouen la sobreexpressió de l'AR, mutacions somàtiques que fan que el receptor adquireixi noves funcions, modificacions post-traduccionals aberrants, esdeveniments de *splicing* alternatiu i desregulació de cofactors (D. Robinson et al., 2015; Yuan et al., 2013). En teixits de PCa s'han identificat més de 1000 mutacions diferents localitzades predominantment a les regions codificants del DBD i l'LBD de l'AR (Tan et al., 2015), responsables d'una elevada activació del receptor en resposta a diversos lligands, incloent molècules no-androgèniques (Buchanan et al., 2001a). Totes les mutacions reportades en PCa es troben catalogades en la base

de dades de les mutacions del gen de l'AR en l'Institut de Recerca Mèdica Lady David, disponible a http://www.mcgill.ca/androgendb/.

#### 8.1.4.2. Altres malalties relacionades amb l'AR

Més d'un miler de mutacions en el gen de l'*AR* han estat reportades en pacients amb el síndrome d'insensibilitat als andròg ens (AIS) (Gottlieb et al., 2012), una malaltia genètica recessiva lligada al cromosoma X. L'AIS es caracteritza per una resistència completa (CAIS) o parcial (PAIS) a l'acció biològica dels andròg ens en un mascle XY amb testicles normals i un nivell de producció d'andròg ens també normal (Mongan et al., 2015). Les mutacions més severes estan generalment associades a un fenotip CAIS, causant una pèrdua completa de la funció de l'AR i donant lloc a un fenotip de dona (Wisniewski et al., 2000). Els fenotips PAIS són menys freqüents i es caracteritzen per diversos graus de masculinització dels genitals externs degut a una resposta parcial als andròg ens i mutacions que atenuen l'activitat del receptor (Quigley et al., 2004). La forma més lleu de l'AIS (MAIS) està causada per mutacions de l'AR en homes infèrtils que no presenten cap anomalia en els genials (Zuccarello et al., 2008).

D'altra banda, l'extensió en el número de repeticions de poliglutamines en l'NTD de l'AR, oscil·lant entre les 40 i les 62 repeticions, causa l'anomenada atròfia muscular espinal i bulbar (SBMA), coneguda també com a malaltia de Kennedy, una enfermetat neuromuscular hereditària degenerativa (Kumar et al., 2011; La Spada et al., 1991). Malgrat s'han proposat diversos models, encara no està clar com contribueixen aquestes amplificacions de les poliglutamines a la patogènesi de la malaltia (Grunseich et al., 2013)

### 8.1.5. El receptor d'andròg ens com a diana terapèutica en el tractament del càncer de pròstata

Els antagonistes d'AR són compostos que interfereixen amb els efectes biològics dels andròg ens i s'utilitzen freqüentment en el tractament de patologies relaciones amb els andròg ens. Fins a dia d'avui, tots els antiandròg ens comercialment

disponibles s'uneixen a l'LBP de l'AR, competint amb les hormones naturals (Mohler et al., 2012).

Els antiandròGens es poden classificar en agents esteroides i no-esteroides. Entre els primers destaca l'acetat de ciproterona (CPA) (Cyprostat®, Androcur®), el primer antiandrogen comercialitzat en el 1989 que actualment té un ús clínic limitat degut als seus efectes adversos. Entre els antiandròGens no-esteroides (NSAAs) comercialment disponibles i utilitzats com a primera línia en el tractament del PCa hi ha la bicalutamida (Casodex®), la flutamida (Eulexin®, Cytomid®), la nilutamida (Nilandron®) i l'enzalutamide (Xtandi®), aquesta última aprovada en el 2012.

Malauradament, com ja s'ha comentat anteriorment, mutacions adquirides en l'AR provoquen que el receptor sigui resistent als fàrmacs. Els antiandròGens donen lloc a una selecció de mutacions que fan que l'AR sigui capaç de respondre a aquests fàrmacs com si d'agonistes es tractés, donant lloc a un creixement incontrolat del tumor, remarcant la necessitat urgent d'antiandròGens de nova generació capaços de superar aquest mecanisme compensatori. A banda de l'LBP, altres superfícies de l'AR, incloent l'AF-2 i el BF-3, han estat proposades com a possibles dianes terapèutiques (Tian et al., 2015). De fet, diversos compostos han demostrat unir-se al BF-3 (Estébanez-Perpiñá et al., 2007; Munuganti et al., 2014), un dels quals ha provat, a més, tenir una gran potència antiandrogènica (Munuganti et al., 2014), evidenciant el potencial farmacològic de la butxaca BF-3.

Un dels majors reptes en el disseny de nous fàrmacs contra l'AR és el de generar compostos que regulin una única o diverses de les múltiples funcions que porta a terme l'AR per tal d'aconseguir l'efecte farmacològic desitjat. Desenvolupaments recents en el nostre coneixement de l'estructura de l'AR i el seu mecanisme d'acció han contribuït en el desenvolupament de moduladors selectius de l'AR (SARMs), lligands del receptor específics de teixits. El gran objectiu dels SARMs és el d'eliminar els efectes secundaris no desitjats a través d'incrementar l'especificitat de l'AR i millorant la selectivitat de les activitats farmacològiques a nivell de teixit (W. Gao and Dalton, 2007). Diversos SARMs per diferents indicacions clíniques es troben actualment en desenvolupament preclínic i clínic (Narayanan et al., 2008).

## 8.2. OBJECTIUS DE LA TESI

(1) **Estudiar la conservació de la butxaca BF-3 entres diferents NRs**. S'ha analitzat la conservació de la superfícies BF-3 en termes d'identitat estructural, mida, forma i profunditat de la butxaca superimposant les estructures disponibles.

(2) **Efecte de mutacions en la butxaca BF-3 en funcions crucials del receptor**. S'ha estudiat els efectes que mutacions en el BF-3 exerceixen en l'activitat i coactivació del receptor, la interacció amb l'NTD i corepressors, així com el seu impacte en la translocació del receptor al nucli.

(3) **Identificació de proteïnes que interaccionen amb el BF-3.** Un dels majors objectius d'aquesta tesi ha estat el d'identificar el paper de la butxaca BF-3 en l'associació macromolecular de l'AR amb proteïnes coreguladores, ja siguin noves o conegudes. Aquests nous interactors del receptor podrien representar noves dianes capaces de modular l'AR sota condicions patològiques.

(4) **Efecte dels antiandrògens en la unió de l'AR LBD WT i mutants de BF-3 a coreguladors d'AR.** Amb l'objectiu de valorar si mutants de BF-3 potencien o reprimeixen el reclutament de coreguladors, es va establir en el laboratori un assaig doble híbrid en llevats en forma de matriu. Aquest sistema va permetre avaluar la interacció entre l'LBD de l'AR, tant del WT com de diferents mutants de BF-3, amb diferents proteïnes coreguladores del receptor en presència de diferents antiandrògens.

## 8.3. RESULTATS

### 8.3.1. BF-3: una superfície conservada en l'LBD dels NRs pel seu control al·lostèric

Per tal de determinar si la butxaca BF-3 està present en altres NRs, les coordenades atòmiques dels LBDs de diversos NRs (AR, PR, MR, GR, ERα, VDR, Nurr1, FXR, PPARγ, i RARα) resolta per cristal·lografia de raigs X han estat superimposades. Per tal de visualitzar la concavitat de l'ERα (PDB 1ERE), els

residus de la seva regió frontissa i de l'H1 es van eliminar manualment ja que impedien la correcta visualització de la superfície BF-3.

Comparant la butxaca BF-3 en la superimposició estructural, es va veure que aquesta superfície està altament conservada en el PR (PDB 1A28), MR (PDB 2AA7), i GR (PDB 1P93), sent les butxaques del PR i MR les més similars a l'AR. La conservació en l'altre membre dels SRs, l'ER, va demostrar ser més discreta.

Els residus de les corresponents butxaques BF-3 que mostren el major grau de conservació entre els SRs són el tàndem d'arginina i asparagina localitzades a la frontera entre l'AF-2 i el BF-3 (R726 i N727), el qual està conservat en tots els SRs excepte l'ER. Ambdós residus han demostrat interaccionar amb inhibidors de superfícies i jugar un paper fonamental en la transmissió d'informació a l'AF-2 (Estébanez-Perpiñá et al., 2007).

Els residus homòlegs a l'AR P723 i G724 es troben estructuralment conservats en pràcticament tots els NRs estudiats, tal i com ja s'havia demostrat anteriorment (Wurtz et al., 1996). La superimposició estructural també va revelar que en la posició equivalent a l'AR R840 hi ha preferiblement una lisina amb càrrega positiva en altres NRs.

Malgrat la baixa conservació que mostra la seqüència primària dels LBDs dels NRs orfes Nurr1, FXR, RXR, RARs, PPARs, VDR i DAX1, les seves butxaques BF-3 mostren un grau de conservació sorprenent a nivell d'aminoàcids, tot i que menor que el que presenten els SRs. El FXR, RARs, PPARs, VDR i Nurr1 posseeixen unes superfícies BF-3 semblants a la de l'AR en termes de forma i profunditat.

El BF-3 de l'AR està dividit per la presència d'una leucina en la posició 830. Curiosament, sembla haver-hi una preferència per una leucina, isoleucina o metionina en les posicions homòlogues dels NRs estudiats, remarcant la naturalesa hidrofòbica d'aquest solc.

El BF-3 de l'AR presenta un nivell de conservació molt baix en comparació amb les dues isoformes del TR i la semblança de les cavitats és també baixa. No obstant, en el TRβ es van identificar superfícies addicionals i, malgrat aquestes superfícies no coincideixen amb el BF-3, alguns dels seus residus es sobreposen amb certs residus clau del BF-3 de l'AR (Marimuthu et al., 2002).

### 8.3.2. Mutacions en la butxaca BF-3 de l'AR altera múltiples funcions del receptor

Per tal de poder identificar un possible paper de la butxaca BF-3 en la funció de l'AR, es va dur a terme un estudi mutagènic per avaluar els efectes funcionals *in vitro* que mutacions del BF-3 podrien exercir en el receptor. Concretament, es va avaluar les capacitats de transactivació i coactivació de l'AR LBD wild-type (WT) i diferents mutants del BF-3, així com les seves interaccions N/C i les seves unions als corepressors NCoR i SMRT. Així mateix, l'activitat i translocació nuclear de certs mutants BF-3 també es va avaluar en el context del receptor sencer. Les mutacions es troben delimitant la superfície BF-3 (I672, F826, N833 i R840) o en la frontera entre l'AF-2 i el BF-3 (R726, N727). Addicionalment, com a control, es va incloure una mutació (V757A) localitzada a l'H5, distant d'ambdues superfícies estudiades. Els residus seleccionats inclouen tant mutacions que han estat associades amb el PCa (R726L i V757A), com mutacions que han estat reportades en pacients diagnosticats d'AIS (N727K i F826L), així com formes que no han estat relacionades amb cap patologia encara.

Avaluant l'activitat de tots els mutants a diferents concentracions d'hormona, es va veure que els mutants I672R, N833R i R840A eren molt més actius que l'AR LBD WT a elevades concentracions de DHT. Curiosament, l'N833R i el R840A van ser els únics variants, juntament amb l'R840E, que eren inactius a concentracions d'hormona d'1 nM. El mutant F826L va manifestar una activitat moderadament elevada a 10 i 100 nM de DHT. Les activacions dels mutants R726L, N727K, V757A i F826R va resultar similar a les del WT a totes les concentracions d'hormona, tot i que el F826R va comprometre significativament l'activitat del receptor a 100 nM de DHT. Finalment, la substitució de l'arginina de la posició 840 per l'àcid glutàmic (R840E) va abolir completament l'activitat del receptor, que es va mostrar inactiu a totes les concentracions d'hormona. El nivell d'expressió de proteïna de l'AR LBD tant de tots els mutants de BF-3 com del WT eren comparables.

Es va analitzar també la coactivació dels tots els LBDs en presència de la proteïna d'unió al receptor glucocorticoide 1 (GRIP1/SRC2), un conegut coactivador de l'AR. El mutant F826R va demostrar un clar increment en l'activitat en presència de GRIP1 en comparació amb el WT. Curiosament, el N833R va manifestar l'increment

més petit, sense arribar als nivells del WT, mentre que la coactivació que GRIP1 va exercir en el mutant mort R840E va ser similar a la del WT. La resta de mutants van mostrar una coactivació similar al WT.

Tanmateix, a través de la tècnica del doble híbrid, es va avaluar la interacció de tots els LBDs amb l'NTD. Sobtadament, els 3 mutants que exhibien l'activitat més elevada, I672R, N833R i R840A, van manifestar l'associació més feble. D'altra banda, el F826R va demostrar la interacció més forta, mentre que els variants R726L i V757A van exhibir una unió similar al WT, i les formes N727L i F826L van manifestar un reclutament lleugerament inferior. Per últim, la substitució de l'arginina a la posició 840 per un àcid glutàmic va impedir completament la seva associació amb l'NTD.

Finalment, es va estudiar la interacció dels mutants BF-3 amb els corepressors SMRT i NCoR. La majoria de mutants van mostrar una unió més feble a tots dos corepressors en comparació amb el WT, indicant que la butxaca BF-3 podria tenir un possible paper corepressor.

D'altra banda, es va analitzar també l'activitat i importació nuclear d'alguns mutants BF-3 (I672R, G724R, N727A, V757A, R840A i R840E) en el context dels receptors formats per tots tres dominis (NTD, DBD i LBD). Mentre els mutants I672R, N727A i V757A van manifestar una activitat similar a la del WT a totes les concentracions d'hormona, els variants G724R, R840 i R840E van exhibir una activitat lleugerament inferior a la del WT a tot el rang de concentracions, destacant aquest últim mutant amb una activació bastant per sota de la del WT. Finalment, pel que fa la translocació nuclear, es va analitzar la importació dels receptors al nucli estimulant les cèl·lules durant 15 minuts, 2 hores i 16 hores. Sota l'estimulació hormonal, en 15 minuts l'AR LBD WT ja havia completat el procés d'importació nuclear, mentre que la translocació dels mutants BF-3 era significativament inferior. L'estimulació de les cèl·lules durant 2 hores, va induir la importació nuclear de tots els mutants BF-3 excepte la del R840E, que encara persistia en el citoplasma. Ni en les cèl·lules tractades durant 16 hores, va aconseguir el mutant R840E completar la translocació, senyalant la necessitat de més temps o major concentració d'hormona per completar el procés.

### 8.3.3. Identificació de noves proteïnes d'unió a l'AR

Per tal d'identificar nous interactors i possibles coreguladors de l'AR, l'LBD i part de la regió frontissa de l'AR (residus 646-919) unit a hormona va ser utilitzat com a esquer per dur a terme set assajos de doble híbrid en llevat (Y2H) de dues llibreries de cDNA tant de cervell humà com de pròstata. En presència de DHT, es van aïllar 4 nous interactors putatius: un clon de l'armadillo repeat containing 9 (ARMC9), tres clons de la proteïna interactora 1 de la quinasa activada per mitogen 8 (MAPK8IP1), dos clons de la proteïna 3 d'interacció a la família Rab11 (Rabb11FIP3), i quatre clons de l'enzima modificador d'activació similar a la ubiqüitina (Uba3), la subunitat catalítica de l'enzim activador E1 específic NEDD8 (NAE). Dels quatre interactors putatius, tres d'ells, l'ARMC9, MAPK8IP3 i Uba3 van ser validats en un sistema Y2H d'alt rendiment (HT-Y2H) en format de matriu posat a punt en el laboratori. Tot i que la interacció de Rab11FIP3 amb l'LBD de l'AR no es va poder confirmar en aquest segon sistema, en assajos *in vitro*, aquesta proteïna va demostrar inhibir l'LBD de l'AR i impedir la seva coactivació en presència de GRIP1, així com impossibilitar la seva unió a l'NTD. Els mateixos resultats es van obtenir amb l'Uba3.

Aquest sistema HT-Y2H també es va utilitzar per avaluar la interacció dels mutants de BF-3 amb les quatre noves proteïnes, així com amb d'altres coreguladors coneguts de l'AR, en absència i presència d'hormona, així com en presència dels antiandrògens hidroxiflutamida, bicalutamida, mifepristona i enzalutamida. Pràcticament cap de les substitucions en els residus que conformen la superfície BF-3 van alterar la unió amb els coreguladors de l'AR en presència de DHT, tot i que hi ha algunes excepcions que val la pena esmentar. Els mutants R726L i N833R no van ser capaços de reclutar cap proteïna en presència de DHT. La substitució de la lisina de la posició 720 (K720) per una alanina va impedir la seva associació amb el coactivador SRC3, i els mutants R840A i R840E van impossibilitar la interacció amb la MAPK8IP1 i l'Uba3.

Pel que fa a les interaccions en presència d'antiandrògens, sorprenentment, el SRC3, ARMC9 i Uba3 van poder associar-se amb l'LBD de l'AR WT en presència de bicalutamida, mifepristona i enzalutamida. Cap LBD va poder unir-se a cap de les proteïnes coreguladores en absència d'hormona ni presència d'hidroxiflutamida.

## 8.4. DISCUSSIÓ

Tot i que tots els antiandròsens que hi ha actualment al mercat s'uneixen a l'LBP de l'AR, hi ha altre superfícies reguladores de l'AR, incloent l'AF-2 i el BF-3, que han atret gran atenció en el desenvolupament de nous fàrmacs. De fet, la viabilitat d'atacar tant l'AF-2 (Tan et al., 2015) com el BF-3 (Estébanez-Perpiñá et al., 2007; Munuganti et al., 2014; 2013) ja ha estat demostrada. Compostos que s'uneixen a BF-3 han demostrat inhibir la proliferació de línies cel·lulars de PCa resistents a l'enzalutamida i reduir l'expressió de gens diana de l'AR (Munuganti et al., 2014), evidenciant el seu potencial farmacològic.

En aquesta tesi demostrem, en primer lloc, que la recent identificada superfície BF-3 està altament conservada entre els SRs, i que mutacions associades a patologia que afecten la funció de diversos NRs *in vitro*, colocalitzen en les respectives butxaques putatives BF-3. Tots els SRs tenen una glicina en la posició 724, en un bloc conservat entres les hèlices 2 i 3 de l'LBD (Matias et al., 2002), assenyalant la importància d'aquest residu per la correcta funció del receptor. El tàndem d'arginina i asparagina en el límit de les superfícies AF-2 i BF-3 (R726 i N727) també es troba altament conservat.

En segon lloc, per tal d'identificar nous possibles interactors de BF-3, vam portar a terme diversos assajos Y2H de llibreries de cDNA tant de cervall humà d'adult com de pròstata. En presència de DHT, quatre nous interactors van ser identificats: ARMC9, MAPK8IP1, Uba3 i Rab11FIP3, els tres primers dels quals van ser validats en el sistema HT-Y2H. Malgrat l'associació entre Rab11FIP3 i l'LBD de l'AR no es va poder confirmar, assajos *in vitro* van demostrar que Rab11FIP3 redueix la capacitat d'activació del receptor, atenua la coactivació de GRIP1 i impedeix la interacció entre els dominis LBD i NTD. Resultats *in vitro* similars es van obtenir amb l'Uba3. Sorprenentment, diverses mutacions en el BF-3 van trencar la unió de l'LBD a determinats coreguladors, recolzant la possible implicació de BF-3 en la interacció de proteïnes.

Per últim, vam realitzar mutagènesi dirigida en residus de la superfícies de BF-3 per tal d'elucidar la seva possible funció en l'activitat de l'AR. Els nostres resultats confirmen i complementen estudis anteriors que demostren que mutants de BF-3 alteren diferents funcions de l'AR (Ahmed et al., 2000; Estébanez-Perpiñá et al.,

2007; Robins, 2012; Tilley et al., 1996; Yong et al., 1994). Demostrem que mutacions en residus de la superfície BF-3 o residus que delimiten l'AF-2 i el BF-3 provoquen una gran varietat d'efectes en la capacitat transactivadora del receptor, des de superactivants a potents inhibidors, i aquests resultats estan influenciats per la concentració d'hormona i el constructe utilitzats (receptor complet versus LBD aïllat). Diferents conseqüències en la coactivació en presència del coregulador GRIP1 i en la interacció funcional amb l'NTD s'han obtingut. Finalment, els mutants BF-3 han exhibit diferents efectes en la unió de corepressors: mentre que la majoria dels mutants van impedir la interacció amb els corepressors NCoR i SMRT, altres van exhibir efectes més lleus o cap efecte, i algun d'ells van semblar distingir entre l'NCoR i l'SMRT. Cal remarcar que tots els mutants BF-3, a excepció de l'R726L i l'N727K, localitzats en la frontera entre l'AF-2 i el BF-3, van mostrar *in vitro* una interacció defectuosa amb l'SMRT. En el HT-Y2H, els variants R726L i N833R van trencar la unió de l'LBD d'AR amb tots els coreguladors, incloent l'SMRT; i la substitució de l'arginina a la posició 840 per una alanina o un àcid glutàmic va impedir que l'LBD de l'AR reclutés tant la MAPK8IP1 com l'Uba3, suggerint la possible implicació d'aquest residu en la interacció amb totes dues proteïnes. Sorprenentment, l'LBD de l'AR va ser capaç d'associar-se al SRC3, l'ARMC9 i l'Uba3 en presència d'antiandrògens, i tant la bicalutamida com la mifepristona va promoure la unió de l'LBD d'AR R726L al SRC3. Aquesta arginina a la posició 726 resideix en un clúster positiu en l'H3 i participa tant en la interacció amb el motiu FxxLF de l'NTD com en l'unió als motius LxxLL presents en els coactivadors SRCs (Dubbink, 2004; Estébanez-Perpiñá et al., 2007; 2005; He et al., 2004). A més a més, el mutants R726L ha demostrat impedir l'associació amb activadors SRC i amb l'NTD (Hay and McEwan, 2012), possiblement explicant per què no vam obtenir cap interacció en el Y2H, tot i que *in vitro*, aquest mutant va demostrar una interacció amb l'NTD similar a la del WT. D'altra banda, aquest mutant està implicat en un augment en el risc a patir PCa, fet que podria explicar la seva associació amb el SRC3 en presència d'antiandrògens.

En la majoria dels casos, és molt difícil correlacionar mutacions específiques en un NR amb el fenotip clínic dels pacients, ja que les condicions *in vitro* (constructe, concentració d'hormona, línia cel·lular) poden canviar dramàticament els resultats. La coactivació en presència de GRIP1, la interacció entre l'LBD i l'NTD i

les capacitats de transactivació obtingudes amb els diferents mutants de BF-3 no poden explicar en tots els casos els fenotips corresponents causats pel mutant en concret. Algunes mutacions donen *in vitro* uns resultats oposats als que un esperaria a partir del fenotip del pacient, on l'entorn de coreguladors o altres factors crucials pel correcte funcionament del receptor estan alterats. No obstant, aquests assajos *in vitro* són àmpliament utilitzats en el camp dels NRs, ja que proporcionen una indicació indirecte de possibles alteracions en el funcionament de l'AR (Bevan et al., 1996).

Considerant tots els resultats, es poden especular dues possibles funcions fisiològiques del BF-3. D'una banda, els mutants de BF-3 han demostrat actuar com a inductors al·lostèrics de canvis conformacionals que es transmeten a l'AF-2, afectant la funció de l'LBD de l'AR (Estébanez-Perpiñá et al., 2007; Grosdidier et al., 2012). D'altra banda, és plausible que mutants de BF-3 alterin contactes directes amb proteïnes com l'Uba3, potenciant o silenciant la funció de l'AR. La superfície de BF-3 podria, inclús, contactar amb el coactivador GRIP1, l'NTD o els corepressors NCoR/SMRT, i aquestes proteïnes podrien interaccionar amb l'LBD de l'AR en una regió que s'estendria més enllà de l'AF-2 cap al BF-3 (Estébanez-Perpiñá et al., 2007).

Per concloure, els NRs són reguladors de la reproducció, el desenvolupament i el metabolisme i, malgrat totes les dificultats associades amb els seus efectes adversos, encara són una major diana en el desenvolupament de nous fàrmacs. De fet, s'estima que el 10% dels fàrmacs que hi ha actualment al mercat actuen via els NRs (Laudet, 2015). No obstant, la majoria dels compostos que actuen sobre l'LBP dels NRs presenten efectes secundaris seriosos degut a l'activitat creuada entre les diferents famílies de NRs. Desenvolupar moduladors de NRs contra superfícies alternatives de l'LBD, com la butxaca BF-3, podria obrir alternatives prometedores a les teràpies actuals. Compostos que podrien modular al·lostèricament la funció dels NRs unint-se a la superfície BF-3 obren noves vies prometedores de desenvolupar moduladors específics de NRs.

## 8.5. CONCLUSIONS

(1) La superfície BF-3 del receptor humà d'andrògens està altament conservat entre la subclasse dels receptors d'esteroides i, malgrat estar també present en altres NRs (amb un nivell de conservació més baix), podria representar una possible diana terapèutica per controlar la funció dels NRs i desenvolupar una nova generació de moduladors.

(2) La superfície BF-3 és un modulador al·lostèrics de la butxaca adjacent AF-2, de manera que compostos que s'uneixin a aquesta superfície podrien modular les associacions proteïna-proteïna de l'AR implicades en patologia.

(3) Mutacions en la butxaca BF-3 i residus situats entre les superfícies AF-2 i BF-3 alteren la transactivació i coactivació de l'AR en presència de GRIP1, així com la interacció de l'LBD de l'AR amb l'NTD i els corepressors NCoR i SMRT.

(4) BF-3 és un lloc d'interacció proteïna-proteïna per proteïnes coreguladores.

(5) ARMC9, MAPK8IP1 i Uba3 són nous interactors putatius d'AR.

(6) Rab11FIP3 i Uba3 inhibeixen la transactivació de l'LBD de l'AR, atenuen la coactivació de GRIP1 i trenquen la interacció entre els dominis N- i C-terminals, suggerint que podrien ser corepressors d'AR no descrits prèviament.

(7) SRC3, ARMC9 i Uba3 s'associent a l'LBD de l'AR en presència d'antiandrògens.

(8) Certes interaccions proteïna-proteïna i formació de complexes d'AR amb coreguladors coneguts tenen lloc en presència d'antiandrògens. Conseqüentment, el model que tots els antiandrògens donen lloc a un AF-2 mal format o no-productiu necessita ser reavaluat.

(9) Les complexes associacions d'AR amb coreguladors tant nous com ja coneguts mantinguts en presència d'antiandrògens indiquen que la butxaca AF-2 i l'hèlix 12 podrien exhibir una major funcionalitat conformacional de la descrita prèviament.

# IX. APPENDIX

# 9. APPENDIX

## 9.1. LIST OF PUBLICATIONS

**Carbó* LR**, Grosdidier* S, Buzón V, Brooke G, Nguyen P, Baxter JD, Bevan C, Webb P, Estébanez-Perpiñá E, Fernández-Recio J (2012) Allosteric conversation in the androgen receptor ligand-binding domain surfaces. Mol Endocrinol 26(7):1078-90.

**Carbó* LR**, Buzón* V, Estruch SB, Fletterick RJ, Estébanez-Perpiñá E (2012) A conserved surface on the ligand binding domain of nuclear receptors for allosteric control. Mol Cell Endo 348(2):394-402.

Estruch SB, Buzón V, **Carbó LR**, Schorova L, Lüders J, Estébanez-Perpiñá E (2012) The oncoprotein BCL11A binds to orphan nuclear receptor TLX and potentiates its transrepressive function. PLoS One 7(6):e37963.

Jehle K, Cato L, Neeb A, Muhle-Goll C, Jung N, Smith EW, Buzon V, **Carbó LR**, Estébanez-Perpiñá E, Schmitz K, Fruk L, Luy B, Chen Y, Cox MB, Bräse S, Brown M, Cato AC (2014) Coregulator control of androgen receptor action by a novel nuclear receptor-binding motif. J Biol Chem. 2014 Mar 28;289(13):8839-51.

## 9.2. SHORT TALKS AND POSTERS PRESENTED AT CONGRESSES

- Androgens (Nov. 2012). Helsinki, Finland. **Poster**.
- European Molecular Biology Organization (EMBO) *Nuclear Receptors: From Molecular Mechanism to Health and Disease* (Sept. 2011). Sitges, Barcelona, Spain. **Poster**.
- XXXIV Congress Biochemistry and Molecular Biology Spanish Society (SEBBM) (Sept. 2011). Barcelona, Spain. **Poster**.
- FEBS Advanced Lecture Course. Spetses Summer School on *Nuclear Receptor Signaling in Physiology and Disease* (Sept. 2011). Island of Spetses, Greece. **Poster** and **short talk**.

- Biochemistry and Molecular Biology Spanish Society (SEBBM) XXXIII Congress (Sept. 2010). Córdoba, Spain. **Poster**.

## 9.3. SHORT STAYS PERFORMED DURING THE PHD

**Imperial College London**, London. Institute of Reproductive and Developmental Biology, Department of Surgery and Cancer. Dra. Charlotte Bevan's laboratory. October-December 2011.

**The Methodist Hospital Research Institute**, Houston. Diabetes and Metabolic Diseases Dept. Dr. Paul Webb's laboratory. February-April 2011.

**University of California, San Francisco (UCSF)**. Biochemistry and Biophysics Dept. Prof. Robert Fletterick's laboratory. November 2009.