

Diverse roles of androgen receptor (AR) domains in AR-mediated signaling

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Androgens control male sexual development and maintenance of the adult male phenotype. They have very divergent effects on their target organs like the reproductive organs, muscle, bone, brain and skin. This is explained in part by the fact that different cell types respond differently to androgen stimulus, even when all these responses are mediated by the same intracellular androgen receptor. To understand these tissue- and cell-specific readouts of androgens, we have to learn the many different steps in the transcription activation mechanisms of the androgen receptor (NR3C4). Like all nuclear receptors, the steroid receptors have a central DNA-binding domain connected to a ligand-binding domain by a hinge region. In addition, all steroid receptors have a relatively large amino-terminal domain. Despite the overall structural homology with other nuclear receptors, the androgen receptor has several specific characteristics which will be discussed here. This receptor can bind two types of androgen response elements (AREs): one type being similar to the classical GRE/PRE-type elements, the other type being the more divergent and more selective AREs. The hormone-binding domain has low intrinsic transactivation properties, a feature that correlates with the low affinity of this domain for the canonical LxxLL-bearing coactivators. For the androgen receptor, transcriptional activation involves the alternative recruitment of coactivators to different regions in the amino-terminal domain, as well as the hinge region. Finally, a very strong ligand-induced interaction between the amino-terminal domain and the ligand-binding domain of the androgen receptor seems to be involved in many aspects of its function as a transcription factor. This review describes the current knowledge on the structure-function relationships within the domains of the androgen receptor and tries to integrate the involvement of different domains, subdomains and motifs in the functioning of this receptor as a transcription factor with tissue- and cell-specific readouts.

Received March 18th, 2008; Accepted May 29th, 2008; Published June 27th, 2008 | **Abbreviations:** AF: activation function; AR: androgen receptor; ARE: androgen response element; CBP: CREB-binding protein; ChIP: chromatin immunoprecipitation; CHIP: carboxy terminal domain of hsp70-interacting protein; CTE: carboxy-terminal extension; DBD: DNA-binding domain; ER: estrogen receptor; FSH: follicle stimulating hormone; GR: glucocorticoid receptor; GRE: glucocorticoid response element; GST: glutathione S transferase; LBD: ligand binding domain; LH: luteinizing hormone; MR: mineralocorticoid receptor; N-CoR: nuclear receptor corepressor; NR: nuclear receptor; NTD: N-terminal domain; p/CAF: p300/CBP-associated factor; PIAS: protein inhibitor of activated STAT; PR: progesterone receptor; PRK1: protein kinase C-related kinase 1; SPARK1: specificity affecting AR knock-in; SRC: steroid receptor coactivator; SUMO: small ubiquitin-like modifier; Tau: transcription activation unit | Copyright © 2008, Claessens et al. This is an open-access article distributed under the terms of the Creative Commons Non-Commercial Attribution License, which permits unrestricted non-commercial use distribution and reproduction in any medium, provided the original work is properly cited.

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Introduction

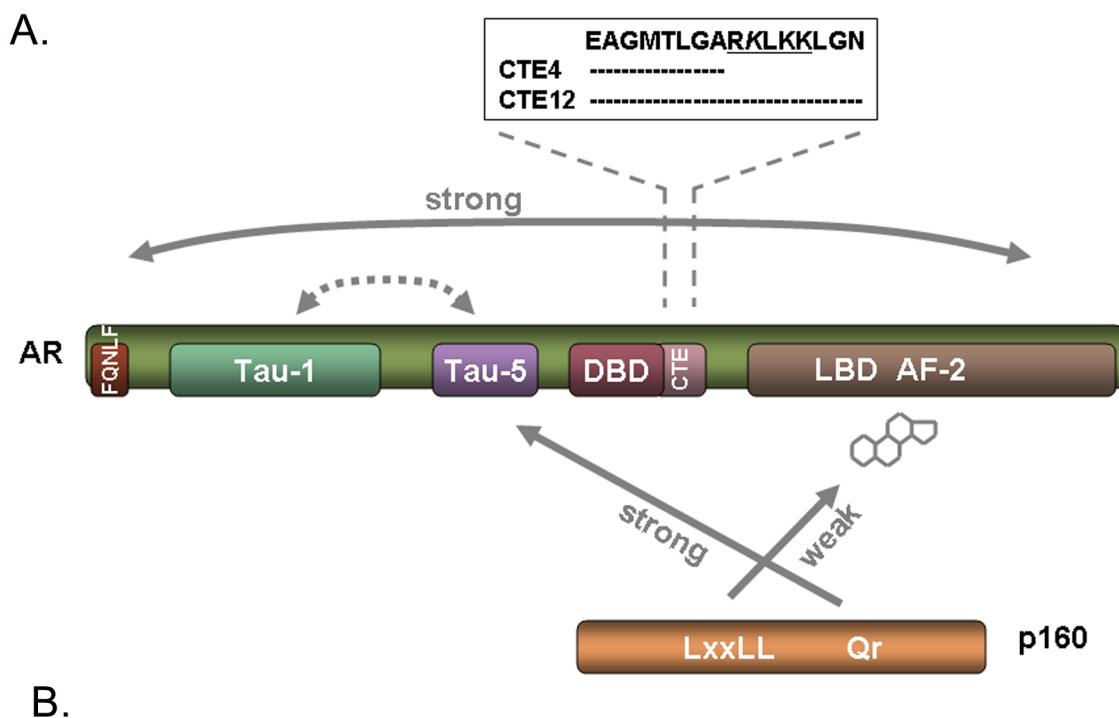
Androgens are the male sex hormones that belong to the steroid hormone family. They are mainly produced in testes, ovaries and adrenals. In early life, testicular androgens induce differentiation processes that lead to the development of the male phenotype. During adulthood, androgens remain essential for the maintenance of the male reproductive function, as well as a number of gender-dependent parameters like bone and muscle mass, hair growth and behavior.

The androgenic steroid testosterone is the precursor for the local synthesis of dihydrotestosterone, as well as estrogens. Each of these hormones has specific functions in the development and maintenance of the male phenotype. Both testosterone and dihydrotestosterone interact with the androgen receptor (AR or NR3C4), which is a member of the nuclear receptor (NR) family [Evans, 1988].

The human AR is a protein of 919 amino acids in length, but this can vary because it contains a poly-glutamine

and a poly-glycine stretch of variable lengths (Figure 1). The protein migrates in a polyacrylamide gel electrophoresis with an apparent molecular weight of 110 kDa. It is encoded by a single copy gene located on the X-chromosome. Like all nuclear receptors, the AR has a centrally-located DNA-binding domain (DBD) consisting of two zinc-coordinated modules (Figure 2). Carboxyl-terminally situated is the ligand-binding domain (LBD), which provides the regulatory switch by which androgens control the activity of the AR as a transcription factor. The hinge region connects the DBD with the LBD. It has different control functions which will be discussed in greater detail. The amino-terminal domain (NTD) is less conserved, both in size and sequence, between the different NRs. Even between the different steroid receptors, homology in this domain is only about 15%, and comparative structural studies revealed little similarity [McEwan et al., 2007].

For several nuclear receptors, a communication between the ligand-binding domain and the amino-terminal domain has been documented [He et al., 1999; Kraus et al., 1995;



AR-specific features of the aminoterminal domain

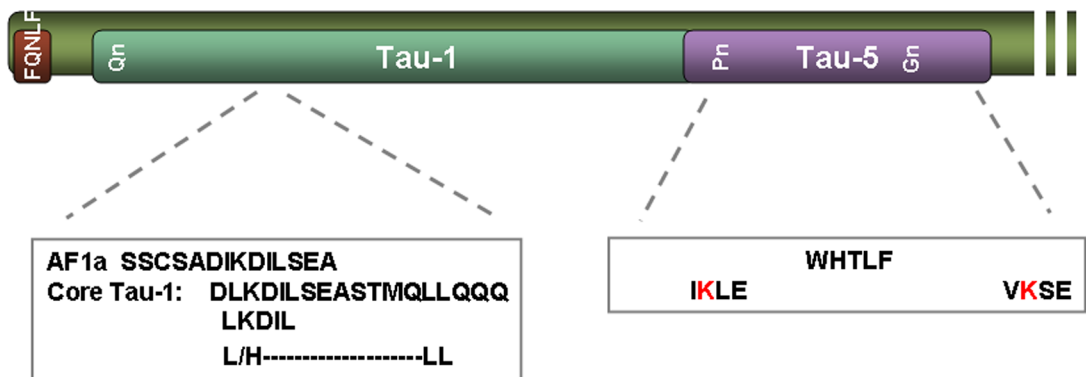


Figure 1. (A) Schematic representation of the androgen receptor with indications of its specific motifs and domains, and (B) the features of the amino-terminus of the androgen receptor. (A) A schematic representation of the AR (top) and of a p160 steroid receptor coactivator (bottom) is given with indications of the nuclear receptor-interacting domain (LxxLL) and the Tau-5-interacting domain (Qr). Arrows indicate possible inter- or intramolecular interactions. The dotted line with arrowheads indicates the interference of Tau-1 on Tau-5. The sequence of the carboxyl-terminal extension (CTE) is given in the box at the top. The acetyltable Lysine 630 is given in italic, the part of the nuclear localization signal is underscored. The location of core Tau-1 is indicated. (B) The relative positions of Tau-1 and Tau-5 are indicated, together with: the ²³FQNLF²⁷-motif, the polyglutamine (Qn), the polyproline (Pn) and the polyglycine (Gn) stretches. The sequences of the core Tau-1 overlapping motifs (see text) are given in the box on the left, the features of Tau-5 discussed in the text are given in the box on the right: the ⁴³³WHTLF⁴³⁷ motif and the SUMO-ylation sites.

Langley et al., 1998; Metivier et al., 2001; Tetel et al., 1999]. For the AR, this interaction is very strong. The current knowledge and questions on its physiological importance will be discussed.

Further clues on structure-function relationships within the AR have been provided by the discovery of the links

between divergent diseases and mutations in the human AR gene (summarized in <http://androgendb.mcgill.ca/> for overview). While germ line mutations have been detected in androgen-insensitivity patients, a number of somatic mutations have been linked with prostate cancer. In this paper, we will discuss the specific roles of the DBD, LBD, NTD and hinge region of the androgen receptor that

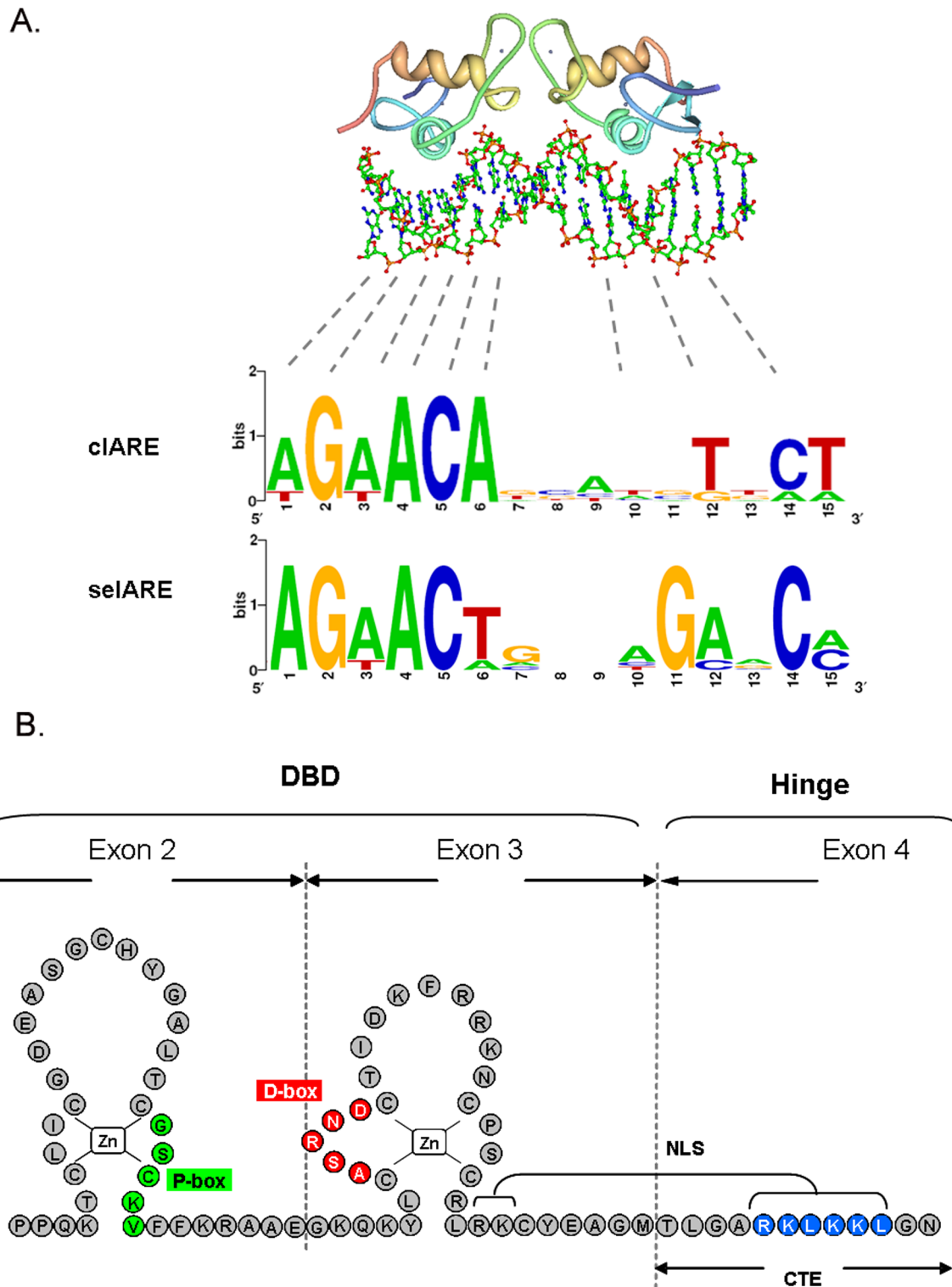


Figure 2. (A) Crystal structure of the AR-DBD and consensus sequences of the classical AREs and selective AREs, and (B) the two Zn finger coordinated modules of the DNA binding domain of the androgen receptor. (A) The top panel shows the crystal structure of the AR-DBD bound to a direct repeat of 5'-TGTCT-3' (PDB ID code 1R4I; Shaffer et al., 2004). This image was generated with the software PDB protein Workshop 1.50. The consensus sequences of the classical AREs and the selective AREs are given in the lower panel. This picture was obtained with the software Weblogo3 (Crooks et al., 2004). Dotted lines indicate the stronger interactions with the 5'-AGAAC-3' hexamer on the left. (B) The single letter code for amino acids is used. The P-box residues are indicated in green, the D-box residues in red and the nuclear localization signal in blue. The fragments that are encoded by exon 2, exon 3 and part of exon 4 are given. CTE indicates the carboxyl terminal extension involved in DNA binding, intracellular trafficking and transactivation.

emerged amongst others from the study of the mutations found in these diseases.

The androgen response elements

Steroid receptors have long been known to bind DNA elements that are organized as inverted repeats of hexameric binding sites separated by three nucleotide spacers. For the estrogen receptors, the consensus hexamer is 5'-TGACCT-3'. For androgen, glucocorticoid, progesterone and mineralocorticoid receptors (AR, GR, PR and MR, respectively), the consensus reads 5'-TGTTCT-3' [Cato et al., 1987; Ham et al., 1988]. Such binding sequences have been described in androgen-responsive genes and will be called here classical androgen response elements (cAREs, Figure 2A).

In view of the high homology between the DNA-binding domains and the similarity of the response elements, it is not surprising, *in vitro* at least, that these response elements are promiscuous for all four receptors. The AR, however, holds a specific position within the group of steroid receptors, since several selective AREs (selAREs, Figure 2A) have been described that are not recognized by the GR. Selective androgen-dependent enhancers/promoters were first discovered near the rat probasin gene, the sex-limited protein gene and the secretory component gene [Adler et al., 1991; Rennie et al., 1993; Verrijdt et al., 1999]. A consensus sequence for these selAREs is given in Figure 2A. Based on the sequence comparison of the selective AREs that were described initially (PB-ARE-2:GGTTCTTGACGACT; SC ARE: GGCTCTTTTCAGTTCT; Slp HRE 2: TGGTCAGCCAGTTCT), we speculated that the selective AREs are organized as partial direct repeats rather than inverted repeats of the same 5'-TGTTCT-3' motif. This seems to be corroborated by the fact that substitutions of e.g. an adenine for a thymine at position 12 (see Figure 2A) result in a loss of specificity of the androgen-selective elements or enhancers [Adler et al., 1993; Kasper et al., 1994; Verrijdt et al., 2000].

Surprisingly, all androgen-selective enhancers contain promiscuous cAREs, as well as selective AREs. This indicates a hierarchy among the AREs, and this hierarchy was shown to depend on the topology of the enhancers, since small insertions or inversions between the receptor binding sites can result in a loss of selectivity [Adler et al., 1993].

Furthermore, mutations in the sequence immediately downstream of an ARE can also affect the functionality of the element, even when little effect on *in vitro* affinity of the AR-DBD was observed. Such experiments show that flanking sequences have direct effects on DNA recognition, as well as indirect, possibly allosteric effects on the transactivation by the AR [Haelens et al., 2003; Ham et al., 1988; Nelson et al., 1999]. This explains for instance the fact that even when in reporter constructs the C3(1) ARE and the SC ARE are active, transferring the two 5'-TGTTCT-3'-like hexamers of the SC ARE into the surrounding sequence of the C3(1) ARE resulted in

a very weak ARE, even when the *in vitro* affinity for the AR-DBD was unaffected [Haelens et al., 2007; Haelens et al., 2003].

From earlier DNA-cellulose competition assays, we learned that AR-binding DNA fragments are enriched in 5'-TGTTCT-3' motifs, but only a few of these were shown to act as AREs in functional assays [Claessens et al., 1990]. These AR binding fragments contain complex enhancers with monomer AR binding sites, but are also enriched for binding sites for NF1, Sp1, Oct1 and Ets-like transcription factors (reviewed in [Claessens et al., 2001]). Since then, transcription factor binding studies have benefited from the development of new techniques like chromatin immunoprecipitation (ChIP), ChIP-on-chip and ChIP-seq assays. An advantage of these techniques is that binding of a transcription factor to its target DNA sequences is studied in a cellular environment. ChIP-on-chip data revealed that the AR binds to genomic regions that contain DNA elements which are very similar to the classical GRE consensus, but also to fragments that contain AR-binding sequences that differ considerably from this consensus and might consist of simple 5'-TGTTCT-3'-like monomer binding sites [Bolton et al., 2007; Massie et al., 2007; Wang et al., 2007]. It has also been possible to subdivide the putative AREs from a ChIP-on-chip analysis into inverted repeat-like and direct repeat-like elements, but further work is needed to prove the functional importance of each of these sequences. The novelty of the ChIP-on-chip and ChIP-seq data resides in the fact that chromatin-embedded AR-binding sites are characterized, while earlier AREs were characterized by *in vitro* binding assays and transient transfection assays. It will be interesting to see how the AR interacts with these newly-described elements, and what the consequences are of DNA/chromatin binding on the functionality of the AR as a transcription factor.

The genomic fragments identified by ChIP-on-chip with antibodies against AR seem to contain complex enhancers with monomer AR binding sites, but are also enriched for binding sites for GATA-2, Oct1 and Ets1, thus enabling the unraveling of the hierarchical regulatory networks that govern androgen-dependent gene expression [Bolton et al., 2007; Massie et al., 2007; Wang et al., 2007]. These networks most likely will also involve indirect DNA recruitment of the AR by other factors like AP1, NFkB and GATA-factors, as well as direct effects of the AR on expression of other transcription factors [Bhardwaj et al., 2008; Heemers et al., 2006; Palvimo et al., 1996]. Most recently, Lupien et al. [Lupien et al., 2008] have shown that FoxA1 binding in the vicinity of AREs primes the chromatin for binding by other transcription factors like the AR and ER.

The DNA-binding domain

The DNA-binding domains of the nuclear receptors are approximately 80 amino acids long and are organized in two zinc fingers or modules in which zinc atoms are coordinating four cysteines (Figure 2B). An α -helix in the first zinc-coordinated module enters the major groove of the hexameric motif (Figure 2A). The P-box residues

within this helix that make the base-specific contacts are identical for AR, GR, MR and PR. The second zinc-coordinated module is involved in the DNA-dependent dimerisation via the so-called D-box residues (Figure 2B). These residues are also conserved between AR, GR, MR and PR [Zilliacus et al., 1995]. In analogy with the other steroid receptors, the DNA-binding domain of the AR binds as a dimer to the classical AREs ([Shaffer et al., 2004] and Figure 1A). This dimerisation fixes the position of the DNA-interacting residues of the P-box, thus explaining how an inverted repeat of 5'-TGTTCT-3'-like motifs separated by exactly three nucleotides is bound with high affinity.

The high evolutionary conservation of the DNA binding by the steroid receptor explains why the DBD backbone structures are superimposable. Why the selective AREs are recognized by AR and not by GR is still a matter of debate. On the one hand, biochemical analysis of the DNA-binding domains has shown the usage of an alternative dimerisation interface, distinct from the one described for receptor binding to classical GRE/AREs (reviewed in [Verrijdt et al., 2003]). On the other hand, in a crystal structure of the AR-DBD on a direct repeat of the 5'-TGTTCT-3', the two DBDs dimerise in a head-to-head conformation similar to what has been observed for GR, PR and ER [Luisi et al., 1991; Roemer et al., 2006; Schwabe et al., 1990; Shaffer et al., 2004]. Interestingly, the AR dimerisation surface, as seen in the crystal data, is enriched by an extended vanderWaals surface and three supplementary hydrogen bonds. However, swapping the residues involved between AR and GR-DBDs did not affect DNA binding specificity, thus contradicting the crystallization data [Verrijdt et al., 2006].

For other nuclear receptors, a dimerisation on direct repeat elements has been documented as well. For the VDR-DBD, an extension of the second zinc finger of one monomer contacts the DBD that binds to the immediately upstream hexamer [Shaffer and Gewirth, 2002]. Earlier biochemical analyses also indicated an important role of the carboxyl-terminal extension (CTE; Figure 2B) of the AR-DBD in the recognition of selAREs [Haelens et al., 2003; Schoenmakers et al., 1999; Schoenmakers et al., 2000]. Unfortunately, as for the GR- and ER-DBDs, the crystal data did not reveal the structure of this CTE. Only for the PR DBD was this fragment visible, and it showed non-specific interaction between an arginine residue in the PR-CTE and the minor groove 3' of the 5'-TGTTCT-3' hexamer [Roemer et al., 2006].

Overall, the AR seems to have the highest affinity for DNA, mainly due to a stronger dimerisation. While the GR and PR have weaker dimerisations, the PR is able to bind monomeric binding sites through an extended DNA-interaction surface [Roemer et al., 2006]. Whether a similarly extended DNA-interacting surface is also present for the AR- and GR-DBD is still unclear, although nucleotide substitutions 3' of AREs can have an effect on binding and transactivation in transient assays [Haelens et al., 2003; Ham et al., 1988].

To evaluate the physiological relevance of the selective AREs, we developed a transgenic mouse model, called SPARK1 for 'Specificity affecting AR Knock-In'. In these mice, the exon encoding the second zinc finger of the AR was replaced by the exon encoding the second zinc finger of the GR. The encoded mutant AR lost high affinity for selective AREs, while recognition of the classical AREs remained unaffected. In male SPARK1 mice, the expression of some of the Sertoli cell and prostate-specific androgen-regulated genes is differentially affected. For the genes for which androgen control in SPARK1 animals is primarily affected, we have been able to define selective AREs, while the less affected genes are controlled by classical AREs [Moehren et al., 2008; Schauwaers et al., 2007].

The phenotype of the SPARK1 mouse model demonstrates that the second zinc finger of the AR, and by extension the selAREs, are not involved in the androgen signaling in muscle and bone, nor does it change circulating FSH, LH and testosterone levels. The mutation severely affects the weight of organs and tissues of the reproductive system like testis, prostate, epididymis and seminal vesicles, while it does not affect the weight of the Levator ani, or the lean body mass or the bone mineral density of the animals. Therefore, the selAREs seem to have clear system-specific effects. Further analyses of the reproductive organs should lead to the further identification of genes regulated via selAREs.

The hinge region

DNA binding and ligand binding involve two distinct receptor domains, separated by a flexible linker, called the hinge region. This hinge region can be defined as the fragment between the last α -helix of the DNA-binding domain and the first α -helix of the ligand binding domain (from 623 to 671 for the human AR). After the cloning of the first steroid receptor cDNAs, it became apparent that the sequence of this hinge domain is poorly conserved, although for all steroid receptors it contains a nuclear localization signal (NLS) ([Evans, 1988; Zhou et al., 1994] and Figure 2B). The AR-NLS binds to importin α , and pathological mutations within the NLS that are associated with prostate cancer and androgen-insensitivity syndrome reduce the binding affinity [Cutress et al., 2008]. Aside from this nuclear translocation signal, several other roles for the hinge region in the control of AR activity are emerging.

Ser 650

The AR hinge contains a serine at position 650 that can be phosphorylated by MEKK-kinases and which seems to be involved in the regulation of receptor translocation [Gioeli et al., 2006; Kesler et al., 2007]. Indeed, a mutation of Serine 650 to Alanine reduced the nuclear export of the AR [Gioeli et al., 2006]. Recently, a germ line Serine to Glycine mutation has been found in a fertility patient with hypogonadism and scrotal hypoplasia [Zuccarello et al., 2008]. A detailed comparative *in vitro* analysis revealed a reduction of the activity of this mutant AR, but the effect on nuclear export has not been evaluated.

PEST sequence

The hinge region harbors a putative PEST sequence. In view of the recent implication of receptor degradation in transcription [Kang et al., 2002; Lin et al., 2002; Metivier et al., 2003], this would be an important function. However, deletion of this putative PEST sequence did not affect AR activity or its steady state levels [Haelens et al., 2007].

Arg 629 and Lys 630

Somatic mutations of arginine 629 and lysine 630 have been detected in prostate cancer biopsies, and these mutations positively affect cell colony formation in xenotransplants [Fu et al., 2003]. What the functions of these residues are and how these mutations could be correlated with prostate cancer development has been studied intensely. The lysine 630 has been reported to be an *in vitro* target for acetylation by CBP/p300, p/CAF and Tip60. These histone acetyltransferases also interact with the AR hinge region and can coactivate the AR [Fu et al., 2003; Fu et al., 2000; Gaughan et al., 2002]. However, the exact functional consequence of AR acetylation is difficult to determine because of the multiple roles of acetyltransferases in transcription and chromatin restructuring on the one hand, and because lysine 630 is part of the nuclear translocation signal on the other. Mutating the lysines in the nuclear localization signal affects the intracellular location of the AR, as well as its DNA binding, and has also been shown to increase or prevent specific coregulator interactions, receptor folding and receptor aggregation [Faus and Haendler, 2006; Haelens et al., 2007; Thomas et al., 2004].

DNA binding

Part of the AR hinge region is also involved in high affinity DNA binding (see above). While binding to classical AREs only involves the two zinc coordinating modules (ending at position 628 in the human AR), binding to selAREs involves a carboxyl-terminal extension (CTE) up to position 636. In addition, from mutation analysis of the hinge region, it becomes increasingly clear that there is no strict correlation between DNA-binding affinity and transactivation [Haelens et al., 2007; Tanner et al., 2004]. The deletion or mutation of the ⁶²⁹RKLKLG⁶³⁶ motif, which is part of the CTE (Figure 1A), results in an apparent loss of *in vitro* DNA binding, as well as an impairment of nuclear translocation, but the mutant AR has an increased activity in ARE-mediated transcriptional control. This apparent paradox certainly merits further investigations, since it might provide new therapeutic targets for prostate cancer treatment.

In summary, the hinge region was first described as merely a flexible linker between the DBD and the LBD, but now it is known to have an important input/output function: it is involved in nuclear import and export, DNA selectivity and affinity, and transactivation potential of the AR. The underlying mechanisms remain elusive, but several proteins have been reported to interact physically or functionally with the hinge region. The group of Jänne and Palvimo reported the interaction with Ubc9 and PIAS

(protein inhibitor of activated STAT) proteins [Poukka et al., 2000]. Both proteins are involved in the sumoylation of the AR-NTD (see below), as well as e.g. the SRC coactivator family. Other coactivator complexes that interact through the hinge region are the BAF57-containing SWI/SNF complex and the p300/PCAF complex [Garcia-Pedrero et al., 2006; Link et al., In Press; Link et al., 2005]. The small glutamine-rich tetrapeptide is a hsp70/hsp90 co-chaperone (SGT-1) that interacts with the AR hinge region. This protein acts on the androgen response outside the nucleus, keeping the AR in the cytoplasm and thus regulating its activity and its responsiveness to ligand [Buchanan et al., 2007]. Similar *in vitro* effects on ligand binding and intracellular trafficking of AR have been described for the other tetrapeptide repeat proteins, FKBP51 and FKBP52. However, knock-out experiments demonstrated an effect of FKBP51 ablation on some, but not all, androgen-regulated tissues, thus demonstrating the tissue-specificity of the action mechanisms of these TRPs [Yong et al., 2007].

Clearly, further structural data of the hinge region, as well as further elucidation of its exact interacting domains within the receptor itself or in coregulatory proteins will help to clear up the exact mechanisms affecting the activity of the androgen receptor.

The ligand-binding domain

The structure of the AR ligand-binding domain complexed with a variety of ligands has been solved. Very similar to what has been reported for other nuclear receptor LBDs, it is organized as a twelve α -helical sandwich with a central ligand-binding cavity [Matias et al., 2000; Sack et al., 2001]. Eighteen residues of helix 3, 5 and 11 of the AR LBD directly contact the bound ligand, but the many mutations described in androgen insensitivity syndrome patients, as well as prostate cancer biopsies, indicate that the integrity of the whole ligand-binding domain is necessary for correct ligand binding.

The general mechanism of nuclear receptor activation by the binding of ligand involves a repositioning of helix 12 in such a way that the ligand-binding pocket is closed, and a hydrophobic cleft is formed on the surface of the LBD [Moras and Gronemeyer, 1998]. This restructured part of the LBD surface is synonymous to the earlier described activation function-2 (AF-2) [Danielian et al., 1992] and serves as a docking site for coactivators. For all nuclear receptors, the hydrophobic cleft will be recognized by the nuclear receptor signature motif-bearing steroid receptor coactivators [Heery et al., 1997]. However, in a comparative study of a series of these LxxLL-motifs derived from the NR coactivators, only one interacted with high affinity with the isolated AR-LBD, illustrating a different sequence-specificity of the AR-LBD [Chang et al., 1999]. This might explain why the AR-AF-2 was so weak when tested in isolation [Jenster et al., 1995].

When analyzed in more detail, the AR-AF-2 cleft differs from that of other nuclear receptors in that it is able to

accommodate aromatic side chains, like those of the ²³FQNLF²⁷-motif in the AR-NTD [Dubbink et al., 2004; Hur et al., 2004]. Moreover, the hydrophobic cleft is surrounded by a charged clamp formed by Lysine 720 and Glutamate 897, which can make backbone contact with such FxxLF-motifs, but not with LxxLL-motifs.

The fact that canonical LxxLL-bearing coactivators have a low affinity for the isolated AR-LBD was contradicted by the strong AR coactivation by the SRC/p160 coactivators that include SRC-1, GRIP1/TIF2, and AIB1/ACTR/TRAM-1. An explanation came from experiments that demonstrated interaction and coactivation of the isolated AR-AF1 by the same SRC/p160s (see below).

The ligand-binding domain not only interacts with testosterone and dihydrotestosterone, but also with a number of steroidal and non-steroidal agonists and antagonists [Gao and Dalton, 2007]. Surprisingly, all agonist-bound structures are nearly superimposable, even when *in vivo* they have distinct effects. Obviously, the *in vivo* differences could be due to ligand metabolism or differential expression of AR-interacting proteins, or alternatively, the reported structural similarities reflect limitations of the crystallization experiments. Alternatively, a more detailed comparison might reveal some minor variations in structure with important allosteric effects on e.g. coactivator binding, like those documented for testosterone and dihydrotestosterone [Askew et al., 2007].

Recently, Estébanez-Perpiñá et al. [Estebanez-Perpina et al., 2007] postulated an alternative binding site for (ant-)agonists at the surface of the AR-LBD, different from the AF-2 forming hydrophobic cleft. How such compounds can affect AR activity is still unclear, but obviously such new features of the AR-LBD can become important targets for alternative and more selective (ant-)agonists.

The amino-terminal domain

The NTDs of the steroid receptors are clearly very divergent in length, amino acid composition, as well as the role they play in transcription transactivation processes. The AR in particular depends on multiple contributions from this NTD, even when the LBD provides the necessary hormone control switch.

In contrast to the DBDs and the LBDs of the nuclear receptors, no crystallographic or NMR-based structure has been obtained for the NTDs, or fragments of it. This is likely due to the lack of a tightly folded structure or the existence of alternative structures [McEwan et al., 2007]. In spite of this, several motifs and structures have been identified as binding sites for interacting partners or sites for posttranslational modifications. An overview of what is known on the AR-NTD is given in the following paragraphs and in Figure 1B.

Transcription activation unit-1

Since the activation potential of the AR-LBD when tested in isolation is weak (see above), the AR-NTD (called AF-1) is considered to be the major activation domain of

the AR. Jenster et al. [Jenster et al., 1995] defined the two transcription activation units, Tau-1 and Tau-5, within AR-AF1. The group of Rennie identified two other transactivation regions in the rat AR-NTD, designated AF-1a and AF-1b, respectively [Chamberlain et al., 1996]. While AF-1b does not seem to be conserved in the human AR, later studies have confirmed AF-1a as part of a functional motif within the boundaries of Tau-1. Different studies have ascribed functionality to motifs that overlap with AF1a. They were named the ¹⁷⁹LKDIL¹⁸³ motif [He et al., 2000], the ¹⁸³L/HX₇LL¹⁹² motif [Zhu et al., 2006] and the core Tau-1 between residues 177 and 203 ([Callewaert et al., 2006] and Figure 2).

The ¹⁷⁹LKDIL¹⁸³-motif was picked up during a search for LxxLL-like motifs that might be involved in the interactions between the AR-NTD and the AR-LBD. However, its affinity for the AR-LBD is very weak and most likely not significant [Alen et al., 1999; Steketee et al., 2002]. By contrast, another LxxLL-like motif is necessary and sufficient to explain this interaction (²³FQNLF²⁷, see below).

Mutation analysis of putative α -helices in Tau-1 resulted in the description of the so-called core Tau-1 between amino acids 173 and 203 in the human AR. Both the hydrophobic side chains and the negative charges of this amphipathic helix are important for the transactivating capacity of the AR. It is also a strong autonomous activation function, for which the coactivator partners remain to be discovered [Callewaert et al., 2006].

The ¹⁸³L/HX₇LL¹⁹² motif has been proposed to serve as a binding site for Tab2, a component of a NCoR repressor complex [Zhu et al., 2006]. Binding of antagonists to the AR triggers the recruitment of the Tab2/NCoR complex and leads to the repression of AR-regulated genes. However, in the presence of IL-1 β , Tab2 is phosphorylated by the MEK1 pathway, which causes the dissociation of the NCoR complex. In this way, the equilibrium between coactivator binding and corepressor binding to the AR is shifted so that the transcriptional outcome can be positive, even when the AR-LBD is bound by antagonist. This suggests a possible mechanism for antagonists gaining characteristics of agonists, which is also observed in prostate cancers after relapse of patients treated with these antagonists. Of course, other mechanisms that might explain such relapses are mutations in the AR that change its ligand-specificity [Taplin and Balk, 2004], overexpression of coactivators or loss of corepressors [Bevan, 2005] or changes in the androgen effects on cell cycle progression [Balk and Knudsen, 2008].

The region around core Tau-1 was also shown to acquire a more folded conformation when interacting with fragments of the SRC coactivator family or of the RAP47 subunit of the general transcription factor TFIIF [Betney and McEwan, 2003; McEwan and Gustafsson, 1997; McEwan et al., 2007]. It is proposed that this induced structure is the active conformation of AR-AF1, because mutations that disrupt the α -helical structure lead to a

decrease in AR-activity and also impair the interaction with RAP47.

Another motif that does not overlap with, but is very close to AF1a, is the ²³⁴AKELCKAVSVSMGL²⁴⁷ motif immediately carboxyl-terminal of core Tau-1. This sequence is highly conserved in the AR from mammals to fish and is the interaction site for the Hsp70-interacting protein E3 ligase CHIP [He et al., 2004a], the overexpression of which downregulates the steady state levels of AR.

Interestingly, mutations in both core Tau-1 and the CHIP-interacting motif have been described in prostate cancer biopsies. The mutation K179R has been described in a primary prostate cancer biopsy, and the A197G mutation was described in a prostate cancer sample of a patient treated with bicalutamide [Tilley et al., 1996]. Moreover, two mutations in the CHIP-interacting motif, A234T and E236G, were detected in tumors that arose in the TRAMP model of prostate cancer [Han et al., 2001]. When tested *in vitro*, all four mutations gave rise to a more active AR [Callewaert et al., 2006; Han et al., 2001], and the E236G mutation induces metastatic prostate cancer very efficiently when introduced in a transgenic model [Han et al., 2005].

Transcription activation unit-5

The size and locations of the activation functions in use by the AR seem to vary depending on the presence of the AR-LBD or the AR-DBD, as well as on the nature of the reporter genes tested [Alen et al., 1999; Callewaert et al., 2006; Jenster et al., 1995]. Tau-5 is the fragment of the AR-AF-1 described by Jenster et al. [Jenster et al., 1995] that retains the activation potential in the absence of the LBD, while Tau-1 was more dependent on the presence of the LBD. Tau-5 covers residues from positions 360 to 528. It is an autonomous activation domain, which is targeted by several proteins. Most importantly, it strongly interacts with a glutamine-rich domain of the SRC/p160 coactivators [Alen et al., 1999; Bevan et al., 1999; Irvine et al., 2000]. Since the affinity of the LxxLL motifs of the SRC/p160s for the AR-AF-2 is low, the Tau-5 region is considered to be the major interaction site for the SRC/p160 coactivators [Bevan et al., 1999; Christiaens et al., 2002].

Tau-5 has also been defined as the target for the RhoA effector protein kinase C-related kinase PRK1 [Metzger et al., 2003]. Stimulation of the PRK1 signaling cascade results in a ligand-dependent superactivation of the AR, which might be the result of an enhanced association with the SRC/p160s.

The integrity of the complete Tau-5 is required for its optimal autonomous activation function, since any deletion affects its transactivation properties and its interaction with the SRC/p160s [Callewaert et al., 2006]. Possibly, Tau-5 is a globular domain rather than a molten globule or an extended domain containing one or more motifs, like what has been described for the AF-1 fragment, which

partly overlaps with Tau-1 and Tau-5 [McEwan et al., 2007].

The only motif that is described thus far in Tau-5 is the ⁴³³WHTLF⁴³⁷ motif that was first proposed as an interaction site for the liganded AR-LBD, again because of its resemblance to the LxxLL-motif [Alen et al., 1999; He et al., 2000]. While this motif is not involved in these N/C interactions, it plays a role in the ligand-independent actions of AR in refractory prostate cancer. Its function in ligand-dependent AR actions seems limited, however, although the AR fragment from 426 to 446 can act as an autonomous activation domain [Dehm and Tindall, 2007].

Several steroid receptors, as well as coactivators and histones, have been reported to be conjugated with SUMO-1. SUMO-ylation of the AR, GR and MR is probably linked with repression of transactivation potential, but the effect appears to be promoter- or enhancer-specific (reviewed in [Faus and Haendler, 2006] and [Leader et al., 2006]). The AR-NTD has two SUMO-1 modification sites in Tau-5 at positions 385 and 511, respectively. AR SUMO-ylation is responsive to agonists, but is not induced by the pure antagonist hydroxyflutamide. Mutation of K385 clearly affects the cooperativity of the receptor on complex hormone response elements [Callewaert et al., 2004; Poukka et al., 2000], similar to what has been shown for other transcription factors [Iniguez-Lluhi and Pearce, 2000], but how the SUMO-ylation at K385 determines the magnitude of the receptor-dependent transcription responses remains obscure.

Interplay between Tau-1 and Tau-5

Both Tau-1 (as described above) and Tau-5 are necessary and sufficient for the intrinsic activity of the AR-NTD and the full activity of the AR [Callewaert et al., 2006]. Indeed, when Tau-1 and Tau-5 mutations are introduced in the full length AR, it becomes completely inactive.

Interestingly, some mutations in Tau-1 affect the recruitment of the SRC/p160 coactivators through Tau-5. Since there is no direct interaction of core Tau-1 with SRC1, and since there is no evidence for direct interactions between Tau-1 and Tau-5, this effect is likely to be indirect, e.g. via induction of a conformational change, or the recruitment of (a) secondary interaction partner(s).

The polymorphic glutamine and glycine repeats of the AR-NTD

The polymorphic CAG repeat in the AR gene encodes a poly-glutamine-stretch (starts at position 57) that can vary in length. The length ranges from 9-36 residues, with a highest frequency of approximately 20. Epidemiological studies have reported weak correlations between the CAG repeat number and the risk of developing prostate cancer, but the evidence for the implication of an extension of the poly-glutamine tract in the etiology of Kennedy's disease is undeniable [Casella et al., 2001; Ferro et al., 2002; La Spada et al., 1991]. The CAG repeat

is also associated with the age of onset and the aggressiveness of this disease. In a mouse model with humanized AR-NTD, an extended glutamine tract resulted in myopathic features similar to those known for Kennedy's disease, which suggests a role for muscle in the non-cell autonomous toxicity of motor neurons [Yu et al., 2006]. This pathologic effect seems unrelated to the general function of the AR as a transcription factor, but rather may be the consequence of a gain-of-function of the mutated receptor.

Mononen et al. [Mononen et al., 2002] described a correlation between CAG repeat length and prostate cancer risk. On the other hand, Sircar et al. [Sircar et al., 2007] described a shortening of the CAG repeats in genomic DNA derived from prostate cancer lesions. This is an important finding, correlating an enhanced AR activity to prostate cancer development. Indeed, earlier *in vitro* studies showed that ARs with shorter or no glutamine stretch are more potent transcription factors. An enhanced interaction of the AR-NTD with the SRC/p160s has been proposed as a possible explanation [Buchanan et al., 2004; Callewaert et al., 2003a; Chamberlain et al., 1994; Irvine et al., 2000]. Again, depending on the reporter gene tested, this effect can be more or less significant.

Besides the glutamine stretch, a glycine stretch (starting at position 449, Figure 1B) within Tau-5 varies in length, ranging from 10 to 30 residues. Its length variations might also be correlated weakly with the incidence of diseases. There are several studies which indicate that a short GGN repeat may be a risk factor for the development of prostate cancer [Ding et al., 2005]. Recently, a combination of a short glycine stretch with a long glutamine stretch, combined with an A645D substitution in the hinge region, has been postulated to contribute to the development of a case of androgen insensitivity [Werner et al., 2006].

The ²³FQNL²⁷-motif and the N/C interactions

Subsequent to the observation that there was a strong N/C interaction between the NTD and the LBD of the AR, several candidate motifs were tested for their possible involvement in this process. The strongest LBD-interacting motif clearly is the ²³FQNL²⁷ motif at position 23, which is highly conserved among the AR of different species [Alen et al., 1999; Berrevoets et al., 1998; He et al., 2004b; Langley et al., 1998; Steketee et al., 2002].

Deletion or mutation of the ²³FQNL²⁷ motif and flanking residues abrogates the N/C interaction, affect transactivation by the AR in transient transfections and change the kinetics of ligand-binding [He et al., 2004b]. Surprisingly, the functional importance of the N/C interactions seems to be dependent on the nature of the enhancer [Callewaert et al., 2003b]. ²³FQNL²⁷-like signature motifs have also been described in some of the AR-interacting proteins such as ARA54 and ARA70 [He et al., 2002; Heinlein and Chang, 2002; van de Wijngaert et al., 2006]. The hormone-dependent interaction of the FxxLF motif-containing ARA70 and ARA54 with the AR

LBD has been demonstrated *in vitro* and *in vivo*, but their role in transcription remains controversial [Alen et al., 1999; Brooke et al., 2008; Toumazou et al., 2007; van Royen et al., 2007].

Several lines of evidence also point to the possibility of indirect N/C interactions mediated by the SRC/p160 steroid receptor coactivators [Berrevoets et al., 1998; Ikonen et al., 1997; Shen et al., 2005]. Similar to what was described for the estrogen receptor- α [Webb et al., 1998], the LBD of the AR has some affinity for LxxLL-like motifs, while the NTD is bound by a glutamine-rich region of the SRC/p160s, suggesting that the p160s act as bridging factors [Ma et al., 1999]. Conversely, some of the AR corepressors seem to inhibit the N/C interactions in the AR, but the exact implication of this on AR action remains unclear [Burd et al., 2005; Liao et al., 2003].

In living cells, the N/C interaction is induced by ligand in the cytoplasm [van Royen et al., 2007]. Once in the nucleus, the AR distributes between a fast moving fraction and fractions retained in speckles. Interestingly, no N/C interaction is detectable in these speckles [van Royen et al., 2007]. Since at least part of the speckles are active in transcription, this indicates that the AR does not have a closed conformation the whole time it occupies the enhancers in the chromatin. By contrast, Klock et al. [Klock et al., 2007] demonstrated intramolecular N/C interactions when the AR is bound to an array of the MMTV promoter in living cells. Also, Wong and co-workers showed that N/C interactions are necessary for full AR activity on chromatinized templates. [Li et al., 2006].

In conclusion, the N/C interactions of the AR, as well as the interactions of the NTD and LBD with their coregulators, seem much more dynamic than originally thought. Most likely, the interactions change during one or more of the steps in the transcription activation cycles, as described by the group of Gannon [Metivier et al., 2003]. When the N/C clamp opens, alternative surfaces on the LBD, as well as the NTD, are expected to become available for the interaction with proteins involved in receptor turnover, nuclear translocation, nuclear mobility, chromatin-modification, transcriptional regulation, etc.

General conclusions

The androgen receptor is a member of the nuclear receptor family. Compared to most other members, the AR has many similarities, but also many differences in its mode of action. Most striking, is the absence of a strong activation function in the ligand binding domain, but this is compensated for by the presence of strong activation functions in the NTD, one of which recruits the same SRC/p160 coactivators as the canonical NR-AF-2.

The current challenges surrounding research on NR action in general, and AR action in particular, are many. Indeed, the action of the AR seems to depend on the interplay between many motifs and domains that all seem to have more than one specific function. For instance, at least *in vitro*, the AR can dimerise through interactions

between DBDs, between LBDs, and through N/C interactions. However, at present it is still unclear whether the N/C interactions happen intra- or intermolecularly, or both. What signals drive the dimerisations and the opening and closing of the N/C interactions of the AR, and when the different alternative interactions take place, remain largely unexplored. As a consequence, knowledge on the spatio-temporal control of the communications of the AR with other coactivators and transcription factors, and their chronological sequence in the transcriptional control by androgens in a cellular environment, is at its infancy. Furthermore, these studies of biochemical and cellular AR behavior will need to be substantiated further by *in vivo* models, like the tissue-specific knock-out models of AR or its coactivators, or specific knock-in models resulting in deletions or mutations of specific AR motifs or domains (cf. SPARK1 model). Ultimately, these should help to establish links between diverse observations, such as the role of FoxA1 in interpretation of the histone code [Lupien et al., 2008], the spatio-temporal control of enhancer binding by NRs in the nucleus [Nunez et al., 2008], and the intranuclear behavior of AR and AREs *in vivo* [Klokk et al., 2007; van Royen et al., 2007].

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