Molecular Mechanisms of Androgen Receptor Functions

Moleculaire mechanismen van androgeenreceptor functies

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In dankbare herinnering aan mijn vader
Voor mijn moeder
Voor Jeroen

Contents

List of abbreviations

aa	amino acids
AD	activation domains
AF-1	transcription activation function 1
AF-2	transcription activation function 2
AIS	androgen insensitivity syndrome
ARA	AR-associated protein
ARE	androgen response element
AR	androgen receptor
ARIP4	AR interaction protein 4
BCA	bicalutamide
BPH	benign prostatic hyperplasia
BRG	Brahma related gene
BAF	BRG1-associated factor
BRM	Brahma
CARM-1	cofactor associated arginine (R) methyltransferase 1
CBP	CREB binding protein
CDK	cyclin dependent kinase
CFP	cyan fluorescent protein
ChIP	chromatin immuno precipitation
CHIP	carboxy terminus of the Hsp70-interacting protein
CoRNRbox	corepressor NR interaction motif
CPA	cyproterone acetate
CTE	carboxyl-terminal extension
DHEA	dehydroepiandrosterone
DBD	DNA-binding domain
DHT	dihydrotestosterone
DRIP	vitamin D receptor–interacting protein
E6-AP	E6 associated protein
EcR	ecdysone receptor
EGF	epidermal growth factor
EMSA	electrophoretic mobility shift assay
ERα	estrogen receptor α
ERβ	estrogen receptor β
ERR	estrogen related receptor
FRAP	fluorescence recovery after photobleaching
FRET	fluorescence resonance emission transfer
GPCR	G protein-coupled receptor
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GRIP-1	glucocorticoid receptor interacting protein-1
HAT	histone acetyltransferase
HDAC	histone deacetylase activity
HER-2	human epidermal growth factor receptor-2
HMT	histone methyltransferase
HRE	hormone response element
IGF-1	insulin-like growth factor-1
IL-6	interleukin-6
ISWI	imitation switch
LBD	ligand-binding domain
LcoR	ligand-dependent corepressor
LNCaP	lymph node derived prostate cancer cell line
MAGE-11	melanoma antigen gene protein 11
MAPK	mitogen-activated protein kinase
NURD	nucleosome remodeling and histone deacetylation

MR	mineralocorticoid receptor
N/C interaction	interaction between the NTD and LBD of an NR
NCoR	NR corepressor
NES	nuclear export signal
NLS	nuclear localization signal
NR	nuclear receptor
NTD	amino-terminal domain
NURSA	Nuclear Receptor Signaling Atlas
OH-Fl	hydroxy-flutamide
PPARα	peroxisome proliferator activated receptor α
PPARβ	peroxisome proliferator activated receptor β
PPARγ	peroxisome proliferator activated receptor γ
PPARδ	peroxisome proliferator activated receptor δ
PAP	prostatic acid phosphatase
p/CAF	p300/CBP-associated factor
PCSC	prostate cancer stem cells
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PRMT1	protein arginine methyltransferase 1
PR	progesterone receptor
PSA	prostate specific antigen
P-TEFb	positive transcription elongation factor b
RARα	retinoic acid receptor α
RARβ	retinoic acid receptor β
RARγ	retinoic acid receptor γ
RD	repression domain
RID	receptor interacting domain
RIP 140	receptor interacting protein 140
RNApolII	RNA polymerase II
RU486	mifepristone
RXRα	retinoic X receptor α
RXRβ	retinoic X receptor β
SARG	specifically androgen regulated gene
Sc	Secretory component
Slp	sex-limited protein
SBMA	spinal and bulbar muscular atrophy
SMRT	silencing mediator for retinoid and thyroid hormone receptor
SRC	steroid receptor coactivator
SRG3	SWI3-related gene product
STAT	signal transducers and activators of transcription
SUMO-1	small ubiquitin-like molecule-1
SWI/SNF	switch/sucrose non-fermenting
T	testosterone
TA	transit amplifying
TAU1	transcription activation unit 1
TBP	TATA binding protein
TFIIF	transcription factor II F
TFIIH	transcription factor II H
TIF2	transcription intermediary factor 2
TRα	thyroid hormone receptor α
TRβ	thyroid hormone receptor β
TRAP	thyroid receptor-associated protein
VDR	vitamin D receptor
YFP	yellow fluorescent protein

Chapter 1

General introduction

INTRODUCTION

The main physiological functions of the androgens testosterone (T) and dihydrotestosterone (DHT) involve the development and maintenance of the male phenotype. T is mainly produced in the testis and converted to DHT in several target organs, including the prostate. Androgens have a hydrophobic cholesterol-derived steroid structure, which allows free traveling between the extra- and intracellular space. The intracellular target of androgens is the androgen receptor (AR). Upon androgen binding, the AR translocates to the nucleus and regulates transcription of androgen target genes.

1.1 NUCLEAR RECEPTORS

The AR belongs to the group of steroid receptors, which is a subgroup of the nuclear receptor (NR) family of transcription factors. In vertebrates and invertebrates, more than 150 members of the NR superfamily have been identified. During the last decades, this family of physiologically highly important proteins has been subject of study by many research groups, resulting in an overwhelming number of publications. In this section a comprehensive overview of general NR functioning is given, based on excellent reviews published on this subject [1-11]. Where appropriate, references to key publications are included in the text. In section 1.2, more detailed information of the AR is presented.

1.1.1 General features of nuclear receptors

Based on phylogenetic studies, the NR family can be subdivided into six different subtypes [12]. The group of type I receptors includes the thyroid hormone receptors (TRα and TRβ), the vitamin D receptor (VDR), the retinoic acid receptors (RARα, RARβ and RARγ), the peroxisome proliferator activated receptors (PPARα, PPARβ and PPARγ), and the *Drosophila* ecdysone receptor (EcR). Members of the type II receptor group are the retinoic X receptors (RXRα and RXRβ) and orphan receptors like COUP-TF, Rev-Erb, TR2, and TR4, for which the ligand has not been identified as yet, or which can function without stimulation by a ligand [13]. The type III receptors comprise the group of steroid receptors, which includes the AR, the glucocorticoid receptor (GR), the mineralocorticoid receptor (MR), the progesterone receptor (PR), and the estrogen receptors (ERα and ERβ). The closely related orphan receptors estrogen related receptors (ERRs) also belong to the type III receptors. The NR subtypes IV, V and VI all include various orphan receptors.

In general, NRs are ligand-activated transcription factors. Upon ligand binding, NRs undergo conformational changes that result in a cascade of events leading to DNA binding and transcription activation or repression of specific target genes. The liganded, and in some cases the unliganded, receptor is able to bind as a homo- or heterodimer to specific sites in the DNA and subsequently recruits coactivators or corepressors, general transcription factors and RNA polymerase II (RNApolII), to ultimately regulate transcription of target genes. Protein-protein interactions play a prominent role in these processes [14-19]. A simplified scheme of these events is depicted in Figure 1A.

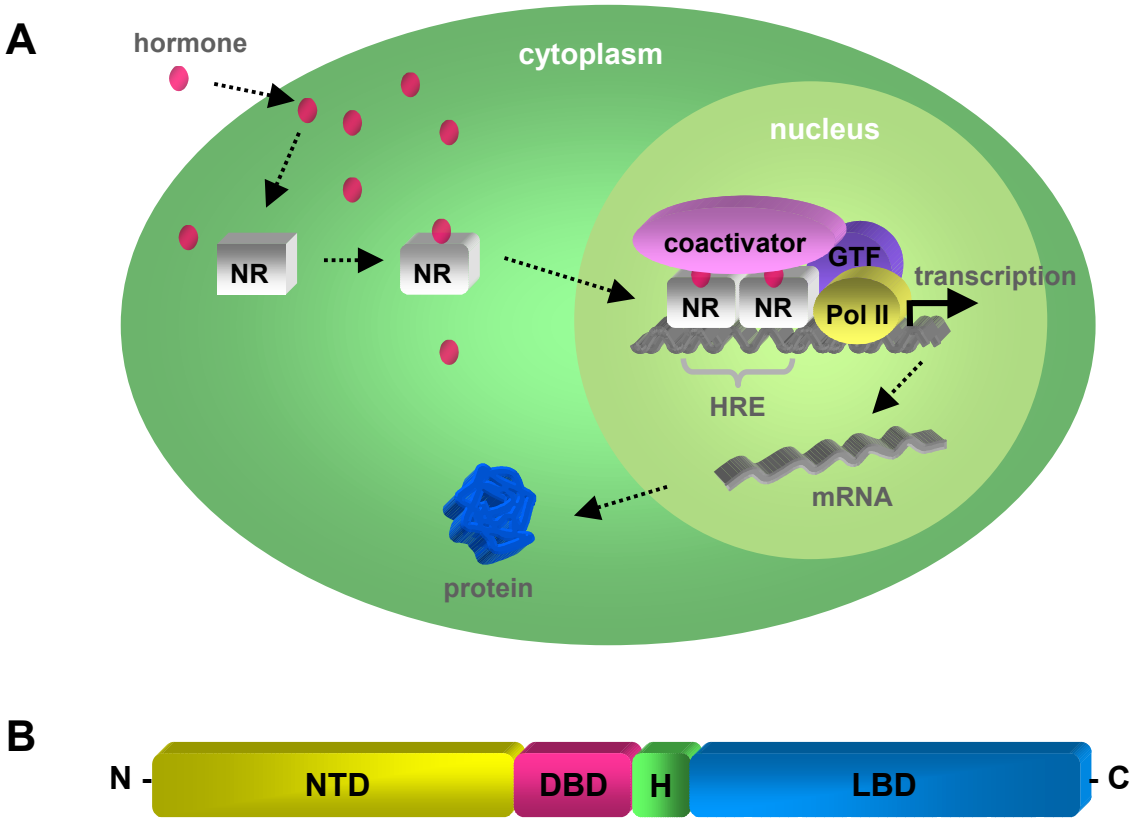

Figure 1. **A**) Schematical overview of intracellular NR functioning. GTF = general transcription factors, PolII = RNA polymerase II, HRE = hormone response element. **B**) Schematical representation of NR functional domains. NTD = N-terminal domain, DBD = DNA binding domain, H = hinge region, LBD = ligand binding domain.

1.1.2 Structure and functional domains of nuclear receptors

NRs have a modular structure composed of a non-conserved amino-terminal domain (NTD), a highly conserved central DNA-binding domain (DBD), a moderately conserved carboxyl-terminal ligand-binding domain (LBD), and a flexible hinge region located between the DBD and the LBD (Figure 1B).

1.1.2.1 The amino-terminal domain

The NTD is the most variable domain both in size and amino acid composition. Until now, for none of the NRs the three-dimensional structure of this domain has been resolved. It is generally assumed that the NTD has an unorganized, flexible structure, which hampers crystallization. Small regions in the NTD might adopt a more structured conformation upon protein-protein interactions in transcription initiating complexes. However, structures of such complexes have not been elucidated as yet.

For most NTDs, a trans-activation function, AF-1, has been established. The AF-1 domains in the different NRs do not show structural homology and are mapped at quite distinct positions in the NTD. AF-1 is promoter and cell dependent, which indicates interactions of the NTD with promoter and cell specific proteins involved in transcriptional regulation [14]. An increasing number of NTD interacting proteins has been identified. The NTD, if bound to a DBD, can autonomously activate expression of target genes, as proven by the constitutive activity of truncated NRs lacking their LBD. The LBD functions as an activation lock, which is opened by binding of its cognate ligand.

1.1.2.2 The DNA binding domain

Ligand-activated NRs bind through their highly conserved DBDs to hormone response elements (HREs) in the regulatory regions of target genes. HREs have specific consensus sequences for high affinity binding to the different NR subgroups, and are composed of an inverted or direct imperfect repeat of two 6 bp half-sites separated by a spacer of variable length. NRs bind to HREs as homo- or heterodimers, each receptor to one HRE half-site [20, 21].

The DBD is composed of two zinc clusters and a carboxyl-terminal extension (CTE). The N-terminal zinc cluster of steroid receptors contains three amino acids, denoted as the P-box, which determine DNA binding specifity [22, 23]. The C-terminal zinc cluster contains a dimerization interface, the D-box (Figure 2A). For several NRs, crystallographic analyses have revealed the DNA-bound DBD dimer structures with detailed data on protein-protein and protein-DNA interaction sites (reviewed in ref. 24). Adjacent to the C-terminal part of each zinc cluster a short α-helical structure can be recognized. The helix flanking the N-terminal zinc cluster enters the major groove in the DNA. This conserved recognition α-helix enables the amino acid residues of the P-box to bind to receptor specificity determining sites in the HREs. Two phenylalanine residues in this helix are also involved in nuclear export of NRs in the presence of ligand [25, 26]. The helix flanking the C-terminal zinc cluster, the CTE, is positioned perpendicular to the other helix, in this way exposing the D-box, facilitating dimerization (Figure 2B) [20].

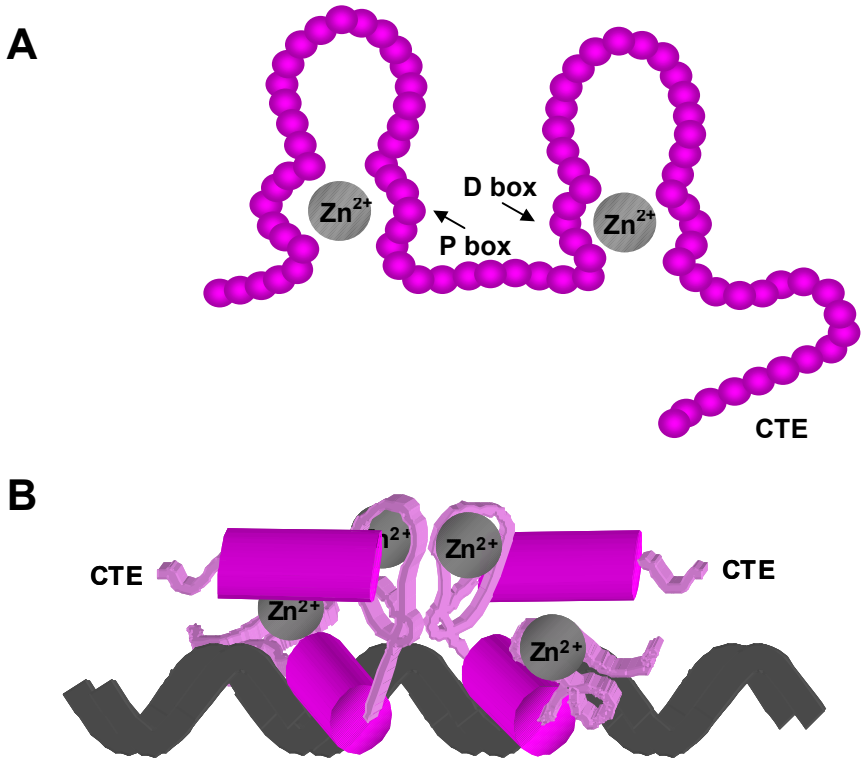

Figure 2. A) Schematic representation of the NR DBD structure. CTE = carboxy-terminal extension. B) Tertiary structure of a DNA bound NR DBD dimer.

1.1.2.3 The hinge region

The hinge region forms a flexible link between the DBD and the LBD, allowing easy rotation of the latter domain. The amino acid sequence of the hinge region is not conserved among NRs. In many NRs, the hinge region contains a bipartite nuclear localization signal (NLS) essential for nuclear import of the liganded receptor [27].

1.1.2.4 The ligand binding domain

Small variations excluded, NR LBDs have a canonical structure consisting of 12 α-helices, folded in a three-layered anti-parallel sandwich conformation [28, 29]. The ligand-binding pocket is lined by many amino acid residues throughout the LBD and varies with the structure of the ligands of the respective receptors. Upon agonist binding, helix 12 is repositioned like a lid over the ligand binding pocket in a manner favoring NR activation. This is illustrated for the RXRα and RARγ in Figure 3. Binding of antagonists induces a different LBD conformation leading to an unfavorable positioning of helix 12, thereby causing blocking of receptor function [30-33].

In addition to ligand binding, the LBD might be involved in homo- or heterodimerization. It also interacts with chaperone proteins like heat shock proteins, and harbors the ligand-dependent transcription activation function 2 (AF-2).

The AF-2 structure is conserved among many NRs. A core region in the AF-2 domain, which is in helix 12, appears essential for ligand-dependent transcriptional activity [34-38]. A general mechanism has been proposed, in which the AF-2 core plays a central role in the generation of an interaction surface, allowing binding of NR coactivators to the LBD [31]. Coactivators can modulate the transcriptional activity of a broad range of NRs (see section 1.1.5). A large number of coactivators that bind to NRs has been described. The interaction with NRs is not limited to the LBDs, but also involves binding to the NTDs, resulting in cooperation or even synergy between AF-1 and AF-2 activities [39-42].

Unliganded or antagonist bound receptors can bind to corepressors by an interaction interface in the LBD that overlaps the coactivator binding site [43]. Recruitment of corepressors inhibits receptor functioning leading to silencing of gene transcription [44].

In addition to the motif found in the DBD, for the MR, ER and AR, a nuclear export signal (NES) has been identified in the LBD. This second NES is, in contrast to the DBD NES, active in the absence of ligand and inhibited when ligand is present [45].

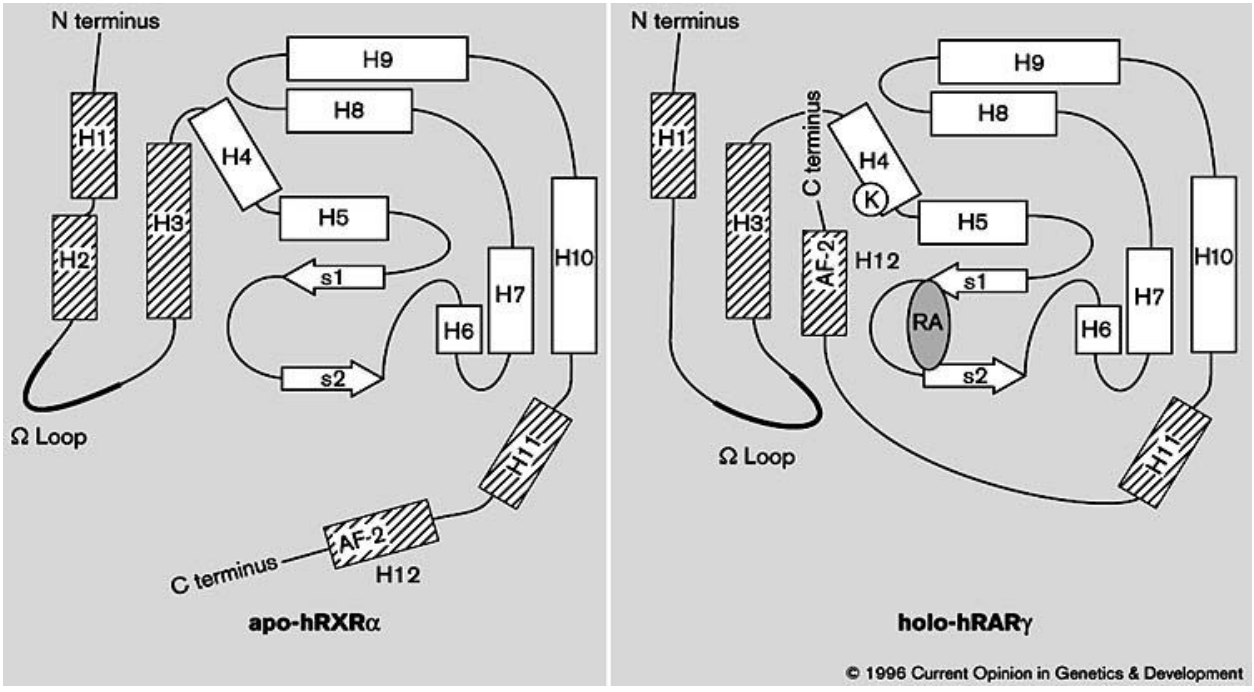

Figure 3. Two-dimensional representation of an NR LBD structure. Left: RXRα LBD without retinoic acid (RA). Right: RARγ LBD bound by RA. From ref. 29.

1.1.3 The nuclear receptor subfamily of steroid receptors

Through binding to their respective receptors, steroid hormones mediate biological processes like cell growth, cell differentiation, and homeostasis. The GR regulates storage, mobilization and metabolism of carbohydrates and fatty acids. The MR plays an important role in maintaining electrolyte levels within a narrow range. ERα, ERβ, and PR mediate development and maintenance of the female phenotype, but also play a role in males. The AR is responsible for the male phenotype, but is also important for female development.

The structures of steroid receptors are highly conserved. The highest percentage homology is present in the DBDs and LBDs. GR, PR, and MR DBDs show up to 80 % homology with the AR DBD. This conservation level is reflected in the HRE preferences of the steroid receptors. GR, MR, PR and AR homodimers all bind with high affinity to a consensus HRE, the inverted repeat AGAACAnnnTGTTCT, generally denoted as glucocorticoid response element (GRE) [46-49]. ERα and ERβ are quite distinct from the other steroid receptors. Their structures deviate more from these receptors and the high-affinity DNA binding site is an inverted repeat of the consensus halfsite AG$^{G}/_{T}$TCA. Most type I and II NRs also bind to this sequence, which might be present as a single half-site or in a direct or inverted tandem repeat orientation in the regulatory sequences of target genes [16, 50]. Because GR, MR, PR, and AR recognize the same high affinity GRE, many genes can be regulated by more than one steroid receptor. However, the physiological function of the different receptors is quite distinct, which can only partly be explained by their tissue specific expression pattern. So, additional mechanisms are required for a hormone specific response in case multiple receptors are simultaneously present in one and the same cell. Several mutually not exclusive mechanisms to explain steroid specific transcriptional regulation include selective interactions of receptors with specific and general transcription factors and coregulators, coregulator levels, ligand availability, local chromatin structure at the regulatory sites of the receptor target genes and, as recently described for the AR, steroid receptor specific HREs (see 1.2.6 and Chapter 5) [4, 7, 51-56].

The AF-1 functions of the steroid receptors have roughly been mapped. For each receptor a region in the NTD has been defined, which is required for optimal transcriptional activity. Within these regions, a core region that is essential for AF-1 activity has been established. The AF-1 regions in different receptors are not identical and map at different parts of the NTD. Two PR isoforms are known, PR-A and PR-B. PR-B has an amino-terminal extension harboring an AF-3 function that enhances AF-1 activity [57]. Like in other NR subgroups, the extent to which the separate AF-1 and AF-2 activities contribute to steroid receptor activity varies among the different receptors. In addition, cell and promoter context determine the relative activities of AF-1 and AF-2. Furthermore, AF-1 and AF-2 activities can act synergistically, as was found for ERα, ERβ, GR, PR and AR [39-42].

Unlike other NRs, in the unliganded inactive state steroid receptors are bound by heat shock protein complexes. These complexes bind preferentially to the LBDs and are supposed to be involved in proper LBD folding, intracellular transport, nuclear import and protection against degradation. Most studies indicate that heat shock protein complexes dissociate from the steroid receptor upon ligand binding, thereby enabling the receptor to dimerize, bind to DNA and activate transcription of its target genes [58].

1.1.4 Nuclear receptors in transcription regulatory complexes

To regulate transcription, the activity of NRs is directly and indirectly modulated by many other proteins, which can be components of large protein complexes. These complexes function in a spatiotemporal sequence, of which the dynamics are not yet completely understood. A model of protein complexes, and protein-DNA and protein-protein interactions involved in transcriptional gene regulation by NRs is depicted in Figure 4. It is based on many studies utilizing a variety of experimental approaches, including electrophoretic mobility shift assays (EMSA), two-hybrid assays, reporter gene assays, chromatin immuno precipitation (ChIP), fluorescence resonance emission transfer (FRET), and real-time movement measuring in living cells by fluorescence recovery after photobleaching (FRAP). The model includes binding of nuclear receptor, coregulators, chromatin remodeling complexes, and Mediator complexes, ultimately leading to activation or inhibition of transcription initiation.

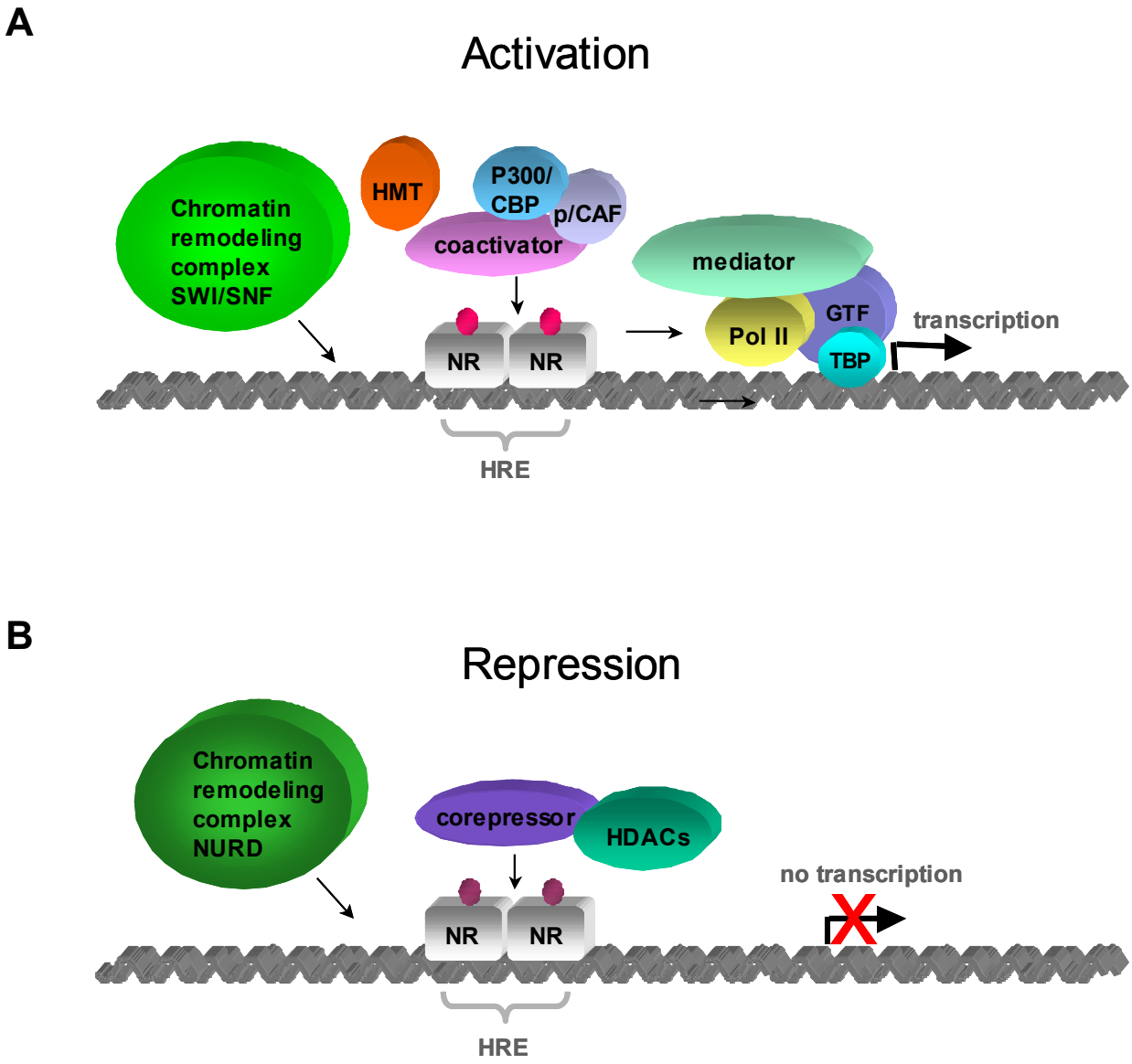

Figure 4. Schematic model of transcriptional regulation by NRs. A) Transcriptional activation, ligand (agonist) is in red. B) Transcriptional repression, ligand (antagonist) is in dark red. For further description see text of this section (1.1.4).

In the model ligand-bound NRs bind as a dimer to their cognate response elements present in target genes. It is known that some NRs have direct access to DNA, which might be dependent on nucleosomal phasing of their cognate recognition sites [59]. Interaction of NRs with DNA might be very transient with tenths of seconds to seconds per cycle [60-62]. For GR and ERα it has been shown that they can be removed from their DNA binding sites by proteasomes. This would fasten cycling and thereby allow the continuous response to fluctuations in hormone levels [63,64]. The chaperone protein hsp90 might stabilize GR binding by inhibiting removal from its response element [63]. Unlike FRAP experiments, ChIP experiments have shown that transcription factors and coregulators can be recruited to promoter/enhancer sequences within minutes [64]. It is possible that proteins present in transcription initiation complexes individually and rapidly exchange multiple times within a binding cycle observed with ChIP.

Binding of a NR dimer to DNA is followed by recruitment of coregulators, which might be coactivators or corepressors. The best known NR coactivators are the p160 proteins, which are described in more detail in section 1.1.5 [60].

Coactivators, in turn, recruit factors with strong histone acetyltransferase (HAT) activity, like the cointegrators CREB binding protein (CBP)/p300 and p300/CBP-associated factor (p/CAF). Some p160 coactivators also have weak intrinsic HAT activity [65, 66]. Subsequently, histones on target genes are acetylated on lysine residues. This decreases histone affinity for DNA, which results in an opened chromatin structure allowing ongoing recruitment of all required protein complexes favoring gene transcription [67-69]. Acetylation also disrupts internucleosomal contacts, thereby disturbing higher order chromatin structures [70]. In case of transcription inhibition, a NR corepressor is recruited, which in turn recruits proteins with histone deacetylase activity (HDACs) through which histones will be deacetylated resulting in tightening of chromatin conformation and thereby preventing transcriptional activation [44, 71-73]. Examples of NR corepressors are described in more detail in section 1.1.6.

Nucleosome disruption and thereby activation of gene transcription is also conferred by the protein arginine methyltransferases: protein arginine methyltransferase 1 (PRMT1) and cofactor associated arginine (R) methyltransferase 1 (CARM-1/PRMT4), which both can methylate histones. These histone methyltransferases (HMTs) are recruited by p160 coactivators and p300 [74-77].

The chromatin structure on transcribed genes is not only modified by HATs and methyltransferases, but also by ATP dependent chromatin remodeling complexes. In fact, ERα, its coactivator SRC-1, and p300, were all found to bind to components of a remodeling complex [78, 79]. Remodeling complexes confer unwinding of DNA and release of histones in a non-covalent and ATP-driven manner (reviewed in refs 80, 81). Three groups of these complexes have been found. The best known is the switch/sucrose non-fermenting (SWI/SNF) complex. It was first identified in yeast; the human homologs are the Brahma related proteins. A second complex is imitation switch (ISWI), which was originally identified in *Drosophila*. However, involvement in NR mediated transcription activation was only established for the SWI/SNF complexes. Other described complexes are Mi-2/ nucleosome remodeling and histone deacetylation (Mi-2/NURD) complexes, which act on chromatin regions that have become deacetylated by HDACs. These contain histone deacetylase activity, which confers further tightening of the chromatin structure of a gene that has to be silenced.

Crosslinking assays have revealed that the GR and the human SWI/SNF complex bind on a tandemly repeated MMTV promoter in an alternating fashion, with a cycle time of approximately 5 min. [62]. This indicates that within one exchange cycle, after conferring the first

step in opening the chromatin by recruitment of HATs, the GR is removed from its DNA binding site, leaving the stage for further chromatin remodeling.

Another large complex involved in transcriptional regulation is the Mediator complex, of which orthologs have been found in mammalians, *Drosophila, C. elegans* and yeast. This complex consists of about 25 subunits. The Mediator complex was initially identified as the thyroid receptor-associated proteins (TRAP) or the vitamin D receptor–interacting proteins (DRIP) or activator-recruited cofactor (ARC) [82-85]. A universal code for each Mediator protein in different species is now available [86]. FRET experiments have shown that NRs can directly interact with components of the Mediator [87]. The Mediator complex binds with its DRIP205/TRAP220 subunit (MED1) to the NR LBD in a ligand-dependent manner, through an LXXLL motif which is also found in many coactivators (see section 1.1.5) [88-92]. ChIP experiments have revealed that the TR first recruits p160 and p300, followed by the Mediator complex. This finding is substantiated by the observation that recruitment of the Mediator is stimulated by inhibition of HDAC activity, which indicates that histone acetylation is required for recruitment of the Mediator complex to chromatin [93]. Consistent with this observation, synergism was observed between the Mediator complex and p300/CBP-SRC for ERα-mediated transcription with chromatin templates, but not with naked DNA [94]. ChIP experiments with the ERα and its recruited proteins showed that the time scale of Mediator cycling is in the same order as that of the receptor and its coregulators, indicating a cooperating role for the Mediator complex in transcriptional regulation [64].

The Mediator complex can bind RNApolII thereby functioning as a scaffold to recruit other components of the transcription machinery [95]. In addition to transcription initiation, the Mediator complex enables re-initiation of transcription by providing access for multiple rounds of transcription [96]. The composite nature of different promoters will influence the conformation of bound NRs, which is ultimately transmitted to the recruited Mediator complex (reviewed in ref. 97). The conformation of the Mediator complex, in turn, will determine the number of RNApolII molecules that can start transcription in a defined period of time.

A summarizing spatiotemporal model of the events occuring to regulate transcription by NRs can be as follows:

After ligand binding, NRs can freely bind to and depart from their cognate DNA binding sites very quickly, giving target genes the opportunity to respond immediately to changes in hormone concentration. This could imply that NRs have left the response element directly following the recruitment of coregulators, which has been proposed as a 'hit and run' mechanism [61]. After binding to chromatin, NRs recruit nucleosome disrupting HATs and HMTs via their coactivators and chromatin remodeling complexes to further disturb chromatin structures at the regulatory sites of the NRs target genes (Figure 4A). In case of transrepression, inhibition of transcription is established by recruitment of HDACs via their corepressors, which may also be guided by chromatin remodeling complexes (Figure 4B). The loosened chromatin enables recruitment of the general transcription factors and RNApolII to the transcription initiation site via the Mediator complex. Many of the spatiotemporal details on transcriptional regulation are not elucidated as yet, therefore the model described above is expected to be extended and modified.

1.1.5 Nuclear receptor coactivators

NRs function in large networks of proteins and protein complexes that dynamically interact to activate or inhibit transcription initiation. An overview of the recent knowledge on these networks was described in section 1.1.4. Proteins that directly or indirectly activate or enhance transcriptional activation of NR target genes are generally considered as coactivators. Examples are: the chromatin remodelling complexes, the Mediator complex, HATs, HMTs, and the group of proteins that recruit all these proteins to the NRs (reviewed in ref. 98). This latter group includes the p160 coactivators, which have been studied in most detail.

Three distinct human p160 steroid receptor coactivator proteins have been identified, SRC-1, TIF2/SRC-2, and SRC-3, also known as ACTR, AIB1, RAC3, or TRAM-1 [65, 99-103]. NCoA-1 is the mouse homolog of SRC-1, GRIP-1/NCoA-2 in the mouse is homologous to TIF-2, and p/CIP is the mouse homolog of SRC-3 [104, 105].

P160 coactivators contain several functional domains (Figure 5). Best studied in this regard is the NR interacting domain, or NR box, which contains three LXXLL motifs (L = leucine, x = any residue) [106]. The leucine residues in these motifs are indispensible for receptor interaction, and flanking residues play a modulating role herein. The LXXLL motifs interact with a hydrophobic cleft in the NR LBD in a ligand-dependent manner. This cleft is lined by a charged clamp formed by a conserved lysine in helix 3 and a conserved glutamic acid in the AF-2 core in helix 12 [107]. Through an amino-terminal glutamine(Q)-rich region and independent of its LXXLL motifs, p160 coactivators can bind to NR NTDs, resulting in synergism of AF-1 and AF-2 activities, which might be necessary for optimal receptor functioning [39, 41, 42, 108, 109].

P160 coactivators contain also binding domains for the HAT containing cointegrators CBP/p300 and p/CAF (see 1.1.4), which play an essential role in gene transcription as has been demonstrated in knock-out models [110, 111]. If fused to a heterologous DBD, p160 coactivators can autonomously transactivate through two activation domains, AD1 and AD2 (see Figure 5). AD1 coincides completely with the CBP/p300 binding domain, and partly with the PCAF binding domain, which also contains the Q-rich region [100, 105, 112]. To some extent, SRC-1 and SRC-3 can acetylate histones by their weak intrinsic HAT domain, formed by the Q-rich region and AD2 [65, 66]. AD2 can bind to the HMTs CARM1 and PMRT1 [74, 76].

Figure 5. Schematical repesentation of a p160 NR coactivator. NID = nuclear receptor interacting domain, AD = activation domain, Q-rich = Q-rich region.

The physiological function of p160 coactivators has been established in knock-out and null mutant mice (reviewed in refs 98, 113). SRC-1 inactivation led to decreased growth of steroid target organs, like uterus, mammae, testes and prostate, indicating a role for SRC-1 in ER, PR and AR function [114]. Pituitary resistance to thyroid hormone was also observed, whereas PPARα regulated genes were unaffected in SRC-1 null mice [114, 115]. These mice showed compensatory overexpression of TIF-2/SRC-2, indicating redundancy between these two coactivators [114]. Furthermore, SRC-1 null mice exhibit moderate motor dysfunction and delayed development of cerebellar Purkinje cells [116].

SRC-2 plays a critical role in reproductive behaviour and function, and is the most prominent SRC family member expressed in the testis. Both male and female SRC-2 knock out mice

showed reduced fertility [117]. An AR mutation, which disrupts the interaction with SRC-2 was reported in some men with oligospermic infertility [118]. SRC-2 is a coactivator of PPARγ as reflected in fat accumulation in mice lacking SRC-2 [119]. In addition, SRC-2 was found to form a fusion oncoprotein, as a result of a translocation causing leukemia [120].

SRC-3 null-mutant mice showed dwarfism, deficiency in mammary gland development, and other abnormalities in the female reproductive system [121, 122]. Very high levels of SRC-3 mRNA were found in many ER-positive breast and ovarian tumors [123, 124]. This indicates an important role in estrogen stimulation of these tumors as substantiated by the observation that SRC-3 depletion in MCF-7 cells inhibited their estrogen-dependent growth [125, 126]. SRC-3 is also thought to facilitate the estrogen mediated vasoprotective effects of estrogens via ER activation [127, 128].

P300 inactivation is lethal in the embryonic stage, indicating potential redundancy of related CBP cannot overcome p300 deficiency [110]. Inactivation of CBP is associated with the severely disabling Rubinstein-Taybi syndrome [129]. As compared with the p160 coactivators, the higher importance of CBP/p300 proteins correlates with their evolutionary strong conservation.

The viability and fertility of SRC-1 and SRC-3 null mutants are indications for redundancy among SRC family members. Indeed, analysis of knock-out mice lacking SRC-1 and SRC-2 revealed that SRC-1 can partially compensate for loss of SRC-2/TIF2. A *TIF2*$^{-/-}$ mutant showed an impaired testicular function, which was further decreased by inactivation of one or both SRC-1 alleles [130]. A *p/CIP*$^{-/-}$*SRC-1*$^{-/-}$ mouse displayed impaired brown fat development and defective adaptive thermogenesis, which are due to absence of activation of certain PPARγ target genes [131].

Based on the data on mutant mice and the tissue specific expression patterns it can be assumed that each member of the SRC family has preferential functions. During recent years, a number of studies has been published that are consisitent with this observation (reviewed in refs 132-134). The individual SRC proteins each preferentially bind to a different subset of NRs. They even can distinguish between different NR subtypes. For example, ERα has a higher affinity for SRC-1 and SRC-3 than ERβ, and PR-A interacts more efficiently with SRC-1 than PR-B. The GR has an extra level of binding preference through a second charge clamp in the LBD. Moreover, the three different NR boxes of SRC proteins show NR binding specificities. The GR shows the highest affinity to NR-box 3 of SRC-2, whereas TRβ prefers NR-box 2. Ligands can specify NR box affinity of a particular NR or even NR subtype. Different peptides, selected by phage-display, were found to bind to ERα activated by different ligands. For several NRs, crystallographic studies have revealed different conformations of the coactivator binding interface in LBDs bound by different ligands. SRC recruitment can be promoter dependent as was found for the MMTV promoter on which the GR recruits SRC-1 and SRC-3, but not SRC-2 [135]. The functionality of the interactions between SRC-1 and the AR NTD or LBD was also found to vary in a promoter-dependent fashion [136].

A large number of other NR coactivating proteins with less defined function has been identified, many of which contain LXXLL motifs. These factors are thought to bind directly to multiple NRs through these motifs in a ligand-dependent manner. In addition to the numerous coactivators that interact with DNA bound NRs, another category of coactivators is formed by proteins that positively influence NR activity prior to or after DNA binding. These proteins are involved in: synthesis, proper folding and stabilization, nuclear trafficking, nuclear import and export, or degradation of the NR. A detailed description of those kinds of coactivators is beyond the scope of this thesis.

1.1.6 Nuclear receptor corepressors

In addition to transcription activation, transcriptional silencing is required for balanced regulation of transcription. Unliganded type I receptors, antagonist bound type III receptors, and orphan receptors, are able to repress transcription, also called transrepression. To inhibit transcription, NRs recruit corepressors, which bind HDACs that tighten nucleosomal structures by their histone deacetylating capacities [44]. In this way a non-permissive environment is created for appropriate transcription initiation.

Several corepressors are known, including the related corepressors NR corepressor (NCoR) and silencing mediator for retinoid and thyroid hormone receptor (SMRT) [72, 137]. NCoR and SMRT show high sequence similarity and share key functional domains (Figure 6).

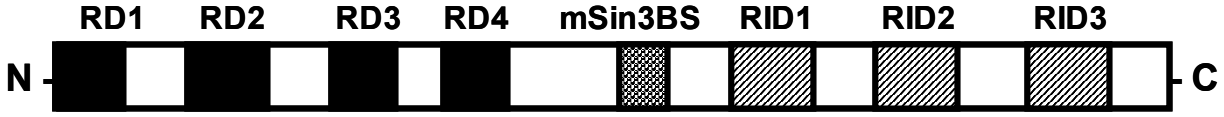

Figure 6. Schematical repesentation of the NR corepressor NCoR. RD = repression domain, mSin3BS = mSin3 binding site, RID = receptor interacting domain.

Corepressors have two or three NR interacting domains (RIDs) through which they bind to NR LBDs. Each RID contains a receptor interaction motif (CoRNR box) with a consensus sequence LxxI/HIxxxI/L. This motif is predicted to adopt an amphipathic α-helical conformation similar to that of the LXXLL motifs in coactivators [43, 138, 139]. Like LXXLL motifs, the hydrophobic residues of the CoRNR boxes are essential in receptor interaction, flanking amino acid residues play a modulating role in this interaction. The coactivator binding cleft in the LBD is also involved in corepressor binding. However, compared to the LXXLL motif, the CoRNR box helix is NH_2-terminally extended, which most likely prevents binding of the corepressor to the hydrophobic cleft in the agonist activated LBD. In the unliganded state of type I receptors or antagonist bound type III receptors, helix 12 of the LBD is thought to be displaced by the extended CoRNR helix [43].

SMRT and NCoR recruit different classes of HDAC subtypes through three or four different repressor domains (RDs), respectively [140-143]. This recruitment might be direct or via the Sin3A protein [144-146]. To deacetylate histones in association with corepressor and DNA bound NRs, HDACs have to gain access to their target site. Therefore, chromatin-remodeling activity is also required. Indeed, the Mi-2/NURD remodeling complexes were found to contain HDAC activity [147-149].

Like for the coactivators, receptor specificity has been established for NCoR and SMRT. The TR prefers to recruit NCoR, whereas the RAR isoforms bind much better to SMRT than to NCoR. PPARα and PPARδ only bind to SMRT, whereas the orphan receptor Reverb exclusively binds to NCoR. In the two corepressors, specific CoRNR box motif residues of individual RIDs have been identified to confer these receptor specificities [150].

The three-dimensional structures of several NR LBDs bound to agonists and antagonists have been elucidated (reviewed in ref. 151). The agonistic and antagonistic forms provide interaction interfaces suitable for binding to coactivators or corepressors, respectively. The interaction interfaces are overlapping, which is illustrated by a single mutation in the TR LBD that changes its interaction interface from a SRC-1-binding to an NcoR-binding interface [152].

Whether a ligand acts as an agonist or antagonist is not only determined by the conformation it induces to an NR LBD, but is also dependent on the coactivators and corepressors present in a

cell. To which extent coactivators and corepressors influence NR function is determined by their stoichiometry, which is cell-type specific and depends on cell activity state [54]. In case of NCoR excess, the vitamin D analog Gemini was found to shift from an agonist to an inverse agonist by disabling coactivator interaction and stimulating corepressor recruitment [153]. This indicates a coregulator interface conformation that is dynamic rather than static, and the equilibrium between the different conformations is dependent on the ligand structure, the cell-specific coactivator/corepressor ratio and presumably other factors like the promoter context of the receptors target genes. This is also substantiated by the fact that corepressors and coactivators, to some extent, can compete for binding to agonist bound RAR and ERβ [154, 155]. Additionally, two proteins, transducin β-like R1 and Ski-interacting protein, were found to mediate exchange of NR corepressors for coactivators [156, 157].

Another kind of corepressors includes receptor interacting protein 140 (RIP 140) and ligand-dependent corepressor (LcoR) [158, 159]. These corepressors resemble the p160 coactivators in that they are recruited by agonist bound NR LBDs through their LXXLL motifs. LCoR has one, and RIP140 even has ten LXXLL motifs. Both corepressors function by HDAC-dependent and -independent mechanisms.

Like for the coactivators, the number of proteins identified to have corepressing activity is still growing. Further details on this subject are beyond the scope of this thesis.

1.1.7 Post-translational modifications of nuclear receptors

In addition to cofactors that modulate the chromatin structure, NR function is also regulated by different kinds of direct post-translational modifications, like acetylation, methylation, ubiquitylation, sumoylation and phosphorylation. These modifications are reviewed in refs 160-162.

P300 and CBP, which are recruited by p160 coativators, can acetylate histones, but also transcription factors, among which the AR and ERα. Through acetylation, activation of ERα is attenuated, whereas AR activation is stimulated [163]. TRβ1 acetylation was found to be associated with p300 recruitment [164].

P160 coactivators recruit, in addition to HATs also HMTs, like CARM1 and PMRT1 (see section 1.1.4) [74, 165]. Although these HMTs can methylate histones and other proteins in the transcription initiation complex, no methylation of NRs and their coregulators by these HMTs has been reported.

Ubiquitin is a well-conserved 76-amino acid protein, which is conjugated to many different substrates, including NRs. Poly-ubiquitylation of proteins leads to their proteasome-mediated degradation, whereas mono-ubiquitylation is involved in transcriptional regulation. The AR, ERα, GR, PPARα, RARγ, RXRα, and TR were all found to be degraded by the ubiquitin-proteasome system [166-171]. However, overall activity of the GR is increased by this system, suggesting that a high turn-over of GR on target promoters favors the opportunity for other GR molecules to start another round of transcription [63].

Sumoylation involves the conjugation of the 101-amino acid small ubiquitin-like molecule-1 (SUMO-1) protein (18% homology with ubiquitin) to lysines of a target protein. Sumoylation influences proteins with respect to their subcellular localization, subnuclear sequestration, stability, or ability to regulate gene transcription. Sumoylation of the GR increases its transcriptional activity probably by stabilization of this receptor, whereas AR-dependent transcription is repressed by sumoylation [172-175]. However, the effect on AR may be caused by sumoylation of GRIP1, which probably impairs their co-localization [176]. In contrast,

sumoylation of SRC-1 was shown to enhance PR-SRC-1 interaction thereby increasing PR activity [177].

NRs can be phosphorylated at serine, threonine and tyrosine residues in their NTDs and LBDs as well as in their DBDs. The kinases involved are mitogen-activated protein kinases (MAPKs), cyclin dependent kinases (CDKs), Akt, protein kinase A (PKA), and protein kinase C (PKC). The number of phosphorylation sites ranges from one in the VDR to at least 13 in the PR. Phosphorylation of ERα, ERβ, PPARα and AR NTDs by MAPKs or Akt stimulates recruitment of coactivators and of the transcription machinery, thereby facilitating chromatin remodelling and transcription initiation [178-183] The GR and PPARγ are inhibited by phosphorylation of their NTDs, probably by promoting the ubiquitin-proteasome mediated degradation [184-189]. Phosphorylation of the ERα and RARα LBDs favors receptor dimerization and coactivator recruitment [183, 190-192]. DBD phosphorylation has been observed for the VDR, ERα, and RARα, which interferes with DNA binding and receptor dimerization [193-196]. Excessive kinase activity may activate NRs in a ligand-independent manner. This was illustrated in breast, ovarian and prostate cancers, which showed estrogen and androgen-independent growth and had abberant MAPK and Akt activities [180, 197-200].

NR coregulators are also subject to the post-transcriptional modifications described above. SRC-3 can be acetylated by CBP, which modulates one of its lysines leading to an inhibition of binding to CBP. This modification may form a negative feedback loop [201]. For the ER, which is not sumoylated and the PR, which can be sumoylated, it has been established that sumoylation of SRC-1, but not of these receptors, increased their binding to this coactivator [177]. Sumoylation of p300 leads to recuitment of a HDAC, which represses its activity. HDACs, in turn are also regulated by sumoylation [202]. P160 family members and CBP/p300 are particularly subject to become phosphorylated by the different kinase pathways [203-206]. Phosphorylation of the corepressor SMRT inhibits its interaction with NRs and causes its redistribution from the nucleus to the cytoplasm [207]. Phosphorylation-dependent ubiquitylation was found for RARγ, indicating a mechanism of initial receptor activation followed by tagged degradation [208]. The liganded PR is also degraded upon phosphorylation, however, its degradation pathway is not clear yet [209, 210]. Taken together, cross-talk with other signal transducing pathways can sophistically modulate NR and cofactor function. This might result in stimulation or inhibition of transcription regulation.

1.1.8 Additional nuclear receptor functions

In addition to the classic ligand-induced transcriptional regulation of target genes NRs can be involved in other pathways, including ligand-independent transactivation, DNA-binding-independent transactivation, and even transcription-independent function. Illustrating examples are described below.

For several NRs ligand-independent functions have been described. Unliganded TR and RAR can bind to HREs and recruit corepressors thereby repressing transcription [211-213]. Ligand binding to these receptors causes dissociation of the corepressors, allowing coactivator binding. Another mode of ligand-independent action involves the orphan receptors (type V NRs). These are constitutively active, have an LBD structure similar to that of agonist-bound transcriptionally active NRs, but have an empty ligand-binding pocket or no ligand-binding pocket at all [214, 215]. Mutations of a conserved tyrosine in the LBD induce constitutive activation of the ERα and ERβ. However, antiestrogens could completely abolish this ligand-independent activation [216, 217].

Several ligand responsive NRs can also be activated in the absence of ligand. For example, in reporter assays the chicken progesterone receptor (cPR) could be activated by 8-Br cAMP [218]. 8-Br cAMP activated p42/p44 MAPK and increased phosphorylation of SRC-1, which then contributed to the ligand-independent activation of the cPR [219]. Dopamine can activate a number of NRs including cPR and ERα in the absence of their cognate ligands [220, 221]. Treatment with dopamine causes PR-dependent induction of mating behavior in female rats and mice, whereas PR null mice are not responsive [222-225]. The ERα is also responsive to cell signaling pathways in the absence of ligand. In cells maintained in phenol red-free, charcoal-stripped serum, the ERα shows substantial basal transcriptional activity. This activity can be inhibited by a pure anti-estrogen such as ICI 182780 [221]. Epidermal growth factor (EGF)-dependent activation of human ERα was observed in transfected hormone depleted HeLa cells, whereby Ser118 was phosphorylated [226]. Substitution of serine by alanine abrogated the hormone-independent activation [227]. In the absence of ligand, AR activity can be induced by EGF, keratinocyte growth factor (KGF), interleukin 6 (IL-6), and forskolin (see for more details section 1.2.5.1.3.4) [228-230]. Aberrant activation of AR by cell signaling pathways is presumed to be involved in many cases of androgen-independent prostate cancer (see section 1.2.5.1.3.4). The GR needs ligand for activation, but can be extra stimulated by activators of PKA and PKC [220, 231-233]. RARα can be activated in the absence of retinoic acid by the catalytic subunit of PKA [191]. In CV1 cells, RARα, RARβ, RXRα, RXRβ, and RXRγ, but not RARγ, could be activated in the absence of ligand and after treatment with the phosphatase inhibitor okadaic acid, which was not observed if the cells were treated with dopamine [234]. The VDR could be acivated by okadaic acid or by dopamine [234].

NRs can also regulate transcription independent of HRE binding, which is referred to as 'transcriptional cross-talk'. For example, in the presence of agonists, GR, TR, RAR, and ERβ inhibit AP-1-dependent transcription, whereas ERα is activating. Antiestrogen bound ERβ can activate AP-1 directed transcription [235, 236].

Non-genomic signaling mechanisms of NRs have also been reported and include recruitment of signaling pathways that are often associated with cell membrane receptors such as G-protein-coupled receptors (GPCRs), ion channels or enzyme-linked receptors [237]. For plasma membrane associated ERs it has been suggested that they are involved in the regulation of cell membrane ion channels, GPCRs, and tyrosine kinases and MAPKs [238-242]. They also can activate adenylate cyclase production, and induce phospholipase C activation [243, 244]. This results in a rapid increase in intracellular Ca^{2+} concentration due to Ca^{2+} mobilization from the endoplasmic reticulum and to the formation of inositol 1,4,5-trisphosphate and diacylglycerol [244]. The ER is also supposed to be involved in the Src/Ras/MAPK signal transduction pathway by interaction with c-Src [238]. In breast cancer cells, the B isoform of the PR stimulates the activated ER to recruit the Src/p21ras/ERK pathway [245]. A specific polyproline motif in the NH_2-terminal domain of PR mediates direct progestin-dependent interaction of PR with SH3 domains of various cytoplasmic signaling molecules, including c-Src tyrosine kinases [246]. Testosterone is also able to regulate the MAPK family of protein kinases. Recent work indicates that, via a cell membrane associated AR, testosterone induces a rapid rise in the intracellular free Ca^{2+} concentration in macrophages [247]. Similar findings were reported for the VDR [248]. In addition to protein kinase signaling pathways, non-transcriptional actions of NRs can be mediated by the recruitment of lipid kinases. E_2-bound ERα interacts with the regulatory subunit of the lipid kinase PI3K, triggering activation of the catalytic subunit and increasing intracellular production of phosphoinositides [249]. Interaction with PI3K was also found for other NRs, such as the AR and the GR [250].

1.2 THE ANDROGEN RECEPTOR

The AR is essential in development of the male phenotype and controls male fertility and sexual behaviour. High AR levels are present in the tissues of the male urogenital tract, including the prostate. Low AR expression was found in many other tissues including mammary gland, kidney, liver brain, genital skin fibroblasts and keratinocytes, hair follicles, cardiac and skeletal muscle, and salivary glands [251, 252]. The androgens, T and DHT are the main AR ligands. T is produced in the Leydig cells of the testis. In target tissues it can be converted into the more active DHT by 5α-reductase [253, 254].

In addition to its role in prostate development and maintenance, the AR is involved in prostate carcinogenesis. Orchiectomy or inhibition of AR activity by anti-androgens causes shrinkage of the normal prostate and of tumor prostatic tissue, including loss of luminal epithelial cells (see for review ref. 255). Therefore, AR signaling is an important subject of research.

1.2.1 General features of the AR

The *AR* gene maps to chromosome band Xq11.2-q12 and is composed of 8 exons spanning 186 kbp [256-258]. Two hAR mRNA species of approximately 8.5 and 11 kb have been identified [256, 259-262]. These transcripts differ only in the sizes of their 3'-UTRs. The AR NTD is encoded by part of exon 1. Exons 2 and 3 code for the AR DBD, each for one zinc cluster domain. The AR LBD is encoded by part of exon 4, exons 5, 6, and 7, and part of exon 8.

The size of the human AR can be variable, due to variation in the length of poly-glutamine and poly-glycine stretches in its NTD (Figure 7) [256, 259]. In the AR from other species these stretches are absent or have a considerably different size and are located in a different part of the NTD. Amino acid numbering in this thesis corresponds to an AR length of 919 amino acids, as employed by The Androgen Receptor Gene Mutations Database (http://www.mcgill.ca/androgendb) [263].

The AR can be phosphorylated in the absence or presence of androgens resulting in isoforms displayed as a 110-112-114 kDa triplet in an SDS-PAGE gel. As a result of alternative translation initiation, a truncated 87 kDa AR has been found in fetal tissues and genital skin fibroblasts [264, 265]. It constitutes 10% of the total AR protein level, but its *in vivo* relevance is not known.

The AR DBD and LBD primary struture are well conserved between species. The AR NTD contains only short conserved regions, the homology of amino acid stretches 1-36, 234-247 and 501-529 are most obvious. These regions are expected to be of functional importance and are discussed in the next section.

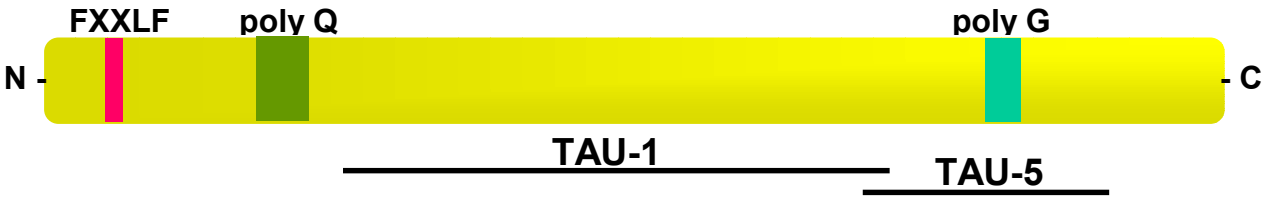

Figure 7. Schematical overview of AR NTD functional subdomains. FXXLF = FXXLF motif, polyQ = poly-glutamine stretch, polyG = poly-glycine stretch, TAU = transactivation unit.

1.2.1.1 The AR NH_2-terminal domain

In the AR NTD several functional subdomains have been identified (Figure 7). The NTD harbours the transcription activation unit 1 (TAU1). TAU1 comprises a region of approx. 250 amino acid (aa) residues (100-370) and is active in the full-length ligand activated AR. However, in a truncated AR lacking the LBD, another region containing aa 360-485, termed TAU5, functions as a constitutively active transactivation domain [266]. Depending on the cellular context, deletion of TAU5 causes a small or more pronounced decrease in activity of the full length AR [266, 267]. In contrast, TAU1 is indispensible for ligand-induced activation of the full-length AR [266]. These findings indicate that the unliganded LBD prevents TAU1 activity, which is reversed upon ligand binding. Indeed, the TAU1 region by itself shows a constitutive activity that is approx. 40% of the activity of the complete NTD [267]. TAU1 in the rat AR was even further narrowed to two regions, AF-1a and AF-1b, which correspond to aa 172-185 and aa 296-360 in the human AR. Deletion of either subdomain caused a minor reduction of AR transcriptional activity, but absence of both AF-1a and AF-1b resulted in a 90% decrease in activity [268]. However, another study indicated that AF-1b was dispensible [267]. The importance of TAU1 and TAU5 in AR activity is further substantiated by the finding that the NTD is essential in p160 coactivator binding. Although the AR LBD can bind to p160 coactivators, the AR NTD also interacts with these coactivators. TAU5 directly interacts with the glutamine-rich region of SRC-1, whereas TAU1 seems to attenuate indirectly this interaction [267].

It has been predicted that the NTD contains several small α-helical structures, but a well-defined structure of the complete AR NTD could not be established. It is presumed that the AR NTD adopts an induced fit conformation in response to binding other proteins, as proposed for binding of the general transcription factor TFIIF [269].

The region aa 234-247, which constitute the most conserved part of AR NTD was found to bind to the carboxy terminus of the Hsp70-interacting protein (CHIP), a negative regulator of AR (see also section 1.2.2.2) [270].

The highly conserved region aa 16-36 is predicted to fold in a long amphipathic α-helix, indicating a protein-protein interaction domain. Aa 23-27 is located within this region and comprise the LXXL-like motif, FQNLF. This motif is essential in the direct ligand-dependent interaction between the AR NTD and AR LBD, the so-called N/C interaction. This interaction will be extensively discussed in section 1.2.3 and Chapter 2. Some AR coregulators have also been found to contain FxxLF motifs, through which they, like the motif in AR NTD, bind to the AR LBD coactivator groove, thereby competing with the N/C interaction (reviewed in ref. 271, see also section 1.2.2.1). Recently, the melanoma antigen gene protein 11 (MAGE-11) has been identified as a protein that interacts with a region in the AR NTD overlapping with the FXXLF motif. Binding of MAGE-11 increases AR activity (see section 1.2.2.1) [272].

The region aa 501-529 is the third part of the NTD that is highly conserved between ARs from different species. However, for this region no specific function has been established as yet.

In addition to the proteins described above, many other proteins, including other coactivators and corepressors, have been found to interact with the AR NTD and influence AR function. An overview of these proteins can be found in The Androgen Receptor Gene Mutations Database (http://www.mcgill.ca/androgendb) [263]. A selection of these proteins is given in section 1.2.2.

1.2.1.2 The AR DNA binding domain

The AR binds with high affinity to the consensus androgen response element (ARE) 5'-AGAACAnnnTGTTCT-3', which is shared with the GR, PR and MR. Also, more or less AR

specific AREs have been reported in the regulary regions of genes that show androgen-specific transcriptional regulation. These AREs contain one perfect or almost perfect half-site, whereas the second half-site can vary considerable from the consensus sequence (reviewed in ref. 55). Based on crystallographic data of the AR DBD bound to an AR specific ARE, the mechanism of AR binding to a specific ARE has been elucidated. One AR binds to a high affinity consensus half-site, the second AR can have a weaker affinity to the second half-site, because the AR DBD, unlike other steroid receptors, contains an additional interface that stabilizes the AR dimer/ARE complex. Dimerization strength of the other steroid receptors would not be sufficient to retain stable binding to an AR-specific ARE. The orientation of the AR dimer on the ARE is in a head-to-head fashion on both non-specific and AR-specific AREs (Figure 8) [24]. More details on this subject are described in section 1.2.4 and Chapter 3.

Like other NRs, the AR DBD was found to contain a non-classical NES, which is necessary and sufficient for nuclear export, indicating a role for the DBD in nucleo-cytoplasmic shuttling of the AR[26]. In addition, a second NES has been postulated in the AR LBD (see section 1.2.1.4) [45].

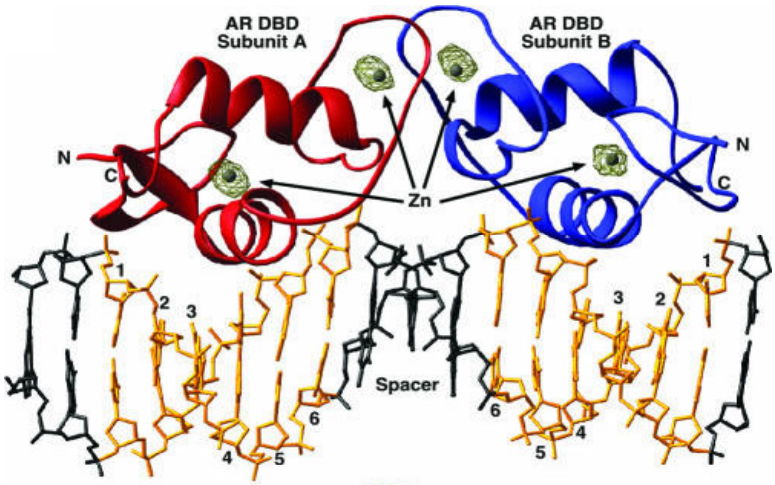

Figure 8. Overall architecture of the AR DBD complexed to an AR-specific ARE. The two AR DBD subunits are in red and blue, repectively, the hexameric ARE half-site is in gold, the spacer and flanking base pairs are black. From ref. 24.

1.2.1.3 The AR hinge region

The hinge region of the androgen receptor contains a bipartite nucleoplasmin-like NLS (aa 605-624), which is essential for nuclear import of the receptor [273, 274]. The NLS is blocked by the unliganded LBD, because only in the presence of androgens the AR is transported to the nucleus. AR mutants lacking the LBD are constitutively localized to the nucleus.

Between aa 628 and 646 a domain has been identified that inhibits AF-2 activity of AR LBD. Mutations in this region enhanced the weak (see 1.2.1.4) AF-2 transactivation by TIF2 indicating that the hinge region plays an inhibitory effect on coactivator-mediated AF-2 function of AR [275, 276]. Alternatively, region 628-646 might bind an inhibitory protein, thereby negatively influencing the overall activity of the AR.

1.2.1.4 The AR ligand binding domain

Crystallographic data have revealed the three-dimensional structure of the AR LBD (Figure 9) [277]. Although no helix 2 has been identified, helix numbering is further consistent with the general NR 12-helical LBD structure, as was previously predicted by comparison modeling [278]. AR LBD helices 10 and 11 actually form one long helix, and helix 12 appeared to be longer than proposed by modeling. The crystallographic structure was elucidated for the AR LBD complexed with DHT and complexed with the synthetic androgen R1881. A dynamic structure was indicated for the AR LBD, because binding of DHT induced a continuous helix 12, which was apparently split into two separate helices upon binding of R1881. Indications for the influence of the ligand on LBD conformation were earlier provided in limited proteolytic digestion experiments, in which the AR bound to either agonists or antagonists showed protease protected fragments of different sizes [279].

The ligand binding pocket in the AR LBD is primarily formed by 18 amino acid residues scattered throughout helices 3, 4, 5, 10 and 11 [277]. The positions of all individual amino acid residues relative to the bound ligand have been identified. Substitutions of amino acids that are in contact with or in the vicinity of the ligand have been found in prostate cancer specimens and in the androgen insensitivity syndrome [280-282]. These substitutions impair AR function or render the AR, also responsive to female sex hormones, glucocorticoids, and even antagonists [283]. More details on AR related diseases and AR ligand specificity can be found in section 1.2.5 and in Chapter 4.

Like was found for other NRs, helix 12 of the AR LBD is folded like a lid over the ligand-binding pocket when occupied with an agonist [277]. The resulting conformation of the LBD provides an interface for coactivator binding. In case of antagonist binding, the resulting conformation might lead to corepressor recruitment and/or inhibition of coactivator binding. However, although the coactivators SRC-1, TIF2 and GRIP1 can bind to the AR LBD through their LXXLL motifs, this binding is not essential for AR function. This is reflected by an AR deletion mutant lacking the NTD, that can itself not be activated by androgens [266]. This mutant needs overexpression of a p160 coactivator to be activated, indicating very weak AR AF-2 activity [40, 284].

Although p160 coactivator binding to the AR LBD might not be essential for full length AR function, different LBD conformations induced by different ligands might influence coactivator binding to the NTD. Indications for this were found in interactions with coregulators. The anti-androgen bicalutamide (BCA) is unable to activate the AR. It cannot induce coregulator recruitment, although a BCA-bound AR is able to bind to DNA [285, 286]. However, as indicated by FRAP experiments the time period of interaction with DNA is very short [287]. Cyproterone acetate (CPA) elicits a transcriptionally productive AR-GRIP1 interaction, reflecting its partial antagonistic character. CPA was also found to induce recruitment of the corepressor SMRT to the AR NTD [288]. So, the outcome of CPA activity may depend on the ratio of coactivators and corepressors present in a cell.

A separate category of AR coregulators, including ARA54, ARA70 and RAD9 (see section 1.2.2), were found to bind to the LBD through their FXXLF motifs, which are related to the LXXLL motifs found in most coactivators [289]. An FXXLF motif (see also section 1.2.1.1), which can bind to the AR LBD, was also found in the NTD. This motif establishes a ligand-dependent interaction between these two domains, the N/C interaction. This interaction might contribute to AR activity, dependent on the promoter context [290].

The AR LBD shows a preference for FXXLF motifs over LXXLL motifs. Computer modeling and crystallographic analyses of AR LBD bound to FXXLF and LXXLL motif containing

peptides, have revealed a larger coactivator groove for the AR LBD compared to the ER-LBD. This larger groove provides suitable contact sites for the bulky phenylalanine side chains of the FXXLF motif, whereas LXXLL motifs have less favorable interactions with the amino acids lining the AR coactivator groove [162, 291, 292]. Detailed information on the N/C interaction and FXXLF motifs can be found in section 1.2.3 and Chapter 2.

An AR NES is localized in the AR LBD between amino acids 742 and 817. In the absence of ligand, this NES is active and dominant over the NLS in the hinge region (see previous section). Ligand binding inhibits the NES and simultaneously releases repression of the NLS, leading to an increase of AR nuclear localization [45]. The AR LBD also plays an important role in stabilization of AR-DNA interaction as revealed by FRAP analysis [293].

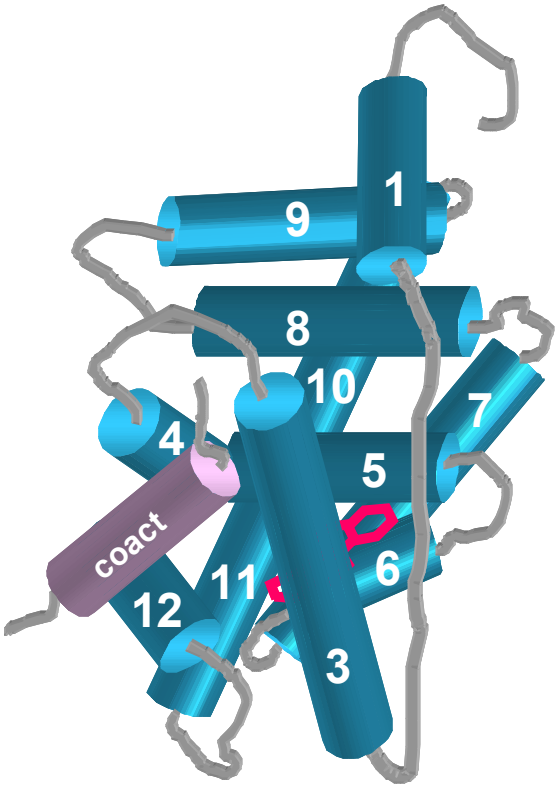

Figure 9. AR LBD helical structure. Numbers of the helices are indicated, coact = helix of a coactivator that interacts with the coactivator groove, androgen molecule is in red.

1.2.2 AR coregulators

Many proteins have been found to interact with the AR. A portion of these proteins was identified as AR coregulators, either coactivator or corepressor. In addition, a number of proteins have been descibed that do not interact directly with the AR, but can indirectly influence receptor activity. These proteins might also be considered as AR coregulators. An overview of AR-interacting proteins is presented in the AR mutations database (http://www.mcgill.ca/androgendb) [263]. In this section, an overview of AR coactivators and corepressors is given, in Tables 1 and 2, respectively. Selected proteins are discussed in sections 1.2.2.1 and 1.2.2.2.

1.2.2.1 AR coactivators

The most studied group of NR coactivators is the family of p160 (SRC) proteins. All three members are capable of enhancing AR activity. SRC-1 and TIF2 (SRC-2) were found to bind to the ligand-activated AR LBD through their LXXLL motifs, but this interaction, as compared to other NRs, is weak. SRC-1 also binds to the AR NTD region 360-494 (TAU5) through its glutamine-rich region, which is essential for its recruitment, whereas the LXXLL motif is dispensable for coactivation of the AR [108]. However, depending on the promoter context, LXXLL-guided SRC-1 binding to the AR LBD contributes to the total AR activity [136].

Male SRC-1 knockout mice do not display a significant androgen-insensitive phenotype [114]. They are fertile, but their testes are smaller than those of wild-type mice. Androgen treatment of castrated SRC-1 knockout mice showed prostate growth albeit less if compared to wild-type castrated mice. These data suggest compensation of the SRC-1 absence by other coactivators. Indeed, in SRC-1 knockout mice the TIF2 mRNA level is elevated [114]. This might explain why the SRC-1 knockouts showed normal fertility. Combinatory knockout models have provided more insight in the redundancy of SRC coactivators. SRC-$1^{-/-}$/$TIF2^{-/-}$ mice, which were not viable after birth, and SRC-$1^{+/-}$/$TIF2^{-/-}$ mice showed severe testis degeneration. The latter were sterile, whereas SRC-$1^{+/+}$/$TIF2^{-/-}$ mice were hypofertile [130]. These findings indicate that SRC-1 can partially compensate for the lack of TIF2 in a dose-dependent manner. Also SRC-3 seems important in androgen-regulated development of the male phenotype, since its glutamine-repeat was found to be shorter in undermasculinized subjects [294]. This observation might indicate absence of redundancy by SRC-1, TIF2 or non-SRC coactivators. However, details of the mechanism of inhibition of AR function by the shortened poly-glutamine stretch in SRC-3 remains to be established. More recently it has been reported that in a transgenic mouse model, in which the AR DBD was swapped by the Gal4_DBD, $AR^{GAL4DBD}$ mice, the AR activity in testes was decreased in a TIF2 +/- background, and even more decreased in TIF2 null mutants compared to wild type. However, SRC-1 +/- mice showed no significant changes in $AR^{GAL4DBD}$ activity. These findings indicate that TIF2 might serve as the preferential coactivator of AR in the testis [295].

The general coactivator CBP, SRC-1, and AR are coexpressed in luminal epithelial cells of the prostate and in a subpopulation of prostate stromal cells. If transfected in CV-1 cells, CBP enhances AR-dependent transcription. Coimmunoprecipitation experiments have revealed that the AR and CBP can be found in the same multi-protein complex. An AR deletion mutant lacking the LBD and region aa 38-296 of the NTD is still capable to bind CBP, indicating a ligand-independent interaction [296].

Tip60 (Tat-interactive protein, 60 kDa), first identified in complex with the Tat protein of the human immunodeficiency virus-1, is an NR type III-specific coactivator [297, 298]. It displays HAT activity and can directly acetylate the AR, which is necessary for Tip60-mediated AR coactivation [299, 300]. PIRH2 can interact with both Tip60 and the AR, and enhances AR activity [301]. PIRH2 also binds to the AR corepressor HDAC1, which leads to inhibition of transcriptional repression [301].

AR-associated protein 70, ARA70, binds to the AR LBD and was shown to enhance androgen-induced AR transcriptional activity [302]. Overexpression of ARA70 changes the AR antagonists OH-FL and BCA into agonists and it also induces AR transcription at physiological E_2 concentrations in females [303-305].

ARA55 binds to the AR LBD and, when hypophosphorylated, is thought to act as an AR coactivator in the nucleus [306, 307]. Like ARA70, it can enhance the AR(T877A) mutant response to OH-FL [305]. The phosphorylated form of ARA55 is found in focal adhesions that are

connected with the extracellular matrix [307, 308]. This property indicates that phosphorylated ARA55 might be involved in regulation of anchorage-dependent growth, differentiation and apoptosis. ARA55 contains LIM domains known as protein-protein interaction sites, but it is not known whether ARA55 recruits other coregulators via these domains [309].

ARA70, ARA55, and ARA54 contain the LXXLL-related motif FXXLF (see sections 1.2.1.1 and 1.2.1.4). The FXXLF peptide motifs of ARA70 and ARA54 specifically bind to the AR LBD. However, the ARA55 FXXLF motif was unable to bind to the AR LBD, indicating that other regions in ARA55 are responsible for AR interaction [289]. FXXLF and related motifs will be discussed extensively in section 1.2.4.

ARA267β, which is homologous to mouse NSD1 (NR-binding SET(Su(var)3-9, Enhancer of Zeste, and Trithorax)-domain-containing protein), and its amino-terminal shortened isoform, ARA267α, can bind both the AR NTD and AR LBD and enhance DHT induced transcription [310]. Both ARA267 proteins contain a SET domain, which is known to interact with components of the SWI/SNF complex. Whether the SET domains of the ARA267 proteins play a role in the recruitment of the SWI/SNF complex to the AR remains to be established. NSD1 contains an FXXLL motif, through which it can bind to the AR LBD [311].

MAGE-11 (see also section1.2.1.1) has been identified as a protein that binds to the region in the AR NTD that contains the FXXLF motif. It stabilizes the unliganded AR and in the presence of an agonist it competes with the N/C interaction, thereby increasing the exposure of the LBD coactivator groove to the recruitment and activation by coactivators [272].

Two related AR coactivators, PIASαx and PIAS1, are members of the protein inhibitor of activated signal transducer and activator of transcription (STAT) family. STATs are transcription factors that are phosphorylated in response to interferon, interleukines, and EGF [312]. So, it is possible that the PIAS proteins mediate cross-talk between cytokines and androgen signalling, but AR activation by these proteins may also be a distinct regulatory process. PIAS1 is a target of androgen receptor-interacting nuclear kinase (ANPK) and is expressed in Sertoli and Leydig cells, and in spermatogenic cells [313, 314]. PIASxα, identified as androgen receptor interacting protein 3 (ARIP3), is also expressed in the testis and was found to facilitate the AR N/C interaction (see section 1.2.4) [315]. PIAS proteins contain amino-terminal LXXLL motifs and bind to the AR in an androgen-dependent manner. The LXXLL motifs of PIASxα were found to interact with GRIP1, but it is not clear as yet whether the motifs are also involved in AR binding [176].

Filamentous actin (F-actin) binding proteins β-catenin, gelsolin, supervillin, and filamin-A determine cell morphology by regulating actin polymerization and depolymerization. β-catenin also acts as a coactivator of downstream transcription factors of the Wnt signalling pathway [316]. Interactions between the Wnt and AR signalling pathways, can both activate or inhibit AR activity [317, 318]. β-catenin was found to function as an AR coactivator in prostate cancer cells [319]. The histone methyltransferase activity of CARM (see section 1.2.2.4) can function in synergy with β-catenin and p300 as a coactivator of the AR. Through β-catenin, CARM1 binds indirectly to the AR, but no methylation of AR has been found [76]. The exact AR coactivating mechanism of CARM1 is still to be elucidated. Recently, β-catenin was found to be involved in insulin-like growth factor 1 (IGF-1) mediated activation of the AR [320]. Gelsolin interacts with the AR LBD by an FXXFF motif in a T-dependent manner and functions as an AR coactivator in the prostate cancer cell line DU145 [321, 322]. Supervillin can coactivate the AR and binds to both the AR NTD and AR LBD [88]. It also activates the GR and is predominantly localized to the plasma membrane at sites of intercellular contacts [323]. This suggests a role for supervillin in transducing signals from cellular adhesion sites to the nucleus, thereby influencing NR function.

Filamin-A is a protein that can cross-link F-actins. It can also interact with the AR hinge region. Mutant filamin-A disables the AR to translocate to the nucleus, thereby inhibiting AR regulated transcription [324].

Androgen receptor trapped clone 27, ART27, is an AR coactivator that binds to the AR NTD. It is expressed in prostate epithelial cells, in muscle, and in breast tissues, but not in AR positive stromal cells of the prostate [325]. ART27 is associated with growth suppression and cell differentiation, which is illustrated by its enhancement of PSA production [326].

The four and a half of LIM-only protein 2 (FHL2) is an AR coactivator that is predominantly expressed in the heart and to a lesser extent in epithelial and stromal prostate cells. FHL2 requires the full-length AR to enhance its activity [327]. It contains an FXXLF motif, but a peptide fragment with this motif is unable to interact with the AR LBD [289]. FHL2 interacts also with β-catenin (see above), but stimulation of AR activity by both FHL2 and β-catenin was found to be additive and not synergistic [328].

The ubiquitin-protein ligase E6 associated protein (E6-AP) (see also section 1.2.3) has an intrinsic transactivation function, and can act as a coactivator for RARα, TR, ER, PR, GR and AR, in a ligand-dependent manner. Coactivation is independent of the ubiquitylation capacity of this enzyme, which indicates that coactivation and ubiquitylation are distinct processes [329]. It could be presumed that the ubiquitinating activity contributes to NR activity by targeting degradation of corepressors, as was found for NCoR, or it could be involved in degradation of components of the preinitiation complex, thereby facilitating reinitiation of transcription. The AR interaction domain of E6-AP has not been identified as yet. Ubc9 (or UBE2I) (see also section 1.2.3) is related to the ubiquitin-conjugating enzymes and can act as a coactivator for the ligand-bound AR. Instead of ubiquitylation, Ubc9 rather is involved in covalent linking of SUMO-1. Similar to E6-AP, the coactivation function of Ubc9 is independent of its sumoylation capacity. Ubc9 binds to the AR DBD-hinge region [172].

Table 1. AR coactivators. *Modified and extended from ref. 271.*

Coactivator	Alternative name	Binding to	Comments	References
SRC-1	NCoA-1, p160	NTD, DBD, LBD	Enhances AR N/C interaction; Interacts with CBP/p300; General nuclear receptor coactivator; Weak acetyltransferase activity.	66, 99, 108, 136, 330
TIF2	GRIP1,NcoA-2, SRC-2	NTD, DBD, LBD	General nuclear receptor coactivator; Facilitates AR N/C interaction.	100, 104, 284, 331
SRC-3	AIB1, NcoA-3, ACTR, p/CIP, Rac3, TRAM1	unknown	Enhances transcription by AR, PR, TR and RAR; Interacts with CBP/p300; Acetyltransferase activity.	65, 101, 303, 332
Tip60	-	hinge-LBD	Acetylates AR; Also coactivates PR and ER; Family member of the MYST/SAS histone acetyltransferases.	298-300
hPIRH2	-	unknown	Binds to AR, Tip60, and HDAC1. Enhances AR by reducing HDAC1 protein levels	301
SNURF	RNF4	DBD	RING finger protein; Interacts with AR, ER and PR; May recruit cromatin remodeling factor HMGI(Y).	313
ARA54	-	LBD	Ring finger protein; Ligand dependent coactivator of AR and PR.	333
ARA55	Hic5	LBD	Contains a LIM domain; Ligand dependent coactivator of AR, PR, GR and ER.	289, 305-307, 309, 334
ARA70	ELE1, RFG	DBD, LBD	Ligand dependent coactivator of AR, GR, ER and PPARγ; Bridging factor to p/CAF and TFIIB.	302-305, 335, 336
FHL2	DRAL, Slim3	unknown	Ligand dependent and prostate-specific coactivator of AR; LIM only protein.	327, 328
ARA160	TMF	NTD	Enhances AR, PR and GR function; Synergistic with ARA70.	337
ARA267β	NSD1	NTD, LBD	Interacts with AR, ER, TR, RAR and RXR; Contains SET and domains ARA267α is N-terminal shortened ARA267β	310, 311
ARIP3	PIASαx	DBD	Facilitates AR N/C interaction; represses transactivation of the probasin promoter at high levels (AR:ARIP3 =	315

Coactivator	Alternative name	Binding to	Comments	References
			1:200)	
PIAS1	-	DBD, LBD	Coactivator of AR and GR, but a corepressor of PR; Expression in rat testes at onset of spermatogenesis	314
E6-AP	-	unknown	Interacts with AR, ER, PR and GR; Ubiquitinylates target protein, which is separate from coactivation.	329
Ubc9	-	DBD-hinge	Interacts with AR and GR; Sumoylates target protein, which is separate from coactivation.	172, 338
Gelsolin	-	LBD	Enhances AR in prostate and muscle cells; Actin filament severing and capping protein.	321
Caveolin-1	-	NTD, LBD	Membrane protein associated with caveoli membrane structures.	339
β-catenin	-	unknown	Ligand dependent coactivator; Also interacts with FHL2 to activate Wnt-responsive genes (independent of AR).	319, 320, 328
Filamin-A	-	hinge	Involved in AR nuclear import; Represses AR function by interfering with the N/C interaction and competing for TIF2.	324, 340
Supervilin	-	NTD, LBD	Interacts with AR and GR; Actin-binding protein.	88
ANPK	PKY	DBD	Stabilizes AR protein	313
ARA24	Ran	NTD	Interacts with AR NTD polyglutamine repeat.	341
BRCA1	-	NTD, LBD	Enhances AR transcription synergistically with ARA55 and ARA70.	342
BRCA2	-	NTD, LBD	Enhances AR function, which may be antiproliferative in (male) breast cancer.	343
Cyclin E	-	NTD	Enhances AR activity independent of cell cycle progression.	344
pRb	-	NTD, DBD	Tumor suppressor; Enhances AR transcription and represses TR by binding to the TR coactivator Trip230 (hMED12).	345
BAG-1L	-	unknown	Enhances AR activity; Regulates Hsp70 function.	345
Hsp40	dnaJ, ydj1p	LBD	Mutation of Hsp40 in yeast reduces AR transactivation; Member of the chaperone heterocomplex.	346, 347
CBP	p300	NTD, DBD	Facilitates AR N/C interaction; interacts with SRC family members; acetyltransferase activity; Coactivator of multiple transcription factors.	296, 330, 348
RIP140	-	NTD, DBD, LBD	Coactivator at low receptor-coactivator ratios, but repressor at high ratios; Influences activity of AR, ER, PPARα and PPARγ	349
ZAC1	-	LBD	Interacts with AR, GR, ER and TR; AR coactivator in HeLa cells (synergistic with TIF2) and AR corepressor in 1471.1 cells.	350
PGC-1	LEM6	unknown	General nuclear receptor coactivator.	351
SRA	-	unknown	Enhances transcription of AR, PR, GR and ER; Functions as a RNA transcript and associates with an SRC-1 containing coregulator complex.	352
RAF	IDE	NTD	Enhances AR and GR DNA binding.	353
HMG-1/-2	-	unknown	Enhances DNA binding of AR, PR, GR and ER; Abundant chromatin-associated protein; Does not recognize a specific DNA binding sequence.	354
MAGE-11	-	NTD	Binds the AR NTD FXXLLF motif; Competes with the AR N/C interaction; Stabilizes the unliganded AR	272
ART-27	-	NTD	Associated with growth suppression and cell differentiation of prostate epithelial cells, low or negligible levels in prostate cancers showing dedifferentiation.	325, 326
CARM1	-	indirect	Functions in synergy with β-catenin and p300; Binds through β-catenin indirectly to the AR; Overexpressed in prostate carcinoma.	355, 356

1.2.2.2 AR corepressors

Although the number of identified AR corepressors is less than that of AR coactivators, the list of AR inhibiting proteins is still growing [357]. The best-studied AR corepressors so far are NCoR and SMRT.

AR transcription activity can be inhibited by cotransfection of small amounts of the corepressor NCoR in the presence of partial antagonists, higher NCoR expression levels can in the presence of agonists even inhibit AR activity [358, 359, 360]. Possibly NCoR can easily compete with coactivators for binding to the agonist-bound AR, because interaction of coactivators with the AR LBD via its coactivator binding groove is not strong [358]. These results indicate that AR activity will not only be determined by the presence of agonists and/or (partial) antagonists, but also by the ratios of coactivators and corepressors in a cell. The partial antagonist CPA and the antagonist OH-FL show agonistic activities for the AR mutant T877A, present in the lymph node derived prostate cancer (LNCaP) cell line. These activities were hardly affected by NCoR. However, cotransfection of the coactivator TIF2 strongly enhanced the activity of the AR-mutant [359]. This indicates that the antagonist-bound T877A mutant preferentially binds to coactivators reflecting its activation by antagonists. NCoR mediated repression of AR seems independent of HDACs, as was shown by deletion of the HDAC-interacting domain of NCoR [358]. Both the AR NTD and LBD are required for NCoR binding, which is dependent on the CoRNR boxes and independent of ligand and helix 12 [358, 360]. The observations that AR and NCoR are present in the same complexes, as shown by co-immunoprecipitation and ChIP analysis of endogenous proteins, is strong evidence that NCoR is of relevance for regulation of AR function under physiological conditions [358, 360].

Like NCoR, SMRT can bind to both the AR NTD and LBD. It interacts with the LBD via the RID2 corepressor motif. This interaction is enhanced by the presence of the AR DBD-hinge region [361]. SMRT binding to the AR can occur in CV1 cells in the presence of R1881 or the partial antagonist CPA, but not with the antagonists BCA or OH-FL [288]. However, in human kidney 293 cells SMRT can be recruited to the AR in the presence of OH-FL or BCA, indicating cell-dependent SMRT function [361, 362]. SMRT weakly decreases AR activition by agonists, but strongly enhances the AR inhibiting activity of (partial) antagonists [288, 361]. In addition, the antagonist mifepristone (RU486) induces binding of the AR to SMRT, and also to NCoR. This interaction seems even stronger than that induced by CPA, OH-FL and BCA [363]. It was also found that the coactivator SRC-1 can compete with SMRT for binding to the AR NTD, indicating a modulating role for relative coregulator levels in AR function [364].

RAD9, a member of the Rad family of checkpoint proteins involved in DNA damage detection, DNA repair, and cell cycle arrest, was found to interact with the AR LBD. An FXXLF motif in the C terminal region of RAD9 mediates this interaction, thereby inhibiting the AR N/C interaction [365]. In this way, cross talk between checkpoint proteins and AR signalling can occur.

Dosage-sensitive sex reversal adrenal hypoplasia congenita critical region on the X-chromosome gene I (DAX1) is an atypical nuclear receptor, lacking a DBD [366]. It binds to the AR LBD and can sequester the AR in the cytoplasm [367].

Short heterodimeric partner (SHP) is an orphan nuclear receptor that can bind to the AR NTD and LBD and inhibits the activation functions of both AR subdomains. Through its LXXI/LL motifs SHP binds to the AR LBD in an androgen-dependent manner. SHP was shown to compete with the AR coactivators TIF2 and FHL2 for AR LBD binding [368]. SHP induced AR repression was found to be mediated by recruitment of HDACs [369].

The highly conserved region aa 234-247 was found to have an AR inhibiting function if bound to CHIP (see also section 1.2.1.1). CHIP binds to the unliganded AR, which might promote AR degradation [270]. Two mutations, A234T and E236G (see also 1.2.5.2.3), detected in a transgenic mouse prostate cancer model, reduced the interaction with CHIP [370].

Table 2. AR corepressors. *Modified and extended from ref. 357.*

Corepressor	Alternative name	Binding to	Comments	References
ARR19	-	DBD	Inhibits AR activity by recruitment of HDAC4; co-translocates in the nucleus with AR	371
DJBP	-	DBD	Recruits a HDAC1 and mSin3A containing complex.	372
HDAC1	-	DBD-LBD	Inhibits AR activity; no effect on AR protein levels.	300, 373
NCoR	-	LBD	Antagonist-dependent corepressor; competes with TIF2; binding to AR depends on corepressor nuclear receptor boxes, but is independent of ligand.	358-360
PIASγ	-	unknown	Represses AR activity independent of its sumoylation activity, probably by recruiting HDACs.	374
SMRT	-	NTD, LBD	Ligand-dependent corepressor by inhibiting AR N/C interaction and competing for p160 coactivators.	288, 361-364
TGIF	-	DBD	Represses AR activity through the HDAC pathway	375
Calreticulin	-	DBD	Corepresses AR, GR, RAR and RXR; inhibits DNA binding and transcription.	376
Tip110	-	LBD	Binds to AR through its LXXLL motif; prevents binding of AR to AREs.	377
DAX1	-	LBD	Inhibits ligand-dependent transactivation and AR N/C interaction; relocalizes AR in nucleus and cytoplasm; inhibits ER and SF1.	367
LATS2	KPM	LBD	Inhibits AR N/C interaction; lower expression in prostate tumors than in normal prostate.	378
RAD9	-	LBD	FXXLF motif within C-terminus; interrupts AR N/C interaction.	365
ARA67	PAT1	NTD (strong), DBD, LBD	Promotes cytoplasmic retention of AR.	379
PAK6	-	hinge, LBD	Inhibits nuclear translocation of the ligand-bound AR; also inhibits ER.	380-382
Akt	-	unknown	Phosphorylates the AR; represses AR-ARA70 binding	383, 384
PTEN	-	DBD	Inhibits AR via a PI3K/Akt independent pathway in early passages of LNCaP cells.	384, 385
CHIP	-	NTD	Binds to the unliganded AR, which is thought to promote AR degradation	270, 370
Cyclin D1	-	unknown	Reduces AR activity independent of cell cycle progression; coactivator of ER.	386, 387
HBO1	-	DBD, LBD	Ligand-dependent corepressor; member of the MYST/SAS protein family.	388
RIP140	-	NTD, DBD, LBD	Coactivator at low receptor-coactivator ratios, but repressor at high ratios; influences activity of AR, ER, PPARα and PPARγ	349
SHP	-	NTD. LBD	LXXLL motif mediated interaction with AR LBD; inhibits AR and ER.	368, 369, 389
SMAD4	-	DBD, LBD	Inhibits SMAD3-enhanced AR activity by decreasing AR-SMAD3 interaction.	390-392
SRY	-	DBD	Inhibits AR activity.	393
TR2	-	unknown	Inhibits androgen-mediated transactivation in PC3 cells; overexpression causes suppressed PSA expression	394
ZAC1	-	LBD	Interacts with AR, GR, ER and TR; AR coactivator in HeLa cells (synergistic with TIF2) and AR corepressor in 1471.1 cells.	350

1.2.2.3 The AR in transcription regulatory complexes

During the last decade many studies have addressed the question of AR function in cooperation with the plethora of transcription regulatory proteins. Data are ranging from direct interactions with coactivators and corepressors through complex participation in chromatin remodeling, Mediator function, and ultimately to recruitment and activation of the general transcription machinery. In the majority of the studies on this subject the *PSA* promoter and enhancer have been used as AR target. A comprehensive overview of the relationship between the AR and transcription regulatory complexes is given below.

AR regulated recruitment of coregulators

In several studies the complex process of recruitment of coregulators to AR regulated genes has been described, however, the outcomes of the different studies are in some cases conflicting. First, it has been shown that androgens induce recruitment of TIF2, SRC-3, and CBP/p300 to the prostate specific antigen (PSA) enhancer. ChIP experiments in LNCaP cells revealed that the coactivators are preferentially recruited to the strong 4 kbp upstream enhancer of the *PSA* gene and much less efficiently to the proximal *PSA* promoter. Remarkably, promoter-independent recruitment of RNApolII to the enhancer and subsequent induction of transcription has been observed. SRC-3 was found to facilitate RNApolII recruitment [395]. A second study revealed that for both *PSA* and the closely related *kalikrein* gene *KLK2* more AR is loaded onto the enhancers than onto the promoters, but the residence time of the AR on the enhancers was more transient [396]. However, in contrast to the previous study, RNApolII was found to be assembled on the promoters. The reason for this discrepancy is not understood. Binding by different anti-androgens resulted in different AR occupation on enhancer/promoter regions: BCA-bound AR is able to bind to the *PSA* promoter, but not to the enhancer, whereas the partial antagonists CPA and RU486 were capable of promoting AR loading on both the *PSA* enhancer and promoter [396]. CPA and RU486, but not BCA, bound AR led to occupation of both the *PSA* enhancer and promoter by GRIP1, p300, and RNApolII. Like BCA, CPA and RU486 were also able to induce recruitment of NCoR to the *PSA* promoter. Consistent with these findings, treatment with the pure anti-androgen BCA does not result in histone acetylation [396].

In an earlier report androgen-induced association of RNApolII with both the *PSA* promoter and enhancer has been described [362]. However, quantitative differences between both AR target regions were not addressed. Based on this study a model was proposed in which protein-protein contact between the *PSA* enhancer and promoter is via RNApolII, thereby looping out the interval DNA. A scenario of a 'facilitated tracking' mechanism was proposed, whereby RNApolII trails from the enhancer along the entire 4 kb *PSA* upstream sequence to the promoter [395]. This would be in accordance with the finding that the AR can reside for a longer time period on the promoter and RNApolII mainly occupies the promoter, indicating an analysis time point at which AR and RNApolII have reached the end point of the 'preinitiation trail' [396].

AR interplay with components of chromatin remodeling complexes

Although many aspects of chromatin remodeling in AR function remain to be elucidated, a few of the large number of components of chromatin remodeling complexes have been identified to be directly involved in AR function.

An interaction of AR with Brahma (BRM), one of the core ATPases of the SWI/SNF chromatin remodeling complex, was found, correlating with the agonistic or antagonistic properties of the tested ligands [397]. The two largest subunits of the human SWI/SNF chromatin remodeling complex, hOsa1, which is identical to BRM related gene 1(BRG1)-associated factor 250 (BAF250) or ARID1A, and hOsa2 , which is similar to hOsa1, were found to stimulate AR and GR transcriptional activity [398]. Both Osa proteins can interact with hBRM and BRG1, which

are necessary for activation of the *PSA* promoter, but addition of the *PSA* enhancer sequence to the reporter gene overruled this requirement. In contrast, the promoter of the *probasin* gene maintained a low activation level in the absence of SWI/SNF. Addition of the *PSA* enhancer to the *probasin* promoter did not bypass the requirement of the chromatin remodeling complex to achieve increased AR induced activation of this promoter [399]. These findings suggest different mechanisms of chromatin remodeling for enhancer and promoter regions and also for different promoters.

BAF57 is an accessory component of SWI/SNF that can directly bind to the AR. It is recruited to endogenous AR targets such as the *PSA* enhancer upon ligand activation, and loss or inhibition of BAF57 inhibits AR activity [400]. BAF57, together with hBRM, was found to be required for the proliferation of androgen-dependent prostate cancer cells [400]. In addition, BAF57 can cooperate with p160 coactivators and ARA70 and ARA55 to activate transcription of AR target genes [400].

SWI3-related gene product (SRG3), a component of the mouse SWI/SNF complex, is a homologue of human BAF155 and recruits SRC-1. Coactivation of the AR by SRG3 is independent of BRG1 and BRM, indicating that the complete SWI/SNF complex is not required [401].

AR interaction protein 4 (ARIP4) is a SNF2-like protein with DNA-dependent ATPase activity. It was found to modestly enhance AR activity on minimal promoters, but not on the *probasin* promoter [402]. It remains to be established whether ARIP4 is needed for activation of AR regulated natural promoters.

AR interactions with the Mediator complex

Ligand-dependent transcription by the AR in LNCaP cells was found to be enhanced by the TRAP220 (MED1, according to the unified nomenclature for Mediator subunits [86]), TRAP170 (MED14), and TRAP100 (MED24) components of the Mediator complex [403]. In lysates from a Hela-derived cell line, E_{19}, pull-down experiments have shown that in the presence of T or DHT the AR directly binds to TRAP220 through its LBD. ChIP assays have revealed that TRAP220 is recruited to the *PSA* promoter in R1881 treated LNCaP cells [403].

AR interactions with the general transcription machinery

Three components of the general transcription machinery have been identified that interact with the AR. These are the general transcription factors II F and H (TFIIF, TFIIH) and the positive transcription elongation factor b (P-TEFb).

AR NTD strongly binds to the TFIIF subunit RAP74 (TFIIF is a tetramer of two RAP74 and two RAP30 subunits), modest binding was found with the TFIIF subunit RAP30 [404]. RAP74 to AR NTD interaction, which is mainly via the C-terminal domain of RAP74, resulted in increased protease resistance of the AR NTD, indicating that the assumed flexible structure of this AR subdomain can fold into a more stable conformation upon interaction with TFIIF [269, 405].

The highly conserved AR NTD region 224-258 contains several conserved hydrophobic residues that are involved in TFIIF binding: M244, L246, and V248 [406]. Outside this region also S159, S162, S340, and S343 were found to be involved in binding to TFIIF [405]. So, interaction sites for TFIIF are scattered throughout TAU1 in the AR NTD. None of the residues involved in TFIIF binding are binding sites for SRC-1, because this coactivator binds to the C-terminal part of the AR NTD, aa 360-494, which includes TAU5 [108]. The distinct binding sites for TFIIF and SRC-1 enable the AR NTD to make multiple protein-protein interactions simultaneously with coactivators and components of the general transcription machinery [405]. TFIIF was found to play a role in cooperation with TFIIE and TFIIH to overcome stalling of RNApolII after the

formation of the initial phosphodiester linkage [407]. So, the AR might be linked to transcription elongation through TFIIF binding.

TFIIH contains a kinase moiety, CAK, that interacts with AR NTD and enhances AR activity. This interaction may provide efficient communication between AR, GTFs and RNApolII. Transfection of CAK in LNCaP and PC3 cells enhanced AR activity in a ligand-dependent manner, whereas in DU145 cells AR activation by CAK was ligand-independent. It was suggested that CAK can phosphorylate the AR, thereby enhancing the activity of the receptor [408].

The AR can interact with PITALRE, a kinase subunit of P-TEFb. Mutation of this kinase resulted in preferential inhibition of AR-mediated transcription activation. A nuclear run-on transcription assay of the *PSA* gene revealed that transcription efficiency of the distal region of this gene was increased after androgen treatment [409]. So, interactions of AR with TFIIF and P-TEFb are both suggested to influence transcription elongation.

AR NTD was also shown to bind to the general transcription factor TATA binding protein (TBP) [404]. The second largest subunit of RNApolII (RPB2) is able to bind to the AR and can enhance AR transactivation [410]. However, more detailed information of these interactions is lacking.

1.2.2.4 Post-translational modifications of the AR

AR function can be regulated by post-translational modifications like phosphorylation, acetylation, ubiquitinylation, and sumoylation.

Phosphorylation mainly occurs at the AR NTD [411]. Serines at positions 16, 81, 256, 308 and 424 are phosphorylated in an androgen-dependent manner, whereas S94 is constitutively phosphorylated. Phosphorylation of S650 in the hinge region is also induced by androgens and necessary for optimal AR activity depending on cell and promoter context. Specific agonists might differentially regulate the AR phosphorylation status. The functional role of AR NTD phosphorylation is not clear yet. PKA, PKC and epidermal growth factor (EGF) up-regulate the androgen-induced phosphorylation of S650, and can also phosphorylate the AR independent of androgen. This strongly suggests that kinase pathways influence AR function by ligand-independent AR activation or by sensitization of the AR to reduced ligand levels [412, 413]. Apparently, cross-talk occurs between androgen and growth factor signaling pathways [414].

E6 associated protein (E6-AP) (see also section 1.2.2.1) is an E3-type ubiquitin-protein ligase with an intrinsic transactivation function. It can function as a coactivator of many NRs, including AR, in a ligand-dependent manner. However, this coactivation is independent of the ubiquitinylation capacity of this enzyme [329]. A defect in the E6-AP ubiquitinylation function causes the Angelman syndrome, a genetic neurological disorder, but a role of disturbed AR ubiquitylation in this disease has not been established [415, 416]. Recently, the proto-oncogene Mdm2 E3 ligase has been shown to catalyze AR ubiquitylation and proteolysis *in vivo*. ChIP analysis has revealed that Mdm2 associates with the *PSA* promoter and is a component of a promoter-bound multi-protein complex containing AR and HDAC1 [417].

Ubc9 (see also section 1.2.2.1) is a homologue of the E2-type ubiquitin-conjugating enzymes and was found to act as a coactivator of the ligand-bound AR. In stead of ubiquitylation, Ubc9 rather is involved in covalent linking of the small ubiquitin-like molecule-1 (SUMO-1). Similar to E6-AP, the coactivation function of Ubc9 is independent of its sumoylation capacity. Ubc9 binds to the AR DBD-hinge region and has been shown to sumoylate the AR at K386 as the principal sumoylation site and to a lesser extent K520 [172]. A corresponding lysine in the PR (K388) can also be sumoylated [177]. Sumoylation defective mutants of AR and PR showed higher

transactivating activity than the wild-type receptors, indicating an attenuating role for sumoylation [172, 177]. Ubc9 can also bind to and sumoylate the GR DBD, but its effect on receptor activity has not been established yet [172]. Potential SUMO-1 acceptor sites in the GR (K277, K293, K703) and AR (K386 and K520) are located in negative control motifs that restrict the transcriptional synergy of these two receptors [418]. Whereas ubiquitylation is often involved in protein degradation, sumoylation plays a role in subcellular localization, especially in intranuclear targeting, protein stability, and gene transcription [419, 420]. Because these protein modifying mechanisms each have lysine residues as their targets, they might compete with each other, depending on the cellular needs [421].

In addition to acetylation of histones, CBP/p300 and p/CAF are also able to acetylate transcription factors [422]. AR acetylation augments its ligand-dependent transcriptional activity. A highly conserved lysine-rich motif, ^{630}KLKK^{633}, is the acetylation target site in the AR [423]. Acetylation neutralizes positively charged lysines, which may lead to conformational changes that affect protein-protein or protein-DNA interactions of the AR. The HAT activity of Tip60 can activate the AR by direct acetylation [299, 300].

Although CARM1 (see section 1.2.2.1) can increase AR activity it was not found to methylate the AR protein [356]. Possibly this activation is mediated by histone or coactivator methylation.

1.2.3 AR LBD interaction with AR NTD and AR coactivators

1.2.3.1 AR N/C interaction

In full-length AR, TAU1 (aa 100-370) has been identified as essential in transcription activity of the AR NTD. However, deletion of AR LBD shifted the main transcription activation unit in AR NTD from TAU-1 to TAU-5 (aa 360-485) [266]. This finding led to the hypothesis of an interaction between the AR NTD and the AR LBD. Indeed, an androgen-dependent functional NTD-LBD interaction, designated AR N/C interaction, has been detected in protein-protein interaction systems. This interaction could be blocked by anti-androgens [424, 425]. The N/C interaction is direct, as assessed by *in vitro* pull down experiments [331].

Initial deletion mapping studies from our group indicated three regions in the AR NTD to be involved in the functional interaction with the AR LBD: aa 3-36, aa 172-185 (AF-1a), and aa 370-494 [268, 284]. The first region appeared to be an essential interaction domain [284]. Mutational analysis of AF-1a decreased the functional N/C interaction as well as AR transactivation, but an AF-1a peptide was not able to interact with the AR LBD, indicating an indirect role for this domain in N/C interaction [40, 267] (and Steketee, unpublished). As region 370-494 encompasses TAU-5, which probably is a p160 coactivator binding site, it contributes to the transcriptional function of the AR NTD/AR LBD protein complex rather than to the N/C interaction itself [267, 284]. Initial studies from others pointed to the aa 14-150 region as the AR N/C interaction domain [425]. Combined, these findings indicated that the most important interaction domain of the AR NTD is constrained to residues 14-36.

For aa 16-36 a remarkably long amphipathic α-helical structure was predicted, suggesting an important protein interaction interface. *In vivo* and *in vitro* protein interaction experiments have shown that aa 16-36 can interact autonomously with the AR LBD and is essential for N/C interaction. Within aa 16-36, the motif ^{23}FQNLF^{27} appeared to be pivotal for direct N/C interaction [426, 427]. Protein interaction assays showed that the hydrophobic residues F23, L26 and F27 each were indispensable. Amino acids flanking the ^{23}FQNLF^{27} motif modulate AR N/C interaction [290, 291, 427-429]. Therefore, aa 23-27 is referred to as an FXXLF motif in which X can be any amino acid. Region aa 370-494 (encompassing TAU5) contains an FXXLF-related motif, ^{433}WHTLF^{437}, which was postulated to affect the ^{23}FQNLF^{27} mediated functional interaction with the LBD [426]. However, a peptide containing this region was not able to interact with the AR LBD, which excluded ^{433}WHTLF^{437} as an autonomous interaction motif in AR NTD (Steketee unpublished results).

Like for the AR NTD, AR LBD amino acids involved in AR N/C interaction have been studied in detail. The AR FXXLF motif ^{23}FQNLF^{27} shows similarities to LXXLL motifs present in NR boxes of p160 coactivators, and therefore it was hypothesized that the FXXLF motif could bind to the coactivator groove in the AR LBD in a similar manner as LXXLL motifs [106, 430, 431]. As described in section 1.2.2, these LXXLL motifs are essential in the interaction with NR LBDs. They bind to a hydrophobic cleft in LBDs, which is lined by the highly conserved charged lysine residue in helix 3 and the glutamic acid residue in helix 12 [106]. In AR, amino acid residues corresponding to this 'charged clamp' are K720 and E897, respectively. Both were shown to be involved in the ligand-dependent interaction between the AR FXXLF motif and AR LBD, but solely K720 is involved in LXXLL-AR LBD binding [40, 284, 291, 292, 331, 432-434]. So, the FXXLF-mediated AR N/C interaction is comparable, but not completely identical to the LXXLL-

mediated coactivator-AR LBD interaction. In section 1.2.4.4, a detailed mechanistic description of the FXXLF-AR LBD interaction is given.

The AR functions as a homodimer to regulate transcription of its target genes. Initial studies aiming at unraveling the AR N/C interaction have been carried out with the FXXLF motif and LBD coactivator groove present in separate AR protein fragments. Therefore it could not be determined from those experiments whether the interaction is intra- or intermolecular or a dynamic combination of both. More recent FRET experiments in living cells with cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) attached to either end of the AR (CFP-AR-YFP) showed that after androgen treatment an N/C interaction occurs both in the cytoplasm and in the nucleus [435]. A FRET signal between single tagged CFP-AR and AR-YFP fusion proteins indicated dimerization of AR (intermolecular interaction). This interaction is preferentially found in the nucleus. Deletion or mutation of the FXXLF motif in both CFP-AR and AR-YFP did not alter the dimerization signal, but clearly diminished the FRET signal of CFP-AR-YFP. These results indicate that the AR N/C interaction is intramolecular in the cytoplasm as well as in the nucleus, and that AR dimerization does not rely on FXXLF-mediated intramolecular interaction.

1.2.3.2 Relevance of AR N/C interaction in receptor function

The exact role of N/C interaction in full-length AR function has not been completely established as yet. However, several studies have given clues that contribute to elucidating the relevance of the interaction.

Ligand-dependent AR N/C interaction was found to slow ligand dissociation thereby increasing AR activity [291, 331, 426, 436]. Several AR LBD mutations in androgen insensitivity syndrome (AIS) patients have been found with a reduced or completely abolished N/C interaction [331, 436-438]. However, not all AIS mutants cause similar conformational changes leading to disturbance of AR N/C interaction. So, disturbance of N/C interaction cannot be used as a general indicator of AIS. However, if disruption of AR N/C interaction is found for an AIS mutant, this may give a clue for the mechanism of that particular AR mutant. An N/C interaction deficient AR mutant can be translocated to the nucleus, which implies that the N/C interaction is not necessary for nuclear import [439].

Both the ligand-dependent N/C interaction and the transactivating function of the AR (T877A) mutant found in LNCaP prostate cancer cells could not only be induced by androgens, but also by non-cognate ligands as estrogens, progestagens and anti-androgens at physiological or therapeutic concentrations [424]. This reflects the broadened ligand-specificity of this AR mutant (see section 1.2.5.1.3.2), and thus indicates a physiological role of the N/C interaction.

The N/C interaction was proposed to be a prerequisite for p160 coactivator recruitment, supporting an important role of AR NTD in p160 interaction [40, 108]. This would also be consistent with the exclusion of TIF2 binding to the AR LBD reported as an effect of the N/C interaction [440]. However, the AR N/C interaction was found to be influenced in a negative or positive manner by different coactivators as well as corepressors [441]. So, there is no clear correlation as yet between the N/C interaction and coactivator/corepressor interaction with AR.

The N/C interaction might play a modulating role in AR-mediated transcription. N/C interaction disrupting AR mutants tended to be less active on non-specific AREs than on androgen-specific AREs (see section 1.2.5.4) [290].

Recently, it was found that an AR deletion mutant lacking the FXXLF motif does not bind to the MMTV promoter and PSA enhancer assembled on chromatin in *Xenopus* or mammalian cells [439]. However, this AR mutant can bind to a consensus ARE *in vitro* and activates the

MMTV promoter in transient transfection, which are supposed to be experimental conditions without proper chromatin structures. The mutant is also diminished in its binding to the ATPase subunit of SWI/SNF, Brg1 [439]. These findings indicate a role of N/C interaction in chromatin embedded AR function. In section 1.2.2.3 a more detailed description of interplay of AR with components of chromatin remodeling complexes has been presented.

An artificial chromatin embedded promoter containing 4 linked AREs could be activated by the AR FXXLF deletion mutant, indicating that multiple AREs can overcome the chromatin constraints for the N/C interaction deficient AR [439]. Although the MMTV promoter also contains 4 AREs, the ARE arrangement might be insufficient to accommodate the AR mutant properly if packaged in chromatin. In transient transfected LNCaP cells an AR deletion mutant lacking aa 1-36 was able to activate an MMTV-driven reporter, but much less efficient the 6 kbpPSA promoter/enhancer (Steketee, unpublished results). Apparently, PSA transcription has specific demands with respect to the AR N/C interaction, which may be chromatin-independent.

1.2.3.3 N/C interaction in other nuclear receptors

Using a mammalian two-hybrid system, N/C interaction has been found for the rat AR, indicating a universal mechanism of AR function in different species [442].

An N/C interaction has also been described for other NRs. The ERα displays a ligand-dependent N/C interaction, which is disrupted by amino acid substitutions that affect receptor function [42, 443]. The ERα N/C interaction is claimed to be direct and can be induced by the agonist estradiol (E_2) and the antagonist hydroxytamoxifen, but not by the antagonist ICI164,384. It was found that the N/C interaction of ERα was required for SRC-1 mediated synergism between AF-1 and AF-2 function [41, 42]. The coactivator TIF2 was also found to synergize AF-1 and AF-2 functions in ERα [41]. Recently, FRET experiments have shown that ERα N/C interaction can occur intramolecularly [435, 444]. Although ERβ AF-1 is able to bind to SRC1 and to TIF2, it is not known whether AF-1 and AF-2 synergize and, so far, a direct N/C interaction has not been reported for this receptor [179, 445].

For both PR isoforms, PR-A and PR-B, an N/C interaction was reported in the presence of an agonist, but not in the presence of an antagonist [446]. This interaction was much stronger for PR-B, which is the strongest transcriptional activator. This activating capacity is attributed to its N-terminal 164 amino acids, which are lacking in the PR-A. An AF-3 function has been mapped to this region and it contains two LXXLL motifs [447, 448]. These motifs are essential for PR-B transcriptional activity. However, they are thought to bind sites in the PR-LBD which are different from the coactivator binding groove.

For a number of other NRs there are indications of communication between their NTD and LBD. Addition of the MR NTD does not restore the diminished ligand induced activity of an NTD-deleted GR[449]. This suggests a GR N/C interaction. For PPARγ communication between its NTD and LBD has been supposed to regulate ligand binding [450]. However, experiments with CFP-PPARγ2 and PPARγ2-YFP did not result in a detectable FRET signal [435]. For TRβ, it has been observed that its NTD can bind p160 coactivators in a ligand-independent manner. Because its LBD binds ligand-dependently to those coactivators, this suggests an indirect N/C interaction bridged by a p160 coactivator [451-453]. Also the NTD and LBD of RARα1 were found to be bridged by SRC-1, which resulted in synergistic transcription activity [109].

So, NTD-LBD communication has been proposed for other NRs, but a direct N/C interaction is most clearly established for AR and might even be unique for this receptor.

1.2.3.4 AR LBD specifically binds to FXXLF motifs in AR NTD and AR coactivators

Overexpression of coactivator TIF2 can enhance AR LBD AF-2 function, but less efficiently full length AR activity [284]. SRC1 does not need its LXXLL motifs for enhancement of full length AR activity [108]. AR NTD can, through its FXXLF motif, compete with p160 coactivators for binding to AR LBD [440]. These findings indicated that coactivation through LXXLL-AR LBD interaction is not essential in AR function. However, the AR candidate cofactors ARA54, ARA55, ARA70, and RAD9 have been identified to enhance AR activity by binding to its LBD [302, 306, 333, 365]. Importantly, these coregulators mediate this function by FXXLF motifs, which bind to the AR LBD [289]. Random peptide phage display experiments using the AR LBD as well as the full-length AR revealed almost exclusively FXXLF or related motifs, as high affinity interaction motifs [292, 429]. Although some LXXLL motifs, i.e. TIF2 motifs 1 and 3, do have affinity for AR LBD that might equal that of the AR FXXLF motif, most LXXLL motifs tested have a low affinity for AR LBD [289, 291, 292, 428, 432-434, 454]. So, FXXLF motifs in cofactors seem to be specifically involved in AR function.

1.2.3.4.1 Structural background of the preference for FXXLF motifs of the AR coactivator groove

The question of high affinity of AR LBD to FXXLF motifs compared to LXXLL motifs has been addressed by elucidation of the structure of AR LBD complexed with FXXLF motifs. Like reported for LXXLL motifs, the FXXLF motif has a short α-helical structure, of which the phenylalanines fit in the hydrophobic coactivator groove [32, 33]. The +1 phenylalanine binds a hydrophobic part of the AR coactivator groove that is formed by L712 (H3), V716 (H3), M734 (H5), I737 (H5), Gln738 (H5), M894 (H12), and I898 (H12). The +5 phenylalanine binding site is formed by V716 (H3), K720 (H3), F725 (H4), V730 (H5), Gln 733 (H5), M734 (H5), and I737 (H5). The +4 leucine contacts L712 (H3) and V716 (H3) [292, 434]. The AR coactivator binding groove is much deeper than that on the surface of ERα LBD (Figure 10) [291, 292, 433]. This space allows phenylalanine residues, which have bulkier side chains than leucine residues, to enter the groove. In turn, although LXXLL motifs can enter the AR groove, they might lack sufficient hydrogen bonding and hydrophobic interaction necessary for tight interaction. F to L substitutions in FXXLF motifs abolished AR LBD interaction, whereas L/F swaps increased interaction with peptide motifs or even induced AR LBD interaction capacity in non-binding LXXLL motifs [432, 454].

The specificity of the AR groove for FXXLF-like motifs is further substantiated by the prostate cancer AR mutant V730M, which showed an increase in binding of LXXLL motifs , whereas it slightly decreased FXXLF binding [455]. The methionine might provide additional interactions to the +5 leucine of an LXXLL motif without condiserably changing the interaction with the corresponding phenylalanine in FXXLF motifs. So, V730 seems important in FXXLF specificity of the AR LBD.

It was expected that, like for LXXLL motifs, the FXXLF motif is stabilized by interaction with the charged clamp lining the AR LBD coactivator groove [32, 33]. Indeed, structural analysis of an FXXLF peptide-AR coactivator groove complex indicated E897 binding to the +1 phenylalanine of the FXXLF motif, and K720 binding to the +5 phenylalanine [291, 292, 433, 434]. In contrast, LXXLL motif binding to the AR LBD needs K720, but E897 is not involved (Figure 11) [270, 292, 433]. This may, together with the larger size of the coactivator groove, explain the lower binding affinity of LXXLL motifs for the AR LBD if compared to FXXLF motifs.

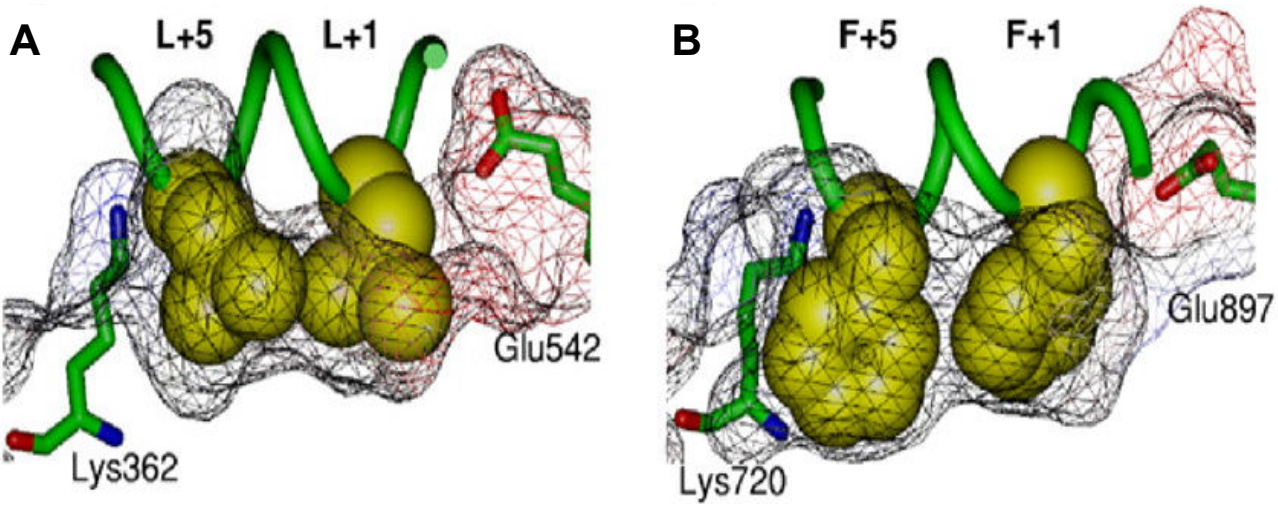

Figure 10. Side views of the coactivator binding grooves/peptide motifs of A, the ER LBD/LXXLL and B, AR LBD/FXXLF. Meshes represent the extent of the ER LBD and AR LBD molecular surfaces, respectively. The LXXLL and FXXLF motifs are represented in *green coils*. Side chains at positions +1 and +5 are in *space-filling* representation and colored in *yellow*. The positions of the charge clamp Lys and Glu are indicated. From ref. 291.

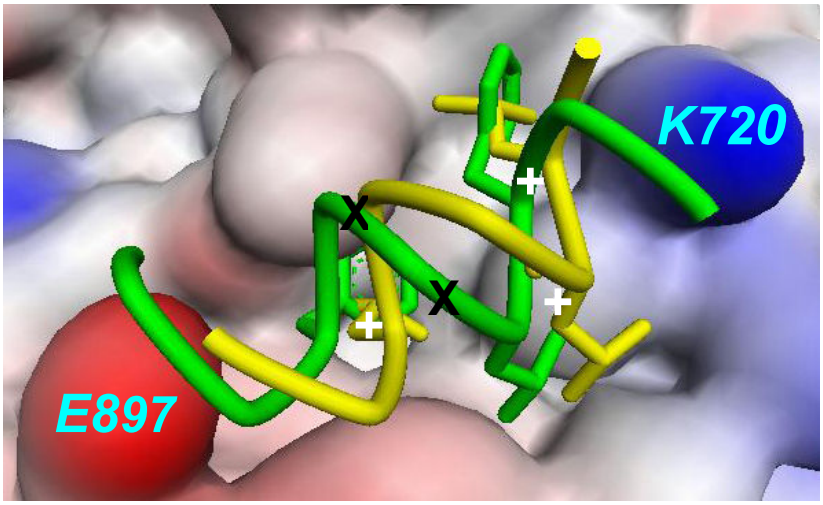

Figure 11. Model of FXXLF (green) and LXXLL (yellow) binding modes to DHT bound AR LBD. +1, +4, and +5 positions as well as the X positions in the motifs are indicated. This picture was kindly provided by H.J. Dubbink.

Like observed for LXXLL motifs, residues flanking FXXLF motifs underlie AR LBD binding specificity [291, 429, 430, 432, 433, 456]. Electrostatic interactions between residues flanking the motif and the charged clamp are supposed to affect this specificity [428]. For example, in addition to binding to the +5 phenylalanine of the AR FXXLF motif, K720 also makes contact with the +8 valine [434]. At the N-terminal side of the motif, E897 binds not only to the +1 phenylalanine, but also to the -1 alanine. This is consistent with a study described in chapter 3, showing that mutations of residues flanking the AR FXXLF motif affected interaction with the AR LBD [427]. The peptides found in phage display studies mostly have positively charged residues N-terminal and negatively charged residues C-terminal to the peptide motif enabling electrostatic interaction with AR LBD [292, 429]. A G21E mutation, N-terminal to the FXXLF motif in AR NTD, impaired the AR N/C interaction [290]. A crystal structure of AR LBD in complex with the

third LXXLL motif of TIF2 has shown that four negatively charged amino acid residues following this motif (DKDD) interact with positively charged patches on the receptor surface. This might explain the selective binding of AR LBD to this TIF2 motif and indicates that certain LXXLL motifs are appreciated by the AR LBD [433].

Residues at positions +2 and +3 in FXXLF motifs may also play a role in affinity for AR LBD. Phage display studies have shown a variety of residues on these positions, which are compatible with strong AR LBD interaction. However, at position +2 E and S residues were found more often, and for position +3 preference for K was reported [432, 457]. Peptides containing the FXXLF motifs of TAFII250 (FLRLF) or TFIIEα (FEDLF) interacted weakly with AR LBD. FXXLF motifs found in CBP (FGSLF) and p300 (FGSLF) could not interact with AR LBD possibly due to a glycine at position +2 [289]. The FETLF motif found in the AR coactivator FHL2 (see also section 1.2.2.1) was also not capable to bind AR LBD [289]. This might be due to the T residue at position +3, but also to sequences flanking the motif. Obviously, not all FXXLF motifs can bind to AR LBD.

Taken together, AR LBD binding preference for FXXLF motifs compared to LXXLL motifs is mainly determined by the hydrophobic phenylalanines, but specific flanking residues may play a role as well.

1.2.3.4.2 Variations of the FXXLF motif

In order to elucidate the mechanism and relevance of FXXLF motifs in AR function and to identify AR specific peptide antagonists, phage display studies and screening of randomized peptide libraries have been carried out using AR LBD or full-length AR as a bait. These experiments have revealed many FXXLF motif variants containing exclusively hydrophobic amino acid substitutions at positions +1, +4, and +5, including F/WXXLF/W, FXXLY/MW, WXXVW, FXXFF, FXXFY, FXXYF, FXXVF, and sporadically LXXLL [291, 292, 429, 457]. Many of these motifs showed a weaker interaction with AR LBD than the AR FXXLF motif. However, like AR FXXLF, many FXXFF, FXXYF, and FXXFY motifs had a high affinity for AR LBD [292, 457]. Crystallographic experiments have revealed that the conformation of FXXYF mimicked closest that of FXXLF if bound to the AR LBD coactivator groove, by making direct backbone interactions with E897. Binding of an FXXFF motif resembled binding of the FXXYF and FXXFY motifs by interaction with its -2 serine residue to E897 [292]. Substitution of the leucine of AR and ARA54 FXXLF motifs by phenylalanine or methionine did not decrease their AR specificity, and the same variants of the less AR-selective ARA70 FXXLF motif even increased its AR specificity [322].

Some FXXLF or related motifs found in the phage display and peptide screening studies have also been found in AR LBD binding proteins. In addition to its FXXLF motif, the AR coactivator FHL2 also contains an FXXLY motif. Although a peptide with its FXXLF motif cannot bind AR LBD on its own (see also section 1.2.4.4.1), both motifs are supposed to be necessary for FHL2 to bind AR LBD [289, 429]. As mentioned before in section 1.2.5.1, the WXXLF motif in the AR NTD only weakly contributes to the AR N/C interaction [289, 428]. An FXXFF and FXXMF motif found in the AR binding proteins gelsolin and PAK6, respectively, showed direct, efficient and specific interaction with AR LBD [322].

Several of the peptides containing FXXLF variant motifs that bind to the AR LBD were able to suppress AR N/C interaction. However, this did not always result in inhibition of AR transactivation [429, 457]. Putative implications of such motifs in inhibiting AR function in order to treat prostate cancer is further discussed in Chapter 5.

1.2.4 Androgen-specific gene transcription

1.2.4.1 General mechanism of androgen regulated gene transcription

For an increasing number of androgen regulated genes, the mechanism of androgen action has been at least partially elucidated [52, 458-497]. Identification of novel androgen regulated genes has been accelerated during the last 5 years, because techniques have evoluated from subtractive hybridization and differential display to the high-throughput expression microarrays. Array technology has been used for gene expression profiling of prostatic tissue and different prostate cancer cell lines in the presence and absence of androgens [498-504] (reviewed in ref. 505). This has resulted in an extensive and still growing catalogue of genes being up- or down-regulated by androgens. Bioinformatics assisted analysis of the transcription regulation mechanisms of this huge number of genes will help to further elucidate the mechanism of action of androgens. In the near future whole genome identification of androgen regulated genes is to be expected, as now already started for ER regulated genes [506].

Basically, androgen induced transcriptional regulation occurs through ligand-activated AR dimers that bind to AREs in the regulatory regions of target genes. AREs have been found in promoters and in enhancers upstream or downstream of the promoter. Many AREs deviate considerably from the inverted repeat high affinity ARE consensus sequence 5'-AGAACAnnnTGTTCT-3' and, in many cases, have a relatively low affinity for the AR. In transfection experiments, those AREs are individually not able to show detectable androgen inducibility, but together act synergistically, thereby conferring very effective androgen induced transcriptional regulation. Cooperatively acting AREs can be located in close vicinity of each other, but can also be separated by long distances.

An illustrative example of androgen regulated transcription is that of the extensively studied prostate specific antigen (PSA) gene. Its proximal core promoter contains two AREs, ARE-I at -170 (AGAACAgcaAGTGCT) and ARE-II at -394 (GGATCAgggAGTCTC) [463, 491]. These AREs act cooperatively, albeit that their total activity is still weak [463]. A third ARE, ARE-III (GGAACAtatTGTATC), which is present in an enhancer region at -4.2 kbp of the PSA gene, is able to confer considerable androgen induced transcriptional activation of a reporter gene. Combination of the enhancer region and the core promoter synergistically results in a strong androgen inducibility [462]. In the vicinity of ARE-III, five additional candidate AREs have been reported: ARE-IIIA, ARE-IIIB, ARE-IV and ARE-VI, which have a low affinity for the AR, and ARE-V with moderate affinity to the AR. These AREs might increase PSA enhancer activity.

1.2.4.2 Androgen-specifically regulated genes

Many androgen regulated genes, like the PSA gene, have been found to be also regulated by other steroids, because GR, MR, PR and AR recognize the same sequences in the DNA. However, during the last decade, genes have also been found that can be specifically regulated by androgens. As mentioned in section 1.1.2, this specificity can be caused by different mechanisms, including tissue specific AR expression, specific coactivator/corepressor expression, ligand availability and local chromatin structure [4, 7, 51, 53-55, 59].

A mechanism leading to receptor specificity can also be found in the hormone response elements. Deviations from the HRE consensus sequence have been found to lead to receptor specific transcriptional regulation by AR, PR, GR, and MR. Flanking sequences can also contribute to

receptor preference [507]. However, for the AR a clear specificity appears to exist. For several genes identified as specifically regulated by androgens, a distinct class of HREs has been found to be highly androgen specific. Most of these AREs deviate considerably in one half-site from a standard ARE [52, 494, 496, 508-512].

Up to date, five genes harboring androgen specific AREs have been identified. Features of these genes and their androgen responsiveness are described below. An overview of their androgen specific AREs is presented in Table 3.

Probasin

The rat probasin gene encodes a nuclear and secreted protein, which is expressed in epithelial cells of the dorsolateral prostate [513]. Its transcription can be induced by both androgens and glucocorticoids, but its androgen inducibility is much stronger than its regulation by glucocorticoids [481, 514-516]. The probasin gene was the first gene described to be preferentially regulated by androgens. Its promoter contains two cooperative AREs, PbAREI and PbAREII, which were initially found not to function individually, but if duplicated in front of a heterologous minimal thymidine kinase promoter, androgen inducibility for PbAREII was observed [52, 481, 490]. PB-ARE1 shows a classical inverted repeat sequence and has a low affinity for the AR. PB-ARE2 (see Table 3) is specifically responsive to androgens [52]. PbAREII can bind to the AR, but not to the GR, and it has a higher AR binding affinity than PbAREI [52, 517]. The left hand half-site of PbAREII, 5'-GGTTCT-3', determines its AR specificity by excluding GR binding, as was deduced from experiments using chimeric response elements with swapped half-sites [52]. In an upstream enhancer fragment of the probasin gene two additional AREs were identified, ARBS3 and ARBS4. Like PbAREII, ARBS3 is also androgen specific and has also one half-site that deviates considerably from a consensus half site (see Table 3) [518]. Two additional low affinity AREs with atypical half-site sequences, G-1 and G-2, are located near ARBS1 and ARBS2. These were postulated to stabilize AR binding to ARBS1 and ARBS2 and result in synergistic transcriptional activity and increased hormone sensitivity [519]. All AREs found in the probasin gene appeared to be necessary for full androgen induction of probasin transcription [518].

Secretory component

The rat Secretory component (Sc) is the transepithelial transporter of immunoglobulins A and M [520]. In many tissues, expression of the Sc gene is constitutive. In the prostate and the lacrimal gland its expression is modulated by androgens [521, 522]. In the -3.5 kbp enhancer region of the *Sc* gene, an androgen specific ARE, designated ScARE1.2, has been found, which has a relatively high affinity for the AR (see Table 3) [496]. Exon 1 of the *Sc* gene contains a second androgen specific ARE, ScARE (see Table 3), which is responsive to glucocorticoids, but, despite of a low AR affinity, its androgen response is much stronger [475]. Mutational analysis of ScARE1.2 and ScARE showed that their left half-sites excluded or decreased GR binding. AR selectivity disappeared by mutating these half-sites to more consensus-like sequences [496, 508, 511].

Slp

The mouse sex-limited protein (*Slp*) gene is a duplicated *C4* gene encoding a liver-specific protein. Its transcription is regulated by androgens. The *Slp* gene contains an enhancer region at -2 kbp, which was introduced by insertion of an ancient provirus [523]. A fragment of this enhancer is specifically responsive to androgens [524]. It contains three candidate AREs. One is not functional, a second one is active with both AR and GR, and the third one, SlpHRE2 (see Table 3), is androgen specific [511, 525]. The SlpHRE2 was initially considered as an ARE of which the flanking sequences could bind other transcription factors, thereby allowing AR binding, but excluding GR binding. However, although additional factors do contribute to its specific

androgen regulation, the SlpHRE2 was proven to confer androgen specificity autonomously with a relatively high AR affinity [511, 525-529]. Like in the probasin and Sc androgen-specific AREs, the left half-site of the SlpHRE2 determines the exclusion of the GR to function via this HRE [511].

Pem

The *Pem* homeobox transcription factor gene is highly conserved between rat and mouse, and is expressed in reproductive organs as well as in muscle and placenta [530]. For its expression the rat Pem gene was shown to use two different promoters. A distal promoter is independent of androgens and active in testis, ovary, muscle, and placenta. A proximal promoter is located in intron 2, close to exon 3 which harbors the translation start codon. This proximal *Pem* promoter is androgen-dependent and controls expression in testis and epididymis [530]. Analysis of the mouse *Pem* proximal promoter revealed that this promoter contains two androgen specific AREs, PemI and PemII (see Table 3), which act cooperatively and are both selectively responsive to androgens [458]. The Pem AREs have a preference for the AR, but are also responsive to GR and PR, albeit to a lesser extent. Both PemI and PemII show a low relative affinity for the AR. In contrast with standard AREs, PemI contains a 5 bp spacing. This does not prevent responsiveness to androgens, but reducing the spacing to three nucleotides increases its activity [458].

SARG

The specifically androgen regulated gene (*SARG*) was identified in the human LNCaP subline LNCaP-1F5, which expresses AR and GR in comparable amounts. In this cell line, transcription of *SARG* can be up-regulated by androgens but not by glucocorticoids, whereas *PSA* transcription is stimulated by both hormones [531]. In intron 1 of the *SARG* gene, at +4.6 kbp, an androgen specific ARE, designated SARG+4.6ARE (see Table 3) is present [494]. SARG+4.6ARE has a lower affinity for AR, compared to probasin PB-ARE2. SARG+4.6ARE cannot bind GR, and in transfection it is not responsive to glucocorticoids. The identification and a detailed analysis of the *SARG* gene is described in Chapter 4 of this thesis.

Table 3. Androgen-specific androgen response elements.

ARE name	ARE sequence -6 -5 -4 -3 -2 -1 +1 +2 +3 +4 +5 +6	References
PbAREII	**GGTTCT** tgg **AGTACT**	52
ARBS3	**AGAACC** tcc **AGTTCC**	518
ScARE1.2	**GGCTCT** ttc **AGTTCT**	496
ScARE	**AGCAGG** ctg **TGTCCC**	475
SlpHRE2	**TGGTCA** gcc **AGTTCT**	511
PemI	**AGATCT**cattc**TGTTCC**	458
PemII	**AGCACA** tcg **TGCTCA**	458
SARG+4.6ARE	**TGTGCT** aac **TGTTCT**	494

1.2.4.3 Mechanism of AR binding to androgen-specific AREs

Like for non-specific AREs, androgen-specific AREs with different AR affinities have been found [52]. SARG+4.6ARE and SC-ARE weakly bind the AR, but are both highly androgen-specific, they cannot bind to the GR. However, the low AR affinity Pem ARE1 and Pem ARE2 show weak glucocorticoid responses. PB-ARE2, Slp HRE2 and SC-ARE1.2 have relatively high affinities for the AR, but also show differences in androgen specificity. PB-ARE2 and Slp HRE2 are not responsive to glucocorticoids, but SC-ARE1.2 can confer weak glucocorticoid induced transcriptional activation. So, it is not their relative affinity for the AR, but their preferential binding to the AR over the GR that may contribute to the androgen specific responsiveness of AREs. Additional indications for this hypothesis were provided by AR-GR chimeric receptor studies which revealed a crucial role for the AR DBD in androgen-specific activation of the Slp HRE2 [532].

For most androgen-specific AREs it has been established that the left half-sites diminish GR binding (see Table 3). It has been postulated that in this half-site a T at position -4 is responsible for GR exclusion [511]. However, Pem-ARE2 and SC-ARE have an A at this position and SARG+4.6ARE a G. In addition, the G at position -3 of the SC-ARE is indispensable for androgen specificity, but no other androgen-specific ARE has a G at this position [508]. A T at position -2 was also supposed to contribute to androgen specificity, but is also not present in all androgen-specific AREs [511]. So, specific nucleotides in the androgen-specific AREs seem not to be individually responsible for androgen specificity.

Since androgen specificity of AREs could not be explained by their relative affinity for the AR or specific nucleotide composition, it was proposed that another characteristic of androgen specific AREs is the determining factor. Most of the natural and synthetic androgen-specific AREs investigated so far can be considered as (im)perfect direct repeats rather than inverted repeats. Several crystallographic studies have shown a head-to-head orientation of NR DBD dimers binding to an inverted repeat response element and a head-to-tail orientation for direct repeat elements (reviewed in ref. 21). Therefore, it was suggested that an AR dimer binds to an androgen specific ARE in a head-to-tail orientation [508, 510, 511]. This would imply an alternative dimerization of the AR DBD when bound to an androgen-specific ARE. In this way, the GR would be more or less excluded from binding to the androgen specific AREs, because it could not form head-to-tail DBD dimers. Unlike the first zinc cluster, the second zinc cluster of a DBD does not contact the DNA, but is an important part of the dimerization interface. Indeed, the second zinc cluster and a C-terminal extension of the AR DBD were previously found to be involved in DNA-specificity [509]. This was substantiated by mutation to GR homologues of second zinc cluster residues Thr585, Gly610, and Leu617, which caused a lower affinity of the AR for PB-ARE2 [510]. Although these findings indicate that specific AR DBD dimerization plays a role in AR binding to androgen specific AREs, AR DBD dimer orientation on these AREs could not be determined from those experiments.

Ultimately, the orientation of AR DBD dimers on androgen-specific AREs was elucidated by a crystallographic study that showed that AR DBDs dimerize in a head-to-head fashion to a synthetic androgen-specific ARE (ADR3) (Figure 8 in section 1.2.1.2) [24]. This head-to-head oriented DBD dimer indicated that an important part of the mechanism underlying androgen-specific transcriptional regulation indeed could be found in the AR protein itself. So, the previously proposed head-to-tail dimerization of AR DBD, compared to that of the GR-DBD, is related to the non-standard ARE character of androgen-specific AREs. All androgen-specific AREs (Table 3) are comprised of one half-site with a complete or almost complete consensus sequence, which is known to bind AR as well as GR with high affinity. The specific half-site

can be designated as non-consensus, and will have a very low affinity for both AR and GR. It is thought that one DBD binds to the consensus half-site and that the second DBD can cooperatively bind to the non-consensus half-site only when there is a strong interaction between the DBDs. Indeed, crystallographic data revealed three extra hydrogen bonds between the second zinc clusters in the AR DBD dimer if compared to the GR DBD dimer (Figure 12) [24, 510]. A hydrogen bond can be formed between S580 of both AR DBD monomers, and T585 of each AR DBD monomer can hydrogen bond to A579 of the opposite monomer. So, the dimerization of AR DBDs is supposed to be strong enough to overcome a low affinity half-site, and as also previously proposed, the GR tends to be excluded from the non-consensus half-site of an androgen specific ARE because of a much weaker dimerization interface.

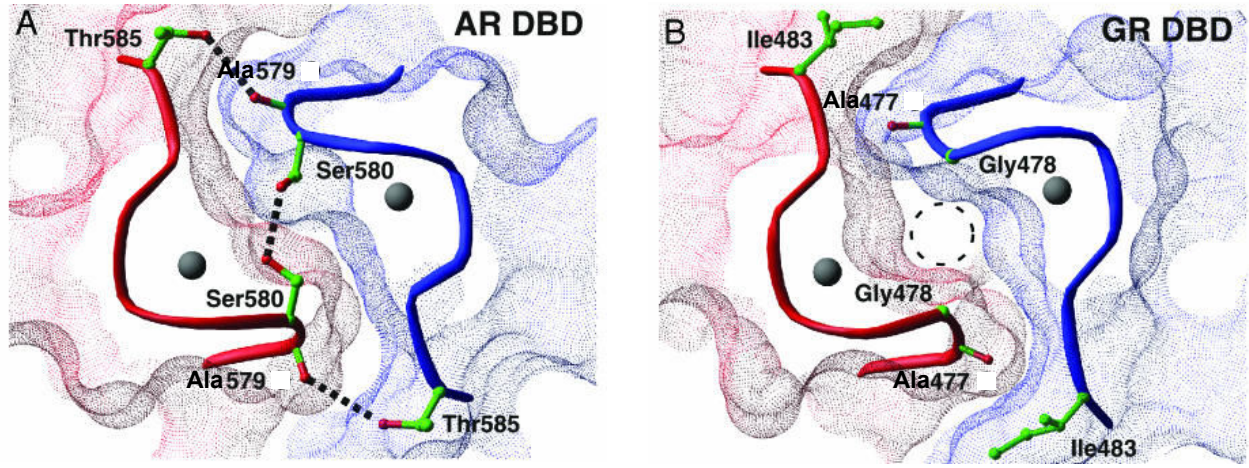

Figure 12. Molecular mechanisms of AR and GR dimer interfaces *A*) The AR DBD dimer interface. The molecular surfaces of the AR subunits are shown in red and blue. Dashed black lines are hydrogen bonds. (*B*) A similar view of the GR DBD dimer interface. A "glycine hole" is noted by the dashed circle. Adapted from ref. 24.

1.2.4.4 Role of androgen-specific AREs

Recently, several studies have revealed possible roles for the androgen-specific AREs. Androgen specific AREs seem to reduce the need for the AR N/C interaction for transcription regulation (see section 1.2.5.1) [290]. The non-specific AREs TAT-GRE, slp-HRE3, and C3 (1)-ARE, are responsive to wild type AR, but not to a N/C interaction defective mutant, whereas the androgen specific Slp HRE2 and sc-ARE1.2 (see section 1.2.6.2) are equally responsive to the wild type and mutant AR. Mutation of the latter AREs to non-specific ones decreased their responses to the AR mutant. However, the androgen-specific Pb-ARE2 required the AR N/C interaction for its androgen responsiveness. The better resemblance of its left half-site to high affinity binding sites may play a role in this regard (see section 1.2.6.3). The N/C interaction-dependent AREs were no longer dependent on the interaction if they were present in their natural enhancer/promoter regions. It was suggested that the higher complexity of these regions might overcome the need for AR N/C interaction in androgen responsiveness.

A second study also described the requirement of N/C interaction for AR activity on non-specific elements [533]. These AREs were all tested in the context of their corresponding enhancer/promoter sequences, like the PSA enhancer/promoter and the probasin enhancer, which is in conflict with the first report in which N/C interaction requirement was only found

for isolated non-specific AREs. In contrast, the same study reported that for the MMTV-promoter, the AR N/C interaction was dispensable, although it contains non-specific AREs.

The function of several AR associated proteins could be differentially influenced by androgen-specific and non-specific AREs. Overexpression of the AR coactivators TIF2 and ARA55 was reported to increase AR activation on androgen specific AREs rather than on non-specific AREs [534]. Two proteins involved in mediating AR SUMO-ylation, (PIAS)xα and Ubc9 (see section 1.2.2), had, if overexpressed, different effects on non-specific and androgen specific AREs [534]. (PIAS)xα repressed AR activity much more on androgen-specific ARE driven genes than on genes regulated by non-specific AREs. Ubc9 showed little effect on androgen-specific AREs, whereas it enhanced AR activity on non-specific elements. Additionally, mutational analysis of an AR SUMO-ylation site revealed that overexpression of SUMO-1 repressed AR-mediated transcription only on non-specific elements [535].

1.2.5 The AR in disease

The AR is, next to its central role in normal development and maintenance of the male phenotype, also an important player in several diseases. AR associated diseases are androgen insensitivity syndrome (AIS), spinal and bulbar muscular atrophy (SBMA) or Kennedy's disease, and prostate cancer.

AIS is a rare inherited defect of male development in which 46, XY individuals have a partial or complete lack of virilization, referred to as partial AIS (PAIS) or complete AIS (CAIS) [536, 537]. In this disorder, over one hundred different amino acid substitutions in the AR in different individuals or families have been documented [263]. These substitutions partially or completely inactivate AR function by affecting its ligand or DNA binding. AIS can also be caused by coactivator defects [538].

SBMA or Kennedy's disease is a spinobulbar motor neuropathy with an adult-onset and associated with mild PAIS [539, 540]. This disease is caused by an expanded CAG repeat in the AR NTD, which results in a longer poly-glutamine stretch [541]. AR containing aggregates are formed in the cytoplasm of motorneurons, but the exact molecular mechanism of this disease is still unknown [542, 543].

In Western countries prostate cancer forms a significant health problem as it is the most frequently diagnosed male cancer and second leading cause of cancer deaths in men [544]. The role of AR in prostate cancer is discussed in the 1.2.5.1 subsection below.

1.2.5.1 The AR in prostate cancer

1.2.5.1.1 Prostate cancer development and progression

The prostate is a walnut-sized gland located below the bladder and surrounding the urethra. It produces approximately 30% of the seminal fluid, which contains secreted proteins like prostatic acid phosphatase (PAP) and the semen liquefying serine protease PSA [545, 546]. Measurement of PSA serum levels is a well known diagnostic tool of prostate cancer. Benign prostatic hyperplasia (BPH), which is a common disorder in men over age 50, arises in the transitional zone of the prostate, whereas most prostate cancers originate in the peripheral zone (see for review ref. 547).

The glandular acini of the prostate are lined by a double-layer of epithelial cells. A basal layer of cuboidal cells is attached to the basal lamina. The basal layer is covered by a layer of columnar mucus-secreting cells lining the lumen of the gland. The luminal epithelial cells express cytokeratins 8 and 18, AR, PAP, and PSA [548]. The basal cells express cytokeratins 5 and 14, p63, and bcl-2 [434, 548, 549]. Most prostate cancers have a predominantly luminal phenotype with expression of keratins 8 and 18, and AR. However, a small percentage of the cells in a prostate tumor might express keratins 5 and 14 indicating a basal cell type. Like in normal tissues, in several cancers tumor-initiating cells have been found, which are called cancer stem cells (CSCs) [550-556]. The keratin 5 and 14 positive prostate tumor cells were suggested to be prostate CSCs (PCSCs). Additionally, cells with an intermediate phenotype have been found to express either keratin 17 or 19, and low levels of keratin 8 and 18 [557-559]. These cells are considered to form an androgen-independent transit amplifying (TA) population. This led to the hypothesis of keratin 5 positive and AR negative PCSCs, which are androgen-independent and give rise to androgen-dependent, fully differentiated luminal cells via a TA population [560]. PCSCs are supposed to arise from normal stem cells, which are believed to reside in the basal layer [561]. Evidence for this is provided by the finding of CD133 and α2β1-integrin, which have

been identified as normal prostate epithelial stem cell markers, in 0.1% of prostate tumor cells, regardless of Gleason grade and metastatic states [562-565]. *In vitro* these cells can, like normal stem cells, give rise to a mixed population of $CD133^+$ and AR^- cells, and more differentiated $CD133^-$ and AR^+ cells [563, 564]. The more differentiated cells develop through subsequent TA and intermediate stages into non-proliferating mature cells [566]. Whether the CD133+ cells indeed can form tumors remains to be established.

Like normal development and maintenance of the prostate, prostate tumor growth depends on continuous stimulation by androgens [567]. Because of this androgen dependence, prostate cancer therapy is generally based on androgen withdrawal, which is achieved by inhibition of testicular androgen production using LHRH agonists, and/or blockade of AR function by antiandrogens such as BCA or OH-Fl. However, after an initial regression, essentially all prostate tumors continue to grow and become androgen-independent, which means independent of androgenic stimulation. Although loss of AR expression has been found in some tumors, studies of prostate tumors of various clinical stages have revealed that most androgen-independent prostate cancers retain AR expression [558]. In androgen-ablated progressive prostate cancer the AR is still primarily located in the nucleus and androgen-regulated genes are expressed [568-571]. That the AR indeed still is playing an important role in androgen-independent prostate cancer is further substantiated by RNAi driven AR inhibiton in androgen-independent prostate cell lines, which decreased PSA expression, cell proliferation and survival [572-576].

Important and challenging questions in prostate cancer research are how prostate tumors develop and how transition from androgen-dependent to androgen-independent prostate cancer occurs and which role the AR plays in these processes.

1.2.5.1.2 Androgen-dependent prostate cancer

Because early stages of prostate tumor growth are dependent on androgens, the underlying mechanisms are expected to involve AR function. Although a large number of androgen regulated genes has been identified in expression array experiments, until recently none of these was specifically identified to play a direct role in the androgen-dependent tumor stage. However, recently, the androgen regulated *TMPRSS2* gene was found to be highly relevant in androgen-dependent prostate cancer [577, 578]. Fusions of the promoter and first exon(s) of the *TMPRSS2* gene with members of the ETS transcription factor family, *ERG* or *ETV1*, have been found in the majority (estimate of 60%) of primary prostate tumors. These gene fusions are caused by interstitial deletions and translocations [577-581]. The fusion genes are androgen-responsive and highly expressed in tumor tissue, and therefore ETS factors are overexpressed if compared to normal prostate tissue [582, 583]. Expression of the *TMPRSS2:ERG* fusion genes was found to correlate with a higher recurrence rate [584]. In addition, different isoforms seem to be associated with clinical and pathological variables of aggressive disease, and therefore might serve as prognostic factors for tumor progression [585, 586].

1.2.5.1.3 Mechanisms of AR activation in androgen-independent prostate cancer

For androgen-independent prostate tumor growth G. Jenster (personal communication) has proposed several mechanisms in which, despite of androgen withdrawal, the AR is still able to stimulate tumor growth (Figure 13). These mechanisms include: AR amplification and/or overexpression, AR mutations, intraprostatic conversion of adrenal androgens to DHT, ligand-independent AR activation, and aberrant AR coregulator expression and function. In addition, several cellular pathways that by-pass the AR have been found to influence prostate tumor growth (See for reviews refs 505, 587-591).

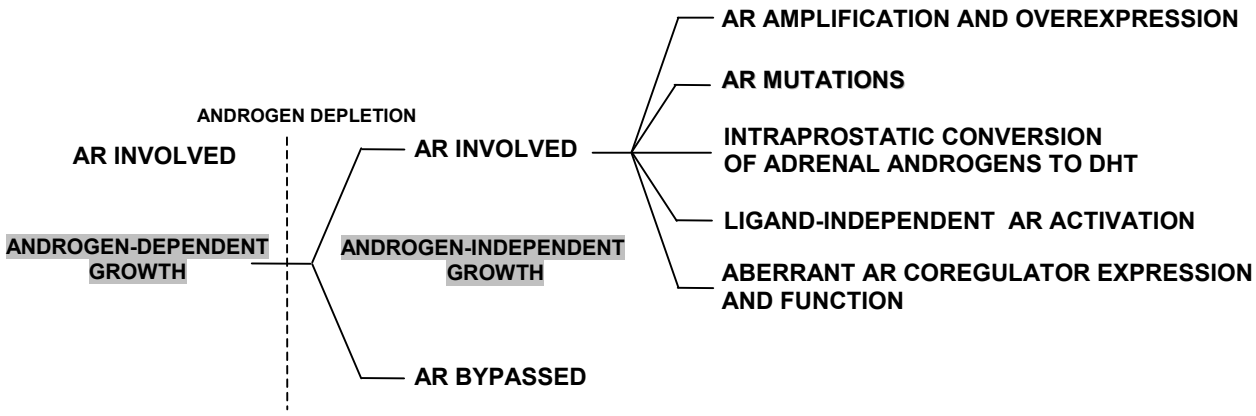

Figure 13. Schematic overview of the role of the AR in prostate tumor growth. Proposed and designed first by G.Jenster

1.2.5.1.3.1 AR amplification and overexpression

AR overexpression, as found in a subset of prostate cancers, can be caused by several mechanisms of which gene amplification is the best studied. In approximately 30% of androgen-independent prostate tumors, *AR* gene amplification has been detected, whereas this is a rare event in untreated primary tumors [592-597]. In a study of matched paired androgen-sensitive and androgen-independent tumors, 80% of androgen-independent tumors with AR gene amplification showed an increase in AR protein [598]. Although AR amplification is not always accompanied by increased AR levels, it was found to correlate with a shorter life expectancy after relapse of prostate cancer [598]. Of androgen-independent tumors without AR amplification, 35% showed increased AR levels [598]. Comparable observations were done in a study of seven paired androgen sensitive and androgen independent prostate cancer xenografts [599]. Increased AR expression was suggested to enable the tumor cells to respond to low androgen levels present during androgen withdrawal therapy. This finding was substantiated by experiments in which overexpression of AR in LNCaP cells by transfection led to a higher sensitivity of these cells to low androgen levels followed by more rapidly progress to hormone-refractoriness [599].

Because not all cases of increased AR expression can be explained by gene amplification, also other mechanisms that regulate AR levels are supposed to be involved in androgen-independence of prostate tumors. These include increased *AR* transcription, stabilization of AR mRNA, and stabilization of the AR protein (reviewed in ref. 588).

1.2.5.1.3.2 AR mutations

AR mutations are rare in primary and locally progressive untreated prostate tumors, but are more common in high grade androgen-independent prostate cancer and distant metastases of patients following endocrine therapy. The relative prevalence of AR mutations found in androgen-independent prostate cancer varies between different studies, but is estimated to be approximately 10% [600]. A proportion of the prostate cancer related AR mutations enhance AR activity, although there are also examples of AR inactivating mutations. Most AR mutations lead to gain-of-function by altering the ligand specificity of the AR allowing adrenal androgens, non-androgen steroids, and even antiandrogens to activate the receptor. The majority of AR mutations found in prostate cancer are confined to 8% of the coding region dispersed over

distinct regions of the receptor, but they are most common in the LBD [601]. AR mutations identified in prostate cancer are deposited in the Androgen Receptor Mutations Database (http://www.mcgill.ca/androgendb)[263].

Mutations in the NTD are rare, but they might affect AR interaction with coregulators [602]. A E236G mutant is thought to increase AR activity through reduced binding of CHIP (see 1.2.2.1) and concomitantly increased responsiveness to coactivators ARA160 and ARA70 [370]. This mutation caused rapid development of intraepithelial neoplasia that progressed to invasive and metastatic prostate carcinoma in a mouse model [370]. Several mutations in the hinge region affect AR activity. S646F substitution was detected in a high grade tumor and increased AR activity [603]. A Q640Stop nonsense mutant, which appears as a truncated AR, is constitutively active in the absence of ligand [604]. A C619Y substitution is thought to inactivate and change localization of the AR [605]. AR mutations at the boundary of the hinge region and LBD show an enhanced response to androgens, but also to other steroidal and nonsteroidal ligands [606]. A748T substitution in the LBD caused rapid degradation, has a higher ligand dissociation rate and a low expression level, if compared to the wild-type AR [607].

Best studied are the mutations in the AR LBD. Often, these mutations considerably alter the ligand-specificity, which includes responsiveness to adrenal androgens, glucocorticoids, progesterone, estrogens, and even antiandrogens. The first AR mutation identified was T877A in the LNCaP cell line, which was derived from a orchidectomized and E_2 treated patient [608, 609]. Subsequently, this mutation was repeatedly found in prostate cancer tissue specimens of patients with advanced disease and treated with OH-Fl [280, 610-614]. Less frequently found mutations are H874Y in the androgen-independent prostate cancer xenograft CWR22 and in a few androgen-independent OH-Fl treated prostate cancer specimens, and T877S, which is found twice in androgen-independent OH-Fl treated tumors [281, 615, 616]. The T877A substitution rendered the AR responsive to progesterone, E_2, DHEA, cortisol, OH-Fl, and cyproterone acetate. Both H874Y and T877S induce, albeit to a lesser extent, similar properties to the AR (see Chapter 4) [280, 281, 494, 613, 615-618]. A double AR mutant, L701H/T877A, found in the MDA PCa 2a cell line, which was derived from a bone metastasis of a orchiectomized prostate cancer patient, has properties similar to that of the T877A mutant, but it binds cortisol with a higher affinity [614, 619, 620]. Incubation of MDA PCa 2a cells with cortisol is associated with PSA expression and stimulation of growth [620]. AR V715M was found in a OH-Fl treated metastasis and V730M was from an organ-confined untreated prostate cancer. Both mutants can be activated by adrenal androgens [621-623]. In a BCA treated prostate cancer, a W741C mutation was found. The same mutation and another one at the same position, W741L, were detected in an LNCaP subline chronically treated with BCA [624, 625]. This mutant could not be activated by OH-Fl [626].

AR T877A mutation seems a hot spot in androgen-independent prostate cancer [280, 610-613]. It is believed that it has arisen by selection of an AR mutant that could be activated by E_2 or OH-FL that have been used as treatment. This selection process was mimicked by screening of AR expression libraries with random mutations at codons 874 or 877 in a yeast read out system in the presence of Pg. This hormone was chosen because it is the best activating non-cognate ligand of the AR H874Y and T877A mutants found sofar. The T877A mutant was obtained most frequently, followed by the T877S and H874Y mutants (see Chapter 4) [494]. When tested in mammalian cells, these mutants showed broadened ligand responses, including responsiveness to OH-FL. This indicates that the mutants found in prostate cancer could be selected with non-cognate ligands that can activate the mutant ARs and therefore prostate tumor growth. It is assumed that therapeutic anti-androgen levels are sufficiently high to confer growth advantage to cells harboring those mutants and thus favor tumor growth.

Homology modeling and crystallographic analysis of wild-type and mutant AR LBD bound to agonists or non-cognate ligands have revealed possible structural mechanisms of the broadened ligand responsiveness of AR mutants identified in prostate cancer [277, 278, 626-630]. The ligand binding pocket of the AR is lined by 18 amino acid residues, of which most are hydrophobic, except N705, Q711, R752, and T877 [278, 630]. N705 and T877 form hydrogen bonds to the 17β hydroxyl group of the D-ring of R1881, R752 forms a hydrogen bond to O-3 in the A-ring of R1881. Q711 binds to O-3 through a H2O molecule. T877 is a very important amino acid in determining ligand specificity, because substitution by a less bulkier amino acid, like in the frequently found T877A mutant, enables binding of other ligands like progesterone, estrogens, adrenal androgens, antiandrogens, and even glucocorticoids [277, 278]. The alanine residue in T877A is not able to form a hydrogen bond to the 17β hydroxyl group of the ligand, either DHT or R1881, but N705 binding is still sufficient for ligand binding (see Figure 14) [277, 278]. In addition, L880 makes van der Waals contact with the 17β oxygen atom of R1881. On its corresponding position, the PR has a threonine (T894), which has a less bulkier side chain. This amino acid is supposed to be responsible for binding of progesterone to the PR and not to the wild-type AR [278].

Homology modeling has predicted that binding of the wild-type AR to non-steroidal antagonists like OH-Fl includes N705, Q711, and R752 [629]. Crystallographic analysis of the wild-type AR LBD bound to agonists that resemble the OH-Fl structure, has shown that hydrogen bonding to T877 is not involved, and that the side chain of this amino acid is rotated 180° compared to its position in the receptor bound to steroidal ligands [627]. This leaves some space between T877 and the non-steroidal ligand, which is increased in the T877A mutant and becomes occupied then by a water molecule that bridges the ketone group of the non-steroidal ligand to the backbone oxygen of L873 [627]. This interaction is thought to increase the agonistic activity of such a ligand. Similarly, crystallographic data on the T877A mutant bound to the partial agonist CPA have shown that this ligand induces movement of the L701 side-chain. This leads to an expansion of the ligand binding pocket that is supposed to enable the bulky CPA molecule to act as a full agonist [628].

Compared to the T877A mutant, in the AR double mutant L701H/T877A there is even more space to accomodate non-cognate ligands with bulkier substituents at position 17. The crystal structure of this mutant complexed with 9α-fluorocortisol showed favorable hydrogen bonding between the C17 and C21 of this synthetic ligand with the mutant protein [631]. This might explain its activation by cortisol.

H874 is not directly lining the ligand binding pocket. In fact, it projects away from it, and is not able to bind the ligand [630, 632]. This suggests a conformational change induced by the H874Y substitution that indirectly affects ligand binding.

In the W741L AR mutant the bulky tryptophan residue is replaced by leucine, which allows accomodation of large molecules like BCA, thereby maintaining the agonist bound LBD structure. In the wild-type AR, M895 is displaced by BCA, whereas in the W741L mutant this residue can be accomodated near L741 [626]. This may explain the presence of this mutation in BCA treated prostate cancer patients and in BCA treated LNCaP cells [624, 625]. Why the W741L mutant can not be activated by OH-Fl is not clear yet, but it is thought that a water-mediated interaction does not occur between OH-Fl and L873 as was found for the T877A mutant [627].

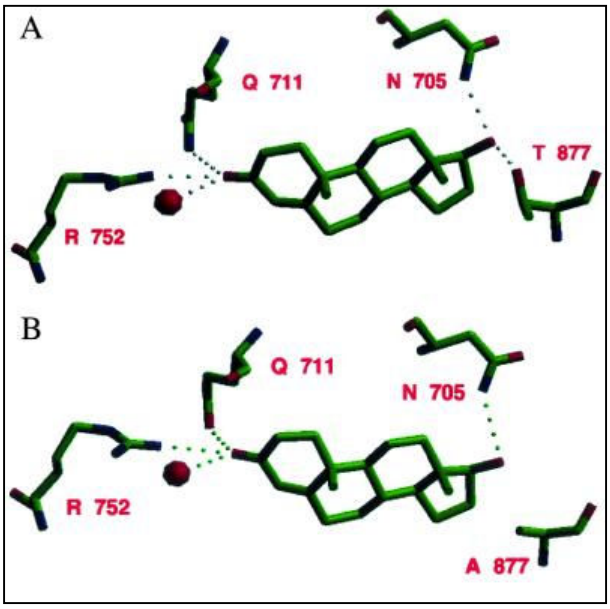

Figure 14. Comparison of (**A**) binding of DHT to AR LBD with (**B**) binding of DHT to the AR LBD T877A mutant. From ref. 277.

In vitro time-resolved FRET analysis of the wild-type and T877A mutant AR LBD revealed that DHT, R1881, E_2, spironolactone, Pg, and cortisol, conferred recruitment of the peptide D11FXXLF and the SRC-3 LXXLL motif 1 in a way that correlated with the rank order potency of these ligands to induce receptor transactivation [633]. These findings indicate that ligand binding and NTD or coactivator binding can be conformationally associated. Also, the extent of partial antagonistic activity of antiandrogen might be reflected by differential motif binding. For example, the usually antagonistic OH-Fl conferred a better binding of the FXXLF and LXXLL peptides to AR T877A LBD than to wild-type AR LBD, whereas the partial antagonist CPA conferred recruitment of the LXXLL motif, but not of the FXXLF motif to the mutant LBD [633]. V715M and AR H874Y showed an increase in both FXXLF and LXXLL motif binding as compared to the wild-type AR [634]. The V730M mutant showed an increase in LXXLL motif binding, and slightly decreases FXXLF binding [455]. Androgen dissociation was not altered for this mutant [634]. These findings suggest that several AR LBD mutations found in prostate cancer could confer aberrant AR activity through conformational changes that influence coactivator recruitment by the non-cognate ligand bound LBD.

1.2.5.1.3.3 Intraprostatic conversion of adrenal androgens to T and DHT

The most common (90%) circulating androgen is T, which is synthesized by the Leydig cells in the testes. The remaining 10% are produced by the adrenals and include androgens like dehydroepiandrosterone (DHEA), androstenediol, and androstenedione. Upon entrance in target cells T can be converted to more potent DHT by 5α-reductase. LHRH agonists used as prostate cancer therapy inhibit production of T, thereby leaving adrenal androgen production unchanged. In normal and prostate cancer tissues, adrenal androgens have been found to be converted *in vitro* and *in vivo* to several different androgens among which T and DHT [635-638]. This might be caused by increased expression of aldo-keto reductase family 1, member C3 (AKR1C3) [639]. This enzyme, also called 17-β hydroxysteroiddehydrogenase type 5, converts androstenedione into T, which in turn can be converted to DHT. An additional mechanism could be that the conversion

of prostatic DHT to 5α-androstane-3α,17β-diol is reversed by a 11-*cis* retinol dehydrogenase like oxidative 3α-hydroxysteroid dehydrogenase (RL-HSD) and/or L-3-hydroxyacyl coenzyme A dehydrogenase. These two enzymes were found to be highly expressed in normal prostate tissue and are suggested to contribute to castrate prostatic DHT levels [636]. Whatever the mechanism after LHRH induced castration there is an intraprostatic reduction of DHT levels by only 50-70%, while serum castrate levels of T are reduced by 90-95% [640-643]. As described in 1.2.5.1.3.2, adrenal androgen levels are thought to be able to activate particular AR mutants found in a subset of prostate cancers, but not wild-type AR. Moreover, prostatic castrate DHT levels are supposed to be sufficient for wild-type AR activation. Therefore a combination of a LHRH agonist and an antiandrogen is often used as prostate cancer therapy.

1.2.5.1.3.4 Ligand-independent activation of the AR through cross-talk with other signaling pathways

An increasing number of manuscripts describes modulation of AR activity by complex cross talk with other signal transduction pathways. Cross-talk with other signaling pathways might be both ligand-dependent or -independent. Here several relevant examples of "alternative activation" of AR are presented.

Overexpression of the epidermal growth factor (EGF) receptor related human epidermal growth factor receptor-2 (HER-2) has been found in a subpopulation of prostate cancer patients. It has been described that HER-2 activates the AR *via* the MAPK pathway [180]. Use of a MAPK inhibitor reduced PSA levels in prostate cells and the antagonistic action of antiandrogens is decreased in the presence of activated MAPK [644]. Constitutively expressed active Ras stimulates MAPK activity and sensitizes prostate cells to low androgen levels, as examplified in the androgen independent C4-2 LNCaP subline where dominant-negative Ras restored sensitivity to BCA [645, 646]. MAPK activity was found to increase during androgen ablation. Its phosphorylation, which is thought to be due to TGF-β action, correlates with prostate tumor metastatic potential [647-650].

Interleukin-6 (IL-6) activates the AR in a ligand-independent manner through p300 and SRC-1 [651]. It also activates signal transducers and activators of transcription (STAT3), MAPK, and phosphatidylinositol 3-kinase (PI3K), which all might activate the AR [652,653].

Recently, it was found that AR can be phosphorylated on Y534 by Src [654, 655]. This phosphorylation increased AR activity at low androgen levels and stimulated prostate tumor growth in castrated mice [655]. Src in turn can be activated by growth factors including EGF, heregulin, and IL-6 [655, 656]. Src is constitutively expressed in an androgen-independent LNCaP subline with a high passage number [657]. Tyrosine phosphorylation of the AR is increased in androgen-independent clinical prostate cancer and correlates with a high Gleason score [655].

Contradictory reports have been published on the effect of the protein kinase Akt on AR activity. It has been described that activation of Akt is associated with advanced prostate cancer and that Akt enhances AR activity [658]. However, inhibition AR activity by Akt has also been described [659]. Possibly, differential phosphorylation of AR by Akt in different cell lines might explain these discrepancies. For example Ser phosphorylated AR was found in LAPC-4 cells, but not in LNCaP cells [660]. HER-2 might activate AR not only through MAPK but also by Akt stimulated phosphorylation of S213 and S791 [198]. The Akt downstream target Forkhead box O 1 (FoxO1) transcription factor activates AR by interaction with the receptor, thereby inhibiting apoptosis [661].

Elevated serum levels of insulin-like growth factor-1 (IFG-1) have been associated with an increased prostate cancer risk and with the transition of prostate cancer xenografts to an

androgen-independent state [662]. IGF-1 can activate the AR by inducing the PI3K/Akt or Ras/MAPK pathways, which results in AR phosphorylation and sensitization to low androgen levels [271]. IGF-1 receptor (IGF-1R) expression in LNCaP cells can be induced by androgens, however, this seems independent of binding of AR to DNA, but dependent on the Src/MAPK pathway [663]. Increased levels of IGF binding proteins IGFBP-2 and IGFBP-5 have been found in prostate cancer patients. These proteins bind to the extracellular matrix thereby maintaining a relatively high IGF-1 concentration in the vicinity of the IGF-1R [664-666]. Taken together, several lines of evidence indicate that the IGF signaling pathway can be involved in androgen-induced cell proliferation causing androgen-dependent prostate tumor growth, moreover it is a good candidate to subsequently promote progression to androgen-independent prostate cancer.

1.2.5.1.3.5 Aberrant AR coregulator expression and function

Although the relative contributions of coregulators to AR function have not been fully determined as yet, several are considered to contribute to the development of androgen-independent prostate cancer. Aberrant expression of coregulators has been described in prostate tumor samples and cell lines, which could lead to stimulation of AR activity in the presence of low androgen levels, or, like some AR mutations do (see section 1.2.5.2.2), alter ligand specificity of the AR (reviewed in ref. 667). Below several examples are described in more detail. It has to be realized, however, that this still is a controversial subject, and that not all data are conclusive.

For all members of the SRC family, SRC-1, TIF2, and SRC-3, overexpression has been reported in subsets of androgen-independent tumors [668, 669]. Microarray analysis of a large patient cohort has revealed that increased SRC-1 expression in prostate cancer is correlated with more aggressive disease [670]. Another important finding is that phosphorylation of SRC-1 by MAPK can activate the AR in the absence of androgens to the same extent as physiological levels of DHT [671, 672]. TIF2 expression and phosphorylation were found to be increased in an androgen-independent xenograft model by EGF stimulation [668, 673]. The partial antagonist CPA and the antagonist OH-FL are agonists for AR mutant T877A (see section 1.2.5.2.2), and these activities were hardly affected by small amounts of the corepressor NCoR. However, TIF2 strongly enhanced antagonist-induced AR activity [359]. This indicates that the antagonist-bound T877A mutant prefers to bind a coactivator. So, AR activity will not only be determined by the ligand (agonist or antagonists), but also by the ratio of coactivator to corepressor ratio in a cell. Some prostate tumors show SRC-3 overexpression [674, 675]. SCR-3 increases PSA levels in the presence of very low adrenal androgen levels [395]. Furthermore there is a correlation between SRC-3 expression and a decreased time of prostate cancer relapse [669].

CBP expression in LNCaP cells is down-regulated by androgens, and seems overexpressed in androgen-independent clinical prostate cancer, possibly due to androgen withdrawal [676]. In addition, the agonistic action of the AR antagonist OH-FL is increased by transiently transfected CBP in prostate cancer cells containing either wild-type or antiandrogen sensitive mutant AR [677]. Increased level of CBP-related p300 also correlated with prostate cancer progression [678].

ARA55 expression was found to be increased in primary prostate tumors, and even more in androgen-independent tumors [679]. It is also associated with shorter recurrence free survival and overall survival in androgen-independent prostate cancer patients [680]. ARA55 is phosphorylated by proline-rich tyrosine kinase-2 (PYK-2), which inhibits its binding to AR. In progressive prostate cancers PYK-2 expression was found to be reduced, which increases AR-ARA55 binding and therefore AR activity [307].

ARA70 is overexpressed in prostate tumors and androgen independent CWR22 xenografts, and enables OH-Fl and BCA to function as AR agonists [569, 681-683]. E_2 levels in the stroma of benign prostatic hypertrophy and in some prostate cancer samples were found to be relatively high by

an increased level of aromatase, which converts T to E_2 [684-687]. *In vitro* increased ARA70 expression was found to activate AR in the presence of low adrenal androgen or E_2 levels [304]. In addition, expression of ARA70 itself is induced by E_2, which can further enhance the AR response to E_2 [688]. Because of these effects of ARA70 on the ligand response of the AR, use of E_2 or antiandrogens as therapy may be disadvantageous in prostate cancers with elevated ARA70.

Like ARA70, other AR coactivators were also found to allow AR activation by adrenal androgens. β-Catenin is mutated in 5% of prostate cancers [689]. This seems to increase AR activation by androstenedione at the physiological nanomolar level [319]. Supervillin is able to enhance AR activity in the presence of 10 nM androstenediol [690].

Upon androgen withdrawal, expression and nuclear localization of Tip60 are increased in LNCaP cells and CWR22 xenografts. In androgen-independent tumors Tip60 was exclusively localized in the nucleus [691]. However, another study revealed that Tip60 is down-regulated in prostate cancer metastases, which indicates that it can have different roles in different tumor stages [692]. Tip60 is also related to PSA expression in androgen-independent cell lines, probably by modulating AR acetylation [300].

Gelsolin, which is up-regulated upon androgen withdrawal in LNCaP cells, LNCaP xenografts, and human prostate tumors, was shown to enhance OH-Fl induced AR activity [321].

ART-27 is supposed to be differentiation-related and is expressed at low or negligible levels in prostate cancer specimens compared to well differentiated normal prostate tissue [326]. However, the AR mutant P340L in prostate cancer showed an increased interaction with ART-27. This indicates that the role of ART-27 switches during prostate cancer progression.

Overexpression of CARM1 has been detected in both early stage prostate carcinoma and in androgen-independent prostate carcinoma [355].

1.2.5.1.3.6 Signaling pathways in prostate cancer cells bypassing the AR

Several cellular signaling pathways that show cross-talk with AR function might also play a role in prostate tumor growth without involvement of AR. These bypass pathways include MAPK, Akt, and PKC signaling.

Members of the MAPK cascade were found amplified in androgen-independent prostate cancer [693]. Transfection experiments have indicated that Ras may induce androgen-independence by increasing MAPK expression and activation [646]. AP1, a c-Jun/c-Fos heterodimer, activated by the MAPK cascade, can increase expression of androgen-regulated genes by binding to its recognition site present in the promoters of some of these genes [694]. In PC3 cells, which are androgen-independent, the levels of c-Jun and c-Fos showed a seven-fold increase as compared to androgen-dependent LNCaP cells [695].

Akt was found to play a role in apoptosis and proliferation of prostate cancer cell lines by suppressing pro-apoptotic processes and stimulating G1 cell-cycle progression. It inactivates the FoxO transcription factors, which has been claimed to decrease p27kip, a cell-cycle regulator [661, 696, 697].

Increased PKC expression is correlated with decreased survival time after development of androgen-independence [695]. PKC has at least 12 isoforms, which activate different pathways, and since it is not known which isoforms are higher expressed in androgen independent prostate cancer it is not possible to determine which PKC induced mechanism is involved [698].

1.3 AIM AND SCOPE OF THIS THESIS

The AR, which is the target protein of androgens, is essential in development and maintenance of the male phenotype, and therefore plays a key role in diseases like AIS, SBMA or Kennedy's disease, and prostate cancer.

Androgens stimulate prostate tumor growth, and a generally applied therapy is androgen ablation and in case of metastatic prostate cancer anti-androgens are subscribed. This treatment initially causes a decrease of tumor size. However, unfortunately, almost all tumors ultimately relapse and have started to grow independent of androgens. In a number of patients the AR seems still involved in androgen-independent prostate tumor growth through an alternative mechanism for activation of the receptor which would not require binding of an androgenic ligand to the AR. Therefore, a thorough knowledge of the mechanisms underlying normal and aberrant AR function is essential for development and improvement of prostate cancer therapies.

Investigation of the molecular mechanisms of AR functions was done at the level of the AR protein itself, AR regulated gene transcription and AR mutations. The study 'Molecular mechanisms of androgen receptor functions' described in this thesis can be subdivided in three topics:

1) interactions between AR subdomains,
2) androgen specific transcriptional gene regulation,
3) aberrant AR function caused by AR mutations found in prostate cancer.

Ad 1) A ligand-dependent functional interaction can occur between the AR NTD and AR LBD. Deletion mapping of the AR NTD has revealed an important interaction domain. This domain contains an interaction motif that is essential in the interaction with the AR LBD. A detailed study of this motif is described in Chapter 2.

Ad 2) The specifically androgen regulated gene *SARG* has been identified in the AR and GR positive LNCaP-1F5 subline. Its transcription can be up-regulated by androgens, but not by glucocorticoids. Chapter 3 describes the characterization of the *SARG* gene, and the bioinformatics-based identification and functional analysis of an androgen specific response element in intron 1.

Ad 3) Chapter 4 describes a random mutagenesis screening and the subsequent isolation of AR mutants, which are not only responsive to androgens, but also to other non-cognate steroids and even antiandrogens and cortisol. This broadened ligand specificity might have implications for prostate cancer patients carrying such AR mutations.

Chapter 5 summarizes the findings described in Chapters 2, 3, and 4, and places these in the context of recently published studies and indicates directions of future research.

1.4 REFERENCES

1 M. Beato, P. Herrlich and G. Schutz (1995) Steroid hormone receptors: many actors in search of a plot *Cell* **83** 6 851-7

2 L. P. Freedman (1999) Increasing the complexity of coactivation in nuclear receptor signaling *Cell* **97** 1 5-8

3 N. J. Mckenna, J. Xu, Z. Nawaz, S. Y. Tsai, M. J. Tsai and B. W. O'malley (1999) Nuclear receptor coactivators: multiple enzymes, multiple complexes, multiple functions *J Steroid Biochem Mol Biol* **69** 1-6 3-12

4 C. K. Glass and M. G. Rosenfeld (2000) The coregulator exchange in transcriptional functions of nuclear receptors *Genes Dev* **14** 2 121-41

5 N. J. Mckenna and B. W. O'malley (2000) From ligand to response: generating diversity in nuclear receptor coregulator function *J Steroid Biochem Mol Biol* **74** 5 351-6

6 D. Robyr, A. P. Wolffe and W. Wahli (2000) Nuclear hormone receptor coregulators in action: diversity for shared tasks *Mol Endocrinol* **14** 3 329-47

7 A. Aranda and A. Pascual (2001) Nuclear hormone receptors and gene expression *Physiol Rev* **81** 3 1269-304

8 K. C. Lee and W. Lee Kraus (2001) Nuclear receptors, coactivators and chromatin: new approaches, new insights *Trends Endocrinol Metab* **12** 5 191-7

9 J. W. Lee, Y. C. Lee, S. Y. Na, D. J. Jung and S. K. Lee (2001) Transcriptional coregulators of the nuclear receptor superfamily: coactivators and corepressors *Cell Mol Life Sci* **58** 2 289-97

10 A. M. Naar, B. D. Lemon and R. Tjian (2001) Transcriptional coactivator complexes *Annu Rev Biochem* **70** 475-501

11 N. J. Mckenna and B. W. O'malley (2002) Combinatorial control of gene expression by nuclear receptors and coregulators *Cell* **108** 4 465-74

12 V. Laudet (1997) Evolution of the nuclear receptor superfamily: early diversification from an ancestral orphan receptor *J Mol Endocrinol* **19** 3 207-26

13 V. Giguere (1999) Orphan nuclear receptors: from gene to function *Endocr Rev* **20** 5 689-725

14 W. H. Evans and J. J. Bergeron (1988) Nuclear receptors: a re-evaluation *Trends Biochem Sci* **13** 1 7-8

15 S. Green and P. Chambon (1988) Nuclear receptors enhance our understanding of transcription regulation *Trends Genet* **4** 11 309-14

16 M. Beato, G. Chalepakis, M. Schauer and E. P. Slater (1989) DNA regulatory elements for steroid hormones *J Steroid Biochem* **32** 5 737-47

17 M. Beato (1989) Gene regulation by steroid hormones *Cell* **56** 3 335-44

18 M. G. Parker (1993) Steroid and related receptors *Curr Opin Cell Biol* **5** 3 499-504

19 K. B. Horwitz, T. A. Jackson, D. L. Bain, J. K. Richer, G. S. Takimoto and L. Tung (1996) Nuclear receptor coactivators and corepressors *Mol Endocrinol* **10** 10 1167-77

20 C. K. Glass (1994) Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers *Endocr Rev* **15** 3 391-407

21 S. Khorasanizadeh and F. Rastinejad (2001) Nuclear-receptor interactions on DNA-response elements *Trends Biochem Sci* **26** 6 384-90

22 S. Green, V. Kumar, I. Theulaz, W. Wahli and P. Chambon (1988) The N-terminal DNA-binding 'zinc finger' of the oestrogen and glucocorticoid receptors determines target gene specificity *Embo J* **7** 10 3037-44

23 K. Umesono and R. M. Evans (1989) Determinants of target gene specificity for steroid/thyroid hormone receptors *Cell* **57** 7 1139-46

24 P. L. Shaffer, A. Jivan, D. E. Dollins, F. Claessens and D. T. Gewirth (2004) Structural basis of androgen receptor binding to selective androgen response elements *Proc Natl Acad Sci U S A* **101** 14 4758-63

25 B. E. Black, J. M. Holaska, L. Levesque, B. Ossareh-Nazari, C. Gwizdek, C. Dargemont and B. M. Paschal (2001) NXT1 is necessary for the terminal step of Crm1-mediated nuclear export *J Cell Biol* **152** 1 141-55

26 B. E. Black and B. M. Paschal (2004) Intranuclear organization and function of the androgen receptor *Trends Endocrinol Metab* **15** 9 411-7

27 X. S. Chen, Y. Y. Zhang and C. D. Funk (1998) Determinants of 5-lipoxygenase nuclear localization using green fluorescent protein/5-lipoxygenase fusion proteins *J Biol Chem* **273** 47 31237-44

28 J. M. Wurtz, W. Bourguet, J. P. Renaud, V. Vivat, P. Chambon, D. Moras and H. Gronemeyer (1996) A canonical structure for the ligand-binding domain of nuclear receptors *Nat Struct Biol* **3** 2 206

29 S. Minucci and K. Ozato (1996) Retinoid receptors in transcriptional regulation *Curr Opin Genet Dev* **6** 5 567-74

30 A. M. Brzozowski, A. C. Pike, Z. Dauter, R. E. Hubbard, T. Bonn, O. Engstrom, L. Ohman, G. L. Greene, J. A. Gustafsson and M. Carlquist (1997) Molecular basis of agonism and antagonism in the oestrogen receptor *Nature* **389** 6652 753-8

31 D. Moras and H. Gronemeyer (1998) The nuclear receptor ligand-binding domain: structure and function *Curr Opin Cell Biol* **10** 3 384-91

32 R. T. Nolte, G. B. Wisely, S. Westin, J. E. Cobb, M. H. Lambert, R. Kurokawa, M. G. Rosenfeld, T. M. Willson, C. K. Glass and M. V. Milburn (1998) Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-gamma *Nature* **395** 6698 137-43

33 A. K. Shiau, D. Barstad, P. M. Loria, L. Cheng, P. J. Kushner, D. A. Agard and G. L. Greene (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen *Cell* **95** 7 927-37
34 N. J. Webster, S. Green, J. R. Jin and P. Chambon (1988) The hormone-binding domains of the estrogen and glucocorticoid receptors contain an inducible transcription activation function *Cell* **54** 2 199-207
35 P. S. Danielian, R. White, J. A. Lees and M. G. Parker (1992) Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors *Embo J* **11** 3 1025-33
36 D. Barettino, M. M. Vivanco Ruiz and H. G. Stunnenberg (1994) Characterization of the ligand-dependent transactivation domain of thyroid hormone receptor *Embo J* **13** 13 3039-49
37 B. Durand, M. Saunders, C. Gaudon, B. Roy, R. Losson and P. Chambon (1994) Activation function 2 (AF-2) of retinoic acid receptor and 9-cis retinoic acid receptor: presence of a conserved autonomous constitutive activating domain and influence of the nature of the response element on AF-2 activity *Embo J* **13** 22 5370-82
38 R. B. Lanz and S. Rusconi (1994) A conserved carboxy-terminal subdomain is important for ligand interpretation and transactivation by nuclear receptors *Endocrinology* **135** 5 2183-95
39 S. A. Onate, V. Boonyaratanakornkit, T. E. Spencer, S. Y. Tsai, M. J. Tsai, D. P. Edwards and B. W. O'malley (1998) The steroid receptor coactivator-1 contains multiple receptor interacting and activation domains that cooperatively enhance the activation function 1 (AF1) and AF2 domains of steroid receptors *J Biol Chem* **273** 20 12101-8
40 P. Alen, F. Claessens, G. Verhoeven, W. Rombauts and B. Peeters (1999) The androgen receptor amino-terminal domain plays a key role in p160 coactivator-stimulated gene transcription *Mol Cell Biol* **19** 9 6085-97
41 A. Benecke, P. Chambon and H. Gronemeyer (2000) Synergy between estrogen receptor alpha activation functions AF1 and AF2 mediated by transcription intermediary factor TIF2 *EMBO Rep* **1** 2 151-7
42 R. Metivier, G. Penot, G. Flouriot and F. Pakdel (2001) Synergism between ERalpha transactivation function 1 (AF-1) and AF-2 mediated by steroid receptor coactivator protein-1: requirement for the AF-1 alpha-helical core and for a direct interaction between the N- and C-terminal domains *Mol Endocrinol* **15** 11 1953-70
43 V. Perissi, L. M. Staszewski, E. M. Mcinerney, R. Kurokawa, A. Krones, D. W. Rose, M. H. Lambert, M. V. Milburn, C. K. Glass and M. G. Rosenfeld (1999) Molecular determinants of nuclear receptor-corepressor interaction *Genes Dev* **13** 24 3198-208
44 X. J. Yang and E. Seto (2003) Collaborative spirit of histone deacetylases in regulating chromatin structure and gene expression *Curr Opin Genet Dev* **13** 2 143-53
45 A. J. Saporita, Q. Zhang, N. Navai, Z. Dincer, J. Hahn, X. Cai and Z. Wang (2003) Identification and characterization of a ligand-regulated nuclear export signal in androgen receptor *J Biol Chem* **278** 43 41998-2005
46 S. K. Nordeen, B. J. Suh, B. Kuhnel and C. D. Hutchison (1990) Structural determinants of a glucocorticoid receptor recognition element *Mol Endocrinol* **4** 12 1866-73
47 P. J. Roche, S. A. Hoare and M. G. Parker (1992) A consensus DNA-binding site for the androgen receptor *Mol Endocrinol* **6** 12 2229-35
48 B. A. Lieberman, B. J. Bona, D. P. Edwards and S. K. Nordeen (1993) The constitution of a progesterone response element *Mol Endocrinol* **7** 4 515-27
49 M. Lombes, N. Binart, M. E. Oblin, V. Joulin and E. E. Baulieu (1993) Characterization of the interaction of the human mineralocorticosteroid receptor with hormone response elements *Biochem J* **292 (Pt 2)** 577-83
50 S. Kato, L. Tora, J. Yamauchi, S. Masushige, M. Bellard and P. Chambon (1992) A far upstream estrogen response element of the ovalbumin gene contains several half-palindromic 5'-TGACC-3' motifs acting synergistically *Cell* **68** 4 731-42
51 U. Strahle, M. Boshart, G. Klock, F. Stewart and G. Schutz (1989) Glucocorticoid- and progesterone-specific effects are determined by differential expression of the respective hormone receptors *Nature* **339** 6226 629-32
52 F. Claessens, P. Alen, A. Devos, B. Peeters, G. Verhoeven and W. Rombauts (1996) The androgen-specific probasin response element 2 interacts differentially with androgen and glucocorticoid receptors *J Biol Chem* **271** 32 19013-6
53 V. G. Thackray, B. A. Lieberman and S. K. Nordeen (1998) Differential gene induction by glucocorticoid and progesterone receptors *J Steroid Biochem Mol Biol* **66** 4 171-8
54 D. Szapary, Y. Huang and S. S. Simons, Jr. (1999) Opposing effects of corepressor and coactivators in determining the dose-response curve of agonists, and residual agonist activity of antagonists, for glucocorticoid receptor-regulated gene expression *Mol Endocrinol* **13** 12 2108-21
55 F. Claessens, G. Verrijdt, E. Schoenmakers, A. Haelens, B. Peeters, G. Verhoeven and W. Rombauts (2001) Selective DNA binding by the androgen receptor as a mechanism for hormone-specific gene regulation *J Steroid Biochem Mol Biol* **76** 1-5 23-30
56 Z. Liu, D. Auboeuf, J. Wong, J. D. Chen, S. Y. Tsai, M. J. Tsai and B. W. O'malley (2002) Coactivator/corepressor ratios modulate PR-mediated transcription by the selective receptor modulator RU486 *Proc Natl Acad Sci U S A* **99** 12 7940-4
57 C. A. Sartorius, M. Y. Melville, A. R. Hovland, L. Tung, G. S. Takimoto and K. B. Horwitz (1994) A third transactivation function (AF3) of human progesterone receptors located in the unique N-terminal segment of the B-isoform *Mol Endocrinol* **8** 10 1347-60
58 D. F. Smith and D. O. Toft (1993) Steroid receptors and their associated proteins *Mol Endocrinol* **7** 1 4-11

59 J. R. Lambert and S. K. Nordeen (1998) Steroid-selective initiation of chromatin remodeling and transcriptional activation of the mouse mammary tumor virus promoter is controlled by the site of promoter integration *J Biol Chem* **273** 49 32708-14
60 J. G. Mcnally, W. G. Muller, D. Walker, R. Wolford and G. L. Hager (2000) The glucocorticoid receptor: rapid exchange with regulatory sites in living cells *Science* **287** 5456 1262-5
61 G. L. Hager, A. K. Nagaich, T. A. Johnson, D. A. Walker and S. John (2004) Dynamics of nuclear receptor movement and transcription *Biochim Biophys Acta* **1677** 1-3 46-51
62 A. K. Nagaich, D. A. Walker, R. Wolford and G. L. Hager (2004) Rapid periodic binding and displacement of the glucocorticoid receptor during chromatin remodeling *Mol Cell* **14** 2 163-74
63 D. A. Stavreva, W. G. Muller, G. L. Hager, C. L. Smith and J. G. Mcnally (2004) Rapid glucocorticoid receptor exchange at a promoter is coupled to transcription and regulated by chaperones and proteasomes *Mol Cell Biol* **24** 7 2682-97
64 R. Metivier, G. Penot, M. R. Hubner, G. Reid, H. Brand, M. Kos and F. Gannon (2003) Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter *Cell* **115** 6 751-63
65 H. Chen, R. J. Lin, R. L. Schiltz, D. Chakravarti, A. Nash, L. Nagy, M. L. Privalsky, Y. Nakatani and R. M. Evans (1997) Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300 *Cell* **90** 3 569-80
66 T. E. Spencer, G. Jenster, M. M. Burcin, C. D. Allis, J. Zhou, C. A. Mizzen, N. J. Mckenna, S. A. Onate, S. Y. Tsai, M. J. Tsai and B. W. O'malley (1997) Steroid receptor coactivator-1 is a histone acetyltransferase *Nature* **389** 6647 194-8
67 A. P. Wolffe and D. Pruss (1996) Targeting chromatin disruption: Transcription regulators that acetylate histones *Cell* **84** 6 817-9
68 K. Struhl (1998) Histone acetylation and transcriptional regulatory mechanisms *Genes Dev* **12** 5 599-606
69 M. Hagmann (1999) How chromatin changes its shape *Science* **285** 5431 1200-1, 1203
70 D. Rhodes (1997) Chromatin structure. The nucleosome core all wrapped up *Nature* **389** 6648 231, 233
71 W. Y. Chen, E. C. Bailey, S. L. Mccune, J. Y. Dong and T. M. Townes (1997) Reactivation of silenced, virally transduced genes by inhibitors of histone deacetylase *Proc Natl Acad Sci U S A* **94** 11 5798-803
72 A. J. Horlein, A. M. Naar, T. Heinzel, J. Torchia, B. Gloss, R. Kurokawa, A. Ryan, Y. Kamei, M. Soderstrom, C. K. Glass and Et Al. (1995) Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor *Nature* **377** 6548 397-404
73 A. J. De Ruijter, A. H. Van Gennip, H. N. Caron, S. Kemp and A. B. Van Kuilenburg (2003) Histone deacetylases (HDACs): characterization of the classical HDAC family *Biochem J* **370** Pt 3 737-49
74 D. Chen, H. Ma, H. Hong, S. S. Koh, S. M. Huang, B. T. Schurter, D. W. Aswad and M. R. Stallcup (1999) Regulation of transcription by a protein methyltransferase *Science* **284** 5423 2174-7
75 Y. Z. Chen, V. Mohan and R. H. Griffey (2000) Spontaneous base flipping in DNA and its possible role in methyltransferase binding *Phys Rev E Stat Phys Plasmas Fluids Relat Interdiscip Topics* **62** 1 Pt B 1133-7
76 S. S. Koh, D. Chen, Y. H. Lee and M. R. Stallcup (2001) Synergistic enhancement of nuclear receptor function by p160 coactivators and two coactivators with protein methyltransferase activities *J Biol Chem* **276** 2 1089-98
77 H. Wang, R. Cao, L. Xia, H. Erdjument-Bromage, C. Borchers, P. Tempst and Y. Zhang (2001) Purification and functional characterization of a histone H3-lysine 4-specific methyltransferase *Mol Cell* **8** 6 1207-17
78 B. Belandia, R. L. Orford, H. C. Hurst and M. G. Parker (2002) Targeting of SWI/SNF chromatin remodelling complexes to estrogen-responsive genes *Embo J* **21** 15 4094-103
79 Z. Q. Huang, J. Li, L. M. Sachs, P. A. Cole and J. Wong (2003) A role for cofactor-cofactor and cofactor-histone interactions in targeting p300, SWI/SNF and Mediator for transcription *Embo J* **22** 9 2146-55
80 H. K. Kinyamu and T. K. Archer (2004) Modifying chromatin to permit steroid hormone receptor-dependent transcription *Biochim Biophys Acta* **1677** 1-3 30-45
81 A. Lusser and J. T. Kadonaga (2003) Chromatin remodeling by ATP-dependent molecular machines *Bioessays* **25** 12 1192-200
82 J. D. Fondell, H. Ge and R. G. Roeder (1996) Ligand induction of a transcriptionally active thyroid hormone receptor coactivator complex *Proc Natl Acad Sci U S A* **93** 16 8329-33
83 C. Rachez, Z. Suldan, J. Ward, C. P. Chang, D. Burakov, H. Erdjument-Bromage, P. Tempst and L. P. Freedman (1998) A novel protein complex that interacts with the vitamin D3 receptor in a ligand-dependent manner and enhances VDR transactivation in a cell-free system *Genes Dev* **12** 12 1787-800
84 S. Malik and R. G. Roeder (2000) Transcriptional regulation through Mediator-like coactivators in yeast and metazoan cells *Trends Biochem Sci* **25** 6 277-83
85 A. M. Naar, P. A. Beaurang, S. Zhou, S. Abraham, W. Solomon and R. Tjian (1999) Composite co-activator ARC mediates chromatin-directed transcriptional activation *Nature* **398** 6730 828-32
86 H. M. Bourbon, A. Aguilera, A. Z. Ansari, F. J. Asturias, A. J. Berk, S. Bjorklund, T. K. Blackwell, T. Borggrefe, M. Carey, M. Carlson, J. W. Conaway, R. C. Conaway, S. W. Emmons, J. D. Fondell, L. P. Freedman, T. Fukasawa, C. M. Gustafsson, M. Han, X. He, P. K. Herman, A. G. Hinnebusch, S. Holmberg, F. C. Holstege, J. A. Jaehning, Y. J. Kim, L. Kuras, A. Leutz, J. T. Lis, M. Meisterernest, A. M. Naar, K. Nasmyth, J. D. Parvin, M. Ptashne, D. Reinberg, H. Ronne, I. Sadowski, H. Sakurai, M. Sipiczki, P. W. Sternberg, D. J. Stillman, R. Strich, K. Struhl, J. Q. Svejstrup, S. Tuck, F. Winston, R. G. Roeder and R. D. Kornberg (2004) A unified nomenclature for protein subunits of mediator complexes linking transcriptional regulators to RNA polymerase II *Mol Cell* **14** 5 553-7
87 J. Llopis, S. Westin, M. Ricote, Z. Wang, C. Y. Cho, R. Kurokawa, T. M. Mullen, D. W. Rose, M. G. Rosenfeld, R. Y. Tsien and C. K. Glass (2000) Ligand-dependent interactions of coactivators steroid receptor coactivator-1 and peroxisome proliferator-activated receptor binding protein with nuclear

hormone receptors can be imaged in live cells and are required for transcription *Proc Natl Acad Sci U S A* **97** 8 4363-8
88 H. J. Baek, S. Malik, J. Qin and R. G. Roeder (2002) Requirement of TRAP/mediator for both activator-independent and activator-dependent transcription in conjunction with TFIID-associated TAF(II)s *Mol Cell Biol* **22** 8 2842-52
89 S. Wang, K. Ge, R. G. Roeder and O. Hankinson (2004) Role of mediator in transcriptional activation by the aryl hydrocarbon receptor *J Biol Chem* **279** 14 13593-600
90 Y. K. Kang, M. Guermah, C. X. Yuan and R. G. Roeder (2002) The TRAP/Mediator coactivator complex interacts directly with estrogen receptors alpha and beta through the TRAP220 subunit and directly enhances estrogen receptor function in vitro *Proc Natl Acad Sci U S A* **99** 5 2642-7
91 S. Malik, A. E. Wallberg, Y. K. Kang and R. G. Roeder (2002) TRAP/SMCC/mediator-dependent transcriptional activation from DNA and chromatin templates by orphan nuclear receptor hepatocyte nuclear factor 4 *Mol Cell Biol* **22** 15 5626-37
92 A. Warnmark, T. Almlof, J. Leers, J. A. Gustafsson and E. Treuter (2001) Differential recruitment of the mammalian mediator subunit TRAP220 by estrogen receptors ERalpha and ERbeta *J Biol Chem* **276** 26 23397-404
93 D. Sharma and J. D. Fondell (2002) Ordered recruitment of histone acetyltransferases and the TRAP/Mediator complex to thyroid hormone-responsive promoters in vivo *Proc Natl Acad Sci U S A* **99** 12 7934-9
94 M. L. Acevedo and W. L. Kraus (2003) Mediator and p300/CBP-steroid receptor coactivator complexes have distinct roles, but function synergistically, during estrogen receptor alpha-dependent transcription with chromatin templates *Mol Cell Biol* **23** 1 335-48
95 L. C. Myers, C. M. Gustafsson, D. A. Bushnell, M. Lui, H. Erdjument-Bromage, P. Tempst and R. D. Kornberg (1998) The Med proteins of yeast and their function through the RNA polymerase II carboxy-terminal domain *Genes Dev* **12** 1 45-54
96 N. Yudkovsky, J. A. Ranish and S. Hahn (2000) A transcription reinitiation intermediate that is stabilized by activator *Nature* **408** 6809 225-9
97 B. A. Lewis and D. Reinberg (2003) The mediator coactivator complex: functional and physical roles in transcriptional regulation *J Cell Sci* **116** Pt 18 3667-75
98 J. Xu and Q. Li (2003) Review of the in vivo functions of the p160 steroid receptor coactivator family *Mol Endocrinol* **17** 9 1681-92
99 S. A. Onate, S. Y. Tsai, M. J. Tsai and B. W. O'malley (1995) Sequence and characterization of a coactivator for the steroid hormone receptor superfamily *Science* **270** 5240 1354-7
100 J. J. Voegel, M. J. Heine, C. Zechel, P. Chambon and H. Gronemeyer (1996) TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors *Embo J* **15** 14 3667-75
101 S. L. Anzick, J. Kononen, R. L. Walker, D. O. Azorsa, M. M. Tanner, X. Y. Guan, G. Sauter, O. P. Kallioniemi, J. M. Trent and P. S. Meltzer (1997) AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer *Science* **277** 5328 965-8
102 H. Li, P. J. Gomes and J. D. Chen (1997) RAC3, a steroid/nuclear receptor-associated coactivator that is related to SRC-1 and TIF2 *Proc Natl Acad Sci U S A* **94** 16 8479-84
103 A. Takeshita, G. R. Cardona, N. Koibuchi, C. S. Suen and W. W. Chin (1997) TRAM-1, A novel 160-kDa thyroid hormone receptor activator molecule, exhibits distinct properties from steroid receptor coactivator-1 *J Biol Chem* **272** 44 27629-34
104 H. Hong, K. Kohli, M. J. Garabedian and M. R. Stallcup (1997) GRIP1, a transcriptional coactivator for the AF-2 transactivation domain of steroid, thyroid, retinoid, and vitamin D receptors *Mol Cell Biol* **17** 5 2735-44
105 J. Torchia, D. W. Rose, J. Inostroza, Y. Kamei, S. Westin, C. K. Glass and M. G. Rosenfeld (1997) The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function *Nature* **387** 6634 677-84
106 D. M. Heery, E. Kalkhoven, S. Hoare and M. G. Parker (1997) A signature motif in transcriptional co-activators mediates binding to nuclear receptors *Nature* **387** 6634 733-6
107 W. Feng, R. C. Ribeiro, R. L. Wagner, H. Nguyen, J. W. Apriletti, R. J. Fletterick, J. D. Baxter, P. J. Kushner and B. L. West (1998) Hormone-dependent coactivator binding to a hydrophobic cleft on nuclear receptors *Science* **280** 5370 1747-9
108 C. L. Bevan, S. Hoare, F. Claessens, D. M. Heery and M. G. Parker (1999) The AF1 and AF2 domains of the androgen receptor interact with distinct regions of SRC1 *Mol Cell Biol* **19** 12 8383-92
109 M. Bommer, A. Benecke, H. Gronemeyer and C. Rochette-Egly (2002) TIF2 mediates the synergy between RARalpha 1 activation functions AF-1 and AF-2 *J Biol Chem* **277** 40 37961-6
110 T. P. Yao, S. P. Oh, M. Fuchs, N. D. Zhou, L. E. Ch'ng, D. Newsome, R. T. Bronson, E. Li, D. M. Livingston and R. Eckner (1998) Gene dosage-dependent embryonic development and proliferation defects in mice lacking the transcriptional integrator p300 *Cell* **93** 3 361-72
111 A. L. Kung, V. I. Rebel, R. T. Bronson, L. E. Ch'ng, C. A. Sieff, D. M. Livingston and T. P. Yao (2000) Gene dose-dependent control of hematopoiesis and hematologic tumor suppression by CBP *Genes Dev* **14** 3 272-7
112 Y. Kamei, L. Xu, T. Heinzel, J. Torchia, R. Kurokawa, B. Gloss, S. C. Lin, R. A. Heyman, D. W. Rose, C. K. Glass and M. G. Rosenfeld (1996) A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors *Cell* **85** 3 403-14
113 J. Xu and B. W. O'malley (2002) Molecular mechanisms and cellular biology of the steroid receptor coactivator (SRC) family in steroid receptor function *Rev Endocr Metab Disord* **3** 3 185-92

114 J. Xu, Y. Qiu, F. J. Demayo, S. Y. Tsai, M. J. Tsai and B. W. O'malley (1998) Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene *Science* **279** 5358 1922-5
115 C. Qi, Y. Zhu, J. Pan, A. V. Yeldandi, M. S. Rao, N. Maeda, V. Subbarao, S. Pulikuri, T. Hashimoto and J. K. Reddy (1999) Mouse steroid receptor coactivator-1 is not essential for peroxisome proliferator-activated receptor alpha-regulated gene expression *Proc Natl Acad Sci U S A* **96** 4 1585-90
116 E. Nishihara, H. Yoshida-Komiya, C. S. Chan, L. Liao, R. L. Davis, B. W. O'malley and J. Xu (2003) SRC-1 null mice exhibit moderate motor dysfunction and delayed development of cerebellar Purkinje cells *J Neurosci* **23** 1 213-22
117 M. Gehin, M. Mark, C. Dennefeld, A. Dierich, H. Gronemeyer and P. Chambon (2002) The function of TIF2/GRIP1 in mouse reproduction is distinct from those of SRC-1 and p/CIP *Mol Cell Biol* **22** 16 5923-37
118 F. J. Ghadessy, J. Lim, A. A. Abdullah, V. Panet-Raymond, C. K. Choo, R. Lumbroso, T. G. Tut, B. Gottlieb, L. Pinsky, M. A. Trifiro and E. L. Yong (1999) Oligospermic infertility associated with an androgen receptor mutation that disrupts interdomain and coactivator (TIF2) interactions *J Clin Invest* **103** 11 1517-25
119 F. Picard, M. Gehin, J. Annicotte, S. Rocchi, M. F. Champy, B. W. O'malley, P. Chambon and J. Auwerx (2002) SRC-1 and TIF2 control energy balance between white and brown adipose tissues *Cell* **111** 7 931-41
120 M. Carapeti, R. C. Aguiar, J. M. Goldman and N. C. Cross (1998) A novel fusion between MOZ and the nuclear receptor coactivator TIF2 in acute myeloid leukemia *Blood* **91** 9 3127-33
121 Z. Wang, D. W. Rose, O. Hermanson, F. Liu, T. Herman, W. Wu, D. Szeto, A. Gleiberman, A. Krones, K. Pratt, R. Rosenfeld, C. K. Glass and M. G. Rosenfeld (2000) Regulation of somatic growth by the p160 coactivator p/CIP *Proc Natl Acad Sci U S A* **97** 25 13549-54
122 J. Xu, L. Liao, G. Ning, H. Yoshida-Komiya, C. Deng and B. W. O'malley (2000) The steroid receptor coactivator SRC-3 (p/CIP/RAC3/AIB1/ACTR/TRAM-1) is required for normal growth, puberty, female reproductive function, and mammary gland development *Proc Natl Acad Sci U S A* **97** 12 6379-84
123 T. Bouras, M. C. Southey and D. J. Venter (2001) Overexpression of the steroid receptor coactivator AIB1 in breast cancer correlates with the absence of estrogen and progesterone receptors and positivity for p53 and HER2/neu *Cancer Res* **61** 3 903-7
124 H. J. List, R. Reiter, B. Singh, A. Wellstein and A. T. Riegel (2001) Expression of the nuclear coactivator AIB1 in normal and malignant breast tissue *Breast Cancer Res Treat* **68** 1 21-8
125 I. T. Cavarretta, R. Mukopadhyay, D. M. Lonard, L. M. Cowsert, C. F. Bennett, B. W. O'malley and C. L. Smith (2002) Reduction of coactivator expression by antisense oligodeoxynucleotides inhibits ERalpha transcriptional activity and MCF-7 proliferation *Mol Endocrinol* **16** 2 253-70
126 H. J. List, K. J. Lauritsen, R. Reiter, C. Powers, A. Wellstein and A. T. Riegel (2001) Ribozyme targeting demonstrates that the nuclear receptor coactivator AIB1 is a rate-limiting factor for estrogen-dependent growth of human MCF-7 breast cancer cells *J Biol Chem* **276** 26 23763-8
127 M. E. Mendelsohn and R. H. Karas (1999) The protective effects of estrogen on the cardiovascular system *N Engl J Med* **340** 23 1801-11
128 Y. Yuan, L. Liao, D. A. Tulis and J. Xu (2002) Steroid receptor coactivator-3 is required for inhibition of neointima formation by estrogen *Circulation* **105** 22 2653-9
129 Y. Tanaka, I. Naruse, T. Maekawa, H. Masuya, T. Shiroishi and S. Ishii (1997) Abnormal skeletal patterning in embryos lacking a single Cbp allele: a partial similarity with Rubinstein-Taybi syndrome *Proc Natl Acad Sci U S A* **94** 19 10215-20
130 M. Mark, H. Yoshida-Komiya, M. Gehin, L. Liao, M. J. Tsai, B. W. O'malley, P. Chambon and J. Xu (2004) Partially redundant functions of SRC-1 and TIF2 in postnatal survival and male reproduction *Proc Natl Acad Sci U S A* **101** 13 4453-8
131 Z. Wang, C. Qi, A. Krones, P. Woodring, X. Zhu, J. K. Reddy, R. M. Evans, M. G. Rosenfeld and T. Hunter (2006) Critical roles of the p160 transcriptional coactivators p/CIP and SRC-1 in energy balance *Cell Metab* **3** 2 111-22
132 H. Greschik and D. Moras (2003) Structure-activity relationship of nuclear receptor-ligand interactions *Curr Top Med Chem* **3** 14 1573-99
133 H. A. Molenda, C. P. Kilts, R. L. Allen and M. J. Tetel (2003) Nuclear receptor coactivator function in reproductive physiology and behavior *Biol Reprod* **69** 5 1449-57
134 R. S. Savkur and T. P. Burris (2004) The coactivator LXXLL nuclear receptor recognition motif *J Pept Res* **63** 3 207-12
135 J. Grenier, A. Trousson, A. Chauchereau, L. Amazit, A. Lamirand, P. Leclerc, A. Guiochon-Mantel, M. Schumacher and C. Massaad (2004) Selective recruitment of p160 coactivators on glucocorticoid-regulated promoters in Schwann cells *Mol Endocrinol* **18** 12 2866-79
136 S. M. Powell, V. Christiaens, D. Voulgaraki, J. Waxman, F. Claessens and C. L. Bevan (2004) Mechanisms of androgen receptor signalling via steroid receptor coactivator-1 in prostate *Endocr Relat Cancer* **11** 1 117-30
137 J. D. Chen and R. M. Evans (1995) A transcriptional co-repressor that interacts with nuclear hormone receptors *Nature* **377** 6548 454-7
138 X. Hu and M. A. Lazar (1999) The CoRNR motif controls the recruitment of corepressors by nuclear hormone receptors *Nature* **402** 6757 93-6
139 L. Nagy, H. Y. Kao, J. D. Love, C. Li, E. Banayo, J. T. Gooch, V. Krishna, K. Chatterjee, R. M. Evans and J. W. Schwabe (1999) Mechanism of corepressor binding and release from nuclear hormone receptors *Genes Dev* **13** 24 3209-16
140 C. M. Grozinger, C. A. Hassig and S. L. Schreiber (1999) Three proteins define a class of human histone deacetylases related to yeast Hda1p *Proc Natl Acad Sci U S A* **96** 9 4868-73

141 M. G. Guenther, W. S. Lane, W. Fischle, E. Verdin, M. A. Lazar and R. Shiekhattar (2000) A core SMRT corepressor complex containing HDAC3 and TBL1, a WD40-repeat protein linked to deafness *Genes Dev* **14** 9 1048-57
142 E. Y. Huang, J. Zhang, E. A. Miska, M. G. Guenther, T. Kouzarides and M. A. Lazar (2000) Nuclear receptor corepressors partner with class II histone deacetylases in a Sin3-independent repression pathway *Genes Dev* **14** 1 45-54
143 H. Y. Kao, M. Downes, P. Ordentlich and R. M. Evans (2000) Isolation of a novel histone deacetylase reveals that class I and class II deacetylases promote SMRT-mediated repression *Genes Dev* **14** 1 55-66
144 L. Alland, R. Muhle, H. Hou, Jr., J. Potes, L. Chin, N. Schreiber-Agus and R. A. Depinho (1997) Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression *Nature* **387** 6628 49-55
145 T. Heinzel, R. M. Lavinsky, T. M. Mullen, M. Soderstrom, C. D. Laherty, J. Torchia, W. M. Yang, G. Brard, S. D. Ngo, J. R. Davie, E. Seto, R. N. Eisenman, D. W. Rose, C. K. Glass and M. G. Rosenfeld (1997) A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression *Nature* **387** 6628 43-8
146 L. Nagy, H. Y. Kao, D. Chakravarti, R. J. Lin, C. A. Hassig, D. E. Ayer, S. L. Schreiber and R. M. Evans (1997) Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase *Cell* **89** 3 373-80
147 P. A. Wade, P. L. Jones, D. Vermaak, G. J. Veenstra, A. Imhof, T. Sera, C. Tse, H. Ge, Y. B. Shi, J. C. Hansen and A. P. Wolffe (1998) Histone deacetylase directs the dominant silencing of transcription in chromatin: association with MeCP2 and the Mi-2 chromodomain SWI/SNF ATPase *Cold Spring Harb Symp Quant Biol* **63** 435-45
148 Y. Xue, J. Wong, G. T. Moreno, M. K. Young, J. Cote and W. Wang (1998) NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities *Mol Cell* **2** 6 851-61
149 P. S. Knoepfler and R. N. Eisenman (1999) Sin meets NuRD and other tails of repression *Cell* **99** 5 447-50
150 A. Makowski, S. Brzostek, R. N. Cohen and A. N. Hollenberg (2003) Determination of nuclear receptor corepressor interactions with the thyroid hormone receptor *Mol Endocrinol* **17** 2 273-86
151 L. Nagy and J. W. Schwabe (2004) Mechanism of the nuclear receptor molecular switch *Trends Biochem Sci* **29** 6 317-24
152 A. Stevens, H. Garside, A. Berry, C. Waters, A. White and D. Ray (2003) Dissociation of steroid receptor coactivator 1 and nuclear receptor corepressor recruitment to the human glucocorticoid receptor by modification of the ligand-receptor interface: the role of tyrosine 735 *Mol Endocrinol* **17** 5 845-59
153 M. M. Gonzalez, P. Samenfeld, M. Perakyla and C. Carlberg (2003) Corepressor excess shifts the two-side chain vitamin D analog Gemini from an agonist to an inverse agonist of the vitamin D receptor *Mol Endocrinol* **17** 10 2028-38
154 Y. C. Sohn, S. W. Kim, S. Lee, Y. Y. Kong, D. S. Na, S. K. Lee and J. W. Lee (2003) Dynamic inhibition of nuclear receptor activation by corepressor binding *Mol Endocrinol* **17** 3 366-72
155 P. Webb, C. Valentine, P. Nguyen, R. H. Price, Jr., A. Marimuthu, B. L. West, J. D. Baxter and P. J. Kushner (2003) ERbeta Binds N-CoR in the Presence of Estrogens via an LXXLL-like Motif in the N-CoR C-terminus *Nucl Recept* **1** 1 4
156 G. M. Leong, N. Subramaniam, L. L. Issa, J. B. Barry, T. Kino, P. H. Driggers, M. J. Hayman, J. A. Eisman and E. M. Gardiner (2004) Ski-interacting protein, a bifunctional nuclear receptor coregulator that interacts with N-CoR/SMRT and p300 *Biochem Biophys Res Commun* **315** 4 1070-6
157 V. Perissi, A. Aggarwal, C. K. Glass, D. W. Rose and M. G. Rosenfeld (2004) A corepressor/coactivator exchange complex required for transcriptional activation by nuclear receptors and other regulated transcription factors *Cell* **116** 4 511-26
158 V. Cavailles, S. Dauvois, F. L'horset, G. Lopez, S. Hoare, P. J. Kushner and M. G. Parker (1995) Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor *Embo J* **14** 15 3741-51
159 I. Fernandes, Y. Bastien, T. Wai, K. Nygard, R. Lin, O. Cormier, H. S. Lee, F. Eng, N. R. Bertos, N. Pelletier, S. Mader, V. K. Han, X. J. Yang and J. H. White (2003) Ligand-dependent nuclear receptor corepressor LCoR functions by histone deacetylase-dependent and -independent mechanisms *Mol Cell* **11** 1 139-50
160 O. Hermanson, C. K. Glass and M. G. Rosenfeld (2002) Nuclear receptor coregulators: multiple modes of modification *Trends Endocrinol Metab* **13** 2 55-60
161 C. Rochette-Egly (2003) Nuclear receptors: integration of multiple signalling pathways through phosphorylation *Cell Signal* **15** 4 355-66
162 S. H. Baek and M. G. Rosenfeld (2004) Nuclear receptor coregulators: their modification codes and regulatory mechanism by translocation *Biochem Biophys Res Commun* **319** 3 707-14
163 M. Fu, M. Rao, C. Wang, T. Sakamaki, J. Wang, D. Di Vizio, X. Zhang, C. Albanese, S. Balk, C. Chang, S. Fan, E. Rosen, J. J. Palvimo, O. A. Janne, S. Muratoglu, M. L. Avantaggiati and R. G. Pestell (2003) Acetylation of androgen receptor enhances coactivator binding and promotes prostate cancer cell growth *Mol Cell Biol* **23** 23 8563-75
164 H. Y. Lin, R. Hopkins, H. J. Cao, H. Y. Tang, C. Alexander, F. B. Davis and P. J. Davis (2005) Acetylation of nuclear hormone receptor superfamily members: thyroid hormone causes acetylation of its own receptor by a mitogen-activated protein kinase-dependent mechanism *Steroids* **70** 5-7 444-9
165 H. Wang, Z. Q. Huang, L. Xia, Q. Feng, H. Erdjument-Bromage, B. D. Strahl, S. D. Briggs, C. D. Allis, J. Wong, P. Tempst and Y. Zhang (2001) Methylation of histone H4 at arginine 3 facilitating transcriptional activation by nuclear hormone receptor *Science* **293** 5531 853-7
166 H. Syvala, A. Vienonen, Y. H. Zhuang, M. Kivineva, T. Ylikomi and P. Tuohimaa (1998) Evidence for enhanced ubiquitin-mediated proteolysis of the chicken progesterone receptor by progesterone *Life Sci* **63** 17 1505-12

167 M. Boudjelal, Z. Wang, J. J. Voorhees and G. J. Fisher (2000) Ubiquitin/proteasome pathway regulates levels of retinoic acid receptor gamma and retinoid X receptor alpha in human keratinocytes *Cancer Res* **60** 8 2247-52
168 A. Dace, L. Zhao, K. S. Park, T. Furuno, N. Takamura, M. Nakanishi, B. L. West, J. A. Hanover and S. Cheng (2000) Hormone binding induces rapid proteasome-mediated degradation of thyroid hormone receptors *Proc Natl Acad Sci U S A* **97** 16 8985-90
169 B. J. Deroo, C. Rentsch, S. Sampath, J. Young, D. B. Defranco and T. K. Archer (2002) Proteasomal inhibition enhances glucocorticoid receptor transactivation and alters its subnuclear trafficking *Mol Cell Biol* **22** 12 4113-23
170 H. K. Lin, L. Wang, Y. C. Hu, S. Altuwaijri and C. Chang (2002) Phosphorylation-dependent ubiquitylation and degradation of androgen receptor by Akt require Mdm2 E3 ligase *Embo J* **21** 15 4037-48
171 G. Reid, M. R. Hubner, R. Metivier, H. Brand, S. Denger, D. Manu, J. Beaudouin, J. Ellenberg and F. Gannon (2003) Cyclic, proteasome-mediated turnover of unliganded and liganded ERalpha on responsive promoters is an integral feature of estrogen signaling *Mol Cell* **11** 3 695-707
172 H. Poukka, U. Karvonen, O. A. Janne and J. J. Palvimo (2000) Covalent modification of the androgen receptor by small ubiquitin-like modifier 1 (SUMO-1) *Proc Natl Acad Sci U S A* **97** 26 14145-50
173 Y. Le Drean, N. Mincheneau, P. Le Goff and D. Michel (2002) Potentiation of glucocorticoid receptor transcriptional activity by sumoylation *Endocrinology* **143** 9 3482-9
174 T. Nishida and H. Yasuda (2002) PIAS1 and PIASxalpha function as SUMO-E3 ligases toward androgen receptor and repress androgen receptor-dependent transcription *J Biol Chem* **277** 44 41311-7
175 S. Tian, H. Poukka, J. J. Palvimo and O. A. Janne (2002) Small ubiquitin-related modifier-1 (SUMO-1) modification of the glucocorticoid receptor *Biochem J* **367** Pt 3 907-11
176 N. Kotaja, U. Karvonen, O. A. Janne and J. J. Palvimo (2002) The nuclear receptor interaction domain of GRIP1 is modulated by covalent attachment of SUMO-1 *J Biol Chem* **277** 33 30283-8
177 A. Chauchereau, L. Amazit, M. Quesne, A. Guiochon-Mantel and E. Milgrom (2003) Sumoylation of the progesterone receptor and of the steroid receptor coactivator SRC-1 *J Biol Chem* **278** 14 12335-43
178 H. Endoh, K. Maruyama, Y. Masuhiro, Y. Kobayashi, M. Goto, H. Tai, J. Yanagisawa, D. Metzger, S. Hashimoto and S. Kato (1999) Purification and identification of p68 RNA helicase acting as a transcriptional coactivator specific for the activation function 1 of human estrogen receptor alpha *Mol Cell Biol* **19** 8 5363-72
179 A. Tremblay, G. B. Tremblay, F. Labrie and V. Giguere (1999) Ligand-independent recruitment of SRC-1 to estrogen receptor beta through phosphorylation of activation function AF-1 *Mol Cell* **3** 4 513-9
180 S. Yeh, H. K. Lin, H. Y. Kang, T. H. Thin, M. F. Lin and C. Chang (1999) From HER2/Neu signal cascade to androgen receptor and its coactivators: a novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells *Proc Natl Acad Sci U S A* **96** 10 5458-63
181 P. M. Barger, A. C. Browning, A. N. Garner and D. P. Kelly (2001) p38 mitogen-activated protein kinase activates peroxisome proliferator-activated receptor alpha: a potential role in the cardiac metabolic stress response *J Biol Chem* **276** 48 44495-501
182 P. H. Driggers, J. H. Segars and D. M. Rubino (2001) The proto-oncoprotein Brx activates estrogen receptor beta by a p38 mitogen-activated protein kinase pathway *J Biol Chem* **276** 50 46792-7
183 M. Watanabe, J. Yanagisawa, H. Kitagawa, K. Takeyama, S. Ogawa, Y. Arao, M. Suzawa, Y. Kobayashi, T. Yano, H. Yoshikawa, Y. Masuhiro and S. Kato (2001) A subfamily of RNA-binding DEAD-box proteins acts as an estrogen receptor alpha coactivator through the N-terminal activation domain (AF-1) with an RNA coactivator, SRA *Embo J* **20** 6 1341-52
184 E. Hu, J. B. Kim, P. Sarraf and B. M. Spiegelman (1996) Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARgamma *Science* **274** 5295 2100-3
185 M. Adams, M. J. Reginato, D. Shao, M. A. Lazar and V. K. Chatterjee (1997) Transcriptional activation by peroxisome proliferator-activated receptor gamma is inhibited by phosphorylation at a consensus mitogen-activated protein kinase site *J Biol Chem* **272** 8 5128-32
186 H. S. Camp, A. Chaudhry and T. Leff (2001) A novel potent antagonist of peroxisome proliferator-activated receptor gamma blocks adipocyte differentiation but does not revert the phenotype of terminally differentiated adipocytes *Endocrinology* **142** 7 3207-13
187 I. Rogatsky, C. L. Waase and M. J. Garabedian (1998) Phosphorylation and inhibition of rat glucocorticoid receptor transcriptional activation by glycogen synthase kinase-3 (GSK-3). Species-specific differences between human and rat glucocorticoid receptor signaling as revealed through GSK-3 phosphorylation *J Biol Chem* **273** 23 14315-21
188 A. D. Wallace and J. A. Cidlowski (2001) Proteasome-mediated glucocorticoid receptor degradation restricts transcriptional signaling by glucocorticoids *J Biol Chem* **276** 46 42714-21
189 Z. E. Floyd and J. M. Stephens (2002) Interferon-gamma-mediated activation and ubiquitin-proteasome-dependent degradation of PPARgamma in adipocytes *J Biol Chem* **277** 6 4062-8
190 A. Migliaccio, M. Di Domenico, S. Green, A. De Falco, E. L. Kajtaniak, F. Blasi, P. Chambon and F. Auricchio (1989) Phosphorylation on tyrosine of in vitro synthesized human estrogen receptor activates its hormone binding *Mol Endocrinol* **3** 7 1061-9
191 J. I. Huggenvik, M. W. Collard, Y. W. Kim and R. P. Sharma (1993) Modification of the retinoic acid signaling pathway by the catalytic subunit of protein kinase-A *Mol Endocrinol* **7** 4 543-50
192 C. Rochette-Egly, M. Oulad-Abdelghani, A. Staub, V. Pfister, I. Scheuer, P. Chambon and M. P. Gaub (1995) Phosphorylation of the retinoic acid receptor-alpha by protein kinase A *Mol Endocrinol* **9** 7 860-71
193 J. C. Hsieh, P. W. Jurutka, S. Nakajima, M. A. Galligan, C. A. Haussler, Y. Shimizu, N. Shimizu, G. K. Whitfield and M. R. Haussler (1993) Phosphorylation of the human vitamin D receptor by protein kinase C. Biochemical and functional evaluation of the serine 51 recognition site *J Biol Chem* **268** 20 15118-26

194 D. Chen, P. E. Pace, R. C. Coombes and S. Ali (1999) Phosphorylation of human estrogen receptor alpha by protein kinase A regulates dimerization *Mol Cell Biol* **19** 2 1002-15
195 M. H. Delmotte, A. Tahayato, P. Formstecher and P. Lefebvre (1999) Serine 157, a retinoic acid receptor alpha residue phosphorylated by protein kinase C in vitro, is involved in RXR.RARalpha heterodimerization and transcriptional activity *J Biol Chem* **274** 53 38225-31
196 C. Solomon, J. H. White and R. Kremer (1999) Mitogen-activated protein kinase inhibits 1,25-dihydroxyvitamin D3-dependent signal transduction by phosphorylating human retinoid X receptor alpha *J Clin Invest* **103** 12 1729-35
197 X. Zhu and J. P. Liu (1997) Steroid-independent activation of androgen receptor in androgen-independent prostate cancer: a possible role for the MAP kinase signal transduction pathway? *Mol Cell Endocrinol* **134** 1 9-14
198 Y. Wen, M. C. Hu, K. Makino, B. Spohn, G. Bartholomeusz, D. H. Yan and M. C. Hung (2000) HER-2/neu promotes androgen-independent survival and growth of prostate cancer cells through the Akt pathway *Cancer Res* **60** 24 6841-5
199 R. A. Campbell, P. Bhat-Nakshatri, N. M. Patel, D. Constantinidou, S. Ali and H. Nakshatri (2001) Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance *J Biol Chem* **276** 13 9817-24
200 B. J. Feldman and D. Feldman (2001) The development of androgen-independent prostate cancer *Nat Rev Cancer* **1** 1 34-45
201 H. Chen, R. J. Lin, W. Xie, D. Wilpitz and R. M. Evans (1999) Regulation of hormone-induced histone hyperacetylation and gene activation via acetylation of an acetylase *Cell* **98** 5 675-86
202 D. Girdwood, D. Bumpass, O. A. Vaughan, A. Thain, L. A. Anderson, A. W. Snowden, E. Garcia-Wilson, N. D. Perkins and R. T. Hay (2003) P300 transcriptional repression is mediated by SUMO modification *Mol Cell* **11** 4 1043-54
203 B. G. Rowan, N. L. Weigel and B. W. O'malley (2000) Phosphorylation of steroid receptor coactivator-1. Identification of the phosphorylation sites and phosphorylation through the mitogen-activated protein kinase pathway *J Biol Chem* **275** 6 4475-83
204 L. W. Yuan and J. E. Gambee (2000) Phosphorylation of p300 at serine 89 by protein kinase C *J Biol Chem* **275** 52 40946-51
205 G. N. Lopez, C. W. Turck, F. Schaufele, M. R. Stallcup and P. J. Kushner (2001) Growth factors signal to steroid receptors through mitogen-activated protein kinase regulation of p160 coactivator activity *J Biol Chem* **276** 25 22177-82
206 N. Vo and R. H. Goodman (2001) CREB-binding protein and p300 in transcriptional regulation *J Biol Chem* **276** 17 13505-8
207 S. H. Hong and M. L. Privalsky (2000) The SMRT corepressor is regulated by a MEK-1 kinase pathway: inhibition of corepressor function is associated with SMRT phosphorylation and nuclear export *Mol Cell Biol* **20** 17 6612-25
208 M. Gianni, A. Bauer, E. Garattini, P. Chambon and C. Rochette-Egly (2002) Phosphorylation by p38MAPK and recruitment of SUG-1 are required for RA-induced RAR gamma degradation and transactivation *Embo J* **21** 14 3760-9
209 C. A. Lange, T. Shen and K. B. Horwitz (2000) Phosphorylation of human progesterone receptors at serine-294 by mitogen-activated protein kinase signals their degradation by the 26S proteasome *Proc Natl Acad Sci U S A* **97** 3 1032-7
210 T. Shen, K. B. Horwitz and C. A. Lange (2001) Transcriptional hyperactivity of human progesterone receptors is coupled to their ligand-dependent down-regulation by mitogen-activated protein kinase-dependent phosphorylation of serine 294 *Mol Cell Biol* **21** 18 6122-31
211 A. Baniahmad, X. Leng, T. P. Burris, S. Y. Tsai, M. J. Tsai and B. W. O'malley (1995) The tau 4 activation domain of the thyroid hormone receptor is required for release of a putative corepressor(s) necessary for transcriptional silencing *Mol Cell Biol* **15** 1 76-86
212 J. Casanova, E. Helmer, S. Selmi-Ruby, J. S. Qi, M. Au-Fliegner, V. Desai-Yajnik, N. Koudinova, F. Yarm, B. M. Raaka and H. H. Samuels (1994) Functional evidence for ligand-dependent dissociation of thyroid hormone and retinoic acid receptors from an inhibitory cellular factor *Mol Cell Biol* **14** 9 5756-65
213 G. X. Tong, M. Jeyakumar, M. R. Tanen and M. K. Bagchi (1996) Transcriptional silencing by unliganded thyroid hormone receptor beta requires a soluble corepressor that interacts with the ligand-binding domain of the receptor *Mol Cell Biol* **16** 5 1909-20
214 E. P. Sablin, I. N. Krylova, R. J. Fletterick and H. A. Ingraham (2003) Structural basis for ligand-independent activation of the orphan nuclear receptor LRH-1 *Mol Cell* **11** 6 1575-85
215 Z. Wang, G. Benoit, J. Liu, S. Prasad, P. Aarnisalo, X. Liu, H. Xu, N. P. Walker and T. Perlmann (2003) Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors *Nature* **423** 6939 555-60
216 G. B. Tremblay, A. Tremblay, F. Labrie and V. Giguere (1998) Ligand-independent activation of the estrogen receptors alpha and beta by mutations of a conserved tyrosine can be abolished by antiestrogens *Cancer Res* **58** 5 877-81
217 R. White, M. Sjoberg, E. Kalkhoven and M. G. Parker (1997) Ligand-independent activation of the oestrogen receptor by mutation of a conserved tyrosine *Embo J* **16** 6 1427-35
218 L. A. Denner, N. L. Weigel, B. L. Maxwell, W. T. Schrader and B. W. O'malley (1990) Regulation of progesterone receptor-mediated transcription by phosphorylation *Science* **250** 4988 1740-3
219 B. G. Rowan, N. Garrison, N. L. Weigel and B. W. O'malley (2000) 8-Bromo-cyclic AMP induces phosphorylation of two sites in SRC-1 that facilitate ligand-independent activation of the chicken progesterone receptor and are critical for functional cooperation between SRC-1 and CREB binding protein *Mol Cell Biol* **20** 23 8720-30

220 R. F. Power, S. K. Mani, J. Codina, O. M. Conneely and B. W. O'malley (1991) Dopaminergic and ligand-independent activation of steroid hormone receptors *Science* **254** 5038 1636-9
221 C. L. Smith, O. M. Conneely and B. W. O'malley (1993) Modulation of the ligand-independent activation of the human estrogen receptor by hormone and antihormone *Proc Natl Acad Sci U S A* **90** 13 6120-4
222 E. M. Apostolakis, J. Garai, C. Fox, C. L. Smith, S. J. Watson, J. H. Clark and B. W. O'malley (1996) Dopaminergic regulation of progesterone receptors: brain D5 dopamine receptors mediate induction of lordosis by D1-like agonists in rats *J Neurosci* **16** 16 4823-34
223 S. K. Mani, J. M. Allen, J. P. Lydon, B. Mulac-Jericevic, J. D. Blaustein, F. J. Demayo, O. Conneely and B. W. O'malley (1996) Dopamine requires the unoccupied progesterone receptor to induce sexual behavior in mice *Mol Endocrinol* **10** 12 1728-37
224 S. K. Mani, J. D. Blaustein, J. M. Allen, S. W. Law, B. W. O'malley and J. H. Clark (1994) Inhibition of rat sexual behavior by antisense oligonucleotides to the progesterone receptor *Endocrinology* **135** 4 1409-14
225 S. K. Mani, A. M. Reyna, J. Z. Chen, B. Mulac-Jericevic and O. M. Conneely (2006) Differential response of progesterone receptor isoforms in hormone-dependent and -independent facilitation of female sexual receptivity *Mol Endocrinol* **20** 6 1322-32
226 S. Kato, H. Endoh, Y. Masuhiro, T. Kitamoto, S. Uchiyama, H. Sasaki, S. Masushige, Y. Gotoh, E. Nishida, H. Kawashima, D. Metzger and P. Chambon (1995) Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase *Science* **270** 5241 1491-4
227 G. Bunone, P. A. Briand, R. J. Miksicek and D. Picard (1996) Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation *Embo J* **15** 9 2174-83
228 Z. Culig, A. Hobisch, M. V. Cronauer, C. Radmayr, J. Trapman, A. Hittmair, G. Bartsch and H. Klocker (1994) Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor *Cancer Res* **54** 20 5474-8
229 A. Hobisch, I. E. Eder, T. Putz, W. Horninger, G. Bartsch, H. Klocker and Z. Culig (1998) Interleukin-6 regulates prostate-specific protein expression in prostate carcinoma cells by activation of the androgen receptor *Cancer Res* **58** 20 4640-5
230 L. V. Nazareth and N. L. Weigel (1996) Activation of the human androgen receptor through a protein kinase A signaling pathway *J Biol Chem* **271** 33 19900-7
231 M. L. Moyer, K. C. Borror, B. J. Bona, D. B. Defranco and S. K. Nordeen (1993) Modulation of cell signaling pathways can enhance or impair glucocorticoid-induced gene expression without altering the state of receptor phosphorylation *J Biol Chem* **268** 30 22933-40
232 S. K. Nordeen, M. L. Moyer and B. J. Bona (1994) The coupling of multiple signal transduction pathways with steroid response mechanisms *Endocrinology* **134** 4 1723-32
233 P. N. Rangarajan, K. Umesono and R. M. Evans (1992) Modulation of glucocorticoid receptor function by protein kinase A *Mol Endocrinol* **6** 9 1451-7
234 T. Matkovits and S. Christakos (1995) Ligand occupancy is not required for vitamin D receptor and retinoid receptor-mediated transcriptional activation *Mol Endocrinol* **9** 2 232-42
235 K. Paech, P. Webb, G. G. Kuiper, S. Nilsson, J. Gustafsson, P. J. Kushner and T. S. Scanlan (1997) Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites *Science* **277** 5331 1508-10
236 M. Pfahl (1993) Nuclear receptor/AP-1 interaction *Endocr Rev* **14** 5 651-8
237 C. S. Watson (1999) Signaling themes shared between peptide and steroid hormones at the plasma membrane *Sci STKE* **1999** 12 PE1
238 A. Migliaccio, M. Di Domenico, G. Castoria, A. De Falco, P. Bontempo, E. Nola and F. Auricchio (1996) Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells *Embo J* **15** 6 1292-300
239 T. Nakajima, T. Kitazawa, E. Hamada, H. Hazama, M. Omata and Y. Kurachi (1995) 17beta-Estradiol inhibits the voltage-dependent L-type Ca2+ currents in aortic smooth muscle cells *Eur J Pharmacol* **294** 2-3 625-35
240 J. Tesarik and C. Mendoza (1995) Nongenomic effects of 17 beta-estradiol on maturing human oocytes: relationship to oocyte developmental potential *J Clin Endocrinol Metab* **80** 4 1438-43
241 M. A. Valverde, P. Rojas, J. Amigo, D. Cosmelli, P. Orio, M. I. Bahamonde, G. E. Mann, C. Vergara and R. Latorre (1999) Acute activation of Maxi-K channels (hSlo) by estradiol binding to the beta subunit *Science* **285** 5435 1929-31
242 J. J. Watters, J. S. Campbell, M. J. Cunningham, E. G. Krebs and D. M. Dorsa (1997) Rapid membrane effects of steroids in neuroblastoma cells: effects of estrogen on mitogen activated protein kinase signalling cascade and c-fos immediate early gene transcription *Endocrinology* **138** 9 4030-3
243 S. M. Aronica, W. L. Kraus and B. S. Katzenellenbogen (1994) Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription *Proc Natl Acad Sci U S A* **91** 18 8517-21
244 V. Le Mellay, B. Grosse and M. Lieberherr (1997) Phospholipase C beta and membrane action of calcitriol and estradiol *J Biol Chem* **272** 18 11902-7
245 A. Migliaccio, D. Piccolo, G. Castoria, M. Di Domenico, A. Bilancio, M. Lombardi, W. Gong, M. Beato and F. Auricchio (1998) Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor *Embo J* **17** 7 2008-18
246 V. Boonyaratanakornkit, M. P. Scott, V. Ribon, L. Sherman, S. M. Anderson, J. L. Maller, W. T. Miller and D. P. Edwards (2001) Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family tyrosine kinases *Mol Cell* **8** 2 269-80
247 Z. Guo, W. P. Benten, J. Krucken and F. Wunderlich (2002) Nongenomic testosterone calcium signaling. Genotropic actions in androgen receptor-free macrophages *J Biol Chem* **277** 33 29600-7

248 S. Bravo, R. Paredes, P. Izaurieta, J. B. Lian, J. L. Stein, G. S. Stein, M. V. Hinrichs, J. Olate, L. G. Aguayo and M. Montecino (2006) The classic receptor for 1alpha,25-dihydroxy vitamin D3 is required for non-genomic actions of 1alpha,25-dihydroxy vitamin D3 in osteosarcoma cells *J Cell Biochem* **99** 4 995-1000
249 T. Simoncini, A. Hafezi-Moghadam, D. P. Brazil, K. Ley, W. W. Chin and J. K. Liao (2000) Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase *Nature* **407** 6803 538-41
250 A. Hafezi-Moghadam, T. Simoncini, Z. Yang, F. P. Limbourg, J. C. Plumier, M. C. Rebsamen, C. M. Hsieh, D. S. Chui, K. L. Thomas, A. J. Prorock, V. E. Laubach, M. A. Moskowitz, B. A. French, K. Ley and J. K. Liao (2002) Acute cardiovascular protective effects of corticosteroids are mediated by non-transcriptional activation of endothelial nitric oxide synthase *Nat Med* **8** 5 473-9
251 J. A. Ruizeveld De Winter, J. Trapman, M. Vermey, E. Mulder, N. D. Zegers and T. H. Van Der Kwast (1991) Androgen receptor expression in human tissues: an immunohistochemical study *J Histochem Cytochem* **39** 7 927-36
252 N. Kimura, A. Mizokami, T. Oonuma, H. Sasano and H. Nagura (1993) Immunocytochemical localization of androgen receptor with polyclonal antibody in paraffin-embedded human tissues *J Histochem Cytochem* **41** 5 671-8
253 S. Andersson, R. W. Bishop and D. W. Russell (1989) Expression cloning and regulation of steroid 5 alpha-reductase, an enzyme essential for male sexual differentiation *J Biol Chem* **264** 27 16249-55
254 S. Andersson, D. M. Berman, E. P. Jenkins and D. W. Russell (1991) Deletion of steroid 5 alpha-reductase 2 gene in male pseudohermaphroditism *Nature* **354** 6349 159-61
255 J. E. Karp, A. Chiarodo, O. Brawley and G. J. Kelloff (1996) Prostate cancer prevention: investigational approaches and opportunities *Cancer Res* **56** 24 5547-56
256 P. W. Faber, G. G. Kuiper, H. C. Van Rooij, J. A. Van Der Korput, A. O. Brinkmann and J. Trapman (1989) The N-terminal domain of the human androgen receptor is encoded by one, large exon *Mol Cell Endocrinol* **61** 2 257-62
257 G. G. Kuiper, P. W. Faber, H. C. Van Rooij, J. A. Van Der Korput, C. Ris-Stalpers, P. Klaassen, J. Trapman and A. O. Brinkmann (1989) Structural organization of the human androgen receptor gene *J Mol Endocrinol* **2** 3 R1-4
258 D. B. Lubahn, T. R. Brown, J. A. Simental, H. N. Higgs, C. J. Migeon, E. M. Wilson and F. S. French (1989) Sequence of the intron/exon junctions of the coding region of the human androgen receptor gene and identification of a point mutation in a family with complete androgen insensitivity *Proc Natl Acad Sci U S A* **86** 23 9534-8
259 C. S. Chang, J. Kokontis and S. T. Liao (1988) Molecular cloning of human and rat complementary DNA encoding androgen receptors *Science* **240** 4850 324-6
260 D. B. Lubahn, D. R. Joseph, M. Sar, J. Tan, H. N. Higgs, R. E. Larson, F. S. French and E. M. Wilson (1988) The human androgen receptor: complementary deoxyribonucleic acid cloning, sequence analysis and gene expression in prostate *Mol Endocrinol* **2** 12 1265-75
261 J. Trapman, P. Klaassen, G. G. Kuiper, J. A. Van Der Korput, P. W. Faber, H. C. Van Rooij, A. Geurts Van Kessel, M. M. Voorhorst, E. Mulder and A. O. Brinkmann (1988) Cloning, structure and expression of a cDNA encoding the human androgen receptor *Biochem Biophys Res Commun* **153** 1 241-8
262 W. D. Tilley, M. Marcelli, J. D. Wilson and M. J. Mcphaul (1989) Characterization and expression of a cDNA encoding the human androgen receptor *Proc Natl Acad Sci U S A* **86** 1 327-31
263 B. Gottlieb, L. K. Beitel, J. H. Wu and M. Trifiro (2004) The androgen receptor gene mutations database (ARDB): 2004 update *Hum Mutat* **23** 6 527-33
264 C. M. Wilson and M. J. Mcphaul (1994) A and B forms of the androgen receptor are present in human genital skin fibroblasts *Proc Natl Acad Sci U S A* **91** 4 1234-8
265 C. M. Wilson and M. J. Mcphaul (1996) A and B forms of the androgen receptor are expressed in a variety of human tissues *Mol Cell Endocrinol* **120** 1 51-7
266 G. Jenster, H. A. Van Der Korput, J. Trapman and A. O. Brinkmann (1995) Identification of two transcription activation units in the N-terminal domain of the human androgen receptor *J Biol Chem* **270** 13 7341-6
267 L. Callewaert, N. Van Tilborgh and F. Claessens (2006) Interplay between two hormone-independent activation domains in the androgen receptor *Cancer Res* **66** 1 543-53
268 N. L. Chamberlain, D. C. Whitacre and R. L. Miesfeld (1996) Delineation of two distinct type 1 activation functions in the androgen receptor amino-terminal domain *J Biol Chem* **271** 43 26772-8
269 J. Reid, S. M. Kelly, K. Watt, N. C. Price and I. J. Mcewan (2002) Conformational analysis of the androgen receptor amino-terminal domain involved in transactivation. Influence of structure-stabilizing solutes and protein-protein interactions *J Biol Chem* **277** 22 20079-86
270 B. He, S. Bai, A. T. Hnat, R. I. Kalman, J. T. Minges, C. Patterson and E. M. Wilson (2004) An androgen receptor NH2-terminal conserved motif interacts with the COOH terminus of the Hsp70-interacting protein (CHIP) *J Biol Chem* **279** 29 30643-53
271 C. A. Heinlein and C. Chang (2002) Androgen receptor (AR) coregulators: an overview *Endocr Rev* **23** 2 175-200
272 S. Bai, B. He and E. M. Wilson (2005) Melanoma antigen gene protein MAGE-11 regulates androgen receptor function by modulating the interdomain interaction *Mol Cell Biol* **25** 4 1238-57
273 G. Jenster, J. Trapman and A. O. Brinkmann (1993) Nuclear import of the human androgen receptor *Biochem J* **293 (Pt 3)** 761-8
274 Z. X. Zhou, M. Sar, J. A. Simental, M. V. Lane and E. M. Wilson (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** 18 13115-23

275 Q. Wang, J. Lu and E. L. Yong (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** 10 7493-9
276 A. Haelens, T. Tanner, S. Denayer, L. Callewaert and F. Claessens (2007) The hinge region regulates DNA binding, nuclear translocation, and transactivation of the androgen receptor *Cancer Res* **67** 9 4514-23
277 J. S. Sack, K. F. Kish, C. Wang, R. M. Attar, S. E. Kiefer, Y. An, G. Y. Wu, J. E. Scheffler, M. E. Salvati, S. R. Krystek, Jr., R. Weinmann and H. M. Einspahr (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** 9 4904-9
278 P. M. Matias, P. Donner, R. Coelho, M. Thomaz, C. Peixoto, S. Macedo, N. Otto, S. Joschko, P. Scholz, A. Wegg, S. Basler, M. Schafer, U. Egner and M. A. Carrondo (2000) Structural evidence for ligand specificity in the binding domain of the human androgen receptor. Implications for pathogenic gene mutations *J Biol Chem* **275** 34 26164-71
279 C. W. Kuil and E. Mulder (1995) Effects of androgens and antiandrogens on the conformation of the androgen receptor *Ann N Y Acad Sci* **761** 351-4
280 J. Veldscholte, C. Ris-Stalpers, G. G. Kuiper, G. Jenster, C. Berrevoets, E. Claassen, H. C. Van Rooij, J. Trapman, A. O. Brinkmann and E. Mulder (1990) A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens *Biochem Biophys Res Commun* **173** 2 534-40
281 M. E. Taplin, G. J. Bubley, T. D. Shuster, M. E. Frantz, A. E. Spooner, G. K. Ogata, H. N. Keer and S. P. Balk (1995) Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer *N Engl J Med* **332** 21 1393-8
282 M. N. Patterson, I. A. Hughes, B. Gottlieb and L. Pinsky (1994) The androgen receptor gene mutations database *Nucleic Acids Res* **22** 17 3560-2
283 J. Veldscholte, M. M. Voorhorst-Ogink, J. Bolt-De Vries, H. C. Van Rooij, J. Trapman and E. Mulder (1990) Unusual specificity of the androgen receptor in the human prostate tumor cell line LNCaP: high affinity for progestagenic and estrogenic steroids *Biochim Biophys Acta* **1052** 1 187-94
284 C. A. Berrevoets, P. Doesburg, K. Steketee, J. Trapman and A. O. Brinkmann (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** 8 1172-83
285 U. Karvonen, O. A. Janne and J. J. Palvimo (2002) Pure antiandrogens disrupt the recruitment of coactivator GRIP1 to colocalize with androgen receptor in nuclei *FEBS Lett* **523** 1-3 43-7
286 D. Masiello, S. Cheng, G. J. Bubley, M. L. Lu and S. P. Balk (2002) Bicalutamide functions as an androgen receptor antagonist by assembly of a transcriptionally inactive receptor *J Biol Chem* **277** 29 26321-6
287 P. Farla, R. Hersmus, J. Trapman and A. B. Houtsmuller (2005) Antiandrogens prevent stable DNA-binding of the androgen receptor *J Cell Sci* **118** Pt 18 4187-98
288 H. Dotzlaw, U. Moehren, S. Mink, A. C. Cato, J. A. Iniguez Lluhi and A. Baniahmad (2002) The amino terminus of the human AR is target for corepressor action and antihormone agonism *Mol Endocrinol* **16** 4 661-73
289 B. He, J. T. Minges, L. W. Lee and E. M. Wilson (2002) The FXXLF motif mediates androgen receptor-specific interactions with coregulators *J Biol Chem* **277** 12 10226-35
290 L. Callewaert, G. Verrijdt, V. Christiaens, A. Haelens and F. Claessens (2003) Dual function of an amino-terminal amphipatic helix in androgen receptor-mediated transactivation through specific and nonspecific response elements *J Biol Chem* **278** 10 8212-8
291 H. J. Dubbink, R. Hersmus, C. S. Verma, H. A. Van Der Korput, C. A. Berrevoets, J. Van Tol, A. C. Ziel-Van Der Made, A. O. Brinkmann, A. C. Pike and J. Trapman (2004) Distinct recognition modes of FXXLF and LXXLL motifs by the androgen receptor *Mol Endocrinol* **18** 9 2132-50
292 E. Hur, S. J. Pfaff, E. S. Payne, H. Gron, B. M. Buehrer and R. J. Fletterick (2004) Recognition and accommodation at the androgen receptor coactivator binding interface *PLoS Biol* **2** 9 E274
293 P. Farla, R. Hersmus, B. Geverts, P. O. Mari, A. L. Nigg, H. J. Dubbink, J. Trapman and A. B. Houtsmuller (2004) The androgen receptor ligand-binding domain stabilizes DNA binding in living cells *J Struct Biol* **147** 1 50-61
294 N. P. Mongan, J. Jaaskelainen, S. Bhattacharyya, R. M. Leu and I. A. Hughes (2003) Steroid receptor coactivator-3 glutamine repeat polymorphism and the androgen insensitivity syndrome *Eur J Endocrinol* **148** 2 277-9
295 X. Ye, S. J. Han, S. Y. Tsai, F. J. Demayo, J. Xu, M. J. Tsai and W. O'malley B (2005) Roles of steroid receptor coactivator (SRC)-1 and transcriptional intermediary factor (TIF) 2 in androgen receptor activity in mice *Proc Natl Acad Sci U S A* **102** 27 9487-92
296 P. Aarnisalo, J. J. Palvimo and O. A. Janne (1998) CREB-binding protein in androgen receptor-mediated signaling *Proc Natl Acad Sci U S A* **95** 5 2122-7
297 J. Kamine, B. Elangovan, T. Subramanian, D. Coleman and G. Chinnadurai (1996) Identification of a cellular protein that specifically interacts with the essential cysteine region of the HIV-1 Tat transactivator *Virology* **216** 2 357-66
298 L. Gaughan, M. E. Brady, S. Cook, D. E. Neal and C. N. Robson (2001) Tip60 is a co-activator specific for class I nuclear hormone receptors *J Biol Chem* **276** 50 46841-8
299 M. E. Brady, D. M. Ozanne, L. Gaughan, I. Waite, S. Cook, D. E. Neal and C. N. Robson (1999) Tip60 is a nuclear hormone receptor coactivator *J Biol Chem* **274** 25 17599-604

300 L. Gaughan, I. R. Logan, S. Cook, D. E. Neal and C. N. Robson (2002) Tip60 and histone deacetylase 1 regulate androgen receptor activity through changes to the acetylation status of the receptor *J Biol Chem* **277** 29 25904-13
301 I. R. Logan, L. Gaughan, S. R. Mccracken, V. Sapountzi, H. Y. Leung and C. N. Robson (2006) Human PIRH2 enhances androgen receptor signaling through inhibition of histone deacetylase 1 and is overexpressed in prostate cancer *Mol Cell Biol* **26** 17 6502-10
302 S. Yeh and C. Chang (1996) Cloning and characterization of a specific coactivator, ARA70, for the androgen receptor in human prostate cells *Proc Natl Acad Sci U S A* **93** 11 5517-21
303 S. Yeh, H. Miyamoto, H. Shima and C. Chang (1998) From estrogen to androgen receptor: a new pathway for sex hormones in prostate *Proc Natl Acad Sci U S A* **95** 10 5527-32
304 H. Miyamoto, S. Yeh, G. Wilding and C. Chang (1998) Promotion of agonist activity of antiandrogens by the androgen receptor coactivator, ARA70, in human prostate cancer DU145 cells *Proc Natl Acad Sci U S A* **95** 13 7379-84
305 S. Yeh, H. Y. Kang, H. Miyamoto, K. Nishimura, H. C. Chang, H. J. Ting, M. Rahman, H. K. Lin, N. Fujimoto, Y. C. Hu, A. Mizokami, K. E. Huang and C. Chang (1999) Differential induction of androgen receptor transactivation by different androgen receptor coactivators in human prostate cancer DU145 cells *Endocrine* **11** 2 195-202
306 N. Fujimoto, S. Yeh, H. Y. Kang, S. Inui, H. C. Chang, A. Mizokami and C. Chang (1999) Cloning and characterization of androgen receptor coactivator, ARA55, in human prostate *J Biol Chem* **274** 12 8316-21
307 X. Wang, Y. Yang, X. Guo, E. R. Sampson, C. L. Hsu, M. Y. Tsai, S. Yeh, G. Wu, Y. Guo and C. Chang (2002) Suppression of androgen receptor transactivation by Pyk2 via interaction and phosphorylation of the ARA55 coregulator *J Biol Chem* **277** 18 15426-31
308 S. M. Thomas, M. Hagel and C. E. Turner (1999) Characterization of a focal adhesion protein, Hic-5, that shares extensive homology with paxillin *J Cell Sci* **112 (Pt 2)** 181-90
309 M. Shibanuma, E. Mochizuki, R. Maniwa, J. Mashimo, N. Nishiya, S. Imai, T. Takano, M. Oshimura and K. Nose (1997) Induction of senescence-like phenotypes by forced expression of hic-5, which encodes a novel LIM motif protein, in immortalized human fibroblasts *Mol Cell Biol* **17** 3 1224-35
310 X. Wang, S. Yeh, G. Wu, C. L. Hsu, L. Wang, T. Chiang, Y. Yang, Y. Guo and C. Chang (2001) Identification and characterization of a novel androgen receptor coregulator ARA267-alpha in prostate cancer cells *J Biol Chem* **276** 44 40417-23
311 N. Huang, E. Vom Baur, J. M. Garnier, T. Lerouge, J. L. Vonesch, Y. Lutz, P. Chambon and R. Losson (1998) Two distinct nuclear receptor interaction domains in NSD1, a novel SET protein that exhibits characteristics of both corepressors and coactivators *Embo J* **17** 12 3398-412
312 J. Briscoe, F. Kohlhuber and M. Muller (1996) JAKs and STATs branch out *Trends Cell Biol* **6** 9 336-40
313 A. M. Moilanen, U. Karvonen, H. Poukka, O. A. Janne and J. J. Palvimo (1998) Activation of androgen receptor function by a novel nuclear protein kinase *Mol Biol Cell* **9** 9 2527-43
314 J. Tan, S. H. Hall, K. G. Hamil, G. Grossman, P. Petrusz, J. Liao, K. Shuai and F. S. French (2000) Protein inhibitor of activated STAT-1 (signal transducer and activator of transcription-1) is a nuclear receptor coregulator expressed in human testis *Mol Endocrinol* **14** 1 14-26
315 A. M. Moilanen, U. Karvonen, H. Poukka, W. Yan, J. Toppari, O. A. Janne and J. J. Palvimo (1999) A testis-specific androgen receptor coregulator that belongs to a novel family of nuclear proteins *J Biol Chem* **274** 6 3700-4
316 K. Willert and R. Nusse (1998) Beta-catenin: a key mediator of Wnt signaling *Curr Opin Genet Dev* **8** 1 95-102
317 S. Terry, X. Yang, M. W. Chen, F. Vacherot and R. Buttyan (2006) Multifaceted interaction between the androgen and Wnt signaling pathways and the implication for prostate cancer *J Cell Biochem* **99** 2 402-10
318 X. Yang, M. W. Chen, S. Terry, F. Vacherot, D. L. Bemis, J. Capodice, J. Kitajewski, A. De La Taille, M. C. Benson, Y. Guo and R. Buttyan (2006) Complex regulation of human androgen receptor expression by Wnt signaling in prostate cancer cells *Oncogene* **25** 24 3436-44
319 C. I. Truica, S. Byers and E. P. Gelmann (2000) Beta-catenin affects androgen receptor transcriptional activity and ligand specificity *Cancer Res* **60** 17 4709-13
320 M. Verras and Z. Sun (2005) Roles and regulation of Wnt signaling and beta-catenin in prostate cancer *Cancer Lett*
321 K. Nishimura, H. J. Ting, Y. Harada, T. Tokizane, N. Nonomura, H. Y. Kang, H. C. Chang, S. Yeh, H. Miyamoto, M. Shin, K. Aozasa, A. Okuyama and C. Chang (2003) Modulation of androgen receptor transactivation by gelsolin: a newly identified androgen receptor coregulator *Cancer Res* **63** 16 4888-94
322 D. J. Van De Wijngaart, M. E. Van Royen, R. Hersmus, A. C. Pike, A. B. Houtsmuller, G. Jenster, J. Trapman and H. J. Dubbink (2006) Novel FXXFF and FXXMF motifs in androgen receptor cofactors mediate high affinity and specific interactions with the ligand-binding domain *J Biol Chem* **281** 28 19407-16
323 K. N. Pestonjamasp, R. K. Pope, J. D. Wulfkuhle and E. J. Luna (1997) Supervillin (p205): A novel membrane-associated, F-actin-binding protein in the villin/gelsolin superfamily *J Cell Biol* **139** 5 1255-69
324 D. M. Ozanne, M. E. Brady, S. Cook, L. Gaughan, D. E. Neal and C. N. Robson (2000) Androgen receptor nuclear translocation is facilitated by the f-actin cross-linking protein filamin *Mol Endocrinol* **14** 10 1618-26
325 S. M. Markus, S. S. Taneja, S. K. Logan, W. Li, S. Ha, A. B. Hittelman, I. Rogatsky and M. J. Garabedian (2002) Identification and characterization of ART-27, a novel coactivator for the androgen receptor N terminus *Mol Biol Cell* **13** 2 670-82
326 S. S. Taneja, S. Ha, N. K. Swenson, I. P. Torra, S. Rome, P. D. Walden, H. Y. Huang, E. Shapiro, M. J. Garabedian and S. K. Logan (2004) ART-27, an androgen receptor coactivator regulated in prostate development and cancer *J Biol Chem* **279** 14 13944-52

327 J. M. Muller, U. Isele, E. Metzger, A. Rempel, M. Moser, A. Pscherer, T. Breyer, C. Holubarsch, R. Buettner and R. Schule (2000) FHL2, a novel tissue-specific coactivator of the androgen receptor *Embo J* **19** 3 359-69

328 Y. Wei, C. A. Renard, C. Labalette, Y. Wu, L. Levy, C. Neuveut, X. Prieur, M. Flajolet, S. Prigent and M. A. Buendia (2003) Identification of the LIM protein FHL2 as a coactivator of beta-catenin *J Biol Chem* **278** 7 5188-94

329 Z. Nawaz, D. M. Lonard, C. L. Smith, E. Lev-Lehman, S. Y. Tsai, M. J. Tsai and B. W. O'malley (1999) The Angelman syndrome-associated protein, E6-AP, is a coactivator for the nuclear hormone receptor superfamily *Mol Cell Biol* **19** 2 1182-9

330 T. P. Yao, G. Ku, N. Zhou, R. Scully and D. M. Livingston (1996) The nuclear hormone receptor coactivator SRC-1 is a specific target of p300 *Proc Natl Acad Sci U S A* **93** 20 10626-31

331 B. He, J. A. Kemppainen, J. J. Voegel, H. Gronemeyer and E. M. Wilson (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** 52 37219-25

332 J. A. Tan, S. H. Hall, P. Petrusz and F. S. French (2000) Thyroid receptor activator molecule, TRAM-1, is an androgen receptor coactivator *Endocrinology* **141** 9 3440-50

333 H. Y. Kang, S. Yeh, N. Fujimoto and C. Chang (1999) Cloning and characterization of human prostate coactivator ARA54, a novel protein that associates with the androgen receptor *J Biol Chem* **274** 13 8570-6

334 M. Shibanuma, J. Mashimo, T. Kuroki and K. Nose (1994) Characterization of the TGF beta 1-inducible hic-5 gene that encodes a putative novel zinc finger protein and its possible involvement in cellular senescence *J Biol Chem* **269** 43 26767-74

335 P. Alen, F. Claessens, E. Schoenmakers, J. V. Swinnen, G. Verhoeven, W. Rombauts and B. Peeters (1999) 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** 1 117-28

336 T. Gao, K. Brantley, E. Bolu and M. J. Mcphaul (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** 10 1645-56

337 P. W. Hsiao and C. Chang (1999) Isolation and characterization of ARA160 as the first androgen receptor N-terminal-associated coactivator in human prostate cells *J Biol Chem* **274** 32 22373-9

338 H. Poukka, P. Aarnisalo, U. Karvonen, J. J. Palvimo and O. A. Janne (1999) Ubc9 interacts with the androgen receptor and activates receptor-dependent transcription *J Biol Chem* **274** 27 19441-6

339 M. L. Lu, M. C. Schneider, Y. Zheng, X. Zhang and J. P. Richie (2001) Caveolin-1 interacts with androgen receptor. A positive modulator of androgen receptor mediated transactivation *J Biol Chem* **276** 16 13442-51

340 C. J. Loy, K. S. Sim and E. L. Yong (2003) Filamin-A fragment localizes to the nucleus to regulate androgen receptor and coactivator functions *Proc Natl Acad Sci U S A* **100** 8 4562-7

341 P. W. Hsiao, D. L. Lin, R. Nakao and C. Chang (1999) The linkage of Kennedy's neuron disease to ARA24, the first identified androgen receptor polyglutamine region-associated coactivator *J Biol Chem* **274** 29 20229-34

342 S. Yeh, Y. C. Hu, M. Rahman, H. K. Lin, C. L. Hsu, H. J. Ting, H. Y. Kang and C. Chang (2000) Increase of androgen-induced cell death and androgen receptor transactivation by BRCA1 in prostate cancer cells *Proc Natl Acad Sci U S A* **97** 21 11256-61

343 S. Shin and I. M. Verma (2003) BRCA2 cooperates with histone acetyltransferases in androgen receptor-mediated transcription *Proc Natl Acad Sci U S A* **100** 12 7201-6

344 A. Yamamoto, Y. Hashimoto, K. Kohri, E. Ogata, S. Kato, K. Ikeda and M. Nakanishi (2000) Cyclin E as a coactivator of the androgen receptor *J Cell Biol* **150** 4 873-80

345 S. Yeh, H. Miyamoto, K. Nishimura, H. Kang, J. Ludlow, P. Hsiao, C. Wang, C. Su and C. Chang (1998) Retinoblastoma, a tumor suppressor, is a coactivator for the androgen receptor in human prostate cancer DU145 cells *Biochem Biophys Res Commun* **248** 2 361-7

346 A. J. Caplan, E. Langley, E. M. Wilson and J. Vidal (1995) Hormone-dependent transactivation by the human androgen receptor is regulated by a dnaJ protein *J Biol Chem* **270** 10 5251-7

347 A. E. Fliss, J. Rao, M. W. Melville, M. E. Cheetham and A. J. Caplan (1999) Domain requirements of DnaJ-like (Hsp40) molecular chaperones in the activation of a steroid hormone receptor *J Biol Chem* **274** 48 34045-52

348 D. Chakravarti, V. J. Lamorte, M. C. Nelson, T. Nakajima, I. G. Schulman, H. Juguilon, M. Montminy and R. M. Evans (1996) Role of CBP/P300 in nuclear receptor signalling *Nature* **383** 6595 99-103

349 E. Treuter, T. Albrektsen, L. Johansson, J. Leers and J. A. Gustafsson (1998) A regulatory role for RIP140 in nuclear receptor activation *Mol Endocrinol* **12** 6 864-81

350 S. M. Huang and M. R. Stallcup (2000) Mouse Zac1, a transcriptional coactivator and repressor for nuclear receptors *Mol Cell Biol* **20** 5 1855-67

351 D. Knutti, A. Kaul and A. Kralli (2000) A tissue-specific coactivator of steroid receptors, identified in a functional genetic screen *Mol Cell Biol* **20** 7 2411-22

352 R. B. Lanz, N. J. Mckenna, S. A. Onate, U. Albrecht, J. Wong, S. Y. Tsai, M. J. Tsai and B. W. O'malley (1999) A steroid receptor coactivator, SRA, functions as an RNA and is present in an SRC-1 complex *Cell* **97** 1 17-27

353 S. R. Kupfer, K. B. Marschke, E. M. Wilson and F. S. French (1993) Receptor accessory factor enhances specific DNA binding of androgen and glucocorticoid receptors *J Biol Chem* **268** 23 17519-27

354 V. Boonyaratanakornkit, V. Melvin, P. Prendergast, M. Altmann, L. Ronfani, M. E. Bianchi, L. Taraseviciene, S. K. Nordeen, E. A. Allegretto and D. P. Edwards (1998) High-mobility group chromatin proteins 1 and 2 functionally interact with steroid hormone receptors to enhance their DNA binding in vitro and transcriptional activity in mammalian cells *Mol Cell Biol* **18** 8 4471-87

355 H. Hong, C. Kao, M. H. Jeng, J. N. Eble, M. O. Koch, T. A. Gardner, S. Zhang, L. Li, C. X. Pan, Z. Hu, G. T. Maclennan and L. Cheng (2004) Aberrant expression of CARM1, a transcriptional coactivator of androgen receptor, in the development of prostate carcinoma and androgen-independent status *Cancer* **101** 1 83-9
356 S. S. Koh, H. Li, Y. H. Lee, R. B. Widelitz, C. M. Chuong and M. R. Stallcup (2002) Synergistic coactivator function by coactivator-associated arginine methyltransferase (CARM) 1 and beta-catenin with two different classes of DNA-binding transcriptional activators *J Biol Chem* **277** 29 26031-5
357 L. Wang, C. L. Hsu and C. Chang (2005) Androgen receptor corepressors: an overview *Prostate* **63** 2 117-30
358 S. Cheng, S. Brzostek, S. R. Lee, A. N. Hollenberg and S. P. Balk (2002) Inhibition of the dihydrotestosterone-activated androgen receptor by nuclear receptor corepressor *Mol Endocrinol* **16** 7 1492-501
359 C. A. Berrevoets, A. Umar, J. Trapman and A. O. Brinkmann (2004) Differential modulation of androgen receptor transcriptional activity by the nuclear receptor co-repressor (N-CoR) *Biochem J* **379** Pt 3 731-8
360 M. C. Hodgson, I. Astapova, S. Cheng, L. J. Lee, M. C. Verhoeven, E. Choi, S. P. Balk and A. N. Hollenberg (2005) The androgen receptor recruits nuclear receptor CoRepressor (N-CoR) in the presence of mifepristone via its N and C termini revealing a novel molecular mechanism for androgen receptor antagonists *J Biol Chem* **280** 8 6511-9
361 G. Liao, L. Y. Chen, A. Zhang, A. Godavarthy, F. Xia, J. C. Ghosh, H. Li and J. D. Chen (2003) Regulation of androgen receptor activity by the nuclear receptor corepressor SMRT *J Biol Chem* **278** 7 5052-61
362 Y. Shang, M. Myers and M. Brown (2002) Formation of the androgen receptor transcription complex *Mol Cell* **9** 3 601-10
363 L. N. Song, M. Coghlan and E. P. Gelmann (2004) Antiandrogen effects of mifepristone on coactivator and corepressor interactions with the androgen receptor *Mol Endocrinol* **18** 1 70-85
364 H. Dotzlaw, M. Papaioannou, U. Moehren, F. Claessens and A. Baniahmad (2003) Agonist-antagonist induced coactivator and corepressor interplay on the human androgen receptor *Mol Cell Endocrinol* **213** 1 79-85
365 L. Wang, C. L. Hsu, J. Ni, P. H. Wang, S. Yeh, P. Keng and C. Chang (2004) Human checkpoint protein hRad9 functions as a negative coregulator to repress androgen receptor transactivation in prostate cancer cells *Mol Cell Biol* **24** 5 2202-13
366 T. P. Burris, W. Guo and E. R. Mccabe (1996) The gene responsible for adrenal hypoplasia congenita, DAX-1, encodes a nuclear hormone receptor that defines a new class within the superfamily *Recent Prog Horm Res* **51** 241-59; discussion 259-60
367 E. Holter, N. Kotaja, S. Makela, L. Strauss, S. Kietz, O. A. Janne, J. A. Gustafsson, J. J. Palvimo and E. Treuter (2002) Inhibition of androgen receptor (AR) function by the reproductive orphan nuclear receptor DAX-1 *Mol Endocrinol* **16** 3 515-28
368 J. Gobinet, G. Auzou, J. C. Nicolas, C. Sultan and S. Jalaguier (2001) Characterization of the interaction between androgen receptor and a new transcriptional inhibitor, SHP *Biochemistry* **40** 50 15369-77
369 J. Gobinet, S. Carascossa, V. Cavailles, F. Vignon, J. C. Nicolas and S. Jalaguier (2005) SHP represses transcriptional activity via recruitment of histone deacetylases *Biochemistry* **44** 16 6312-20
370 G. Han, G. Buchanan, M. Ittmann, J. M. Harris, X. Yu, F. J. Demayo, W. Tilley and N. M. Greenberg (2005) Mutation of the androgen receptor causes oncogenic transformation of the prostate *Proc Natl Acad Sci U S A* **102** 4 1151-6
371 B. C. Jeong, C. Y. Hong, S. Chattopadhyay, J. H. Park, E. Y. Gong, H. J. Kim, S. Y. Chun and K. Lee (2004) Androgen receptor corepressor-19 kDa (ARR19), a leucine-rich protein that represses the transcriptional activity of androgen receptor through recruitment of histone deacetylase *Mol Endocrinol* **18** 1 13-25
372 T. Niki, K. Takahashi-Niki, T. Taira, S. M. Iguchi-Ariga and H. Ariga (2003) DJBP: a novel DJ-1-binding protein, negatively regulates the androgen receptor by recruiting histone deacetylase complex, and DJ-1 antagonizes this inhibition by abrogation of this complex *Mol Cancer Res* **1** 4 247-61
373 C. G. Korkmaz, K. Fronsdal, Y. Zhang, P. I. Lorenzo and F. Saatcioglu (2004) Potentiation of androgen receptor transcriptional activity by inhibition of histone deacetylation--rescue of transcriptionally compromised mutants *J Endocrinol* **182** 3 377-89
374 M. Gross, R. Yang, I. Top, C. Gasper and K. Shuai (2004) PIASy-mediated repression of the androgen receptor is independent of sumoylation *Oncogene* **23** 17 3059-66
375 M. Sharma and Z. Sun (2001) 5'TG3' interacting factor interacts with Sin3A and represses AR-mediated transcription *Mol Endocrinol* **15** 11 1918-28
376 S. Dedhar, P. S. Rennie, M. Shago, C. Y. Hagesteijn, H. Yang, J. Filmus, R. G. Hawley, N. Bruchovsky, H. Cheng, R. J. Matusik and Et Al. (1994) Inhibition of nuclear hormone receptor activity by calreticulin *Nature* **367** 6462 480-3
377 Y. Liu, B. O. Kim, C. Kao, C. Jung, J. T. Dalton and J. J. He (2004) Tip110, the human immunodeficiency virus type 1 (HIV-1) Tat-interacting protein of 110 kDa as a negative regulator of androgen receptor (AR) transcriptional activation *J Biol Chem* **279** 21 21766-73
378 M. Powzaniuk, S. Mcelwee-Witmer, R. L. Vogel, T. Hayami, S. J. Rutledge, F. Chen, S. Harada, A. Schmidt, G. A. Rodan, L. P. Freedman and C. Bai (2004) The LATS2/KPM tumor suppressor is a negative regulator of the androgen receptor *Mol Endocrinol* **18** 8 2011-23
379 Y. Zhang, Y. Yang, S. Yeh and C. Chang (2004) ARA67/PAT1 functions as a repressor to suppress androgen receptor transactivation *Mol Cell Biol* **24** 3 1044-57
380 S. R. Lee, S. M. Ramos, A. Ko, D. Masiello, K. D. Swanson, M. L. Lu and S. P. Balk (2002) AR and ER interaction with a p21-activated kinase (PAK6) *Mol Endocrinol* **16** 1 85-99

381 N. Schrantz, J. Da Silva Correia, B. Fowler, Q. Ge, Z. Sun and G. M. Bokoch (2004) Mechanism of p21-activated kinase 6-mediated inhibition of androgen receptor signaling *J Biol Chem* **279** 3 1922-31
382 F. Yang, X. Li, M. Sharma, M. Zarnegar, B. Lim and Z. Sun (2001) Androgen receptor specifically interacts with a novel p21-activated kinase, PAK6 *J Biol Chem* **276** 18 15345-53
383 H. K. Lin, S. Yeh, H. Y. Kang and C. Chang (2001) Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor *Proc Natl Acad Sci U S A* **98** 13 7200-5
384 B. Nan, T. Snabboon, E. Unni, X. J. Yuan, Y. E. Whang and M. Marcelli (2003) The PTEN tumor suppressor is a negative modulator of androgen receptor transcriptional activity *J Mol Endocrinol* **31** 1 169-83
385 H. K. Lin, Y. C. Hu, D. K. Lee and C. Chang (2004) Regulation of androgen receptor signaling by PTEN (phosphatase and tensin homolog deleted on chromosome 10) tumor suppressor through distinct mechanisms in prostate cancer cells *Mol Endocrinol* **18** 10 2409-23
386 K. E. Knudsen, W. K. Cavenee and K. C. Arden (1999) D-type cyclins complex with the androgen receptor and inhibit its transcriptional transactivation ability *Cancer Res* **59** 10 2297-301
387 C. E. Petre, Y. B. Wetherill, M. Danielsen and K. E. Knudsen (2002) Cyclin D1: mechanism and consequence of androgen receptor co-repressor activity *J Biol Chem* **277** 3 2207-15
388 M. Sharma, M. Zarnegar, X. Li, B. Lim and Z. Sun (2000) Androgen receptor interacts with a novel MYST protein, HBO1 *J Biol Chem* **275** 45 35200-8
389 L. Johansson, J. S. Thomsen, A. E. Damdimopoulos, G. Spyrou, J. A. Gustafsson and E. Treuter (1999) The orphan nuclear receptor SHP inhibits agonist-dependent transcriptional activity of estrogen receptors ERalpha and ERbeta *J Biol Chem* **274** 1 345-53
390 J. E. Chipuk, S. C. Cornelius, N. J. Pultz, J. S. Jorgensen, M. J. Bonham, S. J. Kim and D. Danielpour (2002) The androgen receptor represses transforming growth factor-beta signaling through interaction with Smad3 *J Biol Chem* **277** 2 1240-8
391 S. A. Hayes, M. Zarnegar, M. Sharma, F. Yang, D. M. Peehl, P. Ten Dijke and Z. Sun (2001) SMAD3 represses androgen receptor-mediated transcription *Cancer Res* **61** 5 2112-8
392 H. Y. Kang, H. K. Lin, Y. C. Hu, S. Yeh, K. E. Huang and C. Chang (2001) From transforming growth factor-beta signaling to androgen action: identification of Smad3 as an androgen receptor coregulator in prostate cancer cells *Proc Natl Acad Sci U S A* **98** 6 3018-23
393 X. Yuan, M. L. Lu, T. Li and S. P. Balk (2001) SRY interacts with and negatively regulates androgen receptor transcriptional activity *J Biol Chem* **276** 49 46647-54
394 X. Mu and C. Chang (2003) TR2 orphan receptor functions as negative modulator for androgen receptor in prostate cancer cells PC-3 *Prostate* **57** 2 129-33
395 M. C. Louie, H. Q. Yang, A. H. Ma, W. Xu, J. X. Zou, H. J. Kung and H. W. Chen (2003) Androgen-induced recruitment of RNA polymerase II to a nuclear receptor-p160 coactivator complex *Proc Natl Acad Sci U S A* **100** 5 2226-30
396 Z. Kang, O. A. Janne and J. J. Palvimo (2004) Coregulator recruitment and histone modifications in transcriptional regulation by the androgen receptor *Mol Endocrinol* **18** 11 2633-48
397 T. I. Klokk, P. Kurys, C. Elbi, A. K. Nagaich, A. Hendarwanto, T. Slagsvold, C. Y. Chang, G. L. Hager and F. Saatcioglu (2007) Ligand-specific dynamics of the androgen receptor at its response element in living cells *Mol Cell Biol* **27** 5 1823-43
398 H. Inoue, T. Furukawa, S. Giannakopoulos, S. Zhou, D. S. King and N. Tanese (2002) Largest subunits of the human SWI/SNF chromatin-remodeling complex promote transcriptional activation by steroid hormone receptors *J Biol Chem* **277** 44 41674-85
399 T. W. Marshall, K. A. Link, C. E. Petre-Draviam and K. E. Knudsen (2003) Differential requirement of SWI/SNF for androgen receptor activity *J Biol Chem* **278** 33 30605-13
400 K. A. Link, C. J. Burd, E. Williams, T. Marshall, G. Rosson, E. Henry, B. Weissman and K. E. Knudsen (2005) BAF57 governs androgen receptor action and androgen-dependent proliferation through SWI/SNF *Mol Cell Biol* **25** 6 2200-15
401 C. Y. Hong, J. H. Suh, K. Kim, E. Y. Gong, S. H. Jeon, M. Ko, R. H. Seong, H. B. Kwon and K. Lee (2005) Modulation of androgen receptor transactivation by the SWI3-related gene product (SRG3) in multiple ways *Mol Cell Biol* **25** 12 4841-52
402 N. Rouleau, A. Domans'kyi, M. Reeben, A. M. Moilanen, K. Havas, Z. Kang, T. Owen-Hughes, J. J. Palvimo and O. A. Janne (2002) Novel ATPase of SNF2-like protein family interacts with androgen receptor and modulates androgen-dependent transcription *Mol Biol Cell* **13** 6 2106-19
403 Q. Wang, D. Sharma, Y. Ren and J. D. Fondell (2002) A coregulatory role for the TRAP-mediator complex in androgen receptor-mediated gene expression *J Biol Chem* **277** 45 42852-8
404 I. J. Mcewan and J. Gustafsson (1997) Interaction of the human androgen receptor transactivation function with the general transcription factor TFIIF *Proc Natl Acad Sci U S A* **94** 16 8485-90
405 J. Reid, I. Murray, K. Watt, R. Betney and I. J. Mcewan (2002) The androgen receptor interacts with multiple regions of the large subunit of general transcription factor TFIIF *J Biol Chem* **277** 43 41247-53
406 R. Betney and I. J. Mcewan (2003) Role of conserved hydrophobic amino acids in androgen receptor AF-1 function *J Mol Endocrinol* **31** 3 427-39
407 Q. Yan, R. J. Moreland, J. W. Conaway and R. C. Conaway (1999) Dual roles for transcription factor IIF in promoter escape by RNA polymerase II *J Biol Chem* **274** 50 35668-75
408 D. K. Lee, H. O. Duan and C. Chang (2000) From androgen receptor to the general transcription factor TFIIH. Identification of cdk activating kinase (CAK) as an androgen receptor NH(2)-terminal associated coactivator *J Biol Chem* **275** 13 9308-13
409 D. K. Lee, H. O. Duan and C. Chang (2001) Androgen receptor interacts with the positive elongation factor P-TEFb and enhances the efficiency of transcriptional elongation *J Biol Chem* **276** 13 9978-84

410 D. K. Lee, M. Li and C. Chang (2003) The second largest subunit of RNA polymerase II interacts with and enhances transactivation of androgen receptor *Biochem Biophys Res Commun* **302** 1 162-9
411 G. G. Kuiper and A. O. Brinkmann (1995) Phosphotryptic peptide analysis of the human androgen receptor: detection of a hormone-induced phosphopeptide *Biochemistry* **34** 6 1851-7
412 T. Ikonen, J. J. Palvimo, P. J. Kallio, P. Reinikainen and O. A. Janne (1994) Stimulation of androgen-regulated transactivation by modulators of protein phosphorylation *Endocrinology* **135** 4 1359-66
413 P. Reinikainen, J. J. Palvimo and O. A. Janne (1996) Effects of mitogens on androgen receptor-mediated transactivation *Endocrinology* **137** 10 4351-7
414 D. Gioeli, S. B. Ficarro, J. J. Kwiek, D. Aaronson, M. Hancock, A. D. Catling, F. M. White, R. E. Christian, R. E. Settlage, J. Shabanowitz, D. F. Hunt and M. J. Weber (2002) Androgen receptor phosphorylation. Regulation and identification of the phosphorylation sites *J Biol Chem* **277** 32 29304-14
415 T. Kishino, M. Lalande and J. Wagstaff (1997) UBE3A/E6-AP mutations cause Angelman syndrome *Nat Genet* **15** 1 70-3
416 T. Matsuura, J. S. Sutcliffe, P. Fang, R. J. Galjaard, Y. H. Jiang, C. S. Benton, J. M. Rommens and A. L. Beaudet (1997) De novo truncating mutations in E6-AP ubiquitin-protein ligase gene (UBE3A) in Angelman syndrome *Nat Genet* **15** 1 74-7
417 L. Gaughan, I. R. Logan, D. E. Neal and C. N. Robson (2005) Regulation of androgen receptor and histone deacetylase 1 by Mdm2-mediated ubiquitylation *Nucleic Acids Res* **33** 1 13-26
418 J. A. Iniguez-Lluhi and D. Pearce (2000) A common motif within the negative regulatory regions of multiple factors inhibits their transcriptional synergy *Mol Cell Biol* **20** 16 6040-50
419 M. Biel, V. Wascholowski and A. Giannis (2004) A fatal affair: the ubiquitinylation of proteins *Angew Chem Int Ed Engl* **43** 47 6414-6
420 V. G. Wilson and G. Rosas-Acosta (2005) Wrestling with SUMO in a new arena *Sci STKE* **2005** 290 pe32
421 J. M. Desterro, M. S. Rodriguez and R. T. Hay (1998) SUMO-1 modification of IkappaBalpha inhibits NF-kappaB activation *Mol Cell* **2** 2 233-9
422 M. J. Pazin and J. T. Kadonaga (1997) What's up and down with histone deacetylation and transcription? *Cell* **89** 3 325-8
423 M. Fu, C. Wang, A. T. Reutens, J. Wang, R. H. Angeletti, L. Siconolfi-Baez, V. Ogryzko, M. L. Avantaggiati and R. G. Pestell (2000) p300 and p300/cAMP-response element-binding protein-associated factor acetylate the androgen receptor at sites governing hormone-dependent transactivation *J Biol Chem* **275** 27 20853-60
424 P. Doesburg, C. W. Kuil, C. A. Berrevoets, K. Steketee, P. W. Faber, E. Mulder, A. O. Brinkmann and J. Trapman (1997) Functional in vivo interaction between the amino-terminal, transactivation domain and the ligand binding domain of the androgen receptor *Biochemistry* **36** 5 1052-64
425 E. Langley, Z. X. Zhou and E. M. Wilson (1995) Evidence for an anti-parallel orientation of the ligand-activated human androgen receptor dimer *J Biol Chem* **270** 50 29983-90
426 B. He, J. A. Kemppainen and E. M. Wilson (2000) FXXLF and WXXLF sequences mediate the NH2-terminal interaction with the ligand binding domain of the androgen receptor *J Biol Chem* **275** 30 22986-94
427 K. Steketee, C. A. Berrevoets, H. J. Dubbink, P. Doesburg, R. Hersmus, A. O. Brinkmann and J. Trapman (2002) Amino acids 3-13 and amino acids in and flanking the 23FxxLF27 motif modulate the interaction between the N-terminal and ligand-binding domain of the androgen receptor *Eur J Biochem* **269** 23 5780-91
428 B. He and E. M. Wilson (2003) Electrostatic modulation in steroid receptor recruitment of LXXLL and FXXLF motifs *Mol Cell Biol* **23** 6 2135-50
429 C. L. Hsu, Y. L. Chen, S. Yeh, H. J. Ting, Y. C. Hu, H. Lin, X. Wang and C. Chang (2003) The use of phage display technique for the isolation of androgen receptor interacting peptides with (F/W)XXL(F/W) and FXXLY new signature motifs *J Biol Chem* **278** 26 23691-8
430 B. D. Darimont, R. L. Wagner, J. W. Apriletti, M. R. Stallcup, P. J. Kushner, J. D. Baxter, R. J. Fletterick and K. R. Yamamoto (1998) Structure and specificity of nuclear receptor-coactivator interactions *Genes Dev* **12** 21 3343-56
431 E. M. Mcinerney, D. W. Rose, S. E. Flynn, S. Westin, T. M. Mullen, A. Krones, J. Inostroza, J. Torchia, R. T. Nolte, N. Assa-Munt, M. V. Milburn, C. K. Glass and M. G. Rosenfeld (1998) Determinants of coactivator LXXLL motif specificity in nuclear receptor transcriptional activation *Genes Dev* **12** 21 3357-68
432 H. J. Dubbink, R. Hersmus, A. C. Pike, M. Molier, A. O. Brinkmann, G. Jenster and J. Trapman (2006) Androgen receptor ligand-binding domain interaction and nuclear receptor specificity of FXXLF and LXXLL motifs as determined by L/F swapping *Mol Endocrinol* **20** 8 1742-55
433 E. Estebanez-Perpina, J. M. Moore, E. Mar, E. Delgado-Rodrigues, P. Nguyen, J. D. Baxter, B. M. Buehrer, P. Webb, R. J. Fletterick and R. K. Guy (2005) The molecular mechanisms of coactivator utilization in ligand-dependent transactivation by the androgen receptor *J Biol Chem* **280** 9 8060-8
434 T. N. Alam, M. J. O'hare, I. Laczko, A. Freeman, F. Al-Beidh, J. R. Masters and D. L. Hudson (2004) Differential expression of CD44 during human prostate epithelial cell differentiation *J Histochem Cytochem* **52** 8 1083-90
435 F. Schaufele, X. Carbonell, M. Guerbadot, S. Borngraeber, M. S. Chapman, A. A. Ma, J. N. Miner and M. I. Diamond (2005) The structural basis of androgen receptor activation: intramolecular and intermolecular amino-carboxy interactions *Proc Natl Acad Sci U S A* **102** 28 9802-7
436 E. Langley, J. A. Kemppainen and E. M. Wilson (1998) Intermolecular NH2-/carboxyl-terminal interactions in androgen receptor dimerization revealed by mutations that cause androgen insensitivity *J Biol Chem* **273** 1 92-101

437 J. Jaaskelainen, A. Deeb, J. W. Schwabe, N. P. Mongan, H. Martin and I. A. Hughes (2006) Human androgen receptor gene ligand-binding-domain mutations leading to disrupted interaction between the N- and C-terminal domains *J Mol Endocrinol* **36** 2 361-8
438 J. Thompson, F. Saatcioglu, O. A. Janne and J. J. Palvimo (2001) Disrupted amino- and carboxyl-terminal interactions of the androgen receptor are linked to androgen insensitivity *Mol Endocrinol* **15** 6 923-35
439 J. Li, J. Fu, C. Toumazou, H. G. Yoon and J. Wong (2006) A role of the amino-terminal (N) and carboxyl-terminal (C) interaction in binding of androgen receptor to chromatin *Mol Endocrinol* **20** 4 776-85
440 B. He, N. T. Bowen, J. T. Minges and E. M. Wilson (2001) Androgen-induced NH2- and COOH-terminal Interaction Inhibits p160 coactivator recruitment by activation function 2 *J Biol Chem* **276** 45 42293-301
441 C. L. Hsu, Y. L. Chen, H. J. Ting, W. J. Lin, Z. Yang, Y. Zhang, L. Wang, C. T. Wu, H. C. Chang, S. Yeh, S. W. Pimplikar and C. Chang (2005) Androgen receptor (AR) NH2- and COOH-terminal interactions result in the differential influences on the AR-mediated transactivation and cell growth *Mol Endocrinol* **19** 2 350-61
442 T. Ikonen, J. J. Palvimo and O. A. Janne (1997) Interaction between the amino- and carboxyl-terminal regions of the rat androgen receptor modulates transcriptional activity and is influenced by nuclear receptor coactivators *J Biol Chem* **272** 47 29821-8
443 W. L. Kraus, E. M. Mcinerney and B. S. Katzenellenbogen (1995) Ligand-dependent, transcriptionally productive association of the amino- and carboxyl-terminal regions of a steroid hormone nuclear receptor *Proc Natl Acad Sci U S A* **92** 26 12314-8
444 R. Michalides, A. Griekspoor, A. Balkenende, D. Verwoerd, L. Janssen, K. Jalink, A. Floore, A. Velds, L. Van't Veer and J. Neefjes (2004) Tamoxifen resistance by a conformational arrest of the estrogen receptor alpha after PKA activation in breast cancer *Cancer Cell* **5** 6 597-605
445 P. Webb, P. Nguyen, J. Shinsako, C. Anderson, W. Feng, M. P. Nguyen, D. Chen, S. M. Huang, S. Subramanian, E. Mckinerney, B. S. Katzenellenbogen, M. R. Stallcup and P. J. Kushner (1998) Estrogen receptor activation function 1 works by binding p160 coactivator proteins *Mol Endocrinol* **12** 10 1605-18
446 M. J. Tetel, P. H. Giangrande, S. A. Leonhardt, D. P. Mcdonnell and D. P. Edwards (1999) Hormone-dependent interaction between the amino- and carboxyl-terminal domains of progesterone receptor in vitro and in vivo *Mol Endocrinol* **13** 6 910-24
447 X. Dong, J. R. Challis and S. J. Lye (2004) Intramolecular interactions between the AF3 domain and the C-terminus of the human progesterone receptor are mediated through two LXXLL motifs *J Mol Endocrinol* **32** 3 843-57
448 L. Tung, T. Shen, M. G. Abel, R. L. Powell, G. S. Takimoto, C. A. Sartorius and K. B. Horwitz (2001) Mapping the unique activation function 3 in the progesterone B-receptor upstream segment. Two LXXLL motifs and a tryptophan residue are required for activity *J Biol Chem* **276** 43 39843-51
449 N. Yoshikawa, K. Yamamoto, N. Shimizu, S. Yamada, C. Morimoto and H. Tanaka (2005) The distinct agonistic properties of the phenylpyrazolosteroid cortivazol reveal interdomain communication within the glucocorticoid receptor *Mol Endocrinol* **19** 5 1110-24
450 D. Shao, S. M. Rangwala, S. T. Bailey, S. L. Krakow, M. J. Reginato and M. A. Lazar (1998) Interdomain communication regulating ligand binding by PPAR-gamma *Nature* **396** 6709 377-80
451 T. Iwasaki, A. Takeshita, W. Miyazaki, W. W. Chin and N. Koibuchi (2006) The interaction of TRbeta1-N terminus with steroid receptor coactivator-1 (SRC-1) serves a full transcriptional activation function of SRC-1 *Endocrinology* **147** 3 1452-7
452 C. Oberste-Berghaus, K. Zanger, K. Hashimoto, R. N. Cohen, A. N. Hollenberg and F. E. Wondisford (2000) Thyroid hormone-independent interaction between the thyroid hormone receptor beta2 amino terminus and coactivators *J Biol Chem* **275** 3 1787-92
453 A. Takeshita, P. M. Yen, M. Ikeda, G. R. Cardona, Y. Liu, N. Koibuchi, E. R. Norwitz and W. W. Chin (1998) Thyroid hormone response elements differentially modulate the interactions of thyroid hormone receptors with two receptor binding domains in the steroid receptor coactivator-1 *J Biol Chem* **273** 34 21554-62
454 J. C. Chapman, S. Min, S. Kunaporn, S. Shah, A. Kaiki-Astara and S. D. Michael (2001) The differential effect of injecting estradiol-17beta, testosterone, and hydrocortisone during the immune adaptive period on the fertility of female mice *Am J Reprod Immunol* **46** 4 288-97
455 B. He, R. T. Gampe, Jr., A. J. Kole, A. T. Hnat, T. B. Stanley, G. An, E. L. Stewart, R. I. Kalman, J. T. Minges and E. M. Wilson (2004) Structural basis for androgen receptor interdomain and coactivator interactions suggests a transition in nuclear receptor activation function dominance *Mol Cell* **16** 3 425-38
456 Z. X. Zhou, B. He, S. H. Hall, E. M. Wilson and F. S. French (2002) Domain interactions between coregulator ARA(70) and the androgen receptor (AR) *Mol Endocrinol* **16** 2 287-300
457 C. Y. Chang, J. Abdo, T. Hartney and D. P. Mcdonnell (2005) Development of peptide antagonists for the androgen receptor using combinatorial peptide phage display *Mol Endocrinol* **19** 10 2478-90
458 K. Barbulescu, C. Geserick, I. Schuttke, W. D. Schleuning and B. Haendler (2001) New androgen response elements in the murine pem promoter mediate selective transactivation *Mol Endocrinol* **15** 10 1803-16
459 D. Brochard, L. Morel, G. Veyssiere and C. Jean (1999) Structural and functional analysis of the promoter of a mouse gene encoding an androgen-regulated protein (MSVSP99) *J Mol Endocrinol* **23** 3 287-98
460 W. S. Cheng, V. Giandomenico, I. Pastan and M. Essand (2003) Characterization of the androgen-regulated prostate-specific T cell receptor gamma-chain alternate reading frame protein (TARP) promoter *Endocrinology* **144** 8 3433-40
461 F. Claessens, L. Celis, B. Peeters, W. Heyns, G. Verhoeven and W. Rombauts (1989) Functional characterization of an androgen response element in the first intron of the C3(1) gene of prostatic binding protein *Biochem Biophys Res Commun* **164** 2 833-40

462 K. B. Cleutjens, H. A. Van Der Korput, C. C. Van Eekelen, H. C. Van Rooij, P. W. Faber and J. Trapman (1997) An androgen response element in a far upstream enhancer region is essential for high, androgen-regulated activity of the prostate-specific antigen promoter *Mol Endocrinol* **11** 2 148-61
463 K. B. Cleutjens, C. C. Van Eekelen, H. A. Van Der Korput, A. O. Brinkmann and J. Trapman (1996) Two androgen response regions cooperate in steroid hormone regulated activity of the prostate-specific antigen promoter *J Biol Chem* **271** 11 6379-88
464 M. Crossley, M. Ludwig, K. M. Stowell, P. De Vos, K. Olek and G. G. Brownlee (1992) Recovery from hemophilia B Leyden: an androgen-responsive element in the factor IX promoter *Science* **257** 5068 377-9
465 A. Crozat, J. J. Palvimo, M. Julkunen and O. A. Janne (1992) Comparison of androgen regulation of ornithine decarboxylase and S-adenosylmethionine decarboxylase gene expression in rodent kidney and accessory sex organs *Endocrinology* **130** 3 1131-44
466 J. L. Dai and K. L. Burnstein (1996) Two androgen response elements in the androgen receptor coding region are required for cell-specific up-regulation of receptor messenger RNA *Mol Endocrinol* **10** 12 1582-94
467 A. Devos, F. Claessens, P. Alen, J. Winderickx, W. Heyns, W. Rombauts and B. Peeters (1997) Identification of a functional androgen-response element in the exon 1-coding sequence of the cystatin-related protein gene crp2 *Mol Endocrinol* **11** 8 1033-43
468 A. Devos, N. De Clercq, I. Vercaeren, W. Heyns, W. Rombauts and B. Peeters (1993) Structure of rat genes encoding androgen-regulated cystatin-related proteins (CRPs): a new member of the cystatin superfamily *Gene* **125** 2 159-67
469 J. Y. Dube, P. Chapdelaine, S. Guerin, S. Leclerc, P. S. Rennie, R. J. Matusik and R. R. Tremblay (1995) Search for androgen response elements in the proximal promoter of the canine prostate arginine esterase gene *J Androl* **16** 4 304-11
470 S. Fabre, M. Manin, E. Pailhoux, G. Veyssiere and C. Jean (1994) Identification of a functional androgen response element in the promoter of the gene for the androgen-regulated aldose reductase-like protein specific to the mouse vas deferens *J Biol Chem* **269** 8 5857-64
471 W. Gao, C. E. Bohl and J. T. Dalton (2005) Chemistry and structural biology of androgen receptor *Chem Rev* **105** 9 3352-70
472 N. B. Ghyselinck, I. Dufaure, J. J. Lareyre, N. Rigaudiere, M. G. Mattei and J. P. Dufaure (1993) Structural organization and regulation of the gene for the androgen-dependent glutathione peroxidase-like protein specific to the mouse epididymis *Mol Endocrinol* **7** 2 258-72
473 J. M. Grad, J. L. Dai, S. Wu and K. L. Burnstein (1999) Multiple androgen response elements and a Myc consensus site in the androgen receptor (AR) coding region are involved in androgen-mediated up-regulation of AR messenger RNA *Mol Endocrinol* **13** 11 1896-911
474 J. M. Grad, L. S. Lyons, D. M. Robins and K. L. Burnstein (2001) The androgen receptor (AR) amino-terminus imposes androgen-specific regulation of AR gene expression via an exonic enhancer *Endocrinology* **142** 3 1107-16
475 A. Haelens, G. Verrijdt, E. Schoenmakers, P. Alen, B. Peeters, W. Rombauts and F. Claessens (1999) The first exon of the human sc gene contains an androgen responsive unit and an interferon regulatory factor element *Mol Cell Endocrinol* **153** 1-2 91-102
476 H. Heemers, G. Verrijdt, S. Organe, F. Claessens, W. Heyns, G. Verhoeven and J. V. Swinnen (2004) Identification of an androgen response element in intron 8 of the sterol regulatory element-binding protein cleavage-activating protein gene allowing direct regulation by the androgen receptor *J Biol Chem* **279** 29 30880-7
477 K. C. Ho, K. B. Marschke, J. Tan, S. G. Power, E. M. Wilson and F. S. French (1993) A complex response element in intron 1 of the androgen-regulated 20-kDa protein gene displays cell type-dependent androgen receptor specificity *J Biol Chem* **268** 36 27226-35
478 A. Jain, A. Lam, I. Vivanco, M. F. Carey and R. E. Reiter (2002) Identification of an androgen-dependent enhancer within the prostate stem cell antigen gene *Mol Endocrinol* **16** 10 2323-37
479 H. Jeong, M. S. Kim, J. Kwon, K. S. Kim and W. Seol (2006) Regulation of the transcriptional activity of the tyrosine hydroxylase gene by androgen receptor *Neurosci Lett* **396** 1 57-61
480 H. H. Juang, L. C. Costello and R. B. Franklin (1995) Androgen modulation of multiple transcription start sites of the mitochondrial aspartate aminotransferase gene in rat prostate *J Biol Chem* **270** 21 12629-34
481 S. Kasper, P. S. Rennie, N. Bruchovsky, P. C. Sheppard, H. Cheng, L. Lin, R. P. Shiu, R. Snoek and R. J. Matusik (1994) Cooperative binding of androgen receptors to two DNA sequences is required for androgen induction of the probasin gene *J Biol Chem* **269** 50 31763-9
482 J. J. Lareyre, K. Reid, C. Nelson, S. Kasper, P. S. Rennie, M. C. Orgebin-Crist and R. J. Matusik (2000) Characterization of an androgen-specific response region within the 5' flanking region of the murine epididymal retinoic acid binding protein gene *Biol Reprod* **63** 6 1881-92
483 S. D. Lund, P. M. Gallagher, B. Wang, S. C. Porter and R. E. Ganschow (1991) Androgen responsiveness of the murine beta-glucuronidase gene is associated with nuclease hypersensitivity, protein binding, and haplotype-specific sequence diversity within intron 9 *Mol Cell Biol* **11** 11 5426-34
484 J. A. Magee, L. W. Chang, G. D. Stormo and J. Milbrandt (2006) Direct, androgen receptor-mediated regulation of the FKBP5 gene via a distal enhancer element *Endocrinology* **147** 1 590-8
485 S. H. Mitchell, P. E. Murtha, S. Zhang, W. Zhu and C. Y. Young (2000) An androgen response element mediates LNCaP cell dependent androgen induction of the hK2 gene *Mol Cell Endocrinol* **168** 1-2 89-99
486 P. Murtha, D. J. Tindall and C. Y. Young (1993) Androgen induction of a human prostate-specific kallikrein, hKLK2: characterization of an androgen response element in the 5' promoter region of the gene *Biochemistry* **32** 25 6459-64
487 X. M. Ou, K. Chen and J. C. Shih (2006) Glucocorticoid and androgen activation of monoamine oxidase A is regulated differently by R1 and Sp1 *J Biol Chem* **281** 30 21512-25

488 D. Phan, X. Sui, D. T. Chen, S. M. Najjar, G. Jenster and S. H. Lin (2001) Androgen regulation of the cell-cell adhesion molecule-1 (Ceacam1) gene *Mol Cell Endocrinol* **184** 1-2 115-23
489 J. M. Rae, M. D. Johnson, K. E. Cordero, J. O. Scheys, J. M. Larios, M. M. Gottardis, K. J. Pienta and M. E. Lippman (2006) GREB1 is a novel androgen-regulated gene required for prostate cancer growth *Prostate* **66** 8 886-94
490 P. S. Rennie, N. Bruchovsky, K. J. Leco, P. C. Sheppard, S. A. Mcqueen, H. Cheng, R. Snoek, A. Hamel, M. E. Bock, B. S. Macdonald and Et Al. (1993) Characterization of two cis-acting DNA elements involved in the androgen regulation of the probasin gene *Mol Endocrinol* **7** 1 23-36
491 P. H. Riegman, R. J. Vlietstra, J. A. Van Der Korput, A. O. Brinkmann and J. Trapman (1991) The promoter of the prostate-specific antigen gene contains a functional androgen responsive element *Mol Endocrinol* **5** 12 1921-30
492 Z. Salah, M. Maoz, I. Cohen, G. Pizov, D. Pode, M. S. Runge and R. Bar-Shavit (2005) Identification of a novel functional androgen response element within hPar1 promoter: implications to prostate cancer progression *Faseb J* **19** 1 62-72
493 R. Shen, M. Sumitomo, J. Dai, D. O. Hardy, D. Navarro, B. Usmani, C. N. Papandreou, L. B. Hersh, M. A. Shipp, L. P. Freedman and D. M. Nanus (2000) Identification and characterization of two androgen response regions in the human neutral endopeptidase gene *Mol Cell Endocrinol* **170** 1-2 131-42
494 K. Steketee, A. C. Ziel-Van Der Made, H. A. Van Der Korput, A. B. Houtsmuller and J. Trapman (2004) A bioinformatics-based functional analysis shows that the specifically androgen-regulated gene SARG contains an active direct repeat androgen response element in the first intron *J Mol Endocrinol* **33** 2 477-91
495 P. Val, A. Martinez, I. Sahut-Barnola, C. Jean, G. Veyssiere and A. M. Lefrancois-Martinez (2002) A 77-base pair LINE-like sequence elicits androgen-dependent mvdp/akr1-b7 expression in mouse vas deferens, but is dispensable for adrenal expression in rats *Endocrinology* **143** 9 3435-48
496 G. Verrijdt, E. Schoenmakers, P. Alen, A. Haelens, B. Peeters, W. Rombauts and F. Claessens (1999) Androgen specificity of a response unit upstream of the human secretory component gene is mediated by differential receptor binding to an essential androgen response element *Mol Endocrinol* **13** 9 1558-70
497 D. C. Yu, G. T. Sakamoto and D. R. Henderson (1999) Identification of the transcriptional regulatory sequences of human kallikrein 2 and their use in the construction of calydon virus 764, an attenuated replication competent adenovirus for prostate cancer therapy *Cancer Res* **59** 7 1498-504
498 S. E. Deprimo, M. Diehn, J. B. Nelson, R. E. Reiter, J. Matese, M. Fero, R. Tibshirani, P. O. Brown and J. D. Brooks (2002) Transcriptional programs activated by exposure of human prostate cancer cells to androgen *Genome Biol* **3** 7 RESEARCH0032
499 J. Holzbeierlein, P. Lal, E. Latulippe, A. Smith, J. Satagopan, L. Zhang, C. Ryan, S. Smith, H. Scher, P. Scardino, V. Reuter and W. L. Gerald (2004) Gene expression analysis of human prostate carcinoma during hormonal therapy identifies androgen-responsive genes and mechanisms of therapy resistance *Am J Pathol* **164** 1 217-27
500 F. Jiang and Z. Wang (2003) Identification of androgen-responsive genes in the rat ventral prostate by complementary deoxyribonucleic acid subtraction and microarray *Endocrinology* **144** 4 1257-65
501 P. S. Nelson, N. Clegg, H. Arnold, C. Ferguson, M. Bonham, J. White, L. Hood and B. Lin (2002) The program of androgen-responsive genes in neoplastic prostate epithelium *Proc Natl Acad Sci U S A* **99** 18 11890-5
502 S. T. Pang, K. Dillner, X. Wu, A. Pousette, G. Norstedt and A. Flores-Morales (2002) Gene expression profiling of androgen deficiency predicts a pathway of prostate apoptosis that involves genes related to oxidative stress *Endocrinology* **143** 12 4897-906
503 A. M. Velasco, K. A. Gillis, Y. Li, E. L. Brown, T. M. Sadler, M. Achilleos, L. M. Greenberger, P. Frost, W. Bai and Y. Zhang (2004) Identification and validation of novel androgen-regulated genes in prostate cancer *Endocrinology* **145** 8 3913-24
504 L. L. Xu, Y. P. Su, R. Labiche, T. Segawa, N. Shanmugam, D. G. Mcleod, J. W. Moul and S. Srivastava (2001) Quantitative expression profile of androgen-regulated genes in prostate cancer cells and identification of prostate-specific genes *Int J Cancer* **92** 3 322-8
505 S. M. Dehm and D. J. Tindall (2006) Molecular regulation of androgen action in prostate cancer *J Cell Biochem* **99** 2 333-44
506 J. S. Carroll and M. Brown (2006) Estrogen receptor target gene: an evolving concept *Mol Endocrinol* **20** 8 1707-14
507 C. C. Nelson, S. C. Hendy, R. J. Shukin, H. Cheng, N. Bruchovsky, B. F. Koop and P. S. Rennie (1999) Determinants of DNA sequence specificity of the androgen, progesterone, and glucocorticoid receptors: evidence for differential steroid receptor response elements *Mol Endocrinol* **13** 12 2090-107
508 A. Haelens, G. Verrijdt, L. Callewaert, B. Peeters, W. Rombauts and F. Claessens (2001) Androgen-receptor-specific DNA binding to an element in the first exon of the human secretory component gene *Biochem J* **353** Pt 3 611-20
509 E. Schoenmakers, P. Alen, G. Verrijdt, B. Peeters, G. Verhoeven, W. Rombauts and F. Claessens (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)** 515-21
510 E. Schoenmakers, G. Verrijdt, B. Peeters, G. Verhoeven, W. Rombauts and F. Claessens (2000) Differences in DNA binding characteristics of the androgen and glucocorticoid receptors can determine hormone-specific responses *J Biol Chem* **275** 16 12290-7
511 G. Verrijdt, E. Schoenmakers, A. Haelens, B. Peeters, G. Verhoeven, W. Rombauts and F. Claessens (2000) Change of specificity mutations in androgen-selective enhancers. Evidence for a role of differential DNA binding by the androgen receptor *J Biol Chem* **275** 16 12298-305

512 Z. Zhou, J. L. Corden and T. R. Brown (1997) Identification and characterization of a novel androgen response element composed of a direct repeat *J Biol Chem* **272** 13 8227-35
513 A. M. Spence, P. C. Sheppard, J. R. Davie, Y. Matuo, N. Nishi, W. L. Mckeehan, J. G. Dodd and R. J. Matusik (1989) Regulation of a bifunctional mRNA results in synthesis of secreted and nuclear probasin *Proc Natl Acad Sci U S A* **86** 20 7843-7
514 J. G. Dodd, P. C. Sheppard and R. J. Matusik (1983) Characterization and cloning of rat dorsal prostate mRNAs. Androgen regulation of two closely related abundant mRNAs *J Biol Chem* **258** 17 10731-7
515 N. M. Greenberg, F. J. Demayo, P. C. Sheppard, R. Barrios, R. Lebovitz, M. Finegold, R. Angelopoulou, J. G. Dodd, M. L. Duckworth, J. M. Rosen and Et Al. (1994) The rat probasin gene promoter directs hormonally and developmentally regulated expression of a heterologous gene specifically to the prostate in transgenic mice *Mol Endocrinol* **8** 2 230-9
516 R. J. Matusik, P. A. Cattini, K. J. Leco, P. C. Sheppard, B. E. Nickel, B. L. Neubauer, J. R. Davie, C. Chang, S. Liao, Y. Matuo and W. L. Mckeehan (1991) Regulation of gene expression in the prostate. *Molecular and cellular biology of prostate cancer.* 299-314 J. P. Karr, D. S. Coffey, R. G. Smith and D. J. Tindall New York and London Plenum Press
517 S. Kasper, P. S. Rennie, N. Bruchovsky, L. Lin, H. Cheng, R. Snoek, K. Dahlman-Wright, J. A. Gustafsson, R. P. Shiu, P. C. Sheppard and R. J. Matusik (1999) Selective activation of the probasin androgen-responsive region by steroid hormones *J Mol Endocrinol* **22** 3 313-25
518 J. Zhang, N. Gao, S. Kasper, K. Reid, C. Nelson and R. J. Matusik (2004) An androgen-dependent upstream enhancer is essential for high levels of probasin gene expression *Endocrinology* **145** 1 134-48
519 K. J. Reid, S. C. Hendy, J. Saito, P. Sorensen and C. C. Nelson (2001) Two classes of androgen receptor elements mediate cooperativity through allosteric interactions *J Biol Chem* **276** 4 2943-52
520 K. E. Mostov (1994) Transepithelial transport of immunoglobulins *Annu Rev Immunol* **12** 63-84
521 D. A. Sullivan and M. R. Allansmith (1987) Hormonal influence on the secretory immune system of the eye: endocrine interactions in the control of IgA and secretory component levels in tears of rats *Immunology* **60** 3 337-43
522 H. Vanaken, F. Claessens, I. Vercaeren, W. Heyns, B. Peeters and W. Rombauts (1996) Androgenic induction of cystatin-related protein and the C3 component of prostatic binding protein in primary cultures from the rat lacrimal gland *Mol Cell Endocrinol* **121** 2 197-205
523 J. B. Stavenhagen and D. M. Robins (1988) An ancient provirus has imposed androgen regulation on the adjacent mouse sex-limited protein gene *Cell* **55** 2 247-54
524 F. Loreni, J. Stavenhagen, M. Kalff and D. M. Robins (1988) A complex androgen-responsive enhancer resides 2 kilobases upstream of the mouse Slp gene *Mol Cell Biol* **8** 6 2350-60
525 A. J. Adler, A. Scheller and D. M. Robins (1993) The stringency and magnitude of androgen-specific gene activation are combinatorial functions of receptor and nonreceptor binding site sequences *Mol Cell Biol* **13** 10 6326-35
526 A. J. Adler, M. Danielsen and D. M. Robins (1992) Androgen-specific gene activation via a consensus glucocorticoid response element is determined by interaction with nonreceptor factors *Proc Natl Acad Sci U S A* **89** 24 11660-3
527 S. A. Nelson and D. M. Robins (1997) Regulatory capacity of an androgen-specific enhancer of the mouse Slp gene in transgenic mice *Mol Cell Endocrinol* **133** 2 89-97
528 Y. M. Ning and D. M. Robins (1999) AML3/CBFalpha1 is required for androgen-specific activation of the enhancer of the mouse sex-limited protein (Slp) gene *J Biol Chem* **274** 43 30624-30
529 C. O. Scarlett and D. M. Robins (1995) In vivo footprinting of an androgen-dependent enhancer reveals an accessory element integral to hormonal response *Mol Endocrinol* **9** 4 413-23
530 S. Maiti, J. Doskow, S. Li, R. P. Nhim, J. S. Lindsey and M. F. Wilkinson (1996) The Pem homeobox gene. Androgen-dependent and -independent promoters and tissue-specific alternative RNA splicing *J Biol Chem* **271** 29 17536-46
531 C. B. Cleutjens, K. Steketee, C. C. Van Eekelen, J. A. Van Der Korput, A. O. Brinkmann and J. Trapman (1997) Both androgen receptor and glucocorticoid receptor are able to induce prostate-specific antigen expression, but differ in their growth-stimulating properties of LNCaP cells *Endocrinology* **138** 12 5293-300
532 A. Scheller, E. Hughes, K. L. Golden and D. M. Robins (1998) Multiple receptor domains interact to permit, or restrict, androgen-specific gene activation *J Biol Chem* **273** 37 24216-22
533 B. He, L. W. Lee, J. T. Minges and E. M. Wilson (2002) Dependence of selective gene activation on the androgen receptor NH2- and COOH-terminal interaction *J Biol Chem* **277** 28 25631-9
534 C. Geserick, H. A. Meyer, K. Barbulescu and B. Haendler (2003) Differential modulation of androgen receptor action by deoxyribonucleic acid response elements *Mol Endocrinol* **17** 9 1738-50
535 L. Callewaert, G. Verrijdt, A. Haelens and F. Claessens (2004) Differential effect of small ubiquitin-like modifier (SUMO)-ylation of the androgen receptor in the control of cooperativity on selective versus canonical response elements *Mol Endocrinol* **18** 6 1438-49
536 J. E. Griffin, M. Leshin and J. D. Wilson (1982) Androgen resistance syndromes *Am J Physiol* **243** 2 E81-7
537 G. Pettersson and G. Bonnier (1937) Inherited sex-mosaic in man. *Heriditas* **23** 49-69
538 M. Adachi, R. Takayanagi, A. Tomura, K. Imasaki, S. Kato, K. Goto, T. Yanase, S. Ikuyama and H. Nawata (2000) Androgen-insensitivity syndrome as a possible coactivator disease *N Engl J Med* **343** 12 856-62
539 T. Arbizu, J. Santamaria, J. M. Gomez, A. Quilez and J. P. Serra (1983) A family with adult spinal and bulbar muscular atrophy, X-linked inheritance and associated testicular failure *J Neurol Sci* **59** 3 371-82
540 W. R. Kennedy, M. Alter and J. H. Sung (1968) Progressive proximal spinal and bulbar muscular atrophy of late onset. A sex-linked recessive trait *Neurology* **18** 7 671-80

541 A. R. La Spada, E. M. Wilson, D. B. Lubahn, A. E. Harding and K. H. Fischbeck (1991) Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy *Nature* **352** 6330 77-9
542 D. E. Merry (2001) Molecular pathogenesis of spinal and bulbar muscular atrophy *Brain Res Bull* **56** 3-4 203-7
543 S. Simeoni, M. A. Mancini, D. L. Stenoien, M. Marcelli, N. L. Weigel, M. Zanisi, L. Martini and A. Poletti (2000) Motoneuronal cell death is not correlated with aggregate formation of androgen receptors containing an elongated polyglutamine tract *Hum Mol Genet* **9** 1 133-44
544 A. Jemal, E. Ward, X. Wu, H. J. Martin, C. C. Mclaughlin and M. J. Thun (2005) Geographic patterns of prostate cancer mortality and variations in access to medical care in the United States *Cancer Epidemiol Biomarkers Prev* **14** 3 590-5
545 J. Tremblay, G. Frenette, R. R. Tremblay, A. Dupont, M. Thabet and J. Y. Dube (1987) Excretion of three major prostatic secretory proteins in the urine of normal men and patients with benign prostatic hypertrophy or prostate cancer *Prostate* **10** 3 235-43
546 G. R. Cunha and A. Donjacour (1987) Stromal-epithelial interactions in normal and abnormal prostatic development *Prog Clin Biol Res* **239** 251-72
547 V. Kumar, A. K. Abbas, N. Fausto and (2004) Robbins & Cotran Pathologic Basis of Disease 7th edition, Chapter 21 Saunders
548 A. Y. Liu, L. D. True, L. Latray, P. S. Nelson, W. J. Ellis, R. L. Vessella, P. H. Lange, L. Hood and G. Van Den Engh (1997) Cell-cell interaction in prostate gene regulation and cytodifferentiation *Proc Natl Acad Sci U S A* **94** 20 10705-10
549 T. J. Mcdonnell, P. Troncoso, S. M. Brisbay, C. Logothetis, L. W. Chung, J. T. Hsieh, S. M. Tu and M. L. Campbell (1992) Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer *Cancer Res* **52** 24 6940-4
550 M. Al-Hajj and M. F. Clarke (2004) Self-renewal and solid tumor stem cells *Oncogene* **23** 43 7274-82
551 D. Bonnet and J. E. Dick (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell *Nat Med* **3** 7 730-7
552 M. Florek, M. Haase, A. M. Marzesco, D. Freund, G. Ehninger, W. B. Huttner and D. Corbeil (2005) Prominin-1/CD133, a neural and hematopoietic stem cell marker, is expressed in adult human differentiated cells and certain types of kidney cancer *Cell Tissue Res* **319** 1 15-26
553 R. Galli, E. Binda, U. Orfanelli, B. Cipelletti, A. Gritti, S. De Vitis, R. Fiocco, C. Foroni, F. Dimeco and A. Vescovi (2004) Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma *Cancer Res* **64** 19 7011-21
554 C. Hirschmann-Jax, A. E. Foster, G. G. Wulf, J. G. Nuchtern, T. W. Jax, U. Gobel, M. A. Goodell and M. K. Brenner (2004) A distinct "side population" of cells with high drug efflux capacity in human tumor cells *Proc Natl Acad Sci U S A* **101** 39 14228-33
555 C. F. Kim, E. L. Jackson, A. E. Woolfenden, S. Lawrence, I. Babar, S. Vogel, D. Crowley, R. T. Bronson and T. Jacks (2005) Identification of bronchioalveolar stem cells in normal lung and lung cancer *Cell* **121** 6 823-35
556 S. K. Singh, I. D. Clarke, T. Hide and P. B. Dirks (2004) Cancer stem cells in nervous system tumors *Oncogene* **23** 43 7267-73
557 D. L. Hudson, M. O'hare, F. M. Watt and J. R. Masters (2000) Proliferative heterogeneity in the human prostate: evidence for epithelial stem cells *Lab Invest* **80** 8 1243-50
558 I. V. Litvinov, A. M. De Marzo and J. T. Isaacs (2003) Is the Achilles' heel for prostate cancer therapy a gain of function in androgen receptor signaling? *J Clin Endocrinol Metab* **88** 7 2972-82
559 G. Van Leenders, H. Dijkman, C. Hulsbergen-Van De Kaa, D. Ruiter and J. Schalken (2000) Demonstration of intermediate cells during human prostate epithelial differentiation in situ and in vitro using triple-staining confocal scanning microscopy *Lab Invest* **80** 8 1251-8
560 J. T. Isaacs and D. S. Coffey (1989) Etiology and disease process of benign prostatic hyperplasia *Prostate Suppl* **2** 33-50
561 H. Bonkhoff and K. Remberger (1996) Differentiation pathways and histogenetic aspects of normal and abnormal prostatic growth: a stem cell model *Prostate* **28** 2 98-106
562 A. T. Collins, P. A. Berry, C. Hyde, M. J. Stower and N. J. Maitland (2005) Prospective identification of tumorigenic prostate cancer stem cells *Cancer Res* **65** 23 10946-51
563 A. T. Collins, F. K. Habib, N. J. Maitland and D. E. Neal (2001) Identification and isolation of human prostate epithelial stem cells based on alpha(2)beta(1)-integrin expression *J Cell Sci* **114** Pt 21 3865-72
564 R. Heer, C. N. Robson, B. K. Shenton and H. Y. Leung (2007) The role of androgen in determining differentiation and regulation of androgen receptor expression in the human prostatic epithelium transient amplifying population *J Cell Physiol* **212** 3 572-8
565 G. D. Richardson, C. N. Robson, S. H. Lang, D. E. Neal, N. J. Maitland and A. T. Collins (2004) CD133, a novel marker for human prostatic epithelial stem cells *J Cell Sci* **117** Pt 16 3539-45
566 A. R. Uzgare, Y. Xu and J. T. Isaacs (2004) In vitro culturing and characteristics of transit amplifying epithelial cells from human prostate tissue *J Cell Biochem* **91** 1 196-205
567 C. Huggins, R. E. Stevens and C. V. Hodges (1941) Studies on prostatic cancer II. The effects of castration on advanced carcinoma of the prostate gland *Arch Surg* **43** 209-25
568 E. Corey, J. E. Quinn, K. R. Buhler, P. S. Nelson, J. A. Macoska, L. D. True and R. L. Vessella (2003) LuCaP 35: a new model of prostate cancer progression to androgen independence *Prostate* **55** 4 239-46
569 C. W. Gregory, K. G. Hamil, D. Kim, S. H. Hall, T. G. Pretlow, J. L. Mohler and F. S. French (1998) Androgen receptor expression in androgen-independent prostate cancer is associated with increased expression of androgen-regulated genes *Cancer Res* **58** 24 5718-24
570 J. L. Mohler, C. W. Gregory, O. H. Ford, 3rd, D. Kim, C. M. Weaver, P. Petrusz, E. M. Wilson and F. S. French (2004) The androgen axis in recurrent prostate cancer *Clin Cancer Res* **10** 2 440-8

571 T. H. Van Der Kwast, J. Schalken, J. A. Ruizeveld De Winter, C. C. Van Vroonhoven, E. Mulder, W. Boersma and J. Trapman (1991) Androgen receptors in endocrine-therapy-resistant human prostate cancer *Int J Cancer* **48** 2 189-93
572 I. E. Eder, Z. Culig, R. Ramoner, M. Thurnher, T. Putz, C. Nessler-Menardi, M. Tiefenthaler, G. Bartsch and H. Klocker (2000) Inhibition of LncaP prostate cancer cells by means of androgen receptor antisense oligonucleotides *Cancer Gene Ther* **7** 7 997-1007
573 O. L. Zegarra-Moro, L. J. Schmidt, H. Huang and D. J. Tindall (2002) Disruption of androgen receptor function inhibits proliferation of androgen-refractory prostate cancer cells *Cancer Res* **62** 4 1008-13
574 X. Liao, S. Tang, J. B. Thrasher, T. L. Griebling and B. Li (2005) Small-interfering RNA-induced androgen receptor silencing leads to apoptotic cell death in prostate cancer *Mol Cancer Ther* **4** 4 505-15
575 J. X. Chen, P. F. Fu, T. Wang and X. Y. Cui (2004) Effects of DNA methylation inhibitor on down-regulation of ribonuclease inhibitor expression in cancer cell lines *Ai Zheng* **23** 11 1249-56
576 P. Haag, J. Bektic, G. Bartsch, H. Klocker and I. E. Eder (2005) Androgen receptor down regulation by small interference RNA induces cell growth inhibition in androgen sensitive as well as in androgen independent prostate cancer cells *J Steroid Biochem Mol Biol* **96** 3-4 251-8
577 K. G. Hermans, R. Van Marion, H. Van Dekken, G. Jenster, W. M. Van Weerden and J. Trapman (2006) TMPRSS2:ERG fusion by translocation or interstitial deletion is highly relevant in androgen-dependent prostate cancer, but is bypassed in late-stage androgen receptor-negative prostate cancer *Cancer Res* **66** 22 10658-63
578 S. A. Tomlins, D. R. Rhodes, S. Perner, S. M. Dhanasekaran, R. Mehra, X. W. Sun, S. Varambally, X. Cao, J. Tchinda, R. Kuefer, C. Lee, J. E. Montie, R. B. Shah, K. J. Pienta, M. A. Rubin and A. M. Chinnaiyan (2005) Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer *Science* **310** 5748 644-8
579 J. Clark, S. Merson, S. Jhavar, P. Flohr, S. Edwards, C. S. Foster, R. Eeles, F. L. Martin, D. H. Phillips, M. Crundwell, T. Christmas, A. Thompson, C. Fisher, G. Kovacs and C. S. Cooper (2007) Diversity of TMPRSS2-ERG fusion transcripts in the human prostate *Oncogene* **26** 18 2667-73
580 S. Perner, F. Demichelis, R. Beroukhim, F. H. Schmidt, J. M. Mosquera, S. Setlur, J. Tchinda, S. A. Tomlins, M. D. Hofer, K. G. Pienta, R. Kuefer, R. Vessella, X. W. Sun, M. Meyerson, C. Lee, W. R. Sellers, A. M. Chinnaiyan and M. A. Rubin (2006) TMPRSS2:ERG Fusion-Associated Deletions Provide Insight into the Heterogeneity of Prostate Cancer *Cancer Res* **66** 17 8337-41
581 M. J. Soller, M. Isaksson, P. Elfving, W. Soller, R. Lundgren and I. Panagopoulos (2006) Confirmation of the high frequency of the TMPRSS2/ERG fusion gene in prostate cancer *Genes Chromosomes Cancer* **45** 7 717-9
582 K. Iljin, M. Wolf, H. Edgren, S. Gupta, S. Kilpinen, R. I. Skotheim, M. Peltola, F. Smit, G. Verhaegh, J. Schalken, M. Nees and O. Kallioniemi (2006) TMPRSS2 fusions with oncogenic ETS factors in prostate cancer involve unbalanced genomic rearrangements and are associated with HDAC1 and epigenetic reprogramming *Cancer Res* **66** 21 10242-6
583 S. A. Tomlins, R. Mehra, D. R. Rhodes, L. R. Smith, D. Roulston, B. E. Helgeson, X. Cao, J. T. Wei, M. A. Rubin, R. B. Shah and A. M. Chinnaiyan (2006) TMPRSS2:ETV4 gene fusions define a third molecular subtype of prostate cancer *Cancer Res* **66** 7 3396-400
584 J. M. Mosquera, S. Perner, F. Demichelis, R. Kim, M. D. Hofer, K. D. Mertz, P. L. Paris, J. Simko, C. Collins, T. A. Bismar, A. M. Chinnaiyan and M. A. Rubin (2007) Morphological features of TMPRSS2-ERG gene fusion prostate cancer *J Pathol* **212** 1 91-101
585 R. K. Nam, L. Sugar, Z. Wang, W. Yang, R. Kitching, L. H. Klotz, V. Venkateswaran, S. A. Narod and A. Seth (2007) Expression of TMPRSS2 ERG Gene Fusion in Prostate Cancer Cells is an Important Prognostic Factor for Cancer Progression *Cancer Biol Ther* **6** 1
586 J. Wang, Y. Cai, C. Ren and M. Ittmann (2006) Expression of Variant TMPRSS2/ERG Fusion Messenger RNAs Is Associated with Aggressive Prostate Cancer *Cancer Res* **66** 17 8347-51
587 I. U. Agoulnik and N. L. Weigel (2006) Androgen receptor action in hormone-dependent and recurrent prostate cancer *J Cell Biochem* **99** 2 362-72
588 K. L. Burnstein (2005) Regulation of androgen receptor levels: implications for prostate cancer progression and therapy *J Cell Biochem* **95** 4 657-69
589 Z. Culig and G. Bartsch (2006) Androgen axis in prostate cancer *J Cell Biochem* **99** 2 373-81
590 J. Edwards and J. M. Bartlett (2005) The androgen receptor and signal-transduction pathways in hormone-refractory prostate cancer. Part 2: Androgen-receptor cofactors and bypass pathways *BJU Int* **95** 9 1327-35
591 J. Edwards and J. M. Bartlett (2005) The androgen receptor and signal-transduction pathways in hormone-refractory prostate cancer. Part 1: Modifications to the androgen receptor *BJU Int* **95** 9 1320-6
592 L. Bubendorf, J. Kononen, P. Koivisto, P. Schraml, H. Moch, T. C. Gasser, N. Willi, M. J. Mihatsch, G. Sauter and O. P. Kallioniemi (1999) Survey of gene amplifications during prostate cancer progression by high-throughout fluorescence in situ hybridization on tissue microarrays *Cancer Res* **59** 4 803-6
593 J. Edwards, N. S. Krishna, R. Mukherjee, A. D. Watters, M. A. Underwood and J. M. Bartlett (2001) Amplification of the androgen receptor may not explain the development of androgen-independent prostate cancer *BJU Int* **88** 6 633-7
594 P. Koivisto, J. Kononen, C. Palmberg, T. Tammela, E. Hyytinen, J. Isola, J. Trapman, K. Cleutjens, A. Noordzij, T. Visakorpi and O. P. Kallioniemi (1997) Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer *Cancer Res* **57** 2 314-9
595 P. A. Koivisto, J. Schleutker, H. Helin, C. Ehren-Van Eekelen, O. P. Kallioniemi and J. Trapman (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** 11 3578-82

596 M. J. Linja, K. J. Savinainen, O. R. Saramaki, T. L. Tammela, R. L. Vessella and T. Visakorpi (2001) Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer *Cancer Res* **61** 9 3550-5
597 T. Visakorpi, E. Hyytinen, P. Koivisto, M. Tanner, R. Keinanen, C. Palmberg, A. Palotie, T. Tammela, J. Isola and O. P. Kallioniemi (1995) In vivo amplification of the androgen receptor gene and progression of human prostate cancer *Nat Genet* **9** 4 401-6
598 J. Edwards, N. S. Krishna, K. M. Grigor and J. M. Bartlett (2003) Androgen receptor gene amplification and protein expression in hormone refractory prostate cancer *Br J Cancer* **89** 3 552-6
599 C. D. Chen, D. S. Welsbie, C. Tran, S. H. Baek, R. Chen, R. Vessella, M. G. Rosenfeld and C. L. Sawyers (2004) Molecular determinants of resistance to antiandrogen therapy *Nat Med* **10** 1 33-9
600 M. E. Taplin, B. Rajeshkumar, S. Halabi, C. P. Werner, B. A. Woda, J. Picus, W. Stadler, D. F. Hayes, P. W. Kantoff, N. J. Vogelzang and E. J. Small (2003) Androgen receptor mutations in androgen-independent prostate cancer: Cancer and Leukemia Group B Study 9663 *J Clin Oncol* **21** 14 2673-8
601 G. Buchanan, N. M. Greenberg, H. I. Scher, J. M. Harris, V. R. Marshall and W. D. Tilley (2001) Collocation of androgen receptor gene mutations in prostate cancer *Clin Cancer Res* **7** 5 1273-81
602 E. R. Hyytinen, K. Haapala, J. Thompson, I. Lappalainen, M. Roiha, I. Rantala, H. J. Helin, O. A. Janne, M. Vihinen, J. J. Palvimo and P. A. Koivisto (2002) Pattern of somatic androgen receptor gene mutations in patients with hormone-refractory prostate cancer *Lab Invest* **82** 11 1591-8
603 J. Thompson, E. R. Hyytinen, K. Haapala, I. Rantala, H. J. Helin, O. A. Janne, J. J. Palvimo and P. A. Koivisto (2003) Androgen receptor mutations in high-grade prostate cancer before hormonal therapy *Lab Invest* **83** 12 1709-13
604 J. Ceraline, M. D. Cruchant, E. Erdmann, P. Erbs, J. E. Kurtz, B. Duclos, D. Jacqmin, D. Chopin and J. P. Bergerat (2004) Constitutive activation of the androgen receptor by a point mutation in the hinge region: a new mechanism for androgen-independent growth in prostate cancer *Int J Cancer* **108** 1 152-7
605 L. V. Nazareth, D. L. Stenoien, W. E. Bingman, 3rd, A. J. James, C. Wu, Y. Zhang, D. P. Edwards, M. Mancini, M. Marcelli, D. J. Lamb and N. L. Weigel (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** 12 2065-75
606 G. Buchanan, M. Yang, J. M. Harris, H. S. Nahm, G. Han, N. Moore, J. M. Bentel, R. J. Matusik, D. J. Horsfall, V. R. Marshall, N. M. Greenberg and W. D. Tilley (2001) Mutations at the boundary of the hinge and ligand binding domain of the androgen receptor confer increased transactivation function *Mol Endocrinol* **15** 1 46-56
607 A. J. James, I. U. Agoulnik, J. M. Harris, G. Buchanan, W. D. Tilley, M. Marcelli, D. J. Lamb and N. L. Weigel (2002) A novel androgen receptor mutant, A748T, exhibits hormone concentration-dependent defects in nuclear accumulation and activity despite normal hormone-binding affinity *Mol Endocrinol* **16** 12 2692-705
608 J. S. Horoszewicz, S. S. Leong, T. M. Chu, Z. L. Wajsman, M. Friedman, L. Papsidero, U. Kim, L. S. Chai, S. Kakati, S. K. Arya and A. A. Sandberg (1980) The LNCaP cell line--a new model for studies on human prostatic carcinoma *Prog Clin Biol Res* **37** 115-32
609 J. S. Horoszewicz, S. S. Leong, E. Kawinski, J. P. Karr, H. Rosenthal, T. M. Chu, E. A. Mirand and G. P. Murphy (1983) LNCaP model of human prostatic carcinoma *Cancer Res* **43** 4 1809-18
610 J. P. Gaddipati, D. G. Mcleod, H. B. Heidenberg, I. A. Sesterhenn, M. J. Finger, J. W. Moul and S. Srivastava (1994) Frequent detection of codon 877 mutation in the androgen receptor gene in advanced prostate cancers *Cancer Res* **54** 11 2861-4
611 H. Suzuki, K. Akakura, A. Komiya, S. Aida, S. Akimoto and J. Shimazaki (1996) Codon 877 mutation in the androgen receptor gene in advanced prostate cancer: relation to antiandrogen withdrawal syndrome *Prostate* **29** 3 153-8
612 H. Suzuki, N. Sato, Y. Watabe, M. Masai, S. Seino and J. Shimazaki (1993) Androgen receptor gene mutations in human prostate cancer *J Steroid Biochem Mol Biol* **46** 6 759-65
613 M. E. Taplin, G. J. Bubley, Y. J. Ko, E. J. Small, M. Upton, B. Rajeshkumar and S. P. Balk (1999) Selection for androgen receptor mutations in prostate cancers treated with androgen antagonist *Cancer Res* **59** 11 2511-5
614 X. Y. Zhao, B. Boyle, A. V. Krishnan, N. M. Navone, D. M. Peehl and D. Feldman (1999) Two mutations identified in the androgen receptor of the new human prostate cancer cell line MDA PCa 2a *J Urol* **162** 6 2192-9
615 M. A. Fenton, T. D. Shuster, A. M. Fertig, M. E. Taplin, G. Kolvenbag, G. J. Bubley and S. P. Balk (1997) Functional characterization of mutant androgen receptors from androgen-independent prostate cancer *Clin Cancer Res* **3** 8 1383-8
616 J. Tan, Y. Sharief, K. G. Hamil, C. W. Gregory, D. Y. Zang, M. Sar, P. H. Gumerlock, R. W. Devere White, T. G. Pretlow, S. E. Harris, E. M. Wilson, J. L. Mohler and F. S. French (1997) Dehydroepiandrosterone activates mutant androgen receptors expressed in the androgen-dependent human prostate cancer xenograft CWR22 and LNCaP cells *Mol Endocrinol* **11** 4 450-9
617 J. Veldscholte, C. A. Berrevoets, C. Ris-Stalpers, G. G. Kuiper, G. Jenster, J. Trapman, A. O. Brinkmann and E. Mulder (1992) The androgen receptor in LNCaP cells contains a mutation in the ligand binding domain which affects steroid binding characteristics and response to antiandrogens *J Steroid Biochem Mol Biol* **41** 3-8 665-9
618 J. Veldscholte, C. A. Berrevoets, N. D. Zegers, T. H. Van Der Kwast, J. A. Grootegoed and E. Mulder (1992) Hormone-induced dissociation of the androgen receptor-heat-shock protein complex: use of a new monoclonal antibody to distinguish transformed from nontransformed receptors *Biochemistry* **31** 32 7422-30

619 N. M. Navone, M. Olive, M. Ozen, R. Davis, P. Troncoso, S. M. Tu, D. Johnston, A. Pollack, S. Pathak, A. C. Von Eschenbach and C. J. Logothetis (1997) Establishment of two human prostate cancer cell lines derived from a single bone metastasis *Clin Cancer Res* **3** 12 Pt 1 2493-500
620 X. Y. Zhao, P. J. Malloy, A. V. Krishnan, S. Swami, N. M. Navone, D. M. Peehl and D. Feldman (2000) Glucocorticoids can promote androgen-independent growth of prostate cancer cells through a mutated androgen receptor *Nat Med* **6** 6 703-6
621 Z. Culig, A. Hobisch, M. V. Cronauer, A. C. Cato, A. Hittmair, C. Radmayr, J. Eberle, G. Bartsch and H. Klocker (1993) Mutant androgen receptor detected in an advanced-stage prostatic carcinoma is activated by adrenal androgens and progesterone *Mol Endocrinol* **7** 12 1541-50
622 J. R. Newmark, D. O. Hardy, D. C. Tonb, B. S. Carter, J. I. Epstein, W. B. Isaacs, T. R. Brown and E. R. Barrack (1992) Androgen receptor gene mutations in human prostate cancer *Proc Natl Acad Sci U S A* **89** 14 6319-23
623 H. Peterziel, Z. Culig, J. Stober, A. Hobisch, C. Radmayr, G. Bartsch, H. Klocker and A. C. Cato (1995) Mutant androgen receptors in prostatic tumors distinguish between amino-acid-sequence requirements for transactivation and ligand binding *Int J Cancer* **63** 4 544-50
624 K. Haapala, E. R. Hyytinen, M. Roiha, M. Laurila, I. Rantala, H. J. Helin and P. A. Koivisto (2001) Androgen receptor alterations in prostate cancer relapsed during a combined androgen blockade by orchiectomy and bicalutamide *Lab Invest* **81** 12 1647-51
625 T. Hara, J. Miyazaki, H. Araki, M. Yamaoka, N. Kanzaki, M. Kusaka and M. Miyamoto (2003) Novel mutations of androgen receptor: a possible mechanism of bicalutamide withdrawal syndrome *Cancer Res* **63** 1 149-53
626 C. E. Bohl, W. Gao, D. D. Miller, C. E. Bell and J. T. Dalton (2005) Structural basis for antagonism and resistance of bicalutamide in prostate cancer *Proc Natl Acad Sci U S A* **102** 17 6201-6
627 C. E. Bohl, D. D. Miller, J. Chen, C. E. Bell and J. T. Dalton (2005) Structural basis for accommodation of nonsteroidal ligands in the androgen receptor *J Biol Chem* **280** 45 37747-54
628 C. E. Bohl, Z. Wu, D. D. Miller, C. E. Bell and J. T. Dalton (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** 18 13648-55
629 C. A. Marhefka, B. M. Moore, 2nd, T. C. Bishop, L. Kirkovsky, A. Mukherjee, J. T. Dalton and D. D. Miller (2001) Homology modeling using multiple molecular dynamics simulations and docking studies of the human androgen receptor ligand binding domain bound to testosterone and nonsteroidal ligands *J Med Chem* **44** 11 1729-40
630 N. Poujol, J. M. Wurtz, B. Tahiri, S. Lumbroso, J. C. Nicolas, D. Moras and C. Sultan (2000) Specific recognition of androgens by their nuclear receptor. A structure-function study *J Biol Chem* **275** 31 24022-31
631 P. M. Matias, M. A. Carrondo, R. Coelho, M. Thomaz, X. Y. Zhao, A. Wegg, K. Crusius, U. Egner and P. Donner (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** 7 1439-46
632 S. Mcdonald, L. Brive, D. B. Agus, H. I. Scher and K. R. Ely (2000) Ligand responsiveness in human prostate cancer: structural analysis of mutant androgen receptors from LNCaP and CWR22 tumors *Cancer Res* **60** 9 2317-22
633 M. S. Ozers, B. D. Marks, K. Gowda, K. R. Kupcho, K. M. Ervin, T. De Rosier, N. Qadir, H. C. Eliason, S. M. Riddle and M. S. Shekhani (2007) The androgen receptor T877A mutant recruits LXXLL and FXXLF peptides differently than wild-type androgen receptor in a time-resolved fluorescence resonance energy transfer assay *Biochemistry* **46** 3 683-95
634 B. He, R. T. Gampe, Jr., A. T. Hnat, J. L. Faggart, J. T. Minges, F. S. French and E. M. Wilson (2006) Probing the functional link between androgen receptor coactivator and ligand-binding sites in prostate cancer and androgen insensitivity *J Biol Chem* **281** 10 6648-63
635 H. F. Acevedo and J. W. Goldzieher (1965) Further Studies on the Metabolism of 4-(4-14c)Androstene-3,17-Dione by Normal and Pathological Human Prostate Tissue *Biochim Biophys Acta* **97** 564-70
636 D. R. Bauman, S. Steckelbroeck, M. V. Williams, D. M. Peehl and T. M. Penning (2006) Identification of the major oxidative 3alpha-hydroxysteroid dehydrogenase in human prostate that converts 5alpha-androstane-3alpha,17beta-diol to 5alpha-dihydrotestosterone: a potential therapeutic target for androgen-dependent disease *Mol Endocrinol* **20** 2 444-58
637 M. E. Harper, A. Pike, W. B. Peeling and K. Griffiths (1974) Steroids of adrenal origin metabolized by human prostatic tissue both in vivo and in vitro *J Endocrinol* **60** 1 117-25
638 W. H. Pearlman and M. R. Pearlman (1961) The metabolism in vivo of Delta 4-androstene-3, 17-dione-7-H3; its localization in the ventral prostate and other tissues of the rat *J Biol Chem* **236** 1321-7
639 M. Stanbrough, G. J. Bubley, K. Ross, T. R. Golub, M. A. Rubin, T. M. Penning, P. G. Febbo and S. P. Balk (2006) Increased expression of genes converting adrenal androgens to testosterone in androgen-independent prostate cancer *Cancer Res* **66** 5 2815-25
640 A. Belanger, M. Brochu and J. Cliche (1986) Levels of plasma steroid glucuronides in intact and castrated men with prostatic cancer *J Clin Endocrinol Metab* **62** 5 812-5
641 F. Labrie, A. Dupont, A. Belanger, R. Lachance and M. Giguere (1985) Long term treatment with luteinising hormone releasing hormone agonists and maintenance of serum testosterone to castration concentrations *Br Med J (Clin Res Ed)* **291** 6492 369-70
642 S. T. Page, D. W. Lin, E. A. Mostaghel, D. L. Hess, L. D. True, J. K. Amory, P. S. Nelson, A. M. Matsumoto and W. J. Bremner (2006) Persistent intraprostatic androgen concentrations after medical castration in healthy men *J Clin Endocrinol Metab* **91** 10 3850-6
643 M. A. Titus, M. J. Schell, F. B. Lih, K. B. Tomer and J. L. Mohler (2005) Testosterone and dihydrotestosterone tissue levels in recurrent prostate cancer *Clin Cancer Res* **11** 13 4653-7

644 N. Craft, Y. Shostak, M. Carey and C. L. Sawyers (1999) A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase *Nat Med* **5** 3 280-5
645 R. E. Bakin, D. Gioeli, E. A. Bissonette and M. J. Weber (2003) Attenuation of Ras signaling restores androgen sensitivity to hormone-refractory C4-2 prostate cancer cells *Cancer Res* **63** 8 1975-80
646 R. E. Bakin, D. Gioeli, R. A. Sikes, E. A. Bissonette and M. J. Weber (2003) Constitutive activation of the Ras/mitogen-activated protein kinase signaling pathway promotes androgen hypersensitivity in LNCaP prostate cancer cells *Cancer Res* **63** 8 1981-9
647 T. Chen, R. W. Cho, P. J. Stork and M. J. Weber (1999) Elevation of cyclic adenosine 3',5'-monophosphate potentiates activation of mitogen-activated protein kinase by growth factors in LNCaP prostate cancer cells *Cancer Res* **59** 1 213-8
648 D. Gioeli, J. W. Mandell, G. R. Petroni, H. F. Frierson, Jr. and M. J. Weber (1999) Activation of mitogen-activated protein kinase associated with prostate cancer progression *Cancer Res* **59** 2 279-84
649 B. J. Park, J. I. Park, D. S. Byun, J. H. Park and S. G. Chi (2000) Mitogenic conversion of transforming growth factor-beta1 effect by oncogenic Ha-Ras-induced activation of the mitogen-activated protein kinase signaling pathway in human prostate cancer *Cancer Res* **60** 11 3031-8
650 T. Putz, Z. Culig, I. E. Eder, C. Nessler-Menardi, G. Bartsch, H. Grunicke, F. Uberall and H. Klocker (1999) Epidermal growth factor (EGF) receptor blockade inhibits the action of EGF, insulin-like growth factor I, and a protein kinase A activator on the mitogen-activated protein kinase pathway in prostate cancer cell lines *Cancer Res* **59** 1 227-33
651 J. D. Debes, L. J. Schmidt, H. Huang and D. J. Tindall (2002) p300 mediates androgen-independent transactivation of the androgen receptor by interleukin 6 *Cancer Res* **62** 20 5632-6
652 T. Matsuda, A. Junicho, T. Yamamoto, H. Kishi, K. Korkmaz, F. Saatcioglu, H. Fuse and A. Muraguchi (2001) Cross-talk between signal transducer and activator of transcription 3 and androgen receptor signaling in prostate carcinoma cells *Biochem Biophys Res Commun* **283** 1 179-87
653 S. Xie, H. K. Lin, J. Ni, L. Yang, L. Wang, P. A. Di Sant'agnese and C. Chang (2004) Regulation of interleukin-6-mediated PI3K activation and neuroendocrine differentiation by androgen signaling in prostate cancer LNCaP cells *Prostate* **60** 1 61-7
654 S. Kraus, D. Gioeli, T. Vomastek, V. Gordon and M. J. Weber (2006) Receptor for activated C kinase 1 (RACK1) and Src regulate the tyrosine phosphorylation and function of the androgen receptor *Cancer Res* **66** 22 11047-54
655 Z. Guo, B. Dai, T. Jiang, K. Xu, Y. Xie, O. Kim, I. Nesheiwat, X. Kong, J. Melamed, V. D. Handratta, V. C. Njar, A. M. Brodie, L. R. Yu, T. D. Veenstra, H. Chen and Y. Qiu (2006) Regulation of androgen receptor activity by tyrosine phosphorylation *Cancer Cell* **10** 4 309-19
656 A. Migliaccio, M. Di Domenico, G. Castoria, M. Nanayakkara, M. Lombardi, A. De Falco, A. Bilancio, L. Varricchio, A. Ciociola and F. Auricchio (2005) Steroid receptor regulation of epidermal growth factor signaling through Src in breast and prostate cancer cells: steroid antagonist action *Cancer Res* **65** 22 10585-93
657 E. Unni, S. Sun, B. Nan, M. J. Mcphaul, B. Cheskis, M. A. Mancini and M. Marcelli (2004) Changes in androgen receptor nongenotropic signaling correlate with transition of LNCaP cells to androgen independence *Cancer Res* **64** 19 7156-68
658 S. Lu, C. Ren, Y. Liu and D. E. Epner (2006) PI3K-Akt signaling is involved in the regulation of p21(WAF/CIP) expression and androgen-independent growth in prostate cancer cells *Int J Oncol* **28** 1 245-51
659 H. K. Lin, Y. C. Hu, L. Yang, S. Altuwaijri, Y. T. Chen, H. Y. Kang and C. Chang (2003) Suppression versus induction of androgen receptor functions by the phosphatidylinositol 3-kinase/Akt pathway in prostate cancer LNCaP cells with different passage numbers *J Biol Chem* **278** 51 50902-7
660 S. S. Taneja, S. Ha, N. K. Swenson, H. Y. Huang, P. Lee, J. Melamed, E. Shapiro, M. J. Garabedian and S. K. Logan (2005) Cell-specific regulation of androgen receptor phosphorylation in vivo *J Biol Chem* **280** 49 40916-24
661 P. Li, H. Lee, S. Guo, T. G. Unterman, G. Jenster and W. Bai (2003) AKT-independent protection of prostate cancer cells from apoptosis mediated through complex formation between the androgen receptor and FKHR *Mol Cell Biol* **23** 1 104-18
662 R. Kaaks, A. Lukanova and B. Sommersberg (2000) Plasma androgens, IGF-1, body size, and prostate cancer risk: a synthetic review *Prostate Cancer Prostatic Dis* **3** 3 157-172
663 G. Pandini, R. Mineo, F. Frasca, C. T. Roberts, Jr., M. Marcelli, R. Vigneri and A. Belfiore (2005) Androgens up-regulate the insulin-like growth factor-I receptor in prostate cancer cells *Cancer Res* **65** 5 1849-57
664 J. I. Jones, M. E. Doerr and D. R. Clemmons (1995) Cell migration: interactions among integrins, IGFs and IGFBPs *Prog Growth Factor Res* **6** 2-4 319-27
665 S. Kiyama, K. Morrison, T. Zellweger, M. Akbari, M. Cox, D. Yu, H. Miyake and M. E. Gleave (2003) Castration-induced increases in insulin-like growth factor-binding protein 2 promotes proliferation of androgen-independent human prostate LNCaP tumors *Cancer Res* **63** 13 3575-84
666 V. C. Russo, L. A. Bach, A. J. Fosang, N. L. Baker and G. A. Werther (1997) Insulin-like growth factor binding protein-2 binds to cell surface proteoglycans in the rat brain olfactory bulb *Endocrinology* **138** 11 4858-67
667 R. Chmelar, G. Buchanan, E. F. Need, W. Tilley and N. M. Greenberg (2007) Androgen receptor coregulators and their involvement in the development and progression of prostate cancer *Int J Cancer* **120** 4 719-33

668 C. W. Gregory, B. He, R. T. Johnson, O. H. Ford, J. L. Mohler, F. S. French and E. M. Wilson (2001) A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy *Cancer Res* **61** 11 4315-9
669 M. J. Linja, K. P. Porkka, Z. Kang, K. J. Savinainen, O. A. Janne, T. L. Tammela, R. L. Vessella, J. J. Palvimo and T. Visakorpi (2004) Expression of androgen receptor coregulators in prostate cancer *Clin Cancer Res* **10** 3 1032-40
670 I. U. Agoulnik, A. Vaid, W. E. Bingman, 3rd, H. Erdeme, A. Frolov, C. L. Smith, G. Ayala, M. M. Ittmann and N. L. Weigel (2005) Role of SRC-1 in the promotion of prostate cancer cell growth and tumor progression *Cancer Res* **65** 17 7959-67
671 T. Ueda, N. Bruchovsky and M. D. Sadar (2002) Activation of the androgen receptor N-terminal domain by interleukin-6 via MAPK and STAT3 signal transduction pathways *J Biol Chem* **277** 9 7076-85
672 T. Ueda, N. R. Mawji, N. Bruchovsky and M. D. Sadar (2002) Ligand-independent activation of the androgen receptor by interleukin-6 and the role of steroid receptor coactivator-1 in prostate cancer cells *J Biol Chem* **277** 41 38087-94
673 C. W. Gregory, X. Fei, L. A. Ponguta, B. He, H. M. Bill, F. S. French and E. M. Wilson (2004) Epidermal growth factor increases coactivation of the androgen receptor in recurrent prostate cancer *J Biol Chem* **279** 8 7119-30
674 V. J. Gnanapragasam, H. Y. Leung, A. S. Pulimood, D. E. Neal and C. N. Robson (2001) Expression of RAC 3, a steroid hormone receptor co-activator in prostate cancer *Br J Cancer* **85** 12 1928-36
675 H. J. Zhou, J. Yan, W. Luo, G. Ayala, S. H. Lin, H. Erdem, M. Ittmann, S. Y. Tsai and M. J. Tsai (2005) SRC-3 is required for prostate cancer cell proliferation and survival *Cancer Res* **65** 17 7976-83
676 B. Comuzzi, L. Lambrinidis, H. Rogatsch, S. Godoy-Tundidor, N. Knezevic, I. Krhen, Z. Marekovic, G. Bartsch, H. Klocker, A. Hobisch and Z. Culig (2003) The transcriptional co-activator cAMP response element-binding protein-binding protein is expressed in prostate cancer and enhances androgen- and anti-androgen-induced androgen receptor function *Am J Pathol* **162** 1 233-41
677 B. Comuzzi, C. Nemes, S. Schmidt, Z. Jasarevic, M. Lodde, A. Pycha, G. Bartsch, F. Offner, Z. Culig and A. Hobisch (2004) The androgen receptor co-activator CBP is up-regulated following androgen withdrawal and is highly expressed in advanced prostate cancer *J Pathol* **204** 2 159-66
678 J. D. Debes, T. J. Sebo, C. M. Lohse, L. M. Murphy, D. A. Haugen and D. J. Tindall (2003) p300 in prostate cancer proliferation and progression *Cancer Res* **63** 22 7638-40
679 C. Mestayer, M. Blanchere, F. Jaubert, B. Dufour and I. Mowszowicz (2003) Expression of androgen receptor coactivators in normal and cancer prostate tissues and cultured cell lines *Prostate* **56** 3 192-200
680 Y. Miyoshi, H. Ishiguro, H. Uemura, K. Fujinami, H. Miyamoto, Y. Miyoshi, H. Kitamura and Y. Kubota (2003) Expression of AR associated protein 55 (ARA55) and androgen receptor in prostate cancer *Prostate* **56** 4 280-6
681 Z. Culig, H. Klocker, G. Bartsch and A. Hobisch (2002) Androgen receptors in prostate cancer *Endocr Relat Cancer* **9** 3 155-70
682 Y. C. Hu, S. Yeh, S. D. Yeh, E. R. Sampson, J. Huang, P. Li, C. L. Hsu, H. J. Ting, H. K. Lin, L. Wang, E. Kim, J. Ni and C. Chang (2004) Functional domain and motif analyses of androgen receptor coregulator ARA70 and its differential expression in prostate cancer *J Biol Chem* **279** 32 33438-46
683 H. Miyamoto, S. Yeh, H. Lardy, E. Messing and C. Chang (1998) Delta5-androstenediol is a natural hormone with androgenic activity in human prostate cancer cells *Proc Natl Acad Sci U S A* **95** 19 11083-8
684 M. C. Bosland (2000) The role of steroid hormones in prostate carcinogenesis *J Natl Cancer Inst Monogr* 27 39-66
685 M. Hiramatsu, I. Maehara, M. Ozaki, N. Harada, S. Orikasa and H. Sasano (1997) Aromatase in hyperplasia and carcinoma of the human prostate *Prostate* **31** 2 118-24
686 M. Krieg, R. Nass and S. Tunn (1993) Effect of aging on endogenous level of 5 alpha-dihydrotestosterone, testosterone, estradiol, and estrone in epithelium and stroma of normal and hyperplastic human prostate *J Clin Endocrinol Metab* **77** 2 375-81
687 H. Matzkin and M. S. Soloway (1992) Immunohistochemical evidence of the existence and localization of aromatase in human prostatic tissues *Prostate* **21** 4 309-14
688 S. Tekur, K. M. Lau, J. Long, K. Burnstein and S. M. Ho (2001) Expression of RFG/ELE1alpha/ARA70 in normal and malignant prostatic epithelial cell cultures and lines: regulation by methylation and sex steroids *Mol Carcinog* **30** 1 1-13
689 H. J. Voeller, C. I. Truica and E. P. Gelmann (1998) Beta-catenin mutations in human prostate cancer *Cancer Res* **58** 12 2520-3
690 H. J. Ting, S. Yeh, K. Nishimura and C. Chang (2002) Supervillin associates with androgen receptor and modulates its transcriptional activity *Proc Natl Acad Sci U S A* **99** 2 661-6
691 K. Halkidou, V. J. Gnanapragasam, P. B. Mehta, I. R. Logan, M. E. Brady, S. Cook, H. Y. Leung, D. E. Neal and C. N. Robson (2003) Expression of Tip60, an androgen receptor coactivator, and its role in prostate cancer development *Oncogene* **22** 16 2466-77
692 J. H. Kim, B. Kim, L. Cai, H. J. Choi, K. A. Ohgi, C. Tran, C. Chen, C. H. Chung, O. Huber, D. W. Rose, C. L. Sawyers, M. G. Rosenfeld and S. H. Baek (2005) Transcriptional regulation of a metastasis suppressor gene by Tip60 and beta-catenin complexes *Nature* **434** 7035 921-6
693 J. Edwards, N. S. Krishna, C. J. Witton and J. M. Bartlett (2003) Gene amplifications associated with the development of hormone-resistant prostate cancer *Clin Cancer Res* **9** 14 5271-81
694 N. Sato, M. D. Sadar, N. Bruchovsky, F. Saatcioglu, P. S. Rennie, S. Sato, P. H. Lange and M. E. Gleave (1997) Androgenic induction of prostate-specific antigen gene is repressed by protein-protein interaction between the androgen receptor and AP-1/c-Jun in the human prostate cancer cell line LNCaP *J Biol Chem* **272** 28 17485-94

695 J. Edwards, N. S. Krishna, R. Mukherjee and J. M. Bartlett (2004) The role of c-Jun and c-Fos expression in androgen-independent prostate cancer *J Pathol* **204** 2 153-8
696 N. Gao, Z. Zhang, B. H. Jiang and X. Shi (2003) Role of PI3K/AKT/mTOR signaling in the cell cycle progression of human prostate cancer *Biochem Biophys Res Commun* **310** 4 1124-32
697 M. Sun, L. Yang, R. I. Feldman, X. M. Sun, K. N. Bhalla, R. Jove, S. V. Nicosia and J. Q. Cheng (2003) Activation of phosphatidylinositol 3-kinase/Akt pathway by androgen through interaction of p85alpha, androgen receptor, and Src *J Biol Chem* **278** 44 42992-3000
698 H. J. Mackay and C. J. Twelves (2003) Protein kinase C: a target for anticancer drugs? *Endocr Relat Cancer* **10** 3 389-96

Chapter 2

Amino acids 3-13 and amino acids in and flanking the ^{23}FXXLF^{27} motif modulate the interaction between the amino-terminal and ligand-binding domain of the androgen receptor

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SUMMARY

The NH_2-terminal domain (NTD) and the ligand-binding domain (LBD) of the androgen receptor (AR) exhibit a ligand-dependent interaction (N/C interaction). Amino acids 3 to 36 in the NTD (AR_{3-36}) play a dominant role in this interaction. Previously, it has been shown that a ΦxxΦΦ motif in AR_{3-36}, ^{23}FxxLF^{27}, is essential for LBD interaction. We demonstrate in the current study that AR_{3-36} can be subdivided into two functionally distinct fragments: AR_{3-13} and AR_{16-36}. AR_{3-13} does not directly interact with the AR LBD, but rather contributes to the transactivation function of the AR.NTD-AR.LBD complex. AR_{16-36}, encompassing the ^{23}FxxLF^{27} motif, is predicted to fold into a long amphipathic α-helix. A second ΦxxΦΦ candidate protein interaction motif within the helical structure, ^{30}VREVI^{34}, shows no affinity to the LBD. Within AR_{16-36}, amino acid residues in and flanking the ^{23}FxxLF^{27} motif are demonstrated to modulate N/C interaction. Substitution of Q24 and N25 by alanine residues enhances N/C interaction. Substitution of amino acids flanking the ^{23}FxxLF^{27} motif by alanines are inhibitory to LBD interaction.

INTRODUCTION

The androgen receptor (AR) is a member of the steroid receptor subgroup of the nuclear receptor family of transcription factors. Nuclear receptors have a modular structure, composed of a moderately conserved carboxy-terminal ligand-binding domain (LBD) folded in 12 α-helices, a highly conserved central DNA-binding domain (DBD) and a non-conserved amino-terminal domain (NTD). Most nuclear receptors contain two transactivation functions: AF-1 in the NTD, and AF-2 in the LBD. Ligand-activated nuclear receptors bind as homo- or heterodimers to hormone response elements in the regulatory regions of their target genes. Together with coactivators, general transcription factors and RNA polymerase II they form a stable transcription initiation complex (see for recent reviews [1-4]).

Upon ligand binding the LBD acquires a conformation that facilitates the interaction with coactivators. Best studied in this regard are the interactions with the p160 coactivators SRC1, TIF2/GRIP1 and ACTR/RAC3. The nuclear receptor interaction domains of p160 coactivators contain LxxLL motifs (NR boxes), which bind to a hydrophobic cleft in the agonist-activated LBD. Antagonists induce a different LBD conformation, which inhibits the interaction with coactivators and enables the binding of corepressors [5] (see for review [3]).

P160 coactivators not only bind to the LBD, but also to the NTD [6,7]. This interaction is independent of the NR boxes. As shown for the estrogen receptor α (ERα), simultaneous NTD and LBD binding by one coactivator can confer synergism of AF-1 and AF-2 activities, which might be necessary for optimal functioning [8].

Like shown for other nuclear receptors, p160 coactivators can bind the AR LBD by their LxxLL motifs, and they interact with the AR NTD, independent of these motifs [9-11]. In contrast to AR AF1, which is strong, AF-2 needs overexpression of a p160 coactivator to become manifest [9,10,12-15]. Many other proteins with known or unknown functions have been found to interact with the AR. An overview of AR-interacting proteins is presented in the AR mutations database (http://www.mcgill.ca/androgendb) [16].

Previously, a ligand-dependent functional interaction between the AR subdomains NTD and LBD, has been described [17-19]. This N/C interaction might be intra- or intermolecular [15,17-19]. *In vitro* pull down experiments indicated that AR N/C interaction is direct [11]. The AF-2

core domain in helix 12 of the AR LBD was shown to be involved this interaction [11,15]. In the AR NTD two regions are involved in the functional interaction with the AR LBD: AR_{3-36}, including the $^{23}FxxLF^{27}$ motif, and $AR_{370-494}$, which encompasses a transactivation function and a presumed supplementary protein interaction domain [15,20]. In the present study, AR_{3-36} is subdivided into two fragments: AR_{3-13} and $AR_{16-36,}$ which are further characterized.

EXPERIMENTAL PROCEDURES

Materials and plasmid construction

Dihydrotestosterone (DHT) was purchased from Steraloids (Wilton, NH), R1881 (methyltrienolone) was from NEN (Boston, MA).

Standard procedures were utilized for PCR and molecular cloning [21]. PCR products were inserted in pGEM-T Easy (Promega, Madison, WI). All plasmids were sequenced to verify their correct construction. Primer sequences are shown in Table 1. AR numbering corresponds to a length of 919 amino acids, as employed by The Androgen Receptor Gene Mutations Database (http://www.mcgill.ca/androgendb).

Table 1. Primers for construction of plasmids

Primer name	Primer sequence
pr14	5'-TCTAGATTCCCGGGTCCGCCGTCCAAGACCTACCGAGG-3'
pr1B	5'-CAGCAGCAGCAAACTGGC-3'
pr23/27RR	5'-CTGGGGCCCGGGTTCTGGATCACTTCGCGGACGCTCTGGCGCAGATTCTGGCGAGCTCCT-3'
pr30/33RR	5'-CTGGGGCCCGGGTTCTGGATCCGTTCGCGGCGGCTCTGGAACAGATTCTGGAA-3'
pr24/25AA	5'-CTGGGGCCCGGGTTCTGGATCACTTCGCGGACGCTCTGGAACAGAGCCGCGAAAGCTCC-3'
pr26/27AA	5'-CTGGGGCCCGGGTTCTGGATCACTTCGCGGACGCTCTGGGCCGCATTCTGGAAAGCTCC-3'
pr2-36sense	5'-AATTGGGGATCCGAGAAGTGCAGTTAGGGCTGGGAAGG-3'
pr2-36anti-sense	5'-GATCGAATTCGTTCTGGATCACTTCGCGCACGCTC-3'
pr1-14sense	5'-GATCGAAGTGCAGTTAGGGCTGGGAAGGGTCTACCCTCGGCCGG-3'
pr1-14anti-sense	5'-AATTCCGGCCGAGGGTAGACCCTTCCCAGCCCTAACTGCACTTC-3'
pr16-36sense	5'-GATCTCCAAGACCTACCGAGGAGCTTTCCAGAATCTGTTCCAGAGCGTGCGCGAAGTGATC CAGAACG-3'
pr16-36anti-sense	5'-AATTCGTTCTGGATCACTTCGCGCACGCTCTGGAACAGATTCTGGAAAGCTCCTCGGTAGG TCTTGGA-3'
pr17-32sense	5'-GATCAAGACCTACCGAGGAGCTTTCCAGAATCTGTTCCAGAGCGTGCGCG-3'
pr17-32anti-sense	5'-AATTCGCGCACGCTCTGGAACAGATTCTGGAAAGCTCCTCGGTAGGTCTT-3'
pr24-39sense	5'-GATCCAGAATCTGTTCCAGAGCGTGCGCGAAGTGATCCAGAACCCGGGCCCCG-3'
pr24-39anti-sense	5'-AATTCGGGGCCCGGGTTCTGGATCACTTCGCGCACGCTCTGGAACAGATTCTG-3'
pr172B	5'-CGGAGCAGCTGCTTAAGCCGGGG-3'
pr-242	5'-AAGCTTCTGCAGGTCGACTCTAGG-3'
PDsense	5'-GATCCATATCGATAAGCTTAGATCTGAATTCA-3'
PDanti-sense	5'-AATTCAGATCTAAGCTTATCGATATG-3'

Yeast expression constructs

pGalAD-AR.NTDwt (AR_{3-503}), originally derived from the yeast expression vector pACT2 (Clontech, Palo Alto, CA), and pGalDBD-AR.LBD ($AR_{661-919}$), originally derived from the yeast expression vector pGBT9 (Clontech), were previously described as AR.N8(high) and pGAL4(DBD)AR(LBD), respectively [15,18]. pGalAD-AR.NTDΔ1-13 was obtained by exchange of a 75 bp *Sma*I fragment of pGalAD-AR.NTDwt with a corresponding fragment derived from a PCR product synthesized with primers pr14 and pr1B, utilizing $pSVAR_0$ [22] as template. pGalAD-AR.NTDΔ3-36 was obtained by excision of a 117 bp *Sma*I fragment from pGalAD-AR.NTDwt. For generation of pGalAD-AR.NTD23/27RR, pGalAD-AR.NTD30/33RR, pGalAD-AR.NTD24/25AA and pGalAD-AR.NTD26/27AA, a 117 bp *Sma*I fragment of pGalAD-AR.NTDwt was exchanged with corresponding fragments containing the indicated mutations, which were obtained by PCR on the template pGalAD-AR.NTDwt utilizing primer G4AD1 (Clontech) in combination with one of the following oligonucleotides: pr23/27RR, pr30/33RR, pr24/25AA, and pr26/27AA (mutated codons are underlined in Table1).

The AR peptide construct pGalAD-AR_{2-36} was obtained by insertion of a 117 bp *Bam*HI/*Eco*RI fragment, which was synthesized by PCR on the template $pSVAR_3$ [23], utilizing primers pr2-36sense and pr2-36anti-sense, into the corresponding sites of pACT2 (Clontech). All other pGalAD-ARpeptide constructs were generated by *Bam*HI/*Eco*RI in frame insertion of double-stranded oligonucleotides into the corresponding sites of $pACT_2$ (Clontech), yielding pGalAD-AR_{1-14}, pGalAD-AR_{16-36}, pGalAD-AR_{17-32}, pGalAD-AR_{24-39}, pGalAD-AR_{17-32}(18/19AA), pGalAD-AR_{17-32}(20/21AA), pGalAD-AR_{17-32}(23A), pGalAD-AR_{17-32}(24/25AA), pGalAD-AR_{17-32}(26/27AA), pGalAD-AR_{17-32}(28/29AA) and pGalAD-AR_{17-32}(30/31AA). Oligonucleotides for these AR peptide expression constructs were: pr1-14sense, pr1-14anti-sense, pr16-36sense, pr16-36anti-sense, pr17-32sense, pr17-32anti-sense, pr24-39sense, and pr24-39anti-sense. Primers pr18/19AA, pr20/21AA, pr22A, pr24/25AA, pr26/27AA, pr28/29AA, and pr30/31AA sense and anti-sense oligonucleotides were modified pr17-32 sense and anti-sense oligonucleotides, containing GCTGCA (sense) and TGCAGC (anti-sense) as two adjacent alanine codons at the indicated positions.

Mammalian cell expression constructs

pMMTV-LUC, pSVAR.NTDwt (AR_{1-503}) (originally described as pSVAR(TAD_{1-494})) and pSVAR.DBD.LBD ($AR_{537-919}$) (originally described as pSVAR-104) were previously published [18,23,24]. Insertion of a 1.9 kb *Hind*III fragment from $pSVAR_3$ in *Hind*III digested $pGAD_{424}$ (Clontech) yielded $pGAD_3$. $pGAD_3$.NTDΔ3-13 was obtained by insertion of a 75 bp *Sma*I fragment synthesized by PCR on the $pSVAR_0$ template, utilizing primers pr14 and pr172B, into the *Xba*I(Klenow-filled)/*Sma*I sites of $pGAD_3$. Exchange of a 1.5 kb *Hind*III/*Bst*EII fragment of pSVAR.NTDwt with the corresponding fragment of $pGAD_3$.NTDΔ3-13 yielded pSVAR.NTDΔ3-13. $pGAD_3$Δ3-37 was obtained by excision of a 108 bp fragment from $pGAD_3$ by *Xba*I(Klenow-filled)/*Sma*I digestion. $pSVAR_8$ was obtained by exchange of a 1.8 kb *Hind*III fragment of $pSVAR_3$ with the corresponding fragment of $pGAD_3$Δ3-37. For construction of pSVAR.NTDΔ3-37, a 1.7 kb *Hind*III/*Asp*718 fragment of pSVAR.NTDwt was exchanged with the corresponding fragment of $pSVAR_8$. pSVAR.NTD23/27RR, pSVAR.NTD30/33RR, pSVAR.NTD24/25AA and pSVAR.NTD26/27AA were obtained by exchange of a 348 bp *Hind*III/*Sma*I fragment of pSVAR.NTDwt with corresponding fragments synthesized by PCR on the $pSVAR_0$ template, utilizing primer pr-242 and one of the mutant primers pr23/27RR, pr30/33RR, pr24/25AA or pr26/27AA.

Pull-down constructs

For pSVAR.NTDwt and pSVAR.NTDmutant see *Mammalian cell expression constructs*. pCMV-GST-AR.LBD ($AR_{664-919}$) was generated as follows: pGEX-2TK-CHB was obtained by *Bam*HI/*Eco*RI in frame insertion of a double-stranded oligonucleotide in the corresponding sites of pGEX-2TK (Amersham Biosciences, Uppsala, Sweden). Oligonucleotides were PDsense and PDanti-sense. Insertion of the AR.LBD *Cla*I/*Bgl*II fragment from pAR_{34} [23] into the corresponding sites of pGEX-2TK-CHB yielded pGST-AR.LBD. Insertion of the AR LBD *Bam*HI/*Sal*I fragment of pGST-AR.LBD into the corresponding sites of pCMV-GST [25] yielded pCMV-GST-AR.LBD.

Yeast growth, transformation and β-galactosidase assay

Yeast strain Y190 (Clontech), containing an integrated Gal4 driven UAS_{GAL1}-lacZ reporter gene, was utilized for two-hybrid experiments. Yeast cells were grown in the appropriate selective medium (0.67% w/v yeast nitrogen base without amino acids, 2% w/v glucose, pH5.8), supplemented with the required amino acids. Yeast transformation was carried out according to the lithium acetate method [26]. A yeast liquid β-galactosidase assay was performed to quantify the interaction of GalAD-AR.NTDwt, GalAD-AR.NTDmutant and GalAD-ARpeptide proteins with GalDBD-AR.LBD. In short, stationary phase cultures of Y190 yeast transformants grown in selective medium were diluted in the same medium supplemented with 1 μM DHT or without hormone, and grown until an OD_{600} between 0.7 and 1.2. Next, β-galactosidase activity was determined as described previously [18].

Mammalian cell culture, transfection, and luciferase assay

CHO cells were maintained in DMEM/F12 culture medium, supplemented with 5% dextran-coated charcoal-treated FCS (Life Technologies, Gaithersburg, MD). Cells were plated in 24 wells-plates at a density of 2 x 10^4 cells per well, in a total volume of 0.5 ml. Cells were transfected with MMTV-LUC reporter plasmid (50 ng/well) and pSVAR.DBD.LBD (10 ng/well) together with increasing amounts of pSVAR.NTDwt or pSVAR.NTDmutant (10, 30, 100, 300 ng/well), supplemented with pTZ19 as carrier DNA to a total amount of 300 ng/well, utilizing 0.5 μl FuGENE transfection reagent (Roche Inc, Mannheim, Germany) per well. After overnight incubation with or without 1 nM R1881, cells were harvested and luciferase measurement was performed as described previously [27].

Protein extraction and Western blot analysis

Yeast protein extracts were obtained by direct lysis of yeast cells in 2x SDS gel-loading buffer by a freeze/thawing cycle and boiling, according to Sambrook and Russell (2001) [21]. Western blot analysis for detection of GalAD fusion proteins was performed as previously described, utilizing a GAL4AD monoclonal antibody (Clontech) [18].

CHO cells were plated at a density of 1.5 x 10^6 cells per 80 cm^2 flask and the next day transfected with 1 μg pSVAR.NTDwt or pSVAR.NTDmutant, utilizing 12 μl FuGENE transfection reagent. After overnight incubation, cells were harvested by scraping in 1 ml PBS and centrifugation (5 min. 800xg). Protein extracts were obtained by lysis of the pelleted cells in 60 μl lysis buffer A (20 mM Tris, 1 mM EDTA, 0.1% Nonidet P40, 25% glycerol, 20 mM Na-molybdate, pH 6.8), with addition of 0.3 M NaCl, followed by three cycles of freeze/thawing and centrifugation (10 min. at 400,000xg). Western blot analysis for detection of AR.NTD proteins was performed as previously described, utilizing AR antibody SP061 [18,28].

Pull-down assay

CHO cell plating, transfection, harvesting, and protein extraction were done as described in the previous section, except that 3 µg pCMVGST-AR.LBD and 1 µg pSVAR.NTDwt or pSVAR.NTDmutant were utilized, and that transfection and cell lysis were in the absence or presence of 100 nM R1881. 5 µl protein lysate was directly applied on a 10% SDS-PAGE gel (10% input). 50 µl lysate was mixed with 150 µl buffer A, with or without 100 nM R1881, and rotated for 5 h at 4°C with 25 µl glutathione-agarose beads (Sigma-Aldrich, Deisenhofen, Germany). Next, agarose beads were washed 5 times with buffer A supplemented with 0.1 M NaCl with or without 100 nM R1881, boiled in 30 µl Laemmli sample buffer and 25 µl supernatant was separated over a 10% SDS-PAGE gel. After Western blotting, visualization of input and precipitated AR.NTD proteins was done as described above.

RESULTS

Systems for detection of androgen receptor N/C interaction

The ligand-dependent interaction between AR NTD and AR LBD, N/C interaction, was studied in yeast and mammalian *in vivo* protein interaction systems, and in pull down assays. In the yeast two-hybrid system, vectors encoding the Gal4 transactivating domain (GalAD) fused to AR NTDwt, AR NTDmutant or ARpeptides derived from AR NTD, were transfected to a yeast strain, which expressed the Gal4 DNA-binding domain (GalDBD) linked to AR.LBD (Figure 1A). Upon incubation with DHT, N/C interaction mediated the expression of an integrated UAS_{GAL1}-lacZ reporter gene, which was assessed in a β-galactosidase assay. Note that in this assay the transactivating function is provided by both AR NTD and GalAD.

In the mammalian protein interaction system, vectors encoding wild type or mutated AR NTD, and AR DBD-LBD were cotransfected to CHO cells (Figure 1B). R1881-induced activity of a transiently transfected androgen-inducible MMTV promoter was assessed in a luciferase assay. Note that in this assay the transactivating function is solely contributed by AR NTD.

In pull down assays the fusion protein GST-AR.LBD and wild type or mutated AR.NTD proteins were transiently expressed in CHO cells.

AR_{3-13} modulates the androgen receptor N/C interaction

As assayed in the yeast protein interaction system, deletion of AR_{3-36} (GalAD-AR.NTDΔ3-36) completely abolished the ligand-dependent functional N/C interaction (Figure 2A). Deletion of the amino-terminal 13 amino acids (GalAD-AR.NTDΔ1-13) resulted in a slightly diminished (approximately 20%) N/C interaction. Because GalAD-AR.NTDΔ1-13 was expressed at a higher level than GalAD-AR.NTDwt (Figure 2C), the decrease of AR N/C interaction caused by AR_{1-13} deletion might actually be more than observed.

Similar to the yeast assay, in the mammalian protein interaction assay, deletion of AR_{3-37} completely prevented N/C interaction (Figure 2B). A much more pronounced effect of AR_{3-13} deletion on N/C interaction was observed as compared to the yeast assay. The approximately 90% drop in activity is indicative of an important role of AR_{3-13} in N/C interaction. The diminished interaction was not due to a lower expression level of AR.NTDΔ3-13. In fact, AR.NTDΔ3-13 expression was higher than AR.NTDwt expression (Figure 2C).

To investigate whether AR_{3-13} directly binds to AR LBD, pull down experiments were carried out. The results are presented in Figure 3. In the absence of ligand, none of the AR NTD

proteins showed LBD interaction. However, in the presence of ligand, both AR.NTDwt and AR.NTDΔ3-13 bound to AR LBD with similar affinity (Figure 3). In contrast, AR.NTDΔ3-37 did not interact.

AR_{2-14} cannot autonomously interact with the androgen receptor LBD

To substantiate the modulating role of AR_{2-14} in N/C interaction, as suggested by the experiments described above, the individual peptides AR_{2-36}, AR_{2-14} and AR_{16-36} coupled to GalAD (Figure 4A) were assayed in the yeast protein interaction system (Figure 4B). No substantial interaction with AR.LBD was found for GalAD-AR_{2-14}. Activity was retained for approximately 60% in the GalAD-AR_{16-36}/AR.LBD complex. Because the GalAD-AR_{2-36} expression level was lower than that of GalAD-AR_{16-36} (Figure 4C), the actual difference in activity between GalAD-AR_{2-36} and GalAD-AR_{16-36}, might be larger.

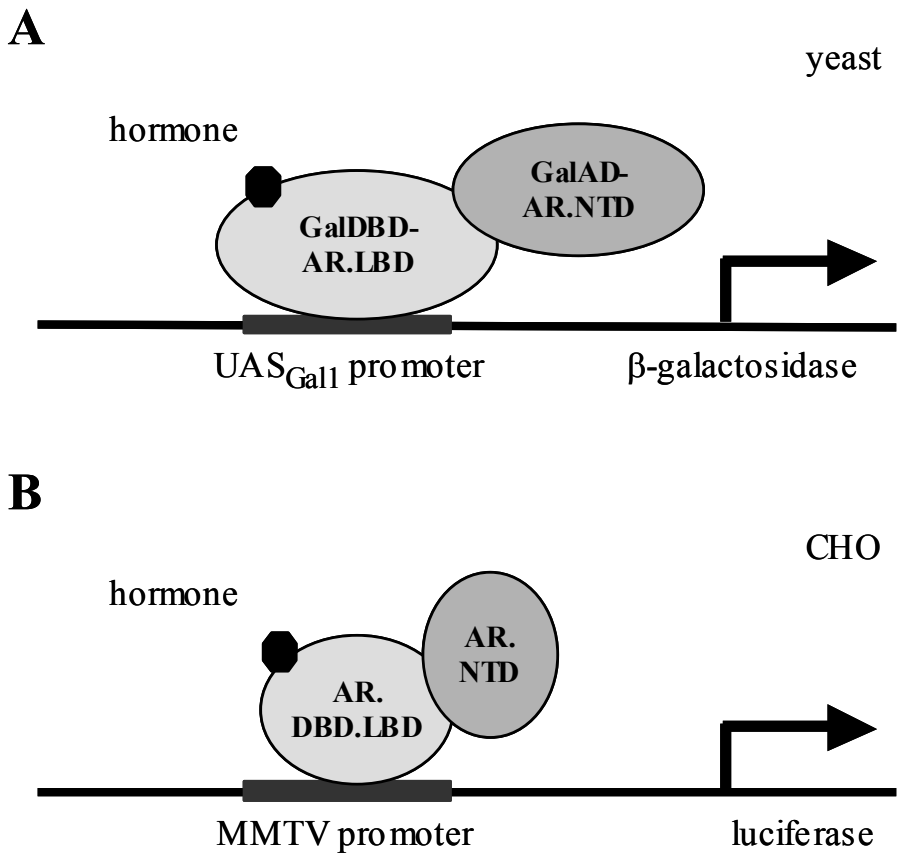

Figure 1. Schematic representation of *in vivo* protein interaction systems utilized in this study. (**A**) Yeast protein interaction (two-hybrid) system. DHT dependent interaction between GalAD-AR.NTD and GalDBD-AR.LBD induces expression of the UAS_{GAL1} regulated lacZ reporter gene. Cotransfection of pGBT9 and pACT2, which encode GalDBD and GalAD, respectively, does not induce reporter gene expression (data not shown). Similarly, individually expressed GalDBD-AR.LBD and GalAD-AR.NTD are not active in this assay. (**B**) Mammalian (CHO cells) protein interaction system. R1881 dependent interaction between AR.NTD and AR.DBD.LBD induces MMTV-promoter driven luciferase expression. Separately expressed AR.DBD.LBD and AR.NTD are unable to activate the MMTV promoter (data not shown).

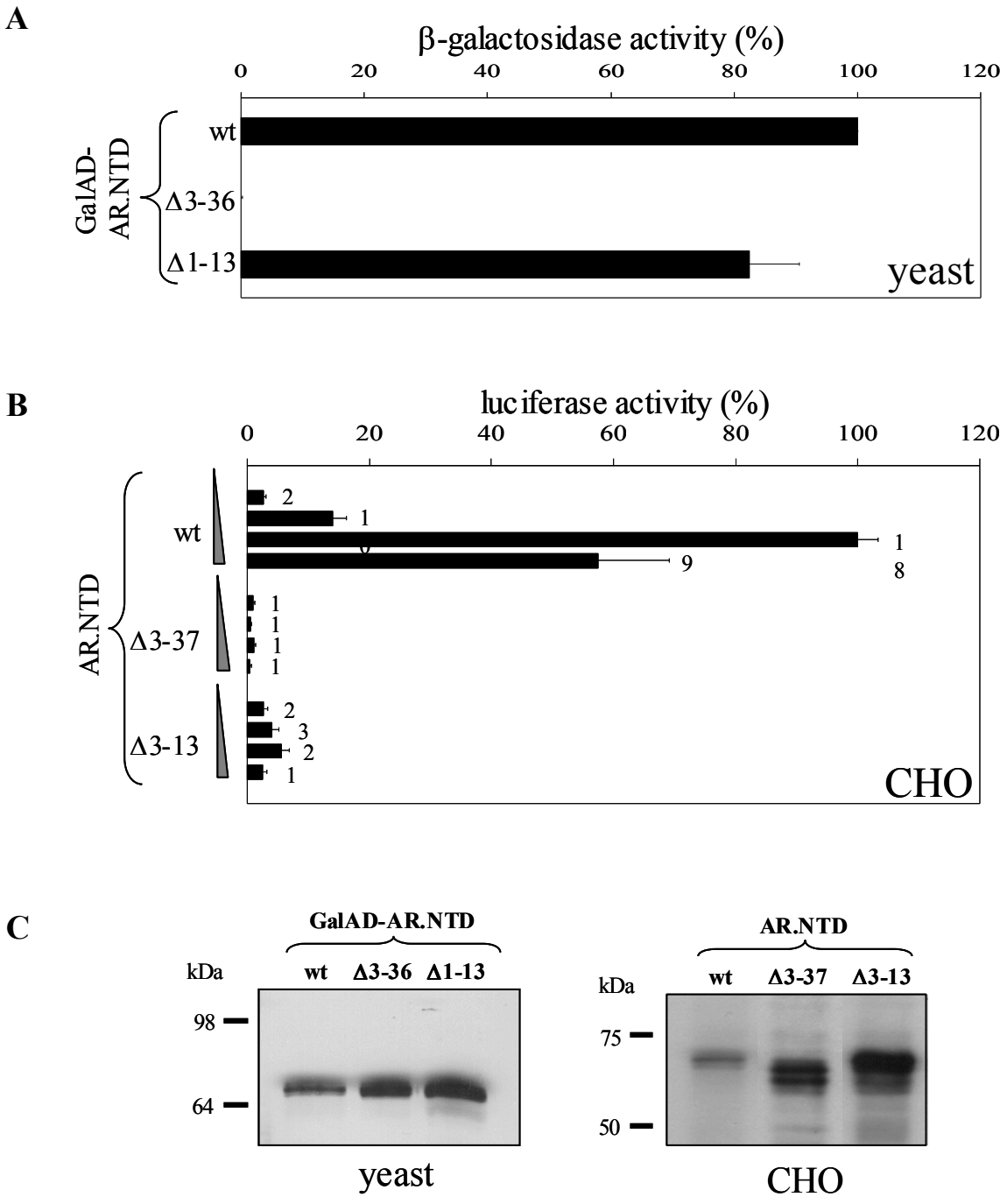

Figure 2. AR_{3-13} modulates androgen receptor N/C interaction.
(**A**) Interaction of AR.NTDwt and NH_2-terminal deletion mutants with AR.LBD in the presence of 1 μM DHT in the yeast protein interaction system. In each experiment the activity of GalAD-AR.NTDwt was set at 100%. Each bar represents the mean (±sem) β-galactosidase activity of three independent experiments. (**B**) Interaction of AR.NTDwt and deletion mutants with AR.LBD in the presence of 1 nM R1881 in the mammalian protein interaction system. pSVAR.DBD.LBD was cotransfected with increasing amounts of pSVAR.NTDwt or mutant (see Experimental Procedures). In each experiment, carried out in triplicate, the mean of the highest AR.NTDwt value was set at 100%. Each bar represents the mean (±sem) luciferase activity of three independent experiments. Fold induction is shown to the right of each bar and represents the ratio of activities determined in the presence and absence of R1881. (**C**) Western analysis of indicated GalAD-AR.NTD proteins in the yeast protein interaction system (left panel) and of indicated AR.NTD proteins in the mammalian protein interaction system (right panel). See Experimental Procedures for details.

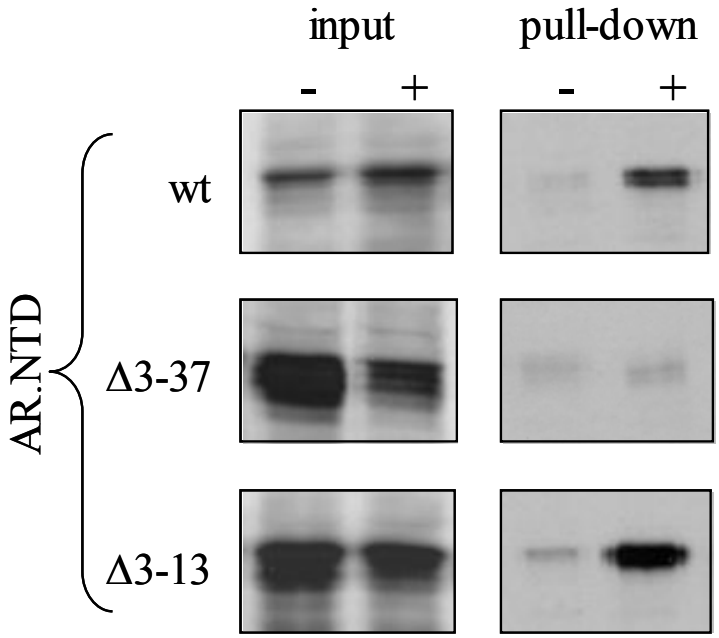

Figure 3. AR_{3-13} is not involved in direct binding of AR NTD to AR LBD.
Interaction of AR.NTDwt and NH_2-terminal deletion mutants with GST-AR.LBD as studied by pull down assays. Proteins were produced in CHO cells by cotransfection of pCMVAR.LBD and pSVAR.NTDwt or indicated deletion constructs. CHO cells were cultured in the absence (-) or presence (+) of 100 nM R1881. Input is 1/10 of the lysate utilized in a pull down experiment. See Experimental Procedures for details.

Analysis of ^{30}VREVI^{34} in androgen receptor N/C interaction

Prediction programs of protein secondary structures (see http://npsa-pbil.ibcp.fr) indicated a long α-helical structure for AR_{20-34}. A helical wheel drawing of this region predicted an amphipathic character of this helical structure (Figure 5A) [29]. At positions 15 and 37, the putative α-helix is flanked by proline residues. Within the helix, two candidate ΦxxΦΦ protein interaction motifs (Φ is any hydrophobic amino acid residue; x is any amino acid residue) are present: ^{30}VREVI^{34} and the previously identified ^{23}FQNLF^{27} motif (Figure 5B) [20,30,31]. To investigate whether like ^{23}FQNLF^{27}, ^{30}VREVI^{34} could contribute to N/C interaction, two constructs were generated, expressing either the complete ^{30}VREVI^{34} or the complete ^{23}FQNLF^{27} motif linked to GalAD (Figure 5B). As expected, in the yeast protein interaction system, ligand-dependent interaction with AR LBD could easily be detected for GalAD-AR_{17-32}. However, the interaction was weak for GalAD-AR_{24-39} (Figure 5C). Low activity was not due to decreased protein expression (Figure 5D).

In a complementary yeast protein interaction experiment, the ^{30}VREVI^{34} motif in GalAD-AR.NTDwt was modified by substitution of two hydrophobic amino acids by arginine residues, resulting in GalAD-AR.NTD30/33RR. These substitutions might cause sterical hindrance in the interaction with the AR LBD surface, change the charge and disrupt the proposed amphipathic α-helical structure of AR_{16-36}. GalAD-AR.NTD23/27RR was utilized as control. Substitution of V30 and V33 partially reduced the interaction, whereas the F23R,F27R mutation completely abolished the interaction (Figure 6A). Expression levels of GalAD-AR.NTDwt and GalAD-AR.NTD30/33RR were similar (Figure 6C).

Results obtained in the mammalian protein interaction system, utilizing the AR.NTD30/33RR mutant and AR.NTD23/27RR, were essentially identical to the observations made in the yeast

system (Figure 6B). A partial inhibition of AR N/C interaction was observed for AR.NTD30/33RR, and an almost complete inhibition for AR.NTD23/27RR.

Pull down experiments confirmed and extended the *in vivo* protein interaction experiments (Figure 6D). AR N/C interaction was diminished due to 30/33RR substitutions, and completely abolished by 23/27RR substitutions.

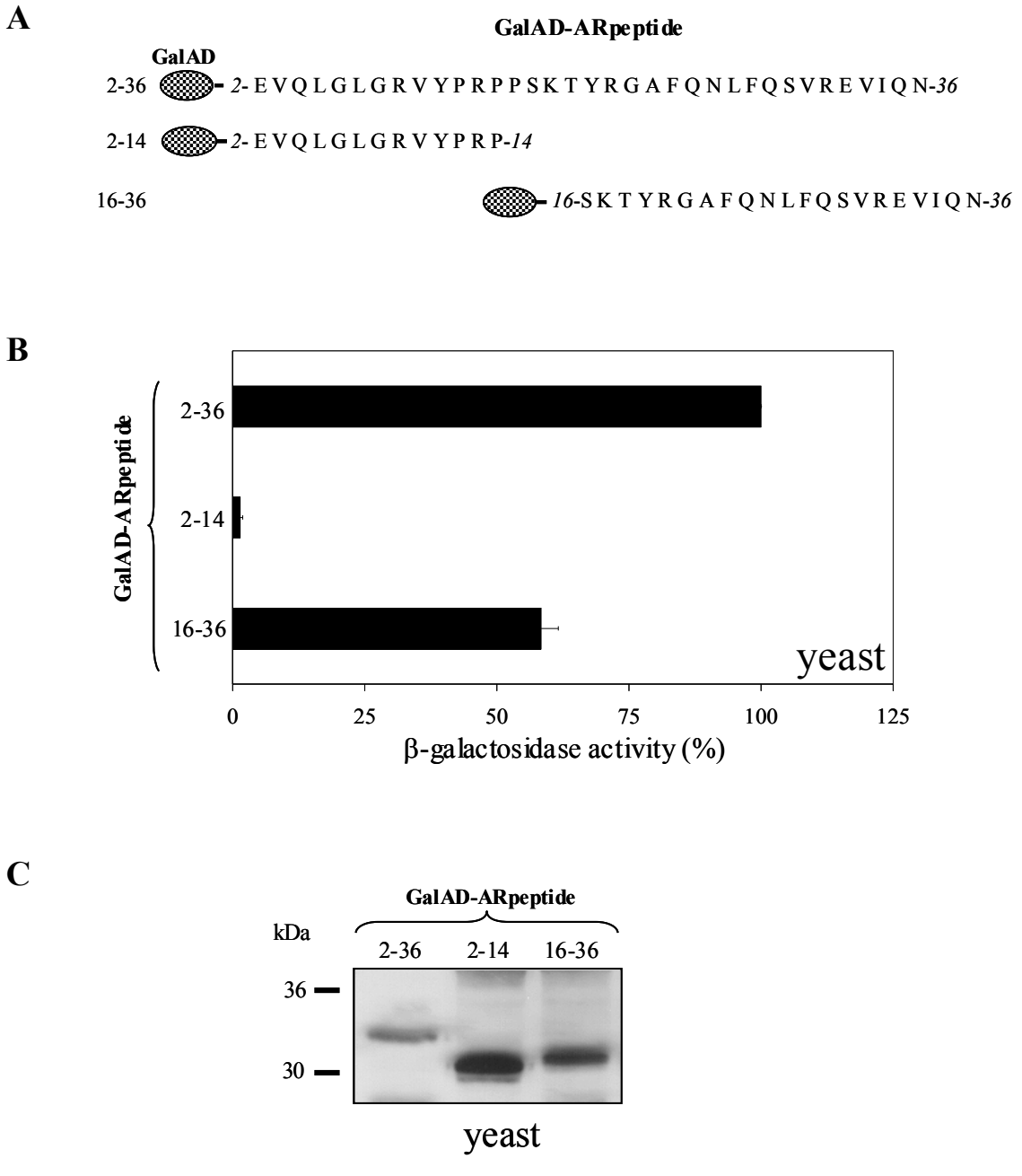

Figure 4. AR_{2-14} cannot autonomously interact with AR LBD.
(**A**) AR peptides utilized in GalAD-ARpeptide fusion proteins in the yeast protein interaction system. (**B**) Interaction of indicated GalAD-ARpeptides with GalDBD-AR.LBD in yeast in the presence of 1 μM DHT. In each experiment the activity of GalAD-AR_{2-36} was set at 100% (see also legend to Figure 2A). (**C**) Western analysis of indicated GalAD-ARpeptide proteins in yeast. For details, see Experimental Procedures.

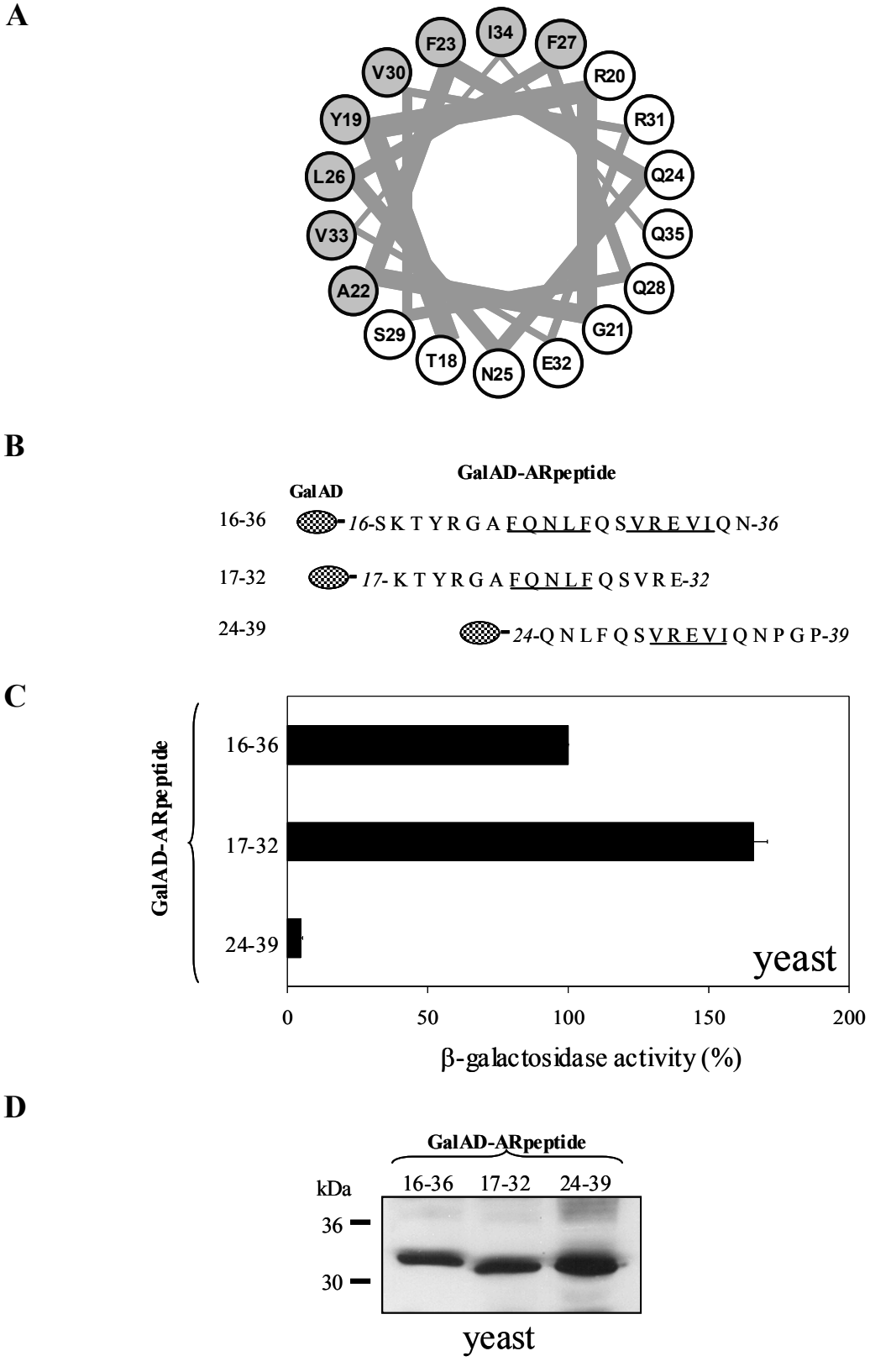

Figure 5. Analysis of a predicted long amphipathic α-helix of AR_{18-35} in AR N/C interaction.
(**A**) A helical wheel drawing of AR_{18-35} predicts a long amphipathic α-helical structure. Grey circles represent hydrophobic amino acids. (**B**)) GalAD-ARpeptide fusion proteins utilized in the yeast protein interaction system. The ΦxxΦΦ motifs ^{23}FQNLF^{27} and ^{30}VREVI^{34} are underlined. (**C**) Interaction of GalAD-ARpeptides with GalDBD-AR.LBD in yeast in the presence of 1 μM DHT. In each experiment the activity of GalAD-AR_{16-36} was set at 100% (see also legend to Figure 2A). (**D**) Western analysis of indicated GalAD-ARpeptide proteins in the yeast system. For details, see Experimental Procedures.

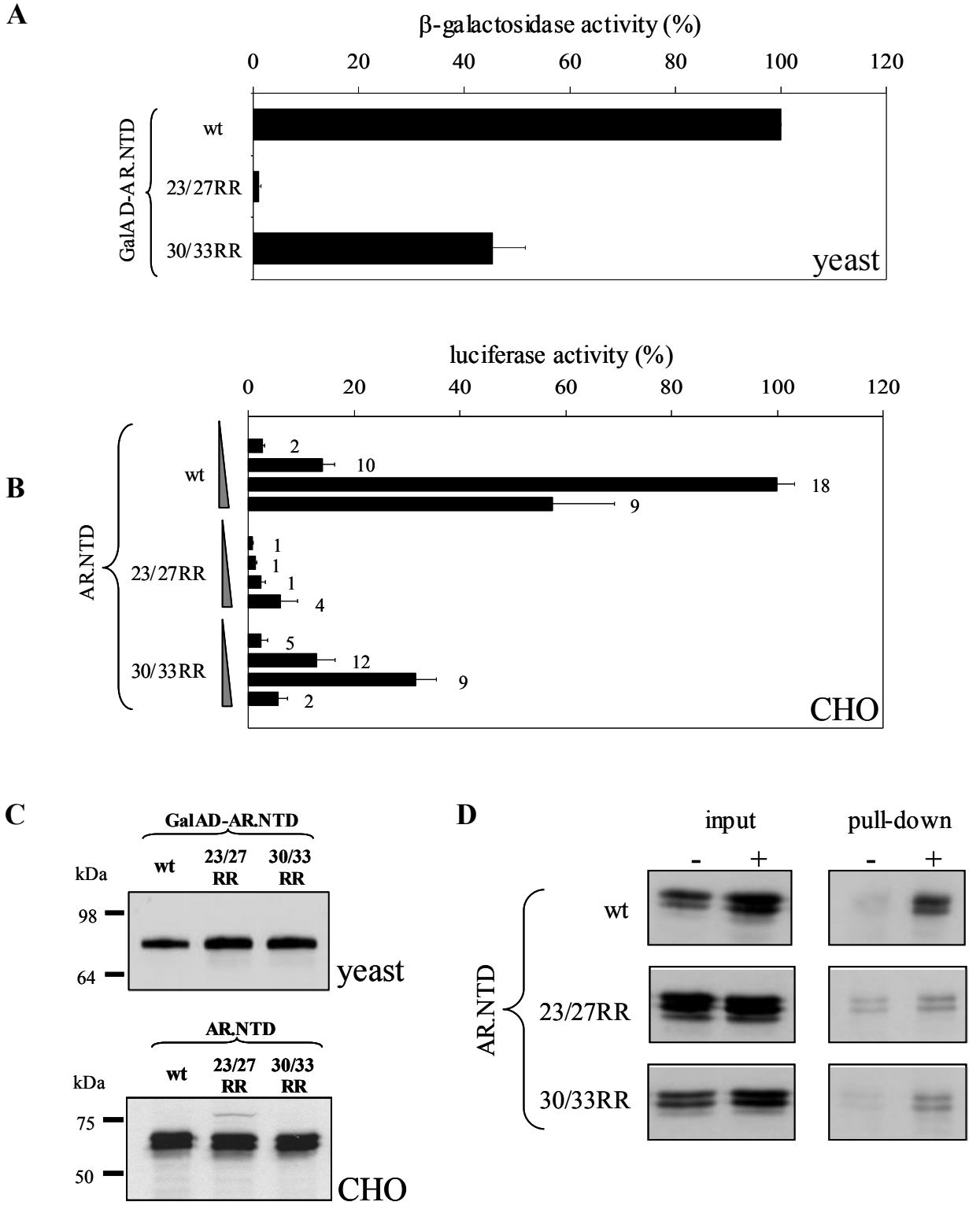

Figure 6. ^{30}VREVI^{34} is not essential for AR N/C interaction.

(**A**) Interaction of GalAD-AR.NTDwt and mutants with AR.LBD in the presence of 1 μM DHT in the yeast protein interaction system. In each experiment GalAD-AR.NTDwt activity was set at 100% (see legend to Figure 2A). (**B**) Interaction of AR.NTDwt and mutants with AR.LBD in the presence of 1 nM R1881 in the mammalian protein interaction system. pSVAR.DBD.LBD was cotransfected with increasing amounts of pSVAR.NTDwt or indicated mutants (see Experimental Procedures and legend to Figure 2B). (**C**) Western analysis of indicated GalAD-AR.NTD proteins in the yeast system (left panel) and indicated AR.NTD proteins in the mammalian system (right panel). See also Experimental Procedures. (**D**) Pull down assays showing interaction of AR.NTDwt and mutants with GST-AR.LBD (see also legend to Figure 3).

Amino acid residues flanking F23, L26 and F27 modulate androgen receptor N/C interaction

To study in more detail the role of 24/25QN in the ^{23}FQNLF^{27} motif in AR N/C interaction, these amino acids were substituted by 24/25AA. In both the yeast and mammalian protein interaction assay, GalAD-AR.NTD24/25AA and AR.NTD24/25AA formed even more active complexes with AR LBD than wild-type AR NTD (Figures 7A and 7B) (note the low expression levels of the 24/25AA mutants in both systems; Figure 7C). As expected, AR.NTD26/27AA was incapable to interact with AR.LBD.

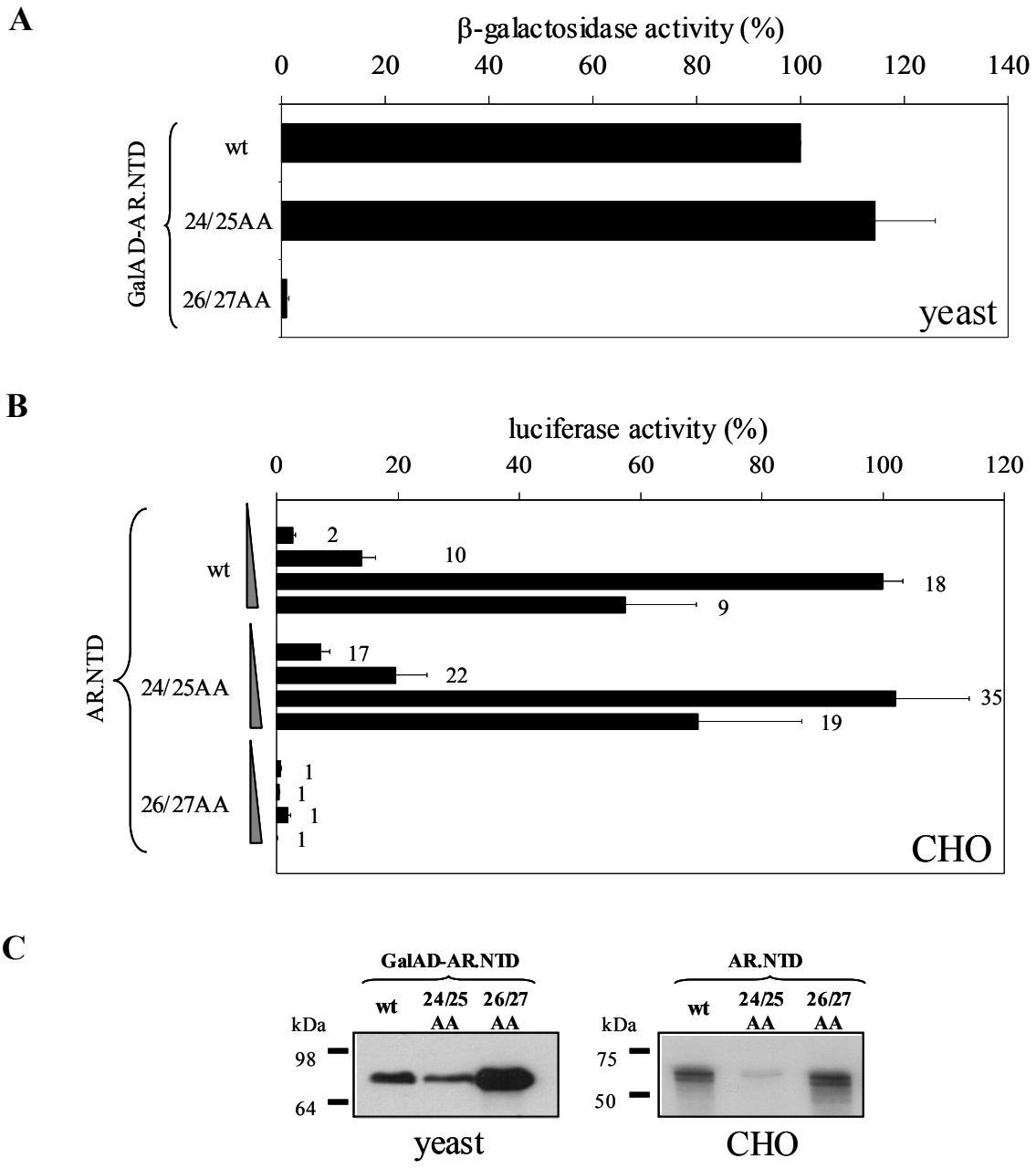

Figure 7. Alanine substitutions of Q24 and N25 stimulate AR N/C interaction.

(**A**) Interaction of GalAD-AR.NTDwt and mutants with GalDBD-AR.LBD in the presence of 1 μM DHT in the yeast protein interaction system. In each experiment GalAD-AR.NTDwt activity was set at 100%. See also legend to Figure 2A. (**B**) Interaction of AR.NTDwt and mutants with AR.LBD in the presence of 1 nM R1881 in the mammalian protein interaction system. pSVAR.DBD.LBD was cotransfected with increasing amounts of pSVAR.NTDwt or mutants (see Experimental Procedures and legend to Figure 2B). (**C**) Western analysis of indicated GalAD-AR.NTD proteins in the yeast protein system (left panel) and indicated AR.NTD proteins in the mammalian system (right panel). For details, see Experimental Procedures.

To extend these findings, an alanine scan was carried out for peptide GalAD-AR_{17-32} (Figure 8A). Results of the yeast protein interaction assay are shown in Figure 8B. Substitution of amino acids 23, 26 and 27 completely abolished interaction with GalDBD-AR.LBD and alanines at positions 24 and 25 increased the interaction capacity. All alanine substitutions of amino acids flanking ^{23}FQNLF^{27} reduced the binding to AR LBD. Most prominent inhibitory effects were found for amino acid residues directly flanking ^{23}FQNLF^{27}. Note that expression levels of the peptide constructs were similar (Figure 8C).

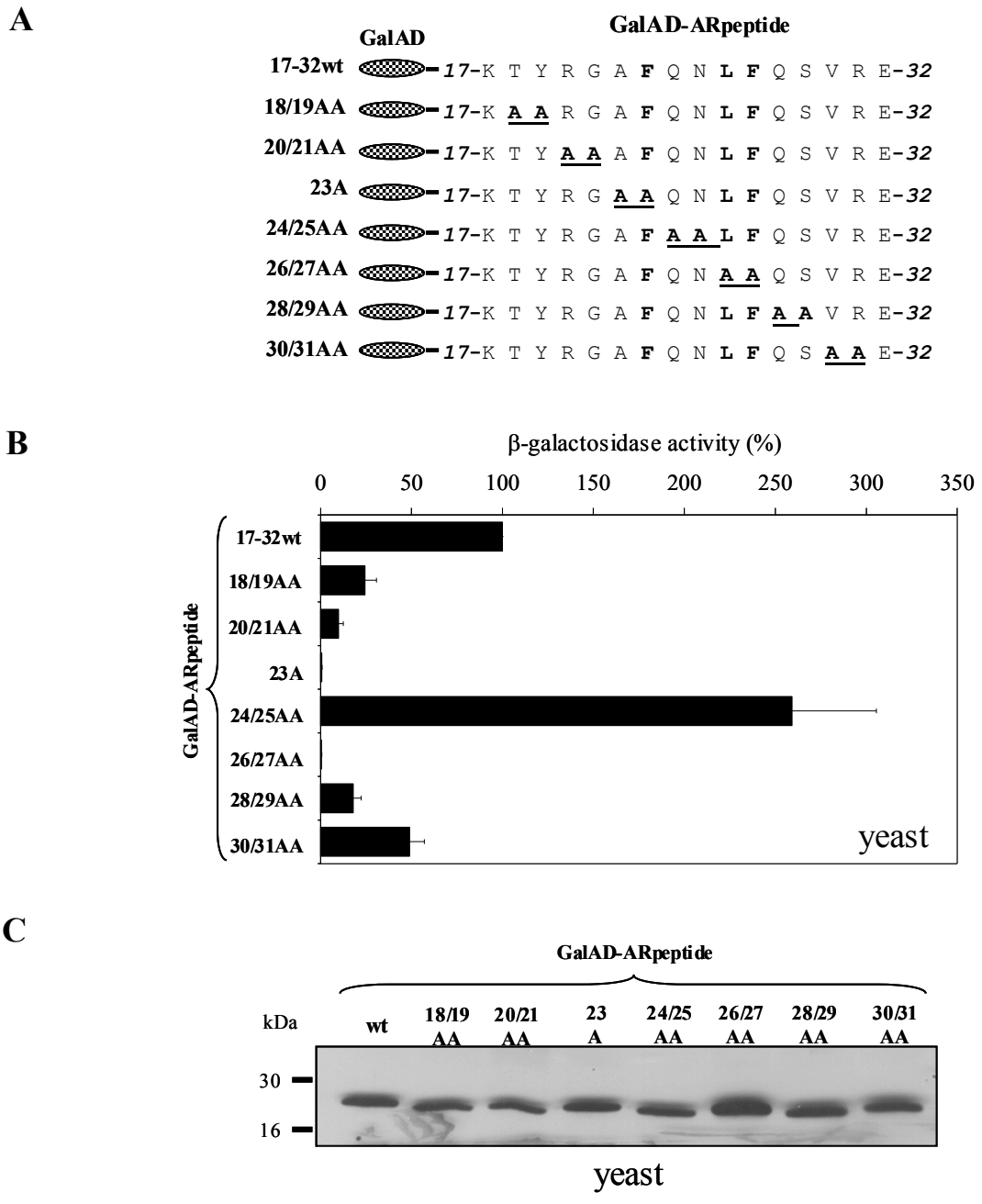

Figure 8. Alanine scanning of AR_{17-32}: amino acids flanking F23, L26 and F27 modulate AR N/C interaction.

(**A**) GalAD-ARpeptide fusion proteins in the yeast protein interaction system**.** (**B**) Interaction of GalAD-ARpeptides with AR.LBD in the presence of 1 μM DHT in the yeast protein interaction system. In each experiment the activity of GalAD-AR_{17-32} was set at 100%. See also legend to Figure 2A. (**C**) Western analysis of indicated GalAD-ARpeptide proteins in the yeast assay. For details, see Experimental Procedures.

DISCUSSION

Previously, we and others demonstrated a ligand-dependent functional interaction between AR NTD and AR LBD. Amino acids 3-36 in the NTD (AR_{3-36}), including the $^{23}FxxLF^{27}$ motif, play a pivotal role in N/C interaction [15,20]. Here we studied the function of the AR_{3-36} subdomain AR_{3-13} in N/C interaction and the role of individual amino acid residues in and flanking the $^{23}FQNLF^{27}$motif in AR_{16-36} in N/C interaction.

Yeast protein interaction assays indicated that AR_{3-13} contributed to the ligand-induced transactivation function of the AR.NTD/AR.LBD complex (Figures 2 and 4). Pull down experiments provided evidence that AR_{3-13} does not directly interact with AR LBD (Figure 3). On first sight, conflicting results were obtained in the yeast and mammalian protein interaction assays (Figure 2). In the yeast assay, reporter gene activity, which monitored the N/C interaction, was partly reduced by AR_{3-13} deletion, whereas in the mammalian assay almost all reporter gene activity was lost. The most obvious difference between both assays is the coupling of AR.NTD to GalAD in the yeast assay, and the absence of a second transactivation domain linked to AR NTD in the mammalian assay. The latter assay completely depends on the intrinsic transactivating function of AR NTD and thus does not allow discrimination between loss of AR.NTD-AR.LBD binding and loss of AR.NTD transactivating function. In the yeast assay, loss of transactivation function of AR NTD mutants, which retain AR LBD interacting capacity, like AR.NTDΔ3-13, will be masked by the GalAD transactivating function. So, AR_{3-13} is not essential, but rather modulates N/C interaction, most likely by affecting the transactivation function of AR.NTD. **A**lternative explanations might be induction of a more favorable NTD conformation or stabilization of the *in vivo* N/C interaction, which are not reflected in the pull down assays and peptide interaction experiments. Unfortunately, the primary structure and the predicted secondary structure of AR_{3-13} do not give a clue to a more precise description of its function (data not shown). However, the fact that, between species, AR_{3-13} is one of the most conserved regions of AR NTD, underscores a presumed important role in AR function [32].

The second domain that was studied, $AR_{16-36,}$ is essential in N/C interaction. The predicted structure indicated that AR_{16-36} can fold in a remarkably long amphipathic α-helical structure, suggesting an important protein interaction interface [29]. AR_{16-36} contains two ΦxxΦΦ putative protein interaction motifs: $^{23}FxxLF^{27}$, which was found to be pivotal for direct N/C interaction [20, this study], and $^{30}VxxVI^{34}$ (Figures 5 and 6). The latter sequence modulates N/C interaction. Amino acid residues in this sequence might contribute to the stability of the predicted α-helix. Alternatively, they might make additional contacts to the LBD surface. This is also true for other amino acid residues flanking the $^{23}FxxLF^{27}$ motif (Figure 8). Remarkably, substitution of Q24 and N25 by alanines increased N/C interaction (Figures 7 and 8).

The AR FxxLF motif shows similarities to LxxLL motifs [5,33,34] present in nuclear receptor interaction domains (NR boxes) of p160 coactivators. LxxLL motifs are essential in the interaction with LBDs [33]. They bind to a hydrophobic cleft in nuclear receptor LBDs, which is marked by a charged clamp composed of a highly conserved lysine and glutamate residue in helix 3 and helix 12 of the LBD, respectively (K720 and E897 in AR) [35-37]. AR K720 and E897 are both involved in the ligand-dependent interaction between AR LBD and the coactivator TIF2 [9,11,15]. However, in the FxxLF-mediated AR N/C interaction, E897 is essential, but K720 can be replaced by many other amino acids, without affecting N/C interaction [9,11,15,38]. So, the AR N/C interaction is comparable, but not identical to LxxLL-mediated coactivator-LBD interaction.

The 3D structures of agonist bound LBD/LxxLL peptide complexes of several nuclear receptors have been elucidated, and interactions of the peptide backbone and its amino acid side chains with the LBD surface have been identified [5,36,37,39]. It is presumed that upon binding to the LBD surface, the LxxLL motif adapts a short α-helical structure, which is stabilized by interaction with the charged clamp [5,36,37]. The first and last leucine residue in the LxxLL motif enter the hydrophobic cleft in the LBD, and directly contact amino acid residues within the cleft. The variable amino acids (xx) in the LxxLL motif point away from the cleft and seem not to interact directly with the LBD surface. Structural data for AR.LBD/LxxLL peptides are not available, but, because AR.LBD/coactivator interaction also depends on K720 and E897, it might be predicted that they will be similar to LBD/LxxLL peptide complexes studied so far [9,11,15]. Because K720 is not essential for $AR^{23}FxxLF^{27}$/AR.LBD interaction, the structure of this complex might be different. A different complex would also explain the stimulation of $AR^{23}FxxLF^{27}$/AR.LBD interaction by substitution of Q24 and N25 by alanine residues. Structural analyses of AR.LBD/AR_{16-36} complexes have to reveal the function of amino acid residues flanking F23, L26 and F27 and answer the question whether or not the entire long amphipathic AR_{16-36} α-helix is required for a stable AR NTD/LBD complex.

The LxxLL-like motifs LxxIL, FxxLL, and L/IxxI/VI, have been found in LBD binding coactivators or corepressors [40-43]. FxxLF motifs that are able to contact AR LBD, have only been found in AR NTD and most recently in the AR coactivators ARA54 and ARA70, suggesting a specific role of these motifs in AR function [44-47]. The increasing number of proteins found to interact with the AR LBD raises the question of the physiological relevance of the many interactions. It remains to be established whether all interactions take place in living cells under physiological conditions, whether interactions with different proteins are simultaneous or consecutive events, and which interactions are most stable and most specific. Recently, a start has been made to the identification of factors, including the AR, present in the transcription initiation complex of the prostate specific antigen enhancer/promoter, using chromatin immunoprecipitation (ChIP) [48].

Another question concerns the interaction of AR_{16-36} with other proteins. One candidate might be the TFIID TATA box-binding protein associated factor 31, $TAF_{II}31$, which has been found to interact with FxxΦΦ motifs in acidic transcription activation domains of p65 (NF-κB), VP16, p53 and related proteins [31,49-51].

AR NTD has previously been proposed to accommodate more than one AR LBD interacting domain [9,15,20]. A candidate second domain is $^{433}WHTLF^{437}$, which was found to modulate $^{23}FxxLF^{27}$ function [20]. Another candidate motif is $^{179}LxxIL^{183}$ [9]. However, peptides containing these motifs were unable to interact with AR LBD in the yeast protein interaction assay, excluding their role as a second autonomous interaction motif in AR NTD (data not shown).

N/C interaction is not unique for the AR, but has also been described for other nuclear receptors. ERα ligand-dependent direct N/C interaction has been demonstrated, which was disrupted by amino acid substitutions that affect receptor function [52,53]. The ERα N/C interaction could be induced by the agonist estradiol (E_2), but not by the antagonist ICI164,384 [53]. Recently, it was found that the ERα N/C interaction was required for SRC-1 mediated synergism between AF-1 and AF-2 function [8,53]. The progesterone receptor (PR) showed direct N/C interaction in the presence of agonist R5020, but not in the presence of antagonist RU486 [54]. LxxLL motifs in the PR-B form were most likely not involved, because the shorter PR-A form, lacking these motifs, also showed N/C interaction [55].

The role of N/C interaction in full-length AR function is not well understood. Ligand-dependent AR N/C interaction affects ligand dissociation [11,20,56]. Whether this is a direct or an indirect effect is unknown. Disruption of the N/C interaction by mutation of the ^{23}FxxLF^{27} motif has a limited effect on full length AR transactivation function [20, Steketee unpublished]. However, several AR LBD mutants with reduced or completely abolished N/C interaction have been found in androgen insensitivity patients [11,56,57]. Additionally, both N/C interaction and transactivating function of the AR prostate cancer mutant T877A can be induced by natural low affinity ligands like progesterone or E_2 or the AR antagonist cyproterone acetate [18].

In conclusion, we propose that AR_{3-36} is involved in a dynamic sequence of protein interaction events, including N/C interaction, in regulation of AR function. Detailed knowledge on the role of the AR N/C interaction would require the elucidation of its function under more physiological conditions, including the study of mouse models carrying AR mutants defective in N/C interaction.

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Abbreviations: AF, transactivation function; AR, androgen receptor; DBD, DNA-binding domain; DHT, dihydrotestosterone; E_2, estradiol; ERα, estrogen receptor α; GalAD, Gal4 transactivating domain; GAlDBD, Gal4 DNA-binding domain; LBD, ligand-binding domain; N/C interaction, interaction between NTD and LBD; NR, nuclear receptor; NTD, NH_2-terminal domain; PR, progesterone receptor; R1881, methyltrienolone.

REFERENCES

1. Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M. & Chambon, P. (1995) The nuclear receptor superfamily: the second decade. *Cell* **83**, 835-839.
2. McKenna, N.J., Lanz, R.B. & O'Malley, B.W. (1999) Nuclear receptor coregulators: cellular and molecular biology. *Endocr. Rev.* **20**, 321-344.
3. Glass, C.K. & Rosenfeld, M.G. (2000) The coregulator exchange in transcriptional functions of nuclear receptors. *Genes Dev.* **14**, 121-141.
4. Lee, K.C. & Lee Kraus, W. (2001) Nuclear receptors, coactivators and chromatin: new approaches, new insights. *Trends Endocrinol. Metab.* **12**, 191-197.
5. 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.
6. Onate, S.A., Boonyaratanakornkit, V., Spencer, T.E., Tsai, S.Y., Tsai, M.J., Edwards, D.P. & O'Malley, B.W. (1998) The steroid receptor coactivator-1 contains multiple receptor interacting and activation domains that cooperatively enhance the activation function 1 (AF1) and AF2 domains of steroid receptors. *J. Biol. Chem.* **273**, 12101-12108.
7. Webb, P., Nguyen, P., Shinsako, J., Anderson, C., Feng, W., Nguyen, M.P., Chen, D., Huang, S.M., Subramanian, S., McKinerney, E., Katzenellenbogen, B.S., Stallcup, M.R. & Kushner, P.J. (1998) Estrogen receptor activation function 1 works by binding p160 coactivator proteins. *Mol. Endocrinol.* **12**, 1605-1618.
8. Benecke, A., Chambon, P. & Gronemeyer, H. (2000) Synergy between estrogen receptor alpha activation functions AF1 and AF2 mediated by transcription intermediary factor TIF2. *EMBO Rep.* **1**, 151-157.
9. Alen, P., Claessens, F., Verhoeven, G., Rombauts, W. & Peeters, B. (1999) The androgen receptor amino-terminal domain plays a key role in p160 coactivator-stimulated gene transcription. *Mol. Cell. Biol.* **19**, 6085-6097.
10. 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
11. 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.
12. 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.
13. Simental, J.A., Sar, M., Lane, M.V., French, F.S. & Wilson, E.M. (1991) Transcriptional activation and nuclear targeting signals of the human androgen receptor. *J. Biol. Chem.* **266**, 510-518.
14. Palvimo, J.J., Kallio, P.J., Ikonen, T., Mehto, M. & Janne, O.A. (1993) Dominant negative regulation of trans-activation by the rat androgen receptor: roles of the N-terminal domain and heterodimer formation. *Mol. Endocrinol.* **7**, 1399-1407.
15. 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.
16. Gottlieb, B., Beitel, L.K. & Trifiro, M.A. (2001) Variable expressivity and mutation databases: The androgen receptor gene mutations database. *Hum. Mutat.* **17**, 382-388.
17. Langley, E., Zhou, Z.X. & Wilson, E.M. (1995) Evidence for an anti-parallel orientation of the ligand-activated human androgen receptor dimer. *J. Biol. Chem.* **270**, 29983-29990.
18. Doesburg, P., Kuil, C.W., Berrevoets, C.A., Steketee, K., Faber, P.W., Mulder, E., Brinkmann, A.O. & Trapman, J. (1997) Functional in vivo interaction between the amino-terminal, transactivation domain and the ligand binding domain of the androgen receptor. *Biochemistry* **36**, 1052-1064.
19. Ikonen, T., Palvimo, J.J. & Janne, O.A. (1997) Interaction between the amino- and carboxyl-terminal regions of the rat androgen receptor modulates transcriptional activity and is influenced by nuclear receptor coactivators. *J. Biol. Chem.* **272**, 29821-29828.
20. 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.
21. Sambrook, J. & Russell, D.W. (2001) *Molecular Cloning a laboratory manual.* Third edition Cold Spring Harbor Laboratory Press Cold Spring Harbor, NY
22. Brinkmann, A.O., Faber, P.W., van Rooij, H.C., Kuiper, G.G., Ris, C., Klaassen, P., van der Korput, J.A., Voorhorst, M.M., van Laar, J.H. & Mulder, E. (1989) The human androgen receptor: domain structure, genomic organization and regulation of expression. *J. Steroid. Biochem.* **34**, 307-310
23. 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.
24. de Ruiter, P.E., Teuwen, R., Trapman, J., Dijkema, R. & Brinkmann, A.O. (1995) Synergism between androgens and protein kinase-C on androgen-regulated gene expression. *Mol. Cell. Endocrinol.* **110**, R1-6.
25. Tsai, R.Y. & Reed, R.R. (1997) Using a eukaryotic GST fusion vector for proteins difficult to express in E. coli. *Biotechniques* **23**, 794-796, 798, 800.

26. Gietz, D., St Jean, A., Woods, R.A. & Schiestl, R.H. (1992) Improved method for high efficiency transformation of intact yeast cells. *Nucleic. Acids. Res.* **20**, 1425.
27. Kuil, C.W., Berrevoets, C.A. & Mulder, E. (1995) Ligand-induced conformational alterations of the androgen receptor analyzed by limited trypsinization. Studies on the mechanism of antiandrogen action. *J. Biol. Chem.* **270**, 27569-27576.
28. Zegers, N.D., Claassen, E., Neelen, C., Mulder, E., van Laar, J.H., Voorhorst, M.M., Berrevoets, C.A., Brinkmann, A.O., van der Kwast, T.H. & Ruizeveld de Winter, J.A. (1991) Epitope prediction and confirmation for the human androgen receptor: generation of monoclonal antibodies for multi-assay performance following the synthetic peptide strategy. *Biochim. Biophys. Acta.* **1073**, 23-32.
29. Segrest, J.P., De Loof, H., Dohlman, J.G., Brouillette, C.G. & Anantharamaiah, G.M. (1990) Amphipathic helix motif: classes and properties. *Proteins* **8**, 103-117
30. 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.
31. Uesugi, M. & Verdine, G.L. (1999) The alpha-helical FXXPhiPhi motif in p53: TAF interaction and discrimination by MDM2. *Proc. Natl. Acad. Sci. U S A* **96**, 14801-14806.
32. Thornton, J.W. & Kelley, D.B. (1998) Evolution of the androgen receptor: structure-function implications. *Bioessays* **20**, 860-869.
33. 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.
34. 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
35. 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.
36. Nolte, R.T., Wisely, G.B., Westin, S., Cobb, J.E., Lambert, M.H., Kurokawa, R., Rosenfeld, M.G., Willson, T.M., Glass, C.K. & Milburn, M.V. (1998) Ligand binding and co-activator assembly of the peroxisome proliferator- activated receptor-gamma. *Nature* **395**, 137-143.
37. 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.
38. Slagsvold, T., Kraus, I., Bentzen, T., Palvimo, J. & Saatcioglu, F. (2000) Mutational analysis of the androgen receptor AF-2 (activation function 2) core domain reveals functional and mechanistic differences of conserved residues compared with other nuclear receptors. *Mol. Endocrinol.* **14**, 1603-1617.
39. Gampe, R.T., Jr., Montana, V.G., Lambert, M.H., Miller, A.B., Bledsoe, R.K., Milburn, M.V., Kliewer, S.A., Willson, T.M. & Xu, H.E. (2000) Asymmetry in the PPARgamma/RXRalpha crystal structure reveals the molecular basis of heterodimerization among nuclear receptors. *Mol. Cell.* **5**, 545-555.
40. Huang, N., vom Baur, E., Garnier, J.M., Lerouge, T., Vonesch, J.L., Lutz, Y., Chambon, P. & Losson, R. (1998) Two distinct nuclear receptor interaction domains in NSD1, a novel SET protein that exhibits characteristics of both corepressors and coactivators. *EMBO J.* **17**, 3398-3412.
41. Li, D., Desai-Yajnik, V., Lo, E., Schapira, M., Abagyan, R. & Samuels, H.H. (1999) NRIF3 is a novel coactivator mediating functional specificity of nuclear hormone receptors. *Mol. Cell. Biol.* **19**, 7191-7202.
42. Johansson, L., Bavner, A., Thomsen, J.S., Farnegardh, M., Gustafsson, J.A. & Treuter, E. (2000) The orphan nuclear receptor SHP utilizes conserved LXXLL-related motifs for interactions with ligand-activated estrogen receptors. *Mol. Cell. Biol.* **20**, 1124-1133.
43. Gobinet, J., Auzou, G., Nicolas, J.C., Sultan, C. & Jalaguier, S. (2001) Characterization of the interaction between androgen receptor and a new transcriptional inhibitor, SHP. *Biochemistry* **40**, 15369-15377.
44. Yeh, S. & Chang, C. (1996) Cloning and characterization of a specific coactivator, ARA70, for the androgen receptor in human prostate cells. *Proc. Nat.l Acad. Sci. U S A* **93**, 5517-5521.
45. Kang, H.Y., Yeh, S., Fujimoto, N. & Chang, C. (1999) Cloning and characterization of human prostate coactivator ARA54, a novel protein that associates with the androgen receptor. *J. Biol. Chem.* **274**, 8570-8576.
46. 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.
47. 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
48. Shang, Y., Myers, M. & Brown, M. (2002) Formation of the androgen receptor transcription complex. *Mol. Cell.* **9**, 601-610.
49. Burley, S.K. & Roeder, R.G. (1996) Biochemistry and structural biology of transcription factor IID (TFIID). *Annu. Rev. Biochem.* **65**, 769-799
50. Uesugi, M., Nyanguile, O., Lu, H., Levine, A.J. & Verdine, G.L. (1997) Induced alpha helix in the VP16 activation domain upon binding to a human TAF. *Science* **277**, 1310-1313.
51. Yoon, J.W., Liu, C.Z., Yang, J.T., Swart, R., Iannaccone, P. & Walterhouse, D. (1998) GLI activates transcription through a herpes simplex viral protein 16- like activation domain. *J. Biol. Chem.* **273**, 3496-3501.
52. Kraus, W.L., McInerney, E.M. & Katzenellenbogen, B.S. (1995) Ligand-dependent, transcriptionally productive association of the amino- and carboxyl-terminal regions of a steroid hormone nuclear receptor. *Proc. Natl. Acad. Sci. U S A* **92**, 12314-12318.

53. Metivier, R., Penot, G., Flouriot, G. & Pakdel, F. (2001) Synergism between ERalpha transactivation function 1 (AF-1) and AF-2 mediated by steroid receptor coactivator protein-1: requirement for the AF-1 alpha-helical core and for a direct interaction between the N- and C-terminal domains. *Mol. Endocrinol.* **15**, 1953-1970.
54. Tetel, M.J., Giangrande, P.H., Leonhardt, S.A., McDonnell, D.P. & Edwards, D.P. (1999) Hormone-dependent interaction between the amino- and carboxyl-terminal domains of progesterone receptor in vitro and in vivo. *Mol. Endocrinol.* **13**, 910-924.
55. Tung, L., Shen, T., Abel, M.G., Powell, R.L., Takimoto, G.S., Sartorius, C.A. & Horwitz, K.B. (2001) Mapping the unique activation function 3 in the progesterone B-receptor upstream segment. Two LXXLL motifs and a tryptophan residue are required for activity. *J. Biol. Chem.* **276**, 39843-39851.
56. Langley, E., Kemppainen, J.A. & Wilson, E.M. (1998) Intermolecular NH2-/carboxyl-terminal interactions in androgen receptor dimerization revealed by mutations that cause androgen insensitivity. *J. Biol. Chem.* **273**, 92-101.
57. 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.

Chapter 3

A bioinformatics-based functional analysis shows that the specifically androgen-regulated gene *SARG* contains an active direct repeat androgen response element in the first intron.

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ABSTRACT

We characterized the specifically androgen-regulated gene *SARG*, which is expressed in the androgen receptor and glucocorticoid receptor positive cell line LNCaP-1F5. SARG mRNA expression can be up-regulated by androgens, but not by glucocorticoids. SARG mRNA expression is high in prostate tissue. *SARG* is composed of 4 exons and spans a region of 14.5 kbp on chromosome 1q32.2. Transcripts of 5.5, 3.3 and 2.3 kb are the result of alternative polyadenylation. SARG mRNA splice variants lack exon 2 and vary in length of exon 1. The SARG protein has a length of 601 amino acids and is located in the cytoplasm. By screening 18 kbp genomic sequence flanking the transcription start site we identified the imperfect direct repeat 5'-TGTGCTaacTGTTCT-3' in intron 1 as an active androgen response element (ARE-SARG+4.6). A 569 bp genomic DNA fragment, containing this element functioned as an androgen-specific enhancer in transiently transfected LNCaP-1F5 cells. ARE-SARG+4.6 cooperated with flanking sequences for optimal activity. Inactivation of ARE-SARG+4.6 completely abolished the androgen response of the enhancer. ChIP experiments showed chromatin structural changes of the enhancer in the presence of R1881. ARE-SARG+4.6 was able to bind to the androgen receptor, but not to the glucocorticoid receptor, correlating with its androgen-specific activity in transfections.

INTRODUCTION

Androgens are essential in the development and maintenance of the male phenotype. They mediate their function by activation of the androgen receptor (AR), which is a member of the nuclear receptor family of ligand-activated transcription factors. Nuclear receptors have a modular structure composed of a moderately conserved carboxyl-terminal ligand-binding domain (LBD), a highly conserved central DNA-binding domain (DBD) and a non-conserved amino-terminal domain (NTD). Most ligand-activated nuclear receptors bind as homodimers or heterodimers to hormone response elements (HREs) in the regulatory regions of their target genes. HREs are composed of an inverted or direct repeat of two 6 bp half-sites separated by a spacer of variable size (Khorasanizadeh and Rastinejad 2001). Together with coactivators, chromatin remodelling complexes, general transcription factors and RNA polymerase II, nuclear receptors initiate the transcription of target genes in a tightly controlled fashion (Glass and Rosenfeld 2000, Lee and Lee Kraus 2001, McKenna and O'Malley 2002).

An important class of nuclear receptors is the family of steroid hormone receptors, which is composed of AR, glucocorticoid receptor (GR), mineralocorticoid receptor (MR), progesterone receptor (PR) and estrogen receptors alpha and beta (ERα and ERβ) (Thornton 2001). Steroid hormone receptors display distinct physiological functions, reflected in their tissue-specific expression pattern and to some extent in their spectrum of target genes. However, AR, GR, MR and PR all bind with high affinity to the same inverted repeat consensus sequence 5'-AGAACAnnnTGTTCT-3' (Nordeen *et al.* 1990, Roche *et al.* 1992, Lieberman *et al.* 1993, Lombes *et al.* 1993). As a result, the activity of several promoters can be regulated by more than one of these receptors. Examples are the MMTV promoter, and the promoters of the *C3*, the *CRP* and the *PSA* gene (Ham *et al.* 1988, Claessens *et al.* 1989, De Vos *et al.* 1994, Cleutjens *et al.* 1997, Devos *et al.* 1997). The consensus high affinity binding site of ERα and ERβ is slightly different, 5'-AGGTCAnnnTGACCT-3' (Klein-Hitpass *et al.* 1989). Therefore, ERα and ERβ direct the expression of a different panel of target genes. Because GR, MR, PR and AR recognize

the same DNA sequence, it has been postulated that additional mechanisms are necessary to explain their specificity. These include differences in expression levels of the various receptors in specific cell types (Strahle *et al.* 1989), selective interaction with specific transcription factors, coactivators and corepressors, and ligand availability (Glass and Rosenfeld 2000, Aranda and Pascual 2001, Heinlein and Chang 2002).

In spite of the identical high-affinity recognition sequence for AR, PR, GR and MR, steroid response elements can also direct receptor specificity. In natural promoters steroid receptor binding sites can deviate considerably from the consensus high-affinity binding site. These sequences might have a different affinity to the various receptors. Additionally, sequences directly flanking the response element can contribute to receptor affinity and preference (Nelson *et al.* 1999, Haelens *et al.* 2003). On top of this, the AR seems to have adopted an exclusive mechanism of specificity. A few genes are known to be preferentially regulated by AR (Claessens *et al.* 2001). The structures of the androgen response elements (AREs) that direct androgen-specificity to these genes more resemble direct repeats of the sequence 5'-TGTTCT-3' than classic inverted repeats of this sequence.

The androgen-sensitive human prostate carcinoma cell line LNCaP expresses AR, but lacks GR and PR (Horoszewicz *et al.* 1983, Berns *et al.* 1986). It was previously shown that growth of LNCaP cells, and PSA mRNA expression in these cells can be stimulated by androgens (Schuurmans *et al.* 1988, Riegman *et al.* 1991, Young *et al.* 1991). In order to compare directly the molecular and biological function of AR and GR, the LNCaP-1F5 subline, containing a stably integrated GR expression vector, was generated (Cleutjens *et al.* 1997). PSA mRNA expression in LNCaP-1F5 can be induced by both androgens and glucocorticoids, but cell growth is selectively induced by androgens. We identified in LNCaP-1F5 cells a novel gene that is specifically regulated by androgens (Cleutjens *et al.* 1997). In the present study an integrated experimental and bioinformatics-based approach was applied to characterize the gene, designated *SARG*, and to decipher the molecular mechanism of androgen-specific regulation of the gene.

MATERIALS AND METHODS

Materials

Methyltrienolone (R1881) was purchased from NEN (Boston, MA), dexamethasone (Dex) was obtained from Steraloids (Wilton, NH). Cell culture media were from Bio Whittaker (Verviers, Belgium), fetal calf serum (FCS) was from Roche Diagnostics (Almere, The Netherlands).

Plasmid construction

pLUC and pPSA-4-LUC have been described previously (Cleutjens *et al.* 1996). pHisXpress-cSARG, expressing $(His)_6$-Xpress-SARG protein, contains the SARG cDNA fragment 209-2579 (SARG ORF is from 251 to 2053) inserted in the eukaryotic expression vector pcDNA3.1His (Invitrogen, Carlsbad, CA).

The *SARG* genomic fragments SARG-8.5, SARG-7.3 and SARG+4.6, with sizes of 510 bp, 476 bp and 569 bp, respectively, were obtained by PCR on PAC90L18 DNA (GenomeSystems, St Louis, MO) as template with the primer sets:

-8.5F: 5'-GATCAGCTGGATCCCAGGGACATGGATGAAGCTG-3'
-8.5R: 5'-GATCAGCTGGATCCTGCCTCAACCTCCCAAGTAG-3'
-7.3F: 5'-GATCAGCTGGATCCGTCATAATGACTTGGCCATG-3'
-7.3R: 5'-GATCAGCTGGATCCTGTCCAACATTTGAGGCCAG-3'

+4.6F: 5'-GATCAGCTGGATCCGTATCGTAGCGGTGGTTGTG-3'
+4.6R: 5'-GATCAGCTGGATCCTGGAGAGGCAGTCTAGTCAG-3'
The resulting amplified fragments were inserted in pGEM-T Easy (Promega, Madison, WI), sequenced and subsequently inserted as *Bam*HI/*Bam*H1 fragments in pPSA-4-LUC, yielding pSARG-8.5-PSA-LUC, pSARG-7.3-PSA-LUC and pSARG+4.6-PSA-LUC, respectively.

The -55 to +168 genomic fragment SARG-S was obtained by PCR on PAC90L18 DNA, utilizing the primers SARG-A: 5'-GCTAAGAGGGAACAGCACCAC-3' and SARG-B: 5'-CCCGGGAGATCTACTAGTCCACTGGGTTG-3'. The PCR product was inserted in pGEM-T Easy, verified by sequencing and inserted as a 240 bp *Pvu*II/*Bgl*II fragment in pLUC, yielding pSARG-S-LUC. To generate pSARG-L-LUC, the –3012 to -1559 *Hind*III/*Pvu*II *SARG* genomic fragment was isolated from PAC90L18 and inserted in the corresponding sites of pSARG-S-LUC, yielding pSARG-LΔ-LUC. Subsequently, the -1559 to –55 *Pvu*II/*Pvu*II genomic *SARG* fragment was inserted into the *Pvu*II site of pSARG-LΔ-LUC. The resulting construct pSARG-L-LUC contains SARG bp -3012 to +168.

SARG+4.6 was inserted as a *Bam*HI/*Bam*H1 fragment upstream of the SARG-S promoter or SARG-L promoter in pSARG-S-LUC and pSARG-L-LUC, respectively, yielding pSARG+4.6-SARG-S-LUC and pSARG+4.6-SARG-L-LUC. pSARG+4.6m-SARG-S-LUC was generated by mutagenesis utilizing the QuikChange Site Directed Mutagenesis Kit (Stratagene, LaJolla, CA) on pSARG+4.6-SARG-S-LUC as template. Primers: mut-4603S: 5'-CAACTAAACTATGATAACTATTATCTCATTTAATC-3' and its complementary strand: mut-4603AS.

SARG+4.6-(+4447/+4659)-S-LUC and SARG+4.6-(+4548/4659)-S-LUC were constructed by insertion of the respective *Bam*H1/*Bam*H1 fragments upstream of the S promoter in pSARG-S-LUC. The fragments SARG+4.6-(+4447/+4659) and SARG+4.6(+4548/+4659) were generated by PCR utilizing the primers

S+4.6-A (5'-GATCAGCTGGATCCCCTTCTTTTCTGAGATCCTG-3') and

S+4.6-B (5'-GATCAGCTGGATCCCTCATGAGGTCTTAGGGTAT-3') as respective forward primers, and S+4.6-C (5'-GATCAGATGGATCCGGCAAATTACTCTGAGTCTG-3') as reverse primer. Amplified fragments were sequenced prior to insertion into pSARG-S-LUC as *Bam*H1/*Bam*H1 fragments.

pRIT2TAR, encoding rat AR DBD, was described previously (De Vos *et al.* 1991). prGR-DBD-PRIT2T, encoding rat GR DBD, was constructed by *Bam*HI/*Sal*I insertion of a PCR fragment, synthesized with primers rGR-DBD-1: 5'-CAGCGGATCCGCAGCCACGGGACCACCTCCC-3' and rGR-DBD-2: 5'-CTATTGTCGACTAAGGATTTTCCGAAGTGTCTTG-3' on pSTC-GR3-795 (Rusconi and Yamamoto 1987) as template, in pRIT2T (Amersham Biosciences, Bucks, UK).

Screening of a prostate cDNA library

Screening of a λgt10 human prostate cDNA library (BD Biosciences Clontech, Palo Alto, CA) was performed according to the manufacturer's protocol. Hybridization probes were the SARG differential display PCR (DD-PCR) fragment (GenBank Accession Number AF007835) and SARG cDNA fragment 855-1957 (see GenBank Accession Number AY352640) .

RACE-PCR

For RACE-PCR we applied the Marathon-Ready prostate cDNA cloning kit (BD Biosciences Clontech). Primers: SARG-RACE: 5'-CCTGAAGTTCTGGCTTCTGGCAATGTG-3' and the standard AP1 primer of the

kit. Amplified cDNA fragments were inserted in pGEM-T Easy and sequenced.

Analysis of alternative splicing of mRNA by RT-PCR

cDNA was synthesized from 1 μg total RNA isolated from LNCaP cells incubated for 24 h in RPMI 1640 supplemented with 5% (v/v) dextran-coated charcoal treated FCS (FCS-DCC), antibiotics and 1 nM R1881. cDNA synthesis was performed at 55°C utilizing M-MuLV reverse transcriptase and an oligo-dT primer. Subsequently, PCR was carried out under standard conditions on the LNCaP cDNA template utilizing either forward primer SARG-F1A: 5'-CCAGGCAGCACAGATGAAGC-3' or SARG-F1B: 5'-AGCCTCTGTCTCCATCTCTGC-3' in combination with the reverse primer SARG-R: 5'-CTTCAGTGGACAGGAAGTCG-3'. RT-PCR products were inserted in pGEM-T Easy and sequenced.

RNA isolation and Northern analysis

Total RNA from LNCaP and LNCaP-1F5 cells was isolated by the guanidinium thiocyanate method (Sambrook and Russell 2001). RNA (10 μg per lane) was separated by electrophoresis on a 1% (w/v) agarose formaldehyde gel in TBE. Following electrophoresis RNA was transferred to a Hybond-N^+ membrane (Amersham Biosciences). The blot was hybridized under standard conditions at 65°C utilizing the ^{32}P-labelled *Hind*III/*Hind*III SARG cDNA fragment (854-1957) as a probe (Sambrook and Russell 2001). Actin cDNA was utilized as a hybridization control. Blots were exposed to X-ray film with intensifying screens at -80°C.

Analysis of tissue specific expression of mRNA by PCR

Tissue specificity of SARG mRNA was assayed by semi-quantitative PCR on Human MTC Panel II cDNA (BD Biosciences, Clontech), containing cDNAs from spleen, thymus, prostate, testis, ovary, small intestine, colon and peripheral blood lymphocytes, essentially according to the procedure described in the User Manual. G3PDH primers from the cDNA kit were used as a control (30 amplification cycles). SARG primers utilized were 5'-AGTCTGAGCCAGCCACAACT-3' (F-ex3) and 5'-TGTGGATATTCCTAGGGAGG-3' (R-ex4) (30 amplification cycles; primer annealing was at 55°C).

Immunocytochemistry

LNCaP cells were seeded at a density of 3 x 10^5 cells per well on sterile micro-slides in four-well tissue culture plates (Heraeus Instruments, Hanau, Germany), cultured until 50% confluence in RPMI 1640, supplemented with 5% (v/v) FCS and antibiotics, and subsequently transfected with 5 μg pHisXpress-cSARG. After overnight incubation, cells were washed twice in phosphate buffered saline (PBS), and fixed in acetone for 10 min. Next, slides were rinsed twice in PBS, followed by overnight incubation in mouse anti-Xpress antibody solution (Invitrogen) diluted 1:500 in PBS at 4°C. Incubation was stopped by four PBS washes. Next, slides were incubated for 30 min at room temperature in goat anti-mouse peroxidase conjugate antibody (DAKO, Glostrup, Denmark) solution (1:100 dilution in PBS). After four PBS washes, immunoreactivity was visualized by diaminobenzidine (DAB) staining. The reaction was stopped in water. Cells were counterstained with Mayers Hematoxylin.

Isolation of genomic DNA fragments

The SARG DD-PCR fragment was randomly ^{32}P-labelled and utilized to screen a genomic human PAC library on gridded filters (GenomeSystems, St. Louis, MO) according to the manufacturer's protocol. DNA was isolated from positive PACs by standard procedure

(Sambrook and Russell 2001). For Southern blot analysis 10 μg PAC90L18 DNA was *Hind*III, *Pst*I or *Eco*RI digested, electrophoresed on a 0.8% TAE-agarose gel and subsequently transferred to a Hybond-N^+ membrane. Filters were hybridized at high stringency with randomly ^{32}P-labelled SARG probes under standard conditions (Sambrook and Russell 2001). *Hind*III, *Pst*I or *Eco*RI digested PAC DNA was shot-gun cloned in the corresponding sites of pBSKS$^{+/-}$ (Stratagene). Clones were utilized for isolation of genomic fragments by screening with randomly ^{32}P-labelled SARG cDNA fragments and for *SARG* gene walking with overlapping *Hind*III, *Pst*I and *Eco*RI fragments. Hybridizing inserts were sequenced.

Search for candidate androgen response elements

The MatInspector professional program (www.genomatix.de/mat_fam) (Quandt *et al.* 1995) was utilized for detection of candidate AREs with queries for the inverted repeat 5'-RGWACANNNTGTTCT-3' (R=A/G, W=G/T) and the direct repeat 5'-TGTTCTNNNTGTTCT-3'. The threshold for candidate AREs was set at 9 out of 12 matches. The Matinspector program searched both the sense and anti-sense strand. Identified sequences were manually further selected according to additional criteria. Candidate inverted repeat AREs should contain G and C at the double-underlined positions in the sequence above, and at least one of either single underlined C or G. Candidate direct repeat AREs should contain three out of four single-underlined C and G residues.

Cell culture, transfection and luciferase assay

LNCaP and LNCaP-1F5 cells were cultured in RPMI 1640 supplemented with 5% (v/v) FCS and antibiotics. Four hour prior to transfection, medium was substituted by Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 5% (v/v) FCS-DCC. Transient transfections were performed according to the calcium phosphate precipitation method (Sambrook and Russell 2001) utilizing 1 x 10^6 cells per 25 cm^2 flask and 5 μg of one of the pLUC-constructs. After 4 h the medium was removed and cells were incubated for 90 sec at room temperature in PBS containing 15% (v/v) glycerol. Next, transfected cells were cultured in DMEM-FCS-DCC medium for 24 h in the absence or presence of 1 nM R1881 or 10 nM Dex. Transfected cells were washed in PBS, and subsequently incubated in 300 μl lysis buffer (25 mM Tris-phosphate, pH 7.8/ 8 mM $MgCl_2$/ 1mM DTT/ 1% (v/v) Triton X-100/ 15% (v/v) glycerol). Next, 100 μl 0.25 mM luciferin (Sigma, St. Louis, MO)/ 0.25 mM ATP in lysis buffer was added to 150 μl lysate, and luciferase activity was measured in a LUMAC 2500 M Biocounter (LUMAC, Landgraaf, The Netherlands).

Electrophoretic mobility shift assay

AR DBD and GR DBD were produced in *Escherichia coli*, and purified as described previously (De Vos *et al.* 1991). AR DBD and GR DBD were expressed from pRIT2TAR and pRIT2TrGR-DBD, respectively.

Oligonucleotide electrophoresis mobility shift assay (EMSA) probes:

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PSA ARE I:   5'-GATCCTTGCAGAACAGCAAGTGCTAGCTG-3'
                    3'-GAACGTCTTGTCGTTCACGATCGACCTAG-5'
Probasin ARE II:     5'-TCGACTAGGTTCTTGGAGTACTTTG-3'
                          3'-GATCCAAGAACCTCATGAAACAGCT-5'
ARE-SARG+4.6:        5′-TCGACACTGTGCTAACTGTTCTCTG-3′
                          3′-GTGACACGATTGACAAGAGACAGCT-5′
DR:                  5′-TCGACACTGTTCTAACTGTTCTCTG-3′
                          3′-GTGACAAGATTGACAAGAGACAGCT-5′
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ARE-mSARG+4.6: 5′-TCGACACTATGATAACTATTATCTG-3′
3′-GTGATACTATTGATAATAGACAGCT-5′

Probes were filled in by standard M-MuLV-RT reaction in the presence of α-^{32}P-dATP, and subsequently purified on a non-denaturing polyacrylamide gel. For EMSA, 50 x 10^3 cpm probe was added to 20 µl reaction mixture, containing 2 µg poly dIdC, 2 µg BSA, 10 µM $ZnCl_2$, 1 mM DTT and 2 µl 10x binding buffer (100 mM Hepes, pH 7.6/ 300 mM KCl/ 62.5 mM $MgCl_2$/ 4% (v/v) ficoll 400), and 5 pmol AR DBD or GR DBD. Incubation was for 30 min on ice. Samples were electrophoresed on a 4% (w/v) polyacrylamide (19:1 mono/bis acryl ratio) gel in a 25 mM Tris.HCl/ 41.5 mM boric acid/ 0.5 mM EDTA buffer for 2 h at 150 V at room temperature. Subsequently, gels were fixed, dried and exposed to X-ray film.

Chromatin immunoprecipitation

Chromatin immunoprecipitations (ChIP) were done essentially according to the method described in the Acetyl-Histone H3 ChIP assay kit (Upstate Biotechnology, Chicago, Il). In short, LNCaP cells were grown for at least 3 days on 5% FCS-DCC supplemented RPMI 1640 medium. To half of the cultures R1881 was added to a final concentration of 10 nM. After 1 h cells were cross-linked with formaldehyde (1% final concentration) at 22^{o}C for 10 min. Cross-linking was stopped by addition of glycine to a final concentration of 125 mM. Next, cells were washed in ice-cold PBS and harvested in PBS supplemented with protease inhibitors (Roche Diagnostics). Cell pellets were resuspended in SDS lysis buffer and sonicated to shear the DNA. Sonicated samples were centrifuged, diluted in Chip Dilution Buffer and precleared by incubation with salmon sperm DNA/Protein A agarose slurry for 1 h at 4^{o}C with rotation. After centrifugation, immunoprecipitation of the supernatant was performed overnight at 4 ^{o}C with Acetyl-Histone H3 antibody. Next, salmon sperm DNA/Protein A agarose slurry was added, and the incubation was continued for another hour. Agarose beads were washed according to the procedure described by the manufacturer. Eluates were heated overnight at 65^{o}C to reverse the cross-linking. DNA fragments were purified with a QIAquick Spin Kit (QIAGEN, Hilden, Germany). One µl from 50 µl DNA solution was used in a standard PCR (35 amplification cycles). Primer sequences were:

-8.5F: 5'-CAGGGACATGGATGAAGCTG-3'
-8.5R: 5'-GAACCCGTCATCTACATTAG-3'
-7.3F: 5'-GTAAGTCCAACACAGCTAGTC-3'
-7.3R: 5'-CTGAGATGCTGAGAGGCTGA-3'
+4.6F: 5'-CAAGTCTACAGTCTCCCATC-3'
+4.6R: 5'-CTCAAATCCCAGTTTAGCCA-3'.

PCR fragments were separated over an agarose gel.

RESULTS

SARG mRNA expression in LNCaP-1F5 cells

Utilizing DD-PCR technology, we previously identified in LNCaP-1F5 prostate cancer cells, which express both AR and GR, a novel androgen-specific regulated gene, denoted 21.1 in the initial study and *SARG* in the present study (Cleutjens *et al.* 1997). SARG mRNA expression was found to be up-regulated by the synthetic androgen R1881, but not by the synthetic glucocorticoid Dex. Utilizing the DD-PCR fragment as hybridization probe, a 5.5 kb transcript was identified in R1881-incubated LNCaP-1F5 cells.

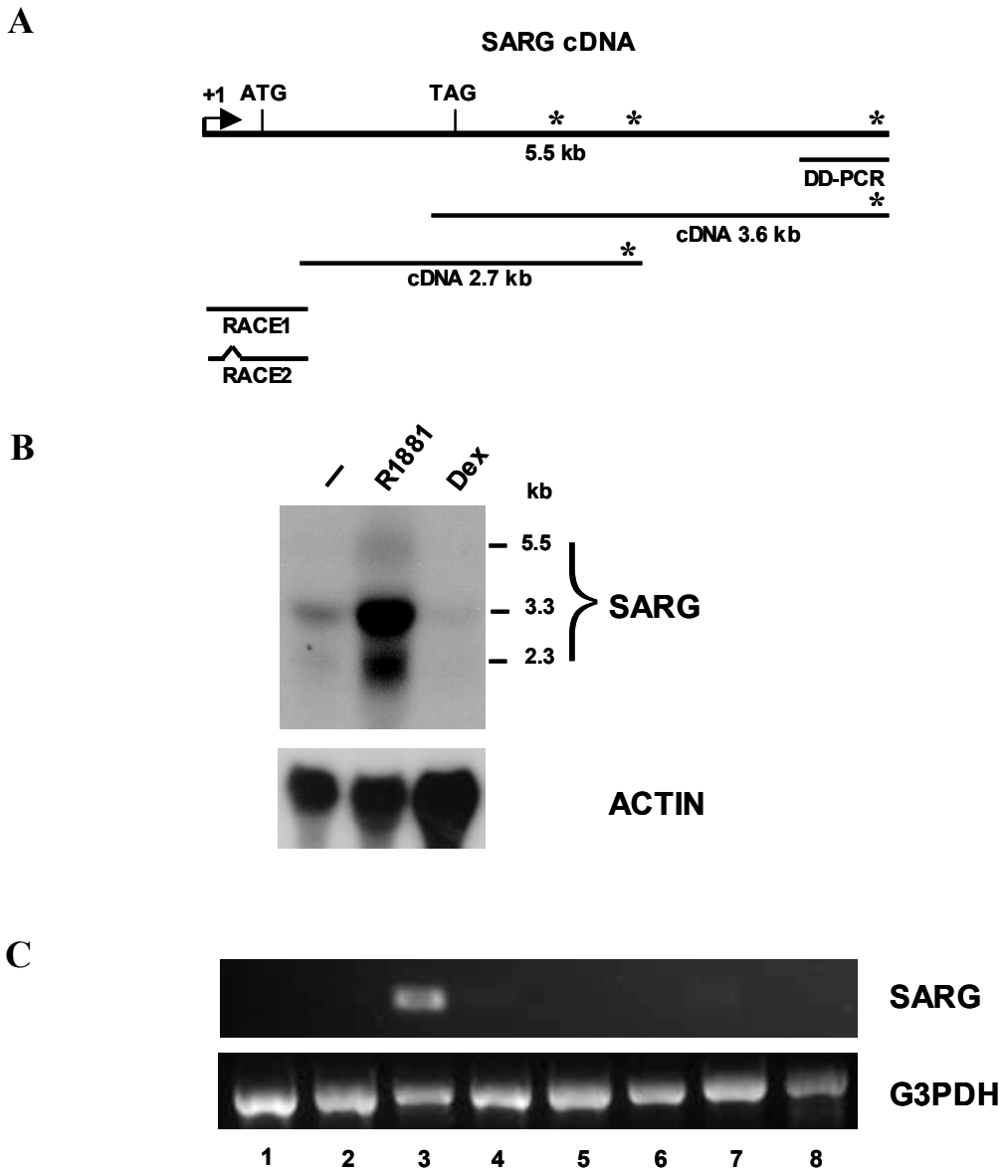

Figure 1. SARG cDNA, and androgen-regulated and prostate specific SARG mRNA expression.
(A) Schematic overview of the isolation of the complete SARG cDNA. The start and stop codons are indicated. An asterisk indicates a polyadenylation signal. DD-PCR indicates the original differential display fragment (Cleutjens *et al.* 1997). RACE1, RACE2 are two related 5' ends of SARG cDNA. (B) Northern blot of SARG mRNA expression in LNCaP-1F5 cells incubated for 24 h without hormone (-), with 1 nM R1881 or 10 nM Dex. Transcript sizes are indicated. Actin was utilized as a loading control. (C) RT-PCR analysis of SARG mRNA expression in spleen (1), thymus (2), prostate (3), testis (4), ovary (5), small intestine (6), colon (7) and lymphocytes (8). cDNAs were normalized for G3PDH expression as loading control.

For further characterization of SARG we first isolated full-length SARG mRNA. Overlapping SARG cDNA fragments were obtained by repeated screening of a human prostate cDNA library. In the first screen the DD-PCR fragment was utilized as hybridization probe (Figure 1A). The longest cDNA, containing a polyadenylation signal and a polyA tail, was 3.6 kbp. Screening of the cDNA library with a 5' fragment of this cDNA as a probe resulted in the detection of an overlapping 2.7 kbp cDNA with a second polyadenylation signal and a polyA tail. This cDNA fragment extended the cDNA sequence to approximately 4.9 kbp. Further 5' SARG cDNA sequence was obtained by RACE-PCR, utilizing a primer in the 2.7 kbp cDNA. Two related 5'

cDNA fragments of 665 bp and 537 bp, respectively, were found. The shorter fragment (RACE2) lacked nucleotides 42-169 of the longer fragment (RACE1) (Figure 1A). The longest SARG cDNA sequence of 5487 bp was deposited in GenBank under Accession Number AY352640.

A BLAST search of the EST database (www.ncbi.nlm.nih.gov/) identified two EST clusters overlapping the SARG cDNA sequence. The first group represented the 3' parts of the 5.5 kb and 3.3 kb transcripts, as detected in the cDNA library (Unigene cluster Hs.32417), the second cluster represented a 2.3 kb transcript, which contained SARG 5' cDNA sequences, and a third polyadenylation sequence and polyA tail (Unigene cluster Hs.223394). We confirmed the presence of three polyadenylation signals in the 5.5 kbp SARG cDNA sequence (Figure 1A). A 1.1 kbp SARG cDNA fragment (nucleotides 854 to 1957) hybridized with all three predicted SARG mRNAs in a Northern blot of LNCaP-1F5 RNA (Figure 1B). The 3.3 kb transcript showed the highest expression. All SARG transcripts were up-regulated by R1881, but their expression could not be induced by Dex. Semi-quantitative RT-PCR indicated high SARG mRNA expression in prostate tissue (Figure 1C, lane 3) as compared to spleen, thymus, testis, ovary, small intestine, colon and lymphocytes.

SARG protein

The SARG open reading frame (ORF) encodes a 601 amino acid protein (Figure 2A). This ORF is identical to that of the hypothetical protein MGC2742 (Genbank). Because Unigene cluster Hs.23417 encompasses the 3' part of the two longest SARG transcripts, a predicted protein from this EST cluster (MGC4309) is unlikely.

To determine its cellular localization, SARG protein was Xpress-tagged and transiently expressed in LNCaP cells. Immunocytochemical staining with anti-Xpress antibody showed that SARG protein was exclusively present in the cytoplasm (Figure 2B).

SARG gene structure and splice variants

The complete *SARG* gene was isolated in one PAC (90L18) by screening of a human genomic PAC library with the SARG DD-PCR fragment (see Figure 1A) as probe. To characterize *SARG*, subcloned overlapping *Hind*III, *Pst*I and *Eco*RI fragments of PAC 90L18 were hybridized with appropriate cDNA fragments. Comparison with the cDNA sequence revealed that *SARG* was composed of 4 exons, and spanned 14.5 kbp (Figure 3A). The two cDNA fragments obtained by RACE-PCR represented two forms of exon 1, the short exon 1A, and the extended exon 1AB. All splice junctions were consistent with the GT/AG rule (Figure 3B). The SARG ORF started in exon 2 and ended in the large exon 4 (Figure 3A). *SARG* is part of BAC RP11-564A8 (Genbank Accession Number AC098935.2). The transcription start site is at position 184,776 in this clone. *SARG* maps at chromosome band 1q32.2.

RACE-PCR and RT-PCR revealed four different SARG splice variants (Figure 3C). The largest variant contained all 4 exons, smaller variants lacked either part B of exon 1AB, exon 2 or both. The splice variants lacking exon 2, which formed a minority, are predicted to encode a protein of 355 amino acids, starting at methionine 247 (Figure 2A). The corresponding ATG codon is in exon 4, in frame with the long SARG ORF (Figure 3C).

A

A

```
251         ATGCCCGAGAGGGAGCTGTGGCCAGCGGGGACTGGCTCAGAACCCGTGACCCGTGTCGGC
1             M   P   E   R   E   L   W   P   A   G   T   G   S   E   P   V   T   R   V   G

311         AGCTGTGACAGCATGATGAGCAGCACCTCCACCCGCTCTGGATCTAGTGATAGCAGCTAC
21            S   C   D   S   M   M   S   S   T   S   T   R   S   G   S   S   D   S   S   Y

371         GACTTCCTGTCCACTGAAGAGAAGGAGTGTCTGCTCTTCCTGGAGGAGACCATTGGCTCA
41            D   F   L   S   T   E   E   K   E   C   L   L   F   L   E   E   T   I   G   S

431         CTGGACACGGAGGCTGACAGCGGACTGTCCACTGACGAGTCTGAGCCAGCCACAACTCCC
61            L   D   T   E   A   D   S   G   L   S   T   D   E   S   E   P   A   T   T   P

491         AGAGGTTTCCGAGCACTGCCCATAACCCAACCCACTCCCCGGGGAGGTCCAGAGGAGACC
81            R   G   F   R   A   L   P   I   T   Q   P   T   P   R   G   G   P   E   E   T

551         ATCACTCAGCAAGGACGAACGCCAAGGACAGTAACTGAGTCCAGCTCATCCCACCCTCCT
101           I   T   Q   Q   G   R   T   P   R   T   V   T   E   S   S   S   S   H   P   P

611         GAGCCCCAGGGCCTAGGCCTCAGGTCTGGCTCCTACAGCCTCCCTAGGAATATCCACATT
121           E   P   Q   G   L   G   L   R   S   G   S   Y   S   L   P   R   N   I   H   I

671         GCCAGAAGCCAGAACTTCAGGAAAAGCACCACCCAGGCTAGCAGTCACAACCCTGGAGAA
141           A   R   S   Q   N   F   R   K   S   T   T   Q   A   S   S   H   N   P   G   E

731         CCGGGGAGGCTTGCGCCAGAGCCTGAGAAAGAACAGGTCAGCCAGAGCAGCCAACCCAGG
161           P   G   R   L   A   P   E   P   E   K   E   Q   V   S   Q   S   S   Q   P   R

791         CAGGCACCTGCCAGCCCCCAGGAGGCTGCCCTTGACTTGGACGTGGTGCTCATCCCTCCG
181           Q   A   P   A   S   P   Q   E   A   A   L   D   L   D   V   V   L   I   P   P

851         CCAGAAGCTTTCCGGGACACCCAGCCAGAGCAGTGTAGGGAAGCCAGCCTGCCCGAGGGG
201           P   E   A   F   R   D   T   Q   P   E   Q   C   R   E   A   S   L   P   E   G

911         CCAGGACAGCAGGGCCACACACCCCAGCTCCACACACCATCCAGCTCCCAGGAAAGAGAG
221           P   G   Q   Q   G   H   T   P   Q   L   H   T   P   S   S   S   Q   E   R   E

971         CAGACTCCTTCAGAAGCCATGTCCCAAAAAGCCAAGGAAACAGTCTCAACCAGGTACACA
241           Q   T   P   S   E   A   M   S   Q   K   A   K   E   T   V   S   T   R   Y   T

1031        CAACCCCAGCCTCCTCCTGCAGGGTTGCCTCAGAATGCAAGAGCTGAAGATGCTCCCCTC
261           Q   P   Q   P   P   P   A   G   L   P   Q   N   A   R   A   E   D   A   P   L

1091        TCATCAGGGGAGGACCCAAACAGCCGACTAGCTCCCCTCACAACCCCTAAGCCCCGGAAG
281           S   S   G   E   D   P   N   S   R   L   A   P   L   T   T   P   K   P   R   K

1151        CTGCCACCTAATATTGTTCTGAAGAGCAGCCGAAGCAGTTTCCACAGTGACCCCCAGCAC
301           L   P   P   N   I   V   L   K   S   S   R   S   S   F   H   S   D   P   Q   H

1211          TGGCTGTCCCGCCACACTGAGGCTGCCCCTGGAGATTCTGGCCTGATCTCCTGTTCACTG
321           W   L   S   R   H   T   E   A   A   P   G   D   S   G   L   I   S   C   S   L

1271        CAAGAGCAGAGAAAAGCACGTAAAGAAGCTCTAGAGAAGCTGGGGCTACCCCAGGATCAA
341           Q   E   Q   R   K   A   R   K   E   A   L   E   K   L   G   L   P   Q   D   Q

1331        GATGAGCCTGGACTCCACTTAAGTAAGCCCACCAGCTCCATCAGACCCAAGGAGACACGG
361           D   E   P   G   L   H   L   S   K   P   T   S   S   I   R   P   K   E   T   R

1391        GCCCAGCATCTGTCCCCAGCTCCAGGTCTGGCTCAGCCTGCAGCTCCAGCCCAGGCCTCA
381           A   Q   H   L   S   P   A   P   G   L   A   Q   P   A   A   P   A   Q   A   S

1451        GCAGCTATTCCTGCTGCTGGGAAGGCTCTGGCTCAAGCTCCGGCTCCAGCTCCAGGTCCA
401           A   A   I   P   A   A   G   K   A   L   A   Q   A   P   A   P   A   P   G   P

1511        GCTCAGGGACCTTTGCCAATGAAGTCTCCAGCTCCAGGCAATGTTGCAGCTAGCAAATCT
421           A   Q   G   P   L   P   M   K   S   P   A   P   G   N   V   A   A   S   K   S

1571        ATGCCAATTCCTATCCCTAAGGCCCCAAGGGCAAACAGTGCCCTGACTCCACCGAAGCCA
441           M   P   I   P   I   P   K   A   P   R   A   N   S   A   L   T   P   P   K   P

1631        GAGTCAGGGCTGACTCTCCAGGAGAGCAACACCCCTGGCCTGAGACAGATGAACTTCAAG
461           E   S   G   L   T   L   Q   E   S   N   T   P   G   L   R   Q   M   N   F   K

1691        TCCAACACTCTGGAGCGCTCAGGCGTGGGACTGAGCAGCTACCTTTCAACTGAGAAAGAT
481           S   N   T   L   E   R   S   G   V   G   L   S   S   Y   L   S   T   E   K   D

1751        GCCAGCCCCAAAACCAGCACTTCTCTGGGAAAGGGCTCCTTCTTGGACAAGATCTCGCCC
501           A   S   P   K   T   S   T   S   L   G   K   G   S   F   L   D   K   I   S   P

1811        AGTGTCTTACGTAATTCTCGGCCCCGCCCGGCCTCCCTGGGCACGGGGAAAGATTTTGCA
521           S   V   L   R   N   S   R   P   R   P   A   S   L   G   T   G   K   D   F   A

1871        GGTATCCAGGTAGGCAAGCTGGCTGACCTGGAGCAGGAGCAGAGCTCCAAGCGCCTGTCC
541           G   I   Q   V   G   K   L   A   D   L   E   Q   E   Q   S   S   K   R   L   S

1931        TACCAAGGACAGAGCCGTGACAAGCTTCCTCGCCCCCCCTGTGTCAGTGTCAAGATCTCC
561           Y   Q   G   Q   S   R   D   K   L   P   R   P   P   C   V   S   V   K   I   S

1991        CCAAAGGGTGTCCCCAATGAACACAGAAGGGAGGCCCTGAAGAAGCTGGGACTGTTGAAG
              P   K   G   V   P   N   E   H   R   R   E   A   L   K   K   L   G   L   L   K
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B

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2051        GAGTAG
601           E   *
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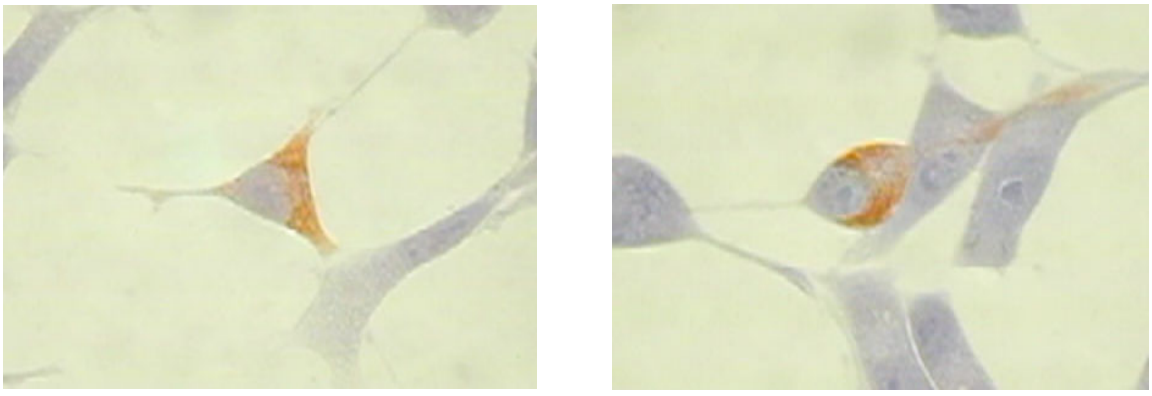


Figure 2. The SARG open reading frame and the cellular localization of the SARG protein.
(A) The SARG ORF encodes a protein of 601 amino acids. Methionine 247 (in bold) is the first amino acid residue of the shorter SARG protein translated from mRNA lacking exon 1B and/or exon 2 sequences. (B) The SARG protein is located in the cytoplasm. LNCaP cells were transfected with pcHisXpress-cSARG encoding Xpress-tagged SARG protein. Xpress-SARG was visualized by immunostaining with anti-Xpress antibody.

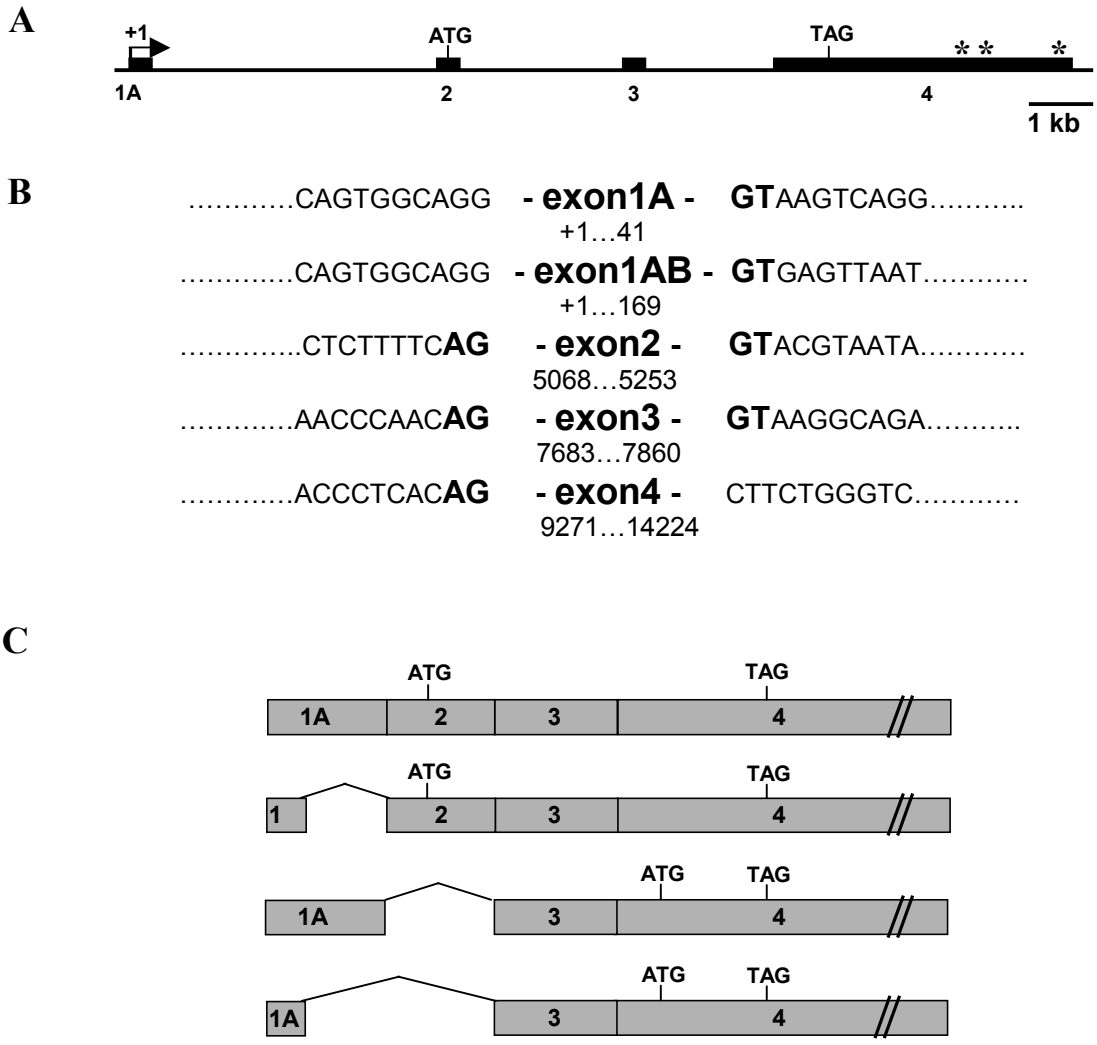


Figure 3. *SARG* gene structure and splice variants.
(A) Schematic presentation of the *SARG* gene. The ATG start codon in exon 2 and the TAG stop codon in exon 4 are indicated. The three asterisks indicate polyadenylation signals. (B) Splice donor and acceptor sites at the exon/intron boundaries in the *SARG* gene. Numbering is according to the genomic *SARG* sequence. (C) Four different splice variants of the *SARG* gene. Start and stop codons are indicated.

Functional and bioinformatics-based selection of candidate androgen response elements

To establish androgen response of *SARG*, the promoter fragments SARG-L (-3012 to +168) and SARG-S (-55 to +168) were inserted in front of the luciferase reporter gene in the constructs pSARG-L-LUC and pSARG-S-LUC, respectively. Transient transfection of these constructs to LNCaP cells showed in both cases a very weak androgen response, indicative of the absence of strong AR binding sites (see Figure 4D). This prompted us to screen for candidate AREs in a region of approximately 18 kbp, from 9 kbp upstream to 9 kbp downstream of the transcription start site, by a bioinformatics-based approach. This sequence is present in BACs AC098935.2 and AC023534. The MatInspector program was applied to search both DNA strands for sequences homologous to the direct repeat 5'-TGTTCTnnnTGTTCT-3' or to the inverted repeat consensus ARE sequence 5'-A/GGA/TACAnnnTGTTCT-3' (see Materials and Methods). We selected sequences that showed at least 9 out of 12 matches in the two half sites. Out of the sequences obtained candidate AREs were further selected manually, based on the criteria of presence of at

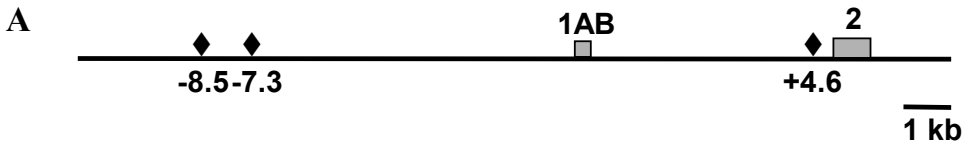
least 3 out of 4 underlined C or G residues in the direct repeat, or presence of the double-underlined C and G residue, and at least one of the two single-underlined C and G residues in the inverted repeat. Utilizing this approach we identified 34 candidate AREs in the 18 kbp region, 12 inverted repeats and 22 direct repeats. None of these was completely identical to the consensus inverted repeat. One was a perfect direct repeat and two sequences deviated at one position from a perfect direct repeat. Four sequences matched the inverted or direct repeat at 10 out of 12 positions. Several candidate AREs clustered in the genome. At approximately –8.5 kbp a cluster of 4 candidate AREs was present, including the imperfect direct repeat 5'-TGAACAatgAGAACA-3' (11/12 matches); at +4.6 kbp a cluster of 5 candidate AREs was detected, including the imperfect direct repeat 5'-TGTGCTaacTGTTCT-3' (11/12 matches). The latter cluster is located in *SARG* intron 1. The perfect direct repeat 5'-TGTTCTcctTGTTCT-3' mapped at -7.3 kbp, one ARE-like sequence was close to this repeat (Figure 4 A,B).

Next, it was investigated whether three genomic fragments containing the indicated direct repeats and flanking candidate AREs could function as enhancer regions. Genomic fragments with a size of approximately 500 bp, SARG-8.5, SARG-7.3 and SARG+4.6, respectively, were coupled to PSA4-LUC, containing the 600 bp promoter of the *PSA* gene. This promoter was weakly responsive to androgens, but combination with an upstream PSA enhancer fragment resulted in a strong androgen-inducible promoter (Cleutjens *et al.* 1997). In transfected LNCaP cells, SARG-8.5 and SARG-7.3 had no significant effect on the weak R1881 induction of the PSA4 promoter. In contrast, SARG+4.6 clearly increased R1881 induced PSA4 activity (Figure 4C). Next, SARG+4.6 was linked to SARG-S (-55 to +168) and SARG-L (-3012 to +168), which both showed, as mentioned above, a very low androgen induction. Similar to the PSA promoter experiment, linkage of SARG+4.6 to both SARG-L and SARG-S showed androgen response in transfected LNCaP cells (Figure 4D).

SARG intron 1 contains a functional direct repeat androgen response element

To determine whether the imperfect direct repeat 5'-TGTGCTaacTGTTCT-3' in SARG+4.6 was responsible for androgen induction, it was mutated to 5'-TATGATaacTATTAT-3'. The mutated fragment was coupled to SARG-S and tested in LNCaP cells for its response to R1881. As shown in Figure 5A, the androgen induction of SARG+4.6 was completely abolished by the mutations.

Next, the direct repeat 5'-TGTGCTaacTGTTCT-3' in SARG+4.6, designated ARE-SARG+4.6, was tested in an EMSA for its ability to bind to AR DBD (Figure 5B). Control AREs were PSA ARE I (5'-AGAACAgcaAGTGCT-3'), which has been shown to bind strongly to the AR DBD, and rat probasin ARE II (5'-AGTACTccaAGAACC-3'), which is considered as a direct repeat, strongly interacting with AR DBD (Riegman *et al.* 1991, Rennie *et al.* 1993, Claessens *et al.* 1996, Cleutjens *et al.* 1996). ARE-SARG+4.6 bound to AR DBD, albeit weaker than the PSA and probasin AREs. The change of ARE-SARG+4.6 into the perfect direct repeat 5'-TGTTCTaacTGTTCT-3' (DR) did not affect its capacity to bind AR DBD. AR DBD was unable to bind inactive mutant ARE-mSARG+4.6 (5'-TATGATaacTATTAT-3').

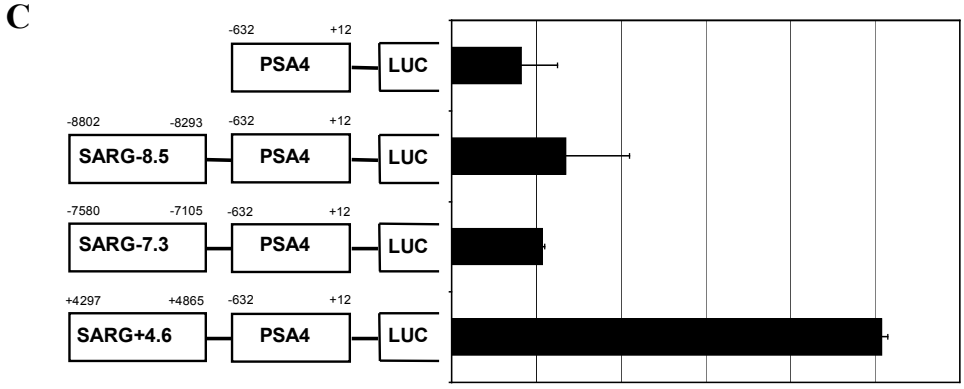


B

SARG -8.5 -8802…..…..**TGAACA**atg**AGAACA**……-8293
(direct repeat 11/12)

SARG -7.3 -7580……..…**TGTTCT**cct**TGTTCT**……..…-7105
(direct repeat 12/12)

SARG +4.6 +4297……**TGTGCT**aac**TGTTCT**……..…..+4865
(direct repeat 11/12)



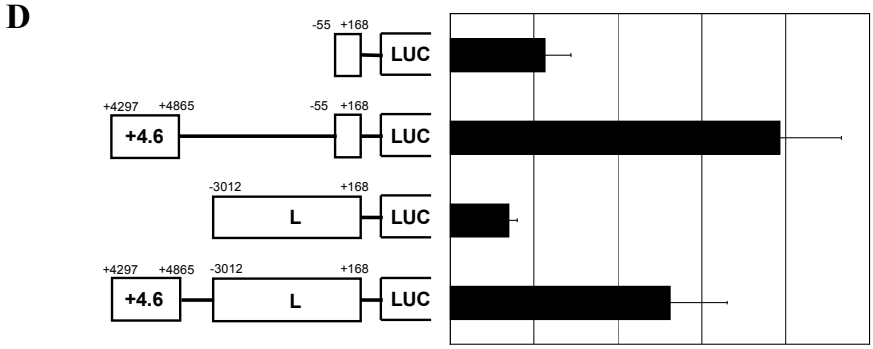
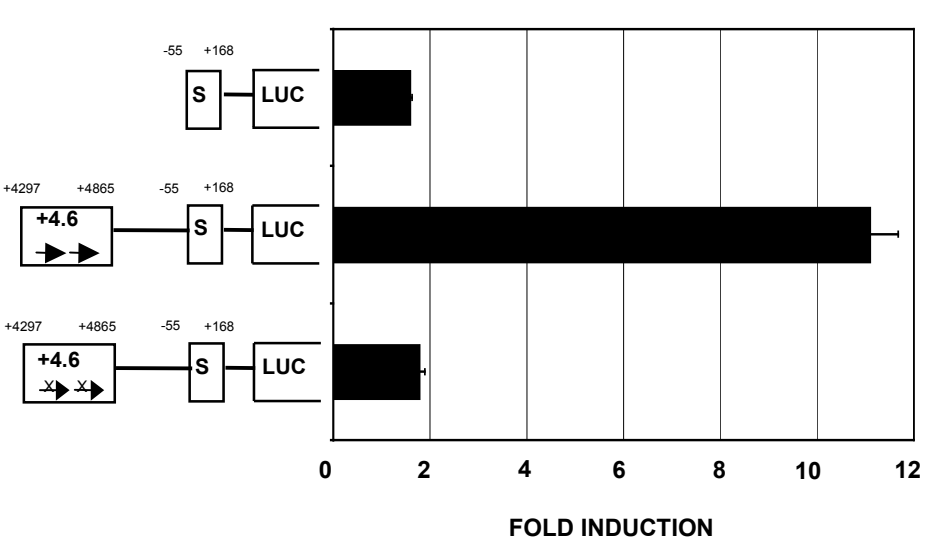


Figure 4. Three direct repeat candidate androgen response elements in the *SARG* gene.
(A) Positions (kbp) of the 3 direct repeat candidate AREs (♦) in the *SARG* gene. (B) Sequences and location of the 3 direct repeat candidate AREs in the *SARG* gene. Numbering is according to the genomic *SARG* sequence. Matching to the direct repeat 5'-TGTTCTnnnTGTTCT-3' is indicated below the sequences. (C) Androgen-induced activity of the *SARG* fragments, containing a direct repeat candidate ARE, coupled to the PSA4 promoter. LNCaP cells were transiently transfected with pPSA-4-LUC, pSARG-8.5-PSA-LUC, pSARG-7.3-PSA-LUC or pSARG+4.6-PSA-LUC. Luciferase activity was measured after 24 h incubation with or without 1 nM R1881. (D) Androgen-induced activity of SARG+4.6 coupled to SARG-S and SARG-L, respectively. LNCaP cells were transfected with pSARG-S-LUC, pSARG+4.6-SARG-S-LUC, pSARG-L-LUC or pSARG+4.6-SARG-L-LUC. After 24 h incubation with or without 1 nM R1881 luciferase activity was measured. Value +/- SEM in (C) and (D) are from two experiments carried out in duplicate. Fold induction is the ratio of luciferase activity measured in the presence and absence of R1881.

A



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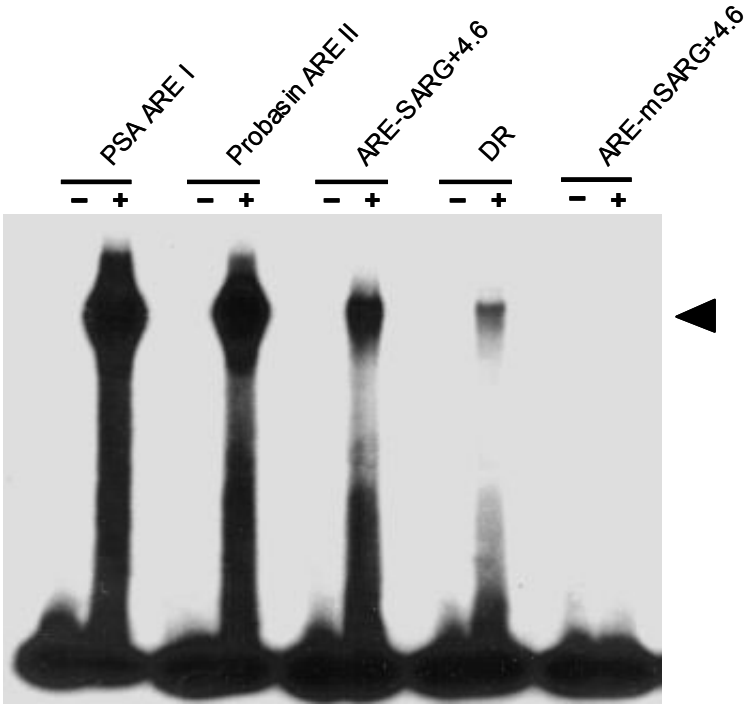


PSA ARE I: AGAACAgcaAGTGCT
Probasin AREII: GGTTCTtggAGTACT
ARE-SARG+4.6: TGTGCTaacTGTTCT
DR: TGTTCTaacTGTTCT
ARE-mSARG+4.6:TATGATaacTATTAT

Figure 5 The direct repeat in SARG+4.6 is a functional androgen response element.
(A) Effect of mutation of ARE-SARG+4.6 on the transcriptional activity of SARG+4.6. LNCaP cells were transfected with pSARG-S-LUC, pSARG+4.6-SARG-S-LUC or pSARG+4.6m-SARG-S-LUC and incubated for 24 h with or without 1 nM R1881. Transcriptional activity was measured in a luciferase assay. Values +/- SEM are from two experiments carried out in duplicate. Fold induction is the ratio of luciferase activity measured in the presence and absence of R1881. (B) EMSA of indicated AREs and AR DBD. ARE sequences are shown below the figure. The arrowhead indicates the position of the AR DBD-ARE complex.

Characterization of enhancer SARG+4.6

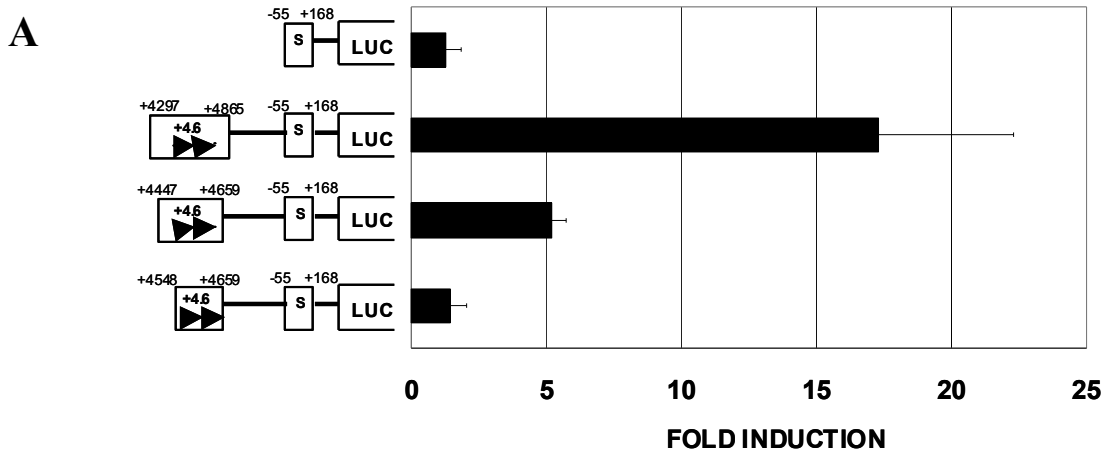
To further decipher the role of the 569 bp enhancer SARG+4.6-(+4297/+4865) in androgen regulation, two deletion constructs of SARG+4.6-S-LUC were generated. Construct SARG+4.6-(+4447/4659) lacked all four candidate weak ARE sequences present in the SARG+4.6 enhancer, but contained the imperfect direct repeat ARE (ARE-SARG+4.6). The 112 bp enhancer fragment SARG+4.6-(+4548/4659) lacked even more upstream sequences, but also still contained ARE-SARG+4.6. In transfection experiments the shortened enhancer SARG+4.6-(4447/4659) was less active than the 569 bp fragment, suggesting that clustering of ARE-SARG+4.6 with weak ARE-like sequences is important for full enhancer activity (Figure 6A). Interestingly, further shortening of the enhancer completely abolished its activity although ARE-SARG+4.6 was still present. However, in the deleted region we could not detect an obvious ARE-like sequence.

We carried out ChIP assays in order to investigate the *in vivo* function of enhancer SARG+4.6. Utilizing an antibody directed against acetylated histone H3 we observed a difference in H3 acetylation over SARG+4.6 between LNCaP cells grown in the presence and in the absence of R1881, showing a difference in chromatin structure on this part of the gene (Figure 6B). The higher signal with AcH3 antibody in the presence of R1881 indicated an active structure of the enhancer region. Such a difference was not detected for the genomic fragments SARG-8.5 and SARG-7.3. These findings were in accordance with the transient transfection studies, as shown in Figure 4C. Unfortunately, ChIP assays with a large series of different antibodies against the AR were not successful, probably due to the low affinity of AR for ARE-SARG+4.6.

ARE-SARG+4.6 is androgen receptor specific

To address the question whether ARE-SARG+4.6 is involved in androgen specificity, SARG+4.6 coupled to both SARG-S-LUC and PSA4-LUC was tested for activation by Dex. The constructs were transfected to LNCaP-1F5 cells cultured in the presence of 1 nM R1881 or 10 nM Dex, or in the absence of hormone (Figure 7A,B). SARG+4.6 did not significantly stimulate Dex induced activity of SARG-S and PSA4. In contrast, R1881 induced activity of these two promoters was clearly increased by SARG+4.6.

ARE-SARG+4.6 was also tested in an EMSA for its ability to bind to GR DBD (Figure 7C). Control PSA ARE I did bind to GR DBD, but rat probasin ARE II did not. Importantly, ARE-SARG+4.6 was also not able to bind to GR DBD, which correlated with the R1881 specificity of SARG+4.6 in the transfection assay.



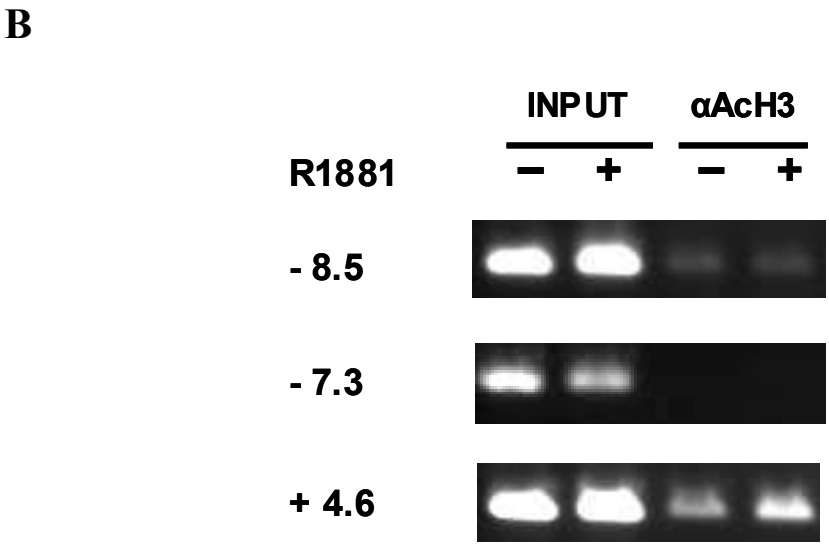


Figure 6. Characterization of enhancer SARG+4.6.
(A) Deletion mapping of enhancer SARG+4.6 in transiently transfected LNCaP cells. See legend to Figure 5A and Materials and Methods for experimental details. (B) ChIP assay of the candidate –8.5 kb, -7.3 kb and +4.6 kb enhancer regions of *SARG* in the presence and absence of R1881. Acetyl-Histone H3 antibody was used for immunoprecipitation. Experimental details are described in Materials and Methods. Input: DNA prior to immunoprecipitation.

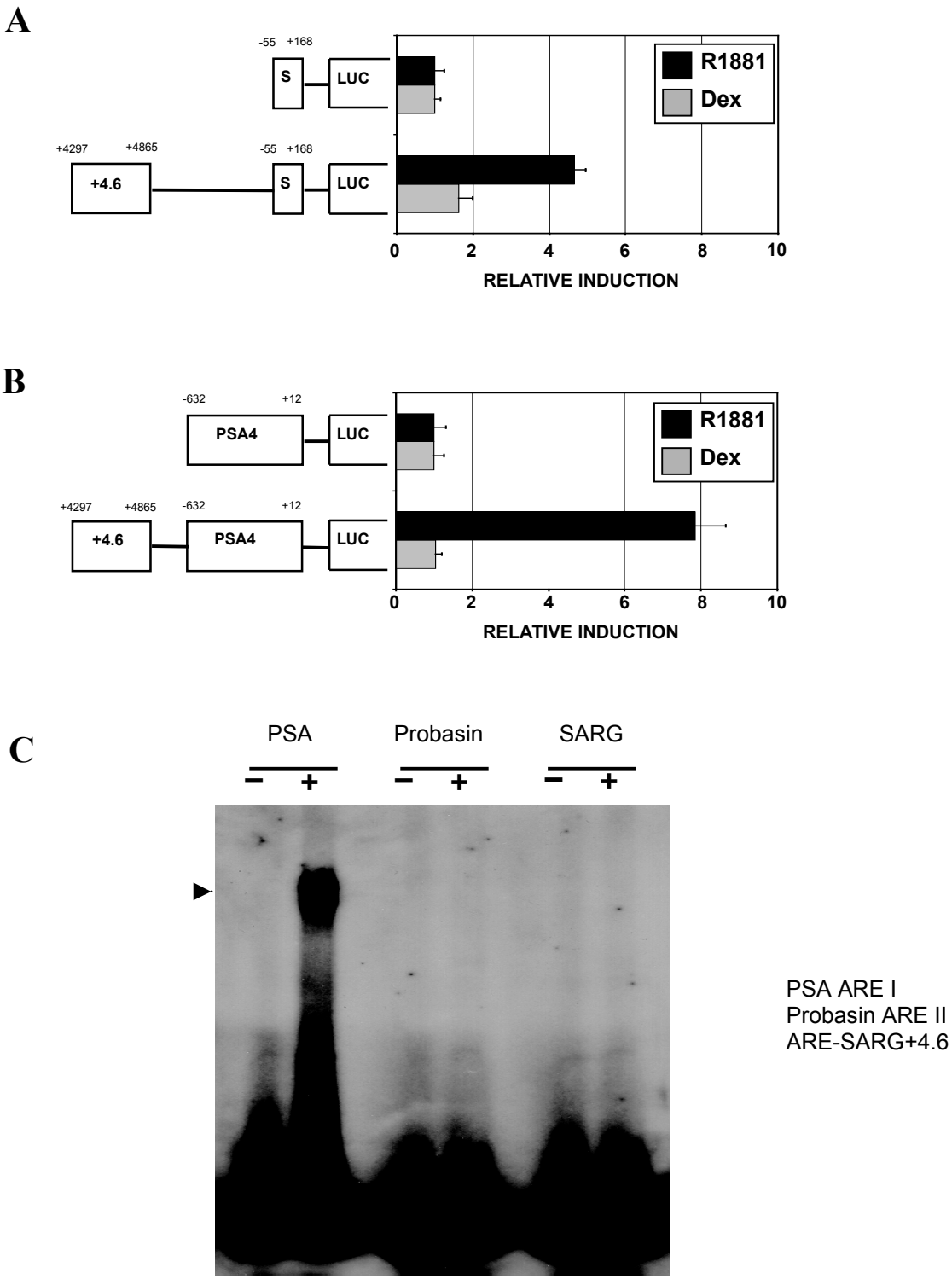


Figure 7. ARE-SARG+4.6 is androgen receptor specific.
LNCaP-1F5 cells were transfected with pSARG-S-LUC and pSARG+4.6-SARG-S-LUC (A) and pPSA4-LUC and pSARG+4.6-PSA-LUC (B). Luciferase activity was measured after 24 h incubation with 1 nM R1881 or 10 nM Dex or without hormone. R1881 and Dex induced activities of SARG-S-LUC (A) and PSA4-LUC (B) were set at 1. Relative inductions +/- SEM are from 2 experiments carried out in duplicate. (C) EMSA of AREs with GR DBD. Sequences of the DNA fragments analysed are shown in figure 5 (B). GR DBD-ARE complexes are indicated by the arrowhead.

DISCUSSION

The androgen-specific regulated *SARG* gene was identified in the LNCaP-1F5 subline that expresses endogenous AR, and GR from a stable-integrated cDNA expression vector (Cleutjens *et al.* 1997). We showed that SARG mRNA expression could be up-regulated by androgens, but not by glucocorticoids. *SARG* is a 4 exon gene of 14.5 kbp mapping to chromosome band 1q32.2. Exon 1 can appear in a short or long form, 1A and 1AB, respectively. The 2.3, 3.3 and 5.5 kb transcripts result from alternative polyadenylation. Splice variants might lack either exon 2, part B of exon 1 or both. The predicted genes *MGC2742* and *MGC4309* are both part of *SARG*. *SARG* is preferentially expressed in the prostate (Figure 1C). Our findings are substantiated by data in the expression profile database GeneNote (http://bioinformatics/weizmann.ac.il/cards/). In this database high *SARG* expression was only documented for prostate and lung.

The SARG ORF encodes a protein of 601 amino acids; splice variants lacking exon 2 are expected to code for a carboxyl-terminal fragment of 355 amino acids of the full-length protein. Transient transfection experiments showed that the SARG protein is located in the cytoplasm. No homology to other proteins was found. Unfortunately, the amino acid composition of SARG does not indicate motifs that could predict its function. The Sarg mouse ortholog is 605 amino acids with a homology to human SARG of 65%. Highest homology is in the amino-terminal and carboxyl-terminal regions of the proteins (data not shown).

To explain androgen specificity of SARG expression we first studied a 3 kbp promoter region in transfection assays. Because these experiments were unsuccessful, we decided to carry out an *in silico* search for candidate AR binding sites in 18 kbp flanking the *SARG* transcription start site. The search criteria were based on three or less deviations from a perfect direct repeat or the consensus high affinity ARE inverted repeat. We identified 34 candidate AREs. Functional studies, based on clustering of candidate AREs indicated that an imperfect direct repeat in intron 1, 5'-TGTGCTgcaTGTTCT-3' (ARE-SARG+4.6) was active and AR-specific. Importantly, ARE-SARG+4.6 cooperated with surrounding sequences in a 569 bp enhancer region for full activity. Part of the cooperating sequences might be weak AR binding sites. However, others might be binding sites for prostate specific and more common transcription factors. The properties of these factors remain to be identified.

Mutation of ARE-SARG+4.6 to a perfect repeat did not affect AR DBD binding. However, SARG-7.3, which contains a perfect direct repeat, did not show any detectable androgen-induction in transfections. Also, linkage of SARG-7.3 to SARG+4.6-SARG-S did not increase the activity of SARG+4.6-SARG-S in transfections (data not shown). In contrast to ARE-SARG+4.6, ARE-SARG-7.3 might lack favourable modulating flanking sequences (Nelson *et al.* 1999) or binding sites for other transcription factors in its close vicinity. This might also be true for inactivity of ARE-SARG-8.5.

The present study shows that a bioinformatics-based search for AR binding sites followed by selected functional studies can successfully identify active regulatory elements. However, it shows also the limitations of such an approach, due to the complexity of the regulation mechanism of gene expression. The functional studies were limited to the two largest clusters of candidate AREs, and to a small cluster containing a perfect direct repeat in an 18 kbp region. Without clustering as a selection criterion, the bioinformatics approach would not have been selective, because of the high density of candidate AREs (1 per 500 bp). We realize that functional AREs in enhancers and promoters might also cluster with binding sites for other transcription factors. Moreover, although less likely, it cannot be excluded completely that some candidate ARE sequences did not pass the selection criteria. One such ARE should be in SARG-S (-55 to +168), which is weakly androgen inducible. A candidate is the sequence 5'-

GGGCCAggcAGCACA-3' (+5 to +17) in exon 1, which deviates at 4 positions from a perfect direct repeat.

A complicating factor in direct repeat ARE search is the lack of a consensus sequence for high-affinity, high-specificity AR binding, due to the limited number of this type of AREs identified so far. Rat probasin ARE II (5'-GGTTCTtggAGTACT-3'), which deviates from a perfect direct repeat at 3 positions, seems at present the specific ARE with highest AR affinity (Rennie *et al.* 1993, Kasper *et al.* 1994, Claessens *et al.* 1996, Kasper *et al.* 1999, Claessens *et al.* 2001). However, a search of the 18 kbp *SARG* sequence did not detect candidate AREs closely resembling this sequence (data not shown). Our data provide the first evidence that an almost perfect direct 5'-TGTTCT-3' repeat as present in SARG+4.6, can function as an AR-specific element in a natural enhancer. Other direct repeat-like functional AREs, with variable AR specificity, as detected in the *SC* (Secretory Component) gene, the mouse *Slp* (Sex limited protein) gene and the *PEM* (placenta and embryo) homeobox gene all deviate at least at three positions from a perfect direct repeat (Verrijdt *et al.* 1999, Verrijdt *et al.* 2000, Barbulescu *et al.* 2001).

Comparison of functional AREs of a large series of preferentially androgen regulated genes should reveal the sequence of a consensus high-affinity, high-specificity AR binding site in a natural context. Such genes might be identified by expression profiling of the AR and GR positive LNCaP-1F5 cell line followed by an unbiased functional study of a large series of overlapping fragments flanking the transcription start sites of these genes. This should also give a better insight in selection criteria for a bioinformatics-based search for functional AR binding sites in novel genes. Such an approach might also include comparison with data from other species.

It may be possible that the composition of an ARE can influence receptor activity by transduction of a particular conformation via the DNA-bound DBD to other AR domains. Recent evidence indicates that binding to different AREs indeed induces different conformational changes (Geserick *et al.* 2003). A different DBD conformation might directly or indirectly affect the association with coactivators, as shown for ER (Wood *et al.* 2001, Hall *et al.* 2002). In addition, it remains to be elucidated whether the ARE sequence is the major molecular determinant of AR specificity, or whether AR protein-protein interactions, including interactions with other specific transcription factors can contribute significantly to receptor specificity (Karvonen *et al.* 1997, Scheller *et al.* 1998).

ACKNOWLEDGEMENT

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Abbreviations

AR, androgen receptor; ARE, androgen response element; ChIP, chromatin immunoprecipitation; DBD, DNA binding domain; DCC, dextran coated-charcoal; DD, differential display; Dex, dexamethasone; DR, direct repeat; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; GR, glucocorticoid receptor; HRE, hormone response element; MR, mineralocorticoid receptor; LBD, ligand binding domain; NTD, amino-terminal domain; PBS, phosphate buffered saline; PR, progesterone receptor; PSA, prostate specific antigen; R1881, methyltrienolone; *SARG*, specific androgen regulated gene.

REFERENCES

Aranda A & Pascual A 2001 Nuclear hormone receptors and gene expression. *Physiological Reviews* **81** 1269-1304.

Barbulescu K, Geserick C, Schuttke I, Schleuning WD & Haendler B 2001 New androgen response elements in the murine pem promoter mediate selective transactivation. *Molecular Endocrinology* **15** 1803-1816.

Berns EM, de Boer W & Mulder E 1986 Androgen-dependent growth regulation of and release of specific protein(s) by the androgen receptor containing human prostate tumor cell line LNCaP. *Prostate* **9** 247-259.

Claessens F, Dirckx L, Delaey B, Decourt JL, Hemschoote K, Peeters B & Rombauts W 1989 The androgen-dependent rat prostatic binding protein: comparison of the sequences in the 5' part and upstream region of the C1 and C2 genes and analysis of their transcripts. *Journal of Molecular Endocrinology* **3** 93-103.

Claessens F, Alen P, Devos A, Peeters B, Verhoeven G & Rombauts W 1996 The androgen-specific probasin response element 2 interacts differentially with androgen and glucocorticoid receptors. *Journal of Biological Chemistry* **271** 19013-19016.

Claessens F, Verrijdt G, Schoenmakers E, Haelens A, Peeters B, Verhoeven G & Rombauts W 2001 Selective DNA binding by the androgen receptor as a mechanism for hormone-specific gene regulation. *Journal of Steroid Biochemistry and Molecular Biology* **76** 23-30.

Cleutjens CB, Steketee K, van Eekelen CC, van der Korput JA, Brinkmann AO & Trapman J 1997 Both androgen receptor and glucocorticoid receptor are able to induce prostate-specific antigen expression, but differ in their growth-stimulating properties of LNCaP cells. *Endocrinology* **138** 5293-5300.

Cleutjens KB, van Eekelen CC, van der Korput HA, Brinkmann AO & Trapman J 1996 Two androgen response regions cooperate in steroid hormone regulated activity of the prostate-specific antigen promoter. *Journal of Biological Chemistry* **271** 6379-6388.

De Vos P, Claessens F, Winderickx J, Van Dijck P, Celis L, Peeters B, Rombauts W, Heyns W & Verhoeven G 1991 Interaction of androgen response elements with the DNA-binding domain of the rat androgen receptor expressed in Escherichia coli. *Journal of Biological Chemistry* **266** 3439-3443.

De Vos P, Claessens F, Celis L, Peeters B, Rombauts W, Heyns W & Verhoeven G 1994 Nuclear extracts enhance the interaction of fusion proteins containing the DNA-binding domain of the androgen and glucocorticoid receptor with androgen and glucocorticoid response elements. *Journal of Steroid Biochemistry and Molecular Biology* **48** 317-323.

Devos A, Claessens F, Alen P, Winderickx J, Heyns W, Rombauts W & Peeters B 1997 Identification of a functional androgen-response element in the exon 1-coding sequence of the cystatin-related protein gene crp2. *Molecular Endocrinology* **11** 1033-1043.

Geserick C, Meyer HA, Barbulescu K & Haendler B 2003 Differential modulation of androgen receptor action by deoxyribonucleic acid response elements. *Molecular Endocrinology* **17** 1738-1750.

Glass CK & Rosenfeld MG 2000 The coregulator exchange in transcriptional functions of nuclear receptors. *Genes and Development* **14** 121-141.

Haelens A, Verrijdt G, Callewaert L, Christiaens V, Schauwaers K, Peeters B, Rombauts W & Claessens F 2003 DNA recognition by the androgen receptor: evidence for an alternative DNA-dependent dimerization, and an active role of sequences flanking the response element on transactivation. *Biochemical Journal* **369** 141-151.

Hall JM, McDonnell DP & Korach KS 2002 Allosteric regulation of estrogen receptor structure, function, and coactivator recruitment by different estrogen response elements. *Molecular Endocrinology* **16** 469-486.

Ham J, Thomson A, Needham M, Webb P & Parker M 1988 Characterization of response elements for androgens, glucocorticoids and progestins in mouse mammary tumour virus. *Nucleic Acids Research* **16** 5263-5276.

Heinlein CA & Chang C 2002 Androgen receptor (AR) coregulators: an overview. *Endocrine Reviews* **23** 175-200.

Horoszewicz JS, Leong SS, Kawinski E, Karr JP, Rosenthal H, Chu TM, Mirand EA & Murphy GP 1983 LNCaP model of human prostatic carcinoma. *Cancer Research* **43** 1809-1818.

Karvonen U, Kallio PJ, Janne OA & Palvimo JJ 1997 Interaction of androgen receptors with androgen response element in intact cells. Roles of amino- and carboxyl-terminal regions and the ligand. *Journal of Biological Chemistry* **272** 15973-15979.

Kasper S, Rennie PS, Bruchovsky N, Sheppard PC, Cheng H, Lin L, Shiu RP, Snoek R & Matusik RJ 1994 Cooperative binding of androgen receptors to two DNA sequences is required for androgen induction of the probasin gene. *Journal of Biological Chemistry* **269** 31763-31769.

Kasper S, Rennie PS, Bruchovsky N, Lin L, Cheng H, Snoek R, Dahlman-Wright K, Gustafsson JA, Shiu RP, Sheppard PC & Matusik RJ 1999 Selective activation of the probasin androgen-responsive region by steroid hormones. *Journal of Molecular Endocrinology* **22** 313-325.

Khorasanizadeh S & Rastinejad F 2001 Nuclear-receptor interactions on DNA-response elements. *Trends in Biochemical Sciences* **26** 384-390.

Klein-Hitpass L, Tsai SY, Greene GL, Clark JH, Tsai MJ & O'Malley BW 1989 Specific binding of estrogen receptor to the estrogen response element. *Molecular and Cellular Biology* **9** 43-49.

Lee KC & Lee Kraus W 2001 Nuclear receptors, coactivators and chromatin: new approaches, new insights. *Trends in Endocrinology and Metabolism* **12** 191-197.

Lieberman BA, Bona BJ, Edwards DP & Nordeen SK 1993 The constitution of a progesterone response element. *Molecular Endocrinology* **7** 515-527.

Lombes M, Binart N, Oblin ME, Joulin V & Baulieu EE 1993 Characterization of the interaction of the human mineralocorticosteroid receptor with hormone response elements. *Biochemical Journal* **292** 577-583.

McKenna NJ & O'Malley BW 2002 Minireview: nuclear receptor coactivators--an update. *Endocrinology* **143** 2461-2465.

Nelson CC, Hendy SC, Shukin RJ, Cheng H, Bruchovsky N, Koop BF & Rennie PS 1999 Determinants of DNA sequence specificity of the androgen, progesterone, and glucocorticoid receptors: evidence for differential steroid receptor response elements. *Molecular Endocrinology* **13** 2090-2107.

Nordeen SK, Suh BJ, Kuhnel B & Hutchison CD 1990 Structural determinants of a glucocorticoid receptor recognition element. *Molecular Endocrinology* **4** 1866-1873.

Quandt K, Frech K, Karas H, Wingender E & Werner T 1995 MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Research* **23** 4878-4884.

Rennie PS, Bruchovsky N, Leco KJ, Sheppard PC, McQueen SA, Cheng H, Snoek R, Hamel A, Bock ME, MacDonald BS & et al. 1993 Characterization of two cis-acting DNA elements involved in the androgen regulation of the probasin gene. *Molecular Endocrinology* **7** 23-36.

Riegman PH, Vlietstra RJ, van der Korput JA, Brinkmann AO & Trapman J 1991 The promoter of the prostate-specific antigen gene contains a functional androgen responsive element. *Molecular Endocrinology* **5** 1921-1930.

Roche PJ, Hoare SA & Parker MG 1992 A consensus DNA-binding site for the androgen receptor. *Molecular Endocrinology* **6** 2229-2235.

Rusconi S & Yamamoto KR 1987 Functional dissection of the hormone and DNA binding activities of the glucocorticoid receptor. *EMBO Journal* **6** 1309-1315.

Sambrook J & Russell DW 2001 Molecular Cloning a laboratory manual, Third edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Scheller A, Hughes E, Golden KL & Robins DM 1998 Multiple receptor domains interact to permit, or restrict, androgen-specific gene activation. *Journal of Biological Chemistry* **273** 24216-24222.

Schuurmans AL, Bolt J, Voorhorst MM, Blankenstein RA & Mulder E 1988 Regulation of growth and epidermal growth factor receptor levels of LNCaP prostate tumor cells by different steroids. *International Journal of Cancer* **42** 917-922.

Strahle U, Boshart M, Klock G, Stewart F & Schutz G 1989 Glucocorticoid- and progesterone-specific effects are determined by differential expression of the respective hormone receptors. *Nature* **339** 629-632.

Thornton JW 2001 Evolution of vertebrate steroid receptors from an ancestral estrogen receptor by ligand exploitation and serial genome expansions. *Proceedings of the National Academy of Sciences U S A* **98** 5671-5676.

Verrijdt G, Schoenmakers E, Alen P, Haelens A, Peeters B, Rombauts W & Claessens F 1999 Androgen specificity of a response unit upstream of the human secretory component gene is mediated by differential receptor binding to an essential androgen response element. *Molecular Endocrinology* **13** 1558-1570.

Verrijdt G, Schoenmakers E, Haelens A, Peeters B, Verhoeven G, Rombauts W & Claessens F 2000 Change of specificity mutations in androgen-selective enhancers. Evidence for a role of differential DNA binding by the androgen receptor. *Journal of Biological Chemistry* **275** 12298-12305.

Wood JR, Likhite VS, Loven MA & Nardulli AM 2001 Allosteric modulation of estrogen receptor conformation by different estrogen response elements. *Molecular Endocrinology* **15** 1114-1126.

Young CY, Montgomery BT, Andrews PE, Qui SD, Bilhartz DL & Tindall DJ 1991 Hormonal regulation of prostate-specific antigen messenger RNA in human prostatic adenocarcinoma cell line LNCaP. *Cancer Research* **51** 3748-3752.

Chapter 4

Broadened ligand responsiveness of androgen receptor mutants obtained by random amino acid substitution of H874 and mutation hot spot T877 in prostate cancer

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ABSTRACT

In a subset of endocrine therapy resistant prostate cancers, amino acid substitutions H874Y, T877A and T877S, which broaden ligand specificity of the ligand binding domain (LBD) of the androgen receptor (AR), have been detected. To increase our knowledge about the role of amino acid substitutions at these specific positions in prostate cancer, codons 874 and 877 were subjected to random mutagenesis. AR mutants were screened in a yeast read out system for responsiveness to 5α-dihydrotestosterone, progesterone and dehydroepiandrosterone. At position 874, only the histidine to tyrosine substitution could broaden AR ligand specificity. At position 877, four ligand specificity broadening substitutions were found: T877A, T877S, T877C and T877G. The latter two were not found in prostate cancer. The AR mutants were tested in mammalian (Hep3B) cells for responsiveness to thirteen different ligands. All mutants displayed their own ligand specificity spectrum. Importantly, AR(H874Y) and AR(T877A) could be activated by cortisol. According to the three-dimensional structure of the AR LBD, T877 interacts directly with the 17β-hydroxyl-group of androgens. All amino acid substitutions identified at position 877 had smaller side chains than the threonine in the wild-type receptor, indicating that increased space in the ligand binding pocket is important in broadened ligand specificity. Because H874 does not interact directly with the ligand, its substitution by a tyrosine is expected to change the ligand binding pocket conformation indirectly. For T877C and T877G substitutions two point mutations are required, and for H874Y, T877A and T877S substitutions, only one point mutation is sufficient. This most likely explains that the latter three have been found in prostate cancer.

INTRODUCTION

Androgens (T and DHT) are essential for development and maintenance of the male phenotype. They mediate their function by activation of the AR, which is a member of the nuclear receptor family of transcription factors. The AR also plays a pivotal role in prostate tumor growth. Because growth of the majority of prostate cancers depends on continuous androgenic stimulation, therapy of metastatic disease is generally based on androgen withdrawal or blockade of AR function by antiandrogens. However, after an initial regression, essentially all tumors continue to grow.

Like other nuclear receptors, the AR displays a modular structure: a carboxy-terminal LBD, a central DBD, and an amino-terminal TAD. Upon ligand binding, the AR regulates transcription by binding to specific androgen response elements in regulatory regions of target genes. Together with coactivators, general transcription factors and RNA polymerase II, a stable transcription initiation complex is formed (see for reviews refs [1-3]). The size of the AR can be variable, due to variation in the length of poly-glutamine and poly-glycine stretches in the TAD. Amino acid numbering in this manuscript corresponds to an AR with a length of 919 amino acids, which is employed by The Androgen Receptor Gene Mutations Database (http://www.mcgill.ca/androgendb).

One of the causes of transition from androgen-dependent to apparent androgen-independent prostate tumor growth is modification of AR functioning. In a proportion of endocrine therapy resistant tumors, AR gene amplification has been detected.[4-6] This can lead to AR overexpression. Another mechanism, which directly affects AR function, can be activation of the AR by aberrant cross talk with other signal transduction pathways.[7-11] A third mechanism is

modification of AR properties by missense mutations. In a subgroup of endocrine therapy resistant prostate cancers, amino acid substitutions in the AR LBD have been found, which result in a broadened ligand response spectrum. The most common substitution, T877A, has first been described in the LNCaP prostate cancer cell line.[12] Subsequently, it was repeatedly found in prostate cancer tissue specimens of patients with advanced disease.[13-18] The T877A substitution renders the AR responsive to natural low affinity ligands and antiandrogens; T877S and H874Y substitutions, which have also been found in prostate cancer, induce similar properties to the AR.[12,17,19-23]

During the last years, the three dimensional structures of many nuclear receptor LBDs have been elucidated.[24-33] The crystallographic data revealed a three-layer structure composed of ten to twelve α-helices. Ligand binding induces a specific conformational change in the helical LBD structure, which makes it accessible to coactivators.[34,35] Antagonists induce a different LBD conformation than agonists, indicatig the importance of the LBD conformation for activation or inhibition of nuclear receptor function.[27,31,34]

Knowledge of the LBD structure is invaluable for explanation of the molecular and the biological effects of specific amino acid substitutions in the AR in prostate cancer.

Homology modeling predicted a three-dimensional structure of the AR LBD that is similar to other nuclear receptors.[36-38] The crystal structures of the DHT and R1881 complexed wild-type AR LBDs and DHT complexed T877A mutant AR LBD have recently been elucidated and confirmed most of the earlier assumptions.[39,40]

In this study we investigated the biological effects of amino acid substitutions at positions 874 and 877, which are both in helix 11 of the AR LBD.[36,37,39,41] We addressed the question whether in addition to H874Y, T877A and T877S other, as yet unidentified, amino acid substitutions at these positions could give rise to similar functional alterations. Previously, we have shown that both wild-type AR and AR(T877A) retained their ligand specificity in yeast.[42] Therefore, AR expression libraries with random mutations at codons 874 or 877 were screened for ligand specificity in a yeast read out system. AR mutants with an altered ligand specificity as identified in the yeast system, were analyzed in mammalian cells for their responsiveness to a large series of sex steroids, antiandrogens and adrenal steroids, including glucocorticoids.

MATERIAL AND METHODS

Hormones

DHT, ASD, Pg, E_2, DHEA, DEX, cortisol, aldosterone and TAA were purchased from Steraloids (Wilton, NH), R1881 (methyltrienolone) was from NEN (Boston, MA). CPA was a gift from Schering AG (Berlin, Germany), OH-Fl from Schering USA (Bloomfield, NJ), and bicalutamide (Casodex) from Zeneca Pharmaceuticals (Macclesfield, UK).

Construction of androgen receptor cDNA libraries with random mutation of codons 874 and 877

The yeast AR cDNA expression vector pG1ARII[42] was used to generate pG1ARIIΔ(863-919) as a cloning vector for the construction of the control AR expression vector pG1ARIII, and the AR expression libraries pG1ARIII(874X) and pG1ARIII(877X). All deletions and mutations were generated essentially as described.[43] First, a PCR fragment was synthesized utilizing pG1ARII as a template, with the forward primer 5'-CACTGAGGAGACAACCCAGAAGCT-3' and the reverse primer 5'-AAGACGTCGACTACGCGGCGCGCAATAGGCTGCACGG-3'. A

SalI restriction site in the reverse primer is boldfaced and underlined; a BssHII site in this primer is underlined. The amplified fragment was TthIII and SalI digested and exchanged with the corresponding AR fragment in pG1ARII, resulting in pG1ARIIΔ(863-919). To generate the pG1ARIII(874X) library, PCR mutagenesis was carried out on the pG1ARII template, utilizing the forward primer 874X: 5'-ATTGCGCGCGAGCTGNNNCAGTTCACTTTTGACCTG-3' (BssHII boldfaced and underlined, codon 874 underlined) combined with the reverse primer RP 5'-AAGACGTCGACCGGATCCGCTTCACTGGGTGTGG-3' (SalI boldfaced and underlined; BamHI underlined; stop codon boldfaced). The amplified fragment was BssHII-SalI digested and inserted in the corresponding sites in pG1ARIIΔ(863-919). The pG1ARIII(877X) AR cDNA library was generated by the same procedure, utilizing the forward primer 877X 5'-ATTGCGCGCGAGCTGCATCAGTTCNNNTTTGACCTGCTAATC-3' and the RP reverse primer. Similarly, PG1ARIII was generated, utilizing forward primer 5'-ATTGCGCGCGAGCTGCATCAGTTCAC-3' and the reverse primer RP, resulting in an AR cDNA expression vector with an internal BssHII site and a BamHI site in the slightly shorter 3'-UTR, as compared to pG1ARII. The internal BssHII site does not result in an altered AR amino acid composition. Random codon representation at codons 874 and 877 in the pG1ARIII(874X) and pG1ARIII(877X) libraries was verified by sequencing of fourteen clones of each library. In both libraries the sequenced clones were unique.

Yeast LacZ-reporter plasmids

The androgen inducible yeast integration vector pGRE3LacZi was constructed by insertion of a 100 bp HindIII-EcoRI fragment of pARE3tkCAT[44], containing a triple arranged repeat of the -174/-152 prostate specific antigen (PSA) promoter region, in the corresponding sites of pLacZi (Clontech, Palo Alto, CA). The androgen inducible yeast LacZ-reporter plasmid pUCΔSS-26X, containing a triple arranged 26 bp GRE oligonucleotide, was provided by Dr. Picard.[45]

Construction of mammalian androgen receptor expression plasmids

Mammalian AR expression plasmids pSVARIII, pSVARIII(H874Y), pSVARIII(T877A), pSVARIII(T877C), pSVARIII(T877G) and pSVARIII(T877S) were constructed by exchanging the TthIII-BamHI fragments of pG1ARIII(mutant) constructs with the corresponding fragment of pSVAR0.[46]

Yeast strains, growth and transformation

Yeast strain YM4271(GRE3LacZ) was utilized for AR cDNA library screening. YM4271(GRE3LacZ) was derived from YM4271 (Clontech, Palo Alto, CA) by integration of NcoI linearized pGRE3LacZi into its non-functional *ura* locus. Yeast strain BJ2168, a gift from Dr. Picard, was used for quantitative measurement of AR activity.[42] Yeast cells were grown in the appropriate selective media (0.67% w/v yeast nitrogen base without amino acids, 2% glucose, pH 5.8) supplemented with the required amino acids. Yeast transformation was carried out according to the lithium acetate method.[47]

Yeast screening of androgen receptor mutants

Approximately four hundred clones of YM4271(GRE3LacZ) transformed with pG1ARIII(874X) or pG1ARIII(877X) were grown on a master plate with the appropriate selective medium. After replica plating on Hybond-N filters (Amersham, Buckinghamshire, UK), colonies were grown for 16 h on the same medium supplemented with different hormones: DHT (10^{-8} M), Pg (10^{-7} M) and DHEA (10^{-6} M) or in the absence of hormone. Yeast colonies

were made permeable by freezing the filters in liquid nitrogen. Next, LacZ expression was visualized by incubation on Whatmann paper soaked in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) supplemented with 0.27% β-mercaptoethanol and 0.1% X-GAL.

AR expression plasmids were isolated from LacZ-positive yeast clones as described.[48] Plasmids were sequenced to identify specific mutations at codons 874 and 877 and to confirm proper PCR amplification of the inserted fragments in the pG1ARIII vectors.

Quantitative analysis of androgen receptor mutants in yeast

A liquid β-galactosidase assay was performed to quantify the activity of selected AR mutants, utilizing yeast strain BJ2168 containing the pUCΔSS-26X LacZ-reporter plasmid. Overnight cultures of yeast transformants grown in selective medium were diluted to OD_{600} of 0.3 in the same medium supplemented with ligand (DHT, Pg or DHEA) or without hormone and grown until an OD_{600} of approximately 1.0. Next, β-galactosidase activity was determined as described previously.[42]

Mammalian cell culture, transfection, and luciferase assay

Hep3B (human liver) cells were maintained in α minimal essential medium (α-MEM) supplemented with 5% fetal calf serum and antibiotics. Cells were seeded at a density of 5 x 10^4 cells/well (1.9 cm^2) and grown for 24 h.. Four h. prior to transfection, the medium was replaced by 250 µl α-MEM, supplemented with 5% charcoal-stripped fetal calf serum, antibiotics and one of the following hormones: DHT, R1881, ASD, Pg, E_2, OH-Fl, CPA, bicalutamide, DHEA, DEX, cortisol, aldosterone or TAA. For transfection, 25 µl α-MEM containing 1 µl Fugene 6 (Boehringer Mannheim, Germany), 0.5 µg AR expression plasmid (pSVARIII constructs), and 1 µg MMTV-LUC reporter plasmid were added per well. Following 24 h incubation, cells were lysed and luciferase activity was assayed as described previously.[49] In the absence of ligand, wild-type and mutant ARs displayed comparable background activities.

RESULTS

Detection of androgen receptor H874 and T877 mutants in a yeast screening system

Using random mutagenesis, two AR cDNA libraries were generated in a yeast expression vector: one with mutations in codon 874, AR(874X), and one with mutations in codon 877, AR(877X). Approximately four hundred independent yeast colonies from each library were screened for activation of an AR-inducible LacZ reporter by DHT, Pg and DHEA (see Materials and Methods). Pg was tested because H874Y, and T877 mutant ARs showed an increased response to this hormone.[12,19-23,50] DHEA was chosen because the H874Y and T877A mutant ARs were known to be responsive to this adrenal androgen.[21] In each library, approximately two hundred out of the four hundred colonies were β-galactosidase positive upon incubation with DHT, indicating that the other half of the colonies contained inactivating AR mutations.

Screening of the AR(874X) library resulted in four yeast colonies, which were positive after DHT, Pg and DHEA incubation. Colonies positive with two tested hormones, or with Pg or DHEA alone were not found. Sequencing revealed that the DHT+/Pg+/DHEA+ colonies contained at codon 874 the sequences TAC or TAT, which both encode a tyrosine residue. From this finding it was concluded that a tyrosine residue at position 874 is unique in the generation of an AR, which can not only be activated by DHT, but also by Pg and by DHEA.

Screening of the AR(877X) library resulted in sixty-three colonies, which were DHT+/Pg+/DHEA+; fourteen colonies were DHT+/Pg+/DHEA-. Sequencing of the DHT+/Pg+/DHEA+ colonies revealed three different amino acid substitutions at 877: T877A, T877G and T877S. The DHT+/Pg+/DHEA- colonies contained a cysteine residue at 877. Interestingly, as described above, H874Y, T877A and T877S have been found in prostate cancer, whereas T877C and T877G substitutions have not been detected in these tumors.

Hormone induced transcriptional activity of the androgen receptor H874Y mutant in yeast and in mammalian cells

Activation of AR(H874Y) by DHT, Pg and DHEA was quantified in a yeast liquid β-galactosidase assay. The results are summarized in Figure 1. AR(H874Y) showed a decrease in AR activation by DHT, as compared to wild-type AR (Fig. 1*a*). At the highest hormone concentrations, Pg and DHEA responses of AR(H874Y) were clearly stronger than that of wild-type AR (Fig. 1*b*,*c*).

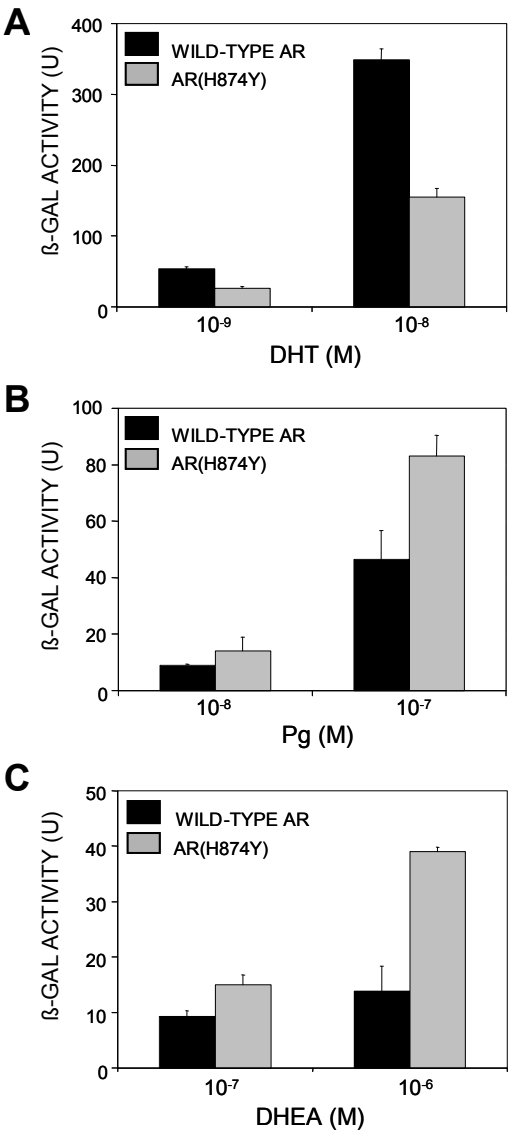

FIGURE 1 — Transcriptional activity of wild-type AR and AR(H874Y) in yeast. Yeast cells were cotransfected with the wild-type AR or AR(H874Y) yeast expression vector and the androgen inducible LacZ reporter pUCΔSS-26X. *(a)* DHT activation. *(b)* Pg activation. *(c)* DHEA activation. Values (±SEM) are the mean of three independent experiments each carried out in duplicate.

For direct comparison with the yeast data, Hep3B mammalian cells were cotransfected with the AR(H874Y) mutant or wild-type AR expression plasmid and a MMTV-luciferase reporter plasmid. Transfected cells were incubated in the absence of hormone or in the presence of serial dilutions of a large set of different ligands: the androgens DHT, and R1881; the steroidal antiandrogen CPA, the non-steroidal antiandrogens OH-Fl and bicalutamide, and the steroids Pg and E_2. R1881 activated wild-type AR and AR(H874Y) equally; bicalutamide was inactive on both wild-type AR and AR(H874Y) (data not shown). The activities of wild-type AR and AR(H874Y) induced by DHT, Pg, E_2, OH-Fl and CPA are shown in Figures 2*a* and 2*b*, respectively. As expected, wild-type AR activation was DHT specific. Only at high concentrations, some agonistic activity of Pg, E_2 and CPA was observed; OH-Fl was unable to activate the wild-type AR at all concentrations tested. Like in yeast, DHT was found to be a less potent activator of AR(H874Y) than of wild-type AR, which was not due to a lower expression level (data not shown). Both E_2 and Pg induced AR(H874Y) activity to almost the same extent as DHT. At high concentrations, OH-Fl showed some agonistic activity; agonistic activity of CPA on AR(H874Y) was as low as on wild-type AR.

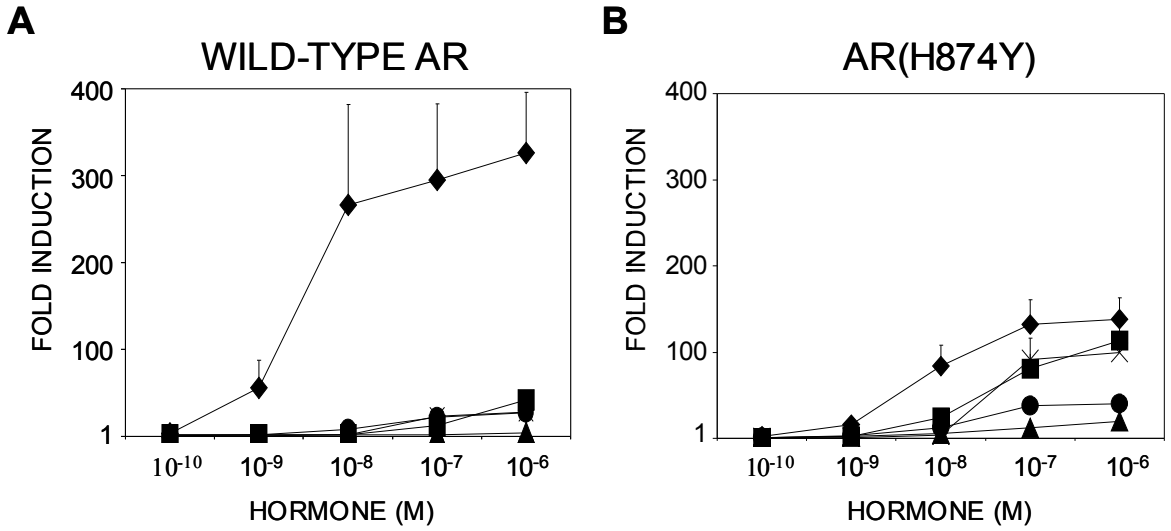

FIGURE 2 — Transcriptional activity of wild-type AR and AR(H874Y) in mammalian cells. Hep3B cells were cotransfected with the wild-type AR or AR(H874Y) mammalian expression vector and a MMTV-LUC reporter, and activated by different hormones. *(a)* wild-type AR. *(b)* AR(H874Y). Ligands: (◆) DHT, (■) Pg, (▯) E_2, (●) CPA, (⬧) OH-Fl. Values (±SEM) represent the mean of three independent experiments each carried out in duplicate.

Hormone induced transcriptional activity of the androgen receptor T877 mutants in yeast and mammalian cells

Activation of the AR 877 mutants by DHT, Pg and DHEA was analyzed in the quantitative yeast assay as described above for AR(H874Y). The results are summarized in Figure 3. DHT activation of all mutants, AR(T877A), AR(T877S), AR(T877G) and AR(T877C), was comparable to wild-type AR (Fig. 3*a*). AR(T877A) and AR(T877S) displayed the most prominent altered ligand specificity at the two Pg and DHEA concentrations tested. Both showed increased activation by Pg and DHEA as compared to wild-type AR (Fig. 3*b*,*c*). AR(T877C) and AR(T877G) were activated by Pg (Fig. 3*b*), but only AR(T877G) was DHEA-inducible (Fig. 3*c*), in agreement with the qualitative yeast screening.

Ligand specificity studies of the four AR 877 mutants were extended to mammalian Hep3B cells, using the same set of ligands as used for the wild-type AR and AR(H874Y) studies shown

in Figure 2. R1881 activation, which was identical for wild-type AR and the four 877 mutants, is not shown. Bicalutamide did not activate any of the 877 mutants (data not shown). The T877A and T877S substitutions introduced the most dramatic alterations in ligand specificity (Fig. 4; see for wild-type AR Fig. 2*a*). Both AR(T877A) and AR(T877S) exhibited a strong activation by Pg, E_2 and the antiandrogen CPA (Fig. 4*a*,*d*). AR(T877A) was more responsive to OH-Fl than AR(T877S) (Fig. 4*a*,*d*). AR(T877C) and AR(T877G) ligand specificity was less altered. Although Pg activation could clearly be established, agonistic activity of other ligands was limited (Fig. 4*b*,*c*). Differences in ligand responses between the AR mutants were not due to different protein levels (data not shown).

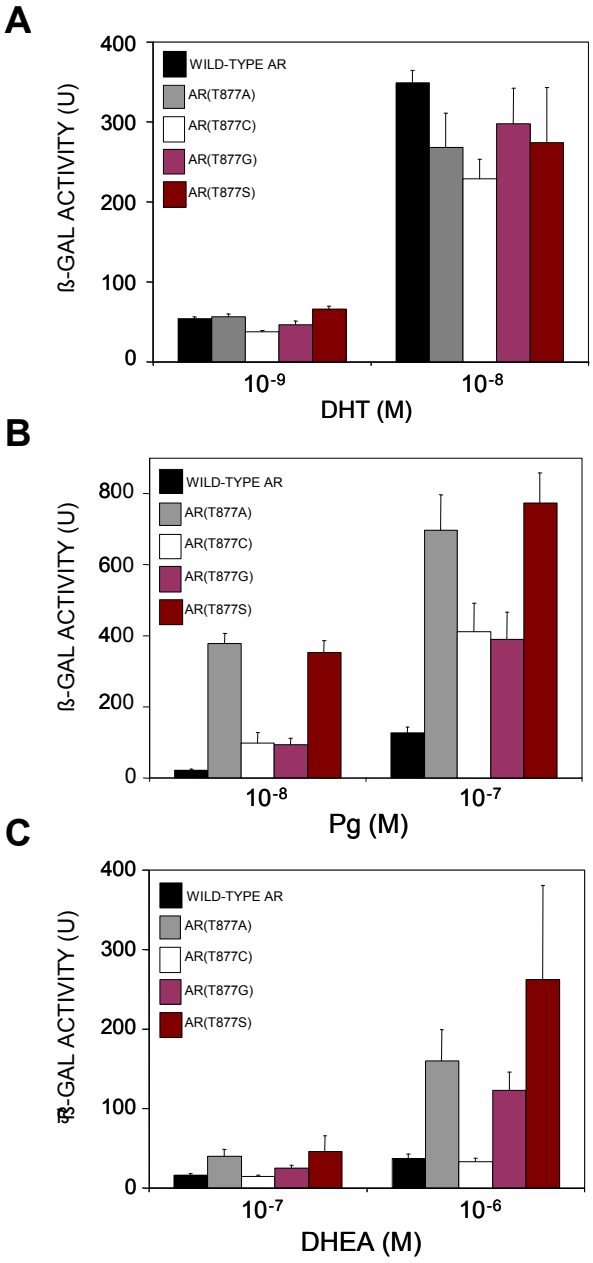

FIGURE 3 — Transcriptional activity of wild-type AR and AR(T877) mutants in yeast. Yeast cells were cotransfected with wild-type AR yeast expression vector, or AR(T877A), or AR(T877C), or AR(T877G), or AR(T877S) mutant yeast expression vectors and the androgen inducible LacZ-reporter pUCΔSS-26X. *(a)* DHT activation. *(b)* Pg activation. *(c)* DHEA activation. Values (±SEM) represent the mean of three independent experiments each carried out in duplicate.

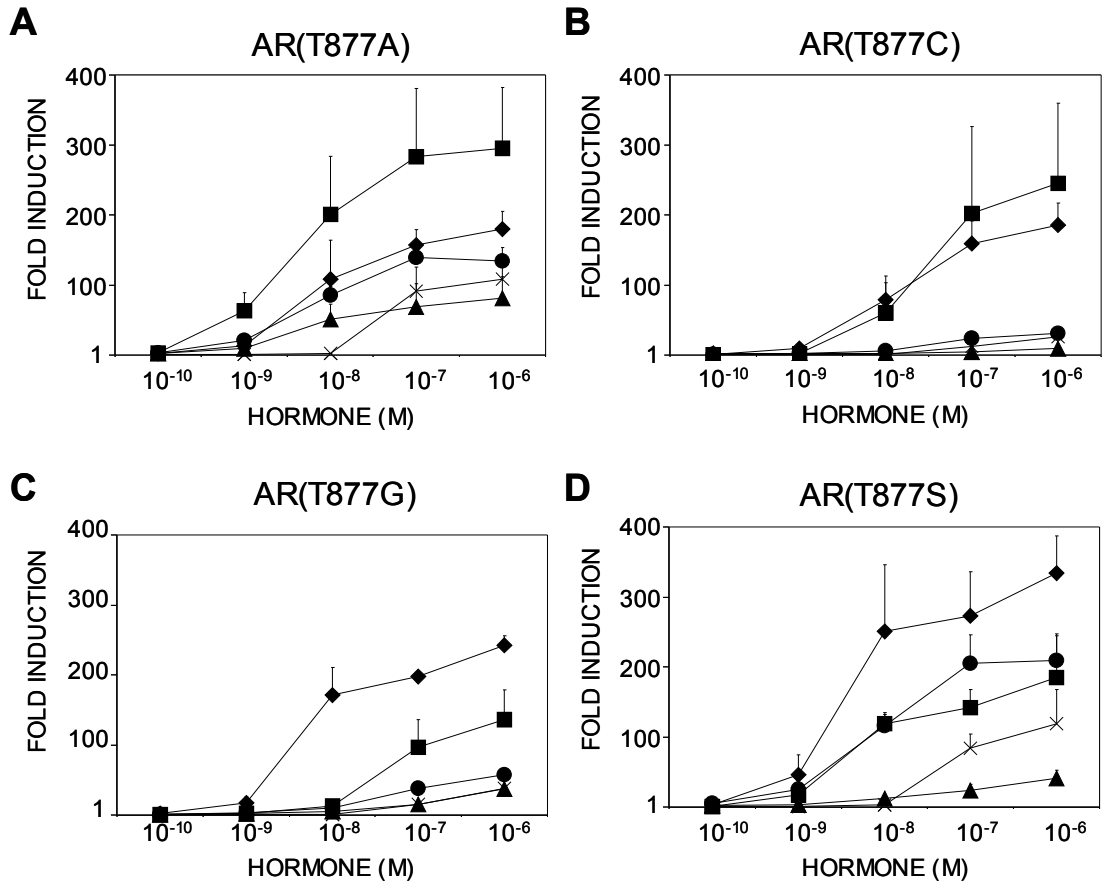

FIGURE 4 — Transcriptional activity of AR(T877) mutants in mammalian cells. Hep3B cells were cotransfected with *(a)* AR(T877A), *(b)* AR(T877C), *(c)* AR(T877G), and *(d)* AR(T877S) mammalian expression vector and a MMTV-LUC reporter. Ligands: (◆) DHT, (■) Pg, (▥) E_2, (●) CPA, (⬧) OH-Fl. Values (±SEM) represent the mean of three independent experiments each carried out in duplicate.

Transcriptional activation of androgen receptor H874 and T877 mutants by adrenal steroids and synthetic glucocorticoids

The AR 874 and AR 877 mutants were also assayed in Hep3B cells for their activation by the adrenal steroids DHEA and ASD (androgens), cortisol (glucocorticoid) and aldosterone (mineralocorticoid), and the synthetic glucocorticoids DEX and TAA. Activation of wild-type AR and all mutant ARs by ASD was identical; TAA was unable to activate wild-type and mutant ARs (data not shown). For the other ligands a remarkable variation in activation patterns of the different mutants was observed. Figure 5*a* displays the activities of wild-type AR and all AR mutants induced by high concentrations DHEA, cortisol, DEX and aldosterone (10^{-6} M). In Figure 5*b-e* the ligand concentration dependent activation of selected mutants is shown. AR(H874Y), AR(T877A) and AR(T877S) were clearly responsive to DHEA (Fig. 5*a*,*b*). Also, a concentration dependent activation of AR(H874Y) and AR(T877A) by aldosterone, cortisol and DEX was observed (see Fig. 5*a*,*c-e*). In contrast, AR(T877S) and AR(T877G) could hardly be activated by these ligands (Fig. 5*a*). AR(T877C) did not respond to any of the ligands (Fig. 5*a*).

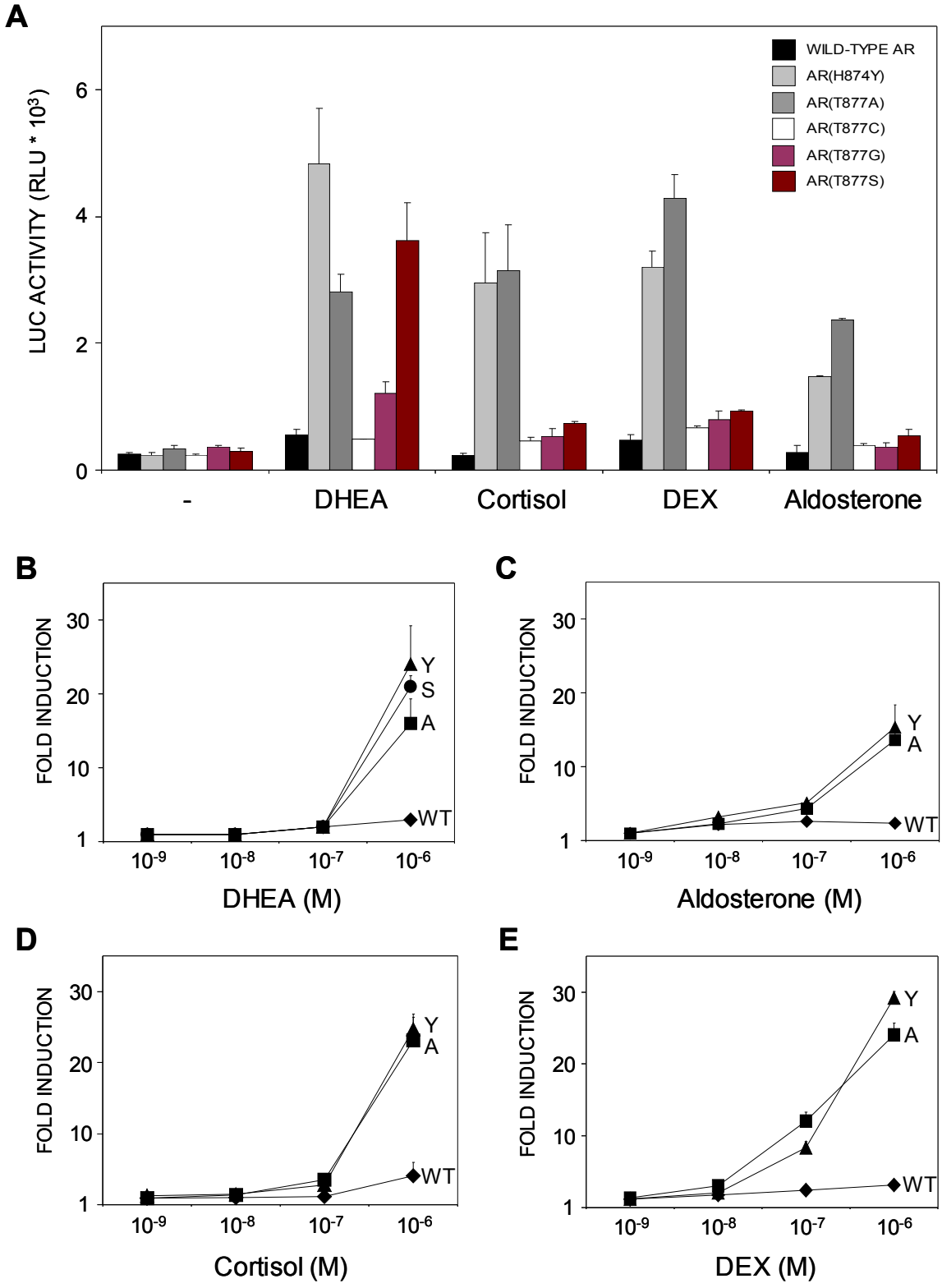

FIGURE 5 — DHEA and corticoid induced transcriptional activity of wild-type AR and AR mutants. Hep3B cells were cotransfected with the wild-type AR, AR(H874Y) or AR(T877) mutant mammalian expression vector and a MMTV-LUC reporter. *(a)* incubation in the absence of hormone, or in the presence of DHEA, Cortisol, DEX or ALD (all at 10^{-6} M). *(b)* DHEA activation. *(c)* ALD activation. *(d)* Cortisol activation. *(e)* DEX activation. (◆)WT: wild-type AR, (♦)Y: AR(T874Y), (■)A: AR(T877A), and (●)S: AR(T877S). Values (±SEM) represent the mean of three independent experiments each carried out in duplicate.

TABLE 1 – LIGAND RESPONSIVENESS[1] OF AR MUTANTS TO THIRTEEN DIFFERENT HORMONES AS TESTED ON A MMTV-LUC REPORTER IN TRANSIENTLY TRANSFECTED MAMMALIAN (HEP3B) CELLS

	wild-type AR	AR (H874Y)	AR (T877A)	AR (T877C)	AR (T877G)	AR (T877S)
androgens						
DHT	+++	+++	+++	+++	+++	+++
R1881	+++	+++	+++	+++	+++	+++
sex steroids						
Pg	+	++	+++	+++	++	+++
E_2	+	++	++	+	+	++
antiandrogens						
CPA	+	+	+++	+	+	+++
OH-Fl	-	+	++	-	+	+
Bicalutamide	-	-	-	-	-	-
adrenal androgens						
DHEA	-	+	+	-	±	+
ASD	++	++	++	++	++	++
glucocorticoids						
Cortisol	-	+	+	-	-	-
DEX	-	+	+	-	-	-
TAA	-	-	-	-	-	-
mineralocorticoid						
Aldosterone	-	+	+	-	-	-

[1]Degree of ligand responsiveness: - no activity; + low activity; ++ moderate acivity; +++ high activity, comparable to wild-type AR responsiveness to DHT.

DISCUSSION

Mutations in the AR have been described in several diseases. In androgen insensitivity, which is an inherited defect of male development, over one hundred amino acid substitutions in the AR LBD have been documented (http://www.mcgill.ca/androgendb).[1,51] These mutations completely or partially inactivate AR function. In Kennedy's disease or SBMA (spinal and bulbar muscular atrophy), an expanded $(CAG)_n$ repeat results in a longer glutamine-stretch in the AR TAD.[52] In prostate cancer, AR mutants are rare in primary and locally progressive tumors, but more frequent in metastatic disease, following endocrine therapy.[16-18,22,53-57] The relevance of most AR mutants in progressive prostate cancer remains to be established. The mutants investigated in more detail are functionally different from AR mutants in androgen insensitivity, and tend to cluster in different regions of the LBD.[57]

The most frequently described AR mutations in prostate cancer are substitutions of H874 and T877, which are both in helix 11 of the AR LBD.[36,37,39,41] AR(T877A), which has originally been detected in the LNCaP cell line, seems to be the preferred amino acid substitution in endocrine therapy resistant prostate cancer.[12,14-17] Like the less common H874Y and T877S amino acid substitutions, T877A broadens AR ligand specificity in such a manner, that not only androgens, but also other sex steroids, and antiandrogens can activate the AR.[12,19-23] The recently identified T877A&L701H AR double mutant exhibited an even broader ligand specificity than the T877A single mutant,[58] adding cortisol to the spectrum of strong activators. There is increasing evidence that the AR LBD mutants with less specific ligand responsiveness are of clinical relevance in a subset of endocrine therapy resistant prostate cancers.[16,17]

In the present study two types of experiments were carried out. First, ARs randomly mutated at positions 874 or 877 were screened for broadened ligand specificity in a yeast read out system. Second, AR mutants with broadened ligand responsiveness were assayed in mammalian cells for activation by thirteen different ligands, including sex steroids, adrenal steroids and antiandrogens. From our findings, several important conclusions can be drawn.

1. In the AR cDNA library randomly mutated at codon 874, AR(H874Y) was the only mutant able to broaden AR ligand specificity. In the 877 AR cDNA library, an alanine, serine, glycine or cysteine residue at position 877 broadened AR ligand response. As pointed out above, AR(H874Y), AR(T877A) and AR(T877S) are well known from prostate cancer; AR(T877G) and AR(T877C) have never been described in prostate cancer. The random mutagenesis system used in this study allowed the screening of all triplets possible for codons 874 and 877. As expected, among the identified AR mutants, one, two and three base deviations from the wild-type codon were detected. In nature, the chance of more than one point mutation within a codon is extremely low. Indeed, all amino acid substitutions found at AR codons 874 and 877 in prostate cancer are due to single point mutations: H874Y: CAT>TAT,[17,21,22] T877A: ACT>GCT,[12-17,22] T877S: ACT>TCT.[17,22] For T877C or T877G substitutions, at least two bases need to be mutated. This can explain their absence in prostate cancer. In conclusion, the H874Y substitution is not only unique at this position in prostate cancer, but is also the only possibility at this position to broaden AR ligand-specificity. The T877A and T877S substitutions in prostate cancer are not unique, in that they are not the only substitutions that can broaden AR ligand-specificity at this position, but seem to be sequence-driven selections of four possible amino acid substitutions.
2. The results of the extensive series of transactivation experiments with wild-type and mutated ARs in mammalian cells are summarized in Table 1. Importantly, each mutant displayed its own characteristic spectrum of ligand responsiveness. Differences in ligand affinities, as well as differences in ligand-induced conformational changes may account for this variation. Most remarkable are the similarities between activation of AR(H874Y) and AR(T877A) by the various ligands. Although completely different, both show identical responses to the glucocorticoids cortisol and DEX, and the mineralocorticoid aldosterone. Zhao *et al.*[58] described activation of the AR double mutant T877A&L701H by cortisol, but they did not find cortisol responsiveness of the single mutant AR(T877A). The apparent discrepancy with our data might be due to a less sensitive assay, or different cell line used for transfection experiments. Our findings warrant a further investigation of the role of glucocorticoids in prostate cancer patients carrying a mutated AR.

Based on the crystal structures of closely related steroid hormone receptor LBDs, homology models of the AR LBD have been constructed.[36-38] These models indicated that T877 is part of the ligand-binding pocket and directly interacts with the ligand, H874 does not participate directly in ligand binding. Recently, the predictions of AR LBD folding and ligand interaction were modified and extended by the elucidation of the crystal structures of the wild-type and T877A mutant AR LBD complexed with androgens.[39,40] For the wild-type AR LBD, eighteen amino acids were found to contact the ligand directly. Importantly, T877 in helix 11 of the LBD, together with N705 in helix 3, form hydrogen bonds to the 17β-hydroxyl group of R1881, which is also present in DHT (Fig. 6). As predicted, H874, which is also in helix 11, projects away from the ligand binding pocket.

The crystal structure of the T877A mutant AR LBD complexed with DHT revealed an increased space in the ligand binding pocket.[40] The amino acid residues serine, glycine and cysteine at position 877, have in common that, like alanine, they all are smaller than the

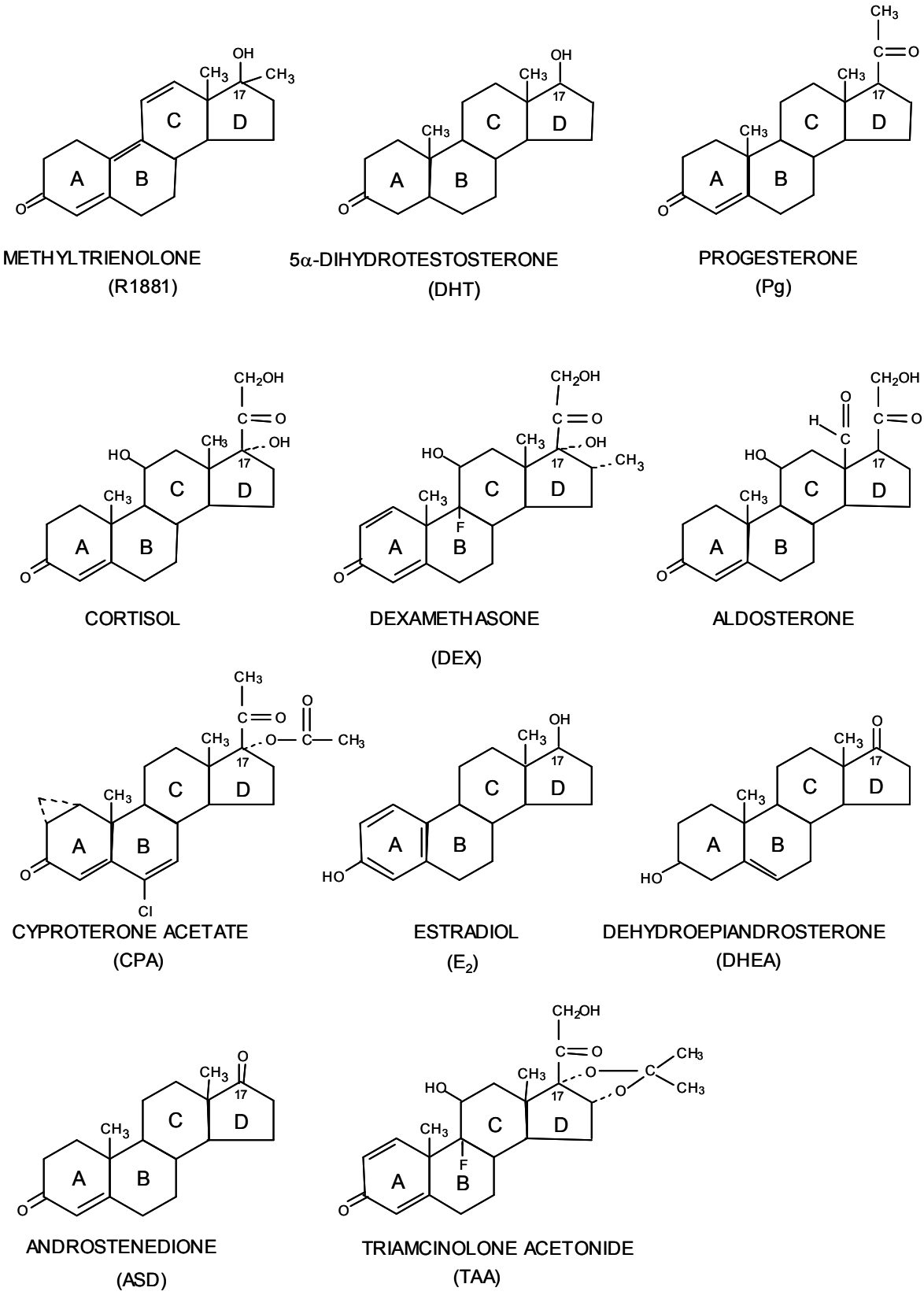

FIGURE 6 — Chemical structures of steroids used in this study.

threonine residue at this position in the wild-type AR.[59] So, substitution of T877 by S, G or C will also increase the space of the ligand binding pocket. This larger space will facilitate appropriate entering by ligands with more bulky side chains at C17 like Pg, cortisol, DEX and aldosterone (Fig. 6). This may allow a conformational change in the LBD, which is favorable for the AR transactivation function. A larger binding pocket may also explain appropriate folding of the LBD induced by the antiandrogen CPA, resulting in agonistic activity. The synthetic glucocorticoid TAA might be too big for proper entering an enlarged AR ligand binding pocket (Fig. 6). Not only the size of the C17 side chain, but also slight differences in overall conformation of a steroid, as determined by the A- to D-ring moities, might contribute to positioning in the ligand binding pocket. Particularly in case of E_2 and DHEA, which both have a small C17 side chain (Fig. 6), but not excluding other ligands, the larger binding pocket might be needed for appropriate binding of these different conformations. Elucidation of the crystal structures of the various AR mutants complexed with different ligands has to prove and extend these hypotheses.

Many other mutations have been described in the AR LBD in prostate cancer, but most of the mutants have not been characterized.[22,55,56,60,61] In contrast to L701 and T877, none of the mutated amino acid residues can be predicted to contact directly the ligand.[39,40] This is also true for mutant V715M, which clearly displays broadened ligand response.[23,61,62] So, like for H874Y, for the V715M mutant a different mechanism of activation can be predicted.

One of the most important questions, which remain to be addressed, is the identification of the physiological ligand of the AR mutants in endocrine therapy resistant prostate cancer. Most AR LBD mutants with a broadened ligand response seem to be induced or selected during antiandrogen therapy.[17] Our findings suggest that in androgen depleted patients following antiandrogen withdrawal, Pg or cortisol might be the physiological ligands for activation of a mutated AR. The concentration of circulating Pg in men (0.3-0.9 nM)[63] seems sufficient for such a function, because of the strong response of especially AR(T877A) and AR(T877S) to this ligand (Fig. 4). Although cortisol is a less potent activator of the mutants (Fig. 5), its high concentration in the circulation (70-550 nM)[63] warrants further investigation of its role in patients carrying a AR(H874Y) or AR(T877A) mutation. Activation of these mutants by cortisol would be in line with activation of the AR(L701H) single and AR(L701H/T877A) double mutant by cortisol.[58] Circulating E_2, DHEA and aldosterone concentrations (73-184 pM, 6-28 nM and 83-832 pM, respectively),[63] seem too low to account for such a function. However, inactive DHEA sulfate, with a serum level of 1-9 μM,[63] can be converted into DHEA in the prostate, and resulting local DHEA concentrations may be sufficient for activation of the various AR mutants (Fig. 5, Table 1).[64]

To obtain relevant data on non-cognate ligands as activators of mutant ARs, extended studies with prostate cell lines containing mutated ARs are needed. In a previous study with DEX incubated LNCaP cells, applied hormone concentrations were too low to be able to observe an effect on the endogenous AR(T877A).[65] Monitoring of the response of LNCaP (T877A) and CWR22 (H874Y)[12,21,66] cells to physiological concentrations of different ligands, including glucocorticoids and DHEA, will give important supportive information about the role of mutated ARs in prostate cancer.

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***Abbreviations**:* AR, androgen receptor; ASD, androstenedione; CPA, cyproterone acetate; DBD, DNA binding domain; DEX, dexamethasone; DHEA, dehydroepiandrosterone; DHT, 5α-dihydrotestosterone; E_2, estradiol; LBD, ligand binding domain; OH-Fl, hydroxy-flutamide; Pg, progesterone; T, testosterone; TAA, triamcinolone acetonide; TAD, transactivation domain.

REFERENCES

1. Quigley CA, De Bellis A, Marschke KB, el-Awady MK, Wilson EM, French FS. Androgen receptor defects: historical, clinical, and molecular perspectives [published erratum appears in Endocr Rev 1995 Aug;16(4):546]. Endocr Rev 1995;16:271-321.
2. Trapman J, Cleutjens KB. Androgen-regulated gene expression in prostate cancer. Semin Cancer Biol 1997;8:29-36.
3. Brinkmann AO, Blok LJ, de Ruiter PE, Doesburg P, Steketee K, Berrevoets CA, Trapman J. Mechanisms of androgen receptor activation and function. J Steroid Biochem Mol Biol 1999;69:307-13.
4. Visakorpi T, Hyytinen E, Koivisto P, Tanner M, Keinanen R, Palmberg C, Palotie A, Tammela T, Isola J, Kallioniemi OP. In vivo amplification of the androgen receptor gene and progression of human prostate cancer. Nat Genet 1995;9:401-6.
5. Koivisto P, Kononen J, Palmberg C, Tammela T, Hyytinen E, Isola J, Trapman J, Cleutjens K, Noordzij A, Visakorpi T, Kallioniemi OP. Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. Cancer Res 1997;57:314-9.
6. Linja MJ, Savinainen KJ, Saramaki OR, Tammela TL, Vessella RL, Visakorpi T. Amplification and overexpression of androgen receptor gene in hormone- refractory prostate cancer. Cancer Res 2001;61:3550-5.
7. Culig Z, Hobisch A, Cronauer MV, Radmayr C, Trapman J, Hittmair A, Bartsch G, Klocker H. Androgen receptor activation in prostatic tumor cell lines by insulin- like growth factor-I, keratinocyte growth factor, and epidermal growth factor. Cancer Res 1994;54:5474-8.
8. Hobisch A, Eder IE, Putz T, Horninger W, Bartsch G, Klocker H, Culig Z. Interleukin-6 regulates prostate-specific protein expression in prostate carcinoma cells by activation of the androgen receptor. Cancer Res 1998;58:4640-5.
9. Craft N, Shostak Y, Carey M, Sawyers CL. A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. Nat Med 1999;5:280-5.
10. Abreu-Martin MT, Chari A, Palladino AA, Craft NA, Sawyers CL. Mitogen-activated protein kinase kinase kinase 1 activates androgen receptor-dependent transcription and apoptosis in prostate cancer. Mol Cell Biol 1999;19:5143-54.
11. Peterziel H, Mink S, Schonert A, Becker M, Klocker H, Cato AC. Rapid signalling by androgen receptor in prostate cancer cells. Oncogene 1999;18:6322-9.
12. Veldscholte J, Ris-Stalpers C, Kuiper GG, Jenster G, Berrevoets C, Claassen E, van Rooij HC, Trapman J, Brinkmann AO, Mulder E. A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. Biochem Biophys Res Commun 1990;173:534-40.
13. Suzuki H, Sato N, Watabe Y, Masai M, Seino S, Shimazaki J. Androgen receptor gene mutations in human prostate cancer. J Steroid Biochem Mol Biol 1993;46:759-65.
14. Gaddipati JP, McLeod DG, Heidenberg HB, Sesterhenn IA, Finger MJ, Moul JW, Srivastava S. Frequent detection of codon 877 mutation in the androgen receptor gene in advanced prostate cancers. Cancer Res 1994;54:2861-4.
15. Kleinerman DI, Troncoso P, Pisters LL, Navone NM, Hsieh J-T, Logothetis CJ, Sleddens HFBM, Van der Kwast TH, Brinkmann AO, Schroder FH, Trapman J. Expression and structure of the androgen receptor in bone metastasis of hormone refractory prostate cancer. Proc. Am. Urol. Assoc. 1996;155:624A.
16. Suzuki H, Akakura K, Komiya A, Aida S, Akimoto S, Shimazaki J. Codon 877 mutation in the androgen receptor gene in advanced prostate cancer: relation to antiandrogen withdrawal syndrome. Prostate 1996;29:153-8.
17. Taplin ME, Bubley GJ, Ko YJ, Small EJ, Upton M, Rajeshkumar B, Balk SP. Selection for androgen receptor mutations in prostate cancers treated with androgen antagonist. Cancer Res 1999;59:2511-5.
18. Zhao XY, Boyle B, Krishnan AV, Navone NM, Peehl DM, Feldman D. Two mutations identified in the androgen receptor of the new human prostate cancer cell line MDA PCa 2a. J Urol 1999;162:2192-9.
19. Veldscholte J, Berrevoets CA, Brinkmann AO, Grootegoed JA, Mulder E. Anti-androgens and the mutated androgen receptor of LNCaP cells: differential effects on binding affinity, heat-shock protein interaction, and transcription activation. Biochemistry 1992;31:2393-9.
20. Veldscholte J, Berrevoets CA, Ris-Stalpers C, Kuiper GG, Jenster G, Trapman J, Brinkmann AO, Mulder E. The androgen receptor in LNCaP cells contains a mutation in the ligand binding domain which affects steroid binding characteristics and response to antiandrogens. J Steroid Biochem Mol Biol 1992;41:665-9.
21. Tan J, Sharief Y, Hamil KG, Gregory CW, Zang DY, Sar M, Gumerlock PH, deVere White RW, Pretlow TG, Harris SE, Wilson EM, Mohler JL, et al. Dehydroepiandrosterone activates mutant androgen receptors expressed in the androgen-dependent human prostate cancer xenograft CWR22 and LNCaP cells. Mol Endocrinol 1997;11:450-9.
22. Taplin ME, Bubley GJ, Shuster TD, Frantz ME, Spooner AE, Ogata GK, Keer HN, Balk SP. Mutation of the androgen-receptor gene in metastatic androgen- independent prostate cancer. N Engl J Med 1995;332:1393-8.
23. Fenton MA, Shuster TD, Fertig AM, Taplin ME, Kolvenbag G, Bubley GJ, Balk SP. Functional characterization of mutant androgen receptors from androgen- independent prostate cancer. Clin Cancer Res 1997;3:1383-8.
24. Bourguet W, Ruff M, Chambon P, Gronemeyer H, Moras D. Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-alpha. Nature 1995;375:377-82.

25. Renaud JP, Rochel N, Ruff M, Vivat V, Chambon P, Gronemeyer H, Moras D. Crystal structure of the RAR-gamma ligand-binding domain bound to all- trans retinoic acid. Nature 1995;378:681-9.
26. Wagner RL, Apriletti JW, McGrath ME, West BL, Baxter JD, Fletterick RJ. A structural role for hormone in the thyroid hormone receptor. Nature 1995;378:690-7.
27. Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson JA, Carlquist M. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 1997;389:753-8.
28. Williams SP, Sigler PB. Atomic structure of progesterone complexed with its receptor. Nature 1998;393:392-6.
29. Uppenberg J, Svensson C, Jaki M, Bertilsson G, Jendeberg L, Berkenstam A. Crystal structure of the ligand binding domain of the human nuclear receptor PPARgamma. J Biol Chem 1998;273:31108-12.
30. Moras D, Gronemeyer H. The nuclear receptor ligand-binding domain: structure and function. Curr Opin Cell Biol 1998;10:384-91.
31. Pike AC, Brzozowski AM, Hubbard RE, Bonn T, Thorsell AG, Engstrom O, Ljunggren J, Gustafsson JA, Carlquist M. Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. Embo J 1999;18:4608-18.
32. Rochel N, Wurtz JM, Mitschler A, Klaholz B, Moras D. The crystal structure of the nuclear receptor for vitamin D bound to its natural ligand. Mol Cell 2000;5:173-9.
33. Bourguet W, Germain P, Gronemeyer H. Nuclear receptor ligand-binding domains: three-dimensional structures, molecular interactions and pharmacological implications. Trends Pharmacol Sci 2000;21:381-8.
34. Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, Greene GL. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 1998;95:927-37.
35. Nolte RT, Wisely GB, Westin S, Cobb JE, Lambert MH, Kurokawa R, Rosenfeld MG, Willson TM, Glass CK, Milburn MV. Ligand binding and co-activator assembly of the peroxisome proliferator- activated receptor-gamma. Nature 1998;395:137-43.
36. McDonald S, Brive L, Agus DB, Scher HI, Ely KR. Ligand responsiveness in human prostate cancer: structural analysis of mutant androgen receptors from LNCaP and CWR22 tumors. Cancer Res 2000;60:2317-22.
37. Poujol N, Wurtz JM, Tahiri B, Lumbroso S, Nicolas JC, Moras D, Sultan C. Specific recognition of androgens by their nuclear receptor. A structure-function study. J Biol Chem 2000;275:24022-31.
38. Marhefka CA, Moore BM, 2nd, Bishop TC, Kirkovsky L, Mukherjee A, Dalton JT, Miller DD. Homology modeling using multiple molecular dynamics simulations and docking studies of the human androgen receptor ligand binding domain bound to testosterone and nonsteroidal ligands. J Med Chem 2001;44:1729-40.
39. Matias PM, Donner P, Coelho R, Thomaz M, Peixoto C, Macedo S, Otto N, Joschko S, Scholz P, Wegg A, Basler S, Schafer M, et al. Structural evidence for ligand specificity in the binding domain of the human androgen receptor. Implications for pathogenic gene mutations. J Biol Chem 2000;275:26164-71.
40. Sack JS, Kish KF, Wang C, Attar RM, Kiefer SE, An Y, Wu GY, Scheffler JE, Salvati ME, Krystek SR, Jr., Weinmann R, Einspahr HM. 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 2001;98:4904-9.
41. Brinkmann AO, Trapman J. Prostate cancer schemes for androgen escape. Nat Med 2000;6:628-9.
42. Doesburg P, Kuil CW, Berrevoets CA, Steketee K, Faber PW, Mulder E, Brinkmann AO, Trapman J. Functional in vivo interaction between the amino-terminal, transactivation domain and the ligand binding domain of the androgen receptor. Biochemistry 1997;36:1052-64.
43. Higuchi R, Krummel B, Saiki RK. A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res 1988;16:7351-67.
44. Riegman PH, Vlietstra RJ, van der Korput JA, Brinkmann AO, Trapman J. The promoter of the prostate-specific antigen gene contains a functional androgen responsive element. Mol Endocrinol 1991;5:1921-30.
45. Schena M, Picard D, Yamamoto KR. Vectors for constitutive and inducible gene expression in yeast. Methods Enzymol 1991;194:389-98.
46. Brinkmann AO, Faber PW, van Rooij HC, Kuiper GG, Ris C, Klaassen P, van der Korput JA, Voorhorst MM, van Laar JH, Mulder E, et al. The human androgen receptor: domain structure, genomic organization and regulation of expression. J Steroid Biochem 1989;34:307-10.
47. Gietz D, St. Jean A, Woods RA, Schiestl RH. Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res 1992;20:1425.
48. Kaiser P, Auer B. Rapid shuttle plasmid preparation from yeast cells by transfer to E. coli. Biotechniques 1993;14:552.
49. Cleutjens KB, van Eekelen CC, van der Korput HA, Brinkmann AO, Trapman J. Two androgen response regions cooperate in steroid hormone regulated activity of the prostate-specific antigen promoter. J Biol Chem 1996;271:6379-88.
50. Ris-Stalpers C, Verleun-Mooijman MC, Trapman J, Brinkmann AO. Threonine on amino acid position 868 in the human androgen receptor is essential for androgen binding specificity and functional activity. Biochem Biophys Res Commun 1993;196:173-80.
51. Brinkmann AO, Trapman J. Genetic analysis of androgen receptors in development and disease. Adv Pharmacol 2000;47:317-41.
52. La Spada AR, Wilson EM, Lubahn DB, Harding AE, Fischbeck KH. Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. Nature 1991;352:77-9.

53. Ruizeveld de Winter JA, Janssen PJ, Sleddens HM, Verleun-Mooijman MC, Trapman J, Brinkmann AO, Santerse AB, Schroder FH, van der Kwast TH. Androgen receptor status in localized and locally progressive hormone refractory human prostate cancer. Am J Pathol 1994;144:735-46.
54. Evans BA, Harper ME, Daniells CE, Watts CE, Matenhelia S, Green J, Griffiths K. Low incidence of androgen receptor gene mutations in human prostatic tumors using single strand conformation polymorphism analysis. Prostate 1996;28:162-71.
55. Tilley WD, Buchanan G, Hickey TE, Bentel JM. Mutations in the androgen receptor gene are associated with progression of human prostate cancer to androgen independence. Clin Cancer Res 1996;2:277-85.
56. Marcelli M, Ittmann M, Mariani S, Sutherland R, Nigam R, Murthy L, Zhao Y, DiConcini D, Puxeddu E, Esen A, Eastham J, Weigel NL, et al. Androgen receptor mutations in prostate cancer. Cancer Res 2000;60:944-9.
57. Buchanan G, Greenberg NM, Scher HI, Harris JM, Marshall VR, Tilley WD. Collocation of androgen receptor gene mutations in prostate cancer. Clin Cancer Res 2001;7:1273-81.
58. Zhao XY, Malloy PJ, Krishnan AV, Swami S, Navone NM, Peehl DM, Feldman D. Glucocorticoids can promote androgen-independent growth of prostate cancer cells through a mutated androgen receptor. Nat Med 2000;6:703-6.
59. Chothia C. Structural invariants in protein folding. Nature 1975;254:304-8.
60. Newmark JR, Hardy DO, Tonb DC, Carter BS, Epstein JI, Isaacs WB, Brown TR, Barrack ER. Androgen receptor gene mutations in human prostate cancer. Proc Natl Acad Sci U S A 1992;89:6319-23.
61. Culig Z, Hobisch A, Cronauer MV, Cato AC, Hittmair A, Radmayr C, Eberle J, Bartsch G, Klocker H. Mutant androgen receptor detected in an advanced-stage prostatic carcinoma is activated by adrenal androgens and progesterone. Mol Endocrinol 1993;7:1541-50.
62. Peterziel H, Culig Z, Stober J, Hobisch A, Radmayr C, Bartsch G, Klocker H, Cato AC. Mutant androgen receptors in prostatic tumors distinguish between amino- acid-sequence requirements for transactivation and ligand binding. Int J Cancer 1995;63:544-50.
63. Greenspan FS, Strewler, G.J. Appendix: Table of normal hormone reference ranges, Basic and clinical endocrinology. London: Prentice-Hall International, 1997
64. Harper ME, Pike A, Peeling WB, Griffiths K. Steroids of adrenal origin metabolized by human prostatic tissue both in vivo and in vitro. J Endocrinol 1974;60:117-25.
65. Cleutjens CB, Steketee K, van Eekelen CC, van der Korput JA, Brinkmann AO, Trapman J. Both androgen receptor and glucocorticoid receptor are able to induce prostate-specific antigen expression, but differ in their growth- stimulating properties of LNCaP cells. Endocrinology 1997;138:5293-300.
66. Schuurmans AL, Bolt J, Voorhorst MM, Blankenstein RA, Mulder E. Regulation of growth and epidermal growth factor receptor levels of LNCaP prostate tumor cells by different steroids. Int J Cancer 1988;42:917-22.

Chapter 5

General discussion and future perspectives

5.1 Aim of AR research

The AR plays a prominent role in development of the male phenotype, and therefore also in diseases like Kennedy's disease, AIS, and prostate cancer. Prostate cancer is the second leading cause of cancer deaths in men in Western countries and therefore it is a major research topic. Normal growth, development, and maintenance of the prostate depends on androgens which act through AR mediated regulation of androgen target genes. Prostate tumor growth also depends on androgens. Androgen withdrawal therapy initially inhibits tumor growth and leads to a decrease of tumor size. However, eventually prostate tumors relapse and become apparantly androgen-independent. The tumor then appears to grow without androgen, although in most cases the AR is still present. In androgen-independent tumors, the receptor might be activated by other mechanisms, like overexpression, mutations, aberrant coactivator function, and cross-talk with other signaling pathways. Even intraprostatic conversion of adrenal androgens to DHT might occur. So, the AR is a key player in both androgen-dependent and -independent prostate cancer and therefore it is a major subject of research in this disease and an important target for therapy.

In this thesis research on several aspects of AR function have been described. These include: 1) the functional interaction between the AR NTD and LBD, also known as N/C interaction, 2) androgen specific regulation of gene expression, and 3) AR LBD mutations. In this Chapter 5 the findings are placed in the general context of AR function, and directions of future research are discussed.

5.2 Molecular mechanisms of AR functions

5.2.1 N/C interaction

In Chapter 2, the androgen-dependent interaction between the amino-terminal AR NTD and the carboxy-terminal AR LBD, the N/C interaction, is described. In this interaction an FXXLF motif (^{23}FQNFL^{27}) in the AR NTD is essential. The phenylalanine and leucine residues are indispensible, but X can be any residue. Residues flanking the AR FXXLF motif have a modulating role in the interaction [1, 2].

Like LXXLL motifs, the FXXLF motif can form an α-helical structure that fits in the coactivator groove of the AR LBD. The groove in the AR LBD is relatively deep compared to that in other steroid receptors and therefore it can harbor phenylalanine residues, which are bulkier than leucines [3, 4]. Several AR coregulators, like ARA54, ARA70, and Rad9, also contain an FXXLF motif that can interact with the AR LBD [5-7]. However, not all FXXLF motifs found in AR coregulators can bind to the AR LBD. For example, the coactivator FHL2 contains an FXXLF motif, but this motif can not bind to the AR LBD [5, 8]. So, residues flanking the motif might prevent interaction.

Indeed, hydrophylic residues surrounding the groove and forming a charged clamp are very important in binding of FXXLF motifs [1]. Like was found for LXXLL motifs, this clamp can interact with the charged residues flanking the AR FXXLF motif [1]. Structural data have revealed that in principle E897, but not K720, is necessary for LXXLL-AR LBD binding, whereas both residues are needed for the N/C interaction [4, 9]. However, crystallographic analysis of various peptides containing either LXXLL or FXXLF motifs indicates a more differentiated induced-fit model in which other residues lining the coactivator groove, like K717 and R726, also can play an important role depending on the residues that flank the motif [9, 10]. This can explain that in

some studies K720 was found to be indispensible for FXXLF motif binding, whereas in experiments whith other peptides, K720 appeared to be less important [1, 4, 9-13]. Similar differences were found for E897.

So, binding of the FXXLF motif is similar, but not identical to LXXLL binding, and most LXXLL motifs bind to the AR LBD with less affinity than FXXLF motifs. However, the third LXXLL motif of TIF2 was found to have C-terminally flanking negatively charged residues that interact with positively charged patches on the AR LBD surface [14]. This might explain the relatively strong binding of this motif to the AR LBD and AR preference for TIF2 over other p160 coactivators [15]. So, AR coactivators that contain an FXXLF or LXXLL motif could compete with the N/C interaction and/or with each other. Therefore, it might also be possible that there is redundancy between p160 coactivators and FXXLF containing coactivators, like the redundancy between SRC-1 and TIF2, and between SRC-1 and SRC-3/p/CIP as observed in p160 coactivator knock-out mice [15-19].

Screening of peptide libraries has revealed that variations of the FXXLF motif are allowed. These include exclusively hydrophobic bulky residues at positions +1, +4, and +5 of the motif, with preference for F at +1, F, M or Y at +4, and F or Y at +5 for strong interaction with AR LBD [20, 21]. However, although in some peptides a W was found at position +1 of the motif, these peptides showed a very weak interaction with the AR [8, 9]. In addition, a peptide containing a WXXLF motif found in the AR NTD that was proposed to be involved in the N/C interaction, is not able to bind to the AR LBD (Steketee unpublished results) [22]. A W residue might not properly fit in the coactivator groove, or does not make sufficient contacts to allow stable binding. Crystallographic analysis has provided evidence for the preference for bulky hydrophobic residues at the +1, +4, and +5 position of AR LBD interacting motifs [9]. The structural observations correspond with the strength of the interaction between the AR LBD and the motifs identified with random screening. Illustrative of the prefered motif residues are an FXXFF motif in gelsolin and an FXXMF motif in PAK6, that show high affinity binding to AR LBD [21]. It may be expected that more AR LBD binding proteins will be found that contain FXXLF or variant motifs that can interact with the AR LBD.

To further unravel the molecular mechanism of AR function it was investigated whether the FXXLF mediated N/C interaction is intra- or intermolecular or both. FRET experiments with CFP-AR-YFP, CFP-AR, and AR-YFP fusion proteins have indicated that the FXXLF mediated N/C interaction can be intramolecular in the cytoplasm as well as in the nucleus, whereas AR dimerization was predominantly found in the nucleus [23]. Mutation of the FXXLF motif affected the FRET signal of the AR-YFP/AR CFP combination, but not the affinity between these two AR monomers, from which it was concluded that the FXXLF motif influences the dimerization, but it is not clear yet whether and to which extent this is contributed by intermolecular N/C interaction. In another study, it was investigated whether the N/C interaction occurs in the DNA bound AR. Wild type AR and the N/C interaction deficient mutant E897A, both double tagged with CFP and YFP, were used for FRET analysis in a cell line containing approximately 200 copies of an MMTV Ras tandem array [24]. In this system an N/C interaction was established on a spot in the nucleus representing the AR bound to the MMTV array. In contrast with this study, FRET-FRAP experiments in wild-type cells with a wild-type AR and a DNA binding deficient mutant, showed a clear N/C interaction in nuclei for the mutant AR, whereas that of the wild type AR was considerably less [25]. This indicated that the N/C interaction does not occur when the AR is bound to DNA, which could imply interactions with coactivators that prevent the N/C interaction. This observation was substantiated by binding of an ARA54 peptide to the wild type AR and not to the DNA binding deficient mutant [25]. Similarly, GST pull-down assays and ChIP

on a MMTV-reporter have shown that ARA54 and ARA70 are able to bind to the AR LBD and to an N/C interaction defective full-length AR, but not to the wild type AR [26]. So, the AR N/C interaction could prevent premature/improper cofactor binding in both the cytoplasm and nucleus. Detection of N/C interaction on the MMTV array might be explained by a short-lived existence of the interaction prior to coactivator binding, that is measurable because of the concentration of many HREs on this array.

Several studies have indicated that the N/C interaction is needed for optimal functioning of the AR [3, 12, 22, 27-33]. Under different experimental conditions FXXLF deletion or mutations partially inhibits AR activity. In mouse mammary adenocarcinoma cells the AR LBD mutant E897A has a clearly impaired N/C interaction and is not functional, whereas in another study with the same mutant tested in monkey kidney (CV1) cells, still AR activity could be measured [24, 34]. It can be speculated that in a specific cellular environment the impaired activity of the E897A mutant is mainly due to an abolished interaction with FXXLF and LXXLL containing coactivators, which need the E897 residue for AR binding, rather than to a disturbed N/C interaction. Indeed, in the mouse mammary cells the E897A mutant was not able to recruit PolII, GRIP1 or CREB [24]. GRIP-1 binds through its LXXLL motif for which E897 is essential, subsequently followed by CREB and PolII. The same might count for a number of AR mutants found in AIS, which show a disturbed N/C interaction [12, 30, 31, 33]. Here also other mechanisms needed for AR function may be affected, including increased ligand dissociation.

The N/C interaction was also found to have variable influence on AR function on different AR target sites in the DNA. This was illustrated by N/C interaction defective AR mutants, which had, compared to the wild-type AR, a decreased activity on a transient transfected reporter driven by a promoter with non-specific AREs, but not with androgen-specific AREs [27]. In addition to DNA sequence requirements of the N/C interaction, the interaction was also proposed to play a role in accessibility of the AR to chromatin embedded DNA. An AR deletion mutant lacking the FXXLF motif does not bind to the MMTV promoter and PSA enhancer when assembled in chromatin in *Xenopus* or mammalian kidney cells [32]. However, on naked DNA the mutant was able to bind the MMTV promoter and the PSA enhancer. The role of the N/C interaction in chromatin access of the AR is further substantiated by the finding that the FXXLF deleted AR mutant is also diminished in its binding to the ATPase subunit of SWI/SNF, Brg1 [32].

The AR FXXLF motif is highly conserved, which indicates that it is an important domain. Although deletion or mutation of this motif only partially inhibits AR activity in functional assays, *in vivo* it might have a more pronounced long term effect on AR function. So far, however, no mutations in the FXXLF motif were found in prostate cancer or other AR related diseases (mcgill.ca/androgendb/).

Inhibition of AR function by androgen depletion and administration of antiandrogens is the standard treatment of metastatic prostate cancer. Another method of blocking AR function could be inhibition of coactivator action. Therefore structural knowledge on interactions between the AR and its coactivators is essential. One target of inhibition could be the coactivator groove by blocking it with AR specific peptides, e.g. a peptide containing the AR FXXLF motif. However, this would not only prevent binding of AR coactivators, but it would also interupt the N/C interaction. Hereby the FXXLF motif in the AR NTD becomes available for interaction with other proteins, like MAGE-11, which can specifically bind to the FXXLF motif , stabilizes the AR in the absence of ligand, and in the presence of an agonist, augments exposure of AF-2 to the recruitment and activation by the SRC/p160 coactivators. [20, 35]. This way, a net inhibition of the AR might not be achieved. For the ER antagonistic peptides were developed that can

succesfully block the LBD coactivator groove [36, 37]. However, in the ER, AF-2 substantially contributes to the receptor transcriptional activity, whereas in the AR, AF-1 is much more important for transactivation than the LBD [11, 38-41]. Therefore it could be an option to combine a peptide antagonist that binds to the AR LBD coactivator groove with an additional AF-1-blocking approach. This way p160 coactivators, other AR NTD interacting coactivators, and FXXLF motif containing coactivators could be inhibited in an AR specific manner.

Taken together, the AR N/C interaction may play a fine-tuning role in stabilizing ligand bound AR, effective traveling of the ligand bound AR from the cytoplasm to the nucleus, access of the AR to chromatin embedded DNA, proper deposition of the AR on its target ARE's, and inhibition of premature cofactor binding. Determination of the physiological role of the AR N/C interaction awaits a mouse model in which the FXXLF motif is deleted. Such a model could also be used for microarray analysis to determine which androgen target genes need the N/C interaction for their optimal regulation and under which cellular conditions. Furthermore, ChIP experiments with an FXXLF deletion mutant could establish the requirement of the FXXLF motif in protein complexes involved in regulation of expression of particular androgen responsive genes.

5.2.2 AR specific target genes

All steroid receptors (except ER) can bind to the same high affinity consensus GRE (5'-AGAACAnnnTGTTCT-3'). Steroid specific gene regulation is conferred by several mechanisms including receptor expression levels, selective interactions of receptors with specific and general transcription factors and coregulators, relative coregulator levels, ligand availability, and local chromatin structure at regulatory sites of target genes. However, although many GREs are recognized by AR, as well as by GR and PR, also AR specific AREs have been identified. These AREs are present in androgen-specifically regulated genes like *SC*, *Slp*, *probasin*, *PEM*, and *SARG* [42-47].

The *SARG* gene was identified in the AR^+/GR^+ LNCaP subline 1F5, using differential display. The function of the SARG protein is not known yet. It does not have any known domains that could predict its function. Recently, differential display data were confirmed and extended by micro-array analysis of 1F5 cells. This revealed several genes of which the expression is highly induced by androgens (van der Korput, unpublished results). Among these was *SARG*, which indicates an important role for this gene in androgen regulation of prostate cells. Together with the observation that 1F5 cells depend on androgens for their growth, *SARG* is a candidate to play a role in prostatic (cancer) cell growth. Elucidation of the *SARG* function awaits RNAi experiments and a mouse knock-out model.

Initially, models of AR DBD bound to AR specific AREs suggested that the direct repeat-like character of the AR specific AREs, like that of *SARG*, determines the mode of AR binding. This interaction was proposed to involve head-to-tail dimerization, similar to the orientation of NR heterodimers that bind to direct-repeat HREs like VDR/RXR. However, it now has been proven by crystallographic analysis that an AR dimer has a head-to-head conformation on both AR specific and non-specific AREs [48]. The mechanism underlying AR specificity of AREs involves strong homo-dimerization between AR DBDs. This interaction is less strong between other steroid receptors. Three extra hydrogen bondings between two AR DBDs, if compared to the GR DBD, confer this stronger binding. In fact, one AR binds to a high-affinity left half-site of the AR specific ARE and the second monomer can bind to the second half-site, which might deviate considerably from the high affinity consensus site sequence and has a low affinity for the AR. Apparently, the dimerization interaction is strong enough to keep the AR dimer intact.

This might also explain why the AR was able to bind to the Pem1 ARE with a 5 bp spacing, while the GR cannot [42]. Although the mechanism of the PemI ARE could be complex, one aspect might be that the strong AR dimerization overcomes the extra spacing and concomittant phase change of the major groove to which the AR binds if compared to a normal 3 bp spacing, by resisting the tension put on it by bending of the spacer in order to accomodate the AR dimer.

The physiological roles of androgen-specific regulated genes are not known, but knowledge on these genes might be important for development of specific AR targeting prostate cancer therapies. Mutations have been found in AIS patients at the positions of the three amino acids involved in the extra hydrogen bondings between AR DBDs. In PAIS patients A596T, S597G, S597T, S597R, and T602P substitutions were identified [49-56]. Some of these were shown to impair AR function [50, 51, 56]. It would be informative to test whether these AR mutants are active on non-specific AR target genes and less active on AR-specific genes. Indeed, one of the mutants, A596T, was tested and showed reduced activity on AR specific AREs, but not on non-specific AREs [57].

Recently, a transgenic mouse model, SPARKI (SPecificity-affecting AR KnockIN), was generated in which the second zinc cluster of the AR was swapped with the second zinc cluster of the GR [58]. This AR mutant showed a significant loss of function on androgen specific AREs, but not on non-specific AREs. SPARKI males have an apparently normal phenotype. However, they are subfertile. The reproductive organs are decreased in weight and spermatogenesis is disturbed. This model can help reveal which pathways are involved in androgen specific gene regulation.

Micro-arrays are very important tools for the identification of androgen-specific regulated genes. To find AREs in those genes a very useful approach can be a bioinformatics based search for AREs. However, to identify a real AR specific ARE, functional studies always will be needed. For example, by using bioinformatics at 7.5 kb upstream of the SARG transcription start site, a candidate ARE was found, which is identical to an artificial AR specific ARE previously tested as an oligonucleotide [59]. In contrast to the artificial ARE, the -7.5 kb candidate ARE appeared to be non-functional and even no AR binding was found [45]. This clearly illustrates that half-site sequences alone are not sufficient, but need flanking and/or more distant sequences to determine the androgen response of a candidate ARE. The concomitant local chromatin structure in which the ARE is embedded, and binding of other transcription factors to the flanking sequences, might also determine whether a candidate ARE is functional or not.

Another difficulty in finding androgen regulated genes by using bioinformatics, is that many known functional AREs differ considerably from the consensus sequence. So, genome wide screening for ARE sequences would involve a huge variety of sequences to be used as search string. This would result in a too high number of candidate AREs to be suitable for further analysis. Searching exclusively for AR specific AREs would even be more problematic. For this, one half site must be a high affinity, mostly near consensus sequence, and one half site should considerably deviate from the consensus sequence. However, this would imply screening with one half-site sequence, which means using a search string with only 6 bases. This would reveal far too many candidate AREs to handle. As the second half-site can be extremely variable, it would be very complicated and may be impossible to define a consensus sequence for AR specific AREs.

An approach to solve the problem of too many candidate AREs in screening of genomic sequences is the ChIP-on-chip technique [60]. This technique combines protein-DNA binding with microarray analysis. A genome wide screening has been done to identify ER binding sequences, which is one step forward in limiting the number of candidate HREs [61]. Recently, ChIP-on-chip

for the AR was performed on a so-called ENCODE chip, which represents approximately 1% of the whole genome [62]. Several novel androgen response genes have been identified. Most AREs appeared to be located in regions other than proximal promoter regions. The intronic androgen specific ARE in the SARG gene is an example of this (Chapter 3) [45]. ChIP-on-chip analysis of the complete chromosomes 21 and 22 has revealed 90 AR binding sites, among which one upstream of the *TMPRSS2* gene [63]. In a ChIP-on-chip approach with more than 24,000 gene promoter regions, 92 genes were identified as strong candidate AR regulated genes, and this corresponded with expression data sets of those genes [64]. In another study, a few hundred androgen-regulated genes were identified with expression profiling and subsequent ChIP-on-chip analysis of the promoter regions of those genes. This revealed more than 500 AR binding regions of which 22 were tested in a reporter gene assay, and 20 of these were androgen inducible [65]. Most of these AR binding regions contained two or more AREs. Clustering of candidate AREs may indicate an androgen regulated region, but a single ARE may have no measurable activity in transfection experiments and therefore can be difficult to identify [66]. Using ChIP-on-chip analysis may also help overcome this problem. Identification of all androgen regulated genes and all existing AREs, both androgen specific and non-specific, awaits a ChIP-on-chip analysis of the complete genome.

5.2.3 AR LBD mutations

AR mutations have been found in pathological conditions as Kennedy's disease and AIS, and in a subset (up to 10%) of endocrine therapy resistant prostate cancers. In prostate cancer most mutations are found in the LBD, which could imply changes in ligand and cofactor binding. Well characterized mutations are T877A, L701H, H874Y, and W741C/L, which cause a broadened ligand specificity of the AR. These mutations render the AR, in addition to T and DHT, also responsive to non-cognate ligands like progesterone, estradiol, adrenal androgens, glucocorticoids, and even anti-androgens [45, 67-77]. For some of these mutants the mechanism of broadened ligand specificity has been elucidated. Crystallographic analysis has shown that the T877A mutant has a modified ligand binding pocket size that can explain accomodation of ligands larger than T and DHT [78]. However, also mutations of residues that do not line the ligand binding pocket, e.g. in AR H874Y, can confer broadened ligand specificity, most likely by structural changes that indirectly influence ligand binding. Crystallographic analyses of the different mutants bound to different non-cognate ligands explain and confirm the mechanisms underlying the broadened ligand specificity, such as the recently elucidated crystal structure of the T877A AR LBD complexed to CPA [79]. In this mutant residue L701 in the LBD is displaced resulting in expansion of the ligand binding pocket. This enables CPA, which is a partial agonist for the wild-type AR and is bulkier than AR steroidal agonists like DHT, T, or R1881, to fit the pocket properly so that it can act as a full agonist for the T877A AR mutant. Similarly, an L701A mutant can also be activated by CPA at the same nanomolar concentrations as the T877A mutant [79]. These kind of structural studies provide insight in ligand-induced conformational changes and could be helpful in structure-based drug design.

Agonistic action of antagonists on the AR mutants could be determined by proper coactivator interactions as a result of the changed ligand accomodation. For example, in the presence of ligands such as estradiol, progesterone, spironolactone, and OH-Fl, the T877A mutant showed an increased ligand potency in recruitment of LXXLL and FXXLF peptides [80]. Likewise, it might be expected that the AR FXXLF motif interacts differently with the mutant LBD as with the wild-type LBD in the presence of an antagonist. So, crystallographic data of wild-type and mutant AR LBDs complexed with peptides in the presence of different ligands could give more

insight in the mechanism of AR function. This could be useful for the design of new approaches to specifically block AR LBD-coactivator interactions in both wild-type and mutant ARs.

Androgen ablation by LHRH agonists used for treatment of metastatic prostate cancer does not reduce serum T levels such that DHT levels in the prostate will become low enough to completely inhibit the AR [81-84]. Therefore, also anti-androgens are used to counteract DHT action. It is believed that use of antiandrogens in prostate cancer treatment can induce androgen-independent tumor growth in a subset of patients. Strong indications for this are provided by experiments in a yeast system and in prostate cancer cell lines, in which AR mutants could be selected for their response to non-cognate ligands, among which anti-androgens [45, 69, 85]. The AR mutations found in these experiments are the same as found in antiandrogen treated patients. Although it is possible that those AR mutants are already present in non-treated tumors, these must be rare as they have not been found in primary tumors. This implies that those mutants could not have growth advantage in the untreated stage when DHT is at physiological levels. This is in accordance with the responses of wild-type and AR mutants to DHT, which are comparable as measured in transfection.

The W741C mutant can be activated by bicalutamide, but not by nilutamide and OH-Fl, whereas the T877A mutant does not respond to bicalutamide, but can be activated by OH-Fl and nilutamide [86]. Structural modeling has revealed the possible underlying mechanisms of these mutants with the respective ligands [79, 86-88]. This has led to the hypothesis that switching of anti-androgens during prostate cancer treatment could abolish the effects of appearing AR mutations. This would also be in line with the phenomenon 'anti-androgen withdrawal syndrome' in which prostate tumors decrease in size when anti-androgen administration is temporarily stopped [89]. However, only a small proportion of the cases of antiandrogen withdrawal syndrome carry AR mutations, which means that also growth pathways other than those regulated by AR mutants must be involved in tumor progression. Nevertheless, it has been argued that a therapy in which use of different anti-androgens and withdrawal of these compounds is alternated, could inhibit progression of at least a subset of prostate cancers [86, 90].

Prostate cancer related AR mutations are not only found in the LBD, but, albeit much less frequent, also in the NTD and DBD. In the transgenic adenocarcinoma of mouse prostate (TRAMP) model, AR LBD mutations were found in the tumors of non-treated mice, whereas in castrated TRAMP mice AR NTD mutations were found [91]. Apparently, the androgen-depleted hormone status leads to mutations in the androgen-independent NTD. However, androgen depletion, mostly by using LHRH agonists, is often combined with antiandrogens. This substantiates why most AR mutations in anti-androgen treated prostate cancers are found in the ligand-dependent LBD as these mutations may appear under selection pressure by the anti-androgens that can activate them. As AR mutations in the TRAMP model developed under physiologically normal hormone conditions, it also raised the question whether AR mutations could induce oncogenesis. Indeed, in all transgenic mice harboring the AR NTD mutant E231G, oncogenic transformation of the prostate was found [92]. So far, the E231G mutation has not been found in human prostate cancer (mcgill.ca/androgendb/). The underlying mechanism could be an increased AR activity caused by a disturbed interaction with the negative AR regulator CHIP. Recently, an AR DBD mutation, K580R, which was found in a lymph node metastasis, was found to be oncogenic in transfection. Activation of the Akt signalling pathway might be involved, because this mutant increases p-Akt and p-p70 S6K levels [93, 94]. These findings indicate that it is important to investigate mutations in all three AR domains, which will lead to a better understanding of the role of the AR in prostate cancer.

5.3 AR in further research and future perspectives

Although there is already an extensive knowledge on the molecular mechanisms of AR functions, many aspects remain to be elucidated, of which a few important ones are discussed below.

One feature of the AR that still is to be established is the structural conformation of the NTD. So far, only an α-helical structure for the ^{23}FXXLF^{27} motif was found [4, 9]. It is thought that the NTD, if not bound to other proteins, is very flexible and that it adopts different induced fit conformations depending on the proteins that bind to this AR domain. So, structural analysis of AR NTD might only be succesful in the context of its interacting proteins like CHIP, MAGE, SHP, SMRT, CBP, and p160 coactivators (see section 1.2.2).

Although AR mutations are found in only a subset of prostate cancer patients, further unraveling the functional consequences of these mutations will highly contribute to general understanding of AR function, which could be useful for development of new or better therapies.

RNAi could be an effective therapy for prostate cancer treatment. As the AR plays a key role in prostate cancer, androgen responsive genes might be selected as targets of RNAi in addition to RNAi of the AR itself. For this it will be important to develop a therapy that can balance expression of androgen up- and down-regulated genes so precisely that cell proliferation is completely shut down in a prostate specific manner. To develop such an approach, more detailed knowledge is needed on (specifically) androgen regulated genes, which has been achieved by using micro- array technology and now also with the recently developed ChIP-on-chip technique [60-62]. A good candidate for RNAi might be the recently identified TMPRSS2:ERG fusion gene. It is highly expressed in a substantial proportion of prostate cancers, androgen responsive, and correlates with recurrence and aggressiveness of the disease [95-99]. This gene is suggested to play a role in the androgen-dependent stage of prostate cancer [96].

The AR can also have a function in mechanisms other than regulation of gene transcription. Recently, the AR was found to be a licensing factor for DNA replication in androgen-dependent prostate cancer cells, but not in normal prostate cells [100, 101]. In the prostate cancer cells, the AR has to be degraded before AR signalling can promote a next round of DNA replication. So, paradoxically, AR stabilizing approaches would inhibit DNA replication and therefore disturb the cell cycle. A new approach for prostate cancer treatment could be intermittent androgen blockade (IMAB). This would anticipate on the effect of selection of AR mutants responding to antiandrogens and inhibit cell proliferation on a more general level by diminishing DNA replication.

In summary, knowledge on the molecular mechanisms of AR functions is important to understand AR function in order to be able to improve existing therapies and to develop new treatments for AR-related diseases like prostate cancer. Our current knowledge still needs further extension to find targets for improvement of current prostate cancer treatment or develop new therapies.

5.4 REFERENCES

1 B. He and E. M. Wilson (2003) Electrostatic modulation in steroid receptor recruitment of LXXLL and FXXLF motifs *Mol Cell Biol* **23** 6 2135-50

2 K. Steketee, C. A. Berrevoets, H. J. Dubbink, P. Doesburg, R. Hersmus, A. O. Brinkmann and J. Trapman (2002) Amino acids 3-13 and amino acids in and flanking the 23FxxLF27 motif modulate the interaction between the N-terminal and ligand-binding domain of the androgen receptor *Eur J Biochem* **269** 23 5780-91

3 H. J. Dubbink, R. Hersmus, C. S. Verma, H. A. Van Der Korput, C. A. Berrevoets, J. Van Tol, A. C. Ziel-Van Der Made, A. O. Brinkmann, A. C. Pike and J. Trapman (2004) Distinct recognition modes of FXXLF and LXXLL motifs by the androgen receptor *Mol Endocrinol* **18** 9 2132-50

4 B. He, R. T. Gampe, Jr., A. J. Kole, A. T. Hnat, T. B. Stanley, G. An, E. L. Stewart, R. I. Kalman, J. T. Minges and E. M. Wilson (2004) Structural basis for androgen receptor interdomain and coactivator interactions suggests a transition in nuclear receptor activation function dominance *Mol Cell* **16** 3 425-38

5 B. He, J. T. Minges, L. W. Lee and E. M. Wilson (2002) The FXXLF motif mediates androgen receptor-specific interactions with coregulators *J Biol Chem* **277** 12 10226-35

6 Y. C. Hu, S. Yeh, S. D. Yeh, E. R. Sampson, J. Huang, P. Li, C. L. Hsu, H. J. Ting, H. K. Lin, L. Wang, E. Kim, J. Ni and C. Chang (2004) Functional domain and motif analyses of androgen receptor coregulator ARA70 and its differential expression in prostate cancer *J Biol Chem* **279** 32 33438-46

7 L. Wang, C. L. Hsu, J. Ni, P. H. Wang, S. Yeh, P. Keng and C. Chang (2004) Human checkpoint protein hRad9 functions as a negative coregulator to repress androgen receptor transactivation in prostate cancer cells *Mol Cell Biol* **24** 5 2202-13

8 C. L. Hsu, Y. L. Chen, S. Yeh, H. J. Ting, Y. C. Hu, H. Lin, X. Wang and C. Chang (2003) The use of phage display technique for the isolation of androgen receptor interacting peptides with (F/W)XXL(F/W) and FXXLY new signature motifs *J Biol Chem* **278** 26 23691-8

9 E. Hur, S. J. Pfaff, E. S. Payne, H. Gron, B. M. Buehrer and R. J. Fletterick (2004) Recognition and accommodation at the androgen receptor coactivator binding interface *PLoS Biol* **2** 9 E274

10 H. J. Dubbink, R. Hersmus, A. C. Pike, M. Molier, A. O. Brinkmann, G. Jenster and J. Trapman (2006) Androgen receptor ligand-binding domain interaction and nuclear receptor specificity of FXXLF and LXXLL motifs as determined by L/F swapping *Mol Endocrinol* **20** 8 1742-55

11 P. Alen, F. Claessens, G. Verhoeven, W. Rombauts and B. Peeters (1999) The androgen receptor amino-terminal domain plays a key role in p160 coactivator-stimulated gene transcription *Mol Cell Biol* **19** 9 6085-97

12 B. He, J. A. Kemppainen, J. J. Voegel, H. Gronemeyer and E. M. Wilson (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** 52 37219-25

13 T. Slagsvold, I. Kraus, T. Bentzen, J. Palvimo and F. Saatcioglu (2000) Mutational analysis of the androgen receptor AF-2 (activation function 2) core domain reveals functional and mechanistic differences of conserved residues compared with other nuclear receptors *Mol Endocrinol* **14** 10 1603-17

14 E. Estebanez-Perpina, J. M. Moore, E. Mar, E. Delgado-Rodrigues, P. Nguyen, J. D. Baxter, B. M. Buehrer, P. Webb, R. J. Fletterick and R. K. Guy (2005) The molecular mechanisms of coactivator utilization in ligand-dependent transactivation by the androgen receptor *J Biol Chem* **280** 9 8060-8

15 X. Ye, S. J. Han, S. Y. Tsai, F. J. Demayo, J. Xu, M. J. Tsai and W. O'malley B (2005) Roles of steroid receptor coactivator (SRC)-1 and transcriptional intermediary factor (TIF) 2 in androgen receptor activity in mice *Proc Natl Acad Sci U S A* **102** 27 9487-92

16 M. Mark, H. Yoshida-Komiya, M. Gehin, L. Liao, M. J. Tsai, B. W. O'malley, P. Chambon and J. Xu (2004) Partially redundant functions of SRC-1 and TIF2 in postnatal survival and male reproduction *Proc Natl Acad Sci U S A* **101** 13 4453-8

17 N. P. Mongan, J. Jaaskelainen, S. Bhattacharyya, R. M. Leu and I. A. Hughes (2003) Steroid receptor coactivator-3 glutamine repeat polymorphism and the androgen insensitivity syndrome *Eur J Endocrinol* **148** 2 277-9

18 J. Xu, Y. Qiu, F. J. Demayo, S. Y. Tsai, M. J. Tsai and B. W. O'malley (1998) Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene *Science* **279** 5358 1922-5

19 Z. Wang, C. Qi, A. Krones, P. Woodring, X. Zhu, J. K. Reddy, R. M. Evans, M. G. Rosenfeld and T. Hunter (2006) Critical roles of the p160 transcriptional coactivators p/CIP and SRC-1 in energy balance *Cell Metab* **3** 2 111-22

20 C. Y. Chang, J. Abdo, T. Hartney and D. P. Mcdonnell (2005) Development of peptide antagonists for the androgen receptor using combinatorial peptide phage display *Mol Endocrinol* **19** 10 2478-90

21 D. J. Van De Wijngaart, M. E. Van Royen, R. Hersmus, A. C. Pike, A. B. Houtsmuller, G. Jenster, J. Trapman and H. J. Dubbink (2006) Novel FXXFF and FXXMF motifs in androgen receptor cofactors mediate high affinity and specific interactions with the ligand-binding domain *J Biol Chem* **281** 28 19407-16

22 B. He, J. A. Kemppainen and E. M. Wilson (2000) FXXLF and WXXLF sequences mediate the NH2-terminal interaction with the ligand binding domain of the androgen receptor *J Biol Chem* **275** 30 22986-94

23 F. Schaufele, X. Carbonell, M. Guerbadot, S. Borngraeber, M. S. Chapman, A. A. Ma, J. N. Miner and M. I. Diamond (2005) The structural basis of androgen receptor activation: intramolecular and intermolecular amino-carboxy interactions *Proc Natl Acad Sci U S A* **102** 28 9802-7

24 T. I. Klokk, P. Kurys, C. Elbi, A. K. Nagaich, A. Hendarwanto, T. Slagsvold, C. Y. Chang, G. L. Hager and F. Saatcioglu (2007) Ligand-specific dynamics of the androgen receptor at its response element in living cells *Mol Cell Biol* **27** 5 1823-43
25 M. E. Van Royen, S. M. Cunha, M. C. Brink, K. A. Mattern, A. L. Nigg, H. J. Dubbink, P. J. Verschure, J. Trapman and A. B. Houtsmuller (2007) Compartmentalization of androgen receptor protein-protein interactions in living cells *J Cell Biol* **177** 1 63-72
26 C. Toumazou, J. Li and J. Wong (2007) Cofactor Restriction by Androgen Receptor N-terminal and C-terminal Interaction *Mol Endocrinol*
27 L. Callewaert, G. Verrijdt, V. Christiaens, A. Haelens and F. Claessens (2003) Dual function of an amino-terminal amphipatic helix in androgen receptor-mediated transactivation through specific and nonspecific response elements *J Biol Chem* **278** 10 8212-8
28 B. He, N. T. Bowen, J. T. Minges and E. M. Wilson (2001) Androgen-induced NH2- and COOH-terminal Interaction Inhibits p160 coactivator recruitment by activation function 2 *J Biol Chem* **276** 45 42293-301
29 C. L. Hsu, Y. L. Chen, H. J. Ting, W. J. Lin, Z. Yang, Y. Zhang, L. Wang, C. T. Wu, H. C. Chang, S. Yeh, S. W. Pimplikar and C. Chang (2005) Androgen receptor (AR) NH2- and COOH-terminal interactions result in the differential influences on the AR-mediated transactivation and cell growth *Mol Endocrinol* **19** 2 350-61
30 J. Jaaskelainen, A. Deeb, J. W. Schwabe, N. P. Mongan, H. Martin and I. A. Hughes (2006) Human androgen receptor gene ligand-binding-domain mutations leading to disrupted interaction between the N- and C-terminal domains *J Mol Endocrinol* **36** 2 361-8
31 E. Langley, J. A. Kemppainen and E. M. Wilson (1998) Intermolecular NH2-/carboxyl-terminal interactions in androgen receptor dimerization revealed by mutations that cause androgen insensitivity *J Biol Chem* **273** 1 92-101
32 J. Li, J. Fu, C. Toumazou, H. G. Yoon and J. Wong (2006) A role of the amino-terminal (N) and carboxyl-terminal (C) interaction in binding of androgen receptor to chromatin *Mol Endocrinol* **20** 4 776-85
33 J. Thompson, F. Saatcioglu, O. A. Janne and J. J. Palvimo (2001) Disrupted amino- and carboxyl-terminal interactions of the androgen receptor are linked to androgen insensitivity *Mol Endocrinol* **15** 6 923-35
34 B. He and E. M. Wilson (2002) The NH(2)-terminal and carboxyl-terminal interaction in the human androgen receptor *Mol Genet Metab* **75** 4 293-8
35 S. Bai, B. He and E. M. Wilson (2005) Melanoma antigen gene protein MAGE-11 regulates androgen receptor function by modulating the interdomain interaction *Mol Cell Biol* **25** 4 1238-57
36 C. Chang, J. D. Norris, H. Gron, L. A. Paige, P. T. Hamilton, D. J. Kenan, D. Fowlkes and D. P. Mcdonnell (1999) Dissection of the LXXLL nuclear receptor-coactivator interaction motif using combinatorial peptide libraries: discovery of peptide antagonists of estrogen receptors alpha and beta *Mol Cell Biol* **19** 12 8226-39
37 J. M. Hall, C. Y. Chang and D. P. Mcdonnell (2000) Development of peptide antagonists that target estrogen receptor beta-coactivator interactions *Mol Endocrinol* **14** 12 2010-23
38 A. Benecke, P. Chambon and H. Gronemeyer (2000) Synergy between estrogen receptor alpha activation functions AF1 and AF2 mediated by transcription intermediary factor TIF2 *EMBO Rep* **1** 2 151-7
39 C. A. Berrevoets, P. Doesburg, K. Steketee, J. Trapman and A. O. Brinkmann (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** 8 1172-83
40 R. Metivier, G. Penot, G. Flouriot and F. Pakdel (2001) Synergism between ERalpha transactivation function 1 (AF-1) and AF-2 mediated by steroid receptor coactivator protein-1: requirement for the AF-1 alpha-helical core and for a direct interaction between the N- and C-terminal domains *Mol Endocrinol* **15** 11 1953-70
41 S. A. Onate, V. Boonyaratanakornkit, T. E. Spencer, S. Y. Tsai, M. J. Tsai, D. P. Edwards and B. W. O'malley (1998) The steroid receptor coactivator-1 contains multiple receptor interacting and activation domains that cooperatively enhance the activation function 1 (AF1) and AF2 domains of steroid receptors *J Biol Chem* **273** 20 12101-8
42 K. Barbulescu, C. Geserick, I. Schuttke, W. D. Schleuning and B. Haendler (2001) New androgen response elements in the murine pem promoter mediate selective transactivation *Mol Endocrinol* **15** 10 1803-16
43 F. Claessens, P. Alen, A. Devos, B. Peeters, G. Verhoeven and W. Rombauts (1996) The androgen-specific probasin response element 2 interacts differentially with androgen and glucocorticoid receptors *J Biol Chem* **271** 32 19013-6
44 A. Haelens, G. Verrijdt, E. Schoenmakers, P. Alen, B. Peeters, W. Rombauts and F. Claessens (1999) The first exon of the human sc gene contains an androgen responsive unit and an interferon regulatory factor element *Mol Cell Endocrinol* **153** 1-2 91-102
45 K. Steketee, A. C. Ziel-Van Der Made, H. A. Van Der Korput, A. B. Houtsmuller and J. Trapman (2004) A bioinformatics-based functional analysis shows that the specifically androgen-regulated gene SARG contains an active direct repeat androgen response element in the first intron *J Mol Endocrinol* **33** 2 477-91
46 G. Verrijdt, E. Schoenmakers, P. Alen, A. Haelens, B. Peeters, W. Rombauts and F. Claessens (1999) Androgen specificity of a response unit upstream of the human secretory component gene is mediated by differential receptor binding to an essential androgen response element *Mol Endocrinol* **13** 9 1558-70
47 J. Zhang, N. Gao, S. Kasper, K. Reid, C. Nelson and R. J. Matusik (2004) An androgen-dependent upstream enhancer is essential for high levels of probasin gene expression *Endocrinology* **145** 1 134-48
48 P. L. Shaffer, A. Jivan, D. E. Dollins, F. Claessens and D. T. Gewirth (2004) Structural basis of androgen receptor binding to selective androgen response elements *Proc Natl Acad Sci U S A* **101** 14 4758-63

49 A. Deeb, C. Mason, Y. S. Lee and I. A. Hughes (2005) Correlation between genotype, phenotype and sex of rearing in 111 patients with partial androgen insensitivity syndrome *Clin Endocrinol (Oxf)* **63** 1 56-62

50 A. Gast, F. Neuschmid-Kaspar, H. Klocker and A. C. Cato (1995) A single amino acid exchange abolishes dimerization of the androgen receptor and causes Reifenstein syndrome *Mol Cell Endocrinol* **111** 1 93-8

51 A. Giwercman, T. Kledal, M. Schwartz, Y. L. Giwercman, H. Leffers, H. Zazzi, A. Wedell and N. E. Skakkebaek (2000) Preserved male fertility despite decreased androgen sensitivity caused by a mutation in the ligand-binding domain of the androgen receptor gene *J Clin Endocrinol Metab* **85** 6 2253-9

52 Y. L. Giwercman, S. A. Ivarsson, J. Richthoff, K. B. Lundin and A. Giwercman (2004) A novel mutation in the D-box of the androgen receptor gene (S597R) in two unrelated individuals Is associated with both normal phenotype and severe PAIS *Horm Res* **61** 2 58-62

53 P. M. Holterhus, G. H. Sinnecker, H. A. Wollmann, D. Struve, N. Homburg, K. Kruse and O. Hiort (1999) Expression of two functionally different androgen receptors in a patient with androgen insensitivity *Eur J Pediatr* **158** 9 702-6

54 K. F. Melo, B. B. Mendonca, A. E. Billerbeck, E. M. Costa, M. Inacio, F. A. Silva, A. M. Leal, A. C. Latronico and I. J. Arnhold (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** 7 3241-50

55 A. Nordenskjold, E. Friedman, M. Tapper-Persson, C. Soderhall, A. Leviav, J. Svensson and M. Anvret (1999) Screening for mutations in candidate genes for hypospadias *Urol Res* **27** 1 49-55

56 S. Zoppi, M. Marcelli, J. P. Deslypere, J. E. Griffin, J. D. Wilson and M. J. Mcphaul (1992) Amino acid substitutions in the DNA-binding domain of the human androgen receptor are a frequent cause of receptor-binding positive androgen resistance *Mol Endocrinol* **6** 3 409-15

57 C. Geserick, H. A. Meyer, K. Barbulescu and B. Haendler (2003) Differential modulation of androgen receptor action by deoxyribonucleic acid response elements *Mol Endocrinol* **17** 9 1738-50

58 K. Schauwaers, K. De Gendt, P. T. Saunders, N. Atanassova, A. Haelens, L. Callewaert, U. Moehren, J. V. Swinnen, G. Verhoeven, G. Verrijdt and F. Claessens (2007) Loss of androgen receptor binding to selective androgen response elements causes a reproductive phenotype in a knockin mouse model *Proc Natl Acad Sci U S A* **104** 12 4961-6

59 F. Claessens, G. Verrijdt, E. Schoenmakers, A. Haelens, B. Peeters, G. Verhoeven and W. Rombauts (2001) Selective DNA binding by the androgen receptor as a mechanism for hormone-specific gene regulation *J Steroid Biochem Mol Biol* **76** 1-5 23-30

60 J. S. Carroll, X. S. Liu, A. S. Brodsky, W. Li, C. A. Meyer, A. J. Szary, J. Eeckhoute, W. Shao, E. V. Hestermann, T. R. Geistlinger, E. A. Fox, P. A. Silver and M. Brown (2005) Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1 *Cell* **122** 1 33-43

61 J. S. Carroll and M. Brown (2006) Estrogen receptor target gene: an evolving concept *Mol Endocrinol* **20** 8 1707-14

62 K. Takayama, K. Kaneshiro, S. Tsutsumi, K. Horie-Inoue, K. Ikeda, T. Urano, N. Ijichi, Y. Ouchi, K. Shirahige, H. Aburatani and S. Inoue (2007) Identification of novel androgen response genes in prostate cancer cells by coupling chromatin immunoprecipitation and genomic microarray analysis *Oncogene* **26** 30 4453-63

63 Q. Wang, W. Li, X. S. Liu, J. S. Carroll, O. A. Janne, E. K. Keeton, A. M. Chinnaiyan, K. J. Pienta and M. Brown (2007) A hierarchical network of transcription factors governs androgen receptor-dependent prostate cancer growth *Mol Cell* **27** 3 380-92

64 C. E. Massie, B. Adryan, N. L. Barbosa-Morais, A. G. Lynch, M. G. Tran, D. E. Neal and I. G. Mills (2007) New androgen receptor genomic targets show an interaction with the ETS1 transcription factor *EMBO Rep* **8** 9 871-8

65 E. C. Bolton, A. Y. So, C. Chaivorapol, C. M. Haqq, H. Li and K. R. Yamamoto (2007) Cell- and gene-specific regulation of primary target genes by the androgen receptor *Genes Dev* **21** 16 2005-17

66 C. B. Cleutjens, K. Steketee, C. C. Van Eekelen, J. A. Van Der Korput, A. O. Brinkmann and J. Trapman (1997) Both androgen receptor and glucocorticoid receptor are able to induce prostate-specific antigen expression, but differ in their growth-stimulating properties of LNCaP cells *Endocrinology* **138** 12 5293-300

67 M. A. Fenton, T. D. Shuster, A. M. Fertig, M. E. Taplin, G. Kolvenbag, G. J. Bubley and S. P. Balk (1997) Functional characterization of mutant androgen receptors from androgen-independent prostate cancer *Clin Cancer Res* **3** 8 1383-8

68 K. Haapala, E. R. Hyytinen, M. Roiha, M. Laurila, I. Rantala, H. J. Helin and P. A. Koivisto (2001) Androgen receptor alterations in prostate cancer relapsed during a combined androgen blockade by orchiectomy and bicalutamide *Lab Invest* **81** 12 1647-51

69 T. Hara, J. Miyazaki, H. Araki, M. Yamaoka, N. Kanzaki, M. Kusaka and M. Miyamoto (2003) Novel mutations of androgen receptor: a possible mechanism of bicalutamide withdrawal syndrome *Cancer Res* **63** 1 149-53

70 J. Tan, Y. Sharief, K. G. Hamil, C. W. Gregory, D. Y. Zang, M. Sar, P. H. Gumerlock, R. W. Devere White, T. G. Pretlow, S. E. Harris, E. M. Wilson, J. L. Mohler and F. S. French (1997) Dehydroepiandrosterone activates mutant androgen receptors expressed in the androgen-dependent human prostate cancer xenograft CWR22 and LNCaP cells *Mol Endocrinol* **11** 4 450-9

71 M. E. Taplin, G. J. Bubley, Y. J. Ko, E. J. Small, M. Upton, B. Rajeshkumar and S. P. Balk (1999) Selection for androgen receptor mutations in prostate cancers treated with androgen antagonist *Cancer Res* **59** 11 2511-5

72 M. E. Taplin, G. J. Bubley, T. D. Shuster, M. E. Frantz, A. E. Spooner, G. K. Ogata, H. N. Keer and S. P. Balk (1995) Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer *N Engl J Med* **332** 21 1393-8
73 J. Veldscholte, C. A. Berrevoets, A. O. Brinkmann, J. A. Grootegoed and E. Mulder (1992) Anti-androgens and the mutated androgen receptor of LNCaP cells: differential effects on binding affinity, heat-shock protein interaction, and transcription activation *Biochemistry* **31** 8 2393-9
74 J. Veldscholte, C. A. Berrevoets, C. Ris-Stalpers, G. G. Kuiper, G. Jenster, J. Trapman, A. O. Brinkmann and E. Mulder (1992) The androgen receptor in LNCaP cells contains a mutation in the ligand binding domain which affects steroid binding characteristics and response to antiandrogens *J Steroid Biochem Mol Biol* **41** 3-8 665-9
75 J. Veldscholte, C. Ris-Stalpers, G. G. Kuiper, G. Jenster, C. Berrevoets, E. Claassen, H. C. Van Rooij, J. Trapman, A. O. Brinkmann and E. Mulder (1990) A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens *Biochem Biophys Res Commun* **173** 2 534-40
76 X. Y. Zhao, B. Boyle, A. V. Krishnan, N. M. Navone, D. M. Peehl and D. Feldman (1999) Two mutations identified in the androgen receptor of the new human prostate cancer cell line MDA PCa 2a *J Urol* **162** 6 2192-9
77 X. Y. Zhao, P. J. Malloy, A. V. Krishnan, S. Swami, N. M. Navone, D. M. Peehl and D. Feldman (2000) Glucocorticoids can promote androgen-independent growth of prostate cancer cells through a mutated androgen receptor *Nat Med* **6** 6 703-6
78 J. S. Sack, K. F. Kish, C. Wang, R. M. Attar, S. E. Kiefer, Y. An, G. Y. Wu, J. E. Scheffler, M. E. Salvati, S. R. Krystek, Jr., R. Weinmann and H. M. Einspahr (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** 9 4904-9
79 C. E. Bohl, Z. Wu, D. D. Miller, C. E. Bell and J. T. Dalton (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** 18 13648-55
80 M. S. Ozers, B. D. Marks, K. Gowda, K. R. Kupcho, K. M. Ervin, T. De Rosier, N. Qadir, H. C. Eliason, S. M. Riddle and M. S. Shekhani (2007) The androgen receptor T877A mutant recruits LXXLL and FXXLF peptides differently than wild-type androgen receptor in a time-resolved fluorescence resonance energy transfer assay *Biochemistry* **46** 3 683-95
81 A. Belanger, M. Brochu and J. Cliche (1986) Levels of plasma steroid glucuronides in intact and castrated men with prostatic cancer *J Clin Endocrinol Metab* **62** 5 812-5
82 F. Labrie, A. Dupont, A. Belanger, R. Lachance and M. Giguere (1985) Long term treatment with luteinising hormone releasing hormone agonists and maintenance of serum testosterone to castration concentrations *Br Med J (Clin Res Ed)* **291** 6492 369-70
83 S. T. Page, D. W. Lin, E. A. Mostaghel, D. L. Hess, L. D. True, J. K. Amory, P. S. Nelson, A. M. Matsumoto and W. J. Bremner (2006) Persistent intraprostatic androgen concentrations after medical castration in healthy men *J Clin Endocrinol Metab* **91** 10 3850-6
84 M. A. Titus, M. J. Schell, F. B. Lih, K. B. Tomer and J. L. Mohler (2005) Testosterone and dihydrotestosterone tissue levels in recurrent prostate cancer *Clin Cancer Res* **11** 13 4653-7
85 R. B. Marques, S. Erkens-Schulze, C. M. De Ridder, K. G. Hermans, K. Waltering, T. Visakorpi, J. Trapman, J. C. Romijn, W. M. Van Weerden and G. Jenster (2005) Androgen receptor modifications in prostate cancer cells upon long-termandrogen ablation and antiandrogen treatment *Int J Cancer* **117** 2 221-9
86 M. Urushibara, J. Ishioka, N. Hyochi, K. Kihara, S. Hara, P. Singh, J. T. Isaacs and Y. Kageyama (2007) Effects of steroidal and non-steroidal antiandrogens on wild-type and mutant androgen receptors *Prostate* **67** 8 799-807
87 C. E. Bohl, W. Gao, D. D. Miller, C. E. Bell and J. T. Dalton (2005) Structural basis for antagonism and resistance of bicalutamide in prostate cancer *Proc Natl Acad Sci U S A* **102** 17 6201-6
88 C. E. Bohl, D. D. Miller, J. Chen, C. E. Bell and J. T. Dalton (2005) Structural basis for accommodation of nonsteroidal ligands in the androgen receptor *J Biol Chem* **280** 45 37747-54
89 H. Miyamoto, M. M. Rahman and C. Chang (2004) Molecular basis for the antiandrogen withdrawal syndrome *J Cell Biochem* **91** 1 3-12
90 J. J. Rambeaud (1999) Intermittent complete androgen blockade in metastatic prostate cancer *Eur Urol* **35 Suppl 1** 32-6
91 G. Han, B. A. Foster, S. Mistry, G. Buchanan, J. M. Harris, W. D. Tilley and N. M. Greenberg (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** 14 11204-13
92 G. Han, G. Buchanan, M. Ittmann, J. M. Harris, X. Yu, F. J. Demayo, W. Tilley and N. M. Greenberg (2005) Mutation of the androgen receptor causes oncogenic transformation of the prostate *Proc Natl Acad Sci U S A* **102** 4 1151-6
93 M. Marcelli, M. Ittmann, S. Mariani, R. Sutherland, R. Nigam, L. Murthy, Y. Zhao, D. Diconcini, E. Puxeddu, A. Esen, J. Eastham, N. L. Weigel and D. J. Lamb (2000) Androgen receptor mutations in prostate cancer *Cancer Res* **60** 4 944-9
94 X. B. Shi, L. Xue, C. G. Tepper, R. Gandour-Edwards, P. Ghosh, H. J. Kung and R. W. Devere White (2007) The oncogenic potential of a prostate cancer-derived androgen receptor mutant *Prostate* **67** 6 591-602
95 J. Clark, S. Merson, S. Jhavar, P. Flohr, S. Edwards, C. S. Foster, R. Eeles, F. L. Martin, D. H. Phillips, M. Crundwell, T. Christmas, A. Thompson, C. Fisher, G. Kovacs and C. S. Cooper (2007) Diversity of TMPRSS2-ERG fusion transcripts in the human prostate *Oncogene* **26** 18 2667-73

96 K. G. Hermans, R. Van Marion, H. Van Dekken, G. Jenster, W. M. Van Weerden and J. Trapman (2006) TMPRSS2:ERG fusion by translocation or interstitial deletion is highly relevant in androgen-dependent prostate cancer, but is bypassed in late-stage androgen receptor-negative prostate cancer *Cancer Res* **66** 22 10658-63

97 S. Perner, F. Demichelis, R. Beroukhim, F. H. Schmidt, J. M. Mosquera, S. Setlur, J. Tchinda, S. A. Tomlins, M. D. Hofer, K. G. Pienta, R. Kuefer, R. Vessella, X. W. Sun, M. Meyerson, C. Lee, W. R. Sellers, A. M. Chinnaiyan and M. A. Rubin (2006) TMPRSS2:ERG Fusion-Associated Deletions Provide Insight into the Heterogeneity of Prostate Cancer *Cancer Res* **66** 17 8337-41

98 M. J. Soller, M. Isaksson, P. Elfving, W. Soller, R. Lundgren and I. Panagopoulos (2006) Confirmation of the high frequency of the TMPRSS2/ERG fusion gene in prostate cancer *Genes Chromosomes Cancer* **45** 7 717-9

99 S. A. Tomlins, D. R. Rhodes, S. Perner, S. M. Dhanasekaran, R. Mehra, X. W. Sun, S. Varambally, X. Cao, J. Tchinda, R. Kuefer, C. Lee, J. E. Montie, R. B. Shah, K. J. Pienta, M. A. Rubin and A. M. Chinnaiyan (2005) Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer *Science* **310** 5748 644-8

100 I. V. Litvinov, D. J. Vander Griend, L. Antony, S. Dalrymple, A. M. De Marzo, C. G. Drake and J. T. Isaacs (2006) Androgen receptor as a licensing factor for DNA replication in androgen-sensitive prostate cancer cells *Proc Natl Acad Sci U S A* **103** 41 15085-90

101 D. J. Vander Griend, I. V. Litvinov and J. T. Isaacs (2007) Stabilizing androgen receptor in mitosis inhibits prostate cancer proliferation *Cell Cycle* **6** 6 647-51

Summary

The androgens testosterone (T) and dihydrotestosterone (DHT) are steroid hormones, which are necessary for development and maintenance of the functions of the male sex organs, including the prostate. Androgens also play an important role in benign abnormalities of the prostate and in the growth of prostate cancer. Prostate tumors, which are not yet metastatic, are treated with radiotherapy or by surgical removal of the complete prostate. Therapy of metastasized prostate cancer aims on inhibition of androgen action, by inhibtion of the production of T in the testis (chemical castration) and by administration of anti-androgens. T and DHT exert their function by specific binding as a ligand to the androgen receptor (AR). The AR is a member of the family of nuclear receptors. It is expressed in androgen target cells, and functions as a ligand induced transcription factor. In this thesis described research project focusses on several molecular mechanisms of AR functions.

Chapter 1 gives an overview of the current knowledge on nuclear receptors in general and different aspects of AR functions in particular. Among the latter are: receptor structure, interaction with other proteins involved in transcription, the ligand-dependent interaction between the N-terminal domain (NTD) and the ligand binding C-terminal domain (LBD) of the AR (N/C interaction), expression of androgen-specific regulated genes, and the role of AR mutations in prostate cancer.

In Chapter 2 a motif in the AR NTD, ^{23}FXXLF^{27}, that is essential for the N/C interaction is described. Mutation of the phenylalanine residues or leucine in this motif completely disturbs the interaction. Flanking residues have a modulating role in the N/C interaction. The FXXLF motif can adopt an α-helix conformation and binds to the LBD surface at the same postition as some coactivators. Coactivators can contain one or more LXXLL or FXXLF motifs. Coactivators with an FXXLF motif are specific for the AR. The function of the AR N/C interaction is not clear as yet. The interaction can play a role in the stability of ligand binding. There are also indications that the N/C interaction is important for expression of endogenic genes, but less important in model systems that transiently express a reporter gene.

In Chapter 3 the characterization of the specifically androgen-regulated gene *SARG* is described. This gene was identified in the LNCaP-1F5 sub cell line, which in addition to the AR also stably expresses the glucocorticoid receptor (GR). In this way it is possible to distinguish between genes that can be activated by more than one steroid receptor and genes that are regulated by one specific receptor. *SARG* transcription in 1F5 cells can be regulated by androgens, but not by glucocorticoids. The *SARG* gene contains 4 exons and alternative splicing results in transcripts missing exon 2 and with an exon 1 that varies in length. Variable polyadenylation leads also to transcripts of different lengths. The SARG protein consists of 601 amino acids and is localized in the cytoplasm. However, its function has not been established yet. A bioinformatics-based screening of the *SARG* gene, including up- and down-stream sequences, revealed a number of candidate AR binding sites (androgen response elements or AREs) on the DNA, of which one that is localized in intron 1, is AR specific. EMSA and ChIP experiments have shown that the GR cannot bind to this ARE, and an enhancer containing this ARE cannot be activated by GR. It is now known that AR specific AREs have one half-site with a high affinity for the AR, and one low affinity half-site. Hydrogen bonds between the DBDs in an AR dimer are responsible for a strong AR-AR interaction, so that a relatively weak binding to one of the half-sites is permitted. These hydrogen bonds are not present in the GR dimer, which can explain why the GR dimer does not bind to an AR-specific ARE.

Prostate tumors initially are dependent on androgens for their growth. In a late stage of tumor growth this is much less the case. Then the tumors are called endocrine therapy resistant. In part of these resistant tumors, amino acid substitutions have been found in the AR through which it cannot only be activated by T or DHT, but also by other steroids and even by anti-androgens. In Chapter 4 research is decribed in which the prostate cancer mutation hot spots H874 and T877 are randomly mutated and mutants are selected for their activation by progesterone. This screen identified the same mutations as found in prostate cancer: H874Y, T877A, and T877S. These mutants are subsequently tested with a variety of hormones and, next to the expected activation by progesterone, estradiol, and hydroxyflutamide, these mutants also appeared to be sensitive to physiological concentrations of cortisol. This could mean that prostate tumors have obtained these mutant ARs by selective pressure by anti-androgens, after which these could also be maintained by cortisol. The broadened ligand-specificity of the mutant ARs caused by a substitution on position 877 can be explained by the substitution of threonine by a smaller amino acid residue, like alanine or serine. The amino acid at position 877 is within the ligand binding pocket and binds to the 17β-hydroxyl-group of androgens, whereby a smaller amino acid residue means that there is more space in this pocket, so that ligands larger than T or DHT, e.g. progesterone, also can bind, which is not possible in the wild-type receptor. Apparently, the interactions left are sufficient for stable binding of the ligand and/or new interactions might be formed. H874 is not located in the ligand binding pocket, but it is presumed that the H874Y substitution changes the conformation of the AR LBD, in such a way that the ligand binding pocket becomes available for non-androgenic hormones.

In Chapter 5 the research which is described in the previous chapters is placed in the broader context of recent literature on molecular mechanisms of AR functions. In this chapter also a perspective for further research on these mechanisms is given.

Samenvatting

De androgenen testosteron (T) en dihydrotestosteron (DHT) zijn steroïdhormonen, die noodzakelijk zijn voor de ontwikkeling en het instandhouden van de functies van de mannelijke geslachtsorganen, waaronder de prostaat. Daarnaast spelen androgenen een belangrijke rol in benigne afwijkingen van de prostaat en bij de groei van prostaatkanker. Prostaattumoren, die nog niet zijn uitgezaaid, worden behandeld door middel van radiotherapie of door chirurgische verwijdering van de gehele prostaat. De therapie van gemetastaseerd prostaatkanker is erop gericht om de werking van androgenen tegen te gaan, door de productie van T in de testis te remmen (chemische castratie) en door het toedienen van anti-androgenen. T en DHT oefenen hun werking uit door specifiek te binden als ligand aan de androgeenreceptor (AR). De AR behoort tot de familie van kernreceptoren. De AR komt tot expressie in doelwitcellen van androgenen en functioneert als een ligand-geïnduceerde transcriptiefactor. Het in dit proefschrift beschreven onderzoek richt zich op een aantal moleculaire mechanismen van AR functies.

Hoofdstuk 1 geeft een overzicht van de huidige kennis van kernreceptoren in het algemeen en verschillende aspecten van de AR functies in het bijzonder. Onder deze laatste zijn: structuur van de receptor, de interactie met andere eiwitten betrokken bij de regulering van transcriptie, de ligand-afhankelijke interactie tussen het N-terminale domein (NTD) en het ligand-bindende of C-terminale domein (LBD) van de AR (N/C interactie), de expressie van androgeen-specifiek gereguleerde genen, en de rol van AR mutaties in prostaat kanker.

In Hoofdstuk 2 is een motief in de AR NTD, ^{23}FXXLF^{27}, beschreven dat essentieel is voor de N/C interactie. Mutatie van de fenylalanine residuen of van leucine in dit motief verstoort de interactie compleet. Omliggende aminozuur residuen hebben een modulerende rol in de N/C interactie. Het FXXLF motief kan een α-helix conformatie aannemen en bindt aan het LBD oppervlak op dezelfde plaats als sommige coactivatoren. Coactivatoren kunnen één of meerdere LXXLL of FXXLF motieven bevatten. Coactivatoren met een FXXLF motief zijn specifiek voor de AR. De functie van de AR N/C interactie is nog niet geheel duidelijk. De interactie kan een rol spelen bij stabiliteit van de binding van een ligand. Ook zijn er aanwijzingen dat de N/C interactie belangrijk is voor de expressie van endogene genen, maar van minder belang in model systemen, waarbij een reportergen transient tot expressie wordt gebracht.

In Hoofdstuk 3 wordt de karakterisering van het specifiek androgeen-gereguleerde gen *SARG* beschreven. Dit gen werd geïdentificeerd in de LNCaP-1F5 subcellijn die naast de AR ook stabiel de glucocorticoidreceptor (GR) tot expressie brengt. Hierdoor is het mogelijk om onderscheid te maken tussen genen die door meerdere steroidreceptoren geactiveerd kunnen worden en genen die door één specifieke receptor gereguleerd worden. *SARG* transcriptie kan in 1F5 cellen wel door androgenen gestimuleerd worden maar niet door glucocorticosteroïden. Het *SARG* gen bevat 4 exonen en alternatieve splicing resulteert in transcripten die exon 2 missen en een exon 1 hebben dat varieert in lengte. Variabele polyadenylering leidt ook tot transcripten van verschillende lengtes. Het SARG eiwit bestaat uit 601 aminozuren en is gelokaliseerd in het cytoplasma. De functie ervan is echter nog niet vastgesteld. Een op bioinformatica gebaseerde screening van het *SARG* gen, inclusief sequenties voor en achter het gen, leverde op het DNA een aantal kandidaat bindingsplaatsen voor de AR (androgeen respons elementen of AREs) op, waarvan er één, die zich in intron 1 bevindt, AR-specifiek is. EMSA, ChIP experimenten hebben laten zien dat deze ARE niet in staat is de GR te binden en ook dat de enhancer die deze ARE bevat niet geactiveerd kan worden door de GR, maar wel door de AR. Van de AR-specifieke AREs is nu bekend dat ze één half-site met hoge affiniteit voor de AR hebben en één

half-site met een veel lagere affiniteit. Waterstofbruggen tussen de twee DBDs in een AR dimeer zorgen voor een sterke AR-AR interactie, waardoor een zwakkere binding aan één van de ARE half-sites gepermitteerd lijkt. Deze waterstofbruggen komen niet voor in een GR dimeer, wat kan verklaren waarom de GR niet goed bindt aan een AR specifieke ARE.

Prostaattumoren zijn in het begin van hun groei afhankelijk van androgenen. Dit is in een laat stadium van tumorgroei veel minder het geval. Deze laatste tumoren zijn dus resistent geworden tegen de endocriene therapie. In een deel van deze resistente tumoren zijn aminozuursubstituties in de AR gevonden waardoor deze niet alleen geactiveerd kan worden door T of DHT, maar ook door andere steroïden en zelfs door anti-androgenen. In Hoofdstuk 4 is een onderzoek beschreven waarin de in prostaatkanker gevonden mutatie hot spots H874 en T877 random gemuteerd zijn en mutanten geselecteerd zijn voor hun activering door progesteron. Hierbij werden dezelfde mutanten gevonden als ook in prostaatkanker voorkomen: H874Y, T877A en T877S. Deze mutanten zijn in vervolgexperimenten getest met een heel scala aan hormonen en naast de verwachte activering door progesteron, estradiol en hydroxyflutamide bleken deze mutanten ook gevoelig voor fysiologische concentraties cortisol. Dit zou kunnen betekenen dat prostaattumoren deze mutante ARs door selectiedruk van anti-androgeen hebben verkregen, waarna deze bovendien in stand gehouden zouden kunnen worden door cortisol. De bredere ligand-specificiteit van de mutante ARs veroorzaakt door een substitutie op positie 877 kan worden verklaard door de substitutie van threonine door een kleiner aminozuur residu, zoals alanine of serine. Positie 877 is onderdeel van de ligand-bindende pocket en bindt de 17β-hydroxyl-groep van androgenen, waarbij een kleiner aminozuur betekent dat er meer ruimte is in deze pocket, zodat liganden groter dan T of DHT, bv. progesteron, ook kunnen binden, wat ze niet kunnen met de wild-type receptor. Blijkbaar zijn overgebleven interacties voldoende voor stabiele binding van het ligand en/of worden nieuwe interacties gevormd. H874 ligt niet in de ligand-bindende pocket, maar verondersteld wordt dat de H874Y substitutie een verandering in de conformatie van het AR LBD teweegbrengt, die de ligand-bindende pocket ook geschikt maakt voor niet-androgene hormonen.

In Hoofdstuk 5 zijn de onderzoeken die beschreven zijn in voorgaande hoofdstukken in de bredere context van recente literatuur over de moleculaire mechanismen van AR functies geplaatst. Ook is in dit hoofdstuk een perspectief aangegeven voor verder onderzoek naar deze mechanismen.

List of publications

A bioinformatics-based functional analysis shows that the specifically androgen-regulated gene SARG contains an active direct repeat androgen response element in the first intron.
Karine Steketee K, Hetty A.G.M. van der Korput, Adriaan B. Houtsmuller and Jan Trapman
J Mol Endocrinol. 2004, 33: 477-91

Amino acids 3-13 and amino acids in and flanking the ^{23}FxxLF^{27} motif modulate the interaction between the amino-terminal and ligand binding domain of the androgen receptor.
Karine Steketee, Cor A. Berrevoets, Erik Jan Dubbink, Paul Doesburg, Remko Hersmus, Albert O. Brinkmann and Jan Trapman
Eur J Biochem. 2002 Dec;269(23):5780-11

Broadened ligand responsiveness of androgen receptor mutants obtained by random amino acid substitution of H874 and mutation hot spot T877 in prostate cancer.
Karine Steketee, Leon Timmerman, Angelique C.J. Ziel-van der Made, Paul Doesburg, Albert O. Brinkmann and Jan Trapman
Int J Cancer. 2002 Jul 20;100(3):309-17

Mechanisms of androgen receptor activation and function.
Brinkmann AO, Blok LJ, de Ruiter PE, Doesburg P, Steketee K, Berrevoets CA, Trapman J.
J Steroid Biochem Mol Biol. 1999 Apr-Jun;69(1-6):307-13. Review

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).
Berrevoets CA, Doesburg P, Steketee K, Trapman J, Brinkmann AO.
Mol Endocrinol. 1998 Aug;12(8):1172-83

Both androgen receptor and glucocorticoid receptor are able to induce prostate-specific antigen expression, but differ in their growth-stimulating properties of LNCaP cells.
Cleutjens CB, Steketee K, van Eekelen CC, van der Korput JA, Brinkmann AO, Trapman J.
Endocrinology. 1997 Dec;138(12):5293-300

Functional in vivo interaction between the amino-terminal, transactivation domain and the ligand binding domain of the androgen receptor.
Doesburg P, Kuil CW, Berrevoets CA, Steketee K, Faber PW, Mulder E, Brinkmann AO, Trapman J.
Biochemistry. 1997 Feb 4;36(5):1052-64

Genomic and cDNA cloning of a novel mouse lipoxygenase gene.
Willems van Dijk K, Steketee K, Havekes L, Frants R, Hofker M.
Biochim Biophys Acta. 1995 Oct 26;1259(1):4-8

Curriculum vitae

Karine Steketee was born on the 20th of November 1967 in 's-HeerHendrikskinderen (in the former municipality of 's-HeerArendskerke, currently Goes). Her secondary education (VWO) was taken at the Buys Ballot College (currently Ostrea Lyceum) in Goes. In 1986/1987 she worked for one year as a nurse trainee. In 1991 she obtained her bachelors degree in Chemistry (specialization Biochemistry) at the Hogeschool Zeeland in Vlissingen. In 1994 she graduated in Biology (specialization Medical Genetics) at Leiden University. In July 1992 she started a minor graduation project, entitled 'Extracellular phosphorylation of adenosine by Dictyostelium discoideum', which was performed at the Department of Cell Biology of Leiden University under supervision of Ir. P.H.A.M. Michielsen. Her major graduation project was started in August 1993 at the Department of Human Genetics, and was supervised by Prof. Dr. R.R. Frants and Dr. Ir. J.A.P. Willems van Dijk. The title of this project was 'Characterization of murine lipoxygenase genes', and it was part of an atherosclerosis research project. She obtained her MSc on August 8th 1994. From August to November 1994 she worked as a research assistant at the same department on a Multiple Tumor Suppressor 1 (MTS1) project under supervision of Dr. N.A. Gruis. In November 1994 she started her PhD project at the at the Department of Pathology (Josephine Nefkens Institute) of the ErasmusMC at the Erasmus University Rotterdam, under supervision of Prof.dr. J. Trapman and Dr. A.O. Brinkmann. From June 1999 to July 2000 she worked as a research assistant, and until recently as a guest worker at the same department. Since August 2003 she has been employed by the Christelijke Scholengemeenschap Walcheren in Middelburg as a teacher in Biology, Chemistry, and General Sciences (Algemene Natuurwetenschappen). In 2004 she was a lecturer at the Department of Chemistry and Aquatic Ecotechnology of the Hogeschool Zeeland. Since August 2005 she has been working as a junior university teacher in the Science Department at the Roosevelt Academy (honors university college) in Middelburg. In November 2006 she obtained her teaching qualification at the ICLON, Leiden University Graduate School of Teaching.

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