“The Role of Ubiquitylation and Acetylation in Androgen Receptor Function”

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Index

Abbreviations

A	Alanine
AF	Activation function
AR	Androgen receptor
ARE	Androgen response elements
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CAIS	Complete androgen insensitivity syndrome
CARM1	Coactivator-associated arginine methyltransferase 1
CBP	CREB binding protein
ChIP	Chromatin immunoprecipitation
CHIP	Carboxy terminus of Hsp70-interacting protein
CMV	Cytomegalovirus
CREB	cAMP responsive element binding protein
DBD	DNA-binding domain
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DUB	Deubiquitylating enzyme
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin-protein ligase
E6-AP	Human papilloma virus E6-associated protein
ECFP	Enhanced cyan fluorescent protein
EFB	Estrogen-responsive finger protein
ER	Estrogen receptor
EtOH	Ethanol
F	Phenylalanine
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
G3BP	GTPase activating protein (SH3 domain) binding protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GR	Glucocorticoid receptor
GST	Glutathione S-transferase
h	Hours
H1-4	Histone 1 - 4
HA	Hemagglutinin
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
HECT	Homologous to the E6-AP C-terminus
HIF-1α	Hypoxia inducible factor 1α
HRP	Horseradish peroxidase
HSP	Heat-shock protein
IP	Immunoprecipitation
ISG5	Interferon-stimulated protein, 15 kDa
K	Lysine
L	Leucine
LBD	Ligand-binding domain

Luc	Luciferase
Mdm2	murine double minute 2
MMTV	Mouse mammary tumour virus
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
N/C	N-terminal / C-terminal
NCoR	Nuclear receptor corepressor
Nedd8	Neural precursor cell expressed, developmentally down-regulated 8
NF-κB	Nuclear factor kappa B
NLS	Nuclear localisation signal
NR	Nuclear receptor
NTD	N-terminal domain
P/CAF	p300/CBP-associated factor
PAGE	Polyacrylamide gel electrophoresis
PAIS	Partial androgen insensitivity syndrome
PCR	Polymerase chain reaction
PIAS	Protein inhibitor of activated STAT
pol II	RNA polymerase II
PR	Progesterone receptor
PRMT1	Protein arginine methyltransferase 1
PSA	Prostate-specific antigen
pVHL	von Hippel-Lindau tumour suppressor protein
R	Arginine
R.L.U.	Relative luciferase unit
R1881	Methyltrienolone
RING	Really interesting new gene
RNA	Ribonucleic acid
S	Serine
SBMA	Spinal/bulbar muscular atrophy
SDS	Sodium dodecyl sulfate
SERPIN	Serine (or cysteine) proteinase inhibitor
siRNA	Small interfering RNA
SIRT	Sirtuin
SMRT	Silencing mediator for retinoid and thyroid hormone receptors
SRC	Steroid receptor coactivator
SRE	Steroid response elements
STAT	Signal transducer and activator of transcription
SUMO	Small ubiquitin-like modifier
TAF	TBP-associated factor
TBP	TATA-binding protein
TFII	Transcription factor II
TIF2	Transcriptional intermediary factor 2
Tip60	Tat interacting protein, 60kDa
TMPRSS2	Transmembrane protease, serine 2
TRITC	Tetramethyl rhodamine isothiocyanate
TSA	Trichostatin A
TSG101	Tumour susceptibility gene 101
Ub	Ubiquitin
UBC	Ubiquitin-conjugating enzyme
Ubc9	Ubiquitin-conjugating enzyme E2I (homologous to yeast UBC9)
USP	Ubiquitin specific protease
W	Tryptophan
wt	Wild type
Zimp10	Zinc finger-containing, Miz1, PIAS-like protein on chromosome 10

1 Introduction

1.1 Regulation of gene expression in eukaryotes

1.1.1 General mechanisms

Almost all cells in a pluricellular organism contain the same genetic material. However, as a result of differential gene expression, they become committed to different lineages during ontogeny, which leads to dramatic changes in their morphology and function. Moreover, cells are able to rapidly modify their gene expression pattern as a response to extracellular or intracellular signals, thus reacting to changes in their environment. The regulatory mechanisms that determine which genes will be transcribed at which time point in the cell are known as the control of gene expression [1].

Virtually each step leading to the generation of mature RNA transcripts is susceptible to be controlled. Different mechanisms regulate the transcription initiation and elongation, the splicing of the pre-mRNA and the stability of the end product. In addition, the translation step and the stability and activity of the resulting protein are controlled by post-translational modifications. For most genes the most important control point is the initiation of transcription. For transcription to take place some crucial steps are needed. First, the DNA compacted in the chromatin must become accessible. This is controlled by the binding of large multi-protein complexes possessing ATP-dependent chromatin-remodelling activities and able to locally modify histone tails, mainly acetylation/deacetylation and methylation/demethylation of H3 and H4, and ubiquitylation/deubiquitylation of H2A and H2B. This modulates the accessibility of transcription factors to gene regulatory regions. Transcription factors are proteins that recognise and bind to specific DNA sequences in the control region of a gene and allow to turn on or off its transcription. They enhance gene expression by binding upstream of the transcription initiation site and promoting the assembly of a complex that serves as a recruitment platform for the RNA polymerase II and the general transcription factors. Recruitment and binding of RNA polymerase II and of the general transcriptional machinery to the promoter regions is also enabled by local changes in chromatin structure and histone modifications. These events culminate in the phosphorylation

of RNA polymerase II at multiple positions leading to activation of its function and start of transcription.

Transcription factors are themselves influenced by signals which can control their activity in a variety of ways. One well-studied activating trigger is the direct binding of an agonistic ligand as is the case for several members of the nuclear receptor superfamily [2, 3]. This large group includes receptors that bind small hydrophobic signalling molecules such as steroid hormones, thyroid hormones, retinoids and vitamin D. The signal molecules diffuse passively through the plasma membrane and bind to their receptor protein in the cytoplasm or the nucleus. This leads to activation and binding to specific DNA response elements and ultimately regulation of the transcription of downstream target genes.

1.1.2 Post-translational modifications in the control of gene expression

The role of covalent protein modifications in the control of gene expression has been known for a long time. The first modifications found were the acetylation and methylation of histones. Since then, a complex modification map of the histone tails has been described to lead to transcriptional activation or repression and therefore it is generally named the histone code [4]. Transcription factors can also be direct targets for a variety of post-translational modifications such as phosphorylation, acetylation, ubiquitylation and sumoylation, which modulate their activity in different ways. Extensive studies carried out for transcription factors such as p53 show that a complicated network of post-translational modifications that cross-talk with each other regulates their activity [5].

1.1.2.1 Ubiquitylation

The ubiquitin-proteasome system

Ubiquitylation is the covalent binding of a 76-residue-long ubiquitin polypeptide to a protein substrate at specific lysine residues. This process requires the sequential action of three enzymes. First, an activating enzyme (E1) forms a thioester with the carboxyl group of the last residue of ubiquitin, glycine 76, in an ATP-dependent reaction. Second, the ubiquitin polypeptide is transferred to a conjugating enzyme (E2) forming also a thioester. Third, a ubiquitin ligase (E3) transfers the activated ubiquitin to a lysine of the substrate to form an isopeptide bond [6]. There are two main types of ubiquitin ligases. The ligases of the RING class bind both the E2 and the protein substrate and facilitate the direct transfer of ubiquitin.

The other family, the HECT-type ligases, takes over the ubiquitin residue from the E2 enzyme at its catalytic site and then transfers it to the substrate [7].

In a following reaction round, a second ubiquitin is attached to an internal lysine of the first one, usually at position 48. Additional rounds eventually result in the formation of a polyubiquitin chain attached to the protein substrate. Polyubiquitin chains linked through K48 target the protein substrate for degradation through the proteasome. The proteasome is a multimeric protease complex composed of the 20S proteolytic core and two 19S regulatory particles [8]. The 20S core consists of two copies of 14 different subunits arranged in four heptamer rings forming a barrel-shaped complex with the proteolytic activity directed to the inside and thus separated from the cellular context. The 19S regulatory cap, bound to either end of the 20S barrel, contains a non-ATPase lid of 12 subunits and a hexameric AAA ATPase.

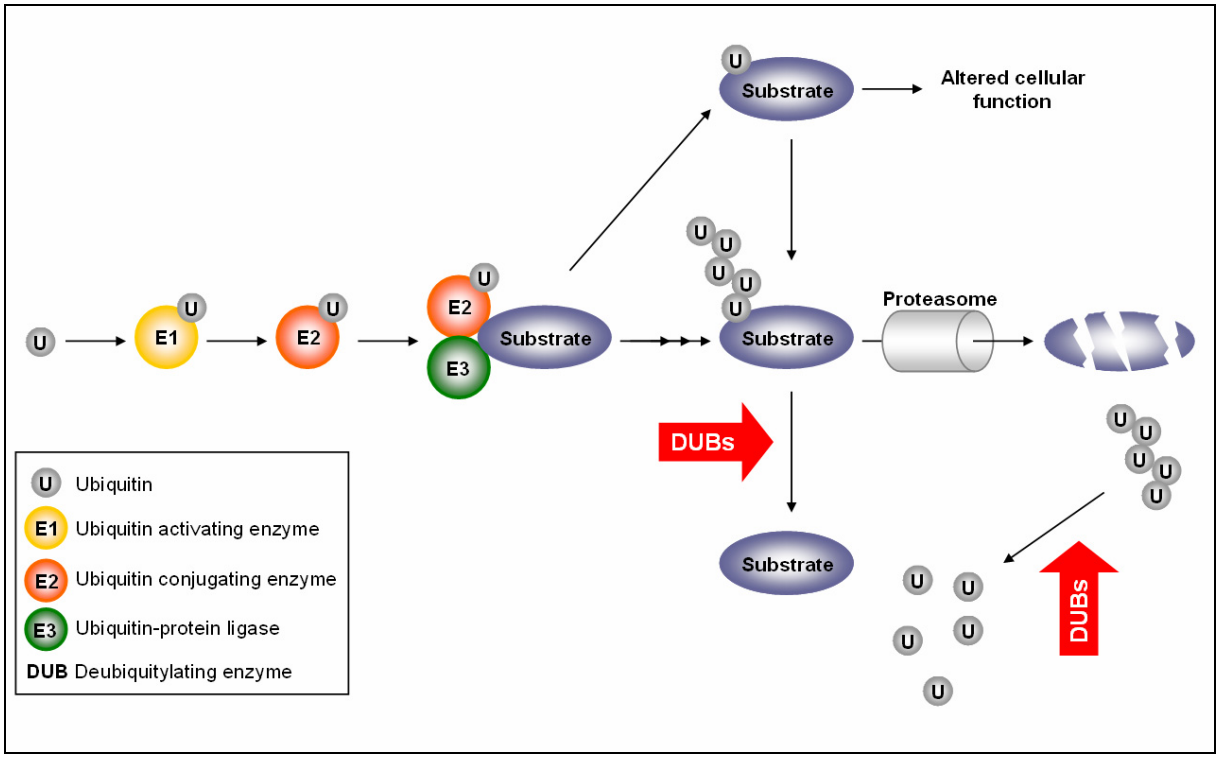

Figure 1

The ubiquitin-proteasome system. Ubiquitin is transferred to a protein substrate in a three-step reaction. Lysine 48-linked polyubiquitin chains target the protein for degradation through the proteasome, whereas monoubiquitylation or alternative polyubiquitin chains modify localisation and/or function of the protein. Ubiquitylation can be reversed by the action of specific proteases (deubiquitylating enzymes).

Besides K48 other lysines can be targeted for the chain formation, for example K6, K11, K29 and K63. With the exception of K29, the other polyubiquitin chains do not target the protein for degradation. The best characterised of them are the K63-linked ubiquitin chains which regulate protein-protein interactions and are involved in processes such as DNA repair and signal transduction [9]. In addition, proteins may also be targets for monoubiquitylation, a modification which leads to modified protein characteristics. Monoubiquitylation of proteins is known to play a role in endocytosis and transcriptional regulation [10]. Interestingly, ubiquitin polymers containing mixed linkages have also been observed in vitro and in vivo, but their prevalence and significance remain unclear [11].

The modification through ubiquitin is a reversible process. A large family of proteases, known as deubiquitylating enzymes (DUB), are responsible for the cleavage of the isopeptide bond between ubiquitin and its substrate [12]. By reverting their ubiquitylation status DUB enzymes may therefore stabilise proteins but also revert the other functional consequences of protein ubiquitylation. Moreover, DUB enzymes are responsible for processing inactive ubiquitin precursors, for removing the ubiquitin chain from the substrate just before degradation and for the disassembly of this chain to recycle the ubiquitin monomers [13]. The DUBs are grouped in five subfamilies according to their sequence similarity [14]. The largest group is the ubiquitin-specific protease (USP) family, which contains cysteine proteases harbouring two conserved motifs named the Cys and His boxes.

It is worth mentioning that a number of related ubiquitin-like proteins have additionally been found to function as protein modifiers as well. They include, among others, SUMO, Nedd8 and ISG15 which regulate many cellular processes such as transcription, DNA repair, and signal transduction [15].

Regulation of transcription factor function through the ubiquitin-proteasome system

Transcription factors are regulated by the ubiquitin-proteasome system in different ways [16]. Ubiquitylation of a transcription factor can regulate its abundance in the cell, its subcellular localisation and its activity by modulating the interaction with partner proteins. The first known activity of the ubiquitin-proteasome system is to target proteins for degradation and this is used by the cell to control the abundance and therefore activity of transcription factors. This is the case for the hypoxia-inducible factor 1a (HIF-1α). In normoxic conditions, HIF-1α is hydroxylated which leads to ubiquitylation and subsequent degradation in the proteasome.

In hypoxic conditions, ubiquitylation does not occur so that HIF-1α rapidly accumulates and function as a transcriptional activator [17]. How ubiquitylation and degradation can control the subcellular localisation of a transcription factor is exemplified by NF-κB, which is found in the cytoplasm bound to the inhibitor protein IκB. During inflammation, IκB is phosphorylated, ubiquitylated and degraded, which allows NFκB to enter the nucleus and carry out its function. As mentioned before, ubiquitylation can control protein-protein interactions independently of the degradation pathway. The ubiquitylation of Myc resulting in K68-linked polyubiquitin chains allows its interaction with the coactivator p300, which is required for its transcriptional activity. Interestingly, for some transcription factors activity and proteolytic destruction seem to be tightly coupled as monoubiquitylation of the transcription factor is required for its activity but at the same time is the marker for polyubiquitylation and degradation [18]. This is documented for the estrogen receptor α (ERα). After ligand binding, ERα transcriptional activity depends on its ubiquitylation by EFP, which subsequently also promotes ERα degradation [19].

Other roles of the ubiquitin-proteasome system in transcription

The role of the ubiquitin-proteasome system in the regulation of transcription is not restricted to the modulation of the activity of transcription factors [16]. Ubiquitylated forms of histones have been reported and can be associated with active or silenced chromatin. Moreover, ubiquitylation influences other histone modifications. For example, ubiquitylation of H2B is required for the methylation of H3 at K4 and K79, contributing to gene silencing [20]. Histone ubiquitylation can be considered, like acetylation and methylation, as an integral part of the histone code. The crosstalk between the transcription and the ubiquitin-proteasome machineries is so tight that they even share several components. Subunits of the 19S cap of the proteasome have been found at the promoters of active genes whereas components of the general transcriptional machinery such as $TAF_{II}250$ have been shown to act as ubiquitin ligases [21].

1.1.2.2 Acetylation

Both non-histone proteins and histones can be acetylated. Concerning histones, multiple acetylated lysines have been identified in their N-terminal tail including H4K5, H4K8, H4K12 and H4K16. The correlation between these modifications and the loosening of chromatin structure that facilitates gene transcription has been known for a long time. The

enzymes responsible for these modifications are the histone acetyl transferases (HATs), of which about 30 members have been found. The acetyl group can be removed by histone deacetylases (HDACs). There are 18 potential deacetylase enzymes belonging either to the HDAC family (HDAC1 to HDAC11) or to the more recently discovered sirtuin family (SIRT1 to SIRT7). Although these enzymes have originally been identified as regulators of histones, recent results have revealed that several other proteins, mainly transcription factors, are also targets for lysine acetylation [22]. The regulation of transcription factors by acetylation is complex and may have several outcomes depending on the context. In most of the cases, acetylation increases transcription factor activity through enhanced DNA binding capacity and protein-protein interactions as is the case for STAT3 [23]. However, acetylation within the DNA-binding motif can also lead to a loss of DNA binding and therefore to inactivation. Moreover, some protein-protein interactions can be disrupted upon acetylation of the transcription factor. For example Tip60 acetylation of Notch1 results in the suppression of its transcriptional activity due to the dissociation of the Notch1-IC-CLS complex [24].

One of the most interesting regulation mechanisms is the control of protein stability through the cross-talk between acetylation and ubiquitylation [25]. The primary interacting point between these modifications is that both have a lysine residue as target. Therefore, acetylation of a lysine will protect it from ubiquitylation. The result will be enhanced protein stability and this is indeed the case for several transcription factors such as p53 and Runx3 where the same lysines are suggested to be subject of both modifications. On the other hand, the acetylation of HIF-1α at lysine 532 has been shown to enhance its interaction with the E3 ligase pVHL mediating its ubiquitylation at a second lysine and its subsequent degradation [26]. The acetylation-induced protein degradation may follow more indirect mechanisms. For instance, lysine acetylation of the chaperone HSP90 may disrupt its interaction with the protected protein leading to its degradation.

1.2 The Androgen Receptor

1.2.1 Structure and function of the AR

The AR is a ligand-dependent transcription factor which belongs to the family of steroid receptors together with the estrogen receptors (ERα and ERβ), the glucocorticoid receptor (GR), the progesterone receptor (PR) and the mineralocorticoid receptor (MR) [27, 28]. As such, the AR is bound by the male sexual hormones, testosterone and its more active metabolite dihydrotestosterone (DHT), and is responsible for the subsequent changes in gene expression. The AR regulates the expression of almost 100 genes, including up-regulated genes such as prostate specific antigen (PSA), probasin, kallikrein 2, keratinocyte growth factor, and TMPRSS2, and also down-regulated genes such as the members of the SERPIN family B5 (maspin) and I1. This knowledge was originally derived from in vivo experiments or from cell culture studies after androgen treatment or depletion for several days or hours. This makes it difficult to distinguish between direct AR target genes and secondary downstream effects. New approaches such as genome-wide chromatin immunoprecipitation (ChIP-on-chip) have already added more information about the DNA motifs that are recognised by the AR and the regulation mechanisms of target genes [29, 30].

In the absence of hormone, the AR is sequestered in the cytoplasm in complex with heat-shock proteins in a transcriptionally inactive form. Upon ligand binding, the AR undergoes a conformational change that exposes the nuclear localisation signal and enables the translocation of the AR to the nucleus. There, the AR binds as a homodimer to specific DNA sequences known as androgen response elements in the promoter and enhancer regions of target genes. The DNA-bound receptor can then exert a positive or negative effect on gene transcription by recruiting either coactivators or corepressors to the targeted promoter [31, 32]. Coactivators positively regulate transcription by forming large protein complexes that modify and remodel the local chromatin structure to make it more accessible. On the other hand, corepressors recruited to the promoter facilitate chromatin condensation and silence transcription [33].

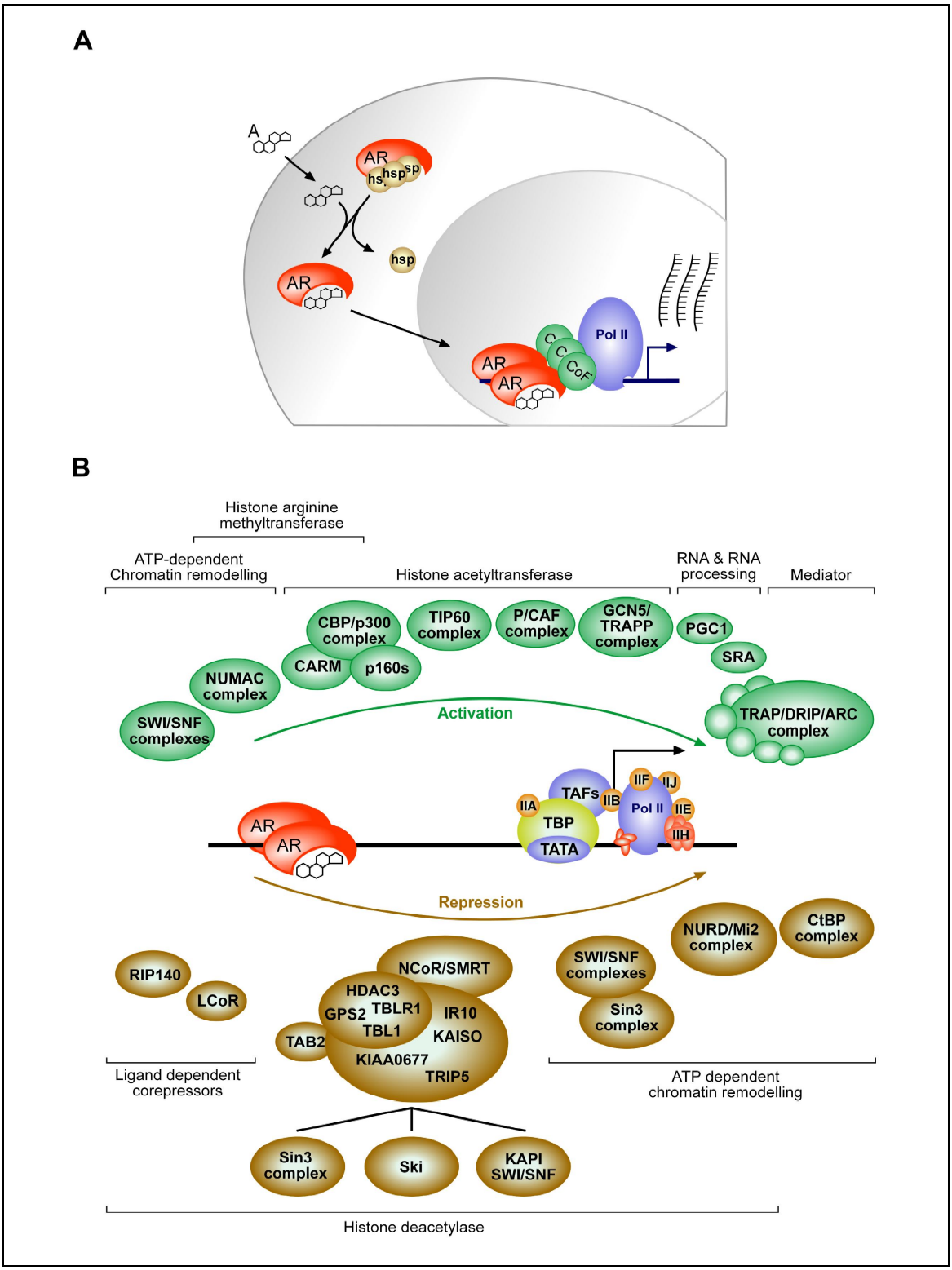

Figure 2

Androgen receptor mode of action. A. Androgens (A) diffuse through the cell membrane and bind to the androgen receptor (AR). Following hormone binding, the AR changes its conformation, dissociates from the heat-shock proteins (hsp) and translocates into the nucleus. There it binds to specific response elements as a homodimer, cofactors (CoF) are then recruited which interact with the general transcriptional machinery (Pol II) thus leading to transcriptional control of target genes. B. The DNA-bound receptor can exert a positive or negative effect on gene transcription by recruiting either coactivators or corepressors to the targeted promoter. Coactivator complexes (green) facilitate chromatin accessibility whereas corepressors (brown) promote chromatin condensation and gene silencing.

To date, more than 200 proteins that modulate AR transcriptional activity have been described [34]. The p160 steroid receptor coactivator family (SRC) contains three of the most important coactivators of the AR and other nuclear receptors, SRC-1, SRC-2 or TIF2 and SRC-3 or AIB1. SRC-1 and SRC-3 possess only a week acetylase activity which is not required for their coactivator function. The main role of the SRC family is to recruit chromatin-modulating complexes containing histone acetylases such as CBP, p300 and P/CAF, and histone methylases such as CARM1 and PRMT1 [35]. The regulation of AR activity through corepressors is less well understood but it has already been shown that the nuclear receptor corepressors NCoR and SMRT interact with the AR, resulting in the recruitment of proteins involved in transcriptional repression such as HDAC3 [36].

The human AR gene is located at the Xq11-12 locus and consists of eight exons. It spans a genomic region of approximately 90 kb. The AR gene codes for a protein containing approximately 919 amino acids with an apparent molecular mass of 110 kDa [37, 38]. The AR protein presents a strong length polymorphism due to two variable repeat regions, a polyglutamine repeat with a normal length varying from 18 to 31 and a polyglycine repeat with a normal length that ranges between 10 and 30 [39].

In contrast with other steroid receptors which present several isoforms resulting from different genes, as is the case for the ERα and ERβ, or from alternative start codon usage in the case of PR-A and PR-B, the concept of AR isoforms has for a long time not been accepted. A shortened form of the AR originating from an internal ATG codon had been described [40], but later data strongly suggested it to be a degradation product [41]. Later, a bona fide AR variant resulting from the use of an alternative exon 1, was described [42]. The resulting protein, AR45, possesses a seven amino acid long N-terminal stretch instead of the long N-terminal domain found in the AR. The role of AR45 in vivo is not yet known, but overexpression studies in cell lines suggest this isoform to act as a repressor of AR activity.

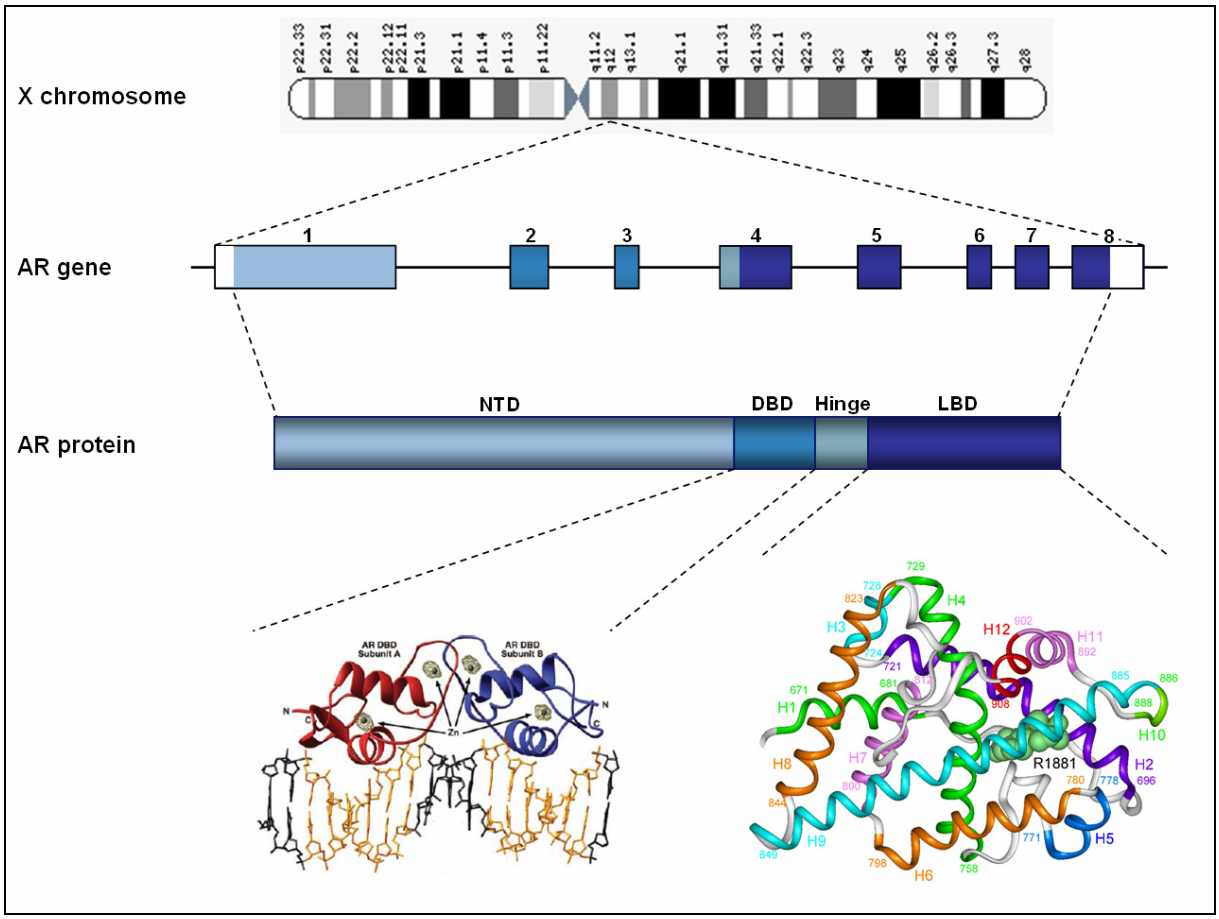

Figure 3

Schematic representation of the AR gene and protein structure. The human AR gene is located at the Xq11-12 locus. It consists of eight exons and spans a genomic region of more than 90 kb. The AR protein contains four domains, the N-terminal domain (NTD), the DNA-binding domain (DBD), a hinge region and the ligand-binding domain (LBD). The 3D-structure of the DBD bound to DNA (modified from [43]), and the LBD bound to the testosterone analogue R1881 (by JH Wu, Molecular Modelling Lab.) are also shown.

Like other nuclear receptors the AR is composed of four domains, a long N-terminal domain (NTD), a highly conserved DNA-binding domain (DBD), a hinge region and the ligand-binding domain (LBD) [32]. While the 3-dimensional structure of the full-length AR could not be determined up to now, the structure of the LBD in complex with diverse agonists and antagonists has been described [44-47]. The AR LBD domain consists of 11 α-helices forming a globular domain which contains the ligand-binding pocket. These helices are numbered from 1 to 12, omitting helix 2 to keep the nomenclature of the other family members that exhibit 12 helices. A total of 18 amino acid residues within the different helices are responsible for the direct interaction with the ligand, with helix 12 acting as a flexible lid to stabilise the ligand in the binding pocket. Binding of ligand to the LBD causes a change in the position of helix 12, as well as an overall conformation change which induces the formation of the activation function 2 (AF-2) The AF-2 forms a coactivator-binding surface

which serves as a docking site for LxxLL motifs present in many nuclear receptor cofactors [48, 49]. However, the isolated AF-2 displays a very low transcriptional activity suggesting that it may play a role in other processes [50]. Indeed, the AF-2 region binds preferably to the FxxLF and WxxLF motifs in the NTD, resulting in the interaction between the N-terminal and the C-terminal domains of the AR protein [51]. This N/C interaction is critical for the transcriptional activity of the AR on several promoters such as PSA, whereas other promoters seem to be independent of this interaction. Furthermore, in the case of the MMTV promoter it has been demonstrated that the N/C interaction is indispensable for the AR to bind the chromatin-integrated promoter but not the non-integrated reporter [52].

The region between the LBD and the DBD was first thought to be only a flexible region without further function. However, it has more recently been found that the hinge region modulates important functions of the AR. It contains part of the bipartite nuclear localisation signal (NLS) which starts in the DBD [53]. Three lysines included in the NLS have been found to undergo acetylation, adding complexity to the role of this region. Furthermore, a PEST motif [54], a sequence found in proteins with short half-lives due to rapid degradation by the proteasome system [55], has also been described within the hinge region. Finally, the hinge region seems to be able to modulate the interaction between the NTD and the LBD.

The AR DBD consists of two C4 zinc fingers characteristic of the nuclear receptor family. These fingers recognise and bind to specific DNA sequences named androgen response elements (ARE) and usually located in the promoter and enhancer regions of androgen-regulated genes. Most DNA response elements recognised by the AR and other steroid receptors are organised as semi-palindromic hexamers, but variant response elements have more recently been found [56]. These are organised as direct repeats and are androgen selective. In the case of the inverted ARE, the first zinc finger of the AR recognises and binds to DNA with further stabilisation of the interaction through the second zinc finger interaction with the phosphate backbone. In the case of the direct repeat ARE, the second zinc finger and a part of the hinge region interact directly with DNA [57, 43]. The second zinc finger contains the D-box which is the region responsible for the dimerisation of the AR. Mutations in this region impair the formation of AR dimers and result in loss of transcriptional activity at simple response elements [58] but not at tandem repeats.

The long N-terminal domain of the AR contains the main regions responsible for mediating transcriptional activity. When expressed as a separate domain, the NTD is a potent transcriptional activator and this activity, generally named AF-1, contrasts with the weak transcriptional activity of the AF-2 in the LBD. The NTD forms the principal surface of interaction with cofactors. Various AR cofactors, such as the members of the p160 family, and the general transcription factors TFIIF and TFIIH have been shown to interact with the AR NTD and to modulate AR trans-activation of target genes [34]. The AR NTD displays intrinsic disorder in solution which has precluded its crystallisation and the elucidation of its three-dimensional structure so far. However, biophysical studies indicate that the NTD exist in a so-called molten globule conformation. In this model, the NTD contain several partially folded intermediary structures that collapse into a stable structure upon cofactor binding [59, 60]. Finally, it should be added that the binding to specific DNA sequences affects the three-dimensional structure not only of the DBD but also of distal domains, as shown by limited proteolytic digestion of the AR [61, 56].

1.2.2 Post-translational modifications in AR transcriptional activity

The transcriptional activity of the AR is mainly governed by ligand binding but more and more studies document that post-translational modifications play an important additional part. Besides the long-known phosphorylation at several serine residues, other covalent additions such as acetylation, ubiquitylation and sumoylation have been found in the AR in recent years. These modifications affect the function of the AR by altering receptor stability, subcellular localisation as well as the interactions with other proteins. Since this work concentrates on AR ubiquitylation and acetylation, the other modifications will be only briefly commented.

AR phosphorylation has been studied for a long time and the existence of several basal and hormone-induced phosphorylated serines has been reported [62, 63]. However, these modifications do not seem to have a major impact on AR activity since mutation of several targeted serines does not affect AR function in reporter gene assays. On the other hand, the recently described phosphorylation-dependent ubiquitylation of the AR has again attracted interest in this modification.

The AR is modified by sumoylation at lysines 386 and 520 [64]. AR sumoylation is hormone dependent and involves Ubc9 and E3 ligases of the PIAS family, mainly PIAS1, PIASxα and Zimp10 [65-68]. The effects of sumoylation are mainly repressive. However, various androgen-controlled promoters respond differently to overexpression of enzymes involved in the sumoylation pathway [69]. Also, mutation of the main SUMO acceptor site in the AR leads to enhanced activity on promoters containing non-selective response elements, but has little effects in the presence of selective androgen response elements [70].

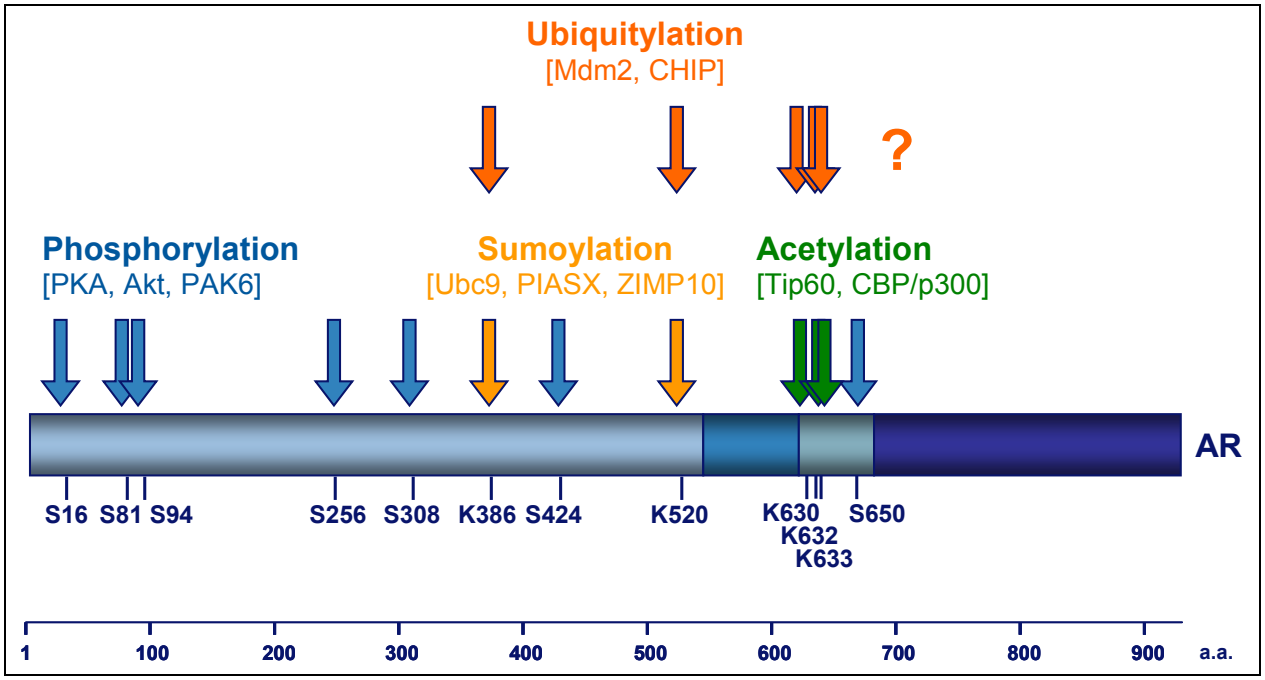

Figure 4

Schematical representation of the AR and location of the sites which undergo post-translational modifications. The enzymes known to be responsible for the modifications are also shown. The exact ubiquitylation site has not been identified.

1.2.2.1 Role of the Ubiquitin-Proteasome system in AR activity

Several lines of evidence indicate that rapid turnover of nuclear receptors after ligand binding is required for sustained transcription at a given promoter. After initiation of the transcription the receptor is degraded, which allows a new transcription cycle to be started. This has been already shown for the ER, GR, and PR [71-74]. Interestingly, the AR seems to be an exception, as the AR protein is stabilised in the presence of androgens. In the last years, however, some contradictory data have appeared. First, it has been shown that the AR also becomes ubiquitylated [54] and this finding has been later extended to the fact that the AR undergoes ligand-dependent ubiquitylation and degradation through the proteasome [75]. To

explain this apparent contradiction a model has been proposed in which only a subpopulation of the AR molecules is degraded after androgen exposure [76]. This explanation is also in agreement with the observation that the same pool of AR molecules is able to perform up to four rounds of transcription but with decreasing efficiency [77]. Only the small AR subpopulation which had formed a transcriptionally active complex would undergo degradation whereas the rest of the AR molecules would stay ready for the next transcription round.

Whereas several AR-interacting proteins exhibit ubiquitylating activity, either as ubiquitin conjugase or ligase, only few of them have been shown to directly modify the AR. The best characterised is the ubiquitin ligase Mdm2 which, following its activation through phosphorylation by Akt, ubiquitylates the AR and reduces its activity by directing it to the proteasome [78]. Interestingly, the AR has to be phosphorylated by Akt as well in order to become ubiquitylated by Mdm2. Furthermore, the AR-Mdm2 complex recruits HDAC1, which has cooperative effects on reducing AR-dependent transcription [75]. CHIP, another E3 enzyme, also limits AR activity by promoting its degradation and in this case as well previous AR phosphorylation probably takes place [79]. However the role of CHIP in AR stability seems to be complex since the reduction in AR levels after CHIP overexpression could not be completely reversed by proteasome inhibitors [80]. In spite of increasing information about AR ubiquitylation, the lysines targeted by this modification have not been identified yet. Another publication shows that androgen treatment results in the generation of monoubiquitylated AR forms [81]. TSG101 then binds to monoubiquitylated AR and prevents its polyubiquitylation, which leads to stabilisation and enhancement of the transcriptional activity. In the case of monoubiquitylation neither the responsible enzyme nor the affected lysines are known at the moment.

The role of ubiquitylation in AR transcription is not limited to the direct modification of the receptor and modulation of its activity and protein levels [82]. Several AR coactivators such as UBCH5, UBCH7 and E6-AP possess E2 or E3 activity. Another interesting example is p300, which has been found to display ubiquitin ligase activity as well as the better-known acetyltransferase function [83]. At the same time, many of the AR cofactors and also members of the general transcription machinery are targets for ubiquitylation and proteasome-mediated degradation. It has been shown that specific combinations of E2 and E3 enzymes are able to degrade coactivators and corepressors of nuclear receptors [84]. Several E2/E3

combinations could degrade the same substrate, while the same E2/E3 combination is also capable of degrading a variety of cofactors, indicating that despite the specificity of the system, redundancy also exists to some extent. The general transcription factors TBP and TAFII135, and RNA polymerase II have also been shown to be targets of the proteasome [85, 86].

Furthermore, the 20S and 19S subunits of the 26S proteasome are involved in the transcriptional process, with the latter involved in transcriptional initiation as well as in elongation [87-89]. The S1 subunit of the 19S proteasome cap is recruited to the PSA promoter and correlates with AR release [90]. Finally, inhibition of the 26S proteasome decreases AR transcriptional activity as demonstrated by using specific inhibitors [91, 92].

Taken together, the components of the ubiquitin-proteasome system play an important role at the promoter of androgen-responsive genes by regulating the turnover of the receptor and its coregulators, by regulating chromatin structure and by serving as bridging factors [82, 76]. In addition, the proteasome activity may be needed for the degradation of the transcription complex in order to clean the promoter and to reinitiate a new round of transcription as is the case for the estrogen receptor. Although some groups have already reported a cyclic recruitment of the AR and its coactivators to the PSA promoter, there are other, contradictory data showing a steady increase in AR loading over time [93].

1.2.2.2 Acetylation of the AR

Acetylation of proteins plays an important regulatory role. Numerous studies have documented that acetylation of the N-terminal chains of histones at specific lysine residues is an essential step in the opening-up of the chromatin structure that makes promoter regions accessible to regulatory proteins [94]. In addition, a variety of transcription factors are known to be directly acetylated, leading to enhanced or reduced activity [22]. The important role of acetylation in steroid receptor function has only recently been appreciated. The AR acetylation sites are clustered in the KXKK motif located in the hinge region at positions 630-633 [95]. Several known histone acetyltransferases, Tip60, P/CAF and p300, have been shown to directly modify the AR at these lysines [95-98]. Mutation of lysine residues to alanine within the acetylation motif dramatically impairs AR function by favouring the binding of corepressors. Conversely, mutations that mimic acetylation stimulate the

expression of AR target genes and promote prostate cancer cell growth [97]. This suggests that AR acetylation may represent a key modification modulating the recruitment of cofactors. However, some groups report an enhanced activity of AR deletion mutants which lack part of the hinge region, including the acetylation motif. The AR mutants Δ629-633, Δ629-636 and Δ628-648 are able to activate both minimal androgen-responsive promoters and native promoters using cell-based reporter-gene assays [65, 99]. Also, removal of the complete hinge region (amino acids 628-669) results in an AR form that is three times more active than the wild-type on different androgen-controlled promoters [100, 101]. The reason for this apparent discrepancy has not yet been clarified.

1.2.3 AR and disease

Androgen insensitivity syndrome

The essential role of the AR in male sex differentiation is well established. Naturally occurring germ line mutations in the AR gene, resulting in different degrees of loss of function of the AR protein, cause a phenotypic spectrum known as the androgen insensitivity syndrome and characterised by incomplete male sexual development [102, 103].Depending on the degree of AR inactivation, the insensitivity syndrome can be complete (CAIS) or partial (PAIS). Male patients with CAIS present female external genitalia, whereas patients with PAIS present a spectrum of defects that vary from near normal male to near normal female phenotypes.

Kennedy's disease

Expansion of the glutamine repeat in the AR NTD to more than forty residues results in X-linked adult onset spinal/bulbar muscular atrophy (SBMA) or Kennedy's disease [104]. Clinical features of this disease are progressive muscle weakness, muscle atrophy associated with loss of lower motor and primary sensor neurons, and partial androgen insensitivity. The severity of the symptoms increases with longer repeats. The expanded polyglutamine stretch disrupts the normal degradation process and generates insoluble protein aggregates in the nucleus resulting in cellular toxicity [105], similar to what is observed for other diseases caused by trinucleotide repeat expansions such as Huntington's disease and spino-cerebellar ataxia type 1.

Prostate cancer

Prostate cancer is the third most common cancer among men worldwide [106]. One essential factor for prostate cancer is the AR, since an active AR is required for carcinoma development and growth. Based on this fact, complete androgen ablation therapy by treatment with GnRH analogs and antiandrogens, which bind and block AR activity, has been applied to prostate cancer patients with non-confined disease [107]. Although this therapy is very efficient for about 18-24 months, tumour growth usually sets on again, and this resistant phenotype is then referred to as androgen-independent prostate cancer. Interestingly, recent data from several groups support the fact that androgen-independent prostate cancer remains AR-dependent [108, 109]. Numerous mechanisms that explain AR-dependent cancer growth in the absence of androgens have been described [110]. AR overexpression due to gene amplification or increased transcription rates has been found in patients with androgen-independent prostate cancer. Also, the altered expression of coactivators and corepressors may result in growth advantage. The incidence of AR mutations in prostate cancer is in the range of 10 - 40 % depending on previous exposure to therapeutic compounds. Point mutations resulting in mutated AR forms which can be activated by other steroids or even by anti-androgens have also been described. For instance, AR mutated at codon 877 is activated by progesterone, estradiol, cyproterone acetate, and by the antiandrogens nilutamide and hydroxy-flutamide. The AR W741C mutant, found in a patient with androgen-independent prostate cancer after bicalutamide treatment, is activated by this antiandrogen. Finally, growth factors such as insulin-like growth factor and keratinocyte growth factor can also activate the AR. These factors are ligands for receptor tyrosine kinases, and activation of one or several kinase pathways may promote AR activation in low-androgen environments via post-translational modifications of the AR and/or its cofactors.

1.3 Aim of this work

The androgen receptor (AR) is necessary for the proper development and function of male reproductive organs. At the same time it plays a key role in the formation and growth of prostate cancer. Although the basic mechanisms of AR activation by androgens have been known for a long time, new aspects of its regulation have become apparent in the last years. Post-translational modifications of the AR and its cofactors can significantly modulate androgen action. The AR has been shown to be modified at several residues by phosphorylation, sumoylation, ubiquitylation and acetylation, but the role of these various modifications is only starting to be understood. The objective of this work was therefore to broaden our knowledge about the role of ubiquitylation and acetylation in the modulation of the activity of the AR.

The ubiquitylation sites of the AR are not known so far, making it difficult to analyse the role of this modification. Although proteasome inhibitors are available, its use alters too many processes in the cell to obtain specific information about one protein. Therefore, the chosen approach was to analyse the effect of enzymes of the ubiquitin-proteasome pathway on the AR. The ubiquitin-specific protease 10 (USP10) had just been identified as an AR-interacting protein and the first goal of this work was its characterisation as a potential AR cofactor.

Further, the role of AR acetylation was analysed. Since in this case the three lysines target for acetylation were known, it was possible to generate loss-of-function mutants and use them to study the effect of acetylation on the regulation of AR target genes.

2 Materials and Methods

2.1 Materials

Cell culture

RPMI 1640	BIOCHROM AG
RPMI 1640 w/o phenol red	BIOCHROM AG
PBS w/o Ca^{2+}, Mg^{2+}	BIOCHROM AG
Trypsin-EDTA	PAA Laboratories
FCS	BIOCHROM AG
L-Glutamine	GIBCO (Invitrogen)
Penicillin/Streptomycin	GIBCO (Invitrogen)
Geneticin®	GIBCO (Invitrogen)
Puromycin	SIGMA
Cell culture flasks	COSTAR (Corning)
Tissue culture dishes	FALCON (Becton Dickinson Labware)
Test plates 96/24/6 wells	TPP
Centrifuge tubes	TPP
BBD 6220 incubator	Heraeus
HERAsafe cleanbench	Heraeus

Cloning and mutagenesis

pSG5 vector	Stratagene
pECFP-N1 vector	BD Biosciences
pCMV-HA vector	BD Biosciences
Primers	MWG
PfuTurbo DNA polymerase	Stratagene
HotStarTaq Master Mix	QIAGEN
Restriction enzymes and buffers	Roche
Shrimp alkaline phosphatase	Roche
T4 Polynucleotide kinase	New England BioLabs
PCR purification / Gel extraction kits	QIAGEN
Rapid DNA ligation kit	Roche
Agarose electrophoresis grade	GIBCO (Invitrogen)
DNA ladder	GIBCO (Invitrogen)
QuikChange XL SDM kit	Stratagene
XL1-Blue supercompetent cells	Stratagene
XL10-Gold ultracompetent cells	Stratagene
Ampicillin, Kanamycin, Chloramphenicol	Roche
Plasmid Purification Mini, Maxi kits	QIAGEN
Sub-Cell GT Gel electrophoresis system	BIO-RAD
Power Pack P25	Biometra

Transfections and reporter assays

pGL3 basic and promoter vectors	Promega
Block-iT fluorescent oligo	Invitrogen
FuGENE 6 transfection reagent	Roche
Lipofectamine 2000 reagent	Invitrogen
Nucleofector Solution V	Amaxa Biosystems
OPTI-MEM	GIBCO (Invitrogen)
Steadylite HTS	PerkinElmer Life Sciences
CulturPlate-96	PerkinElmer Life Sciences
TopSeal-A film	PerkinElmer Life Sciences
LumiCount	Packard
VICTOR^3	PerkinElmer Life Sciences
Nucleofector	Amaxa Biosystems

RNA extraction and reverse transcription

QIAshredder	QIAGEN
RNeasy Mini Kit	QIAGEN
RNase-Free DNase Set	QIAGEN
SuperScript III	Invitrogen
RNA 6000 Nano LabChip kit	Agilent Technologies
Agilent 2100 Bioanalyzer	Agilent Technologies

Quantitative PCR

SYBR Green Master Mix	Eurogentec
Primers	MWG
Optical 96-Well Reaction Plates	Applied Biosystems
Optical Adhesive Film	Applied Biosystems
ABI PRISM 7000	Applied Biosystems

Protein extraction, PAGE and western blot

M-PER	Pierce Biotechnology
Complete Mini EDTA-free tablets	Roche
Benzonase	Merck
NuPAGE LDS Sample Buffer	Invitrogen
Precision Plus Protein Standards	BIO-RAD
NuPAGE 4-12% Bis-Tris gels	Invitrogen
NuPAGE MOPS SDS Running Buffer	Invitrogen
XCell II SureLock Mini-Cell	Invitrogen
PVDF Membrane	Invitrogen
NuPAGE Transfer Buffer	Invitrogen
XCell II Blot Module	Invitrogen
Milk powder	ROTH
Western Lightning Reagent	PerkinElmer
Hyperfilm ECL	Amersham Biosciences
Hyperfilm cassette	Amersham Biosciences

Re-blot Plus Strong Solution	CHEMICON International

Protein and plasmid immunoprecipitations

Protein A/G-Agarose	Santa Cruz Biotechnology
Protein A Agarose/Salmon Sperm DNA	Upstate
RNase A	QIAGEN
Proteinase K	QIAGEN
DNeasy kit	QIAGEN
Sonoplus HD2070	Bandelin
Antibodies	see below

Immunofluorescent staining

Lab-Tek II Chamber Slide System	NalgeNunc Int.
BSA	Sigma
Hoechst 33258	Molecular Probes
Fluorescent Mounting Medium	DakoCytomation
Axiophot microscope	Carl Zeiss
Axiowert 25 microscope	Carl Zeiss
Antibodies	see below

Antibodies

AR 441	Mouse	Santa Cruz Biotechnology
AR C-19	Rabbit	Santa Cruz Biotechnology
USP10	Rabbit	Bethyl Laboratories
GAPDH	Mouse	Advanced ImmunoChemical
Ubiquitin (P4D1)	Mouse	Cell Signaling Technology
RGS-His	Mouse	QIAGEN
HA	Mouse	BD Biosciences
HRP-conjugated anti-mouse	Sheep	Amersham Biosciences
HRP-conjugated anti-rabbit	Donkey	Amersham Biosciences
FITC/TRITC-labelled anti-mouse	Goat	Jackson Immunoresearch
FITC/TRITC-labelled anti-rabbit	Goat	Jackson Immunoresearch

Chemicals

R1881 (methyltrienolone)	Dupont NEN
MG132	CALBIOCHEM (Merck)
Cycloheximide	CALBIOCHEM (Merck)
Trichostatin A	Sigma
Others	Sigma and Merk

2.2 Methods

2.2.1 Cell culture

Cells were grown at 37 °C in a 5% CO_2 atmosphere in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The medium for PC-3/AR cells additionally contained 600 µg/ml geneticin®, the medium for PC-3/AR/MMTV-Luc cells geneticin® at the same concentration plus 10 µg/ml puromycin.

Table 1: Cell lines used in this work

Name	Description	Origin
PC-3	Human prostate carcinoma cell line derived from a bone metastasis. Does not express the AR.	ATCC[a]
PC-3/AR	PC-3 cell line stably transfected with the AR.	A. Cato[b]
PC-3/AR/MMTV-Luc	PC-3/AR cell line stably transfected with a MMTV-Luciferase reporter construct.	K. Parczyk[c], M. Klotz[c]
22Rv1	Human prostate carcinoma cell line derived from the CWR22R xenograft. Expresses the AR with the mutation H874Y	ATCC
CV-1	African green monkey kidney cell line. Lacks all steroid receptors.	ATCC
HeLa	Human cervix carcinoma cell line. Does not express the AR.	ATCC

[a]American type culture collection; [b]Forschungszentrum Karlsruhe; [c]Bayer Schering Pharma AG

2.2.2 Cloning and site-directed mutagenesis

USP10 in pSG5

The pOTB7-USP10 clone, which included the USP10 sequence corresponding to the BC000263 entry of the NCBI database, was purchased from the IMAGE consortium via the RZPD (Ressourcenzentrum für Genomforschung, Berlin). The coding sequence of USP10 was amplified from this plasmid with the primers USP10 s. and USP10 as. (see table), which additionally contained the *Bgl*II restriction site. The PCR product and the pSG5 vector were appropriately digested and purified. The vector was dephosphorylated to prevent religation and the PCR product was phosphorylated to facilitate cloning. The ligation reaction was performed with the Rapid DNA ligation kit for 5 min. The product was then used to transform XL1-Blue supercompetent cells following the manufacturer´s protocol. Bacterial colonies were allowed to grow on LB-Agar plates supplemented with 100 µg/ml ampicillin. The

colonies were tested for the presence of the insert by PCR using USP10-specific primers. Positive clones were then amplified, the plasmid purified and the insert sequenced.

USP10 in pECFP

The USP10 coding sequence was amplified from the pSG5-USP10 plasmid with a sense primer containing a *Bgl* II restriction site and with an antisense primer ignoring the stop codon and including a *Sac* II restriction site. The PCR product and the pECFP-N1 vector were digested with both enzymes, purified and ligated as before. The resulting plasmid contained the USP10 sequence upstream of and in-frame with the sequence of the ECFP gene. This was confirmed by DNA sequencing.

USP10 N-term / C-term in pSG5

For the subcloning of the N-terminal domain of USP10, the first four exons were amplified from pSG5-USP10 using the primers USP10/Nt s. and USP10/Nt as., which binds to the end of exon 4 and includes a Stop codon and a *Bgl* II restriction site. The amplification of the C-terminal domain was performed with the primers USP10/Ct s., which binds to the beginning of exon 5 and included a *Bgl* II restriction site and a Start codon, and USP10 as. Both PCR products and the pSG5 vector were digested with *Bgl* II, purified and ligated as before. The sequence of the resulting constructs was verified by DNA sequencing.

USP10 in pCMV-HA

The pSG5-USP10 plasmid was digested with *Bgl* II and the fragment corresponding to the USP10 coding sequence was isolated and subcloned into the *Bgl* II site of the pCMV-HA vector. In the resulting plasmid the USP10 sequence was downstream of and in-frame with the sequence of the HA tag.

RGSHis-Ubiquitin in pSG5

For the cloning of the His-tagged ubiquitin monomer, the sequence of the RGSHis tag was first constructed by annealing the primers RGSHis s. and RGSHis as. (see table). This resulted in a DNA fragment where the sequence of the tag was flanked by an *EcoR* I compatible end at the 5' position and an *EcoR* I end at the 3' position. The pSG5P vector, which was derived from the pSG5 vector by inserting additional cloning sites, was digested with *EcoR* I and dephosphorylated. The annealed fragment was phosphorylated and cloned into the vector. The coding sequence of the ubiquitin monomer was then amplified with the primers Ub s. and Ub as., which included an *EcoR* I site and a *Kpn* I site respectively, and cloned into the pSG5-RGSHis plasmid.

Table 2: Primers used for cloning.

Name	Sequence (5' - 3')
USP10 s.	TGAGAGATCTCCACCATGAAACGGGCAGCCATGGCCCTCC
USP10 as.	CTTAAGATCTTTACAGCAGGTCCACTCGGCGGTAATACAGGAGGTAG
USP10/ECFP s.	TATTAAAGATCTATGAAACGGGCAGCCATGGCCCTCCACAGCC
USP10/ECFP as.	TAATAACCGCGGCAGCAGGTCCACTCGGCGGTAATACAGGAGG
USP10/Nt s.	TATTAAAGATCTCAGCCATGGCCCTCCACAGCCCGCAG
USP10/Nt as.	TAAATTAGATCTCTATGCAATCTTTATGGCTACAGGATC
USP10/Ct s.	AAAATTAGATCTCCGCCATGTTGCTGGAGAATGTAACCCTAATCC
RGSHis s.	AATTACCACCATGAGAGGATCGCATCACCATCACCATCACG
RGSHis as.	AATTCGTGATGGTGATGGTGATGCGATCCTCTCATGGTGGT
Ub s.	GCGCGAATTCCAGATCTTCGTGAAGACTCTGAC
Ub as.	GCGCGGGTACCCTACCCACCTCTGAGACGGAGTACC

Site directed-mutagenesis was carried out with the QuickChange XL kit following the recommended protocol. Briefly, the complete template plasmid was amplified using primers containing the desired change (see table). The extension time was of 1 min per kb of plasmid length except for mutagenesis of the AR, where 2 min/kb were needed. After PCR the template plasmid was eliminated by *Dpn* I digestion and the product was used to transform XL1-Gold ultracompetent cells. Plasmids were prepared from selected colonies and the presence of the desired mutation was confirmed by sequencing.

Several mutations were introduced into the pSG5-USP10 plasmid. The USP10 sequence originally cloned in pSG5 (BC000263) had a Proline and a Leucine at positions 203 and 204. These did not correspond to the genomic sequence, which codes for a Serine and a Valine residue at these positions. The pSG5-USP10 plasmid was therefore first back-mutated to be in accordance with the genomic sequence and was used in this work as the wild-type form. To create the enzymatically inactive form of USP10, the codon corresponding to the catalytic Cysteine was substituted by GCC, coding for Alanine. For the NR-box mutant, Lysines 545 and 546 were mutated to Alanine.

In the pRGSHis-Ubiquitin plasmid, the codon for Lysine 48 was substituted for an Arginine codon to give the pRGSHis-Ub K48R plasmid, coding for a ubiquitin form unable to form polyubiquitin chains at this position.

To generate the acetylation-defective forms of the AR the Lysines at positions 630, 631 and 632 were mutated to Alanine. Three different plasmids resulting from mutating one (K630A), two (KK631, 632AA) or the three Lysines were generated and named AxKK, KxAA and AxAA, respectively.

The AR N/C interaction-defective mutants were obtained by mutating the motifs FXXLF (amino acids 23-27) and WXXLF (amino acids 429-433) to FXXAA and WXXAA respectively.

Table 3 Primers used for site-directed mutagenesis.

Name	Sequence (5' - 3')
USP10 MPPSV s	GGGTGACATGCCTCCGTCAGTTACGCCCAGGACTTG
USP10 MPPSV as	CAAGTCCTGGGCGTAACTGACCGGAGGCATGTCACCC
USP10 C424A s.	GATCAATAAAGGGAACTGGGCCTACATTAATGCTACACTG
USP10 C424A as.	CAGTGTAGCATTAATGTAGGCCCAGTTCCCTTTATTGATC
USP10 LxxAA s.	GAAATGTTGAACCTAAAGAAGGCTGCCTCACCAAGTAATGAAAAAC
USP10 LxxAA as.	GTTTTTCATTACTTGGTGAGGCAGCCTTCTTTAGGTTCAACATTTC
Ub K48R s.	GTTGATCTTTGCCGGAAGACAGCTGGAAGATGGTCG
Ub K48R as.	CGACCATCTTCCAGCTGTCTTCCGGCAAAGATCAAC
AR AxKK s.	CTCTGGGAGCCCGGGCGCTGAAGAAACTTGG
AR AxKK as.	CCAAGTTTCTTCAGCGCCCGGGCTCCCAGAG
AR KxAA s.	CTCTGGGAGCCCGGAAGCTGGCAGCGCTTGGTAATCTGAAACTACAGGAG
AR KxAA as.	CTCCTGTAGTTTCAGATTACCAAGCGCTGCCAGCTTCCGGGCTCCCAGAG
AR AxAA s.	GATGACTCTGGGAGCCCGGGCGCTGGCGGCACTTGGTAATCTGAAACTAC
AR AxAA as.	GTAGTTTCAGATTACCAAGTGCCGCCAGCGCCCGGGCTCCCAGAGTCATC
AR FxxAA s.	CCGAGGAGCTTTCCAGAATGCAGCTCAGAGCGTGCGCGAAGTG
AR FxxAA as.	CACTTCGCGCACGCTCTGAGCTGCATTCTGGAAAGCTCCTCGG
AR WxxAA s.	CTTCCTCATCCTGGCACACTGCAGCTACAGCCGAAGAAGGCCAGTTG
AR WxxAA as.	CAACTGGCCTTCTTCGGCTGTAGCTGCAGTGTGCCAGGATGAGGAAG

2.2.3 Transfection

Plasmid transfections were generally carried out with FuGENE 6. The cells were seeded as required in 96-, 24-, 6-well plates, 10 cm dishes or 8-well chamber slides and the transfection was performed the day after using 3 µl of reagent per 1µg of plasmid DNA. A transfection reaction with a plasmid coding for EGFP was always included to control efficiency.

For larger amounts of PC-3 and PC-3/AR cells, transfections were performed by electroporation with the AMAXA technology. For each transfection reaction $1x10^6$ to $1x10^7$ cells were pelleted and resuspended in 100 µl of the cell line solution V. Immediately after, 2 to 5 µg of DNA were added and the cells were electroporated in the Amaxa Nucleofector using the program A23.

Transfection with siRNA and cotransfection with siRNA and plasmid were performed with Lipofectamin 2000 following the recommended protocol. In order to control the transfection efficiency, a fluorophore-linked, non-targeting RNA (Block-IT) was routinely used.

2.2.4 Luciferase gene reporter assays

For the transactivation assays cells were seeded into 96-well plates in RPMI 1640 media without phenol red supplemented with 5% charcoal-stripped FCS and 2 mM L-glutamine. The seeding concentration was 7500 cells/well for the PC-3/AR and HeLa lines and 5000 cells/well for the PC-3 and CV-1 lines. Transfections were carried out 18 h later using FuGENE 6 diluted in OPTI-MEM. Expression plasmids and luciferase-based reporter vectors were cotransfected. The total amount of transfected plasmids was kept constant by adding the appropriate concentrations of the pSG5 vector containing a neutral insert. The amount of transfected expression plasmids varied between 10 and 75 ng/well and the amount of transfected reporter was 50 or 75 ng/well. Induction of the AR transactivation was performed 5 h later by adding to the media the synthetic androgen analogue R1881 (methyltrienolone) at a final concentration of 1 nM, unless indicated otherwise. After 24 h, 100 µl of the SteadyLite Plus reagent were added and measurement of the luciferase activity was carried out in a Lumicount luminometer or in a VICTOR^3 multilabel reader. The activity of a reporter plasmid where the luciferase gene is under control of a constitutively active promoter was determined in parallel to assess transfection efficiency. For all points, the average value of six wells treated in parallel was taken. The experiments were repeated at least three times independently.

Table 4 Reporter plasmids used in the luciferase assays.

Name	Description
ARE-1	4 copies of the Pem ARE-1: 5′-AGATCTcattcTGTTCC-3′
ARE-2	4 copies of the Pem ARE-2: 5′-AGCACAtcgTGCTCA-3′
SRE-1	4 copies of the CRISP-1 SRE: 5′-GGTACAtctTGTTCA-3′
PSA	-6000 to +12 fragment of the human PSA promoter
MMTV	Fragment of the MMTV promoter
Pem	-444 to -36 fragment of the mouse Pem promoter

2.2.5 Gene knock-down

For the knock-down of USP10 expression, siRNA technology was used. Three different siRNAs targeting different positions of the USP10 transcript and the mismatch control of the siRNA1 were purchased from Invitrogen. PC3/AR/MMTV-Luc cells seeded in 6-well plates were transfected with the siRNAs and a fluorescent, non-targeting RNA as transfection control, at a final concentration of 40 nM. After 1, 2 or 3 days, the cells were harvested and the extent of the RNA knock-down was measured by quantitative PCR (see below). For the

determination of luciferase activity, cells were harvested after 2 days, seeded in 96-well plates and treated as indicated with R1881. 24 h later luciferase activity was measured as described before.

For the knock-down of HA-tagged USP10, cells seeded in a 24-well plate (5 x 10^4cells/well) were cotransfected with 150 ng of pCMV-HA-USP10 expression plasmid and 20 pmol of the different siRNAs (40 nM final concentration), using Lipofectamine 2000. The cells were harvested 24 h later, lysed and HA-USP10 content was analysed by western blot.

Table 5: siRNAs used for gene knock-down.

Name	Sequence
USP10_siRNA1	GGUGGCCUAUGUGGAAACUAAGUAU
USP10_siRNA2	GCAGGUUGAAGUCAAAGAAGGGCUU
USP10_siRNA3	CCCUAAUCCAUAAACCAGUGUCGUU
USP10_siRNA1 control	GGUAUCCGUGUAAAGAAUCGGGUAU

2.2.6 RNA purification and reverse-transcription

Cells were transfected or treated as indicated for each experiment and total RNA was then extracted using the RNeasy mini kit including the DNaseI digestion on column. The RNA concentration was measured in a spectrophotometer and its quality was determined using the Agilent Bioanalyzer.

First-strand cDNA synthesis was performed starting with 1 to 4 µg of total RNA with the Oligo$(dT)_{20}$ primer using the SuperScript III reverse transcriptase.

2.2.7 Quantitative PCR

Real-time PCR quantification was performed on an ABI PRISM 7000 Sequence Detection System using the standard temperature program (50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 sec followed by 60 °C for 1 min). The MasterMix Plus for SYBR Green from Eurogentec was used and as template 20 to 50 ng of cDNA per reaction were added. Specific primers for detection of USP10, AR and cyclophilin H transcripts were designed with help of the Primer Express software so that, if possible, the generated amplicon included an exon-exon junction. The dissociation protocol at 60 °C was run for each primer pair to confirm that a unique population of amplicons had been generated.

Serial dilutions of cDNA were first used for validation of the primer pairs. The obtained Ct values were plotted against the starting cDNA concentration in logarithmic units. The slope of

the curve was used to calculate the PCR efficiency ($E=10^{(-1/slope)}$ x 100) for each primer pair. Comparable values were obtained, which allowed the use of the ΔΔCt method for comparison between transcript levels.

For the analysis of the results, the Ct value of USP10 or AR obtained for each sample was normalised to the Ct value of cyclophilin H (ΔCt) and then to the control sample (ΔΔCt). Results are expressed as fold ($2^{-\Delta\Delta Ct}$) or as percentage ($2^{-\Delta\Delta Ct}$ x 100) as compared to the control.

Table 6: Primers used in the qPCR.

Name	Sequence (5' - 3')
USP10 s.	GGGCTTGTTCCGGTTTCAG
USP10 a.s.	CAACGACACTGGTTTATGGATTAGG
AR s.	TGTCAACTCCAGGATGCTCTACTTC
AR a.s.	GCTGTACATCCGGGACTTGTG
Cyclophilin H s.	GAAGTTGGCCGCATGAAGA
Cyclophilin H a.s.	GCCTAAAGTTCTCGGCCGT

2.2.8 Protein extraction, PAGE and western blot

Total protein extracts were prepared from transfected cells using M-Per reagent supplemented with protease inhibitor mix and benzonase.

For the separation of cytoplasmic and nuclear fractions, PC-3 cells were grown in 6-well plates, transfected and stimulated or not with 1 nM R1881. After 24 h, the cells were harvested and treated with lysis buffer (50 mM Tris pH 7.5, 10 mM NaCl, 5 mM EDTA, 0.5% Nonidet P40, protease inhibitor tablets). Following centrifugation for 5 min at 13,000 x g, the supernatant was kept as cytoplasmic fraction and the pelleted nuclei were lysed with M-Per buffer supplemented as above.

For Western blot analysis the protein extracts were separated in NuPAGE gradient gels for 1h at 200 V and transferred onto polyvinylidene fluoride membranes for at least 1h at 30 V. The membranes were blocked in 5% milk powder in PBS/Tween and incubated overnight at 4 °C with the specified antibodies. Blots were washed with PBS/Tween, incubated with the corresponding secondary antibodies for 1 h at room temperature, washed again and developed using the Western Lightning chemiluminescence reagent.

2.2.9 Protein immunoprecipitation

PC-3/AR cells were transfected and treated as required. At the end of the experiment cells were harvested, washed with cold PBS and resuspended in lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, protease inhibitors tablets). After sonicating twice for 15 sec, cell extracts were centrifuged at 13000 rpm for 15 min and supernatants were transferred to a new tube. 20 µl of protein A/G-Agarose beads were added and incubated for 1 h at 4 °C. After centrifugation, protein concentration of the precleared lysates was measured and adjusted with lysis buffer. An aliquot was taken at this step to be used as the input fraction. AR immunoprecipitation was carried out with the AR-441 monoclonal antibody at a concentration of 2µg per 1 ml cell lysate. After 1 h, 30 µl protein A/G-Agarose beads were added and incubation was performed overnight. The immunoprecipitates were collected by centrifugation and extensively washed as follows: once with lysis buffer, twice with high salt wash buffer (20 mM Tris pH 7.5, 500 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40) once with low salt wash buffer (20 mM Tris pH 7.5, 10 mM NaCl, 5 mM EDTA) for 15 min each. Elution was performed by adding 50µl of 2x PAGE sample buffer and heating to 95 °C for 3 min. Then 10 to 25 µl of the eluates were separated by SDS-PAGE and analysed by western blotting using the AR-19 rabbit polyclonal antibody to confirm the success of the immunoprecipitation, the GAPDH antibody to confirm equal loading of the input fractions and absence of GAPDH in the eluates, the anti-ubiquitin-protein conjugate antibody to detect AR ubiquitylation or the anti-RGSHis antibody to detect monoubiquitylation.

2.2.10 Plasmid immunoprecipitation

PC-3 cells were plated on 10 cm-dishes and transfected with either 5 µg of pSG5-ARwt or pSG5-AR-AxAA together with 1 µg of the Pem reporter plasmid. Four hours later, the cells were treated with 1 nM R1881. After 24 h, the cells were cross-linked with 1% formaldehyde for 5 min and the reaction was stopped by adding glycine to a final concentration of 125 mM. Cells were harvested, resuspended in lysis buffer (50 mM Tris pH 8, 10 mM EDTA, 1% SDS, protease inhibitor mix) and sonicated on ice four times during 15 s at 70% power using a Sonopuls HD2070 device. Cell lysates were diluted in dilution buffer (20 mM Tris pH 8, 150 mM NaCl, 1% Triton X-100, protease inhibitor mix), precleared and incubated at 4 °C overnight with 2 µg of the anti-AR antibody C-19 or without antibody as mock control. 60 µl of Protein A agarose/salmon sperm DNA were then added and the incubation was continued for 2 h. Washing was performed with buffer I (20 mM Tris pH 8, 2 mM EDTA, 150 mM

NaCl, 1% Triton X-100, 0.1% SDS), buffer II (20 mM Tris pH 8, 2 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.1% SDS) and buffer III (10 mM Tris pH 8, 1 mM EDTA, 250 mM LiCl, 1% Nonidet P40, 1% Sodium deoxycholate) followed by two washes with TE (10 mM Tris pH 8, 1 mM EDTA). Each washing step was performed for 15 min at 4 °C.

The immunocomplexes were eluted with 0.1 M NaHCO3, 1% SDS and the cross-link was reverted at 65 °C for 5 h. The eluates were sequentially treated with RNase H (1 h) and proteinase K (1 h). The DNA was then purified using the DNeasy kit. For the PCR amplification 5 µl of each sample were used as template. This was performed at an annealing temperature of 55 °C for 35 cycles using primers designed to amplify the whole Pem promoter fragment cloned in the reporter plasmid. Primer sequences were: 5'-TGCCAGAACATTTCTCTATCG-3' (forward) and 5'-CTTTATGTTTTTGGCGTCTTCC-3' (reverse).

2.2.11 Immunofluorescent staining

HeLa cells were seeded in chamber slides at a concentration of 50000 cells/500µl/well and transfected or treated as described for each experiment. The culture medium was then aspirated and cells were fixed with 3.7% formaldehyde in PBS for 10 min and permeabilised with 0.5% Triton X-100 in PBS for 2 min. After washing, unspecific binding was blocked with 1% BSA for 30 min. Cells were then incubated for 1 h with specific antibodies against AR or USP10 diluted in 1% BSA, followed by incubation with FITC- or TRITC-conjugated secondary antibodies in 1% BSA solution for 1h in the dark. Nuclei were stained by treating the cells with Hoechst dye at 0.1 µg/ml in PBS for 2 min in the dark. Between the incubations, cells were washed with PBS 3 times for 5 min. Slides were then mounted, sealed and imaged using a Zeiss Axiophot microscope coupled to an AxioCam MRc CCD camera. Images were taken with the help of the AxioVision LE 4.5 software.

3 Results

3.1 The Ubiquitin-Specific Protease 10

USP10 was identified as part of a complex isolated from nuclei of PC-3/AR cells. This complex contained a specific DNA response element, the AR and associated cofactors. The interaction of USP10 with the AR was substantiated by GST pull-down experiments [111].

3.1.1 USP10 subcellular localisation

USP10 had been identified in nuclear extracts which does not imply that it is confined to this compartment. Indeed an earlier report describes USP10 as an interaction partner of G3BP, a cytoplasmic protein. To clarify this possible contradiction, subcellular localisation experiments were needed. Since at that time point no commercial antibodies against USP10 were available, a fluorescent fusion protein was generated in order to be able to perform fluorescent microscopy. The coding sequence of USP10 was amplified and cloned into the pECFP vector upstream of the sequence coding for the cyan fluorescent protein. This plasmid was then transfected into proliferating PC-3/AR cells cultured in growth media supplemented or not with 1nM of R1881. After 24h the localisation of the fusion protein was determined in living cells using fluorescence microscopy. USP10-CFP was found both in the nucleus and in the cytoplasm after R1881 treatment (Figure 5) as well as in the absence of androgen (not shown).

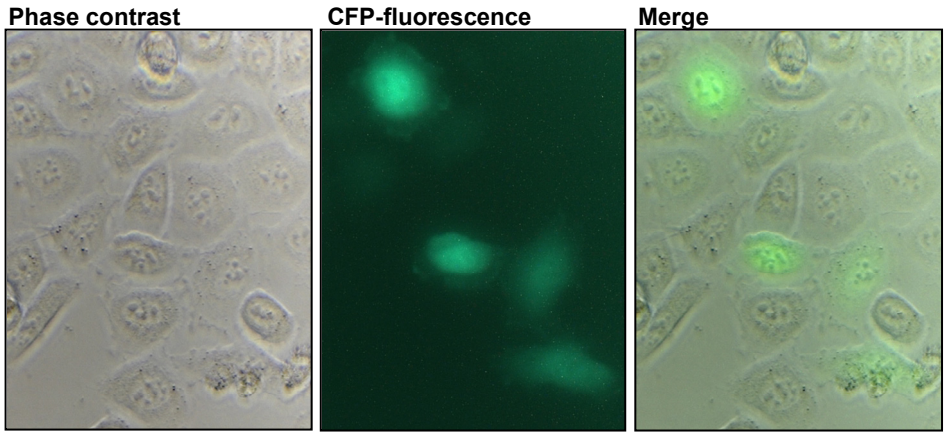

Figure 5

USP10-CFP is localised in both the cell nucleus and the cytoplasm. PC3/AR cells were seeded in 24-well plates, transfected with 200 ng of an USP10-CFP expression construct and treated with 10 nM R1881. After 24 h cells were examined by light microscopy (left) and fluorescence microscopy (middle). The right panel corresponds to the merged picture.

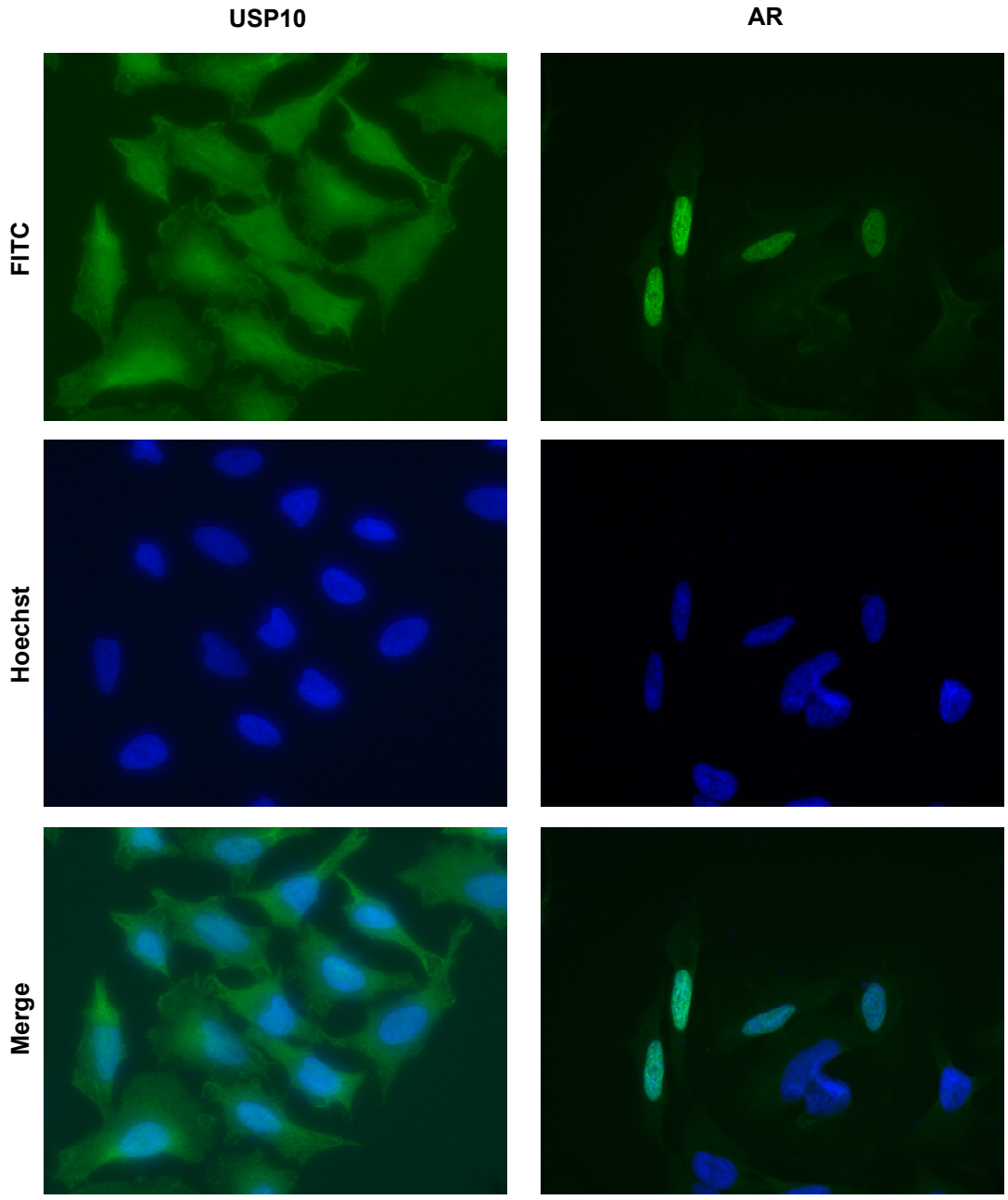

Figure 6

Subcellular localisation of USP10 and AR. For USP10 staining, HeLa cells were fixed, permeabilised and incubated with anti-USP10 antibody followed by a FITC-labelled anti-rabbit antibody. For AR localisation, HeLa cells were first transfected with an expression plasmid for the AR and treated for 24 h with R1881. Then cells were immunostained with an anti-AR antibody followed by a FITC-labelled anti-mouse antibody. Nuclei were visualised with Hoechst.

An anti-USP10 antibody became available later, opening the possibility to perform immunofluorescent staining. HeLa cells seeded in chamber slides were incubated with a rabbit anti-USP10 antibody and a FITC-labelled goat anti-rabbit antibody. Nuclei were

visualised with Hoechst. Again USP10 was localised in both nucleus and cytoplasm (Figure 6).

The immunostaining of the AR was also tested. For that, HeLa cells were transfected with an expression plasmid coding for the AR and incubated for 24h in the presence of 1 nM R1881. Cells were then fixed, incubated with a mouse monoclonal antibody specific for AR and developed with a FITC-labelled goat anti-mouse antibody. As expected, the AR was clearly visualised in the nucleus in the presence of androgen (Figure 6).

This allowed to perform a double staining of USP10 and AR. HeLa cells were transfected and treated as before. After fixation they were sequentially incubated with anti-USP10 followed by TRITC-labelled goat anti-rabbit, and with anti-AR followed by FITC-labelled goat anti-mouse. Nuclei were visualised with Hoechst staining. As shown in Figure 7, USP10 and the AR colocalised in the nucleus.

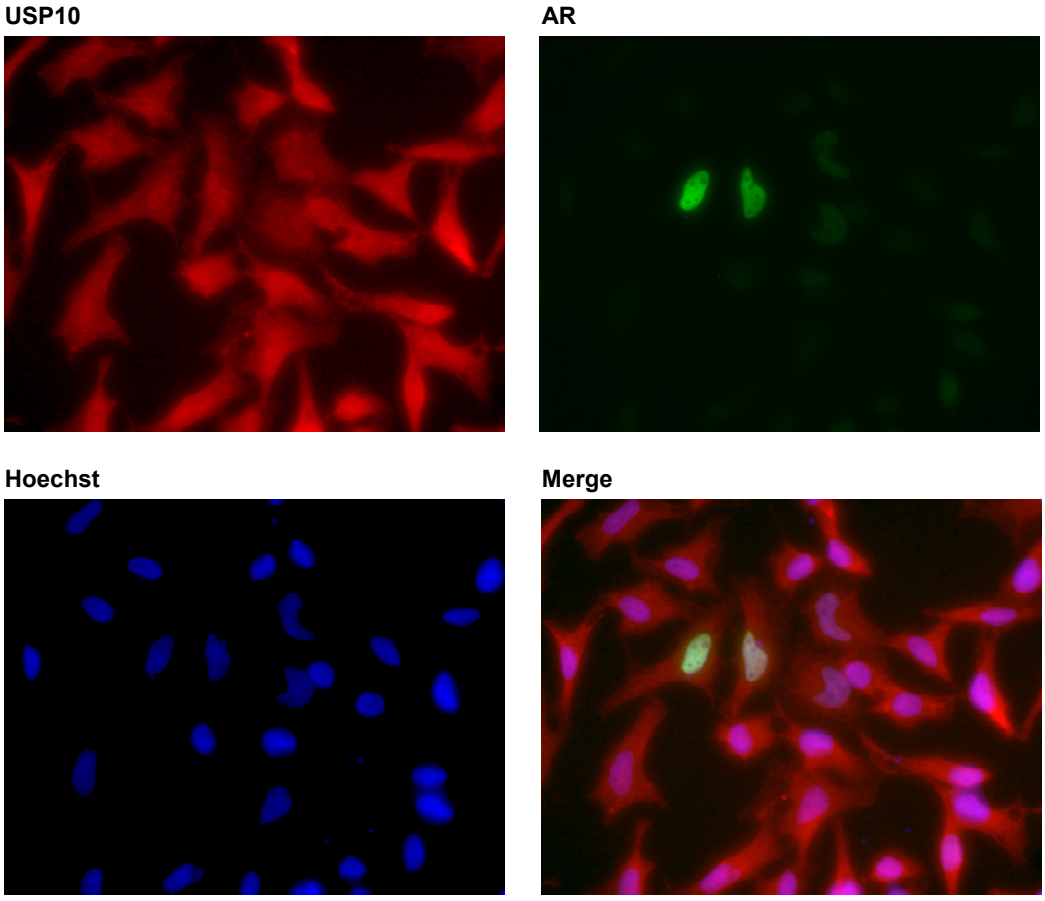

Figure 7

Coimmunolocalisation of USP10 and AR. HeLa cells were transfected with pSG5-AR and treated for 24 h with R1881. Fluorescent staining was performed with anti-USP10 followed by TRITC-labelled goat anti-rabbit treatment, and with anti-AR followed by FITC-labelled goat anti-mouse treatment. Nuclei were also visualised with Hoechst.

The anti-USP10 antibody also allowed performing western blot analyses with the PC-3/AR cell line in which the AR-USP10 interaction had originally been found. Nuclear and cytoplasmic extracts of PC-3/AR cells grown in the presence of R1881 or vehicle were prepared and analysed by western blotting. As shown in Figure 8, USP10 was present in both fractions. Again its localisation was not affected by the presence of androgen, whereas the AR translocated to the nucleus upon androgen treatment, as expected. The glycolytic enzyme GAPDH was clearly restricted to the cytoplasmic fraction demonstrating that there was no remaining cytoplasmic contamination in the nuclear fraction.

Together these results show that USP10 is present in both the nucleus and the cytoplasm. This is compatible with the previous reports and also in line with the diverse functions of the yeast ortholog Ubp3p both in the nucleus and in the cytoplasm.

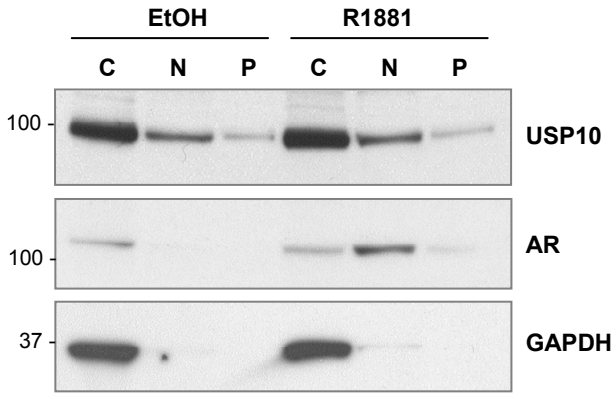

Figure 8

Subcellular localisation of USP10 in PC-3/AR cells. Cells incubated with hormone (R1881) or with vehicle (EtOH) were fractionated in cytoplasmic (C), nuclear (N) and insoluble (P) fractions. Western blot analyses were performed with anti-USP10, anti-AR or anti-GAPDH antibodies, as indicated.

3.1.2 Role of USP10 in AR function

3.1.2.1 Effect of USP10 overexpression on AR transcriptional activity

In order to determine whether the interaction of USP10 with the AR affected its transcriptional function, luciferase gene reporter assays were performed. The coding sequence of USP10 was cloned into the pSG5 mammalian expression vector. PC-3/AR cells were transfected with this plasmid together with reporter plasmids where the luciferase gene is

under control of androgen-responsive promoters. These reporter constructs harboured four copies of either the selective response elements ARE-1 or ARE-2 found in the Pem promoter, or of a non-selective steroid response element (SRE) from the CRISP-1 gene promoter. Selective AREs have been shown to be preferentially stimulated by the AR whereas the non-selective SREs are additionally responsive to the progesterone receptor, glucocorticoid receptor and mineralocorticoid receptor.

Overexpression of USP10 enhanced the R1881-dependent response of all three tested minimal promoters, but had no significant effects on the unstimulated activity (Figure 9A and B).

In order to find out whether this effect was due to the enzymatic function of USP10, a point mutation destroying the active site of the cysteine protease was introduced to generate the C424A form (numbering is according to GenBank entry NM_005153). This mutation has previously been shown to eliminate the deubiquitylation activity of USP10 in vitro [112]. The introduction of this mutation abolished the stimulating effect of USP10 on AR transactivation of the ARE-1 promoter (Figure 9C). The expression of TIF2, a well-characterised cofactor of steroid receptors, enhanced androgen effects as expected and was used as comparison.

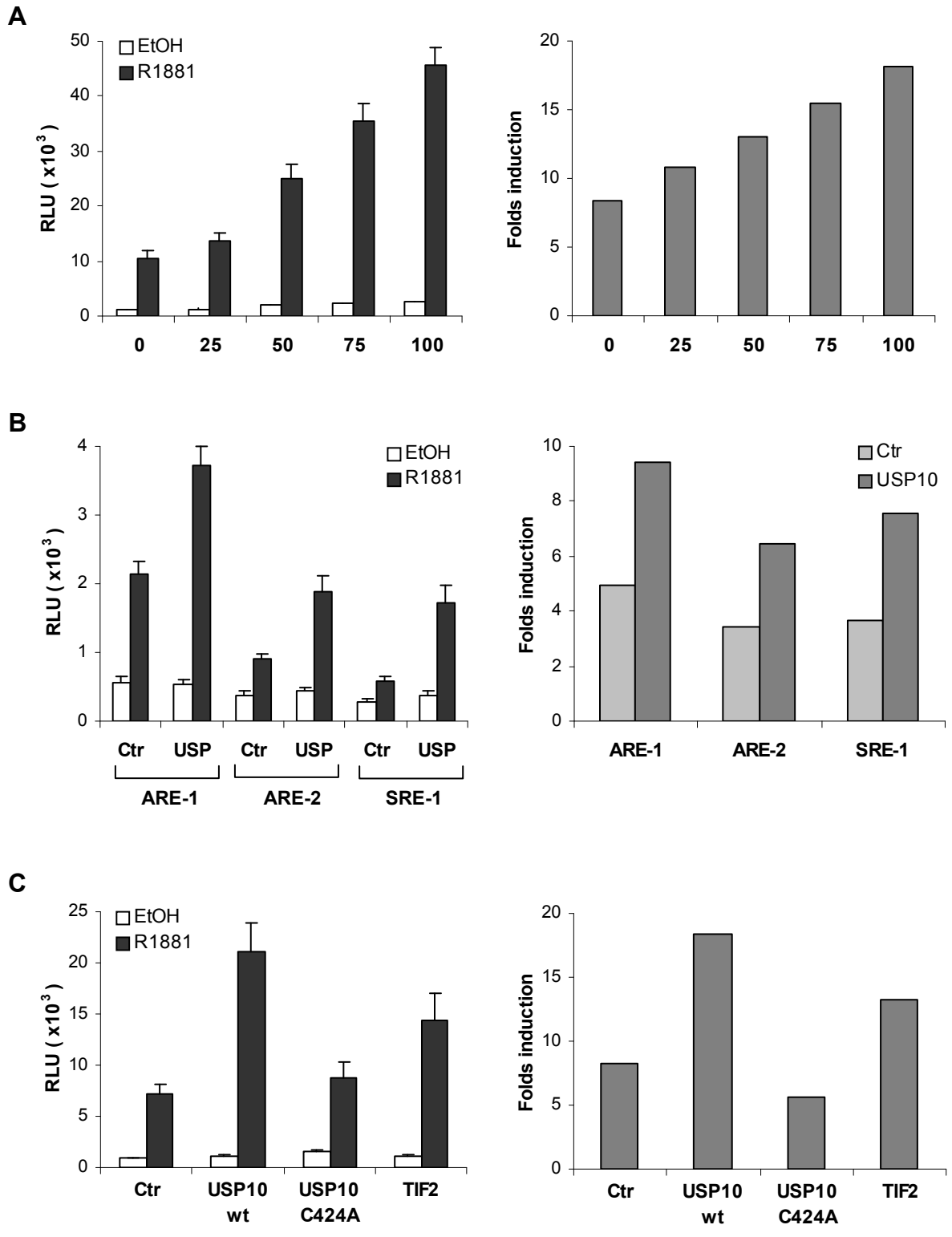

Figure 9

(See next page)

The effect of USP10 overexpression was furthermore tested on a natural androgen-responsive promoter, the mouse mammary tumour virus (MMTV) promoter. Again an enhancement of the androgen-stimulated response in presence the wild-type USP10, but not of the mutated form was measured (Figure 10A).

USP10 contains one LXXLL motif also called NR box, which has been found to be important in other cofactors for the interaction with nuclear receptors [113]. In order to find out whether this was also the case for USP10, this motif was mutated to LXXAA and reporter gene assays were performed. The enhancement of AR activity after USP10 LXXAA overexpression was equivalent to the one observed for the wild-type, indicating that this motif was not essential for the function of USP10 as an AR coactivator (Figure 10A). This may be due to the existence of only one LXXLL motif in USP10, as opposed to the several motifs seen in other steroid receptor cofactors.

The protease domain is located at the C-terminal half of USP10 whereas the long N-terminal sequence has no known function. In order to obtain some information about the relevance of this domain, two different expression plasmids were constructed, the first containing exons 1 to 4 (USP10 Nt) and the second containing exons 5 to 14 (USP10 Ct) and tested in the reporter assays. The N-terminal domain of USP10 alone had no effect on AR function demonstrating again the importance of the catalytic activity. On the other side, the C-terminal domain containing the enzymatic activity was sufficient to recapitulate the effects of the full-length protein (Figure 10B).

Together these results document that USP10 acts as an AR cofactor and that this function necessitates an intact enzymatic activity.

Figure 9 (previous page)
USP10 overexpression stimulates AR function in luciferase gene reporter assays. PC-3/AR cells were transfected with 50 ng of an androgen-dependent reporter construct and 75 ng of an expression plasmid coding for wild-type USP10 (wt) or an inactive form (C424A), or for TIF2. An expression plasmid with a neutral insert was used in the controls. Treatment was with 10 nM R1881 or with vehicle (EtOH). (A) Transfection with increasing amounts of an expression vector coding for USP10 as indicated in ng/well together with a reporter vector harbouring four copies of the Pem ARE-1. (B) Transfection with pSG5-USP10 and reporter constructs containing four copies of the Pem ARE-1, Pem ARE-2 or CRISP-1 SRE. (C) Transfection with pSG5-USP10 wt or an inactive mutant, or with pSG5-TIF2 together with the Pem ARE-1 reporter construct. The results in the left panel are given in relative luminescence units (RLU) and are the mean ± S.D. of sextuplicate values. In the right panel the corresponding folds induction for each experiment are shown.

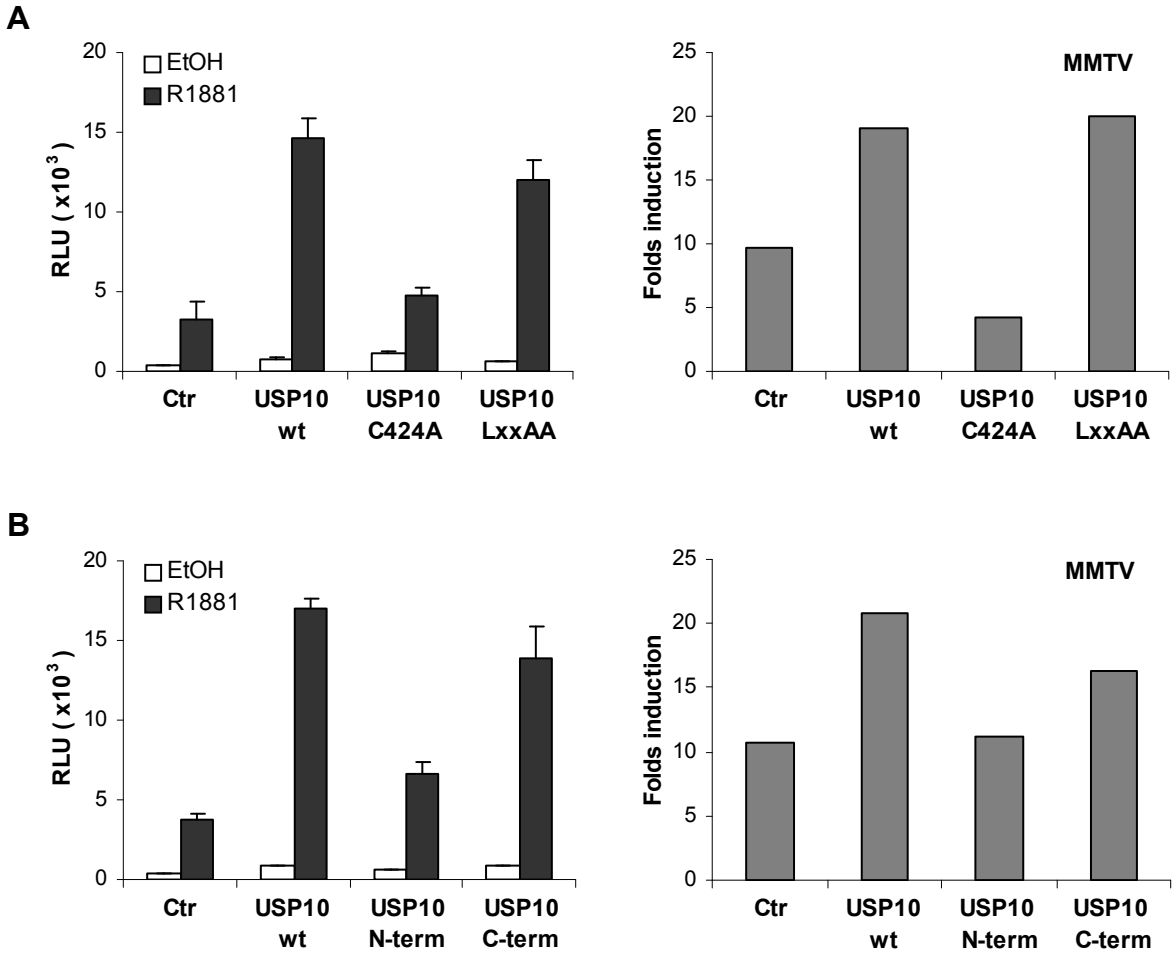

Figure 10

Effect of USP10 wt and mutant forms on AR activity. PC-3/AR cells were transfected with a reporter construct containing the androgen-responsive promoter MMTV and with expression plasmids coding for (A) USP10 wt, inactive mutant C424A or NR box mutant LxxAA, or for (B) USP10 wt, USP10 N-terminal fragment or USP10 C-terminal fragment. Treatment was with androgen (R1881) or vehicle (EtOH) for 24 h. The results in the left panel are given in relative luminescence units (RLU) and are the mean ± S.D. of sextuplicate values. In the right panel the corresponding folds induction for each experiment are shown.

3.1.2.2 Effect of USP10 knock-down on AR transcriptional activity

For further substantiation of the coactivator function, USP10 expression was down-regulated by RNA interference. In order to circumvent the problems linked to a double transfection procedure, a PC-3/AR cell line stably expressing a luciferase reporter gene under control of the MMTV promoter was used. Three siRNAs directed against different regions of the USP10 transcript were transfected into these cells. First the extent of USP10 expression knock-down was determined by quantitative PCR at days 1 and 2 post-transfection. USP10 transcript

levels were found to be down-regulated to less than 20% of control levels for the siRNA2, and to less than 10% for the siRNAs 1 and 3 even (Figure 11A). Since no antibodies for USP10 detection were available at the time these experiments were performed, expression knock-down was monitored at the protein level by cotransfecting an expression vector for HA-tagged USP10. Western blot analysis revealed that an efficient suppression of USP10 expression was observed in the presence of the three specific siRNAs, but not with the mismatch control or in absence of siRNA (Figure 11B).

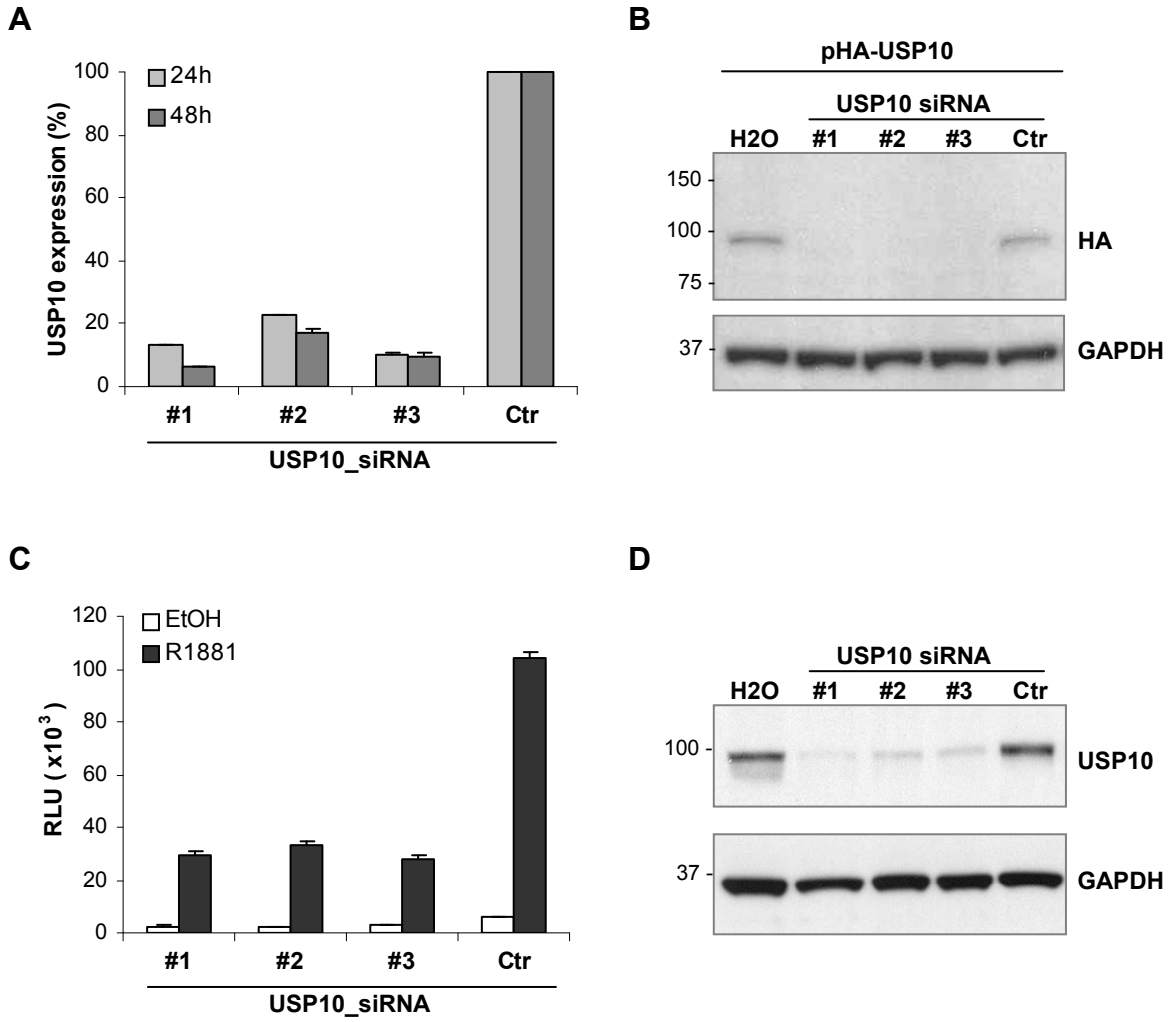

Figure 11

Effect of USP10 knock-down on AR activity. (A) PC-3/AR cells were transfected with three different siRNAs against USP10 or with a mismatch control and after 24 or 48 h the amounts of USP10 transcript were measured by real-time PCR. (B) PC-3/AR cells were transfected with an expression plasmid coding for HA-tagged USP10 together with the siRNAs against USP10 or control siRNA. After 24 h cell extracts were analysed by western blotting using specific antibodies for the HA-tag or for GAPDH as loading control. (C) PC-3/AR/MMTV-Luc cells were transfected with the USP10 siRNAs or control, two days later they were treated with R1881 or vehicle (EtOH) for 24 h and luciferase activity was then determined. (D) PC-3/AR cells were transfected with the USP10 siRNAs and control and 72 h later USP10 protein content was analysed by western blotting.

The effect of USP10 down-regulation by siRNA on androgen-stimulated MMTV activity was then determined by measuring luciferase activity. A marked diminution of reporter gene activity was found for all siRNAs in comparison to the mismatch control (Figure 11C). These data indicate that the decrease of USP10 expression is paralleled by a loss of MMTV promoter response to androgen treatment, and are in line with a function of this ubiquitin-specific protease as a coactivator of the AR.

Later on, as an antibody against USP10 became available, it was possible to determine the extent of the knock-down of the endogenous USP10 protein. PC-3/AR cells were transfected as before with three different siRNAs against USP10, with the mismatch control or mock transfected (H_2O). Cells were harvested 48 and 72 h later and USP10 protein content was analysed by western blotting with the specific antibody. After 48 h there was only a light reduction of USP10 protein levels (not shown) whereas after 72 h a clear reduction of the USP10 signal in the cells treated with siRNA was observed as compared to both controls (Figure 11D).

3.1.3 Effect of USP10 on AR protein levels

The next step was to find out whether USP10 affected the amount of AR protein in the cells. If USP10 is able to decrease AR polyubiquitylation, this should lead to stabilisation of the AR. Conversely, USP10 depletion should lead to an increase of ubiquitylation and thereby to destabilisation of the AR.

Using western blot analysis, a stronger AR signal was detected in the cells overexpressing USP10 wild-type form, compared to the cells overexpressing the mutated form, or to the cells transfected with the empty vector or mock transfected (Figure 12A).

The results were less clear-cut after USP10 knock-down. PC-3/AR cells were transfected with the three siRNAs against USP10, harvested after 48 h and the AR content was analysed by western blot. A reduction in AR protein levels was found in the cells transfected with siRNA 1 as compared to the mismatch control lane, but also for the siRNA mismatch control lane compared with other negative controls (Figure 12B). This experiment was performed again and the cells were harvested 48 and 72 h after transfection. The observed differences in AR levels remained stable (not shown).

If the observed differences were due to different ubiquitylation states and therefore to different degradation rates of the AR, they should disappear when blocking the activity of the proteasome. Transfections were therefore performed in presence of 5µM of the proteasome

inhibitor MG132. The absolute amount of AR protein increased upon MG132 treatment but the relative differences in AR protein were not affected by proteasome inhibition (Figure 12C) indicating that they were not related to the ubiquitylation status of the AR.

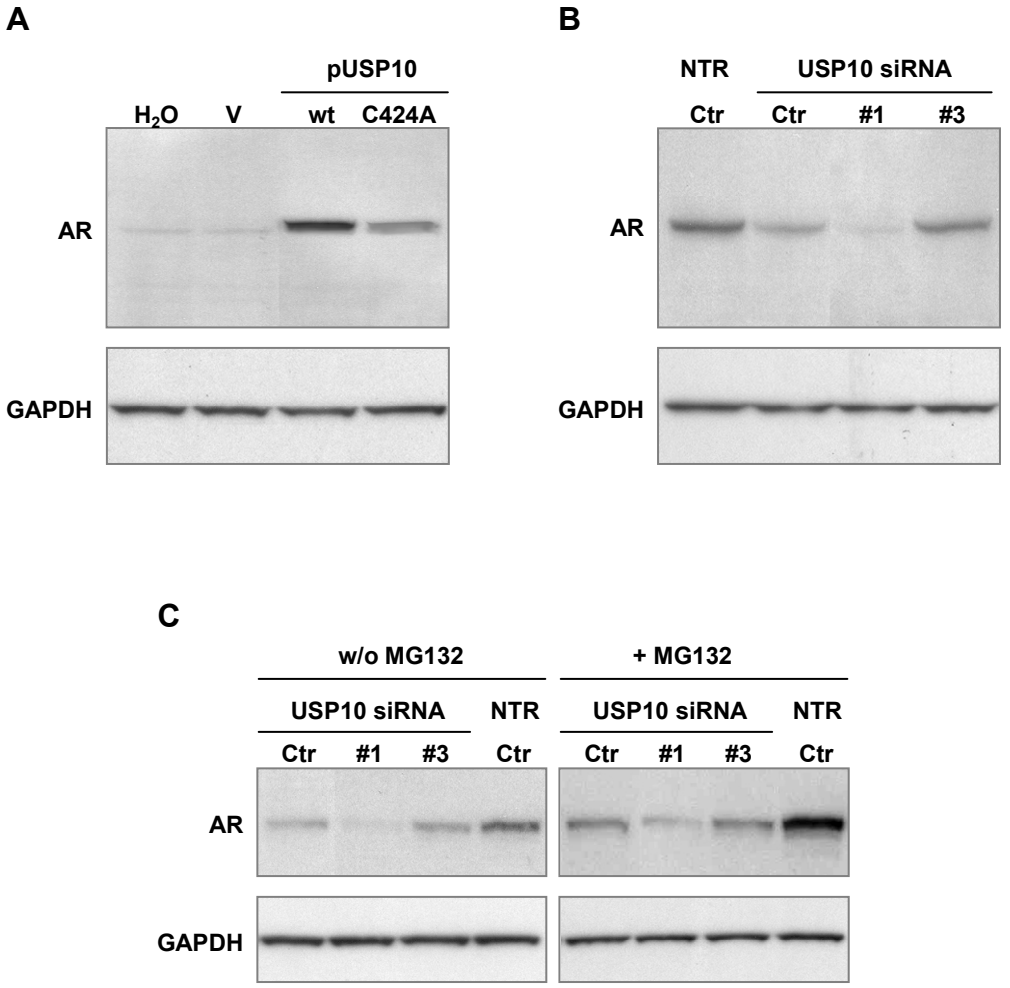

Figure 12

Effect of USP10 on AR protein stability. (A) PC-3/AR cells were transfected with expressions plasmids for USP10 wt or the inactive mutant C424A, with empty vector (V) or mock transfected (H_2O). AR content was analysed 48 h later by western blotting. (B) PC-3/AR cells were transfected with 2 siRNAs directed against USP10, a mismatch control and a non-targeting control (NTR). AR content was analysed 48 h later by western blotting. (C) PC-3/AR cells were transfected as in (B), treated or not with MG132 and analysed for AR content by western blotting.

3.1.4 Effect of USP10 on AR ubiquitylation

Because the effects of USP10 on AR stability were not conclusive, the effect of USP10 on AR ubiquitylation was assessed directly.

In order to obtain an accumulation of ubiquitylated proteins, the proteasome function has to be blocked. PC3/AR cells were therefore treated with the proteasome inhibitor MG132 for 2 or 6 hours. Proteins were extracted and western blot analysis was performed with an anti-AR antibody or an antibody directed against protein-ubiquitin conjugates. As shown in Figure 13A, an accumulation of ubiquitylated species could be detected already after 2 h treatment and even more so after 6 h. As expected, an accumulation of AR protein was also observed. For the detection of AR ubiquitylation, the AR was immunoprecipitated from cells treated with 5µM MG132 for 6h or mock treated. After extensive washing the AR was eluted, resolved by SDS-PAGE and the ubiquitylated forms were detected on western blot using an anti-ubiquitin specific antibody. The characteristic smear of polyubiquitylated proteins could be detected only after MG132 treatment and not in the negative control, where the immunoprecipitation was performed with a non-specific isotype antibody (Figure 13B) indicating that this could correspond to AR ubiquitylated forms.

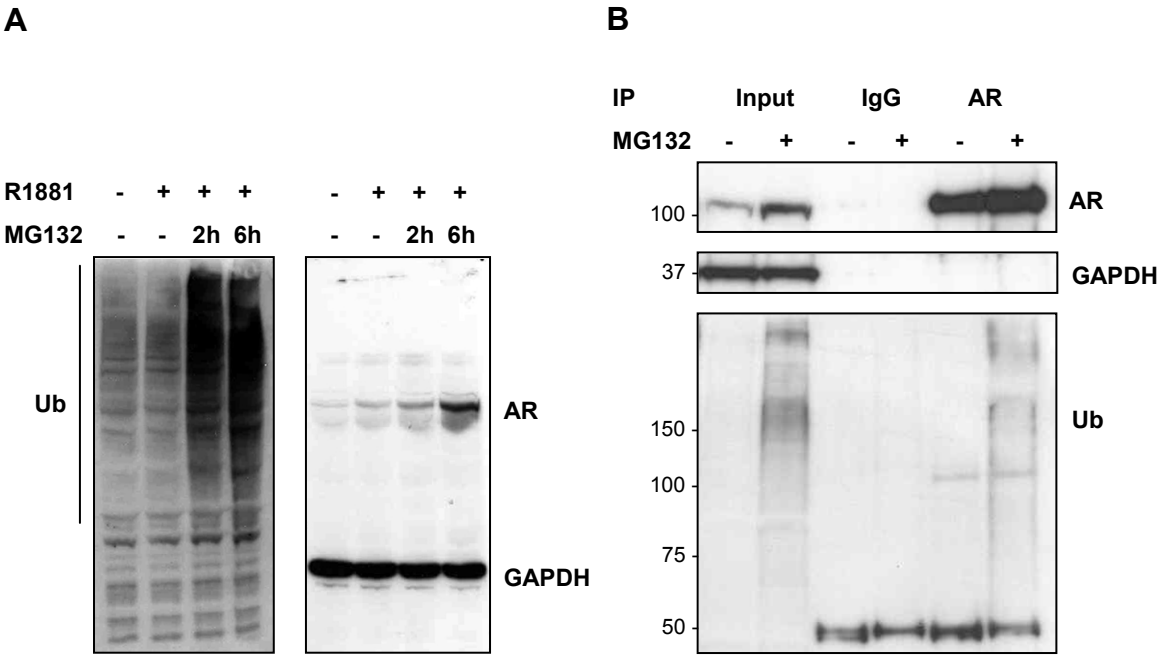

Figure 13

Detection of AR ubiquitylation. (A) PC-3/AR cells were treated for 2 or 6 h with the proteasome inhibitor MG132. Cell extracts were then analysed by western blotting with an anti-ubiquitin antibody to detect total ubiquitylated proteins and with an anti-AR antibody to detect AR accumulation. GAPDH served as loading control. (B) PC-3/AR cells treated or not with MG132 were immunoprecipitated with an anti-AR 441 monoclonal antibody or an unspecific antibody as negative control. The eluates were analysed on western blot with anti-AR C-19, anti-GAPDH and anti-ubiquitin antibodies.

Finally, the effect of USP10 on AR polyubiquitylation was analysed by overexpressing the wild-type form or the inactive mutant in PC3/AR cells prior to the immunoprecipitation. In this experiment however no significant changes in AR ubiquitylation levels could be detected when USP10 was overexpressed (Figure 14A).

Proteins can also be modified by monoubiquitylation and this modification does not lead to protein degradation. To explore this possibility, His-tagged ubiquitin was cloned into the pSG5 expression vector and its lysine 48, the main site for polyubiquitin chain formation, was mutated to arginine to promote the accumulation of monoubiquitylated forms. This expression construct was then transfected into PC-3/AR cells together with USP10 wt or mutant. After 24 h, AR immunoprecipitations were carried out in this case without treating with proteasome inhibitor. The expression of the His-tagged ubiquitin monomer could be detected on western blot in the input fractions but not in the immunoprecipitated fraction (Figure 14B). This suggests that no monoubiquitylation of the AR had taken place under the tested conditions. The role of USP10 in regulating the ubiquitylation of AR therefore remains unclear.

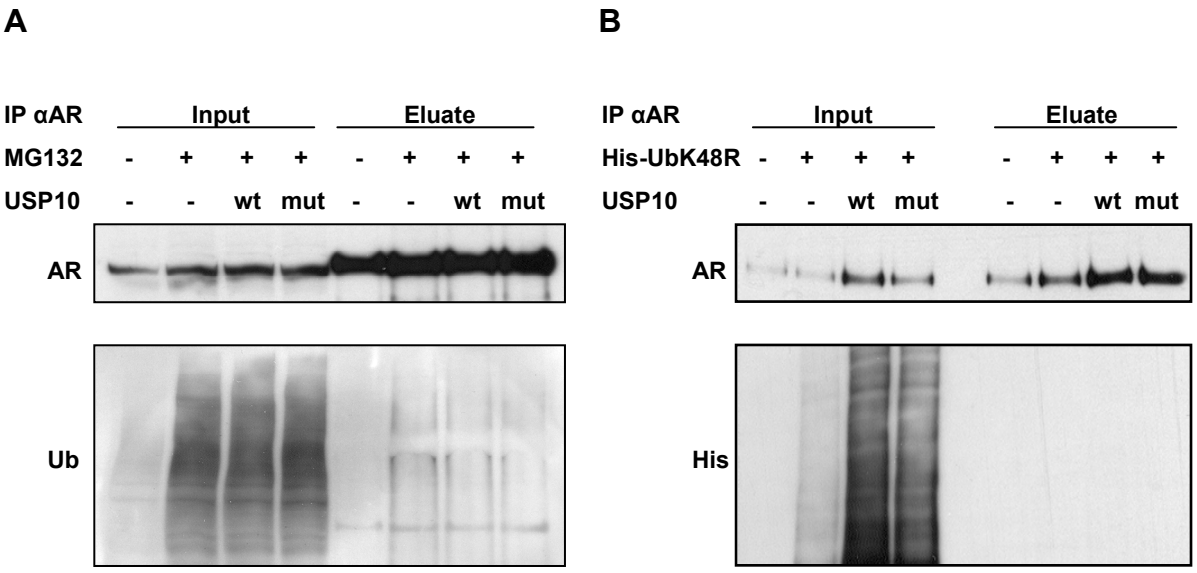

Figure 14

Effect of USP10 overexpression on AR ubiquitylation. (A) PC-3/AR cells were transfected with USP10 wt or inactive mutant forms or mock transfected, treated or not with MG132 for 6 h and immunoprecipitated with anti-AR 441. Imput fractions and eluates were analysed on western blot with anti-AR C19 and with anti-ubiquitin. (B) PC-3/AR cells were transfected with USP10 wt or inactive mutant forms or mock transfected together with a plasmid coding for His-tagged ubiquitin mutated at lysine 48. Immunoprecipitation was performed with anti-AR 441 and input fractions and eluates were analysed on western blot with anti-AR C19 and with anti-His antibodies.

3.2 Androgen Receptor Acetylation

3.2.1 Role of acetylation in AR protein stability

It has been already described for several proteins that acetylation can modulate their stability by promoting or interfering with the ubiquitylation process. It was therefore interesting to explore whether this could also be the case for the AR. The first hint was the strong accumulation of the AR observed following inhibition of cellular histone deacetylase (HDAC) activity. PC-3/AR cells were treated with two different HDAC inhibitors for 8 or 24 hours, cell extracts were prepared and the AR content was analysed by western blotting. As seen in Figure 15A, a strong AR accumulation was detected after 8 h and even more after 24 h treatment. However, when performing quantitative PCR, an equivalent increase of the AR transcript could be measured, indicating that the observed protein accumulation could be solely explained by an increased AR mRNA expression (Figure 15B). In the stably transfected PC-3/AR cells the expression of the AR is under control of the CMV promoter and so this activation lacks physiological significance. The same experiment was therefore performed in 22Rv1 cells which endogenously express the AR. In this case no stimulation of AR expression could be measured, showing that the natural AR promoter was not activated by the HDAC inhibitor treatment (Figure 15D). There was also no accumulation of the AR protein (Figure 15C) and this fact suggests that the stability of the AR is probably not affected by its acetylation status.

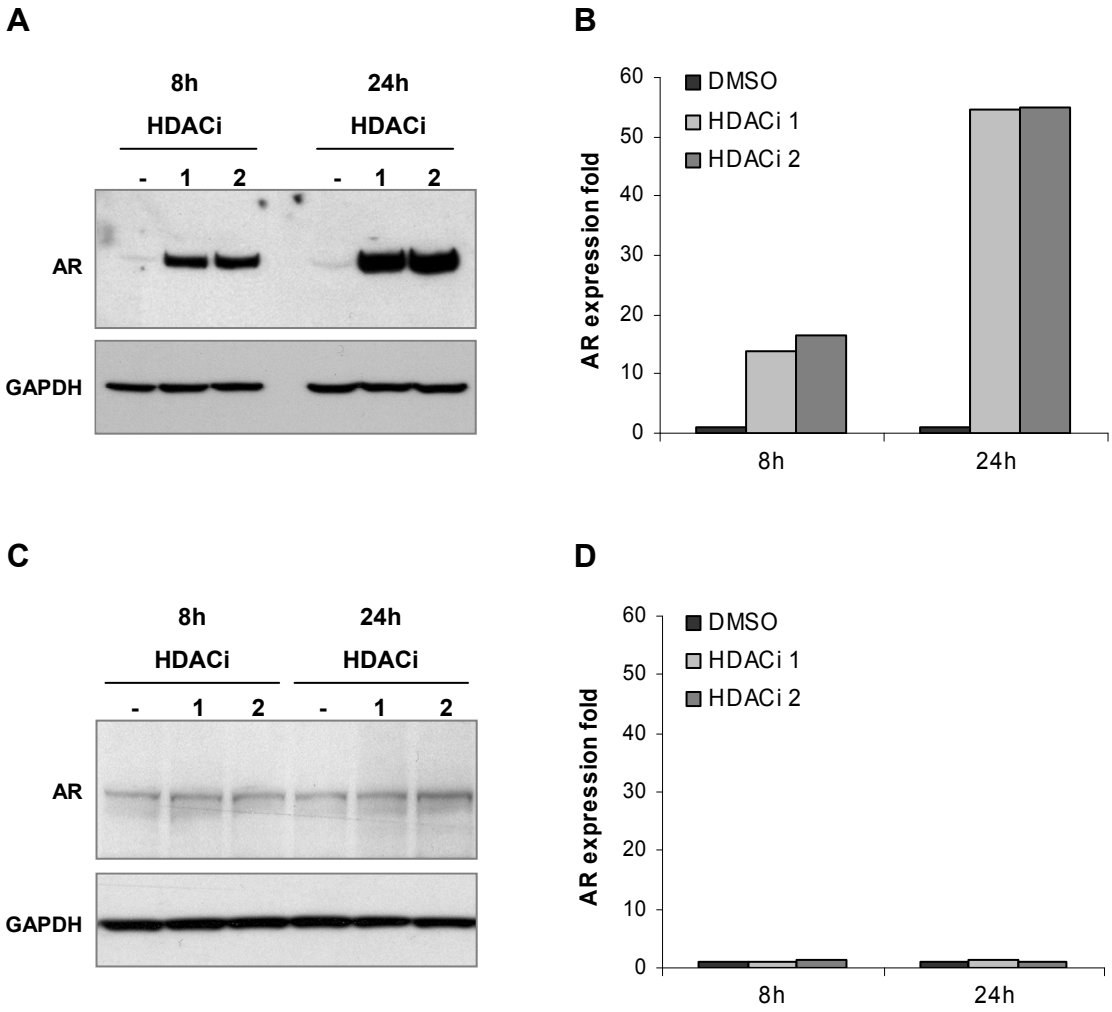

Figure 15

Effect of histone deacetylase inhibitors on AR expression. PC-3/AR cells were treated with two different histone deacetylase inhibitors (HDACi) for 8 or 24 h as indicated. Then AR protein levels were determined by western blot (A) and AR mRNA levels were measured by quantitative PCR (B). In (C) and (D) the same experiments were performed using 22Rv1 cells.

The acetylation sites in the AR have been identified previously. The three lysines targeted by acetylation were substituted by alanine in the pSG5-AR expression vector by site-directed mutagenesis of the corresponding codons. This allowed a direct comparison between the stability of the AR wt and the acetylation defective mutant AxAA.

For this purpose PC-3 cells were transfected with AR wt or mutant form and after 24h they were treated with cycloheximide to stop new protein synthesis thus allowing to asses AR protein decay. 24h later a similar reduction of protein levels of both wt and mutant could be observed (Figure 16) indicating that in PC-3 cells AR protein stability was not regulated by acetylation.

Together these data suggest that in the case of the AR protein acetylation and stability are not linked.

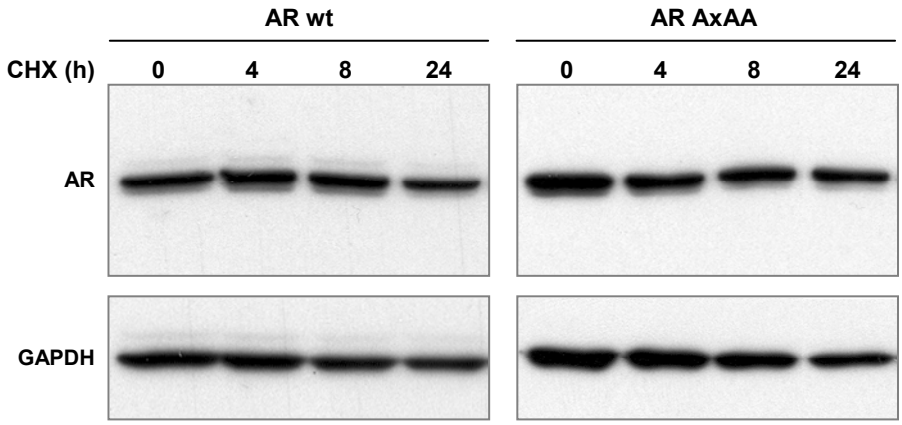

Figure 16

Effect of acetylation site mutations on AR protein stability. PC-3 cells were transfected with expression plasmids coding for AR wt or acetylation defective mutant (AxAA). 24 h after transfection cells were treated with cycloheximide for the time indicated and AR protein levels were analysed by western blot.

3.2.2 Role of acetylation in AR activity

3.2.2.1 Activity of the AR acetylation-defective mutants

Three lysine acetylation sites belonging to the consensus acetylation motif KXKK exist in the AR hinge region, just C-terminal of the DNA-binding domain (DBD). In order to better understand the role of acetylation in the transcriptional activity of the AR, these sites were substituted by alanine to generate loss-of-function mutants. Expression constructs for K630A (single mutant AxKK), K632A/K633A (double mutant KxAA) and K630A/K632A/K633A (triple mutant AxAA) were generated by site-directed mutagenesis (Figure 17A).

Cell-based transactivation assays were performed by transfecting the corresponding expression vectors in PC-3 cells, which do not express endogenous AR, together with a reporter vector containing four repeats of the androgen response element 1 (ARE-1). All three mutants were able to stimulate the expression of the reporter gene. Transfection with the reporter construct together with the empty expression vector confirmed the lack of AR and of any endogenous activity in PC-3 cells (Figure 17B).

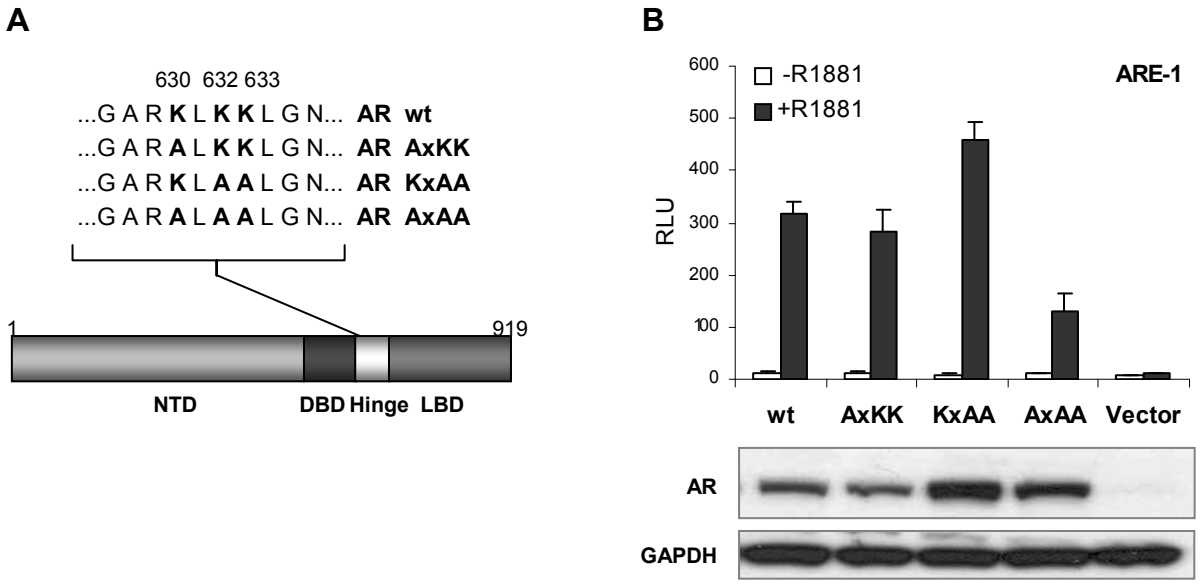

Figure 17

Activity of the AR acetylation-defective mutants. (A) Schematic representation of the human AR and localisation of the motif involved in acetylation. The four AR domains are indicated: N-terminal domain (NTD), DNA-binding domain (DBD), hinge and ligand-binding domain (LBD). The amino acid residues that were modified in the mutant forms are highlighted in boldface. (B) PC-3 cells were transfected with an androgen-dependent reporter vector and expression plasmids for wt AR or mutant forms, or with empty vector. Treatment was with 1 nM R1881 (black bars) or with vehicle (white bars). The reporter activity measured is given in relative light units (R.L.U.). The results are a representative of three separate experiments and the bars are the mean ±SD of sextuplicate values. Western blot analysis of AR levels is shown for each transfection.

These results were surprising since the single and double mutants had already been described as inactive in previous reports. Additional androgen-responsive promoters were therefore tested. The reporter constructs used contained the mouse Pem promoter which is selectively responsive to androgens; or the promoter of the gene for human PSA, an often used biomarker for prostate cancer; or the highly androgen-responsive promoter of the mouse mammary tumour virus (MMTV). When testing the Pem promoter, a similar androgen-dependent stimulation was observed after treatment with 1 nM R1881 in presence of the wild-type (wt) AR and the KxAA forms, and slightly less activity was seen in presence of the AxKK mutant. Conversely, no stimulation was seen for the AxAA triple mutant. The situation was different when using the PSA promoter as reporter. Here the AxKK and especially the KxAA mutant had stronger activity than wt AR whereas the triple mutant was as active. Next, the MMTV promoter was tested. Here, wt AR, the AxKK and KxAA forms had similar activities whereas the AxAA mutant conveyed the strongest effect. Western blot analysis showed that the KxAA and AxAA forms were generally expressed at higher levels than wt AR or the AxKK form, but this did not parallel the differences seen in transactivation efficiencies at the different promoters (Figure 18).

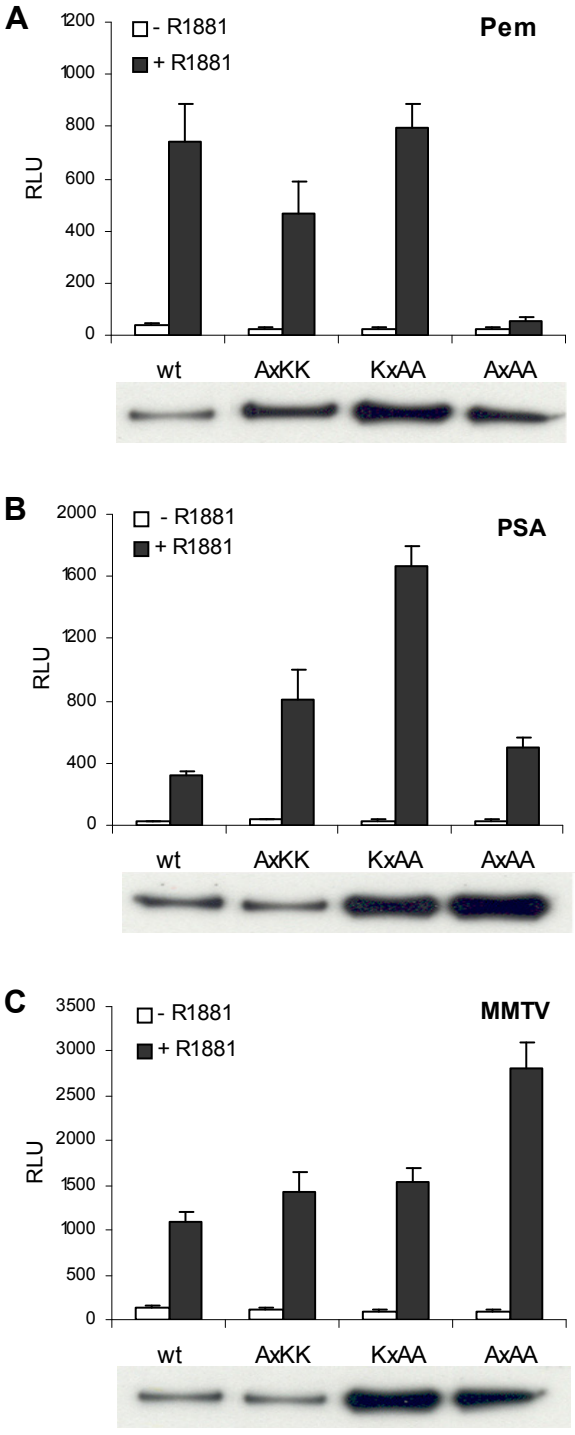

Figure 18

Differential effects of acetylation site mutations on AR function. PC-3 cells were transfected with an androgen-dependent reporter vector and a pSG5-based expression plasmid for wt AR or a mutant form. Treatment was with 1 nM R1881 (black bars) or with vehicle (white bars). The reporter activity measured is given in relative light units (R.L.U.). The results are a representative of three separate experiments and the bars are the mean ±SD of sextuplicate values. Western blot analysis of AR levels is shown for each experiment. (A) Transfection with pSG5-AR (wt or mutant) and the mouse Pem promoter reporter. (B) Transfection with pSG5-AR (wt or mutant) and the human PSA promoter reporter. (C) Transfection with pSG5-AR (wt or mutant) and the MMTV promoter reporter.

These experiments point out that despite the elimination of its three acetylation sites, the AR still retains transactivation potential, at least on some promoters. In several cases the alteration of acetylation sites even potentiates AR function.

For a more precise comparison of the activities of the different AR forms, the androgen concentrations used for stimulation were varied. When giving increasing R1881 amounts to PC-3 cells transfected with the Pem promoter-based reporter vector (Figure 19A), reporter gene activity was measurable for wt AR starting at 0.01 nM and reaching a maximum at 1 nM. For the KxAA mutant, a stronger stimulation was seen. For the AxKK and more so the AxAA form, little or no stimulatory effects of androgen were observed, even at the highest hormone concentration used. Concerning the MMTV reporter (Figure 19B), comparable induction profiles were seen in presence of wt AR and the AxKK mutant, with 0.01 nM R1881 being sufficient to achieve the maximal effect. Higher induction values were seen for the KxAA and more so for the AxAA mutants. Interestingly, the higher efficacy seen for the triple mutant was accompanied by a loss of potency, as a ten-fold higher hormone concentration was needed to achieve the EC_{50} value.

The use of different androgen concentrations confirms that the acetylation defective AR triple mutant has lost its transactivation potential for the Pem promoter. Conversely, this mutant is more active than wt AR in the presence of the MMTV promoter.

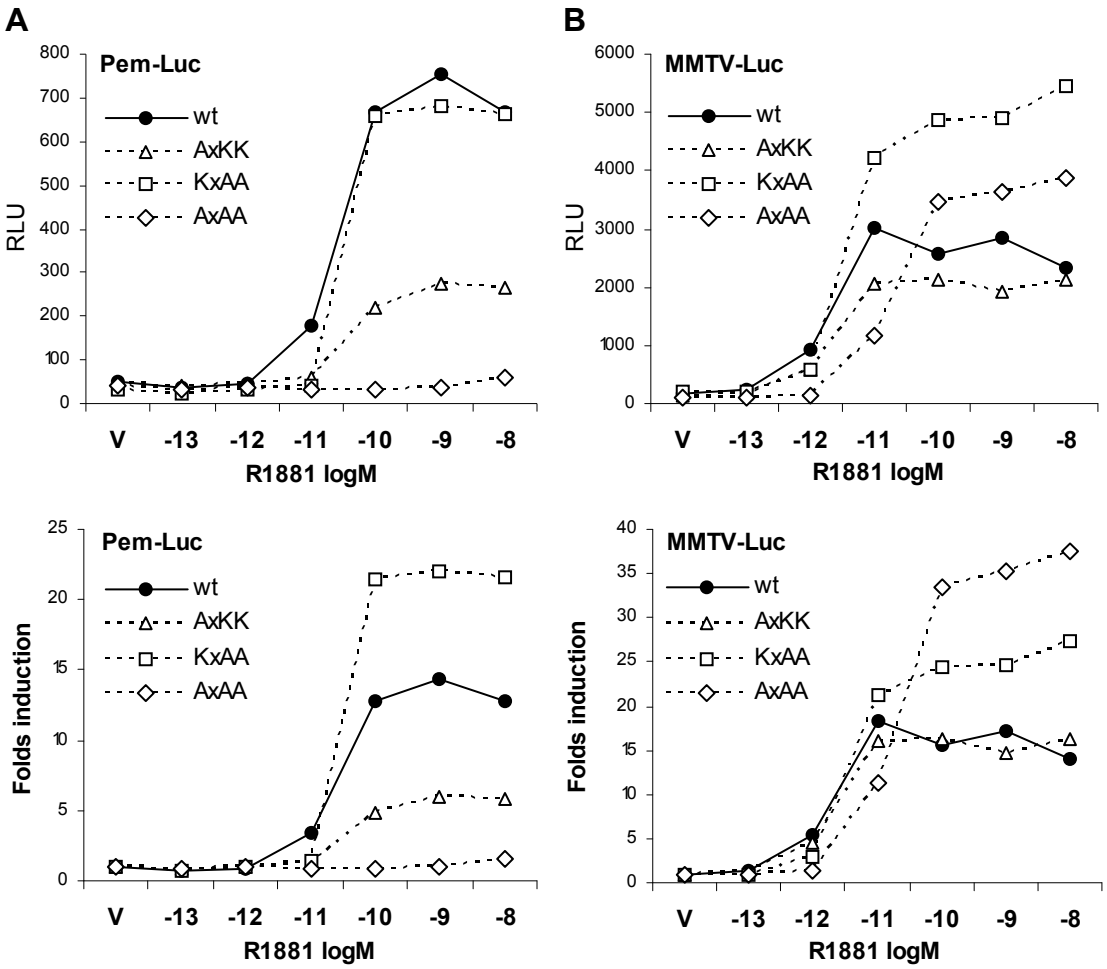

Figure 19

Hormone-dependent activity of AR mutants. PC-3 cells were transfected with an androgen-dependent reporter vector and a pSG5-based expression plasmid for wt AR or a mutant form. Treatment was with different R1881 concentrations as indicated. The reporter activity measured is given in relative luciferase units in the upper panels and as fold induction at the bottom. The results are a representative of three separate experiments. (A) Transfection with pSG5-AR wt or mutant forms and the mouse Pem promoter reporter. (B) Transfection with pSG5-AR wt or mutant forms and the MMTV promoter reporter.

In order to further substantiate these findings, the ratio between the wt and mutated AR forms in the transactivation experiments was varied. The focus was on the AR triple mutant and on the Pem and MMTV promoters, as these combinations had given the most differentiated responses.

Decreasing amounts of plasmids coding for wt AR and increasing amounts of the construct expressing the AxAA mutant were transfected into PC-3 cells, while maintaining the total DNA concentration constant (Figure 20A). In presence of the Pem promoter, increasing the AxAA/ARwt ratio led to a complete loss of androgen-dependent activity. The situation was different for the MMTV promoter. Here, the hormone-dependent reporter activity remained the same, regardless of the AxAA/ARwt ratio.

In order to find out if this finding could be extended to another cell line, similar experiments were performed with CV-1 cells, which do not express the AR (Figure 20B). Here also, increasing the amounts of the AxAA form led to a complete loss of androgen stimulation of the Pem promoter. Conversely, the response of the MMTV promoter was not affected by the AxAA/ARwt ratio. A comparison of the respective inductions clearly showed that both in PC-3 and in CV-1 cells, the Pem promoter was not responsive at all to the AxAA mutant. Conversely, the MMTV promoter despite giving comparable signals for all tested mutant to wt AR ratios, was actually more strongly induced by the AxAA form. This was due to the lower basal activity of the MMTV promoter in presence of the AxAA form.

When determining the total AR protein levels some variation was found, but this could not explain the differences in activities seen. For instance, in CV-1 cells no stimulation of the Pem promoter was observed in presence of the AxAA form, even though this mutant was expressed at higher levels than wt AR in the same experiment.

The results further document that the Pem and MMTV promoters differentially respond to obliteration of the AR acetylation sites.

Figure 20 (next page)
Dose-dependent effects of AR acetylation site mutants. Cells were transfected with the indicated amounts of expression vector for wt AR or for the triple mutant form, while keeping the total DNA amount constant, and with different reporter constructs. Treatment was with 1 nM R1881 (black bars) or with vehicle (white bars). The reporter activity measured is given in relative light units (R.L.U.) in the upper panels and in fold induction in the bottom panels. Western blot analysis of AR levels is shown for each experiment. (A) Transfection of PC-3 cells with the mouse Pem promoter or MMTV promoter reporters and comparison of inductions seen for both promoters. (B) The same experiment as in (A) was performed in CV-1 cells.

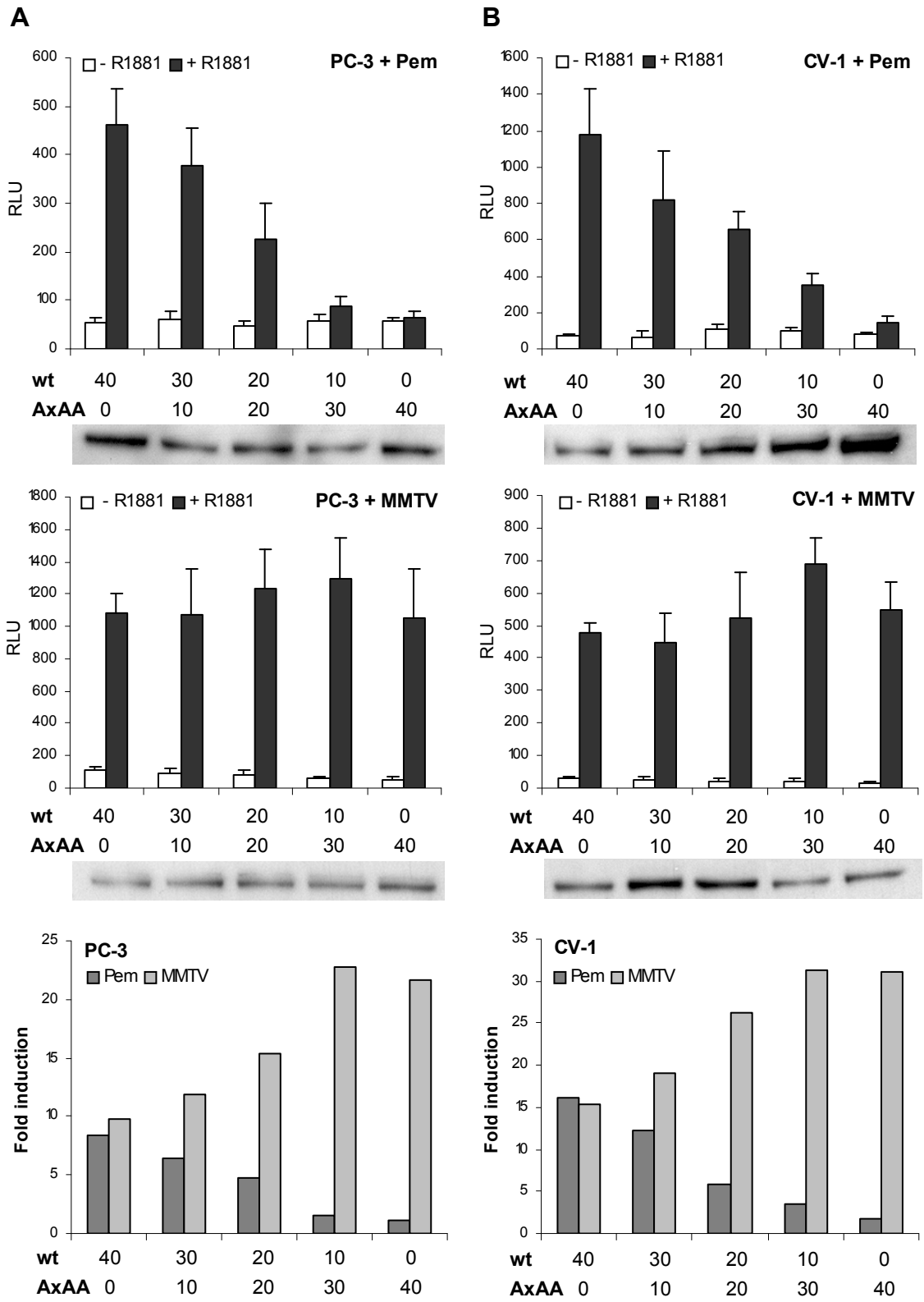

Figure 20

(See previous page)

3.2.2.2 Subcellular localisation and DNA binding of the AxAA mutant

The KLKK acetylation motif is part of the bipartite nuclear localisation signal of the AR (Figure 21A). Indeed several studies report that mutations in this region impair AR transport to the nucleus. In order to find out whether the triple mutation introduced into this motif affected subcellular localisation, AR wt or mutant fusions with GFP were cloned and transfected into PC-3 cells. After hormone treatment, the ARwt-GFP fusion protein was found as expected in the nucleus (not shown) whereas the ARAxAA-GFP protein showed a very heterogeneous distribution (Figure 21B).

With the intention to quantify the distribution of the AR and to circumvent the use of GFP fusion proteins, PC-3 cells expressing wt AR or the AxAA mutant were fractionated and Western blot analysis was performed (Figure 21C). As expected most of the AR was located in the cytoplasm in the absence of hormone. Following R1881 treatment, a sizeable fraction of the wt AR pool was translocated into the nucleus. When looking at the AxAA mutant, a stronger accumulation in the cytoplasm of hormone-treated cells was found. A significant amount was however also present in the nucleus.

These fractionation experiments indicate that alteration of the AR acetylation sites still allows nuclear translocation after androgen treatment.

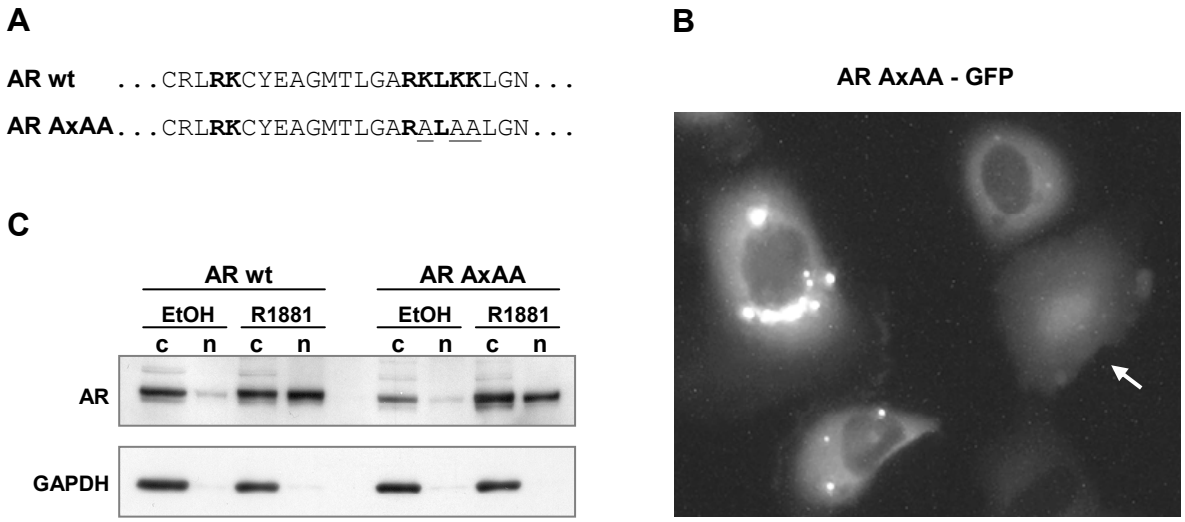

Figure 21

Subcellular localisation of the AR triple mutant. (A) Representation of the bipartite nuclear localisation signal of the AR (bold) and amino acids modified in the acetylation defective triple mutant (underlined) (B) PC-3 cells were transfected with an expression vector coding for AR AxAA-GFP fusion protein. After 24h treatment with 1 nM R1881 the fusion protein was localised by fluorescent microscopy of the living cells. (C) PC-3 cells transfected with an expression vector for wt AR or the AxAA form were treated or not with 1 nM R1881. Cytoplasmic (c) and nuclear (n) fractions were prepared. Western blot analysis was performed with antibodies specific for AR or for GAPDH.

In order to further understand the reason for the non-responsiveness of the Pem promoter to the AR triple mutant, plasmid immunoprecipitation was performed. PC-3 cells transfected with expression vectors for wt AR or the AxAA form and with the Pem promoter construct were treated for 24h with R1881. They were then crosslinked by formaldehyde and sonicated. The AR-DNA complexes were precipitated with a specific anti-AR antibody and after reversing the crosslinking, the purified DNA was analyzed by PCR using primers designed to amplify the whole Pem promoter region. Signals of comparable intensities were generated from complexes purified from the PC-3 cells expressing wt AR or the mutated form (Figure 22A). The mock-precipitated samples showed only a very weak background signal.
These data show that the AxAA form binds to the Pem promoter as well as the wt form does, suggesting that impaired activity is not linked to reduced promoter recognition.

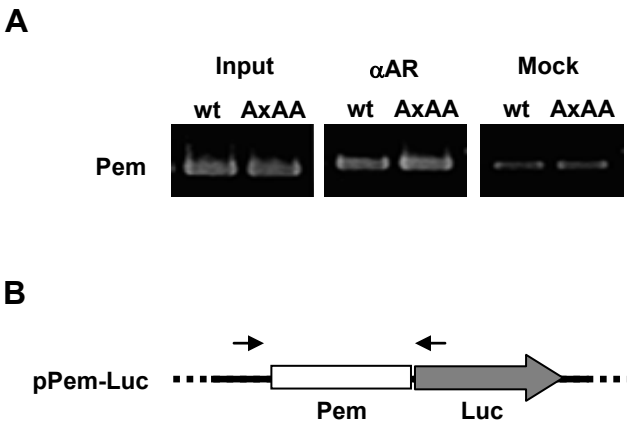

Figure 22

(A) PC-3 cells transfected with an expression vector for wt AR or the triple mutant form and with the Pem reporter construct were treated with 1 nM R1881 for 24 h. The cells were cross-linked with formaldehyde, extracts were prepared and the AR was immunoprecipitated. After de-crosslinking, the Pem promoter was amplified by PCR using specific primers (αAR, middle panel). The input samples (cell extract prior to immunoprecipitation, left panel) served as positive control and extracts incubated without antibody (mock, right panel) as negative control. (B) Schematic representation of the Pem promoter cloned into the reporter vector. The position of the primers used in (A) is represented by the arrows.

3.2.2.3 Role of the N/C interaction in the phenotype of the AxAA mutant

Recent data indicate that some androgen-responsive promoters depend on the interaction between the AR N-terminal domain (NTD) which contains the important transactivation function 1 and the C-terminal ligand-binding domain (LBD) for full response. This N/C interaction is mainly mediated by the FXXLF motif located at positions 23-27 and less so by the WXXLF motif located at positions 433-437.

As the AR hinge region in which the acetylation sites are located may play a role in modulating the interdomain communication, the activities of AR forms mutated in regions involved in the N/C interaction were compared. The FXXLF motif was mutated to FXXAA (AR FxxAA) and the WXXLF to WXXAA (AR WxxAA) in the AR sequence (Figure 23A). Transactivation experiments were then performed with these constructs in PC-3 cells (Figure 23B). When assaying the Pem promoter, the FxxAA form showed similar induction levels to those of wt AR whereas the WxxAA form was more than twice as active. Concerning the MMTV promoter, all expression constructs gave similar inductions, with possibly a stronger effect of the WxxAA mutant, as previously reported.

The fact that the AR mutants with impaired N/C interaction are still able to activate the Pem promoter shows that a loss of N/C interaction in the AxAA mutant is probably not responsible to this lack of activity.

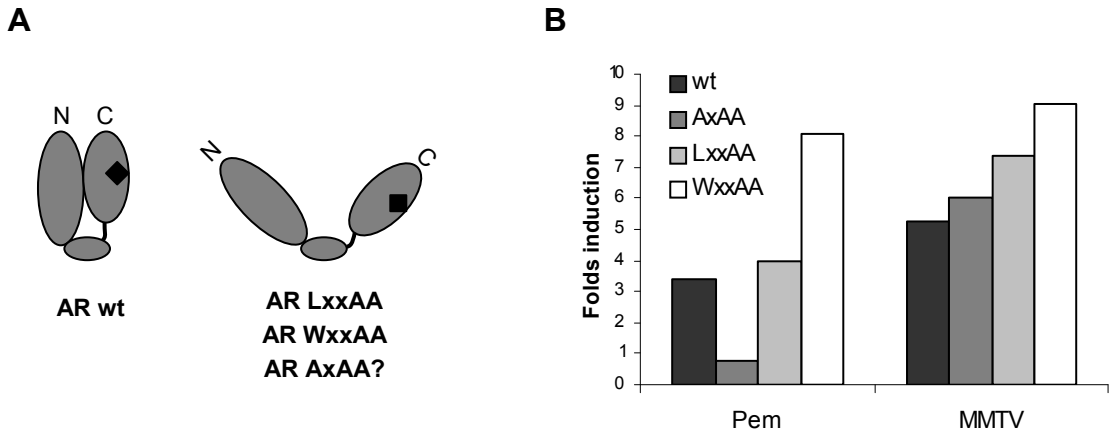

Figure 23

Mutations of acetylation motif and N/C interaction motif differentially affect AR activity. (A) Schematic representation of N/C interaction. (B) PC-3 cells were transfected with 50 ng of expression vector for wt AR (black bars), acetylation defective mutant (dark grey bars) or N/C interaction mutants (light grey and white bars), and with Pem or MMTV promoter reporter constructs, as indicated. Treatment was with 1 nM R1881. The reporter activity measured is given as fold inductions. The results are a representative of three separate experiments.

3.2.2.4 Activity of the AxAA mutant on the androgen response elements ARE-1 and ARE-2

The molecular basis for the differential response of the Pem promoter to the acetylation-defective AR was further analyzed. The androgen response of the Pem promoter is mainly mediated by two potent DNA response elements named ARE-1 and ARE-2 (Figure 24A). The role of these elements was therefore analyzed in transactivation assays using reporter constructs containing four copies of each. The activity of Pem ARE-1 in presence of the AxAA mutant was half of that seen with wt AR. In contrast, the response of Pem ARE-2 was the same in presence of wt AR and the AxAA mutant. As before, the Pem promoter was not stimulated by the AxAA form (Figure 24B).

These results establish that two related DNA elements exhibit different responses to the AR AxAA mutant. They furthermore suggest that Pem ARE-1, but not ARE-2, is directly involved in the non-responsiveness of the Pem promoter to the AxAA form.

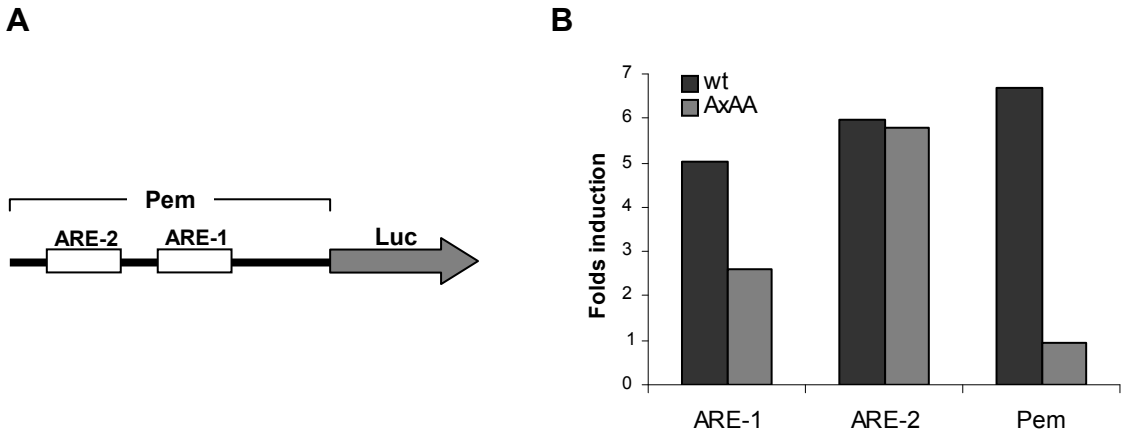

Figure 24

AR acetylation site mutation has different effects on Pem ARE-1 and ARE-2. (A) Schematic representation of the mouse Pem promoter. (B) PC-3 cells were transfected with an expression vector for wt AR (black bars) or triple mutant form (grey bars) and with Pem promoter or minimal reporter constructs, as indicated. Treatment was with 1 nM R1881. The reporter activity measured is given as fold inductions. The results are a representative of three separate experiments.

4 Discussion

4.1 The Ubiquitin-Specific Protease 10

4.1.1 Characterisation of the USP10 as an AR cofactor

USP10 had been found in native complexes of the AR bound to DNA response elements purified from prostate cancer cell nuclei. A direct interaction between both proteins had furthermore been demonstrated in GST pull-down experiments [111]. The aim of this work was to analyse USP10 in terms of its functional relationship with the AR.

USP10 had originally been reported to interact with Ras-GTPase-activiting protein SH3 domain-binding protein (G3BP), a multi-functional protein first identified as a binding protein of Ras GTPase Activating Protein [112, 114]. However G3BP did not appear to be a direct substrate of USP10 but rather acted as an inhibitor of USP10 deubiquitylating activity. The USP10-G3BP interaction occurs in the cytoplasm leading to a possible contradiction with the finding that USP10 interacted with the AR in nuclear complexes. The first step was therefore to determine the subcellular localisation of USP10. A fusion protein between USP10 and the cyan fluorescent protein was therefore generated. It localised to both the nucleus and the cytoplasm of PC-3/AR living cells, a result which was subsequently confirmed in HeLa cells by immunofluorescent staining and by western blot analysis of subcellular fractions. The dual localisation of the USP10 protein was not affected when treating the cells with the testosterone analogue R1881 and was also independent of the presence of the AR. These results are compatible with the previous reports and also in line with the different functions in the nucleus and the cytoplasm known for the yeast orthologue Ubp3p.

The next step was to investigate the possible role of USP10 in modulating AR transcriptional activity. Using luciferase reporter gene assays, USP10 was found to act as a coactivator of the AR. USP10 overexpression enhanced androgen-mediated AR activity whereas overexpression of the catalytic inactive mutant had no significant effect. In line with these results, USP10 depletion by siRNA transfection diminished AR activity after hormone induction. The coactivating effects were observed with selective and non-selective response elements as well as with native promoters, indicating that USP10 was a broad coactivator of the AR.

The exact domains involved in the AR-USP10 interaction could not be mapped. USP10 contains an NR-Box, which is a conserved motif found in several coactivators and important for their interaction with nuclear receptors [113]. However, the mutation of this motif did not influence USP10 coactivator function, suggesting that the NR-box was not responsible for the interaction or, alternatively, that a direct interaction of USP10 with the AR was not required for its role as coactivator. Furthermore, deletion of the 400 amino acid-long N-terminal region of USP10 only marginally affected its function, whereas the C-terminal domain containing the ubiquitin protease motif was sufficient to recapitulate the effects of the full-length protein. Moreover, no conserved motif could be found in this region and also no strongly ordered structure could be predicted. The role of the N-terminal region therefore remains unclear.

The related yeast ubiquitin protease Ubp3p shares 46% similarity and 27% identity with human USP10 [115]. Disruption of the Ubp3 gene leads to an accumulation of large ubiquitin-protein conjugates but not of polypeptide precursors, which points to a function in removing ubiquitin chains from protein substrates before destruction by the proteasome [116]. For Ubp3p a role in transcriptional regulation has been already documented. Ubp3p, together with the HECT E3 ligase TOM1p, regulates the activity of the ADA/SAGA multi-factor complex which is implicated in the transcription of about 10% of yeast genes [117]. In addition, Ubp3p was found in a purified TFIID complex with transcriptional activity in vitro, which is also in line with a regulatory role in gene expression control [118]. Altogether these results indicate that Ubp3p plays an important role in modulating gene transcription events and the data presented in this work extend these findings to the human form USP10 in the case of androgen-dependent gene expression.

4.1.2 The AR is not a substrate of USP10 enzymatic activity

As is the case for many other proteins, the stability of the AR is controlled by polyubiquitylation which leads to degradation by the proteasome. Inhibition of the proteasome pathway results in increased AR levels in tumour cell lines [54] and several E3 ligases have been shown to influence AR ubiquitylation and degradation. The best studied example is probably the E3 RING finger ligase MDM2, whose ubiquitylating activity is linked to other post-translational modifications of the AR, namely phosphorylation by Akt and deacetylation by histone deacetylase 1 [78, 75]. The E3 U box ligase CHIP increases the

levels of ubiquitylated AR and promotes its degradation [80, 119]. The E3 HECT ligase E6-AP is a coactivator of several steroid receptors, including the AR, however this effect is independent of its enzymatic activity [120]. Interestingly, decreased levels of E6-AP are paralleled by up-regulation of the AR in prostate tumours [121]. Finally, recent data show that the E2 ubiquitin conjugase UBCH7 is a coactivator of the AR and of other steroid receptors [122]. It was therefore conceivable that reversion of ubiquitylation by USP10 could lead to stabilisation of the AR and thereby increase the stimulation of androgen-dependent promoters.

Western blot analysis showed AR protein levels to be increased in cells overexpressing USP10. However, after USP10 knock-down by siRNAs, the results were not consistent. The three different siRNAs used had a different effect on AR protein levels and even the two RNAs used as controls seemed to regulate them. These differences in AR levels after USP10 down-regulation did not correlate with the activity data from the luciferase assays, where all three siRNA had similar effects. Moreover, inhibition of the proteasome by MG132 treatment did not abolish the observed differences in AR levels indicating that these were not related to the ubiquitylation status of the AR. Changes in the levels of non-targeted proteins due to siRNA transfection have already been reported [123] and this may also have taken place in this case. Altogether these data suggested that USP10 did not affect AR stability in a specific way.

Because the results obtained for AR protein stability were not conclusive, a possible role of USP10 in controlling the polyubiquitylation status of the AR was directly investigated. After inhibiting proteasome function, the AR was immunoprecipitated from cells overexpressing USP10 wild-type or mutant and the ubiquitylated forms were detected by western blot analysis. However, no effect of USP10 on AR polyubiquitylation could be observed in these experiments.

Polyubiquitin chains where the ubiquitin monomers are linked through lysine 48 provide the signal for degradation of the targeted protein, but polyubiquitin chains linked through other lysine residues have a different function. The best characterised are K63-linked chains which have a major role in regulating protein-protein interactions and influence processes such as DNA repair, translation and endocytosis [9, 124]. For the AR, there are no data on alternative polyubiquitin chains so far. Also, in the experiments described above, high molecular weight

forms could be observed only after inhibiting proteasome activity. Other polyubiquitylated forms of the AR, if they exist, could therefore not be investigated.

The ubiquitylation status also plays an important role in regulating gene expression independently of proteolysis. It was for instance found that treatment with proteasome inhibitors affects AR transcriptional activity by impairing nuclear translocation and the interaction with cofactors such as TIF2 [91]. Also, inhibition of the proteasome prevents the release of AR bound to the PSA promoter and thereby interferes with the cyclic assembly and disassembly of transcriptionally active complexes [90]. In line with this, mono- and triubiquitylation of the AR have been linked to transcriptional activity at the PSA promoter. Monoubiquitylation of the AR is enhanced by TSG101, an E2-like enzyme deprived of ubiquitin conjugase activity and possibly acting as a dominant-negative inhibitor of polyubiquitylation, and this correlates with increased transactivation potential [81]. USP10 could therefore also act by shifting the balance of poly- towards mono- or triubiquitylated AR, the latter forms possibly being the most stable and transcriptionally active ones.

In order to address this possibility, PC-3/AR cells were transfected with a His-tagged ubiquitin mutant where lysine 48 had been mutated to arginine. After immunoprecipitating the AR, the ubiquitin moieties should be detectable by western blot. Unfortunately, no monoubiquitylated forms of the AR could be visualised under the tested conditions, and a possible role of USP10 in this process remains therefore unclear. AR monoubiquitylation has until now only been shown in LNCaP and MCF-7 cells and it is possible that other cell lines such as the ones we tested show lower levels of this modified form of the AR, making it difficult to detect.

Altogether, USP10 does not appear to directly modify the ubiquitylation status of the AR. Since USP10 was first found in large complexes of DNA-bound AR it is possible that its protease activity is directed towards AR cofactors. Indeed, the stability of important coactivators of the AR is governed by the ubiquitin/proteasome system. It is the current view that for efficient transcription by nuclear receptors, the transcription complex must undergo proteasome-mediated degradation (Figure 25). This is for instance the case for the three SRC family members and for CBP [84]. Also, several AR-interacting proteins that are involved in the ubiquitin-proteasome pathway are themselves target for ubiquitylation. This applies to several ubiquitin ligases such as E6-AP [84], ARA54 [125] and Mdm2 [126]. USP10 could

therefore exert its coactivator function by stabilising or modifying one or more of these proteins and further efforts will therefore be needed to discover the actual substrate of its enzymatic activity.

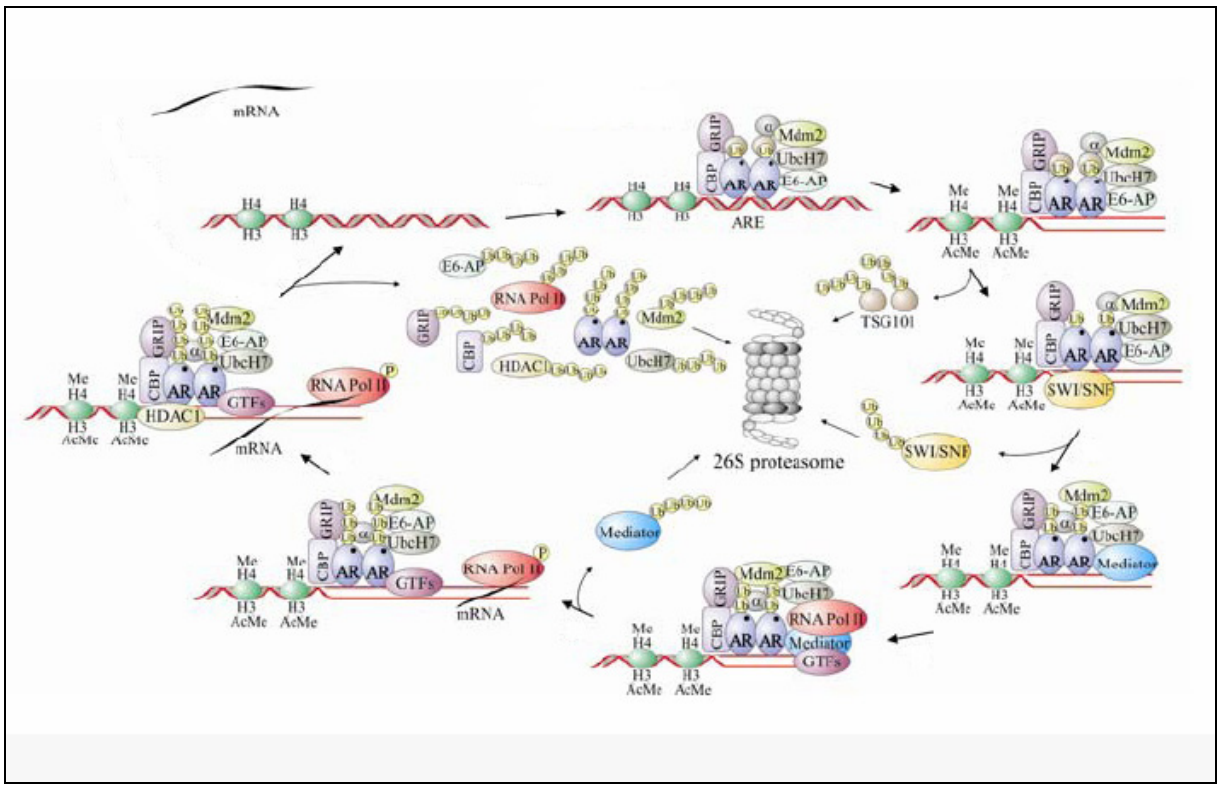

Figure 25

Model for the cycling of AR on androgen-responsive promoters. The ordered recruitment and release of cofactors and members of the general transcriptional machinery rely on their sequencial degradation by the proteasome (modified from [76]).

In summary, this part of the work reinforce the concept that the ubiquitin-proteasome system plays an important role in modulating the function of the AR. Up to now only enzymes involved in the ubiquitylation process have been shown to act as AR coregulators and the results presented here indicate that the deubiquitylation side has a regulatory role as well. Considering the general importance of the system in regulating gene expression, it is possible that a role for additional USP family members in controlling nuclear receptor activity will be identified in the near future. Indeed, very recently, the deubiquitylating enzyme MYSM1 has also been found to indirectly modulate AR-regulated transcription through deubiquitylation of histone 2A [127].

4.2 Acetylation of the Androgen Receptor

AR modification by acetyl groups has been directly demonstrated by in vitro acetylation assays and by immunoprecipitation using antibodies specific for acetylated lysines. The precise sites were mapped by mass spectrometry leading to the identification of three modified lysines belonging to the KLKK sequence located at positions 630-633 in the hinge region. This motif has previously been described in other proteins subject to this post-translational modification [22, 128].

4.2.1 Role of acetylation in AR stability

It has already been described for several proteins that acetylation can modulate their stability by promoting or interfering with the ubiquitylation process [25]. It was therefore interesting to explore whether this could also be the case for the AR. The first hint is the strong AR accumulation observed following inhibition of cellular histone deacetylase (HDAC) activity. However, this effect is due to enhanced expression of the AR transcript and not to increased protein stability. Furthermore, when testing a cell line with endogenous AR expression, no enhancement in transcript or protein levels is observed, indicating that inhibition of the HDAC activity and therefore induction of a hyper-acetylated status has no dramatic effect on AR protein stability. Because treatment with HDAC inhibitors is a very unspecific approach, further experiments were however needed to clarify the role of acetylation in AR function. Three lysines of the AR hinge region located at positions 630, 632 and 633 are part of the acetylation consensus KLKK motif and have previously been shown to undergo this post-translational modification. They were each substituted for alanine residues which allowed a direct comparison between the stability of wild-type and acetylation-defective forms of the AR. However, no significant difference in the stability of the wild-type and mutant forms could be detected by western blot analyses.

Together these data suggest that in the tested cell line, the acetlyation status and the stability of the AR are not linked.

4.2.2 Role of acetylation in AR transcriptional activity

In order to understand the role of acetylation in AR function, the lysine to alanine mutants were analysed in cell-based transactivation assays. AR forms in which one, two or all three lysines were exchanged for alanine residues were tested by comparing their ability to stimulate natural promoters originating from three highly androgen-regulated genes.

Surprisingly, elimination of one or two acetylation sites either reduced or increased AR activity, depending on the reporter system. On the other hand, the triple acetylation mutant form behaved quite differently from the single or double mutant. Strong activity was seen in the presence of the PSA and the MMTV promoter whereas no activity could be measured when using the Pem promoter in the reporter assays. This implied that the role of the mutated lysines was not equivalent and suggested a complex mechanism in which single, double and triple acetylation sites elicited different effects.

In contrast with this, several reports relate that elimination of AR acetylation sites is followed by a dramatic loss of activity. Cell-based transactivation experiments using mutants defective at only one or two acetylation sites, namely the AxKK and KxAA forms, showed that in DU145, HEK and COS cells, the androgen-dependent AR activity was much reduced when using MMTV-Luc, PSA-Luc or ARE-Luc as reporter vectors [95, 129]. Conversely, the QxKK and TxKK mutants which mimic acetylation exhibit stronger activity on the PSA-Luc and MMTV-Luc promoters [97]. In line with this, the HAT Tip60, which acetylates the AR, stimulates its activity, whereas HDAC1 has opposite effects [96].

On the other hand, other groups found that deletion of a region comprising the KLKK motif was still compatible with strong hormone-stimulated AR activity. The AR-Δ629-633 mutant is conducive to higher stimulation of the $(ARE)_2$-TATA-Luc reporter in COS and HeLa cells, in comparison to wt AR [65]. When testing a probasin promoter-based reporter plasmid, similar effects are elicited by this deletion mutant and by the wt AR [99]. Deletion of the larger 629-636 or 628-648 regions also leads to increased AR transactivation potential, as seen in presence of different minimal androgen-responsive promoters in HeLa cells [101]. Finally, removal of the complete hinge region (amino acids 628-669) results in an AR form that is three times more active on different androgen-controlled promoters, when tested in COS and HeLa cells [100].

Up to now, there is no explanation for the discrepancy between these reports. It is unlikely that the variations are due to cell-specific factors, since the different groups worked in part with the same COS cell line. A possible explanation is that despite the fact that the same promoters were used, different subregions were present in the reporter vectors. This is however only a supposition as the exact regions used are not mentioned. Hence, the presence or absence of important control motifs (for instance in enhancer regions) may lead to a

differential effect of AR acetylation. Another unknown factor is the length of the polyglutamine stretch present in the human AR N-terminal region. A polymorphism for this region has been described and it is known that the activity of the AR is inversely related to the length of this stretch. Elimination of the AR acetylation sites may have more or less dramatic effects on the AR, depending on the length of its polyglutamine tract. Taking into consideration the results of this work which showed that the same AR AxAA mutant behaved differently on two reporter constructs, the promoter-dependent explanation may be more likely.

4.2.3 Molecular basis of the promoter-selective effects of AR acetylation site mutations

Additional analyses were carried out to try to understand the basis of the strong discriminatory effects seen for the AR triple mutant on the Pem and MMTV promoters. These differences were not due to changes in expression levels as shown by western blot after cotransfecting different ratios of AR wt and AxAA mutant. To test the hypothesis that the mutant could have an impaired ligand recognition, AR activity was measured after stimulating cells with varying hormone concentrations. The AR AxAA form remained inactive on the Pem promoter even at the highest hormone concentration used. Interestingly, when testing the MMTV promoter, AR AxAA showed an enhanced activity as seen before but a reduced sensitivity to very low hormone levels. This fact, together with the observation that the triple mutant had a lower basal activity in the absence of hormone, is in line with the studies which relate the important role of acetylation in AR activation in the presence of suboptimal hormone levels [98].

The acetylation motif is included in the bipartite nuclear localisation signal of the AR and inactivating mutations in this region have been shown to reduce transport to the nucleus [130, 131]. The subcellular localisation of the AR triple mutant was assessed in this work by fluorescent microscopy of living cells expressing the AR mutant fused to GFP and by western blot after fractionation of cells expressing the mutated AR. An amount of AR mutant compatible with transcriptional activation was found in the nucleus, even though a considerable fraction was retained in the cytoplasm where it formed clusters similar to those described in previous reports.

The next step was to determine whether the lack of AR activity on the Pem promoter was due to impaired DNA binding. This was not the case as an equivalent binding of AR wt and AxAA mutant to the Pem promoter could be demonstrated using plasmid immunoprecipitation assays.

Promoter-specific effects have been reported for AR mutants defective in their N/C interaction [132, 133]. Mutation of the FxxLF motif which is essential for this interaction has no effect on the activation of the MMTV and Slp promoters but only permits partial response of the PSA and probasin promoters. The deletion of this motif does not influence the transcriptional activation via selective AREs, i.e. those that are only stimulated by the AR, but reduces that mediated by promiscuous response elements, which are stimulated by the AR, GR, PR and MR. It was therefore pertinent to compare mutants deficient in N/C interaction and acetylation mutants. Little difference was observed in presence of the MMTV promoter, as expected. In contrast, the N/C interaction mutant was fully active on the Pem promoter. This extends the above-mentioned findings that selective AREs, as found in the Pem promoter, are not dependent on N/C interaction for their stimulation by the AR. In sharp contrast, the acetylation-deficient AR did not activate the Pem promoter. This strongly suggests that preventing the N/C communication does not hinder acetylation at the KxKK motif. A link between N/C interaction and enzymes involved in AR acetylation has however previously been reported. Opposite roles of CBP and sirtuin 1 in increasing and decreasing N/C interaction respectively, have been documented [134]. However, even though these enzymes directly modify the acetylation status of the AR, it is not clear whether this was the immediate cause for the changes in the N/C interaction.

The molecular basis for the differential response of the Pem promoter to the acetylation-defective AR was further analysed. The androgen response of the Pem promoter is mainly mediated by two potent and selective DNA response elements named ARE-1 and ARE-2 [135]. Surprisingly, when analysing these elements separately, both were activated by the AR mutant. The activity displayed by the reporter containing ARE-1 elements in the presence of the AxAA mutant was however half of that seen with the wt AR, whereas the response of the ARE-2 reporter was the same in the presence of the wt or mutant forms of the AR. These results show that two related DNA elements exhibit different responses to the AR acetylation-defective mutant and suggest that the ARE-1 element but not the ARE-2 element is involved in the non-responsiveness of the Pem promoter to the AR AxAA form.

In summary, the loss of activity of the AR acetylation-defective mutant on the Pem promoter was not due to changes in protein levels, in ligand recognition, in nuclear translocation, in promoter binding or to defective N/C interaction. An attractive possibility is the altered recognition by cofactors. Indeed, several cofactors that bind to the AR hinge region, such as Ubc9, silencing mediator for retinoic and thyroid hormone receptor, small nuclear ring finger protein, activating signal cointegrator-1, filamin A, AR corepressor-19, Pod-1 and glycogen synthase kinase-3α, have been described [65, 136-143]. As acetylation of lysines neutralises their positive charge, this might regulate the interaction of the hinge region with a distinct subset of proteins. This is exemplified by the K630Q and K630T mutations which are better recognised by p300 and by the K630R modification which is preferentially bound by the N-CoR complex [129]. Assuming that different androgen responsive promoters recruit, among shared cofactors, also others that are promoter-specific, the loss of AR interaction with a specific coactivator, or an enhanced interaction with a corepressor may lead to the differential effects observed.

A crosstalk between acetylation and other post-translational modifications may also happen. This could occur by competition for the same lysine residue, as has been observed for ERα K266 and K268 which can be either acetylated or sumoylated [144, 145]. Whether this also takes place in the AR KLKK region remains to be determined. Lysine acetylation may also influence other post-translational modifications. This is the case for the AR phosphorylation events that come about in response to activating signaling pathways. Here AR acetylation is essential for activation by the AKT, PKA, and JNK but not by the MAPK pathways [146]. Finally, interplay between acetylation/deacetylation by Tip60/HDAC1 and ubiquitylation by Mdm2 for control of AR stability has been reported [96].

Promoter-selective effects of post-translational modifications have already been found in the case of sumoylation. A differential impact of the sumoylation E2 and E3 enzymes PIASxα and Ubc9 on AR function has been reported [69]. Overexpression of either of them reduces AR activity on minimal promoters containing selective AREs but not on those with promiscuous response elements. Altogether this shows that a subset of androgen target genes, mostly those harboring selective AREs, respond differently to hormone stimulation due to regulatory mechanisms involving the hinge region and N/C interaction. Interestingly, the genes controlled by selective AREs are mainly implicated in reproductive functions, as

evidenced by the generation of a transgenic mouse model expressing a mutated AR that can not activate ARE-dependent genes [147].

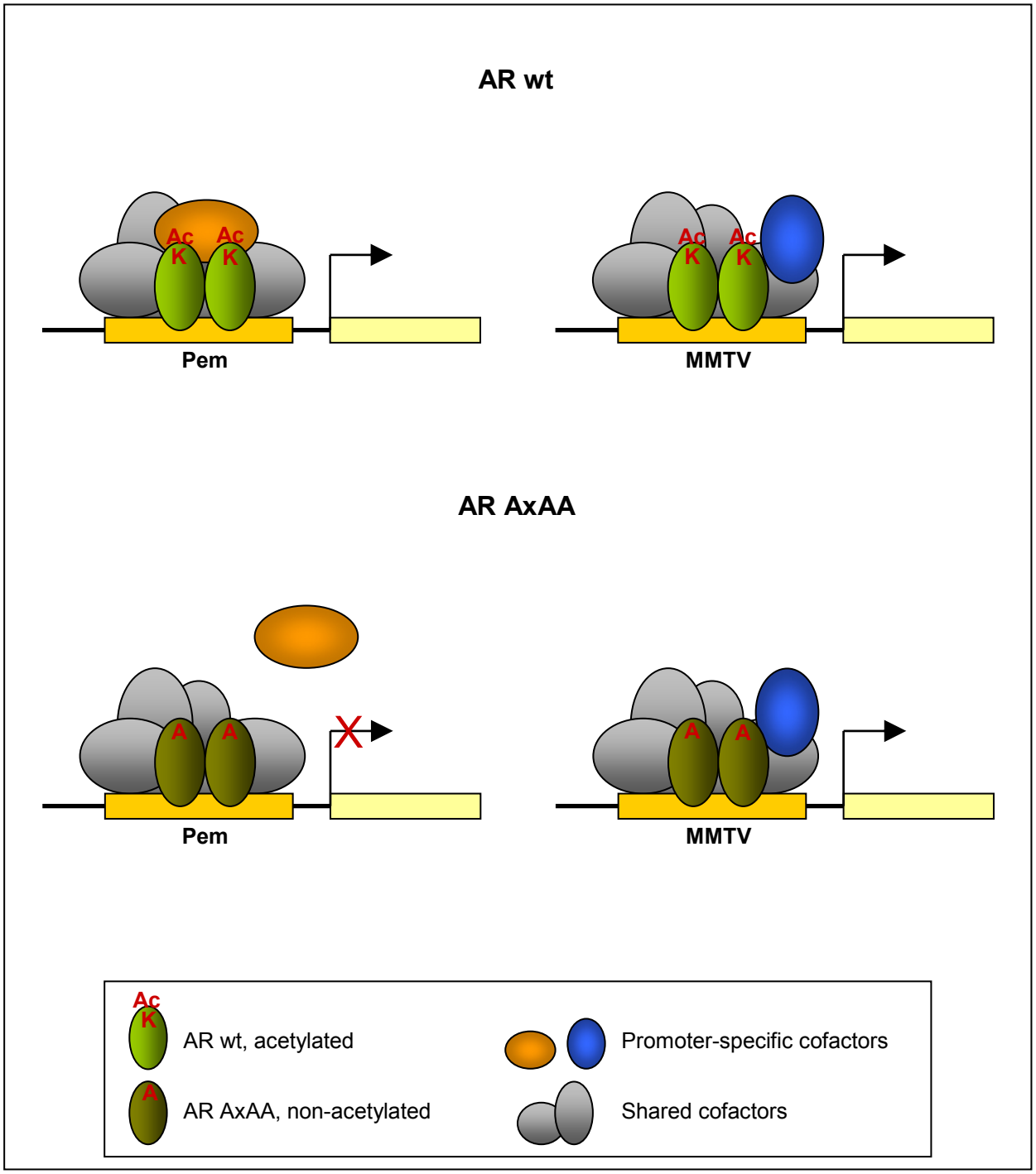

Figure 26

Model for the discriminatory effects of the AR acetylation-defective mutant in the induction of the Pem and the MMTV promoters. The AR wt recruits cofactors shared by both promoters together with promoter-specific cofactors. The existence of a coactivator essential for induction of the Pem promoter and that binds to the AR only in its acetylated form is postulated. Loss of AR acetylation will therefore results in an inactive Pem promoter. On the other hand, this factor in not required for induction of the MMTV promoter whereas other MMTV-specific cofactors are not dependent on AR acetylation. The acetylation-defective AR mutant is therefore able to stimulate the MMTV promoter.

The AR is a clinically validated target for the treatment of non-confined prostate cancer [110]. Multiple mechanisms of resistance to anti-androgen therapy have however been described, many of which allow AR functionality in a very low hormone environment, thus leading to re-expression of a subset of androgen target genes. In this regard, the description in prostate cancer patients of several mutations affecting the hinge region suggests an important role for this domain [148-150]. Concerning the KxKK motif, one mutation which confers growth advantage and resistance against apoptosis has been identified, suggesting that AR acetylation may play an important pathophysiological role [97]. Further studies are now needed to understand how the balance between AR acetylation and deacetylation selectively controls gene transcription and to find out if this post-translational modification plays a role in prostate tumour progression.

5 Summary

The androgen receptor (AR) is essential for proper development and function of the male reproductive system as well as for secondary male traits. It also plays a key role in the origin and growth of prostate cancer. Although the basic mechanisms of AR activation by androgens are known, new aspects such as the regulation by post-translational modifications have recently emerged.

The role of ubiquitylation in androgen signalling was studied by an indirect approach. Ubiquitin-specific protease 10 (USP10) had been identified in a purified, DNA-bound AR complex and its role in modulating AR activity was examined. By overexpressing USP10 it was found to act as a coactivator of the AR, dependently on its enzymatic activity. Confirming these results, USP10 expression knock-down impaired AR function. However, no changes in the ubiquitylation status of the AR could be evidenced, suggesting that the AR was not the direct substrate of USP10. In view of the interconnection between the ubiquitin-proteasome system and transcription, USP10 may act by stabilising AR cofactors or members of the general transcriptional machinery, or by modifying histones. This part of the work supports the concept that the ubiquitin-proteasome system plays an important role in modulating transcriptional events and the results presented here indicate that the deubiquitylation side has a regulatory role as well.

In the second part, the role of AR acetylation was analysed. Mutation of various acetylation sites had remarkably different effects, depending on the tested promoter. The most interesting data were obtained when eliminating all three AR acetylation sites. This led to a total loss of stimulation of the Pem promoter whereas an activity stronger than that of the wild-type form was seen when testing the MMTV promoter. The loss of activity on the Pem promoter was not due to changes in protein level, in ligand recognition, in nuclear translocation, in promoter binding or to defective N/C interaction. A possible explanation is the altered interaction of acetylation-defective AR mutants with promoter-specific cofactors.

In summary, this work emphasises the role of post-translational modifications in modulating AR function. More experiments will be required to better delineate how these modifications fine-tune androgen action in vivo since they are expected to have promoter-selective effects and also to be dependent on the cellular context.

5.1 Zusammenfassung

Der Androgenrezeptor ist essentiell für die richtige Entwicklung und Funktion des männlichen Fortpflanzungssystems sowie für die sekundären männlichen Merkmale. Er spielt auch eine entscheidende Rolle in der Entstehung und dem Wachstum von Prostatakrebs. Obwohl die grundlegenden Mechanismen der Aktivierung des AR durch Androgene bekannt sind, sind kürzlich neue Aspekte, wie die Regulation durch posttranslationale Modifikationen herausgefunden worden.

Die Rolle der Ubiquitylierung in dem Androgensignalweg wurde durch einen indirekten Ansatz untersucht. Die Ubiquitin spezifische Protease 10 (USP10) war in einem AR-DNA-Komplex identifiziert worden. Deswegen wurde die mögliche Rolle von USP10 in der Regulation der AR-Aktivität geprüft. USP10 wirkte als Coaktivator des AR und diese Wirkung war abhängig von der enzymatischen Aktivität des USP10. Darüber hinaus beeinträchtigte das knock-down von USP10 die Funktion des AR. Allerdings konnte keine Änderungen in der Ubiquitylierung des AR gezeigt werden und das deutet an, dass der AR nicht das Substrat von USP10 ist. Ausgehend von der engen Verbindung zwischen dem Ubiquitin-Proteasom-System und der Transkription könnte USP10 durch die Stabilisierung von Cofaktoren des AR oder von Elementen des Transkriptionsapparates oder durch die Modifizierung der Histone wirken. Dieser Teil der Arbeit unterstützt die Idee, dass das Ubiquitin-Proteasom-System eine wichtige Rolle in der Modulation der Transkription spielt und die Ergebnisse zeigen, dass die Deubiquitylierung auch an der Regulation beteiligt ist.

Im zweiten Teil dieser Arbeit wurde die Bedeutung der Acetylierung des AR untersucht. Die Mutagenese verschiedener Acetylierungsstellen verursachte unterschiedliche Effekte, abhängig vom verwendeten Promotor. Die interessantesten Ergebnisse wurden gefunden als alle drei Acetylierungsstellen mutiert waren. Diese AR-Mutante zeigte einen vollständigen Verlust der Aktivierung des Pem Promotors während ihre Aktivität auf dem MMTV Promotor stärker war als die des wild-type AR. Der Verlust der AR-Aktivität auf dem Pem Promotor wurde weder bei Unterschieden in der Proteinmenge, in der Ligandbindung, in der Kernlokalisierung oder in der Promotorbindung noch bei gestörter N/C Interaktion verursacht. Eine mögliche Erklärung ist eine fehlende Interaktion der Mutante mit promotorspezifischen Cofaktoren.

Diese Arbeit betont die Rolle von posttranslationalen Modifikationen in der Regulation der Funktion des AR. Es wird mehr Arbeit nötig sein, um die Feinabstimmung des

Androgensignalwegs durch diese Modifikationen besser beschreiben zu können, da zu erwarten ist, dass diese promotor- und zellkontextabhängige Effekte zeigen.

6 References

1. Alberts, B., D. Bray, and J. Lewis, *Molecular Biology of the Cell*. 4th ed. 2002: Taylor & Francis.

2. Kishimoto, M., R. Fujiki, S. Takezawa, Y. Sasaki, T. Nakamura, K. Yamaoka, H. Kitagawa, and S. Kato, *Nuclear receptor mediated gene regulation through chromatin remodeling and histone modifications.* Endocr J, 2006. 53(2): p. 157-72.

3. Bain, D.L., A.F. Heneghan, K.D. Connaghan-Jones, and M.T. Miura, *Nuclear receptor structure: implications for function.* Annu Rev Physiol, 2007. 69: p. 201-20.

4. Bernstein, B.E., A. Meissner, and E.S. Lander, *The mammalian epigenome.* Cell, 2007. 128(4): p. 669-81.

5. Toledo, F. and G.M. Wahl, *Regulating the p53 pathway: in vitro hypotheses, in vivo veritas.* Nat Rev Cancer, 2006. 6(12): p. 909-23.

6. Hershko, A. and A. Ciechanover, *The Ubiquitin System.* Annual Review of Biochemistry, 1998. 67(1): p. 425-479.

7. Pickart, C.M., *Mechanisms Underlying Ubiquitination.* Annual Review of Biochemistry, 2001. 70(1): p. 503-533.

8. Baumeister, W., J. Walz, F. Zuhl, and E. Seemuller, *The Proteasome: Paradigm of a Self-Compartmentalizing Protease.* Cell, 1998. 92(3): p. 367-380.

9. Pickart, C.M. and D. Fushman, *Polyubiquitin chains: polymeric protein signals.* Current Opinion in Chemical Biology, 2004. 8(6): p. 610-616.

10. Sigismund, S.S.P., and P P Di Fiore, *Signaling through monoubiquitination.* Curr Top Microbiol Immunol, 2004. 286: p. 149–185.

11. Kirkpatrick, D.S., N.A. Hathaway, J. Hanna, S. Elsasser, J. Rush, D. Finley, R.W. King, and S.P. Gygi, *Quantitative analysis of in vitro ubiquitinated cyclin B1 reveals complex chain topology.* Nat Cell Biol, 2006. 8(7): p. 700-710.

12. Guterman, A. and M.H. Glickman, *Deubiquitinating Enzymes are IN(Trinsic to Proteasome Function).* Current Protein and Peptide Science, 2004. 5: p. 201-210.

13. Soboleva, T.A. and R.T. Baker, *Deubiquitinating Enzymes: Their Functions and Substrate Specificity.* Current Protein and Peptide Science, 2004. 5: p. 191-200.

14. Nijman, S.M.B., M.P.A. Luna-Vargas, A. Velds, T.R. Brummelkamp, A.M.G. Dirac, T.K. Sixma, and R. Bernards, *A Genomic and Functional Inventory of Deubiquitinating Enzymes.* Cell, 2005. 123(5): p. 773-786.

15. Kerscher, O., R. Felberbaum, and M. Hochstrasser, *Modification of Proteins by Ubiquitin and Ubiquitin-Like Proteins.* Annual Review of Cell and Developmental Biology, 2006. 22(1): p. 159-180.

16. Muratani, M. and W.P. Tansey, *How the ubiquitin-proteasome system controls transcription.* Nat Rev Mol Cell Biol, 2003. 4(3): p. 192-201.

17. Maxwell, P.H., M.S. Wiesener, G.-W. Chang, S.C. Clifford, E.C. Vaux, M.E. Cockman, C.C. Wykoff, C.W. Pugh, E.R. Maher, and P.J. Ratcliffe, *The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis.* Nature, 1999. 399(6733): p. 271-275.

18. Kodadek, T., D. Sikder, and K. Nalley, *Keeping Transcriptional Activators under Control.* Cell, 2006. 127(2): p. 261-264.

19. Nakajima, A., S. Maruyama, M. Bohgaki, N. Miyajima, T. Tsukiyama, N. Sakuragi, and S. Hatakeyama, *Ligand-dependent transcription of estrogen receptor [alpha] is mediated by the ubiquitin ligase EFP.* Biochemical and Biophysical Research Communications, 2007. 357(1): p. 245-251.

20. Sun, Z.-W. and C.D. Allis, *Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast.* Nature, 2002. 418(6893): p. 104-108.

21. Pham, A.-D. and F. Sauer, *Ubiquitin-Activating/Conjugating Activity of TAFII250, a Mediator of Activation of Gene Expression in Drosophila.* Science, 2000. 289(5488): p. 2357-2360.

22. Glozak, M.A., N. Sengupta, X. Zhang, and E. Seto, *Acetylation and deacetylation of non-histone proteins.* Gene, 2005. 363: p. 15-23.

23. Yuan, Z.-l., Y.-j. Guan, D. Chatterjee, and Y.E. Chin, *Stat3 Dimerization Regulated by Reversible Acetylation of a Single Lysine Residue.* Science, 2005. 307(5707): p. 269-273.

24. Kim, M.-Y., E.-J. Ann, J.-Y. Kim, J.-S. Mo, J.-H. Park, S.-Y. Kim, M.-S. Seo, and H.-S. Park, *Tip60 Histone Acetyltransferase Acts as a Negative Regulator of Notch1 Signaling by Means of Acetylation.* Mol. Cell. Biol., 2007. 27(18): p. 6506-6519.

25. Caron C., B.C., Khochbin S.,, *Regulatory cross-talk between lysine acetylation and ubiquitination: role in the control of protein stability.* BioEssays, 2005. 27(4): p. 408-415.

26. Jeong, J.-W., M.-K. Bae, M.-Y. Ahn, S.-H. Kim, T.-K. Sohn, M.-H. Bae, M.-A. Yoo, E.J. Song, K.-J. Lee, and K.-W. Kim, *Regulation and Destabilization of HIF-1[alpha] by ARD1-Mediated Acetylation.* Cell, 2002. 111(5): p. 709-720.

27. Evans, R.M., *The steroid and thyroid hormone receptor superfamily.* Science, 1988. 240(4854): p. 889-95.

28. Beato, M., *Gene regulation by steroid hormones.* Cell, 1989. 56(3): p. 335-44.

29. Takayama, K., K. Kaneshiro, S. Tsutsumi, K. Horie-Inoue, K. Ikeda, T. Urano, N. Ijichi, Y. Ouchi, K. Shirahige, H. Aburatani, and S. Inoue, *Identification of novel androgen response genes in prostate cancer cells by coupling chromatin immunoprecipitation and genomic microarray analysis.* Oncogene, 2007. 26(30): p. 4453-4463.

30. Wang, Q., W. Li, X.S. Liu, J.S. Carroll, O.A. Janne, E.K. Keeton, A.M. Chinnaiyan, K.J. Pienta, and M. Brown, *A Hierarchical Network of Transcription Factors Governs Androgen Receptor-Dependent Prostate Cancer Growth.* Molecular Cell, 2007. 27(3): p. 380-392.

31. Lee, D.K. and C. Chang, *Endocrine mechanisms of disease: Expression and degradation of androgen receptor: mechanism and clinical implication.* J Clin Endocrinol Metab, 2003. 88(9): p. 4043-54.

32. Dehm, S.M. and D.J. Tindall, *Androgen Receptor Structural and Functional Elements: Role and Regulation in Prostate Cancer.* Mol Endocrinol, 2007: p. me.2007-0223.

33. Perissi, V. and M.G. Rosenfeld, *Controlling nuclear receptors: the circular logic of cofactor cycles.* Nat Rev Mol Cell Biol, 2005. 6(7): p. 542-54.

34. Heinlein, C.A. and C. Chang, *Androgen receptor (AR) coregulators: an overview.* Endocr Rev, 2002. 23(2): p. 175-200.

35. Xu, J. and Q. Li, *Review of the in Vivo Functions of the p160 Steroid Receptor Coactivator Family.* Mol Endocrinol, 2003. 17(9): p. 1681-1692.

36. Yoon, H.C., DW; Huang, ZQ; Li, J; Fondell, JD; Qin, J; Wong, J *Purification and functional characterization of the human N-CoR complex: the roles of HDAC3, TBL1 and TBLR1.* Embo J. , 2003. 22: p. 1336–46.

37. Lubahn, D.B., D.R. Joseph, P.M. Sullivan, H.F. Willard, F.S. French, and E.M. Wilson, *Cloning of human androgen receptor complementary DNA and localization to the X chromosome.* Science, 1988. 240(4850): p. 327-30.

38. Trapman, J., 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, *Cloning, structure and expression of a cDNA encoding the human androgen receptor.* Biochem Biophys Res Commun, 1988. 153(1): p. 241-8.

39. Edwards, S.M., M.D. Badzioch, R. Minter, R. Hamoudi, N. Collins, A. Ardern-Jones, A. Dowe, S. Osborne, J. Kelly, R. Shearer, D.F. Easton, G.F. Saunders, D.P. Dearnaley, and R.A. Eeles, *Androgen receptor polymorphisms: association with prostate cancer risk, relapse and overall survival.* Int J Cancer, 1999. 84(5): p. 458-65.

40. Wilson, C.M. and M.J. McPhaul, *A and B forms of the androgen receptor are present in human genital skin fibroblasts.* Proc Natl Acad Sci U S A, 1994. 91(4): p. 1234-8.

41. Gregory, C.W., B. He, and E.M. Wilson, *The putative androgen receptor-A form results from in vitro proteolysis.* J Mol Endocrinol, 2001. 27(3): p. 309-319.

42. Ahrens-Fath, I., O. Politz, C. Geserick, and B. Haendler, *Androgen receptor function is modulated by the tissue-specific AR45 variant.* FEBS Journal, 2005. 272(1): p. 74-84.

43. Shaffer, P.L., A. Jivan, D.E. Dollins, F. Claessens, and D.T. Gewirth, *Structural basis of androgen receptor binding to selective androgen response elements.* Proceedings of the National Academy of Sciences, 2004. 101(14): p. 4758-4763.

44. Matias, P.M., 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, *Structural Evidence for Ligand Specificity in the Binding Domain of the Human Androgen Receptor. Implications for pathogenic gene mutations.* J. Biol. Chem., 2000. 275(34): p. 26164-26171.

45. Sack, J.S., 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, *Crystallographic structures of the ligand-binding domains of the androgen receptor and its T877A mutant complexed with the natural agonist dihydrotestosterone.* PNAS, 2001. 98(9): p. 4904-4909.

46. Bohl, C.E., W. Gao, D.D. Miller, C.E. Bell, and J.T. Dalton, *Structural basis for antagonism and resistance of bicalutamide in prostate cancer.* PNAS, 2005. 102(17): p. 6201-6206.

47. Bohl, C.E., Z. Wu, D.D. Miller, C.E. Bell, and J.T. Dalton, *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., 2007. 282(18): p. 13648-13655.

48. He, B., R.T. Gampe, A.J. Kole, A.T. Hnat, T.B. Stanley, G. An, E.L. Stewart, R.I. Kalman, J.T. Minges, and E.M. Wilson, *Structural Basis for Androgen Receptor Interdomain and Coactivator Interactions Suggests a Transition in Nuclear Receptor Activation Function Dominance.* Molecular Cell, 2004. 16(3): p. 425-438.

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

50. He, B., J.A. Kemppainen, J.J. Voegel, H. Gronemeyer, and E.M. Wilson, *Activation Function 2 in the Human Androgen Receptor Ligand Binding Domain Mediates Interdomain Communication with the NH2-terminal Domain.* J. Biol. Chem., 1999. 274(52): p. 37219-37225.

51. He, B., J.A. Kemppainen, and E.M. Wilson, *FXXLF and WXXLF Sequences Mediate the NH2-terminal Interaction with the Ligand Binding Domain of the Androgen Receptor.* J. Biol. Chem., 2000. 275(30): p. 22986-22994.

52. Li, J., J. Fu, C. Toumazou, H.-G. Yoon, and J. Wong, *A Role of the Amino-terminal (N) and Carboxyl-terminal (C) Interaction in Binding of Androgen Receptor to Chromatin.* Mol Endocrinol, 2005: p. me.2005-0298.

53. Jenster G, v.d.K.J., Trapman J, Brinkmann AO, *Functional domains of the human androgen receptor.* J Steroid Biochem Mol Biol, 1992(41): p. 671-5.

54. Sheflin, L., B. Keegan, W. Zhang, and S.W. Spaulding, *Inhibiting proteasomes in human HepG2 and LNCaP cells increases endogenous androgen receptor levels.* Biochem Biophys Res Commun, 2000. 276(1): p. 144-50.

55. Rechsteiner, M. and S.W. Rogers, *PEST sequences and regulation by proteolysis.* Trends Biochem Sci, 1996. 21(7): p. 267-71.

56. Geserick, C., H.-A. Meyer, and B. Haendler, *The role of DNA response elements as allosteric modulators of steroid receptor function.* Molecular and Cellular Endocrinology, 2005. 236(1-2): p. 1-7.

57. Schoenmakers, E., P. Alen, G. Verrijdt, B. Peeters, G. Verhoeven, W. Rombauts, and F. Claessens, *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., 1999. 341(3): p. 515-521.

58. Gast, A., F. Neuschmid-Kaspar, H. Klocker, and A.C.B. Cato, *A single amino acid exchange abolishes dimerization of the androgen receptor and causes Reifenstein syndrome.* Molecular and Cellular Endocrinology, 1995. 111(1): p. 93-98.

59. Reid, J., S.M. Kelly, K. Watt, N.C. Price, and I.J. McEwan, *Conformational Analysis of the Androgen Receptor Amino-terminal Domain Involved in Transactivation. Influence of structure-stabilizing solutes and protein-protein interactions.* J. Biol. Chem., 2002. 277(22): p. 20079-20086.

60. Lavery, D.N. and I.J. McEwan, *The human androgen receptor AF1 transactivation domain: interactions with transcription factor IIF and molten-globule-like structural characteristics.* Biochem. Soc. Trans., 2006. 34(Pt 6): p. 1054-1057.

61. Brodie, J. and I.J. McEwan, *Intra-domain communication between the N-terminal and DNA-binding domains of the androgen receptor: modulation of androgen response element DNA binding.* J Mol Endocrinol, 2005. 34(3): p. 603-615.

62. Gioeli, D., 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, *Androgen Receptor Phosphorylation. Regulation and Identification of the Phosphorylation Sites.* J. Biol. Chem., 2002. 277(32): p. 29304-29314.

63. Wong, H.Y., J.A. Burghoorn, M. Van Leeuwen, P.E. De Ruiter, E. Schippers, L.J. Blok, K.W. Li, H.L. Dekker, L. De Jong, J. Trapman, J.A. Grootegoed, and A.O. Brinkmann, *Phosphorylation of androgen receptor isoforms.* Biochem J, 2004. 383(Pt 2): p. 267-76.

64. Poukka, H., U. Karvonen, O.A. Janne, and J.J. Palvimo, *Covalent modification of the androgen receptor by small ubiquitin-like modifier 1 (SUMO-1).* PNAS, 2000. 97(26): p. 14145-14150.

65. Poukka, H., P. Aarnisalo, U. Karvonen, J.J. Palvimo, and O.A. Janne, *Ubc9 Interacts with the Androgen Receptor and Activates Receptor-dependent Transcription.* J. Biol. Chem., 1999. 274(27): p. 19441-19446.

66. Kotaja, N., U. Karvonen, O.A. Janne, and J.J. Palvimo, *PIAS Proteins Modulate Transcription Factors by Functioning as SUMO-1 Ligases.* Mol. Cell. Biol., 2002. 22(14): p. 5222-5234.

67. Nishida, T. and H. Yasuda, *PIAS1 and PIASxalpha Function as SUMO-E3 Ligases toward Androgen Receptor and Repress Androgen Receptor-dependent Transcription.* J. Biol. Chem., 2002. 277(44): p. 41311-41317.

68. Sharma, M., X. Li, Y. Wang, M. Zarnegar, C.Y. Huang, J.J. Palvimo, B. Lim, and Z. Sun, *hZimp10 is an androgen receptor co-activator and forms a complex with SUMO-1 at replication foci.* Embo J, 2003. 22(22): p. 6101-14.

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

70. Callewaert, L., G. Verrijdt, A. Haelens, and F. Claessens, *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, 2004. 18(6): p. 1438-1449.

71. Nawaz, Z., D.M. Lonard, A.P. Dennis, C.L. Smith, and B.W. O'Malley, *Proteasome-dependent degradation of the human estrogen receptor.* Proceedings of the National Academy of Sciences, 1999. 96(5): p. 1858-1862.

72. Lange, C.A., T. Shen, and K.B. Horwitz, *Phosphorylation of human progesterone receptors at serine-294 by mitogen-activated protein kinase signals their degradation by the 26S proteasome.* PNAS, 2000. 97(3): p. 1032-1037.

73. Wallace, A.D. and J.A. Cidlowski, *Proteasome-mediated Glucocorticoid Receptor Degradation Restricts Transcriptional Signaling by Glucocorticoids.* J. Biol. Chem., 2001. 276(46): p. 42714-42721.

74. Deroo, B.J., C. Rentsch, S. Sampath, J. Young, D.B. DeFranco, and T.K. Archer, *Proteasomal inhibition enhances glucocorticoid receptor transactivation and alters its subnuclear trafficking.* Mol Cell Biol, 2002. 22(12): p. 4113-23.

75. Gaughan, L., I.R. Logan, D.E. Neal, and C.N. Robson, *Regulation of androgen receptor and histone deacetylase 1 by Mdm2-mediated ubiquitylation.* Nucl. Acids Res., 2005. 33(1): p. 13-26.

76. Jaworski, T., *Degradation and beyond: control of androgen receptor activity by the proteasome system.* Cell Mol Biol Lett, 2006. 11(1): p. 109-31.

77. Tyagi, R.K., Y. Lavrovsky, S.C. Ahn, C.S. Song, B. Chatterjee, and A.K. Roy, *Dynamics of Intracellular Movement and Nucleocytoplasmic Recycling of the Ligand-Activated Androgen Receptor in Living Cells.* Mol Endocrinol, 2000. 14(8): p. 1162-1174.

78. Lin, H.K., L. Wang, Y.C. Hu, S. Altuwaijri, and C. Chang, *Phosphorylation-dependent ubiquitylation and degradation of androgen receptor by Akt require Mdm2 E3 ligase.* Embo J, 2002. 21(15): p. 4037-48.

79. Rees, I., S. Lee, H. Kim, and F.T.F. Tsai, *The E3 ubiquitin ligase CHIP binds the androgen receptor in a phosphorylation-dependent manner.* Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics, 2006. 1764(6): p. 1073-1079.

80. Cardozo, C.P., C. Michaud, M.C. Ost, A.E. Fliss, E. Yang, C. Patterson, S.J. Hall, and A.J. Caplan, *C-terminal Hsp-interacting protein slows androgen receptor synthesis and reduces its rate of degradation.* Arch Biochem Biophys, 2003. 410(1): p. 134-40.

81. Burgdorf, S., P. Leister, and K.H. Scheidtmann, *TSG101 Interacts with Apoptosis-antagonizing Transcription Factor and Enhances Androgen Receptor-mediated Transcription by Promoting Its Monoubiquitination.* J. Biol. Chem., 2004. 279(17): p. 17524-17534.

82. Dennis, A.P. and B.W. O'Malley, *Rush hour at the promoter: How the ubiquitin-proteasome pathway polices the traffic flow of nuclear receptor-dependent transcription.* The Journal of Steroid Biochemistry and Molecular Biology, 2005. 93(2-5): p. 139-151.

83. Grossman, S.R., M.E. Deato, C. Brignone, H.M. Chan, A.L. Kung, H. Tagami, Y. Nakatani, and D.M. Livingston, *Polyubiquitination of p53 by a ubiquitin ligase activity of p300.* Science, 2003. 300(5617): p. 342-4.

84. Yan, F., X. Gao, D.M. Lonard, and Z. Nawaz, *Specific ubiquitin-conjugating enzymes promote degradation of specific nuclear receptor coactivators.* Mol Endocrinol, 2003. 17(7): p. 1315-31.

85. Mitsui, A. and P.A. Sharp, *Ubiquitination of RNA polymerase II large subunit signaled by phosphorylation of carboxyl-terminal domain.* Proc Natl Acad Sci U S A, 1999. 96(11): p. 6054-9.

86. Perletti, L., E. Kopf, L. Carre, and I. Davidson, *Coordinate regulation of RARgamma2, TBP, and TAFII135 by targeted proteolysis during retinoic acid-induced differentiation of F9 embryonal carcinoma cells.* BMC Mol Biol, 2001. 2: p. 4.

87. Makino, Y., T. Yoshida, S. Yogosawa, K. Tanaka, M. Muramatsu, and T.A. Tamura, *Multiple mammalian proteasomal ATPases, but not proteasome itself, are associated with TATA-binding protein and a novel transcriptional activator, TIP120.* Genes Cells, 1999. 4(9): p. 529-39.

88. Ferdous, A., F. Gonzalez, L. Sun, T. Kodadek, and S.A. Johnston, *The 19S regulatory particle of the proteasome is required for efficient transcription elongation by RNA polymerase II.* Mol Cell, 2001. 7(5): p. 981-91.

89. Gonzalez, F., A. Delahodde, T. Kodadek, and S.A. Johnston, *Recruitment of a 19S proteasome subcomplex to an activated promoter.* Science, 2002. 296(5567): p. 548-50.

90. Kang, Z., A. Pirskanen, O.A. Janne, and J.J. Palvimo, *Involvement of Proteasome in the Dynamic Assembly of the Androgen Receptor Transcription Complex.* J. Biol. Chem., 2002. 277(50): p. 48366-48371.

91. Lin, H.K., S. Altuwaijri, W.J. Lin, P.Y. Kan, L.L. Collins, and C. Chang, *Proteasome activity is required for androgen receptor transcriptional activity via regulation of androgen receptor nuclear translocation and interaction with coregulators in prostate cancer cells.* J Biol Chem, 2002. 277(39): p. 36570-6.

92. Ikezoe, T., Y. Yang, T. Saito, H.P. Koeffler, and H. Taguchi, *Proteasome inhibitor PS-341 down-regulates prostate-specific antigen (PSA) and induces growth arrest and apoptosis of androgen-dependent human prostate cancer LNCaP cells.* Cancer Science, 2004. 95(3): p. 271-275.

93. Wang, Q., J.S. Carroll, and M. Brown, *Spatial and Temporal Recruitment of Androgen Receptor and Its Coactivators Involves Chromosomal Looping and Polymerase Tracking.* Molecular Cell, 2005. 19(5): p. 631-642.

94. Kouzarides, T., *Acetylation: a regulatory modification to rival phosphorylation?* Embo J, 2000. 19(6): p. 1176-9.

95. Fu, M., C. Wang, A.T. Reutens, J. Wang, R.H. Angeletti, L. Siconolfi-Baez, V. Ogryzko, M.-L. Avantaggiati, and R.G. Pestell, *p300 and p300/cAMP-response Element-binding Protein-associated Factor Acetylate the Androgen Receptor at Sites Governing Hormone-dependent Transactivation.* J. Biol. Chem., 2000. 275(27): p. 20853-20860.

96. Gaughan, L., I.R. Logan, S. Cook, D.E. Neal, and C.N. Robson, *Tip60 and Histone Deacetylase 1 Regulate Androgen Receptor Activity through Changes to the Acetylation Status of the Receptor.* J. Biol. Chem., 2002. 277(29): p. 25904-25913.

97. Fu, M., 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, *Acetylation of Androgen Receptor Enhances Coactivator Binding and Promotes Prostate Cancer Cell Growth.* Mol. Cell. Biol., 2003. 23(23): p. 8563-8575.

98. Gong, J., J. Zhu, O.B. Goodman, Jr., R.G. Pestell, P.N. Schlegel, D.M. Nanus, and R. Shen, *Activation of p300 histone acetyltransferase activity and acetylation of the androgen receptor by bombesin in prostate cancer cells.* Oncogene, 2006. 25(14): p. 2011-21.

99. Karvonen, U., O.A. Janne, and J.J. Palvimo, *Androgen receptor regulates nuclear trafficking and nuclear domain residency of corepressor HDAC7 in a ligand-dependent fashion.* Exp Cell Res, 2006. 312(16): p. 3165-83.

100. Tanner, T., F. Claessens, and A. Haelens, *The hinge region of the androgen receptor plays a role in proteasome-mediated transcriptional activation.* Ann N Y Acad Sci, 2004. 1030: p. 587-92.

101. Haelens, A., T. Tanner, S. Denayer, L. Callewaert, and F. Claessens, *The Hinge Region Regulates DNA Binding, Nuclear Translocation, and Transactivation of the Androgen Receptor.* Cancer Res, 2007. 67(9): p. 4514-4523.

102. Brown, T.R., *Human androgen insensitivity syndrome.* J Androl, 1995. 16(4): p. 299-303.

103. Avila, D.M., S. Zoppi, and M.J. McPhaul, *The androgen receptor (AR) in syndromes of androgen insensitivity and in prostate cancer.* The Journal of Steroid Biochemistry and Molecular Biology, 2001. 76(1-5): p. 135-142.

104. Merry, D.E., *Molecular pathogenesis of spinal and bulbar muscular atrophy.* Brain Res Bull, 2001. 56(3-4): p. 203-7.

105. Bailey, C.K., I.F. Andriola, H.H. Kampinga, and D.E. Merry, *Molecular chaperones enhance the degradation of expanded polyglutamine repeat androgen receptor in a cellular model of spinal and bulbar muscular atrophy.* Hum Mol Genet, 2002. 11(5): p. 515-23.

106. Delongchamps, N.S., A; Haas, GP, *The role of prevalence in the diagnosis of prostate cancer.* Cancer Control, 2006.

107. Tammela, T., *Endocrine treatment of prostate cancer.* The Journal of Steroid Biochemistry and Molecular Biology, 2004. 92(4): p. 287-295.

108. Cheng, H., R. Snoek, F. Ghaidi, M.E. Cox, and P.S. Rennie, *Short Hairpin RNA Knockdown of the Androgen Receptor Attenuates Ligand-Independent Activation and Delays Tumor Progression.* Cancer Res, 2006. 66(21): p. 10613-10620.

109. Compagno, D., C. Merle, A. Morin, C. Gilbert, J.R. Mathieu, A. Bozec, C. Mauduit, M. Benahmed, and F. Cabon, *SIRNA-Directed In Vivo Silencing of Androgen Receptor Inhibits the Growth of Castration-Resistant Prostate Carcinomas.* PLoS ONE, 2007. 2(10): p. e1006.

110. Taplin, M.E., *Drug insight: role of the androgen receptor in the development and progression of prostate cancer.* Nat Clin Pract Oncol, 2007. 4(4): p. 236-44.

111. Faus, H., H.A. Meyer, M. Huber, I. Bahr, and B. Haendler, *The ubiquitin-specific protease USP10 modulates androgen receptor function.* Mol Cell Endocrinol, 2005. 245(1-2): p. 138-46.

112. Soncini, C., I. Berdo, and G. Draetta, *Ras-GAP SH3 domain binding protein (G3BP) is a modulator of USP10, a novel human ubiquitin specific protease.* Oncogene, 2001. 20(29): p. 3869-79.

113. Heery, D.M., E. Kalkhoven, S. Hoare, and M.G. Parker, *A signature motif in transcriptional co-activators mediates binding to nuclear receptors.* Nature, 1997. 387(6634): p. 733-6.

114. Irvine, K., R. Stirling, D. Hume, and D. Kennedy, *Rasputin, more promiscuous than ever: a review of G3BP.* Int J Dev Biol, 2004. 48(10): p. 1065-77.

115. Baker, R.T., J.W. Tobias, and A. Varshavsky, *Ubiquitin-specific proteases of Saccharomyces cerevisiae. Cloning of UBP2 and UBP3, and functional analysis of the UBP gene family.* J Biol Chem, 1992. 267(32): p. 23364-75.

116. Baxter, B.K. and E.A. Craig, *Isolation of UBP3, encoding a de-ubiquitinating enzyme, as a multicopy suppressor of a heat-shock mutant strain of S. cerevisiae.* Curr Genet, 1998. 33(6): p. 412-9.

117. Saleh, A., M. Collart, J.A. Martens, J. Genereaux, S. Allard, J. Cote, and C.J. Brandl, *TOM1p, a yeast hect-domain protein which mediates transcriptional regulation through the ADA/SAGA coactivator complexes.* J Mol Biol, 1998. 282(5): p. 933-46.

118. Auty, R., H. Steen, L.C. Myers, J. Persinger, B. Bartholomew, S.P. Gygi, and S. Buratowski, *Purification of active TFIID from Saccharomyces cerevisiae. Extensive promoter contacts and co-activator function.* J Biol Chem, 2004. 279(48): p. 49973-81.

119. He, B., S. Bai, A.T. Hnat, R.I. Kalman, J.T. Minges, C. Patterson, and E.M. Wilson, *An Androgen Receptor NH2-terminal Conserved Motif Interacts with the COOH Terminus of the Hsp70-interacting Protein (CHIP).* J. Biol. Chem., 2004. 279(29): p. 30643-30653.

120. Nawaz, Z., D.M. Lonard, C.L. Smith, E. Lev-Lehman, S.Y. Tsai, M.-J. Tsai, and B.W. O'Malley, *The Angelman Syndrome-Associated Protein, E6-AP, Is a Coactivator for the Nuclear Hormone Receptor Superfamily.* Mol. Cell. Biol., 1999. 19(2): p. 1182-1189.

121. Gao, X., S.K. Mohsin, Z. Gatalica, G. Fu, P. Sharma, and Z. Nawaz, *Decreased expression of e6-associated protein in breast and prostate carcinomas.* Endocrinology, 2005. 146(4): p. 1707-12.

122. Verma, S., A. Ismail, X. Gao, G. Fu, X. Li, B.W. O'Malley, and Z. Nawaz, *The Ubiquitin-Conjugating Enzyme UBCH7 Acts as a Coactivator for Steroid Hormone Receptors.* Mol. Cell. Biol., 2004. 24(19): p. 8716-8726.

123. Scacheri, P.C., O. Rozenblatt-Rosen, N.J. Caplen, T.G. Wolfsberg, L. Umayam, J.C. Lee, C.M. Hughes, K.S. Shanmugam, A. Bhattacharjee, M. Meyerson, and F.S. Collins, *Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells.* Proc Natl Acad Sci U S A, 2004. 101(7): p. 1892-7.

124. Mukhopadhyay, D. and H. Riezman, *Proteasome-independent functions of ubiquitin in endocytosis and signaling.* Science, 2007. 315(5809): p. 201-5.

125. Ito, K., S. Adachi, R. Iwakami, H. Yasuda, Y. Muto, N. Seki, and Y. Okano, *N-Terminally extended human ubiquitin-conjugating enzymes (E2s) mediate the ubiquitination of RING-finger proteins, ARA54 and RNF8.* Eur J Biochem, 2001. 268(9): p. 2725-2732.

126. Fang, S., J.P. Jensen, R.L. Ludwig, K.H. Vousden, and A.M. Weissman, *Mdm2 Is a RING Finger-dependent Ubiquitin Protein Ligase for Itself and p53.* J. Biol. Chem., 2000. 275(12): p. 8945-8951.

127. Zhu, P., W. Zhou, J. Wang, J. Puc, K.A. Ohgi, H. Erdjument-Bromage, P. Tempst, C.K. Glass, and M.G. Rosenfeld, *A Histone H2A Deubiquitinase Complex Coordinating Histone Acetylation and H1 Dissociation in Transcriptional Regulation.* Molecular Cell, 2007. 27(4): p. 609-621.

128. Faus, H. and B. Haendler, *Post-translational modifications of steroid receptors.* Biomed Pharmacother, 2006. 60(9): p. 520-8.

129. Fu, M., C. Wang, J. Wang, X. Zhang, T. Sakamaki, Y.G. Yeung, C. Chang, T. Hopp, S.A.W. Fuqua, E. Jaffray, R.T. Hay, J.J. Palvimo, O.A. Janne, and R.G. Pestell, *Androgen Receptor Acetylation Governs trans Activation and MEKK1-Induced Apoptosis without Affecting In Vitro Sumoylation and trans-Repression Function.* Mol. Cell. Biol., 2002. 22(10): p. 3373-3388.

130. Zhou, Z.X., M. Sar, J.A. Simental, M.V. Lane, and E.M. Wilson, *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, 1994. 269(18): p. 13115-23.

131. Thomas, M., N. Dadgar, A. Aphale, J.M. Harrell, R. Kunkel, W.B. Pratt, and A.P. Lieberman, *Androgen Receptor Acetylation Site Mutations Cause Trafficking Defects, Misfolding, and Aggregation Similar to Expanded Glutamine Tracts.* J. Biol. Chem., 2004. 279(9): p. 8389-8395.

132. He, B., L.W. Lee, J.T. Minges, and E.M. Wilson, *Dependence of Selective Gene Activation on the Androgen Receptor NH2- and COOH-terminal Interaction.* J. Biol. Chem., 2002. 277(28): p. 25631-25639.

133. Callewaert, L., G. Verrijdt, V. Christiaens, A. Haelens, and F. Claessens, *Dual function of an amino-terminal amphipatic helix in androgen receptor-mediated transactivation through specific and nonspecific response elements.* J Biol Chem, 2003. 278(10): p. 8212-8.

134. Fu, M., M. Liu, A.A. Sauve, X. Jiao, X. Zhang, X. Wu, M.J. Powell, T. Yang, W. Gu, M.L. Avantaggiati, N. Pattabiraman, T.G. Pestell, F. Wang, A.A. Quong, C. Wang, and R.G. Pestell, *Hormonal Control of Androgen Receptor Function through SIRT1.* Mol. Cell. Biol., 2006. 26(21): p. 8122-8135.

135. Barbulescu, K., C. Geserick, I. Schuttke, W.D. Schleuning, and B. Haendler, *New androgen response elements in the murine pem promoter mediate selective transactivation.* Mol Endocrinol, 2001. 15(10): p. 1803-16.

136. Poukka, H., U. Karvonen, N. Yoshikawa, H. Tanaka, J.J. Palvimo, and O.A. Janne, *The RING finger protein SNURF modulates nuclear trafficking of the androgen receptor.* J Cell Sci, 2000. 113(17): p. 2991-3001.

137. Lee, Y.S., H.J. Kim, H.J. Lee, J.W. Lee, S.Y. Chun, S.K. Ko, and K. Lee, *Activating signal cointegrator 1 is highly expressed in murine testicular Leydig cells and enhances the ligand-dependent transactivation of androgen receptor.* Biol Reprod, 2002. 67(5): p. 1580-7.

138. Liao, G., L.Y. Chen, A. Zhang, A. Godavarthy, F. Xia, J.C. Ghosh, H. Li, and J.D. Chen, *Regulation of androgen receptor activity by the nuclear receptor corepressor SMRT.* J Biol Chem, 2003. 278(7): p. 5052-61.

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

140. Jeong, B.C., C.Y. Hong, S. Chattopadhyay, J.H. Park, E.Y. Gong, H.J. Kim, S.Y. Chun, and K. Lee, *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, 2004. 18(1): p. 13-25.

141. Liao, X., J.B. Thrasher, J. Holzbeierlein, S. Stanley, and B. Li, *Glycogen Synthase Kinase-3{beta} Activity Is Required for Androgen-Stimulated Gene Expression in Prostate Cancer.* Endocrinology, 2004. 145(6): p. 2941-2949.

142. Salas, T.R., J. Kim, F. Vakar-Lopez, A.L. Sabichi, P. Troncoso, G. Jenster, A. Kikuchi, S.-Y. Chen, L. Shemshedini, M. Suraokar, C.J. Logothetis, J. DiGiovanni, S.M. Lippman, and D.G. Menter, *Glycogen Synthase Kinase-3{beta} Is Involved in the Phosphorylation and Suppression of Androgen Receptor Activity.* J. Biol. Chem., 2004. 279(18): p. 19191-19200.

143. Hong, C.Y., E.Y. Gong, K. Kim, J.H. Suh, H.M. Ko, H.J. Lee, H.S. Choi, and K. Lee, *Modulation of the expression and transactivation of androgen receptor by the basic helix-loop-helix transcription factor Pod-1 through recruitment of histone deacetylase 1.* Mol Endocrinol, 2005. 19(9): p. 2245-57.

144. Wang, C., M. Fu, R.H. Angeletti, L. Siconolfi-Baez, A.T. Reutens, C. Albanese, M.P. Lisanti, B.S. Katzenellenbogen, S. Kato, T. Hopp, S.A. Fuqua, G.N. Lopez, P.J. Kushner, and R.G. Pestell, *Direct acetylation of the estrogen receptor alpha hinge region by p300 regulates transactivation and hormone sensitivity.* J Biol Chem, 2001. 276(21): p. 18375-83.

145. Sentis, S., M. Le Romancer, C. Bianchin, M.-C. Rostan, and L. Corbo, *Sumoylation of the Estrogen Receptor {alpha} Hinge Region Regulates Its Transcriptional Activity.* Mol Endocrinol, 2005. 19(11): p. 2671-2684.

146. Fu, M., M. Rao, K. Wu, C. Wang, X. Zhang, M. Hessien, Y.-G. Yeung, D. Gioeli, M.J. Weber, and R.G. Pestell, *The Androgen Receptor Acetylation Site Regulates cAMP and AKT but Not ERK-induced Activity.* J. Biol. Chem., 2004. 279(28): p. 29436-29449.

147. Schauwaers, K., K. De Gendt, P.T. Saunders, N. Atanassova, A. Haelens, L. Callewaert, U. Moehren, J.V. Swinnen, G. Verhoeven, G. Verrijdt, and F. Claessens, *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, 2007. 104(12): p. 4961-6.

148. Buchanan, G., N.M. Greenberg, H.I. Scher, J.M. Harris, V.R. Marshall, and W.D. Tilley, *Collocation of androgen receptor gene mutations in prostate cancer.* Clin Cancer Res, 2001. 7(5): p. 1273-81.

149. Shi, X.-B., A.-H. Ma, L. Xia, H.-J. Kung, and R.W. de Vere White, *Functional Analysis of 44 Mutant Androgen Receptors from Human Prostate Cancer.* Cancer Res, 2002. 62(5): p. 1496-1502.

150. Thompson, J., E.R. Hyytinen, K. Haapala, I. Rantala, H.J. Helin, O.A. Janne, J.J. Palvimo, and P.A. Koivisto, *Androgen receptor mutations in high-grade prostate cancer before hormonal therapy.* Lab Invest, 2003. 83(12): p. 1709-13.

7 Appendix

7.1 Publications

Publications in reviewed journals

H. Faus, B. Haendler. *Androgen receptor acetylation sites differentially regulate gene control*. J Cell Biochem. 2008 May 15;104(2):511-24.

B. Weiss, H. Faus, B. Haendler. *Phylogenetic conservation of the androgen receptor AR45 variant form in placental mammals*. Gene 2007. 399(2):105-11.

H. Faus, B. Haendler. *Post-translational modifications of steroid receptors*. Biomed. & Pharmacother. 2006. 60(9):520-8.

H. Faus, HA. Meyer, M. Huber, I. Bahr, B. Haendler. *The ubiquitin-specific protease USP10 modulates androgen receptor function*. Mol. Cell. Endocrinol. 2005. 245(1-2):138-46.

J. Hahn, HM. Strauss, FT. Landgraf, H. Faus, G. Lochnit, P. Schmieder, J. Hughes. *Probing protein-chromophore interactions in Cph1 phytochrome by mutagenesis*. FEBS J. 2006. 273(7):1415-29.

KB. Sandau, H. Faus, B. Brüne. *Induction of Hypoxia-Inducible-Factor 1 by Nitric Oxide is mediated via the PI 3K Pathway*. Biochem. Biophys. Res. Commun. 2000. 278(1):263-7.

P. Villa, F. Ferrando, J. Serra, H. Faus, Y. Mira, A. Vaya, J. Aznar. *Quantification of D-dimer using a new fully automated assay: its application for the diagnosis of deep vein thrombosis*. Haematologica 2000. 85(5):520-4.

Oral presentations

Post-translational modifications modulate androgen receptor activity – Androgens 2006 Symposium. September 2006. Cambridge, UK.

Post-translational regulation of androgen receptor activity – Molecular Andrology International Workshop. October 2005. Giessen, Germany.

7.2 Curriculum Vitae

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7.3 Ehrenwörtliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig durchgeführt und verfasst habe. Dabei wurden keine anderen, als die angegebenen Quellen und Hilfsmittel verwendet.

Berlin, den 30.10.2007

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Hortensia Faus Giménez