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Sanna Kaikkonen

Regulation of Androgen Receptor Signaling by SUMO Modifications in Prostate Cancer Cells

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UNIVERSITY OF EASTERN FINLAND

SANNA KAIKKONEN

Regulation of Androgen Receptor Signaling by SUMO Modifications in Prostate Cancer Cells

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ABSTRACT

Male hormones, androgens, mediate their effects *via* the androgen receptor (AR) at the level of gene regulation. Not only hormones, but also coregulatory proteins, chromatin structure and post-translational modifications (PTMs) are involved in the control of androgen-responsive genes. The AR is modified by different forms of PTMs, e.g. ubiquitylation and phosphorylation, which affect AR stability and activity of AR target genes. The AR plays several key roles in regulating both the normal growth of prostate and the development of prostate cancer. Since the AR is a pivotal factor in prostate cancer, it represents a drug target of interest. This study aimed at exploring SUMO (small ubiquitin-like modifier) modifications of the AR and their roles as modulators of AR activity in prostate cancer cells. The AR showed SUMO paralog selectivity towards SUMO-2/3, and the SUMO conjugation, SUMOylation, could be enhanced by androgens. Two SUMO specific proteases, SENP1 and SENP2, were potent in reversing AR SUMOylation in intact cells and *in vitro*. Based on coexpression and gene silencing analyses, SENP1 acted also as an effective AR coactivator. However, gene expression analyses of cells stably expressing SUMOylated or SUMOylation-defective AR demonstrated that the effect of receptor SUMOylation was target gene selective. Diverse types of cell stress, including elevated temperature and electrophilic stress evoked by the anti-inflammatory prostaglandin, 15d-PGJ_2, induced a rapid and massive conjugation of SUMO to the AR. The stress-triggered SUMOylation was reversible, as AR-SUMO conjugates were no longer detected when the stress was alleviated. The cell stress had dynamic and reversible effects on the AR action as assessed by chromatin immunoprecipitation (ChIP) assays and quantitative PCR: AR-chromatin interactions were disrupted and the accumulation of AR target gene mRNAs was repressed in the stressed cells, but both AR chromatin loading and transcriptional activity recovered after stress. Biochemical cell fractionation and microscopic imaging assays revealed that the AR intranuclear distribution had been altered during the stress conditions. SUMOylation-defective AR showed retarded mobility in relation to the SUMOylated receptor as measured by fluorescence recovery after photobleaching (FRAP) assays. These results suggest that SUMOylation is involved in the modulation of AR mobility and cycling of the receptor between different intanuclear compartments during transcription activity cycles, while stress-induced SUMOylation is likely to sustain AR solubility. The study also proved that prostate cancer cell growth was dependent on an intact SUMOylation pathway, since silencing of SENP1 retarded proliferation of androgen-treated cells. To conclude, this study produced new information concerning AR kinetics in the nucleoplasm and the effect of SUMOylation in modulating AR-regulated gene expression in prostate cancer cells. This thesis also provides interesting prospects for prostate cancer therapy, i.e. identifying novel ways to restrict the AR activity and the growth of prostate cancer cells.

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Medical Subject Headings: Receptors, Androgen; Protein Processing, Post-Translational; Small Ubiquitin-Related Modifier Proteins; Sumoylation; Activity Cycles; Cell Nucleus; Prostaglandin D2/analogs & derivatives; Gene Expression; Transcription, Genetic; Cell Line, Tumor; Prostatic Neoplasms

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TIIVISTELMÄ

Androgeenit eli miessukuhormonit vaikuttavat geenisäätelyyn androgeenireseptorin (AR) välityksellä. AR:n kanssa vuorovaikuttavat koregulaattorit eli säätelyproteiinit, kromatiinirakenne sekä proteiinien synteesin jälkeiset muokkausreaktiot, proteiinimodifikaatiot, vaikuttavat myös geeniluennan aktiivisuuteen. Ubikitinaatio ja fosforylaatio ovat esimerkkejä proteiinimodifikaatioista, jotka vaikuttavat AR:n stabiilisuuteen ja aktiivisuuteen geeniluennassa. AR:n toiminta säätelee eturauhasen normaalia kasvua sekä eturauhassyövän kehittymistä ja etenemistä, minkä vuoksi AR on merkittävä lääkinnällisen terapian ja lääkekehitystyön kohde. Tämän väitöskirjatyön tavoitteena oli tutkia AR:n SUMO (small ubiquitin-like modifier) modifikaatioita ja niiden vaikutusta reseptorin toimintaan eturauhassyöpäsoluissa. AR:n havaittiin muodostavan konjugaatteja enimmäkseen SUMO-2/3:n kanssa, ja androgeenit lisäsivät SUMO-konjugaatiota, SUMOlaatiota. SENP-entsyymit katalysoivat reaktion palautuvuutta. SENP1- ja SENP2- entsyymien todettiin olevan tehokkaita vähentämään AR:n SUMOlaatiota. Lisäksi ne voimistivat AR:n aktiivisuutta geeniluennan säätelijänä. Työssä kuitenkin todettiin AR-säädeltyjen geenien olevan erilailla riippuvaisia reseptorin SUMOlaatiosta. Solustressi, kuten kohonnut lämpötila ja prostaglandiini 15d-PGJ_2, lisäsivät nopeasti ja voimakkaasti AR:n SUMO-modifikaatiota. Solustressillä oli dynaamisia vaikutuksia AR:n toimintaan, sillä solustressi esti AR:n sitoutumisen kromatiiniin ja androgeenisäädeltyjen geenien ilmentymisen. Lisäksi stressitekijät muuttivat AR:n tumansisäistä sijaintia. Solustressin vaikutukset AR:iin olivat palautuvia. SUMO-muokatun AR:n havaittiin liikkuvan tumassa nopeammin kuin SUMOlaatioon kyvyttömän reseptorin. Tutkimuksen johtopäätöksenä todettiin, että SUMOlaatio vaikuttaa AR:n liikkumiseen tumassa, millä taas on vaikutusta reseptorin aktiiviseen kierrätykseen geeniluennan eri vaiheissa. Solustressin aiheuttama SUMOlaatio puolestaan vaikuttaa tärkeältä AR:n liukoisuuden ylläpitämisessä. Työssä myös osoitettiin SUMO-muokkausten säätelyn olevan tärkeää eturauhassyöpäsolujen kasvun kannalta, sillä *SENP1*-geenin hiljentäminen vähensi solukasvua. Tutkimus tuotti uutta tietoa AR:n tumansisäisestä kinetiikasta sekä sen vaikutuksesta AR-säädeltyjen geenien aktiivisuuteen. Tutkimustuloksilla on merkitystä eturauhassyövän solu- ja molekyylibiologisen taustan ymmärtämisen kannalta. Lisäksi tulokset voivat edesauttaa syövän uudentyyppisten hoitomuotojen kehittämistyötä.

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Kuopio, February 2013

Sanna Kaikkonen

List of the original publications

This thesis is based on the following original publications referred to in the text by their corresponding Roman numerals (I-III). In addition, unpublished results are presented.

I Kaikkonen S, Jääskeläinen T, Karvonen U, Rytinki MM, Makkonen H, Gioeli D, Paschal BM, Palvimo JJ. SUMO-specific protease 1 (SENP1) reverses the hormone-augmented SUMOylation of androgen receptor and modulates gene responses in prostate cancer cells. *Mol Endocrinol* 23(3):292-307, 2009

II Rytinki M*, Kaikkonen S*, Sutinen P, Paakinaho V, Rahkama V, and Palvimo JJ. Dynamic SUMOylation is linked to the activity cycles of androgen receptor in the cell nucleus. *Mol Cell Biol* 32(20):4195-205, 2012 *) equal contribution

III Kaikkonen S, Paakinaho V, Sutinen P, Levonen A-L, Palvimo JJ. Prostaglandin 15d-PGJ_2 inhibits androgen receptor signaling in prostate cancer cells. *Mol Endocrinol* 27(2):212-223, 2013

The publications were adapted with the permission of the copyright owners. The original publications listed above have not been included in other theses.

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Abbreviations

AF	activation function
AR	androgen receptor
ARE	androgen response element
C	carboxy terminus of a protein
CARM1	coactivator-associated arginine methyltransferase 1
CBP	CREB (cAMP response element-binding protein)-binding protein
ChIP	chromatin immunoprecipitation
COS-1	African green monkey kidney cells
CTE	carboxy terminal extension
DR3	direct repeat separated by three nucleotides
CRPC	castration-resistant prostate cancer
DAXX	death-domain associated protein
DBD	DNA-binding domain
E1	ubiquitin/SUMO-activating enzyme
E2	ubiquitin/SUMO-conjugating enzyme
E3	ubiquitin/SUMO ligase
EGFP	enhanced green fluorescent protein
ETS	E-twenty-six (E26) transformation-specific
FOXA1	forkhead box A1
FRAP	fluorescence recovery after photobleaching
GRIP1	glucocorticoid receptor-interacting protein 1
HIPK	homeodomain-interacting protein kinase
HDAC	histone deacetylase
HEK293	human embryonal kidney cells 293
HSP	heat shock protein
IR3	inverted repeat separated by three nucleotides
LBD	ligand binding domain
LNCaP	PC derived from lymph node metastasis
MAPK	mitogen-activated protein kinase
MDM2	mouse homologue of double minute 2 protein
N	amino terminus of a protein
NES	nuclear export signal
NLS	nuclear localization signal
NR	nuclear receptor
NTD	N (amino)-terminal domain
PC	prostate cancer

15d-PGJ_2	15-deoksi-$\Delta^{12,14}$-prostaglandin J_2
PIAS	protein inhibitor of activated STAT
PML	promyelocytic protein
PR	progesterone receptor
PTM	post-translational modification
R1881	methyltrienolone, synthetic AR agonist
RanBP2	Ran binding protein 2
RanGAP1	Ran GTPase activating protein 1
RNF	ring finger protein
ROS	reactive oxygen species
SAE	SUMO-activating enzyme
SATB1	AT-rich sequence-binding protein 1
SENP	SUMO-specific protease
SET9	SET domain-containing protein 9
SF-1	steroidogenic factor 1
SIM	SUMO-interacting motif
S100P	S100 calcium-binding protein
SR	steroid receptor
SRC	steroid receptor coactivator
STAT	signal transducer and activator of transcription
SUMO	small ubiquitin-like modifier
SUMOylation	conjugation of SUMO
TAU	transactivation unit
TF	transcription factor
TMPRSS2	transmembrane serine protease 2
TSA	trichostatin A
UBC	ubiquitin/SUMO-conjugating enzyme
USP	ubiquitin specific protease
VCaP	vertebral-cancer of the prostate

1 Introduction

Androgen receptor (AR) is a hormone-activated transcription factor that belongs to the steroid receptor subclass of the nuclear receptor superfamily. Androgens, male sex hormones testosterone and 5α-dihydrotestosterone, are natural agonists for the AR. Once the hormone binds to the AR in the cytosol, the receptor is released from chaperone proteins, its conformation changes and the receptor-hormone complex enters the nucleus. AR homodimers bind to chromatin at palindromic androgen response elements and regulate expression of target genes. In addition to the basal transcription machinery, chromatin remodelling and interactions with coregulator proteins contribute to the AR function.

Many cellular proteins, including the AR, are modified by different post-translational modifications (PTMs). PTMs act as tags that are conjugated to the amino acid side chains in their targets in a reversible manner. In addition to androgens, also PTMs regulate the activity of AR. PTMs are dynamically adjusted according to the changes in the cellular environment, such as cell stress. PTMs can affect protein stability and cellular localization. Furthermore, they modify protein activity and the interactions occurring with other proteins. In summary, PTMs confer wide variation onto protein structure and function. PTMs can be small molecule modifications, such as phosphorylation and acetylation, or alternatively the modifiers can be small proteins, like ubiquitin and SUMO (small-ubiquitin like modifier). It is noteworthy that there are many factors, such as chromatin structure and coregulators, which play a role in AR-dependent transcription, and these too can be modified by various PTMs.

The development and progression of prostate cancer is strongly dependent on AR signaling. Thus, the receptor represents a significant drug target. Currently, AR antagonists, antiandrogens, are being used in therapeutic protocols to restrict the AR activity and prevent progression of the cancer. Unfortunately, hormonal therapy often fails, since the disease eventually converts into a castration-resistant stage, i.e. it no longer is responsive to antihormones. Therefore, alternative approaches for AR inhibition are needed.

The AR is modified by SUMO, which modulates the transcriptional activity of the receptor. Moreover, several components of the SUMOylation pathway function as coregulators of AR signaling, and interestingly, these are often over-expressed in prostate cancer cells. Thus, SUMOylation pathway may appear as a potential target for medical intervention to inhibit AR function in the prostate tissue. Therefore, the formation of AR-SUMO conjugates and the reversibility of the modification will be addressed here. In summary, the study described in this doctoral thesis aims at improving the understanding of the regulation of AR function by SUMO modifications in prostate cancer cells.

2 *Review of Literature*

2.1 ANDROGEN RECEPTOR

Androgen receptor (AR) is expressed to some degree almost in every tissue, with the highest expression level observed in the male reproductive organs and adrenal gland (Keller et al. 1996). The physiological function of the AR is to mediate the effects of the male hormones, i.e. androgens, to the level of the genes. Testosterone and its more potent metabolite, 5α-dihydrotestosterone (DHT), are the endogenous high-affinity ligands for the AR. Testosterone is a steroid hormone that is primarily synthesized from cholesterol in Leydig cells in the testes. In addition, the adrenal glands and the ovaries synthesize dehydroepiandrosterone (DHEA) that can be converted into both testosterone and estrogen. Testosterone is further processed by the 5α-reductase enzyme in target tissues to yield DHT (Gao et al. 2005). Hormone-binding to the receptor results in expression of AR target genes to control the development, differentiation and function of male reproductive tissues, including prostate. Moreover, AR action is involved in the promotion and maintenance of the male phenotype. Androgens are responsible for male sexual differentiation, pubertal changes, and spermatogenesis. In the absence of AR activity men present a testicular feminization syndrome and express a female phenotype (Jääskeläinen 2012). The hormone-activated AR acts also in brain and skin and participates in maintaining both the mass and strength of muscle and bone. Thus, a functional AR is an important factor in both developmental and differentiation processes in health and disease (Matsumoto et al. 2012).

The gene encoding the human AR is located on the X chromosome long arm and it consists of eight exons that encode a protein of 919 amino acids. AR is a member of the steroid receptor (SR) family within the broad nuclear receptor (NR) superfamily. AR is structurally and functionally related to the other SRs, such as the glucocorticoid receptor (GR), mineralocorticoid receptor (MR), progesterone receptor (PR), and estrogen receptor (ER) (Aranda & Pascual 2001). In common, SRs are modular in structure, and they function as hormone-activated transcription factors (TFs) capable of regulating the expression of complex gene networks. However, SRs exhibit several differences in their structural properties and mode of action. The specific features of the AR will be discussed in the following sections.

2.1.1 Domain structure

As with other SRs, the AR was cloned over 20 years ago (Chang et al. 1988, Lubahn et al. 1988). SRs proved to share many structural similarities, i.e. they are organized into functional domains (Mangelsdorf et al. 1995, Huang et al. 2010). In general termes, AR consists of four distinct domains listed in order from the amino terminus (N) to the carboxy (C) terminus: the amino terminal domain (NTD), the DNA-binding domain (DBD), the hinge region and the ligand-binding domain (LBD) (Gao et al. 2005, Claessens et al. 2008). AR domain structure is illustrated in Fig. 1.

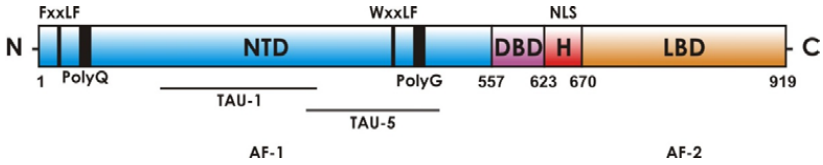

Figure 1. Domain structure of the full-length human AR (919 amino acids). N, the amino terminus; C, carboxy terminus; NTD, the amino-terminal domain; DBD, the DNA-binding domain; H, the hinge region; LBD, the ligand-binding domain, AF, activation function; TAU, transcription activation unit; PolyQ and PolyG, the polyglutamine and the polyglycine stretch, respectively; NLS, the nuclear localization signal; FxxLF and WxxLF, the motifs that are involved between interactions of the NTD with the AF-2 in the LBD.

2.1.1.1 Amino-terminal domain

In contrast to the other domains, the AR NTD is encoded in a single exon. The NTD is the most variable, non-conserved, domain among the SRs. The AR NTD accounts for more than 60% of the AR protein, mediates the majority of AR transcriptional activity and is an active interaction surface for AR-interacting proteins. However, it shares only 20% sequence similarity with PR, and thus, it is believed to contribute to the specificity of the steroid hormone/receptor response (Gao et al. 2005). Moreover, the AR NTD is polymorphic in itself, since it contains variable polyglutamine (polyQ) and polyglycine (polyG) extensions. Thus, the major difference between AR variants in different individuals lies within the length of the NTD. The polyQ and polyG repeats are commonly 9-36 and 10-30 residues in length, respectively (Palazzolo et al. 2008). An atypical extension of the polyQ tract has been found to play a role in the neuromuscular Kennedy's disease, where the repeat ranges from 40 to 52 residues. A naturally occurring 45-kDa receptor isoform (AR45) has been reported to exist as a splice variant of human AR (Ahrens-Fath et al. 2005). The AR45 contains the entire AR DBD and LBD and a seven amino acid sequence at the N terminus in place of the wild-type AR NTD.

The AR NTD contains the ligand-independent activation function-1 (AF-1) and it is considered as the major activation domain of the AR that consists of two transcription activation units, TAU-1 and TAU-5 (Jenster et al. 1995). TAU-5 contains an LBD-independent activation potential, whereas TAU-1 activity requires the presence of LBD. Importantly, these TAUs interact with several coregulatory proteins. The NTD contains highly conserved FxxLF-like motifs, 23-FQNLF-27 and 433-WHTLF-437 that mediate interactions between the NTD and the C-terminal LBD of the receptor (He et al. 1999, He et al. 2000). The androgen-induced intramolecular interaction, termed the N/C interaction, is essential for AR function (Ikonen et al. 1997). Intriguingly, this kind of property has not been identified in the other NRs (Schaufele et al. 2005). In summary, the variable and relatively unstructured AR NTD is an important regulatory domain of the receptor. The AR NTD has been postulated to serve as a flexible platform for the recruitment and assembly of coregulators and transcription machinery to mediate the cell and gene specific effects of androgens (Dehm & Tindall 2007, Claessens et al. 2008).

2.1.1.2 DNA-binding domain and hinge region

In contrast to the NTD, the DBD is well conserved in NRs (Helsen et al. 2012). The DBD consists of two tandem cysteine-rich zinc finger motifs, where one zinc ion coordinates four cysteine residues within each zinc finger structure (Fig. 2). The function of DBD is to dock the receptor to the hexanucleotide androgen response elements (AREs)

which are located within AR target genes. A five-amino acid P-box region in the first zinc finger is responsible for sequence specific DNA recognition and binding to the major groove of DNA. The second zinc finger contains a D-box region that is involved in DNA-dependent dimerization of AR monomers. In addition to the P- and D-box primary motifs that are identical for AR, GR, MR and PR, the carboxy terminal extension (CTE) in the hinge region also participates in the formation of AR dimers and high-affinity DNA-binding (Dehm & Tindall 2007).

The AR hinge region is a short flexible linker domain between the DBD and the LBD of the receptor (Clinckemalie et al. 2012). However, the hinge is not simply a structural part of the receptor as it contains the main nuclear localization signal (NLS) of the AR (Poukka et al. 1999). Interestingly, the hinge is poorly conserved among SRs even though it contains an NLS as is also the case in other SRs. A mutation of lysine residues in the NLS impairs intracellular localization of the AR, DNA-binding, coregulator interactions, and receptor folding emphasizing the importance of the short hinge region (Haelens et al. 2007, Tanner et al. 2010). The AR hinge harbors a putative PEST sequence, i.e. a peptide sequence, containing proline (P), glutamate (E), serine (S), and tyrosine (T), that is involved in receptor degradation during transcription cycles (Rechsteiner & Rogers 1996, Sheflin et al. 2000, Kang et al. 2002).

In contrast to the AR NTD that is encoded within a single exon, the two zinc fingers and the hinge region are encoded in different exons (exons 2, 3 and 4). A splice variant named AR23 has been found in specimens taken from prostate cancer (PC) patients. The misspliced receptor contains a 69-bp insertion in the frame that creates a 23-amino acid extension between the two zinc fingers (Steinkamp et al. 2009). In addition, several AR splice variants in which the hinge region is deleted or truncated have been described in PC tissues (Haile & Sadar 2011).

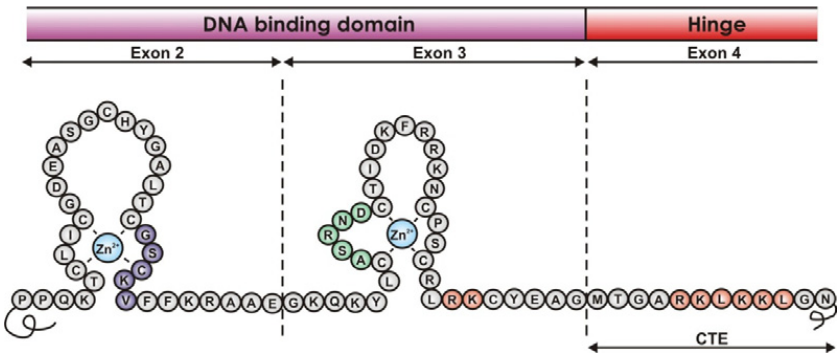

Figure 2. Structure of the human AR DBD and a part of the hinge region. Amino acids comprising the P-box are in purple, D-box in green and the bipartite nuclear localization signal (NLS) in orange. CTE, carboxy terminal extension.

2.1.1.3 Ligand-binding domain

Similar to the C-terminal LBDs of other NRs, the AR LBD consists of twelve α-helixes that form a hydrophobic pocket for high-affinity hormone-binding (Moras & Gronemeyer 1998). Upon androgen binding, the helix 12 forms a lid above the ligand pocket providing a cleft-like interaction surface for coregulatory proteins. The surface is termed the ligand-dependent activation function-2 (AF-2). However, the AF-2 of the AR LBD interacts only

weakly with LxxLL motifs of general NR coregulators, but it rather contacts FxxLF motif in the NTD to mediate the N/C interaction of the AR (Ikonen et al. 1997). As the AR is transcriptionally inactive in the absence of androgen, the LBD might actually inhibit the strong ligand-independent AF-1 activity of the AR NTD, and therefore, suppress activation of AR target genes in the absence of hormone (Brinkmann et al. 1999).

The AR LBD binds natural androgens, synthetic androgens and different antiandrogens. Ligand binding often alters the cellular distribution of the AR (Fig. 3). AR ligands can be classified as agonists or antagonists based on their ability to activate or inhibit transcription of AR target genes. The classification can also be based on the structure of the ligand, and hence, named as steroidal or non-steroidal hormones (Gao et al. 2005).

Several mutations have been found in the AR LBD (The Androgen Receptor Gene Mutations Database World Wide Web Server, http://www.androgendb.mcgill.ca/). Almost two thirds of the mutations are missense substitutions of single amino acids. These kinds of mutations in the LBD commonly affect both ligand-binding affinity and specificity. Thus, many mutations of the LBD are associated with diseases, like PC and androgen insensitivity syndrome (Heinlein & Chang 2004, Jääskeläinen 2012). In addition, LBD-truncated splice variants of the receptor have been identified in clinical PC samples (Dehm & Tindall 2011).

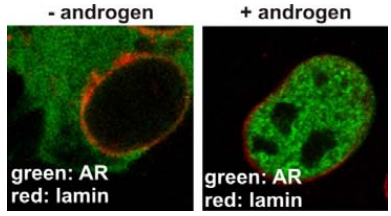

Figure 3. The effect of androgen on the cellular distribution of the AR as visualized by confocal microscopy. COS-1 cells transiently expressing enhanced green fluorescent protein (EGFP) tagged AR were treated with (+) synthetic R1881 or vehicle (-) for 1 h. Anti-lamin immunostaining depicts the nuclear envelope. The images were collected using Zeiss LSM 700 confocal microscope (Kaikkonen S).

2.1.2 Function in transcriptional regulation

The regulation of AR-dependent transcription is a tightly controlled process involving several co-ordinated functions. The AR is a ligand-activated TF, and high-affinity androgen binding to the receptor LBD induces AR-dependent transactivation processes (Shang et al. 2002). Like other NRs, the AR shuttles between cytoplasmic and nuclear compartments of the cell (Black et al. 2004, Shank & Paschal 2005, Marcelli et al. 2006). In the absence of hormone, the apo-AR is incorporated into a chaperone/immunophilin complex, containing e.g. heat-shock protein 90 (HSP90) and the receptor is localized mainly in the cytosol (Pratt & Toft 1997). The androgen-occupied holo-AR becomes concentrated in the nucleus. Ligand-binding launches a cascade where the receptor monomer releases the chaperone proteins, undergoes a conformational change allowing the AR dimerize, and the dimer then enters the nucleus ready to interact with both with chromatin and coregulatory proteins, initiating activation of AR target genes (Fig. 4).

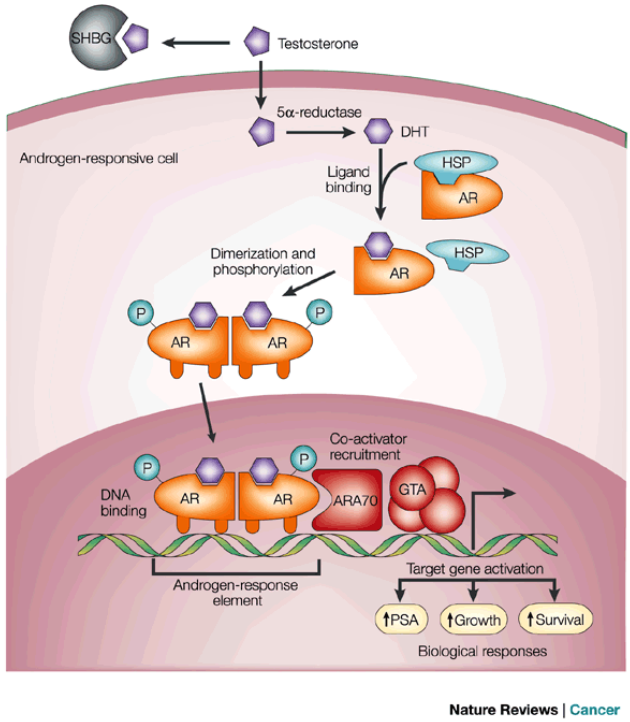

Figure 4. A schematic presentation of AR signaling. SHBG, sex-hormone-binding globulin; DHT, dihydrotestosterone; AR, androgen receptor; HSP, heat-shock protein; P, phosphorylation; ARA70, an AR coregulator; GTA, general transcription apparatus; PSA, prostate-specific antigen. (Reprinted from Feldman & Feldman 2001 with permission of Nature Publishing Group.)

After nuclear entry of the holo-AR, the receptor recognizes and binds specific DNA motifs, AREs, in a DBD-directed manner to start transcription of androgen-responsive genes (Riegman et al. 1991). Classically, SR homodimers bind head-to-head in hexameric inverted-repeat (IR3) response elements 5′-AGAACAnnnTGTTCT-3′, where n represents any nucleotide. However, AR dimers are also able to bind chromatin head-to-head and head-to-tail in direct-repeat (DR3) elements 5′-AGAACAnnnAGAACA-3′ (Claessens et al. 2001, Shaffer et al. 2004, Denayer et al. 2010). Multiple AREs are often found in the regulatory regions of AR target genes, and the elements display co-operativity in enhancing AR-dependent transcription (Geserick et al. 2005). Binding of AR dimer to the canonical AREs involves the two zinc finger motifs, while binding to selective AREs is distinguished by the involvement of the second zinc finger and the CTE (Schoenmakers et al. 1999). Since the second zinc finger is responsible for DNA-dependent receptor dimerization, it has been postulated that the discrimination between canonical and selective AREs is determined by alternative receptor dimerization with a head-to-tail conformation (Shaffer et al. 2004). It is noteworthy that the best-characterized androgen-regulated genes are not regulated by perfect AREs (Dehm & Tindall 2007), suggesting that AR DNA-binding properties may be highly adjustable. AR-binding on some genes depends on the surrounding binding sites for the other cell-specific TFs such as the members of the Forkhead, GATA-binding protein (GATA), octamer-binding protein (Oct) and E-twenty-six transformation-specific (ETS)

families (Heemers & Tindall 2007, Wang et al. 2007). For instance, the forkhead box A1 protein (FOXA1; also HNF-3α, hepatocyte nuclear factor 3α) may act as a pioneering or licenzing factor for AR binding. FOXA1 may participate in AR target gene recognition, or it may mask AR-binding sites (Gao et al. 2003, Sahu et al. 2011). Recent genome-wide studies (chromatin immunoprecipitation followed by DNA sequencing, ChIP-seq) have proved that most of the AR-binding sites are located on distal enhancers, far away from the transcription start sites (Wu et al. 2011).

The biological function of the AR and androgens is to alter expression of AR target genes. Gene products which are expressed in an AR-dependent manner are involved in several cellular functions including cell survival, induction of proliferation and suppression of apoptosis (Lamont & Tindall 2010). Moreover, AR is involved in the regulation of steroid biosynthesis and fatty acid metabolism (Ngan et al. 2009). In addition to the activation of transcription, the AR is also capable of evoking repression (Grosse et al. 2012). However, AR-regulated repression of transcription has not been so extensively studied as the AR-activated processes. The repressing effect of the AR is not mediated solely *via* direct AR-binding to chromatin. Instead, it is believed that the AR can interact with another transcription factor, such as activator protein 1 (AP-1), that is in direct contact with DNA (Kallio et al. 1995). Furthermore, other "non-genomic" actions of the AR have also been described and these kinds of transcription-independent, very rapidly occurring actions of the AR have been identified in neuroendocrine cells (Foradori et al. 2008).

2.1.2.1 The role of chromatin structure and coregulators

In addition to hormone-binding and specific interactions with chromatin, the AR function is controlled by coregulators that are also often called cofactors (Heinlein & Chang 2004, Rosenfeld et al. 2006, O'Malley 2008). Basically, a coregulator is defined as a factor that interacts with a particular TF and affects the activity of the TF in a reporter gene-based assay. Thus, the coregulators are subdivided into coactivators and corepressors depending on the transcriptional outcome. A total of, more than 200 coregulators have been identified for AR (van de Wijngaart et al. 2012). Transcriptional coregulators directly interact with TFs and the basal transcriptional machinery, and thus interfere with chromatin remodeling. Importantly, the primary event in AR-directed transcription in the nucleus is the modulation of local chromatin, since the chromatin structure is a principal factor that controls the activity of gene expression. Therefore, tightly packed chromatin has to be loosened by mechanisms that alter the arrangement of nucleosomes and covalently modify nucleosomal histones. Non-covalent modulation of nucleosome arrangement requires the presence of chromatin remodeling complexes, such as switch mating type/sucrose non-fermenting (SWI/SNF), that consist of multiple subunits. Furthermore, covalent modifications of the N-terminal histone tails (also termed histone marks) participate in regulating the access of transcriptional initiators to chromatin (Kouzarides 2007). For instance, acetylation of histones has long been known to loosen chromatin packing. The acetylation is catalysed by histone acetylases (HATs) and histone deacetylases (HDACs). In addition, methylation is an important modification of histones, which is regulated by methyltransferases and demethylases (Heemers & Tindall 2007).

AR coregulators have often been identified by yeast-two-hybrid screens as direct interaction partners of the receptor (Jänne et al. 2000, van de Wijngaart et al. 2012). The AR-interacting coregulators play major roles during the AR-dependent gene transcription, since they can influence a number of functional properties of the receptor, including its ligand-binding selectivity and DNA-binding capacity. In contrast to the other NRs, the coactivators involved in transcriptional activation of the AR are recruited into different regions in the AR NTD and the hinge region (Heemers & Tindall 2007). Ligand-independent AF-1 in the AR NTD is the major interaction surface for AR coregulators, as it binds the LxxLL motif commonly present in coactivators. However, AR-specific ARA70

(AR-associated protein of 70 kDa) is one exception, since it prefers to interact *via* the FxxLF motif with the AF-2 in the AR LBD (He et al. 2002). There are many important AR coregulators e.g. p160-family members, steroid receptor coactivators 1-3 (SRC1-3; also transcriptional intermediary factors 1-3, TIF1-3). They facilitate the recruitment of histone modifing enzymes such as p300, cAMP response element-binding protein (CREB)-binding protein (CBP), p300/CBP-associated factor (P/CAF), and coactivator-associated arginine methyltransferase 1 (CARM1) (Heemers & Tindall 2007). In addition to chromatin modulating processes and interactions with coactivators listed as examples above, also a multi-subunit bridging factor, termed the Mediator complex, between the AR and RNA polymerase II machinery is involved in the transcriptional activation of the AR.

Since transcription is a well controlled multi-step event, transcriptional corepressors work to suppress the process. Antiandrogen binding to the AR LBD directs the helix 12 to a position different from that involved in the agonist-bound receptor recruiting transcriptional corepressors (Gao et al. 2005). Nuclear corepressor 1 (NCoR1), silencing mediator of retinoic and thyroid receptors (SMRT, also NCoR2), and nucleosome remodeling and deacetylase (NuRD) complexes are examples that contain multiple HDACs to change the chromatin structure. In addition, they harbor also other enzymatic activities that catalyze epigenetic changes to modulate the histone marks to transcriptionally inactive stage (Kouzarides 2007).

2.2 POST-TRANSLATIONAL MODIFICATIONS OF THE AR

The human genome comprises ≥20 000 genes encoding the synthesis of proteins that are important structural components of cells or which function as biologically active factors, such as enzymes and transcriptional regulators. Post-translational modifications (PTMs) provide huge opportunities for the regulation of the properties of these proteins. The PTMs are also involved in the transcriptional activity of AR (Anbalagan et al. 2012, Coffey & Robson 2012, Gioeli & Paschal 2012). PTMs can be roughly divided into two categories: small molecule modifiers, such as phosphate (phosphorylation), acetyl (acetylation), methyl (methylation), nitric oxide (nitrosylation), and modifiers that are proteins in themselves, like ubiquitin (ubiquitylation) and ubiquitin-like modifiers (e.g. SUMOylation, NEDDylation, ISGylation). Moreover, protein structures can also be modulated by conjugation with carbohydrates (glycosylation) and by ADP ribosylation. PTMs play key roles in many biological functions, such as the regulation of gene expression and cellular differentiation, protein degradation, and protein-protein interactions (Geiss-Friedlander & Melchior 2007, Grotenbreg & Ploegh 2007). Furthermore, a wide pattern of diverse PTMs has been identified in mediating the rapid responses to changes in cell environment, such as cell stress (Deribe et al. 2010). Thus, PTMs can influence numerous processes in both normal cell biology and in pathogenesis.

PTMs are commonly identified by mass-spectrometric analyses. Mutation analysis is also often utilized, since some PTMs occur in consensus sites that can be predicted *in silico* and subsequently verified in a biological model. Moreover, specific antibodies have been developed to detect the modified species of a particular substrate. Interestingly, numerous target sites of different PTMs found in SRs are well conserved among the receptor family members, indicating the PTMs may have common regulatory effects on SR activity (Faus & Haendler 2006). Hormone binding affects the kinetics of diverse PTMs targeting the SRs. Not only hormones, but also PTMs are likely to regulate the transcriptional activity of SRs, including the AR. Thus, the receptor, as well as transcriptional coregulators and chromatin are targeted by PTMs in their actions to modulate the activity of transcription. However,

the following discussion will focus on the most important modifiers directly binding to the AR (Fig. 5).

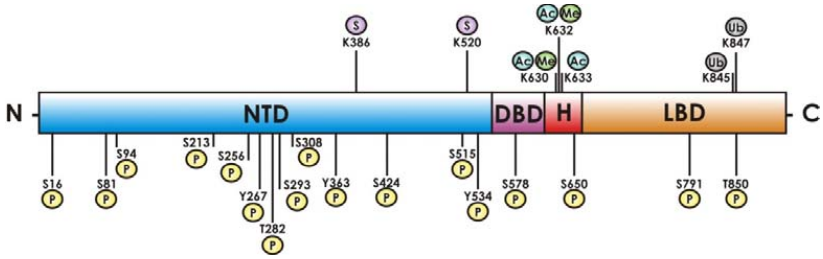

Figure 5. Major sites for PTMs in the human AR: A linear presentation of the AR polypeptide, where lysine modifications (SUMOylation, ubiquitylation, acetylation, methylation) and phosphorylation sites are depicted above and below the functional domains of the receptor, respectively. The numbering is based on the accession number P10275 in National Center for Biotechnology Information. S, SUMO; Ub, ubiquitin; Ac, acetyl; Me, methyl; P, phosphate.

2.2.1 Phosphorylation

In protein phosphorylation, a phosphate group from ATP is transferred to the hydroxyl group in the side chain of serine (S), threonine (T), or tyrosine (Y) residue. The activities of numerous cellular proteins are controlled by phosphorylation. In addition, phosphorylation of the AR is a significant modification in relation to the activity of the receptor. At present, the AR is modified by phosphate altogether on 17 amino acid residues (Fig. 4). The majority of the AR phosphorylation sites (phosphosites) are serine residues (12 of 17), but also threonine (2 of 17) and tyrosine (3 of 17) residues are putative phosphosites of AR. Most of the AR phosphosites reside in the NTD. Furthermore, phosphosites are also located in other functional domains: S578 in the DBD, S650 in the hinge, and S791 and T850 in the LDB. Typically, androgens induce phosphorylation of the AR. The phosphorylated AR can be detected as a slightly slower migrating bands in immunoblot analysis (van Laar et al. 1990, Kuiper et al. 1991). Phosphospecific anti-AR antibodies have also been generated (Gioeli et al. 2006). In addition to androgen-induced modification, constitutively phosphorylated sites, such as S94, have been identified (Gioeli et al. 2002). In addition to androgens, also growth factors are able to induce phosphorylation of the AR. For instance, epidermal growth factor (EGF) signaling leads to activation of several downstream kinases and increased phosphorylation of the AR (Gioeli et al. 2002, Guo et al. 2006, Ponguta et al. 2008).

Phosphorylation is a reversible reaction: kinases phosphorylate proteins and phosphatases reverse this modification. Generally, androgen-binding is thought to induce a conformational change in the receptor, which renders the phosphosites more accessible to the kinases. Alternatively, in the absence of androgen, the phosphosites may be well available to kinases, but androgen-binding can impair phosphatase interactions, which results in increased phosphorylation of the AR (Yang et al. 2007). Overall, the localization of the AR is a major determinant of its phosphorylation state. It is known that the AR shuttles between cytoplasmic and nuclear compartments, and AR-targeting kinases and phosphatases are likely to be enriched in different cellular localisations. A study using AR fused with a nuclear localization signal (NLS) or a nuclear export signal (NES) demonstrated that phosphorylation of specific amino acid residues is differently modulated in each cellular compartment, since kinases and phosphatases have their own abundances in each cellular location (Kesler et al. 2007).

Different kinases can phosphorylate AR in a target site-specific manner. Stress kinases, such as mitogen-activated protein kinase 1 (MAPK1, also p38), that belong to the family of serine/threonine kinases, can transfer phosphate to S650 in the hinge domain of the AR resulting in nuclear export of the receptor and blunted AR transcription (Gioeli et al. 2006). Moreover, cyclin-dependent kinases (CDKs) that are widely known as regulators of the cell cycle, and hence modulators of cell growth, are emerging as serine kinases of the AR. Thus far, CDK1, -7, -9, and -11 have been identified to be able to phosphorylate the AR (Chen et al. 2006, Zong et al. 2007, Gordon et al. 2010, Chymkowitch et al. 2011). In contrast to the many kinases identified, only two protein phosphatases, PP1 and PP2A, have been found to dephosphorylate the AR (Yang et al. 2007, Chen et al. 2009).

2.2.2 Acetylation

Acetylation was initially found to enhance AR transcription, since the HDAC inhibitor trichostatin A (TSA) increased the expression of AR target genes (List et al. 1999). Thereafter, the lysine (K) residues within the motif 630-KLKK-633 in the AR hinge region have been identified as direct acetylation target sites in the receptor. In addition, the acetyl moiety can also be attached to the side chain of arginine (R). However, in the AR, only acetylation of lysine has been described. Interestingly, the lysine-rich acetylation motif is conserved with different TFs, including NRs and the tumor suppressor p53 (Sterner & Berger 2000). Acetyltransferases p300/CBP, Tat-interacting protein 60 kDa (TIP60) and N-acetyltransferase arrest-defect 1 protein (ARD1) acetylate the AR and increase its activity (Fu et al. 2000, Gaughan et al. 2002, Wang Z. et al. 2012).

In addition to processing histones, HDAC1 deacetylates AR and suppresses AR activity (Gaughan et al. 2002). Moreover, silent information regulator 1 (also sirtuin 1, SIRT1) reverses AR acetylation (Fu et al. 2006). In contrast to the situation with the kinases, (de)acetylases are not thought to discriminate between different acetylation sites in the AR. Acetylation regulates protein-protein interactions by modulating the affinity of the AR for its coregulators. The AR acetylation sites modulate transcription in a promoter-selective manner, providing fine-tuning effects on the expression of different AR target genes (Faus & Haendler 2008). As a consequence, acetylation of NRs, including the AR, affects cellular growth and apoptosis, biological functions that are typically dysregulated in cancer. Acetylation appears to be a physiologically important process that integrates hormone signaling and intracellular metabolism (Fu et al. 2004, Popov et al. 2007).

2.2.3 Methylation

Methylation has been known to modulate NR-regulated transcription, because several components of chromatin, i.e. histones and cytosine nucleotides in DNA, are methylated. Interestingly, methylation of the AR has only recently been discovered (Gaughan et al. 2011, Ko et al. 2011). Like acetylation, methylation can occur at lysine or arginine residues, but only K630 and K632 in the AR have been proposed to be putative methylation sites (Gaughan et al. 2011, Ko et al. 2011). The AR hinge region contains a motif (named above as an acetylation motif) that is similar to the sites in proteins modified by the methyltransferase SET domain-containing protein 9 (SET9). SET9 has been identified as an AR-interacting protein that can methylate the receptor (Gaughan et al. 2011, Ko et al. 2011). Furthermore, these two reports published in tandem showed that SET9 could function as a coactivator of the AR, enhance the N/C interaction of the receptor and be overexpressed in malignant epithelial cells in PC patients (Gaughan et al. 2011, Ko et al. 2011).

CARM1 and protein arginine methyltransferase 2 (PRMT2) have also been postulated to be AR-interacting coactivators (Hong et al. 2004, Meyer et al. 2007). In addition, CARM1 overexpression has been associated with PC (Hong et al. 2004, Kim et al. 2010). Thus far, it is not known whether CARM1 and PRMT2 can directly methylate arginine residues of the AR. Moreover, demethylases targeting the AR remain to be found. Lysine specific histone

demethylase 1 (LSD1) promotes AR-dependent transcription by demethylating repressive histone marks. LSD1 has been found to be up-regulated in high grade prostate tumors (Metzger et al. 2005, Kahl et al. 2006). However, it remains to be determined, whether LSD1 can directly demethylate the AR. Since methylation is the most recently found PTM of the AR, further studies are likely to clarify the role of AR methylation in the control of AR target genes.

2.2.4 Ubiquitylation

Protein modification by ubiquitin is a similarly reversible PTM as those discussed above. However, the major difference is that the modifier is a small protein itself; a 76-amino acid polypeptide binds *via* a covalent isopeptide bond to the ε-amino group of a lysine in a substrate. This is a bulky modification, since ubiquitin is also able to form both linear and branched polymers. Conjugation of ubiquitin, ubiquitylation, is a three-step enzymatic cascade requiring specific enzymatic activities: E1 activation → E2 conjugation → E3 ligation. Human cells express two different E1 enzymes, ~30 and over 300 E2s and E3s, respectively (Bergink & Jentsch 2009). First, an E1 ubiquitin activating enzyme binds ubiquitin, consuming energy from ATP. Then, the E1-bound ubiquitin is transferred to an E2 conjugating enzyme. Finally, an E3 ubiquitin ligase transfers ubiquitin from the E2 to the target protein. Deconjugation of ubiquitin is carried out by deubiquitinating enzymes (DUBs) which belong either to the family of cysteine proteases or that of metalloproteases (Komander et al. 2009, Katz et al. 2010). The human genome expresses ~100 different DUB enzymes (Bergink & Jentsch 2009).

The ubiquitin was discovered almost 40 years ago (Goldstein et al. 1975, Schlesinger et al. 1975), and in the early 1980s (poly-) ubiquitylation was identified as a pathway sorting proteins to proteasomal degradation (Ciechanover et al. 1980, Hershko et al. 1980). The significance of the finding culminated in the award of the Nobel Prize in chemistry in 2004. Histone H2 was the first protein identified to form ubiquitin conjugates (Goldknopf & Busch 1977, Hunt & Dayhoff 1977). Dynamic monoubiquitylation of H2A and H2B has been found to regulate chromatin structure and recruitment of transcriptional coregulators (Weake & Workman 2008). Ubiquitin-proteasome system (UPS) participates in controlling gene regulatory mechanisms, and importantly, both proteolytic and non-proteolytic activities are involved (Geng et al. 2012, Hammond-Martel et al. 2012). Non-proteolytic ubiquitylation can regulate TF binding with chromatin and coregulators, whereas proteolytic ubiquitylation modulates functional activity of TFs, e.g. by facilitating the turnover of a particular TF on chromatin (Reid et al. 2003, Le Cam et al. 2006).

The divergent outcome of ubiquitin conjugation is likely to arise from the ability of the modifier to conjugate target lysines as monoubiquitin or to form polymers. All seven conserved lysine residues within the ubiquitin can mediate the formation of ubiquitin polymers (Kim H.T. et al. 2007). Interestingly, each type of linear or branching chains conjugated at different lysine residues in the substrate have their own distinct biological effects. For instance, the K48-branched polyubiquitin chains function classically as tags for proteasomal degradation, whereas the K6-linked chains can protect the substrate from proteolysis (Shang et al. 2005). Ubiquitin K27 and K63 linkages have mainly non-proteolytic functions. Polyubiquitylation of H2A by K63 linkages is encountered at DNA double strand breaks (Ben-Saadon et al. 2006, Yan & Jetten 2008).

SRs are degraded *via* conjugation of polyubiquitin, and the ubiquitin pathway is known to regulate SR action, including the AR activity (Nawaz & O'Malley 2004, Kinyamu et al. 2005, Alarid 2006). However, the components of the ubiquitylation machinery responsible for transferring ubiquitin to the AR and the sites of ubiquitylation in the receptor have not yet been definitely established. The ubiquitin E3 ligases the mouse double minute 2 homolog (MDM2) and the carboxy terminus of HSP70-interacting protein (CHIP) mediate the AR ubiquitylation and proteasomal degradation (Lin et al. 2002b,

Chymkowitch et al. 2011). At present, the only putative sites for direct ubiquitin binding in the AR are lysines 845 and 847 in the LBD (Xu et al. 2009). Ubiquitin E3 ligase ring finger protein 6 (RNF6) that is overexpressed in castration-resistant PC, has been reported to mediate K6/K27-linked ubiquitylation to these AR sites promoting the transcriptional activity of the receptor. AR ubiquitylation by mixed branching chains has been suggested to function as a scaffolding factor promoting the recruitment of coactivators (Xu et al. 2009). In addition, the ubiquitin E2 conjugating enzyme, UBCH7, functions as a coactivator of the AR and several other SRs in a conjugating activity-dependent manner, probably modulating the exchange of coactivator complexes (Verma et al. 2004). In addition, ubiquitin protease USP10 can function as a coactivator of the AR, even though its role in the regulation of AR-ubiquitin conjugates is not fully clarified (Faus et al. 2005). Moreover, proteasome activity is required for AR function in PC cells *via* modulating the cyclic formation of AR transcription complexes (Kang et al. 2002, Lin et al. 2002a). Taken together, the components of the ubiquitylation pathway and the proteasome mediate remodelling of the AR transcription complexes, particularly regulating dynamic exchange of coregulators, which further highlights the role of multiple SR-interacting coregulators in achieving accurate gene regulation.

2.2.5 SUMO modifications

SUMOylation is a covalent and reversible modification which is characterized by binding of a small ubiquitin-like modifier, SUMO, to a lysine residue of a target protein (Wilkinson & Henley 2010). The stoichiometric ratio of SUMOylated proteins is usually very low because of the rapid reversibility of this modification (Bawa-Khalfe & Yeh 2010). Therefore, SUMO conjugates may be difficult to detect. SUMO was identified in 1996 in a variety of independent studies, explaining why it also appears as sentrin, PIC1 and UBL1 in the literature (Boddy et al. 1996, Okura et al. 1996, Matunis et al. 1996, Shen et al. 1996, Mahajan et al. 1997). Subsequently increasing number of proteins have been identified as SUMO substrates particularly due to advanced mass spectrometric methods (Gocke et al. 2005, Rosas-Acosta et al. 2005, Vertegaal et al. 2006, Matic et al. 2010, Tatham et al. 2011). Functional categories of SUMOylated proteins include TFs, DNA repair and stress-related proteins, and a variety of metabolic enzymes. Thus, the modification affects many important cellular processes including the control of genomic stability and signal transduction, cell division and differentiation (Gareau & Lima 2010, Lomeli & Vazquez 2011). Moreover, SUMO modification has effects on intra- and intermolecular interactions, as well as on protein transitions between different cellular and nuclear compartments. For instance, Ran GTPase activating protein 1 (RanGAP1) was the first identified SUMO target, and its modification has been found to alter the spatial distribution of the protein. Once RanGAP1 is modified by SUMO-1, it can interact with the Ran binding protein 2 (RanBP2; also Nup358, nucleoporin 358), and it becomes localized to the nucleus (Matunis et al. 1996, Mahajan et al. 1997). SUMOylation machinery is not only present in both the nuclear and the cytoplasmic compartments, but also in the plasma membrane (Rajan et al. 2005, Wilson & Rosas-Acosta 2005, Takahashi et al. 2008). However, SUMO conjugates are enriched in the nucleus.

AR was the very first SR identified as a target for SUMO conjugation (Poukka et al. 2000a). As discussed above, the NTD is the most variable domain among SRs, and the AF-1 sequence of the AR shows weak conservation (<15%) across the SR family. Despite this, the SUMOylation sites are highly conserved in the NTDs of SRs, pointing to an important function for SUMO modification. The SUMOylation sites of the AR, the lysines 386 and 520, are located within the ligand-independent transactivation domain. SUMOylation has been often linked to transrepression. For example, the SUMOylation-deficient AR is transcriptionally more active than the wild type receptor as assessed by reporter gene assays (Poukka et al. 2000a). However, the molecular mechanism is not totally evident yet,

even though the formation of different chromatin modifing repression complexes has been suggested to be involved in the suppression of SUMOylated TFs (Stielow et al. 2008, Ouyang & Gill 2009, Ouyang et al. 2009). It is significant that SUMOylation regulates transcription not only by targeting DNA-binding of TFs, but it also modifies coregulatory proteins and chromatin. For instance, SUMOylation can modulate p300 interaction with HDAC6 (Girdwood et al. 2003), and it can modify the N-terminal tails of histone H4, which leads to formation of repressive chromatin structure (Shiio & Eisenman 2003).

2.2.5.1 SUMO conjugation

Human cells express four different SUMO isoforms, SUMO-1-4. SUMOs resemble ubiquitin in terms of their size (~12 kDa) and three-dimensional structure. However, the amino acid sequences differ significantly from ubiquitin. For example, SUMO-1 shares only ~18% amino acid homology with ubiquitin. Furthermore, SUMO-2 and SUMO-3 are almost identical with each other, whereas they share ~50% sequence homology with SUMO-1. The endogenous expression of SUMOs also differs. SUMO-1, -2 and -3 are ubiquitously expressed in multiple tissues, while SUMO-4 is expressed mainly in the kidney, lymph node and spleen (Bohren et al. 2004, Guo et al. 2004).

Conjugation of SUMO to its target proteins is an ATP consuming, multi-step pathway similar to that of ubiquitin, but it requires specific enzyme activities which are distinct from those involved in the ubiquitylation pathway. Firstly, pre-SUMO is maturated by SUMO-specific proteases (SENPs) to yield a double-glycine at the C-terminal end of the molecule. The major difference between SUMO-2 and -3 is the C-terminal fragment that is cleaved during the maturation/conjugation process (Fig. 6). These SUMO paralogs cannot be distinguished by the currently available antibodies and hence are described in the literature as SUMO-2/3. The SUMO-4 isoform has been suggested to be incapable of performing conjugation, since the C-terminal proline residue proximal to the double-glycine prevents the action SENP (Owerbach et al. 2005). After SUMO maturation, a heterodimer of SUMO activating enzyme 1 and 2 (SAE1/SAE2) contacts the C-terminal carboxy group of SUMO *via* a thioester bond (Gong et al. 1999). Activated SUMO is further transferred to the catalytic cysteine residue of SUMO conjugating enzyme 9 (UBC9) that provides SUMOs to the target sites in the substrate proteins (Desterro et al. 1997, Gong et al. 1997, Sampson et al. 2001). Finally, a ligation step is mediated by a limited number of SUMO ligases (E3), such as RanBP2, polycomp protein 2 (Pc2) and protein inhibitor of activated STATs (signal transducer and activator of transcription) proteins (PIAS). Endogenous SUMO E3 ligases are likely to function as enhancers of the conjugation process by regulating the interactions between substrate proteins, SUMO and UBC9 (Kotaja et al. 2002b, Pichler et al. 2002, Kagey et al. 2003). The SUMO E3 ligases may also play a role in SUMO-paralog selective modifications. Structural analyses have revealed that RanBP2 forms a more stable complex with UBC9/RanGAP1-SUMO-1 than with UBC9/RanGAP1-SUMO-2, which shields the SUMO-1 conjugated RanGAP1 from the deconjugating activities of SUMO proteases (Zhu et al. 2009, Gareau et al. 2012,).

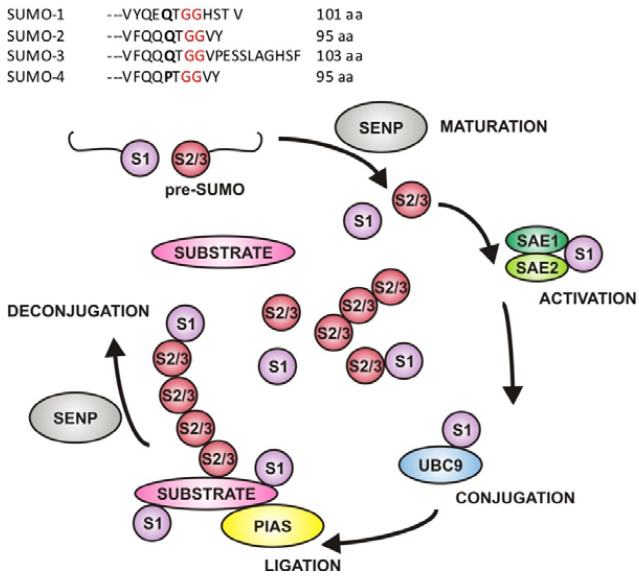

Figure 6. Reversible SUMO modification pathway. The upper part of the figure depicts the double glycine maturation site of human SUMOs.

SUMO acceptor lysines in target proteins are often, also in the AR, located within a consensus sequence ΨKXE/D, where Ψ is a hydrophobic amino acid (leucine, isoleucine, valine) and X is any amino acid followed by an acidic amino acid. An isopeptide bond is formed between the C-terminal glycine of SUMO and the ε-amino group of lysine. A mutation in the consensus sequence leads to impaired SUMOylation. However, SUMO acceptor lysines have also been identified at non-consensus sequences (Blomster et al. 2009). Similarly to ubiquitin, SUMO-2/3 is able to form polymeric chains (polySUMOylation) *via* the K11 residue located within the N-terminal SUMOylation motif (10-VKTE-13), a residue that is not present in the SUMO-1 sequence (Tatham et al. 2001). The formation of SUMO heteropolymers is possible, since SUMO-1 may be conjugated at the end of the SUMO-2/3 chain terminating the polymerization (Matic et al. 2008). SUMO-2/3 conjugation, but not SUMO-1, has been shown to be increased during cellular stress (Saitoh & Hinchey 2000, Vertegaal et al. 2006). Ubiquitin forms branched polymeric chains, but such properties are not reported for SUMOs, even though there are eleven and eight lysine residues in total in SUMO-1 and SUMO-2/3, respectively. Proteins can be modified in a SUMO paralog-selective manner. The charge of the surrounding amino acids close to the SUMO consensus motif and SUMO E3 ligases are believed to function as specificity factors (Tatham et al. 2005, Yang et al. 2006, Matic et al. 2010).

Distinct from the ubiquitin pathway, human cells express merely one SUMO E1 and SUMO E2 enzymes, and at present only a limited number of SUMO E3s have been identified. The physiological significance of UBC9 and intact SUMOylation pathway has been demonstrated with *Ubc9* knockout mice, as the deletion is embryonally lethal (Nacerddine et al. 2005). The mammalian family of PIAS proteins includes five members: PIAS1, PIAS3, PIAS4 (also PIASy), PIASxα (also ARIP3, androgen receptor interacting protein 3), and PIASxβ (also Miz1, Msx-interacting zinc finger protein 1) (Rytinki et al. 2009). The biological importance of PIAS proteins has been illustrated with knockout mice (Liu et al. 2004, Roth et al. 2004, Wong et al. 2004, Santti et al. 2005). However, in contrast to

the single SUMO E2, deletion of one PIAS protein has not severe consequences, apparently because PIAS family members may substitute for each other's functions. In contrast, *Drosophila melanogaster* contains only one PIAS ortholog (Zimp), and deletion in the genomic locus leads to severe chromosomal abnormalities and embryonic lethality (Hari et al. 2001).

PIAS proteins were originally identified as negative regulators of Janus kinase/STAT (JAK/STAT) pathway, and thus, they were named as protein inhibitors of activated STATs. However, today, in addition to cytokine signaling, PIAS proteins are emerging as important regulators of many cellular functions, including AR-dependent transcription. Interestingly, UBC9 and PIAS proteins, such as PIAS1, PIASxα/ARIP3 and PIASxβ/Miz1, have been identified as AR-interacting proteins, and coregulators of SR signaling (Poukka et al. 1999, Kotaja et al. 2000). In addition to their SUMO E3 ligase activity, the coregulatory effect of PIAS proteins in some cases is dependent on their ability to interact with DNA *via* amino terminal scaffold attachment factor-A/B, acinus and PIAS (SAP) domain. Therefore, PIAS proteins are considered to act as multiple scaffolding factors, as they are able to form both protein-protein interactions and protein-DNA interactions. PIAS proteins enhance SUMOylation *via* a cysteine rich Siz/PIAS RING (SP-RING) domain. In addition, PIAS proteins harbor a conserved SUMO-interacting motif (SIM) that is a hydrophobic motif rich in valine and isoleucine residues, interacting with covalently conjugated SUMOs (Kerscher 2007, Rytinki et al. 2009). SIMs and SUMOylation seem to mediate interaction networks between cellular proteins. In addition to PIAS proteins, also other AR coregulators contain SIMs (Moilanen et al. 1998a, Moilanen et al. 1998b, Tatham et al. 2008, de la Vega et al. 2011). Thus, the formation of transcription complexes that regulate the expression of AR target genes may be mediated by SUMOylation-dependent interactions.

2.2.5.2 Reversal of SUMO conjugates

SUMO modification is a dynamic and reversible reaction. Deconjugation of SUMOs is catalyzed by SUMO specific proteases (SENPs) (Mukhopadhyay & Dasso 2007, Yeh 2009, Kolli et al. 2010). Additionally, a newly identified DeSumoylating Isopeptidase 1 (DeSI-1) has been postulated to form a second class of SUMO proteases (Shin et al. 2012). Mammalian genome encodes six SENP enzymes: SENP1, -2, -3, -5, -6, and -7. The SENPs belong to the family of cysteine proteases sharing conserved C-terminal domains. In contrast, the N-terminal domains of SENPs vary in size and sequence and show no conservation. The N-terminal domain is likely to be responsible for substrate specificity and/or localization of SENPs, as at least SENP1 and -2 are known to contain the N-terminal NLS and NES (Bailey & O'Hare 2004). Moreover, in common with factors participating in the SUMO pathway, such as PIAS proteins, also SENPs contain SIMs. However, the SIMs have not been identified in SENP3 and -5, suggesting that properties such as the ability to form non-covalent interactions may contribute to the specificity of SENP action. Thus, the important role of SIMs is not only emerging in SUMO conjugation, but also in deSUMOylation processes, since SUMO conjugates that are bound to other SIM-containing proteins appear to be protected from SENPs (Zhu et al. 2009).

In addition to isopeptidase activity in deconjugating SUMOs, SENPs are involved in the maturation of SUMO precursors and edit polySUMO chains in modified substrates *via* their endopeptidase activity (Fig. 6 and Table 1). SENPs also display preferences in SUMO maturation and deconjugation of different SUMO paralogs. Accordingly, SENPs can be classified into three groups: (i) SENP1 and -2 show no preferences in processing different SUMO paralogs and they also are involved in the deconjugation of all SUMO paralogs (Gong et al. 2000, Hang & Dasso 2002, Zhang et al. 2002). (ii) SENP3 and -5 favour clearly SUMO-2/3 over SUMO-1 (Nishida et al. 2000, Di Bacco et al. 2006, Gong & Yeh 2006). (iii) SENP6 and -7 have also a preference for SUMO-2/3, but they are not involved in the

maturation process of SUMO precursors and they show minimal activity in deSUMOylation of substrate proteins. Instead, SENP6 and -7 efficiently edit polySUMO-2/3 chains (Mukhopadhyay et al. 2006, Shen et al. 2009). Moreover, SENPs vary in their cellular distribution and represent diverse endogenous expression patterns in different tissues. The differences in the major features of SENPs are summarized in Table 1.

Table 1. Summary of SENP characteristics.

	SENP1	**SENP2**	**SENP3**	**SENP5**	**SENP6**	**SENP7**
Aliases	SuPr-2	SuPr-1 AXAM2 SMT3IP2	SSP3 SMT3IP1	FKSG45	SUSP1 SSP1	
Size (aa)	643	589	574	755	1112	984
Subcellular localization	nuclear pore nuclear speckles	nuclear pore	nucleolus	nucleolus	nucleoplasm	nucleoplasm
High expression	testes				testes	
Activity						
maturation	S1>S2/3	S>S1>S3		S3		
deconjugation	S1, S2/3	S1, S2/3	S2/3	S2/3	S1	
chain editing					S2/3	S2/3

Similar to SUMO conjugation, the reversal of SUMOs by SENPs is essential in normal physiology. Knock-down of *Senp1* in mice impairs developmental processes (Yamaguchi et al. 2005). On the other hand, overexpression of SENP1 has been linked to the development of human PC (Cheng et al. 2006). The altered expression of different SENPs has been observed also in several other carcinomas: SENP1 in thyroid oncocytic adenocarcinoma (Jacques et al. 2005), SENP3 in prostate, ovarian, lung, rectum, and colon carcinomas (Han et al. 2010), and SENP6 in breast tumor tissue (Mooney et al. 2010). Furthermore, differential expression of SENP7 variants has been recently associated with breast cancer (Bawa-Khalfe et al. 2012). Thus, these findings highlight the importance of accurate balance in SUMO conjugation and deconjugation.

Because of the critical role of SENPs in normal physiology, it is believed that they are likely to be under stringent cellular control. However, the factors regulating SENP expression and activity have not yet been fully clarified. Transcription of SENP1 has been reported to be induced by the AR (Bawa-Khalfe et al. 2007). The activity of several SENPs has recently been shown to be diminished after heat stress, without there being alterations in the steady-state levels of SENPs (Pinto et al. 2012). Pinto and coworkers (2012) suggested that SENPs are intrinsically heat-sensitive and this property emerges from the catalytic domains of SENPs. Thus, it may be possible that catalytic cysteine residues in the SENPs participate also in sensing functions, e.g. responses to oxidative stress. On the other hand, SENP3 protein levels have been reported to be induced by reactive oxygen species (ROS) (Huang et al. 2009). Due to their deregulation in different cancers, SENPs are considered as potential drug targets. In particular, SENP1 inhibitors are being investigated in the development of new types of anticancer agents (Albrow et al. 2011, Qiao et al. 2011, Uno et al. 2012).

2.3.6 Cross-talk between different PTMs

PTMs have mostly been studied as individual events. The interplay between different modifications in multiple signaling pathways is emerging (Seet et al. 2006). As discussed above, different lysine modifications of the AR seem to be enriched in different functional domains of the receptor: SUMOylation in the NTD, acetylation and methylation in the hinge domain, and ubiquitylation in the LBD (Fig. 5). However, different PTMs are not likely to function in isolation, but rather there is cross-talk with each other establishing molecular diversity. For instance, phosphorylation can modify serine/threonine/tyrosine residues along the whole polypeptide in an androgen-enhanced manner. Androgens also enable receptor folding and promote the N/C interaction, which places the sites for different PTMs and their enzymatic machineries in close proximity with each other. Therefore, the biological three-dimensional structure is far more complex than can be visualized from a linearized model. Furthermore, different modifications may target distinct populations of the particular substrate. In other words, a single molecule may not be concomitantly modified by several PTMs. Different populations of a potential substrate, like the AR, may be modified according to its current localization and function.

The hinge region in the AR is the shortest domain. In relation to its size, the hinge might be the most heavily modified domain of the receptor, as the lysine residues in the hinge are in principle targets for both acetylation and methylation. Thus, these PTMs would seem to be the most obvious modifications to occur in a sequential order as they target the same sites in the AR. However, it has not been reported whether these two modifications compete for the same lysine and whether they are regulated in conjunction with each other. Androgen-induced AR acetylation by TIP60 has been linked to the nuclear import of the receptor (Shiota et al. 2010). Since the nucleus is rich in methyltransferases including SET9, the AR could be subsequently methylated. However, it is not known whether the other lysine modifiers may also target the same sites.

The AR has been proposed to be ubiquitylated and degraded in a phosphorylation-directed manner. The receptor is polyubiquitylated by the ubiquitin E3 ligase MDM2 and thereafter degraded in the proteasomes, but the exact site for conjugation of the polyubiquitin has not been identified. Akt (also protein kinase B, PKB) and provirus integration site for Moloney murine leukemia virus 1 (PIM-1) kinases have been shown to enhance serine phosphorylation of the AR, increasing the turnover rate of the receptor *via* the recruitment of MDM2 (Lin et al. 2002b, Linn et al. 2012). Furthermore, Linn and coworkers (2012) suggested that another PIM-1 isoform was also capable of modifing T850, which in turn recruited the ubiquitin E3 ligase RNF6 resulting in stabilization of the receptor. Both studies suggested that the S213 phosphorylation in the AR is important for the ubiquitylation by MDM2. Interestingly, there is a lysine residue in the +7 position in very close proximity to that particular phosphorylation site in the AR. Moreover, the T850 phosphosite is in the immediate vicinity of K845 and K847 ubiquitin sites which have been reported to be ubiquitylated by RNF6 (Xu et al. 2009). Another study showed that TFIIH, *via* its Cdk7 kinase subunit, phosphorylates the AR at the S515, which directed the receptor towards ubiquitylation by MDM2 (Chymkowitch et al. 2011). Here too, there is a neighboring lysine residue present close to the proposed phosphosite, and interestingly, the K520 has been identified as a SUMO consensus site in the AR (Poukka et al. 2000a). However, it is noteworthy that the target sites for different PTMs do not need to be adjacent in order to orchestrate the regulation of the same substrate.

Phosphorylation has been linked to SUMOylation in several studies. It may either induce conjugation of SUMO or inhibit SUMOylation machinery to interact with the substrate. Hietakangas and coworkers identified a phosphorylation-dependent SUMOylation motif (PDSM) that is present in several TFs and their coregulators (Hietakangas et al. 2006). However, concominant phosphorylation and SUMOylation of the AR has not been reported. Similarly, there are no reports of cross-talk between AR

SUMOylation and ubiquitylation. The discovery of RING finger protein 4 (RNF4; also small nuclear RING finger protein, SNURF) as a SUMO-targeted ubiquitin ligase (STUbL) has stimulated research interest in studying concomitant SUMOylation and ubiquitylation (Praefcke et al. 2012). RNF4 is a ubiquitin E3 ligase that recognizes SUMO moieties *via* SIMs and subsequently polyubiquitinates the SUMOylated substrate (Lallemand-Breitenbach et al. 2008, Tatham et al. 2008). RNF4 was originally identified as an AR-interacting coactivator (Moilanen et al. 1998b), but it has not been reported to polyubiquitylate AR-SUMO conjugates.

As discussed above, the PTMs directly targeting the AR are believed to engage in cross-talk with each other. It is noteworthy that many AR-interacting coregulators are also targets for PTMs, suggesting that the resulting modifications may act as bridging factors in forming transcriptional protein complexes. For instance, PIAS1 has been identified not only as a SUMO E3 ligase but also as an AR coactivator (Kotaja et al. 2000, Kotaja et al. 2002b); its SIM-dependent interactions are promoted by the phosphorylation of an extended SIM module (Stehmeier & Müller 2009). On the other hand, protein-protein interactions mediated by interactions between SUMO and SIM can be impaired by acetylation of the SUMO moiety (Ullmann et al. 2012). Thus, phosphorylation of a SIM may promote the SUMO-SIM interaction, whereas acetylation of SUMO can impair the formation of certain non-covalent interactions.

Dynamic PTMs are thought to function in the creation of a relay system that responds to alterations in the cellular environment, such as cellular stress (Deribe et al. 2010). Oxidative stress has been reported to modulate the SUMOylation/acetylation switch of the homeodomain-interacting protein kinase 2 (HIPK2) (de la Vega et al. 2012). HIPK3 (also androgen receptor-interacting nuclear protein kinase, ANPK) is a serine/threonine kinase family member of HIPK2 which acts as an AR coactivator without directly phosphorylating the AR (Moilanen et al. 1998a). HIPK1, -2 and -3 contain a conserved C-terminal SIM that colocalizes with the AR interaction domain found in HIPK3 (Moilanen et al. 1998a, de la Vega et al. 2011). In addition, similarly to HIPK2, HIPK3 contains a consensus SUMOylation motif in the N-terminal domain preceding the kinase domain. However, SUMOylation of HIPK3 has not been reported. It may be possible that these homeodomain-interacting proteins can modulate AR action in a SUMOylation-mediated manner during cell stress. In conclusion, dynamic PTMs are likely to optimize the activity of many biological processes, including AR-regulated gene expression, to guarantee the maintenance of cellular homeostasis.

2.3 PROSTATE CANCER

Prostate hyperplasia is a common benign disorder in elderly men and it can transform into invasive cancer. However, the development of prostate cancer (PC), which is the most prevalent carcinoma among western men, is not always either age-related or preceded by hyperplasia. The linkage of PC to advanced age presumably reflects the interplay of environmental, physiological, and molecular influences with the normal consequences of aging (Shen & Abate-Shen 2010). The development of PC is linked to chronic inflammation, which causes oxidative stress (Haverkamp et al. 2008, Klein & Silverman 2008). One of the major aging-associated influences on prostate carcinogenesis is the production of ROS, as a consequence of oxidative stress, which has a cumulative impact on DNA damage (Khandrika et al. 2009, Minelli et al. 2009). The application of next-generation sequencing methods has demonstrated a spectrum of DNA alterations in advanced PC. In particular, a high incidence of genomic alterations occurs in key genes important for DNA repair (Beltran et al. 2012). PC is a heterogenous disease that displays inherent molecular and

biological complexity. For instance, PC exhibits many phenotypical attributes, morphological heterogeneity and substantial changes in its genetic makeup both within and among prostate tumor foci. However, a dysfunction in the AR-mediated gene regulation remains the common denominator of divergent prostatic carcinomas (Culig et al. 2002, Heinlein & Chang 2004). Therefore, the following discussion will focus on the role of the AR in the development and progression of PC.

2.3.1 The role of AR

The systemic structure of cancer has been described by Hanahan & Weinberg (2011). Briefly, the development and progression of cancer is due to self-sufficience in growth signals, insensitivity to antigrowth signals, evading apoptosis, sustained angiogenesis, limitless replicative potential, and tissue invasion and metastasis. In the case of the prostate, the growth of the organ is dependent on androgens that are also the key components in the development of PC. Thus, altered steroid biosynthesis and androgen metabolism are likely to provide opportunities for pathological growth of prostatic cells (Sharifi & Auchus 2012, Green et al. 2012). Chromosomal translocations, such as transmembrane serine protease 2 / v-ets erythroblastosis virus E26 oncogene homolog (*TMPRSS2-ERG*) gene fusions, contribute to enhanced growth of PC cells by promoting the expression of an oncogene in an AR/androgen-dependent manner (Tomlins et al. 2005, Kumar-Sinha et al. 2008). Inhibition of the AR remains the key target in the treatment of advanced PC, and suppression of the AR also holds great potential for preventing the development or progression of early stage PC. Since the 1940s, endocrine therapy of prostate cancer has been directed toward the reduction of circulating androgens and consequently inhibition of AR transcriptional activity. Surgical or medical castration often supplemented with antiandrogen treatment is a common therapeutic strategy. After hormone deprivation therapy, however, the disease turns into a castration-resistant prostate cancer (CRPC), which is a major clinical challenge. In CRPC, the cancer cells have no need for testicular androgens to survive and proliferate, since the tumor tissue may express enzymes for androgen synthesis (Feldman & Feldman 2001, Montgomery et al. 2008, Shen & Abate-Shen 2010).

The mechanisms behind the transition of PC to castration resistant phase are likely to include the development of AR hypersensitive to low concentrations of circulating androgens, mutations in the AR leading to altered ligand-binding and coregulator interactions or receptor activation without androgens. In addition, activation of alternative survival pathways may be involved. Late-stage hormone-independent PC almost invariably retains the expression of the AR, despite the near absence of circulating androgens (Hobisch et al. 1995). Furthermore, an amplification of the *AR* gene has been commonly identified in CRPC (Visakorpi et al. 1995, Koivisto et al. 1997, Linja et al. 2001). Elevated expression of the receptor sensitizes the cancer cells to the growth-stimulating effects of low androgen concentrations (Gregory et al. 2001b). Moreover, AR overexpression contributes to the conversion of the antiandrogen bicalutamide from antagonist to agonist (Culig et al. 1999, Chen et al. 2004).

In addition to altered AR expression, AR mutations are common findings in PC patients. Altered AR splicing patterns have been proposed as a mechanism of prostate carcinogenesis and resistance to androgen ablation therapy (Dehm & Tindall 2011, Haile & Sadar 2011). For instance, the ARΔLBD isoform frequently expressed in PC contains intact NTD and DBD, but lacks ligand-binding ability. The LBD-truncated AR isoform is constitutively nuclear and binds DNA in a manner which is independent of androgens (Tepper et al. 2002, Libertini et al. 2007). Furthermore, single amino acid substitutions in the AR are associated with the risk of PC. For instance, the replacement of threonine 877 with alanine in AR LBD is a frequent mutation found in PC patients (Taplin et al. 1995). The LNCaP cell line also contains the T877A substitution, which allows the AR to be activated

by binding to other steroids such as cortisol, and even in the response to antiandrogens which start to promote PC cell growth (Taplin et al. 1999, Zhao et al. 2000). Similarly, a single amino acid substitution V715M in the AR allows receptor activation not only by testicular but also by adrenal androgens and progesterone (Culig et al. 1993). AR mutants T877S and H874S have been reported to be activated by flutamide (Fenton et al. 1997) and W741L/C by bicalutamide (Hara et al. 2003).

Altered expression or aberrant activity of the AR coregulators due to mutations may also be a contributing factor in the progression of PC (Heinlein & Chang 2004). For instance, overexpression of SRC-family members SRC1 and SRC2 (also glucocorticoid receptor-interacting protein 1, GRIP1) have been detected by immunohistochemical analysis (Gregory et al. 2001a). Interestingly, GRIP1 is a highly SUMOylated coactivator of the AR (Kotaja et al. 2002a, Kotaja et al. 2002c). Altered expression of SUMOylation pathway components have also been found in PC. Decreased expression of PIAS1 may be involved in the progression of PC, as the amount of PIAS1 mRNA was significantly lower in castration-resistant prostate tumors than in untreated tumors (Linja et al. 2004). In another study, PIAS1 protein levels have been reported to be significantly higher in malignant areas of clinical PC specimens than in normal tissues, which may enhance proliferation of PC cells through inhibition of p21 (Hoefer et al. 2012). Moreover, overexpression of SENP1 has been detected in both precancerous prostate lesions and PC tissue samples (Cheng et al. 2006). A recent study by the same researchers further indicated that SENP1 promotes PC progression and metastasis *via* regulation of hypoxia inducible factor 1α (HIF-1α)-induced expression of matrix metalloproteinase 2 and 9 (MMP2 and -9) (Wang Q. et al. 2012). Thus, components of the SUMOylation pathway may contribute to the proliferation of cancer cells and tumor invasiveness.

Even though prostate carcinogenesis may be independent of circulating androgens, it is not independent of the AR. The AR is involved in all stages of prostate tumorigenesis including initiation, progression, and treatment resistance. Therefore, the AR is a significant drug target for the development of novel therapeutics to the disease. Conceivable approaches to restrict PC progression by inhibition of the AR include, not only androgen ablation, but also restrain of AR levels, increasing nuclear export of the AR, and inhibition of chromatin binding of the holo-AR. Glucocorticoids have been provided to CRPC patients, because these agents inhibit AR expression by repressing the action of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) that enhances AR expression and the growth of PC cells (Zhang et al. 2009). In addition, glucocorticoids are able to decrease adrenal androgen production (Fakih et al. 2002, Kassi & Moutsatsou 2011). Today, androgen biosynthesis can be effectively inhibited by abiraterone that functions as an inhibitor of the cytochrome P450 family member CYP17A1 (Attard et al. 2009, Stein et al. 2012). Cyproterone acetate was the first antiandrogen identified. It is steroidal in structure and functions as a competitive AR antagonist similar to non-steroidal compounds flutamide and bicalutamide. Modern types of antiandrogens, RD162 and MDV3100 (enzalutamide), impair AR interactions with coregulators and chromatin but also the nuclear import of the receptor/ligand complex (Tran et al. 2009, Scher et al. 2010, Haendler & Cleve 2012). Therapies that target the AR LBD have no effects on constitutively active splice variants of the AR, which are often C-terminally truncated. Therefore, small-molecule inhibitors that bind to the AR NTD have been developed, such as EPI-001 (Andersen et al. 2010). In addition, there is on-going development of the AR ligands with tissue-selective effects, which are classified as specific androgen receptor modulators (SARMs) (Haendler & Cleve 2012). Nonetheless, new pharmacological compounds that target the AR with improved properties in terms of efficacy, differentiation and side-effects are needed to prevent PC progression.

3 *Aims of the Study*

Receptor interactions with androgens, chromatin and coregulatory proteins are essential for the transcriptional activity of the AR. Different PTMs are emerging as being important in regulating the expression of AR target genes, since they can modify several transcriptional components. However, the role of SUMO modifications in the modulation the AR action is still not fully established. Therefore, this doctoral thesis was aimed at examining the impact of SUMO modifications on AR-dependent transcription. The study explores whether SUMOylation pathway could be targeted to restrict the AR activity and the cell growth of PC cells. Thus far, studies investigating SUMOylation of the AR have concentrated on the transient expression of proteins using ectopically expressed reporter genes to measure the effect of SUMOylation on the AR activity. Here, the major aims were to analyze SUMOylation of endogenous AR in PC cells and to study AR function in the chromatin landscape of PC cells.

The specific aims of the study were:

- To uncover cellular signals that affect SUMO modifications of the AR, particularly the endogenous AR in PC cells.
- To examine the reversibility of AR SUMOylation by SUMO specific proteases (SENPs).
- To characterize the effect of SUMOylation on the AR-chromatin interactions and the expression of AR target genes, and thus, the growth of PC cells.

4 Materials and Methods

A wide range of cell and molecular biology methods was utilized in this thesis (Table 2). Detailed experimental procedures have been described in the original articles I-III. In addition, detailed protocols for analysis of AR activity and the receptor SUMOylation have been described by our group (Makkonen et al. 2011, Rytinki et al. 2011).

Table 2. Summary of the methods used in this thesis.

Method	**Original article**
Cell culture	I, II, III
Construction of plasmids and site-directed mutagenesis	I, II
Transient transfection assays	I, II, III
Immunoprecipitation	I, II, III
Immunoblotting	I, II, III
Biotin pull down assay	III
In vitro SUMOylation and deSUMOylation assays	I
Reporter gene assays	I, III
Isolation of RNA	I, II, III
Quantitative RT-PCR	I, II, III
RNA interference	I
Immunofluorescence and confocal microscopy	I, II, III
Fluorescence recovery after photobleaching (FRAP)	II
Chromatin immunoprecipitation (ChIP)	I, II, III
Electrophoretic mobility shift assay (EMSA)	III
Cell proliferation assay	I
Generation of cell lines stably expressing AR	II, III

5 Results and Discussion

5.1 SUMO PARALOG-SELECTIVE MODIFICATIONS OF THE AR

Human cells express three SUMO isoforms capable of conjugation to target proteins: SUMO-1 and the nearly identical forms SUMO-2 and -3, which are collectively named as SUMO-2/3. AR has been previously shown to be modified by SUMO-1 (Poukka et al. 2000a), but the formation of AR-SUMO-1 and AR-SUMO-2/3 has not been compared previously. Fig. 1A in original article I illustrates that transiently expressed AR was modified in a SUMO paralog-selective manner by ectopically expressed SUMOs in COS-1 cells: AR was modified to a greater extent by SUMO-1 than SUMO-2. However, when the relative expression of the endogenous SUMOs was studied, it was found that SUMO-2/3 was more abundant than SUMO-1 in the AR positive vertebral-cancer of the prostate (VCaP) cells (Appendix Fig. A1 in II). A similar SUMO expression pattern has been observed in different mouse tissues (Zhang et al. 2008). Endogenous SUMOylation of the AR was analyzed by co-immunoprecipitation assays with anti-AR antibody followed by subsequent immunoblotting against SUMOs. Interestingly, AR-SUMO-2/3 conjugates dominated in VCaP cells (Fig. 1A in II). SUMO paralog-selective modification has been shown to be determined at the level of deconjugation (Zhu et al. 2009). It appeared probable that AR-SUMO-2/3 conjugates might be processed more rapidly than SUMO-1 modified forms of AR. In line with this concept, AR SUMOylation assay using deconjugation defective forms of SUMO-1 and -2 (SUMO-1P and SUMO-2P) showed that AR-SUMO-2 conjugates are, indeed, more prone to deconjugation than AR-SUMO-1 conjugates (Appendix Fig. A2 in II).

As previously shown (Poukka et al. 2000a), the formation of AR-SUMO-1 conjugates is dependent on the intact SUMOylation consensus lysines (K) 386 and 520 in the AR, the former being the major one. Mutation of these lysines to arginines (R) impairs AR SUMOylation (Poukka et al. 2000a). Interestingly, promyelocytic leukemia protein (PML) is modified by SUMOs at three lysine residues, which are modified by SUMO-1 or SUMO-2/3 to different extents (Kamitani et al. 1998, Gong & Yeh 2006), suggesting that SUMO acceptor sites may independently show a preference for different SUMO paralogs. However, the discrete SUMOylation consensus sites in the AR showed similar SUMO paralog-selectivity (Fig. 7).

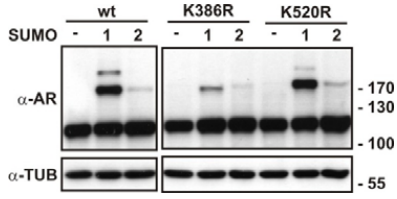

Figure 7. Disruption of the single SUMOylation lysine (K386R or K520R) in the AR shows similar SUMO paralog-selectivity as the wild-type (wt) receptor. COS-1 cells were cotransfected with expression vectors encoding wtAR, ARK386R or ARK520R with or without SUMO-1 or SUMO-2 as indicated. After 40 h transfection, cells were treated with 100 nM testosterone for 2 h and lysed in denaturing SDS buffer containing N-ethylmaleimide (NEM, 10 mM). The cell extracts were analyzed by immunoblotting with anti-AR and anti-tubulin (TUB) antibodies.

Thus far, no one has reported that any modifiers other than SUMOs would target K386 and/or K520 in the AR. However, this remains a potential possibility. Therefore, the SUMO consensus motifs in the AR were mutated by replacing the glutamate (E) residues 388 and 522 to alanine (A), while K386 and K520 remained intact. As shown in Fig. 8A, SUMO modifications of the E388,522A-mutated receptor were impaired similarly to those of the ARK386,520R (Poukka et al. 2000a). In addition, the E388,522A-mutated AR was significantly ($p \leq 0.001$) more active than wtAR (Fig. 8B), which is in line with the ARK386,520R data of Poukka and coworkers (2000a). Importantly, K-to-R mutations preserve the net-charge of the receptor, and additionally, the size of the particular amino acid residue is not significantly altered. In contrast, E-to-A mutations lead to a change both in the charge and the length of the amino acid side chain. Based on results of the mutation analysis, it is not likely that K386 and K520 were modified by other PTMs. Thus, the ARK386,520R was used as a model to study SUMOylation-deficient AR in this thesis.

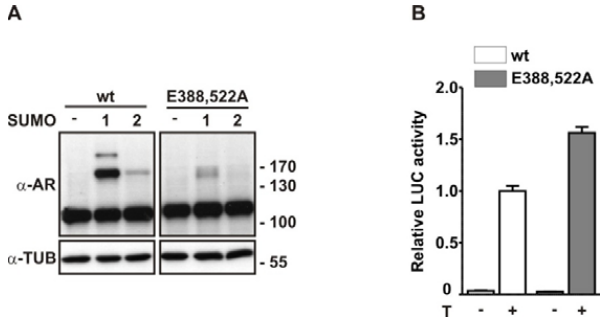

Figure 8. Mutation of the SUMOylation consensus motifs in the AR impairs receptor SUMOylation and enhances the AR transcriptional activity. A. COS-1 cells were cotransfected with expression vectors encoding wtAR or the E388,522A-mutated receptor with or without SUMO-1 or SUMO-2 as indicated. After 40 h transfection, cells were treated with 100 nM testosterone for 2 h. The cell extracts were prepared and analyzed as described in Fig. 7. B. COS-1 cells were cotransfected with a pARE2-TATA-luc together with expression vectors encoding wtAR or ARE388,522A. One day after transfection, cells were incubated in the presence or absence of testosterone (T, 100 nM) for 18 h. LUC and β-galactosidase (a control assessing for transfection efficiency) activities were measured. Relative LUC activity of wtAR in the presence of T is set as 1 and the other values are in relation to that value. The columns represent the mean ± SD values of a representative experiment with triplicate samples.

5.2 SIGNALS AFFECTING AR SUMOYLATION

5.2.1 The role of ligand and subcellular localization

Androgen binding induces a conformational change, nuclear translocation and transcriptional activation of the AR. In addition, several PTMs, the small-molecule modifiers in particular, are known to modulate AR action. Modulation of the AR by a small-protein modifier, SUMO, was poorly understood at the start of this thesis project. AR is a modular protein, with the receptor conformation being altered by hormone binding. It was interesting to study whether agonistic/antagonistic properties of the AR ligands could influence conjugation of SUMOs to the NTD of the receptor. Different AR ligands were compared for their abilities in promoting the ectopic SUMO-1 modification of the AR in intact cells. Agonist-induced conformation of the AR favoured receptor SUMOylation (Fig. 1E in I). Androgen-enabled SUMO modification of the endogenous AR was also detected in

VCaP cells reflecting agonist induced formation of AR-SUMO conjugates in PC cells (Fig. 1A II).

The androgen-bound AR is concentrated into the nucleus. It could be postulated that nuclear translocation is a premise for receptor SUMOylation. However, AR occupied by antagonist bicalutamide was also efficiently localized to nuclei (Poukka et al. 2000b), but after nuclear localization, it was poorly SUMOylated (Fig. 1E in I). The cellular localization of the AR was evoked by either the androgen-independent nuclear localization signal (NLS) or the nuclear export signal (NES); these two forms displayed only negligible differences in the extent of receptor SUMOylation (Fig. 1D in I). In addition, LBD-deficient AR (AR1-657), where the SUMO consensus sites in the NTD were intact, displayed no SUMOylation (Fig. 1C in I), even though it is constitutively found in the nucleus (Poukka et al. 2000b). Forced expression of PIAS1 did not rescue the lack of ARΔLBD SUMOylation suggesting that deletion of the LBD may disrupt interaction surfaces for SUMO E3 ligases. AR LBD contains two hydrophobic sequences, 713-VxVV-716 and 815-IIxV-818, that may be possible SIMs. Intact SIMs in a particular SUMO substrate have been shown to be important for SUMOylation of the molecule (Lin et al. 2006, Karvonen et al. 2008). Interestingly, the AR mutation V715M has been detected in advanced PC (Culig et al. 1993).

5.2.2 Phosphorylation and acetylation

In addition to increased nuclear accumulation and SUMO modifications of the AR, androgens can induce phosphorylation of the receptor (Kuiper et al. 1991). The hormone-induced kinetics of AR phosphorylation (Gioeli et al. 2002) are similar to that of SUMOylation (Fig. 1B in I). Previously it has been demonstrated that phosphorylation regulates SUMOylation of heat-shock factor 1 (HSF1) and STAT1 (Hietakangas et al. 2003, Hietakangas et al. 2006, Vanhatupa et al. 2008). The effect of several AR phosphosites on receptor SUMOylation was examined as described in original article I, but these sites proved to have no major impact on the SUMOylation of the AR (Fig. 2A in I and data not shown). MAPK signaling is known to regulate AR S650 phosphorylation (Gioeli et al. 2006). As shown in Fig. 2A in I, co-transfection of p38 MAPK and a constitutively active form of MAPK kinase 6 (MKK6E) impaired AR SUMOylation. However, this effect was indirect because also the extent of SUMOylation of the phosphorylation-deficient receptor mutant ARS81,94,256,308,424A (AR5A) was reduced (Fig. 2A in I). These results demonstrated for the first time that the same AR molecule can be concomitantly SUMOylated at K386 and phosphorylated at S650 (Fig. 2B in I).

It was also studied whether the acetylation sites in the hinge region of the receptor could regulate AR SUMOylation, since acetylation may orchestrate with SUMOylation (Yang & Sharrocks 2004). Point mutation of the lysines 630, 632, and 633 to arginines did not alter the extent of receptor SUMOylation, whereas deletion of the residues 629-633 blunted AR SUMOylation (Fig. 2C in I). This deletion may disrupt not only nuclear import of the AR (Poukka et al. 2000b), but also contacts with interaction partners of the receptor, like PIAS1 and UBC9 (Poukka et al. 1999, Tan et al. 2002). The intact hinge region is likely to confer flexibility on the receptor to undergo ligand-induced folding, which may enable the SUMOylation to occur. HDACs have been suggested to stimulate SUMOylation, particularly the conjugation of SUMO-2 (Gregoire & Yang 2005, Zhao et al. 2005). HDAC4 has been reported to enhance the formation of AR-SUMO-1 conjugates (Yang et al. 2011). In the experimental conditions employed in this study, co-expression of HDAC4 did not influence the formation of either AR-SUMO-1 or AR-SUMO-2 conjugates in COS-1 cells (data not shown). In conclusion, even though acetylation sites in the AR and HDAC4 did not affect the SUMOylation of the AR, it is evident that the hinge region of the AR is involved in the regulation of receptor SUMOylation.

5.2.3 Proteasome inhibition and cell stress

The steady-state level of endogenous SUMO conjugates is very low, and this extents also to SUMOylated AR (Fig. 1A in I). SUMOylation has been also proposed to label cellular proteins for ubiquitylation and degradation (Uzunova et al. 2007). Inhibition of proteasomes could lead to stabilization of SUMO conjugated proteins, also the AR in PC cells. As shown in Appendix Fig. A3 in II, a massive accumulation of slowly migrating AR-immunoreactive species was seen in VCaP cells treated with the proteasome inhibitor, MG132. Furthermore, other type of proteasome inhibitors, lactacystin and epoxomycin, had the same effect (data not shown). The tested compounds belong to different classes of proteasome inhibitors. MG132 is a member of the group of peptide aldehydes that are reversible inhibitors. Lactacystin and epoxomicin are irreversible inhibitors with the former being a β-lactone and the latter an epoxyketone. They all share the preference for inhibition of proteasome subunit β5 over β1 or β2. However, they are not specific proteasome inhibitors. For instance, MG132 is known as an inhibitor of calpain and other cysteine proteases (Moore et al. 2008). Blockage of the proteasome function leads to accumulation of protein aggregates, which activate cellular stress-responsive machinery to dampen proteotoxic stress (Anckar & Sistonen 2011). The cell stress is also likely to lead to enhanced SUMO conjugation (Saitoh & Hinchey 2000, Hong et al. 2001).

To test the effect of diverse types of cell stress on AR SUMOylation, VCaP cells were exposed to heat stress, osmotic stress, heavy metal ions, oxidative stress, and electrophilic stress. Interestingly, all these stresses provoked AR SUMOylation, but of slightly different magnitudes (Fig. 1B and Appendix Fig. A3 in II, and Fig. 1A in III). Immunoprecipitation of the AR with anti-AR antibody and immunoblotting the eluates with anti-SUMO and anti-ubiquitin antibodies indicated that the AR conjugates contained mainly SUMO-2/3 and less SUMO-1 or the closely related ubiquitin (Fig. 1D in II, and Fig. 1B in III). Stress-induced accumulation of AR-SUMO-2/3 conjugates was largely dependent on K386 and K520 in the AR as revealed in isogenic HEK293 cell lines stably expressing wtAR or ARK386,520R (Fig. 2A in II). ARK386,520R still displayed some signs of SUMO-2/3 modifications under heat stress, suggesting that the AR may contain secondary SUMO acceptor lysines. Furthermore, the data with the SUMOylation-deficient AR indicated that ubiquitin is not likely to conjugate at K386 and K520, since ARK386,520R displayed increased ubiquitylation relative to that found with wtAR. In agreement with this concept, ARK386,520R underwent faster degradation than wtAR after addition of the protein synthesis inhibitor, cycloheximide (CHX) (Fig. 2B in II). The AR mutant containing a deletion in the hinge region appeared to be unable to undergo in SUMOylation (Fig. 2C in I). A similar AR mutant has been shown to be more sensitive to proteasome-mediated degradation than the wtAR (Tanner et al. 2004). To conclude, SUMOylation does not prime the AR for degradation.

Stress-triggered protein modifications by SUMO-2/3 have recently been reported to occur in different cell types. For example, hypothermia has been shown to induce nuclear accumulation of SUMO-2/3 conjugates in neurons. This reversible phenomenon has been postulated to act as an endogenous neuroprotective stress response (Datwyler et al. 2011, Wang L. et al. 2012). In addition, cellular stress has been shown to induce SUMO-2/3 modifications in testes (Shrivastava et al. 2010). Excessive SUMO-2/3 conjugation has also been found in sperm as a marker of defective spermatozoa (Vigodner et al. 2013). These observations indicate that dynamic SUMOylation may have protective quality-control functions in diverse cell types, which is particularly important in germ cells.

Stress kinase signaling has been shown to increase phosphorylation of the AR (Gioeli et al. 2006). As discussed above, the effect of forced MAPK signaling on AR SUMOylation was studied only with ectopic SUMO-1. Since it was found that cell stress enhanced particularly accumulation of the SUMO-2/3-modified AR species, SUMO-2 modification of the AR5A mutant was compared to that of the wtAR in transfected COS-1 cells. However, these phosphosites had no effect on the accumulation of AR-SUMO-2 conjugates in cells

exposed to 30-min heat stress (data not shown). Since new phosphorylation sites in the AR have been recently identified, the stress-induced interplay between phosphorylation and SUMOylation of the AR cannot be excluded.

5.2.4 SUMO proteases

Deconjugation of SUMO, deSUMOylation, is catalyzed by SUMO specific proteases (SENPs) that belong to the family of cysteine proteases. Firstly, the isopeptidase activities of five mammalian SENPs in deconjugating different SUMO paralogs were examined (Fig. 3A in I). The effects of SENPs were demonstrated to be dependent on intact catalytic domains of the enzymes by mutating the specific cysteine residues into serines. In line with the findings published by other research groups (Gong & Yeh 2006, Shen et al. 2006), SENP1 and SENP2 were potent in deconjugating both SUMO-1 and SUMO-2 from cellular proteins, while SENP3 and SENP5 showed clear preference towards SUMO-2-conjugated proteins. Secondly, the isopeptidase activity of the SENPs toward AR-SUMO-1 conjugates was compared in intact cells and *in vitro* (Fig. 3B and C in I). Similar to total cellular deSUMOylation, SENP1 and SENP2 were found to be efficient in reversing AR-SUMO-1 conjugates. These proteases interacted with the AR in co-immunoprecipitation assays (data not shown), and they also showed nuclear colocalization with the holo-AR as visualized by fluorescent confocal microscopy (Fig. 4 in I).

Stress-triggered AR SUMOylation was found to be reversible in VCaP cells, i.e. alleviation of the stress led to the disappearance of AR modifications. When heat-stressed cells were transferred back to 37°C, the AR conjugates were no longer detected (Fig. 1C in II). The findings indicated that endogenous SUMO modifications of the AR are dynamic and readily adjusted in response to changes in cellular environment. As discussed, the activity of SENPs is important for the reversal of AR-SUMO conjugates. MG132-induced accumulation of endogenous AR-SUMO conjugates in VCaP cells may be partly due to the inhibition of cysteine proteases, including SENPs. Thus, cellular stress (heat stress was used as a model stress in the study) could inhibit SENP activities allowing SUMOylated AR to accumulate in VCaP cells. An assay employing a hemagglutinin-tagged SUMO-2 conjugated to vinyl sulfone backbone (HA-SUMO-2-VS) was performed. Briefly, this assay is based on the ability of HA-SUMO-2-VS to covalently and specifically react with the nucleophilic cysteine residue within the active sites of cellular SENPs (explained in detail in: Mukhopadhyay et al. 2006, Wang Y. et al. 2009). Vinyl sulfone reaction products are detected by immunoblotting with an anti-HA antibody. As shown in Fig. 9, the signal of reaction products indicating SENP activities was weaker in heat-stressed cells than in control cells. The massive increase in AR SUMOylation evoked by cellular stress is not likely solely due to inhibition of SENP activity, since AR modifications by deconjugation-deficient SUMO-2 (SUMO-2P) were not further enhanced in transfected COS-1 cells upon heat stress (data not shown). There may be specific differences between SENP activities in cell stress (Yan et al. 2010, Pinto et al. 2012). In addition, SUMO E1 activity has been proposed to be modulated upon heat shock (Truong et al. 2012). The study by Truong and coworkers (2012) demonstrated that the SAE2 subunit is autoSUMOylated under normal conditons, which prevents E1-E2 interaction and the transfer of SUMO from E1 to E2 and the overall SUMO conjugation to target proteins. Heat shock reduced SUMOylation of SAE2 leading to an increase in global SUMOylation. SUMOylation of SAE2 was proposed as a mechanism for storing a population of E1 that can be readily activated in response to environmental changes. SUMOylated SAE2 may also act as a reservoir for SUMOs, since endogenous non-conjugated SUMOs are poorly observable within immunological detection limits (Appendix Fig. A1 in II).

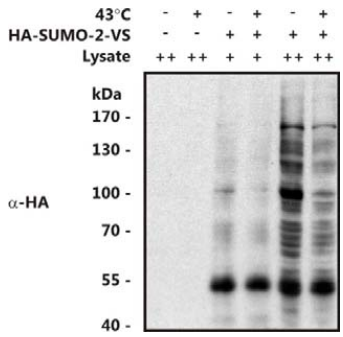

Figure 9. Heat stress inhibits SENPs in VCaP cells as studied by vinyl sulfone (VS) assay. The cells were exposed to 43°C for 1 h as indicated and lysed. Different amounts of lysates (+ and ++ with 5-fold difference) were incubated with 5 μM final concentration of HA-SUMO-2-VS (Boston Biochemicals). After a 15-min incubation at 20°C the reactions were stopped and analyzed by immunoblotting with anti-HA antibody.

5.3 REVERSIBLE SUMO MODIFICATIONS MODULATE TRANSCRIPTIONAL ACTIVITY OF THE AR

AR signaling is mechanistically similar to that occurring in the other SRs. SUMOylation consensus sites in the NTDs of SRs are well conserved, and many components of the SUMOylation pathway have been identified as coregulators of SR signaling (Poukka et al. 1999, Kotaja et al. 2000, Abdel-Hafiz & Horwitz 2012). It is likely that SUMOylation functions as a common regulator of the SR activity. PIAS1 is the major PIAS protein in VCaP cells, and it is up-regulated by androgens as assessed by analysis of mRNA expression (Appendix Fig. A5 in II). Endogenous AR and PIAS1 interact in VCaP cells, and PIAS1 enhances SUMOylation of the holo-AR (unpublished data). The activating effect of PIAS1 on AR-regulated transcription is dependent on intact SUMOylation sites in the receptor, as well as on the E3 SUMO ligating and SUMO interacting properties of PIAS1. An intact SUMOylation pathway may be important in the modulation of AR-dependent transcription, since overexpression of PIAS1 and SENP1 have been found in PC (Cheng et al. 2006, Hoefer et al. 2012).

The AR containing the single K386R mutation showed similar activity as ARK386,520R (Poukka et al. 2000a), but the SUMOylation deficient AR showed enhanced activity compared to wtAR only on a promoter gene driven by more than one AREs (Fig. 5B in I). These results suggest that multiple AR contacts with DNA are more important for transcriptional inhibition than the presence of several SUMO modified sites in the receptor. Similar results have been shown for the GR that contains a protein motif called the synergy control motif (Iniguez-Lluhi & Pearce 2000, Tian et al. 2002). Interestingly, the particular sequence found in the GR was identified at the same time as a SUMO consensus motif in the AR (Poukka et al. 2000a). Thus, mutations in the SUMO consensus sites have been initially shown by two independent studies to derepress the synergistic function of SR homodimers binding to compound response elements. The synergism has been demonstrated to depend on the nature of the response elemets: AR dimers binding to compound response elements display synergism only on canonical IR3 AREs but not on selective AREs organized as DR3 (Callewaert et al. 2004).

SENP1 and SENP2 are efficient enzymes in the abilities to deconjugate SUMOylated AR. Thus, the effect of SENPs on the AR-regulated transcription was studied. As shown in Fig. 5A in I, both SENPs activated wtAR, while the effect of SENPs was only modest on the transcriptional activity of the SUMOylation-deficient AR. In line with the SUMOylation data, the activity of ARΔLBD was not affected by SENP1 (Fig. 5C in I). The enhanced AR activation was dependent on the presence of intact catalytic domains of the SENPs and compound AREs in the reporter gene (Fig. 5A and B in I). The SENP-enhanced synergistic action of the AR dimers was seen only on canonical AREs (Fig. 10), which is in accordance with the SUMOylation site data by Callewaert & coworkers (2004).

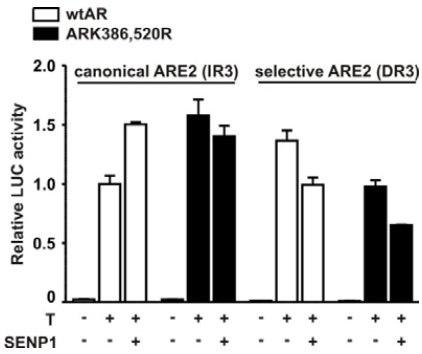

Figure 10. SENP1 enhances the synergism of AR dimers only on canonical AREs. COS-1 cells were cotransfected with LUC reporter vectors containing either canonical IR3 AREs or selective DR3 AREs (Callewaert et al. 2004) together with the expression vectors encoding wtAR, ARK386,520R and SENP1 as indicated. One day after transfection, cells were grown in the presence or absence of testosterone (T, 100 nM) for 18 h. LUC and β-galactosidase activities were measured. Relative LUC activity of wtAR in the presence of T and in the absence of SENP1 on canonical AREs is set as 1 and the other values are in relation to that value. The columns represent the mean ± SD values of a representative experiment with triplicate samples.

SENPs increased also the transcriptional activity of endogenous AR in LNCaP prostate cancer cells (Fig. 6A-D in I). Conversely, silencing of SENPs by specific siRNAs (Fig. 7A in I) attenuated the expression of endogenous AR target genes in LNCaPs (Fig. 7B-D in I). The effects of ectopically expressed SENP1 and SENP2 on AR activity in LNCaPs differed in their magnitude depending on the reporter construct being examined. Both SENP1 and SENP2 clearly induced expression of a reporter gene driven by two synthetic AREs (pARE2-TATA-luc), whereas the SENP1 had only a modest and the SENP2 had no effect on a reporter gene harbouring a natural promoter of the rat probasin gene [pPB(-285/+32)-luc] (Fig. 6B and D in I). Ectopic SENPs showed cell line-specific features in their activities (cf. panels A in Fig. 5 and Fig. 6 in I).

SENP1 has previously been postulated to enhance the AR activity through deSUMOylation of HDAC1 (Cheng et al. 2004). The data in original article I indicates that the SENP1-enhanced AR activity is due to SENP1's direct catalytic mode of action on AR. The HDAC inhibitor, TSA, activated wtAR and a similar stimulation was observed with ARK386,520R (Fig. 5D in I). In addition, the coexpressed SENP1 still enhanced the activity of the wtAR but not that of the SUMOylation-deficient AR with TSA exposure. If the SENP1 was to influence the AR activity indirectly *via* deSUMOylation and regulation of

HDAC1 deacetylase activity, administration of TSA would be predicted to block the stimulatory effect of SENP1 on the AR. Similar results have been shown for ETS-like transcription factor 4 (ELK4) during TSA exposure (Kaikkonen et al. 2010).

It is noteworthy that not only the AR but also its coregulators can be SUMOylated. AR SUMOylation may be involved in the recruitment of transcriptional coregulators. Different cell lines display their own distinct compositions of coregulators, which might account for the cell-specific outcome of AR activity modulated by SENPs. For instance, AR repression has been proposed to be mediated by DAXX (death-domain associated protein) (Lin et al. 2004). In addition, the formation of repressive chromatin structure by the nucleosome remodeling ATPase Mi2α and the histone methyltransferase SETDB1 has been postulated to be involved in the inhibition of SUMOylated TFs (reviewed by Ouyang & Gill 2009). However, this is likely not to be the case in AR-dependent transcription. Both DAXX and Mi2α inhibited AR in reporter gene assays, but the inhibition was not dependent on the SUMOylation of the AR, while SETDB1 had no effect on wtAR or ARK386,520R (data not shown). Similar DAXX-independent results have been reported for SUMO-mediated inhibition of GR activity (Holmstrom et al. 2008). In contrast to previously published data (Lin et al. 2006), the repressing effect of DAXX on AR was not dependent on intact SIM of DAXX.

Based on the results discussed above, it is obvious that SUMO modifications regulate AR action. However, the exact molecular mechanism is unclear. It is important that intact SUMOylation sites do not inhibit DNA binding of the receptor as analyzed by different assays. Promoter interference assay proved that ARK386,520R does not show increased binding to canonical AREs in intact cells (Poukka et al. 2000a). Electrophoretic mobility shift assay (EMSA) showed that both the SUMOylation-deficient AR and the receptor which had been strongly modified by SUMO-2P both display similar abilities to bind a synthetic ARE oligomer (data not shown). In addition, the ChIP assay indicated that the activity of SENPs does not alter the occupancy of the AR on chromatin, since silencing of SENP1 did not alter loading of the AR onto the regulatory regions of endogenous AR target genes in LNCaP cells (Fig. 7E-G in I). However, SUMOylation is evidently involved in the dynamics of AR-chromatin interactions, since the holo-AR and components of the SUMOylation pathway co-occupied the regulatory regions of the AR target genes in VCaP cells (Fig. 6 in II).

In an attempt to study the action of wtAR and SUMOylation-deficient receptor in chromatin lansdcape, isogenic HEK293 cell lines stably expressing wtAR or ARK386,520R were utilized. Analysis of these cell lines showed the receptor SUMOylation had a target gene specific effect on AR chromatin loading and androgen induction of different AR-regulated genes (Fig. 7 in II). Importantly, SUMOylation-deficient AR did not invariably display higher transcriptional activity than wtAR as was the case in the assays based on the reporter genes containing synthetic AREs. The modulatory effect of SUMOylation on the AR activity in the chromatin environment is in line with the observations noted in SUMOylated TFs GATA-1 and microphthalmia-associated transcription factor (MITF), the first expressed in hematopoietic cells and the latter in both melanoma and renal carcinoma (Lee et al. 2009, Bertolotto et al. 2011, Yokoyama et al. 2011). These studies concerning the different TFs operating in different types of tissues reveal that various genes, in addition to being located in different nuclear territories and regulated in an individual manner, are also distinctly dependent on SUMOylation. For instance, genes shown to be sensitive to SUMOylation of the NR family member steroidogenic factor 1 (SF-1) include hedgehog signaling pathways (Lee et al. 2011). PR SUMOylation modulates the expression of genes involved in proliferative and pro-survival signaling pathways (Knutson et al. 2012). It remains unclear whether genes affecting cell growth could also be sensitive to SUMOylation in other NRs. That might be an interesting issue in the context of AR-regulated growth of PC cells.

SUMOylation modulates co-operation of GATA-1 with its coregulator friend of GATA-1 (FOG-1) in a target gene-selective manner (Lee et al. 2009). The difference between wtAR and ARK386,520R in transcription may arise from the gene-dependent vicinity of the other cell-specific TFs, such as FOXA1. FOXA1 is known to interact with the AR contributing to the expression of AR-regulated genes (Gao et al. 2003, Lee et al. 2008). The expression of FOXA1 is cell lineage-specific, and the factor has a dual role in AR action either increasing or decreasing AR chromatin binding (Sahu et al. 2011). The cell- and gene-specific effects of SUMOylation on the AR activity may be partly due to FOXA1-mediated regulation. FOXA1 is thought to contribute to PC progression, since its activity is essential for AR-dependent expression of important genes in cell proliferation and survival (Wang Q. et al. 2009).

An intact SUMOylation pathway was also demonstrated to be important for PC cell growth (Fig. 6H in I). The growth of SENP1-silenced LNCaP cells was significantly retarded in the presence of androgen in comparison with the control siRNA-transfected cells. SENP1 silencing had no significant effect on the cell growth in the absence of the androgen. Taken together, these results emphasize the importance of an intact SUMO modification pathway and tightly controlled expression of AR target genes in the regulation of PC cell growth.

5.4 EFFECT OF CELL STRESS ON THE AR ACTIVITY

As presented in this study, signals that affected AR SUMOylation modulated AR activity. The effect of cell stress on AR-dependent transcription was also analyzed in VCaP cells. Heat stress suppressed accumulation of AR target gene mRNAs (Appendix Fig. A4 in II), and similarly to the formation of AR-SUMO conjugates, the attenuation in mRNA expression was restored at 37°C. Consistent with mRNA expression, loading of AR onto regulatory regions of AR target genes was altered in a reversible manner by heat shock as assessed by quantitative-ChIP (Fig. 5 in II). The accumulation of *S100P* mRNA showed faster recovery after a 30-min heat shock as compared to *TMPRSS2* and *SPOCK1*. The relative amount of *S100P* mRNA in heat-stressed VCaPs recovered within 2 hours to the expression level measured in non-stressed cells (Appendix Fig. A4 in II). The analysis of the AR loading onto the regulatory region of *S100P* after heat shock was indicative of an enhanced AR occupancy as compared to that before the stress (Fig. 5 in II). That may be associated with the importance of *S100P* for cancer cell survival and proliferation. Indeed, overexpression of *S100P* has been described to promote tumorigenesis and metastasis in diverse cancer models including PC (Averboukh et al. 1996, Parkkila et al. 2008). Intriguingly, expression of *S100P* is highly sensitive to AR SUMOylation (Fig. 7A in II).

In addition to heat stress, also the prostaglandin, 15d-PGJ_2 inhibited DNA-binding of the AR both on naked-DNA and chromatin (Fig. 6B and C in III). Accordingly, the expression of AR target gene mRNAs was attenuated in VCaPs (Fig. 3 in III) and in LNCaPs (data not shown) by the prostaglandin. The function of the wtAR appeared to be more prone to inhibition by 15d-PGJ_2 than ARK386,520R both in the reporter gene assays (Fig. 4 in III) and on the endogenous target genes in PC-3 prostate cancer cells (Fig. 5 in III). The results suggest that SUMOylation can modulate the responsiveness of the AR to this compound. In addition, heat stress altered AR-chromatin interactions in a target gene-selective manner in the wtAR- and ARK386,520R-expressing HEK293 cells (Fig. 7B in II). The novel results indicate that SUMOylation can modulate AR function in the chromatin landscape, which has not been formerly reported, since previous studies were based on ectopically expressed proteins and reporter genes containing synthetic response elements.

As discussed, also other factors besides the AR in the AR-transcriptional complex are plausibly SUMOylated in cell stress. It is likely that multiple SUMO/SIM-dependent

protein-protein interactions are altered. In addition to diverse PTMs, cell stress triggers several other pathways, such as heat shock response, so that cells can respond to changes in their microenvironment. Heat shock response is an ordered stress-response pathway that activates DNA-binding TFs, named as heat shock factors (HSFs), to control the expression of stress-protective proteins, such as heat shock proteins (HSPs) that function as molecular chaperones (Richter et al. 2010). A typical feature of heat shock response is the drastic repression of general transcription and translation pathways. Instead, the gene programs involved in acute cell survival are activated. HSPs function as chaperones also under normal cellular conditions. For example, HSP90 and HSP70 bind the apo-AR in cytoplasm. Molecular chaperones appear to be important factors throughout the lifespan of the AR, since they also interact with the holo-AR and mediate receptor cycling on/off target chromatin during transcription cycles (Prescott & Coetzee 2006).

A non-lethal heat dose has been described to induce temporary resistance against a subsequent lethal heat shock, i.e. the development of thermotolerance, which is contributed by elevated levels of HSPs, including HSP70 (Roti Roti et al. 1998, Kim H.J. et al. 2007). Intriguingly, mild heat stress induced the expression of HSP70 in VCaP cells, but repeated heat stress blunted the formation of AR-SUMO-2/3 conjugates (unpublished observations). HSPs are lysine-rich proteins that lack SUMO consensus motif, but contain hydrophobic sequences of valine and isoleucine similar to motifs identified as SIMs. According to proteomics analyses, HSP70 and HSP90 are, indeed, targets for SUMO-2 conjugation (Blomster et al. 2009, Ouyang et al. 2009). Mild stress-enhanced levels of HSP70 may bind and reserve the SUMO moeities modulating AR SUMOylation in VCaPs exposed to repeated stress. The findings suggest that stress-triggered system of chaperone proteins and SUMO-2/3 conjugation may co-operate in modulating AR-dependent transcription in PC cells.

Original article III contains the novel observation that an electrophilic stressor prostaglandin 15d-PGJ_2 can induce substrate-specific SUMOylation. The prostaglandin binds covalently and irreversibly to the cysteine residues in target proteins (Kim & Surh 2006). Prostaglandin 15d-PGJ_2 bound also to AR and induced SUMO conjugation as assessed in VCaP cells (Figs. 1-2 in III). Furthermore, 15d-PGJ_2 inhibited AR-dependent transcription (Fig. 3 in III). Receptor SUMOylation was likely to modulate AR responsiveness to the compound (Figs. 4-5 in III). In addition to disruption of the receptor DNA binding (Fig. 6 in III), the inhibition was due to a defect in AR folding, since the receptor N/C interaction was prevented by 15d-PGJ_2 (Fig. 7A in III). Prostaglandin was likely to target the AR LBD (Fig. 7B-D in III). Binding of 15d-PGJ_2 to the AR did not prevent ligand binding, because bicalutamide and 15d-PGJ_2 had an additive effect on AR inhibition in VCaP cells (Fig. 8 in III). These findings may represent new approaches for pharmacological research to design modern drugs targeting the AR in PC.

Interestingly, arsenic trioxide, used as a drug in ancient Chinese medicine, can induce SUMOylation of PML-RARα oncoprotein in acute promyelocytic leukemia (Lallemand-Breitenbach et al. 2008, Tatham et al. 2008). Similar to 15d-PGJ_2, arsenic trioxide can directly bind to cysteine residues in PML and increase production of ROS (Jeanne et al. 2010, Zhang et al. 2010). Arsenic binding enhances PML oligomerization, which results in an increased interaction with UBC9 and enhanced PML SUMOylation (Zhang et al. 2010). PIAS1 has been recently suggested to mediate arsenic-triggered SUMOylation of PML (Rabellino et al. 2012). The prostaglandin 15d-PGJ_2 increased interaction of endogenous AR and PIAS1 in VCaP cells, while it also displayed binding to SENPs (unpublished observations). Arsenic has been shown to repress AR activity by inhibiting receptor N/C interaction and recruitment of the AR to target gene enhancer in PC cells (Rosenblatt & Burnstein 2009). Thus, arsenic and 15d-PGJ_2 may share similar properties in enhancing SUMOylation of cysteine rich proteins. Numerous SUMO substrates are zinc finger proteins which are involved in transcription (Vertegaal et al. 2006). Proteins rich in cysteine are susceptible in

sensing changes in the cellular redox status and may appear as primary targets for stress-induced SUMOylation.

5.5 STRESS-TRIGGERED INTRANUCLEAR RELOCALIZATION OF THE AR

The agonist-bound AR shows a punctuate distribution pattern while the antagonist-bound receptor is evenly distrubuted throughout the nucleus (Tyagi et al. 2000, Marcelli et al. 2006, van Royen et al. 2007). The AR speckles have been proposed being as potential sites for the formation of precomplexes between the receptor and its coactivators (Saitoh et al. 2002) and as hot spots of active transcription (van Royen et al. 2007). Many SUMOylated and/or SIM-containing AR coregulators, such as PIAS proteins (Kotaja et al. 2002b), GRIP1 (Kotaja et al. 2002c) and HIPK3 (Moilanen et al. 1998a) also display granular nuclear distribution. AR shows dynamic nuclear mobility (van Royen et al. 2009), which is likely to permit AR-coregulator-interactions in specific nuclear substructures. Furthermore, AR SUMOylation may be involved in regulating the receptor's intranuclear movements.

The overall cellular distribution and the hormone-induced nuclear translocation of the wtAR and ARK386,520R are similar as originally discussed by Poukka and coworkers (Poukka et al. 2000a). The intranuclear mobility of the wtAR and SUMOylation-deficient receptor was measured by fluorescence recovery after photobleaching (FRAP) assays. Both the agonist-bound wtAR and ARK386,520R displayed retarded mobility compared to the antagonist-bound receptors in HEK293 (Fig. 4 in II) and COS-1 cells (Fig. 11). The agonist-bound wtAR was ~20% more mobile than the ARK386,520R in both cellular environments. The results suggest that both agonists and antagonists may translocate the AR into different subnuclear compartments. SUMOylation may modulate receptor solubility, since agonists induced SUMOylation of the AR, while the antagonist bicalutamide had only a minor effect on the formation of AR-SUMO conjugates (Fig. 1E in I).

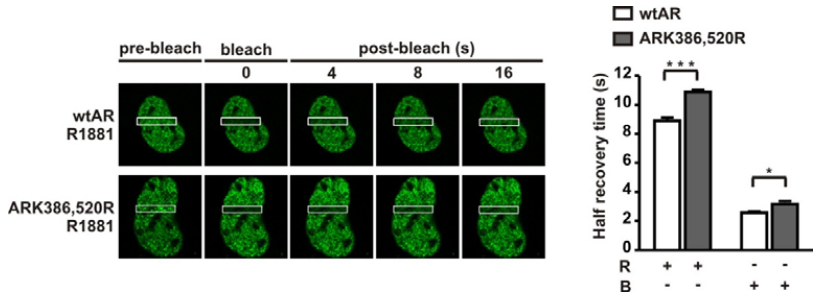

Figure 11. SUMOylation modulates the nuclear mobility of the AR in COS-1 cells. The cells grown on Ibidi 8-well chambers (Integrated BioDiagnostics) were transiently transfected with expression vectors encoding EGFP-AR or EGFP-ARK386,520R and treated with R1881 (R) or bicalutamide (B). FRAP analyses were performed using Zeiss LSM 700 Confocal Microscope with Zen software 2009. Bleach pulses were performed with maximal laser intensity in 2.8 µm × 15.8 µm ROIs, and serial images were collected over 90 s period. Background fluorescence and general bleaching during acquisitions were measured and the fluorescence intensity in the ROI was normalized to these values. Fifty nuclei were analyzed to calculate average recovery times with standard error of mean (SEM). ***, $p \leq 0.001$ for the difference between wtAR and ARK386,520R in the presence of androgen (R), *, $p \leq 0.05$ for the difference between wtAR and ARK386,520R in the presence of antiandrogen (B).

Stress kinase signaling has been shown to mediate phosphorylation of S650 in the hinge region of the AR, which further induced receptor movement from the nucleus to the

cytoplasm and inhibition of the expression of AR target genes (Gioeli et al. 2006). Repression of AR activity by 15d-PGJ_2 was not attributable to the nuclear export of the receptor in VCaP cells (Fig. 6A in III), suggesting that cell stress may influence the intranuclear distribution of the holo-AR. Endogenous holo-AR and SUMO-2/3 showed increased colocalization in nuclear granules in heat-stressed VCaP cells (Fig. 3A in II). The subnuclear structures corresponded to the PML bodies (Fig. 3A in II) that are in contact with the nuclear matrix and are rich in components of the SUMOylation pathway.

The nuclear matrix is biochemically defined as the residual nuclear structure that remains after extraction of most of the chromatin and all of the soluble and loosely bound components. The nuclear matrix consists of the nuclear lamina and the internal nuclear network of subassemblies that are joined and come into contact with other nuclear substructures (Nickerson 2001). The nuclear matrix has been reported to be a target for protective effects of HSPs in stressed cells. For example, HSP70 binds to the nuclear matrix in a heat stress-induced manner. HSPs associate with denatured proteins, protecting the cell from the more deleterious consequences of thermal denaturation of nuclear matrix proteins (Roti Roti et al. 1998). The nuclear matrix is also involved in maintaining chromatin organization to ensure the regulation of gene expression (Lanctot et al. 2007). Defects in the nuclear architecture may have pathological consequences leading to dysregulation in gene expression programs. The SUMOylation pathway may contribute to maintaining an intact nuclear organization. For instance, the special AT-rich sequence-binding protein 1 (SATB1) is the best known nuclear matrix attachment region (MAR)-binding factor. SATB1 is not only a TF but it is also a global chromatin organizer, acting as a linker between chromatin and the nuclear matrix territories (Galande et al. 2007). SATB1 is a target for SUMOylation and it interacts with PIAS1. Furthermore, SUMOylation of SATB1 is involved in the subnuclear relocalization of the factor (Tan et al. 2008, Tan et al. 2010). Thus, impairment in the SUMOylation system may evoke severe defects in the nuclear structure and transcriptional processes.

The AR is a mobile protein that shuttles between different cellular compartments. Upon androgen exposure, the AR becomes enriched in the nucleus, where it further distributes into different subcompartments. In accordance, cellular fractionation of VCaP cells showed that the holo-AR was enriched in the nuclear matrix fraction to a greater extent than the apo-AR (Fig. 3B in II). Heat stress induced accumulation of SUMOylated AR almost completely into the nuclear matrix compartment, and the compartmentalization of the receptor was reversible (Fig. 3B and C in II). In addition to biochemical assays, the dynamic relocalization of the holo-AR was studied in microscopic analyses of fixed (Fig. 3E in II) and living cells (Fig. 12) exposed to either heat stress or osmotic stress.

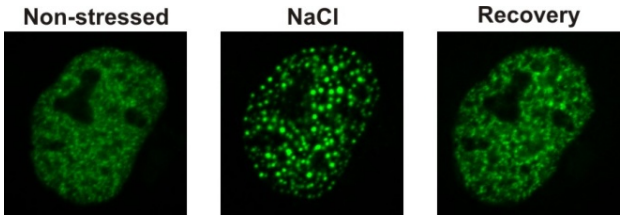

Figure 12. Osmotic stress induces a dynamic and reversible change in the nuclear distribution of the holo-AR as visualized by live cell imaging. EGFP-tagged AR was transiently expressed in COS-1 cells grown on Ibidi 8-well chambers (Integrated BioDiagnostics) and treated with androgen (R1881). The green fluorescence derived from the AR was visualized by confocal microscopy (Zeiss LSM 700) in a representative cell nucleus before stress, 15 min after exposure to osmotic stress (0.3 M NaCl added to growth medium), and 15 min after recovery from the stress (NaCl-containing medium aspirated and substituted with normal medium). The conditions of living cells were controlled by a heatable imaging stage and CO_2 cover during the experiments.

This study showed that both agonist binding and cell stress increased AR SUMOylation that affected receptor solubility. Thus, the results imply that changes in the AR SUMOylation and distribution may be linked to different subnuclear structures. Furthermore, the hinge region of the AR has been reported to be involved in targeting the receptor to the nuclear matrix compartment (van Steensel et al. 1995), and the AR harboring the hinge-deletion was poorly SUMOylated (Fig. 2C in I). Analysis of HEK293 cells showed that the relative amount of wtAR was increased in the nuclear matrix upon androgen exposure, while the level of ARK386,520R in the nuclear matrix was not influenced by androgen (Fig. 3D in II). The findings are in line with the mobility data in FRAP assays. Heat stress resulted in increased accumulation of both receptor forms into the nuclear matrix fraction. Interestingly, the relative amount of ARK386,520R was more pronounced than the levels of wtAR in the nuclear matrix of heat-stressed cells, both in the absence and presence of androgen (Fig. 3D in II). In summary, androgen-induced SUMOylation is likely to modulate the intranuclear mobility and distribution of the AR, while stress-forced hyperSUMOylation may be involved in sustaining the solubility of the receptor (summarized in Fig. 13).

SUMOylation may modulate the AR turnover in chromatin. In addition, SUMOylation appears to have a modulatory effect on the nucleoplasmic cycling of the AR. Interestingly, constitutively active LBD-truncated AR variants have been found in clinical PC samples. This study showed that LBD-deficient AR is not SUMOylated. Therefore, ARΔLBD variants may escape SUMOylation-adjusted activity cycles, which may result in abnormal activity of AR-controlled genes and contribute to progression of PC.

In agreement with the concept that SUMOylation may influence protein solubility, the SUMOylation-defective mutant of CREB binding protein (CBP) has been reported to show reduced mobility compared to its wild-type counterpart (Ryan et al. 2010). Taken together, these results indicate that SUMOylation can regulate the mobility of TFs and their coregulators in the nucleoplasm. SUMOylation may regulate the residency time of TFs on active chromatin regions and cycling of the factors between chromatin and other subnuclear structures to mediate transcriptional homeostasis.

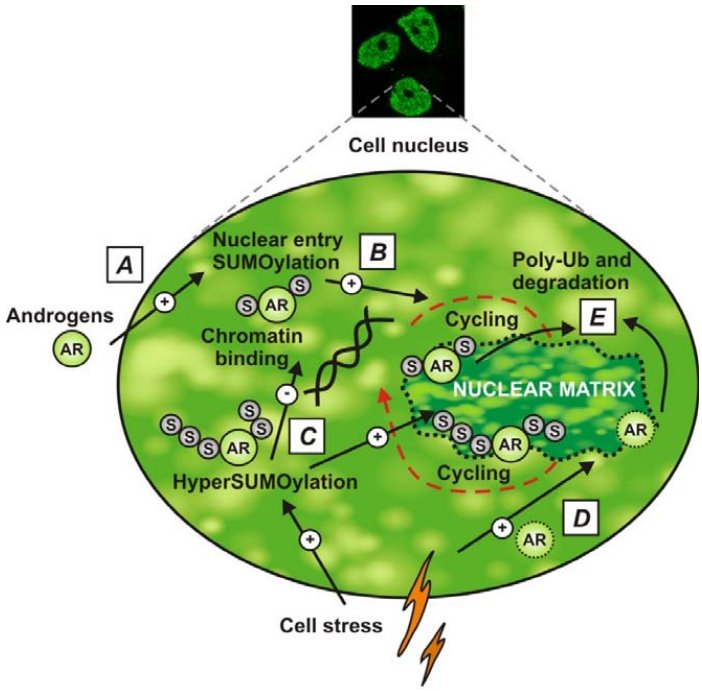

Figure 13. A schematic presentation of the dynamic AR movements in the nucleoplasm. The upper part of the figure shows a confocal image of prostate cancer cell nuclei, where green fluorescence depicts immunostained holo-AR. A) Androgens induce AR nuclear translocation and SUMOylation. B) SUMOylated AR binds to chromatin, and is cycled on/off chromatin and the nuclear matrix during transcription cycles. C) Cell stress induces massive hyperSUMOylation of the AR, which prevents AR-chromatin interactions and induces accumulation of SUMOylated AR in the nuclear matrix. SUMOylation sustains AR solubility that enables receptor cycling. D) SUMOylation-defective AR is translocated into the nuclear matrix in an androgen-enhanced manner in cell stress. E) SUMOylation-defective AR is less soluble compared to the SUMO-conjugated AR and it may be poorly cycled. Polyubiquitylation and proteasomal degradation of the SUMOylation-defective AR is faster than the SUMOylated AR.

6 Summary and conclusions

SUMO modifications are essential for the normal physiology of living organisms. An example of the importance of SUMOylation is that different human pathogens target the host cell SUMOylation pathway to dampen the host response to infection (reviewed by (Wimmer et al. 2012). Furthermore, dysfunctional SUMOylation pathway is linked to development of neurogenerative diseases and tumorigenesis. This study aimed at understanding the role of SUMO modifications in the regulation of AR-dependent transcription in PC cells. The results discussed in this thesis have important implications with regard to both AR function and PC biology. The main findings are:

- SUMOylation of the AR modulates androgen-regulated gene expression in a target gene-specific fashion, i.e. androgen-responsive genes display different SUMO-sensitivities.

- The AR shows a preference for SUMO-2/3 conjugation. Androgens enhance the conjugation, while cell stress produces AR hyperSUMOylation in PC cells.

- Cell stress detaches the AR from chromatin repressing AR-dependent transcription in a reversible manner. AR SUMOylation seems to be involved in the cycling of the receptor between different subnuclear compartments.

- SENPs, particularly SENP1, reverse the SUMO modifications of the AR and modulate expression of AR target genes.

- Silencing of *SENP1* retarded proliferation of androgen-treated PC cells, suggesting that SUMOylation may affect cell survival and proliferation.

This study provides new information about the role of SUMO modifications in the regulation of AR transcriptional activity. Androgens enhance the AR SUMOylation. SUMO conjugation stabilizes the AR, since SUMOylation-defective AR shows higher ubiquitylation and faster decay than SUMOylated receptor. Due to SUMOylation, the AR is divided into distinct subpopulations within a cell, i.e. pools of AR and AR-SUMO, which display different properties in intranuclear kinetics and distribution. The AR pools are likely to be recruited onto the chromatin AREs of different genomic regions in a SUMOylation-modulated manner. AR SUMOylation is suggested to be involved in receptor cycling between chromatin and the nuclear matrix compartments during transcription activity cycles. Therefore, intact nuclear structure that regulates chromatin arrangement seems to be important for the expression of SUMO-sensitive AR target genes.

Modern genome-wide DNA-sequencing and gene expression technologies enable analysis of the AR-chromatin occupancy and expression of androgen-regulated genes. Such genome-wide studies in animal model systems would expand the current comprehension of the AR function and SUMOylation. In particular, analysis of tissues from knockin animals harboring the SUMOylation-defective AR will be of great value. As an example, analysis of SF-1 SUMOylation (Lee et al. 2011) showed that the *in vivo* approach produced novel findings that would not have been probably discovered in cell culture-based studies only. Detailed molecular mechanisms of the SUMOylation in the AR signaling need to be resolved in order to develop modern therapies. Comparison of gene expression profiles in

PC samples between individuals displaying different grades of prostatic growth would assist to discover novel PC markers. SENP1 has already been suggested as a potential prognostic factor in PC (Li et al. 2012). In addition, the role of SUMOylation in different stages of carcinogenesis could be explored. Thus, medical intervention targeting the SUMOylation system could be directed in an appropriate cancer phase, possibly as a future therapy against cancerous cell growth. In conclusion, the results presented in this thesis improve understanding of the interplay between the SUMOylation system and the AR signaling, which implies novel prospects to restrict the AR activity in PC cells.

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Regulation of Androgen Receptor Signaling by SUMO Modifications in Prostate Cancer Cells

Androgen receptor (AR) is a pivotal transcription factor in the development of prostate cancer. In addition to male sex hormones, also post-translational modifications can affect AR function and thus regulate the expression of androgen-responsive genes. This study proves that reversible modifications by a small ubiquitin-like modifier (SUMO) are important in the regulation of AR function in prostate cancer cells. The results may provide novel prospects for targeting the AR in prostate cancer cells.

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