ALFONSO URBANUCCI

Overexpression of Androgen Receptor in Prostate Cancer

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ACADEMIC DISSERTATION
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ABBREVIATIONS

AcH3	acetylation of histone 3/acetylated histone 3
AD	androstenedione
ADT	androgen deprivation therapy
AF1	activation function 1
AF2	activation function 2
AKT	Official name: AKT1 (v-akt murine thymoma viral oncogene homolog 1)
ANOVA	analysis of variance
AR	androgen receptor
ARA24	24 kDa AR-activator (Official name RAN (Ras-like GTPase))
ARBS	androgen receptor binding site
ARE	androgen response element
AZGP1	zinc-alpha 2-glycoprotein
BPH	benign prostatic hyperplasia
BRCA2	breast cancer 2, early onset
BTG2	BTG family, member 2
CBP	official name: CREBBP (CREB binding protein)
CCND1	Cyclin D1
ChIP	chromatin immunoprecipitation
ChIP-seq	ChIP technique coupled with massive high-throughput sequencing
CRPC	castration resistant prostate cancer
CYP17	cytochrome P450 (CYP)17 (steroid 17α-hydroxylase/C17,20 lyase)
DBD	DNA binding domain
DHEA	dehydroepiandrosterone
DHT	dihydrotestosterone
DTL	denticleless homolog (Drosophila)
EP300	E1A binding protein p300
ER	estrogen receptor
ERG	v-ets erythroblastosis virus E26 oncogene homolog (avian)
ES	embryonic stem
ETS	E26 transformation-specific
EZH2	enhancer of zeste homolog 2 (Drosophila)
FEN1	flap structure-specific endonuclease 1
GO	gene ontology
GR	glucocorticoid receptor
GSTP1	glutathione S-transferase class π gene
H	Histone
HAT	histone acetyltransferase
HDAC	histone deacetylase
HSP	heat-shock protein
K	Lysine
KLK2	kallikrein-related peptidase 2
LBD	ligand binding domain
LHRH	luteinizing hormone releasing hormone
MAGEA11	Melanoma antigen gene protein (alias MAGE11)

MAK	male germ cell-associated kinase
miR	microRNA
NTD	N-terminal domain
PC	prostate cancer
Pol2	polymerase 2
PSA	prostate specific antigen (alias KLK3 (kallikrein 3))
PTEN	phosphatase and tensin homolog
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RB1	retinoblastoma 1
RE	response elements
SHR	steroid hormone receptor
SKP2	S-phase kinase-associated protein 2 (p45)
SNAI2	snail homolog 2 (Drosophila)
SRC	Steroid receptor coactivator
SRD5A	5α-reductases (steroid-5α-reductase)
TMPRSS2	transmembrane protease, serine 2
TP53	tumor suppressor gene
TPX2	TPX2, microtubule-associated, homolog (Xenopus laevis)
TSS	transcription start site
ZWINT	ZW10 interactor
β-catenin	Official name: CTNNB1 (catenin (cadherin-associated protein), beta 1, 88kDa

ABSTRACT

Castration-resistant prostate cancer (CRPC) is a deadly disease. The mechanism by which CRPC develops is only partially understood. The best understood alteration in CRPC is the overexpression of the androgen receptor (AR). Prostate cells rely on AR for growth and differentiation as much as prostate cancer (PC) cells rely on it for disease progression. Thus, AR is one of the major contributors to the development of CRPC.

This study aimed to investigate the molecular changes occurring in AR-overexpressing PC cells, particularly at the transcriptional level. Furthermore, this study also aimed to identify AR target genes that are important for PC progression that could be used as potential drug targets or biomarkers.

The data demonstrated that the overexpression of AR enhances the binding of the receptor to chromatin in the presence of relatively low concentrations of androgens. Furthermore, under the same conditions, AR overexpression also altered the dynamics of chromatin binding of the receptor and the binding of basic components of the transcriptional machinery, such as RNA Pol II. The recruitment occurred earlier and more powerfully in AR-overexpressing cells. These changes seemed to translate into an increased acetylation of positioned histones. Furthermore, the data suggested that the chromatin is more open in AR-overexpressing cells that are already in hormone-deprived conditions. Thus, AR overexpression favors chromatin accessibility, which results in enhanced gene transcription. Interestingly, the AR and RNA Pol II binding pattern, and the transcriptional enhancement of the target genes differ from gene to gene.

In addition, several new AR target genes were identified and characterized. *SKP2*, *ZWINT* and *FEN1* were found to be overexpressed in CRPC specimens, whereas *SNAI2* expression was reduced. Functional studies showed that ZWINT, SNAI2 and FEN1 may be important in PC cell growth. FEN1 overexpression was also associated with a shorter time to progression. Thus, FEN1 may be important in the progression of PC to CRPC and may represent a potential drug target.

Altogether, these results offer a mechanism for how overexpression of AR leads to resistance to androgen ablation.

SINTESI

Iper-espressione del recettore degli androgeni nel cancro alla prostata

L'incidenza del cancro alla prostata in Europa é del 20 %. L'inizio della malattia é subdolo ma fortunatamente, anche grazie al test del "prostate specific antigen" (PSA), meno del 5 % di questi casi presenta metastasi al momento della diagnosi. Nonostante ció, il cancro alla prostata é tra le prime cause di morte legate a tumori maschili nei paesi occidentali: in Europa la mortalitá è stimata tra 8 e 12 uomini ogni 100.000. Nella maggior parte dei casi la malattia é curabile, ma circa un quarto dei pazienti presenta recidiva dopo circa 2-3 anni dalla prostatectomia iniziale. Infatti, le terapie in uso selezionano forme resistenti.

Il carcinoma prostatico metastatico refrattario alla castrazione (CRPC) é una malattia mortale per la quale attualmente non ci sono cure efficaci. I meccanismi attraverso i quali queste forme refrattarie di CRPC si sviluppano sono noti solo in parte. Tra le diverse alterazioni studiate, la piú frequente (il 95 % dei casi) é l'iper-espressione del recettore degli androgeni (AR). AR é un fattore di trascrizione in grado di controllare l'espressione di circa il 2 % del genoma. Infatti, in condizioni fisiologiche le cellule prostatiche ne hanno bisogno per la crescita e per il differenziamento. Tuttavia, anche le cellule di tumore prostatitco ne traggono vantaggio in tutti gli stadi della malattia, dal tumore primario al CRPC, per questo motivo, il recettore é molto importante nello sviluppo della malattia. Le terapie piú usate intendono impedire l'attivazione di AR nelle cellule cancerogene di prostata, sopprimendo, per esempio, i livelli di testosterone e altri androgeni, tra i quali il diidrotestosterone, che sono in grado di attivare il recettore. Tuttavia, numerosi studi hanno evidenziato come l'iper-espressione di AR è in grado di eludere gli effetti delle terapie.

Questo studio si propone di chiarire i meccanismi molecolari legati all'iper-espressione di AR nelle cellule di tumore prostatico, in particolare a livello trascrizionale, e di identificare i geni bersaglio del recettore implicati nella progressione del tumore. Tali geni rappresentano potenziali target terapeutici e prognostici.

I dati ottenuti dimostrano che l'iper-espressione di AR incrementa il legame dello stesso recettore alla cromatina, in presenza di concentrazioni relativamente basse di androgeni. Nelle stesse condizioni, l'iper-espressione di AR altera la dinamica del suddetto legame e del legame di fattori base di trascrizione, quali l'RNA Polimerasi II, nelle regioni regolatorie dei geni bersaglio, enhancers e promotori. Il

recrutamento di tali fattori é potenziato ed é piú veloce in cellule che iper-esprimono il recettore. Inoltre, si osserva un incremento di acetilazione degli istoni. Questi cambiamenti si riflettono anche sulla struttura della cromatina, in particolar modo sul posizionamento dei nucleosomi. La cromatina é infatti piú aperta giá in assenza di androgeni. Quindi si evince che l'iper-espressione del recettore favorisce l'accesso alla cromatina, che risulta in un aumento dei livelli di trascrizione. É anche interessante notare come la dinamica dei legami con la cromatina e l'incremento dei livelli trascrizionali, sia potenzialmente diverso di gene in gene. I dati ottenuti hanno anche portato all'identificazione di nuovi geni bersaglio di AR, che sono stati caratterizzati. *SKP2*, *ZWINT* e *FEN1* sono risultati iper-espressi in campioni di CRPC mentre *SNAI2* é risultato ipoespresso. Studi sulla funzione di questi geni hanno rivelato che ZWINT, SNAI2 e FEN1 potrebbero essere coinvolti nella crescita delle cellule tumorali di prostata. In particolare, l'espressione di FEN1 é anche risultata essere associata con il tempo di progressione e recidiva della malattia. Dunque, FEN1 potrebbe essere importante nello sviluppo del CRPC e perció un potenziale target terapeutico.

In conclusione, questi risulati offrono una spiegazione meccanistica dei processi che portano alla resistenza delle terapie ormonali nel contesto dell'iper-espressione del recettore per gli androgeni.

INTRODUCTION

The best-recognized risk factor for prostate cancer (PC) is age. Several studies have reported that ethnicity and family history also contribute to the risk (Grönberg et al., 2003; Patel and Klein, 2009). Genome-wide association studies continue to report genetic factors that may be involved in the onset of the disease. However, although many low penetrance risk loci have been identified, most have not yet been conclusively functionally validated (Freedman et al., 2011). Migration studies have shown that immigrants acquire the risk of their new location, indicating that environmental factors contribute to the risk of PC (Patel and Klein, 2009). However, none of the environmental risk factors have conclusively been associated with the onset of PC (Giovannucci and Platz, 2006; Patel and Klein, 2009).

Due to the increasing quality of life in Western countries, the overall population ages and faces an increasing number of PC cases every year (Scardino and Kelman, 2005). In Europe, the incidence rate is 1 case per 5 men (Heidenreich et al., 2011). Fortunately, the increasing incidence of PC no longer leads to increased mortality. The mortality rates grew until 1995 but have now been decreasing (Bosetti et al., 2011). Currently, fewer than 5 % of men with newly diagnosed PC have distant metastases at diagnosis compared to 25 % more than 20 years ago (Ryan and Small, 2005). However, PC is still among the top causes of cancer-related deaths in men in Western countries (Ferlay et al., 2010; Jemal et al., 2010). Estimated age-adjusted PC mortality rates in 2011 vary between 8.1 and 12.6 for every 100,000 men in Europe (Malvezzi et al., 2011).

PC is asymptomatic in the early phase of the disease and is often diagnosed by screening based on the measurement of serum levels of prostate specific antigen (PSA) (see also Figure 2A) accompanied by a digital rectal examination and needle biopsy (Heidenreich et al., 2011). PSA screening increases the diagnostic sensitivity and decreases mortality by 20-30 % (Schröder et al., 2009) but leads to problems such as high cost and overdiagnosis (Borofsky and Makarov, 2011) and subsequent overtreatment (Klotz and Thompson, 2011; Froehner and Wirth, 2011; Shigeta et al., 2011).

Although most patients are initially diagnosed with a curable disease, approximately 20 % of the cases progress within 5 years after the initial prostatectomy (Bianco et al., 2005). For the past 70 years, the standard care for advanced PC has been chemical or surgical castration (Huggins and Hodges, 1941). However, the treatment is only palliative and, although most of the patients respond to treatment, the disease eventually progresses (Gittes, 1991) (see also Figure 2A). Such recurrent cases are called castration-resistant prostate cancers (CRPCs). Only

recently have new treatment options for CRPC been approved by the Food and Drug Administration (FDA), but these options show only a few months benefit in the median survival (Vasani et al., 2011).

AR is overexpressed in 95 % of the CRPC cases (Linja et al., 2001; Chen et al., 2004). Thus, understanding how the overexpression of AR is advantageous for this type of cancer and the mechanisms it drives is critical and serves as the basis for identifying new drug targets and biomarkers for this disease.

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original publications referred to in the text by the Roman numerals I-III:

I **Alfonso Urbanucci**, Biswajyoti Sahu, Janne Seppälä, Antti Larjo, Kati K. Waltering, Robert L. Vessella, Harri Lähdesmäki, Olli A. Jänne and Tapio Visakorpi. Overexpression of androgen receptor increases the binding sensitivity of the receptor to the chromatin. Oncogene, In press

II **Alfonso Urbanucci**, Saara Marttila, Olli A. Jänne, and Tapio Visakorpi. Androgen receptor overexpression alters binding dynamics of the receptor to chromatin and chromatin structure. The Prostate, In press

III **Alfonso Urbanucci**, Kati K. Waltering, Hanna Suikki, Merja A. Helenius and Tapio Visakorpi. Androgen regulation of the androgen receptor coregulators. BMC Cancer, 2008 8:219.

1. REVIEW OF THE LITERATURE

1.1 Prostate cancer

1.1.1 Natural history of prostate cancer

The prostate is a walnut-sized exocrine gland that surrounds the prostatic urethra, the ejaculatory ducts and the neck of the urinary bladder. The prostate is an accessory reproductive organ formed by small glands (acini) that open into excretory ducts (Figure 1A). The prostate produces approximately 30 % of the total volume of the ejaculate (Pahuja et al., 2006). The function of prostatic fluid is to support the spermatozoa, and this fluid is comprised of many components, including acid phosphatases, albumin, zinc, magnesium and calcium. The ductal epithelium is responsible for the production and the excretion of PSA in the seminal plasma. PSA is a protease that normally functions in the liquefaction of the seminal coagulum (Lilja, 2003). The basement membrane in the prostate forms a barrier that prevents the escape of secreted PSA into the blood circulation. In PC or other pathological conditions of the prostate, this barrier is disrupted and the PSA can enter the circulation (Ward et al., 2001). For this reason, PSA is generally used as a diagnostic biomarker for prostate disorders (Stamey et al., 1987), including PC (Partin et al., 2001; Graff et al., 2007).

Anatomically, the prostate can be divided according to two classifications: by zone or by lobes. However, because the prostate does not have a clear lobular structure, the "zone" classification is used more frequently (McNeal, 1981; Myers et al., 2010). According to this classification, the prostate gland is divided into four glandular zones, two of which arise from different segments of the prostatic urethra:

- The peripheral zone (PZ), which surrounds the distal urethra and represents up to 70 % of the volume in young men. Approximately 70-80 % of PCs originate from this portion of the gland (McNeal et al., 1988).
- The central zone (CZ), which surrounds the ejaculatory ducts, represents approximately 25 % of the total volume. Roughly 2.5 % of PCs arises from the CZ. These cancers tend to be more aggressive and more likely to invade the seminal vesicles (Cohen et al., 2008).
- The transition zone (TZ), which represents 5 % of the prostate volume at puberty, surrounds the proximal urethra and is responsible for benign prostatic hyperplasia (BPH) (McNeal, 1978). Approximately 10-20 % of PCs originate from this zone (McNeal et al., 1988).
- Anterior fibro-muscular zone (or stroma) accounts for approximately 5 % of the volume (McNeal, 1981).

Three cells types form the epithelial compartment of the prostate. These cell types are the basal cells, neuroendocrine and luminal cells. Basal and neuroendocrine cells are AR negative, whereas luminal and stromal cells are AR positive (Klocker, 2006). PC is thought to arise from the epithelial cellular

compartment and is thus classified as adenocarcinoma. However, from which type of epithelial cells cancer arises is not known (Maitland and Collins 2008, Wang and Shen 2011).

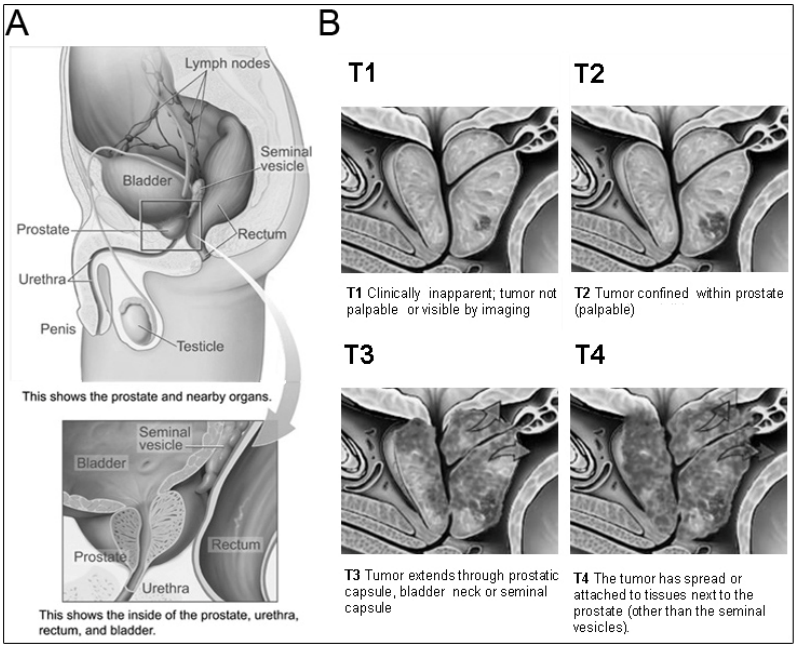

Figure 1. Anatomy of the prostate and prostate cancer. (A) Anatomy of the prostate gland in relation to the urinary bladder, rectum, seminal vesicles and urethra. (B) Staging of prostate cancer according to the American Joint Committee on Cancer TNM System (N and M staging are omitted) (Adapted from http://www.cancer.gov/).

Prostatic intraepithelial neoplasia (PIN) is considered to be a premalignant stage but is not a prerequisite for PC development. Another possible premalignant lesion of the prostate is proliferative inflammatory atrophy (PIA) (DeMarzo et al., 2003). PIA is often located in the PZ of the prostate. The proliferative nature of PIA has led to the hypothesis that PIA could also be a precursor of PC (Woenckhaus and Fenic, 2008), but the evidence is still not strong. Once the tumor escapes out of the prostate capsule, it invades surrounding tissues and eventually metastasizes, primarily to the local lymph nodes and bone (Porkka and Visakorpi, 2006) (Figure 1B).

1.1.2 Androgen dependency and treatment options for prostate cancer

Androgens, primarily testosterone and dihydrotestosterone (DHT), drive the growth and development of the prostate. The production of testosterone is regulated by luteinizing hormone (LH) and luteinizing hormone releasing hormone (LHRH). The hypothalamus secretes the LHRH in a pulsatile manner, which in turn stimulates the secretion of the LH by the anterior pituitary gland. LH induces the production of the testosterone by Leydig cells in the testicles. A negative feedback

loop prevents LHRH release (Burger, 2002). In the blood stream, only 1 to 2 % of testosterone is free. The majority is bound to albumin and sex hormone binding globulin (SHBG). Testosterone can enter the cell by diffusion or, when bound to SHBG, can be actively transported via a special receptor.

The effect of androgens is mediated by AR (Klocker, 2006). In the cytoplasm, testosterone binds to AR as such or after conversion into DHT by the enzyme 5α-reductase. DHT is considered the most active form of androgens because DHT can bind to AR with high affinity (So et al., 2003).

In 1941, Huggins and Hodges (1941) first reported the dependency of metastatic PC on androgens. Today, an increasing amount of evidence has shown that PC is dependent on the androgen/AR signaling pathway in all stages of the disease (Isaacs, 1994; Linja and Visakorpi, 2004; Chen et al., 2004). Consequently, the therapies for PC mostly rely on blocking this signaling axis by either preventing androgen production from the testes and/or blocking the function of AR with antiandrogens. Androgen-deprivation therapy (ADT) (hormonal therapy), through bilateral orchiectomy (castration) or treatment with LHRH agonists or antagonists, is the standard first-line therapy for advanced PC (Heidenreich et al., 2011). ADT is often also used for other indications, such as neoadjuvant or adjuvant therapy to surgery or radiotherapy, biochemical relapse after surgery or radiation and even primary therapy for localized disease in elderly men (Kram et al., 2011).

LHRH agonists, such as goserelin and leupropelin, downregulate the receptor for LHRH, desensitizing the pituitary gland. This downregulation results in inhibition of LH release. In contrast, LHRH antagonists, such as abarelix and degarelix, directly suppress the production of LH by acting on the anterior pituitary gland (Tammela, 2004). Surgical or chemical castration can be combined with antiandrogens to achieve a maximum androgen blockade (Mottet et al., 2011). Commonly used antiandrogens include flutamide, bicalutamide and nilutamide, which are non-steroidal compounds that bind the receptor and inhibit its activity. Thus, these compounds directly block the actions of testosterone and DHT within the cells. However, the clinical benefit of maximal androgen blockade is still marginal compared to castration alone (Tammela, 2004). On the other hand, in men with non-metastatic locally advanced PC, the survival has been shown to be similar in patients treated with bicalutamide monotherapy compared to castration (Iversen et al., 1998; Boccardo et al., 1999). Furthermore, an increasing body of evidence supports the use of bicalutamide in favor of LHRH agonist to preserve bone mineral density (Smith et al., 2004; Wadhwa et al., 2011). For these reasons, the use of bicalutamide monotherapy is widely approved in PC patients with locally advanced disease (Wadhwa et al., 2011).

1.1.3 Castration-resistant prostate cancer and treatment options

Most patients initially respond to hormonal therapies, but the disease eventually relapses and the tumor becomes castration-resistant (Sadar, 2011). Treatments for

CRPC include second-line hormonal therapy (Mottet et al., 2011) or chemotherapy with docetaxel (Petrylak et al., 2004; Tannock et al., 2004). In 2010, the FDA also approved the use of the sipuleucel-T for the treatment of patients with asymptomatic or minimally symptomatic CRPC (Longo, 2010; Kantoff et al., 2010). Sipuleucel-T is a form of immunotherapy in which the patient's immune system is *ex vivo* stimulated to a host antitumor response (Longo, 2010). More recently, the FDA approved abiraterone acetate for use in combination with prednisone for the treatment of patients with metastatic CRPC who have received chemotherapy containing docetaxel (de Bono et al., 2011a; Attard and de Bono, 2011). Abiraterone is an inhibitor of one of the enzymes, cytochrome P450 (CYP)17 (steroid 17α-hydroxylase/C17,20 lyase) (CYP17A), involved in the steroidogenesis.

Other agents, including the novel antiandrogen MDV3100, are in phase III clinical trials (Scher et al., 2010). None of the therapies for CRPC are curative (Antonarakis and Armstrong, 2011a). Thus, CRPC is still associated with poor prognosis, and the median survival is 18 to 24 months (Hussain and Dawson, 2000; Small et al., 2004) (Figure 2A). Therefore, new drug targets are clearly needed.

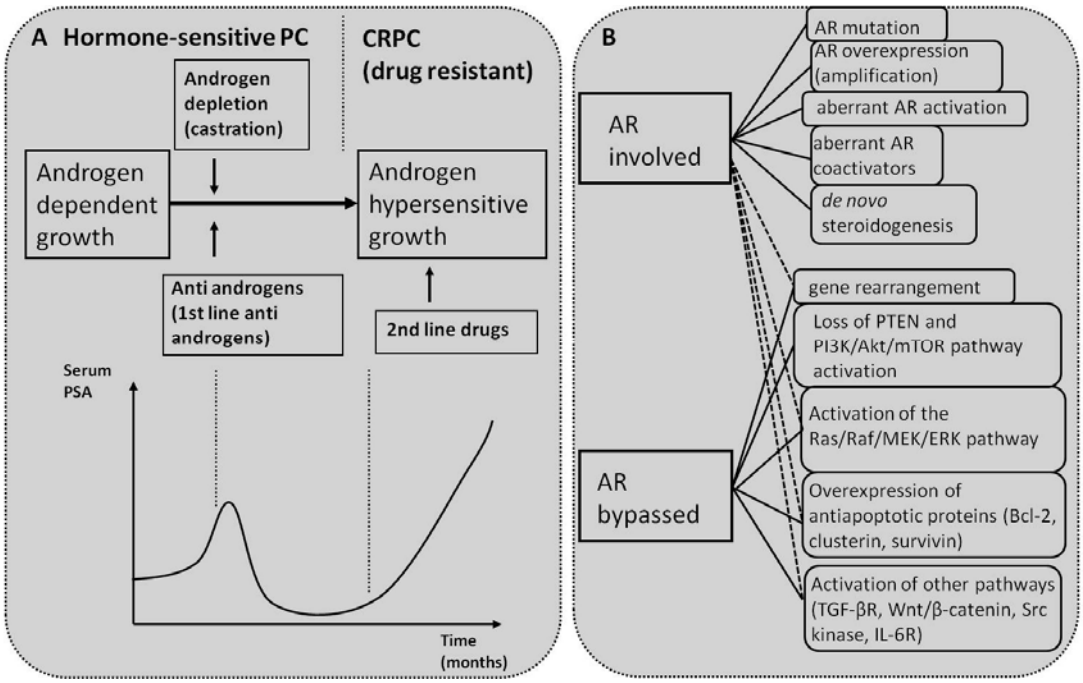

Figure 2. Prostate cancer: an overview. (A) Disease progression and interventions. Serum PSA levels are used for diagnosis and to monitor progression of the disease (B) Molecular mechanisms of PC involving AR and not involving AR. Dashed lines indicate interplay between the pathways.

1.1.4 Molecular pathology of prostate cancer

1.1.4.1 PC susceptibility

PC is a heterogeneous disease, and several molecular mechanisms can contribute to its development (Figure 2B). According to a Scandinavian twin study (Lichtenstein et al., 2000), up to 40 % of the PC risk can be inherited. Still, no common high-risk, PC susceptibility genes seem to exist (Foulkes, 2008). One candidate is BRCA2 since mutations in BRCA2 are associated with aggressive PC (Tryggvadóttir et al., 2007; Agalliu et al., 2007). However, BRCA2 mutations are rare in men with PC (Agalliu et al., 2007). Furthermore, despite considerable collaborative efforts, no recurrent risk loci have yet been conclusively identified by linkage analysis (Schleutker, 2011). Genome-wide association studies have identified several low risk candidate genes and loci (Easton and Eeles, 2008; Kote-Jarai et al., 2011; Freedman et al., 2011). Nevertheless, we are currently able to explain only approximately 25 % of the inherited risk (Kote-Jarai et al., 2011; Schleutker, 2011).

1.1.4.2 GSTP1

The glutathione S-transferase class π (GSTP1) protein is a member of the GST family of multifunctional enzymes and is involved in cell detoxification by catalyzing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione (Li et al., 2011a). To date, the most common somatic alteration in PC is the silencing of the tumor suppressor *GSTP1* expression by hypermethylation of the promoter region of the gene. Hypermethylation has been detected in 90–95 % of PC, suggesting that it is an early event in prostate tumorigenesis (Lee et al., 1994; Porkka and Visakorpi, 2004). The mechanism of hypermethylation is not well understood, but a recent study has suggested that the process is dependent on the histone deacetylase 1 (HDAC1). The silencing of *GSTP1* leads to oxidative stress–induced DNA damage and the expression of hypoxia-induced factor 1α (HIF-1α) (Li et al., 2011a).

1.1.4.3 NKX3.1

NKX3.1 encodes a homeodomain transcription factor that is regulated by androgens and is expressed specifically in luminal epithelial cells of the prostate. One of the most frequent genetic alteration in PC is the somatic loss of heterozygosity (LOH) in chromosome 8p (Taylor et al., 2010), which harbors the NK3 homeobox 1 (NKX3.1) gene. *NXK3.1* is associated with initiation and

progression of PC and is a well-known prostate tumor suppressor gene (Hughes et al., 2005; Gelmann, 2003). *NKX3.1* expression is decreased or absent in approximately 50 % of PIN lesions and primary prostate tumors and in as many as 80 % of metastatic tumors (Taylor et al., 2010). The role of *NKX3.1* loss in PC initiation is not well understood, but a recent study found that NKX3.1 activates the cellular response to DNA damage and has a protective effect against DNA-damaging agents, although it does not directly influence apoptosis (Bowen and Gelmann, 2010). However, the role of NKX3.1 is becoming more controversial, due to the discovery of rare castration-resistant NKX3.1-expressing cells (CARN) in a murine model. These cells can self-renew *in vivo* and can reconstitute prostate ducts in renal grafts using single-cell transplantation assays. Moreover, the concomitant deletion of the phosphatase and tensin homolog (*PTEN*) tumor suppressor gene in CARNs results in rapid formation of carcinoma following androgen-mediated regeneration (Wang et al., 2009a). These characteristics would make CARNs the putative PC stem cells (Wang and Shen 2011).

1.1.4.4 ETS rearrangement

A recent study by Taylor and colleagues (2010), which screened for genetic alterations in a large cohort of primary PC and metastatic PC, confirmed that although mutations in known tumor suppressors and oncogenes exist, they are rare (Taylor et al., 2010). However, in approximately 50 % of PC cases analyzed by Taylor and colleagues (2010), the genomic region 21q22.2-3 between v-ets erythroblastosis virus E26 oncogene homolog (avian) (ERG) and transmembrane protease, serine 2 (TMPRSS2) was lost. This area is of particular importance in PC because Tomlins and colleagues (2005) have shown that, due to the deletion, *ERG* is fused with the androgen-regulated gene *TMPRSS2*. The TMPRSS2:ERG fusion gene is thus androgen-regulated (Tomlins et al., 2005). ERG is a member of the E26 transformation-specific (ETS) family of transcription factors. These transcription factors are involved in embryonic development, cell proliferation, differentiation, angiogenesis, inflammation, and apoptosis (Seth and Watson, 2005). The identification of TMPRSS2:ERG broke the paradigm that fusion transcripts are rare in solid tumors (Helgeson et al., 2008; Rickman et al., 2009). Additionally, less common non-ETS fusion genes have recently been found (Palanisamy et al., 2010; Pflueger et al., 2010). Interestingly, all non-ETS fusion genes occur in the context of the TMPRSS2:ERG rearrangement, suggesting that particular fusions may predispose cancer to rearranging events (Pflueger et al., 2010).

Haffner et al. (2010) have suggested that the TMPRSS2:ERG gene fusion event is AR dependent. According to these authors, AR induces the proximity of the *ERG* and *TMPRSS2* genes and triggers topoisomerase-2b (TOP2B)-mediated gene fusion formation (Haffner et al., 2010).

The formation of the TMPRSS2:ERG fusion seems to be an early event in the development of PC because this fusion is found in approximately 20 % of PIN

lesions (Lawson & Witte, 2007). However, in a transgenic mouse model, ERG is not able to drive PC alone, and a possible cooperation with *PTEN* loss and PI3K pathway activation has been suggested (Zong et al., 2009; Carver et al., 2009; King et al., 2009).

TMPRSS2:ERG is found with the same frequency in PC and CRPC (Saramaki et al., 2008). The data regarding the prognostic value of the fusions are still controversial, and investigations to understand the mechanisms that these fused transcription factors regulate are ongoing (Saramaki et al., 2008; Barwick et al., 2010; Leinonen et al., 2010; Rubio-Briones et al., 2010; Minner et al., 2011; Tomlins et al., 2011).

Recently, a tumor suppressor, ring finger and WD repeat domain 2 (RFWD2 alias COP1) was shown to cause degradation of some ETS family members. The expression of COP1 is low in PC specimens showing concomitant overexpression of ETS transcription factors but not in specimens with ETS rearrangement (Vitari et al., 2011; McCarthy, 2011). Another study by Brenner et al. (2011) has found that the fusion product of the TMPRSS2:ERG gene physically interacts with and is dependent upon for its oncologic activity, the enzyme poly (ADP-ribose) polymerase 1 (PARP1) and the catalytic subunit of DNA protein kinase (DNA-PKcs). PARP1 is also upregulated in response to DNA damage (Brenner et al., 2011). The authors showed that inhibition of PARP1 has an antitumor activity (Brenner et al., 2011), and clinical evaluation in combination with chemotherapy is already ongoing in advanced sporadic PC (de Bono et al., 2011b; Sandhu et al., 2011).

1.1.4.5 PTEN

PTEN targets primarily phosphatidylinositol 3,4,5-triphosphate (PIP3), downregulating the PI3K/AKT signaling pathway, which is crucial for cell survival and apoptosis (Li et al., 1997).

Taylor and colleagues have also confirmed deletions targeting *PTEN* on 10q23.31 (Taylor et al., 2010). The PI3K/AKT signaling pathway has been shown to be altered in 42 % and up to 100 % of primary and metastatic PC, respectively (Taylor et al., 2010).

Deletions and mutations of the PTEN gene are more frequent in metastatic than in primary PC. Because the loss of one allele (*i.e.,* LOH) at the PTEN locus occurs in approximately 40 % of cases, haploinsufficiency has been suggested to be enough to trigger PC initiation/progression (Vlietstra et al., 1998; Davies et al., 2002; Fernandez and Eng, 2002).

As consequence of PTEN expression downregulation, PI3K/AKT pathway activation is very common in late stage tumors (Li et al., 1997; Taylor et al., 2010). Furthermore, PTEN expression inversely correlates with AR expression, and direct downregulation of PTEN by AR has been proposed (Wang et al., 2011a). A recent report has suggested that PC can progress to the CRPC stage independently of the

AR axis via the PI3K/AKT signaling pathway; these data suggest that there is crosstalk between AR and the PI3K/AKT pathway (Mulhoand et al., 2011). Subsequently, Carver and colleagues (2011) have confirmed that this crosstalk between AR and PI3K/AKT signaling exists, suggesting that the concomitant inhibition of AR and PI3K/AKT signaling could be beneficial (Mulhoand et al., 2011; Carver et al., 2011). The crosstalk is dependent on the AR target gene androgen-regulated FK506 binding protein 5 (FKBP5), which encodes for the chaperon protein of the AKT phosphatase PHLPP (PH domain and leucine rich repeat protein phosphatase 1) (Pei et al., 2009). The data suggest that AR negatively regulates AKT activity and that therapeutic inhibition of AR may destabilize PHLPP1 because FKBP5 would no longer be produced. The destabilization of PHLPP1 leaves the activation of the AKT pathway intact, especially in the context of PTEN loss, leading to castration-resistant growth (Mulhoand et al., 2011; Carver et al., 2011). Subsequently, Chen and colleagues (2011a) have identified *PHLPP1* as tumor suppressor that is significantly co-deleted with *PTEN* in metastatic PC.

Another regulator of the PI3/AKT signaling pathway is the membrane associated guanylate kinase, WW and PDZ domain containing 2 (MAGI2) (Wu et al., 2000; Vazquezet al., 2001). Berger and colleagues (2011) have sequenced seven primary PC (three harboring ETS rearrangements) and their matched normal controls. The authors found several rearrangements occurring within genes, especially in the context of the TMPRSS2:ERG fusion. Specifically, they confirmed that PTEN signaling may be disrupted by these microrearrangements. They further characterized rearrangements impeding the function of MAGI2. Loss of function of MAGI2 may also deregulate the PI3/AKT signaling pathway (Berger et al., 2011), suggesting that the PI3/AKT pathway is subjected to diverse activating forces. In the context of PTEN loss, SMAD family member 4 (SMAD4) was recently shown to be downregulated in advanced compared to localized PC and to exert metastatic potential in a mouse model (Ding et al., 2011). This finding links cytokine pathways, such as the transforming growth factor–β (TGFβ) signaling pathway, to PC progression (Ding et al., 2011).

1.1.4.6 TP53

The tumor suppressor gene *TP53* is a key regulator of the cell cycle that controls the transition from the G1 phase to the S phase. Although *TP53* is the most commonly mutated gene in human cancers, mutations are rare in early PC but are found in approximately 20-40 % of advanced PCs cases (Porkka and Visakorpi, 2004), suggesting a role in tumor recurrence and metastasis. Interestingly, p53 inhibition has been proposed to upregulate AR transcription in cellular models (Alimirah et al., 2007). Chen and colleagues (2011a) have also found that concomitant loss of *PTEN* and *PHLPP* is associated with the loss of *TP53* in advanced PC (Chen et al., 2011a). Taken together, these data suggest that p53 is not

involved in PC initiation but rather in progression (Chen et al., 2011a; Shiota et al., 2011).

1.1.4.7 RAS/RAF signaling

The activated RAS/RAF pathway mediates expression of genes involved in cell proliferation and prevention of apoptosis via the activation of several transcription factors, such as ETS and MYC, and the modulation of mitochondrial localized proteins (McCubrey et al., 2007).

The frequency of RAS mutations varies with ethnicity; in the Western population, mutations are found in 5 % of the tumors, whereas in the Japanese population, mutated RAS can be found in 26 % of the tumors (Konishi et al., 2005). However, Taylor and colleagues (2010) have reported that 43 % and 90 % of primary and advanced PCs, respectively, harbor activating alterations in genes involved in the RAS/RAF signaling pathway (Taylor et al., 2010).

1.1.4.8 RB1

RB1 is a well-known tumor suppressor gene that encodes for a protein that controls the cell cycle at the level of the G1/S phase by repressing the transcriptional activity of the activator class of E2F transcription factors (E2F1–E2F3) (Burkhart and Sage, 2008). E2F1 has been shown to upregulate AR transcriptional activity as a consequence of RB1 depletion. This finding was confirmed in the clinical setting by data showing that tumors that do not express an RB1 gene expression signature also show upregulation of AR (Sharma et al., 2010). The *RB1* gene is lost in up to 20 % of early stage PCs, and mutated forms of pRB are found in up to 50 % of advanced PCs (Konishi et al., 2005, Taylor et al., 2010). Furthermore, alterations in the RB signaling pathway or loss or mutation of *RB1* occur in 34 % and 74 % of primary and advanced PCs, respectively (Taylor et al., 2010).

1.1.4.9 Non-coding RNAs

Non-coding RNAs (ncRNAs) have been implicated in cancer initiation and progression (Di Leva and Croce, 2010). MicroRNAs (miRs) are small ncRNAs that regulate protein translation by targeting specific messenger RNAs (Bartel, 2004). A general down-regulation of miRs has been shown in CRPC compared to PC (Porkka et al., 2007); these data have been confirmed just recently (Martens-Uzunova et al., 2011). Several miRs, such as miR-15 and miR-16-1, miR-193b and miR-34a, have also been suggested to be tumor suppressor genes (Cimmino et al., 2005; Bonci et al., 2008; Rauhala et al., 2010; Liu et al., 2011). These miRs modulate various

cellular processes, such as cell cycle regulation and apoptosis, invasion and migration, androgen-independent growth and epigenetic changes (Coppola et al., 2010). More recently, miR expression signatures have also suggested to be both diagnostic and prognostic for PC (Martens-Uzunova et al., 2011). Waltering et al. (2011) have also demonstrated that a small number of miRs are also androgen-regulated.

In addition, a recent report has shown that long intergenic ncRNAs can also be involved in PC progression. Prostate cancer-associated ncRNA transcript 1 (PCAT1) has been implicated in disease progression (Prensner et al., 2011). The expression of PCAT1 is mutually exclusive with the expression of enhancer of zeste homolog 2 (Drosophila) (EZH2) (Prensner et al., 2011). Overexpression of EZH2 is a marker of advanced and metastatic disease in many solid tumors, including prostate and breast cancer (Chase and Cross, 2011). Furthermore, EZH2 is an enzymatic component of the polycomb repressive complex 2 (PRC2), which has recently been found to regulate the expression of a substantial number of miRs involved in PC progression together with the PRC1 (Cao et al., 2011).

1.1.4.10 Amplification and overexpression of MYC

MYC is a transcription factor that plays a key role in regulating a number of cellular processes, including cell-cycle progression, metabolism, ribosome biogenesis, protein synthesis, mitochondrial function, and stem cell self-renewal (Dang et al., 2006).

One of the most common amplified loci in PC includes v-myc myelocytomatosis viral oncogene homolog (avian) (MYC) at 8q24.21 (Taylor et al., 2010). MYC is a well-known oncogene and is overexpressed in many cancers (Nesbit et al., 1999). The amplification of the 8q24 locus and MYC in PC has been known for a long time (Visakorpi et al., 1995; Jenkins et al., 1997; Nupponen et al., 1998). The overexpression of MYC protein has been difficult to evaluate for technical reasons (Koh et al., 2010). Nupponen et al. (1998) have found that gains of copy number in the same loci are even more common than the amplification and occur in up to 70 % of advanced tumors. However, MYC is not overexpressed according to these studies (Nupponen et al., 1999; Savinainen et al., 2004).

A murine model harboring prostate-specific overexpression of MYC (Hi-MYC model) has shown that MYC is sufficient to induce PC (Ellwood-Yen et al., 2003). Moreover, the most recent data suggest that nuclear MYC protein overexpression is an early alteration in PC that also persists to late stage disease (Gurel et al., 2008). MYC was also recently found to cooperate with the AKT pathway and altering the sensitivity to mTOR inhibitors (Balakumaran, et al., 2009; Clegg et al., 2011), probably through the MYC target eukaryotic translation initiation factor 4E binding protein 1 (4EBP1) (Balakumaran, et al., 2009). Other MYC-associated mechanisms have also been implicated in PC initiation and progression. Among these are NKX3.1 downregulation, upregulation of EZH2 (Koh et al., 2010), and interplay of

regulation with TMPRSS2:ERG fusion (see chapter 1.1.4.4) (Sun et al., 2008; Koh et al., 2010). Furthermore, MYC has been proposed to upregulate AR levels in PC cells through the MYC target endothelin 1 (ET-1) (Lee et al., 2009).

1.2 Androgen receptor-mediated mechanisms

1.2.1 Androgen receptor: structure and function

The nuclear receptor AR is a member of the steroid hormone receptors (SHRs) superfamily. SHRs are sensors of the intra- and extracellular physiology and control several processes, such as development, differentiation, metabolic homeostasis and reproduction (Perissi and Rosenfeld, 2005). The SHRs consists of at least the following three distinct domains: a ligand binding domain (LBD), which is at the C-terminal side of the protein, a DNA binding domain (DBD), and an N-terminal domain (NTD) (see also Figure 5 A&B).

The LBD and DBD share a high degree of homology among the members of the family. The activation of the SHRs involves conformational changes in the LBD as a consequence of ligand binding. The LBD differs only to account for the properties of the various ligands. Thus, the volume of the ligand pocket can vary dramatically. Furthermore, the LBD contains amino acid motifs that can be recognized by other proteins, which are able to modulate the transactivation of the receptors; these proteins are named coregulatory proteins or coregulators and can be coactivators or corepressors. In addition, the LBD also contains residues that form a surface involved in dimerization and a region called activation function 2 (AF2) that is essential for specific ligand binding, dimerization, and transactivation. Some nuclear receptors, such as the retinoid X receptor, can form heterodimers, while others, such as AR, can form homodimers. The LBD is linked to the DBD by a hinge region. The DBD is needed for the SHRs to perform their functions (Reviewed by McEwan, 2009). The activated SHRs bind to DNA response elements (REs) in relative proximity to target genes (see chapter 1.2.4.1.1). The DBD also acts as an allosteric modulator by regulating the dimerization and the structure of the NTD and its protein interactions (Lefstin and Yamamoto, 1998). The NTD is the domain that varies the most among the family members. The NTD can vary in length and amino acid sequence and does not show great homology among the members of the family. Additionally, the structure of the NTD of SHRs is modulated by the binding to DNA and coregulatory proteins. The NTD contains a region named activation function 1 (AF1), which is required for transactivation (Reviewed by McEwan, 2009).

Inactive AR is found in the cytoplasm bound to chaperone proteins from the family of the heat-shock proteins (HSPs) (Reviewed by McEwan, 2009). The LBD has been shown to exert inhibitory properties on the receptor to prevent its

inappropriate activation (Jenster et al., 1991). When the ligand, testosterone or DHT, binds in the pocket of the LBD, a conformational change is induced that causes the release of AR from the HSPs. AR is then able to interact with other receptor molecules or with itself intramolecularly (Reviewed by McEwan, 2009). The activated receptor translocates into the nucleus via a particular portion of its amino acid sequence that is functionally similar to a nucleoplasmin nuclear localization signal (Jenster et al., 1993). In the nucleus, AR dimerizes (Gelmann et al., 2002; McEwan, 2004) and interacts with other transcription factors and proteins, which may drive the recognition of specific target regions in the chromatin (Massie et al., 2007; Lupien et al., 2008; McEwan, 2009; Wang et al., 2009b; Lupien and Brown, 2009; Wang et al., 2011b; Robinson et al., 2011).

Once bound to the promoter and/or enhancer elements of target genes, the SHRs activate transcription via AF1 and/or AF2 by recruiting protein complexes with enzymatic activities with the final scope of opening the chromatin structure and components of the general transcription machinery, resulting in the formation of the transcription initiation complex (see chapter 1.2.4.2) (Reviewed by Perissi and Rosenfeld, 2005; McEwan, 2009; He et al., 2010).

Much evidence has led to the conviction that the assembly and disassembly of the protein complexes on a target gene regulatory region in response to a hormone signal follows a cyclical pattern. The mechanism may be shared among the SHRs (Hager et al., 2004; Perissi and Rosenfeld, 2005; Stavreva et al., 2009; Carlberg et al., 2010;) and is dependent on the proteasome (Kang et al., 2002; Jia et al., 2006) and HSP activity (Conway-Campbell et al., 2011). The former dependency may explain why AR constantly shuttles from the nucleus to the cytoplasm in response to androgen stimulation (Black et al., 2001; Gioeli et al., 2006; Kesler et al., 2007).

1.2.2 Androgen receptor and castration-resistant prostate cancer

Given the dependency of the prostate on androgens/AR signaling in physiological and pathological conditions, AR has been extensively studied. The importance of AR signaling in the progression of this disease is increasingly evident, and AR is currently recognized to be an important driver and a validated drug target in advanced PC (Ryan and Tindall, 2011).

Lowering tissue levels of DHT with 5α-reductase (steroid-5α-reductase (SRD5A) inhibitors, such as finasteride or dutasteride, decreases the risk of PC (Thompson et al., 2003; Andriole et al., 2010). Furthermore, several recent clinical studies on CRPC have reported successful results in targeting the AR axis by either preventing steroidogenesis with the CYP17 inhibitor abiraterone (deBono et al., 2011a) or inhibiting AR activity with the novel super antiandrogen MDV3100 (Tran et al., 2009; Scher et al., 2010).

During progression to CRPC, AR signaling is maintained through several mechanisms (Attar et al., 2009; Antonarakis and Armstrong, 2011b) (Figure 2B). These mechanisms include:

- AR overexpression;
- steroidogenesis;
- AR mutations;
- alterations in AR interacting proteins;
- ligand-independent activation of AR signaling and AR variants.

1.2.2.1 Androgen receptor overexpression

AR overexpression has been suggested to be associated with poor prognosis (Henshall et al., 2001; Donovan et al., 2010). In 1995, Visakorpi and colleagues found recurrent amplification of the *AR* gene locus resulting in AR overexpression in one third of CRPCs (Visakorpi et al., 1995; Linja et al., 2001). Amplification of AR (Xq12) occurs in approximately 30 % of patients, but it is found only in CRPCs (Visakorpi et al., 1995; Koivisto et al., 1997; Taylor et al., 2010), suggesting that the ADT selects for this genetic alteration (Porkka and Visakorpi, 2004). Furthermore, patients with AR gene amplification respond better to a second line of androgen blockade than patients without the amplification (Palmberg et al., 2000), suggesting that the relapse is driven by the amplification. However, other mechanisms leading to AR overexpression must exist because AR is overexpressed in approximately 80-90 % of advanced PCs (Edwards et al., 2003; Linja et al., 2004; Chen et al., 2004; Tamura et al., 2007; Liu et al., 2009). The finding of AR gene overexpression has led to the hypothesis that CRPCs might be androgen-hypersensitive (Nupponen and Visakorpi, 1999). Indeed, in a tumor xenograft model, modest AR overexpression was demonstrated to be sufficient to drive androgen independence and convert androgen antagonists, such as bicalutamide and flutamide, into agonists (Chen et al., 2004).

All of the mechanisms leading to the AR overexpression in CRPCs are still not known. In addition to AR gene amplification, loss of the tumor suppressor *RB1* or regulation through other transcription factors may partially explain the AR overexpression (see chapter 1.1.4.8). Another mechanism involving the loss of purine-rich element binding protein A (PURA), part of a transcriptional repressor complex, has been proposed (Wang et al., 2008). This repressor complex was shown to bind immediately after the AR gene's transcription start site (TSS) and repress AR mRNA transcription. Moreover, in CRPC specimens, diminished expression of PURA has been observed, which correlates with AR overexpression (Wang et al., 2008).

Thus, understanding the molecular advantage that AR overexpression provides to the therapy-resistant disease is important.

1.2.2.2 Steroidogenesis

Castration does not affect androgen synthesis in the adrenal glands. This lack of effect is important because the increased level of AR suggests that the tumor may become hypersensitive to residual androgens, which are not abolished by castration (Nupponen and Visakorpi, 1999). Several studies support the idea that CRPCs can be maintained by low levels of androgens produced by the tumor cells or by the adrenal gland through the conversion of a steroid precursor (Yamaoka et al., 2010; Cai and Balk, 2011). Specifically, the adrenal gland produces approximately 10–30 % of serum androgens, which may play a significant role in activating AR signaling (Bruno et al., 2007).

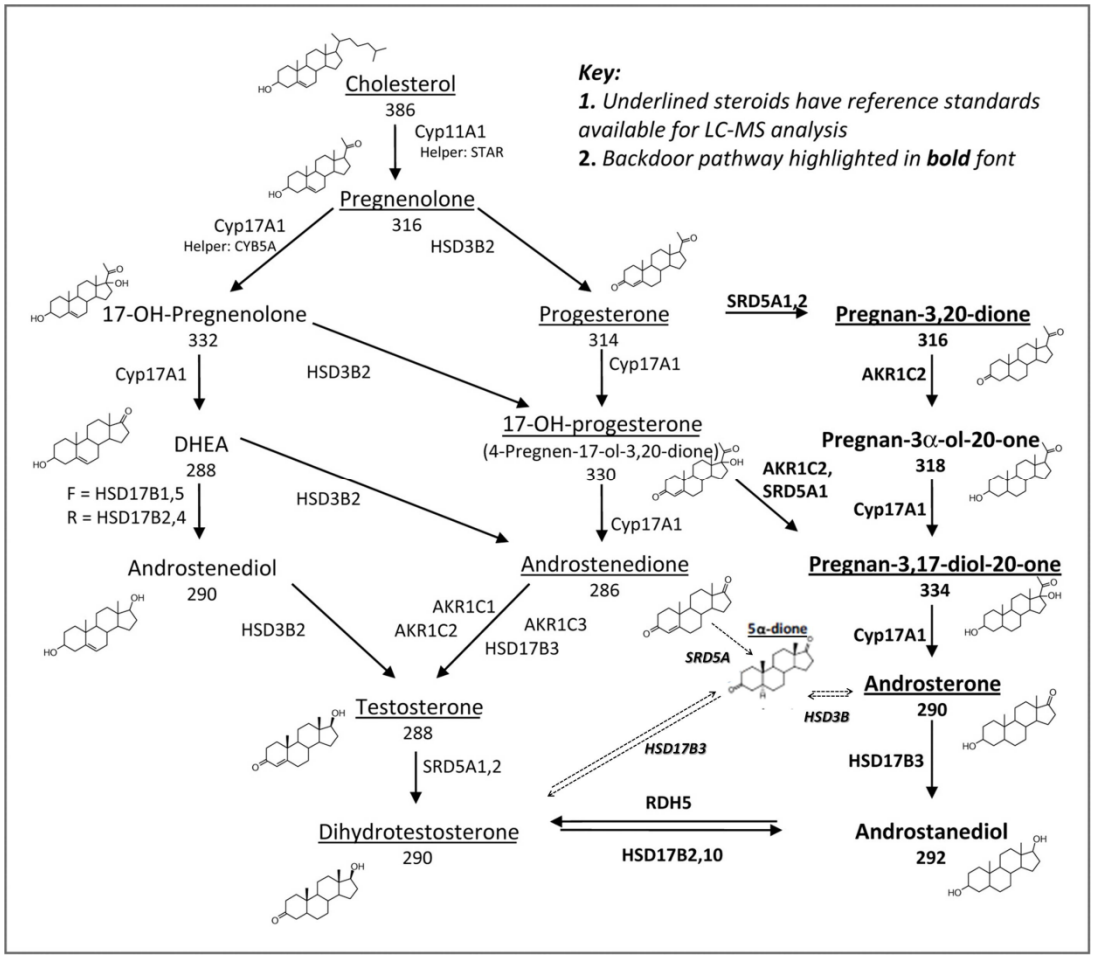

Figure 3. Enzymes involved in both classical (left/middle) and backdoor (far right, bold font) steroidogenesis pathways. Many of the steroidogenic enzymes catalyze more than one step in the pathway. Standards available for underlined steroids. Adapted from Lubik and colleagues (2011), the image now also shows the alternative *de novo* pathway (dashed arrows) as described in Chang et al. (2011). The enzymes involved in the *de novo* pathway are written in Italic. Finasteride inhibits SRD5A1/2, while abiraterone inhibits CYP17A1 (Adapted from Lubik et al. Insulin increases *de novo* steroidogenesis in prostate cancer cells. Cancer Res. 2011;71:5754-64 with permission by American Association for Cancer Research and from Auchus RJ. The backdoor pathway to dihydrotestosterone. Trends Endocrinol Metab. 2004 Nov;15(9):432-8 with permission from Elsevier.).

LHRH agonists fail to prevent synthesis of androgens by the adrenal glands, and patients developing resistance to antiandrogen therapy have been shown to maintain sufficient intratumoral levels of testosterone and DHT to activate AR (Chen et al., 2004). Furthermore, adipose tissue has also been shown to be able to produce androgens (Puche et al., 2002). Dehydroepiandrosterone (DHEA) and other steroid precursors secreted by the adrenal glands, which represents 5-10 % of the total androgens in the bloodstream, can be converted into potent androgens (Labrie et al., 1987). In addition, CRPCs may be able to synthesize testicular androgens through intracrine production from adrenal androgens and cholesterol (Titus et al., 2005) (Figure 3). Therefore, the adrenal androgens DHEA and androstenedione (AD) can be transported to CRPC tissue and converted to testosterone and DHT. DHEA and AD can also be synthesized intratumorally in CRPC from cholesterol tissues and converted to testosterone and DHT via the *de novo* route (DHEA→AD→Testosterone→DHT). In fact, compared to primary PC and normal prostate (*e.g.,* BPH), CRPC has been shown to overexpress enzymes needed during the conversion of adrenal androgens to DHT and enzymes needed for the *de novo* synthesis of DHT (Stanbrough et al., 2006; Mostaghel et al., 2007; Locke et al., 2008; Locke et al., 2010). Pathways that enable CRPCs to synthesize androgens offer the tumor an escape route from ADT (Montgomery et al., 2008) (Figure 3).

Intratumoral enzymes involved in androgen biosynthesis, such as CYP17A1 and CYP19A1 (which also produce DHEA), are upregulated in CRPCs (Montgomery et al., 2008), and their expression seems to correlate with high stage, high Gleason score and short recurrence-free time (Stigliano et al., 2007). Therefore, these enzymes are now considered promising drug targets (Antonarakis and Armstrong, 2011).

The importance of steroidogenesis in PC progression and as a mechanism of resistance has today been confirmed by successful trials with a CYP17 inhibitor, abiraterone, which has shown improved survival in CRPC (deBono et al., 2011a). However, resistance to abiraterone has already been reported (Attard et al., 2008), suggesting that androgen steroidogenesis is a labile process that can rely on several mechanisms to escape these therapeutic approaches. For instance, treatment with abiraterone has recently been demonstrated to select for tumor cells overexpressing CYP17A1, providing a mechanism for the development of resistance to CYP17A1 inhibitors (Cai et al., 2011). Furthermore, CRPC cells have also been shown to be able to use a novel synthetic pathway through which DHT is produced in tumor cells from the adrenal precursor AD via upregulation of steroid 5α-reductase isoenzyme-1 (SRD5A1), bypassing testosterone production (Chang et al., 2011) (Figure 3). This observation suggests alternative mechanisms of recurrence upon treatment with other drugs, such as dutasteride and other primary therapies.

1.2.2.3 Androgen receptor mutations

In 1995, the first study reported AR somatic mutations in the androgen-receptor gene in CRPCs that were not present in the primary tumors of the same patients (Taplin et al., 1995). However, in general, AR mutations are rare (Wallén et al., 1999; Culig et al., 2001; Taylor et al., 2010). Mutations are found in approximately 20-30 % of patients treated with antiandrogens and play a role in the resistance to such drugs (Taplin et al., 1995; Haapala et al., 2001). These mutations are most commonly found in the LBD and are known to affect the specificity of AR toward its ligand (Veldscholte et al., 1992; Sack et al., 2001). These mutations allow other nuclear hormones (estrogen and progestin), corticosteroids (cortisol and cortisone) and, paradoxically, antiandrogens such as cyproterone, hydroxyflutamide and bicalutamide to activate AR (Newmark et al., 1992; Suzuki et al., 1993; Culig et al., 1993; Taplin et al., 1995; Culig et al., 1999; Thin et al., 2002; Hara et al., 2005).

Mutations affecting the NTD of AR have also been reported (Tilley et al., 1996). Although mutations in the NTD may affect the ability of AR to bind its coregulators (Bergerat & Céraline, 2009), no clear effect on AR activity could be predicted due to lack of an NTD crystal structure (McEwan, 2009). The tumors used in these studies were rare forms of already metastasized late stage PC (Tilley et al. 1996), and such mutations have not been found in other studies (Wallen et al., 1999). Mutations in the DBD may also interfere with the nuclear localization signal and thus affect transactivation and transrepression abilities or favor binding to unspecific promoters, affecting the set of target genes regulated by AR (Bergerat & Céraline, 2009).

1.2.2.4 Androgen receptor interacting proteins

More than 300 proteins have now been reported to interact with AR. Approximately 75 % interact directly with the receptor. Approximately half of the proteins are coactivators, and approximately 30 % are corepressors. The rest are of unknown function or do not seem to elicit a clear activity on the receptor. For the majority of these proteins, it is not clear with which domain of the receptor they interact. An updated list of coregulators is available on-line (http://androgendb.mcgill.ca/). In general, the definition of AR coregulators is proteins that either enhance (coactivator) or decrease (corepressor) the transactivation of AR, meaning that they do not modify the AR basal transcriptional activity (Heemers and Tindall, 2007). However, this vision is arguable, given the number of proteins with unknown function that interact with AR.

According to the most recent classification by Heemers and Tindall (2007), the AR interacting proteins can be divided into the following 3 distinct classes:

- components of the general transcription machinery;

- functionally diverse proteins with AR coactivating or corepressing properties (coregulators modifying chromatin status and coregulators with other functions);
- specific transcription factors (see chapter 1.2.4.2).

1.2.2.4.1 Components of the general transcription machinery

It is increasingly evident that AR can modulate the transcription of target genes by regulating both transcription initiation and transcription elongation (Heemers and Tindall, 2007). For instance, AR is able to interact with two proteins of the basal transcription machinery, general transcription factor IIF, polypeptide 1 (GTF2F1) and general transcription factor IIF, polypeptide 2 (alias GTF2F2), which are subunits of the TFIIF (transcription factor IIF) complex and with TFIIH (transcription factor IIH). These complexes are recruited to the RNA polymerase to facilitate strand preparation. The interaction of AR with TFIIF is thought to induce more open chromatin via the further recruitment of other transcriptional activators, while the interaction with TFIIH has been demonstrated to result in the phosphorylation of the RNA polymerase, which is essential in transcription initiation (Lee and Chang, 2003). Transcription elongation is, on the other hand, activated by polymerase (RNA) II (DNA directed) polypeptide B (POLR2B), which activates the RNA polymerase. Again, AR also interacts with this protein, resulting in transcription elongation (Lee et al., 2003).

1.2.2.4.2 Coregulators modifying chromatin status

The AR coregulators have been recently classified according to their function (Heemers and Tindall, 2007). Because transcription occurs on the chromatin, it is not surprising that a vast number of AR coregulators function as components of the chromatin remodeling complex or are histone modifiers.

The local chromatin structure is influenced, in part, by the presence or absence of the nucleosomes, but principally by the covalent modifications on the histone tails and DNA methylation (Li et al., 2007). To date, the most commonly identified histone modifications are acetylation, methylation, phosphorylation, ubiquitination, biotinylation, SUMOylation or poly-ADP-ribosylation (Heemers and Tindall, 2007). These modifications affect the physical properties of the histones and, thus, of the nucleosomes, which in turn affect the chromatin and the accessibly of the genome (Felsenfeld and Groudine, 2003; Cosgrove and Wolfberger, 2005). The great variety of histone modifications has led to the hypothesis of the "histone code" (Santos-Rosa and Caldas, 2005).

Acetylation is the most studied histone modification. The general consensus is that hyperacetylation favors gene expression, whereas hypoacetylation is associated

with genes that are not actively transcribed. For example, the tail of H3 contains 13 positively charged lysine (K) residues and only one to four of these residues become acetylated, which decreases the positive charge by only 10-30 %. These modifications offer a platform for other non-histone proteins that either activate or repress transcription (Munshi et al., 2009). For example, acetylation of H4K8, H3K9 and H3K14 have an important role in the recruitment of the 11 subunit SWI/SNF chromatin remodeling complex and TFIID during transcription initiation (Fukuda et al., 2006). The SWI/SNF complex has been shown to be capable of disrupting, repositioning or displacing histones *in vitro* and *in vivo* (Côté et al., 1994; Owen-Hughes et al., 1996; Whitehouse et al., 1999; Becker and Horz, 2002) (Figure 4).

AR has been found to interact deeply with the components of the chromatin remodeling complex such as SWI/SNF. Coactivators, such as AR interacting protein 4 (ARIP4), BRG1 (alias SMARCA4: SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4) and BAF57 (alias SMARCE1: SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1), all bridge AR activation and are all SWI/SNF dependent (Heemers and Tindall, 2007).

The first SHR coactivator was identified in 1995 by Oñate and colleagues (1995) and was revealed to be a member of the family of the p160 SRC (steroid receptor coactivator) gene family named SRC1 (the official name was then changed to nuclear receptor coactivator 1 (NCOA1)) (Oñate et al., 1995). Subsequently, other members of the p160 SRC family have been identified: SRC2 (alias nuclear receptor coactivator 2 (NCOA2) or glucocorticoid receptor-interacting protein-1 (GRIP1) or transcriptional intermediary factor 2 (TIF2)) and SRC3 (alias p300/CBP interacting protein (p/CIP) or receptor-associated coactivator-3 (RAC3) or amplified in breast cancer 1 (AIB1)). Moreover, SRCs have been shown to recruit more activators, such as E1A binding protein p300 (EP300) and CREB binding protein (CREEBP alias CBP). All of these coactivators have histone acetyltransferase (HAT) activity and others, such as K (lysine) acetyltransferase 5 (KAT5 or Tat interactive protein 60 kDa (TIP60)) and KAT7, have been identified to belong in this group (Heemers and Tindall, 2007). The activity of HAT proteins is counterbalanced by histone deacetylases (HDACs). Among them, Sirtuin1 (SIRT1) (Fu et al., 2006) and HDAC7 (Karvonen et al., 2006) have been shown to directly interact with AR and repress AR activity. However, only SIRT1 (but not HDAC7) has been shown to directly deacetylate AR (Fu et al., 2006; Karvonen et al., 2006). Other HDACs have been shown to indirectly repress AR activity through the association with other subunits of the nuclear receptor corepressor complex (NCoR) (Perissi et al., 2010). Although the NCOR1 and NCOR2 complexes have been shown to comprehend only proteins with HDAC activity, other complexes have been identified, such as the nucleosome remodeling and histone deacetylation (NURD) complex, which combines HDAC activity and histone demethylase activity. These complexes include the AR coregulator lysine specific histone demethylase 1 (LSD1) (Perissi et al., 2010) (Figure 4).

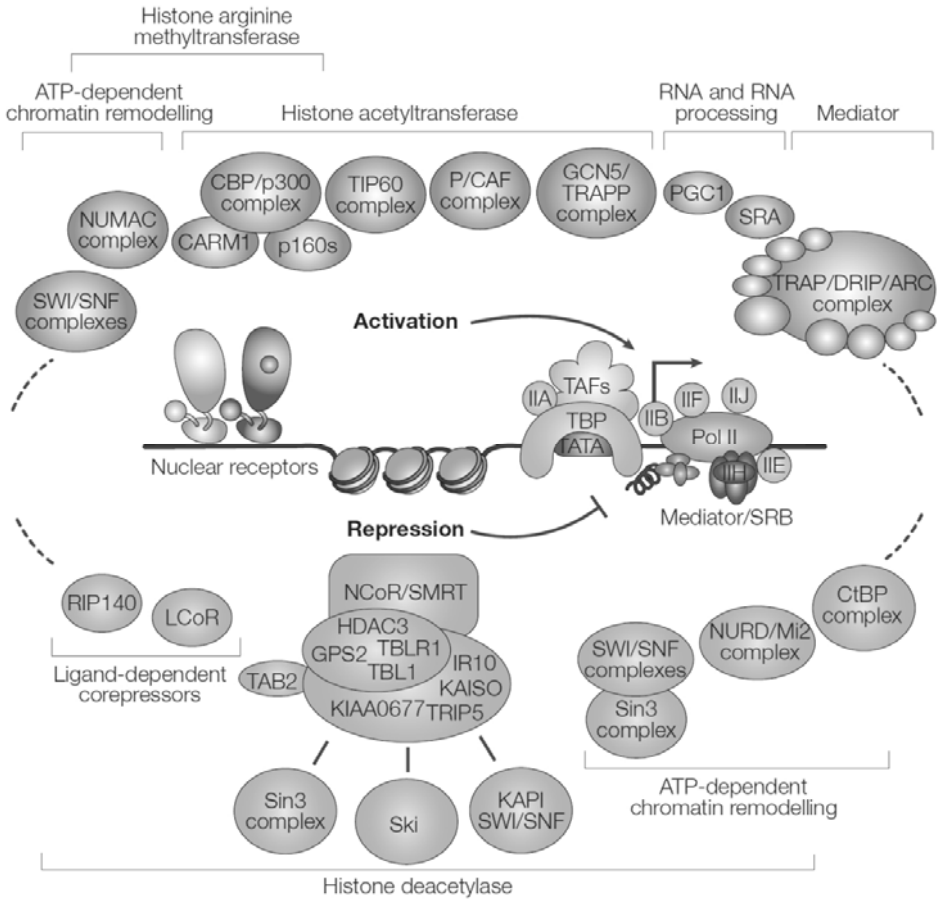

Figure 4. Coactivator and corepressor complexes required for nuclear-receptor-mediated transcriptional regulation. The regulation of target genes by nuclear receptors requires a vast number of coregulatory complexes with various functions and enzymatic activities. Coactivator complexes (top side of the chromatin filament) include factors that contain ATP-dependent chromatin remodeling activity, histone arginine methyltransferases histone acetyltransferases and factors that are involved in RNA processing and components of the so-called Mediator complex that mediate the interaction with the RNA Pol II machinery. Conversely, corepressors (bottom side of the chromatin filament) include ATP-dependent chromatin remodeling complexes basal corepressors, such as NCoR and SMRT, which function as platforms for the recruitment of several subcomplexes that often contain histone deacetylase activity, and specific corepressors, such as LCoR and RIP140, which are able to recruit general corepressors. The recruitment of these complexes is not simultaneous, and this schematic representation does not illustrate the dynamics of their recruitment. IIA, IIB, IIE, IIF, IIH, IIJ, general transcription factors A, B, E, F, H, J; HDAC, histone deacetylase; LCoR, ligand-dependent nuclear-receptor corepressor; NCoR, nuclear-receptor corepressor; RIP140, receptor-interacting protein-140; SMRT, silencing mediator of retinoic acid and thyroid hormone receptors; TAF, TBP-associated factor; TBP, TATA-binding protein. (Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Molecular Cell Biology] (Perissi and Rosenfeld, 2005), copyright (2005)).

The methylation of the histones tails is the least understood histone modification. H3 and H4 lysines residues can be mono-, di-, or trimethylated, which increases the variety of possible modification patterns. Unlike acetylation and phosphorylation, methylation is thought to be a stable histone modification. Methylation can be a sign

of both transcriptional activity and inactivity according to the specific modified residue (Munshi et al., 2009).

Demethylation at distinct lysine residues in histone H3 by LSD1 causes either gene repression (Shi et al., 2004) or activation (Metzger et al., 2005). Furthermore, LSD1 may selectively act as a component of co-repressor complexes, removing mono- and dimethyl marks from H3K4 or, during AR mediated gene transcription, removing mono- and dimethyl marks from H3K9. In AR-dependent gene activation, LSD1 cooperates with a trimethyl demethylase, the Jumonji C (JMJC) 5 domain-containing protein JMJD2C (also known as KDM4C), in removing repressive histone marks from H3K9 (Wissmann et al., 2007). Recently, Metzger and colleagues (2010) found that during AR-dependent gene transcription, LSD1 activity is subject to the proof-reading activity of another enzyme, which catalyzes the phosphorylation of H3T6: protein kinase C beta I (PKCβI, also known as PRKCβ) (Metzger et al., 2010). Other demethylases have also been shown to be involved in AR transcription. One is the lysine (K)-specific demethylase 3A (KDM3A alias JHMD2A or JMJD1A), which has been shown to be an AR coactivator (Yamane et al., 2006). As it is easy to imagine, AR transcription depends not only on demethylases but also on methylases acting in a concerted manner to tightly regulate AR-mediated transcription (Heemers and Tindall, 2007). For instance, coactivator-associated arginine methyltransferase 1 (CARM1, alias protein arginine methyltransferase 5 (PRMT5)), which methylates H3R17, and PRMT1, which methylates H4R3, have been shown to be recruited in an androgen-dependent manner to activated transcription complexes of AR target genes. However, their activity was also shown to be dependent on SRC proteins (Figure 4) (Chen et al., 1999; Wang et al., 2001).

1.2.2.4.3 Coregulators with other functions

As previously mentioned, the AR-mediated transcription of target genes is dependent on proteasome activity (Lin et al., 2002, Kang et al., 2002; Jia et al., 2006). The proteasome is a large protein complex, in which polyubiquitinated proteins are degraded (Groll et al., 2011). Several AR interacting proteins have been shown to have E3 ligase activity, ligating ubiquitin groups to target proteins. Thus, AR and AR coregulators are also targets for ubiquitination (Heemers and Tindall, 2007). The ring finger protein 14 (RNF14 alias ARA54) is one of the E3 ligases (Kang et al., 1999).

Proteins involved in DNA repair are also recruited to the AR-mediated transcription, which suggests that AR-mediated transcription may also involve DNA disrupting events as demonstrated in cancer cells (Mani et al., 2009). BRCA1, with E3 ubiquitin ligase activity, and BRCA2, a component of the homologous recombination machinery, are both AR coactivators (Park et al., 2000; Yeh et al., 2000; Shin et al., 2003).

In absence of ligand, AR is kept stably in the cytoplasm by HSPs. Specifically, a variant of BCL2-associated athanogene (BAG1), BAG1L (which is an HSP associated protein), has been demonstrated to physically interact with AR, to be recruited to AR target genes and to function as coactivator (Knee et al., 2001). AR trafficking into the nucleus is regulated not only by HSPs, but also by cytoskeletal proteins, such as supervillin and gelsolin. These proteins interact with AR and enhance androgen-dependent AR activity (Nishimura et al., 2003; Ting et al., 2004).

Furthermore, AR shows a quite extensive interplay with several signal integrators and transducers, scaffolds and adaptor proteins. For instance, AR has been shown to interact with tyrosine kinase, non-receptor, 2 (TNK2, Alias: activated Cdc42-associated tyrosine kinase (ACK1)), which is able to enhance AR-mediated gene transcription in the nucleus in an androgen-independent manner. Another Cdc42/Rac interactive protein p21 protein (Cdc42/Rac)-activated kinase 6 (PAK6) is activated by AR and translocates into the nucleus where it represses AR-mediated transcription. RanBPM (Ran (member RAS oncogene family)-binding protein in the microtubule-organizing center) and RAN (alias ARA24), another AR coactivator, have also been shown to be involved in AR nuclear translocation.

Finally, AR is in the middle of interplay with several adaptors for multiple signal transduction events, such as the STAT (signal transducer and activator of transcription) signaling pathway via STAT3 and PIAS3 interaction, inhibition of TGFβ (transforming growth factor, beta 1) signaling via repression of SMAD family member 3 (SMAD3), ErbB (v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)), and the Notch and Wnt signaling pathways (reviewed by Heemers and Tindall, 2007). The most critical component of the Wnt signaling pathway is β-catenin (CTNNB1 catenin (cadherin-associated protein), beta 1, 88 kDa), which has been shown to bind AR in a ligand-dependent manner and scaffold to the nucleus with AR enhancing its target genes transcription (Yardy and Brewster, 2005).

AR activity is also enhanced by several cell cycle regulators, such as RB1 and various cyclins (reviewed by Heemers and Tindall, 2007). Interestingly, Cyclin D1 (CCND1), independently of its function in cell cycle progression, has been shown to repress AR activity in a ligand-dependent manner (Knudsen et al., 1999). However, a genome-wide approach has more recently shown that cyclin D1 represses AR-mediated transcription on selected AR target genes (Comstock et al., 2011).

AR activation may also occur by post-translational modifications, such as phosphorylation. AR has been shown to be phosphorylated at several sites in response to the binding of the ligand and nuclear localization (Gioeli and Paschal, 2011). Furthermore, several kinases have also been shown to enhance AR-mediated transcription (Heemers and Tindall, 2007). For instance, male germ cell-associated kinase (MAK) physically interacts with AR and is recruited to the chromatin, enhancing AR activity (Ma et al., 2006). Some phosphatases, such as protein phosphatase 2A activator, regulatory subunit 4 (PPP2R4 alias PP2A), can directly dephosphorylate sites in the NTD of the activated AR and repress its activity (Yang et al., 2006).

Several other not as well studied AR coregulators have been identified, such as NCOA6 (alias AIB3) (coactivator) (Goo et al., 2004), amino-terminal enhancer of split (AES) (corepressor) (Yu et al., 2001), RAD54-like 2 (S. cerevisiae) (RAD54L2 alias ARIP4) (coactivator) (Rouleau et al., 2002), NCOR1 (Zamir et al., 1997) and the cell cycle regulator, BTG family, member 2 (BTG2) (corepressor) (Hu et al., 2011). One of these coregulators, the melanoma antigen gene protein (MAGEA11 alias MAGE11), has been shown to be an AR coactivator. MAGE11 can specifically bind the AR NTD, resulting in stabilization of the ligand-free AR. It was also shown that MAGE11 may favor interdomain interaction in presence of AR agonists (Bai et al., 2005).

1.2.2.5 Androgen receptor coregulators and prostate cancer

Studies in knock-out mice of several coregulators have revealed only mild phenotypes (Reyes et al., 1998; Xu et al., 1998; Gehin et al., 2002; Cheung-Flynn et al., 2005; Yong et al., 2007), suggesting that coregulators can supplement the function of the absent proteins. This suggestion leads to the hypothesis that the stoichiometry of the coregulators may be most important for the AR physiological functions, and the deregulation of such stoichiometry may lead to pathological conditions such as PC. However, it has been shown that deregulation of specific coregulators may derive from tissue-specific splice variants (Tao et al., 2006) or by variants with gained functions (Burd et al., 2006; Desai et al., 2006). Mutations in coactivators that provide a means for antiandrogens to activate AR could represent another mechanism for how coregulators may deregulate AR activity. For example, some AR mutations, together with the interaction with coactivators such as NCOA4 (alias ARA70) and ARA54, can alter the receptor response to antiandrogens, such hydroxyflutamide, or render the receptor promiscuous to 17β-estradiol (Rahman et al., 2004). In contrast, some evidence has suggested that AR mutations may enhance the binding of AR coactivators, such as AIB1 (Zhou et al., 2010).

In light of such a stoichiometric mechanism of the activity of the coregulators, many groups have investigated the abundance of the coregulators in PC. The most systematic and comprehensive studies investigating the expression profile of the AR coregulators in PC were those by Linja et al. (2004) and, to some extent, the more recent by Taylor et al. (2010). Linja et al. (2004) have found that the levels of AR coregulators do not change dramatically in PC, excluding their involvement in disease progression. The study by Taylor and colleagues (2010) also seems to support this observation because of the coregulators, only NCOA2 is upregulated in a proportion of PCs. However, other studies have found coactivators that are overexpressed in PC compared with matched normal tissues (Li et al., 2002; Mestayer et al., 2003; Culig et al., 2004). Experiments in PC cell lines and studies of human clinical tumors have suggested that the overexpression of SRCs (NCOAs) may be involved in PC progression (Desai et al., 2006; Shi et al., 2008; Xu et al., 2009). However, studies on NCOA1 have reported discordant results. Linja et al.

(2004) found that NCOA1 mRNA levels are upregulated in primary PC but not in CRPC specimens. Subsequently Mäki et al. (2006) showed that the protein levels of NCOA1 are only mildly elevated in CRPC. Thus, NCOA1 is not likely to play an important role in PC progression.

Altered levels of coactivators, including NCOA2, 3 and 4, MAGE11 and NFκB p100 subunit, have been found in PC specimens (Gregory et al., 2001; Comuzzi et al., 2003; Léotoing et al., 2008; Karpf et al., 2009; Nadiminty et al., 2010). As mentioned above, the NCOA2 expression level correlates with early biochemical recurrence in PC patients (Agoulnik et al., 2006), and NCOA2 has recently been reported to function as an oncogene in a subset of PCs. The chromosomal region 8q13.3, which harbors the NCOA2 gene, is the most commonly amplified locus in PC (Taylor et al., 2010). NCOA3 overexpression has been found to correlate with the grade of PC (Zhou et al., 2005). NCOA4 protein levels are elevated in high grade PCs and in CWR22 xenograft tumors that have become castration-resistant (Hu et al., 2004).

ARA24/Ran was found to be upregulated in cancer tissue compared with adjacent benign tissue isolated from PC patients (Li et al., 2002). Tip60 was also found to be overexpressed upon androgen ablation in both xenograft and cell line models (Halkidou et al., 2003). BAG1L has been shown to be amplified and overexpressed in a subset of CRPC specimens (Mäki et al., 2007). The levels of p300 correlate with larger tumor volumes, extra-prostatic growth and tumor progression (Debes et al., 2003). The levels of CBP have been shown to be high in advanced PC and, specifically, in tissues from patients that failed endocrine therapy (Comuzzi et al., 2004), although these results are discordant with the study by Linja et al. (2004). Additionally, the levels of histone demethylases, such as KDM1A (alias LSD1), KDM3A and KDM4C have been investigated in PC. KDM3A and KDM4C levels have been shown to be increased in primary PC, and KDM4C is also overexpressed in CRPC (Suikki et al., 2010).

Finally, the downregulation of AR corepressors may also be involved in the development of CRPC. For example, the corepressor PIAS1 was found to be under-repressed in CRPC specimens by Linja et al. (2004). Additionally, the recently identified AR corepressor BTG2 (Hu et al., 2011) is frequently downregulated in PC and is associated with PC aggressiveness (Ficazzola et al., 2001, Hu et al., 2011), while the abundance of the corepressor arrestin, beta 2 (ARRB2) inversely correlates with the AR activity in prostate tissues (Lakshmikanthan et al., 2009).

1.2.3 Ligand independent activation of the androgen receptor

AR signaling may also be activated in the absence of the ligand via other pathways. Several signaling cascades influence AR activation by phosphorylating AR or AR coregulators. Growth factors that may stimulate AR signaling include IGF-I, TGF-β, EGF and IL6 (Heinlein & Chang, 2004). IL-6 regulates

hematopoiesis, immune responses, inflammation, and bone metabolism and has the ability to stimulate the proliferation of PC cells. IL-6 levels are elevated in patients with CRPC. It has been demonstrated that IL-6 activates the Jak/STAT pathway and MAPK pathway, both mediating a cross-talk that leads to AR activation (Culig and Puhr, 2011).

1.2.3.1 Androgen receptor variants

The *AR* gene spans almost 180 kb of DNA and is composed of 8 exons that encode a 4314 bp mature mRNA (Figure 5A). The exons of the *AR* gene encode for functionally distinct regions of the gene that are similar to other SHR genes. The long exon 1 encodes for the AR NTD, which is approximately 60 % of the AR protein but is variable in length because it contains polymorphic (CAG)n and (GGN)n repeat units (Ding et al., 2004; 2005; Ferro et al., 2002; www.ncbi.nlm.nih.gov/gene). The lengths of such repeats have been associated with an increased risk of PC in the African black population (Gu et al., 2011). An alternative exon 1 has been identified, and the resulting naturally occurring splice variant (Figure 5B) was characterized to be expressed in several tissues (Ahrens-Fath et al., 2005). However, the significance of this splice variant and the abundance relative to the main variant remain to be defined (Dehm and Tindall, 2011). Exon 2 and exon 3 of AR encode the two zinc-fingers in the DBD, which are also responsible for dimerization in the presence of two adjacent androgen responsive elements (AREs) half-site (Shaffer et al. 2004) or other DNA binding site motifs of other transcription factors (Massie et al., 2007) (see chapter 1.2.4). Exons 4-8 encode the hinge region and the LBD (Sack et al., 2001). Because the LBD has inhibitory effects on AR activation (Jenster et al., 1991), the absence of this particular domain may lead to aberrant AR activation. Several truncated AR isoforms that lack the LBD have now been reported, and some of these isoforms are constitutively active (Figure 5C). Furthermore, the truncated isoforms are found primarily in advanced CRPC tumors and PC cell lines that represent late stage disease, offering a further explanation for the resistance of PC cells to endocrine therapies (Dehm and Tindall, 2011; Mostaghel et al., 2011).

Tepper et al. (2002) first described a novel AR protein product present in the CWR22Rv1 cell line that contains intact transactivation and DBDs but lacks the LBD. More recently, several independent reports have characterized the alternative splicing that results in up to seven different AR splice variants in the same CWR22Rv1 cell line (Dehm et al., 2008, Hu et al., 2009, Guo et al., 2009), some of which were also found in VCaP cells (Dehm et al., 2008, Hu et al., 2009) and in some of the LuCaP xenografts (Dehm et al., 2008, Sun et al., 2010). These AR splice variants share the feature of being constitutively active because all of these variants lack the LBD (Dehm et al., 2008, Hu et al., 2009, Guo et al., 2009, Sun et al., 2010). The transcripts contain exon 1, exon 2 and one of the several cryptic exon 3 discovered in the intronic region between the normally occurring exon 3 and exon

4 (Figure 5A). Some variants have been described to include a duplication of exon 3 and others to be the result of exon 5, 6 and 7 skipping (Dehm and Tindall, 2011). More recently, another splice variant lacking the DBD has been described as being associated with the plasma membrane (Yang et al., 2011). Finally, Watson et al., (2010) have sequenced AR mRNAs. They confirmed the previous data and identified several additional AR variants (Watson et al., 2010). The molecular mechanisms that lead to differential AR splicing are not known, although they could be attributed to a misregulation of the cellular "splicing code" (Rajan et al., 2009). However, the AR variants in the CWR22Rv1 cell line were found to be produced as consequence of an approximately 35 kb intragenic tandem duplication in the AR locus that was induced by androgen ablation (Li et al., 2011b). These data suggest that mechanisms other than the splicing machinery may also influence the appearance of AR variants in PC.

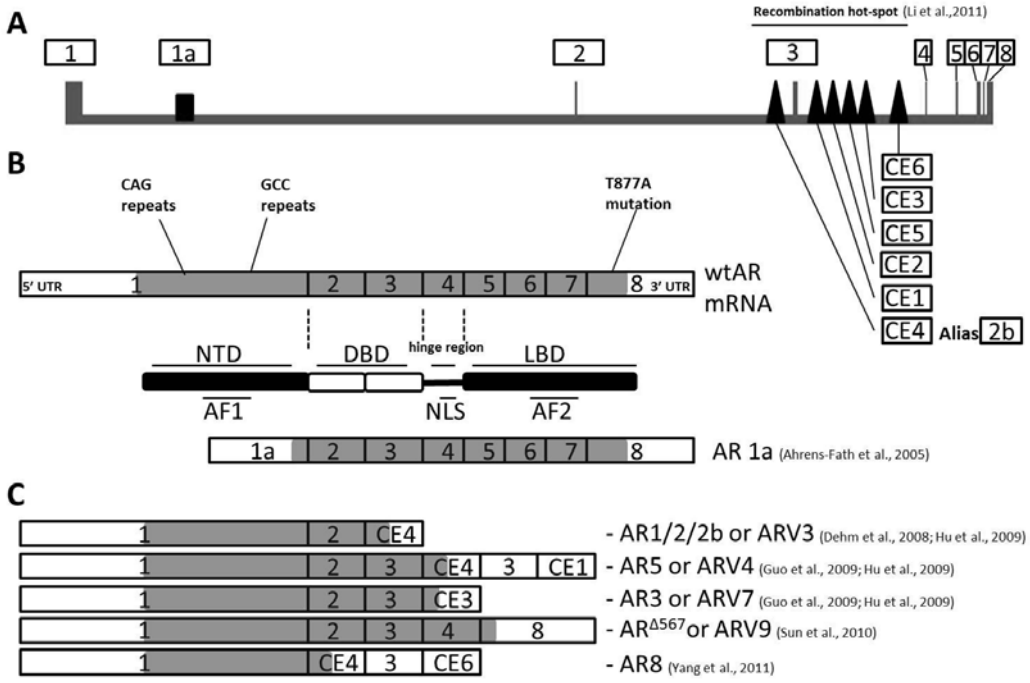

Figure 5. Androgen receptor and prostate cancer. (A) Androgen receptor gene genomic structure. (B) mRNA structure of normally occurring androgen receptor variants and corresponding (indicated by dashed lines) protein domain structure. In prostate cancer, the most frequently found point mutation in the androgen receptor ligand binding domain is the T877A originally characterized in the LNCaP cell line (Veldscholte et al., 1990). (C) Known constitutively active androgen receptor mRNA variants in advanced prostate cancer. Exon 1 is 2731 bp, while exon 8 is longer than represented (the 3'UTR is approximately 7 kb long). Darker shades indicate translated portion of the mRNAs. Numbers inside the squares represent exon numbers, AR = androgen receptor, CE = cryptic exon, UTR = untranslated region, NTD = N-terminal Domain, DBD = DNA-binding Domain, NLS = Nuclear Localization Signal, LBD = Ligand-binding Domain, AF1 & 2 = Activation Function 1 & 2. The numbering of the cryptic exons was adapted for this illustration, and the names of the splice variants are as in the original publications in the absence of a systematic classification.

The clinical relevance of these AR variants has yet to be fully established. These variants have also been found in androgen independent LuCaPs (Dehm et al., 2008), PC metastasis (Sun et al., 2010) and CRPC specimens (Hu et al., 2009), where they correlate with worse clinical outcome (Hu et al., 2009). Guo et al. (2009) have reported three truncated AR isoforms lacking the LBD (designated as AR3, AR4, and AR5) and have performed an immunohistochemical analysis of 429 PC tissues; their data indicate that AR3 is significantly up-regulated during CRPC progression. AR3 expression levels correlate with the risk of tumor recurrence after radical prostatectomy (Guo et al., 2009). Castration in mice has also been shown to induce the expression of AR variants in xenografted tumors (Watson et al., 2010).

Recently, abiraterone treatment in mice was shown to upregulate several AR splice variants in xenografted tumors (Mostaghel et al., 2011). These findings suggest that androgen deprivation therapy may select for expression of these variants in the tumor, possibly contributing to the emergence of castration resistance.

Gene expression profile studies have shown that particular AR splice variants may activate different gene transcription programs (Hu et al., 2009; Guo et al., 2009). For example, unlike the full length AR, AR3 was shown to directly increase AKT1 expression (Guo et al., 2009). An alternatively spliced AR form selectively posttranscriptionally controlled *EGFR* and *ERBB2* expression in 22Rv1 cells (Pignon et al., 2009). Taken together, these data suggest that these alternatively spliced AR forms may play an overlapping but distinct role in prostate cells by regulating different target genes. As previously mentioned, the abundance of AR splice variants relative to the full length AR form remains to be assessed in a large cohort of clinical samples. However, a recent study by Hörnberg et al. (2011) has assessed the protein expression of three of the most abundant AR variants in 10 primary tumors and 40 CRPC bone metastasis specimens. Interestingly, the authors found that together with the full length AR, some of the variants were detectable in primary tumors. However, the variants displayed substantially higher expression in the CRPC samples. Furthermore, in a subgroup of the patients, the levels of the variants were comparable with the full length AR. The expression of these variants was associated with a high nuclear AR immunostaining score, disturbed cell cycle regulation and short patient survival (Hörnberg et al., 2011). It has also been shown that not all the splice variants are active, and even the active variants may require the full length AR to be functional (Watson et al., 2010). These data suggest that the potent super antiandrogen MDV3100 (Tran et al., 2009), which is now in a phase III clinical trial (Payton, 2010; Scher et al., 2010), may still be effective in counteracting the activity of these splice variants, even though the drug targets the LBD (Watson et al., 2010).

1.2.4 Androgen receptor-mediated transcription

1.2.4.1 Formation of the transcription initiation complex

AR mediated transcription initiation has been extensively studied for the androgen-regulated *PSA* gene using chromatin immunoprecipitation (ChIP), quantitative real-time PCR (qPCR), chromosome conformation capture (3C) assays, and fluorescence recovery after photobleaching (FRAP) (Carlberg and Seuter, 2010).

Ligand-bound and activated AR is recruited to both enhancer and promoter regulatory regions (Shang et al., 2002). AR binding leads to the recruitment of RNA polymerase II (Pol II) and AR coregulators. The recruitment of AR and its coactivators is stronger at the enhancer than at the promoter (Wang et al., 2005). From a temporal point of view, there are two recruitment patterns. The short-term recruitment pattern of AR is cyclical. AR is recruited to the *PSA* regulatory regions and disposed in approximately 90 minute cycles (Figure 6).

The same cyclic behavior can also be observed in the promoter of kallikrein-related peptidase 2 (KLK2), another androgen-regulated gene, indicating that the cyclical recruitment is not unique to *PSA*. Proteasome activity has been shown to be needed for maintaining this cyclical behavior. In fact, when the activity of the proteasome is inhibited pharmacologically, AR cannot be released from the promoter after the first cycle (Kang et al., 2002). More recently, HSPs have also been shown to be essential in such dynamic binding (Conway-Campbell et al., 2011). The cyclicity of this short recruitment pattern has also been shown for other SHRs and their coregulatory proteins (Carlberg and Seuter, 2010; Perissi et al., 2010). Upon androgen stimulation, the long-term AR recruitment to the *PSA* enhancer and promoter increases for 16 hours and declines after 96 hours (Wang et al., 2005) (Figure 7). The spatial arrangement of the AR-mediated transcription complex is yet to be defined. When AR binds an antagonist (*e.g.,* bicalutamide), AR is recruited to the promoter along with corepressor proteins, including NCoR and SMRT, but not to the enhancer. In this case, RNA Pol II is not recruited (Kang et al., 2002; Shang et al., 2002), supporting the hypothesis that chromatin looping is needed for gene transcription activation.

This looping/sliding model suggests that AR, which is recruited to both the promoter and enhancer, favors the interaction between these two elements via a common coactivator complex. This complex slides onto DNA, bringing RNA Pol II from the enhancer to the promoter. This model is supported by the finding that the enhancer and promoter recruit coactivators and RNA Pol II in the same way (Wang et al., 2005). Wang et al. (2005) also showed, using ChIP-3C, that upon androgen stimulation, the enhancer and the promoter are in direct contact (Wang et al., 2005). The transcription regulation by chromosomal looping is not unique to *PSA* and has been shown to occur in various genes (reviewed by Kadauke and Blobel, 2009). Today, there is increasing evidence of the importance of chromosomal looping in

gene transcription regulation (Naumova and Dekker, 2010). Other AR target genes, such as *FKBP5*, have also been shown to be transcribed via a similar looping mechanism, which involves several AR binding sites across the gene locus (Makkonen et al., 2009). *FKBP5* has also been shown to be transcribed, most likely by a similar mechanism, by the glucocorticoid receptor (GR) (Paakinaho et al., 2011).

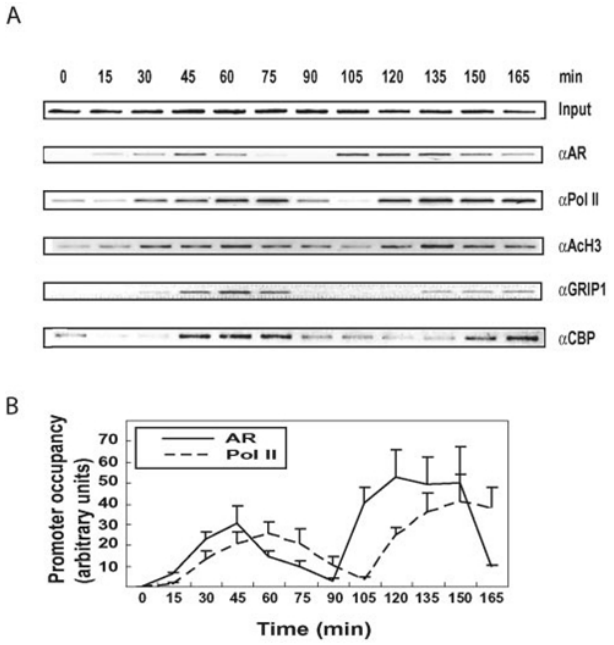

Figure 6. Transient loading of holo-AR and recruitment of RNA Pol II and coactivators to the *PSA* promoter in response to testosterone treatment of LNCaP cells. (A) LNCaP cells were incubated with 100 nM testosterone for the indicated times before harvesting for the ChIP assay. Chromatin samples were immunoprecipitated with the indicated antibodies prior to PCR with *PSA* promoter-specific primers followed by agarose gel electrophoresis and ethidium bromide staining. Input (DNA prior to immunoprecipitation). (B) AR and RNA Pol II occupancy of the *PSA* promoter after androgen treatment of LNCaP cells (This research was originally published in the Journal of Biological Chemestry. Kang Z, Pirskanen A, Jänne OA, Palvimo JJ. Involvement of proteasome in the dynamic assembly of the androgen receptor transcription complex. J Biol Chem. 2002;277(50):48366-71 © the American Society for Biochemistry and Molecular Biology.).

AR-driven chromatin looping is believed to be responsible for bringing *TMPRSS2* and *ERG* in spatial proximity (Mani et al., 2009). This androgen-induced process leads to the formation of the fusion gene (Rubin et al., 2011). Furthermore, the newly established ChIP technique coupled with massive high-throughput sequencing (ChIP-seq) has allowed mapping of AR binding across the entire genome. The majority of AR binding occurs in non-promoter regions, which suggests that such looping must be common (Wu et al., 2011). A recent work by Chen et al. (2011b) has suggested that this process is mediated by a selective PI3K/AKT-induced phosphorylation of the coactivator Mediator 1(MED1) (Chen et al., 2011b).

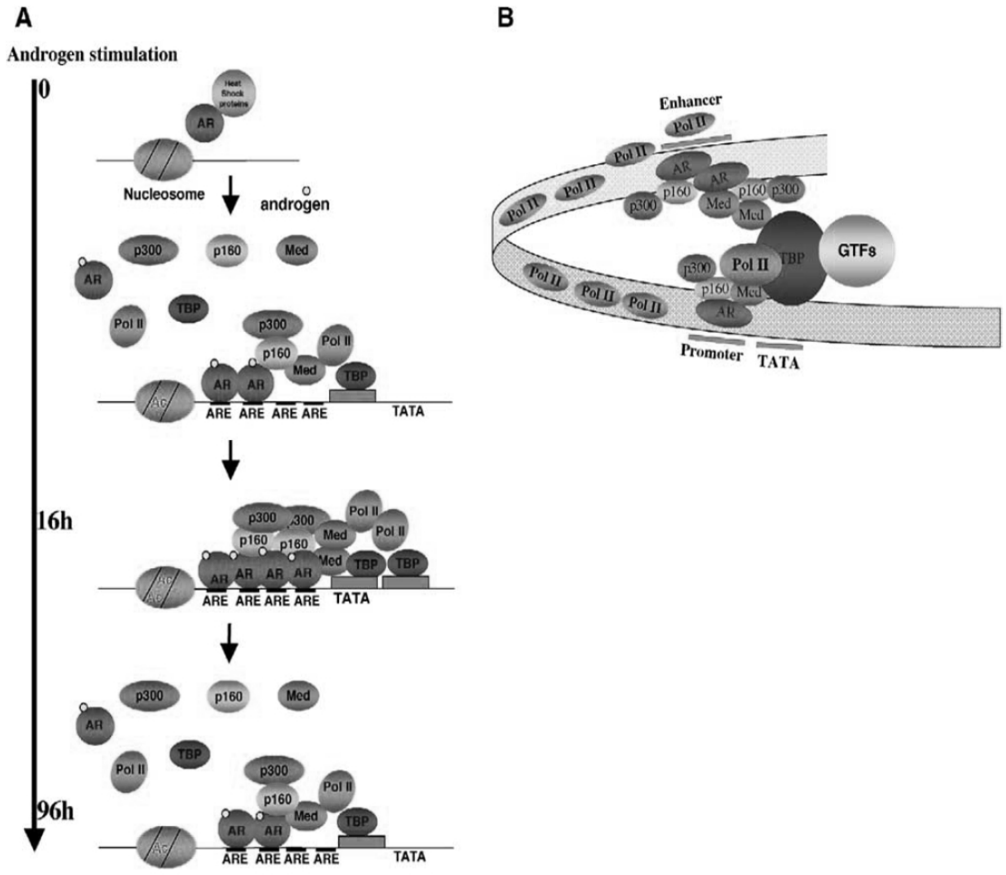

Figure 7. Models of AR coactivator complex assembly on *PSA* regulatory regions. (A) From a temporal view, the level of *PSA* regulatory regions bound to AR complexes gradually increases after androgen treatment, peaks at 16 hours, and then gradually declines following longer treatment. (B) From a spatial view, the AR coactivator complex is predominantly recruited to the *PSA* enhancer, which communicates with the AR transcription complex weakly associated with the *PSA* promoter through the 4 kb intervening DNA through which RNA Pol II tracks. (Reprinted from Wang et al. Spatial and temporal recruitment of AR and its coactivators involves chromosomal looping and polymerase tracking. Mol Cell 2005;19:631-42 with permission from Elsevier, copyright 2005).

1.2.4.1.1 Androgen responsive elements

Like other members of the SHRs family, AR binds to the chromatin at specific consensus sequences known as AREs (Luke and Coffey, 1994). In general, the DNA REs are formed by half-sites arranged as monomeric direct or inverted repeats. The consensus sequences for the SHRs' DNA REs are 5'AGAACA3' or 5'AGGTCA3'(Reviewed by McEwan, 2009). AREs can be non-canonical, with no specific sequence, and they are thought to be present when AR binds indirectly to the DNA through interaction with other transcription factors. However, canonical AREs are present when AR binds in the form of a homodimer to DNA. AREs are composed of 2 hexanucleotide repeats with a 3 nucleotide spacer in between. The

AREs in different promoter areas vary in sequence, but the consensus sequence motif for a canonical ARE is GGTACAnnnTGTTCT (Beato, 1989). Although these consensus motifs are quite conserved, the specific interaction between the receptor and the DNA can also be achieved through protein-protein interactions with other monomers or with coregulators. Additionally, specific interactions with other transcription factors, such as AP-1, ETS proteins, and forkhead transcription factors, may lead to deviations from the canonical 15 bp ARE (Massie et al., 2007; Lupien et al., 2008; McEwan, 2009; Wang et al., 2009b; Lupien and Brown, 2009; Wang et al., 2011b; Robinson et al., 2011). *In vitro* assays of single AREs show a low AR affinity for the canonical sequence. However, *in vivo*, the presence of multiple AREs in tandem causes promoter binding and stabilizes AR, leading to AR-mediated gene transcription activation.

This indicates that some of the specificity of the hormone response derives from other regions of the promoter and interacting proteins (Gelmann, 2002). However, recent studies have shown that only a small proportion of AREs are canonical. On the other hand, 80 % of the AR binding sites contains a six bp ARE "half-site" or a binding site with a non-canonical sequence (Massie et al., 2007; Yu et al., 2010).

1.2.4.2 Modulation of androgen receptor transcriptional activity by other transcription factors

Interestingly, the canonical responsive elements for AR, GR and other SHRs are similar to each other. Because the physiological functions of these hormones are different, the binding of the different SHRs to different target genes must be regulated by other factors (Gao et al., 2011). The mechanism for this discrimination remained unknown until recently when several studies showed that the deregulation of these mechanisms might contribute to cancer initiation and progression (Wang et al., 2007; Lupien et al., 2008; Wang et al., 2009b; Hurtado et al., 2011; Wang et al., 2011b). One of such mechanisms is linked to the activity of the transcription factor forkhead box A1 (FOXA1) overexpression. FOXA1 was originally hypothesized to be a potential drug target in breast cancer because it was found to be overexpressed in most luminal cells (Nakshatri and Badve, 2007). More recently, FOXA1 has also been suggested to play a role in prostate development (Gao et al., 2011). High expression of FOXA1 has been found in metastatic PC (Jain et al., 2011), although low expression has been associated with poor prognosis (Wang et al., 2011b; Jain et al., 2011). Most importantly, FOXA1 has been demonstrated to be a major pioneer factor in AR and ER transcriptional program regulation (Lupien et al., 2008; Wang et al., 2009b). The most striking and intriguing evidence is that suppressing FOXA1 expression in breast cancer cells leads to a decrease in the number of ER binding sites (Hurtado et al., 2011). In contrast, silencing FOXA1 in PC cells leads to increased AR binding to the chromatin and substantial variation of the AR binding sites (Wang et al., 2011b; Sahu et al., 2011). This evidence supports the idea that

FOXA1 translates epigenetics marks into lineage-specific transcription programs (Lupien et al., 2008; Hurtado et al., 2011). This hypothesis has recently been confirmed by the finding that activation of FOXA1 and FOXA1 binding are dependent and associated with DNA hypomethylation, H3K4 dimethylation, and the presence of histone variant H2AZ at the enhancers. These modifications are cell lineage-specific markers (Sérandour et al., 2011). Thus, it is not surprising to find FOXA1 binding sites adjacent to AR binding sites, which was first demonstrated using a ChIP-seq approach (Wang et al., 2009b). Using the same approach, Yu et al. (2010) have discovered another cooperative transcription factor of AR in PC. They proposed that the TMPRSS2:ERG fusion, which leads to ERG upregulation, facilitates AR and ERG cooperation. They suggest that ERG is able to negatively regulate AR activity and to activate the polycomb protein EZH2, facilitating a stem-cell-like dedifferentiation program (Yu et al., 2010).

1.2.5 Androgen receptor target genes and prostate cancer

In prostate cells, the AR transcriptional program regulates the development of the prostate and drives genes related to the prostate function, such as PSA and prostate acid phosphatase (ACPP alias PAP). Both PSA and PAP are known serum markers for PC (Gutman and Gutman, 1938; Hugging and Hodge, 1941; Heidenreich et al., 2011). The gene encoding PSA is kallikrein 3 (KLK3), which is a member of the human kallikrein gene family. All of the 15 members of the kallikrein family encode serine proteases, and many of them are highly expressed in the prostate. Most of these family members are also regulated by androgens (Lilja, 2003; Yousef & Diamandis, 2001).

Riegman et al. (1988) described first the androgen regulation of *PSA*. Several AREs upstream of the *PSA* TSS have been identified. ARE I is located at -170 to -156 from the TSS (Riegman et al., 1991), and a low affinity AR binding site is located also -400 bp from the TSS (Cleutjens et al., 1996). In addition to these, there is also a highly androgen responsive enhancer at -4 kb from the TSS. This enhancer consists of ARE III (-4148 to -4134 bp), which contains several AREs and other non-canonical motifs that are able to bind AR (Schuur et al., 1996; Huang et al., 1999). In addition to its role as a diagnostic marker for PC, PSA has also been hypothesized to be implicated in the development and progression of the disease (Williams et al., 2007), although the data are still poor.

TMPRSS2 is also a well-known AR target gene (Wang et al., 2007). TMPRSS2 is known as epitheliasin and belongs to the family of type II transmembrane serine proteases (TTSP). TTSP genes are found in all vertebrates, and the human TTSP family consists of 17 members (Bugge et al., 2009). However, the function of TMPRSS2 is not yet known because a knockout mouse model failed to show a clear phenotype (Kim et al., 2005), suggesting redundancy in the TTSP family. In the prostate, TMPRSS2 expression is localized to basal and luminal cells (Lin et al., 1999). A downstream promoter element that is needed for recognition of RNA Pol

II has been identified upstream of the TSS. At position -535 bp from the TSS, there is also an ARE (Jacquinet et al., 2001), and 13.5 kb upstream of the TSS are AREs that are thought to form an enhancer element. The enhancer region is the primary site of AR binding in the *TMPRSS2* regulatory region (Wang et al., 2007).

Several microarray studies screening for new PC-associated genes have profiled PC specimens at various stages of the disease. These studies have yielded an enormous amount of information on the different cellular processes controlled by AR in PC (Dhanasekaran et al., 2001; Varambally et al., 2002; Lapointe et al., 2004; Holzbeierlein et al., 2004; Glinsky et al., 2004; Yu et al., 2004; Nanni et al., 2006; True et al., 2006; Tomlins et al., 2007; Chandran et al., 2007; Tamura et al., 2007; Kim et al., 2007; Nakagawa et al., 2008). Furthermore, it is currently reasonable to speculate that 2-4 % of the entire transcriptome is androgen regulated (Dehm and Tindall, 2006).

To date, the best known and characterized AR target gene in PC is the TMPRSS2:ERG fusion gene (Tomlins et al., 2005) (see chapter 1.1.4.4). Recent studies have also demonstrated that the AR transcriptional program can be cell lineage-specific. For example, different genes can be activated in androgen-sensitive cells compared to cells that have been grown for a long time in hormone-deprived conditions. In the last cells, AR selectively upregulates M-phase cell-cycle genes. Among these genes, ubiquitin-conjugating enzyme E2 C (UBE2C) is a gene that inactivates the M-phase checkpoint and is overexpressed in CRPC tissues (Wang et al., 2009b). The overexpression of AR is also able to increase gene expression of cell cycle and metabolism related genes (Waltering et al., 2009).

Furthermore, a recent study by Massie et al. (2011) has found that the AR transcriptional program is directed to regulate aerobic glycolysis and anabolism in PC cells. This work identified Calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK2) as an androgen-regulated gene. CAMKK2 is a metabolic master that has been implicated in the stimulation of glycolysis, biosynthesis and proliferation in PC cells (Massie et al., 2011).

2. AIMS OF THE STUDY

CRPC continues to be a deadly disease for which there is no effective cure and for which there is a strong need for more sensitive and more specific prognostic biomarkers. This study aimed to investigate the molecular consequences of AR overexpression in CRPC.

More specifically, we aimed:

1) To investigate the effect of AR overexpression on the genome-wide recruitment of AR to the chromatin;
2) To investigate the effect of AR overexpression on the initiation of AR-mediated transcription and the dynamics of the recruitment of AR and RNA Pol II to AR-target gene loci;
3) To identify AR coregulators and AR target genes affected by AR overexpression and involved in PC progression.

3. MATERIALS AND METHODS

The materials and methods used in this study are listed below. However, a more detailed description can be found in the original communications I-III.

3.1 Cell lines and cell culture procedure (Studies I-III)

The LNCaP cells overexpressing AR have been described in the original communication I and in Waltering et al. (2009). Briefly, the parental LNCaP (ATCC, Rockville, MO, USA) were transfected with pcDNA3.1(+) empty expression vector (Invitrogen Inc., Carlsbad, CA, USA) and pcDNA3.1(+)with the AR coding region inserted. The cells were stably transfected with Lipofectamine-Plus transfection reagent (Invitrogen Inc., Carlsbad, CA, USA). Transfected clones were selected with 400 µg/ml Geneticin (G418) for 2 weeks. The mRNA and protein levels of AR were measured from purified clones, leading to the establishment of the following 3 stable cell lines:

-LNCaP-pcDNA3.1 (transfected with empty pcDNA3.1-vector as a control);

-LNCaP-ARmo (transfected with AR cloned into pcDNA3.1+ vector);

-LNCaP-ARhi (transfected with AR cloned into pcDNA3.1+ vector).

LNCaP-ARmo overexpresses AR 2-3 fold more than LNCaP-pcDNA3.1, while LNCaP-ARhi overexpresses AR 4-5 fold more.

These cell lines were cultured according to the ATCC protocol with the addition of 200 µg/ml Geneticin (Invitrogen Inc., Carlsbad, CA, USA) and divided in 1:4 ratio plates when in the exponential growing phase. When DHT treatment was performed, the media was changed to RPMI1640 phenol-free medium with 5 % charcoal/dextran-treated (CCS) FBS (Hyclone Inc., South Logan, UT, USA) and 1 % Glutamine (Invitrogen Inc.) for 4 days. The medium was then changed to phenol-free RPMI1640 including 10 % CCS-FBS (Hyclone Inc.), 1 % Glutamine (Invitrogen Inc.), and 0, 0.1, 1, 10 or 100 nM DHT. The cells were maintained in such treatment media according to the time and use specified in the different studies.

In study III, only LNCaP-pcDNA3.1 and LNCaP-ARhi were used, while in studies I and II, LNCaP-ARmo cells were also used.

In study I, LNCaP, VCaP and LAPC4 cells were also used. The cells were purchased from ATCC and maintained according to the manufacturer's instructions.

3.2 Xenografts material (Study I)

Two PC xenografts, LuCaP69 and LuCaP73, were grown in intact male immunocompromised mice. These xenografts were provided by Prof. R. Vessella (University of Washington Medical Center, Seattle, WA, USA).

3.3 Clinical samples (Study I)

Eight BPH and 27 untreated primary PC samples from prostatectomies and 7 BPH and 15 CRPC specimens from transurethral resection of the prostate (TURP) -treated patients were snap-frozen in liquid nitrogen, and total RNA was isolated with Trizol™-Reagent (Invitrogen Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. Tumor samples contained at least 70 % cancer cells.

Tissue microarrays (TMAs) contained 185 formalin-fixed paraffin-embedded prostatectomy and 92 CRPC (TURP) specimens obtained from Tampere University Hospital. For the prostatectomy-treated patients, detectable PSA values (≥0.5 ng/ml) in two consecutive measurements or the emergence of metastases were considered to be signs of progression. The use of the clinical material and the TMAs was approved by the ethical committee of the Tampere University Hospital and the National Authority for Medicolegal Affairs.

3.4 Chromatin immunoprecipitation (Studies I and II)

The ChIP protocol is described in detail in study I. The antibody used to immunoprecipitate AR-DNA complexes is a well-characterized anti-AR polyclonal antibody (AR3) (Karvonen et al., 1997, Thompson et al., 2006, Sahu et al., 2011) and was provided by Prof. Olli A. Jänne (University of Helsinki, Helsinki, Finland).

3.5 ChIP-seq assays (Study I)

The libraries of ChIPped DNA were prepared and sequenced with Genome Analyzer II (Illumina Inc., San Diego, CA, USA) according to the manufacturer's protocol. Briefly, the immunoprecipitated DNA was blunt-ended, and the sequencing adapters were ligated. The DNA was run on agarose gels. Fragments ranging from 150-350 bp were excised and purified with a Qiagen Gel Extraction kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. Eluted DNA from the Qiagen columns was PCR-amplified (15 cycles). Amplified DNA was run on an agarose gel and purified with a QIAquick Gel Extraction kit

(Qiagen Inc., Valencia, CA, USA). Eluted DNA was quantified using a Nanodrop (Thermo Fisher Scientific Inc. Wilmington, DE USA) and stored at -20°C before sequencing.

3.6 ChIP-seq data analysis, Motif overrepresentation analysis, Gene Ontology analysis and mRNA expression profiling (Study I)

These methods are well-described in the supplementary information provided in study I. The raw reads alignment was performed with Bowtie (Langmead et al., 2009), and the reads were mapped on the human genome version 19 (hg19). The peak detection was performed with the tool MACS (Zhang et al., 2008).

To assess the presence of motifs of other transcription factors (TFs) in the ARBSs, overrepresented transcription factor motifs in AR-bound sequences were searched. The prediction of transcription factor binding was performed using a probabilistic method (Lähdesmäki et al., 2008). The binding probability for each human-associated TF with a motif in the TRANSFAC (Matys et al., 2006) database (version 2009.3) was calculated for both strands on each ARBS sequence.

Genes bound by AR at most 25 kb upstream of the transcription start site (TSS) or downstream of the 3'UTR were collected into separate gene lists. The gene lists were then used in an overrepresentation analysis performed by GeneTrail (Keller et al., 2008).

Raw data from the study by Waltering et al. (2009) were retrieved and reanalyzed to obtain the mRNA expression profile of the cell lines treated at various concentrations of DHT for 4 and 24 hours.

3.7 qRT-PCR (Studies I-III)

The cells were harvested into TRIZOL reagent (Life Technologies Inc., Gaithersburg, MD, USA) after the indicated time in each study, followed by total RNA isolation according to the manufacturer's protocol. The cDNA was synthesized from RNA with AMV Reverse Transcriptase and oligo(dT)12-18 primers (Study III) or random examer (Study I and II) according to the manufacturer's protocol (Finnzymes, Espoo Finland). The standards were prepared by mixing total RNA from untreated LNCaP cells and universal RNA (Clontech Laboratories, Inc., Mountain View, CA, USA) in a ratio of 1:5. After first strand cDNA synthesis, serial dilutions corresponding to 1000, 200, 40, 8, 1.6, 0.32, and 0.064 μg of the RNA pool were prepared and stored in aliquots. The PCR reactions were performed in a LightCycler apparatus (Roche Inc.) using an LC Fast Start DNA SYBR Green I Kit (Roche Diagnostics, Mannheim, Germany) (Study III) or

Maxima™ SYBR Green (Fermentas Inc., Burligton, Ontario, Canada) and a CFX96 real-time PCR detection system (Bio-Rad Laboratories Inc., Hercules, California, USA) (Studies I and II). The final volume of each reaction was 20 µl containing 2 µl of cDNA sample (or standard). The expression levels were normalized to the expression levels of TBP.

For the clinical samples (Study I), the relative expression of each gene was measured against the average value of TBP, G3PDH2 and β-actin reference genes. Standards used for gene expression measurements in the clinical samples consisted of an RNA mix from the prostate cancer cell lines prepared as above.

The primers were designed for amplifying regions of the mRNAs derived from different exons to avoid amplification of genomic DNA. A list of the primers used can be found in the respective studies.

3.8 ChIPqPCR (Studies I and II)

This method is described in the study I. The enrichment relative to input chromatin was calculated based on the delta Ct method with the percentages calculated using the formula $2^{-\Delta Ct}$, where ΔCt is Ct(ChIP-template)-Ct(Input).

A standard curve from one of the diluted inputs was included in the run to control that the efficiency of the reaction was maintained in the range between 95 % and 105 %. A qPCR on a control region in which AR is not supposed to bind (between the PSA enhancer and promoter (middle region)) was performed for each ChIP assay. The ChIP assay was considered specific if the enrichment in the control region was not above the enrichment of the non-specific immunoprecipitated sample generated with normal rabbit IgG.

3.9 Statistical analysis (Studies I-III)

In study III, Grubb's test was used to detect the outlier values in the repetitive PCR runs for each gene, and the one-way analysis of variance (ANOVA) test with Bonferroni post-test was used to evaluate the statistical significance of the changes in expression levels.

In study I, hypergeometric distribution computing enrichment p-values for differentially expressed genes and AR bound genes was used to test whether the DHT concentrations used to stimulate the cells directly controlled the differential expression by AR at the 4-hour time point and 24-hour time point. Furthermore, to test the hypothesis that not only the androgens but also the amount of AR in the cells has an effect on gene regulation, a two-way ANOVA analysis was performed. The analysis tested the interaction between the effect on the variance of both AR amount and concentration of DHT.

In study II, one-way ANOVA was used to compare differences in AR binding and to test the influence of the AR level on the variance.

3.10 Western blot (Studies I and III)

In study III, Western blot was performed from cytoplasmic and nuclear protein fractions. The primary antibodies used were anti-AR (Ab-1 AR441 Neomarkers Inc., Fremont, CA, USA); anti-β-catenin (BD Transduction Laboratories, Inc. Franklin Lakes, NJ, USA); anti-MAK (C-term) (ABGENT, San Diego, CA, USA); anti-CBP (R&D Systems, Inc. Minneapolis, MN, USA) and anti-pan-actin (pan AB-5, clone ACTN05, Neomarkers, Fremont, CA, USA).

In study I, Western Blot was performed from total cell lysates. The primary antibodies used were anti-FEN1 (clone 4E7; LifeSpan Biosciences Inc., Seattle, Washington, USA) anti-ZWINT purified polyclonal antibody (clone RB19982; Abgent Inc., San Diego, California, USA), anti-SNAI2 (clone 3C12; Sigma-Aldrich Inc., Saint Louis, Missouri, USA), and anti-ATP synthase subunit alpha (clone 15H4C4; MitoSciences Inc., Eugene, Oregon, USA) monoclonal antibodies. A detailed description of the Western blot procedure can be found in the original communications I and III.

3.11 Immunohistochemistry (Study I)

This method is described in study I. The mouse anti-FEN1 antibody (mAb clone 4E7; LifeSpan Biosciences Inc., Seattle, Washington, USA) was used with the Power Vision+ Poly-HRP IHC kit (ImmunoVision Technologies Co., Burlingame, California, USA) according to the manufacturer's instructions.

3.12 siRNA transfections and Growth curves (Study I)

Ambion (Applied Biosystems/Ambion, Austin, Texas, USA) Silencer® selected siRNAs against *FEN1*, *ZWINT* and *SNAI2* mRNAs were used. Cells were transfected with INTERFERin transfection reagent (Polyplus-transfection, Illkirch, France) according to the manufacturer’s protocol. The expression levels of the genes relative to *TBP* were measured by qRT-PCR (2.5 days after transfection), and protein levels were measured by Western blot analysis (3 days after transfection).

Growth curve measurements were started 1 day after siRNA transfection and marked as day 1. Images of the same growth area in each well were acquired every day using a Retiga-2000R FAST Cooled Mono 12-bit camera (QImaging Inc.,

Surrey, BC Canada) mounted on a Motorized Inverted Research Microscope IXT1 (Olympus America Inc.) and a 10X objective. The total growth area occupied by the cells (area percent) in each well was determined each day of measurement with ImageJ software (Abramoff et al., 2004) and normalized against the growth area of the respective well at day 1.

4. RESULTS AND DISCUSSIONS

4.1 AR overexpression enhances AR mediated signaling (Studies I-III).

CRPC overexpresses AR (Linja et al., 2001; Visakorpi et al., 1995) and maintains dependence upon the AR signaling pathway (Tran et al., 2009; deBono et al., 2011a). In an attempt to mimic the conditions in which CRPC arises and progresses, a LNCaP-based AR overexpression model was developed (Waltering et al., 2009). LNCaP-ARmo expresses a 2-4 times higher level of AR protein than the control cells LNCaP-pcDNA3.1 and LNCaP-ARhi expresses a 5-6 times higher level. Furthermore, the LNCaP-ARhi cells grow faster in the presence of low levels of androgens than the control cells (Waltering et al., 2009).

To test the model, the expression of *PSA* was measured in LNCaP-ARhi and control cells after 4 and 24 hours of stimulation with increasing concentrations of DHT. As expected, *PSA* was strongly induced by the DHT (see Figure 2 in Study III). However, the *PSA* response was significantly stronger in LNCaP-ARhi cells (one-way ANOVA $p<0.0001$).

4.1.1 Binding of AR to chromatin in low androgen concentration is enhanced in AR-overexpressing cells (Study I)

To study the effect of AR overexpression on the chromatin binding of the receptor, the LNCaP-based model was utilized. ChIP-seq in LNCaP-pcDNA3.1, -ARmo, and -ARhi, treated for 2 hours with 0, 1, and 100 nM of DHT was used to profile AR binding to the chromatin (Table 1). The binding profile revealed that after treatment with a relatively low concentration of androgens (1 nM of DHT), the number of AR binding sites (ARBSs) was higher in AR-overexpressing cells. In contrast, when the cells were treated with higher concentrations of androgens (100 nM DHT), the binding to chromatin seemed to be lower in AR-overexpressing cells (Table 1).

To confirm the association between the AR level and the ARBSs, an independent AR overexpression model was used. LuCaP69 cells harbor AR gene amplification, whereas LuCaP73 cells do not (Linja et al., 2001). Consequently, the expression of AR is approximately 10-fold higher in LuCaP69 compared to LuCaP73 according to qRT-PCR (Linja et al., 2001). ChIP-seq analysis revealed approximately 19000 and

7000 ARBSs in LuCaP69 and LuCaP73, respectively, confirming that an association exists between the AR level and the number of ARBSs *in vivo*.

Table 1. AR ChIP-seq analysis of the LNCaP AR overexpression model. The cell lines and the DHT concentrations to which each cell line was exposed for 2 hours before ChIP assay, the antibody used in the ChIP assay and the number of raw reads produced with Solexa GA II run are listed. A pooled sample of LNCaP-ARhi and LNCaP–ARmo treated with ethanol for 2 hours and ChIPped with normal rabbit IgG was sequenced as the control sample for peak calling and further ARBSs analysis.

cell line	Treatment	ChIP antibody	Raw reads	Sample abbreviation	Number of ARBSs[a]	Number of ARBSs[b]
LNCaP-pcDNA3.1	ethanol	AR	9997482	pcDNA3.1_0M	925	2
LNCaP-ARmo	ethanol	AR	10429984	ARmo_0M	812	---
LNCaP-ARhi	ethanol	AR	8146731	ARhi_0M	467	---
LNCaP-pcDNA3.1	1nM DHT	AR	10044966	pcDNA3.1_1nM	1600	680
LNCaP-ARmo	1nM DHT	AR	11159217	ARmo_1nM	2296	1303
LNCaP-ARhi	1nM DHT	AR	9926387	ARhi_1nM	2399	1689
LNCaP-pcDNA3.1	100nM DHT	AR	10898431	pcDNA3.1_100nM	3326	3269
LNCaP-ARmo	100nM DHT	AR	10780058	ARmo_100nM	1359	395
LNCaP-ARhi	100nM DHT	AR	8614715	ARhi_100nM	1207	---
LNCap-ARhi/ARmo 1:1	1nM DHT	Rabbit IgG	9725044	----------	---	---

The number of ARBSs in each sample is given according to peak detection with a p-value threshold of 0.00001 (a) and also controlled for a false discovery rate (FDR) (b) at 5 %.

The ChIP-seq data were validated by the presence of ARBSs in the well-characterized enhancers of *PSA* (Shuur et al., 1996; Cleutjens et al., 1996) and *TMPRSS2* (Wang et al., 2007). Traditional ChIPqPCR was used to study the strength of the AR binding to the *PSA* enhancer and promoter in the LNCaP model in the same treatment conditions (2 hours DHT treatment with 0, 1, and 100 nM DHT). One hundred times less ligand was needed to achieve the same AR recruitment to the *PSA* enhancer in AR-overexpressing cells compared to control cells. Thus, AR overexpression sensitized AR binding by 100-fold. ChIPqPCR on the *PSA* enhancer in the xenografts also showed that the AR binding is stronger in LuCaP69 compared to LuCaP73, confirming that the strength of the AR binding is also associated with the AR level.

These data indicated that both the ligand concentration and the amount of receptor together affect the chromatin binding of AR. A modest overexpression of AR enhances the chromatin binding of the receptor by sensitizing the cells to 100-fold lower ligand concentration. The data are concordant with the results of a recent work by Massie and colleagues (2011), in which almost 5-times more ARBSs in the strongly AR-overexpressing cell line VCaP compared to LNCaP cells was found. Furthermore, a study by Makkonen et al. (2011) has confirmed that the binding at regulatory regions at the single gene level is enhanced in AR-overexpressing cells, such as VCaP cells compared to LNCaP (Makkonen et al., 2011).

The AR binding profiles in the different cell lines upon stimulation with different DHT concentrations were reproducible because overlap was observed. Therefore, a high-confidence ARBSs map representing the most frequently overlapping ARBSs was constructed. This high-confidence map included 1833 ARBSs.

The map was first used to confirm that AR overexpression sensitizes the binding potency of the receptor to lower concentrations of ligand. The number of sequenced reads above background level, which can be assumed to be proportional to the binding strength, was reanalyzed in each single sample, and the average was normalized against the average binding strength in hormone-deprived conditions (0 M). Indeed, the binding strength at 1 nM DHT was greater in LNCaP-ARmo and LNCaP-ARhi compared to control cells. In contrast, in cells treated with 100 nM DHT, the data indicated that a slight decrease in the binding strength may occur in these cells. The data are similar to those obtained at the *PSA* enhancer via ChIPqPCR and also with the results by Makkonen et al. (2011).

The ARBSs of the high confidence map localized mostly in intronic (43 %) and distal intergenic (49 %) regions, which most probably accounts for distal enhancers. Two percent of the ARBSs were located in exons, and the remaining 6 % of ARBSs were located within the 1.5 kb region upstream of the TSS or downstream from the 3'UTR of genes. The data indicated that AR activity is tightly linked to distal enhancer regions, confirming this finding in several previous studies (Lupien et al., 2008; Jia et al.,2008; Yu et al., 2010; Makkonen et al., 2009; Massie et al., 2011).

The high confidence ARBS map was also used to compare the binding profiles of cell lines and the xenograft models. The poor overlap (from 4 to 31 %) of the high-confidence ARBS map between the cell lines and the xenografts emphasizes that AR binding to chromatin varies significantly between samples, suggesting that genetic or other intrinsic differences, such as binding of other transcription factors, could strongly contribute to the AR binding. This suggestion is supported by the finding that AR regulates gene transcription in cooperation with other transcription factors, such as FOXA1 (Wang et al., 2009b; Wang et al., 2011b; Sahu et al., 2011) and ETS1 (Massie et al., 2007; Yu et al., 2010). To test this hypothesis, motif analyses of the high confidence ARBS map was performed. The analysis showed that binding motifs of, especially, FOXA1 and ETS family of transcription factors are enriched in the vicinity of ARBSs, which is in an agreement with previously published data (Massie et al., 2007; Lupien et al., 2008; Wang et al., 2009b; Yu et al., 2010; Wei et al., 2010).

The re-analysis of the publicly available data from the work by Yu et al. (2010) showed that Yu and colleagues obtained 58 % overlap of ARBSs between LNCaP, and only 28.7 % overlap between VCaP and a tumor sample. The same tumor tissue sample showed 44.1 % overlap with the LNCaP-model high confidence ARBSs, while the overlap with the LuCaP69 ARBSs was only 17.9 %. Although the two xenografts overlapped poorly within each other and also with the high confidence ARBSs map of LNCaP, the ARBSs in the xenografts often localized close to the genes that showed ARBSs and androgen regulation in the LNCaP-model. These data suggest that androgen-regulated genes may have alternative ARBSs. The poor overlap between the cell lines and xenografts could also be due to the microenvironmental (cell culture versus mouse) differences.

4.1.2 AR binding occurs earlier and more powerfully in AR-overexpressing cells grown in low concentrations of androgens (Study II)

The majority of the previously reported ChIP-seq and ChIP-chip experiments (Jia et al., 2008; Wang et al., 2009b; Yu et al., 2010; Takayama et al., 2010; Sahu et al., 2011) have compared the binding of AR at the saturating concentration of androgens, and thus, missed the dynamics of AR binding. The short term recruitment of AR to the *PSA* enhancer and promoter is cyclical (Kang et al., 2002; Welsbie et al., 2009), and this cyclicity has also been observed at the promoter of another AR target gene: kallikrein 2 (KLK2). Therefore, the effect of AR overexpression on the chromatin binding dynamics of the receptor was studied in androgen-regulated *PSA* and *TMPRSS2* genes, for which ARBSs at the promoter and enhancer regions are known.

ChIPqPCR was used to profile the AR binding to the *PSA* and *TMPRSS2* enhancer and promoter upon stimulation with a low (1 nM) and high (100 nM) concentration of androgens (DHT) over a time period of 4 hours (0 M 0 min, 30, 60, 80, 120, and 240 min). At the *PSA* locus, recruitment to the *PSA* TSS, a portion of exon 3 and a downstream region less than 1 kb away from the 3' UTR was also tested.

As expected, AR is recruited more abundantly to the enhancers than to other regions. The dynamic recruitment pattern of AR to the *PSA* enhancer was also confirmed for the enhancer of the *TMPRSS2* gene. However, the binding profile at the *TMPRSS2* enhancer was different than for the *PSA* enhancer (Figure 8). Thus, different target genes may exhibit different binding profile dynamics. This possibility should be taken into account when planning or analyzing ChIP-seq data and suggests that several time points should be ChIP-sequenced for a more comprehensive understanding of the dynamic AR binding.

When the cells were treated with 1 nM DHT, the peak of AR binding was achieved 30 minutes (at the *PSA* enhancer) or 1 hour (at the *TMPRSS2* enhancer) earlier in AR-overexpressing cells than in control cells. The AR recruitment was also significantly stronger in LNCaP-ARhi cells after 30 min and after 1 hour (t-test $p<0.05$ and $p<0.001$ for *PSA* and *TMPRSS2* enhancers, respectively). Furthermore, the potency of AR recruitment in the time period of 4 hours was significantly higher in AR-overexpressing cells, at least at the *PSA* enhancer and promoter ($p<0.05$).

Interestingly, when the cells were treated with 100 nM DHT, the overall AR binding to the *PSA* and *TMPRSS2* enhancers was greater and peaked differently than in cells stimulated with 1 nM DHT. However, the dynamics of AR recruitment was not significantly different between the cell lines (see Supplementary Figure 4 in Study II).

Altogether, these data indicate that the binding dynamic is affected by the concentrations of androgens to which the cells are exposed and by the AR level. This relationship should be taken into account while planning *in vitro* experiments. The dynamicity of the AR binding to the chromatin may be able to partially explain

the low degree of overlap between the AR binding profiles in the cell lines *in vitro* compared to the LuCaP xenografts *in vivo* because ChIP-seq analyses are relative to binding to a single time point (see chapter 4.1.1).

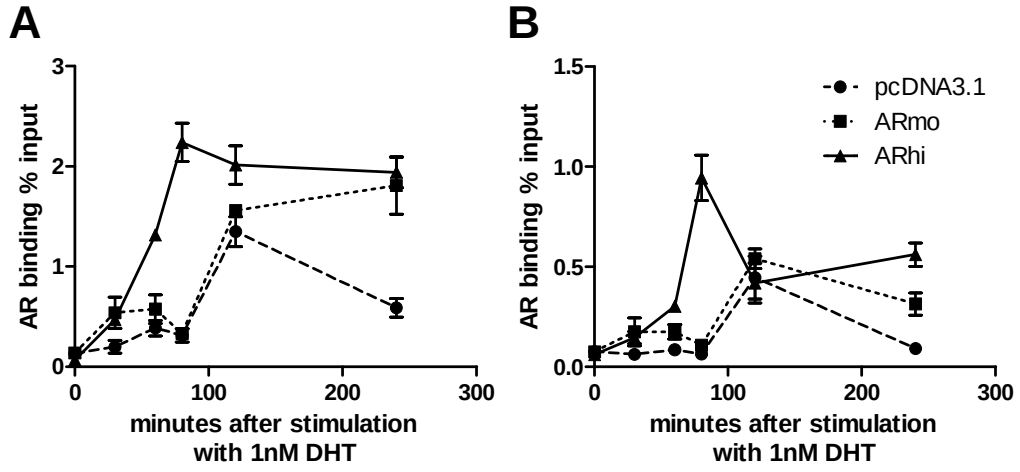

Figure 8. Dynamics of AR binding to AR target gene enhancers in AR-overexpressing cells. AR binding to the enhancer of *PSA* (A) and *TMPRSS2* (B) in the LNCaP-model. LNCaP-pcDNA3.1, -ARmo and –ARhi cells were hormone-starved for 4 days and treated for the indicated time with 1 nM DHT or ethanol (0 h 0 M). ChIPqPCR was performed to assess AR recruitment. The mean and S.E.M. of three biological replicates are shown.

Several groups have investigated the dynamics of the recruitment of RNA polymerase II (Pol II) to the *PSA* (Shang et al., 2002, Kang et al., 2004, Jia et al., 2006) and *TMPRSS2* regulatory regions (Shang et al., 2002, Jia et al., 2006). In fact, binding of AR is known to recruit coregulators, including GRIP1, CBP, and ultimately RNA Pol II (Wang et al., 2007; Heemers and Tindall, 2007). Therefore, because AR overexpression affects its own dynamic recruitment, it is likely to also affect the recruitment of components of the basic transcription machinery. Thus, the effect of AR overexpression on the recruitment of RNA Pol II to the *PSA* and *TMPRSS2* loci was investigated.

The RNA Pol II recruitment profile across the three cell lines was similar to the AR recruitment, which is concordant with the results by Wang et al. (2005). Furthermore, RNA Pol II is also recruited to the TSS, exon 3 and to a locus downstream of the *PSA* gene, although with a certain delay in respect to the promoter and the enhancer.

In AR-overexpressing cells, the RNA Pol II recruitment over the 4 hour time period was enhanced at the *PSA* enhancer, promoter and exon 3 regions (one-way ANOVA $p<0.05$). Furthermore, in all *PSA* loci tested (except for exon 3 and the downstream genomic area immediately at the 3' end of the *PSA* gene), more RNA Pol II was present in LNCaP-ARhi cells at the 4 hour time point (t-test $p<0.05$) (see Figure 4 in Study II).

TMPRSS2 displayed a different RNA Pol II recruitment dynamic. However, consistent with the AR recruitment, RNA Pol II is recruited earlier in AR-

overexpressing cells. Thus, the binding pattern of RNA Pol II, which differs from gene to gene, is also affected by AR overexpression.

4.1.3 Chromatin is more open in AR-overexpressing cells (Study II)

The lineage-specific binding to chromatin by transcription factors, such as AR, is known to be modulated also by other transcription factors, such as FOXA1, which translates epigenetics marks (Wang et al., 2008a; Sahu et al., 2011). For example, the status of histone acetylation is critical for androgen receptor-mediated transcriptional activation of genes (Nakayama et al., 2000). In addition, in PC, several therapeutic approaches have shown efficacy targeting HDACs (Welsbie et al., 2009; Antonarakis and Armstrong, 2011).

Global histone modification patterns have been shown to be able to predict the risk of PC recurrence. In low grade PC patients, a certain pattern of histone acetylation and methylation can be used to predict whether the patient has a low or high risk of PC recurrence (Seligson et al., 2005). Chen and colleagues (2004) have found that modest overexpression of AR can alter the abundance of AR coregulators recruited to the promoters of AR target genes, many of which have histone acetylation activity (Rahman et al., 2004; Chmelar et al., 2006). The activation of gene expression is marked by histone 3 (H3) acetylation. Acetylated H3K9 and H3K9K14 are necessary marks to render accessibility of the regulatory regions to the basic transcription factors (Strahl and Allis, 2000; Verdone et al., 2006; Ito, 2007).

To test whether AR overexpression causes epigenetic changes to the chromatin, the status of the acetylation of the H3 (AcH3) tail was also profiled. The data indicated that the AcH3 increases upon time of stimulation with DHT, with a certain variability but it does not seem to vary in a cyclic manner as AR and RNA Pol II. In agreement with a recent report (Voss et al., 2011), these results suggest a progressive rather an intermittent model of gene activation. Voss and colleagues (2011) have recently found that the dynamic, cyclic binding of the GR causes continuous increased accessibility to chromatin, which favors the transient binding of other factors (Voss et al., 2011). Indeed, this principle could also be applied to the AR.

The data indicated that the AR-overexpressing cells display some sites in which the level of acetylation increases more than in the control cells. For instance, the AcH3K9 at the *PSA* enhancer was significantly higher in LNCaP-ARhi compared to control cells, although it still peaked at the 2 hour time point in both cell lines (see Supplementary Figure 5 in Study II). At the *TMPRSS2* enhancer, the AcH3K9 increased only in the ARhi cells (see Supplementary Figure 6 in Study II). Overall, the AcH3 increases upon the time of stimulation with DHT, and the increases are more pronounced in LNCaP-ARhi cells. The data suggest that the changes in AcH3

and, potentially, the general changes in chromatin marks are sensitive to androgen levels and are locus-specific.

Interestingly, while the recruitment of AR and RNA Pol II did not vary significantly under hormone-deprived conditions across the cell lines, AcH3 differed in the different cell lines upon hormone depletion. Initially, the amount of AcH3 in the regulatory regions seemed lower in LNCaP-ARhi cells compared to ARmo and control cells. However, quantifying the amount of total H3 at the same loci, which is an indirect measure of the amount of nucleosomes, showed that the ratio between AcH3 and pan-H3 increases in AR-overexpressing cells. According to our hypothesis, fewer nucleosomes are present at such sites. However, the remaining histones are more acetylated. These results are in accord with a previous report by Jia et al. (2006) that showed that increased histone acetylation is associated with the development of androgen independence *in vivo*. More importantly, He et al. (2010) have found that in PC cells, androgen treatment dismisses a central nucleosome present at the ARBSs flanked by a pair of marked nucleosomes.

The mechanism for how the histone loss occurs in AR-overexpressing cells and how AR affects this process requires further investigation. However, AR overexpression may enhance such nucleosome dismissing process. Furthermore, given the fact that the progressive loss of nucleosomes may maintain a status of gene activation, this could provide evidence that AR overexpression may favor phenomena of genomic imprinting (Ferguson-Smith, 2011). In PC, epigenetic alterations are important. For instance, silencing of the *GSTP1* gene by hypermethylation of the promoter is found in 90 % of cases (Lee et al., 1994). Yet the molecular features which cause such silencing are not well understood. These results suggest that mechanisms leading to epigenetic memories of nucleosome positioning may be involved in PC progression and deserve more attention. Moreover, a recent study by John and colleagues (2011) pinpointed that chromatin accessibility pre-determines GR binding patterns. Another study by the same group has linked the DNA methylation status to cell type-specific enhancer activity (Wiench et al., 2011).

Given the effect that the AR overexpression has on the dynamic of recruitment of the receptor, and RNA Pol II and given that the chromatin structure is also affected, the dynamic of production of mRNA of the target genes *PSA* and *TMPRSS2* over 16 hours of stimulation with 1 nM DHT was profiled. The mRNA dynamics was measured in the 16 hour period assuming that the effect on chromatin binding within 4 hours is associated with a certain delay to the production of mRNA.

Indeed, after only 4 hours, *PSA* mRNA was significantly more produced in LNCaP-ARhi cells (t-test $p<0.05$) compared to control cells, while the *TMPRSS2* mRNA transcription was significantly higher in LNCaP-ARhi cells by 1 hour after treatment (t-test $p<0.05$). The effect of AR overexpression was stronger in enhancing *TMPRSS2* expression compared to *PSA* during the 16 hours of hormone stimulation, suggesting that the expression of different genes is affected differently

by the AR overexpression. Thus, intrinsic properties may define the degree of enhancement of mRNA transcription in the context of AR overexpression.

4.1.4 AR overexpression enhances androgen regulation of AR coactivators (Studies I and III)

Given the profound relationship between AR activity and AR coregulators (Heemers and Tindall, 2007), the hypothesis that AR coregulators would be regulated by AR was investigated. The aim was to identify AR coregulators whose expression is regulated by androgens and/or affected by the AR receptor level.

The expression of 25 AR coregulators was measured in LNCaP-pcDNA3.1 and –ARhi cells after 4 and 24 hours of hormone stimulation with increasing concentrations of DHT (0, 0.1, 1, 10, 100 nM). The AR coregulators included 17 coactivators, 6 corepressors and 2 coregulators with reported dual functions on AR transcriptional activity (Table 2). The androgens significantly regulated 13 coregulators (52 %) in control cells, seven of which were upregulated more than 2-fold over the vehicle-treated cells (0 M DHT) (see Figure 3 and 4 in Study III). Most of the upregulated genes were coactivators (*AIB1*, *CBP*, *MAK*, *BRCA1*, *β-catenin*, and g*elsolin*), with the exception of the corepressor *cyclin D1* (Table 2). In the LNCaP-ARhi cells, the majority of the androgen-induced coregulators remained under androgen regulation. However, the induction of c*yclin D1*, *gelsolin* and *β-catenin* expression by DHT was reduced compared to control cells. In contrast, the DHT-induced expression of the coactivators *AIB1*, *CBP*, *MAK* and *BRCA1* was further enhanced in AR-overexpressing cells. Moreover, AR overexpression seemed to sensitize the coactivators *AIB3*, *ARA24*, and *JMJD1A* expression because they were upregulated greater than 2-fold by DHT in LNCaP-ARhi cells (Table 2).

The Western blot analysis of CBP, MAK and β-catenin was in concordance with the qRT-PCR results.

Coactivators, such as *AIB1*, *CBP*, *MAK* and *BRCA1,* showed particularly enhanced upregulation in LNCaP-ARhi cells compared to control cells. Certainly, all of these coactivators displayed ARBSs in a putative enhancer region (Table 2; unpublished data). Furthermore, *AIB1* also had an ARBS in the promoter. However, attributing this effect to the presence of ARBSs in the proximity of the loci of these genes is difficult because non-androgen-regulated AR coregulators also displayed ARBSs.

AIB1 is known to have HAT activity and also recruits CBP, with HAT activity, to the transcription multisubunit coactivation complex that favors chromatin accessibility to other transcription factors (Heemers and Tindall, 2007). Thus, these results are concordant with the data on the enhanced chromatin opening in LNCaP-ARhi cells. MAK is a kinase that has been found to associate with AR and enhance its transcriptional activity (Ma et al., 2006), while BRCA1 enhances AR activity through its E3 ubiquitin ligase activity (Park et al., 2000). More recently, Heemers et al. (2009) have profiled the expression and activity of 186 AR coregulators.

Table 2. AR coregulator expression in PC. List, official names and function of the 25 AR coregulators studied in LNCaP-pcDNA3.1 and –ARhi cells upon stimulation with increasing concentrations of DHT for 4 and 24 hours according to study III. The table also contains information retrieved from study I on whether the gene locus of a particular AR coregulator displays ARBSs in the 250 kb bit.

Gene name as in the original publication	Official symbol and name	Androgen-regulated (p value) at		AR overexpression effect (p value) at		ARBSs in the 250 kb bit (Study I)	coactivator or corepressor function
		4 h	24 h	4 h	24 h		
AIB1	NCOA3 (nuclear receptor coactivator 3)	<0.001*	<0.0001*	<0.001*	<0.0001*	Intronic + promoter	Coactivator
CBP	CREBBP (CREB binding protein)	<0.05*	<0.05*	<0.01*	<0.05*	Enhancer	Coactivator
MAK	MAK (male germ cell-associated kinase)		<0.01*	<0.01*		Enhancer	Coactivator
BRCA1	BRCA1 (breast cancer 1, early onset)	<0.001*	<0.0001*	<0.01*	<0.0001*	Enhancer	Coactivator
β-Catenin	CTNNB1 (catenin (cadherin-associated protein), beta 1, 88kDa))	<0.05	<0.0001*	<0.0001	<0.0001		Coactivator
gelsolin	GSN (gelsolin)	<0.05	<0.0001*	<0.05		Enhancer	Coactivator
cyclin D1	CCND1 (cyclin D1)	<0.01*	<0.01*				Corepressor
prohibitin	PHB (prohibitin)	<0.05	<0.01	<0.05	<0.01	Enhancer	Corepressor
AIB3	NCOA6 (nuclear receptor coactivator 6)	<0.0001	<0.05	<0.0001*			Coactivator
ARA24	RAN (RAN, member RAS oncogene family)	<0.001	<0.05	<0.001*	<0.01*		Coactivator
JMJD1A	KDM3A (lysine (K)-specific demethylase 3A)	<0.05	<0.001	<0.0001*		Enhancer	Coactivator
JMJD2C	KDM4C (lysine (K)-specific demethylase 4C)		<0.01	<0.05			Coactivator
BAG-1L	BAG1 (BCL2-associated athanogene (transcript variant 1))		<0.01	<0.05	<0.05	Enhancer	Coactivator
SRC1	NCOA1 (nuclear receptor coactivator 1)					Intronic + promoter	Coactivator
LSD1	KDM1A (lysine (K)-specific demethylase 1A)						Coactivator
ARIP4	RAD54L2 (RAD54-like 2 (S. cerevisiae))					Intronic	Coactivator
p300	EP300 (E1A binding protein p300)					Enhancer	Coactivator
TIF2	NCOA2 (nuclear receptor coactivator 2)					Intronic	Coactivator
MAGE11	MAGEA11 (melanoma antigen family A, 11 (transcript variant 1))						Coactivator
STAT1	STAT1 (signal transducer and activator of transcription 1, 91kDa)					Enhancer	coA/coR
PIASx	PIAS2 (protein inhibitor of activated STAT, 2(α and β isoforms))						coA/coR
NCoR1	NCOR1 (nuclear receptor corepressor 1)					Intronic	Corepressor
PAK6	PAK6 (p21 protein (Cdc42/Rac)-activated kinase 6)						Corepressor
PIAS1	PIAS1 (protein inhibitor of activated STAT, 1)					Promoter	Corepressor
AES	AES (amino-terminal enhancer of split)						Corepressor

***** Over 2-fold upregulated by androgens

Similar to the results presented above, 30 % of the coregulators were androgen-regulated (Heemers et al., 2009). CBP was one of the AR coactivators upregulated in the LNCaP cell line derivative LNCaP-Rf, which was established by long-term androgen ablation of LNCaP cells, mimicking the transition toward CRPC (Comuzzi et al., 2004; Heemers et al., 2009).

These data suggest the existence of a positive feedback loop directed to enhance AR activity. The presence of a positive feedback loop, which enhances a particular signal upon misregulation or loss of degrading proteins, is not new in PC.
For instance, ETS transcription factor may be stabilized or overexpressed upon loss of COP1 (McCarthy, 2011). Additionally, ERG rearrangement positively regulates its own expression, increasing the oncogenic stimulus (Mani et al., 2011). The *AR* gene locus shows ARBSs that may explain further positive regulation (Sahu et al., 2011).

The levels of coregulators in advanced PC did not give conclusive evidence for their implication in CRPC development. However, the coregulators may contribute to such processes (Chmelar et al., 2006; Heemers and Tindall, 2007).

Altogether, the data support the idea that AR overexpression tends to enhance AR-mediated signaling in low concentrations of androgens, maintaining AR to be able to activate gene transcription. The activation of the gene transcription is very likely to be supported by an increased amount of linker DNA, which facilitates AR access to genes regulatory regions, even in hormone-deprived conditions. A positive feedback loop, such as increased transcription of AR coactivators and decreased transcription of AR corepressors, may also concur to maintain and possibly potentiate the signaling pathway.

4.2 Identification of AR target genes in PC (Study I)

In the attempt to identify AR target genes implicated in the progression of PC to CRPC, previously published microarray gene expression profiling data from the LNCaP-based model from the Waltering et al. (2009) study and the ARBSs maps produced in study I were combined.

4.2.1 Gene ontology

First, a gene ontology (GO) analysis of genes located within a 25 kb window of the ARBS maps (see chapter 4.1.1) was performed. Only the ARBS maps of LNCaP-pcDNA3.1 treated with 1 nM, -ARmo treated with 1 nM, -ARhi treated with 1 nM and -pcDNA3.1 treated with 100 nM DHT were used. The analysis revealed that more GO categories were overrepresented in LNCaP-ARhi cells (31) compared to -ARmo (22) and/or control cells (5), suggesting that the AR level is associated with the number of activated biological processes. Cell-cell adhesion

(GO:0016337) and regulation of locomotion (GO:0040012) were the biological processes exclusively enriched in -ARhi cells. Compared with the GO of genes located in proximity of the ARBS map generated in LNCaP-pcDNA3.1 treated with 100 nM, the same processes were enriched in AR-overexpressing cells (see Supplementary Table 3 in Study I). This finding suggests that due to modest AR overexpression, low concentrations of androgens are able to activate processes that are normally activated by higher concentrations.

The GO analysis was consistent with previous findings based on the expression profiling of the LNCaP model (Waltering et al., 2009).

4.2.2 AR target genes identified solely using ARBSs data (unpublished data)

Next, the ARBS maps were analyzed in further detail. The two xenografts, LuCaP69 and LuCaP73, had only 157 ARBSs in common. By sorting them according to the peak height in LuCaP69, the ARBSs showing highest peaks were located on chromosome 8q24. The genes in close proximity to these sites include the non-coding protein oncogene *PVT1* (Guan et al., 2007) and *MYC*.

Table 3. Genes located in a region less than 25 kb to high-confidence ARBSs in the cell lines as well as in the common ARBSs in the two xenografts. Of the 157 ARBSs in common between the two xenografts ARBSs, only 47 were also in common with the high-confidence ARBSs map in the cell lines. Most of the genes close to these ARBSs have already been suggested to be involved in PC or other cancers.

Gene symbol	Official gene name	From the literature
ZBTB16	zinc finger and BTB domain containing 16	Also known as Promyelocytic leukemia zinc finger protein (PLZF) (Chen et al., 1993), it is an androgen-regulated (Waltering et al., 2009; Jiang and Wang, 2004) transcription factor known to be involved in cell growth and apoptosis regulation (Wasim et al., 2010; Hobbs et al., 2010).
STK39	serine threonine kinase 39	It is known to be androgen-regulated (Qi et al., 2001) and involved in PC progression (Hendriksen et al., 2006).
FAM134B	family with sequence similarity 134, member B	It was recently found to encode for a Golgi protein (Kurth et al., 2009) with oncogenic properties (Tang et al., 2007).
RASSF3	the RAS association domain (RalGDS/AF-6) family 3	It is a member of RAS effectors and a tumor suppressor (Jacquemart et al., 2009).
ATAD2	ATPase family, AAA domain containing 2	It is known to be androgen-regulated (Zou et al., 2009). It is a cofactor of MYC oncogene (Cirò et al., 2009) and AR (Zou et al., 2009). It was recently found to predict poor prognosis in breast and lung cancers (Caron et al., 2010).
KCNMA1	potassium large conductance calcium-activated channel, subfamily M, alpha member 1	It is associated with breast cancer invasion (Khaitan et al., 2009).
EMP2	epithelial membrane protein 2	It is involved in endometrial and ovarian cancer development (Wadehra et al., 2006; Shimazaki et al., 2008; Fu et al., 2010; Habeeb et al., 2010).
SFRS1	serine/arginine-rich splicing factor 1	It was found to be a protooncogene (Karni et al., 2007).
PLCB1	phospholipase C, beta 1 (phosphoinositide-specific)	It was found to be implicated in edelfosine resistance in Small cell lung carcinoma (Strassheim et al., 2000).

Interestingly, this region also contains single nucleotide polymorphisms that have been associated with the risk of several malignancies, including prostate cancer

(Ghoussaini et al., 2008). Of these 157 ARBSs, only 47 overlapped with our high confidence ARBSs derived from the LNCaP model. Among the ARBSs, there were several close to cancer associated genes (Table 3). For example, the ARBSs in the intron of *ZBTB16* and immediately upstream of the *RASSF3* gene showed high peaks in both xenografts and in the LNCaP-ARhi cells.

4.2.3 AR target genes identified using ARBSs and microarray expression data (Study I)

To combine ChIP-seq data and expression profiling data, the raw hybridization data from the work by Waltering et al. (2009) were reanalyzed.

The data included expression profiles of LNCaP-pcDNA3.1, -ARmo and –ARhi cells stimulated for 4 and 24 hours with increasing concentrations of DHT (0, 1, and 100 nM). The direct effect of androgens on gene expression was assessed by integrating AR binding data, which was obtained after 2 hours of DHT stimulation in cells treated in the same DHT concentrations. Using hypergeometric distribution to compute enrichment p-values (AR binding information) for differentially expressed genes revealed that the direct androgen regulation at the 4 hour time point also continues at 24 hours ($p < 0.05$). These data indicate that AR target genes are still under tight control of the AR network.

Furthermore, because LNCaP-ARhi cells are known to have a growth advantage over -ARmo and control cells when grown in 1 nM DHT, the binding profiles of the cells treated for 2 hours and the expression profiling at 4 and 24 hours in 1 nM DHT were combined to identify AR downstream genes exclusively upregulated more than 1.5-fold in LNCaP-ARmo or -ARhi cell but not in control cells. The number of genes upregulated was positively associated with the overexpression AR. A total number of 346 genes had ARBSs in the 250 kb window and was upregulated in LNCaP-ARmo and -ARhi cells but not in control cells at 4 or 24 hour time points after stimulation with 1 nM DHT (Figure 9 A&B).

To shorten the list and narrow the search, 14 previously published independent studies that had performed microarray analysis on clinical PC specimens (see Table 4 for references) were interrogated. Among the 346 genes, 38 had shown overexpression in PC compared to BPH or normal adjacent material in at least one of the studies (Table 4).

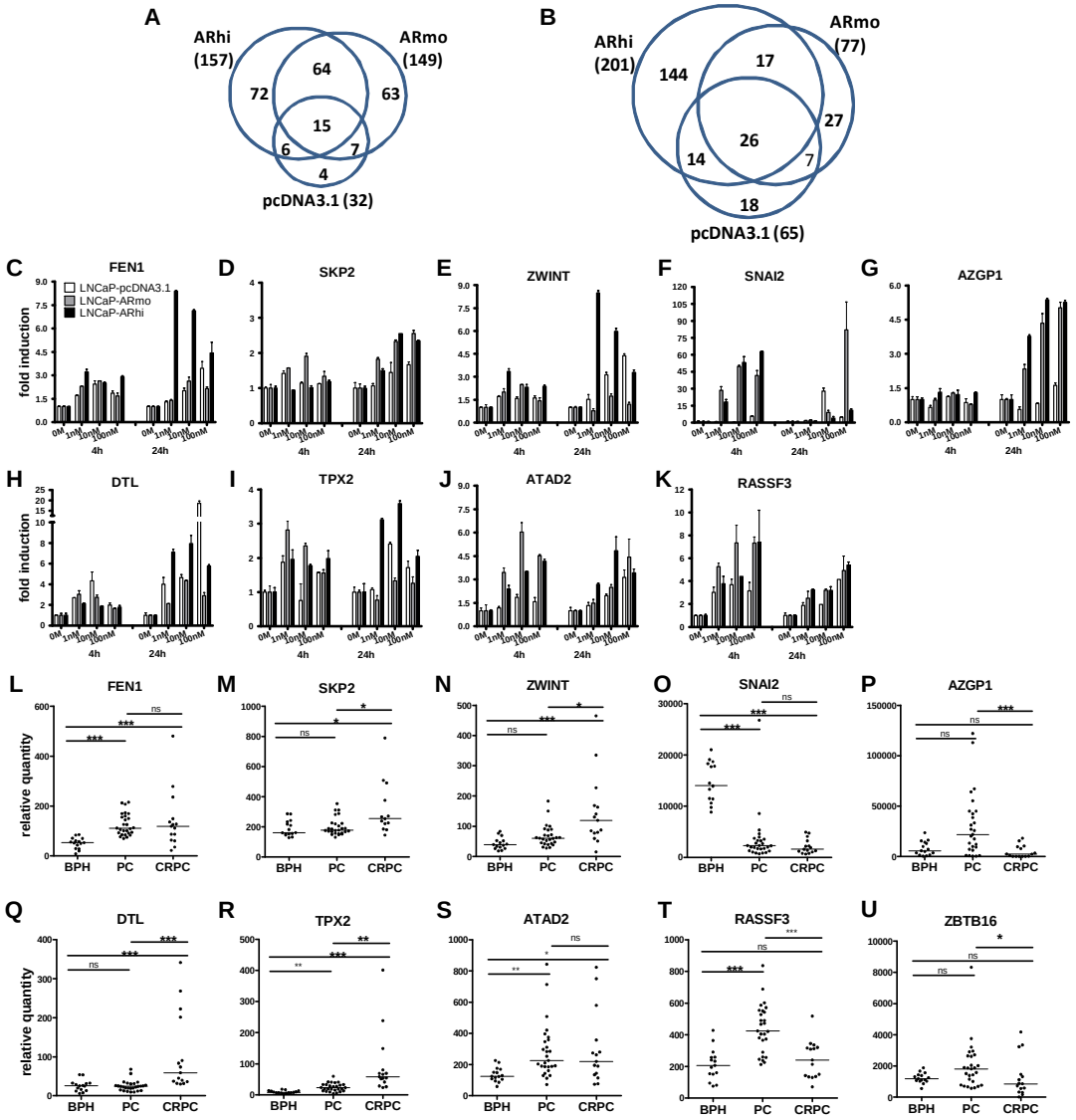

Figure 9. Identification of androgen-regulated AR target genes that are overexpressed in CRPC. The Venn diagrams showing the number of genes that are located in a window of 250 kb around the high confidence ARBSs in LNCaP-pcDNA3.1, -ARmo and -ARhi cells and that showed at least 1.5-fold differential expression upon 1 nM DHT stimulation for 4 hours (A) and 24 hours (B) according to the microarray data. Androgen regulation of *FEN1* (C), *SKP2* (D), *ZWINT* (E), *SNAI2* (F), *AZGP1* (G), *DTL* (H), *TPX2* (I), *ATAD2* (J) and *RASFF3* (K) AR target genes and the effect of AR overexpression on their expression. LNCaP-pcDNA3.1, -ARmo and -ARhi cells were hormone-starved for 4 days and subsequently treated with the indicated concentration of DHT or with vehicle (0 M). The expression of the genes was measured by qRT-PCR. The mean and S.E.M. of each gene against *TBP* values normalized against the 0 M time point are shown. Expression of *FEN1* (L), *SKP2* (M), *ZWINT* (N), *SNAI2* (O), *AZGP1* (P), *DTL* (Q), *TPX2* (R), *ATAD2* (S), *RASFF3* (T) and *ZBTB16* (U) AR target genes relative to the average of 3 housekeeping genes (*TBP*, *β-actin*, *G3PDH2*) in BPH (n=15), PC (n=27) and CRPC (n=13) according to qRT-PCR. Kruskall-Wallis with Dunn post-test results are shown (*** < 0.001; * 0.01 to 0.05; ns. not significant).

Table 4. Androgen-regulated genes overexpressed in PC. Genes that showed expression change exclusively in LNCaP-ARhi and –ARmo cells but not in –pcDNA3.1 cells and were also upregulated in CRPC compared to PC or BPH in at least one of the 14 independent studies that performed gene expression array analysis on clinical samples.

Study	Gene Symbol	Gene name
Dhanasekaran *et al*. (2001)	MYC	v-myc myelocytomatosis viral oncogene homolog (avian)
	FKBP5	FK506 binding protein 5
Varambally *et al*. (2002)	PPIL5	peptidylprolyl isomerase (cyclophilin)-like 5
	LAMC1	laminin, gamma 1 (formerly LAMB2)
Lapointe *et al*. (2004)	AZGP1	alpha-2-glycoprotein 1, zinc-binding
	GUCY1A3	guanylate cyclase 1, soluble, alpha 3
Holzbeierlein *et al*. (2004)	N/A	N/A
Glinsky *et al*. (2004)	N/A	N/A
Yu *et al*. (2004)	ST7	suppression of tumorigenicity 7
	SNAI2	snail homolog 2 (Drosophila)
	STMN4	stathmin-like 4
	PSMA6	proteasome (prosome, macropain) subunit, alpha type, 6
Nanni *et al*. (2006)	CDK2	cyclin-dependent kinase 2
	RAD21	RAD21 homolog (S. pombe)
	CDC25A	cell division cycle 25 homolog A (S. pombe)
	ZWINT	ZW10 interactor
	FEN1	flap structure-specific endonuclease 1
True *et al*. (2006)	AZGP1	alpha-2-glycoprotein 1, zinc-binding
	MAOA	monoamine oxidase A
Iljin *et al*. (2006)	PEX10	peroxisome biogenesis factor 10
	DSC2	desmocollin 2
Tomlins *et al*. (2006)	SLC25A3	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3
	MYC	v-myc myelocytomatosis viral oncogene homolog (avian)
	MRPL47	mitochondrial ribosomal protein L47
	AZGP1	alpha-2-glycoprotein 1, zinc-binding
	SLC22A3	solute carrier family 22 (extraneuronal monoamine transporter), member 3
	MCM4	minichromosome maintenance complex component 4
	MRPL18	mitochondrial ribosomal protein L18
	ABCC4	ATP-binding cassette, sub-family C (CFTR/MRP), member 4
Chandran *et al*. (2007)	KCTD3	potassium channel tetramerisation domain containing 3
	TM9SF3	transmembrane 9 superfamily member 3
Tamura *et al*. (2007)	RAD21	RAD21 homolog (S. pombe)
	ANLN	anillin, actin binding protein
	DTL	denticleless homolog (Drosophila)
Kim *et al*. (2007)	MYC	v-myc myelocytomatosis viral oncogene homolog (avian)
	LCP1	lymphocyte cytosolic protein 1 (L-plastin)
	DDEF2	development and differentiation enhancing factor 2
	MRPL47	mitochondrial ribosomal protein L47
	SKP2	S-phase kinase-associated protein 2 (p45)
	LIFR	leukemia inhibitory factor receptor alpha
	HIPK2	homeodomain interacting protein kinase 2
	GRHL2	grainyhead-like 2 (Drosophila)
Nakagawa *et al*. (2008)	CCNA1	cyclin A1
	RAD21	RAD21 homolog (S. pombe)
	RICS	Rho GTPase-activating protein
	TPX2	TPX2, microtubule-associated, homolog (Xenopus laevis)
	GLRX2	glutaredoxin 2
	AZGP1	alpha-2-glycoprotein 1, zinc-binding
	EDG7	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 7

4.3 Validation and characterization of AR target genes and significance in PC (Study I)

4.3.1 Androgen regulation and expression in clinical material

Among the 38 genes in Table 4, five were selected based on information retrieved from the literature. To confirm their androgen regulation, their expression was measured in LNCaP-ARhi, -ARmo and control cells after 4 and 24 hours of stimulation with increasing concentrations of DHT. The androgen regulation of *FEN1*, *ZWINT*, *SKP2*, *SNAI2*, and *AZGP1* was first confirmed (Figure 9 C-G). Subsequently, *DTL* and *TPX2* genes were also selected for follow-up analyses, and their androgen regulation was confirmed (Figure 9 H&I; unpublished data).

Based on the literature search, 3 genes were also selected from Table 3: *ZBTB16*, *RASSF3* and *ATAD2*. *ZBTB16* has previously been shown to be an AR target gene (Waltering et al., 2009). To confirm the androgen regulation of *ATAD2* and *RASSF3*, their expression was also measured. These genes were strongly induced by DHT (Figure 9 J&K; unpublished data).

A two-way ANOVA analysis was used to test the interaction between the effect of the androgens and AR overexpression. The analysis indicated that both androgens and AR level affect the gene expression significantly ($p<0.0001$), except for *RASFF3*, in which there was not significant interaction between the two effects. However, for all of the genes, the response to DHT was significantly stronger ($p<0.0001$) in LNCaP-ARhi and –ARmo compared to control cells.

The expression of these genes was also measured in BPH, PC and CRPC specimens.

SKP2, *ZWINT* and *FEN1* transcripts were significantly overexpressed in CRPC compared to PC and/or BPH (Figure 9 L-N), whereas the expression of SNAI2 was reduced in CRPC and PC (Figure 9 O). *AZGP1* was not significantly overexpressed in primary PC (Figure 9 P). In the follow-up analysis, *DTL* and *TPX2* were also significantly overexpressed in CRPC compared to primary PC or BPH (Figure 9 Q&R; unpublished data).

The finding that 5 out of 7 genes showed overexpression in CRPC compared to PC or BPH supports the concept that the AR-overexpressing cell line model mimics CRPC-associated AR overexpression.

SKP2 (S-phase kinase-associated protein 2 (p45)) is known to have oncogenic properties and to be overexpressed in many cancers (Nakayama and Nakayama, 2006). SKP2 has previously been shown to be androgen-regulated (Waltregny et al., 2001; Wang et al., 2008b) and to be essential for AR-mediated cell proliferation (Wang et al., 2008b). The expression of *SKP2* has also been associated with a short biochemical recurrence following prostatectomy (Nguyen et al., 2011). A recent study by Lin and colleagues (2010) has suggested SKP2 as a potential target in

cancer treatment and prevention because the inhibition of SKP2 triggered cellular senescence in p53/PTEN-deficient PC-3 cells and tumor regression in mice.

ZWINT (ZW10 interactor) is a kinetochore protein (Starr et al., 2000) that has been suggested to have a role in the development of some malignancies (Obuse et al., 2004; Kops et al., 2005; Lin et al., 2006).

SNAI2 (snail homolog 2 (Drosophila)) is a zinc-finger transcriptional repressor involved in the epithelium to mesenchyme transition (EMT) (Thiery, 2002). EMT is an important concept in cancer, including PC, because increasing Gleason grade is associated with a progressive loss of epithelial glandular architecture, which may be observed as EMT. The involvement of SNAI2 in such process was proposed but the data are still lacking (Nauseef and Henry, 2011). However, PC-3, an AR-negative PC cell line that expresses very high levels of SNAI2, is also dependent on SNAI2 for cell proliferation and invasion (Emadi Baygi et al., 2010). This finding may partially explain why in CRPC, the level of this gene is low and indicates that expression of AR and SNAI2 may be mutually exclusive.

TPX (TPX2, microtubule-associated, homolog (Xenopus laevis)) has been found to be a coactivator of AURKA (aurora kinase A). AURKA is necessary for spindle microtubules assembly in G2 and M phases (Gautschi et al., 2008). Recently, TPX2 was shown to stabilize AURKA and protect it from degradation (Giubettini et al., 2011), and TPX2 is potentially implicated in resistance to DNA damage (Bhatia et al., 2010). Thus, TPX2 plays a critical role in mitotic cells. Although the role of TPX2 in PC is yet to be investigated, both TPX2 and AURKA are overexpressed in many malignancies (Asteriti et al., 2010; Gautschi et al., 2008), including PC (Waltering et al., 2009).

DTL (denticleless homolog (Drosophila)) is also essential for the correct cell cycle regulation at the early G2/M checkpoint that promotes genome stability (Sansam et al., 2006; Abbas and Dutta, 2011). DTL has not been previously studied in PC. However, DTL has been found to be overexpressed in different histological subtypes of breast cancers (Ueki et al., 2008) and, more recently, overexpression of DTL in colon cancer was shown to be a consequence of the deregulation of miR-215, which is under-expressed in tumor cells (Karaayvaz et al., 2011). Thus, DTL may be another potential AR target gene that deserves further investigation.

FEN1 (flap structure-specific endonuclease 1) is a structure-specific metallonuclease that interacts with several other proteins involved in DNA replication, apoptosis, DNA repair and telomere stability (Zheng et al., 2011). Although mutations of *FEN1* in PC are yet to be investigated, somatic mutations of *FEN1* have been reported in several common cancers (Zheng et al., 2007). These mutations abolish the exonuclease activity but retain the flap endonuclease activity (Zheng et al., 2007), which is consistent with the finding that mice carrying mutations show higher chemically induced cancer incidence (Xu et al., 2011).

The expression of *ATAD2*, *RASSF3* and *ZBTB16* has also been measured in clinical specimens. Increased expression of all of these genes was observed in primary PC compared to BPH (Figure 9 S-U; unpublished data), although *ZBTB16* was not significantly increased. Concomitantly, all of these genes showed lower

expression in CRPC than in PC, although the difference was not significant for *ATAD2*. These findings suggest that the use of both expression profiles and binding analysis is a better approach for the identification of AR target genes compared to the binding information alone.

ATAD2 is essential for entry into the S-phase of the cell cycle and has previously been shown to be androgen-regulated (Zou et al., 2009). ATAD2 has been reported to be a coactivator of both the MYC oncogene (Ciró et al., 2009) and AR (Zou et al., 2009). Furthermore, ATAD2 was recently found to be overexpressed in several malignancies (Ciró et al., 2009) and to predict poor prognosis in breast and lung cancers (Ciró et al., 2009; Caron et al., 2010). The data show that ATAD2 levels are consistently high in PC in all stages. Thus, further investigation into its role in PC initiation is warranted. RASSF3 is a tumor suppressor (Jacquemart et al., 2009) and accordingly to its function, it is under-expressed in CRPC specimens. ZBTB16 can also oppose cellular transformation through multiple targets, such as inhibition of MYC. Furthermore, ZBTB16 is a transcription factor with yet undetermined targets that can recruit transcriptional corepressors and HDACs (Hobbs et al., 2010). Accordingly with these findings, *ZBTB16* levels also decrease in CRPC. The data on *RASSF3* and *ZBTB16* suggest that although a selective pressure may boost their levels in PC to contrast the oncogenic activity, an opposing selective pressure tends to suppress their expression in CRPCs. Although strongly androgen-regulated, *ATAD2*, *RASSF3* and *ZBTB16* genes are probably not involved in the emergence of the CRPC phenotype.

4.3.2 ARBSs validation (Study I)

The ARBSs in the proximity of the genes overexpressed in CRPCs (*SKP2*, *ZWINT*, *TPX2*, *DTL* and *FEN1*) were confirmed by regular ChIPqPCR in LNCaP-pcDNA3.1 and –ARhi cells stimulated for 2 hours with 1 nM DHT. Interestingly, the AR binding in –ARhi cells was enhanced compared to control cells, suggesting that the overexpression of these genes in CRPC may be mediated by the increased AR binding. Figure 10 shows examples of the validation of the ARBSs in the proximity of the *DTL* (A&B) and *TPX2* (C&D) genes (unpublished data).

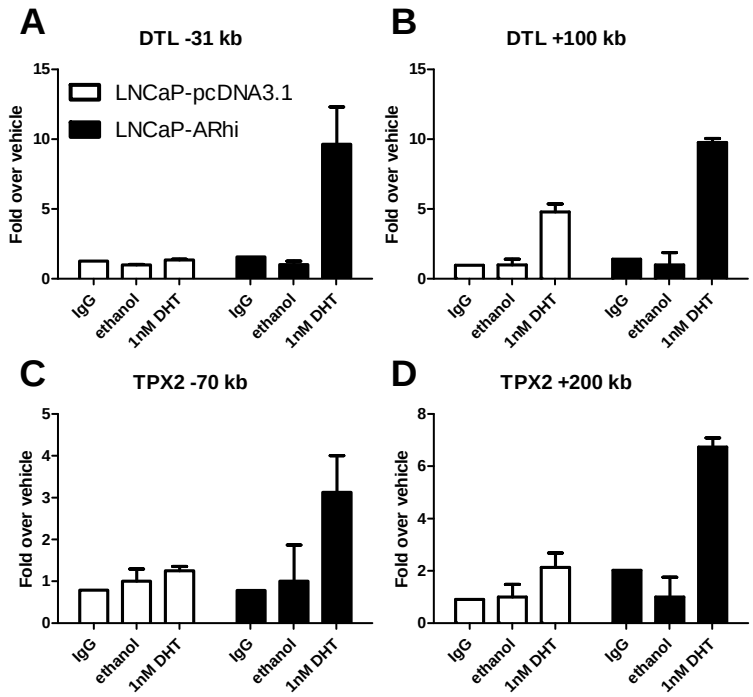

Figure 10. AR binding on putative enhancers of AR target genes. ChIPqPCR on LNCaP-pcDNA3.1 and –ARhi cells, which were hormone-starved for 4 days and treated for 2 hours with 1 nM DHT or ethanol (0 M), was performed to assess the AR recruitment on the putative enhancers of *DTL* at -31 kb (A) and + 100 kb (B) from the TSS and the AR recruitment in the putative enhancers of *TPX2* at -70 kb (C) and +200 kb (D), according to the ChIP-seq data. The data are presented as fold over percentage of input of the ethanol (vehicle)-treated sample. The mean ± S.E.M. are shown.

4.3.3 Functional significance of FEN1, ZWINT and SNAI2 for PC cells growth (Study I)

To study the functional significance of FEN1, ZWINT and SNAI2 in PC cells, a siRNA approach was used. Inhibition of *FEN1* expression in LNCaP-pcDNA3.1 and –ARhi cells resulted in reduced growth of both cell lines, suggesting that FEN1 is important in PC cell growth. In contrast, both *ZWINT*- and *SNAI2*-depleted LNCaP-pcDNA3.1 showed a growth advantage compared to control-transfected cells. For *SNAI2,* this result is in agreement with the clinical data showing decreased *SNAI2* expression in CRPCs compared to PCs. However, inhibition of either of these two genes in -ARhi cells did not lead to any effect on growth.

4.3.4 FEN1 expression predicts clinical outcome

Finally, Western blotting in the cell line model demonstrated increased protein expression in LNCaP-ARhi and -ARmo compared to control cells. FEN1

immunohistochemistry of 185 primary PCs and 92 CRPC specimens on tumor microarray slides demonstrated increased FEN1 nuclear staining in CRPC samples ($p < 0.0001$). The small proportion of PC patients (n=5) treated with prostatectomy with high FEN1 immunostaining also had shorter time to progression.

Overexpression of FEN1 in PC has previously been reported, where it was shown to correlate with high Gleason score (Lam et al., 2006). However, *FEN1* expression has not previously been studied in CRPC specimens. The data suggest that FEN1 is associated with an aggressive form of the disease. Thus FEN1 could be a potential biomarker, at least in a subset of PC patients.

5. CONCLUDING REMARKS

AR overexpression sensitizes cells to low androgen concentrations. A mechanistic explanation for such sensitization is that genome-wide chromatin binding of AR is enhanced in AR-overexpressing cells, and the binding to the regulatory regions of AR target genes is faster and more powerful. Chromatin binding by AR seems to be dependent on both the level of the receptor and the androgen concentrations to which the cells are exposed. Different DHT concentrations are able to alter the dynamics of the AR recruitment to the regulatory region of AR target genes depending on the level of the receptor.

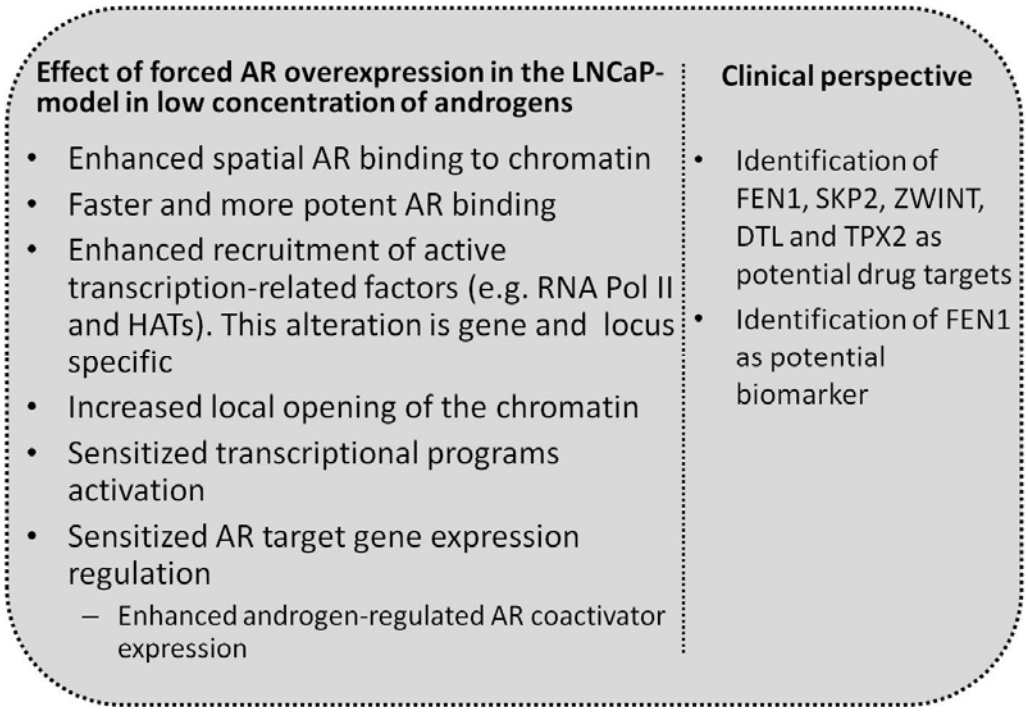

Figure 11. Summary of results. AR overexpression in CRPC cells allows the maintenance and enhancement of AR signaling at lower androgen concentrations through different mechanisms that involve epigenetic, transcriptional and stoichiometric changes.

The binding of AR induces basic transcription machinery to be recruited faster, as demonstrated by the recruitment of RNA Pol II. H3 acetylation during androgen stimulation seems to not be affected by AR levels per se. However, AR overexpression seems to predispose AR binding by opening the chromatin via displacement of nucleosomes and increased acetylation of flanking H3. Although increased chromatin accessibility in AR-overexpressing cells was observed, how the

histone loss occurs is unclear. These changes translate into an enhanced gene transcription of the AR target genes, which may be different from gene to gene and result from intrinsic biological properties of such genes.

AR overexpression also enhances the expression of coactivators, such as *AIB1*, *CBP*, *MAK* and *BRCA1,* but attenuates the expression of corepressors, such as *cyclin D1*. This selective process may potentially result in a positive feedback loop, which sustains the AR activation in low androgen concentrations.

Altogether, these results indicate that the overexpression of AR in CRPC cells allows these cells to maintain and potentiate the AR signaling at lower androgen concentrations through several different mechanisms that involve epigenetic, transcriptional and stoichiometric changes (Figure 11).

By combining ChIP-seq and expression data, genes whose expression are directly regulated by AR and are transcriptionally upregulated in PC and CRPC were identified. Several AR target genes were characterized, and *TPX2*, *DTL*, *ZWINT*, *SKP2* and *FEN1* were found to be androgen-regulated genes that are overexpressed in CRPCs. In concordance with the higher expression in CRPC, the ARBSs in the proximity of their loci were more occupied in AR-overexpressing cells. Functional studies revealed FEN1 to be implicated in PC cell proliferation, and the protein expression was also associated with advanced stage and poor prognosis. As an androgen-regulated gene that is overexpressed and associated with an aggressive phenotype of the disease, *FEN1* could be an important AR downstream gene and, therefore, a putative drug target in PC. Furthermore, *FEN1* could potentially be used as a biomarker in a subset PCs.

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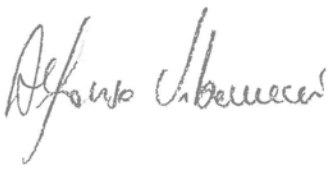
Infine, dedico questo lavoro allo zio Piero e all'amico Salvatore che ci hanno lasciato a causa del cancro.

Tampere, December 2011



ORIGINAL COMMUNICATIONS

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ORIGINAL ARTICLE

Overexpression of androgen receptor enhances the binding of the receptor to the chromatin in prostate cancer

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Androgen receptor (AR) is overexpressed in the majority of castration-resistant prostate cancers (CRPCs). Our goal was to study the effect of AR overexpression on the chromatin binding of the receptor and to identify AR target genes that may be important in the emergence of CRPC. We have established two sublines of LNCaP prostate cancer (PC) cell line, one overexpressing AR 2–3-fold and the other 4–5-fold compared with the control cells. We used chromatin immunoprecipitation (ChIP) and deep-sequencing (seq) to identify AR-binding sites (ARBSs). We found that the number of ARBSs and the AR-binding strength were positively associated with the level of AR when cells were stimulated with low concentrations of androgens. In cells overexpressing AR, the chromatin binding of the receptor took place in 100-fold lower concentration of the ligand than in control cells. We confirmed the association of AR level and chromatin binding in two PC xenografts, one containing *AR* gene amplification with high AR expression, and the other with low expression. By combining the ChIP-seq and expression profiling, we identified AR target genes that are upregulated in PC. Of them, the expression of *ZWINT*, *SKP2* (S-phase kinase-associated protein 2 (p45)) and *FEN1* (flap structure-specific endonuclease 1) was demonstrated to be increased in CRPC, while the expression of *SNAI2* was decreased in both PC and CRPC. FEN1 protein expression was also associated with poor prognosis in prostatectomy-treated patients. Finally, the knock-down of *FEN1* with small interfering RNA inhibited the growth of LNCaP cells. Our data demonstrate that the overexpression of AR sensitizes the receptor binding to chromatin, thus, explaining how AR signaling pathway is reactivated in CRPC cells.

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Introduction

The development of prostate cancer (PC) is strongly dependent on androgens as evidenced by the finding that men castrated early in their life will not develop PC (Isaacs, 1994), and by trials indicating that lowering tissue levels of 5α-dihydrotestosterone (DHT) with 5α-reductase inhibitors, reduces the risk of PC (Thompson *et al*., 2003; Andriole *et al*., 2010). The efficacy of androgen deprivation in the treatment of PC was demonstrated >50 years ago (Huggins and Hodges, 2002) and castration still remains the main form of treatment for advanced PC. Despite the initial positive response, the castration-resistant PC (CRPC) phenotype will eventually emerge during the therapy. Earlier it was believed that PCs progressing during castration are androgen-independent (Thompson *et al*., 2003). Subsequently, the emergence of CRPC has been associated with increased expression of androgen receptor (AR), partly due to the amplification of the *AR* gene (Linja *et al*., 2001; Chen *et al*., 2004). Recently, it has been suggested that also a loss of *RB* gene could lead to AR overexpression (Sharma *et al*., 2010). In addition, mutations in *AR* altering transactivation properties of the receptor, expression of constitutively active AR splice variants and re-expression of androgen-regulated genes have been demonstrated in CRPC (Seruga *et al*., 2011). It has also been suggested that CRPC cells could themselves synthesize low levels of androgens from cholesterol (Seruga *et al*., 2011). Finally, recent phase II trials of CRPC with novel superantiandrogen, MDV3100, and CYP17 inhibitor, abiraterone, have directly demonstrated that CRPC cells are actually still androgen sensitive (Tran *et al*., 2009; Reid *et al*., 2010).

We showed more than a decade ago that one-third of CRPCs contain amplification of *AR* (Visakorpi *et al*., 1995). In addition, we have demonstrated by quantitative reverse transcriptase (qRT)–PCR that almost all CRPCs overexpress *AR* compared with hormone-naïve PC (Linja *et al*., 2001). However, expression of AR protein by immunohistochemistry seems to be variable in CRPC (Roudier *et al*., 2004). Later, Chen and co-workers (2004) showed that in a xenograft model system overexpression of *AR* is necessary and sufficient to

transform the androgen-dependent growth to an independent
one. To study the consequences of *AR* overexpression in PC cells, we have a stable transfected androgen-sensitive LNCaP PC cell line with wild-type *AR* and established two sublines. LNCaP-ARmo expresses 2–4 and LNCaP-ARhi 5–6 times higher level of AR protein than the control cells, LNCaP-pcDNA3.1 (Waltering *et al*., 2009). The LNCaP-ARhi cells grow faster in the presence of low levels of androgens than the control cells and the androgen-regulated genes are induced, on average, at 10-fold lower concentrations of DHT in the *AR* overexpressing compared with control cells. As the sublines share the same genomic background, the model is especially suitable for studying how AR promotes, maintains and drives the PC progression.

AR is a transcription factor that regulates the expression of hundreds of genes. Nevertheless, only one AR target gene that is commonly involved in the development of the disease, *TMPRSS2:ERG* fusion gene, has so far been identified (Tomlins *et al*., 2005). However, the fusion seems not to explain the phenotypical heterogeneity, including hormone responsiveness, of PC (Leinonen *et al*., 2010). The identification of such downstream genes could potentially provide new biomarkers and means to develop novel therapies.

Here, we utilized the LNCaP-based model as well as LuCaP xenografts to study the effect of the AR overexpression on the chromatin binding of AR by using chromatin immunoprecipitation and deep sequencing (ChIP-seq). In addition, we combined the ChIP-seq data with expression profiling to identify AR downstream genes that could be important in the progression of PC, and demonstrated overexpression of *ZWINT*, *SKP2* (S-phase kinase-associated protein 2 (p45)) and *FEN1* (flap structure-specific endonuclease 1) and reduced expression of SNAI2 in clinical samples of CRPC.

Results

In order to map AR-binding sites (ARBSs) across the genome, we first performed ChIP-seq for a total of nine samples: LNCaP-pcDNA3.1, -ARmo and -ARhi, treated for 2 h with 0, 1 and 100 nM of DHT (Supplementary Table S1). We found higher number of ARBSs in LNCaP-ARhi and -ARmo compared with control cells on stimulation with low concentration (1 nM) of DHT (Figure 1a). To confirm the association between AR level and the number of ARBSs in another model system, we utilized two PC xenografts, LuCaP69 and LuCaP73. They derive from castration-resistant tumors and have been grown in intact mice. We have previously demonstrated that LuCaP69 contains *AR* amplification, whereas LuCaP73 cells do not (Linja *et al*., 2001). The expression of AR is about 10-fold higher in LuCaP69 compared with LuCaP73 according to qRT–PCR. We found approximately 19 000 and 7000 ARBSs in LuCaP69 and LuCaP73, respectively, verifying that the level of AR is associated with the number of ARBSs.

To confirm the ChIP-seq data, we used traditional ChIP–qPCR. We studied the well-characterized enhancer and promoter regions of a known AR target gene, *PSA* (Shuur *et al*., 1996; Cleutjens *et al*., 1997), in the cell lines (Figure 1b) and xenografts (Supplementary Figure S1). The ChIP–qPCR data reproduced the ChIP-seq data. Furthermore, we were able to confirm previously identified ARBSs in AR target genes, such as *TMPRSS2* (Wang *et al*., 2007) (Supplementary Figure S1).

In the further analysis of the LNCaP-model, LNCaP-ARmo and -ARhi displayed high overlap of ARBSs in cells exposed to 1 nM DHT. On the contrary, in cells exposed to 100 nM DHT the LNCaP-ARmo and -ARhi cells showed less ARBSs overlap than -ARmo and control cells (Figure 1c and Supplementary Table S2). We then investigated the average peak heights that we assumed to represent the strength of the AR binding (Supplementary Table S2). The peak height was defined as the number of tags present in the specified loci of the ARBSs. The average peak height was higher in -ARhi and -ARmo cells compared with control cells at 1 nM DHT and lower at 100 nM DHT (Figure 1d and Supplementary Table S2).

As we used cells with various levels of AR and ligand concentrations, we were able to make comparison of the effect of AR and ligand on the binding profiles and on the chromatin loading of AR at the ARBSs (Supplementary Table S2). LNCaP-ARmo and -ARhi grown in 1 nM DHT had 985 common ARBSs, whereas pcDNA3.1 at 100 nM and ARmo at 1 nM had 1209, and pcDNA3.1 at 100 nM and ARhi at 1 nM 1323 ARBSs (Supplementary Table S2). By combining these ARBS maps, we constructed a high-confidence ARBS map of 1833 binding sites. The high-confidence ARBSs map includes all the binding sites with high reproducibility in the three samples mentioned above. We then re-analyzed the chromatin binding at these high-confidence ARBSs by computing the binding strength at each of the 1833 ARBSs. In this way, we were able to obtain normalized (against vehicle-treated cells) data on the AR-binding strength (that is, peak height) (Figure 1e). In cells stimulated with 1 nM DHT, the average peak height was greater in LNCaP-ARmo and -ARhi than in control cells. Whereas in cells stimulated with 100 nM DHT there seems to be a slight decrease in the peak height in LNCaP-ARhi cells.

Next, we analyzed the genomic localizations of ARBSs. About 40% of the high-confidence ARBSs were located in intronic regions and about 50% in distal intergenic regions. Thus, those regions probably include most of the enhancer elements (Figure 1f). Similar results were obtained if the ARBSs maps in every individual samples were taken into account.

We then performed a motif analysis by searching for the motifs deposited in TRANSFAC database (Matys *et al*., 2006). We found AR (canonical and 6-bp half site), and HNF3A (alias FOXA1) motifs to be the most significantly overrepresented ($P<10^{-6}$). Furthermore, by categorizing the ARBSs according to their genomic locations, also ETS family of transcription factor motifs

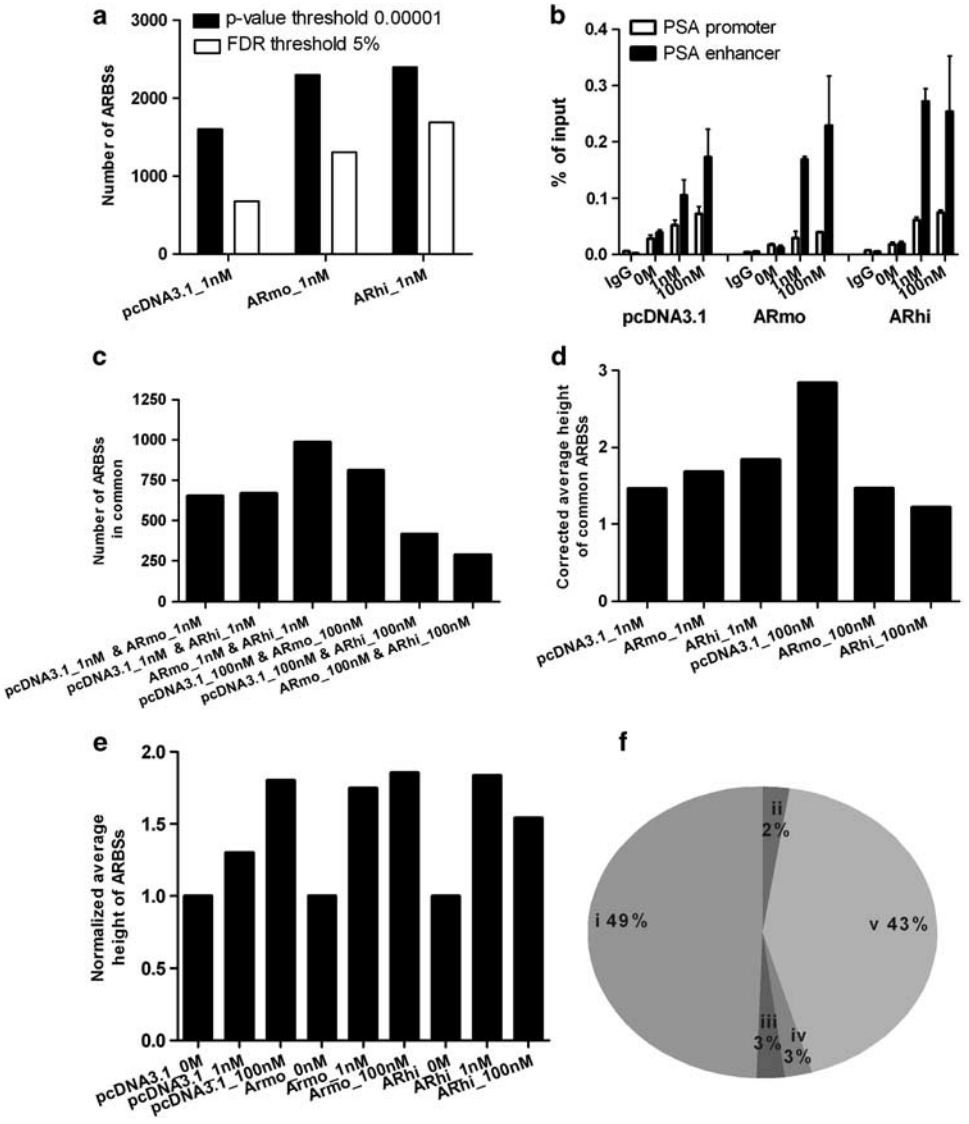


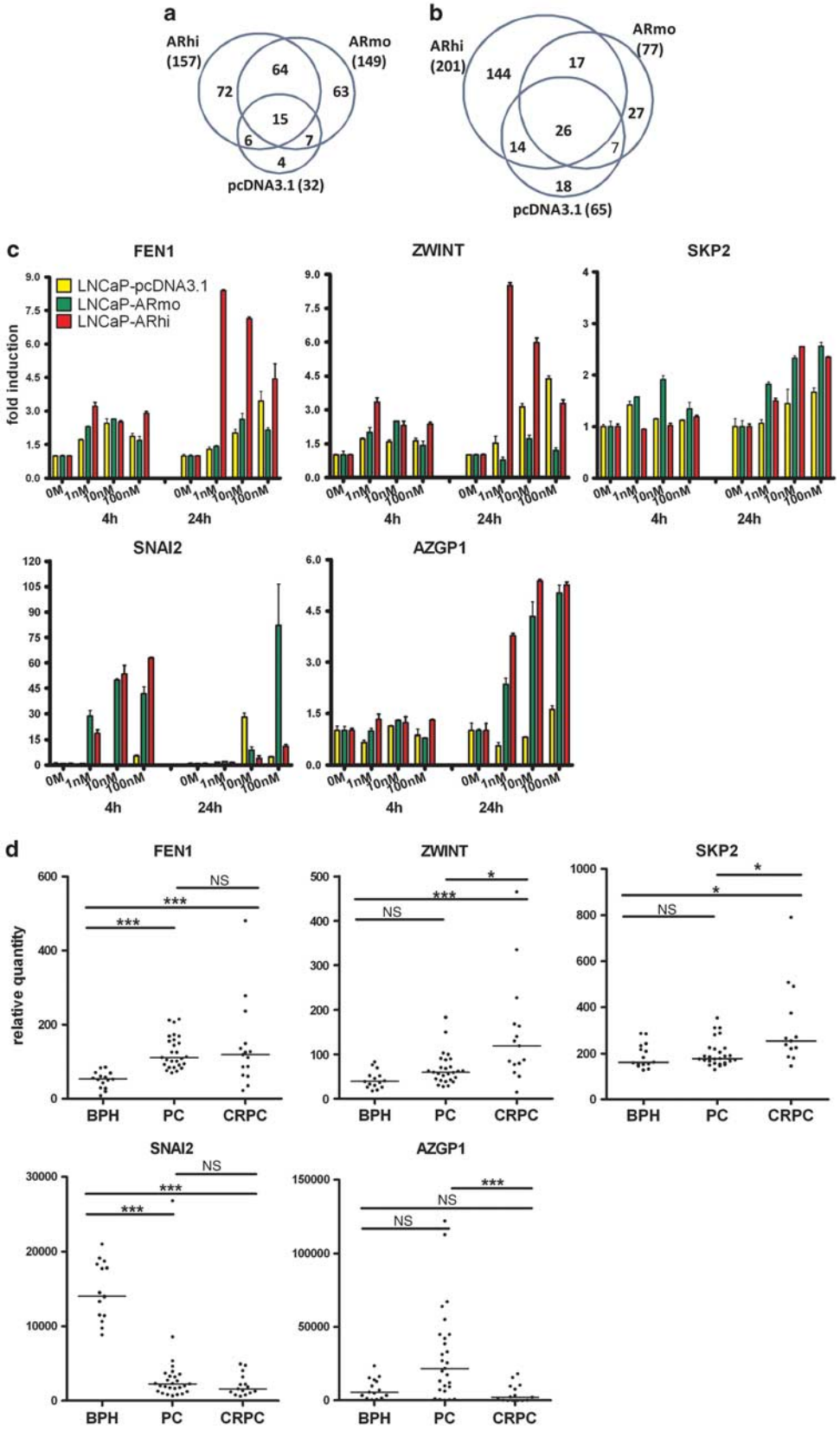
Figure 1 ChIP-seq data analyses. (**a**) Comparison of number of ARBSs between cell lines treated with 1 nM DHT according to peak detection with a *P*-value threshold of 0.00001 (black bar) and controlling also for false discovery rate (FDR) (white bar) at 5%. (**b**) AR binding to PSA promoter and enhancer in LNCaP-model. LNCaP-pcDNA3.1, -ARmo and -ARhi cells were hormone-starved for 4 days and treated for 2 h with DHT or ethanol (0 M). ChIP–qPCR was performed to assess the AR recruitment. Mean and s.e.m. are shown. (**c**) Number of ARBSs in common between the ChIP-seq samples. (**d**) Average background subtracted height of ARBSs in common between samples treated with 1 nM DHT (526 ARBSs in total) and between samples treated with 100 nM DHT (274 ARBSs in total) corrected according to the corresponding amount of raw reads (in millions) obtained in the sequencing (see Supplementary Table S1). (**e**) Normalized (against no DHT) average peak height of all high-confidence ARBSs in cell lines treated with different concentrations of DHT. (**f**) The genomic location of the 1833 high-confidence ARBSs. The high-confidence ARBSs were divided according to their location in distal intergenic regions (i), in exons (ii), within 1.5 kb upstream of transcription start site (TSS) (iii), within 1.5 kb downstream of 3′UTR (iv) and in introns (v).

were found to be enriched in promoters ($P = 3.3 \times 10^{-3}$), and within 1500-bp downstream of transcription start site ($P = 4 \times 10^{-4}$) as well as in exons ($P = 1.1 \times 10^{-2}$).

Next, we performed Gene Ontology (GO) enrichment analysis of genes located within a 25 kb window in the ARBS maps generated by pcDNA3.1 grown at 100 and 1 nM, -ARmo at 1 nM, as well as -ARhi at 1 nM DHT, by using GeneTrail (Keller *et al*., 2008). In 1 nM DHT-treated cells, the number of overrepresented GO (biological processes) categories increased from 5 in pcDNA3.1 to 22 in -ARmo, and 31 in ARhi. The processes that showed enrichment exclusively in -ARhi included, for example, cell–cell adhesion (GO:0016337), and regulation of locomotion (GO:0040012). Generally, in cells overexpressing AR, the same category of genes was enriched than in control cells, except in lower androgen concentration (Supplementary Table S3).

We then investigated the overlap of the high-confidence ARBSs map between the cell lines and the ARBSs maps of the xenografts. The overlap was surprisingly low. LuCaP69 showed 31% overlap with the high-confidence ARBS map in the cell lines, whereas

LuCaP73 only about 4%. When comparing individual samples, LNCaP-ARhi at 1 nM, -ARmo at 100 nM and -pcDNA3.1 at 100 nM showed the highest percentage of overlap with the LuCaP69 ARBS map. In order to confirm our finding, we compared LuCaP69 and LuCaP73 ARBS maps with the publicly available



LNCaP ARBS map from the work by Yu *et al*. (2010). We found even less overlap. The overlap with LuCaP69 was 6.5% and with LuCaP73 0.4%, whereas we found 86.8% overlap between our high-confidence ARBSs in LNCaP-model and the ARBSs of LNCaP cell line published by (Yu *et al*., 2010).

Finally, we integrated the high-confidence ARBS maps and expression profiles of mRNAs (Waltering *et al*., 2009) obtained from the LNCaP-model.

We used first hypergeometric distribution to compute enrichment *P*-values for differentially expressed genes and AR bound genes. When DHT concentrations were used to stimulate the cells, we observed that the differential expression, which was controlled directly by AR at the 4-h time point, still continued at the 24-h time point ($P<0.05$). Thus, we used both time points to identify direct targets of AR. Since we have previously shown that LNCaP-ARhi cells grow significantly faster in 1 nM DHT than control cells (Waltering *et al*., 2009), we focused on genes that showed AR binding and differential expression in that DHT concentration. The Venn diagrams in Figures 2a and b show that there are more such genes in LNCaP-ARhi and -ARmo than in control cells. The lists of 346 genes that were AR bound and androgen-regulated only in LNCaP-ARhi and -ARmo at 4- and/or 24-h time points are given in Supplementary Tables S4 and S5, respectively. Next, we interrogated the expression of these genes in clinical PC specimens by retrieving data from 14 independent array-based studies (see Supplementary Table S6 for references). We found that 38 out of these 346 genes (Supplementary Table S6) were overexpressed in PC according to, at least, one of the studies. Subsequently, of these we selected five putative target genes (*FEN1*, *ZWINT*, *SKP2*, *SNAI2* and *AZGP1*) based on information retrieved from the literature, and confirmed their androgen regulation (Figure 2c). Furthermore, in order to test the hypothesis that not only the androgens but also the amount of AR in the cells has an effect on such regulation, we performed a two-way analysis of variance in which the interaction between the effect on the variance of both AR amount and concentration of DHT was tested. The analysis indicated highly significant ($P<0.0001$) interaction and that both AR amount and the DHT concentration affect the gene expression significantly ($P<0.0001$).

Next, we used qRT–PCR to measure the expression of the genes in benign prostate hyperplasia (BPH), untreated PC and CRPC (Figure 2d). *SKP2*, *ZWINT* and *FEN1* transcripts were significantly overexpressed in CRPC when compared with PC and/or BPH, whereas the expression of *SNAI2* was reduced in CRPC and PC compared with BPH. The expression of *AZGP1* was significantly lower in CRPC than in PC. For *SKP2*, *ZWINT* and *FEN1*, we also confirmed the closest high-confidence ARBSs using ChIP–qPCR (Figure 3), and showed that AR binding is stronger in *AR* overexpressing than in control cells. To investigate the function of *FEN1*, *ZWINT* and *SNAI2* in PC, we suppressed them using small interfering RNAs (siRNAs) in both LNCaP-pcDNA3.1 and LNCaP-ARhi cells. *FEN1* depletion reduced significantly cell growth of both cell lines (Figure 4), while *SNAI2* and *ZWINT* depletions seem to give a growth advantage in control cells, but not in LNCaP-ARhi cells (Supplementary Figure S2). To study protein expression of FEN1, we first used western blotting in the cell line model and demonstrated increased protein expression in LNCaP-ARhi

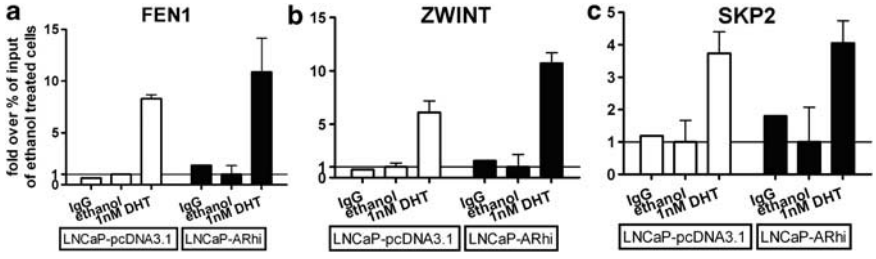


Figure 3 AR binding on putative enhancers of AR target genes. ChIP–qPCR on LNCaP-pcDNA3.1 and -ARhi cells, hormone starved for 4 days and treated for 2 h with 1 nM DHT or ethanol (0 M), was performed to assess the AR recruitment on the putative enhancers of *FEN1* (**a**), *ZWINT* (**b**) and *SKP2* (**c**) genes located 100 kb upstream (in the first intron of the gene DAGLA), 23 kb upstream and 120 kb upstream the above mentioned genes, respectively, according to our ChIPseq data. The data are presented as fold over percentage of input of the ethanol-treated sample. Mean ± s.e.m. are shown.

Figure 2 Identification of androgen-regulated AR target genes that are overexpressed in CRPC. The Venn diagrams showing the number of genes that are located in a window of 250 kb around the high-confidence ARBSs in LNCaP-pcDNA3.1, -ARmo and -ARhi cells and that showed at least 1.5-fold differential expression on 1 nM DHT stimulation for 4 h (**a**) and 24 h (**b**) (see Supplementary Tables S4 and S5). (**c**) Androgen regulation of AR target genes and effect of AR overexpression on their expression. LNCaP-pcDNA3.1, -ARmo and -ARhi cells were hormone starved for 4 days and subsequently treated with the indicated concentration of DHT or with vehicle (0 M). The expression of the genes was measured with qRT–PCR. Mean and s.e.m. of each gene against TBP values, normalized against the 0 M of each time point are shown. (**d**) Expression of the indicated AR target genes relative to average of three housekeeping genes (*TBP*, *β-actin* and *G3PDH2*) in BPH ($n=15$), PC ($n=27$) and CRPC ($n=13$) according to qRT–PCR. Kruskall–Wallis with Dunn *post-test* results are shown (***<0.001; *0.01 to 0.05; NS, not significant).

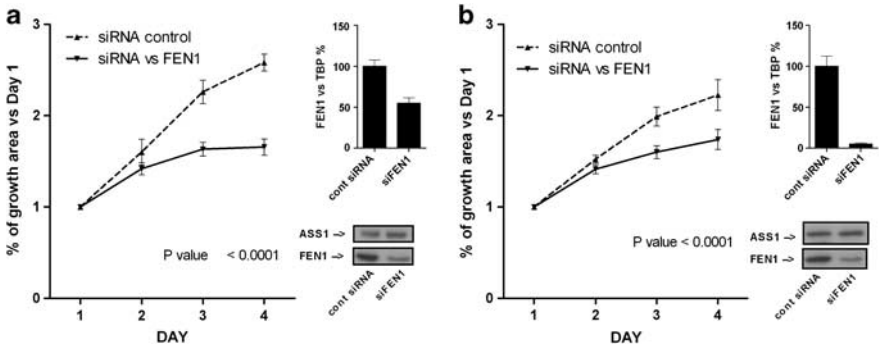


Figure 4 Functional significance of FEN1. Growth curve of siFEN1-transfected LNCaP-pcDNA3.1 (**a**) and LNCaP-ARhi (**b**) cells. Mean and ± s.d. are shown on different days. Statistical significance against control siRNA-transfected cells growth was assessed at day 4 by *t*-test. qRT–PCR at day 2.5 and western blot analysis at day 3 after transfection are also shown in each experiment confirming the FEN1 knockdown.

and -ARmo compared with control cells (Figure 5a). Next, we immunostained 185 untreated prostatectomy specimens as well as 92 CRPC samples (Figures 5b–d). Although cytoplasmic FEN1 staining was equal in PC and CRPC, the strong nuclear staining was observed significantly ($P<0.0001$) more often in CRPC than PC samples (Figure 5e). Only 5/185 (3%) prostatectomy samples showed nuclear staining in >10% of malignant cells. Interestingly, these cases had short time for biochemical recurrence (Figure 5f).

Discussion

Overexpression of AR is a common feature in CRPC (Linja *et al*., 2001), and it has been shown to sensitize cells to low levels of androgens (Kokontis *et al*., 1998; Chen *et al*., 2004; Waltering *et al*., 2009). Here, we utilized our previously established LNCaP-based model (Waltering *et al*., 2009), expressing different levels of AR to interrogate the effect of both ligand and the receptor on chromatin binding of AR. The data here indicated that both the ligand concentration and the amount of receptor affect together the chromatin binding of AR. A modest overexpression of AR enhances the chromatin binding of the receptor by sensitizing the cells to 100-fold lower ligand concentration. The majority of the previously reported ChIP-seq and ChIP-chip experiments (Jia *et al*., 2008; Wang *et al*., 2009; Takayama *et al*., 2010; Yu *et al*., 2010) have compared the binding of AR at the saturating concentration of androgens, and thus, missed the dynamics of AR binding. However, our data are in line with a recent work by Massie *et al*. (2011), in which they found almost five times more ARBSs in the strongly AR-overexpressing cell line VCaP compared with LNCaP cells. The data here are also consistent with our previous finding (Waltering *et al*., 2009) that androgen-regulated genes are induced in lower ligand concentrations in cells overexpressing AR. Thus, the increased chromatin-binding capacity of the receptor because of the overexpression of the receptor provides also a mechanistic explanation to the progression of PC in the presence of only low levels of androgens.

We confirmed the effect of AR levels on chromatin binding of the receptor also in another independent model system. There were almost three times more ARBSs in LuCaP69 than in LuCaP73 PC xenografts verifying that the level of AR is associated with the number of ARBSs. LuCaP69 contains *AR* gene amplification and 10-fold higher expression of *AR* than LuCaP73 (Linja *et al*., 2001). The reliability of the ChIP-seq data was, on the other hand, confirmed by traditional ChIP–qPCR of *PSA*, and our ARBS maps were able to confirm previously reported ARBS like for *TMPRSS2* enhancer. Furthermore, we obtained a high degree of ARBSs overlap with previously published data set on the same cell line.

Although the ChIP–qPCR data confirmed increased binding in the *PSA* enhancer in AR-overexpressing compared with control cell, no such loading difference was seen in the promoter region (Figure 1b). Previous studies (Kang *et al*., 2004; Wang *et al*., 2005) have pinpointed the importance of the dynamics of the AR recruitment in the *PSA* regulatory regions. Here, we studied the AR recruitment only 2 h after DHT stimulation. Thus, we cannot exclude the possibility that there are differences in the AR recruitment at the promoter of *PSA* between the cell lines at later time points.

Unlike most of the previous AR ChIP-chip and ChIP-seq studies (Massie *et al*., 2007; Wang *et al*., 2007, 2009; Jia *et al*., 2008; Takayama *et al*., 2010; Yu *et al*., 2010), we used several ligand concentrations and several LNCaP derivative cell lines. Thus, we were able to produce highly reproducible ARBSs data, which we called high-confidence ARBSs map. We utilized that map in comparison of the cell line model and the xenograft as well as in ARBS localizations, motif and ontology analyses, and also in identification of the critical AR target genes. The poor overlap (from 4 to 31%) of the high-confidence ARBSs map between the cell lines and the xenografts emphasizes that AR binding to chromatin varies significantly between tissue samples suggesting that genetic or other intrinsic differences,

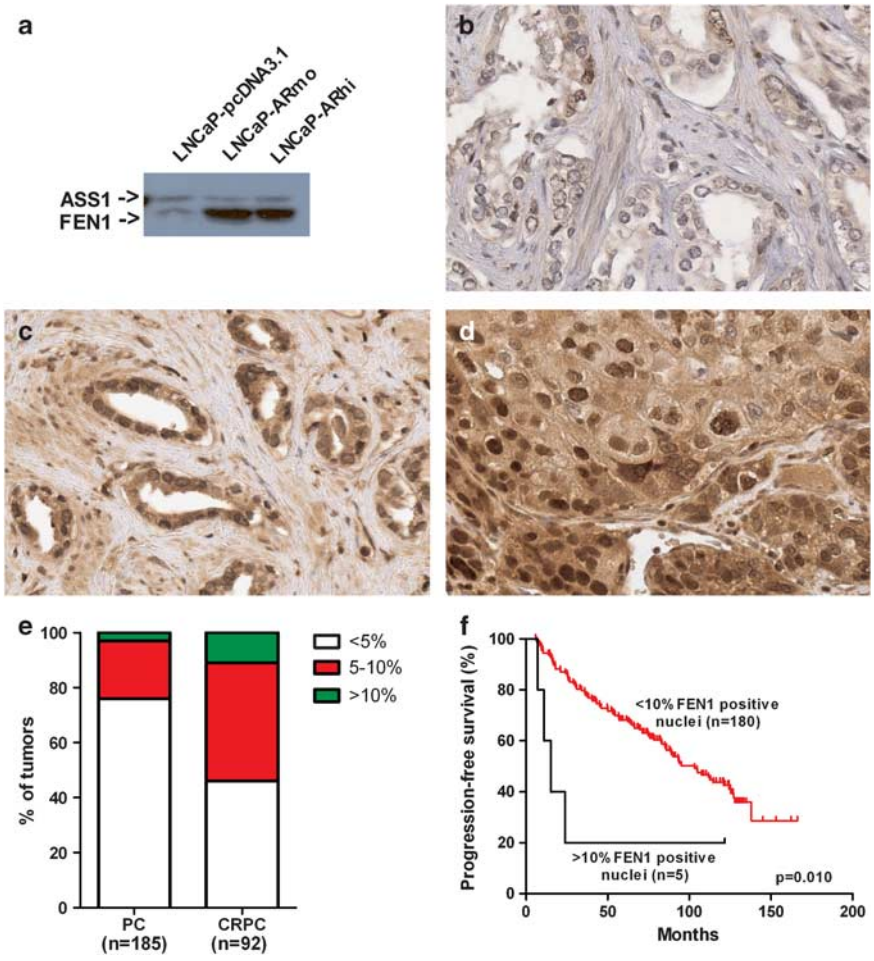


Figure 5 AR overexpression increases FEN1 protein. (**a**) AR overexpression increases FEN1 protein production. Western blot analysis of LNCaP-pcDNA3.1, -ARmo and -ARhi cells grown in normal medium showing FEN1 protein being overexpressed in AR-overexpressing cells compared with control cells. Anti-ATP synthase subunit alpha (ASS1) antibody was used as loading control. Immunohistochemical staining of (**b**) untreated PC with no cytoplasmic or nuclear staining, (**c**) untreated PC, and (**d**) CRPC specimen with strong nuclear staining in almost all malignant cells with monoclonal anti-FEN1 antibody. (**e**) The percentage of tumors according to percentage of positive nuclei in PC ($n = 185$) and CRPC ($n = 92$) specimens ($P < 0.0001$ according to χ^2 test). (**f**) Kaplan–Meier analysis of biochemical progression-free survival in prostatectomy-treated patients according to the percentage of FEN1 positive nuclei. Five patients with high frequency of FEN1-positive nuclei had very short progression-free time. The *P*-value was calculated with Mantel–Cox test.

such as binding of other transcription factors, in the cells could contribute strongly to the AR binding (Zinzen *et al*., 2009; Kasowski *et al*., 2010). This notion is also supported by our re-analysis of the publicly available data from the work by Yu and co-authors (2010). According to the re-analysis, Yu *et al*. obtained 58.1% overlap of ARBSs between LNCaP and only 28.7% overlap between VCaP and a tumor sample. The same tumor tissue sample showed 44.1% overlap with our high-confidence ARBSs, while the overlap with the LuCaP69 ARBSs was only 17.9%. Although the two xenografts overlapped poorly within each other and also with the high-confidence ARBSs map of LNCaP, the ARBSs in the xenografts, localized often close to the genes that showed ARBSs and androgen regulation in the LNCaP-model. This suggest that androgen-regulated genes may have alternative ARBSs. The poor overlap between the cell lines and xenografts may obviously also be due to the microenvironmental (cell culture versus mouse) differences.

The genomic localization of the ARBSs indicated that most of the androgen regulation is mediated by binding of AR to the distal intergenic elements and intronic regions as previously suggested (Jia *et al*., 2008; Lupien *et al*., 2008; Yu *et al*., 2010), instead of, for example, to promoter regions. Also, the motif analyses were in concordance with previously published findings (Massie *et al*., 2007; Lupien *et al*., 2008; Wang *et al*., 2009; Wei *et al*., 2010; Yu *et al*., 2010) showing that binding sites of, especially, FOXA1 and ETS family of transcription factors are enriched in the vicinity of ARBSs. FOXA1 has previously been suggested to be a pioneering factor for binding of other transcription factors to chromatin (Lupien *et al*., 2008, Wang *et al*., 2011), which is in line

with our data. Also, the ontology analysis of genes located within a 25 kb window of the ARBS maps was consistent with our previous findings based on the expression profiling of the LNCaP model (Waltering *et al*., 2009). Cell–cell adhesion and regulation of locomotion were among the most enriched ontologies.

Finally, to identify AR target genes that could be important in the progression of PC, we combined the high-confidence ARBS map with expression profiling of the LNCaP model. The analyses indicated that there are more genes, which show ARBSs in LNCaP-ARhi and -ARmo than in control cells. The data are consistent with our previously published findings on the expression of AR target genes in this model (Waltering *et al*., 2009). Thus, in cells overexpressing AR, less ligand is needed for induction of target gene expression. We then further studied the expression of *FEN1*, *ZWINT*, *SKP2*, *SNAI2* and *AZGP1* in clinical PC first by data mining publicly available microarray data, and subsequently by using qRT–PCR to measure the expression in BPH, PC and CRPC. Of those, the expression of *SKP2*, *ZWINT* and *FEN1* transcripts were significantly overexpressed in CRPC when compared with PC and/or BPH while the expression of *SNAI2* was reduced in cancer compared with BPH. Also, recently published data in advanced PC confirm the overexpression of these genes (Taylor *et al*., 2010). In addition, we confirmed the androgen regulation and the ARBSs of these genes by qRT–PCR, and ChIP–qPCR, respectively.

SKP2 is known to have oncogenic properties and to be overexpressed in many cancers (Nakayama and Nakayama, 2006). It has previously been shown to be androgen regulated (Waltregny *et al*., 2001) and the expression being associated with a short biochemical recurrence following prostatectomy (Nguyen *et al*., 2011). However, to our knowledge, this is the first report showing elevated levels of *SKP2* transcripts in CRPCs. A recent study by Lin and co-authors (2010) suggested *SKP2* as a potential target in cancer treatment and prevention, because inhibition of SKP2 triggered cellular senescence in p53/PTEN-deficient PC-3 cells and tumor regression in mice. *ZWINT* (ZW10 interactor), on the other hand, encodes a kinetokore protein (Starr *et al*., 2000) that has been suggested to have a role in the development of some malignancies (Obuse *et al*., 2004; Kops *et al*., 2005; Lin *et al*., 2006). We have previously shown *ZWINT* to be an androgen-regulated gene (Waltering *et al*., 2009). Depletion of ZWINT in LNCaP-pcDNA3.1 cells resulted in faster growth, whereas there was no significant effect on LNCaP-ARhi cells. Thus, the functional data are discordant with the finding of overexpression of the gene in cancer. SNAI2 (snail homolog 2 (*Drosophila*)) is a zinc-finger transcriptional repressor involved in epithelium to mesenchyme transition (Thiery, 2002). Depletion of also SNAI2 in LNCaP-pcDNA3.1, but not in LNCaP-ARhi, cells resulted in faster growth, which is concordant with the reduced expression in cancer.

FEN1 encodes a structure-specific metallonuclease that interacts with several other proteins involved in DNA replication, apoptosis, DNA repair and telomere stability (Zheng *et al*., 2010). Somatic mutations of *FEN1* have been reported in several common cancers (Zheng *et al*., 2007). These mutations abolish the exonuclease activity but retain the flap endonuclease activity (Zheng *et al*., 2007), which is consistent with the finding that mice carrying mutations show higher chemically induced cancer incidence (Xu *et al*., 2011). The mutations of *FEN1* in PC are yet to be investigated, however, FEN1 has been reported to be overexpressed in PC, especially in high Gleason score tumors (Lam *et al*., 2006). Here, we showed that the high frequency of nuclear staining was found significantly ($P<0.0001$) more often in CRPC than in PC, and that the staining was associated with poor prognosis in prostatectomy-treated patients. Thus, FEN1, as an androgen-regulated, overexpressed and associated with aggressive phenotype of the disease, could be an important AR downstream gene and, therefore, a putative drug target. Also, our preliminary functional data further suggest that FEN1 could promote the growth of PC cells, since the knockdown of FEN1 significantly reduced the growth of both control and AR-overexpressing cells.

In conclusion, we demonstrated that chromatin binding of AR is dependent not just on the ligand concentration, but also on the level of the receptor. Thus, the overexpression of *AR* in CRPC cells allows these cells to activate the AR signaling even in low androgen concentrations. By combining ChIP-seq and expression data, we were able to identify genes whose expression is directly regulated by AR, and are transcriptionally upregulated in PC and CRPC. These genes could be important in the progression of PC.

Materials and methods

Cell line and cell culture procedure

The establishment of LNCaP cells overexpressing AR has been described previously (Waltering *et al*., 2009). The cells were maintained under geneticin 250 μg/ml (Invitrogen Inc., Carlsbad, CA, USA). The hormone treatments and RNA extractions were performed as previously described (Waltering *et al*., 2011). LNCaP, VCaP and LAPC4 cells were purchased from ATCC (LGC/ATCC, Rockville, MD, USA) and maintained according to the manufacturer's instructions.

Xenografts material

Two PC xenografts, LuCaP69 and LuCaP73, grown in intact male mice, were provided by one of the investigators (RLV).

Clinical samples

Freshly frozen 8 BPH and 27 untreated primary PC samples from prostatectomies, as well as 7 BPH and 15 CRPC specimens from transurethral resection of the prostate-treated patients were used in the study. The samples were snap frozen in liquid nitrogen and total RNA was isolated with Trizol-Reagent (Invitrogen Inc.) according to the manufacturer's instructions. Tumor samples contained, at least, 70% of cancer cells. The use of the clinical material has been approved by the ethical committee of the Tampere University Hospital.

Tissue microarrays contained 185 formalin-fixed paraffin-embedded prostatectomy and 92 CRPC (transurethral resection of the prostate) specimens obtained from Tampere University Hospital. For the prostatectomy-treated patients, detectable PSA values ($\geqslant$0.5 ng/ml) in two consecutive measurements or the emergence of metastases were considered as signs of progression. The use of tissue microarrays has been approved by the ethical committee of Tampere University Hospital and the National Authority for Medicolegal Affairs.

ChIP, ChIP-seq assays and data analysis
Four million cells were plated and hormone-deprived for 4 days and treated with DHT at different concentrations for 2 h. Cells were fixed by adding formaldehyde (Merck KGaA, Darmstadt, Germany) in 1% final concentration for 10 min at room temperature and lysed in 1% sodium dodecyl sulfate, 10 mM EDTA, 50 mM Tris–HCl containing 2X protease inhibitor (Roche Inc., Mannheim, Germany). To perform tissue ChIP, 3 ml of phosphate-buffered saline containing 2X protease inhibitor (Roche Inc.) were added to 40×20 µm sections of freshly frozen xenograft specimens. They were first vigorously mixed three times with syringe and 14G needle, then four times with 25G needle. The cells were fixed for 10 min in room temperature by adding 1/10 volume of fixation solution (11% formaldehyde, 0.1 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM HEPES). Fixation was stopped by adding 1/20 volume of 2.5 M glycine for 5 min at room temperature. The cells were pelleted, washed twice in phosphate-buffered saline containing 2X protease inhibitor (Roche Inc.) and lysed as above. The chromatin was immunoprecipitated with 10 µg of normal rabbit immunoglobulin G (Santa Cruz Inc., Santa Cruz, CA, USA) or 10 µl of anti-AR polyclonal antibody (AR3) (provided by one of the investigators: OAJ) (Karvonen *et al*., 1997, Thompson *et al*., 2006). Supplementary Figure S3 shows a validation of the AR3 antibody in western blot and ChIP assay. The libraries of ChIP DNA were prepared and sequenced with Genome Analyzer II (Illumina Inc., San Diego, CA, USA) according to the manufacturer's protocol. Detailed descriptions of the ChIP procedure, sequencing and detailed data analysis are included in Supplementary Information.

mRNA expression profiling
mRNA expression data with Illumina platform (including RefSeq genes) (Illumina Inc.) were retrieved from the studies by Waltering *et al*. (2009). A detailed description of the raw data analysis is provided in the Supplementary Information.

Quantitative PCR assays
For mRNA expression analyses, first-strand complementary DNA synthesis was performed from total RNA using AMV reverse transcriptase (Finnzymes Inc., Espoo, Finland) according to the manufacturer's instructions. The relative expression of each gene against the average value of *TBP, G3PDH2* and *β-actin* reference genes was measured with Maxima SYBR Green (Fermentas Inc., Burlington, Ontario, Canada) and CFX96 real-time PCR detection system (Bio-Rad Laboratories Inc., Hercules, CA, USA) essentially as previously described (Urbanucci *et al*., 2008). For the ChIP–qPCR analysis, the enrichment relative to input chromatin was calculated according to the delta Ct method with the percentages been calculated using the formula $2^{-\Delta Ct}$, where ΔCt is Ct(ChIP-template)-Ct(Input). A standard curve from one of the diluted input was included in the run to control that the efficiency of the reaction would be maintained in the range between 95 and 105%. A qPCR on a control region in which AR is not supposed to bind, between PSA enhancer and promoter (middle region) was performed for each ChIP assay. The ChIP assay was considered specific if in the control region the enrichment was not above the enrichment of the non-specific immunoprecipitated sample made with normal rabbit immunoglobulin G. The primers used are listed in Supplementary Table S7.

Western blot
Western Blot was performed from total cell lysates. The primary antibodies used were anti-FEN1 (clone 4E7; LifeSpan Biosciences Inc., Seattle, WA, USA) and anti-ATP synthase subunit alpha (clone 15H4C4; MitoSciences Inc., Eugene, OR, USA) monoclonal antibodies. A detailed description of the western blot procedure is provided in the Supplementary Information.

Immunohistochemistry
Mouse anti-FEN1 (mAb clone 4E7; LifeSpan Biosciences Inc.) was used with Power Vision + Poly-HRP IHC kit (Immuno-Vision Technologies Co., Burlingame, CA, USA) according to the manufacturer's instructions. The protocol has previously been described (Leinonen *et al*., 2010).

siRNA transfections
Silencer selected siRNAs from Ambion (Applied Biosystems/Ambion, Austin, TX, USA) were used. Cells were transfected with INTERFERin transfection reagent (Polyplus-transfection, Illkirch, France) according to the manufacturer's protocol. Briefly, 20 000 cells/24-well plate were seeded and transfected with 20 nM of siFEN1 (s5103), 20 nM each of siZWINT (s21949 and s21951), 20 nM each of siSNAI2 (s13127 and s13128) or equal concentration of *Silencer* negative control siRNA #1. Expression levels of *FEN1*, *ZWINT* and *SNAI2* relative to *TBP* were measured by qRT–PCR (2.5 days after transfection) and protein levels by Western blot analysis (3 days after transfection).

Growth curves
Growth curve measurements were started 1 day after siRNA transfection and marked as day 1. Images of the same growth area in each well were acquired every day using a Retiga-2000R FAST Cooled Mono 12-bit camera (QImaging Inc., Surrey, BC Canada) mounted on a Motorized Inverted Research Microscope IXT1 (Olympus America Inc., Center Valley, PA, USA) and a 10X objective. The total growth area occupied by cells (area percent) in each well was determined each day of measurement with ImageJ software (Abramoff *et al*., 2004) and normalized against the growth area of the relative well at day 1. Four replicates were used in each siRNA experiment. *T*-test was used to assess significance of differences in growth curves at day 4.

Conflict of interest

The authors declare no conflict of interest.

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Author contributions: TV and AU designed the study, analyzed the data and wrote the paper. AU and BS performed experiments and edited the paper. JS, AL, HL performed the bioinformatic analyses and edited the paper. LML performed experiments and edited the paper. KKW provided the expression data and edited the paper. OAJ contributed new reagents and edited the paper. TLT provided clinical material and edited the paper. RLV provided the xenografts material and edited the paper.

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Androgen receptor overexpression alters binding dynamics of the receptor to chromatin and chromatin structure

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Abstract

BACKGROUND. Castration-resistant prostate cancers (CRPCs) overexpress often androgen receptor (AR). Here, we investigated the effect of AR overexpression on the dynamics of AR loading and RNA polymerase II (RNA Pol II) recruitment to chromatin. Acetylation of histone 3 (AcH3) on lysines 9 and 14 (K9 and K14) was also studied.

METHODS. We used an LNCaP-based AR overexpression cell line model that includes a control line and two sublines, LNCaP-ARmo and LNCaP-ARhi, which overexpress AR 2–3- and 4–5-fold, respectively. Cells were exposed to 1 nM or 100 nM of dihydrotestosterone (DHT). Chromatin immunoprecipitation (ChIP) on the promoters and enhancers of prostate specific antigen (*PSA*) and transmembrane protease, serine 2 (*TMPRSS2*) genes was performed. qRT-PCR was used to measure the levels of *PSA* and *TMPRSS2* transcripts.

RESULTS. Upon stimulation with 1 nM DHT, AR and RNA Pol II were recruited onto *PSA* and *TMPRSS2* enhancer regions to a greater extent ($p<0.05$) in AR-overexpressing cells compared to control cells. The difference in AR loading between the control and AR-overexpressing cells was abolished by a higher DHT concentration. The ratio of AcH3/H3 was increased in AR-overexpressing cells. The induction of transcription of *PSA* and *TMPRSS2* occurred earlier in the AR-overexpressing cells.

CONCLUSIONS. Our findings suggest that the levels of AR potentiate the recruitment of the AR, as well as components of the basic transcription machinery, to chromatin and affect the acetylation of histones in the presence of low levels of androgens. These changes result in enhanced gene transcription of AR target genes.

Keywords: prostatic neoplasia, PSA, TMPRSS2, AR, RNA polymerase 2, histone 3 lysine acetylation.

INTRODUCTION

The mechanisms of the development of castration-resistant prostate cancer (CRPC) are incompletely understood, hampering the treatment of this highly lethal form of the disease. Overexpression of androgen receptor (AR) has been found in the majority of CRPCs [1]. Overexpression is partially explained by amplification of the *AR* gene [1,2]. Mutations in the *AR* gene, which allow activation of the receptor by ligands other than androgens, have also been demonstrated, particularly in CRPCs treated with antiandrogens [3]. It was recently discovered that CRPCs may express splice variants of AR that lack the ligand-binding domain (LBD) of the receptor [4]. Given the inhibitory function of the unliganded LBD on the activation of the receptor [5], such receptors are constitutively active [4]. Although several studies reported an association of AR splice variants with advanced PC [4,6] and their role in abiraterone resistance has been recently hypothesized [7], the clinical significance of splice variants is not yet clear. In fact, suppression of the wild-type AR is enough to reduce AR signaling even in the presence of the constitutively active form of AR [8]. Also, alterations in the levels of AR coregulators [9,10] and cross-talk with other signaling pathways may be involved in the emergence of CRPC [10,11]. Finally, it has been suggested that CRPC cells could produce low levels of androgens themselves [12,13]. In recent clinical trials, abiraterone, an inhibitor of androgen biosynthesis [14], and MDV3100, a novel antiandrogen [15], have shown efficacy in the treatment of CRPC, directly demonstrating that AR signaling is still active in CRPC.

AR is a member of the steroid hormone nuclear receptor (SHR) family and shares several features with other members of the family. The SHRs are transcription factors activated by steroid hormones and mediate cell-fate events, including cell metabolism, organ development, cell growth, and differentiation [16]. Like other members of the SHR family, AR binds to chromatin through specific consensus sequences (*cis*-elements) that, for the AR, are called androgen response elements (AREs) [17]. In general, transcription factors act in a cyclical fashion in order to favor transcription [18,19], and there is some evidence that SHRs cycle onto chromatin [20]. Periodicity and detailed features of the binding have been clearly demonstrated for the estrogen receptor [21,22] and other members of the family [23,24,25]. A proteasome activity-dependent periodic binding has also been shown for the AR [26,27]. Finally, Welsbie et al. [28] have shown that AR cycles onto the enhancer of an AR target gene, the prostate specific antigen (*PSA*).

We recently demonstrated that overexpression of AR sensitizes cells to lower concentrations of androgens [29]. In cells with high levels of AR, cell growth, induction of target genes, and AR binding to chromatin occur at lower dihydrotestosterone (DHT) concentrations than in cells expressing lower levels of AR [29,30,31]. Here, we investigated the effect of AR overexpression on the binding dynamics of the AR and RNA polymerase II (RNA Pol II) to chromatin, as well as the effects on acetylation of histone 3 (AcH3). We utilized two well characterized AR target genes, *PSA* and *TMPRSS2*. We show that in AR-overexpressing cells exposed to low concentrations of androgens, the AR loading and the RNA Pol II recruitment dynamics to chromatin are altered compared to cells expressing lower levels of AR. Under the same conditions, the level of histone acetylation is also altered.

MATERIAL AND METHODS

Cell line and cell culture procedure

The establishment of LNCaP cells overexpressing AR was previously described [29]. The cells were stably transfected with pcDNA3.1(+) empty expression vector (Invitrogen Inc., Carlsbad, CA, USA) and pcDNA3.1(+) inserted with wild-type AR coding region and clonally expanded [29]. The cells were maintained under 250µg/ml geneticin (Invitrogen Inc., Carlsbad, California, USA). Exposure to DHT at the indicated concentrations was performed as previously described [30,31] after 4 days of hormone starvation.

Chromatin immunoprecipitation (ChIP)

The ChIP procedure was carried out as described in Urbanucci et al. [31]. Chromatin was immunoprecipitated with 10 µg of normal rabbit IgG, 13 µg of anti-RNA Pol II (N-20) (Santa Cruz Inc., Santa Cruz, California, USA), 10 µl of anti-AR polyclonal antibody (AR3) [31,32,33,34], 2.5 µl of anti-AcH3K9, 3 µg of anti-AcH3K9K14 or 2.5 µl of anti-H3 pan C-terminal (Millipore Inc., Billerica, Massachusetts, USA). Primer sequences for the *PSA* and *TMPRSS2* loci examined in this work are shown in Supplementary Table 1.

RNA extraction and quantitative PCR

RNA was isolated as previously described [31]. First-strand cDNA was synthesized from total RNA using AMV reverse transcriptase (Finnzymes Inc., Espoo, Finland) according to the manufacturer's instructions. ChIP-qPCR and mRNA expression analysis of each gene

normalized against the average value of the *TBP* reference gene were performed with Maxima™ SYBR Green (Fermentas Inc., Burlington, Ontario, Canada) and a CFX96 real-time PCR detection system (Bio-Rad Laboratories Inc., Hercules, California, USA) as previously described [31]. The primers used are listed in Supplementary Table 1.

Statistical analyses

Data were analyzed with one-way analysis of variance (ANOVA) with a Bonferroni post-test.

RESULTS

We previously established cell lines that have moderate (2–3-fold) or high (4-5-fold) AR overexpression compared to LNCaP-pcDNA3.1 control cells, named LNCaP-ARmo and LNCaP-ARhi, respectively [29]. We have demonstrated 86 % overlap between the chromatin binding of AR in the parental LNCaP and the LNCaP-pcDNA3.1cells. [31]. In addition, we have demonstrated similar AR loading on the PSA enhancer in LNCaP-pcDNA3.1 and LNCaP-1F5 cells (Suppl. Fig. 1). LNCaP-1F5 cells are glucocorticoid receptor transfected LNCaP cells, which have been shown to retain similar AR activity as well as growth properties than parental LNCaP [35]. These data suggest that the empty vector transfected LNCaP cells share similar AR binding properties with the parental LNCaP cells.

First, we measured mRNA levels of AR in LNCaP-pcDNA3.1 and in -ARhi cells upon stimulation with 1 nM DHT within a 16 h time interval. The results show a mild (non-significant) upregulation of AR level at 16 h in control transfected cells and a more substained upregulation already after 10 h in LNCaP-ARhi cells (t-test $p<0.05$) (Suppl. Fig. 2). However, in earlier time points there was no effect on the AR expression.

Next, we investigated how the AR overexpression affects the loading of AR and recruitment of RNA Pol II onto well-known regulatory regions of *PSA* and *TMPRSS2* genes upon stimulation with DHT, as well as the acetylation status of the H3. For *PSA*, in addition to the AREs located 131 and 150 bp from the transcription start site (TSS), an enhancer region 4 kb upstream of the TSS has been documented [36,37]. For the *TMPRSS2* gene, the AREs in the regulatory region are located 13.5 kb [27,38] and 700 bp upstream of the TSS [38]. We

performed ChIP-qPCR analyses on these selected genomic sites of *PSA* (Fig. 1A) and *TMPRSS2* (Fig. 1B) following DHT exposure from 30 min to 4 h. For PSA, we also looked at three additional genomic sites: the TSS, exon 3, and a site downstream of the gene locus.

In cells exposed to a low DHT concentration (1 nM), more AR was loaded onto the *PSA* enhancer after 30 min in AR-overexpressing cells than in control cells ($p<0.05$, t-test). A comparable level of AR loading was observed in control and LNCaP-ARmo cells at 1 h. However, LNCaP-ARhi cells loaded significantly ($p<0.05$) more AR than the other cell line at 1 h and also after 80 minutes (Fig. 2A). Taking all time points and cell lines into account, the higher AR level in the AR-overexpressing cells had a significant effect ($p<0.05$, one-way ANOVA) on receptor loading onto the *PSA* enhancer (Fig 2A). Also, more AR was loaded onto the promoter region ($p<0.05$, one-way ANOVA) in the LNCaP-ARhi cells than in –ARmo or control cells, especially after 80 minutes of androgen exposure (Fig. 2B). However, the difference in AR loading between AR-overexpressing cells and the control cells was not as clear as compared with the difference observed in the enhancer region. For the other chromosomal regions (TSS, exon 3, and the downstream site), AR loading was generally similar to background levels (Fig. 2C-E & Suppl. Fig. 3).

At the 1 h time point, AR loading onto the *TMPRSS2* enhancer (Fig. 3A) was already significantly ($p<0.001$, t-test) higher in LNCaP-ARhi cells than in the control cells. However, the *TMPRSS2* promoter did not significantly recruit AR, at least in the 4 h time (Fig. 3B).

When all cell lines were exposed to 100 nM DHT (Suppl. Fig. 4A&C), loading of AR onto *PSA* and *TMPRSS2* enhancers was more abundant than in cells treated with 1 nM DHT (Fig. 2A & 3A) especially at the 4 h time point. However, there were no significant differences amongst the cell lines when treated with 100 nM DHT. At the promoters of the genes, comparable levels of AR were loaded in all cell lines treated with 100 nM DHT (Suppl. Fig. 4B&D) and 1 nM DHT (Fig. 2B&3B).

Next, we profiled recruitment of RNA Pol II onto the same genomic loci (Fig. 1) in cells exposed to 1 nM DHT. The *PSA* enhancer and promoter regions recruited increasing amounts of RNA Pol II over time, and the recruitment was higher in AR-overexpressing cells (Fig. 4A&B) ($p<0.05$, one-way ANOVA). More RNA Pol II was also recruited onto exon 3 in AR-overexpressing cells than in control cells ($p<0.05$, one-way ANOVA). RNA Pol II

recruitment to the *TMPRSS2* enhancer (Fig. 5A) was, on average, equal in the three cell lines. However, in the LNCaP-ARhi cells, RNA Pol II was recruited onto the enhancer already after 30 min ($p<0.05$, t-test), and RNA Pol II recruitment onto the *TMPRSS2* promoter was significantly higher at the 30 min and 4 h time points ($p<0.01$ and $p<0.001$, t-test, respectively) compared to the other cell lines (Fig. 5B).

Next, we profiled the changes in AcH3 on lysine 9 (H3K9) and on both lysines 9 and 14 (H3K9K14) in the *PSA* (Suppl. Fig. 5) and in the *TMPRSS2* regions (Suppl. Fig 6) upon treatment with 1 nM DHT. In the AR-overexpressing cells, acetylation in the *PSA* enhancer region (Suppl. Fig. 5 A&B) peaked at the 2 h time point but remained at the same level until 4 h, while in the control cells, acetylation of H3K9 was significantly lower ($p<0.05$, t-test), although it still peaked at 2 h time point. AcH3 in the *PSA* promoter (Suppl. Fig. 5 C&D) increased significantly upon stimulation with DHT only in the LNCaP-ARhi cells and only after 4 h ($p<0.05$, t-test). Upon examination of the other genomic sites, the level of acetylation increased significantly on H3K9 only at the TSS (t-test $p < 0.01$) in the AR-overexpressing cells at 4 h (Suppl. Fig 5 E-J). Furthermore, the H3K9 acetylation increased significantly also at exon 3 at 4 h time point in LNCaP-ARhi compared to control and –ARmo cells (t-test $p < 0.05$). The acetylation of H3K9 at the *TMPRSS2* enhancer in the LNCaP-ARhi cells increased significantly ($p<0.05$, t-test) after 2 h compared to control and LNCaP-ARmo cells (Suppl. Fig. 6A). Acetylation of H3K9K14 was higher at the 4 h time point in LNCaP-ARhi cells compared to the other sublines (t-test $p < 0.05$) (Suppl. Fig. 6B). However, the acetylation of *TMPRSS2* promoter did not vary significantly from the basal level in all cell lines (Suppl. Fig. 6C&D).

After 4 days of hormone starvation, the basal loading of AR and recruitment of RNA Pol II to the genomic loci was similar across the three cell lines. However, the level of basal acetylation varied across the cell lines (Suppl. Fig. 7), especially in the *TMPRSS2* enhancer and promoter. The level of H3 acetylation was lower in the LNCaP-ARhi cells compared to the LNCaP-ARmo or control cells. Thus, we hypothesized that the differences across the cell lines may be due to the abundance of nucleosomes in the loci studied. We assumed that the total level of H3 could be used as indirect measure of the nucleosome abundance at a given genomic locus. This approach was previously described and proved to be consistent with nucleosomal DNA mapping [39]. Therefore, we normalized the level of H3K9 at the basal level (in cells hormone starved for 4 days) in the enhancer and promoter regions of *PSA* and

TMPRSS2 and in the control region (ARE mid) to the signal from the ChIP assays with an anti-histone 3 antibody (H3 pan) (Fig. 6). Except for the PSA enhancer, the ratios H3K9/H3pan in these loci were significantly higher in LNCaP-ARhi cells compared to LNCaP-ARmo or control cells ($p < 0.01$, t-test), which is consistent with a more nuclesome free region in those cells.

Finally, we measured accumulation of *PSA* and *TMPRSS2* mRNAs within a 16 h time interval after exposure to 1 nM DHT in LNCaP-pcDNA3.1 and LNCaP–ARhi cells (Fig. 7). *PSA* mRNA accumulated to comparable levels in the two cell lines until 3.5 h. After 4 h, *PSA* mRNA accumulation was significantly ($p<0.05$, t-test) higher in LNCaP-ARhi cells (Fig. 7A), whereas *TMPRSS2* mRNA levels were significantly ($p<0.05$, t-test) higher as early as 1 h after androgen exposure (Fig. 7B). The fold increase in *TMPRSS2* mRNA accumulation was also greater than that of *PSA* mRNA at the end of the time interval investigated (16 h).

DISCUSSION

AR is a nuclear receptor recognized as a major driver of prostate tumorigenesis [40] and is overexpressed in the majority of CRPCs [41]. We developed a cell line model to simulate the AR overexpression in CRPCs [29]. With this model, we previously demonstrated that the overexpression of AR sensitizes the cells to low levels of androgens [29]. The cell line LNCaP-ARhi grows faster compared to the control cell line at low androgen concentrations (1 nM DHT). Likewise, the expression of many androgen-regulated genes is upregulated at lower androgen concentrations in LNCaP-ARhi cells compared to control cells. Using ChIP-sequencing technology and the same cell line model, we recently showed that AR expression levels affect the receptor's genome-wide chromatin binding [31]. Increased levels of AR results in enhanced AR loading to chromatin compared to control cells upon stimulation with low concentration of ligand [31].

Here, we focused on the dynamics of AR loading and recruitment of RNA Pol II onto two AR target genes. Ligand-bound AR is loaded onto both the enhancer and promoter regions of *PSA* [41,42]. Loading of AR and recruitment of its coactivators onto the *PSA* enhancer is stronger than loading onto the promoter [42,43]. AR loading and RNA Pol II recruitment onto the *PSA* enhancer and promoter are initially cyclical [26,28,42] and then increase gradually [43]. Proteasome activity is needed to maintain the initial cyclical behavior [26].

However, all of the previous studies determined the dynamics of AR loading onto and the RNA Pol II occupancy of chromatin following exposure of the cells to high concentrations of natural or synthetic ligand [26,27,28,42,43].

Using low DHT (1 nM), we showed that the AR binding profile is different in the promoter and enhancer regions of both *PSA* and *TMPRSS2* genes than the profiles following high DHT treatment. There was more AR enrichment in the enhancer in AR-overexpressing cells compared to control cells. This finding that the enhancer region is the primary AR-loading site is in agreement with previous studies [37,42,43]. *PSA* and *TMPRSS2* also showed different AR-binding profiles, suggesting that various androgen target genes have locus-specific binding properties. Overexpression of AR also affected the AR-binding profile. In AR-overexpressing cells in the presence of low levels of DHT, the receptor not only loaded faster but loaded to higher levels onto the enhancers and promoters. However, high androgen concentrations abolished the effects of AR levels. This is to our knowledge, the first study profiling the AR loading on target genes' regulatory regions from a dynamic perspective and using low concentration (1nM) of DHT to stimulate the cells.

In order to compare our AR binding profile to previous studies, we chose to determine AR loading also at 80 minutes time point. In the work by Welsbie et al. [28] the AR binding dynamics upon stimulation of LNCaP with 1 nM R1881 shows that AR is strongly recruited to the *PSA* enhancer after 80 min. In our study, using a less potent ligand, the binding was reduced, in the case of PSA enhancer (Fig. 2A) or abolished, in the case of the *TMPRSS2* enhancer (Fig. 3A) in control transfected and LNCaP-ARmo cells but not in -ARhi cells. At 2 h time point the AR loading onto the enhancer of *PSA* and *TMPRSS2* was comparable across the cell lines. However, at 4 h time point, more AR was loaded onto these regions in AR-overexpressing cells. These findings could be due to kinetic variations in the cyclic loading of AR onto the chromatin in the differently AR-expressing cell lines. Taken together, these data further support the idea that AR level alters the binding dynamics of AR in presence of low concentration of androgens.

By measuring *AR* mRNA levels accumulation, we demonstrated that the hormone stimulation upregulates only mildly the AR transcripts in control transfected cells and the AR-overexpression enhances such upregulation. However, this seems to take place after 8 hours of androgen stimulation. Since, the ChIP measurements were done, at latest, 4 hour time

point, the increase of AR levels upon DHT stimulation at later time points should not affect to the AR-ChIP data. However, we have previously demonstrated that nuclear AR protein levels are slightly increased in LNCaP-ARhi cells compared to empty vector transfected cells in hormone deprived conditions [29,44]. Thus, we cannot completely rule out that different nuclear AR protein levels could influence to the AR loading to chromatin in these cells. Interestingly, LNCaP-ARhi cells seemed to load less AR in hormone-deprived conditions especially at the PSA enhancer compared to -ARmo or control cells (see Fig. 2A). This difference may certainly be due to technical reasons. However, we cannot rule-out that differences in the composition of the AR complex at the different genomic loci may have an effect on the loading.

Several groups have investigated the recruitment dynamics of the RNA Pol II onto the *PSA* [27,42,43] and *TMPRSS2* regulatory elements under high androgen concentrations [27,42]. AR loading initiates recruitment of coregulators, including GRIP1 and CBP, and ultimately RNA Pol II [45]. We investigated whether AR overexpression affects recruitment of the key component in the basic transcription machinery, RNA Pol II, under low androgen concentrations. The recruitment of RNA Pol II onto the promoter and enhancer regions of *PSA* and *TMPRSS2* across the three cell lines was, similarly to the AR loading, more enriched in AR-overexpressing cells compared to control cells. However, the recruitment of RNA Pol II at the promoter was significantly higher than for AR. Thus, the binding pattern of RNA Pol II is affected by the level of AR. Furthermore, the RNA Pol II was also recruited to the TSS, exon 3, and to a locus downstream of the PSA gene, although with a delay with respect to the promoter and the enhancer. These results are in concordance with those of Wang et al. [45], whereas Kang et al. [43] have reported a significant difference in the RNA Pol II recruitment between enhancer and promoter regions of the PSA gene. This discrepancy could be due to the difference in the androgen concentrations used.

Chen and colleagues [41] found that a modest overexpression of AR can alter the abundance of AR coregulators, many of which have histone acetylation activity, recruited onto the promoters of AR target genes [46,47]. Therefore, we investigated the pattern of acetylation of recognized markers of active transcription, H3K9 and H3K9K14. These markers are necessary to render regulatory regions accessible to the basic transcription factors [48,49,50]. AcH3 increases upon time of stimulation with DHT, and increases are more pronounced in LNCaP-ARhi cells, but acetylation does not seem to vary as dynamically as AR and RNA Pol

II recruitment. The changes in H3K9 and H3K9K14 were more modest compared to those in the study by Kang et al. [43], in which the cells were exposed to higher androgen concentrations. Thus, the data suggest that the dynamics of chromatin structure are sensitive to androgen levels.

Interestingly, we found that the acetylation of H3 was different between the cell lines under hormone deprivation. We initially found that the amount of AcH3 in the regulatory regions was lower in LNCaP-ARhi cells compared to LNCaP-ARmo and control cells. However, the amount of total H3 was also lower in the LNCaP-ARhi cells. The amount of total H3 has been used as a measure of nucleosome density [39]. Our preliminary results showed that the ratio between AcH3 and H3 pan was higher in LNCaP-ARhi cells. Thus, we speculate that there is a reduced number of histone octamers in the regulatory regions of *PSA* and *TMPRSS2* in these cells. As consequence, the chromatin is more open in those regions in the AR-overexpressing cells. Since we studied the ratio of AcH3K9 and H3 pan, the H3 that are still in those regions seemed to be more acetylated in the AR-overexpressing cells. The acetylation of H3 is known to relieve transcriptional repression given by nucleosomal positioning, without influencing the mobility of histone octamers [51]. Our data are consistent with two recent reports showing how modification of histone tails such as H3 or H4 influence and are tightly connected to the chromatin remodeling factors [52,53]. In a recent study by He and colleagues [54], the androgen treatment in PC cells was found to induce the release of a central nucleosome flanked by a pair of marked nucleosomes present at the AR-binding sites [54]. In the same study, the locations of the positioned nucleosomes were concordant between DHT treatment and vehicle control [54], suggesting that the nucleosome positioning is pre-determined and independent of AR binding. The chromatin opening seems to be dependent on chromatin remodeling factors, such as FOXA1 [55]. Our results are concordant with these reports. However, the mechanism by which the nucleosome depletion occurs in AR-overexpressing cells and how AR affects this process needs further investigation. Our results are also consistent with a previous report by Jia et al. [27] showing that increased histone acetylation is associated with the development of androgen independence *in vivo*.

Given the effect that AR overexpression has on the dynamics of AR recruitment, RNA Pol II recruitment, and chromatin structure, we measured accumulation of *PSA* and *TMPRSS2* transcripts in cells stimulated with 1 nM DHT. We have previously demonstrated that upon

stimulation with different concentrations of DHT, at 4 hours time point, LNCaP-ARmo and -ARhi, show comparable induction of *PSA* and *TMPRSS2* mRNA, and more abundant than in LNCaP-pcDNA3.1 [29]. At 24 hours time point -ARmo shows intermediate induction of both genes [29]. Therefore, we measured the expression of these genes only in the control-transfected and LNCaP-ARhi cells. The expression levels of PSA and TMPRSS2 transcripts were higher in AR-overexpressing cells compared to the control cells at the endpoint of our measurement [29]. The induction kinetics were faster in AR-overexpressing cells, which is consistent with the observed earlier recruitment of AR and RNA Pol II to the enhancer and promoter regions of *PSA* and *TMPRSS2*. The effect of AR overexpression was stronger on *TMPRSS2* compared to *PSA*, suggesting that the AR overexpression differentially affects the expression of target genes.

CONCLUSIONS

Our findings suggest that the level of AR affects the dynamics of receptor binding to chromatin, potentiating the loading of AR and recruitment of RNA Pol II, especially when low levels of androgens are present. Furthermore, AR overexpression seemed to favor chromatin accessibility by modifying the acetylation status of H3 and nucleosome occupancy at the AR-binding sites. Altogether, these changes in the dynamics of chromatin modifications result in enhanced transcription of AR target genes, providing a mechanistic explanation of how AR signaling pathways are reactivated in CRPC.

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FIGURES

Fig. 1.

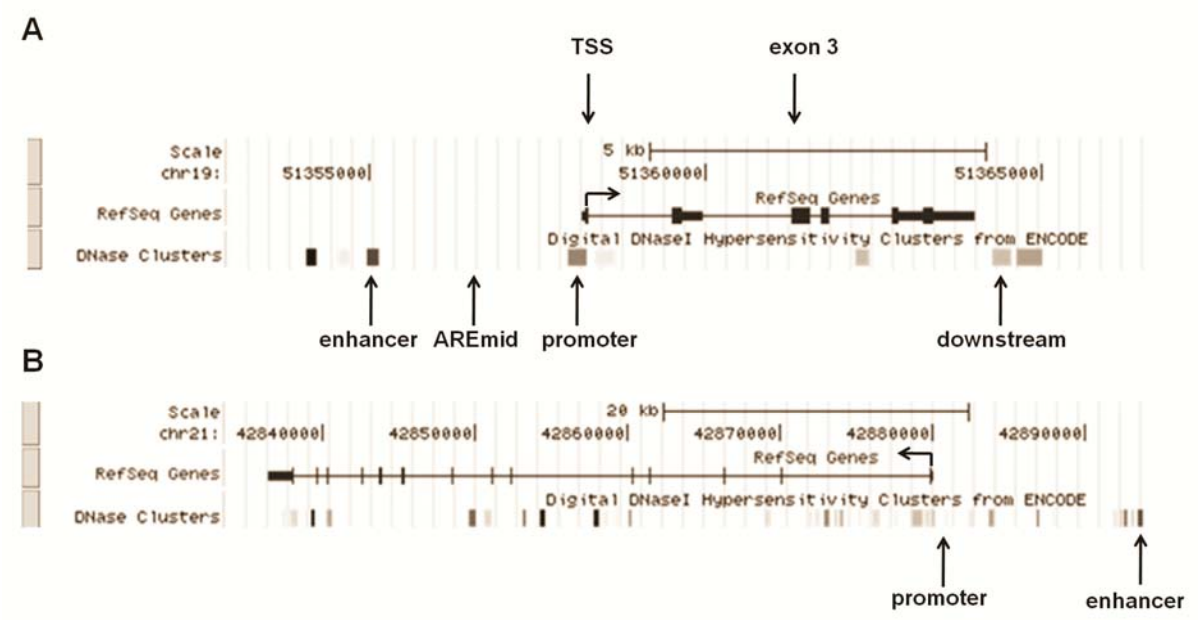

Fig. 1. Location of the primers used on the *PSA* and *TMPRSS2* genes. Structure of *PSA* (A) and *TMPRSS2* (B) genes and the locations of the primers used on the UCSC genome browser showing also DNase clusters for the LNCaP cell line.

Fig. 2.

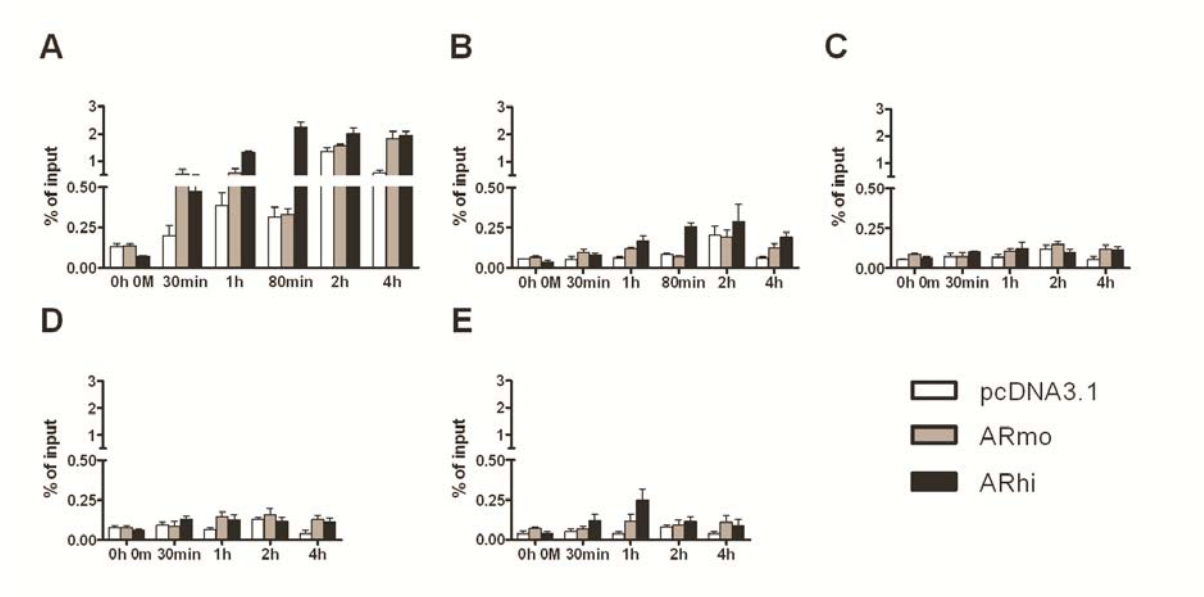

Fig. 2. Loading of AR onto the *PSA* gene regions upon treatment with 1 nM DHT. AR binding to *PSA* enhancer (A), promoter (B), TSS (C), exon 3 (D) and a downstream site (E) in the LNCaP model. LNCaP-pcDNA3.1, -ARmo and –ARhi cells were hormone-starved for 4 days and then exposed for the indicated time periods to 1 nM DHT or ethanol (0h 0M). ChIP-qPCR was performed to assess the AR loading. Mean and S.E.M. of three biological replicates are shown.

Fig. 3.

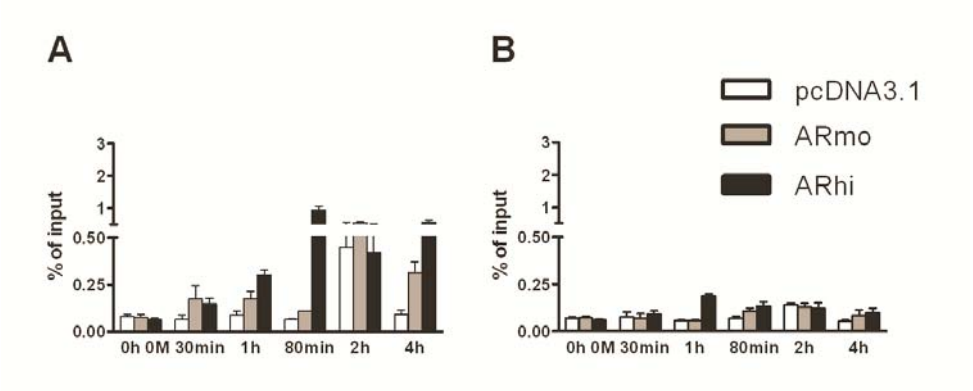

Fig. 3. Loading of AR onto the *TMPRSS2* gene regulatory regions upon treatment with 1 nM DHT. AR binding to the *TMPRSS2* enhancer (A) and promoter (B) in the LNCaP model. LNCaP-pcDNA3.1, -ARmo and –ARhi cells were hormone-starved for 4 days and exposed for the indicated time periods to 1 nM DHT or ethanol (0h 0M). ChIP-qPCR was performed to assess the AR loading. Mean and S.E.M. of three biological replicates are shown.

Fig. 4.

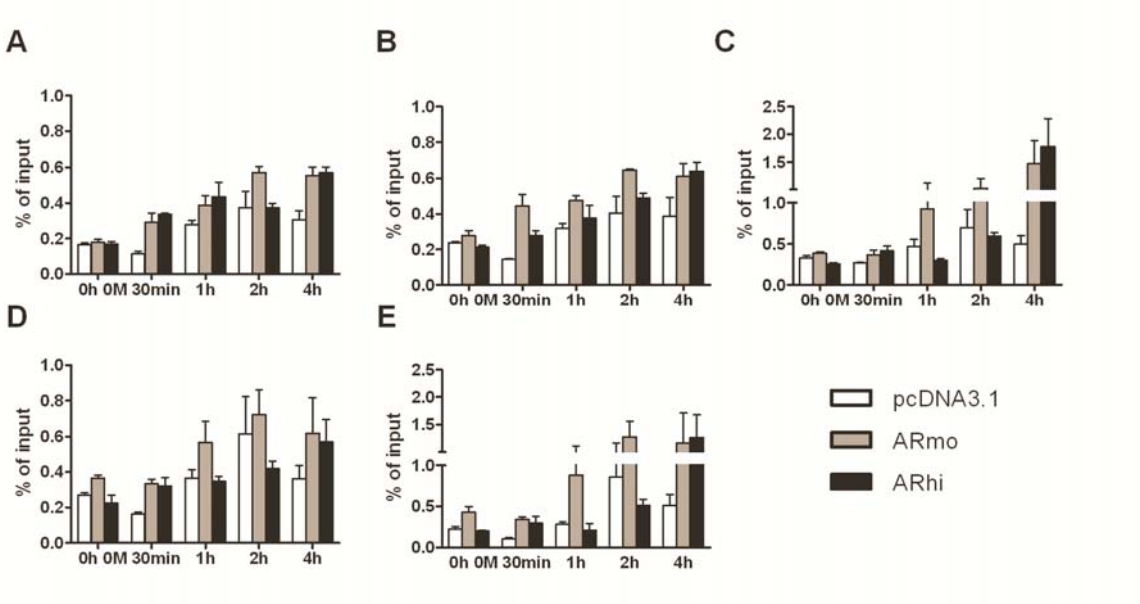

Fig. 4. Recruitment of RNA Pol 2 on the *PSA* gene regions upon treatment with 1 nM DHT. RNA Pol II recruitment onto PSA enhancer (A), promoter (B), TSS (C), exon 3 (D) and a downstream site (E) in the LNCaP model. LNCaP-pcDNA3.1, -ARmo and -ARhi cells were hormone-starved for 4 days and exposed for the indicated time periods to 1 nM DHT or ethanol (0h 0M). ChIP-qPCR was performed to assess the RNA Pol II recruitment. Mean and S.E.M. of two biological replicates are shown.

Fig. 5.

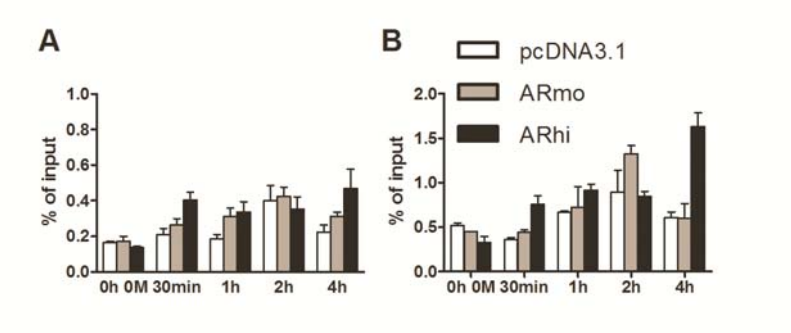

Fig. 5. Recruitment of RNA Pol II onto the *TMPRSS2* gene regions upon treatment with 1 nM DHT. RNA Pol II recruitment to the *TMPRSS2* enhancer (A) and promoter (B) in the LNCaP model. LNCaP-pcDNA3.1, -ARmo and -ARhi cells were hormone-starved for 4 days and then exposed for the indicated time periods to 1 nM DHT or ethanol (0h 0M). ChIP-qPCR was performed to assess RNA Pol II recruitment. Mean and S.E.M. of two biological replicates are shown.

Fig.6.

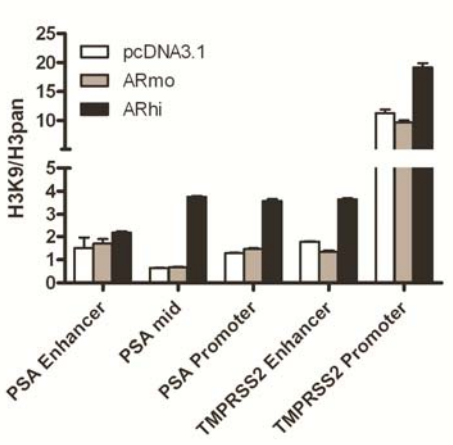

Fig.6. Ratio of acetylated histone 3 in lysine 9 versus the histone 3 in hormone-depleted condition. The acetylation status of H3 in lysine 9 in the given loci is presented as ratio of % of input values.

Fig.7.

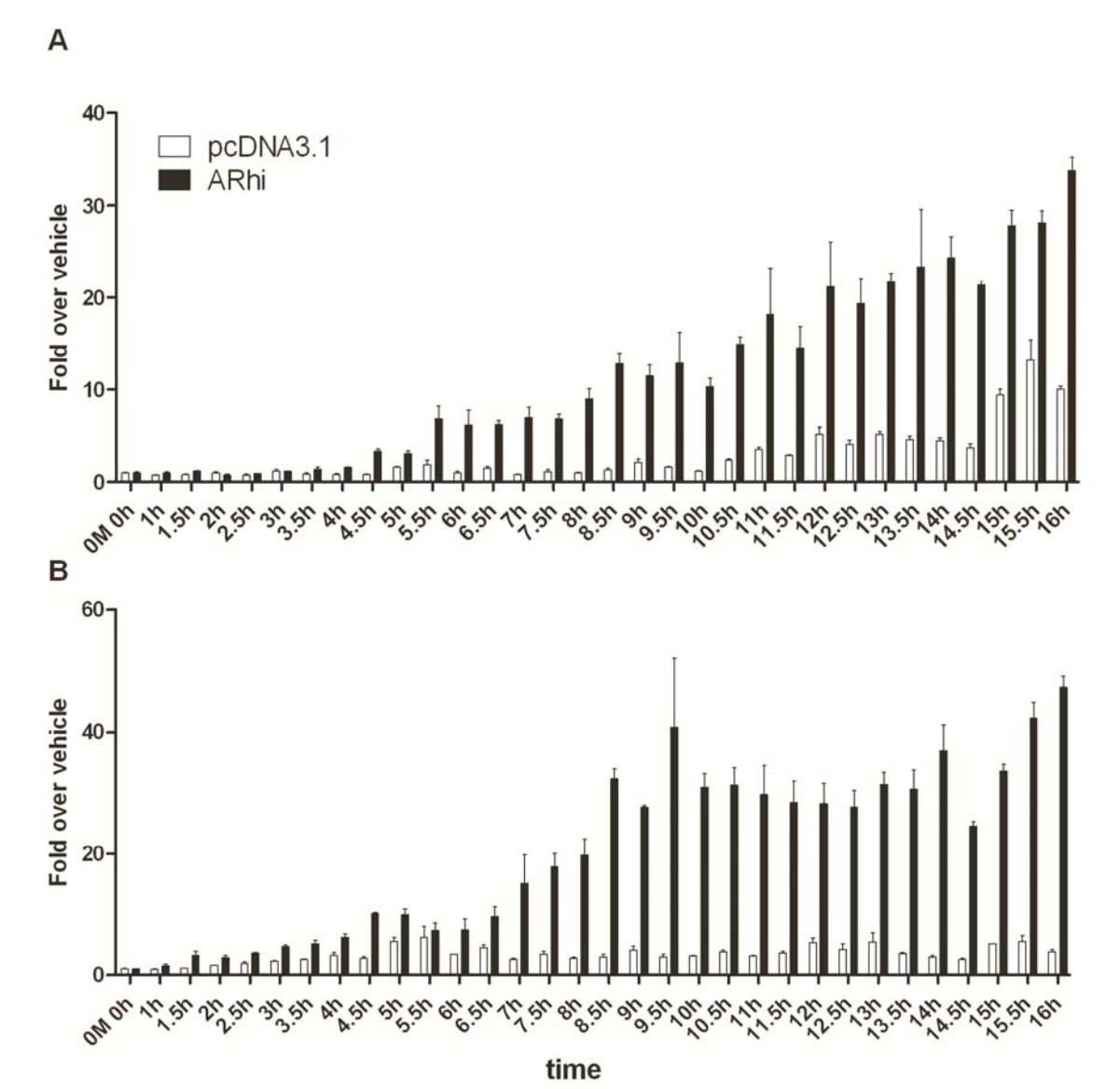

Fig.7. Accumulation of *PSA* and *TMPRSS2* mRNAs in response to androgen exposure. Normalized (against 0 M 0 h) accumulation of *PSA* (A) and *TMPRSS2* (B) mRNAs in LNCaP-pcDNA3.1 and –ARhi cells as measured by RT-PCR. The cells were hormone starved for 4 days and the exposed to 1 nM DHT for the indicated time periods. The data are presented as PSA or TMPRSS2 versus TBP values normalized against the 0h 0M. Mean and S.E.M. of three biological replicates are shown.

Supplementary Information

Androgen receptor overexpression alters binding dynamics of the receptor to chromatin and chromatin structure

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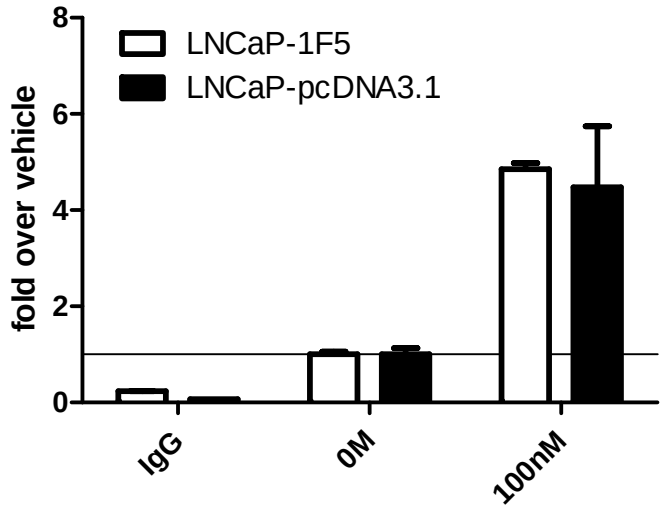
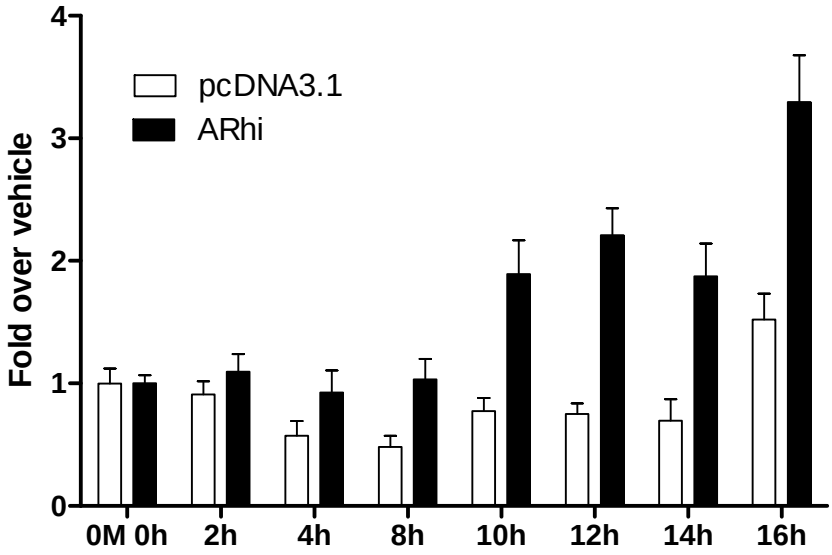
Supplementary Figure 1.



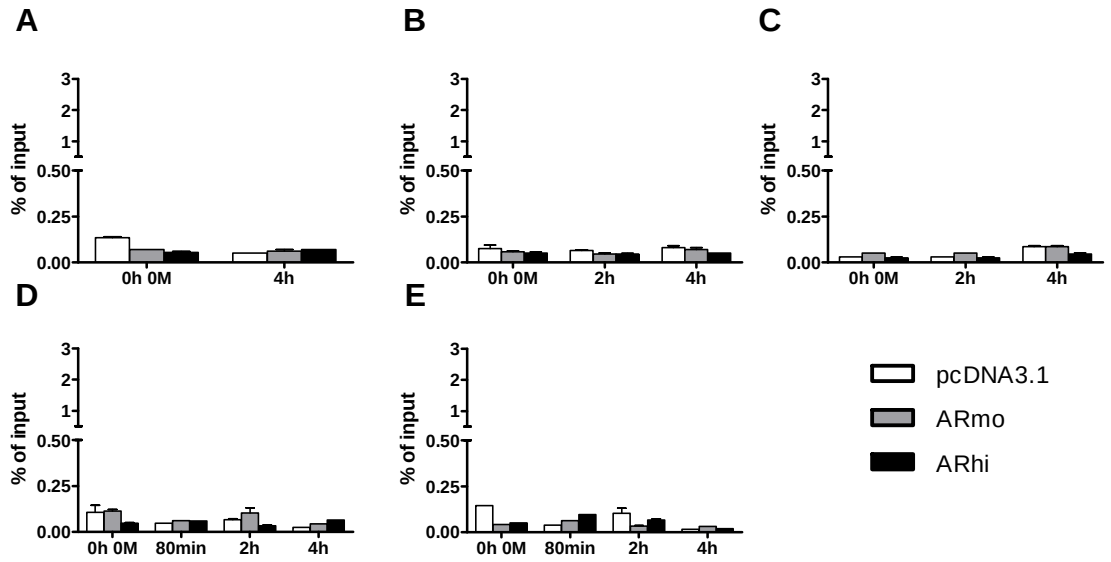
Supplementary Figure 1. Loading of AR on PSA enhancer in LNCaP derivative cell lines. Re-analysis of ChIP-qPCR data from Urbanucci et al. [1] showing loading of AR on PSA enhancer (See Figure 1A) upon 2 hours stimulation of LNCaP-1F5 and LNCaP-pcDNA3.1 cells with 100 nM DHT. ChIP-qPCR with the unspecific control antibody (normal rabbit IgG) in the same genomic regions shows the background binding level. The data are presented as fold over vehicle (0M) of % of input. Mean and S.E.M. are shown.

Supplementary Figure 2.



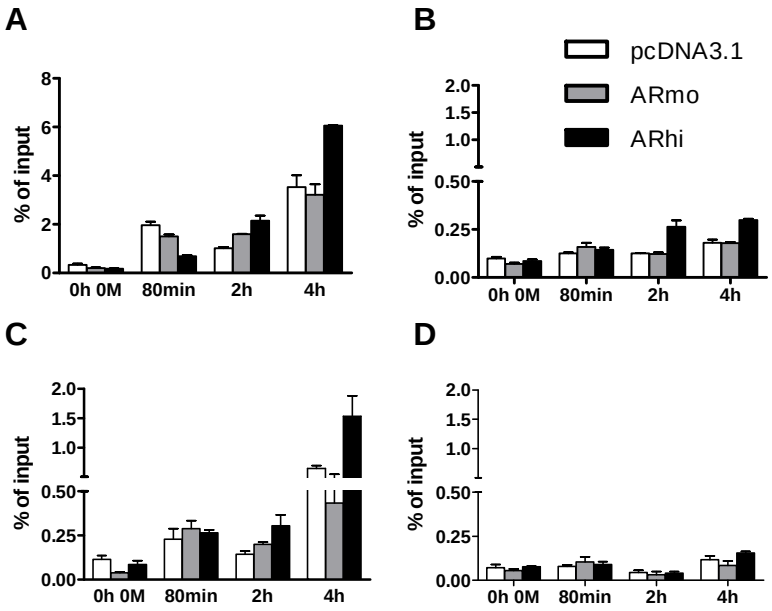
Supplementary Figure 2. Accumulation of *AR* mRNAs in response to androgen exposure. Normalized (against 0 M 0 h) accumulation of *AR* mRNAs in LNCaP-pcDNA3.1 and –ARhi cells as measured by qRT-PCR. The cells were hormone starved for 4 days and exposed to 1 nM DHT for the indicated time periods. The data are presented as AR versus TBP values normalized against the 0h 0M. Mean and S.E.M. of three biological replicates are shown.

Supplementary Figure 3.



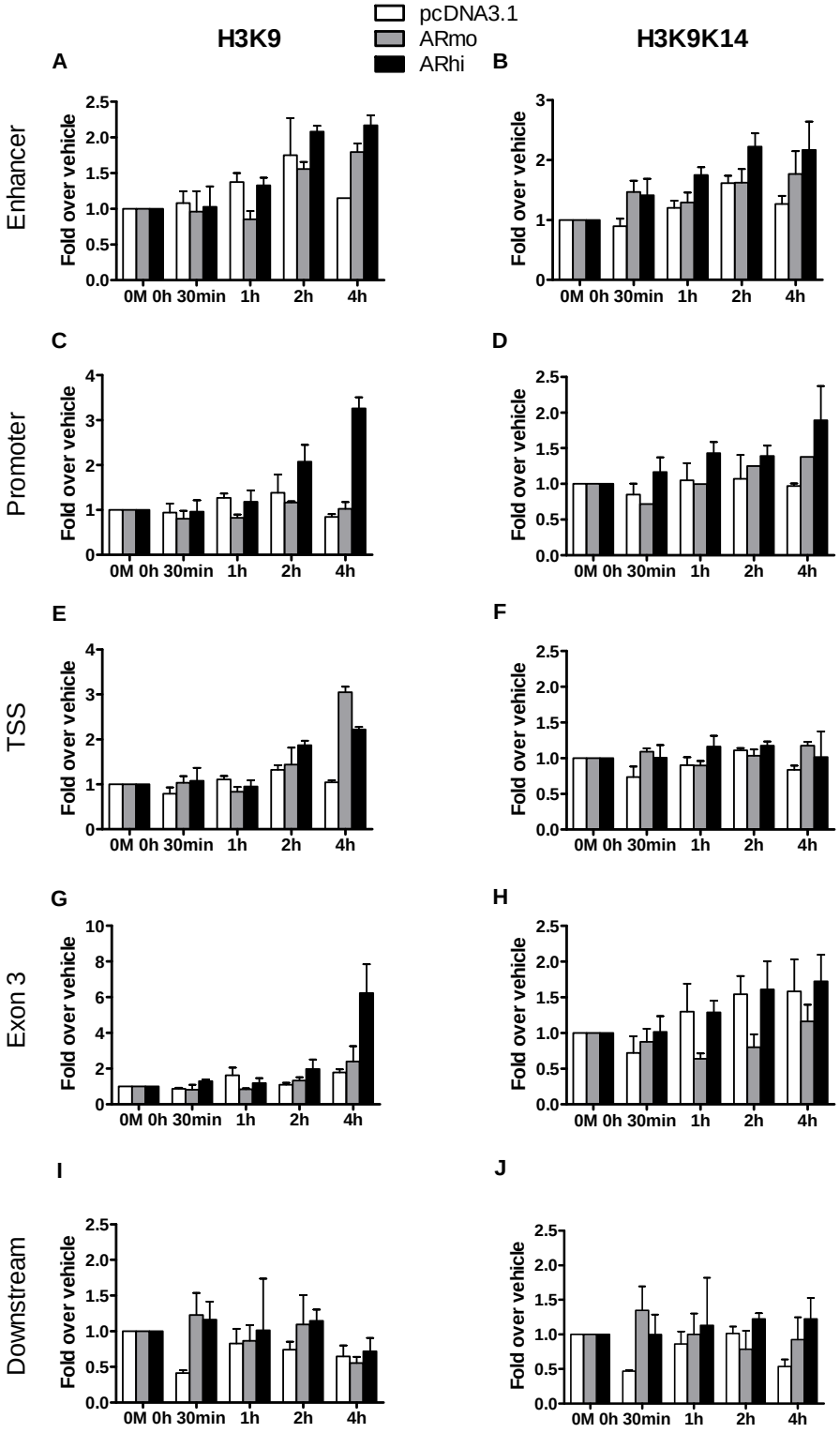
Supplementary Figure 3. Background levels of the ChIP assays. ChIP-qPCR showing the loading of AR to ARE mid (See Figure 1A) upon stimulation of LNCaP model cells with 1 nM DHT (A) or 100 nM DHT (B) for the indicated time periods. ChIP-qPCR with the unspecific control antibody (normal rabbit IgG) in cells are treated with 100 nM DHT to ARE mid (C), *TMPRSS2* enhancer (D) and *TMPRSS2* promoter (E) for the indicated time periods. Mean and S.E.M. of three biological replicates are shown.

Supplementary Figure 4.



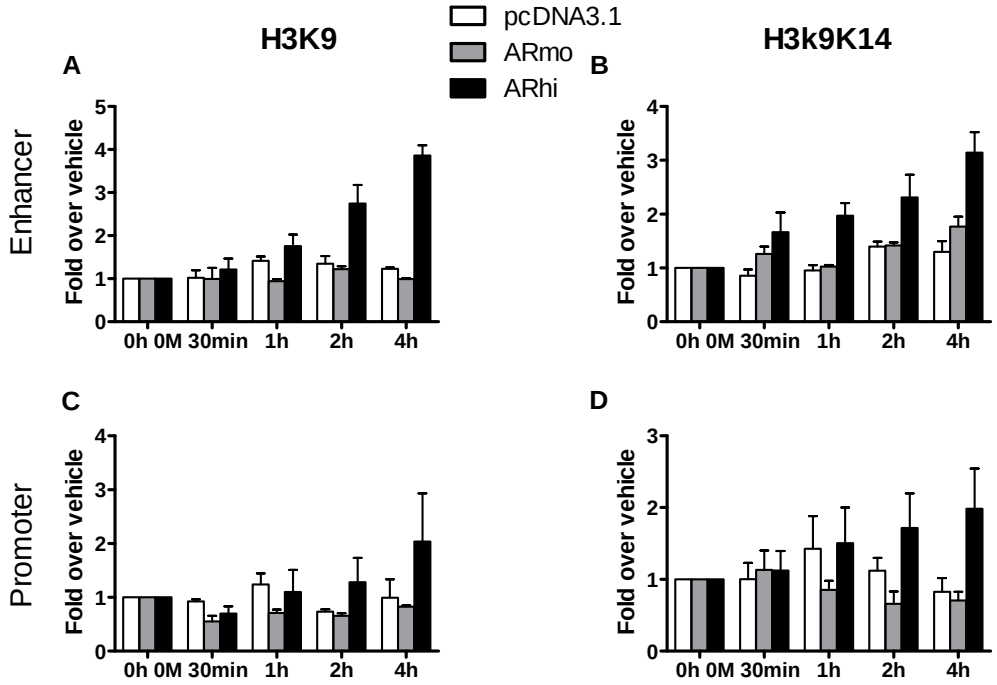
Supplementary Figure 4. Loading of AR on the *PSA* and *TMPRSS2* gene regions upon treatment with 100 nM DHT. AR binding onto the *PSA* enhancer (A) and promoter (B) and to the *TMPRSS2* enhancer (C) and promoter (D) in the LNCaP model cells. LNCaP-pcDNA3.1, -ARmo and -ARhi cells were hormone-starved for 4 days and then exposed for the indicated time periods to 100 nM DHT or ethanol (0h 0M). ChIP-qPCR was performed to assess AR loading. Mean and S.E.M. of three biological replicates are shown.

Supplementary Figure 5.



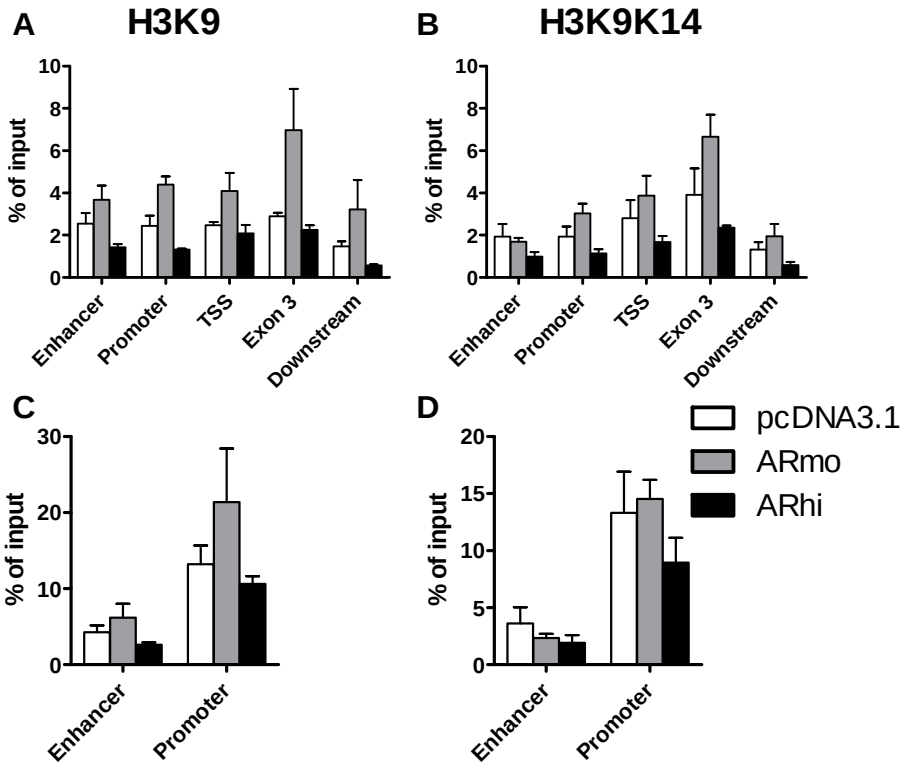
Supplementary Figure 5. Acetylation of H3 in the *PSA* locus. ChIP-qPCR results showing acetylation of H3 on lysine 9 (A, C, E, G, I) and on both lysine 9 and lysine 14 (B, D, F, H, J) upon stimulation with 1 nM DHT for the indicated time periods in the loci shown in Figure 1A: enhancer (A&B), promoter (C&D), TSS (E&F), exon 3 (G&H) and downstream site (I&J). The % of input of each time point is normalized against the 0M 0h. Mean and S.E.M. of two biological replicates are shown.

Supplementary Figure 6.



Supplementary Figure 6. Acetylation of H3 in the *TMPRSS2* locus. ChIP-qPCR results showing acetylation of H3 on lysine 9 (A&C) and on both lysine 9 and lysine 14 (B&D) upon stimulation with 1 nM DHT for the indicated time periods in the loci shown in Figure 1B: enhancer (A&B) and promoter (C&D). The % of input of each time point is normalized against the 0M 0h. Mean and S.E.M. of two biological replicates are shown.

Supplementary Figure 7.



Supplementary Figure 7. Acetylation status of H3 in the *PSA* and *TMPRSS2* loci under hormone deprived condition. ChIP-qPCR at the *PSA* (A&B) and *TMPRSS2* (C&D) loci showing the acetylation of H3 lysine 9 (A&C) and H3 lysine 9 and 14 (B&D) after 4 days of hormone starvation. Mean and S.E.M. of two biological replicates are shown.

Supplementary Table 1. Primers used in ChIPqPCR and qPCR.

AR mRNA	5'-CTCACCAAGCTCCTGGACTC-3' 5'-CAGGCAGAAGACATCTGAAAG-3'	
PSA		
Enhancer	5'-TGGGACAACTTGCAAACCTG-3' 5'-CCAGAGTAGGTCTGTTTTCAATCC-3'	Wang et al. [2]
ARE mid	5'-CAGTGGCCATGAGTTTTGTTTG-3' 5'-AACCAATCCAACTGCATTATACACA-3'	Jia et al. [3]
Promoter	5'-CCTAGATGAAGTCTCCATGAGCTACA-3' 5'-GGGAGGGAGAGCTAGCACTTG-3'	Wang et al. [2]
TSS	5'-GGGGCGGAGTCCTGGGGAAT-3' 5'-GTGCAGCACCAATCCACGTCA-3'	
Exon 3	5'-CACACCCGCTCTACGATATGAG-3' 5'-GAGCTCGGCAGGCTCTGA-3'	Jia et al. [4]
Downstream	5'-TCATCATGAATCGCACTGTTAGC-3' 5'-GCCCAAGTGCCTTGGTATACC-3'	Jia et al. [4]
PSA mRNA	5'-GCAGCATTGAACCAGAGGAG-3' 5'-AGAACTGGGGAGGCTTGAGT-3'	
TMPRSS2		
Enhancer	5'-TCCAGGCAGAGGTGTGGC-3' 5'-CGTATGTCTCCCTGCACCACT-3'	Wang et al. [5]
Promoter	5'-GCTCGAGTTTGGGTTAAGGAA-3' 5'-TACAGGAGCTCGTGAGGTAGCA-3'	Jia et al. [4]
TMPRSS2 mRNA	5'-CCAGGAGTGTACGGGAATGT-3' 5'-CAGCCCCATTGTTTTCTTGT-3'	

Supplementary References.

1. Urbanucci A, Sahu B, Seppälä J, Larjo A, Latonen LM, Waltering KK,Tammela TLJ, Vessella RL, Lähdesmäki H, Jänne OA, Visakorpi T. Overexpression of androgen receptor enhances the binding of the receptor to the chromatin in prostate cancer. Oncogene 2011; In press.

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4. Jia L, Shen HC, Wantroba M, Khalid O, Liang G, Wang Q, Gentzschein E, Pinski JK, Stanczyk FZ, Jones PA, Coetzee GA. Locus-wide chromatin remodelling and enhanced androgen receptor-mediated transcription in recurrent prostate tumor cells. Mol Cell Biol 2006;26:7331-7341.

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Androgen regulation of the androgen receptor coregulators

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Abstract

Background: The critical role of the androgen receptor (AR) in the development of prostate cancer is well recognized. The transcriptional activity of AR is partly regulated by coregulatory proteins. It has been suggested that these coregulators could also be important in the progression of prostate cancer. The aim of this study was to identify coregulators whose expression is regulated by either the androgens and/or by the expression level of AR.

Methods: We used empty vector and AR cDNA-transfected LNCaP cells (LNCaP-pcDNA3.1, and LNCaP-ARhi, respectively), and grew them for 4 and 24 hours in the presence of dihydrotestosterone (DHT) at various concentrations. The expression of 25 AR coregulators (*SRC1*, *TIF2*, *PIAS1*, *PIASx*, *ARIP4*, *BRCA1*, *β-catenin*, *AIB3*, *AIB1*, *CBP*, *STAT1*, *NCoR1*, *AES*, *cyclin D1*, *p300*, *ARA24*, *LSD1*, *BAG1L*, *gelsolin*, *prohibitin*, *JMJD2C*, *JMJD1A*, *MAK*, *PAK6* and *MAGE11*) was then measured by using real-time quantitative RT-PCR (Q-RT-PCR).

Results: Five of the coregulators (*AIB1*, *CBP*, *MAK*, *BRCA1* and *β-catenin*) showed more than 2-fold induction and 5 others (*cyclin D1*, *gelsolin*, *prohibitin*, *JMJD1A*, and *JMJD2C*) less than 2-fold induction. Overexpression of AR did not affect the expression of the coregulators alone. However, overexpression of AR enhanced the DHT-stimulated expression of *MAK*, *BRCA1*, *AIB1* and *CBP* and reduced the level of expression of *β-catenin*, *cyclinD1* and *gelsolin*.

Conclusion: In conclusion, we identified 5 coactivators whose expression was induced by androgens suggesting that they could potentiate AR signaling. Overexpression of AR seems to sensitize cells for low levels of androgens.

Background

Prostate cancer is the most and second most common male malignancy in the USA, and Europe, respectively [1,2]. The androgen-dependence of the growth of prostate cancer has been known for a long time [3]. The importance of the androgens was recently, once again, demonstrated in a large randomized study. In the study the use of finasteride, an inhibitor of 5α-reductase, which converts testosterone into more potent 5α-dihydrotestosterone, was used for prevention of prostate cancer [4]. Almost 25% reduction in the prevalence of prostate cancer was observed in the treatment compared to a placebo arm. Due to the androgen dependence, the golden standard treatment for advanced prostate cancer has been

androgen withdrawal (i.e. castration) for the last half century [5]. The treatment is palliative and although most patients respond to it, the disease will eventually progress [6]. Such tumors, emerging during the androgen withdrawal, are called hormone-refractory ones.

The molecular mechanisms by which prostate cancer cells become resistant to endocrine therapy remain incompletely known. However, the key role of androgens and androgen receptor (AR), not just in early development but also in the progression of prostate cancer has now been demonstrated [7]. The recent finding on genetic rearrangement leading to formation of *TMPRSS2:ERG* fusion gene provides a model for molecular mechanisms how androgens act in promoting the early development of prostate cancer. Due to the rearrangement, the putative oncogene *ERG* becomes androgen regulated [8,9]. It has now also become apparent that the emergence of hormone-refractory prostate cancer is due to reactivation of AR-mediated signaling. It has been experimentally shown that overexpression on AR is required and it is sufficient to transform the growth of prostate cancer cells from androgen-dependence to -independence [10]. Furthermore, it has been shown that hormone-refractory tumors overexpress AR, and one-third of them contains amplification of the *AR* gene [11]. Mutations in the coding region of *AR* have also been found in antiandrogen treated prostate cancers [12,13]. And at least some of the mutations alter the sensitivity of the receptor to other steroid hormones or anti-androgens, such as 17 β-estradiol (E2) or hydroxyflutamide (HF) [14,15].

The transactivation of AR involves several coregulatory proteins [16,17]. Large number of coregulatory proteins have already been identified [18-20]. It has been suggested that changes in the expression of the coregulators may be involved in the development and progression of prostate cancer [18,21,22]. Since androgens and AR are known to be important in the prostate cancer tumorigenesis, it is possible that they also regulate the expression of the coregulators.

In order to identify AR coregulators whose expression is regulated by androgens, we measured the expression of 20 putative coactivators: *SRC1* (alias *NCOA1* nuclear receptor coactivator 1), *TIF2* (alias *NCOA2* nuclear receptor coactivator 2), *PIAS1* (protein inhibitor of activated *STAT, 1*), *PIASx* (alias *PIAS2* protein inhibitor of activated STAT, 2), *ARIP4* (alias *RAD54L2 RAD54*-like 2 (*S. cerevisiae*)), *BRCA1* (breast cancer 1, early onset), *β-catenin* (alias *CTNNB1* catenin (cadherin-associated protein), beta 1, 88 kDa), *AIB3* (alias *NCOA6* nuclear receptor coactivator 6), *AIB1* (alias *NCOA3* nuclear receptor coactivator 3), *CBP* (alias *CREBBP CREB* binding protein (Rubinstein-Taybi syndrome)), *STAT1* (signal transducer and activator of transcription 1, 91 kDa), *p300* (alias *EP300* E1A binding protein p300), *ARA24* (alias *RAN*, member *RAS* oncogene family), *LSD1* (lysine-specific demethylase 1, alias *AOF2* amine oxidase (flavin containing) domain 2), *BAG1L* (*BCL2*-associated athanogene, isoform 1L), *gelsolin* (*GSN* (amyloidosis, Finnish type)), *JMJD2C* (alias JHDM3C/GASC1 jumonji domain containing 2C), *JMJD1A* (alias JHMD2A jumonji domain containing 1A), *MAK* (male germ cell-associated kinase), *MAGE11* (alias *MAGEA11* melanoma antigen family A, 11) and 5 putative corepressors: *NCoR1* (nuclear receptor co-repressor 1), *AES* (amino-terminal enhancer of split), *cyclin D1* (alias *CCND1*), *prohibitin* (*PHB*) and *PAK6* (*p21*(*CDKN1A*)-activated kinase 6) by using quantitative reverse transcription-PCR (Q-RT-PCR) in AR positive prostate cancer cell line LNCaP treated with different concentrations of DHT. To mimic the common overexpression of AR in hormone-refractory prostate cancer, we also stable transfected AR to LNCaP cell line. The LNCaP-ARhi cells express about 10-folds more AR mRNA and 3 to 4-folds more AR protein than that of the control (empty vector-transfected cell line: LNCaP-pcDNA3.1). The cell model was used to study whether the level of AR has an effect on the mRNA expression of the coregulators.

Methods

Cell culture protocols and transfections

pcDNA3.1(+) empty expression vector (Invitrogen Inc., Carlsbad, CA, USA) and pcDNA3.1(+) inserted with AR coding region were stable transfected into LNCaP (ATCC, Manassas, VA, USA) with Lipofectamine-Plus transfection reagent (Invitrogen Inc.). Transfected clones were selected with 400 μg/ml geneticin (G418) for 2 weeks. The mRNA level of AR was examined from purified clones using Northern blotting and Q-RT-PCR with Light-Cycler (Roche Inc., Mannheim, Germany). The protein level of AR was analyzed using Western blotting with anti-AR antibody (441, NeoMarkers-Lab Vision Corporation, Fremont, CA, USA). LNCaP-pcDNA3.1 (transfected with empty pcDNA3.1-vector as a control) and LNCaP-ARhi (transfected with AR cloned into pcDNA3.1+ vector) cell lines were cultured according to the ATCC protocol with the addition of Geneticin 200 μg/ml (Invitrogen Inc.). LNCaP-ARhi has about 10 times higher mRNA level of AR and 3 to 4 times higher protein level of AR than that of the LNCaP-pcDNA3.1 (Figure 1).

LNCaP-pcDNA3.1 and LNCaP-ARhi cells in the end of the exponential growing phase were divided in 1:4 ratio plates in RPMI1640 phenol free medium with 5% charcoal/dextran-treated (CCS) FBS (Hyclone Inc., South Logan, UT, USA), 1% glutamine (Invitrogen Inc.) and 1% penicillin-streptomycin (BioWhittaker Inc., Walkersville, MD, USA) for 4 days. The medium was then changed to phenol free RPMI1640 including 10% CCS-FBS (Hyclone

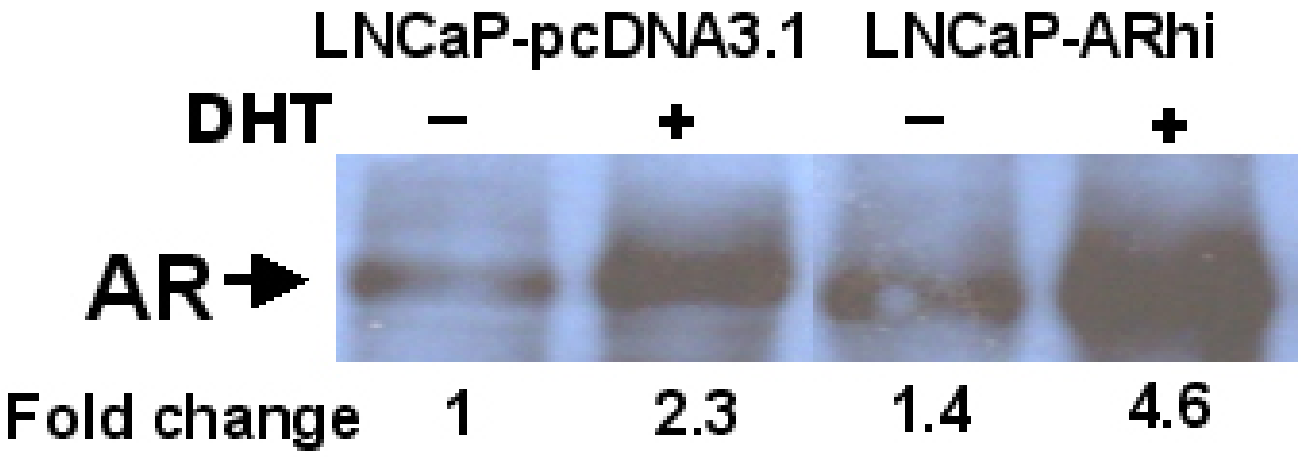

Figure 1
Western blot analysis of AR in empty vector transfected (LNCaP-pcDNA3.1) and AR-cDNA transfected (LNCaP-ARhi) LNCaP cells. Cells were grown either in the absence or presence of DHT. Nuclear fraction of LNCaP-ARhi shows clearly higher AR expression than the control LNCaP-pcDNA3.1. The quantification of the bands by ImageJ is given below the bands.

Inc.), 1% Glutamine (Invitrogen Inc.), 1% Pest (Invitrogen Inc.) and 0, 0.1, 1.0, 10 or 100 nM DHT. The cells were harvested into TRIZOL reagent (Life Technologies Inc., Gaithersburg, MD, USA) after 4 h and 24 h, followed by total RNA isolation according to the manufactures protocol.

RT-PCR

The cDNA was synthesized with AMV Reverse Transcriptase and oligo(dT)12–18 primer according to the manufacturer's protocol (Finnzymes, Espoo Finland). The standards were prepared mixing total RNA from untreated LNCaP cells and universal RNA (Clontech Laboratories, Inc., Mountain View, CA, USA) in a ratio of 1:5. After first strand cDNA synthesis, serial dilutions corresponding to 1000, 200, 40, 8, 1.6, 0.32, 0.064 μg of RNA pool were prepared and stored in aliquots. The PCR reactions were performed in LightCycler apparatus (Roche Inc.) using an LC Fast Start DNA SYBR Green I Kit (Roche Diagnostics, Mannheim, Germany). The final volume of each reaction was 20 μl containing 2 μl of cDNA sample (or standard), 4 mM $MgCl_2$ (except for *PAK6* (5 mM) and *JMJD1A* (3 mM)), 0.5 μM each primer, and 1× ready-to-use SYBR Green I reaction mix including Taq DNA polymerase, reaction buffer and a deoxynucleotide triphosphate mix. The cycling conditions were designed according to the manufacturer's guidelines which are given in additional file 1: primers' table. The primers were designed for amplifying regions of the mRNAs derived from different exons to avoid amplification of genomic DNA. The melting curve analysis with the LightCycler together with 1.5% agarose gel electrophoresis of the products were used to ensure that right size product without significant background was amplified in the reaction. The expression levels of the coregulators were normalized to the expression levels of TATA-box binding protein (*TBP*) as previously described [23].

Western analysis

Cytoplasmatic and nuclear proteins were isolated from subconfluent cells of LNCaP-ARhi. Proteins were separated in 10% SDS-PAGE gel followed by transfer to a membrane (Immobilon-P, Millipore Corp., Billerica, MA) using BIORAD transblot® semi-dry transfer cell (Bio-Rad Laboratories, Inc). After blocking in PBS 0.1% Tween 5% BSA (or 5% non-fat dry milk), the membranes were incubated with primary antibody (AR: dilution 1: 200 Androgen Receptor Ab-1 (AR441) Neomarkers, Fremont, CA; β-catenin: dilution 1:2000 BD Transduction Laboratories, Inc.; MAK: dilution 1:500 MAK antibody (C-term), ABGENT San Diego, CA; CBP: dilution 1:1000, R&D Systems, Inc.) over night at +4°C. After washes and incubation with secondary antibody (DAKO A/S, Denmark) the bound antibody was visualized on autogradiography film using Western Blotting Luminol Reagent (Santa Cruz, Inc.) according to the manufacturer's protocol. Pan-actin antibody (dilution 1:1000 pan AB-5, clone ACTN05, Neomarkers, Fremont, CA;) was used as a reference.

Statistical analysis

Grubb's test was used to detect the outlier values in the repetitive runs for each gene. One-way ANOVA (Parametric) test with Bonferroni post-test was used to evaluate the statistical significance of the expression level's changes.

Results

To identify androgen regulated coregulators, empty vector transfected LNCaP (LNCaP-pcDNA3.1) was grown in different concentrations of DHT. The total RNA was then collected at two time points (4 h and 24 h). To confirm the success of the DHT stimulation, the expression of prostate-specific antigen (*PSA*), known androgen regulated gene was first measured (Figure 2). *PSA* was strongly induced by DHT ($p < 0.0001$ at 4 h and 24 h).

Next, we screened expression of *SRC1*, *TIF2*, *PIAS1*, *PIASx*, *ARIP4*, *BRCA1*, *β-catenin*, *AIB3*, *AIB1*, *CBP*, *STAT1*, *NCoR1*, *AES*, *cyclin D1*, *p300*, *ARA24*, *LSD1* and *BAG1L* coregulators in triplicates (three independent Q-RT-PCRs' runs). Of the coregulators, *PIASx* ($p = 0.0601$ at 4 h and $p = 0.6488$ at 24 h), *BRCA1* ($p = 0.4172$ at 4 h and $p = 0.0377$ at 24 h), *β-catenin* ($p = 0.0272$ at 4 h and $p = 0.4158$ at 24 h), *AIB3* ($p = 0.0690$ at 4 h and $p = 0.1455$ at 24 h), *AIB1* ($p = 0.0401$ at 4 h and $p = 0.2541$ at 24 h), *CBP* ($p = 0.0184$ at 4 h and $p = 0.0588$ at 24 h), *cyclin D1* ($p = 0.2039$ at 4 h and $p = 0.4432$ at 24 h), *ARA24* ($p = 0.0220$ at 4 h and $p = 0.2502$ at 24 h) and *BAG1L* ($p = 0.0359$ at 4 h and $p = 0.3981$ at 24 h), showed dose dependent response to androgens, whereas *SRC1*, *TIF2*, *PIAS1*, *ARIP4*, *STAT1*, *NCoR1*, *AES*, *p300* and *LSD1* did not.

PIASx, *BRCA1*, *β-catenin*, *AIB3*, *AIB1*, *CBP*, *cyclin D1*, *ARA24* and *BAG1L* were selected for additional Q-RT-PCR runs in triplicates. In addition, expression of *gelsolin*, *prohibitin*, *JMJD2C*, *JMJD1A*, *MAK*, *PAK6* and *MAGE11* was measured in triplicates. Figures 3 and 4 show the expression pattern of these 16 coregulators upon DHT stimulation. The genes that showed significant dose dependent expression were *AIB1*, *CBP*, *MAK*, *BRCA1*, *β-catenin*, *cyclin D1*, *gelsolin*, *prohibitin*, *JMJD1A*, and *JMJD2C*. Table 1 indicates the p-values of the sixteen genes. *Gelsolin* showed reduced and increased expression at 4 and 24 hour time points, respectively. Others exhibited an androgen-dependent increase in the expression of the mRNAs. However, only *AIB1*, *CBP*, *MAK*, *BRCA1*, and *β-catenin* showed more than 2-folds induction after stimulation with DHT.

In order to evaluate the effect of the level of AR expression on the expression of the coregulators, we utilized LNCaP-based overexpression model (LNCaP-ARhi). The PSA response in the LNCaP-ARhi cell line at 24 hours was significantly ($p < 0.0001$) stronger than in the empty vector transfected cell line (LNCaP-pcDNA3.1) (Figure 2). PSA

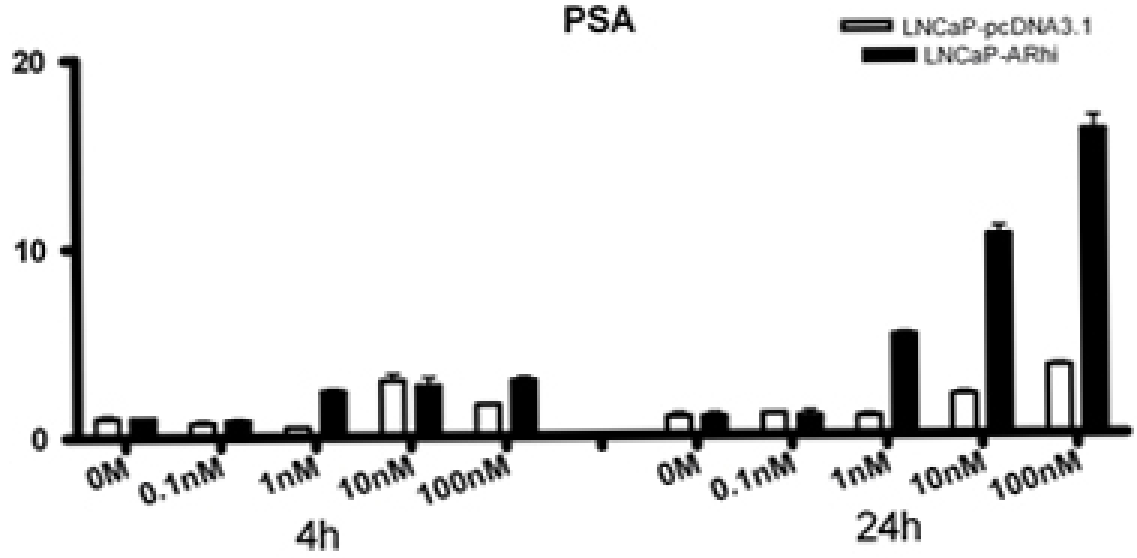

Figure 2
Expression of PSA in LNCaP-pcDNA3.1 and LNCaP-ARhi according to Q-RT-PCR. The cells were cultured in presence of DHT at different concentrations. After 4 and 24 hours, the cells were collected and expression of *PSA* and *TBP* mRNA was measured in triplicates by Q-RT-PCR. The bars and whiskers represent mean + S.E.M. of *PSA*/*TBP* values normalized against the 0 M of each time point.

Table 1: p-values of the One-way ANOVA (Parametric) test

Gene	LNCaP-pcDNA3.1		LNCaP-ARhi	
	4 h	24 h	4 h	24 h
AIB1	0.0019	< 0.0001	0.0023	< 0.0001
CBP	0.0478	0.0103	0.0035	0.0205
MAK	0.1385	0.0014	0.0090	0.1625
BRCA1	0.0007	< 0.0001	0.0022	< 0.0001
B-catenin	0.0336	< 0.0001	< 0.0001	< 0.0001
Cyclin D1	0.0094	0.0094	0.6382	0.4697
Gelsolin	0.0016	< 0.0001	0.0029	0.4211
Prohibitin	0.0291	0.0084	0.0114	0.0053
JMJD1A	0.0276	0.0009	< 0.0001	0.4592
JMJD2C	0.0588	0.0064	0.0259	0.6450
BAG-1L	0.0029	0.1467	0.0244	0.0150
PIASx	0.9371	0.8551	0.8224	0.7616
PAK6	0.1075	0.4876	0.3359	0.6410
MAGE11	0.7256	0.5952	0.6438	0.6014
AIB3	< 0.0001	0.0135	< 0.0001	0.3818
ARA24	0.0016	0.0276	0.0007	0.0013

p-values of the 16 genes were measured in triplicate in the second round analysis.

expression was stimulated already in 1 nM concentration in the LNCaP-ARhi, whereas the stimulation was evident in 10 nM in the LNCaP-pcDNA3.1.

None of the coregulators showed significant alterations in the expression in 0 M DHT between the LNCaP-pcDNA3.1 and LNCaP-ARhi. However, induction of expression in lower concentrations of DHT in LNCaP-ARhi compared to LNCaP-pcDNA3.1 was found in *MAK*, *CBP*, *AIB1*, and *BRCA1*. In contrast, the androgen regulation of *β-catenin*, *cyclin D1* and *gelsolin*, which was apparent in LNCaP-pcDNA3.1 at 24 h was less strong in LNCaP-ARhi (Figures 3 and 4).

Western blot analyses of β-catenin, CBP and MAK were in concordance with the Q-RT-PCR results (see additional file 2: WB analysis).

Discussion

We systematically studied the androgen regulation of the expression of twenty-five AR coregulators. 5 (20%) coregultators showed statistically significant over 2-fold induction by DHT. These were *AIB1*, *CBP*, *MAK*, *BRCA1* and *β-catenin*. In addition, expression of five (20%) other coregulators (*cyclin D1*, *gelsolin*, *prohibitin*, *JMJD1A*, and *JMJD2C*) was significantly, but less than 2-fold, induced by androgens.

AIB1 (Amplified in Breast Cancer 1) encodes for a well characterized protein with histone acetyltransferase activity. It is thought to be a nuclear receptor coactivator that interacts with various nuclear hormone receptors enhancing their transcriptional activity in a hormone dependent fashion. It is a member of the p160/steroid receptor coactivator (SRC) family and recruits p300/CBP-associated factor, with histone acetylation activity, and CBP (CREB binding protein) as a part of the transcription multisubunit coactivation complex [19,24]. *AIB1* has been found to be amplified in about 10% of breast cancers [25]. Previous studies have indicated that estrogens might have a suppressive role in respect to the expression of AIB1 [26,27]. Here we found that at 4 hours time point *AIB1* is nearly 2-fold induced by any concentration of DHT while at 24 hours time point a 4-fold induction was observed. The highest induction was at 1 nM DHT while at 10 nM the expression was declined. We used web-based TESS (Transcription Element Search Software) program and TRANSFAC database [28] to search for canonical binding sites for AR in the 2 kb upstream the transcription start site (TSS) of *AIB1*. The program identified several glucocorticoid receptor (GR) responsive elements (GREs) and two putative androgen responsive elements (AREs) at -527 and at -1291 bp from the TSS.

Similarly to *AIB1*, *CBP* was nearly 2-folds induced at 4 hours time point by any concentration of DHT while at 24 hours time point a 4-folds induction can be observed, although such induction can be seen also in 10 nM DHT concentrated media. In contrast with our results, Comuzzi et al. have suggested that *CBP* expression is down-regulated after treating LNCaP cells with synthetic androgen methyltrienolone (R1881) for 48 hours at concentrations of 0.01 nM and 1 nM [29].

MAK (Male Germ Cell-Associated Kinase) is a recently identified AR coactivator [30] that initially has been found to be strongly androgen-regulated in mice kidney [31]. In addition, it has previously been demonstrated that *MAK* is transcriptionally induced by DHT in LNCaP cells showing 9-fold induction by 10 nM DHT at 24 hours [32]. This is confirmed by our finding of 6.5-fold induction at 24 hours time point by 10 nM DHT. MAK has been found to be associated with AR and being corecruited in the transcriptional complex enhancing AR activity. Furthermore the modulation of its expression strongly alters the vitality of the cells affecting AR pathway mediated signaling [30]. Jia et al. have shown that *MAK* promoter is a direct target of AR and identified putative ARE sites within the promoter of *MAK* (about -3500 bp from the TSS), and that AR is recruited to the *MAK* promoter after DHT stimulation according to chromatin immunoprecipitation (ChIP) analysis [33]. Altogether the data indicates that the expression of *MAK* is regulated by androgens and since it functions as an AR co-activator, it forms a putative feedback loop augmenting the effects of androgens.

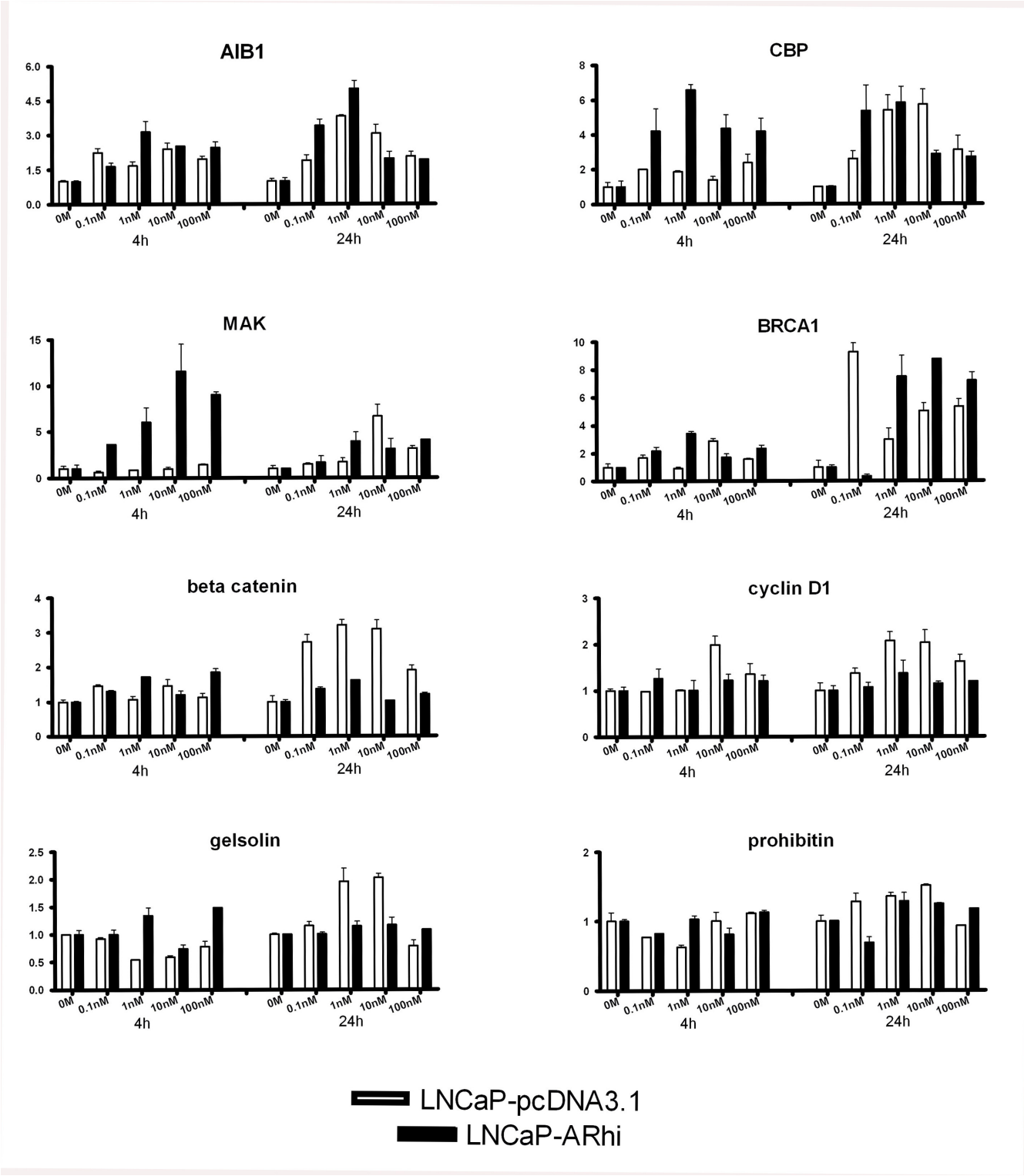

Figure 3
The expression of *AIB1*, *CBP*, *MAK*, *BRCA1*, *β-catenin*, *cyclin D1*, *gelsolin* and *prohibitin* in LNCaP-pcDNA3.1 and LNCaP-ARhi at 4 and 24 hours according to Q-RT-PCR. The measurements were done in triplicates. The bars and whiskers represent mean + S.E.M. of each gene against the TBP, normalized against the 0 M of each time point. p-values are given in Table 1.

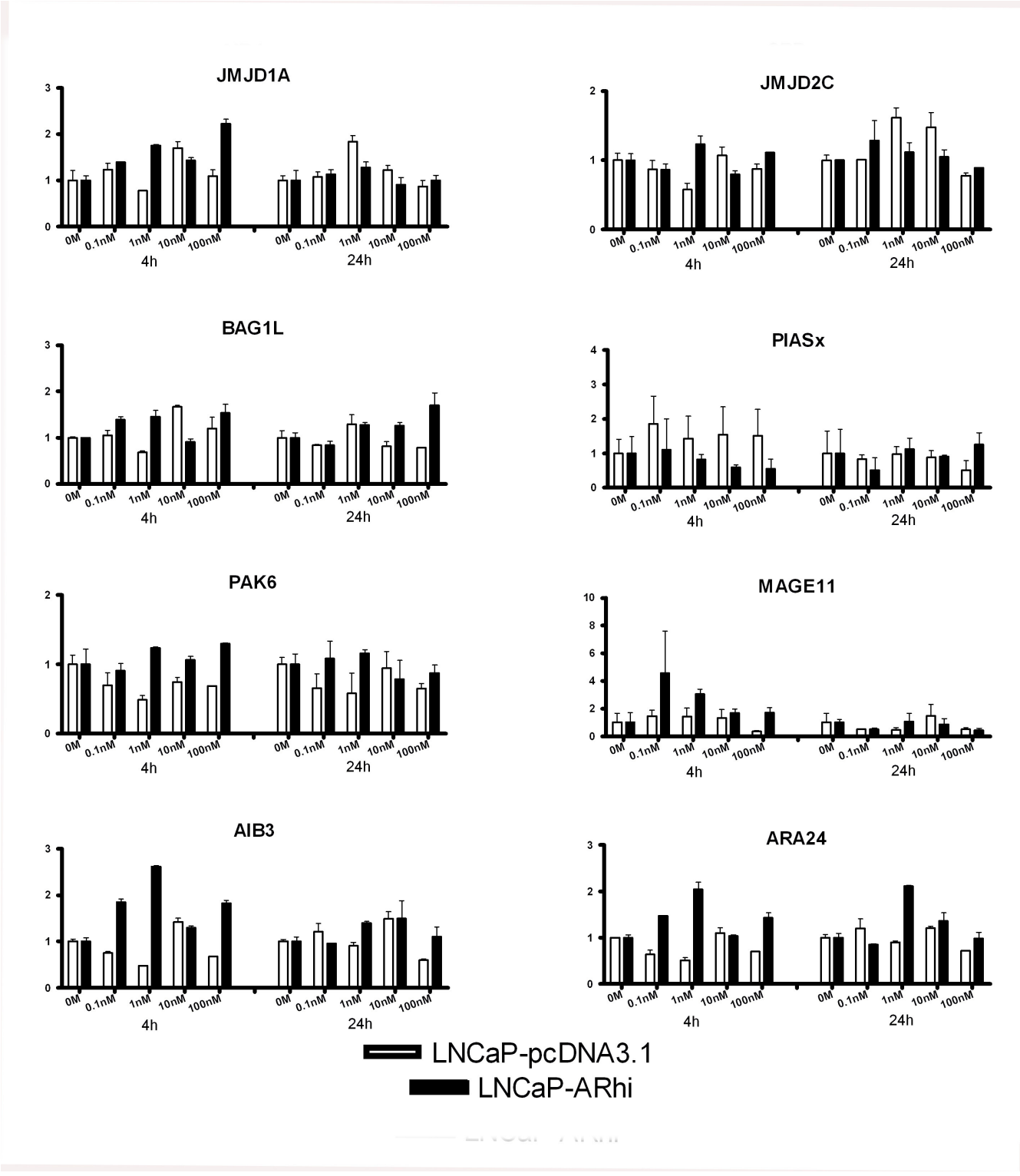

Figure 4
The expression of *JMJD1A*, *JMJD2C*, *BAG1L*, *PIASx*, *PAK6*, *MAGE11*, *AIB3*, and *ARA24* in LNCaP-pcDNA3.1 and LNCaP-ARhi at 4 and 24 hours according to Q-RT-PCR. The measurements were done in triplicates. The bars and whiskers represent mean + S.E.M. of each gene against the TBP, normalized against the 0 M of each time point. p-values are given in Table 1.

BRCA1 (breast cancer 1) is a well characterized gene in breast cancer since mutations in this gene are responsible of at least 40% of inherited breast cancers and more than 80% of inherited ovarian cancers [34,35]. BRCA1 is also an AR coactivator that has been demonstrated to enhance AR transactivation in prostate and breast cancer cell lines with a synergistic effect with other p160 coactivators [36]. Our data shows an androgen-mediated induction of the expression of *BRCA1*. It has previously been demonstrated that *BRCA1* is an estrogen induced gene in breast and ovarian cancer cells [37,38].

β-catenin is an adherens junction protein with altered expression in various tumor types. Its fundamental role as a multifunctional oncoprotein has been established [39]. The gene encodes for a protein that interacts with AR in close proximity with p160 coactivator family and it is thought to be important for the formation of the AR transcription complex [40,41]. There is some evidence that AR could also have an important role as a scaffolding protein in β-catenin translocation from the cytoplasm into the nucleus [42-44]. β-catenin has been shown to augment AR transcriptional function in a ligand-dependent fashion [43,45]. Here we show that *β-catenin* is induced up to 2.5-folds at 24 hours time point, already at a concentration of 0.1 nM of DHT. The induction reaches then the 3-folds and declines at 2-folds at 100 nM showing a significant androgen regulation.

In addition to the above mentioned coregulators, *cyclin D1*, *gelsolin*, *prohibitin*, *JMJD1A* and *JMJD2C* showed a significant dose dependent effect in their expression level mainly at 24 hours time point. However, the induction was very mild, less than 2-fold, suggesting either only weak androgen effect or secondary mechanisms for the induction.

It has been demonstrated that AR is commonly overexpressed in hormone-refractory prostate cancer [11,46,47]. In addition, it has been shown that the overexpression is capable to transform androgen dependent growth of prostate cancer cell to independence [9]. To study the effect of AR overexpression, we stable transfected AR to LNCaP cell line leading to 3 to 4-fold increased expression of AR. The growth of LNCaP-ARhi was stimulated on lower concentrations of androgens than the growth of LNCaP-pcDNA3.1 (unpublished data). None of the coregulators were up- or downregulated by the level of AR alone since there was no significant difference in the expression of the coregulators at 0 M of DHT. Instead, the overexpression of AR seems to sensitize cells to androgens. This was evident *e.g.* for *PSA*, which was significantly upregulated more and in lower concentrations of DHT in LNCaP-ARhi than in LNCaP-pcDNA3.1 cells. Similarly, the expression of *MAK*, *CBP*, and *BRCA1* was increased more in LNCaP-ARhi than in LNCaP-pcDNA3.1, and, at least, a 10-fold sensitization was observed. For *MAK* and *CBP* this was apparent in 4 h time point, whereas for *BRCA1* at 24 h time point. Interestingly, the increased expression of AR did not affect all androgen-regulated coregulators. *β-catenin*, *cyclin D1* and *gelsolin*, which showed androgen-regulation in LNCaP-pcDNA3.1 at 24 hours were not regulated by androgens in LNCaP-ARhi. The data suggest that the AR overexpression has distinct effects on different target genes. It should be recognized that during the androgen withdrawal, there are still adrenal androgens left. For example, the DHT concentration is only partially depleted [48]. Thus, the genes that are regulated in reduced DHT concentrations due to AR overexpression might be important in the progression of prostate cancer during the hormonal therapy.

The effects of androgens on gene expression were measured at two time points, 4 h and 24 h. The 4 h time points represent a time interval where direct effects of androgens administration should be seen, although the effect can be mild. On the other hand, also secondary mechanisms can already be activated at 24 hours time point. All coregulators (*AIB1*, *CBP*, *MAK*, *BRCA1*, *β-catenin*) that were induced more than 2-fold by DHT, showed the induction already at 4 time point in one or both cells lines (LNCaP-pcDNA3.1 and LNCaP-ARhi) suggesting direct regulation. Whereas, coregulators with lower-level of induction showed the effect variably in 4 and 24 hour points, which may also suggest secondary mechanisms of androgen regulation.

Conclusion

In conclusion, by systematic measurement of 25 AR coregulators, we identified 5 coregulators whose expression seems to be significantly androgen-regulated indicating that they could be a part of positive feedback mechanisms potentiating the AR signaling. Overexpression of AR seems to sensitize cells in terms of androgen regulation but the effect is gene-specific since not all androgen-regulated genes were up-regulated in AR overexpressing model.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AU: acquired the data through PCR, prepared the manuscript, analyzed and interpreted the data. KW: made the cell-culture work. HM: supported the PCR work. HS: established the AR overexpression cell lines. TV: designed the study, helped preparing the manuscript and analyzing and interpreting the data. All authors read and approved the final manuscript.

Additional material

Additional file 1

Additional Table 1 – primers' sequences and cycling conditions. primers' sequences and cycling conditions.
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[http://www.biomedcentral.com/content/supplementary/1471-2407-8-219-S1.pdf]

Additional file 2

Additional Figure 1. Western blot analysis. Western analysis of β-catenin, CBP, and MAK in LNCaP-ARhi cells treated with different concentrations of DHT.
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