IDENTIFICATION AND CHARACTERIZATION OF PROTEINS THAT INTERACT WITH THE ANDROGEN RECEPTOR TO MODULATE ITS ACTIVITY

by

LATIF A. WAFA

Honours B.Sc., University of British Columbia, 2000

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Pathology and Laboratory Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

August 2007

Abstract

Prostate cancer is among the most frequently diagnosed malignancies and one of the leading causes of cancer-related death in Canadian men. Prostate tumours are initially dependent on androgens for growth and survival. At this early stage of disease, androgen ablation therapy is an effective treatment but ultimately results in progression to the lethal androgen independence (AI) phase. Androgens exert their effects through the androgen receptor (AR), which is a ligand-activated transcription factor that plays a central role in prostate cancer development. Therefore, proteins that interact with AR and modify its activity may also be associated with the progression of disease to AI.

The overall goal of the research described in this thesis is to gain a more comprehensive understanding of the mechanism by which AR regulates gene transcription during prostate cancer progression. Initial studies focused on the identification of proteins that interact with the unique N-terminal domain (NTD) of AR, which may confer receptor-specific responses. Due to the challenges of utilizing the AR-NTD for analysis in conventional yeast two-hybrid assays, the repressed transactivator (RTA) system was employed as a novel approach to identify AR-binding proteins. Several previously unknown AR-protein interactions were found, which were confirmed through *in vitro* biochemical assays and in prostate cancer cells.

The role of the most frequently detected AR-binding protein from the RTA system, L-dopa decarboxylase (DDC), in modulating AR activity was assessed using transactivation assays. DDC was found to be a coactivator of AR that enhanced receptor transcriptional activity *in vitro* and *in vivo*. Further mechanistic studies revealed that DDC facilitates AR ligand binding and requires its enzymatic activity for coactivation

function. The relevance of DDC to disease was then examined using a clinical prostate cancer progression model. DDC was found to be co-expressed with AR in neuroendocrine-phenotype adenocarcinoma cells and its expression increased in hormone-treated prostate tumours, as well as in AI disease, suggesting that this coactivator may sustain aberrant AR activity during prostate cancer progression. Overall, the research presented in this study furthers our understanding of the role of coregulators in mediating AR activity and their potential importance in progression of disease.

Table of Contents **Page**

List of Tables **Page**

List of Figures **Page**

List of Abbreviations

17β-HSD	17 β-hydroxysteroid dehydrogenase
3β-HSD	3 β-hydroxysteroid dehydrogenase
5-FOA	5-fluoroorotic acid
5-HT	5-hydroxytryptamine (serotonin)
5-HTP	L-5-hydroxytryptophan
ACTH	adrenocorticotropic hormone
AD	activation domain
AF	activation function
AI	androgen independence
AIB1	amplified in breast cancer 1
ANPK	AR-interacting nuclear protein kinase
APC	anaphase-promoting complex
APC7	anaphase-promoting complex subunit 7
AR	androgen receptor
ARA	AR-associated protein
ARE	androgen response element
ARR	androgen response region
ART-27	AR-trapped clone-27
ATP	adenosine triphosphate
BAG-1L	bcl-2 associated athagene-1
BB	bombesin
Bcl-2	B cell lymphoma-2
B_{max}	maximum androgen binding capacity
BPH	benign prostatic hyperplasia
BRCA1	breast cancer susceptibility gene 1
BSA	bovine serum albumin
CAK	cdk-activating kinase
cAMP	cyclic adenosine monophosphate
CBP	CREB binding protein
CgA	chromogranin A
CHIP	C-terminus of hsp70-interacting protein
CK	cytokeratins
CMV	cytomegalovirus
CREB	cAMP response element binding protein
CRH	corticotropin-releasing hormone
DA	dopamine
DAPI	diamidino-2-phenylindole
DAX-1	dosage-sensitive sex reversal adrenal hypoplasia congenita critical region on the X chromosome gene 1
DBD	DNA binding domain
DDC	L-dopa decarboxylase
Dex	dexamethasone
DHEA	dehydroepiandrosterone
DHT	dihydrotestosterone

Dox	doxycycline hyclate
DTT	dithiothreitol
E_2	estradiol
EDTA	ethylenediamine tetraacetic acid
eEF1A	eukaryotic translational elongation factor 1A
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EGFR	EGF receptor
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
ERE	estrogen response element
Erk-2	extracellular signal-regulated kinase 2
FBS	fetal bovine serum
FITC	fluorescein
FSH	follicle-stimulating hormone
GAK	cyclin G-associated kinase
GFP	green fluorescent protein
GnRH	gonadotropin-releasing hormone
GPCR	G-protein-coupled receptor
GR	glucocorticoid receptor
GSK3β	glycogen synthase kinase 3β
GST	glutathione S-transferase
GTF	general transcription factor
H&E	haematoxylin and eosin
HAP	hydroxylapatite
HAT	histone acetyltransferase
HBO1	histone acetyltransferase binding to ORC-1 subunit protein
HDAC	histone deacetylase
HNF-3α	hepatocyte nuclear factor-3α
Hop	hsp organizer protein
Hsp	heat shock protein
IAS	intermittent androgen suppression
IGF-1	insulin-like growth factor-1
IGF-1R	IGF-1 receptor
IHC	immunohistochemical
IL	interleukin
IPP	image pro plus
KGF	keratinocyte growth factor
LBD	ligand binding domain
LC-MS/MS	liquid chromatograpy-tandem mass spectrometry
L-Dopa	L-3,4-dihydroxyphenylalanine
LH	lutenizing hormone
LHRH	lutenizing hormone-releasing hormone
m/z	mass-to-charge ratio
MAK	male germ cell-associated kinase

MAPK	mitogen-activated protein kinase
MOI	multiplicity of infection
MR	mineralocorticoid receptor
NADP	nicotinamide-adenine dinucleotide phosphate
NcoR	nuclear receptor corepressor
NE	neuroendocrine
NGS	normal goat serum
NHT	neo-adjuvant hormone therapy
Ni-NTA	nickel-nitrilotriacetic acid
NLS	nuclear localization signal
NSB	non-specific binding
NSD-1015	3-hydroxybenzylhydrazine
NTD	N-terminal domain
ORC	origin recognition complex
p/CAF	p300/CBP-associated factor
$P\text{-}450_{SCC}$	P-450 side-chain-cleavage enzyme
PAF400	p/CAF-associated factor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PFKM	phosphofructokinase; muscle isoform
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLP	pyridoxal 5'-phosphate
PMSF	phenylmethylsulfonyl fluoride
Poly-G	polyglycine
Poly-P	polyproline
Poly-Q	polyglutamine
PR	progesterone receptor
PSA	prostate-specific antigen
PTEN	phosphatase and tensin homolog deleted on chromosome 10
PTI-1	prostate tumour-inducing gene 1
PTM	post-translational modificiation
Q-TOF	quadrupole time-of-flight
RanGTPase	ras-related nuclear G protein
Rb	retinoblastoma
RD	repression domain
RLU	relative luciferase units
RTA	repressed transactivator
RXR	retinoic acid X receptor
SDS	sodium dodecyl sulfate
SHBG	sex hormone-binding globulin
SHP	short heterodimer partner
siRNA	small interference RNA
SMRT	silencing mediator for retinoid and thyroid hormone receptors
SR	steroid receptor

SRC	steroid receptor coactivator
SRE	steroid response element
STAT3	signal transducer and activator of transcription 3
SUMO-1	small ubiquitin-like modifier-1
T	testosterone
$TAF_{II}250$	TBP-associated factor 250
Tau	transcriptional activation unit
TBP	TATA binding protein
TIF2	transcriptional intermediary factor 2
Tip60	TAT-interactive protein
TK	thymidine kinase
TMA	tissue microarray
TPR	tetratrico peptide repeat
TR	Tet repressor
TRRAP	tranformation/transcription domain-associated protein
TSG101	tumour susceptibility gene product
TURP	trans-urethral prostate resections
UAS	upstream activation sequence
USP10	ubiquitin-specific protease
ZPR1	zinc finger protein 1

Acknowledgements

Many people have contributed to my progress in carrying out the research presented in this study. First of all, I would like to thank my supervisor, Dr. Paul Rennie, who has guided me and always maintained an optimistic view of my work throughout the duration of this thesis...a nicer protein biochemist, I do not know. Several other people have also been instrumental in helping me perform this work. Helen Cheng was the first person to show me around the lab and train me in using the RTA yeast-two hybrid assay. Helen's nurturing nature and dedication to performing high quality work has made working with her a very pleasant experience. Thanks also to Drs. Rob Snoek, Jason Read and Mira Ray for their helpful discussions and willingness to collaborate with myself over the years. During the latter part of my PhD program, I have had the opportunity to pass on some knowledge and expertise onto some of the newer graduate students in our lab; I would like to thank Dr. Peyman Tavassoli and Kim Emsley-Leik for carrying on the AR-coregulator torch.

A special thanks to Dr. Michael Cox who has not only taught me a great deal about neuroendocrine differentiation but has also played a major role at many levels in my development as a basic science researcher. I would also like to thank Dr. Colleen Nelson for her constructive critical discussions regarding my work. Many thanks to other members of my graduate supervisory committee, including Dr. Ivan Sadowski and Dr. John Hill, for their guidance on this project.

I have also had the benefit of collaborating with individuals from Dr. Martin Gleave's lab, including Dr. Ladan Fazli, who was the first person that helped me perform some actual pathology studies. Of course, I cannot leave without thanking the veterans;

Robert Shukin, John Cavanagh and Steve Hendy for their useful insight over the years. Thanks also to the many other wonderful and friendly people at the Prostate Centre. Finally, I would like to thank my family and friends for their support and understanding during the course of completing this thesis.

Co-Authorship Statement

The publications presented in this thesis are based on the work that I have carried out for the completion my PhD program. In cases of collaboration with other researchers at the University of British Columbia and the Prostate Centre, names of the individuals are included in the publication citations, following the titles of each chapter. Further specific contributions of individuals that played an important role in the research are outlined in the section preceding the Introduction of chapters.

CHAPTER 1. LITERATURE REVIEW, HYPOTHESIS AND OBJECTIVES

A version of this chapter is being prepared for publication of a book chapter.

Wafa LA, Snoek R, Rennie PS. Androgen receptor coregulators and their role in prostate cancer progression. In: Tindall D, Mohler J, eds. Androgen Action in Prostate Cancer. New York, Pa: Springer Science Press (*in preparation*).

The content of the above publication predominantly incorporates **Sections 1.4** and **1.5.3** of the following literature review, which I have prepared for this thesis.

Prostate cancer is the most commonly diagnosed non-skin cancer in Canadian men and one of the leading causes of cancer-related death. In 2006, an estimated 20,700 newly diagnosed cases of prostate cancer were expected in Canadian men, resulting in approximately 4,200 deaths (1). While often curable in its early stages, the primary treatment for advanced prostate cancer is androgen ablation therapy. Due to the dependency of prostate growth and development on androgens, this treatment is initially effective in reducing tumour burden but ultimately results in progression of disease to androgen independence (AI). There is currently no effective treatment for this lethal phase of prostate cancer. Androgens exert their effects through the androgen receptor (AR), which is a ligand-activated transcription factor that regulates many of the genes involved in prostate growth and death. Therefore, it is likely that modifications in the

activity of AR and/or the proteins that interact with the receptor are closely related to progression of disease to AI.

The goal of this PhD dissertation is to gain a better understanding of the mechanism by which AR regulates gene transcription in prostate cancer, possibly providing new therapeutic targets for treatment of disease. The central hypothesis studied in this project is that AR-specific protein interactions, which modify receptor transcriptional activity, and the inappropriate expression of these AR-binding proteins, are involved in prostate cancer progression to AI. Accordingly, we pursued the initial identification of unique AR-interacting proteins using a novel yeast-two hybrid system. Several previously unknown AR-binding proteins were identified and subsequent experimentation confirmed that these interactions also occurred in prostate cancer cells. The project further focused on the detailed analysis of the most frequently detected AR-interacting protein from our initial assay, L-dopa decarboxylase (DDC); an enzyme whose key function is regulation of catecholeamine and serotonin neurotransmitter synthesis. DDC was shown to substantially increase AR transcriptional activity and facilitate ligand binding to the receptor. The enzymatic activity of DDC was also shown to be required for increasing AR transactivation. Importantly, DDC expression was found to be elevated in hormone-treated prostate tumours, suggesting that it may play an important role in activation of AR during prostate cancer progression.

Due to the importance of AR action in prostate cancer, it is imperative to understand the factors that are associated with the function of the receptor. These include the production and role of androgens, the structure and function of AR and the events that regulate AR activity. The chapter presented here addresses these topics.

1.1 Androgen Action in the Prostate

1.1.1 Biology and Function of the Prostate

The prostate gland is an unpaired accessory structure of the male reproductive system that surrounds the urethra in the pelvic cavity. Anatomically, it lies immediately inferior to the bladder, posterior to the pubic symphysis and anterior to the rectum. A healthy human prostate is slightly larger than a walnut and its structure consists of three zones; a peripheral zone that comprises about 70 % of the normal prostate gland, a central zone constituting approximately 25 % of the gland and a transition zone that accounts for the remaining 5 % of the prostate volume (2, 3). Male sex hormones or androgens are responsible for growth, development and differentiation of the prostate (2, 4). Prostatic growth from its pre-pubertal size of 1-2 grams to its adult size, weighing approximately 20 grams, occurs during puberty between the ages of 10 and 20 years (5). After puberty, the main function of the prostate is to store and secrete a slightly alkaline fluid that constitutes up to one third of the volume of the semen, which is composed of spermatozoa and seminal fluid. Secretions of the prostate is generally composed of simple sugars with a protein content of less than 1 %, which includes proteolytic enzymes, acid phosphatase and prostate-specific antigen (PSA) (6, 7). The prostate also contains smooth muscle cells that help expel semen during ejaculation.

The prostate is composed of multiple secretory acini that are lined by epithelial cells. These acini drain into epithelial ducts that end in the prostatic urethra. The functional unit of the prostate is the glandular acinus, which is composed of two major cellular compartments; the fibromuscular stroma and glandular epithelial compartments,

as shown in **Figure 1.1**. A well-developed basement membrane separates the epithelial layer from the surrounding stromal tissue, which is composed of fibroblasts, smooth muscle cells and endothelial cells (8). Hence, the prostatic epithelial compartment receives circulatory nutrients and androgens that cross the endothelial and stromal cells, as well as the extracellular matrix and basement membrane. Consequently, the stromal compartment plays an important role in modifying the epithelial cell microenvironment *via* a paracrine manner to maintain balance between proliferation, differentiation and apoptosis.

The prostate epithelial compartment is comprised of basal, secretory luminal and neuroendocrine (NE) cells (9). Basal cells of the prostate epithelium are believed to be the proliferative component and responsible for the self-renewing capacity of the gland (10, 11). These cells are androgen-independent and characterized by expression of high molecular weight cytokeratins (CK), such as CK5 and CK14 (12, 13). In contrast, luminal secretory cells require androgens for survival and express low molecular weight cytokeratins, including CK8 and CK18 (15). These cells express AR and are responsible for the production of PSA and prostatic acid phoshphatase that are secreted as part of the seminal fluid (16, 17). NE cells are sparsely scattered between the luminal and basal cell layers (18). In the normal prostate, NE cells are androgen-independent and are considered to be non-proliferating terminally differentiated cells that do not express AR (19, 20). NE cells are characterized by the expression of chromogranin A and other markers such as serotonin, bombesin and calcitonin (21-26). Based on their morphology NE cells can be described as two types; the "open" type, which possesses long luminal extensions that make contact with the prostatic lumen and the "closed" type, which

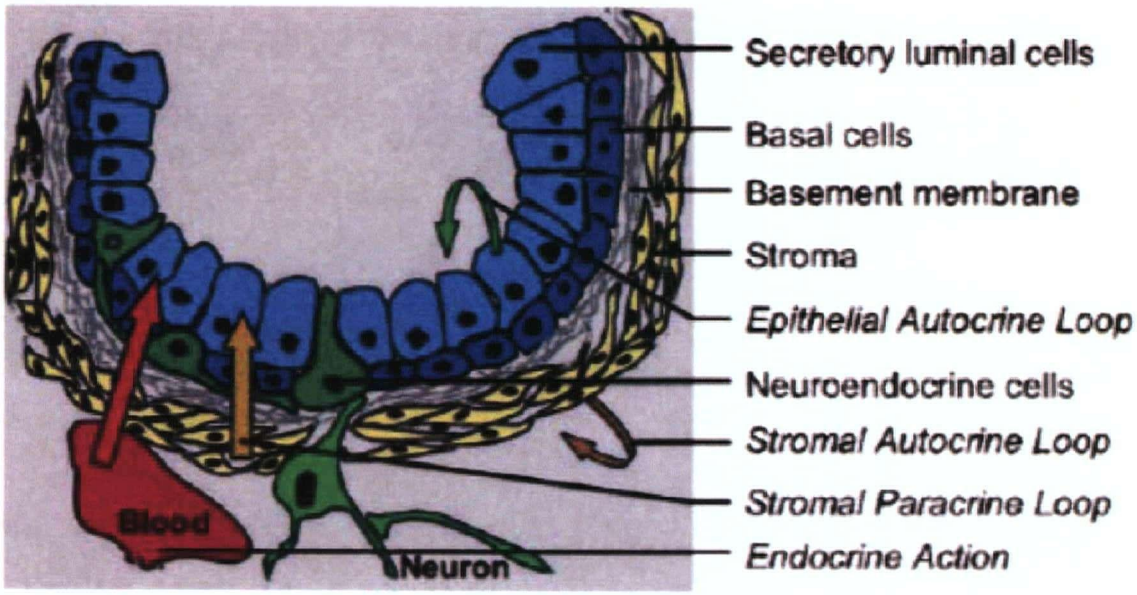

Figure 1.1 - The Major Cellular Compartments of the Prostate Gland. The prostate gland is comprised of the fibromuscular stroma and glandular epithelial compartments. Stromal tissue is made up of fibroblasts, smooth muscle cells and endothelial cells. The prostate epithelial compartment consists of basal, secretory luminal and neuroendocrine cells, which are separated from the surrounding stroma by a basement membrane. Androgens are delivered to the prostate gland *via* the circulatory endocrine system. Growth factors produced by the stroma regulate development and growth of the stroma itself (autocrine) and epithelial cells (paracrine), which can also modulate their own growth. Modified from Evangelou *et al.* (14).

lacks these neuronal extensions (27). The exact role of NE cells is not completely understood, but it is believed that they play a role in the growth and development of the prostate, as well as in carcinogenesis, *via* the paracrine effects of their neurosecretory products (28).

Over 90 % of prostate cancers are adenocarcinomas that arise from the glandular epithelium compartment of the prostate (8). Isaacs and Coffey have hypothesized that the basal layer of the prostatic epithelium also contains a population of stem-like cells that are responsible for the development of all epithelial cell types in the prostate (29). Upon mitosis, the stem-like cell gives rise to two cells that includes another stem cell and a daughter progenitor cell that differentiates into a secretory luminal cell (30, 31). Classically, it was thought that prostate cancer cells originate from the luminal cells of the prostate epithelium since tumour cells express luminal cell markers such as AR, PSA and specific cytokeratins (12, 15, 32). However, it has also been shown that most androgen-independent prostate cancers also express basal cell markers, such as the B cell lymphoma-2 (bcl-2) proto-oncogene, which contributes to the apoptotic-resistant phenotype of the tumour (33). Hence, it can be suggested that prostate cancer cells may either acquire basal cell characteristics or may be unable to lose some basal cell traits during tumour growth and development.

The contribution of NE cells of the prostate epithelium to prostate cancer and their origin is less well understood and controversial. Although it is apparent that NE cells are present at least focally in all cases of prostatic adenocarcinoma, reports on the percent of tumours containing these cells vary from 10 % to 99 % (34-38). It is unknown whether NE cells originate from the neural crest during embryogenesis or whether they

share a common origin with other epithelial cell types from stem cells within the basal layer of the prostate gland (39, 40). In addition, a third source of NE cells has been attributed to the process of transdifferentiation of epithelial-based adenocarcinomas into the NE phenotype (41, 42). This theory is supported by the observation that the increased NE cell population seen in prostatic malignancies, express both NE markers and AR, a luminal cell indicator (43, 44). Thus, in contrast to the normal prostate that contains AR-negative NE cells, prostate tumours can contain AR expressing NE cells that may be partially responsive to androgens for growth.

1.1.2 The Hypothalamic-Pituitary-Gonadal Hormone Axis

The organs involved in the regulation of androgen production include the hypothalamus, pituitary gland, testes and adrenal glands, as shown in **Figure 1.2** (45). Activation of the androgen-signalling pathway is initiated at the hypothalamus where the peptide hormones, lutenizing hormone-releasing hormone (LHRH), also known as gonadotropin-releasing hormone (GnRH), and corticotropin-releasing hormone (CRH) are produced. These releasing hormones reach the anterior pituitary through the hypothalamic-pituitary vascular network, where LHRH stimulates the release of lutenizing hormone (LH) and follicle-stimulating hormone (FSH), while CRH induces the release of adrenocorticotropic hormone (ACTH) into the blood. Through circulation, LH/FSH and ACTH stimulate the testes and adrenal glands, respectively. In the presence of LH/FSH, the Leydig cells of the testes produce 95 % of the circulating testosterone (T).

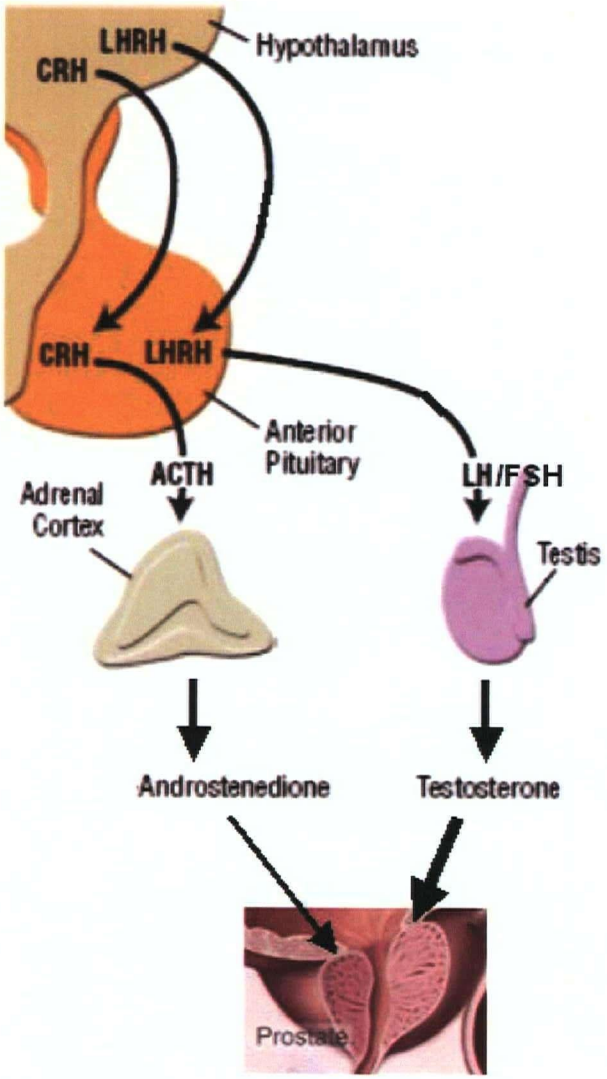

Figure 1.2 - Hypothalamic-Pituitary-Gonadal Hormone Axis. The endocrine pathways involved in androgen synthesis and release are illustrated. Although both gonadal and adrenal androgens can exert effects on the prostate, testosterone produced by the testes is the main source of androgen for growth and development of the gland. Adapted from Denmeade *et al.* (45).

Additionally, the adrenal gland under the influence of ACTH produces androstenedione and dehydroepiandrosterone, which can then be converted into more active androgens in the prostate (45). Importantly, feedback loops regulate the secretions of the hypothalamus and anterior pituitary. Serum T exerts a negative feedback to inhibit the release of LHRH and LH, while adrenal androgens are quite weak and do not appear to exert any negative feedback on the hypothalamic-pituitary axis. Overall, T produced by the testes is the predominant source of androgen necessary for the growth and maintenance of the prostate. The hypothalamic-gonadal endocrine pathway can therefore be exploited for the treatment of prostate cancer using androgen ablation therapy, which can include surgical removal of the testes and adrenal glands, suppression of LH/FSH release and other direct means of inhibiting androgen action in prostate cancer cells; discussed in detail in **Section 1.2.3**.

1.1.3 Androgen Biosynthesis and Metabolism

Androgens are responsible for development of male secondary sexual characteristics and the maturation and differentiation of sexual organs, including growth and maintenance of the prostate gland. Although in men the testes are the predominant source for androgens, several other tissues such as adipose, brain, muscle, skin, adrenal cortex and the prostate itself, can also produce low levels of androgens (46). As can be seen in **Figure 1.3**, cholesterol is the obligate precursor for testosterone synthesis, as well as other steroids including estrogen, progesterone, cortisol (glucocorticoid) and aldosterone (mineralocorticoid). Testosterone production occurs primarily in the Leydig cells of the testes, where synthesis begins in the mitochondria by

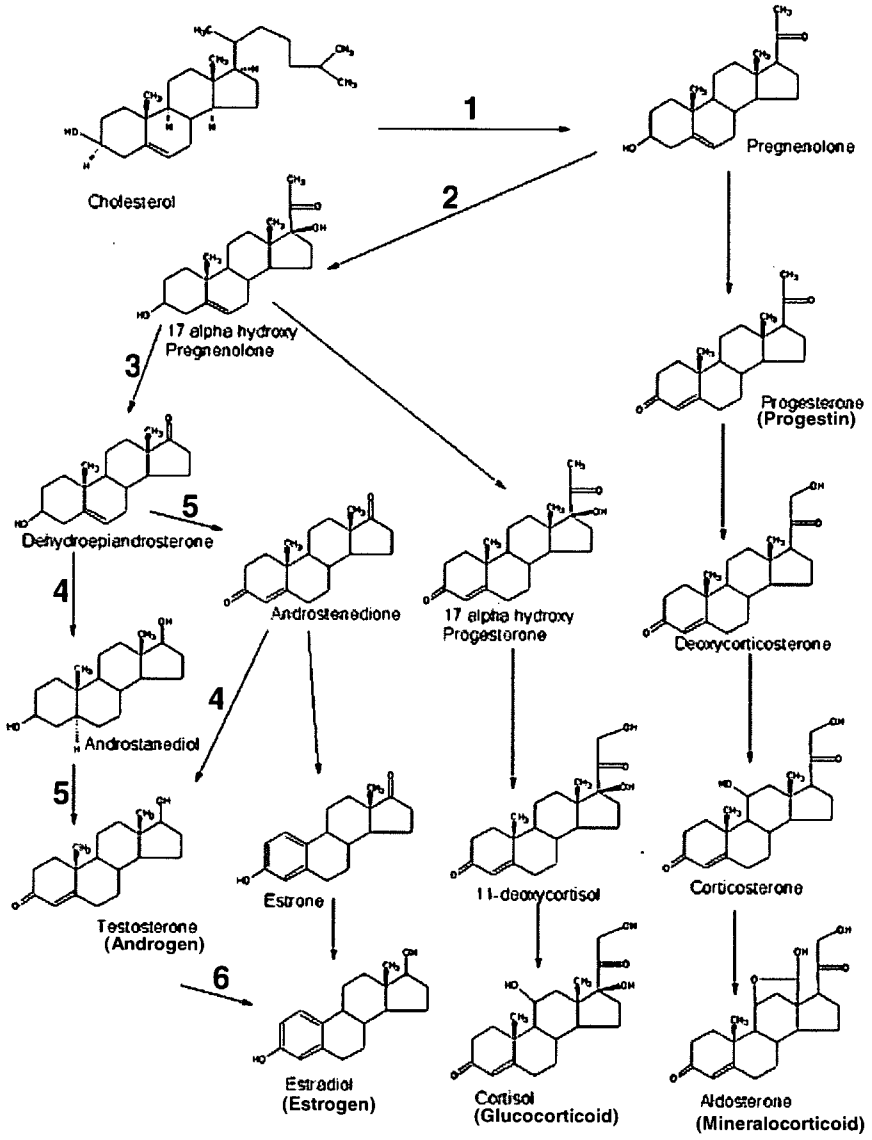

Figure 1.3 – Steroid Hormone Biosynthesis. Cholesterol is the common precursor for multiple steroid hormones, including testosterone, estradiol, cortisol, progesterone and aldosterone. The enzymes involved are: 1) cholesterol P-450 side-chain-cleavage enzyme ($P\text{-}450_{SCC}$); 2) 17 α-hydroxylase ($P\text{-}450_{c17a}$); 3) 17,20-desmolase ($P\text{-}450_{c17b}$); 4) 17 β-hydroxysteroid dehydrogenase (17β-HSD); 5) 3 β-hydroxysteroid dehydrogenase (3β-HSD); 6) aromatase. Reproduced from Boron *et al.* (46) and Voet *et al.* (47).

the P-450 side-chain-cleavage enzyme ($P\text{-}450_{SCC}$) that converts cholesterol into pregnenolone. This initial reaction is stimulated by LH and is the rate-limiting step in the biosynthesis of T, as well as other steroid hormones (46). Pregnenolone is then converted into dehydroepiandrosterone (DHEA) in two steps with additional P-450 enzymes located in the smooth endoplasmic reticulum of Leydig cells. The conversion of DHEA to T is carried out by 17 β-hydroxysteroid dehydrogenase (17β-HSD), producing androstenediol, which is then converted into T by 3 β-hydroxysteroid dehydrogenase (3β-HSD). These final two steps can also occur through production of androstenedione from DHEA, catalyzed by 3β-HSD, followed by conversion into T by 17β-HSD (48). In this manner, Leydig cells produce approximately 95 % of circulating testosterone in males. However, the adrenal gland can also serve as an alternative source of androgen production in both males and females. Normal human adrenal glands synthesize and secrete the pro-androgens DHEA, conjugated DHEA sulfate and androstenedione, all of which can be converted into T in other tissues (49). Overall, only about 2 % of the total testosterone in the blood is derived from an adrenal source.

Once in circulation, T binds specific proteins such as albumin and sex hormone-binding globulin (SHBG). Approximately 54 % of T binds to albumin with low affinity, while 44 % is bound to SHBG with high affinity (50). A small fraction (1-2 %) of total circulating T also exists in a free state, which equates to a serum concentration of 1 nM (51). It is the free form of T that diffuses into target cells and subsequently exerts biological actions or undergoes metabolism (52). The quantity of testosterone entering a cell is determined by the plasma concentration and by the intracellular milieu of enzymes and androgen binding proteins. Within the prostate, T is irreversibly converted into

dihydrotestosterone (DHT) by the membrane-bound 5α-reductase type 2 enzyme, as shown in **Figure 1.4**. There are two distinct 5α-reductase genes in humans, each encoding a biochemically distinct isozyme. The type 1 isozyme is present at low levels in the prostate but is mainly expressed in skin and liver, whereas the type 2 isozyme is the predominant 5α-reductase in androgen target tissues, including the prostate (53, 54). DHT is the most potent ligand for the androgen receptor and is much more effective than T in promoting growth of the prostate (55). The concentration of DHT in prostatic tissue is several times higher than that of T, suggesting that DHT is the more important androgen for development of both the normal prostate and cancer (56). Hence, targeting of androgen signalling axis is the major means of therapy for advanced prostate cancer.

Androgens mediate their activity through binding with high affinity and specificity to the intracellular transcription factor called the androgen receptor (AR). Activation of AR by DHT, and to a lesser extent T, is responsible for regulation of gene expression that stimulates growth and inhibits cell death of androgen-dependent normal prostatic cells, as well as prostate tumours. In comparison to testosterone, DHT has up to a 5-fold greater affinity for AR and is approximately 2.5-times more active as an androgen (51, 57). Therefore, the use of 5α-reductase enzymatic inhibitors that prevent the conversion of T into DHT has been adopted as one mode of treatment for prostate diseases (45, 58). Use of chemical compounds and other means of blocking the androgen signalling pathway, including surgical removal of tissues producing androgens and direct targeting of AR, is discussed in detail in **Section 1.2.3**.

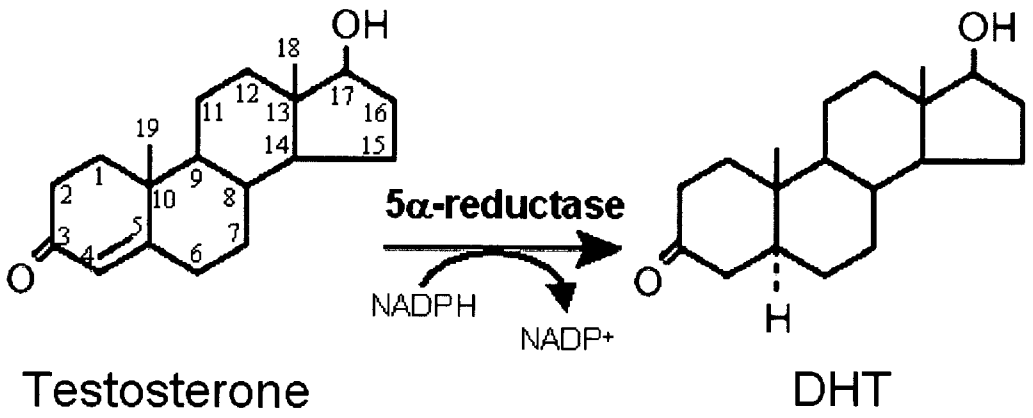

Figure 1.4 – Conversion of Testosterone into DHT. The 5α-reductase isozymes 1 and 2 catalyze the conversion of testosterone into DHT in an irreversible reaction. This reaction involves a sequential series of steps, initiating with the reduced nicotinamide-adenine dinucleotide phosphate (NADPH) cofactor binding to 5α-reductase to form an enzyme-NADPH complex. The T substrate then binds to this complex and electrons are stereo specifically transferred from NADPH to reduce the A4 double bond of T, producing an enzyme-$NADP^+$-DHT complex. The DHT product is then released, along with the oxidized $NADP^+$ cofactor, to regenerate the active 5α-reductase enzyme. Adapted from Patrin *et al.* (51).

1.2 Prostate Cancer

1.2.1 Progression of Prostate Cancer to Androgen Independence

The prostate gland is dependent on androgens for growth and maintenance of normal structure and function. Hence, testosterone and DHT are the main regulators of both prostatic cell proliferation and cell death (57). Like other carcinomas, development of prostate cancer depends on the ratio of these two events. Therefore, androgens also play a crucial role in prostate cancer growth and survival. In fact, tumour growth is initially almost completely androgen-dependent.

If detected at an early stage, prior to escape or spread of adenocarcinoma cells from the prostate capsule, this disease can be cured through radical prostatectomy or radiation therapy (59, 60). However, patients diagnosed with advanced prostate cancer are primarily treated with androgen ablation (withdrawal) therapy, which results in apoptosis of adenocarcinoma cells and ultimately regression of androgen-dependent tumours. Unfortunately, this androgen dependent phase of disease is temporary, lasting a median time of about 21 months, during which surviving tumour cells progress to androgen independence (AI) (61, 62). At the AI stage, adenocarcinoma cells do not require gonadal androgens for growth and proliferate uncontrollably, resulting in an increase of tumour burden. The recurrence of AI prostate cancer eventually results in metastasis and is lethal, with a median survival time of approximately 19 months using even the most effective chemotherapy regimens (63, 64). The best molecular marker for monitoring the progression of prostate cancer through all stages of disease is prostate-specific antigen (PSA). Serum PSA levels increase and decrease proportionally with changes in tumour burden (65, 66) (**Figure 1.5**).

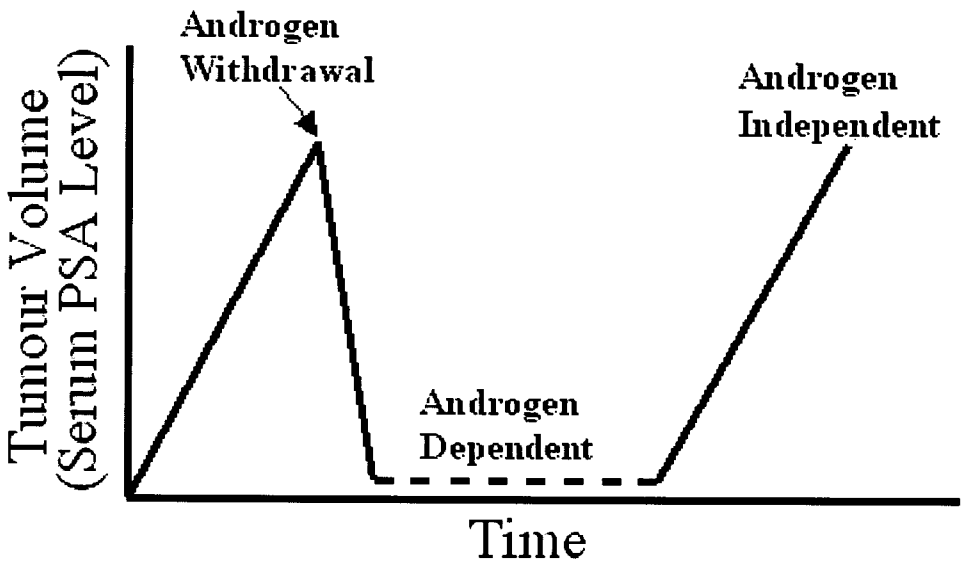

Figure 1.5 – Progression of Prostate Cancer to Androgen Independence. In prostate cancer, tumour burden is monitored by measuring serum PSA levels. After carcinoma detection, androgen withdrawal therapy is initiated, which dramatically reduces tumour burden in the androgen-dependent phase of disease. During this stage, the tumour regresses since adenocarcinoma cells are still dependent on androgens for growth and survival. However, a population of prostate cancer cells are able to bypass the requirement of gonadal androgens for growth and continue to proliferate, resulting in the transition to an androgen-independent phase of disease. At this stage of cancer recurrence, tumour burden and serum PSA levels rise again. Notably, androgen withdrawal therapy does not result in complete androgen ablation. Thus, the AI phase of disease can be more precisely defined as ablation-resistant carcinoma, in which tumours are not entirely androgen-independent and can survive in an androgen-depleted environment.

1.2.2 Gleason Grading of Prostate Cancer

The Gleason grading system for prostatic carcinoma is currently the best histological standard of diagnosis and prognosis. This grading system is based entirely on the glandular architecture of the two most prevalent histological components of a prostate tumour section, stained with haematoxylin and eosin (H&E) (67). Gleason grade determines the extent of gland differentiation and stromal invasion using a scoring system of 1 to 5, which is assigned for the most predominant staining pattern and the second most common pattern. The sum of these two grades is referred to as the Gleason score (or Gleason sum) and can range from 2 to 10. Generally, a well-differentiated tumour is assigned a low score of 2-4, a moderately differentiated tumour is designated as score 5-7 and a score of 8-10 denotes a poorly differentiated tumour. Patients with higher Gleason scores have a poorer clinical outcome, with a greater risk of progression of cancer to metastatic disease.

1.2.3 Androgen Ablation Therapy

Androgen ablation has been the main therapeutic intervention for treatment of prostate cancer ever since pioneering studies by Huggins and Hodges in the early 1940's demonstrated that prostate tumour growth was dependent on androgens (4). For his pioneering work, Charles B. Huggins was awarded the Noble Prize for Medicine in 1966 (68). The ultimate goal of androgen ablation therapy is to deprive androgen-dependent prostate tumour cells of androgenic growth signals. This result can be achieved through three mechanisms; i) elimination of testicular androgens through a bilateral orchiectomy,

ii) inhibition of testosterone production by the testes through suppression of pituitary LH/FSH release and iii) direct inhibition of androgenic action within the androgen-dependent prostate adenocarcinoma cells.

The surgical removal of the testes is an efficient form of androgen ablation reducing circulating testosterone levels by 95 % (45). However, due to the disadvantages associated with this treatment, which include erectile impotence and psychological side effects, it is not commonly used at the current time. As an alternative, there are two types of pharmacological agents that can be used to achieve androgen ablation. First, LHRH agonists, such as leuprolide acetate, reduce the extracellular blood supply of androgens to prostate cancer cells by constantly stimulating LHRH receptors in the pituitary gland, resulting in a decrease of their expression (69, 70). Subsequently, there is a reduction in the secretion of LH/FSH from the pituitary, which results in cessation of T synthesis. Although, this form of endocrine therapy reduces plasma testosterone levels by about 90 %, low levels of adrenal androgens are still secreted into circulation (71).

The second group of pharmacological agents cause androgen ablation by direct inhibition of the intracellular response to androgen within prostatic cancer cells. These agents include 5α-reductase inhibitors, such as finasteride, which inhibit the conversion of testosterone into DHT and AR antagonists that bind to the receptor (72, 73). AR antagonists can be steroidal, such as cyproterone acetate, or non-steroidal in nature, as are flutamide and bicalutamide. Steroid-based antagonists compete with androgens for binding to the AR-LBD, while non-steroidal compounds facilitate the assembly of inactive AR protein complexes to DNA (74). Antiandrogen antagonists are also used in

combination with LHRH agonists and 5α-reductase inhibitors to achieve more effective androgen ablation (75, 76).

Another recent approach to treating prostate cancer is the use of intermittent androgen suppression (IAS) therapy (59, 77). IAS subjects patients to successive rounds of androgen ablation therapy that are separated by intervals of no treatment. The basis of this approach is that periods of no treatment allow tumour cells to maintain some level of androgen sensitivity and can thus undergo cell death upon re-treatment with androgen ablation. IAS therapy has been shown to delay but not prevent progression of prostate cancer to AI (78). Overall, despite the availability and use of a combination of treatments, progression of advanced disease to AI is inevitable. The mechanism through which prostate adenocarcinoma cells transition from an androgen-dependent state to AI is not clearly understood. Thus, in order to develop novel treatments for advanced prostate cancer, a better understanding of the molecular processes that take place during disease progression is necessary.

1.3 Structure-Function Properties and Transcriptional Activity of the Androgen Receptor

1.3.1 Steroid Receptors

The AR is a ligand-dependent transcription factor that belongs to the steroid receptor (SR) family subgroup of the nuclear receptor superfamily. Based on the manner through which they dimerize and bind to DNA, nuclear receptors can be sub-classified into four groups; class I receptors are steroid receptors that bind as homodimers to DNA inverted repeats, class II receptors form heterodimers with the retinoic acid X receptor (RXR) and bind direct DNA repeats, class III receptors form homodimers and bind DNA direct

repeats and class IV receptors do not dimerize and bind DNA as monomers (79). In addition to AR, members of the SR family also include the glucocorticoid (GR), progesterone (PR), mineralocorticoid (MR) and estrogen (ER) receptors. All these SRs share common mechanisms of action and functional domain structures, which includes an N-terminal domain (NTD) that contains the transcriptional activation function 1 (AF1), a centrally located DNA binding domain (DBD), a hinge region and a C-terminal ligand binding domain (LBD) that contains the transcriptional activation function 2 (AF2) (80-83). A comparison of the common domain structures of SRs is shown in **Figure 1.6**.

Among the members of the SR family in humans, the NTD has the lowest degree of amino acid sequence homology (< 15 %) and varies considerably in length (**Figure 1.6**), suggesting that this region of SRs may be important in receptor-specific transcriptional regulation (83) by allowing for specific receptor-protein interactions (discussed in **Section 1.3.3** and **Section 1.4**). In contrast to the NTD, the DBD and LBD of steroid receptor family members are highly and moderately conserved, respectively. The DBD of AR shares up to 80 % amino acid sequence homology with PR and greater than 70 % with that of GR and MR (84). The LBD of AR shares 50-55 % amino acid homology with most other steroid receptors (81). Despite the high degree of similarity among SRs, these receptors are able to specifically and precisely regulate transcription of unique gene sets.

As shown for AR in **Figure 1.7**, steroid receptors also share a common mechanism of action. In the absence of ligand, like other SRs, AR exists in the cytoplasm as a transcriptionally inactive complex consisting of heat shock proteins (hsp),

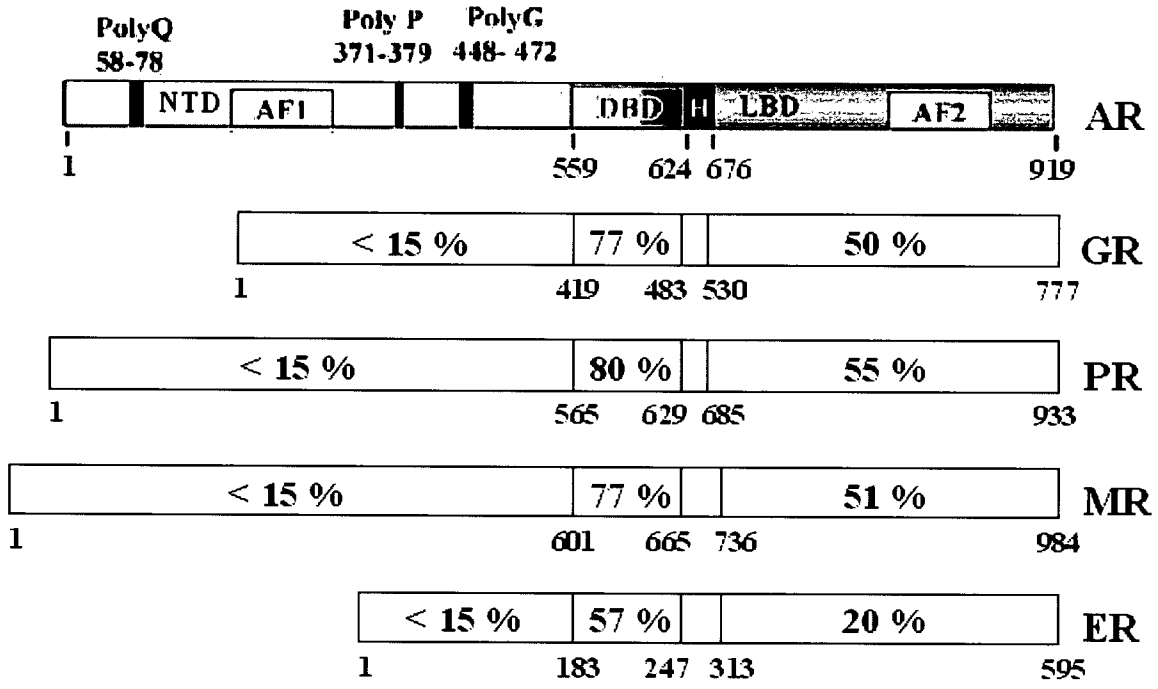

Figure 1.6 – Common Functional Domain Structures and Sequence Homology of Steroid Receptors. All steroid receptors contain common functional domains that include an N-terminal domain (NTD), a DNA binding domain (DBD), a hinge region (H) and a ligand binding domain (LBD). Transcriptional activation functions 1 and 2 (AF1 and AF2) are located in the NTD and LBD, respectively. The level of sequence homology between human androgen receptor (AR) and glucocorticoid (GR), progesterone (PR), mineralocorticoid (MR) and estrogen (ER) receptors is indicated as a percentage relative to the corresponding AR functional domains (set at 100 %). The size of domains is denoted by amino acid number. Unique features of the AR-NTD are also indicated; polyglutamine (poly-Q), polyproline (poly-P) and polyglycine (poly-G) tracts. Modified from Agoulnik *et al.* (83).

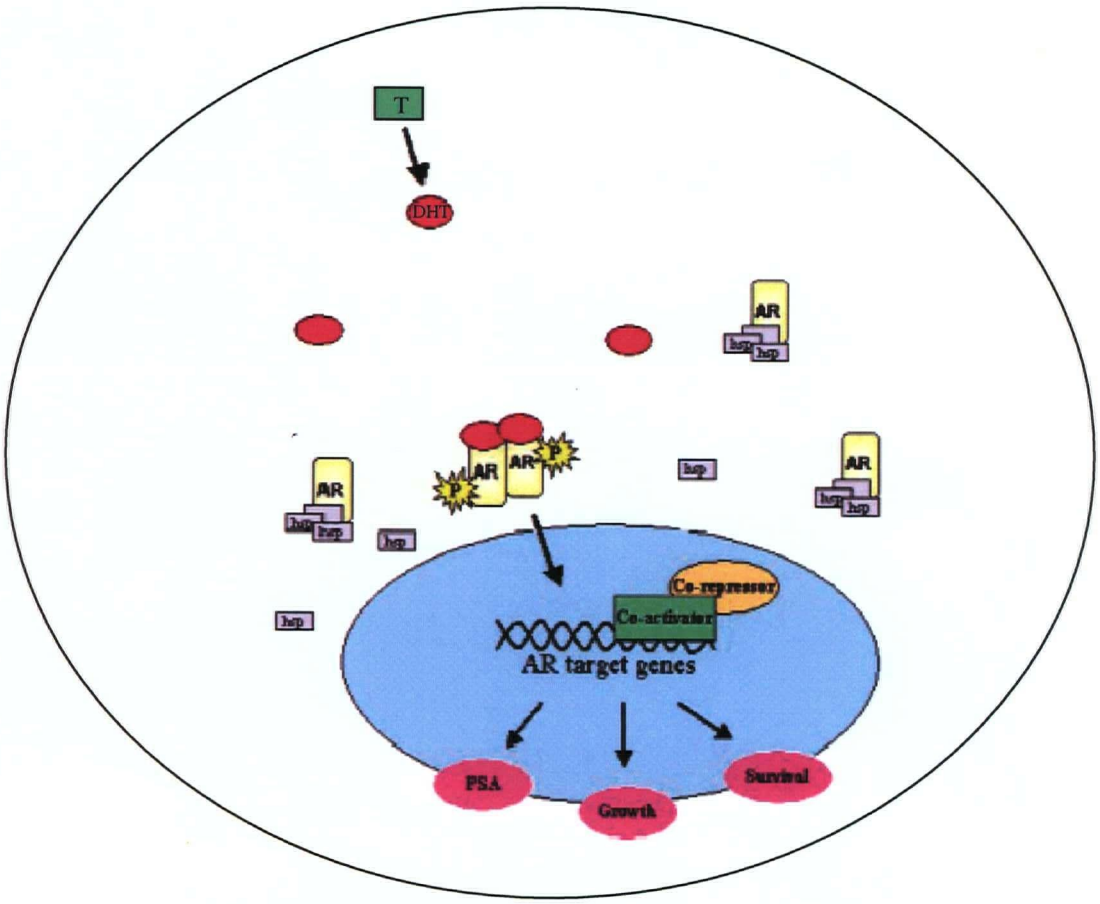

Figure 1.7 – AR-Mediated Gene Transcription. Testosterone (T) is converted to dihydrotestosterone (DHT) by 5-α-reductase inside the cell. Activation of AR is achieved through binding of cognate DHT ligand to the AR-LBD, which induces a cascade of events that include conformational changes and phosphorylation (P) of the receptor, as well as dissociation from heat shock proteins, receptor dimerization, nuclear translocation and binding to DNA androgen response elements within the promoters/enhancers of target genes, such as PSA. At all stages of AR action, the receptor can interact with coregulator (coactivators and corepressors) proteins that modulate its transcriptional activity. Modified from Javidan *et al.* (86).

such as hsp70, hsp90 and hsp40 (85, 86). Upon binding to androgen, AR undergoes conformational changes, dissociates from heat shock proteins and translocates into the nucleus where it binds as a homodimer to androgen response elements upstream of AR-target genes, such as PSA (86). Notably, the ER (α and β isoforms) is an exception among steroid receptors, with respect to its mechanism of action. ER is predominantly localized to the nucleus in the absence and presence of its cognate ligand and does not undergo nuclear translocation (87). Not surprisingly, the amino acid sequence of ER is also the most distinct from other steroid receptors (**Figure 1.6**). However, upon activation by estrogen in the nucleus, ER binds to DNA and regulates transcription in the same manner as other steroid receptors. The active DNA-bound SR, including AR, can recruit coregulator proteins and basal transcriptional machinery to regulate gene-specific expression that leads to changes in the growth and survival of target cells (86, 88).

1.3.2 Androgen Receptor Gene

The human AR gene is located as a single copy on the X chromosome in the q11-12 position and is over 90 kb in length (89). The AR gene consists of 8 exons, which code for a 10.6 kb mRNA that includes a 1.1 kb 5'-untranslated region, a 6.9 kb 3'-untranslated region and a coding region of 2.7 kb (90-92). The AR cDNA codes for a 919 amino acid protein with an apparent molecular weight of 110 KDa. The entire NTD of AR is encoded by the first exon of the AR gene, while exons 2 and 3 encode for the AR-DBD, and exons 4 through 8 encode for the hinge region and AR-LBD (86).

1.3.3 N-Terminal Domain of AR

The N-terminal domain (NTD) of AR (amino acids 1-559) occupies more than half of the receptor's primary sequence of 919 amino acids (93, 94). The AR-NTD contains the transcriptional activation function 1 (AF1), which is actually composed of a combination of two transcriptional activation units (Tau) referred to as Tau1 and Tau5 (95). The Tau1 region (amino acids 141-338) of AF1 is required for ligand-dependent transactivation activity of full-length AR (96, 97), whereas the Tau5 region (amino acids 360-528) is capable of ligand-independent constitutive transcriptional activation of the AR-NTD when the LBD of AR is deleted (98, 99). The AR-NTD also contains two distinct motifs, the ^{23}FQNLF^{27} and ^{433}WHTLF^{437} sequences, which interact with the LBD of AR to allow for the N-/C-terminal interaction of the receptor (100, 101). This intramolecular interaction of AR is unique among steroid receptor family members and may be at least partly responsible for AR-specific action (102). The N-/C-terminal interaction of AR has also been shown to be facilitated by several coactivators and is important in stabilization of bound ligand, as well as increasing receptor protein stability (103-105). Due to the presence of two distinct activation units within the AR-NTD, it is possible that each of these regions interacts with different coregulatory proteins and transcription factors to allow for more precise control of AR activity (98). Moreover, recent studies indicate that the AR-NTD changes in structure upon binding to proteins, after the receptor associates with DNA (106, 107). This suggests that the AR-NTD can serve as a flexible platform for the assembly of coregulators and may be the primary mediator of androgen-specific gene expression (108).

Another unique feature of the AR-NTD in comparison to other steroid receptors is the presence of several homopolymeric stretches of amino acids (**Figure 1.6**). These include the polyglutamine (poly-Q) tract initiating approximately at amino acid 59 (spans 17-29 residues), the polyproline (poly-P) tract at amino acid 372 (spans 9 residues) and the polyglycine (poly-G) tract at amino acid 449 (spans 24 residues) (83, 91). Although the precise function of these repeating tracts is not known, an abnormal extension of the poly-Q tract to 40 or more residues is associated with X-linked spinal and bulbar muscular atrophy or Kennedy's disease (109, 110), while shorter poly-Q repeats have been associated with increased AR activity and prostate cancer (111, 112). Expansion of the poly-Q tract has also been shown to decrease AR protein expression (113) and reduce transactivation of the receptor as a result of both AR protein degradation and decreased interaction with transcriptional coactivator proteins (114, 115). Shortening of the poly-G tract to 14 amino acids or less has also been associated with prostate cancer (116).

As mentioned previously (**Section 1.3.1**), the NTD of steroid receptors has the highest degree of amino acid sequence variability and may therefore allow for receptor-specific action. The unique features of the AR-NTD, including the presence of dual activation regions, distinct motifs that bind to the LBD of AR and the existence of several homopolymeric amino acid tracts, suggests that this region of AR may confer AR-specific responses. One mechanism by which this specificity may be achieved is through interaction with unique coregulator proteins (discussed in detail in **Section 1.4**) that allow for precise and specific AR transactivation. Nevertheless, due to the strong intrinsic transactivation activity of the AR-NTD, most conventional protein interaction assays in yeast have been limited to using other regions of AR for screening (discussed in **Chapter**

2). However, isolating novel AR NTD-binding proteins that alter receptor transcriptional activity could lead to further determination of how AR expressing cells achieve androgen-specific gene regulation.

1.3.4 DNA Binding Domain/Hinge Region of AR

The DNA binding domain (DBD) of AR directly follows after the AR-NTD and consists of amino acids 560-623 (82). The AR-DBD is essential for binding of AR to DNA consensus sequences located in the regulatory elements of AR-regulated genes, as well as for dimerization of AR upon activation and nuclear translocation of the activated receptor. The DBD of AR and other steroid receptors is composed of two zinc finger motifs, each of which utilizes 4 cysteine residues to coordinate a single zinc ion. These motifs interact with steroid response element (SRE) regulatory sequences in the major groove of DNA (117-119). The first (N-terminal) zinc finger of the DBD is responsible for recognizing and binding to specific SREs (120, 121), while the second (C-terminal) zinc finger stabilizes receptor-DNA interactions and mediates dimerization between steroid receptor monomers (122-124). The AR-DBD has also been shown to be important for nuclear localization of AR (125).

In the nucleus, AR binds onto androgen response elements (AREs) composed of inverted palindromic sequences containing two half-sites, separated by a 3 nucleotide spacer. The preferred consensus ARE sequence is 5'-GGA/TACANNNTGTTCT-3', comprised of two 6 bp asymmetrical elements (126). This response element is the same as that described for the GR and other steroid receptors, confounding the problem of how SRs bind specifically to their SREs (80, 127). It has been suggested that the specific

interaction of AR and GR with their response elements may be partly due to receptor binding with unique spacer and flanking nucleotides (127). Moreover, AR transactivation activity has been shown to increase when the receptor binds to two or more adjacent AREs (128, 129). This unique ability of AR, as compared to GR, to cooperatively bind multiple response elements may also contribute to AR-specific gene regulation (130). The interaction of the AR-DBD with coregulator proteins (discussed in detail in **Section 1.4**) may represent an additional level of control to allow for AR-specific transcription. However, as described previously (**Section 1.3.1**), the DBD of steroid receptors is the most conserved region among family members. Therefore, coregulator proteins that interact directly with the AR-DBD are likely to also interact with other steroid receptors.

The hinge region of AR is located between the DBD and LBD of the receptor, consisting of amino acids 624-676 (82). In common with other SRs, AR has a ligand-dependent bipartite nuclear localization signal (NLS), located at the C-terminus of the DBD and in the hinge region (amino acids 617-633), which targets the activated receptor to the nucleus (125, 131). The AR NLS is composed of two basic amino acid clusters separated by ten amino acid residues. The hinge region of AR also contains important sites for post-translational modificiations (PTMs) of the receptor, including phosphorylation, acetylation and ubiquitylation (132-136). For example, the evolutionary conserved ^{630}KLKK^{633} motif of the hinge region is a well-studied target for acetylation by coactivators of AR (108). The regulation of AR activity through PTMs is described in further detail below (**Section 1.3.6**).

1.3.5 Ligand Binding Domain of AR

The ligand binding domain (LBD) of AR is located at the C-terminal end of the receptor, composed of amino acids 676-919 (137). The AR-LBD, in addition to forming the ligand-binding pocket and transcriptional activation function 2 (AF2), also interacts with the AR N-terminus to stabilize bound androgen and mediates the interaction between the receptor and heat shock proteins (104, 138, 139). The crystal structure of the AR-LBD and other steroid receptors has revealed the presence of a conserved ligand binding pocket that is formed by the ordered arrangement of 12 α-helices into a bundle with a hydrophobic centre (140, 141). The binding of agonists induces a conformational change in the LBD and causes helix 12 to fold back across the ligand binding pocket, capping the structure (140, 142, 143). These alterations result in the formation of the AF2 surface, which interacts with coactivator proteins (144, 145). Not surprisingly, binding of antagonists to the AR-LBD positions helix 12 away from the ligand binding pocket, interfering with the binding of the receptor to coactivators (146).

Upon agonist binding, activated AR dissociates from heat shock proteins, dimerizes and binds onto AREs in the nucleus to initiate transcription of androgen-regulated genes, all the while interacting with coregulator proteins that modulate its activity (**Figure 1.7**). The AR-LBD is the predominant site of interaction for heat shock proteins that can stabilize AR protein and maintain the receptor in a high affinity ligand binding conformation (108, 138, 147) (discussed in detail in **Section 1.4**). The dimerization of AR, a crucial event necessary for effective receptor-DNA binding, is also partly mediated by the AR ligand binding domain. Receptor monomer dimerization

results from the interaction of the LBD of one AR molecule with the DBD of another, a process that also increases receptor protein stability (102, 148).

Of the two transcriptional activation functions of AR, N-terminal AF1 and C-terminal AF2, the latter one formed by the LBD of AR is weaker (149, 150). However, both AF1 and AF2 activities of the receptor are important for the full transactivation activity of AR. Like other steroid receptors, ligand binding exposes the active AF2 surface of AR and allows interaction with the LXXLL motifs of classical p160 family member coactivators (151, 152) (discussed in **Section 1.4**). However, unlike other steroid receptors, binding of ligand to the AR-LBD also exposes sites within this region that interact with WXXLF and LXXLF motifs of the AR-NTD to allow the N-/C-terminal interaction (100, 153). This intramolecular interaction has been suggested to position or stabilize helix 12 of the AR-LBD across the ligand binding pocket, which results in a reduced dissociation rate of bound androgen (104). The competition of NTD WXXLF and LXXLF motifs with LXXLL mediated coactivator interactions for the AR-LBD also regulates coregulator binding in a manner that is unique among steroid receptors and can therefore provide a possible specific mechanism by which AR mediates transcription (104, 154, 155).

The AR-LBD is an important site of interaction for coregulator proteins and has thus been heavily targeted for identification of AR-binding protein partners. The search for AR-LBD interacting coregulators has been predominantly carried out using conventional protein interaction assays in yeast. This was made possible due to the fact that the AR-LBD contains a weak, almost negligible, AF2 transactivation activity in the absence of the AR-NTD AF1 and thus does not interfere with screening for positive

interactions in these assays (149) (described in **Chapter 2**). However, as previously described for the AR-DBD, due to the moderate level of homology in LBD amino acid sequence among steroid receptors (50-55 % in most cases; **Section 1.3.1**), use of the AR-LBD for screening of protein interactions drastically reduces the chances of identifying binding partners that are specific for AR. Expectantly, most of the coregulator proteins that have been identified as a result of binding to AR-LBD have also been shown to interact other steroid receptors (139). A comprehensive discussion of AR coregulator proteins is included in **Section 1.4**.

1.3.6 Role of Post-Translational Modifications in Regulation of AR Activity

In addition to being regulated by cognate ligand binding and through interactions with coregulatory proteins, the transcriptional activity of steroid receptors, including AR, is also modulated by a myriad of post-translational modifications (PTMs). These covalent changes can alter subcellular localization, receptor stability, interactions with other coregulator proteins and transactivation activity of steroid receptors (156). The documented PTMs for AR include phosphorylation, acetylation, sumoylation and ubiquitylation.

Phosphorylation of AR has been reported for both the unliganded and activated receptor (133, 157). However, there is a general increase in the number of phosphorylated sites on AR upon binding of hormone. Specifically, there are six serine residues of AR (Ser16, 81, 256, 308, 424 and 650) that are phosphorylated after binding to androgen, while one residue (Ser94) is constitutively phosphorylated (133). Protein kinases that have been shown to directly phosphorylate AR include Akt (protein kinase

B) and the mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase 2 (Erk-2) (108). Akt has been shown to directly phosphorylate AR at Ser213 and Ser791, which results in an increase in receptor transactivation activity (158). However, other studies have shown that phosphorylation of Ser213 by Akt actually represses AR activity and that the effect of Akt on AR transactivation is dependent on the type and passage number of the cells used (159, 160). On the other hand, Erk-2 phosphorylates AR at Ser514, which results in enhanced AR transcriptional activity and an increase in interaction with coactivator proteins (161-164). Activation of protein kinase A (PKA) has also been shown to increase AR activity, but the direct phosphorylation of the receptor by PKA has yet to be demonstrated (66, 165). The work presented in the current study has indentified an additional AR-binding protein with Ser/Thr kinase activity, known as cyclin G-associated kinase (GAK), that may be involved in phosphorylation of AR (166) (discussed in **Chapter 2**).

Acetylation of AR occurs in the hinge region of the receptor (amino acids 630-633) at the KXKK motif (167). Three coactivators of AR, cAMP response element binding protein (CREB) binding protein/p300 (CBP/p300), p300/CBP-associated factor (p/CAF) and TAT-interactive protein (Tip60) (discussed in **Section 1.4**), have been reported to acetylate the receptor (167-169). Mutational analysis of the KXKK motif lysine residues results in inhibition of AR nuclear translocation in the presence of ligand and reduced receptor transcriptional activity (135). In addition, these mutations blunt the enhanced transactivation effect of AR coactivators, while increasing the recruitment of corepressors (135, 170). On the other hand, acetylation of wt AR at the KXKK motif increases coactivator protein interactions and enhances AR transactivation (134). More

recently, the inhibition of AR deacetylation by histone deacetylase-1 (HDAC1) has been shown to stimulate AR activity (171).

The AR can also be modified by the small ubiquitin-like modifier-1 (SUMO-1) through sumoylation of lysine residues embedded in conserved KXE motifs (156, 172). Sumoylation is a reversible process that is similar to ubiquitylation of proteins, but instead of degradation alters interaction with other proteins or the sub-cellular localization of the target. For AR, sites of sumoylation have been identified in the NTD at lysines 386 and 520 (172). Sumoylation of AR results in reduction of receptor transcriptional activity by modulating interactions with coregulator proteins (173, 174). The SUMO-1 modification of AR is thought to allow fine-tuning of receptor activity and govern gene-selective transcription (175).

Accumulating evidence indicates that AR is targeted for degradation *via* the ubiquitin-proteosome pathways (156). Ubiquitylation of proteins is a three-step process involving an E1-activating enzyme, an E2-conjugating enzyme and an E3 ligase (176). In humans, only one E1 protein and 18 E2 proteins have been identified, while the number E3 family members exceed 200. Notably, ubiquitylation does not necessarily result in protein degradation. Poly-ubiquitylation usually targets a protein for degradation through the proteosome, while mono- and bi-ubiquitylation affect the subcellular distribution of proteins, as well as interactions with other proteins (177). For AR, the highly conserved PEST sequence that targets proteins for ubiquitylation and degradation is located in the hinge region (178). The Mdm2 E3 ligase promotes AR poly-ubiquitylation, after the receptor is phosphorylated by Akt, and directs AR to the proteosome for degradation (179). The C-terminus of hsp70-interacting protein (CHIP)

has also been shown to interact with the NTD of AR and induce protein degradation (180).

The tumour susceptibility gene product (TSG101) has been shown to regulate ubiquitin conjugation of AR by increasing mono-ubiquitylation of the receptor, resulting in enhanced transactivation (181). The ubiquitin-specific protease (USP10), which reverses the ubiquitylation process, also binds to AR and stimulates the androgen response of target promoters (182). Furthermore, the UbcH7 E2 conjugase has been shown to coactivate several steroid receptors, including AR, possibly by allowing for rapid turnover to achieve full receptor activity (183). My research has also led to the identification of a novel interaction between a subunit of the E3 ubiquitin-ligase anaphase-promoting complex (APC) (184) and the NTD of AR (discussed in **Chapter 2**).

1.3.7 Mechanisms for AR-Specific Regulation of Gene Transcription

Similarities in the chemical and physical properties of steroid hormone ligands (**Figure 1.3**), the mechanism of action of steroid receptors, and their common protein structures (**Section 1.3.1**) confound the issue of how receptors regulate transcription of specific gene sets in target tissues. Although binding of SRs to specific steroid response elements may seem as the most obvious means of controlling receptor activity, multiple SRs have been shown to bind to the same DNA consensus sequence (80). For example, the receptors AR, GR and PR all bind as homodimers to the canonical 5'-GGTACANNNTGTTCT-3' steroid response element (80). To address this problem, several mechanisms have been proposed for explaining the specificity of the AR-response; i) availability of certain receptors and their ligands, ii) DNA target recognition,

iii) cooperative binding of receptors to two or more DNA-binding sites and iv) interaction with unique combinations of coregulators.

The *first* mechanism may explain AR-specific responses in cells that do not express any other steroid receptor except AR. However, this scenario is probably not a major discriminate for SR-specific responses in hormone target tissues such as the prostate, since in many cell types, two or more SRs are co-expressed with access to their cognate ligands (185-187). More specifically, the steroid receptors AR and GR co-exist in prostate epithelial cells (185). The *second* mechanism for SR-specific responses requires the existence of sequence variations in the canonical SRE of natural genomic promoters to allow for specific steroid receptor binding. As described previously (**Section 1.3.4**), subtle deviations in the nucleotide sequence of the consensus SRE region can allow a significant level of specificity among steroid receptors (127). The *third* mechanism implies that particular configurations of DNA binding sites act cooperatively for one type of receptor specifically. This has been demonstrated for AR binding to multiple sites on several androgen-regulated promoters, including that of the PSA gene (130, 188-190). Although the proximal PSA promoter encodes two androgen response elements (ARE I and II) that bind to both AR and GR (128), the enhancer region of the PSA gene contains an additional four AREs (III, IIIA, IV and V), which can bind AR in a coordinated fashion and allow receptor-specific responses (129, 130, 191).

The *fourth* mechanism proposed to elicit SR-specific responses is the interaction of the receptor with unique combinations of both coactivator and corepressor coregulator proteins. In addition to direct interaction with basal transcription factors and chaperone proteins, AR binds to an array of coregulators to enhance or inhibit transcription of target

genes. This topic is the focus of my research and is therefore discussed in detail in **Section 1.4** below.

1.4 Coregulators of Androgen Receptor Activity

1.4.1 Overview of Androgen Receptor Coregulator Proteins

It has become clear that transcriptional activity of AR, as well as other members of the nuclear receptor superfamily, is modulated by coregulatory proteins. Generally, coregulators can be defined as proteins that interact directly with nuclear receptors to enhance (coactivators) or reduce (corepressors) transactivation of target genes, without significantly altering the basal transcriptional rate (192). Notably, coregulator proteins are not considered to possess specific DNA binding ability (193). Since coactivators use multiple mechanisms to influence AR transcription, they can be categorized, based on their functional characteristics, into two major types; classical or type I and non-classical or type II coactivators (139). Type I coactivators exert their function on AR while the receptor is at the target gene promoter to facilitate chromatin remodelling, DNA occupancy or the recruitment of basal transcription factors associated with the RNA polymerase II holocomplex. Many classical coactivators for AR have been identified, which are discussed in **Section 1.4.2**.

Type II coactivators primarily function by modulating the appropriate folding of AR, ligand binding or facilitating the N-terminal/C-terminal interaction of the receptor. These events can contribute to AR protein stability, alter ligand binding capability or influence the subcellular distribution of AR, which ultimately leads to an increase in AR transcriptional activity. Notably, non-classical coactivators can also alter the post-

translational modification status of AR. As discussed in **Section 1.4.3**, a wide array of type II coactivators have also been found for AR, which can act at many stages of receptor activation. In contrast to coactivators, corepressors suppress nuclear receptor transcriptional activity. Originally, corepressors were identified as nuclear receptor binding proteins that repressed transcription through the formation of non-productive interactions with basal transcriptional machinery or through chromatin remodelling (139). More recently, several proteins that function as corepressors for AR have been reported, each with unique mechanisms of inhibiting receptor activity (**Section 1.4.4**). Overall, a subset of 130-plus coregulators have been identified for AR (108). A partial list of Type I/II AR coactivators and corepressors discussed here is provided in **Tables 1.1** and **1.3** (**Section 1.4.4**), respectively. An additional list of important AR coactivators not clearly classified as type I or II is also included in **Table 1.2**.

1.4.2 Type I Classical Coactivators of AR

i) SRC/p160 Coactivators

The first identified and most extensively characterized of AR coregulators is the steroid receptor coactivator (SRC) family consisting of three 160-kDa proteins. Members of this family include steroid receptor coactivator 1 (SRC1), transcriptional intermediary factor 2 (TIF2) or SRC2 and amplified in breast cancer 1 (AIB1) or SRC3 (154, 194, 195). The SRC/p160 coactivators have been shown to interact with the AR-NTD and AF2 surface of the AR-LBD to enhance ligand-dependent transactivation of the receptor (99, 103, 234, 235). SRC1, SRC2 and SRC3 share similar structural organization that includes an N-terminal tandem basic helix-loop-helix domain, a C-terminal glutamine

Name	Interacting Domain of AR	Selected References
	Type I Classical Coactivators	
SRC/p160 Family		
SRC1/NCoA-1	NTD, DBD, LBD	(139, 194)
SRC2/TIF2/GRIP1	NTD, DBD	(139, 195)
SRC3/AIB1/ACTR	LBD and other domains	(139, 196)
Chromatin Remodelling		
SRC1 and SRC3	NTD, DBD, LBD	(197, 198)
CBP/p300	NTD, DBD	(199)
p/CAF	NTD, DBD	(200)
SWI/SNF	Precise domain unknown	(201)
Link to Basal Transcriptional Machinery*		
TFIIB	Interacts with SRC1	(202)
TFIIF	NTD	(203)
TFIIH	NTD	(204)
RNA Pol II subunit (RBP-2)	LBD	(205)
	Type II Non-Classical Coactivators	
Molecular Chaperones*		
Hsp70	LBD	(206-208)
Hsp90	LBD	(138, 209)
Hsp40 (Ydj1)	LBD	(210, 211)
Hop (p60)	Precise domain unknown	(108, 212)
p23	Precise domain unknown	(212, 213)
Modulation of Ligand Binding and AR Stabilization		
BAG-1L(hsp70 cochaperone)	NTD, LBD	(208, 214)
ARA70	DBD, LBD	(139, 215)
ANPK (PKY)	DBD	(216)
Cellular Trafficking of AR		
Filamin	Hinge domain	(217)
Supervillin	NTD, DBD	(218)
Gelsolin	DBD, LBD	(219)
Ran/ARA24	NTD	(220)
Caveolin-1	NTD, LBD	(221)

Table 1.1 - Type I and II AR Coactivators. The organization of coactivators is based on their known functions and the mechanism by which they can enhance AR activity. *Note that these subcategories are not typically classified as coactivators but have been included here due to the close nature of their effects on AR with Type I and II coactivators.

Name	Description (Selected References)
ARA54	Interacts with the AR-LBD and enhances AR transcription in response to DHT (222)
ARA160	Binds to AR-NTD and can enhance AR transactivation cooperatively with ARA70 coactivator in prostate cancer cells (223)
ARA267	Interacts with both N-/C-termini of AR to increase AR activity and can synergize with other coactivators, such as Ran/ARA24 and p/CAF (224)
Breast cancer susceptibility gene 1 (BRCA1)	Tumour suppressor that binds to AR N-/C-termini and synergistically enhances AR transcription with ARA70 (225)
Cdk-activating kinase (CAK)	An AR-NTD interacting coactivator of AR and kinase moiety of of the general transcription factor complex TFIIH (204)
Retinoblastoma protein (Rb)	Tumour suppressor that binds with the NTD and LBD of AR and enhances receptor transactivation in prostate cancer cells (226)
TAT-interactive protein (Tip60)	Interacts with the LBD and hinge domain of AR and can increase ligand-dependent receptor transcription (227)
β-Catenin	Binds to AR-DBD and LBD to enhance AR transactivation, possibly by increasing receptor nuclear translocation (228, 229)
Hepatocyte nuclear factor-3α (HNF-3α)	DNA binding protein that interacts with AR-DBD/hinge domain to promote assembly of AR-dependent transcription complexes, resulting in increased receptor activity (230)
AR-trapped clone-27 (ART-27)	Binds to the NTD of AR and enhances transcription in prostate cancer cells (231)
Ku70 and Ku80	Interact directly with AR-LBD, enhance AR transcription and can bind to prostate-specific antigen promoter in an androgen-dependant manner (232)
Male germ cell-associated kinase (MAK)	Enhances AR transactivation potential in an androgen- and kinase-dependent manner in prostate cancer cells and can synergize with SRC3 (233)

Table 1.2 - Other Important AR Coactivators. This list includes AR coactivators that have also been implicated in enhancing receptor transactivation through unique or unknown mechanisms.

rich region and three LXXLL motifs in the central portion of the protein, which are necessary for interaction with nuclear receptors (192). Notably, binding of the LXXLL motifs of SRCs to the AR-LBD is weaker than with the LBD AF2 of other steroid receptors (GR and ER), possibly due to competition with the AR-NTD for binding with the C-terminal AF2 of AR (104, 105). However, SRC coactivator LXXLL motif binding does play an important role for regulation of AR activity and has recently been suggested to induce conformational changes in the AF2 of AR to impact ligand binding kinetics (236). The AR-LBD can also bind to FXXLF motifs contained in some AR coregulators, such as the type II ARA70 coactivator (see below), but this sequence is also present in the NTD of AR, which further adds to the competition between coactivators and the NTD for binding to the LBD of the receptor (237, 238).

SRCs can increase AR transactivation *via* their intrinsic histone acetyltransferase (HAT) activity, which allows maintenance of a transcriptionally open chromatin structure at the promoter of target genes (197). More recently, SRC1 was shown to actually enhance the direct binding of AR to chromatin presumably due to its HAT activity (239). In addition, SRCs can act as platforms for the recruitment of secondary coactivators, such as CBP/p300 and p/CAF [see (ii) below], that possess chromatin remodelling capabilities (240, 241) and can also bridge nuclear receptors to basal transcriptional machinery [see (iii) below] (202).

ii) Chromatin Remodelling Coactivators

Chromatin remodelling protein complexes act upon the nucleosome by disrupting the histone-DNA interaction or through controlling the acetylation status of histones.

This disruption of chromatin structure allows transcription factors to bind more readily to DNA and thus facilitates transcriptional activation (242). More specifically, acetylation of the N-terminal histone tails reduces their positive charge, which leads to a reduction in nucleosome-nucleosome contacts and more sites of active transcription (243). AR co-activators that contain intrinsic HAT activity include CREB (cAMP response element binding protein) binding protein (CBP) or its homolog p300 (CBP/p300), p300/CBP-associated factor (p/CAF), as well as members of the SRC/p160 family, SRC1 and SRC3 (197, 198, 241, 244, 245). CBP/p300 has been shown to increase AR-mediated transcription by 3-5 fold (246) and p/CAF can reduce Cyclin D1-mediated repression of AR transactivation (200). Also, members of the SRC family, such as SRC2, can be simultaneously recruited with CBP/p300 by AR to the PSA promoter/enhancer for activation of transcription (247, 248).

The SWI/SNF multi-protein complex, which can perturb the conformation of the nucleosome resulting in diminished interaction between DNA and histones, can also enhance transcription of AR at the PSA promoter (201). The proper N/C-terminal interaction of AR is required to recruit the SWI/SNF complex, which in turn remodels chromatin to allow AR to bind to androgen response elements (249). It has been suggested that both SWI/SNF and CBP/p300 HAT activity are required for hormone-dependent activation of AR. SWI/SNF can be targeted to chromatin by CBP/p300, which itself is recruited through interaction with SRC coactivators (250). Thus, this indicates that multiple cofactors required for activation are not all recruited through their direct interactions with steroid receptors but may involve a coordination of cofactor-cofactor interactions. Nevertheless, neither CBP/p300, p/CAF or SWI/SNF are unique to AR

since they can act as coactivators of other steroid receptors as well (201, 251-253). Similar to these steroid receptor-associated proteins, the work presented here demonstrates the novel interaction of a transcriptional adapter protein, tranformation/transcription domain-associated protein (TRRAP) or p/CAF-associated factor (PAF400) (254, 255), with the NTD of AR (discussed in **Chapter 2**).

iii) Role of Basal Transcriptional Machinery in Coactivation of AR

The transcriptional activation of steroid receptors ultimately requires the recruitment of RNA polymerase (pol) II to the promoter of target genes (256). RNA pol II is recruited through the assembly of the transcriptional preinitiation complex, formed by general transcription factors (GTFs). Transcription is initiated through binding of TATA binding protein (TBP; binds consensus TATA DNA sequence in gene promoters) to the transcriptional start site. TBP is part of the transcription factor IID (TFIID) multiprotein complex, which also includes TBP-associated factors (TAF proteins). Binding of TBP to DNA induces bending of chromatin to allow interaction between GTFs and steroid receptor-coactivator complexes (139). TFIIB then binds to TBP and recruits the TFIIF-RNA pol II complex. Subsequently, the kinase TFIIE and helicase TFIIH are recruited to RNA pol II to facilitate DNA strand separation and allow initiation of transcription.

Coactivators of steroid receptors, such as SRC1, CBP/p300 and p/CAF, can also exert their function by facilitating communication between the receptor and basal transcriptional machinery (139). SRC1 interacts with TBP and TFIIB and also recruits CBP/p300 to the transcriptional start site (202). In turn, CBP/p300, along with p/CAF, can interact directly with subunits of RNA pol II. AR can also interact directly with

GTFs, such as TFIIF and TFIIH, through its NTD (203, 204). Notably, GTFs themselves are not considered coregulator proteins since they can alter the basal transcription rate (192). Analyses of AR-mediated transcription suggest that the orchestrated interaction of AR with TFIIF and TFIIH may increase efficiency of transcriptional elongation from androgen target genes, such as PSA (257). Based on studies so far, AR may regulate transcription by enhanced assembly of the transcriptional initiation complex, by regulating promoter clearance and during elongation of transcription (257, 258). Enhanced communication of AR with the basal transcriptional machinery, through other coactivators or direct GTF interactions, allows for more efficient transcription and an additional level of control in regulating AR activity. The work performed in the current study has also identified a previously unreported interaction between the AR-NTD and a component of the TFIID GTF complex, known as TBP-associated factor 250 ($TAF_{II}250$ or TAF1) (259, 260) (discussed in **Chapter 2**).

1.4.3 Type II Non-Classical Coactivators of AR

i) Role of Molecular Chaperones in Coactivation of AR

Chaperones prevent the irreversible aggregation of unfolded or partially folded proteins through recognition of and binding to their hydrophobic regions (261). Although, chaperone proteins are not usually designated as coactivators, they do interact with steroid receptors and modulate their activity. In the absence of ligand in the cytoplasm, the structural conformation of the AR-LBD necessary for receptor activation is attained *via* multiple cycles of binding and release with components of a multichaperone complex, consisting of heat-shock proteins (hsp) and cochaperone

molecules (262). The minimal complex believed to be crucial for ligand responsive signalling of steroid receptors consists of hsp70 (hsc70), hsp40 (Ydj1), hop (p60; hsp organizer protein), hsp90 and p23 (262-265). The interaction of AR with these proteins can maintain the receptor in a stable, partially unfolded conformation that is primed for high affinity binding with androgens (266).

Upon binding of AR to ligand, the chaperone heterocomplex mediates trafficking of the receptor into the nucleus, possibly through facilitating interaction with dynein (motor protein that moves along microtubules into the nucleus), a cytoplasmic protein that can drive active nuclear transport of the receptor along the cytoskeleton (267, 268). The unfolding and refolding of AR by hsp70 and hsp40 has also been implicated in the facilitation of receptor translocation across intranuclear membranes (269, 270). Although within the nucleus the chaperone complex dissociates from AR, it can still continue to modulate receptor activity by promoting the dissociation of hormone from the receptor, which returns AR into a primed state that can be reactivated if ligand becomes available (108). However, in the absence of nuclear androgen, AR action is rapidly terminated and the receptor is degraded through the ubiquitin-proteosome pathway (262). The search for novel AR interacting proteins in this study has also confirmed the interaction of the receptor with the hsp70 chaperone protein (206-208) (discussed in **Chapter 2**).

ii) Coactivators of AR that Modulate Ligand Binding and Receptor Stability

The stability of AR protein is dependent on the ability of the AR-LBD to bind ligand (271). As discussed above [see (i) of **Section 1.4.3**], the binding of androgen to AR requires the proper folding of the receptor, which is regulated by the chaperone

heterocomplex. Upon ligand binding, AR dimerizes allowing the N- and C-termini of the receptor to interact. The occurrence of the N/C-terminal interaction of AR can result in decreased ligand dissociation and increased AR protein stability (139). Hence, coactivators that can alter the folding of AR protein, receptor ligand binding or the ability of the N- and C-termini to interact, can potentially regulate AR transcriptional activity.

Several proteins have been shown to coactivate AR transactivation through these mechanisms. A component of the hsp70 chaperone complex, Bcl-2 associated athagene-1 (BAG-1L), can increase AR transcription by interacting with the NTD and LBD of the receptor to facilitate proper folding (208, 214). Hsp90 itself can participate in the activation of AR by maintaining the receptor in a high affinity ligand binding conformation (138). The serine/threonine kinase, AR-interacting nuclear protein kinase (ANPK), interacts with the AR-DBD and enhances AR-dependent transcription through stabilization of receptor protein levels (216). The most established coactivator of AR that modulates receptor function through stabilization of ligand-bound receptor is the AR-associated protein 70 (ARA70) (215, 272). Recently, ARA70 has been implicated in changing the conformation of cytosolic AR so that it can bind and/or retain androgen more easily, leading to a faster rate of nuclear translocation (273). ARA70 has also been shown to specifically retard the dissociation of steroid hormones, like estrogen (17 beta-estradiol), which is also known to enhance AR activity, without affecting association of hormone with AR. ARA70 is a key example of how coactivators can regulate AR activity in the cytoplasm, prior to or during nuclear translocation. The work presented in the current study has also led to the identification of a novel AR coactivator, L-dopa decarboxylase (DDC), that enhances ligand-dependent receptor transactivation (274) and

increases ligand binding affinity, as well as total androgen binding capacity (discussed in detail in **Chapters 2, 3, 4, 5** and **6**).

iii) AR Coactivators Involved in Cellular Trafficking of the Receptor

The transactivation of steroid receptors can also be enhanced by coactivators that modulate cellular transport of the ligand-bound receptor. Both an increase in the rate of nuclear translocation and retention of AR in the nucleus can result in higher levels of transcription. Major structural components of cells, such as filamentous actin (f-actin), play an important role in the cellular trafficking of proteins along the cytoskeleton (275). Proteins that bind to f-actin mediate the process of actin-bundling, which defines cellular morphology and regulates actin polymerization and depolymerization. Several actin-binding proteins have been shown to alter AR function, including filamin, supervillin and gelsolin (217-219). The f-actin cross-linking protein, filamin, interacts with the hinge domain of AR and facilitates receptor nuclear translocation (217). Notably, the exact effect of filamin on AR activity is not clear since this protein has also been implicated in repression of AR transactivation (276). Supevillin binds to the NTD and DBD of AR, while gelsolin interacts with the AR DBD and LBD but both increase AR transactivation (218, 219). The association of AR with these actin-binding proteins is thought to serve as a mechanism by which the receptor can migrate along the cytoskeleton. My research has also led to the isolation of a previously unknown interaction between the AR-NTD and translational elongation factor 1A (eEF1A), which is also an actin-binding protein involved in cytoskeleton reorganization and can undergo nuclear translocation with transcription factors (277, 278) (discussed in further detail in **Chapter 2**).

The scaffolding protein, caveolin-1, is a principal component of caveolae membranes involved in many signal transduction pathways and is also another co-activator of AR that modulates transport of the receptor into the nucleus (221). High levels of caveolin-1 expression results in increased AR transactivation and nuclear localization of phosphorylated AR (279). In contrast to the mechanism of AR coactivation by caveolin-1, the Ras-related nuclear G protein (RanGTPase)/AR-associated protein 24 (ARA24) has been suggested to enhance AR trancription by maintaining higher receptor levels in the nucleus (139, 220). Ran/ARA24 is responsible for the nuclear export of importin proteins, which interact with NLS-containing cargo proteins to dock protein complexes onto nuclear pores and allow for nuclear import (280). Hence, an increase in levels of Ran/ARA24 may result in rapid return of importins to the cytoplasm, which can result in more efficient translocation of proteins into the nucleus (139).

1.4.4 Corepressors of AR

Although the majority of AR coregulators identified have been coactivators, in recent years several corepressor proteins have also been reported. The mechanisms by which corepressors suppress AR transcription seem to be as variable as that of coactivators. These mechanisms can include corepressors recruiting histone deacetylases, inhibiting the formation of nuclear active AR, inhibiting AR intramolecular interactions or binding to coactivators and by other unique processes (281). In addition to coactivators, corepressors may also be critical for regulation of AR transcription in a

precise and efficient manner. A partial list of AR corepressors discussed here is provided in **Table 1.3**.

i) Histone Deacetylase Recruitment

As discussed previously for coactivators (**Section 1.4.2**), the acetylation status of histones plays a critical role in the regulation of transcription. In contrast to coactivators that utilize HAT activity to disrupt chromatin structure and activate transcription, histone deacetylation of core histones can re-stabilize chromatin to suppress transcription by nuclear receptors (193). The silencing mediator for retinoid and thyroid hormone receptors (SMRT) corepressor and nuclear receptor corepressor (NcoR) are the best characterized nuclear receptor binding proteins that suppress AR transcription, presumably through recruitment of histone deacetylases (HDACs) and by competing with coactivators for interaction with the receptor (281). SMRT has been shown to interact with both the NTD and LBD of AR (282, 283), while NCoR only binds to the AR-LBD (284). Moreover, histone deacetylase-1 (HDAC1) itself has been shown to bind with the AR-LBD/DBD and down-regulate AR transcription (169). More recently, it was demonstrated that use of small interference RNAs to knock down SMRT and NCoR in prostate cancer cells enhances the recruitment of the coactivators SRC1 and CBP/p300 by ligand-bound AR, suggesting that these corepressors can also compete with coactivators for binding to the active receptor (297).

ii) Inhibition of AR Nuclear Translocation and DNA-Binding

AR can be targeted by corepressor proteins at multiple stages during receptor activation. The dosage-sensitive sex reversal adrenal hypoplasia congenita critical region

Name	Interacting Domain of AR	Selected References
Histone Deacetylase Recruitment		
SMRT	NTD, LBD	(282, 283)
NCoR	LBD	(284)
HDAC1	DBD, LBD	(169)
SHP	NTD, LBD	(285)
Inhibition of AR Nuclear Translocation and DNA-Binding		
Calreticulin	DBD	(286)
DAX-1	LBD	(287)
Disruption of AR N-/C-Terminal Interaction and Coactivator Binding		
Cyclin D1	NTD,Hinge domain	(200, 288, 289)
SHP	NTD, LBD	(290)
Akt	Precise domain unknown	(291)
GSK3β	NTD, LBD	(292)
p53	Precise domain unknown	(293)
Unique Corepressors of AR		
HBO1	DBD, LBD	(294)
PTEN	DBD, LBD	(295)
Alien	NTD	(296)

Table 1.3 - Corepressors of AR. The organization of corepressors is based on the suggested mechanisms by which they can enhance AR activity.

on the X chromosome gene 1 (DAX-1) corepressor interacts directly with the AR-LBD and sequesters the receptor in the cytoplasm (287). Conversely, calreticulin, a calcium binding protein, has been shown to interact with the AR-DBD and inhibit AR binding to androgen response elements and reduce receptor transactivation (286).

iii) Disruption of AR N-/C-Terminal Interaction and Coactivator Binding

The N-/C-terminal interaction of AR plays an important role in stabilizing AR protein and facilitation of androgen binding, which can lead to enhanced transactivation of the receptor (139). Glycogen synthase kinase 3β (GSK3β) is a serine/threonine kinase that phosphorylates a wide range of substrates, including several transcription factors and the NTD of AR (292). Through a direct interaction with the AR-NTD, GSK3β is thought to suppress AR transcription by inhibiting the N-/C-terminal interaction of the receptor. The tumour suppressor protein, p53, can also function as a corepressor of AR through disruption of the N-/C-terminal interaction and potentially inhibition of receptor homodimerization (293).

Several corepressors have been found to alter AR transactivation through inhibiting its association with coactivators. Cyclin D1, known to phosphorylate the cellular proliferation promoting retinablastoma (Rb) protein, binds AR through its hinge domain and suppresses transactivation activity, possibly through disrupting interaction of AR with the p/CAF coactivator (200, 288, 298). More recently, cyclin D1 has been shown to bind HDAC3, indicating a possible role for this corepressor in chromatin remodelling (299). The short heterodimer partner (SHP) protein is an orphan nuclear

receptor that can repress AR transcriptional activity through competing with the SRC2 coactivator for binding to the receptor (290). More recently, SHP has also been shown to inhibit AR transactivation *via* recruitment of HDAC1 (285). The role of some AR corepressor proteins, such as Akt, is complex and controversial. Akt, an oncoprotein, is a serine/threonine kinase that plays a critical role in the phosphatidylinositol 3-kinase (PI3K) mediated pathways. While some studies have shown Akt to be a positive modulator of AR transcriptional activity (158, 160, 300), others have reported that Akt suppresses AR transactivation by binding and phosphorylating the receptor, as well as inhibiting the interaction between AR and the coactivator ARA70 (159, 291). These differences have been attributed to the cell type and passage number of the cells used for assessing AR transcriptional activity.

iv) Unique Corepressors of AR

Although the mechanism by which they suppress AR transactivation is not clear, several other corepressors of AR have also been reported. The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) tumour suppressor protein can interact with AR and inhibit nuclear translocation, while promoting AR protein degradation, which results in inhibition of receptor transactivation (295). The histone acetyltransferase binding to the origin recognition complex (ORC) 1 subunit protein (HBO1) also binds to the DBD and LBD of AR to repress receptor transcription (294). Recently, the nuclear receptor corepressor, Alien (301), was shown to bind to the N-terminus of AR and inhibit endogenous PSA expression in LNCaP prostate cancer cells (296).

As outlined above (**Section 1.4**), the search for proteins that interact with and regulate the transactivation of AR has lead to the identification of a vast number of coactivators and corepressors, a list that continues to expand. However, the relative *in vivo* importance of modifying AR function *via* these coregulators and the relevance to pathological conditions remains the subject of future studies. Nevertheless, the expression profile and cell growth effects of a number of AR coactivators and corepressors in prostate cancer has been recently reported and will be discussed in **Section 1.5.3**.

1.5 Prostate Cancer Progression to Androgen Independence: Mechanisms of Continual AR Activation

Androgens play a critical role in the development and progression of prostate cancer, as discussed in **Section 1.2** (86). For this reason, advanced disease is treated with androgen withdrawal therapy, which is only temporarily effective since after initial tumour regression, surviving adenocarcinoma cells eventually progress to an androgen-independent phenotype. Because androgens mediate their effect on target cells through their receptor, it is of no surprise that AR is also thought to be important in the development of AI. The fact that most AI prostate tumours express the AR-regulated PSA gene indicates that receptor transcriptional activity remains intact (65, 86). This activation of AR may occur through low-levels of adrenal androgens or non-androgenic factors. Several mechanisms have been proposed to explain the reactivation of AR in AI disease, after the temporary suppression of receptor activity during hormone ablation therapy. These include an increase in AR expression and disruption of normal androgen

binding (**Section 1.5.1**), ligand-independent (or ligand-reduced) activation of AR through cross talk with alternate signal transduction pathways (**Section 1.5.2**) and the inappropriate expression/activity of AR coregulator proteins (**Section 1.5.3**). All of the above mechanisms can potentially enable AR-expressing prostate cancer cells or the receptor itself to become sensitive to the reduced levels of androgens present after androgen withdrawal therapy.

1.5.1 Overexpression of AR and Promiscuous Ligand Binding

Amplification of the AR gene has been studied in both untreated prostate cancers, which rarely exhibit AR amplification, and in tumours subjected to androgen ablation therapy, of which 20-30 % contain an amplified AR gene (302-304). Thus, AR amplification may be an adaptive response of prostate cancer cells to castrate levels of androgens. Importantly, amplification of AR in androgen-independent tumours also results in higher levels of AR protein expression (305, 306). Over 80 % of locally advanced, androgen-independent prostate cancers, also exhibit high levels of nuclear AR, suggesting that the receptor is active (307, 308). Overall, AR gene amplification and elevated receptor protein expression is believed to be a possible mechanism that sensitizes prostate cancer cells to low levels of androgens, allowing their escape from androgen ablation therapy (86).

Several studies have reported the existence of mutations in the LBD of AR that result in ligand binding promiscuity, enabling the receptor to bind steroids other than androgens and even non-steroidal antagonists (57). The majority of these mutations in prostate cancers affect the ligand binding pocket and are clustered in three main areas of

the LBD, which include amino acids 670-678, 710-730 and 874-910 (143, 309). One of the best-characterized AR mutants is expressed endogenously in the androgen-sensitive LNCaP prostate cancer cell line and in tumours. A single point mutation (Thr877Ala) in the LBD of AR in LNCaP cells allows the receptor to be activated by cognate ligands (T and DHT), as well as estrogen, progesterone, cortisone and anti-androgens (141, 310). The interaction of AR with the p160/SRC coactivators is also facilitated by mutations in the AR-LBD that increase ligand binding promiscuity (311). Additionally, certain coactivators, such as ARA70, have been shown to reduce the ligand specificity of AR, allowing the receptor to be activated by estradiol and the anti-androgen hydroxyflutamide (273). Overall, AR ligand binding promiscuity can sensitize the receptor to adrenal androgens, permit binding to other steroid ligands and allow antagonists to act as agonists for activation of AR in an androgen-depleted environment.

1.5.2 Ligand-Independent/Ligand-Reduced Activation of AR by Growth Factors and Cytokines

A plethora of studies indicate that one of the main mechanisms for progression of prostate cancer to AI involves ligand-independent, or ligand-reduced, activation of AR through the convergence of cell signalling pathways (309, 312). Several growth factors and cytokines have been shown to increase AR transcriptional activity either directly or indirectly through participation of kinases such as MAPK, as well as *via* the PI3K/Akt pathways.

Growth factors that can activate AR independent of androgens, include the epidermal growth factor (EGF), keratinocyte growth factor (KGF) and insulin-like

growth factor-1 (IGF-1) (313). Although the alterations in the expression of the EGF receptor (EGFR) in prostate cancer remain unclear, another EGFR family member, Her2/ErbB2/neu, is overexpressed in AI prostate carcinomas (314, 315). The Her2/neu-positive tumours from these patients also express AR and PSA, suggesting that elevation of Her2/neu levels may allow AR transcription in an androgen-depleted environment (316). In prostate cancer cells, Her2/neu overexpression has been shown to stimulate AR transcriptional activity at low concentrations of androgen, partly through the MAPK pathway (161). Moreover, this increase in AR activity resulted in an enhanced interaction between the ARA70 coactivator and AR, which can further elevate receptor activation. IGF-1 also increases AR transactivation, by binding to the IGF-1 receptor (IGF-1R), presumably through the activation of the PI3K/Akt and MAPK signalling pathways (313, 316).

The cytokine interleukin-6 (IL6) has also been shown to increase AR activity in the absence of androgens, which is thought to occur through the MAPK and STAT3 (signal transducer and activator of transcription 3) signalling pathways (317, 318). The elevated serum levels of IL-6 in patients with AI prostate cancer further suggest that this cytokine may play an important role in disease progression (319). IL-4 has also been shown to increase ligand-independent AR activity and PSA production through Akt activation in LNCaP prostate cancer cells (320). Moreover, IL-4 sensitized AR to low levels of androgen. Additionally, protein kinase A (PKA) and protein kinase C (PKC) have been shown to increase both ligand-dependent and ligand-independent AR transactivation, but how the signalling pathways of these kinases cross-talk with those of growth factors/cytokines is unclear (165, 321, 322). Overall, the activation of AR by

mitogenic signalling cascades in the complete absence of or at low androgen levels suggests that growth factors and cytokines may have a significant effect on prostate cancer progression to AI.

1.5.3 Role of AR Coregulators in Prostate Cancer Progression

Coregulator proteins play a critical role in regulation of AR transcriptional activity, being able to both enhance (coactivator) and suppress (corepressor) AR transactivation (discussed in detail in **Section 1.4**). Hence, in prostate cancer an increase in expression of coactivators or loss of expression of corepressors can both lead to aberrant AR activity that may promote tumour growth and disease progression.

Given the potent role of coactivators in enhancing AR function, it has long been suspected that their expression may be deregulated in prostate tumours, resulting in possible growth promoting effects on AR expressing prostate cancer cells. Recently, it has been shown that increased SRC1 expression in clinically localized androgen-dependent cancer is associated with increased tumour aggressiveness (323). In the same study, reduction of SRC1 expression significantly reduced growth in androgen-sensitive LNCaP prostate cancer cells, whereas this had no effect on growth of AR-negative PC3 and DU145 prostate cancer cell lines. SRC3 has also been shown to be required for proliferation of androgen-dependent prostate cancer cells and for tumour growth through controlling the expression of key cell cycle genes (324). Moreover, SRC3 is overexpressed in prostate cancer patients and its expression correlates inversely with apoptosis of tumour cells (325). Animal studies have shown that genetic disruption of SRC1 in mice results in slightly decreased growth and development of the prostate, but

knockout of SRC2 has no effect on the gland (326-328). The single and combinatorial knockout targeting of these coactivators suggest that they have partial functional redundancy *in vivo*, whereby the absence of one SRC can be compensated for by another family member or by non-SRC coactivators. Clearly, since coactivators increase AR transcriptional activity, their overexpression can potentially promote progression of prostate cancer by sensitizing the receptor to lower concentrations of androgens, after androgen withdrawal therapy (316).

The expression profile and tumour cell growth effects of several other AR coactivators have also been reported recently. CBP/p300 expression has been found to be up-regulated during androgen ablation therapy in patients with prostate carcinoma (329). The disruption of CBP/p300 transcripts through small interfering RNA also inhibits prostate cancer cell proliferation (330). The core subunit of the SWI/SNF coactivator that mediates direct interactions with AR has been shown to be required for the proliferation of AR-dependent prostate cancer cells (331). Although, basal transcriptional machinery components (TFIIF and TFIIH) that are involved in AR activation have not been studied in prostate tumours, molecular chaperones of AR, such as heat shock proteins, have been targeted in order to abrogate AR activity. Currently, hsp90 is a therapeutic target in prostate cancer clinical drug trials, where inhibition of hsp90 has been shown to lead to increased degradation of AR (332, 333). Expression of the ARA70 coactivator has also been reported to increase in high-grade prostate carcinomas and in hormone-deprived prostate cancer cells (273, 334). In addition, expression of gelsolin, another AR coactivator, has been found to increase in LNCaP xenografts and human prostate tumours after androgen-depletion (219). Recently, the

Tip60 AR coactivator was shown to increase in nuclear accumulation in 87 % of hormone-refractory prostate cancer specimens as compared to benign prostatic hyperplasia samples (335). From these studies, it is becoming clear that coactivators of AR not only facilitate receptor transactivation in cells but can also promote tumour cell growth and may play a crucial role in activation of AR in disease.

Although effects of the expression of the original AR corepressors, SMRT and NCoR, in prostate cancer is not clear, their ability to inhibit activated AR in prostate cancer cells suggests that loss of expression of these corepressors could facilitate tumour growth (108, 336). Similarly, expression of cyclin D1 has been found to inhibit cell-cycle progression specifically in AR-dependent prostate cancer cells, suggesting that loss of this corepressor may promote proliferation of these cells (337). Akt has been shown to suppress AR activity as well as activate the receptor but the relevance of its corepressor function in prostate cancer is not clear. However, activation of AR by Akt through the PI3K mediated pathway has been implicated in prostate cancer progression. Increased Akt activity can synergize with AR signalling to promote the initiation and progression of prostate cancer to AI in xenograft tumour models (338, 339). Furthermore, investigations of human prostate cancer tissues has demonstrated that although there is neither Akt gene amplification nor enhanced protein expression in prostate cancer compared to normal tissue, poorly differentiated tumours exhibit increased expression of a phosphorylated (activated) Akt compared to normal tissue. This activated form of Akt may promote androgen-independent survival of prostate tumour cells through the activation of AR (340).

Mutation or loss of the tumour suppressor proteins, p53 and PTEN, both of which corepress AR activity, has been well documented in advanced prostate cancer. PTEN is a critical regulator of prostate cancer growth and progression (289). In fact, loss of PTEN is associated with a decrease in survival rates, being lost at a frequency of approximately 20-27 % in prostate cancer (341, 342). More recently, it was shown that the deletion of PTEN specifically in the prostate gland induces metastatic prostate cancer (343). Since the ability of PTEN to induce apoptosis in prostate cancer cells is reversible with androgen treatment, it has been suggested that PTEN loss may result in enhancement of AR transactivation and promote resistance to cell death for tumour cells (344). Nevertheless, because PTEN plays an important role in regulation of many cell survival pathways, such as PI3K/Akt, it is difficult to conclude that its effects on prostate cancer growth are entirely due to the loss of PTEN corepressor function for AR (345). Mutations and loss of the p53 tumour suppressor protein may also be crucial in cancer development and progression (289). In prostate cancer, approximately 45 % of tumours have been found to contain p53 mutations (346). It has been reported that there is a balance of AR and p53 expression during the androgen-dependent growth of prostate cancer, which is obliterated during progression of the disease (347). Hence, similar to PTEN, it is tempting to speculate that loss of p53 function can result in increased tumour growth through an elevation in AR activity due to loss of corepressor function. However, currently it is difficult to separate this effect from the normal tumour suppressor role of p53. Nevertheless, it is possible that loss of AR corepressors, such as PTEN, p53 and possibly others, is at least in part responsible for increased activation of AR, and may in turn contribute to prostate cancer growth.

As discussed above (**Section 1.5**), the androgen receptor is a critical regulator of prostate cancer development and progression. Since recurrent AI prostate cancer maintains AR activity, inhibition of AR function is the central focus of disease treatment. Due to the importance of AR in prostate cancer, a plethora of studies have tried to identify the coregulator proteins that modify AR action and possibly allow for bypassing therapeutic intervention. It has become clear that coactivators of AR can contribute to aberrant AR activation under androgen-deprived conditions. In addition, compromised AR corepressor function could also contribute to deregulated AR activity during prostate cancer progression. Therefore, AR coregulator proteins may serve as targets for therapy, in addition to the targeting of AR directly and ligand-independent/ligand-reduced signalling pathways that activate the receptor. The clinical relevance of AR coregulator proteins will be the subject of future studies, which will provide additional insights into the mechanism by which prostate tumour cells escape therapy, as well as possible novel methods of treating prostate cancer.

1.6 Scope of Thesis

1.6.1 Hypothesis

The level and specificity of AR transcriptional activity is in part due to interaction of the receptor with unique coregulator proteins. In addition, the abnormal expression or activity of these AR-interacting proteins contributes to progression of prostate cancer to androgen independence.

1.6.2 Rationale and Specific Objectives

The AR functions as an androgen-activated transcription factor that regulates genes involved in growth and development of prostate cancer. Continual and aberrant activation of AR in advanced disease most probably promotes the growth of adenocarcinoma cells under androgen-deprived conditions, following androgen withdrawal. As discussed in detail above (**Section 1.4** and **1.5.3**), the interaction of coregulator proteins with AR play an important role in enhancing or suppressing AR transactivation and both the overexpression of coactivators or loss of expression of corepressors can lead to elevated AR activation. Moreover, coregulator proteins can modulate AR-specific responses, such as expression of the PSA gene. Hence, AR-coregulators may play a crucial role in specific activation of AR during progression of prostate cancer.

The majority of SR coregulator proteins studied to date, including those of the AR, have been identified as a consequence of their binding to the DNA and ligand binding domains. These regions share the highest degree of amino acid sequence homology among receptor family members. Thus, it is of no surprise that these proteins have been found to be promiscuous with respect to the variety of receptors with which they interact. Since the N-terminal domain of SRs is the most variable, it is expected that protein interactions with this region may confer receptor-specific responses. Accordingly, to test the above hypothesis (**Section 1.6.1**), the following specific objectives were devised: 1) to isolate and identify proteins that interact with the unique N-terminal domain of AR, 2) to characterize these AR-binding proteins with respect to coregulation capacity and receptor specificity, 3) to probe into the mechanism by which

novel AR-coregulators modify receptor transactivation and 4) to determine whether inappropriate expression of newly identified AR-coregulators is related to the androgen-independent phenotype of prostate cancer.

The *first* objective of this project was completed by using a form of the reverse yeast-two hybrid system (repressed transactivator; RTA) that is specifically designed for bait proteins possessing intrinsic transactivation activity. A prostate cancer cell line cDNA library was screened using the AR-NTD as bait and a wide range of AR-interacting proteins were identified (**Chapter 2**). Upon confirmation of a subset of these interactions *in vitro* and in prostate cancer cells, the most frequently detected AR-binding protein, L-dopa decarboxylase (DDC), was selected for further analysis. To complete the *second* goal of this study, the effect of DDC overexpression on AR transactivation and that of other SRs was determined (**Chapter 3**). In prostate cancer cells, DDC strongly enhanced ligand-dependent AR and GR transcriptional activity, while ER activity was minimally affected. This suggests that DDC can at least partially act as a receptor-specific coactivator.

The next set of studies focused on utilization of established mechanisms of coactivator action and the known functions of DDC to achieve the *third* aim of this project (**Chapter 4**). DDC was shown to sensitize AR activity to limiting concentrations of androgen. *In vitro* and cell line-based ligand-binding assays demonstrated that DDC can actually increase the apparent androgen binding affinity for AR and the maximum androgen-binding capacity. Moreover, a newly generated LNCaP-DDC tetracycline inducible prostate cancer cell line used in xenograft analysis revealed that induction of

DDC expression increased PSA production specifically in non-castrated mice, indicating the androgen-dependency of DDC coactivation function *in vivo*.

In regards to the known functions of DDC, it was determined that the enhancement of AR activity by DDC is independent of the production of its known enzymatic products (dopamine/serotonin). Importantly, mutational analysis of the DDC pyridoxal phosphate co-factor binding site, necessary for decarboxylation of the α-carboxyl group on aromatic amino acid substrates, completely abrogates the coactivator effect of DDC on AR activity, without altering binding to the receptor. However, liquid chromatography tandem mass spectrometry analysis performed on purified AR, expectedly revealed that DDC does not directly decarboxylate the α-carboxyl group of the AR C-terminal peptide. Overall, these data demonstrate that DDC increases *in vitro* and *in vivo* AR transcriptional activity through an androgen-dependent mechanism that requires its enzymatic activity.

To complete the *fourth* and final objective of this project, the expression profile of DDC was studied as a neuroendocrine cell marker of prostate cancer (**Chapter 5**). Increased prostatic neuroendocrine cell density has been implicated in promoting progression of prostate cancer. The association of NE differentiation with neo-adjuvant hormone therapy and Gleason grade was determined, using tissue microarrays to monitor the expression profile of DDC and known NE markers. DDC was identified as a novel NE marker of prostate cancer. Immunohistochemical analysis of DDC with established NE markers, such as chromogranin-A, revealed a significant increase in NE differentiation (and hence DDC expression) after long-term hormone therapy and after progression to androgen-independence, but no apparent correlation with Gleason grade.

In addition, dual immunofluorescence analysis revealed that a subset of DDC expressing NE cells continue to express AR. Taken together, these results suggest that the increase of NE differentiation and DDC expression in prostate cancers depends specifically on duration of hormone therapy.

Overall, DDC is a potent ligand-dependent coactivator of AR and neuroendocrine marker that co-expresses with the receptor in prostate adenocarcinoma cells with a NE-phenotype. Hence, DDC may play an important role in sustaining AR activation in the androgen-depleted environment that persists during hormone ablation therapy and in progression of prostate cancers to androgen independence.

1.7 References

1. National Cancer Institute of Canada (NCIC): Canadian Cancer Statistics 2006, Toronto, Canada.

2. Cunha GR, Ricke W, Thomson A, Marker PC, Risbridger G, Hayward SW, et al. Hormonal, cellular, and molecular regulation of normal and neoplastic prostatic development. J Steroid Biochem Mol Biol 2004;92(4):221-36.

3. Laczko I, Hudson DL, Freeman A, Feneley MR, Masters JR. Comparison of the zones of the human prostate with the seminal vesicle: morphology, immunohistochemistry, and cell kinetics. Prostate 2005;62(3):260-6.

4. Huggins C, Hodges CV. Studies on prostatic cancer: I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. 1941. J Urol 2002;168(1):9-12.

5. Isaacs JT. Role of androgens in prostatic cancer. Vitam Horm 1994;49:433-502.

6. Fung KY, Glode LM, Green S, Duncan MW. A comprehensive characterization of the peptide and protein constituents of human seminal fluid. Prostate 2004;61(2):171-81.

7. Constantinou J, Feneley MR. PSA testing: an evolving relationship with prostate cancer screening. Prostate Cancer Prostatic Dis 2006;9(1):6-13.

8. Long RM, Morrissey C, Fitzpatrick JM, Watson RW. Prostate epithelial cell differentiation and its relevance to the understanding of prostate cancer therapies. Clin Sci (Lond) 2005;108(1):1-11.

9. Hansson J, Abrahamsson PA. Neuroendocrine pathogenesis in adenocarcinoma of the prostate. Ann Oncol 2001;12 Suppl 2:S145-52.

10. Bonkhoff H, Stein U, Remberger K. The proliferative function of basal cells in the normal and hyperplastic human prostate. Prostate 1994;24(3):114-8.

11. Huss WJ, Gray DR, Werdin ES, Funkhouser WK, Jr., Smith GJ. Evidence of pluripotent human prostate stem cells in a human prostate primary xenograft model. Prostate 2004;60(2):77-90.

12. Sherwood ER, Berg LA, Mitchell NJ, McNeal JE, Kozlowski JM, Lee C. Differential cytokeratin expression in normal, hyperplastic and malignant epithelial cells from human prostate. J Urol 1990;143(1):167-71.

13. Yang Y, Hao J, Liu X, Dalkin B, Nagle RB. Differential expression of cytokeratin mRNA and protein in normal prostate, prostatic intraepithelial neoplasia, and invasive carcinoma. Am J Pathol 1997;150(2):693-704.

14. Evangelou AI, Winter SF, Huss WJ, Bok RA, Greenberg NM. Steroid hormones, polypeptide growth factors, hormone refractory prostate cancer, and the neuroendocrine phenotype. J Cell Biochem 2004;91(4):671-83.

15. Sherwood ER, Theyer G, Steiner G, Berg LA, Kozlowski JM, Lee C. Differential expression of specific cytokeratin polypeptides in the basal and luminal epithelia of the human prostate. Prostate 1991;18(4):303-14.

16. McNeal JE. Normal histology of the prostate. Am J Surg Pathol 1988;12(8):619-33.

17. Rittenhouse HG, Finlay JA, Mikolajczyk SD, Partin AW. Human Kallikrein 2 (hK2) and prostate-specific antigen (PSA): two closely related, but distinct, kallikreins in the prostate. Crit Rev Clin Lab Sci 1998;35(4):275-368.

18. Noordzij MA, van Steenbrugge GJ, van der Kwast TH, Schroder FH. Neuroendocrine cells in the normal, hyperplastic and neoplastic prostate. Urol Res 1995;22(6):333-41.

19. Abrahamsson PA. Neuroendocrine differentiation and hormone-refractory prostate cancer. Prostate Suppl 1996;6:3-8.

20. Krijnen JL, Janssen PJ, Ruizeveld de Winter JA, van Krimpen H, Schroder FH, van der Kwast TH. Do neuroendocrine cells in human prostate cancer express androgen receptor? Histochemistry 1993;100(5):393-8.

21. Huttner WB, Gerdes HH, Rosa P. The granin (chromogranin/secretogranin) family. Trends Biochem Sci 1991;16(1):27-30.

22. Abrahamsson PA, Wadstrom LB, Alumets J, Falkmer S, Grimelius L. Peptide-hormone- and serotonin-immunoreactive tumour cells in carcinoma of the prostate. Pathol Res Pract 1987;182(3):298-307.

23. Abdul M, Anezinis PE, Logothetis CJ, Hoosein NM. Growth inhibition of human prostatic carcinoma cell lines by serotonin antagonists. Anticancer Res 1994;14(3A):1215-20.

24. Aprikian AG, Tremblay L, Han K, Chevalier S. Bombesin stimulates the motility of human prostate-carcinoma cells through tyrosine phosphorylation of focal adhesion kinase and of integrin-associated proteins. Int J Cancer 1997;72(3):498-504.

25. Ritchie CK, Thomas KG, Andrews LR, Tindall DJ, Fitzpatrick LA. Effects of the calciotrophic peptides calcitonin and parathyroid hormone on prostate cancer growth and chemotaxis. Prostate 1997;30(3):183-7.

26. Shah GV, Rayford W, Noble MJ, Austenfeld M, Weigel J, Vamos S, et al. Calcitonin stimulates growth of human prostate cancer cells through receptor-mediated increase in cyclic adenosine 3',5'-monophosphates and cytoplasmic Ca2+ transients. Endocrinology 1994;134(2):596-602.

27. di Sant'Agnese PA. Neuroendocrine differentiation in human prostatic carcinoma. Hum Pathol 1992;23(3):287-96.

28. Sciarra A, Mariotti G, Gentile V, Voria G, Pastore A, Monti S, et al. Neuroendocrine differentiation in human prostate tissue: is it detectable and treatable? BJU Int 2003;91(5):438-45.

29. Isaacs JT, Coffey DS. Etiology and disease process of benign prostatic hyperplasia. Prostate Suppl 1989;2:33-50.

30. Collins AT, Maitland NJ. Prostate cancer stem cells. Eur J Cancer 2006;42(9):1213-8.

31. Lam JS, Reiter RE. Stem cells in prostate and prostate cancer development. Urol Oncol 2006;24(2):131-40.

32. Nagle RB, Ahmann FR, McDaniel KM, Paquin ML, Clark VA, Celniker A. Cytokeratin characterization of human prostatic carcinoma and its derived cell lines. Cancer Res 1987;47(1):281-6.

33. McDonnell TJ, Troncoso P, Brisbay SM, Logothetis C, Chung LW, Hsieh JT, et al. Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. Cancer Res 1992;52(24):6940-4.

34. Aprikian AG, Cordon-Cardo C, Fair WR, Reuter VE. Characterization of neuroendocrine differentiation in human benign prostate and prostatic adenocarcinoma. Cancer 1993;71(12):3952-65.

35. Theodorescu D, Broder SR, Boyd JC, Mills SE, Frierson HF, Jr. Cathepsin D and chromogranin A as predictors of long term disease specific survival after radical prostatectomy for localized carcinoma of the prostate. Cancer 1997;80(11):2109-19.

36. Abrahamsson PA. Neuroendocrine differentiation in prostatic carcinoma. Prostate 1999;39(2):135-48.

37. Bostwick DG, Qian J, Pacelli A, Zincke H, Blute M, Bergstralh EJ, et al. Neuroendocrine expression in node positive prostate cancer: correlation with systemic progression and patient survival. J Urol 2002;168(3):1204-11.

38. Wafa LA, Palmer J, Fazli L, Hurtado-Coll A, Bell RH, Nelson CC, et al. Comprehensive expression analysis of L-dopa decarboxylase and established neuroendocrine markers in neoadjuvant hormone-treated versus varying Gleason grade prostate tumors. Hum Pathol 2007;38(1):161-70.

39. Abrahamsson PA. Neuroendocrine cells in tumour growth of the prostate. Endocr Relat Cancer 1999;6(4):503-19.

40. Bonkhoff H. Neuroendocrine cells in benign and malignant prostate tissue: morphogenesis, proliferation, and androgen receptor status. Prostate Suppl 1998;8:18-22.

41. Cox ME, Deeble PD, Lakhani S, Parsons SJ. Acquisition of neuroendocrine characteristics by prostate tumor cells is reversible: implications for prostate cancer progression. Cancer Res 1999;59(15):3821-30.

42. Wang Q, Horiatis D, Pinski J. Inhibitory effect of IL-6-induced neuroendocrine cells on prostate cancer cell proliferation. Prostate 2004;61(3):253-9.

43. Nakada SY, di Sant'Agnese PA, Moynes RA, Hiipakka RA, Liao S, Cockett AT, et al. The androgen receptor status of neuroendocrine cells in human benign and malignant prostatic tissue. Cancer Res 1993;53(9):1967-70.

44. Roudier MP, True LD, Vessella RL, Higano CS. Metastatic conventional prostatic adenocarcinoma with diffuse chromogranin A and androgen receptor positivity. J Clin Pathol 2004;57(3):321-3.

45. Denmeade SR, Isaacs JT. Principles of endocrine therapy. In: Kufe DW, Pollock RE, Weichselbaum RR, Bast RC Jr, Gansler TS, Holland JF, Frei III E, eds. Cancer Medicine. 6th ed. 2003. Hamilton, Pa: BC Decker Inc.; Chapter 15.

46. Jones EE, DeCherney AH. The male reproductive system. In: Boron WF, Boulpaep EL, eds. Medical Physiology. Updated ed. 2005. Philadelphia, Pa: Saunders; Chapter 53.

47. Voet D, Voet JG. Biochemistry. 1990. New York, Pa: J Wiley & Sons; Chapter 23.

48. Miller WL. Androgen biosynthesis from cholesterol to DHEA. Mol Cell Endocrinol 2002;198(1-2):7-14.

49. Burger HG. Androgen production in women. Fertil Steril 2002;77 Suppl 4:S3-5.

50. Bruchovsky N, Wilson JD. The intranuclear binding of testosterone and 5-alpha-androstan-17-beta-ol-3-one by rat prostate. J Biol Chem 1968;243(22):5953-60.

51. Partin AW, Rodriguez R. The molecular biology, endocrinology and physiology of the prostate and seminal vesicles. In: Walsh PC, Retik AB, Vaughn ED Jr, Wein AJ, eds. Campbells's Urology. 8th ed. 2002. Philadelphia, Pa: Saunders; Chapter 37.

52. Andreassen TK. The role of plasma-binding proteins in the cellular uptake of lipophilic vitamins and steroids. Horm Metab Res 2006;38(4):279-90.

53. Thigpen AE, Davis DL, Milatovich A, Mendonca BB, Imperato-McGinley J, Griffin JE, et al. Molecular genetics of steroid 5 alpha-reductase 2 deficiency. J Clin Invest 1992;90(3):799-809.

54. Jenkins EP, Andersson S, Imperato-McGinley J, Wilson JD, Russell DW. Genetic and pharmacological evidence for more than one human steroid 5 alpha-reductase. J Clin Invest 1992;89(1):293-300.

55. So AI, Hurtado-Coll A, Gleave ME. Androgens and prostate cancer. World J Urol 2003;21(5):325-37.

56. Deslypere JP, Young M, Wilson JD, McPhaul MJ. Testosterone and 5 alpha-dihydrotestosterone interact differently with the androgen receptor to enhance transcription of the MMTV-CAT reporter gene. Mol Cell Endocrinol 1992;88(1-3):15-22.

57. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. Nat Rev Cancer 2001;1(1):34-45.

58. Higgins B, Thompson IM. The Prostate Cancer Prevention Trial: current status. J Urol 2004;171(2 Pt 2):S15-7; discussion S8.

59. Gleave M, Goldenberg SL, Bruchovsky N, Rennie P. Intermittent androgen suppression for prostate cancer: rationale and clinical experience. Prostate Cancer Prostatic Dis 1998;1(6):289-296.

60. Albertsen PC, Hanley JA, Fine J. 20-year outcomes following conservative management of clinically localized prostate cancer. Jama 2005;293(17):2095-101.

61. Gleave M, Miyake H, Chi K. Beyond simple castration: targeting the molecular basis of treatment resistance in advanced prostate cancer. Cancer Chemother Pharmacol 2005;56 Suppl 1:47-57.

62. So A, Gleave M, Hurtado-Col A, Nelson C. Mechanisms of the development of androgen independence in prostate cancer. World J Urol 2005;23(1):1-9.

63. Tannock IF, de Wit R, Berry WR, Horti J, Pluzanska A, Chi KN, et al. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. N Engl J Med 2004;351(15):1502-12.

64. Petrylak DP. Future directions in the treatment of androgen-independent prostate cancer. Urology 2005;65(6 Suppl):8-12.

65. Miller JI, Ahmann FR, Drach GW, Emerson SS, Bottaccini MR. The clinical usefulness of serum prostate specific antigen after hormonal therapy of metastatic prostate cancer. J Urol 1992;147(3 Pt 2):956-61.

66. Sadar MD. Androgen-independent induction of prostate-specific antigen gene expression via cross-talk between the androgen receptor and protein kinase A signal transduction pathways. J Biol Chem 1999;274(12):7777-83.

67. Humphrey PA. Gleason grading and prognostic factors in carcinoma of the prostate. Mod Pathol 2004;17(3):292-306.

68. Mouraviev V, Gleave ME. A meaningful legacy: urologists as Nobel Prize laureates. Can J Urol 2003;10(1):1737-42.

69. Labrie F, Dupont A, Belanger A, St-Arnaud R, Giguere M, Lacourciere Y, et al. Treatment of prostate cancer with gonadotropin-releasing hormone agonists. Endocr Rev 1986;7(1):67-74.

70. Tammela T. Endocrine treatment of prostate cancer. J Steroid Biochem Mol Biol 2004;92(4):287-95.

71. Belanger A, Brochu M, Cliche J. Levels of plasma steroid glucuronides in intact and castrated men with prostatic cancer. J Clin Endocrinol Metab 1986;62(5):812-5.

72. Andriole G, Bostwick D, Civantos F, Epstein J, Lucia MS, McConnell J, et al. The effects of 5alpha-reductase inhibitors on the natural history, detection and grading of prostate cancer: current state of knowledge. J Urol 2005;174(6):2098-104.

73. Culig Z, Bartsch G, Hobisch A. Antiandrogens in prostate cancer endocrine therapy. Curr Cancer Drug Targets 2004;4(5):455-61.

74. Masiello D, Cheng S, Bubley GJ, Lu ML, Balk SP. Bicalutamide functions as an androgen receptor antagonist by assembly of a transcriptionally inactive receptor. J Biol Chem 2002;277(29):26321-6.

75. Klotz L. Combined androgen blockade: an update. Urol Clin North Am 2006;33(2):161-6, v-vi.

76. Oh WK, Manola J, Bittmann L, Brufsky A, Kaplan ID, Smith MR, et al. Finasteride and flutamide therapy in patients with advanced prostate cancer: response to subsequent castration and long-term follow-up. Urology 2003;62(1):99-104.

77. Sato N, Gleave ME, Bruchovsky N, Rennie PS, Goldenberg SL, Lange PH, et al. Intermittent androgen suppression delays progression to androgen-independent regulation of prostate-specific antigen gene in the LNCaP prostate tumour model. J Steroid Biochem Mol Biol 1996;58(2):139-46.

78. Pether M, Goldenberg SL, Bhagirath K, Gleave M. Intermittent androgen suppression in prostate cancer: an update of the Vancouver experience. Can J Urol 2003;10(2):1809-14.

79. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, et al. The nuclear receptor superfamily: the second decade. Cell 1995;83(6):835-9.

80. Truss M, Beato M. Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors. Endocr Rev 1993;14(4):459-79.

81. Danielian PS, White R, Lees JA, Parker MG. Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. Embo J 1992;11(3):1025-33.

82. Olefsky JM. Nuclear receptor minireview series. J Biol Chem 2001;276(40):36863-4.

83. Agoulnik IU, Weigel NL. Androgen receptor action in hormone-dependent and recurrent prostate cancer. J Cell Biochem 2006;99(2):362-72.

84. Chang CS, Kokontis J, Liao ST. Molecular cloning of human and rat complementary DNA encoding androgen receptors. Science 1988;240(4850):324-6.

85. Tsai MJ, O'Malley BW. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 1994;63:451-86.

86. Javidan J, Deitch AD, Shi XB, de Vere White RW. The androgen receptor and mechanisms for androgen independence in prostate cancer. Cancer Invest 2005;23(6):520-8.

87. Greene GL, Press MF. Structure and dynamics of the estrogen receptor. J Steroid Biochem 1986;24(1):1-7.

88. Leo C, Chen JD. The SRC family of nuclear receptor coactivators. Gene 2000;245(1):1-11.

89. Brown CJ, Goss SJ, Lubahn DB, Joseph DR, Wilson EM, French FS, et al. Androgen receptor locus on the human X chromosome: regional localization to Xq11-12 and description of a DNA polymorphism. Am J Hum Genet 1989;44(2):264-9.

90. Kuiper GG, Faber PW, van Rooij HC, van der Korput JA, Ris-Stalpers C, Klaassen P, et al. Structural organization of the human androgen receptor gene. J Mol Endocrinol 1989;2(3):R1-4.

91. Faber PW, Kuiper GG, van Rooij HC, van der Korput JA, Brinkmann AO, Trapman J. The N-terminal domain of the human androgen receptor is encoded by one, large exon. Mol Cell Endocrinol 1989;61(2):257-62.

92. Tilley WD, Marcelli M, McPhaul MJ. Expression of the human androgen receptor gene utilizes a common promoter in diverse human tissues and cell lines. J Biol Chem 1990;265(23):13776-81.

93. Lubahn DB, Joseph DR, Sullivan PM, Willard HF, French FS, Wilson EM. Cloning of human androgen receptor complementary DNA and localization to the X chromosome. Science 1988;240(4850):327-30.

94. McEwan IJ. Molecular mechanisms of androgen receptor-mediated gene regulation: structure-function analysis of the AF-1 domain. Endocr Relat Cancer 2004;11(2):281-93.

95. Chamberlain NL, Whitacre DC, Miesfeld RL. Delineation of two distinct type 1 activation functions in the androgen receptor amino-terminal domain. J Biol Chem 1996;271(43):26772-8.

96. Simental JA, Sar M, Lane MV, French FS, Wilson EM. Transcriptional activation and nuclear targeting signals of the human androgen receptor. J Biol Chem 1991;266(1):510-8.

97. Jenster G, van der Korput HA, van Vroonhoven C, van der Kwast TH, Trapman J, Brinkmann AO. Domains of the human androgen receptor involved in steroid binding, transcriptional activation, and subcellular localization. Mol Endocrinol 1991;5(10):1396-404.

98. Jenster G, van der Korput HA, Trapman J, Brinkmann AO. Identification of two transcription activation units in the N-terminal domain of the human androgen receptor. J Biol Chem 1995;270(13):7341-6.

99. Callewaert L, Van Tilborgh N, Claessens F. Interplay between two hormone-independent activation domains in the androgen receptor. Cancer Res 2006;66(1):543-53.

100. He B, Kemppainen JA, Wilson EM. FXXLF and WXXLF sequences mediate the NH2-terminal interaction with the ligand binding domain of the androgen receptor. J Biol Chem 2000;275(30):22986-94.

101. He B, Wilson EM. The NH(2)-terminal and carboxyl-terminal interaction in the human androgen receptor. Mol Genet Metab 2002;75(4):293-8.

102. Zhou ZX, Lane MV, Kemppainen JA, French FS, Wilson EM. Specificity of ligand-dependent androgen receptor stabilization: receptor domain interactions influence ligand dissociation and receptor stability. Mol Endocrinol 1995;9(2):208-18.

103. Ikonen T, Palvimo JJ, Janne OA. Interaction between the amino- and carboxyl-terminal regions of the rat androgen receptor modulates transcriptional activity and is influenced by nuclear receptor coactivators. J Biol Chem 1997;272(47):29821-8.

104. He B, Kemppainen JA, Voegel JJ, Gronemeyer H, Wilson EM. Activation function 2 in the human androgen receptor ligand binding domain mediates interdomain communication with the NH(2)-terminal domain. J Biol Chem 1999;274(52):37219-25.

105. He B, Bowen NT, Minges JT, Wilson EM. Androgen-induced NH2- and COOH-terminal Interaction Inhibits p160 coactivator recruitment by activation function 2. J Biol Chem 2001;276(45):42293-301.

106. Kumar R, Betney R, Li J, Thompson EB, McEwan IJ. Induced alpha-helix structure in AF1 of the androgen receptor upon binding transcription factor TFIIF. Biochemistry 2004;43(11):3008-13.

107. Brodie J, McEwan IJ. Intra-domain communication between the N-terminal and DNA-binding domains of the androgen receptor: modulation of androgen response element DNA binding. J Mol Endocrinol 2005;34(3):603-15.

108. Chmelar R, Buchanan G, Need EF, Tilley W, Greenberg NM. Androgen receptor coregulators and their involvement in the development and progression of prostate cancer. Int J Cancer 2007;120(4):719-33.

109. La Spada AR, Wilson EM, Lubahn DB, Harding AE, Fischbeck KH. Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. Nature 1991;352(6330):77-9.

110. Walcott JL, Merry DE. Trinucleotide repeat disease. The androgen receptor in spinal and bulbar muscular atrophy. Vitam Horm 2002;65:127-47.

111. Nelson KA, Witte JS. Androgen receptor CAG repeats and prostate cancer. Am J Epidemiol 2002;155(10):883-90.

112. Ferro P, Catalano MG, Dell'Eva R, Fortunati N, Pfeffer U. The androgen receptor CAG repeat: a modifier of carcinogenesis? Mol Cell Endocrinol 2002;193(1-2):109-20.

113. Choong CS, Kemppainen JA, Zhou ZX, Wilson EM. Reduced androgen receptor gene expression with first exon CAG repeat expansion. Mol Endocrinol 1996;10(12):1527-35.

114. Lieberman AP, Harmison G, Strand AD, Olson JM, Fischbeck KH. Altered transcriptional regulation in cells expressing the expanded polyglutamine androgen receptor. Hum Mol Genet 2002;11(17):1967-76.

115. Ding D, Xu L, Menon M, Reddy GP, Barrack ER. Effect of a short CAG (glutamine) repeat on human androgen receptor function. Prostate 2004;58(1):23-32.

116. Hakimi JM, Schoenberg MP, Rondinelli RH, Piantadosi S, Barrack ER. Androgen receptor variants with short glutamine or glycine repeats may identify unique subpopulations of men with prostate cancer. Clin Cancer Res 1997;3(9):1599-608.

117. Alroy I, Freedman LP. DNA binding analysis of glucocorticoid receptor specificity mutants. Nucleic Acids Res 1992;20(5):1045-52.

118. Freedman LP. Anatomy of the steroid receptor zinc finger region. Endocr Rev 1992;13(2):129-45.

119. Schoenmakers E, Alen P, Verrijdt G, Peeters B, Verhoeven G, Rombauts W, et al. Differential DNA binding by the androgen and glucocorticoid receptors involves the second Zn-finger and a C-terminal extension of the DNA-binding domains. Biochem J 1999;341 (Pt 3):515-21.

120. Umesono K, Evans RM. Determinants of target gene specificity for steroid/thyroid hormone receptors. Cell 1989;57(7):1139-46.

121. Hard T, Kellenbach E, Boelens R, Maler BA, Dahlman K, Freedman LP, et al. Solution structure of the glucocorticoid receptor DNA-binding domain. Science 1990;249(4965):157-60.

122. Gewirth DT, Sigler PB. The basis for half-site specificity explored through a non-cognate steroid receptor-DNA complex. Nat Struct Biol 1995;2(5):386-94.

123. Dahlman-Wright K, Grandien K, Nilsson S, Gustafsson JA, Carlstedt-Duke J. Protein-protein interactions between the DNA-binding domains of nuclear receptors: influence on DNA-binding. J Steroid Biochem Mol Biol 1993;45(4):239-50.

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

125. Zhou ZX, Sar M, Simental JA, Lane MV, Wilson EM. A ligand-dependent bipartite nuclear targeting signal in the human androgen receptor. Requirement for the DNA-binding domain and modulation by NH2-terminal and carboxyl-terminal sequences. J Biol Chem 1994;269(18):13115-23.

126. Roche PJ, Hoare SA, Parker MG. A consensus DNA-binding site for the androgen receptor. Mol Endocrinol 1992;6(12):2229-35.

127. Nelson CC, Hendy SC, Shukin RJ, Cheng H, Bruchovsky N, Koop BF, et al. Determinants of DNA sequence specificity of the androgen, progesterone, and glucocorticoid receptors: evidence for differential steroid receptor response elements. Mol Endocrinol 1999;13(12):2090-107.

128. Cleutjens KB, van Eekelen CC, van der Korput HA, Brinkmann AO, Trapman J. Two androgen response regions cooperate in steroid hormone regulated activity of the prostate-specific antigen promoter. J Biol Chem 1996;271(11):6379-88.

129. Zhang J, Gao N, Kasper S, Reid K, Nelson C, Matusik RJ. An androgen-dependent upstream enhancer is essential for high levels of probasin gene expression. Endocrinology 2004;145(1):134-48.

130. Verrijdt G, Schauwaers K, Haelens A, Rombauts W, Claessens F. Functional interplay between two response elements with distinct binding characteristics dictates androgen specificity of the mouse sex-limited protein enhancer. J Biol Chem 2002;277(38):35191-201.

131. Jenster G, Trapman J, Brinkmann AO. Nuclear import of the human androgen receptor. Biochem J 1993;293 (Pt 3):761-8.

132. Zhou ZX, Kemppainen JA, Wilson EM. Identification of three proline-directed phosphorylation sites in the human androgen receptor. Mol Endocrinol 1995;9(5):605-15.

133. Gioeli D, Ficarro SB, Kwiek JJ, Aaronson D, Hancock M, Catling AD, et al. Androgen receptor phosphorylation. Regulation and identification of the phosphorylation sites. J Biol Chem 2002;277(32):29304-14.

134. Fu M, Rao M, Wang C, Sakamaki T, Wang J, Di Vizio D, et al. Acetylation of androgen receptor enhances coactivator binding and promotes prostate cancer cell growth. Mol Cell Biol 2003;23(23):8563-75.

135. Thomas M, Dadgar N, Aphale A, Harrell JM, Kunkel R, Pratt WB, et al. Androgen receptor acetylation site mutations cause trafficking defects, misfolding, and aggregation similar to expanded glutamine tracts. J Biol Chem 2004;279(9):8389-95.

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

137. Jenster G, van der Korput JA, Trapman J, Brinkmann AO. Functional domains of the human androgen receptor. J Steroid Biochem Mol Biol 1992;41(3-8):671-5.

138. Fang Y, Fliss AE, Robins DM, Caplan AJ. Hsp90 regulates androgen receptor hormone binding affinity in vivo. J Biol Chem 1996;271(45):28697-702.

139. Heinlein CA, Chang C. Androgen receptor (AR) coregulators: an overview. Endocr Rev 2002;23(2):175-200.

140. Matias PM, Donner P, Coelho R, Thomaz M, Peixoto C, Macedo S, et al. Structural evidence for ligand specificity in the binding domain of the human androgen receptor. Implications for pathogenic gene mutations. J Biol Chem 2000;275(34):26164-71.

141. Sack JS, Kish KF, Wang C, Attar RM, Kiefer SE, An Y, et al. Crystallographic structures of the ligand-binding domains of the androgen receptor and its T877A mutant complexed with the natural agonist dihydrotestosterone. Proc Natl Acad Sci U S A 2001;98(9):4904-9.

142. Tahiri B, Auzou G, Nicolas JC, Sultan C, Lupo B. Participation of critical residues from the extreme C-terminal end of the human androgen receptor in the ligand binding function. Biochemistry 2001;40(29):8431-7.

143. Gelmann EP. Molecular biology of the androgen receptor. J Clin Oncol 2002;20(13):3001-15.

144. Lanz RB, Rusconi S. A conserved carboxy-terminal subdomain is important for ligand interpretation and transactivation by nuclear receptors. Endocrinology 1994;135(5):2183-95.

145. Maeda M. The conserved residues of the ligand-binding domains of steroid receptors are located in the core of the molecules. J Mol Graph Model 2001;19(6):543-51, 601-6.

146. Ishioka T, Tanatani A, Nagasawa K, Hashimoto Y. Anti-androgens with full antagonistic activity toward human prostate tumor LNCaP cells with mutated androgen receptor. Bioorg Med Chem Lett 2003;13(16):2655-8.

147. Vanaja DK, Mitchell SH, Toft DO, Young CY. Effect of geldanamycin on androgen receptor function and stability. Cell Stress Chaperones 2002;7(1):55-64.

148. Nemoto T, Ohara-Nemoto Y, Shimazaki S, Ota M. Dimerization characteristics of the DNA- and steroid-binding domains of the androgen receptor. J Steroid Biochem Mol Biol 1994;50(5-6):225-33.

149. Snoek R, Rennie PS, Kasper S, Matusik RJ, Bruchovsky N. Induction of cell-free, in vitro transcription by recombinant androgen receptor peptides. J Steroid Biochem Mol Biol 1996;59(3-4):243-50.

150. Moilanen A, Rouleau N, Ikonen T, Palvimo JJ, Janne OA. The presence of a transcription activation function in the hormone-binding domain of androgen receptor is revealed by studies in yeast cells. FEBS Lett 1997;412(2):355-8.

151. Chang CY, McDonnell DP. Evaluation of ligand-dependent changes in AR structure using peptide probes. Mol Endocrinol 2002;16(4):647-60.

152. Estebanez-Perpina E, Moore JM, Mar E, Delgado-Rodrigues E, Nguyen P, Baxter JD, et al. The molecular mechanisms of coactivator utilization in ligand-dependent transactivation by the androgen receptor. J Biol Chem 2005;280(9):8060-8.

153. Langley E, Zhou ZX, Wilson EM. Evidence for an anti-parallel orientation of the ligand-activated human androgen receptor dimer. J Biol Chem 1995;270(50):29983-90.

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

155. He B, Lee LW, Minges JT, Wilson EM. Dependence of selective gene activation on the androgen receptor NH2- and COOH-terminal interaction. J Biol Chem 2002;277(28):25631-9.

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

157. Wong HY, Burghoorn JA, Van Leeuwen M, De Ruiter PE, Schippers E, Blok LJ, et al. Phosphorylation of androgen receptor isoforms. Biochem J 2004;383(Pt 2):267-76.

158. Wen Y, Hu MC, Makino K, Spohn B, Bartholomeusz G, Yan DH, et al. HER-2/neu promotes androgen-independent survival and growth of prostate cancer cells through the Akt pathway. Cancer Res 2000;60(24):6841-5.

159. Taneja SS, Ha S, Swenson NK, Huang HY, Lee P, Melamed J, et al. Cell-specific regulation of androgen receptor phosphorylation in vivo. J Biol Chem 2005;280(49):40916-24.

160. Lin HK, Hu YC, Yang L, Altuwaijri S, Chen YT, Kang HY, et al. Suppression versus induction of androgen receptor functions by the phosphatidylinositol 3-kinase/Akt pathway in prostate cancer LNCaP cells with different passage numbers. J Biol Chem 2003;278(51):50902-7.

161. Yeh S, Lin HK, Kang HY, Thin TH, Lin MF, Chang C. From HER2/Neu signal cascade to androgen receptor and its coactivators: a novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells. Proc Natl Acad Sci U S A 1999;96(10):5458-63.

162. Bakin RE, Gioeli D, Sikes RA, Bissonette EA, Weber MJ. Constitutive activation of the Ras/mitogen-activated protein kinase signaling pathway promotes androgen hypersensitivity in LNCaP prostate cancer cells. Cancer Res 2003;63(8):1981-9.

163. Rochette-Egly C. Nuclear receptors: integration of multiple signalling pathways through phosphorylation. Cell Signal 2003;15(4):355-66.

164. LaFevre-Bernt MA, Ellerby LM. Kennedy's disease. Phosphorylation of the polyglutamine-expanded form of androgen receptor regulates its cleavage by caspase-3 and enhances cell death. J Biol Chem 2003;278(37):34918-24.

165. Nazareth LV, Weigel NL. Activation of the human androgen receptor through a protein kinase A signaling pathway. J Biol Chem 1996;271(33):19900-7.

166. Ray MR, Wafa LA, Cheng H, Snoek R, Fazli L, Gleave M, et al. Cyclin G-associated kinase: a novel androgen receptor-interacting transcriptional coactivator that is overexpressed in hormone refractory prostate cancer. Int J Cancer 2006;118(5):1108-19.

167. Fu M, Wang C, Reutens AT, Wang J, Angeletti RH, Siconolfi-Baez L, et al. p300 and p300/cAMP-response element-binding protein-associated factor acetylate the androgen receptor at sites governing hormone-dependent transactivation. J Biol Chem 2000;275(27):20853-60.

168. Gong J, Zhu J, Goodman OB, Jr., Pestell RG, Schlegel PN, Nanus DM, et al. Activation of p300 histone acetyltransferase activity and acetylation of the androgen receptor by bombesin in prostate cancer cells. Oncogene 2006;25(14):2011-21.

169. Gaughan L, Logan IR, Cook S, Neal DE, Robson CN. Tip60 and histone deacetylase 1 regulate androgen receptor activity through changes to the acetylation status of the receptor. J Biol Chem 2002;277(29):25904-13.

170. Fu M, Rao M, Wu K, Wang C, Zhang X, Hessien M, et al. The androgen receptor acetylation site regulates cAMP and AKT but not ERK-induced activity. J Biol Chem 2004;279(28):29436-49.

171. Korkmaz CG, Fronsdal K, Zhang Y, Lorenzo PI, Saatcioglu F. Potentiation of androgen receptor transcriptional activity by inhibition of histone deacetylation--rescue of transcriptionally compromised mutants. J Endocrinol 2004;182(3):377-89.

172. Poukka H, Karvonen U, Janne OA, Palvimo JJ. Covalent modification of the androgen receptor by small ubiquitin-like modifier 1 (SUMO-1). Proc Natl Acad Sci U S A 2000;97(26):14145-50.

173. Nishida T, Yasuda H. PIAS1 and PIASxalpha function as SUMO-E3 ligases toward androgen receptor and repress androgen receptor-dependent transcription. J Biol Chem 2002;277(44):41311-7.

174. Lin DY, Fang HI, Ma AH, Huang YS, Pu YS, Jenster G, et al. Negative modulation of androgen receptor transcriptional activity by Daxx. Mol Cell Biol 2004;24(24):10529-41.

175. Callewaert L, Verrijdt G, Haelens A, Claessens F. Differential effect of small ubiquitin-like modifier (SUMO)-ylation of the androgen receptor in the control of cooperativity on selective versus canonical response elements. Mol Endocrinol 2004;18(6):1438-49.

176. Ciechanover A. The ubiquitin proteolytic system: from a vague idea, through basic mechanisms, and onto human diseases and drug targeting. Neurology 2006;66(2 Suppl 1):S7-19.

177. Haglund K, Dikic I. Ubiquitylation and cell signaling. Embo J 2005;24(19):3353-9.

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

179. Lin HK, Wang L, Hu YC, Altuwaijri S, Chang C. Phosphorylation-dependent ubiquitylation and degradation of androgen receptor by Akt require Mdm2 E3 ligase. Embo J 2002;21(15):4037-48.

180. He B, Bai S, Hnat AT, Kalman RI, Minges JT, Patterson C, et al. An androgen receptor NH2-terminal conserved motif interacts with the COOH terminus of the Hsp70-interacting protein (CHIP). J Biol Chem 2004;279(29):30643-53.

181. Burgdorf S, Leister P, Scheidtmann KH. TSG101 interacts with apoptosis-antagonizing transcription factor and enhances androgen receptor-mediated transcription by promoting its monoubiquitination. J Biol Chem 2004;279(17):17524-34.

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

183. Verma S, Ismail A, Gao X, Fu G, Li X, O'Malley BW, et al. The ubiquitin-conjugating enzyme UBCH7 acts as a coactivator for steroid hormone receptors. Mol Cell Biol 2004;24(19):8716-26.

184. Yu H, Peters JM, King RW, Page AM, Hieter P, Kirschner MW. Identification of a cullin homology region in a subunit of the anaphase-promoting complex. Science 1998;279(5354):1219-22.

185. Rundlett SE, Miesfeld RL. Quantitative differences in androgen and glucocorticoid receptor DNA binding properties contribute to receptor-selective transcriptional regulation. Mol Cell Endocrinol 1995;109(1):1-10.

186. Rennie PS, Bowden JF, Freeman SN, Bruchovsky N, Cheng H, Lubahn DB, et al. Cortisol alters gene expression during involution of the rat ventral prostate. Mol Endocrinol 1989;3(4):703-8.

187. Freeman SN, Rennie PS, Chao J, Lund LR, Andreasen PA. Urokinase- and tissue-type plasminogen activators are suppressed by cortisol in the involuting prostate of castrated rats. Biochem J 1990;269(1):189-93.

188. Rennie PS, Bruchovsky N, Leco KJ, Sheppard PC, McQueen SA, Cheng H, et al. Characterization of two cis-acting DNA elements involved in the androgen regulation of the probasin gene. Mol Endocrinol 1993;7(1):23-36.

189. Kasper S, Rennie PS, Bruchovsky N, Sheppard PC, Cheng H, Lin L, et al. Cooperative binding of androgen receptors to two DNA sequences is required for androgen induction of the probasin gene. J Biol Chem 1994;269(50):31763-9.

190. Scheller A, Scheinman RI, Thompson E, Scarlett CO, Robins DM. Contextual dependence of steroid receptor function on an androgen-responsive enhancer. Mol Cell Endocrinol 1996;121(1):75-86.

191. Huang W, Shostak Y, Tarr P, Sawyers C, Carey M. Cooperative assembly of androgen receptor into a nucleoprotein complex that regulates the prostate-specific antigen enhancer. J Biol Chem 1999;274(36):25756-68.

192. McKenna NJ, Lanz RB, O'Malley BW. Nuclear receptor coregulators: cellular and molecular biology. Endocr Rev 1999;20(3):321-44.

193. Xu L, Glass CK, Rosenfeld MG. Coactivator and corepressor complexes in nuclear receptor function. Curr Opin Genet Dev 1999;9(2):140-7.

194. Onate SA, Tsai SY, Tsai MJ, O'Malley BW. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 1995;270(5240):1354-7.

195. Ding XF, Anderson CM, Ma H, Hong H, Uht RM, Kushner PJ, et al. Nuclear receptor-binding sites of coactivators glucocorticoid receptor interacting protein 1 (GRIP1) and steroid receptor coactivator 1 (SRC-1): multiple motifs with different binding specificities. Mol Endocrinol 1998;12(2):302-13.

196. Ozers MS, Marks BD, Gowda K, Kupcho KR, Ervin KM, De Rosier T, et al. The androgen receptor T877A mutant recruits LXXLL and FXXLF peptides differently than wild-type androgen receptor in a time-resolved fluorescence resonance energy transfer assay. Biochemistry 2007;46(3):683-95.

197. Spencer TE, Jenster G, Burcin MM, Allis CD, Zhou J, Mizzen CA, et al. Steroid receptor coactivator-1 is a histone acetyltransferase. Nature 1997;389(6647):194-8.

198. Chen H, Lin RJ, Schiltz RL, Chakravarti D, Nash A, Nagy L, et al. Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. Cell 1997;90(3):569-80.

199. Fronsdal K, Engedal N, Slagsvold T, Saatcioglu F. CREB binding protein is a coactivator for the androgen receptor and mediates cross-talk with AP-1. J Biol Chem 1998;273(48):31853-9.

200. Reutens AT, Fu M, Wang C, Albanese C, McPhaul MJ, Sun Z, et al. Cyclin D1 binds the androgen receptor and regulates hormone-dependent signaling in a p300/CBP-associated factor (P/CAF)-dependent manner. Mol Endocrinol 2001;15(5):797-811.

201. Marshall TW, Link KA, Petre-Draviam CE, Knudsen KE. Differential requirement of SWI/SNF for androgen receptor activity. J Biol Chem 2003;278(33):30605-13.

202. Takeshita A, Yen PM, Misiti S, Cardona GR, Liu Y, Chin WW. Molecular cloning and properties of a full-length putative thyroid hormone receptor coactivator. Endocrinology 1996;137(8):3594-7.

203. McEwan IJ, Gustafsson J. Interaction of the human androgen receptor transactivation function with the general transcription factor TFIIF. Proc Natl Acad Sci U S A 1997;94(16):8485-90.

204. Lee DK, Duan HO, Chang C. From androgen receptor to the general transcription factor TFIIH. Identification of cdk activating kinase (CAK) as an androgen receptor NH(2)-terminal associated coactivator. J Biol Chem 2000;275(13):9308-13.

205. Lee DK, Li M, Chang C. The second largest subunit of RNA polymerase II interacts with and enhances transactivation of androgen receptor. Biochem Biophys Res Commun 2003;302(1):162-9.

206. Veldscholte J, Berrevoets CA, Zegers ND, van der Kwast TH, Grootegoed JA, Mulder E. Hormone-induced dissociation of the androgen receptor-heat-shock protein complex: use of a new monoclonal antibody to distinguish transformed from nontransformed receptors. Biochemistry 1992;31(32):7422-30.

207. Smith DF, Toft DO. Steroid receptors and their associated proteins. Mol Endocrinol 1993;7(1):4-11.

208. Shatkina L, Mink S, Rogatsch H, Klocker H, Langer G, Nestl A, et al. The cochaperone Bag-1L enhances androgen receptor action via interaction with the NH2-terminal region of the receptor. Mol Cell Biol 2003;23(20):7189-97.

209. Marivoet S, Van Dijck P, Verhoeven G, Heyns W. Interaction of the 90-kDa heat shock protein with native and in vitro translated androgen receptor and receptor fragments. Mol Cell Endocrinol 1992;88(1-3):165-74.

210. Caplan AJ, Langley E, Wilson EM, Vidal J. Hormone-dependent transactivation by the human androgen receptor is regulated by a dnaJ protein. J Biol Chem 1995;270(10):5251-7.

211. Fan CY, Ren HY, Lee P, Caplan AJ, Cyr DM. The type I Hsp40 zinc finger-like region is required for Hsp70 to capture non-native polypeptides from Ydj1. J Biol Chem 2005;280(1):695-702.

212. Freeman BC, Felts SJ, Toft DO, Yamamoto KR. The p23 molecular chaperones act at a late step in intracellular receptor action to differentially affect ligand efficacies. Genes Dev 2000;14(4):422-34.

213. Thomas M, Harrell JM, Morishima Y, Peng HM, Pratt WB, Lieberman AP. Pharmacologic and genetic inhibition of hsp90-dependent trafficking reduces aggregation and promotes degradation of the expanded glutamine androgen receptor without stress protein induction. Hum Mol Genet 2006;15(11):1876-83.

214. Froesch BA, Takayama S, Reed JC. BAG-1L protein enhances androgen receptor function. J Biol Chem 1998;273(19):11660-6.

215. Yeh S, Chang C. Cloning and characterization of a specific coactivator, ARA70, for the androgen receptor in human prostate cells. Proc Natl Acad Sci U S A 1996;93(11):5517-21.

216. Moilanen AM, Karvonen U, Poukka H, Janne OA, Palvimo JJ. Activation of androgen receptor function by a novel nuclear protein kinase. Mol Biol Cell 1998;9(9):2527-43.

217. Ozanne DM, Brady ME, Cook S, Gaughan L, Neal DE, Robson CN. Androgen receptor nuclear translocation is facilitated by the f-actin cross-linking protein filamin. Mol Endocrinol 2000;14(10):1618-26.

218. Ting HJ, Yeh S, Nishimura K, Chang C. Supervillin associates with androgen receptor and modulates its transcriptional activity. Proc Natl Acad Sci U S A 2002;99(2):661-6.

219. Nishimura K, Ting HJ, Harada Y, Tokizane T, Nonomura N, Kang HY, et al. Modulation of androgen receptor transactivation by gelsolin: a newly identified androgen receptor coregulator. Cancer Res 2003;63(16):4888-94.

220. Hsiao PW, Lin DL, Nakao R, Chang C. The linkage of Kennedy's neuron disease to ARA24, the first identified androgen receptor polyglutamine region-associated coactivator. J Biol Chem 1999;274(29):20229-34.

221. Lu ML, Schneider MC, Zheng Y, Zhang X, Richie JP. Caveolin-1 interacts with androgen receptor. A positive modulator of androgen receptor mediated transactivation. J Biol Chem 2001;276(16):13442-51.

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

223. Hsiao PW, Chang C. Isolation and characterization of ARA160 as the first androgen receptor N-terminal-associated coactivator in human prostate cells. J Biol Chem 1999;274(32):22373-9.

224. Wang X, Yeh S, Wu G, Hsu CL, Wang L, Chiang T, et al. Identification and characterization of a novel androgen receptor coregulator ARA267-alpha in prostate cancer cells. J Biol Chem 2001;276(44):40417-23.

225. Yeh S, Hu YC, Rahman M, Lin HK, Hsu CL, Ting HJ, et al. Increase of androgen-induced cell death and androgen receptor transactivation by BRCA1 in prostate cancer cells. Proc Natl Acad Sci U S A 2000;97(21):11256-61.

226. Yeh S, Miyamoto H, Nishimura K, Kang H, Ludlow J, Hsiao P, et al. Retinoblastoma, a tumor suppressor, is a coactivator for the androgen receptor in human prostate cancer DU145 cells. Biochem Biophys Res Commun 1998;248(2):361-7.

227. Brady ME, Ozanne DM, Gaughan L, Waite I, Cook S, Neal DE, et al. Tip60 is a nuclear hormone receptor coactivator. J Biol Chem 1999;274(25):17599-604.

228. Truica CI, Byers S, Gelmann EP. Beta-catenin affects androgen receptor transcriptional activity and ligand specificity. Cancer Res 2000;60(17):4709-13.

229. Mulholland DJ, Cheng H, Reid K, Rennie PS, Nelson CC. The androgen receptor can promote beta-catenin nuclear translocation independently of adenomatous polyposis coli. J Biol Chem 2002;277(20):17933-43.

230. Gao N, Zhang J, Rao MA, Case TC, Mirosevich J, Wang Y, et al. The role of hepatocyte nuclear factor-3 alpha (Forkhead Box A1) and androgen receptor in transcriptional regulation of prostatic genes. Mol Endocrinol 2003;17(8):1484-507.

231. Taneja SS, Ha S, Swenson NK, Torra IP, Rome S, Walden PD, et al. ART-27, an androgen receptor coactivator regulated in prostate development and cancer. J Biol Chem 2004;279(14):13944-52.

232. Mayeur GL, Kung WJ, Martinez A, Izumiya C, Chen DJ, Kung HJ. Ku is a novel transcriptional recycling coactivator of the androgen receptor in prostate cancer cells. J Biol Chem 2005;280(11):10827-33.

233. Ma AH, Xia L, Desai SJ, Boucher DL, Guan Y, Shih HM, et al. Male Germ Cell-Associated Kinase, a Male-Specific Kinase Regulated by Androgen, Is a Coactivator of Androgen Receptor in Prostate Cancer Cells. Cancer Res 2006;66(17):8439-8447.

234. Needham M, Raines S, McPheat J, Stacey C, Ellston J, Hoare S, et al. Differential interaction of steroid hormone receptors with LXXLL motifs in SRC-1a depends on residues flanking the motif. J Steroid Biochem Mol Biol 2000;72(1-2):35-46.

235. Wang Q, Lu J, Yong EL. Ligand- and coactivator-mediated transactivation function (AF2) of the androgen receptor ligand-binding domain is inhibited by the cognate hinge region. J Biol Chem 2001;276(10):7493-9.

236. He B, Gampe RT, Jr., Hnat AT, Faggart JL, Minges JT, French FS, et al. Probing the functional link between androgen receptor coactivator and ligand-binding sites in prostate cancer and androgen insensitivity. J Biol Chem 2006;281(10):6648-63.

237. He B, Minges JT, Lee LW, Wilson EM. The FXXLF motif mediates androgen receptor-specific interactions with coregulators. J Biol Chem 2002;277(12):10226-35.

238. Dubbink HJ, Hersmus R, Verma CS, van der Korput HA, Berrevoets CA, van Tol J, et al. Distinct recognition modes of FXXLF and LXXLL motifs by the androgen receptor. Mol Endocrinol 2004;18(9):2132-50.

239. Li J, Zhang D, Fu J, Huang Z, Wong J. Structural and Functional Analysis of Androgen Receptor in Chromatin. Mol Endocrinol 2007.

240. Chakravarti D, LaMorte VJ, Nelson MC, Nakajima T, Schulman IG, Juguilon H, et al. Role of CBP/P300 in nuclear receptor signalling. Nature 1996;383(6595):99-103.

241. Blanco JC, Minucci S, Lu J, Yang XJ, Walker KK, Chen H, et al. The histone acetylase PCAF is a nuclear receptor coactivator. Genes Dev 1998;12(11):1638-51.

242. Rosenfeld MG, Glass CK. Coregulator codes of transcriptional regulation by nuclear receptors. J Biol Chem 2001;276(40):36865-8.

243. Rhodes D. Chromatin structure. The nucleosome core all wrapped up. Nature 1997;389(6648):231, 233.

244. Ogryzko VV, Schiltz RL, Russanova V, Howard BH, Nakatani Y. The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell 1996;87(5):953-9.

245. Heery DM, Kalkhoven E, Hoare S, Parker MG. A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature 1997;387(6634):733-6.

246. Aarnisalo P, Palvimo JJ, Janne OA. CREB-binding protein in androgen receptor-mediated signaling. Proc Natl Acad Sci U S A 1998;95(5):2122-7.

247. Black BE, Vitto MJ, Gioeli D, Spencer A, Afshar N, Conaway MR, et al. Transient, ligand-dependent arrest of the androgen receptor in subnuclear foci alters phosphorylation and coactivator interactions. Mol Endocrinol 2004;18(4):834-50.

248. Kim J, Jia L, Stallcup MR, Coetzee GA. The role of protein kinase A pathway and cAMP responsive element-binding protein in androgen receptor-mediated transcription at the prostate-specific antigen locus. J Mol Endocrinol 2005;34(1):107-18.

249. Li J, Fu J, Toumazou C, Yoon HG, Wong J. A role of the amino-terminal (N) and carboxyl-terminal (C) interaction in binding of androgen receptor to chromatin. Mol Endocrinol 2006;20(4):776-85.

250. Huang ZQ, Li J, Sachs LM, Cole PA, Wong J. A role for cofactor-cofactor and cofactor-histone interactions in targeting p300, SWI/SNF and Mediator for transcription. Embo J 2003;22(9):2146-55.

251. Chiba H, Muramatsu M, Nomoto A, Kato H. Two human homologues of Saccharomyces cerevisiae SWI2/SNF2 and Drosophila brahma are transcriptional coactivators cooperating with the estrogen receptor and the retinoic acid receptor. Nucleic Acids Res 1994;22(10):1815-20.

252. Ratajczak T. Protein coregulators that mediate estrogen receptor function. Reprod Fertil Dev 2001;13(4):221-9.

253. Lambert JR, Nordeen SK. CBP recruitment and histone acetylation in differential gene induction by glucocorticoids and progestins. Mol Endocrinol 2003;17(6):1085-94.

254. McMahon SB, Van Buskirk HA, Dugan KA, Copeland TD, Cole MD. The novel ATM-related protein TRRAP is an essential cofactor for the c-Myc and E2F oncoproteins. Cell 1998;94(3):363-74.

255. Yanagisawa J, Kitagawa H, Yanagida M, Wada O, Ogawa S, Nakagomi M, et al. Nuclear receptor function requires a TFTC-type histone acetyl transferase complex. Mol Cell 2002;9(3):553-62.

256. Roeder RG. The role of general initiation factors in transcription by RNA polymerase II. Trends Biochem Sci 1996;21(9):327-35.

257. Lee DK, Chang C. Molecular communication between androgen receptor and general transcription machinery. J Steroid Biochem Mol Biol 2003;84(1):41-9.

258. Choudhry MA, Ball A, McEwan IJ. The role of the general transcription factor IIF in androgen receptor-dependent transcription. Mol Endocrinol 2006;20(9):2052-61.

259. Tora L. A unified nomenclature for TATA box binding protein (TBP)-associated factors (TAFs) involved in RNA polymerase II transcription. Genes Dev 2002;16(6):673-5.

260. Albright SR, Tjian R. TAFs revisited: more data reveal new twists and confirm old ideas. Gene 2000;242(1-2):1-13.

261. Bukau B, Horwich AL. The Hsp70 and Hsp60 chaperone machines. Cell 1998;92(3):351-66.

262. Prescott J, Coetzee GA. Molecular chaperones throughout the life cycle of the androgen receptor. Cancer Lett 2006;231(1):12-9.

263. Pratt WB, Toft DO. Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocr Rev 1997;18(3):306-60.

264. Dittmar KD, Banach M, Galigniana MD, Pratt WB. The role of DnaJ-like proteins in glucocorticoid receptor.hsp90 heterocomplex assembly by the reconstituted hsp90.p60.hsp70 foldosome complex. J Biol Chem 1998;273(13):7358-66.

265. Kosano H, Stensgard B, Charlesworth MC, McMahon N, Toft D. The assembly of progesterone receptor-hsp90 complexes using purified proteins. J Biol Chem 1998;273(49):32973-9.

266. Bohen SP, Kralli A, Yamamoto KR. Hold 'em and fold 'em: chaperones and signal transduction. Science 1995;268(5215):1303-4.

267. Owens-Grillo JK, Czar MJ, Hutchison KA, Hoffmann K, Perdew GH, Pratt WB. A model of protein targeting mediated by immunophilins and other proteins that bind to hsp90 via tetratricopeptide repeat domains. J Biol Chem 1996;271(23):13468-75.

268. Davies TH, Ning YM, Sanchez ER. A new first step in activation of steroid receptors: hormone-induced switching of FKBP51 and FKBP52 immunophilins. J Biol Chem 2002;277(7):4597-600.

269. Rassow J, Voos W, Pfanner N. Partner proteins determine multiple functions of Hsp70. Trends Cell Biol 1995;5(5):207-12.

270. Caplan AJ, Douglas MG. Characterization of YDJ1: a yeast homologue of the bacterial dnaJ protein. J Cell Biol 1991;114(4):609-21.

271. Krongrad A, Wilson CM, Wilson JD, Allman DR, McPhaul MJ. Androgen increases androgen receptor protein while decreasing receptor mRNA in LNCaP cells. Mol Cell Endocrinol 1991;76(1-3):79-88.

272. Miyamoto H, Yeh S, Wilding G, Chang C. Promotion of agonist activity of antiandrogens by the androgen receptor coactivator, ARA70, in human prostate cancer DU145 cells. Proc Natl Acad Sci U S A 1998;95(13):7379-84.

273. Hu YC, Yeh S, Yeh SD, Sampson ER, Huang J, Li P, et al. Functional domain and motif analyses of androgen receptor coregulator ARA70 and its differential expression in prostate cancer. J Biol Chem 2004;279(32):33438-46.

274. Wafa LA, Cheng H, Rao MA, Nelson CC, Cox M, Hirst M, et al. Isolation and identification of L-dopa decarboxylase as a protein that binds to and enhances transcriptional activity of the androgen receptor using the repressed transactivator yeast two-hybrid system. Biochem J 2003;375(Pt 2):373-83.

275. Stossel TP, Chaponnier C, Ezzell RM, Hartwig JH, Janmey PA, Kwiatkowski DJ, et al. Nonmuscle actin-binding proteins. Annu Rev Cell Biol 1985;1:353-402.

276. Loy CJ, Sim KS, Yong EL. Filamin-A fragment localizes to the nucleus to regulate androgen receptor and coactivator functions. Proc Natl Acad Sci U S A 2003;100(8):4562-7.

277. Edmonds BT, Wyckoff J, Yeung YG, Wang Y, Stanley ER, Jones J, et al. Elongation factor-1 alpha is an overexpressed actin binding protein in metastatic rat mammary adenocarcinoma. J Cell Sci 1996;109 (Pt 11):2705-14.

278. Gangwani L, Mikrut M, Galcheva-Gargova Z, Davis RJ. Interaction of ZPR1 with translation elongation factor-1alpha in proliferating cells. J Cell Biol 1998;143(6):1471-84.

279. Li L, Ren CH, Tahir SA, Ren C, Thompson TC. Caveolin-1 maintains activated Akt in prostate cancer cells through scaffolding domain binding site interactions with and inhibition of serine/threonine protein phosphatases PP1 and PP2A. Mol Cell Biol 2003;23(24):9389-404.

280. Dasso M. Running on Ran: nuclear transport and the mitotic spindle. Cell 2001;104(3):321-4.

281. Wang L, Hsu CL, Chang C. Androgen receptor corepressors: an overview. Prostate 2005;63(2):117-30.

282. Dotzlaw H, Moehren U, Mink S, Cato AC, Iniguez Lluhi JA, Baniahmad A. The amino terminus of the human AR is target for corepressor action and antihormone agonism. Mol Endocrinol 2002;16(4):661-73.

283. Liao G, Chen LY, Zhang A, Godavarthy A, Xia F, Ghosh JC, et al. Regulation of androgen receptor activity by the nuclear receptor corepressor SMRT. J Biol Chem 2003;278(7):5052-61.

284. Cheng S, Brzostek S, Lee SR, Hollenberg AN, Balk SP. Inhibition of the dihydrotestosterone-activated androgen receptor by nuclear receptor corepressor. Mol Endocrinol 2002;16(7):1492-501.

285. Gobinet J, Carascossa S, Cavailles V, Vignon F, Nicolas JC, Jalaguier S. SHP represses transcriptional activity via recruitment of histone deacetylases. Biochemistry 2005;44(16):6312-20.

286. Dedhar S, Rennie PS, Shago M, Hagesteijn CY, Yang H, Filmus J, et al. Inhibition of nuclear hormone receptor activity by calreticulin. Nature 1994;367(6462):480-3.

287. Holter E, Kotaja N, Makela S, Strauss L, Kietz S, Janne OA, et al. Inhibition of androgen receptor (AR) function by the reproductive orphan nuclear receptor DAX-1. Mol Endocrinol 2002;16(3):515-28.

288. Knudsen KE, Cavenee WK, Arden KC. D-type cyclins complex with the androgen receptor and inhibit its transcriptional transactivation ability. Cancer Res 1999;59(10):2297-301.

289. Burd CJ, Morey LM, Knudsen KE. Androgen receptor corepressors and prostate cancer. Endocr Relat Cancer 2006;13(4):979-94.

290. Gobinet J, Auzou G, Nicolas JC, Sultan C, Jalaguier S. Characterization of the interaction between androgen receptor and a new transcriptional inhibitor, SHP. Biochemistry 2001;40(50):15369-77.

291. Lin HK, Yeh S, Kang HY, Chang C. Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor. Proc Natl Acad Sci U S A 2001;98(13):7200-5.

292. Wang L, Lin HK, Hu YC, Xie S, Yang L, Chang C. Suppression of androgen receptor-mediated transactivation and cell growth by the glycogen synthase kinase 3 beta in prostate cells. J Biol Chem 2004;279(31):32444-52.

293. Shenk JL, Fisher CJ, Chen SY, Zhou XF, Tillman K, Shemshedini L. p53 represses androgen-induced transactivation of prostate-specific antigen by disrupting hAR amino- to carboxyl-terminal interaction. J Biol Chem 2001;276(42):38472-9.

294. Sharma M, Zarnegar M, Li X, Lim B, Sun Z. Androgen receptor interacts with a novel MYST protein, HBO1. J Biol Chem 2000;275(45):35200-8.

295. Lin HK, Hu YC, Lee DK, Chang C. Regulation of androgen receptor signaling by PTEN (phosphatase and tensin homolog deleted on chromosome 10) tumor suppressor through distinct mechanisms in prostate cancer cells. Mol Endocrinol 2004;18(10):2409-23.

296. Moehren U, Papaioannou M, Reeb CA, Hong W, Baniahmad A. Alien interacts with the human androgen receptor and inhibits prostate cancer cell growth. Mol Endocrinol 2007.

297. Yoon HG, Wong J. The corepressors silencing mediator of retinoid and thyroid hormone receptor and nuclear receptor corepressor are involved in agonist- and antagonist-regulated transcription by androgen receptor. Mol Endocrinol 2006;20(5):1048-60.

298. Knudsen KE, Weber E, Arden KC, Cavenee WK, Feramisco JR, Knudsen ES. The retinoblastoma tumor suppressor inhibits cellular proliferation through two distinct mechanisms: inhibition of cell cycle progression and induction of cell death. Oncogene 1999;18(37):5239-45.

299. Petre-Draviam CE, Williams EB, Burd CJ, Gladden A, Moghadam H, Meller J, et al. A central domain of cyclin D1 mediates nuclear receptor corepressor activity. Oncogene 2005;24(3):431-44.

300. Gregory CW, Whang YE, McCall W, Fei X, Liu Y, Ponguta LA, et al. Heregulin-induced activation of HER2 and HER3 increases androgen receptor transactivation and CWR-R1 human recurrent prostate cancer cell growth. Clin Cancer Res 2005;11(5):1704-12.

301. Dressel U, Thormeyer D, Altincicek B, Paululat A, Eggert M, Schneider S, et al. Alien, a highly conserved protein with characteristics of a corepressor for members of the nuclear hormone receptor superfamily. Mol Cell Biol 1999;19(5):3383-94.

302. Visakorpi T, Hyytinen E, Koivisto P, Tanner M, Keinanen R, Palmberg C, et al. In vivo amplification of the androgen receptor gene and progression of human prostate cancer. Nat Genet 1995;9(4):401-6.

303. Koivisto P, Kononen J, Palmberg C, Tammela T, Hyytinen E, Isola J, et al. Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. Cancer Res 1997;57(2):314-9.

304. Wallen MJ, Linja M, Kaartinen K, Schleutker J, Visakorpi T. Androgen receptor gene mutations in hormone-refractory prostate cancer. J Pathol 1999;189(4):559-63.

305. Linja MJ, Savinainen KJ, Saramaki OR, Tammela TL, Vessella RL, Visakorpi T. Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. Cancer Res 2001;61(9):3550-5.

306. Edwards J, Krishna NS, Grigor KM, Bartlett JM. Androgen receptor gene amplification and protein expression in hormone refractory prostate cancer. Br J Cancer 2003;89(3):552-6.

307. Tilley WD, Lim-Tio SS, Horsfall DJ, Aspinall JO, Marshall VR, Skinner JM. Detection of discrete androgen receptor epitopes in prostate cancer by immunostaining: measurement by color video image analysis. Cancer Res 1994;54(15):4096-102.

308. Hobisch A, Culig Z, Radmayr C, Bartsch G, Klocker H, Hittmair A. Distant metastases from prostatic carcinoma express androgen receptor protein. Cancer Res 1995;55(14):3068-72.

309. Taplin ME, Balk SP. Androgen receptor: a key molecule in the progression of prostate cancer to hormone independence. J Cell Biochem 2004;91(3):483-90.

310. Steketee K, Timmerman L, Ziel-van der Made AC, Doesburg P, Brinkmann AO, Trapman J. Broadened ligand responsiveness of androgen receptor mutants obtained by random amino acid substitution of H874 and mutation hot spot T877 in prostate cancer. Int J Cancer 2002;100(3):309-17.

311. Duff J, McEwan IJ. Mutation of histidine 874 in the androgen receptor ligand-binding domain leads to promiscuous ligand activation and altered p160 coactivator interactions. Mol Endocrinol 2005;19(12):2943-54.

312. Culig Z, Bartsch G. Androgen axis in prostate cancer. J Cell Biochem 2006;99(2):373-81.

313. Culig Z, Hobisch A, Cronauer MV, Radmayr C, Trapman J, Hittmair A, et al. Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. Cancer Res 1994;54(20):5474-8.

314. Sadasivan R, Morgan R, Jennings S, Austenfeld M, Van Veldhuizen P, Stephens R, et al. Overexpression of Her-2/neu may be an indicator of poor prognosis in prostate cancer. J Urol 1993;150(1):126-31.

315. Signoretti S, Montironi R, Manola J, Altimari A, Tam C, Bubley G, et al. Her-2-neu expression and progression toward androgen independence in human prostate cancer. J Natl Cancer Inst 2000;92(23):1918-25.

316. Heinlein CA, Chang C. Androgen receptor in prostate cancer. Endocr Rev 2004;25(2):276-308.

317. Hobisch A, Eder IE, Putz T, Horninger W, Bartsch G, Klocker H, et al. Interleukin-6 regulates prostate-specific protein expression in prostate carcinoma cells by activation of the androgen receptor. Cancer Res 1998;58(20):4640-5.

318. Ueda T, Bruchovsky N, Sadar MD. Activation of the androgen receptor N-terminal domain by interleukin-6 via MAPK and STAT3 signal transduction pathways. J Biol Chem 2002;277(9):7076-85.

319. Drachenberg DE, Elgamal AA, Rowbotham R, Peterson M, Murphy GP. Circulating levels of interleukin-6 in patients with hormone refractory prostate cancer. Prostate 1999;41(2):127-33.

320. Lee SO, Lou W, Hou M, Onate SA, Gao AC. Interleukin-4 enhances prostate-specific antigen expression by activation of the androgen receptor and Akt pathway. Oncogene 2003;22(39):7981-8.

321. Culig Z, Klocker H, Bartsch G, Hobisch A. Androgen receptors in prostate cancer. Endocr Relat Cancer 2002;9(3):155-70.

322. Darne C, Veyssiere G, Jean C. Phorbol ester causes ligand-independent activation of the androgen receptor. Eur J Biochem 1998;256(3):541-9.

323. Agoulnik IU, Vaid A, Bingman WE, 3rd, Erdeme H, Frolov A, Smith CL, et al. Role of SRC-1 in the promotion of prostate cancer cell growth and tumor progression. Cancer Res 2005;65(17):7959-67.

324. Zou JX, Zhong Z, Shi XB, Tepper CG, deVere White RW, Kung HJ, et al. ACTR/AIB1/SRC-3 and androgen receptor control prostate cancer cell proliferation and tumor growth through direct control of cell cycle genes. Prostate 2006;66(14):1474-86.

325. Zhou HJ, Yan J, Luo W, Ayala G, Lin SH, Erdem H, et al. SRC-3 is required for prostate cancer cell proliferation and survival. Cancer Res 2005;65(17):7976-83.

326. Xu J, Qiu Y, DeMayo FJ, Tsai SY, Tsai MJ, O'Malley BW. Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene. Science 1998;279(5358):1922-5.

327. Gehin M, Mark M, Dennefeld C, Dierich A, Gronemeyer H, Chambon P. The function of TIF2/GRIP1 in mouse reproduction is distinct from those of SRC-1 and p/CIP. Mol Cell Biol 2002;22(16):5923-37.

328. Mark M, Yoshida-Komiya H, Gehin M, Liao L, Tsai MJ, O'Malley BW, et al. Partially redundant functions of SRC-1 and TIF2 in postnatal survival and male reproduction. Proc Natl Acad Sci U S A 2004;101(13):4453-8.

329. Comuzzi B, Nemes C, Schmidt S, Jasarevic Z, Lodde M, Pycha A, et al. The androgen receptor co-activator CBP is up-regulated following androgen withdrawal and is highly expressed in advanced prostate cancer. J Pathol 2004;204(2):159-66.

330. Debes JD, Sebo TJ, Lohse CM, Murphy LM, Haugen DA, Tindall DJ. p300 in prostate cancer proliferation and progression. Cancer Res 2003;63(22):7638-40.

331. Link KA, Burd CJ, Williams E, Marshall T, Rosson G, Henry E, et al. BAF57 governs androgen receptor action and androgen-dependent proliferation through SWI/SNF. Mol Cell Biol 2005;25(6):2200-15.

332. Neckers L, Ivy SP. Heat shock protein 90. Curr Opin Oncol 2003;15(6):419-24.

333. Harashima K, Akimoto T, Nonaka T, Tsuzuki K, Mitsuhashi N, Nakano T. Heat shock protein 90 (Hsp90) chaperone complex inhibitor, radicicol, potentiated radiation-induced cell killing in a hormone-sensitive prostate cancer cell line through degradation of the androgen receptor. Int J Radiat Biol 2005;81(1):63-76.

334. Chang HC, Chen SC, Chen J, Hsieh JT. In vitro gene expression changes of androgen receptor coactivators after hormone deprivation in an androgen-dependent prostate cancer cell line. J Formos Med Assoc 2005;104(9):652-8.

335. Halkidou K, Gnanapragasam VJ, Mehta PB, Logan IR, Brady ME, Cook S, et al. Expression of Tip60, an androgen receptor coactivator, and its role in prostate cancer development. Oncogene 2003;22(16):2466-77.

336. Miyamoto H, Rahman MM, Chang C. Molecular basis for the antiandrogen withdrawal syndrome. J Cell Biochem 2004;91(1):3-12.

337. Burd CJ, Petre CE, Morey LM, Wang Y, Revelo MP, Haiman CA, et al. Cyclin D1b variant influences prostate cancer growth through aberrant androgen receptor regulation. Proc Natl Acad Sci U S A 2006;103(7):2190-5.

338. Graff JR, Konicek BW, McNulty AM, Wang Z, Houck K, Allen S, et al. Increased AKT activity contributes to prostate cancer progression by dramatically accelerating prostate tumor growth and diminishing p27Kip1 expression. J Biol Chem 2000;275(32):24500-5.

339. Xin L, Teitell MA, Lawson DA, Kwon A, Mellinghoff IK, Witte ON. Progression of prostate cancer by synergy of AKT with genotropic and nongenotropic actions of the androgen receptor. Proc Natl Acad Sci U S A 2006;103(20):7789-94.

340. Ghosh PM, Malik S, Bedolla R, Kreisberg JI. Akt in prostate cancer: possible role in androgen-independence. Curr Drug Metab 2003;4(6):487-96.

341. McMenamin ME, Soung P, Perera S, Kaplan I, Loda M, Sellers WR. Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high Gleason score and advanced stage. Cancer Res 1999;59(17):4291-6.

342. Halvorsen OJ, Haukaas SA, Akslen LA. Combined loss of PTEN and p27 expression is associated with tumor cell proliferation by Ki-67 and increased risk of recurrent disease in localized prostate cancer. Clin Cancer Res 2003;9(4):1474-9.

343. Wang S, Gao J, Lei Q, Rozengurt N, Pritchard C, Jiao J, et al. Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer. Cancer Cell 2003;4(3):209-21.

344. Li P, Nicosia SV, Bai W. Antagonism between PTEN/MMAC1/TEP-1 and androgen receptor in growth and apoptosis of prostatic cancer cells. J Biol Chem 2001;276(23):20444-50.

345. Paez J, Sellers WR. PI3K/PTEN/AKT pathway. A critical mediator of oncogenic signaling. Cancer Treat Res 2003;115:145-67.

346. Shi XB, Gandour-Edwards R, Beckett LA, Deitch AD, de Vere White RW. A modified yeast assay used on archival samples of localized prostate cancer tissue improves the detection of p53 abnormalities and increases their predictive value. BJU Int 2004;94(7):996-1002.

347. Cronauer MV, Schulz WA, Burchardt T, Ackermann R, Burchardt M. Inhibition of p53 function diminishes androgen receptor-mediated signaling in prostate cancer cell lines. Oncogene 2004;23(20):3541-9.

CHAPTER 2. IDENTIFICATION OF NOVEL ANDROGEN RECEPTOR-INTERACTING PROTEINS USING THE REPRESSED TRANSACTIVATOR YEAST TWO-HYBRID SYSTEM

A version of part of this chapter has been published and the remainder will be submitted for a second publication.

Wafa LA, Cheng H, Cox ME, Sadowski I, Rennie PS. Identification of novel androgen receptor-interacting proteins using the repressed transactivator yeast two-hybrid system. Submission to *Biochimica et Biophysica Acta* (Molecular Cell Research).

Ray MR*, **Wafa LA***, Cheng H, Snoek R, Fazli L, Gleave M, Rennie PS (2005). Cyclin G-associated kinase: a novel androgen receptor-interacting transcriptional coactivator that is over-expressed in hormone refractory prostate cancer. Int. J. Cancer 118 (5): 1108-1119.
*Co-first authors (both contributed equally)

Dr. Ivan Sadowski at the department of Biochemistry and Molecular Biology (UBC) founded the repressed transactivator yeast two-hybrid system and collaborated with our lab to use this methodology for identifying AR-NTD interacting proteins. Helen Cheng performed yeast two-hybrid screening and trained me to use the RTA system. Dr. Mira Ray (former PhD student in our lab) performed the co-immunoprecipitation assay of AR with cyclin G-associated kinase (one of the detected AR-binding proteins) shown in **Figure 2.3C**. Dr. Michael Cox provided critical comments.

2.1 Introduction

The androgen receptor (AR) is a ligand-activated transcription factor, of the steroid hormone receptor family, that is critical for growth and development of the normal prostate, as well as cancer (1, 2). Steroid receptors have similar functional domain structures that include an N-terminal domain (NTD), a DNA binding domain

(DBD), a hinge region and a ligand binding domain (LBD). In the absence of androgen, AR remains in the cytoplasm as an inactive complex, which includes heat shock proteins (hsps). The LBD of AR, in addition to forming the ligand binding pocket, mediates the interaction between AR and hsps (3), and also interacts with the AR N-terminus to stabilize bound androgen (4). After AR binds to its cognate ligand, the receptor-ligand complex translocates to the nucleus, where it can bind to specific androgen response elements found in the promoter of androgen-regulated genes (5). Similar to other members of the steroid receptor family, AR contains two transcriptional activation functions (AF); a constitutively active AF1 located in the NTD of the receptor and a ligand-dependent AF2 within the LBD (6). The structural and functional properties of AR and role of the receptor in transcription have been discussed in detail in **Chapter 1 (Section 1.3)**.

The underlying mechanism through which AR specifically and differentially regulates gene expression remains uncertain. The DBD of AR is highly conserved among steroid receptors and recognizes the same steroid response element (SRE) consensus sequence as the glucocorticoid receptor (GR) (7). Although subtle differences in the response element may dictate steroid receptor specificity, more recent evidence suggests that AR-specific gene regulation may also occur through interactions with unique coregulatory proteins (discussed in **Chapter 1, Section 1.4**). These proteins can enhance (coactivators) or inhibit (corepressors) the transcriptional activity of steroid receptors (8). A number of coregulators have been shown to interact with AR. These include SRC/p160 family members (9, 10) and CBP/p300 (11), which facilitate the access of steroid receptors and basal transcriptional machinery to their target DNA

sequences through chromatin remodelling and histone acetyltransferase activity. In addition, ARA70 (12), cyclin D1 (13), caveolin-1 (14), β-catenin (15) and several other proteins have also been found to interact with AR. Most of these coregulator proteins have been isolated as a consequence of their binding to the DBD and LBD of steroid receptors. These domains share a high amount of sequence homology, and hence the proteins associated with these regions have been found to be indiscriminate in their ability to interact with and affect steroid receptor activity (16).

Since the NTD of steroid receptors is the least conserved, protein interactions in this region may dictate receptor-specific coregulation capacity. There are several known AR-NTD protein partners, which include the classical coactivators of the SRC/p160 family (9, 10), CBP/p300 (11), ART-27 (17), the transcription factor signal transducers and activators of transcription (STAT3) (18), and the general transcription factor TFIIF (19). However, the interaction of AR with SRCs and CBP/p300 can occur through both the NTD and LBD of the receptor and is not unique, as these coactivators are known to interact with multiple steroid receptors. In contrast, ART-27 has been shown to interact exclusively with the NTD of AR in yeast but also binds the N-terminus of GR and ER. Furthermore, STAT3 can enhance the transactivation of AR, GR, PR and ER (20), while TFIIF studies with AR have been confined to the AR-NTD (19, 21), still allowing the possibility that it may also interact with the LBD of the receptor. Hence, it remains to be seen whether there are coregulator proteins that interact strictly with the unique amino acid sequence of the AR-NTD and specifically modify AR transcriptional activity. Although the list of proteins proposed to bind to the AR N-terminus is expanding, it is likely that additional AR-binding partners remain to be found.

To identify novel AR-interacting proteins, its NTD was used as bait for yeast two-hybrid analysis. However, a major limitation of the AR-NTD is that it contains intrinsic transactivation activity and therefore cannot be utilized as a bait for conventional yeast two-hybrid screening. To circumvent this problem, the repressed transactivator (RTA) system (22), specifically designed for transactivator bait proteins, was used to screen a prostate carcinoma cell line cDNA library for AR-NTD protein partners.

2.2 Materials and Methods

Plasmids and Yeast Strain

Three different regions of the human AR (AR_{1-559}, $AR_{233-559}$ and AR_{1-646}) were cloned downstream of the GAL4 DBD in pGBT9 (Clontech) to produce fusion proteins comprising of GAL4 DBD and AR regions (23). The vector used for construction of the LNCaP cDNA library was modified from pGADT7 (Clontech) in which the GAL4 activation domain (AD) was replaced with the N-terminal 200 residues of TUP1 (pGADT7-TUP1), without disrupting the NLS, HA epitope tag and T7 RNA polymerase promoter. The first step involved PCR amplification of the NLS containing a 5' *Hind*III site using primers (5'-ACTCC*AAGCTT*TGCAAAGATGGATAAA and 5'-GCGGCGGTACCCAATTCGACCTT) on template pGADT7. This fragment was ligated to the PCR product of TUP1, amplified using primers that provide a *Bgl*II site at the 3'end (5'ATGACTGCCAGCGTTTCGAATACG and 5'ATTAA*AGATCT*CTGCCACGGAAACCTGGGGAGGTGG) on template pBD2 (22). The ligated DNA was then cloned into *Hind*III and *Bgl*II sites of pGADT7. The expression of TUP1 was confirmed using Western blotting, probed with an antibody to

the HA epitope. The yeast strain used was MAV103 (*MATα, gal4, gal80, leu2, trp1, his3, SPAL10::URA3)* (24).

Construction of the TUP1-LNCaP cDNA Fusion Library

Total RNA was extracted from the LNCaP prostate cancer cell line using TRIzol according to the manufacturer's protocol (Gibco, BRL). Poly A^+ RNA was purified 3 times over oligo (dT) cellulose columns (Boehringer Mannheim) and cDNA with Eco*RI* and Xho*I* ends was synthesized using the Stratagene cDNA Synthesis Kit, which was ligated into appropriately digested pGADT7 vector with the TUP1 sequence. Ligated DNA was electroporated into ElectroMAX DH10B cells (Gibco, BRL). The library was estimated to contain 6×10^6 independent clones, of which about 80 % contained inserts.

GAL4-AR-NTD RTA Yeast Two-Hybrid Screen

Yeast strain MAV103 was co-transformed with one of the three pGBT9-hAR-NTD baits (AR_{1-559}, $AR_{233-559}$ and AR_{1-646}) and TUP1-LNCaP cDNA library plasmid. Cells were plated on minimal media (-Leu, -Trp) in the presence of 5-fluoroorotic acid (5-FOA). The concentration of 5-FOA used was determined by co-transforming yeast with bait and either TUP1-LNCaP cDNA library or empty pGADT7-TUP1 vector and plating on media with increasing 5-FOA concentration (0.01 % to 0.1 % at 0.005 % intervals). The minimum amount of 5-FOA that caused 100 % cell kill was chosen for screening the library. Colonies that survived selection were collected at days 4, 5, 6 and 7 and were passaged once onto fresh 5-FOA plates. An additional full-length AR bait (AR_{1-919} fused to GAL4 DBD as for AR-NTD baits) was also used for screening but

100 nM DHT androgen was included in the 5-FOA plates to induce receptor transactivation activity. Isolated colonies were expanded in 200 µl of minimal media and DNA was extracted using the Yeast DNA Extraction Reagent (Y-DER) Kit (Pierce); cDNA inserts were amplified by PCR using a TUP1 internal primer and a vector specific primer. PCR products were purified over MinElute columns (Qiagen) and sequenced using the Big Dye Terminator Sequencing Kit (ABI Prism). The sequence was compared to known sequences available from GenBank™. Positive clones that had appeared multiple times were passaged into HB101 bacteria (carry selectable marker for leucine) for cDNA plasmid isolation. Positive interactions with baits were re-confirmed in yeast.

GST-Pulldown Assays

Three fragments of the rat AR ($AR_{234-665}$, $AR_{541-665}$, $AR_{541-919}$) were expressed as GST-fusion proteins in BL21 *Escherichia coli* cells. Equimolar normalization of GST-AR domain fusion proteins was performed through Coomassie Blue staining and a BSA standard curve. The procedure for performing semi-quantitative GST-pulldown assays is described in detail in **Section 3.2** of **Chapter 3.** The same protocol was followed here using the Quick Coupled T7 Promoter TnT *in vitro* transcription/translation kit (Promega) to radiolabel ([^{35}S]-methionine) proteins coded by the pGADT7-TUP1-LNCaP library clone vectors.

Co-immunoprecipitation and Western Blotting

The detailed procedure for cell culture, co-immunoprecipitation assays and performing Western blot analysis is described in **Section 3.2** of **Chapter 3.** Briefly,

human prostate cancer LNCaP cells were seeded onto 10 cm dishes and grown to 70 % confluency in RPMI with 5 % fetal bovine serum (FBS). Media was changed to RPMI containing 5 % dextran-coated charcoal stripped FBS and cells were grown for an additional 16 hours at 37 °C to deplete cells of bio-available hormone. Cells were then treated with or without 10 nM R1881 for 4 hours before harvest and lysis in either 0.5 % NP-40 buffer (150 mM NaCl, 50 mM Tris-Cl pH 7.5, 2 mM EDTA, 0.5% NP-40) or RIPA buffer (150 mM NaCl, 50 mM Tris-Cl pH 7.5, 2 mM EDTA, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS). In this chapter, the immunoprecipitation of AR was carried out with either a rabbit polyclonal anti-AR_{LBD} (C19) or a mouse monoclonal anti-AR_{NTD} (441) antibody (Santa Cruz Biotechnology, Inc.). All Western blot detection of AR was performed using the anti-AR_{NTD} (441) antibody. Immunoprecipitation and Western blot detection of muscle phosphofructokinase (PFKM) was carried out using a goat polyclonal anti-PFKM antibody (Chemicon). Western blot detection of eukaryotic translation elongation factor 1A (eEF1A) was performed with a mouse monoclonal anti-EF1A antibody (Upstate). Cyclin G-associated kinase (GAK) Western blot analysis was carried out with a rabbit polyclonal anti-GAK antibody (Santa Cruz Biotechnology, Inc.). In all immunoprecipitations, 1 μg of antibody was used for 1 mg of protein from whole cell extracts and the same amount of normal IgG of the corresponding species was used for negative controls. The final concentration of all primary antibodies used for Western blot analysis was 2 μg/mL. Further details of co-immunoprecipitation assays and Western blotting is included in **Chapter 3.**

2.3 Results

AR-NTD is a Suitable Bait for the RTA System

To identify proteins that bind to the AR N-terminal domain, we screened a LNCaP cDNA library for interacting proteins using a yeast two-hybrid system. The N-terminus of AR has been shown to contain strong intrinsic transactivation activity in yeast when fused to the GAL4 DNA-binding domain. For this reason, we used a reverse yeast two-hybrid screen that employs the repressed transactivator (RTA) system specifically designed for transactivator bait proteins (22). As with the conventional yeast two-hybrid system, the AR-NTD is fused to a GAL4 DBD domain to form the bait. However, the prey is created by fusion of the cDNA library with a TUP1 repressor protein rather than with an activation domain. Since the N-terminus of TUP1 is sufficient to cause transcriptional repression, only the first 200 amino acid residues were fused to the cDNA library (22, 25). When there is no interaction between the bait and TUP1-library fusion protein, the bait activator allows constitutive expression of the URA3 reporter gene (**Figure 2.1 A**). This permits growth in the absence of uracil, but causes sensitivity to 5-FOA, which is converted to a toxic 5-fluorouracil product through the enzyme encoded by the URA3 gene. Thus, transcriptional repression can be genetically monitored by using a counterselectable URA3 reporter (26). In a situation where there is an interaction between the AR-NTD and a library clone, TUP1 will cause repression of URA3 expression, which can be detected by growth of yeast in the presence of 5-FOA (**Figure 2.1 B**). Therefore, viability of the MAV103 strain, or colony growth, on 5-FOA media represents a positive interaction between the AR-NTD bait and prey.

Detection of AR-NTD Interacting Proteins in Yeast

The RTA system has not been previously used to identify novel AR interacting proteins. We examined three overlapping regions of the human AR (aa 1-559, 233-559 and 1-646) as separate baits in this system. All three baits contain the AR N-terminal region (aa 360-528) that is responsible for the intrinsic transactivation activity of AR when its LBD is deleted (27). A full-length AR bait was also used to screen for AR protein partners but DHT androgen was included in the plating media to allow receptor activation. Initial toxicity tests were done to generate dose response curves to 5-FOA and determine the optimal concentration for screening. The minimum amount of 5-FOA that caused approximately 100 % cell kill in absence of the library and a significant increase in colony survival with the library, was chosen as the optimal concentration for screening with that particular bait (increased by an increment of 0.01 % to ensure maximal stringency). Optimal 5-FOA concentration for screening with the AR full N-terminus (AR_{1-559}) bait was determined to be 0.055% (**Figure 2.2**). Similarly, concentrations of 5-FOA used for the $AR_{233-559}$, AR_{1-646} and full-length AR baits were found to be in the 0.05 – 0.08 % range.

Approximately 3×10^6 transformants were screened with all the full-length AR and three AR-NTD baits. Positive clones were sequenced and compared to known sequences available in GenBank™. A total of 40 unique in-frame positive clones were detected from the LNCaP cDNA library, some of which were picked up multiple times. Several of these clones encoded proteins of unknown function or were not detected more than once. In this study, I have decided to focus on a subset of these isolated clones based on multiplicity of detection and established roles of the encoded proteins. The

most frequently isolated clone was L-dopa decarboxylase (DDC or aromatic L-amino acid decarboxylase, E.C. 4.1.1.28); GenBank™ accession number NM 000790 (28), which was detected a total of 6 times, with all three AR NTD baits. In addition to DDC, other detected clones included GAK, eEF1A, PFKM, TRRAP/PAF400, $TAF_{II}250$, APC7 and the known steroid receptor chaperone hsp70 (defined in **Table 2.1**).

RTA Yeast Two-Hybrid Clones Interact with AR *in Vitro*

To confirm protein interactions revealed with the yeast-two hybrid screen, *in vitro* GST-pulldown assays were performed for each AR-interacting protein listed in **Table 2.1**. Multiple domains of AR were tested for direct binding with detected clones. The results presented in **Table 2.2** confirm that AR interacts with the mix of cytosolic and nuclear proteins detected by the RTA system. The strength of interaction with AR domains varied widely among the binding partners. Moreover, certain proteins, such as DDC, exhibited selective strength of binding for different domains of AR, interacting more strongly with the ligand binding domain than the N-terminus region. In contrast, eEF1A interacted with all AR domains with the same strength.

Notably, all AR protein partners bound the AR N-terminus region (fused with DBD; $AR_{234\text{-}665}$) that possesses the constitutively active intrinsic transactivation activity of AR (27), except for $TAF_{II}250$ and hsp70, which only interacted with the AR-DBD and LBD. This lack of interaction is not surprising since these proteins were only detected during screening with the $AR_{1\text{-}646}$ bait (NTD+DBD), suggesting that hsp70 and $TAF_{II}250$ were isolated as a result of their binding with the AR-DBD. However, the isolated clone for $TAF_{II}250$ only coded for a partial C-terminal region of the full-length protein, which

may interact with the NTD of AR. Similarly, for all other detected clones that were incomplete C-terminal coding regions (DDC, GAK, eEF1A, PFKM, TRRAP), the full-length proteins may have an entirely different pattern of interaction with AR domains. Since DDC was detected more often than any other protein in the yeast two-hybrid screen, an in-depth analysis of its direct interaction with AR domains was performed using full-length and truncated DDC protein (described in detail in **Chapter 3**).

In Vivo Association of RTA System Proteins with AR in Prostate Cancer Cells

In order to determine whether the newly detected AR protein partners can bind the receptor *in vivo*, co-immunoprecipitation assays were performed using LNCaP prostate cancer cells, which express high levels of endogenous AR. Whole cell extracts from untreated or androgen-treated (10 nM R1881) LNCaP cells were subjected to immunoprecipitation with either anti-AR antibodies or ones raised against the AR-interacting proteins. The co-immunoprecipitation of DDC with AR is presented in **Chapter 3**. The binding of eEF1A, PFKM and GAK with AR are included in the current chapter (**Figure 2.3**). Notably, due to the low level expression of PFKM, this protein was immunoprecipitated instead of AR for clear detection by Western blot analysis. The binding of GAK and PFKM with AR were stronger in the presence of androgen. The association of nuclear AR-interacting proteins, TRRAP, $TAF_{II}250$ and APC7, will be the subject of future studies using purified nuclei from LNCaP cells for co-immunoprecipitation assays. Overall, the binding of several of the detected AR-interacting proteins with the receptor in a prostate cancer cell line suggests that these proteins may have important functional effects on AR.

Library Clone	Bait	Number of Islolated Clones	Known Functions (References)
L-Dopa decarboxylase (DDC)/aromatic L-amino acid decarboxylase	AR_{1-646}, AR_{1-559} and $AR_{232-559}$	6	Enzyme responsible for catecholamine and serotonin synthesis (28)
Cyclin G-associated kinase (GAK)	AR_{1-646}, AR_{1-559}	3	Ser/Thr kinase associated with clatherin-coated vesicles (29)
Heat shock protein 70 (hsp70)	AR_{1-646}	2	Steroid receptor chaperone protein (30, 31)
Eukaryotic translation elongation factor 1A (eEF1A)	Full-length AR	2	Translation: aminoacyl-tRNA carrier/several moonlighting functions (32)
Phosphofructokinase; muscle type (PFKM)	Full-length AR	1	Glycolysis: fructose-6-phosphate phosphorylation (33)
Transformation/transcription domain-associated protein or p/CAF-associated factor (TRRAP/PAF400)	Full-length AR	1	Transcriptional adapter protein: associates with histone acetyltransferases (34, 35)
TBP-associated factor 250 ($TAF_{II}250$)	AR_{1-646}	1	Component of general transcription factor TFIID (36, 37)
Anaphase-promoting complex subunit 7 (APC7)	$AR_{232-559}$	2	Component of APC E3 ubiquitin ligase (38, 39)

Table 2.1 – Identification of Novel AR-Interacting Proteins in RTA Yeast-Two Hybrid Screen. The RTA yeast two-hybrid system was used to screen a LNCaP prostate cancer cell line cDNA library with three AR-NTD baits (AR_{1-559}, $AR_{233-559}$ and AR_{1-646}) and one full-length AR bait (AR_{1-919}). From the total of 40 unique in-frame positive clones, the above list was selected for further analysis based on the number of times clones were isolated and known protein functions. All 8 identified clones encoded the C-terminal region of proteins, except for hsp70 and APC7, for which the full-length cDNA clones were detected.

Protein	*GST-Pulldown Assays		
	T-NTD+DBD	DBD	DBD+LBD
Cytosol			
DDC	++	+	+++
Hsp70	-	++	+++
eEF1A	+	+	+
PFKM	++	-	+++
Cytosol and Nucleus			
GAK	+	+	++
Nucleus			
TRRAP	+	-	++
$TAF_{II}250$	-	+	+
APC7	+	++	+++

Table 2.2 - *In Vitro* Interaction of AR with Isolated Clones from the RTA System. Three GST-AR domain fusion proteins ($AR_{234-665}$/Truncated-NTD+DBD, $AR_{541-665}$/DBD and $AR_{541-919}$/DBD+LBD) and control GST protein were expressed in bacteria and coupled to glutathione-agarose beads at equimolar levels. All detected clones were expressed by *in vitro* transcription/translation and radiolabeled with [^{35}S]-methionine, using the pGADT7-TUP1-LNCaP library vectors isolated in the screen. After incubation of radiolabeled proteins and GST-AR domains, bound protein was eluted for SDS-PAGE and autoradiography analysis. Proteins are categorized based on their known cellular localization. A detailed procedure of GST-pulldown assays is included in **Section 3.2** of **Chapter 3**.

*Note that strength of interaction denoted by (–)/no binding, (+)/weak, (++)/moderate and (+++)/strong is not a comparison between different AR-interacting proteins since band intensities on autoradiograms were normalized to loaded input for each radiolabeled protein. For example, the (++) interaction of DDC with the AR T-NTD+DBD was many times stronger than the (++) interaction of GAK with the AR-DBD+LBD.

2.4 Figures

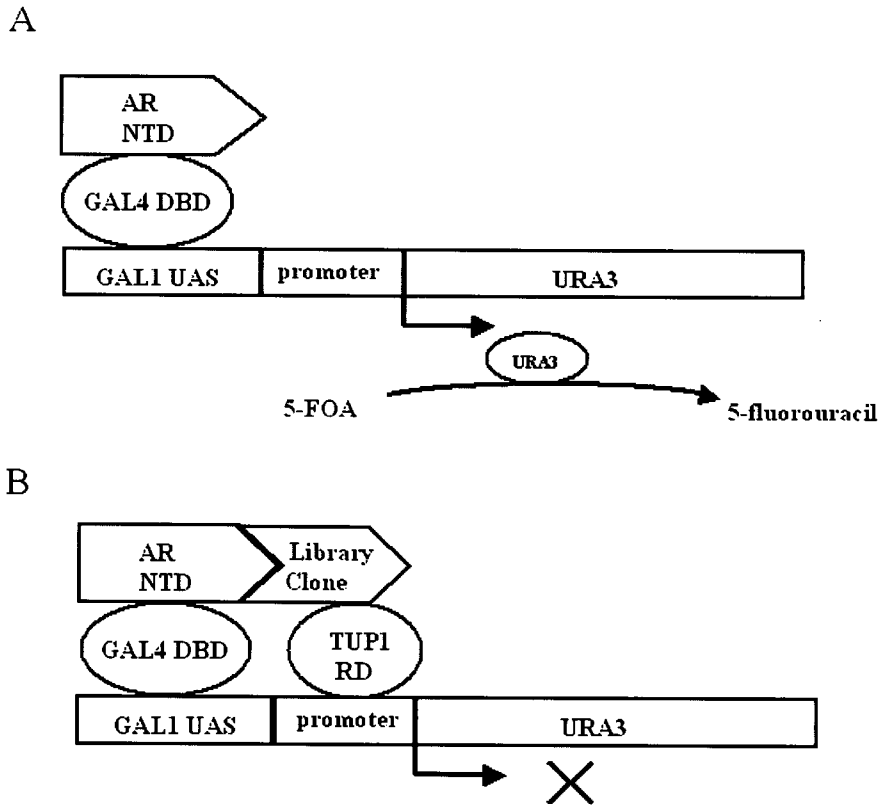

Figure 2.1 - AR-NTD as Bait in the Repressed Transactivator Yeast Two-Hybrid System. The yeast strain used was MAV103, which contains multiple GAL4 binding sites (UAS) upstream of a URA3 reporter gene. The repression domain (RD) of TUP1 is fused to the LNCaP-cDNA library. A) Activation of the GAL1-URA3 reporter gene by GAL4 DBD-AR NTD fusion activator bait protein causes constitutive expression of orotidine-5'-phosphate decarboxylase, leading to 5-FOA sensitivity. B) Interaction of a TUP1-LNCaP cDNA library fusion prey with the AR-NTD activator bait causes transcriptional repression of the URA3 reporter, resulting in resistance to 5-FOA.

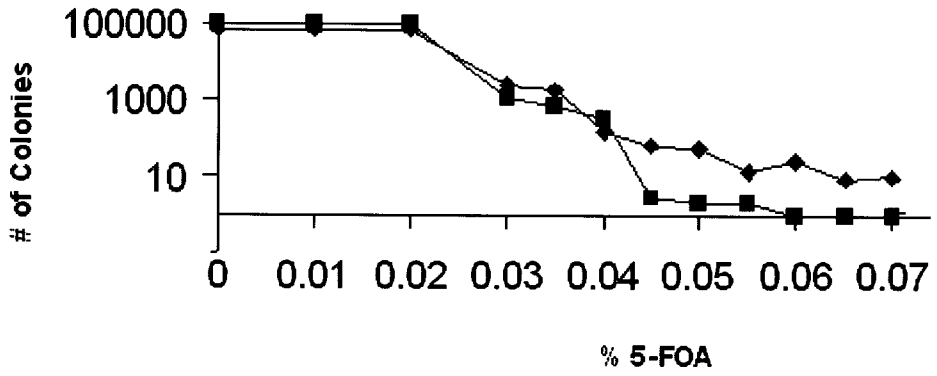

Figure 2.2 – Toxicity of 5-FOA. Concentration of 5-FOA used for screening was determined by co-transformation of yeast with bait (GAL4 DBD-AR_{1-559}) and TUP1-LNCaP cDNA library (♦) or empty pGADT7-TUP1 vector (■) before plating on media with increasing 5-FOA concentrations.

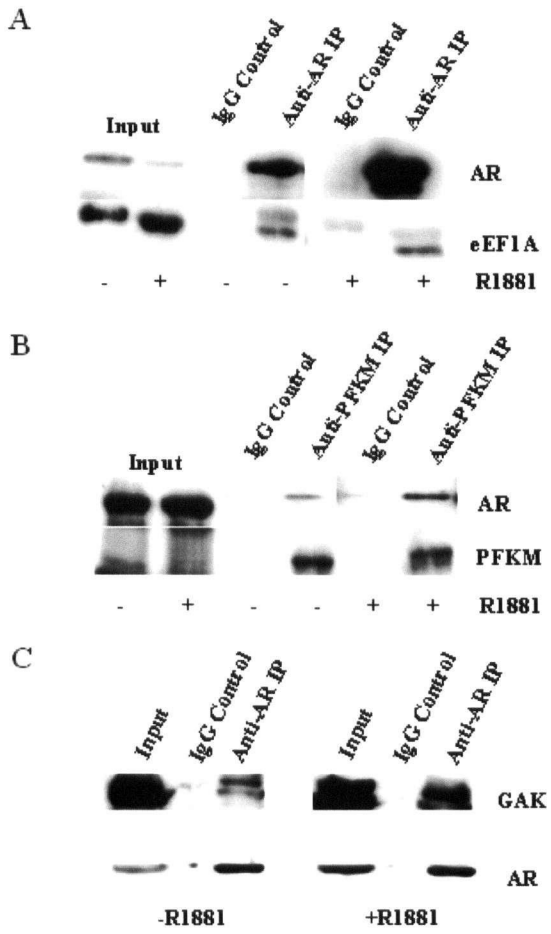

Figure 2.3 - Co-immunoprecipitation of AR with Proteins Isolated from the RTA System. LNCaP cells were grown in the absence or presence of 10 nM R1881 for 4 hours prior to harvest. A) eEF1A; 0.5 % NP-40 buffer was used for cell lysis. AR was immunoprecipitated using a rabbit anti-AR_{LBD} antibody and Western blots were performed with anti-eEF1A and anti-AR_{NTD} antibodies. B) PFKM; cells were lysed with 0.5 % NP-40. PFKM was immunoprecipitated and detected with the same goat anti-PFKM antibody, while AR was identified with the anti-AR_{NTD} antibody. C) GAK; RIPA buffer was used to extract cell lysate. The mouse anti-AR_{NTD} antibody was used to immunoprecipitate and detect AR, while an anti-GAK antibody was used to identify this kinase. Normal IgG protein of corresponding species was used for negative controls. Protein complexes were pulled down using rProtein G-agarose (eEF1A and PFKM) or Protein A/G-agarose (GAK) beads prior to SDS-PAGE and Western blot analysis. Proteins were detected at their expected molecular weights of approximately 110 kDa (AR), 50 kDa (eEF1A), 85 kDa (PFKM) and 150 kDa (GAK).

2.5 Discussion

Traditionally, identification of AR interacting proteins has been carried out with conventional yeast two-hybrid assays, such as the GAL4 system, using the AR DNA binding and ligand binding domains as bait (40-42). In fact, one of the most established type II coactivators of AR, ARA70, was detected using the GAL4 system with the AR-DBD+LBD as bait (12). Previous studies have been limited to using these regions of AR to screen for receptor protein partners since they do not possess strong intrinsic transcriptional activity. Even though the AR-LBD contains transcriptional AF2 of AR, in the absence of the AR-NTD this transactivation activity is extremely weak as compared to that of full-length AR and the constitutively active AR-NTD (43). Hence, in the conventional GAL4 system, use of AR-DBD and AR-LBD baits do not result in constitutive transcription of the β-galactosidase reporter gene (12, 23). Conversely, the AR-NTD possesses a strong intrinsic transactivation activity that prevents its use as bait in conventional screening assays. However, the AR-NTD is a crucial interaction site of coregulators and may even confer receptor-specific binding to other proteins, due to its unique amino acid sequence among steroid receptor family members (discussed in **Section 1.3** of **Chapter 1**).

In order to identify potentially AR-specific protein interactions, we therefore have employed the RTA system as a novel method of performing yeast two-hybrid assay screening with the AR-NTD as bait. In contrast to our approach, other studies have also reported use of a modified conventional LexA yeast two-hybrid system to identify AR-NTD protein partners (17, 44). In these cases, the AR-NTD is expressed as a fusion protein linked to a B42 activation domain, while the cDNA library is fused to a Lex A

DNA binding domain. However, this screening method can result in a high false positive detection rate from library clones that possess intrinsic transcriptional activation potential. Also, fusion of the cDNA library to a DBD, such as that of GAL4 or Lex A, may alter its ability to bind the upstream activation sequence, introducing another level of variation in the two-hybrid assay. Alternatively, using the AR-NTD as bait in the RTA system, allows detection of clones that contain intrinsic transcriptional activity and does not require fusion of the cDNA library to a heterologous DBD.

Surprisingly, in our screen, we did not identify known NTD binding partners of AR, such as SRC family members (9, 10), CBP/p300 (11), ART-27 (17), STAT3 (18) and TFIIF (19). It is possible that these interactions are too weak to be identified at the 5-FOA concentration used or that fusion of these proteins to TUP1 may lead to toxicity in yeast. Furthermore, detection of these AR-NTD interacting proteins is dependent on the presence of the proper sequence in the cDNA inserts of the library, which are not full-length for many clones. However, using the RTA system, we were able to identify several novel AR-interacting proteins that have similar functions to those of classical type I and non-classical type II coactivators of AR (discussed in detail in **Chapter 1, Section 1.4**). These include, DDC, GAK, eEF1A, PFKM, $TAF_{II}250$, TRRAP/PAF400, APC7 and the established AR protein chaperone hsp70 (**Table 2.1**). The interaction of these proteins with AR seem to be direct since *in vitro* transcription/translation products of the cDNA inserts did bind to the AR-NTD and other domains in GST-pulldown studies (**Table 2.2**). Moreover, the co-immunoprecipitation of a subset of these proteins with AR, in LNCaP prostate cancer cells, demonstrates that they can associate with the

receptor in the cellular environment, where they may regulate AR activity through their established biological functions (**Figure 2.3**).

The most often isolated clone with AR-NTD baits in the RTA system was L-dopa decarboxylase. This enzyme is responsible for the decarboxylation step in both the catecholamine (dopamine) and 5-hydroxytryptamine (serotonin) synthetic pathways (28). DDC is expressed in neuronal tissue, where it plays a well-established role in the synthesis of neurotransmitters (dopamine and serotonin), and in peripheral tissues, in which the function of the enzyme is unknown. The role of DDC as an AR-interacting protein and coregulator of AR, as well as its expression profile in prostate cancer was analyzed extensively and is the subject of the remaining studies of this project (**Chapters 3, 4, 5** and **6**).

GAK is a Ser/Thr kinase that has multiple functional domains, which include an N-terminal kinase domain, a central auxilin/tensin homology domain and a C-terminal J-domain (29). Although GAK was initially found through its association with cyclin G, subsequent studies have shown that it also has a role in clathrin-mediated endocytosis (45). GAK is present in clathrin-coated vesicles and its Ser/Thr kinase activity has been found to be directed towards the μ2 component of these structures (46). GAK phosphorylation of μ2 has been suggested to regulate clathrin-coated vesicle trafficking (47). In this study, GAK was found to associate with endogenous AR in prostate cancer LNCaP cells (**Figure 2.3 C**), suggesting that this kinase may act as a bridging protein between AR and clatherin-coated vesicles. This taken together with the fact that GAK can localize to the cytoplasm and nucleus suggests a role for this kinase in AR cellular trafficking. The J-domain of GAK is known to interact with a constitutively expressed

form of the molecular chaperone hsp70, referred to as hsc70 (48), which also interacts directly with AR to regulate receptor protein folding and ligand binding (30, 31). Moreover, recently GAK has been shown to interact directly with the classical AR coactivator CBP (49). Overall, these studies suggest that GAK may regulate AR function by either acting directly on the receptor or receptor chaperones and coactivators. Whether GAK can phosphorylate AR or receptor coregulators is an ongoing project in our laboratory.

The detection of hsp70 as an AR-interacting protein in our yeast two-hybrid assay confirmed that the RTA system can identify known AR-binding protein partners. The hsp70 chaperone protein is a key component of the multichaperone complex. This complex is responsible for configuration of the LBD of steroid receptors, including that of AR, into a stable partially unfolded conformation that has a high affinity for cognate ligand (50). Previous studies have shown that several components of this complex including hsp70, hsp40 and hsp90 can all interact with the AR-LBD (3, 30, 31, 51, 52). In our screen for AR-NTD interacting proteins (**Table 2.1**), the full-length hsp70 protein was detected only with the AR_{1-646} bait that contains both the NTD and DBD of AR (not isolated with AR_{1-559} and $AR_{232-559}$ baits). This suggests that hsp70 does not interact with the AR-NTD but can bind to the AR-DBD, which is located closer to the C-terminal ligand binding domain that associates with chaperone proteins in the cytosol.

eEF1A catalyzes the first step of the elongation cycle of protein synthesis (53, 54). During protein translation, eEF1A carries the aminoacyl-tRNA on the A site of the ribosome, which contains the extending polypeptide chain as peptidyl-tRNA. In addition to its central role in translation, eEF1A has also been shown to be involved in other

cellular processes that include oncogenic transformation, cellular proliferation and organization of the cytoskeleton (32, 55). There are two known eEF1A isoforms, eEF1A1 and eEF1A2, which share approximately 95 % nucleotide and protein sequence homology (56). Both isoforms are believed to have the same enzymatic function in protein translation. In this study, detection of co-immunoprecipitated eEF1A with AR from LNCaP cell lysate was carried out using a general anti-eEF1A antibody, due to the unavailability of an isoform specific antibody. The strong association of endogenous eEF1A with AR (presence or absence of androgen) and the almost identical sequences for eEF1A1 and eEF1A2, suggest that both isoforms most likely bind with AR (**Figure 2.3A**). Notably, a third truncated form of eEF1A (amino acids 1-67 deleted) encodes for the prostate tumour-inducing gene 1 (PTI-1) oncoprotein that is expressed in prostate tumour cells but absent in normal cells (57). Due to a 98 % sequence homology of the PTI-1 coding region with eEF1A (58), it would be expected that AR can also interact with this protein, possibly linking the receptor to the oncogenic function of PTI-1.

In addition to binding with translational machinery components in the cytosol, eEF1A also interacts with a wide variety of other proteins, including the zinc finger protein 1 (ZPR1) transcription factor (59), and filamentous actin (f-actin) (60). Although the exact role of ZPR1 is not known, it has been shown to undergo nuclear translocation with eEF1A upon treatment of cells with mitogens such as EGF, suggesting that this transcription factor functions as a signalling molecule that transmits mitogenic signals to the nucleus (59, 61). Notably, the binding of eEF1A with ZPR1 was found to be necessary for the cellular proliferation effect of this transcription factor. Since AR is also

a zinc finger transcription factor which is activated and undergoes nuclear translocation in cells treated with EGF (62), it is possible that eEF1A binding to AR may be involved in the transmission of mitogenic signals that modulate receptor activity.

The role of eEF1A as an actin binding protein has been documented in many species (32, 60). Cellular co-localization and binding of eEF1A with f-actin has been shown to be involved in reorganization of the actin cytoskeleton. Several f-actin binding proteins, including filamin, supervillin and gelsolin, have also been shown to interact directly with AR and alter its transcriptional activity (63-66). The dual interaction of eEF1A with f-actin and AR, suggests that it may also associate with these known AR coregulator proteins as part of a multi-protein complex tethered to the cytoskeleton. Another AR interacting protein that has been shown to associate with eEF1A is calmodulin, which can bind and induce calcium-dependent AR protein degradation in LNCaP prostate cancer cells (67, 68). Moreover, eEF1A can also form protein complexes with the hsp70 steroid receptor chaperone (69). Taken together, these studies suggest that eEF1A may modulate AR function directly or through binding with known coregulators of the receptor. The fact that we detected two hsp70 binding proteins, eEF1A and GAK, along with hsp70 itself as protein partners of AR in our yeast-two hybrid screen, suggests that all of these proteins may interact with the receptor as part of a multi-protein complex. I am currently pursuing the role of eEF1A as an AR coregulator.

PFKM is the muscle isoform of phosphofructokinase 1 (PFK1; EC 2.7.1.11), which catalyzes the phosphorylation of fructose-6-phosphate to form fructose-1,6-bisphosphate (33). This reaction is the rate-limiting step in glycolysis, which is therefore critically dependent on the activity of PFK1. The three main human isozymes of PFK1

are the muscle type (PFKM), liver type (PFKL) and platelet type (PFKP) (70). Although it is difficult to speculate how the interaction of PFKM with AR may regulate receptor activity, the detection of PFKM as an AR-binding protein provides a possible novel link between androgen action on muscle and energy metabolism (71). Animal studies have shown androgen-induced increases of phosphofructokinase activity in the rat prostate, which was attributed in part to higher levels of enzyme production (72). The positive interaction observed in GST-pulldown assays (**Table 2.2**) and association of endogenous PFKM with AR in LNCaP cells, suggest that AR may directly regulate PFK1 activity in the cytosol (**Figure 2.3 B**).

Notably, a change in the rate of glycolysis has been demonstrated to be important for tumour growth in many cancers, including prostate cancer (73-75). The interaction of AR with phosphofructokinase may play a crucial role in this context. PFKM has also been reported to associate with caveolin-3 (76), another family member of which, caveolin-1, has been shown to bind and coactivate AR (14). Caveolins participate in many important cellular processes, including vesicular transport, cholesterol homeostasis and signal transduction (77). Interaction of PFKM with a member of this family implies that this kinase may form a protein complex with AR and caveolins.

TRRAP is a large nuclear transcriptional adapter protein that associates with macromolecular complexes containing histone acetyltransferases (HATs) (34, 35). TRRAP contains eight LXXLL motifs, which are also found in the p160/SRC family of coactivators and used to bind with the LBD of nuclear receptors (78). TRRAP has been shown to associate with ERα as part of a large coactivator complex in which the receptor interacts directly with three LXXLL motifs of the transcriptional adapter protein in a

ligand-dependent manner (79). TRRAP was shown to enhance the transactivation of ERα/β and other non-steroid receptor nuclear receptors. In this study, we also observe a direct interaction between TRRAP and the LBD of AR (**Table 2.2**). TRRAP is known to associate with p/CAF and Tip60 (35, 80), both of which are coactivators of AR that utilize their intrinsic HAT activities to enhance AR transactivation (81, 82). The interaction of TRRAP with AR suggests that this transcriptional adapter protein may play a role in increasing AR activity by possibly acting as a bridge between AR and p/CAF or Tip60.

$TAF_{II}250$ (also known as TAF1) is one of 12 TATA-binding protein (TBP)-associated factors that form the TFIID general transcription factor (GTF) complex in the nucleus (36, 37, 83). Of the six essential GTFs (TFIID, TFIIA, TFIIB, TFIIE, TFIIF and TFIIH) required by RNA polymerase II to recognize, bind and clear the core promoter of genes, the TBP containing TFIID is the first to bind promoters and facilitate assembly of the transcription initiation machinery (37). $TAF_{II}250$ possesses multiple enzymatic domains that include two Ser/Thr kinase domains at its N- and C-terminals (NTK and CTK), a HAT domain and a ubiquitin-activating/conjugating domain (E1/E2) (84). The interaction of $TAF_{II}250$ with AR observed here (**Tables 2.1** and **2.2**), opens up the possibility that the receptor may be phosphorylated, acetylated or targeted for ubiquitylation by this protein partner. Also, since previously the interaction of AR has only been demonstrated with GTFs TFIIF and TFIIH (19, 85), it has been suggested that AR may regulate gene expression only during the elongation and promoter clearance stages of transcription (86). The interaction of AR with the $TAF_{II}250$ component of TFIID in this study suggests that AR may also regulate transcription during assembly of

the preinitiation transcription complex. Overall, the interaction of AR with GTFs may increase the efficiency of transcription at androgen target gene promoters. The role of $TAF_{II}250$ in the regulation of AR activity is currently being studied in our laboratory.

APC is a nuclear E3 ubiquitin ligase composed of at least 11 subunits, which targets cell cycle regulatory proteins for degradation by the proteosome, thereby allowing progression through mitosis (39, 87). APC targets substrate proteins through two main adapter molecules, cdc20 and cdh1, which bind substrates with specific recognition sequence motifs termed D-box (destruction box; RXXL) and KEN-box, respectively (88, 89). The APC7 subunit detected as an AR-binding protein in this study contains tetratrico peptide repeats (TPRs), which are known to mediate protein-protein interactions (38, 90). The direct interaction of APC7 with AR may occur through these TPR motifs (**Table 2.2**). Notably, analysis of AR amino acid sequence revealed the presence of a D-box motif (^{386}RIKL^{389}), which may be a potential target recognition sequence used by APC. Targeting of AR for degradation *via* the ubiquitin-proteosome pathway has been previously reported in several studies (91). For example, the Mdm2 E3 ligase has been shown to promote AR ubiquitylation and direct the receptor to the proteosome for degradation (92). Similarly, the interaction of AR with APC may lead to ubiquitylation and degradation of the receptor in the nucleus. Recently, the APC7 subunit has been shown to interact with the AR coactivator CBP/p300, suggesting that APC may also target AR coregulators to the proteosome (93).

In conclusion, we have demonstrated that the RTA yeast two-hybrid system can be used as a reliable method of detecting AR-interacting proteins. A wide variety of AR-binding coregulator proteins have been shown to modulate AR function at different

stages of receptor activation (discussed in **Chapter 1, Section 1.4**). These coregulators of AR activity may contribute to progression of prostate cancer during which receptor transcriptional activity remains intact. The identification of several additional previously unknown AR-binding partners in this study potentially expands the already impressive diversity of mechanisms by which AR activity is modulated. These now include possible regulation of AR by a neurotransmitter synthesis enzyme (DDC), alternative phosphorylation of the receptor or its coregulators (GAK), a link to protein translational machinery (eEF1A), association with previously unknown general transcription factors ($TAF_{II}250$ of TFIID) or adapter proteins (TRRAP) and a possible novel route of mediating AR protein degradation (APC7). Future studies will focus on determining the coregulation capacity of these AR-interacting proteins, delineating the mechanism by which they may modulate AR function and analyzing their expression profile in prostate cancer (discussed in **Chapter 6**). Of these novel AR protein partners, DDC was the most frequently detected in the RTA yeast two-hybrid assay and was thus studied in further detail with respect to its coregulation of AR activity and expression profile in prostate tumours (**Chapters 3, 4, 5** and **6**).

2.6 References

1. Green S, Chambon P. Nuclear receptors enhance our understanding of transcription regulation. Trends Genet 1988;4(11):309-14.

2. Heinlein CA, Chang C. Androgen receptor in prostate cancer. Endocr Rev 2004;25(2):276-308.

3. Fang Y, Fliss AE, Robins DM, Caplan AJ. Hsp90 regulates androgen receptor hormone binding affinity in vivo. J Biol Chem 1996;271(45):28697-702.

4. He B, Kemppainen JA, Voegel JJ, Gronemeyer H, Wilson EM. Activation function 2 in the human androgen receptor ligand binding domain mediates interdomain communication with the NH(2)-terminal domain. J Biol Chem 1999;274(52):37219-25.

5. Kuil CW, Berrevoets CA, Mulder E. Ligand-induced conformational alterations of the androgen receptor analyzed by limited trypsinization. Studies on the mechanism of antiandrogen action. J Biol Chem 1995;270(46):27569-76.

6. Beato M, Chavez S, Truss M. Transcriptional regulation by steroid hormones. Steroids 1996;61(4):240-51.

7. Nelson CC, Hendy SC, Shukin RJ, Cheng H, Bruchovsky N, Koop BF, et al. Determinants of DNA sequence specificity of the androgen, progesterone, and glucocorticoid receptors: evidence for differential steroid receptor response elements. Mol Endocrinol 1999;13(12):2090-107.

8. Horwitz KB, Jackson TA, Bain DL, Richer JK, Takimoto GS, Tung L. Nuclear receptor coactivators and corepressors. Mol Endocrinol 1996;10(10):1167-77.

9. Bevan CL, Hoare S, Claessens F, Heery DM, Parker MG. The AF1 and AF2 domains of the androgen receptor interact with distinct regions of SRC1. Mol Cell Biol 1999;19(12):8383-92.

10. Heinlein CA, Chang C. Androgen receptor (AR) coregulators: an overview. Endocr Rev 2002;23(2):175-200.

11. Fronsdal K, Engedal N, Slagsvold T, Saatcioglu F. CREB binding protein is a coactivator for the androgen receptor and mediates cross-talk with AP-1. J Biol Chem 1998;273(48):31853-9.

12. Yeh S, Chang C. Cloning and characterization of a specific coactivator, ARA70, for the androgen receptor in human prostate cells. Proc Natl Acad Sci U S A 1996;93(11):5517-21.

13. Petre CE, Wetherill YB, Danielsen M, Knudsen KE. Cyclin D1: mechanism and consequence of androgen receptor co-repressor activity. J Biol Chem 2002;277(3):2207-15.

14. Lu ML, Schneider MC, Zheng Y, Zhang X, Richie JP. Caveolin-1 interacts with androgen receptor. A positive modulator of androgen receptor mediated transactivation. J Biol Chem 2001;276(16):13442-51.

15. Truica CI, Byers S, Gelmann EP. Beta-catenin affects androgen receptor transcriptional activity and ligand specificity. Cancer Res 2000;60(17):4709-13.

16. Rosenfeld MG, Glass CK. Coregulator codes of transcriptional regulation by nuclear receptors. J Biol Chem 2001;276(40):36865-8.

17. Markus SM, Taneja SS, Logan SK, Li W, Ha S, Hittelman AB, et al. Identification and characterization of ART-27, a novel coactivator for the androgen receptor N terminus. Mol Biol Cell 2002;13(2):670-82.

18. Ueda T, Bruchovsky N, Sadar MD. Activation of the androgen receptor N-terminal domain by interleukin-6 via MAPK and STAT3 signal transduction pathways. J Biol Chem 2002;277(9):7076-85.

19. McEwan IJ, Gustafsson J. Interaction of the human androgen receptor transactivation function with the general transcription factor TFIIF. Proc Natl Acad Sci U S A 1997;94(16):8485-90.

20. De Miguel F, Lee SO, Onate SA, Gao AC. Stat3 enhances transactivation of steroid hormone receptors. Nucl Recept 2003;1(1):3.

21. Choudhry MA, Ball A, McEwan IJ. The role of the general transcription factor IIF in androgen receptor-dependent transcription. Mol Endocrinol 2006;20(9):2052-61.

22. Hirst M, Ho C, Sabourin L, Rudnicki M, Penn L, Sadowski I. A two-hybrid system for transactivator bait proteins. Proc Natl Acad Sci U S A 2001;98(15):8726-31.

23. Rao MA, Cheng H, Quayle AN, Nishitani H, Nelson CC, Rennie PS. RanBPM, a nuclear protein that interacts with and regulates transcriptional activity of androgen receptor and glucocorticoid receptor. J Biol Chem 2002;277(50):48020-7.

24. Vidal M, Brachmann RK, Fattaey A, Harlow E, Boeke JD. Reverse two-hybrid and one-hybrid systems to detect dissociation of protein-protein and DNA-protein interactions. Proc Natl Acad Sci U S A 1996;93(19):10315-20.

25. Tzamarias D, Struhl K. Functional dissection of the yeast Cyc8-Tup1 transcriptional co-repressor complex. Nature 1994;369(6483):758-61.

26. Boeke JD, Trueheart J, Natsoulis G, Fink GR. 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. Methods Enzymol 1987;154:164-75.

27. Jenster G, van der Korput HA, Trapman J, Brinkmann AO. Identification of two transcription activation units in the N-terminal domain of the human androgen receptor. J Biol Chem 1995;270(13):7341-6.

28. Zhu MY, Juorio AV. Aromatic L-amino acid decarboxylase: biological characterization and functional role. Gen Pharmacol 1995;26(4):681-96.

29. Kanaoka Y, Kimura SH, Okazaki I, Ikeda M, Nojima H. GAK: a cyclin G associated kinase contains a tensin/auxilin-like domain. FEBS Lett 1997;402(1):73-80.

30. Veldscholte J, Berrevoets CA, Zegers ND, van der Kwast TH, Grootegoed JA, Mulder E. Hormone-induced dissociation of the androgen receptor-heat-shock protein complex: use of a new monoclonal antibody to distinguish transformed from nontransformed receptors. Biochemistry 1992;31(32):7422-30.

31. Shatkina L, Mink S, Rogatsch H, Klocker H, Langer G, Nestl A, et al. The cochaperone Bag-1L enhances androgen receptor action via interaction with the NH2-terminal region of the receptor. Mol Cell Biol 2003;23(20):7189-97.

32. Ejiri S. Moonlighting functions of polypeptide elongation factor 1: from actin bundling to zinc finger protein R1-associated nuclear localization. Biosci Biotechnol Biochem 2002;66(1):1-21.

33. Dunaway GA. A review of animal phosphofructokinase isozymes with an emphasis on their physiological role. Mol Cell Biochem 1983;52(1):75-91.

34. McMahon SB, Van Buskirk HA, Dugan KA, Copeland TD, Cole MD. The novel ATM-related protein TRRAP is an essential cofactor for the c-Myc and E2F oncoproteins. Cell 1998;94(3):363-74.

35. Vassilev A, Yamauchi J, Kotani T, Prives C, Avantaggiati ML, Qin J, et al. The 400 kDa subunit of the PCAF histone acetylase complex belongs to the ATM superfamily. Mol Cell 1998;2(6):869-75.

36. Goodrich JA, Tjian R. TBP-TAF complexes: selectivity factors for eukaryotic transcription. Curr Opin Cell Biol 1994;6(3):403-9.

37. Nogales E. Recent structural insights into transcription preinitiation complexes. J Cell Sci 2000;113 Pt 24:4391-7.

38. Yu H, Peters JM, King RW, Page AM, Hieter P, Kirschner MW. Identification of a cullin homology region in a subunit of the anaphase-promoting complex. Science 1998;279(5354):1219-22.

39. Peters JM. The anaphase-promoting complex: proteolysis in mitosis and beyond. Mol Cell 2002;9(5):931-43.

40. Moilanen AM, Poukka H, Karvonen U, Hakli M, Janne OA, Palvimo JJ. Identification of a novel RING finger protein as a coregulator in steroid receptor-mediated gene transcription. Mol Cell Biol 1998;18(9):5128-39.

41. Rigas AC, Ozanne DM, Neal DE, Robson CN. The scaffolding protein RACK1 interacts with androgen receptor and promotes cross-talk through a protein kinase C signaling pathway. J Biol Chem 2003;278(46):46087-93.

42. Wang X, Yeh S, Wu G, Hsu CL, Wang L, Chiang T, et al. Identification and characterization of a novel androgen receptor coregulator ARA267-alpha in prostate cancer cells. J Biol Chem 2001;276(44):40417-23.

43. Snoek R, Rennie PS, Kasper S, Matusik RJ, Bruchovsky N. Induction of cell-free, in vitro transcription by recombinant androgen receptor peptides. J Steroid Biochem Mol Biol 1996;59(3-4):243-50.

44. Zhao Y, Goto K, Saitoh M, Yanase T, Nomura M, Okabe T, et al. Activation function-1 domain of androgen receptor contributes to the interaction between subnuclear splicing factor compartment and nuclear receptor compartment. Identification of the p102 U5 small nuclear ribonucleoprotein particle-binding protein as a coactivator for the receptor. J Biol Chem 2002;277(33):30031-9.

45. Greener T, Zhao X, Nojima H, Eisenberg E, Greene LE. Role of cyclin G-associated kinase in uncoating clathrin-coated vesicles from non-neuronal cells. J Biol Chem 2000;275(2):1365-70.

46. Korolchuk VI, Banting G. CK2 and GAK/auxilin2 are major protein kinases in clathrin-coated vesicles. Traffic 2002;3(6):428-39.

47. Flett A, Semerdjieva S, Jackson AP, Smythe E. Regulation of the clathrin-coated vesicle cycle by reversible phosphorylation. Biochem Soc Symp 2005(72):65-70.

48. Lindquist S. The heat-shock response. Annu Rev Biochem 1986;55:1151-91.

49. Yin X, Warner DR, Roberts EA, Pisano MM, Greene RM. Identification of novel CBP interacting proteins in embryonic orofacial tissue. Biochem Biophys Res Commun 2005;329(3):1010-7.

50. Prescott J, Coetzee GA. Molecular chaperones throughout the life cycle of the androgen receptor. Cancer Lett 2006;231(1):12-9.

51. Fan CY, Ren HY, Lee P, Caplan AJ, Cyr DM. The type I Hsp40 zinc finger-like region is required for Hsp70 to capture non-native polypeptides from Ydj1. J Biol Chem 2005;280(1):695-702.

52. Marivoet S, Van Dijck P, Verhoeven G, Heyns W. Interaction of the 90-kDa heat shock protein with native and in vitro translated androgen receptor and receptor fragments. Mol Cell Endocrinol 1992;88(1-3):165-74.

53. Moldave K. Eukaryotic protein synthesis. Annu Rev Biochem 1985;54:1109-49.

54. Kaziro Y, Itoh H, Kozasa T, Nakafuku M, Satoh T. Structure and function of signal-transducing GTP-binding proteins. Annu Rev Biochem 1991;60:349-400.

55. Lamberti A, Caraglia M, Longo O, Marra M, Abbruzzese A, Arcari P. The translation elongation factor 1A in tumorigenesis, signal transduction and apoptosis: review article. Amino Acids 2004;26(4):443-8.

56. Thornton S, Anand N, Purcell D, Lee J. Not just for housekeeping: protein initiation and elongation factors in cell growth and tumorigenesis. J Mol Med 2003;81(9):536-48.

57. Shen R, Su ZZ, Olsson CA, Fisher PB. Identification of the human prostatic carcinoma oncogene PTI-1 by rapid expression cloning and differential RNA display. Proc Natl Acad Sci U S A 1995;92(15):6778-82.

58. Mansilla F, Hansen LL, Jakobsen H, Kjeldgaard NO, Clark BF, Knudsen CR. Deconstructing PTI-1: PTI-1 is a truncated, but not mutated, form of translation elongatin factor 1A1, eEF1A1. Biochim Biophys Acta 2005;1727(2):116-24.

59. Gangwani L, Mikrut M, Galcheva-Gargova Z, Davis RJ. Interaction of ZPR1 with translation elongation factor-1alpha in proliferating cells. J Cell Biol 1998;143(6):1471-84.

60. Edmonds BT, Wyckoff J, Yeung YG, Wang Y, Stanley ER, Jones J, et al. Elongation factor-1 alpha is an overexpressed actin binding protein in metastatic rat mammary adenocarcinoma. J Cell Sci 1996;109 (Pt 11):2705-14.

61. Galcheva-Gargova Z, Konstantinov KN, Wu IH, Klier FG, Barrett T, Davis RJ. Binding of zinc finger protein ZPR1 to the epidermal growth factor receptor. Science 1996;272(5269):1797-802.

62. Culig Z, Hobisch A, Cronauer MV, Radmayr C, Trapman J, Hittmair A, et al. Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. Cancer Res 1994;54(20):5474-8.

63. Ozanne DM, Brady ME, Cook S, Gaughan L, Neal DE, Robson CN. Androgen receptor nuclear translocation is facilitated by the f-actin cross-linking protein filamin. Mol Endocrinol 2000;14(10):1618-26.

64. Loy CJ, Sim KS, Yong EL. Filamin-A fragment localizes to the nucleus to regulate androgen receptor and coactivator functions. Proc Natl Acad Sci U S A 2003;100(8):4562-7.

65. Ting HJ, Yeh S, Nishimura K, Chang C. Supervillin associates with androgen receptor and modulates its transcriptional activity. Proc Natl Acad Sci U S A 2002;99(2):661-6.

66. Nishimura K, Ting HJ, Harada Y, Tokizane T, Nonomura N, Kang HY, et al. Modulation of androgen receptor transactivation by gelsolin: a newly identified androgen receptor coregulator. Cancer Res 2003;63(16):4888-94.

67. Pelley RP, Chinnakannu K, Murthy S, Strickland FM, Menon M, Dou QP, et al. Calmodulin-androgen receptor (AR) interaction: calcium-dependent, calpain-mediated breakdown of AR in LNCaP prostate cancer cells. Cancer Res 2006;66(24):11754-62.

68. Kaur KJ, Ruben L. Protein translation elongation factor-1 alpha from Trypanosoma brucei binds calmodulin. J Biol Chem 1994;269(37):23045-50.

69. Marchesi VT, Ngo N. In vitro assembly of multiprotein complexes containing alpha, beta, and gamma tubulin, heat shock protein HSP70, and elongation factor 1 alpha. Proc Natl Acad Sci U S A 1993;90(7):3028-32.

70. Vora S. Isozymes of phosphofructokinase. Isozymes Curr Top Biol Med Res 1982;6:119-67.

71. Tindall DJ. Androgen receptors in prostate and skeletal muscle. Mayo Clin Proc 2000;75 Suppl:S26-30; discussion S30-1.

72. Singhal RL, Valadares JR. Metabolic control mechanisms in mammalian systems. Hormonal regulation of phosphofructokinase in the rat prostate and seminal vesicles. Biochem J 1968;110(4):703-11.

73. Warburg O. On the origin of cancer cells. Science 1956;123(3191):309-14.

74. Liu Y. Fatty acid oxidation is a dominant bioenergetic pathway in prostate cancer. Prostate Cancer Prostatic Dis 2006;9(3):230-4.

75. Baron A, Migita T, Tang D, Loda M. Fatty acid synthase: a metabolic oncogene in prostate cancer? J Cell Biochem 2004;91(1):47-53.

76. Scherer PE, Lisanti MP. Association of phosphofructokinase-M with caveolin-3 in differentiated skeletal myotubes. Dynamic regulation by extracellular glucose and intracellular metabolites. J Biol Chem 1997;272(33):20698-705.

77. Williams TM, Lisanti MP. The caveolin proteins. Genome Biol 2004;5(3):214.

78. Heery DM, Kalkhoven E, Hoare S, Parker MG. A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature 1997;387(6634):733-6.

79. Yanagisawa J, Kitagawa H, Yanagida M, Wada O, Ogawa S, Nakagomi M, et al. Nuclear receptor function requires a TFTC-type histone acetyl transferase complex. Mol Cell 2002;9(3):553-62.

80. Frank SR, Parisi T, Taubert S, Fernandez P, Fuchs M, Chan HM, et al. MYC recruits the TIP60 histone acetyltransferase complex to chromatin. EMBO Rep 2003;4(6):575-80.

81. Reutens AT, Fu M, Wang C, Albanese C, McPhaul MJ, Sun Z, et al. Cyclin D1 binds the androgen receptor and regulates hormone-dependent signaling in a p300/CBP-associated factor (P/CAF)-dependent manner. Mol Endocrinol 2001;15(5):797-811.

82. Gaughan L, Logan IR, Cook S, Neal DE, Robson CN. Tip60 and histone deacetylase 1 regulate androgen receptor activity through changes to the acetylation status of the receptor. J Biol Chem 2002;277(29):25904-13.

83. Tora L. A unified nomenclature for TATA box binding protein (TBP)-associated factors (TAFs) involved in RNA polymerase II transcription. Genes Dev 2002;16(6):673-5.

84. Wassarman DA, Aoyagi N, Pile LA, Schlag EM. TAF250 is required for multiple developmental events in Drosophila. Proc Natl Acad Sci U S A 2000;97(3):1154-9.

85. Lee DK, Duan HO, Chang C. From androgen receptor to the general transcription factor TFIIH. Identification of cdk activating kinase (CAK) as an androgen receptor NH(2)-terminal associated coactivator. J Biol Chem 2000;275(13):9308-13.

86. Lee DK, Chang C. Molecular communication between androgen receptor and general transcription machinery. J Steroid Biochem Mol Biol 2003;84(1):41-9.

87. Harper JW, Burton JL, Solomon MJ. The anaphase-promoting complex: it's not just for mitosis any more. Genes Dev 2002;16(17):2179-206.

88. Pfleger CM, Kirschner MW. The KEN box: an APC recognition signal distinct from the D box targeted by Cdh1. Genes Dev 2000;14(6):655-65.

89. Vodermaier HC. Cell cycle: Waiters serving the Destruction machinery. Curr Biol 2001;11(20):R834-7.

90. Lamb JR, Tugendreich S, Hieter P. Tetratrico peptide repeat interactions: to TPR or not to TPR? Trends Biochem Sci 1995;20(7):257-9.

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

92. Lin HK, Wang L, Hu YC, Altuwaijri S, Chang C. Phosphorylation-dependent ubiquitylation and degradation of androgen receptor by Akt require Mdm2 E3 ligase. Embo J 2002;21(15):4037-48.

93. Turnell AS, Stewart GS, Grand RJ, Rookes SM, Martin A, Yamano H, et al. The APC/C and CBP/p300 cooperate to regulate transcription and cell-cycle progression. Nature 2005;438(7068):690-5.

CHAPTER 3. CHARACTERIZATION OF L-DOPA DECARBOXYLASE AS A PROTEIN THAT BINDS TO AND ENHANCES TRANSCRIPTIONAL ACTIVITY OF THE ANDROGEN RECEPTOR

A version of this chapter has been published.

Wafa LA, Cheng H, Rao MA, Nelson CC, Cox ME, Hirst M, Sadowski I, Rennie PS (2003). Isolation and identification of L-dopa decarboxylase as a protein that binds to and enhances transcriptional activity of the androgen receptor using the repressed transactivator yeast two-hybrid system. Biochem J. 375 (Pt 2): 373-383.

Dr. Michael Cox and Dr. Colleen Nelson from the Prostate Centre provided critical comments and reviewed this work.

3.1 Introduction

L-dopa decarboxylase (DDC), also known as aromatic L-amino acid decarboxylase (E.C. 4.1.1.28), is a homodimeric enzyme that belongs to the α-family of pyridoxal 5'-phosphate (PLP)-dependent enzymes (1, 2). DDC is responsible for the synthesis of the important neurotransmitters dopamine (DA) and serotonin (5-hydroxytryptamine, 5-HT) *via* decarboxylation of L-3,4-dihydroxyphenylalanine (L-dopa) and L-5-hydroxytryptophan (5-HTP), respectively (**Figure 3.1**) (1). DDC has been implicated in Parkinson's disease, which is a chronic progressive neurological disorder characterized by massive neuronal degeneration, tremors, bradykinesia and postural instability (3). Although the exact cause is not known, Parkinson's disease is thought to be the result of degeneration of dopamine-producing cells in the midbrain (4, 5). Oral administration of L-dopa remains the most effective treatment for the symptoms of this disorder. Since L-dopa is rapidly converted into dopamine in the blood stream, treatment

with this drug is routinely combined with a DDC inhibitor, such as carbidopa, which allows greater amounts of L-dopa to reach the brain for conversion into dopamine (6). The role of DDC in neurotransmitter synthesis and Parkinson's disease has been examined extensively and is a subject outside of the focus of the current study (1, 7-9). However, it is important to review the molecular biology, enzyme structure/function and tissue expression profile of DDC, as these topics are relevant to our finding that this protein can also interact with AR to possibly modify its activity (**Chapter 2**).

The single copy human DDC gene consists of 15 exons spanning more than 85 kb and is located on chromosome band 7p12.1-p12.3 (10). The 5' untranslated region of DDC mRNA consists of exons 1 and 2 (11). Alternative splicing of the first exon in the 5'-untranslated region is known to produce two types of tissue-specific DDC mRNAs. These include the neuronal (pheochromocytoma tissue) and non-neuronal (liver tissue) DDC transcripts, which have identical coding regions that generate a single 480 amino acid protein isoform (11, 12). The existence of a third alternative splice isoform of DDC, missing the exon 3 coding region, has also been reported (13). The normal physiological function of this truncated 442 amino acid DDC protein is not known. However, it is expressed in both neuronal and non-neuronal tissues but lacks the catalytic decarboxylation activity of the enzyme (14).

The active DDC enzyme has been reported to be a homodimer that binds a single PLP cofactor molecule (1). The cofactor binding site, with amino acid sequence Asn-Phe-Asn-Pro-His-Lys-Trp, is highly conserved among species and has been shown to be identical in humans, *Drosophila*, and pigs. DDC catalytic activity is dependent on PLP, which binds tightly to the ε-amino group of the lysine residue in the cofactor binding site.

In humans, PLP binds to Lys303, making this amino acid a necessity for DDC decarboxylation activity (**Figure 3.2**). The active site of DDC is located near the monomer-monomer interface, where PLP binds to Lys303, allowing decarboxylation of amino acid substrates into amines through a Schiff base mechanism (described in further detail in **Section 4.1** of **Chapter 4**) (15). This results in release of CO_2 from the substrate α-carbon, which becomes protonated (**Figure 3.1**). Although, DDC predominantly utilizes the above mechanism for production of DA and 5-HT, it has also been considered to be involved in the synthesis of trace amines from other amino acids. These include conversion of tyrosine into tyramine, phenylalanine into 2-phenylethylamine, and tryptophan into tryptamine (1, 2). Notably, DDC has not been previously reported to interact with steroid receptors or other transcription factors.

The expression of DDC has been investigated in various brain regions as well as several peripheral tissues. It is widely distributed in neural tissues, where it plays a neuron-specific role as a neurotransmitter biosynthetic enzyme, and in non-neuronal tissues (adrenals, kidney, liver, gastrointestinal tract and lungs), where it acts as a non-specific decarboxylating enzyme or may have other so far undetermined functions (16). High levels of DDC expression/activity and its role as a neuroendocrine (NE) marker have been reported in several peripheral cancers, including small cell lung carcinoma, bowel cancer, and neuroblastoma (17-21). More recently, we have also found DDC to be a prostate cancer NE marker that is co-expressed with AR in a sub-population of prostate NE-phenotype adenocarcinoma cells (discussed in **Chapter 5**) (22). Moreover, DDC has been shown to be a core component of the transcript signature for tumour cells in a NE-transformed transgenic prostate cancer mouse model (23). DDC and corresponding

polyamine metabolites were suggested to be biomarkers of poor-prognosis NE tumours and mediators of cellular communication within NE tumour microenvironments. One of the unknown functions of DDC in peripheral tissues may be to interact with AR and possibly regulate its activity in prostatic NE-phenotype cells.

In the previous study (**Chapter 2**), the repressed transactivator (RTA) yeast two-hybrid system was used to screen a prostate carcinoma cell line cDNA library for AR-NTD protein partners. One of the clones detected multiple times in this screen coded for a partial sequence of DDC, demonstrating a novel role for this enzyme as an AR binding protein. Here we demonstrate that DDC interacts with AR *in vitro* and *in vivo,* as well as increases the transcriptional activity of the receptor in prostate cancer cell lines. This enhancement is inhibited when transactivation assays are performed in the presence of bicalutamide (anti-androgen). Taken together, these results suggest that DDC may play an important role in AR gene regulation during prostate cancer progression.

3.2 Materials and Methods

Plasmids and Cloning of Full-length DDC

The full-length DDC expression vector, pDEST12.2-DDC, was constructed by RT-PCR on LNCaP RNA and Gateway™ cloning (Gibco BRL). Primers used (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAGGAGATAGAACC**ATG AACGCAAGTGAATTCCGAAGG** and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTA**CTCCCTCTCTGCTCG CAGCAC**) contained shine-dalgarno, kozak and DDC gene-specific (in bold) sequences, along with the *att*B recombination sites allowing incorporation of the PCR product into

pDONR201 to generate the pENTR-DDC entry vector. After frame and sequence verification, the entry vector was used in a second recombination site reaction with pDEST12.2 (contains CMV promoter for mammalian expression and SP6 promoter for *in vitro* transcription/translation) to produce the pDEST12.2-DDC expression plasmid. Only the coding region of DDC was cloned, excluding any differences due to neuronal versus non-neuronal mRNA (11, 12).

The full-length rat AR was expressed from the pRC/CMV mammalian expression vector, pAR_6 (24). The rat glucocorticoid receptor (pGR) and human estrogen receptor (pERα) mammalian expression vectors have been described previously (25, 26).

GST-Pulldown Assays

Various fragments of the rat AR ($AR_{234-665}$, $AR_{541-665}$, $AR_{541-919}$) and a N-terminal fragment of the human AR (AR_{1-559}), lacking the DNA binding domain, were cloned into the pGEX vector for expression of GST-fusion proteins in BL21 *Escherichia coli* cells, as described previously (27). For semi-quantitative analysis of binding affinity between regions of DDC and AR, the volume of resuspended GST-AR domain fusion protein bound beads corresponding to an equimolar amount of each fusion protein was initially determined. Fusion protein bound bead volumes were titrated, eluted with sample buffer (2 % SDS and 5 % β-mercaptoethanol) and analyzed by SDS-PAGE/Coomassie Blue staining. The eluent in each case was run along side known amounts of bovine serum albumin (BSA), ranging from 250-1000 ng, to generate a standard curve for protein concentrations. Band intensities were measured (Gel Doc 2000, Quantity One, BIO-RAD) and the amount of GST-fusion protein corresponding to specific bead volumes

(fusion protein bound) was calculated from the BSA standard curve (data not shown). The estimated volume of fusion protein bound-beads containing the proper amount of protein was then re-analysed using SDS-PAGE, as above. This was done in triplicate to ensure that bead volumes used in the GST-pulldown assays, contained an equimolar concentration of each GST-AR domain fusion protein.

The pDEST12.2-DDC vector was used to prepare [^{35}S]-radiolabeled full-length DDC with the Quick Coupled SP6 TnT *in vitro* transcription/translation kit (Promega). The same vector was also used for *Eco*57I digestion (single cut site at nucleotide 1071 of DDC cDNA) and preparation of the radiolabeled DDC N-terminal fragment (DDC_{1-333}). The C-terminal DDC fragment ($DDC_{328-480}$) was translated (T7 promoter kit) from the pGADT7-$DDC_{328-480}$ yeast two-hybrid clone vector.

Equimolar amounts of GST-AR fusion protein coupled to glutathione beads were incubated with radiolabeled full-length or fragments of DDC at 4 °C for 2 hours in binding buffer (20 mM HEPES pH 7.6, 150 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.05% NP-40). Beads were washed four times with binding buffer and bound proteins were eluted in sample buffer for SDS-PAGE and autoradiography visualization. Dried gels were also analyzed using a phosphorimaging screen and the Molecular Imager FX (BIO-RAD). Quantity One software was used to obtain [CNT*mm^2] values (counts × area) for radiolabeled protein bands. GST-pulldown assays were performed in triplicate and the mean (± SEM) bound [CNT*mm^2] was normalized to [CNT*mm^2] values of the loaded input in order to determine the percentage of total input bound.

Mammalian Cell Culture and Transfection

Human prostate cancer PC3 and DU145 cells were maintained in Dulbecco's modified Eagle's medium, DMEM (Sigma, MO), supplemented with 5 % fetal bovine serum (FBS) (GibcoBRL) at 37 °C in 5 % CO_2. LNCaP prostate carcinoma cells were cultured in RPMI media containing 5 % FBS. Transactivation assay transient transfections were carried out in 6-well plates. Cells were seeded at a density of 3 x 10^5 cells/well and transfected the following day using Lipofectin Reagent (Invitrogen) according to the manufacturer's protocol (28). Cells were incubated with transfection mix for 16 hours at 37 °C and subsequently re-fed with 5 % dextran-coated charcoal stripped FBS containing either 1 nM R1881, 10 nM dexamethasone (Dex), 10 nM 17β-estradiol (E_2) or vehicle alone. For anti-androgen studies cells were re-fed with 1 nM R1881 and varying concentrations of bicalutamide (provided by Astra-Zenaca, Wilmington, DE) or vehicle alone. The cells were then incubated for 24 hours at 37 °C before lysis in passive lysis buffer (Promega) for luciferase assay and Western blot analysis.

Co-immunoprecipitation and Western Blotting

LNCaP cells (2×10^6) were plated on 10 cm dishes and grown to 70 % confluency in RPMI with 5 % FBS. Cells were then transfected with pDEST12.2-DDC vector (5 μg/dish) using Lipofectin Reagent (Invitrogen). Media was changed to RPMI containing 5 % dextran-coated charcoal stripped FBS and incubated for a further 16 hours at 37 °C to deplete cells of bio-available hormone. Cells were then treated with or without 10 nM R1881 for 4 hours before harvest. Washed cells were resuspended in

0.5 % NP-40 buffer (150 mM NaCl, 50 mM Tris-Cl pH 7.5, 2 mM EDTA, 0.5 % NP-40) for lysis. Cell extracts (1 mg protein, as quantitated by BCA assay) were incubated with a monoclonal mouse anti-AR_{DBD} antibody (BD Biosciences, PharMingen) or with an equivalent amount (1 μg) of normal mouse IgG (negative control). rProtein G-agarose (Invitrogen) beads were used to immunoprecipitate antibody-protein complexes. Beads were washed four times with lysis buffer and resuspended in 2X SDS sample buffer. Associated proteins were resolved by SDS-PAGE and transferred to a PVDF membrane (Millipore, MA), as previously described (24). Membranes were blocked in 5 % skim milk in TBS (20 mM Tris-HCl PH 7.6, 140 mM NaCl) prior to incubation with the appropriately diluted primary antibody (final concentration of 1 μg/mL). AR and DDC were detected using rabbit polyclonal antibodies obtained from Affinity BioReagents and Chemicon, respectively. Blots were developed using horseradish peroxidase-conjugated secondary antibody and the ECL chemiluminescence kit (Amersham). To verify specificity of the anti-$DDC_{N\text{-terminal}}$ antibody (Chemicon), a competition assay was carried out using Ni-NTA (Qiagen) affinity purified 6×His-tagged DDC protein (see **Section 4.2** of **Chapter 4** for detailed protocol of His-DDC protein production). This purified protein was incubated with primary antibody solution before performing the Western blot (10:1 molar ratio of 6×His-DDC to antibody). Antibodies for GR and ERα were obtained from Transduction Laboratories (BD Biosciences) and Santa Cruz Biotechnology, respectively. The rabbit polyclonal anti-β-actin antibody was purchased from Sigma.

Transcriptional Assays

The pARR3-tk-Luc reporter construct (27), which has three tandem androgen response regions (ARRs), was used for assaying AR and GR transcriptional activity.

ERα activity was monitored with the ERE-Luc reporter, which contains a single vitellogenin estrogen response element upstream of a thymidine kinase promoter. PC3 and LNCaP cells, transfected in 6-well plates, had total DNA amounts adjusted to 3 μg/well using pRC/CMV vector. Cells were treated with or without hormone for 24 hours at 37 °C prior to analysis. The renilla luciferase vector, pRL-TK (Promega), was used for normalization of transfection efficiency. The Dual Luciferase Assay kit (Promega) and MicroLumiat*Plus* luminometer (EG&G Berthold) were used to assay firefly and renilla luciferase activities. Firefly luciferase values were normalized to renilla luciferase and expressed as relative luciferase units (RLU) ± SEM. All assays were done in triplicate, with at least three independent trials.

Statistical Analysis

Statistical analyses for transcriptional assay RLU values and for GST-pulldown assays (percentage of total input bound values) were performed using the Student's t-test (two-sample equal variance) with JMPIN statistical software (Version 4.0.2, SAS Institute Inc.). All calculated p-values were two-sided and those less than 0.05 were considered statistically significant.

3.3 Results

Detection of DDC as an AR-NTD Interacting Protein in Yeast

As outlined in **Chapter 2**, screening of a LNCaP cDNA library in the RTA system, using AR-NTD baits, detected six clones that coded for the C-terminal region of L-dopa decarboxylase. Due to its high frequency of detection, DDC was characterized

extensively with respect to its interaction with AR and coregulation capacity on the receptor (current **Chapter 3**), mechanism of AR activity coregulation (**Chapter 4**) and expression profile in prostate cancer (**Chapter 5**). The six library clones isolated with the RTA yeast two-hybrid assay were all approximately 900 bp, one of which coded for amino acids 328-480 of DDC (pGADT7-DDC$_{328-480}$) and the 3'-untranslated region (**Figure 3.2**). The full-length DDC coding region was then cloned into the Gateway^{TM} (Gibco, BRL) pDEST12.2 mammalian expression vector (pDEST12.2-DDC) using RT-PCR on RNA from LNCaP prostate cancer cells.

DDC Interacts with LBD and NTD of AR Through its C-terminus

To confirm results of the two-hybrid screen, GST-pulldown assays were performed using a series of GST-fused AR fragments (**Figure 3.3**). For quantitative assessment of binding affinity between various domains of AR and radiolabeled DDC, initial equimolar normalization of GST-AR domain fusion proteins was carried out, as described in detail under Materials and Methods (**Section 3.2**). With this type of analysis, differences in binding between AR and DDC can be attributed to actual affinity rather than differential expression or binding ability of the GST-AR domain fusion proteins to the glutathione beads. **Figure 3.3B** shows the Coomassie stain used for normalization of the GST-AR domains ($AR_{234-665}$, $AR_{541-665}$, $AR_{541-919}$, AR_{1-559}) and a representative autoradiogram of one GST-pulldown assay with radiolabeled DDC. Full-length DDC protein does not interact significantly with the AR_{1-559} (NTD) fragment, which was one of three baits used in the RTA yeast two-hybrid screen. The strongest interaction of DDC occurs with the $AR_{541-919}$ (DBD/LBD) fragment. Since the $AR_{541-665}$

(DBD) interaction is also extremely weak, the strong binding of full-length DDC protein with AR can be largely attributed to its affinity for the LBD of the receptor.

To determine the region of DDC responsible for interaction with AR, N-terminal (DDC_{1-333}) and C-terminal ($DDC_{328-480}$) fragments of the protein were radiolabeled and used for GST-AR domain pulldown analysis (**Figure 3.3B**). A summary of the quantified GST-pulldown data (% total input bound) for these DDC truncations and the full-length protein is shown in **Figure 3.3C**. Similar to the full-length protein, $DDC_{328-480}$ interacts most strongly with the LBD containing fragment ($AR_{541-919}$) of AR, but has greater than 5-times higher affinity for this region (11 % versus 2 % total input bound). The difference in binding affinity for the AR_{1-559} NTD fragment is even more drastic. A negligible percentage of full-length DDC total input binds to AR_{1-559}, as compared to the 5 % input bound for $DDC_{328-480}$. This strong affinity of the DDC C-terminal region for the N-terminus of AR may explain its high incidence as a positive clone in the RTA screen, where all six detected DDC clones were incomplete C-terminal cDNAs of similar length. The N-terminal fragment of DDC (residues 1-333) does not interact with any domains of AR *in vitro.*

DDC is Expressed in Prostate Cancer Cell Lines

A polyclonal antibody (Chemicon) raised against an N-terminal peptide of human DDC, was used to determine protein expression in three different prostate cancer cell lines. Western blot analysis of LNCaP, PC3 and DU145 cell lysates show that this antibody recognizes a single endogenous protein at the expected molecular weight of ~ 50 kDa in LNCaP cells (**Figure 3.4A**, lane 1), but in PC3 and DU145 cells a lower

~ 35 kDa band is also detected (lanes 2 and 3). The endogenous protein migrates at the same apparent molecular weight as ectopically expressed DDC (LNCaP cells transfected with pDEST12.2-DDC vector, lane 4). An immuno-competition assay was carried out to determine specificity of the antibody using purified 6×His-DDC protein. As can be seen from **Figure 3.4A** (lanes 5-8), incubation of antibody solution with 6×His-DDC protein, prior to immunoblotting, selectively prevents detection of the ~ 50 kDa band for all cell lines. Thus, the ~35 kDa band seen in PC3 and DU145 cells is non-specific. This confirms that endogenous DDC protein is present in these prostate cancer cells. A β-actin antibody was used to check loading efficiency in all lanes.

DDC Interacts with AR *in Vivo*

The presence of DDC protein in LNCaP cells and the strong *in vitro* interaction of this protein with AR, suggested the possibility that DDC may be able to interact with the receptor in intact cells. Since LNCaP cells express high levels of endogenous AR and are androgen sensitive, this prostate carcinoma cell line was used to assess the association of DDC with AR *in vivo* in the presence and absence of androgen. Cell extracts from untreated or 10 nM R1881-treated LNCaP cells (transfected with pDEST12.2-DDC) were subjected to immunoprecipitation with an anti-AR_{DBD} antibody and analyzed by Western blot with antibodies raised against the N-termini of DDC and AR. DDC co-immunoprecipitated with AR in the presence and absence of R1881 (compare lanes 4 and 6 in **Figure 3.4B**), but the association was stronger with androgen treatment.

DDC Enhances AR Transcriptional Activity

To examine the biological impact of the DDC-AR interaction, transient transfection transactivation assays were carried out using PC3 cells. This prostate cancer cell line does not express endogenous AR, allowing the titration of receptor and DDC expression levels. PC3 cells were transfected with a constant amount of AR vector (pAR_6) and increasing amounts of DDC expression vector (pDEST12.2-DDC), along with the pARR3-tk-Luc reporter plasmid. The ratio of AR:DDC was varied from 1:0, with no DDC transfected, to 1:10, where 10 times more DDC was transfected than AR. As can be seen in **Figure 3.5**, ligand-dependent AR activity was enhanced with increasing DDC expression with a maximum of ~ 15 fold at the 1:10 co-transfection ratio. This enhancement of AR transactivation did not result from increased AR protein production since receptor levels remained constant with DDC co-expression, as determined by Western blot analysis. The effect of DDC on AR activity was not restricted to a single cell type. Similar transient transfection assays in LNCaP cells also enhanced ligand-dependent AR transcriptional activity 2-3 fold for the 1:10 ratio (data not shown). In the absence of ligand, AR activity was negligible and did not change with DDC co-transfection.

DDC Enhances Transcriptional Activity of Other Steroid Receptors

To determine whether the biological effect of DDC on AR is receptor-specific, the transient transfection assays were repeated in PC3 cells with GR and ERα (pGR and pERα). **Figure 3.6A** shows that DDC co-transfection increased GR ligand-dependent transcriptional activity up to 20 fold (1:10 ratio) in the presence of 10 nM Dex. Similar

to AR, GR activity remained unchanged in the absence of ligand. Therefore, DDC co-expression seems to enhance GR-mediated transcription in a manner similar to that of AR in PC3 cells. However, the effect of DDC on ERα activity in these cells was much weaker. In the presence of 10 nM E_2, there is only about a 2-fold increase in ERα activity even with high levels (1:10 ratio) of DDC expression (**Figure 3.6B**). Western blot analysis was used to monitor GR and ERα expression. Similar to AR, after normalization with the β-actin loading control, these receptor protein levels remained constant. The ability of DDC to enhance transactivation of other steroid receptors is not surprising since the full-length protein interacts strongly with the LBD of AR *in vitro.* This domain is well conserved among members of the steroid receptor family and may be the region through which DDC generally binds to enhance transcriptional activity.

Bicalutamide (Anti-Androgen) Inhibits the Enhanced AR Transcriptional Activity Observed with DDC Over-Expression

The association of DDC with AR and the resultant enhancement of ligand-dependent AR activity in prostate cancer cells suggest that this protein may play an important role in the regulation of receptor transactivation during disease progression. To investigate the possible effect of DDC on AR activation in hormone refractory prostate cancer treated with anti-androgens, the above transactivation assays were performed using PC3 (AR transfected) and LNCaP (express endogenous AR) cells in the presence and absence of the pure anti-androgen bicalutamide. As shown in **Figure 3.7A**, in PC3 cells, bicalutamide at a concentration of 10 μM could drastically reduce the AR

transcriptional activity stimulated by 1 nM R1881. At 50 μM bicalutamide, AR activity was almost completely blocked. In contrast, DDC transfected PC3 cells treated with 1 nM R1881 and 10 μM bicalutamide had a partial inhibition in AR activity and had lower, but still significant, activity at 50 μM bicalutamide. As expected (29, 30), we were able to substantially block the activity of the endogenous AR in LNCaP cells using bicalutamide (**Figure 3.7B**). The transfection of DDC in LNCaP cells augmented ligand-dependent AR transcriptional activity and similar to PC3 cells, the activity of AR was higher at both concentrations of bicalutamide when DDC was over-expressed. Nevertheless, the enhanced AR activity seen with DDC transfection was significantly reduced with bicalutamide in both PC3 and LNCaP cells, even though this required higher concentrations of the anti-androgen. These results suggest that DDC functions by increasing the transcriptional activity of AR through a ligand-mediated mechanism.

3.4 Figures

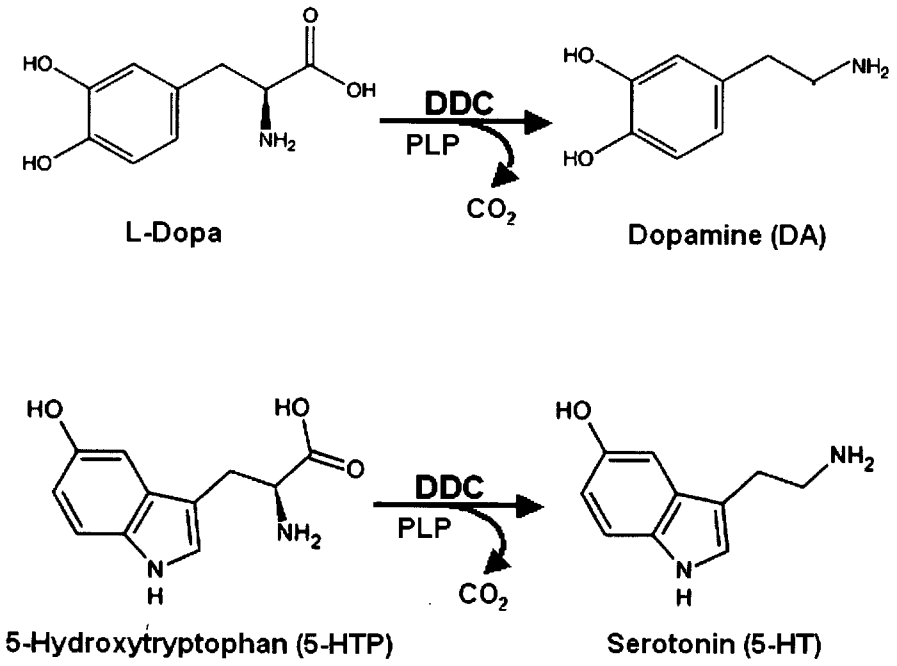

Figure 3.1 - Decarboxylation Activity of DDC. L-Dopa decarboxylase catalyzes the decarboxylation step that converts L-3,4-dihydroxyphenylalanine (L-Dopa) and L-5-hydroxytryptophan (5-HTP) into the neurotransmitters dopamine (DA) and serotonin (5-hydroxytryptamine, 5-HT), respectively. In both reactions the CO_2 group of the amino acid substrate α-carbon is replaced with a proton (H) to produce the amine product. The pyridoxal 5'-phosphate (PLP) cofactor is necessary for enzymatic function of DDC.

1 mnasefrrrg kemvdyvany megiegrqvy pdvepgylrp lipaaapqep dtfediindv

61 ekiimpgvth whspyffayf ptassypaml admlcgaigc igfswaaspa cteletvmmd

121 wlgkmlelpk aflnekageg ggviqgsase **atlvallaar** tkvihrlqaa speltqaaim

181 eklvayssdq ahssveragl iggvklkaip sdgnfamras alqealerdk aaglipffmv

241 atlgtttccs fdnllevgpi cnkediwlhv daayagsafi cpefrhllng vefadsf*n*f*n*

301 *phk*wllvnfd csamwvkkrt dltgafrldp tylkhshqds glitdyrhwq iplgrrfrsl

361 kmwfvfrmyg vkglqayirk hvqlshefes lvrqdprfei cvevilglvc frlkgsnkvn

421 eallqrinsa kkihlvpchl rdkfvlrfai csrtvesahv qrawehikel aadvlraer*

Figure 3.2 - Detection of DDC as an AR-NTD Interacting Protein. The amino acid sequence of DDC (GenBank™ accession number NM000790) is shown, with an asterisk representing the stop codon. Six independent clones coding for DDC were detected using the RTA yeast two-hybrid system. Underlined region shows the protein encoded by one of the cDNA sequences isolated ($DDC_{328-480}$). Italicized residues represent the pyridoxal phosphate cofactor binding site of DDC containing lysine residue 303 (*k*). Bolded residues show the single LXXLL motif (amino acids 153-157) found in the classical p160/SRC family of steroid receptor coactivators. The entire coding region of full-length DDC protein consists of 480 amino acids.

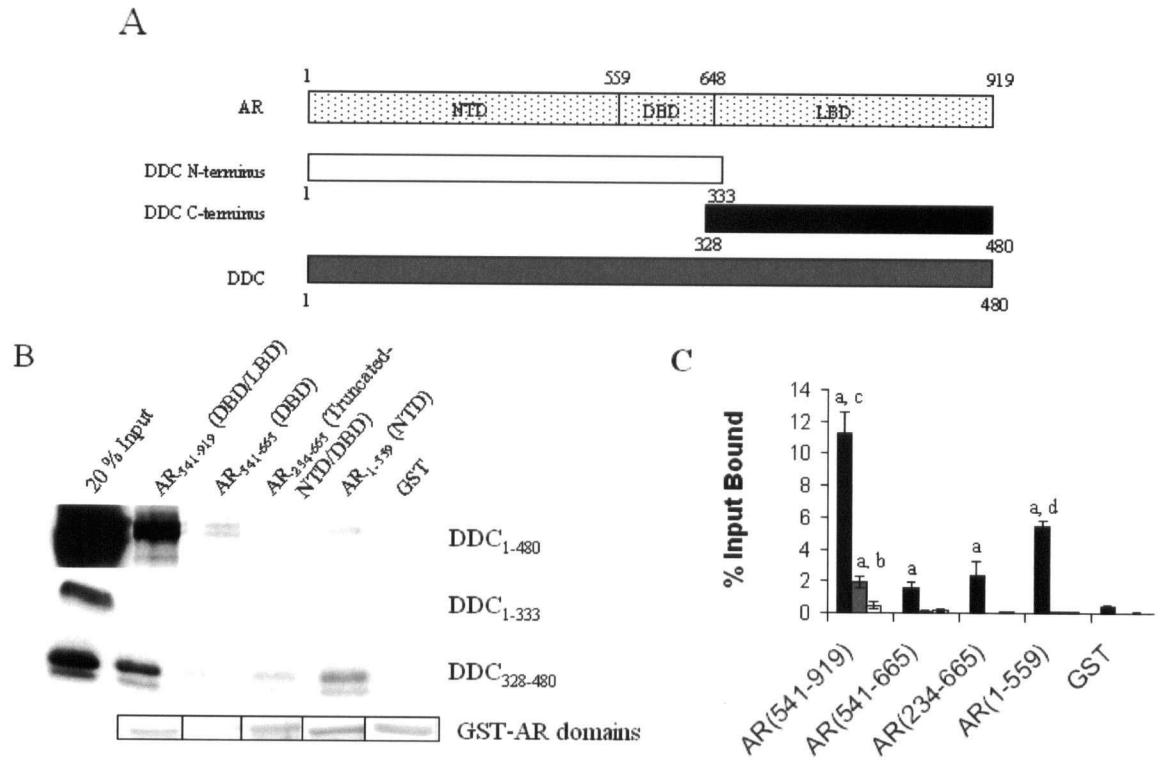

Figure 3.3 - DDC Interacts with AR *in Vitro* Through its C-terminus (amino acids 328-480). A) Diagram of AR functional domains and DDC regions used in GST-pulldown assays (open, closed and shaded bars correspond to **Figure 3.3C**). B) GST protein and four GST-AR domain fusion proteins ($AR_{234-665}$, $AR_{541-665}$, $AR_{541-919}$ and AR_{1-559}) were expressed in bacteria and coupled to glutathione-agarose beads at equimolar levels, as determined by Coomassie Blue staining (GST-AR domains). Full-length DDC (DDC_{1-480}) and fragments (DDC_{1-333} and $DDC_{328-480}$) were expressed by *in vitro* transcription/translation and radiolabeled using [^{35}S]-methionine. After incubation of radiolabeled proteins and GST-AR domains, bound protein was eluted for SDS-PAGE and autoradiography analysis. C) Phosphorimager and Quantity One software were used to quantitate bound protein measured in [CNT*mm^2] values. All GST-pulldowns were done in triplicate and the mean bound values were normalized to loaded input for determination of percentage of total input bound (±SEM); $^{a}p < 0.05$ as compared to GST control, $^{b}p < 0.05$ as compared to all other GST-AR domains for full-length DDC protein, $^{c}p < 0.05$ as compared to all other GST-AR domains for the $DDC_{328-480}$ fragment and full-length DDC protein, $^{d}p < 0.05$ as compared to $AR_{234-665}$ and $AR_{541-665}$ domains for the $DDC_{328-480}$ fragment and $AR_{541-919}$ domain for full-length DDC protein.

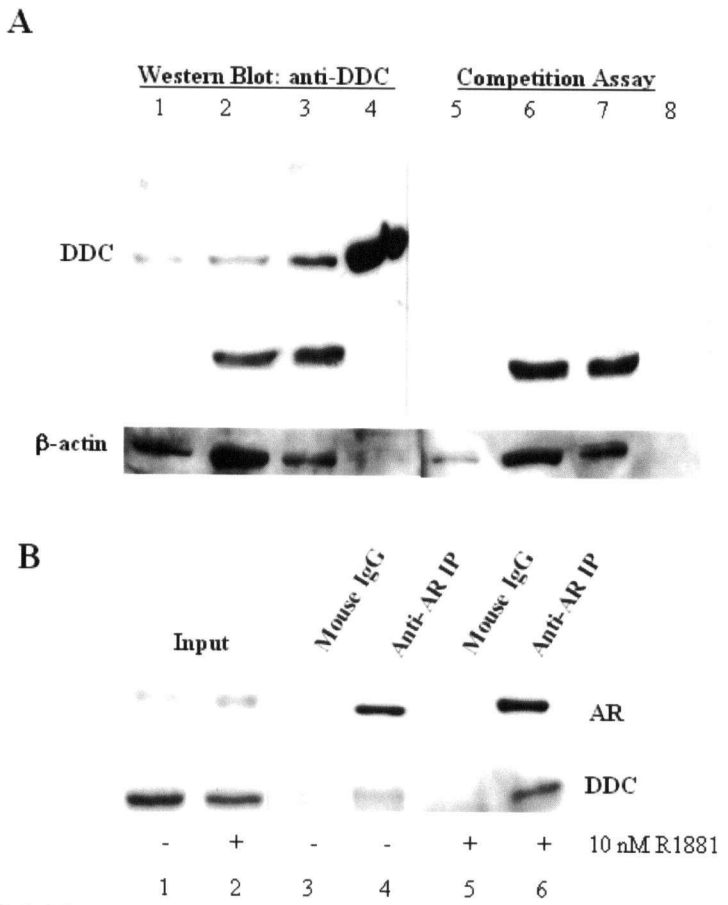

Figure 3.4 - DDC is Expressed in Prostate Cancer Cell Lines and Co-immunoprecipitates with AR *in Vivo*. A) DDC protein expression was analyzed in three prostate cancer cell lines. Equal amounts (50 μg) of cell extract (0.5 % NP-40 lysis) prepared from LNCaP (lanes 1 and 5), PC3 (lanes 2 and 6) and DU145 (lanes 3 and 7) cells were analyzed by immunoblotting with an anti-$DDC_{N\text{-terminal}}$ antibody. A positive control (2 μg of cell extract from pDEST12.2-DDC transfected LNCaP cells) was also included (lanes 4 and 8). Purified 6×His-DDC protein was used for the immuno-competition assay (lanes 5-8). Protein loading efficiency was normalized by β-actin. B) Co-immunoprecipitation; LNCaP cells were transfected with pDEST12.2-DDC vector and grown in the absence (lanes 1, 3, 4) or presence (lanes 2, 5, 6) of 10 nM R1881 for 4 hours prior to lysis in 0.5 % NP-40 buffer. Cell extracts were incubated with an anti-AR_{DBD} antibody (lanes 4 and 6) or normal mouse IgG control (lanes 3 and 5). Input protein samples were loaded in lanes 1 and 2. Protein complexes were pulled down using rProtein G-agarose beads prior to SDS-PAGE and Western blot analysis, with rabbit polyclonal antibodies raised against the N-termini of both DDC and AR.

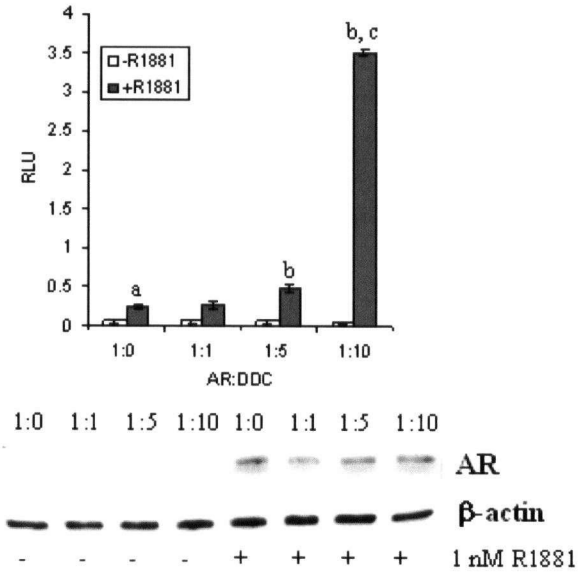

Figure 3.5 - DDC Enhances AR Transcriptional Activity. PC3 cells were transfected with AR (250 ng/well), pARR3-tk-Luc (167 ng/well), pRL-TK-renilla (83 ng/well) and increasing amounts of pDEST12.2-DDC vector (CMV promoter). These amounts included 0, 250, 1250 or 2500 ng/well and correspond to the 1:0, 1:1, 1:5 and 1:10 AR:DDC ratios, respectively. Total DNA was kept constant at 3 μg/well with addition of empty pRC/CMV vector. Cells were induced in the presence (filled bars) or absence (open bars) of 1 nM R1881 for 24 hours before harvest and luciferase assay. Transfection efficiency was normalized with the renilla luciferase pRL-TK vector. RLU values are the mean of triplicates (±SEM); $^{a}p < 0.05$ as compared to –R1881 control (at all AR:DDC ratios), $^{b}p < 0.05$ as compared to +R1881 empty vector control (1:0 ratio) and $^{c}p < 0.05$ as compared to +R1881 1:5 AR:DDC ratio. Each graph is representative of 3 independent trials. To determine receptor expression levels, 10 μg of protein lysate from each triplicate was combined (30 μg total protein) and subjected to SDS-PAGE/Western blot analysis. AR was detected using the anti-AR_{NTD} antibody. β-Actin was used for protein loading control.

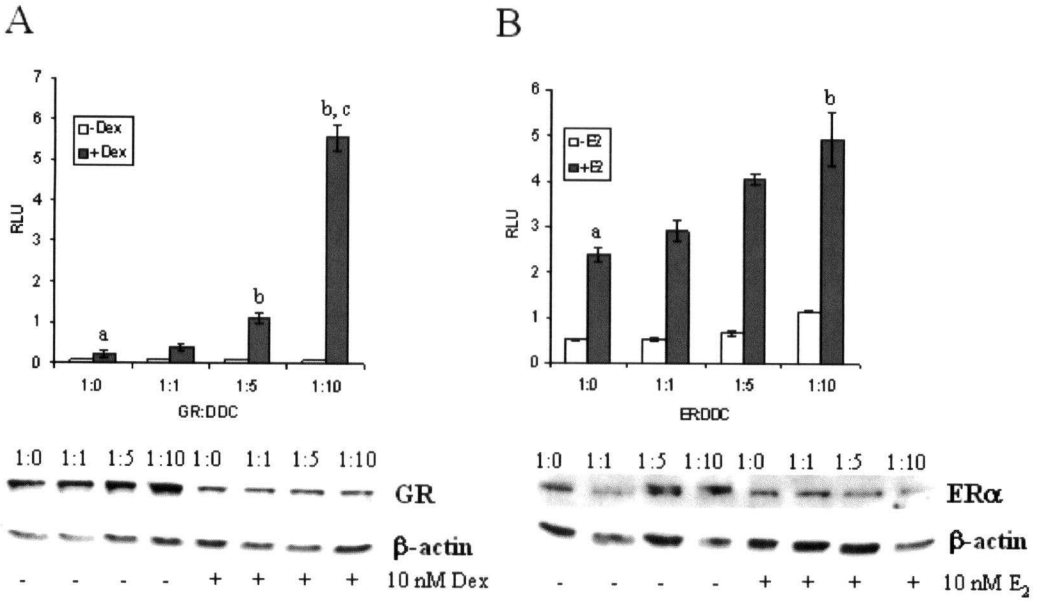

Figure 3.6 - DDC Strongly Enhances GR Transactivation and Mildly Increases ERα Activity. PC3 cells were transfected with (A) GR (250 ng/well) or (B) ERα (250 ng/well) and the corresponding reporter; pARR3-tk-Luc and pERE-Luc, respectively. The ratio of steroid receptor to DDC was varied as outlined in **Figure 3.5**. Cells were treated with or without 10 nM Dex (GR) or 10 nM E_2 (ERα) for 24 hours prior to harvest and luciferase assay. Transfection efficiency was normalized with the renilla luciferase pRL-TK vector. RLU values are the mean of triplicates (±SEM); $^{a}p < 0.05$ as compared to -Dex/-E_2 control (at all GR:DDC and ERα:DDC ratios), $^{b}p < 0.05$ as compared to +Dex/+E_2 empty vector control (1:0 ratio for GR and ERα) and $^{c}p < 0.05$ as compared to +Dex 1:5 GR:DDC ratio. Each graph is representative of 3 independent trials. SDS-PAGE and Western blot analysis was carried out as described in **Figure 3.5** using anti-GR and anti-ERα antibodies, with β-actin as loading control.

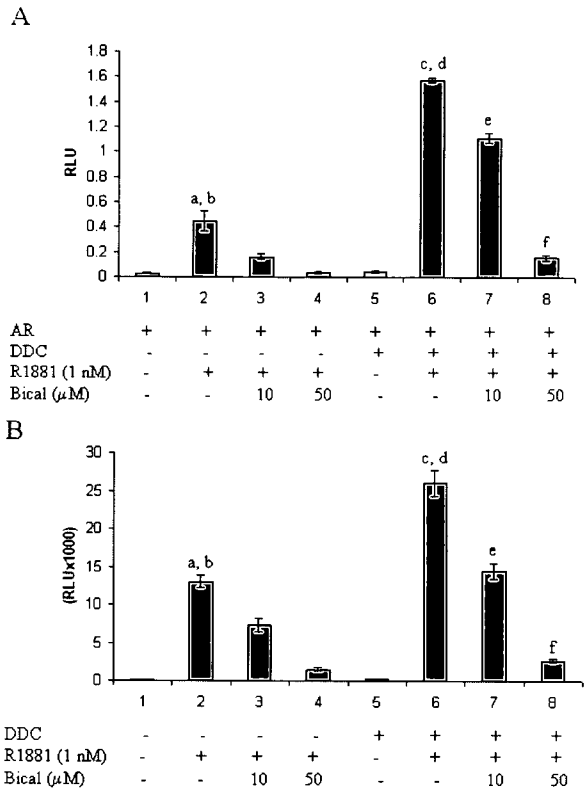

Figure 3.7 - Effect of DDC on Transactivation Activity of AR in the Presence of Bicalutamide in Prostate Cancer Cells. A) PC3 cells were co-transfected with AR (250 ng/well), pARR3-tk-Luc (167 ng/well), pRL-TK-renilla (83 ng/well) and either 1 μg of pDEST12.2-DDC or empty vector. After transfection, cells were treated with 1 nM R1881 and bicalutamide or 1 nM R1881 and vehicle alone for 24 hours, before harvest and luciferase assay. B) LNCaP cells were co-transfected with the same plasmids as above, except 2.5 μg of pDEST12.2-DDC was used and AR was excluded. Transfection efficiency was normalized with the renilla luciferase pRL-TK vector for PC3 cells and for LNCaP cells RLU values were obtained by normalizing to total protein concentration. For both PC3 and LNCaP cells RLU values are the mean of triplicates (±SEM); $^{a}p < 0.05$ as compared to –R1881 control (lanes 1 and 5), $^{b}p < 0.05$ as compared to +bicalutamide (lanes 3 and 4, -DDC), $^{c}p < 0.05$ as compared to +R1881 empty vector control (lane 2), $^{d}p < 0.05$ as compared to +bicalutamide (lanes 7 and 8, +DDC), $^{e}p < 0.05$ as compared to empty vector control with +10 μM bicalutamide (lane 3) and $^{f}p < 0.05$ as compared to empty vector control with +50 μM bicalutamide (lane 4). Each graph is representative of 3 independent trials.

3.5 Discussion

Using the RTA system, L-dopa decarboxylase (DDC) was initially identified as a protein that interacts with the AR-NTD in yeast. Surprisingly, *in vitro* GST-pulldown analysis with various AR domains suggest that the main region of interaction between full-length DDC protein and AR occurs at the C-terminus (amino acids 328-480) of DDC and the LBD of AR (**Figure 3.3**). The initial detection of DDC as an AR-NTD interacting protein could be a consequence of the strong affinity that the truncated $DDC_{328-480}$ region has for the AR_{1-559} domain. Indeed, all six isolated DDC clones from the screen were incomplete C-terminal cDNAs of similar length. Therefore, use of the AR-NTD as bait in the RTA screen can detect the library clones that interact strongly with the AR N-terminus. Coincidentally, the same DDC C-terminal region was found to interact twice as strongly with the LBD of AR (**Figure 3.3**). The dual interaction of $DDC_{328-480}$ with the LBD and NTD of AR was not surprising since several other AR protein partners also interact with the receptor in a similar manner (31, 32).

Compared to the $DDC_{328-480}$ fragment, the full-length protein has a lower binding affinity for all AR domains and exhibits almost a complete loss of interaction with the NTD (**Figure 3.3**). This indicates that the N-terminal of DDC (amino acids 1-333) may cause inhibition of binding to AR, especially with the receptor's N-terminus. Thus, the presence of amino acids 1-333 could drastically lower the affinity of full-length DDC protein for the AR-NTD bait in yeast, which may explain the absence of DDC clones with full-length cDNA insert in the screen. Also, the lack of an interaction between the DDC_{1-333} fragment and all AR domains suggests that the C-terminal region of DDC is solely responsible for interaction with both the NTD and LBD of AR. Although the

DDC_{1-333} fragment contains a LXXLL motif (amino acids 153-157, **Figure 3.2**), which plays an essential role in coactivator interaction and coactivation function with steroid receptors, it does not appear to be involved in the DDC-AR interaction *in vitro*. However, *in vivo*, this motif may serve as an important means of directing the binding of DDC with AR (discussed in **Section 4.5** of **Chapter 4**).

The AR NH_2/COOH-terminal interaction is facilitated by several coactivators and is important for stabilization of bound ligand (33-35). In addition to a strong interaction with the AR-LBD, DDC binding actually increases with the AR-NTD in the presence of the first 233 amino acid residues of the receptor (**Figure 3.3**). The ability of DDC to interact with both the N- and C-termini of AR suggests that it may play a role in modulating the folding of the receptor or facilitating the NH_2/COOH-terminal interaction. Examples of proteins that modulate AR activity in this manner, include ARA70 (36), which stabilizes the ligand-bound receptor, and filamin, which facilitates the translocation of AR to the nucleus (37). One component of the hsp70 chaperone heterocomplex, BAG-1L, also enhances ligand dependent AR transactivation, probably through the appropriate folding of AR (38). DDC, generally considered to be a cytosolic protein (1, 39), probably increases AR transcriptional activity through one of these cytoplasmic processes. Also, since AR NH_2/COOH- terminal interactions may stabilize the AR dimer and promote its activity (40), the dual interaction of DDC with both these domains could play a role in receptor dimerization. Through these events DDC may contribute to stabilize ligand binding or influence the sub-cellular distribution of AR, which would result in an overall increase in AR transcriptional activity. The expression of DDC in prostate cancer cells and its co-immunoprecipitation with AR in LNCaP cells

demonstrates that the DDC-AR interaction is relevant *in vivo* (**Figure 3.4**). Notably, the association of DDC with AR was enhanced in the presence of R1881, suggesting that DDC binds more strongly with ligand-bound AR in the cytosol.

The enhancement of AR transactivation seen with DDC co-transfection was significantly reduced when PC3 and LNCaP cells were treated with bicalutamide (**Figure 3.7**), suggesting that DDC exerts its effect on AR through a ligand-dependent pathway. Also, the increase in ligand-dependent AR activity seen with DDC co-transfection is not accompanied with an elevation in AR protein levels (**Figure 3.5**). Further experimentation also showed that DDC co-expression does not alter AR protein levels at earlier time intervals (3, 6 and 12 hours after ligand treatment) in AR transfected PC3 and LNCaP cells (data not shown). Therefore, stabilization of AR protein can be ruled out as a possible mechanism for the observed increase in AR activity. The strong enhancement of transactivation for both AR and GR compared to the minimal increase in ERα activity (**Figures 3.5** and **3.6**) may be due to the latter receptor's predominant nuclear localization even in the absence of its ligand (41). Hence, any effect that DDC may have on steroid receptor protein in the cytoplasm would not alter ERα activity profoundly.

DDC has been well characterized for its role as a pyridoxal 5'-phosphate (PLP) requiring enzyme that catalyzes the synthesis of dopamine (DA) from L-dopa and serotonin from L-5-hydroxytryptophan. Whether enzymatic activity of DDC is directly involved in enhancing transcriptional activity of AR in PC3 and LNCaP cells is a crucial mechanistic question and is presented in **Chapter 4**. *In vitro*, the PLP cofactor binding site (amino acids 298-304), required for the enzymatic activity of DDC, is not necessary

for binding with AR, as the N-terminal DDC fragment (amino acids 1-333) does not interact with any AR domains (**Figure 3.3**). However, *in vivo* the active enzyme, believed to consist of two ~ 50 kDa homodimeric subunits (1), may be important for indirectly regulating steroid receptor activity. One of the products of DDC, DA, has been shown to activate receptors such as ERα and the progesterone receptor in a ligand-independent manner in cultured cells. DA has no detectable affinity for intracellular steroid receptors. However, after DA binds to its membrane bound receptor, it may act by altering phosphorylation of either the steroid receptor itself or a specific transcription cofactor through a PKA or other unknown pathway (42). This possible indirect route of AR activation by DDC is further explored in detail in **Chapter 4**.

Notably, our observation that higher concentrations of bicalutamide were required to block androgen-stimulated AR transcriptional activity in PC3 and LNCaP cells overexpressing DDC may have important implications for prostate cancer therapy (**Figure 3.7**). During progression of disease, increased expression of DDC could diminish the efficacy of anti-androgen treatment in blocking residual AR activity. The expression profile of DDC in hormone-treated prostate tumours and its role as a neuroendocrine marker is analyzed extensively in **Chapter 5**.

In conclusion, we have demonstrated that L-dopa decarboxylase can bind directly to AR and enhance its transcriptional activity. DDC also enhanced GR transactivation, suggesting that it may play a role in regulating the activity of other steroid receptors. However, the lack of a robust increase in ERα transactivation by DDC suggests that it is a partially selective steroid receptor coactivator. Further studies in **Chapter 4** probe into the mechanism by which DDC enhances AR transactivation.

3.6 References

1. Zhu MY, Juorio AV. Aromatic L-amino acid decarboxylase: biological characterization and functional role. Gen Pharmacol 1995;26(4):681-96.

2. Bertoldi M, Borri Voltattorni C. Reaction and substrate specificity of recombinant pig kidney Dopa decarboxylase under aerobic and anaerobic conditions. Biochim Biophys Acta 2003;1647(1-2):42-7.

3. Mercuri NB, Bernardi G. The 'magic' of L-dopa: why is it the gold standard Parkinson's disease therapy? Trends Pharmacol Sci 2005;26(7):341-4.

4. Feany MB, Bender WW. A Drosophila model of Parkinson's disease. Nature 2000;404(6776):394-8.

5. Masliah E, Rockenstein E, Veinbergs I, Mallory M, Hashimoto M, Takeda A, et al. Dopaminergic loss and inclusion body formation in alpha-synuclein mice: implications for neurodegenerative disorders. Science 2000;287(5456):1265-9.

6. Diederich C, Milakofsky L, Hare TA, Hofford JM, Dadmarz M, Vogel WH. Effects of L-DOPA/carbidopa administration on the levels of L-DOPA, other amino acids and related compounds in the plasma, brain and heart of the rat. Pharmacology 1997;55(3):109-16.

7. Hauser RA, Zesiewicz TA. Advances in the Pharmacologic Management of Early Parkinson Disease. Neurologist 2007;13(3):126-132.

8. Nyholm D, Aquilonius SM. Levodopa infusion therapy in Parkinson disease: state of the art in 2004. Clin Neuropharmacol 2004;27(5):245-56.

9. Swoboda KJ, Saul JP, McKenna CE, Speller NB, Hyland K. Aromatic L-amino acid decarboxylase deficiency: overview of clinical features and outcomes. Ann Neurol 2003;54 Suppl 6:S49-55.

10. Sumi-Ichinose C, Ichinose H, Takahashi E, Hori T, Nagatsu T. Molecular cloning of genomic DNA and chromosomal assignment of the gene for human aromatic L-amino acid decarboxylase, the enzyme for catecholamine and serotonin biosynthesis. Biochemistry 1992;31(8):2229-38.

11. Ichinose H, Sumi-Ichinose C, Ohye T, Hagino Y, Fujita K, Nagatsu T. Tissue-specific alternative splicing of the first exon generates two types of mRNAs in human aromatic L-amino acid decarboxylase. Biochemistry 1992;31(46):11546-50.

12. Ichinose H, Kurosawa Y, Titani K, Fujita K, Nagatsu T. Isolation and characterization of a cDNA clone encoding human aromatic L-amino acid decarboxylase. Biochem Biophys Res Commun 1989;164(3):1024-30.

13. Chang YT, Mues G, Hyland K. Alternative splicing in the coding region of human aromatic L-amino acid decarboxylase mRNA. Neurosci Lett 1996;202(3):157-60.

14. O'Malley KL, Harmon S, Moffat M, Uhland-Smith A, Wong S. The human aromatic L-amino acid decarboxylase gene can be alternatively spliced to generate unique protein isoforms. J Neurochem 1995;65(6):2409-16.

15. Burkhard P, Dominici P, Borri-Voltattorni C, Jansonius JN, Malashkevich VN. Structural insight into Parkinson's disease treatment from drug-inhibited DOPA decarboxylase. Nat Struct Biol 2001;8(11):963-7.

16. Berry MD, Juorio AV, Li XM, Boulton AA. Aromatic L-amino acid decarboxylase: a neglected and misunderstood enzyme. Neurochem Res 1996;21(9):1075-87.

17. Watanabe H, Imaizumi M, Ojika T, Abe T, Hida T, Kato K. Evaluation of biological characteristics of lung cancer by the human 28 kDa vitamin D-dependent calcium binding protein, calbindin-D28k. Jpn J Clin Oncol 1994;24(3):121-7.

18. Gilbert JA, Bates LA, Ames MM. Elevated aromatic-L-amino acid decarboxylase in human carcinoid tumors. Biochem Pharmacol 1995;50(6):845-50.

19. Bozzi F, Luksch R, Collini P, Gambirasio F, Barzano E, Polastri D, et al. Molecular detection of dopamine decarboxylase expression by means of reverse transcriptase and polymerase chain reaction in bone marrow and peripheral blood: utility as a tumor marker for neuroblastoma. Diagn Mol Pathol 2004;13(3):135-43.

20. Lan MS, Russell EK, Lu J, Johnson BE, Notkins AL. IA-1, a new marker for neuroendocrine differentiation in human lung cancer cell lines. Cancer Res 1993;53(18):4169-71.

21. Gazdar AF, Helman LJ, Israel MA, Russell EK, Linnoila RI, Mulshine JL, et al. Expression of neuroendocrine cell markers L-dopa decarboxylase, chromogranin A, and dense core granules in human tumors of endocrine and nonendocrine origin. Cancer Res 1988;48(14):4078-82.

22. Wafa LA, Palmer J, Fazli L, Hurtado-Coll A, Bell RH, Nelson CC, et al. Comprehensive expression analysis of L-dopa decarboxylase and established neuroendocrine markers in neoadjuvant hormone-treated versus varying Gleason grade prostate tumors. Hum Pathol 2007;38(1):161-70.

23. Ippolito JE, Xu J, Jain S, Moulder K, Mennerick S, Crowley JR, et al. An integrated functional genomics and metabolomics approach for defining poor prognosis in human neuroendocrine cancers. Proc Natl Acad Sci U S A 2005;102(28):9901-6.

24. Rennie PS, Bruchovsky N, Leco KJ, Sheppard PC, McQueen SA, Cheng H, et al. Characterization of two cis-acting DNA elements involved in the androgen regulation of the probasin gene. Mol Endocrinol 1993;7(1):23-36.

25. Miesfeld R, Rusconi S, Godowski PJ, Maler BA, Okret S, Wikstrom AC, et al. Genetic complementation of a glucocorticoid receptor deficiency by expression of cloned receptor cDNA. Cell 1986;46(3):389-99.

26. Smith CL, Conneely OM, O'Malley BW. Modulation of the ligand-independent activation of the human estrogen receptor by hormone and antihormone. Proc Natl Acad Sci U S A 1993;90(13):6120-4.

27. Snoek R, Rennie PS, Kasper S, Matusik RJ, Bruchovsky N. Induction of cell-free, in vitro transcription by recombinant androgen receptor peptides. J Steroid Biochem Mol Biol 1996;59(3-4):243-50.

28. Sato N, Sadar MD, Bruchovsky N, Saatcioglu F, Rennie PS, Sato S, et al. Androgenic induction of prostate-specific antigen gene is repressed by protein-protein interaction between the androgen receptor and AP-1/c-Jun in the human prostate cancer cell line LNCaP. J Biol Chem 1997;272(28):17485-94.

29. Veldscholte J, Ris-Stalpers C, Kuiper GG, Jenster G, Berrevoets C, Claassen E, et al. A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. Biochem Biophys Res Commun 1990;173(2):534-40.

30. Veldscholte J, Berrevoets CA, Brinkmann AO, Grootegoed JA, Mulder E. Anti-androgens and the mutated androgen receptor of LNCaP cells: differential effects on

binding affinity, heat-shock protein interaction, and transcription activation. Biochemistry 1992;31(8):2393-9.

31. Bevan CL, Hoare S, Claessens F, Heery DM, Parker MG. The AF1 and AF2 domains of the androgen receptor interact with distinct regions of SRC1. Mol Cell Biol 1999;19(12):8383-92.

32. Fronsdal K, Engedal N, Slagsvold T, Saatcioglu F. CREB binding protein is a coactivator for the androgen receptor and mediates cross-talk with AP-1. J Biol Chem 1998;273(48):31853-9.

33. He B, Kemppainen JA, Voegel JJ, Gronemeyer H, Wilson EM. Activation function 2 in the human androgen receptor ligand binding domain mediates interdomain communication with the NH(2)-terminal domain. J Biol Chem 1999;274(52):37219-25.

34. Moilanen AM, Karvonen U, Poukka H, Yan W, Toppari J, Janne OA, et al. A testis-specific androgen receptor coregulator that belongs to a novel family of nuclear proteins. J Biol Chem 1999;274(6):3700-4.

35. Ikonen T, Palvimo JJ, Janne OA. Interaction between the amino- and carboxyl-terminal regions of the rat androgen receptor modulates transcriptional activity and is influenced by nuclear receptor coactivators. J Biol Chem 1997;272(47):29821-8.

36. Yeh S, Chang C. Cloning and characterization of a specific coactivator, ARA70, for the androgen receptor in human prostate cells. Proc Natl Acad Sci U S A 1996;93(11):5517-21.

37. Ozanne DM, Brady ME, Cook S, Gaughan L, Neal DE, Robson CN. Androgen receptor nuclear translocation is facilitated by the f-actin cross-linking protein filamin. Mol Endocrinol 2000;14(10):1618-26.

38. Froesch BA, Takayama S, Reed JC. BAG-1L protein enhances androgen receptor function. J Biol Chem 1998;273(19):11660-6.

39. Poulikakos P, Vassilacopoulou D, Fragoulis EG. L-DOPA decarboxylase association with membranes in mouse brain. Neurochem Res 2001;26(5):479-85.

40. Ting HJ, Yeh S, Nishimura K, Chang C. Supervillin associates with androgen receptor and modulates its transcriptional activity. Proc Natl Acad Sci U S A 2002;99(2):661-6.

41. Greene GL, Press MF. Structure and dynamics of the estrogen receptor. J Steroid Biochem 1986;24(1):1-7.

42. Mani SK, Allen JM, Clark JH, Blaustein JD, O'Malley BW. Convergent pathways for steroid hormone- and neurotransmitter-induced rat sexual behavior. Science 1994;265(5176):1246-9.

CHAPTER 4. ANDROGEN RECEPTOR TRANSACTIVATION IS ENHANCED BY L-DOPA DECARBOXYLASE VIA AN ANDROGEN-DEPENDENT MECHANISM AND ITS ENZYMATIC ACTIVITY

A version of this chapter will be submitted for publication.

Wafa, LA, Snoek R, Read J, Cheng H, Rogalski J, Nelson CC, Kast J, Cox ME, Rennie PS. Androgen receptor transactivation is enhanced by L-dopa decarboxylase *via* an androgen-dependent mechanism and its enzymatic activity. Submission to the Journal of Biological Chemistry.

Dr. Jeurgen Kast from the department of Chemistry (UBC) collaborated with our laboratory to perform the liquid chromatography tandem mass spectrometry analysis. Dr. Jason Read aided with the purification of FLAG-tagged AR protein and carrying out mass spectrometry experiments. Dr. Colleen Nelson provided critical comments for ligand binding assays. Dr. Michael Cox reviewed the entire body of work in this chapter and provided critical comments.

4.1 Introduction

The androgen receptor (AR) functions as an androgen-responsive transcription factor that regulates genes involved in normal development of the prostate and cancer progression (1). As a steroid receptor family member, AR contains common functional domain structures (2, 3), including an N-terminal domain (NTD) that harbours activation function 1 (AF1), a central DNA binding domain (DBD) and a C-terminal ligand binding domain (LBD) that contains activation function 2 (AF2) (4-6). The AF2 of the AR-LBD, formed by helices 3, 3', 4 and 12, is a highly conserved hydrophobic surface that is stabilized by ligand-binding and required for coactivator recruitment (7, 8). In the presence of androgens, the AR-LBD undergoes conformational changes in the cytoplasm

leading to dissociation from heat shock proteins, including hsp70, 90 and 40 (4). Some hsp chaperones, such as hsp90 have also been shown to increase the ligand binding capacity of AR (9). Upon activation and nuclear translocation, AR binds to specific androgen response elements to initiate target gene expression (10). The mechanism of AR action has been discussed is detail in **Section 1.3** of **Chapter 1**.

Classical nuclear coactivators, such as SRC1, SRC2, SRC3 and CBP/p300, facilitate AR transcription by several mechanisms including histone modifications, chromatin remodelling and bridging of the receptor to other basal transcriptional machinery (11, 12). Steroid receptors can also directly interact with basal transcriptional components, like TBP (13), TFIIB (14), TFIIF (15), and TFIIH (16) to facilitate transcription. In addition to classical coregulators of AR, other cytosolic proteins, such as ARA70 (17, 18), β-catenin (19) and caveolin-1 (20) can also function to increase receptor transactivation. These AR coactivators have been suggested to modulate receptor transcription *via* alternative mechanisms that involve stabilization of bound-androgen/AR protein and facilitation of receptor nuclear translocation (18). Nevertheless, the detailed mechanisms underlying the coactivation properties of these proteins remain to be elucidated. A comprehensive discussion of the role of coactivators in modifying AR function has been included in **Section 1.4** of **Chapter 1**.

In the previous study (**Chapter 3**), we demonstrated that L-dopa decarboxylase (DDC) can interact with and enhance AR transcriptional activity in prostate cancer cells. The traditional role of DDC has been well established as the enzyme that catalyzes decarboxylation of L-3,4-dihydroxyphenylalanine (L-dopa) into dopamine (DA) and 5-hydroxytryptophan (5-HTP) into serotonin (5-HT) (21). The cell surface G-protein-

coupled receptors (GPCRs) for DA and 5-HT are known to modulate a plethora of signal transduction pathways. The five DA receptor subtypes (D1 through D5) have been grouped into two classes, the D1-like and D2-like receptors (22). DA activation of the D1-like receptors, D1 and D5, stimulates adenylyl cyclase activity, elevation of intracellular cAMP and PKA activation (23). Activation of D2-like receptors, D2, D3 and D4, mediate inhibition of adenylyl cyclase, reduction of cAMP and inhibition of PKA (24, 25). MAPK and Akt are also possible downstream effectors of both D1- and D2-like DA receptor stimulation (24, 26, 27). Serotonin receptors have been divided into seven subfamilies by convention. These include 5-HT1 through 5-HT7 GPCRs, except for 5-HT3 receptors, which are serotonin-gated ion channels. 5-HT GPCRs can modulate PKA, PKC, MAPK, PI3-kinase and many other signal transduction pathways (28).

Activation of AR transcription in prostate cancer has partly been attributed to a complex array of cell signalling events (discussed in **Section 1.5** of **Chapter 1**) that involve many of the same pathways modulated by DA and 5-HT. These include AR activation by growth factors (EGF, IGF-1, KGF) and cytokines (IL-6, IL-4) through pathways that directly or indirectly involve MAPK, PKA and PKC signalling (29-33). In addition, modulation of AR activity by the PI3-kinase and Akt pathway has been demonstrated (34, 35). Of note, the DDC enzymatic product, DA, has been shown to activate steroid receptors, such as ER and PR, through DA D1-subtype GPCRs (36-39). This ligand-independent steroid receptor activation has been suggested to occur *via* elevation of cAMP levels and PKA activation (40). The stimulation of ligand-independent AR activity by DA has not been reported. The effect of 5-HT GPCR

activation on steroid receptor transcription has also not been previously studied. Therefore, whether the increase in AR activity seen with overexpression of DDC in prostate cancer cells results from the synthesis of its neurotransmitter products, DA and 5-HT, which might then stimulate their receptors in an autocrine fashion and lead to indirect activation of AR, is unknown.

The mechanism of DDC enzymatic activity has been studied extensively. Catalytic activity of DDC is dependent on the pyridoxal 5'-phosphate (PLP) cofactor molecule, which binds DDC at Lys residue 303 and allows decarboxylation of amino acid substrates through a Schiff base mechanism (21, 41). In this reaction, the enzyme-bound PLP binds the substrate, after which PLP forms a covalent bond with Lys303. The PLP-protein complex is then cleaved, and the lysine residue binds the substrate amino acid. A quinonoid intermediate is formed, CO_2 is released from the substrate α-carbon and the same carbon is protonated. The holoenzyme is recovered after the amino acid is converted into an amine (42). Mutational analysis performed on DDC has highlighted the importance of numerous residues for enzymatic activity, with one of the most essential residues being Lys303 (43-46). Using the above mechanism, DDC catalyzes the synthesis of DA and 5-HT but has been suggested to also synthesize trace amines from other amino acids, such as tyrosine, phenylalanine and tryptophan (21). However, whether DDC can act on the α-carbon of C-terminal amino acid residues of protein substrates has not been reported.

In the present study, we investigate the mechanism by which DDC can act as a coactivator of AR. Briefly, we demonstrate that DDC can enhance PSA promoter activity and AR transcription *in vivo* through an androgen-dependent mechanism.

DDC sensitizes AR to limiting androgen concentrations and increases the apparent affinity of AR for ligand, as well as receptor androgen-binding capacity. Furthermore, the coactivation function of DDC was found to be completely dependent on its catalytic activity, but independent of its known enzymatic products (DA and 5-HT).

4.2 Materials and Methods

Plasmid Construction

The DDC expression plasmid, pDEST12.2-DDC, used for mammalian cells and for *in vitro* transcription/translation was cloned previously (**Section 3.2** of **Chapter 3**). The His-tagged-DDC bacterial expression plasmid was produced *via* RT-PCR on LNCaP RNA and Gateway™ cloning (Invitrogen). The forward (5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTA**AACGCAAGTGAATTCCGA AGGAGA**-3') and reverse (5'GGGGACCACTTTGTACAAGAAAGCTGGGTCCTA**CTCCCTCTCTGCTCGCA GCAC**-3') primers contained a stop codon, but lacked the ATG start site to allow for a 6x-His-tagged-DDC N-terminal fusion. Primers also included DDC gene-specific coding sequence (in bold) and *att*B1/B2 recombination sites allowing incorporation of the PCR product into pDONR201 for generation of the pENTR-His-DDC vector. Upon frame and sequence verification, this entry vector was used for a recombination reaction with pDEST17 to produce the pDEST17-His-DDC bacterial expression plasmid (T7 promoter). This vector was transformed into BL21-SI competent cells (Invitrogen) for optimal fusion protein expression.

To generate a DDC-lentiviral expression construct, pENTR-DDC plasmid (containing coding region of DDC cloned into pDONR201, as described previously in **Section 3.2** of **Chapter 3**) was used for a recombination reaction with pLenti4/TO/V5-DEST vector (Gateway™ Technology, Invitrogen). The resulting pLenti4/TO/V5-DEST-DDC plasmid was transformed into One Shot Stbl3 *E. coli* optimized for use in cloning of unstable lentiviral DNA. Due to the presence of a stop codon in pENTR-

DDC, the C-terminus of DDC was not V5-tagged in the DDC-lentiviral construct. Full-length human AR was cloned into the pcDNA3.1 mammalian expression plasmid (pcDNA3.1-hAR) under control of a cytomegalovirus (CMV) promoter (47).

Mammalian Cell Culture

PC3 and LNCaP human prostate cancer cells were maintained in DMEM and RPMI media containing 5 % FBS, as described previously (**Section 3.2** of **Chapter 3**). HeLa human cervical cancer cells that express a stably transfected FLAG-tagged AR (HeLa-AR) were obtained from Dr. M. Carey (48) and cultured in DMEM supplemented with 5 % FBS. PC12 rat adrenal pheochromocytoma cells were grown in DMEM media containing 5 % FBS and 10 % horse serum (GibcoBRL).

Transactivation Assays

PC3 and LNCaP cells were seeded onto 6-well plates at a density of 3×10^5 cells/well and transfected the following day using Lipofectin Reagent (Invitrogen), as described previously (**Section 3.2** of **Chapter 3**). Human AR expression plasmid (pcDNA3.1-hAR) and androgen-regulated luciferase reporters, pARR3-tk-Luc and pPSA-630bp-Luc (-630/+12 bp of the 5' PSA flanking region) (49), were cotransfected in PC3 cells, along with DDC expression plasmid (pDEST12.2-DDC) or pDEST12.2 control empty vector. Transfected cells were incubated with or without 1 nM R1881, in 5 % dextran-coated charcoal stripped FBS, at 37 °C for 24 hours prior to cell lysis and luciferase assays. Firefly luciferase values were normalized to renilla

luciferase (pRL-TK reporter; Promega Corp.) and expressed as relative luciferase units (RLU). All assays were performed in triplicate with at least three independent trials.

Similar transcriptional assays were carried out for determining the ligand sensitization effect of DDC on AR using varying concentrations of R1881 (0-10 nM) in PC3 and LNCaP cells. Transactivation assays for AR were also performed in PC3 cells treated with dopamine (DA) and serotonin (5-HT, 5-hyroxytyrptamine), which were purchased from Sigma. As described above, cells were transfected with pcDNA3.1-hAR, pRL-TK-renilla and pARR3-tk-Luc reporter but DDC was excluded. Cells were then treated with or without 1 nM R1881 and varying concentrations of pure DA or 5-HT in 5 % dextran-coated charcoal stripped FBS for 24 hours before harvest and luciferase assay.

***In Vitro* Ligand Binding Assays**

Recombinant rat thioredoxin-fused AR ligand binding domain (PanVera, 1.390 mg/mL, 48.4 kDa), which is identical to the human AR-LBD, was used for hydroxylapatite (HAP) pulldown ligand binding assays at R1881 concentrations of 0 to 1000 nM. Initially, his-tagged-DDC protein was expressed in BL21-SI bacteria, containing the pDEST17-His-DDC plasmid, and purified using a nickel-nitrilotriacetic acid (Ni-NTA)-agarose column according to the manufacturer's protocol (Qiagen). Purified AR-LBD was diluted to a final concentration of 1 μg/mL in binding buffer (50 mM Tris pH 7.5, 10 % glycerol, 0.8 M NaCl, 1 mg/mL BSA and 2 mM dithiothreitol), containing tracer 20 nM [^{3}H]-R1881 (NEN Life Science Products, 75.2 Ci/mmol, 1 mCi/mL). In order to minimize the use of radioactive ligand, supplemental cold R1881 was added to the assay mix to achieve the high 100 nM and 1000 nM total R1881

concentrations. Assays were carried out in the absence and presence of 0.5 μg of His-DDC protein, which was added directly to the 1 mL assay mix. Following overnight incubation at 4 °C, a 50 % HAP slurry (Calbiochem Fast Flow hydroxylapatite in 10 nM Tris pH 8.0 and 1 mM EDTA) was added to the assay, pellets were incubated on ice for 10 minutes, and then washed four times with wash buffer (40 mM Tris pH 7.5, 100 mM KCl, 1 mM EDTA and 1 mM EGTA). HAP pellets were resuspended in ethanol and bound [^{3}H]-R1881 was measured (disintegrations per minute, dpm) using a Beckman LS 6500 scintillation counter.

To determine non-specific binding (NSB) to the HAP matrix, control pulldown assays were carried out using an assay mix that lacked AR-LBD. In addition, to assess whether His-DDC can bind non-specifically to the HAP matrix and R1881, pulldown assays were performed with 0.5 μg of His-DDC protein, in the absence of AR-LBD (NSB due to His-DDC protein was minimal; 90 % of NSB was due to HAP matrix). NSB values were subtracted from sample measurements for determination of specific binding. The total dpm per assay was determined by measuring a known amount of [^{3}H]-R1881 and normalizing to the total assay volume (1 mL). All assays were carried out in triplicate and the following equation was used to for calculation of,

pmole bound [^{3}H]-R1881 per mg AR-LBD protein =
[dpm sample – (dpm non-specific binding of HAP + dpm non-specific binding of His-DDC)] × [(AR-LBD protein concentration in mg/mL)/(total dpm per 1 mL assay) × ([^{3}H]-R1881 concentration in nM)]

The above calculated values, determined using measured dpm of tracer 20 nM [^{3}H]-R1881, were extrapolated to determine total ligand bound at the 10 nM, 100 nM and 1000 nM R1881 concentrations. Results were expressed as the mean total pmole bound R1881/mg AR-LBD protein (±SEM).

Cell-Based Ligand Binding Assays

HeLa-AR cells were cultured for 24-48 hours in DMEM containing 5 % dextran-coated charcoal-stripped FBS and seeded onto 6 cm plates at a density of 1×10^6 cells/plate. After 5 hours of incubation under standard conditions, cells were transfected with either pDEST12.2-DDC or pDEST12.2 control vector (5 μg/plate) using Lipofectin Reagent (Invitrogen). The next day, transfection mix was changed to DMEM supplemented with 5 % dextran-coated charcoal-stripped FBS containing increasing concentrations of [^{3}H]-R1881 (0 – 10 nM). To determine non-specific binding, hormone treatments were carried out in the presence (NSB) and absence (total binding) of excess cold 1000 nM R1881. After 24 hours of incubation with hormone, cells were washed and harvested by scraping in phosphate-buffered saline (PBS) containing 1 mM EDTA. Cytosolic fractions were prepared by resuspending cell pellets in 400 μL (per 6 cm plate cell pellet) of Buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol/DTT, 0.5 mM phenylmethylsulfonyl fluoride/PMSF) and incubating on ice for 15 minutes (50). After addition of 25 μL 10 % NP-40, the cell suspension was vortexed for 10 seconds and centrifuged for 1 minute (9.3 g-force). The supernatant cytosolic fractions were stored at – 80 °C.

Nuclear pellets were resuspended in 100 µL (per 6 cm plate nuclear pellet) of ice-cold Buffer C (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) and samples were vigorously rocked at 4 °C for 15 minutes on a shaking platform (50). The nuclear extracts were centrifuged for 5 minutes (9.3 g-force) and supernatants were frozen at – 80 °C. Cytosolic and nuclear extracts were then thawed on ice and incubated for 6 hours at 4 °C with 20 µL of agarose beads conjugated to FLAG monoclonal antibody (Sigma, USA). Lysate aliquots (10 %) were also used for SDS-PAGE/Western blot analysis to determine the expression level of FLAG-tagged AR protein (used for normalization of bound ligand; see below). Beads were washed three times with Buffer D (20 mM HEPES pH 7.9, 20 % glycerol, 0.3 M KCl, 0.2 mM EDTA, 0.05 % NP-40, 0.5 mM DTT, 0.5 mM PMSF) and then resuspended in the same buffer (48). Specifically bound ligand (total binding – NSB) to the immunopreciptated FLAG-tagged AR protein was determined by measuring the levels of tritium (dpm) in bead resuspensions using a Beckman LS 6500 scintillation counter. Results were expressed as the relative (normalized to the level of AR protein) mean bound ligand of three replicates (±SEM).

FLAG-tagged AR protein levels at all [^{3}H]-R1881 concentrations (0 – 10 nM) was determined for both cytosolic and nuclear extracts by Western blotting, as previously described (**Section 3.2** of **Chapter 3**). Briefly, AR protein was detected using the anti-AR441 mouse monoclonal antibody (Santa Cruz Biotechnology). Protein band intentities (INT*mm^2; average intensity of band × measured area) were quantitated using PDI Quantity One Software (Version 4.2.1, BioRad). All measured bound dpm values were normalized (relative bound ligand) to corresponding AR protein band intensities.

For cellular ligand-uptake assays, HeLa-AR cells were seeded onto 6-well plates at a density of 3×10^5 cells/well as done above for cell-based ligand binding assays. Cells were transfected using either pDEST12.2-DDC or pDEST12.2 control vector (1 μg/well) using Lipofectin Reagent (Invitrogen), followed by incubation with increasing concentrations of [^{3}H]-R1881 (0 – 10 nM) for 24 hours. Non-specific cellular androgen uptake was determined by incubating with excess cold 1000 nM R1881. Cells were then washed, harvested by scraping in PBS containing 1 mM EDTA and lysed using RIPA buffer (50 mM Tris-C1 pH 7.5, 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS). Whole cell extracts were used for scintillation counting to determine cellular [^{3}H]-R1881 accumulation. Results were expressed as the mean specific cellular [^{3}H]-R1881 uptake (total uptake – non-specific uptake) of three replicates (±SEM).

Generation of Tetracycline-Inducible DDC LNCaP and HeLa-AR Cells

Stable cell lines that expressed DDC under tetracycline control were generated using the ViraPower™ T-Rex™ Lentiviral Expression System, Version A (Invitrogen), according to the manufacturer's protocol. Briefly, the pLenti4/TO/V5-DEST-DDC plasmid, pLenti4/TO/V5-DEST empty control vector (contain zeocin selection) and the pLenti6/TR Tet repressor plasmid (contains Blasticidin selection) were co-transfected individually with the lentiviral ViraPower™ Packaging Mix plasmids (pLP1, pLP2, pLP/VSVG) into 293FT cells using Lipofectamine 2000 reagent (Invitrogen). After 48 hours, conditioned medium containing virus was passed through a 0.2 μm filter and stored at -70 °C. To assess infection efficiency, a control lentiviral pHR-CMV-EGFP

plasmid (51) was also used with the pLP1, pLP2 and pLP/VSVG packaging plasmids for production of EGFP (enhanced green fluorescent protein) retroviral particles. Viral concentration for all utilized lentiviral vectors was determined using an ELISA assay for the p24 viral protein, as previously reported (51). LNCaP and HeLa-AR cells were co-transduced with TR virus and either DDC or Lenti4/TO/V5-DEST control vector viral particles. Lentiviral infection was also carried out with the EGFP virus and EGFP-positive cells were counted using a fluorescent microscope (Carl Zeiss) for determination of infection efficiency.

The multiplicity of infection (MOI) used for LNCaP cells was 50-60, whereas a higher MOI of 400-500 was needed for HeLa-AR cells to infect the maximum cell population. Cells were selected with varying concentrations of Zeocin and Blasticidin (Invitrogen). LNCaP cells were treated with 50 μg/mL of Zeocin and 2.5 μg/mL of Basticidin with intermittent weekly selection, while HeLa-AR cells were maintained under constant selection using 200 μg/mL of Zeocin and 5 μg/mL of Blasticidin. Induction of DDC protein expression was initially verified by Western blot analysis after treating cells for 48 hours with 1 μg/mL of the tetracycline analogue, doxycycline hyclate (Dox; Sigma). Subsequently, LNCaP-DDC and LNCaP-Vector control stable cell lines were used for xenograft analysis, while HeLa-AR/DDC stable cells were utilized to purify AR protein for mass spectrometry analysis (see below).

LNCaP-DDC Tumour Xenografts

LNCaP-DDC and LNCaP-Vector control cells were inoculated subcutaneously (s.c.), after resuspension in 0.1 mL of Matrigel (Becton Dickinson Labware), into two

flank regions (2.5×10^6 cells/site) of 6 to 8 week old male athymic nude mice (Harlan Sprague Dawley, Inc.) *via* a 27-gauge needle under halothane anesthesia. Tumour volume and serum PSA measurements (blood collected from the tail vein) were performed once per week after tumours became palpable. PSA levels were measured by ELISA (ClinPro International) and tumour size was calculated by the formula; length × width × depth × 0.5236 (52). Once serum PSA values reached 75–100 ng/mL, half of the mice in both the LNCaP-DDC and LNCaP-Vector control groups were randomly selected for castration and given Dox-treated water (200 μg/mL), while the remaining animals only received Dox-treated water. Overall, each of these four treatment groups contained a minimum of 3 mice for every measured time point, with a range of 3-6 mice, for the duration of the entire experiment over 53 days post-treatment. Volume measurements were used to determine the tumour growth rate for all groups with linear regression slope analysis (see Statistical Analysis below). Tumour growth rate was defined as the increase in tumour volume (normalized to pre-treatment value set at 100 %) divided by total duration (53 days) of the experiment. All animal procedures were performed according to the guidelines of the Canadian Council of Animal Care and with appropriate institutional certification.

Immunofluorescence Analysis

Xenograft tissues were formalin-fixed and paraffin-embedded prior to sectioning onto glass slides. Mounted tissues were deparaffinized, rehydrated with ethanol, permeabilized, steamed in citrate buffer (pH 6), and then incubated in 3 % hydrogen peroxide for antigen retrieval, as described in **Section 5.2** of **Chapter 5**. After blocking,

slides were incubated at 4 °C overnight with a rabbit polyclonal antibody to DDC (Chemicon Inc., used at 1:200 dilution). Slides were incubated with rabbit biotinylated-secondary antibody, followed by fluorescein-conjugated streptavidin treatment (Vector Laboratories Inc.) and counterstaining with Hoechst (Bisbenzimide) nuclear stain (Sigma). Slides were covered using cover slips and mounting media (Vector Laboratories), prior to microscopy analysis.

HeLa-AR/DDC stable cells were seeded onto glass cover slips at low density (1×10^5 cells/well of 6-well plate) in 5 % FBS DMEM media and grown overnight. The media was then changed to DMEM containing 5 % dextran-coated charcoal stripped FBS and Dox (1 μg/mL) or vehicle control. The next day, cells were treated with or without 1 nM R1881 and grown for a further 24 hours, with a total of 48 hours Dox treatment. Cells were then fixed with cold methanol for 2 minutes, air dried for 10 minutes and reconstituted in blocking buffer (0.1 % Tween 20 and 0.1 % BSA in PBS with 4 % normal goat serum/NGS) for 20 minutes. Cells were incubated with primary antibodies (mouse monoclonal anti-AR441/Santa Cruz and rabbit polyclonal anti-DDC/Chemicon) at dilutions of 1:100 for 2 hours. Secondary antibodies conjugated to fluorophores (mouse-FITC and rabbit-Texas Red, Jackson Laboratories) were used at a 1:200 dilution with a 1 hour incubation period. Cover slips were mounted onto glass slides with mounting media containing DAPI nuclear stain (Vector Laboratories). Positive staining for both xenograft tissues and HeLa-AR/DDC stable cells was visualized through fluorescent microscopy (Carl Zeiss) with Northern Eclipse imaging software (Empix Imaging).

cAMP Assays

PC3 and LNCaP cells were seeded in 12-well plates at a density of 1.5 x 10^5 cells/well using DMEM and RPMI media, containing 5 % FBS, respectively. The next day, cells were gently washed three times with serum-free media to remove excess FBS and grown for a further 24 hours in the absence of serum. Cells were then stimulated with various concentrations of DA for 15 minutes or 5-HT for 10 minutes at 37 °C. Cells were lysed and intracellular cAMP levels were determined using the cAMP Biotrak Enzymeimmunoassay System (Amersham Biosciences) following the manufacturer's instructions. Assays were performed in triplicate with two independent trials.

Dopamine ELISAs

PC3 cells were seeded in 12-well plates at a density of 1.5 x 10^5 cells/well and transfected the following day with pcDNA3.1-AR and pDEST12.2-DDC or pDEST12.2 empty vector control, as described above for transactivation assays. The next day, media was changed to 5 % dextran-coated charcoal stripped FBS containing 1 nM R1881. Conditioned culture media was removed 24 hours later and stored at – 80 °C. A 100 µL volume of media was used for the competitive enzyme-linked immunosorbent assay (ELISA). Dopamine was assayed using the Dopamine Research Enzyme Immunoassay kit (Rocky Mountain Diagnostics) following the manufacturer's instructions. PC12 cells, used as a positive control (53), were grown to 70 % confluency (12-well plates) in DMEM containing 5 % FBS and 10 % horse serum. Conditioned media was collected and assayed 24 hours later, as above. Secreted DA levels from PC3 and PC12 cells were determined in the presence and absence of 1 µM NSD-1015 (3-hydroxybenzylhydrazine)

DDC enzymatic inhibitor (Sigma). All ELISAs were performed in triplicate with two independent trials.

Generation of Mutant DDC

The QuikChange™ Site-Directed Mutagenesis kit was used to introduce a single nucleotide mutation in the PLP cofactor binding site of DDC that converted Lys303 into Ile. PCR-based mutagenesis was performed on the pENTR-DDC vector (**Section 3.2** of **Chapter 3**) using forward (5'-ATTCAACTTTAATCCCCACA**T**ATGGCTATTGGTGAATTTTG-3') and reverse (5'-CAAAATTCACCAATAGCCAT**A**TGTGGGGATTAAAGTTGAAT-3') primers that replaced the AAA (Lys303) codon with A**T**A (Ile303). After sequence verification, the mutated pENTR-DDC vector was used for an LR recombination reaction with pDEST12.2 (Gateway™ Technology, Invitrogen) to produce the pDEST12.2-mt-DDC vector. This mt DDC expression plasmid was used to perform transactivation assays (see above) and GST-pulldown assays (see below), as described for wt DDC with the pDEST12.2-DDC vector.

GST-Pulldown Assays

GST protein and three rat GST-AR domain fusion proteins ($AR_{234-665}$/Truncated-NTD+LBD, $AR_{541-665}$/DBD, $AR_{541-919}$/DBD+LBD) were coupled to glutathione-agarose beads at equimolar levels, as described previously (**Section 3.2** of **Chapter 3**). The pDEST12.2-DDC (wt DDC) and pDEST12.2-mt-DDC (mt DDC) vectors were used for *in vitro* transcription/translation with [^{35}S]-methionine. After incubation of radiolabeled

proteins with GST-AR domains, bound protein was eluted for SDS-PAGE and autoradiography analysis. GST-pulldown assays were also carried out with the purified His-DDC protein (0.5 μg/assay) used for *in vitro* ligand binding assays (see above). His-DDC protein was incubated with GST-AR domains as done for radiolabeled proteins and bound protein was detected *via* Western blot analysis (described below).

Western Blot Analysis

All SDS-PAGE and Western blot analyses were performed as previously described (**Section 3.2** of **Chapter 3**). Briefly, detection of His-DDC protein in GST-pulldown assays was carried out using the rabbit polyclonal anti-DDC antibody (Chemicon). Detection of AR, DDC and β-actin for PC3/LNCaP cell transactivation assays and for HeLa-AR/DDC stable cells was performed with the mouse monoclonal anti-AR441 (Santa Cruz), rabbit polyclonal anti-DDC (Chemicon) and rabbit polyclonal anti-β-actin (Sigma) antibodies, respectively. The final concentration of all primary antibodies was 1 μg/mL. These antibodies were also used for detection of DDC and β-actin in LNCaP-DDC and LNCaP-Vector control tumour xenografts. Tumour protein extracts were prepared as reported previously (54). Briefly, tumour tissue was removed from hosts at various times after treatment, flash frozen in liquid nitrogen and Dounce homogenized on ice using RIPA lysis buffer. Protein extracts were quantified by BCA assay (Pierce Biotechnology, Inc.) and used for SDS-PAGE/Western blot analysis.

Liquid Chromatography-Tandem Mass Spectrometry Analysis of AR

HeLa-AR/DDC stable cells, which express an N-terminal FLAG-tagged AR constitutively and DDC under tetracycline regulation, were plated in twenty 15 cm dishes and grown to 70 % confluency in DMEM containing 5 % FBS. Media was then changed to 5 % dextran-coated charcoal stripped FBS containing either Dox (1 μg/mL) or vehicle control (ten 15 cm dishes/condition). The following day, 10 nM R1881 was added to the media in all plates and cells were grown for a further 24 hours (total of 48 hours Dox treatment). Cells were harvested by scraping in ice-cold PBS containing 10 nM R1881. Whole cell lysates were prepared by re-suspending cell pellets in 8 mL (for ten 15 cm plates) of ice-cold Buffer C (20 mM HEPES pH 7.9, 0.4 M NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 25 % glycerol) and incubating with rotation at 4 °C for 2 hours (50). Lysates were then centrifuged for 10 minutes (9.3 g-force) at 4 °C and supernatants were frozen at – 80 °C. The following day, cell extracts were dialyzed for 2 hours at 4 °C using dialysis buffer (20 mM HEPES pH 7.9, 0.1 M NaCl, 0.2 mM EDTA, 0.5 mM PMSF, 20 % glycerol, 10 nM R1881). Each cell extract preparation (± Dox) was then incubated for 2 hours at 4 °C with 200 μL of agarose beads conjugated to FLAG monoclonal antibody (Sigma). Spun down beads were washed eight times with dialysis buffer containing increasing concentrations of NP-40 that ranged from 0 to 0.5 %. FLAG-tagged AR was eluted by incubating the beads for 30 minutes (4 °C) in 200 μL of dialysis buffer supplemented with FLAG peptide (1 mg/mL; Sigma). Elution samples were used for SDS-PAGE and proteins were visualized with SYPRO-Ruby (Molecular Probes) staining.

Protein bands corresponding to the approximate molecular weight of AR (110 kDa) were excised from the SDS-PAGE gel and subjected to in-gel trypsinization, as previously described (55). Briefly, digestions were carried out overnight at 37 °C with sequencing grade trypsin (Promega). Peptides were extracted from gel samples, brought to final volume of 20 µL with 5 % formic acid and analyzed by liquid chromatograpy-tandem mass spectrometry (LC-MS/MS). Nanoflow LC was performed using an Ultimate HPLC system (LC Packings) with a 75 µm × 150 mm C-18 Pepmap (reverse phase) analytical column and water/acetonitrile/formic acid gradients. The LC eluent was electrosprayed into the sampling orifice of a QSTAR XL quadrupole time-of-flight (Q-TOF) mass spectrometer (Applied Biosystems/MDS Sciex) and MS/MS spectra data was collected using Analyst QS control software. MASCOT search engine was used to analyze and match MS/MS data to all mammalian protein sequences in the Swiss-Prot database.

Statistical Analysis

LNCaP-DDC and LNCaP-Vector control xenograft tumour volume values were used for linear regression slope analysis to determine tumour growth rates with GraphPad InStat, version 3.06. Tumour growth rate differences were determined by one-way ANOVA and the Tukey-Kramer multiple comparisons test. All other statistical analysis was performed using the Student's t-test (two-sample equal variance) with JMPIN statistical software (Version 4.0.2, SAS Institute Inc.). All calculated p-values were two-sided and those less than 0.05 were considered statistically significant.

4.3 Results

DDC Enhances AR Transactivation from the Androgen-Regulated PSA Promoter and Sensitizes the Receptor to Limiting Ligand Concentrations

Having previously shown that DDC enhances AR transactivation from a synthetic ARR3-tk promoter (**Chapter 3**) (56), we investigated whether DDC has a similar effect on a promoter containing androgen response regions (ARRs) derived from the naturally occurring PSA gene. The proximal PSA promoter and ARR3-tk promoters were tested simultaneously in PC3 cells transfected with human AR and DDC expression vectors. In the presence of 1nM R1881 and with DDC overexpression (1:4 ratio of AR:DDC), AR transactivation of the proximal PSA and ARR3-tk promoters were increased 2.5-fold and 9-fold, respectively (**Figure 4.1A**). These results suggest that DDC can enhance AR activity from a physiologically relevant promoter, although not as strongly as from artificial androgen responsive elements.

Due to the stronger enhancement of AR transactivation seen with the ARR3-tk promoter, this reporter was used to determine the effects of DDC overexpression on AR activity under limiting androgen conditions in androgen-independent and -sensitive prostate cancer cell lines. PC3 cells, transfected with AR, and LNCaP cells that express endogenous AR were used to measure receptor transactivation at varying concentrations of R1881. In both PC3 and LNCaP cells, activation of AR transcription required a minimum concentration of 0.1 nM R1881. Overexpression of DDC enhanced AR activity by 2.5-fold in LNCaP cells and approximately 5.5-fold in PC3 cells (**Figures 4.1B** and **4.1C**) at this threshold androgen concentration. At higher R1881 levels of 1 nM and 10 nM, we observed the usual high 8-fold (1 nM) and 6-fold (10 nM) increases in AR

transactivation for PC3 cells and a 2-fold increase of endogenous AR activity in LNCaP cells. The substantial enhancement of AR transcription seen with DDC overexpression at 0.1 nM R1881 demonstrates that this coactivator can render AR more responsive to low concentrations of androgen.

DDC Increases the Apparent Affinity of AR for Androgen *in Vitro*

Since DDC sensitizes AR to limiting amounts of androgen and has previously been shown to predominantly interact directly with the receptor's ligand-binding domain (**Chapter 3**), we next tested the ability of DDC to influence the androgen-binding ability of AR, using purified AR-LBD and His-tagged-DDC protein. Nickel NTA-affinity chromatography was used to purify His-DDC from bacteria. His-DDC was detected at the known molecular weight of ~ 50 kDa and showed limited degradation, with a purity of greater than 90 %, as determined using Coomassie Blue staining (data not shown). Initial testing for AR-binding activity, using GST-pulldown and Western blot analysis, revealed that His-DDC protein binds strongly to the AR-LBD, while its interaction with the DBD is minimal (**Figure 4.2A**).

Each *in vitro* ligand binding assay was subsequently performed using 1μg of AR-LBD protein and the same amount of His-DDC protein (0.5 μg) used for GST-pulldown assays (1:0.5 mass ratio of AR-LBD:DDC). Total bound ligand to the AR-LBD was determined at increasing concentrations of R1881 (0 - 1000 nM) using [^{3}H]-R1881 as tracer (**Figure 4.2B**). Non-specific binding of ligand to His-DDC protein interacting with the AR-LBD was negligible at the tracer concentration of 20 nM [^{3}H]-R1881 (AR-LBD control/895 ± 93 versus AR-LBD + His-DDC/937 ± 111 pmole bound R1881/mg AR-

LBD). In the absence of His-DDC, an R1881 concentration of 883 nM was required to 50 % saturate total AR-LBD protein, while in the presence of His-DDC a lower androgen amount of 425 nM was necessary to reach the same level of saturation. This corresponds to a 2.1-fold higher apparent affinity for androgen by the AR-LBD, when His-DDC is present (**Figure 4.2B**).

Although the incubation of His-DDC with AR-LBD protein resulted in a 1.75-fold (75 %) and 1.71-fold (71 %) respective increase in bound R1881 at the 100 nM and 1000 nM concentrations, total saturation of AR-LBD protein was not achieved. Due to the inefficiency of ligand binding in this *in vitro* assay, an exact maximum androgen binding capacity (B_{max}) could not be calculated for the AR-LBD. Since a known amount of both AR-LBD protein and R1881 was used in this assay, we were able to calculate the efficiency of androgen-binding. In the absence of cold ligand, at the tracer concentration of 20 nM [^{3}H]-R1881, the number of androgen molecules per assay was equal to the number of AR-LBD molecules (1 μg AR-LBD protein = 1.2×10^{13} AR-LBD molecules = number of [^{3}H]-R1881 molecules). However, in the absence and presence of His-DDC protein, only about 5 % of all available ligand remained bound to the pulled down AR-LBD at this R1881 concentration. In the absence of DDC, higher R1881 concentrations of 100 nM and 1000 nM increased ligand-binding efficiency to 14 % and 54 %, respectively. The total amount of available ligand that bound to the AR-LBD increased to 24.5 % and 92 % upon addition of His-DDC protein for the 100 nM and 1000 nM R1881 concentrations, respectively. Overall, these data suggest that the direct association of DDC with the ligand-binding domain of AR can increase the apparent affinity of AR-

LBD molecules for androgen, which leads to a higher binding efficiency and potentially an elevated B_{max} for the receptor.

DDC Increases AR Androgen-Binding Capacity in Mammalian Cells

Having observed a direct effect of DDC on AR ligand-binding in a cell-free system, we next tested whether overexpression of DDC has an effect on AR androgen-binding capacity in cells. HeLa cells, stably transfected with FLAG-tagged AR (HeLa-AR), were used since these cells express high levels of wild-type human AR and allow for repetitive and consistent immunprecipitation of the receptor. Importantly, FLAG-tagged AR in these cells has been shown to translocate to the nucleus and is transcriptionally active in the presence of 1 nM R1881 (48). HeLa-AR cells were transfected with a DDC expression plasmid or vector control, treated with increasing concentrations of [^{3}H]-R1881 and assessed for the amount of androgen bound to immunprecipitated AR in cytosolic and nuclear fractions. Specifically bound-androgen values were normalized to the level of AR protein to account for variations in receptor protein stability due to [^{3}H]-R1881. Overexpression of DDC does not affect AR protein stability, as determined by Western blot analysis using whole cell lysate from HeLa-AR, AR-transfected PC3 and LNCaP cells (see **Figures 4.8** and **4.10** below). Notably, AR was still detectable in the cytosol at the 1 nM and 10 nM R1881 concentrations, suggesting that in HeLa-AR cells, not all receptor molecules bind ligand even at high androgen levels.

For immunoprecipitated AR from cytosolic fractions, DDC overexpression resulted in a 1.71-fold (71 %) and 1.66-fold (66 %) increase in the maximum androgen

binding capacity at the 1 nM and 10 nM saturation concentrations of [^{3}H]-R1881, respectively (**Figure 4.3A**). Not surprisingly, a significant increase in B_{max} was also detected in nuclear fractions with DDC overexpression at the 1 nM and 10 nM ligand concentrations, presumably due to increased androgen-binding and translocation of AR to the nucleus (**Figure 4.3B**). In addition, similar to the *in vitro* AR-LBD ligand binding assay (**Figure 4.2B**), we observed a 1.79-fold increase in the apparent affinity of AR for androgen when DDC was overexpressed in the cytosol (**Figure 4.3A**). This effect was observed in the 0.1 nM to 1 nM R1881 concentration range where minimal to 100 % saturation occurred, respectively. Within this concentration range, in the presence of DDC a significantly lower amount of R1881 (0.56 nM) was required to reach the control B_{max}, which occurred at 1 nM R1881 (left shift determined by linear regression analysis as in **Figure 4.2B**). Since it is possible that DDC overexpression in HeLa-AR cells may indirectly modulate transport of ligand, cellular androgen-uptake assays were performed at varying concentrations of [^{3}H]-R1881. The specific cellular-uptake of androgen did not change with DDC overexpression (**Figure 4.3C**). Overall, these data suggest that overexpression of DDC in the cytosol can increase the affinity of AR for androgen and possibly increase the population of AR molecules that can bind ligand, resulting in the observed higher B_{max} for the receptor.

DDC Increases *In Vivo* AR Transactivation and Tumour Growth Rate *Via* an Androgen-Dependent Mechanism

To determine whether DDC can also enhance *in vivo* androgen-dependent receptor transactivation, doxycycline-inducible DDC LNCaP cells were generated for

xenograft analysis. LNCaP-DDC and LNCaP-Vector control stable cells were s.c. injected in to mice and monitored for long-term PSA production/tumour growth under castrated and non-castration conditions. After PSA levels reached 75-100 ng/mL, mice were treated with either doxycycline (+Dox) or castrated and then given doxycycline (Cx + Dox). LNCaP tumour xenografts were used for immunofluorescence and Western blot analysis to determine the stability of DDC expression over the 53 day duration of the experiment (**Figure 4.4**). More than 90 % of infected cells maintained DDC expression (**Figure 4.4A**) and the ~ 50 kDa DDC protein was detected after long-term *in vivo* growth only in the LNCaP-DDC cells (**Figure 4.4E**). Castration of mice did not affect DDC protein levels. An additional control study using LNCaP-DDC stables in non-castrated mice was also carried out to determine leakiness of DDC expression in the absence of doxycycline (-Dox). Western blot analysis using tumours even after 32 days of *in vivo* growth showed minimal expression of DDC in the absence of Dox (**Figure 4.4E**). Overall, the lentiviral generated LNCaP-DDC cell line showed robust and consistent DDC expression, tightly regulated by doxycycline within the duration of the entire xenograft study.

To circumvent the problem of non-specific effects of Dox on tumour growth and the residual effects of low-level leaky expression of DDC in the absence of Dox, we focused on the analysis of four groups of mice; LNCaP-DDC (+Dox, Cx + Dox) and LNCaP-Vector control stable cells (+Dox, Cx + Dox). In this LNCaP tumour xenograft model, we monitored the effect of DDC overexpression on the rate of progression to androgen-independence (AI), defined as the time required after castration for PSA levels

to return to or increase above pre-castrate levels (**Figure 4.5A**). As expected, castration of the vector control group resulted in a decrease of serum PSA by day 4 (25 ± 14 % of pre-treatment), before rising to almost pre-treatment levels at day 32 (91 ± 64 %). However, DDC overexpression in castrated mice (Cx + Dox DDC group) did not alter PSA levels significantly from the castrated control group, with a low of 44 ± 31 % of pre-treatment at day 4 and reaching AI by day 32 (112 ± 61 %).

Under non-castration conditions, serum PSA for the control group rose to 236 ± 69 % of pre-treatment levels by day 18 and for the DDC non-castrated group this increase was 363 ± 253 % of pre-treatment (**Figure 4.5A**). Coincidentally, a significant difference in PSA levels only began to appear by day 32, which corresponded to the beginning of AI in castrated mice. At this point, serum PSA levels were 3 times higher for the DDC +Dox group than that of the vector control +Dox group ($p < 0.05$). The significant increase in PSA levels with DDC overexpression was maintained under non-castration conditions up to 53 days of tumour growth, at which point the DDC +Dox group exhibited a PSA level of 905 ± 133 % of pre-treatment, while that of the vector control +Dox group was 453 ± 114 % (2-fold increase with DDC overexpression). Overall, these data suggest that DDC can enhance *in vivo* AR transcriptional activity, as measured by the endogenous PSA marker, under sustained long-term (32 days or longer) overexpression conditions of the coactivator.

In addition to increasing serum PSA levels, DDC also had a significant effect on the rate of tumour growth in LNCaP xenografts (**Figure 4.5B**). Comparison of tumour volumes for the four groups using linear regression slope analysis with ANOVA ($p =$

0.0025) and the Tukey-Kramer multiple comparisons test, revealed that DDC overexpression in non-castrated mice significantly increased tumour growth rate by 2.3-fold from 7.1 ± 0.9 for the vector control group to 16.5 ± 3.6 for the DDC group ($p < 0.05$). In castrated mice, DDC overexpression did not alter tumour growth rate (control/7.3 ± 1 and DDC/6.7 ± 0.8). Overall, for DDC non-castrated mice the tumour volume increased from pre-treatment volume by approximately 12-fold over 53 days, whereas for vector control non-castrated, DDC castrated, and control castrated animals, this increase was only 5-fold. Again, as previously seen for serum PSA levels, the increase in tumour growth rate with DDC overexpression was only observed in non-castrated mice, suggesting that its effects on both AR activity and tumour growth occur through an androgen-dependent mechanism.

DDC Enhancement of AR Transactivation is Independent of its Neurotransmitter Products

Whether the enzymatic products of DDC are also involved in the enhancement of AR transactivation, seen with overexpression of DDC, is not known. To test for this possibility, we next studied the role of known DDC catalytic products and their cell surface receptors in prostate cancer cells. Prior to determining whether dopamine (DA) and serotonin (5-HT), are involved in the activation of AR, initial detection of both DA and 5-HT responsive GPCRs was carried out in PC3 and LNCaP cells, using cAMP assays. In PC3 cells, DA stimulated a dose-dependent increase in intracellular cAMP to levels that were ~ 52-fold higher than untreated cells, at the maximum 200 μM

concentration (**Figure 4.6A**). In LNCaP cells, DA did not induce a significant cAMP response at all concentrations. Although basal levels of cAMP in PC3 cells were greater than those of LNCaP cells, 5-HT treatments, varying from 0.01 nM to 100 nM, did not induce a cAMP response in either prostate cancer cell line (**Figure 4.6B**). Overall, these data suggest that functional and robust DA D1-like (cAMP elevating) GPCRs are present in PC3 cells and that 5-HT receptors, previously shown to be expressed in PC3 and LNCaP cells (57-61), do not significantly activate intracellular signalling pathways *via* cAMP elevation after short-term exposure to 5-HT.

Since we previously observed the most profound AR transactivation enhancement effect by DDC in PC3 cells (8-9 fold compared to 2-fold in LNCaP cells, **Figure 4.1**), which contain functional DA receptors, we next determined whether overexpression of DDC in these cells results in production of DA (in the presence of 1 nM R1881 and AR) that may indirectly activate AR. Hence, ELISAs for DA were carried out using conditioned medium (CM) from PC3 cells transfected with AR and DDC or vector control plasmid, mimicking overexpression levels (1:0 versus 1:4 AR:DDC ratio) used for previous transactivation assays (**Figure 4.1A**). PC12 rat adrenal pheochromocytoma cells (53, 62), known to express high levels of active DDC enzyme that produces DA, were used for comparison. ELISAs were performed in the presence and absence of a DDC inhibitor, NSD-1015 (1 μM), to demonstrate that DA production is due to direct activity of the enzyme (**Figure 4.7A**). With DDC overexpression in PC3 cells, we observed extremely low levels of DA production (522 ± 431 pg/mL CM) as compared to the PC12 positive control cell line (9106 ± 688 pg/mL CM). Addition of NSD-1015

reduced this level of DA production to basal levels in DDC overexpressing PC3 cells (220 ± 85 pg/mL CM), while reducing PC12 DA levels by 2.6-fold (3561 ± 2032 pg/mL CM). To determine whether the almost negligible levels of DA produced with DDC overexpression could activate the robust DA receptors in PC3 cells, cAMP assays were repeated in the presence of 1 nM R1881 and transfected AR, with or without DDC. We could not detect elevation in intracellular cAMP levels in PC3 cells that overexpressed DDC to the same extent as previous transactivation assays (**Figure 4.1A**) and DA ELISAs (**Figure 4.7A**), suggesting that activation of DA GPCRs does not occur in these cells in the presence of DDC (data not shown).

Nevertheless, in order to determine the possible long-term (24 hours) effects of DA and 5-HT on receptor transactivation, as previously tested for DA with other steroid receptors (36, 39), PC3 cells transfected with AR and ARR3-tk-Luc reporter plasmids were treated with the neurotransmitters using the same concentrations as for cAMP assays (**Figure 4.6**). DA treatment resulted in a reduction of ligand-dependent AR transcription, with a 2.2-fold repression at the highest 200 μM DA concentration (**Figure 4.7B**). Ligand-independent activation of AR was not affected by DA, suggesting that the effect of this neurotransmitter on AR activity is not due to a general cytotoxic effect on PC3 cells. In contrast, 5-HT treatment of PC3 cells (0-100 nM) did not alter AR activity in the absence or presence of ligand (data not shown). Overall, the combination of the above data strongly suggest that DA and 5-HT production and autocrine activation of their GPCRs are not responsible for the enhancement of AR activity observed with DDC overexpression in PC3 and LNCaP cells.

Enhancement of AR Transactivation is Dependent on DDC Enzymatic Activity

Although the known enzymatic products of DDC do not appear to be involved in activation of AR, we assessed whether DDC catalytic activity was necessary for its enhancement of AR transcription. PC3 cells were transfected with increasing amounts of wt and mt (Lys303Ile) DDC expression vectors relative to a constant amount of AR plasmid (1:0 to 1:4 ratio of AR:DDC), along side the ARR3-tk-luciferase reporter. LNCaP cells were transfected using the same amounts of DDC and reporter plasmids as for PC3 cells, but exogenous AR was excluded (0:0 versus 0:4 ratio of AR:DDC). Maximal overexpression of wt DDC resulted in the usual approximate 10-fold increase of ligand-dependent AR transactivation in PC3 cells (**Figure 4.8A**) and a 2-fold increase of endogenous AR activity in LNCaP cells (**Figure 4.8C**). However, the same level of overexpressed mt DDC did not affect the magnitude of R1881-induced AR transcription in PC3 or LNCaP cells. Hence, the single amino acid mutation of Lys303Ile completely abrogates the coactivation function of DDC on AR in prostate cancer cells. Importantly, the lack of coactivator activity with mt DDC is not due to lower expression or protein stability, as determined by Western blot analysis (**Figures 4.8B** and **4.8D**). Overall, this suggests that the enzymatic decarboxylation activity of DDC is necessary for its enhancement of AR transcription.

In order to determine whether mutation of the DDC PLP cofactor binding site (Lys303Ile) also affects direct binding of DDC to AR, *in vitro* GST-pulldown assays were performed using wt and mt DDC (**Figure 4.9**). The strongest interaction for wt and mt DDC protein occurred with the AR-LBD+DBD fragment as compared to AR-truncated-NTD+DBD. Since interaction with the AR-DBD is almost negligible, binding

of DDC with AR can be largely attributed to its affinity for the LBD of AR. Overall, the loss of decarboxylase activity had no effect on the direct interaction of DDC with AR, suggesting that the single point mutation of Lys303 is not sufficient to significantly alter conformation of the enzyme *in vitro*.

DDC does not Directly Decarboxylate the Terminal Amino Acid (Gln919) of AR

Having found that the catalytic activity of DDC is necessary for enhancement of AR transactivation and that DDC interacts with the receptor, we next used mass spectrometry to test whether DDC can potentially utilize AR as a direct substrate. In order to generate sufficient amounts of AR for LC-MS/MS analysis, a robust system that allowed for DDC overexpression and large-scale purification of AR protein was required. Hence, HeLa-AR cells that express high levels of FLAG-tagged AR were used to generate DDC-lentiviral stables under tetracycline-inducible regulation. HeLa-AR/DDC stable cells were initially used for immunofluoresence and Western blot analysis to confirm inducible expression of DDC (**Figure 4.10**). Addition of Dox resulted in induced-expression of the ~ 50 kDa DDC protein and did not alter AR protein levels (**Figure 4.10E**). As expected, in the presence of of 1 nM R1881, AR translocated to the nucleus with detectable levels still remaining in the cytoplasm (**Figures 4.10A** and **4.10C**). DDC co-localized with AR in the cytoplasm in the absence of androgen and did not translocate into the nucleus with 1 nM R1881 treatment (**Figures 4.10B** and **4.10D**).

FLAG-tagged affinity purified AR preparations from HeLa-AR/DDC stable cells, treated with 10 nM R1881 and incubated with or without Dox, were resolved by SDS-PAGE and stained with SYPRO-Ruby (data not shown). Visible protein bands that

corresponded to the approximate molecular weight (110 kDa) of AR were excised, digested with trypsin and peptides were analyzed using a quadrupole-TOF mass spectrometer. MS/MS analysis and database searching of sequenced peptides identified AR as the predominant protein with a total score of 746 in the absence of Dox and 942 in the presence of Dox (statistically significant threshold score was > 32, according to MASCOT). Overall, approximately 25 % of the AR amino acid sequence was covered with the list of detected peptides. Differences in the mass-to-charge ratio (m/z) of detected peptides corresponding to loss of a $-CO_2$ group (42 - 44 Da reduction in m/z), or multiples of it, were not observed with DDC overexpression. However, in our initial analysis, the extreme C-terminal AR peptide, containing residue Gln919 with the only free α-COOH of AR, was not be detected.

To enrich for this medium-length peptide, size-based gating was performed during LC-MS/MS analysis to increase detection of peptides corresponding to a molecular weight of 10-15 amino acids. A doubly charged $[M + 2H]^+$ peptide ion at m/z of 566.8, corresponding to the size of a 9 amino acid C-terminal AR peptide, was detected and fragmented *via* collision-induced dissociation. This peptide was identified as amino acid residues 911 to 919 of AR (VKPIYFHTQ), formed by trypsin cleavage on the C-terminal side of Lys910. However, addition of Dox to induce DDC expression in HeLa-AR/DDC stable cells did not result in any detectable modifications of this peptide corresponding to the loss of a $-CO_2$ group, which would have been detected at an m/z of 544.8 (566.8 - 44 Da/$2H^+$). The MS/MS spectrum of the unmodified extreme C-terminal AR peptide ion (Val911 - Gln919) in the absence and presence of Dox is shown in **Figures 4.11A** and **4.11B**, respectively. Overall, as expected these data demonstrate that

in HeLa-AR cells, DDC does not modify amino acid residues 911-919 of AR and does not decarboxylate the α-COOH group on the terminal Gln919 residue of the receptor.

4.4 Figures

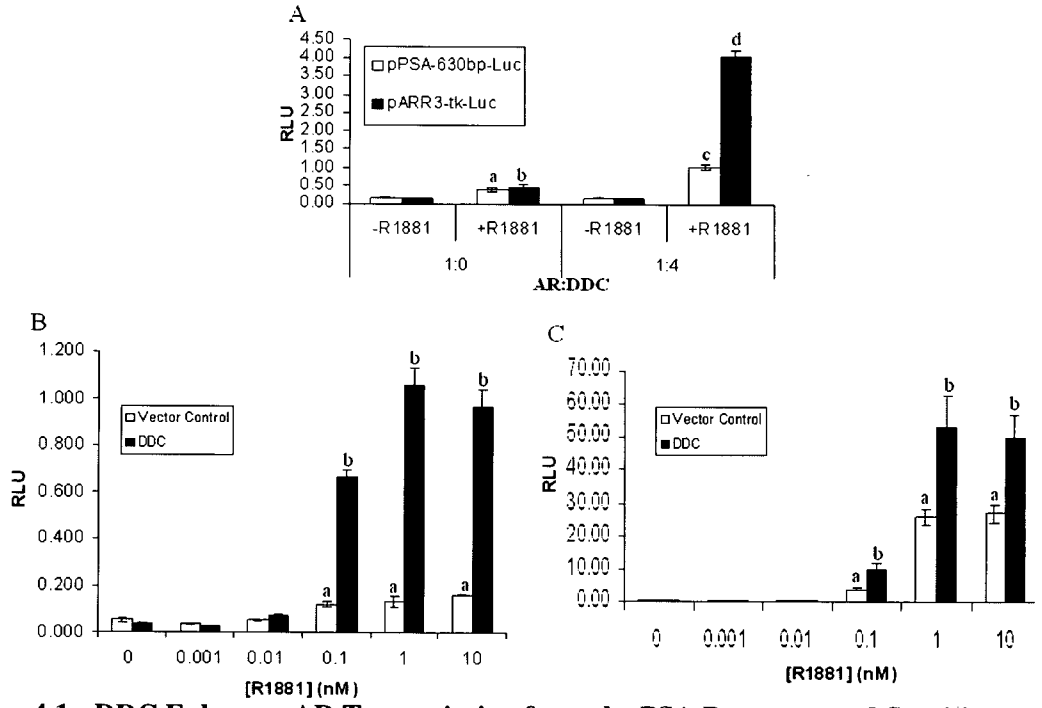

Figure 4.1 - DDC Enhances AR Transcription from the PSA Promoter and Sensitizes the Receptor to Limiting Androgen Concentrations. A) PC3 cells were transfected with human AR expression plasmid (250 ng/well), pRL-TK-renilla (83 ng/well) and pPSA-630bp-Luc or pARR3-tk-Luc synthetic reporter for comparison (167 ng/well). The amount of transfected pDEST12.2-DDC plasmid (1 μg/well) was four times that of AR (AR:DDC ratio of 1:4). Total DNA was kept constant at 1.5 μg/well using pDEST12.2 empty vector control (1:0 ratio). Cells were treated with or without 1 nM R1881 for 24 hours before harvest and luciferase assay. Transfection efficiency was normalized with the renilla luciferase pRL-TK vector. RLU values are the mean of triplicates (±SEM); $^{a}p < 0.05$ as compared to –R1881 control for pPSA-630bp-Luc reporter, $^{b}p < 0.05$ as compared to –R1881 control for pARR3-tk-Luc reporter, $^{c}p < 0.05$ as compared to +R1881 empty vector control (1:0 ratio) for pPSA-630bp-Luc and $^{d}p < 0.05$ as compared to +R1881 empty vector control (1:0 ratio) for pARR3-tk-Luc. Each graph is representative of 3 independent trials. B) PC3 and (C) LNCaP cells were transfected as above using the pARR3-tk-Luc reporter, except AR was not included for LNCaP cells. After transfection, cells were incubated for 24 hours in absence or presence of increasing concentrations of R1881. Firefly luciferase values were normalized to renilla luciferase and the mean RLU values of triplicates (±SEM) are shown; $^{a}p < 0.05$ as compared to –R1881, 0.001 nM and 0.01 nM R1881 and $^{b}p < 0.05$ as compared to 0.1 nM, 1 nM and 10 nM R1881 of empty vector control.

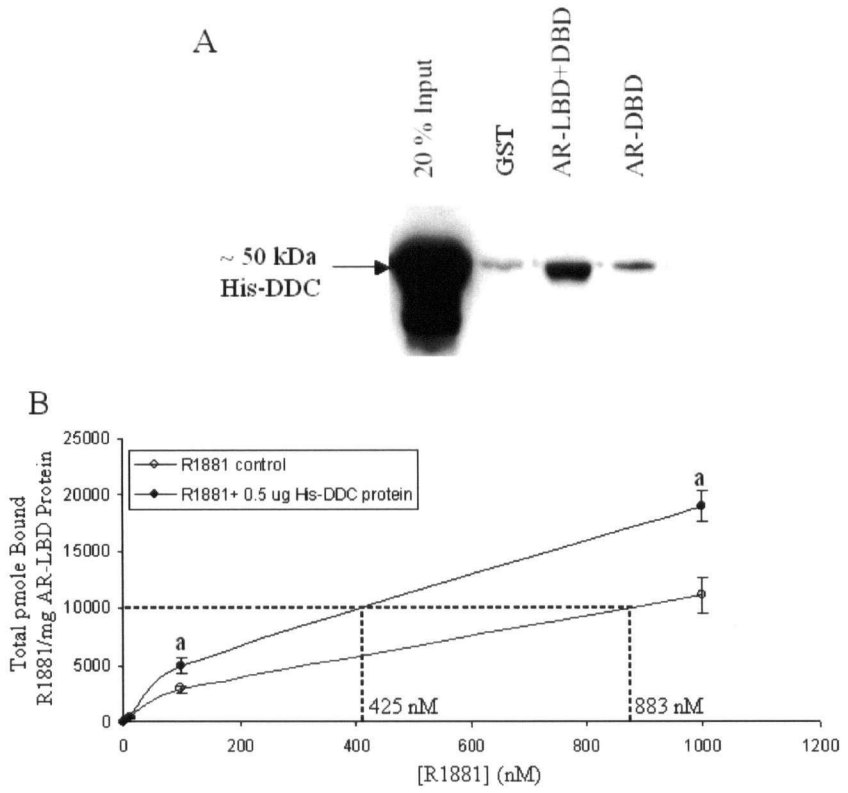

Figure 4.2 – Purified His-DDC Protein Interacts with the AR-LBD and Enhances Apparent Affinity of AR for Androgen *in Vitro*. A) GST protein and GST-AR domain fusion proteins (DBD+LBD and DBD alone) were coupled to glutathione-agarose beads at equimolar levels (see **Figure 9** for Coomassie Blue stain of GST-AR domains). Purified His-DDC protein was incubated (0.5 µg/assay) with GST-AR domains and bound protein was detected *via* SDS-PAGE/Western blot analysis using an anti-DDC antibody. B) Ligand binding assays were performed using AR-LBD protein (1µg/assay) and increasing R1881 concentrations, with 20 nM [^{3}H]-R1881 as tracer. Assays were carried out in the absence (R1881 control) and presence of His-DDC protein (0.5 µg/assay). Results are shown for the 10 nM, 100 nM and 1000 nM concentrations of R1881, expressed as the mean total pmole bound R1881/mg AR-LBD protein from three replicates (±SEM); $^a p < 0.05$ as compared to corresponding 100 nM and 1000 nM R1881 concentrations of the R1881 control. The concentrations of R1881 required, in the absence and presence of His-DDC, to 50 % saturate total AR-LBD protein (equivalent of 10, 000 pmole bound R1881/mg AR-LBD protein) was determined using linear regression analysis (GraphPad InStat, Version 3.06).

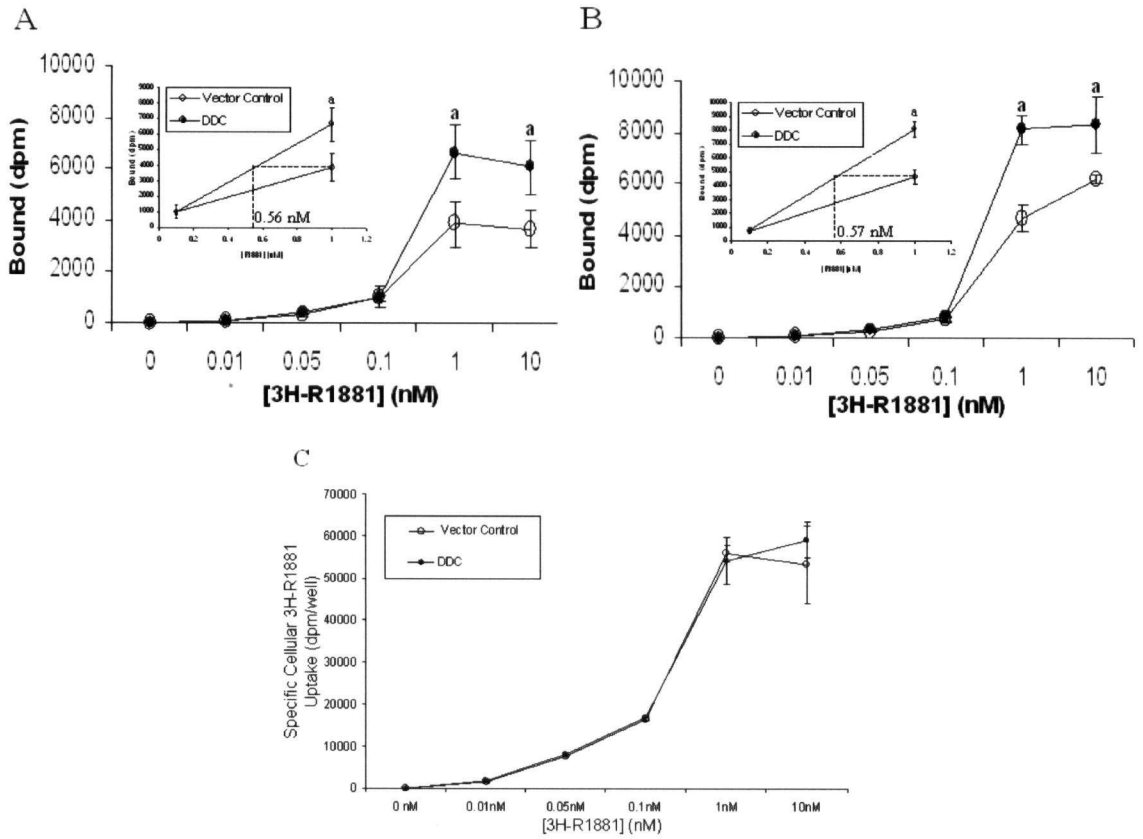

Figure 4.3 – DDC Increases Maximum Androgen-Binding Capacity of AR in HeLa-AR Cells. A) Cytosolic and B) nuclear fraction cell extracts were prepared from HeLa-AR cells that were transfected with pDEST12.2-DDC or pDEST12.2 control vector and treated with increasing concentrations of [^{3}H]-R1881 for 24 hours. FLAG-tagged AR was immunoprecipitated from protein lysates using agarose beads conjugated to a FLAG monoclonal antibody and the amount of specifically bound [^{3}H]-R1881 to AR was measured (dpm) using a scintillation counter. Aliquots (10 %) of cytosolic and nuclear lysates were also subjected to Western blotting (anti-AR antibody) to determine FLAG-tagged AR protein levels, which was used for normalization of measured dpm values (described in detail in **Section 4.2**). Results are expressed as the relative mean bound ligand (dpm) of three replicates (±SEM); $^a p < 0.05$ as compared to corresponding 1 nM and 10 nM [^{3}H]-R1881 concentrations of vector control for both cytosolic and nuclear fractions. C) Androgen cellular-uptake assays were performed using HeLa-AR cells that were transfected with either pDEST12.2-DDC or pDEST12.2 control vector and incubated for 24 hours with increasing concentrations of [^{3}H]-R1881. Whole cell extracts were prepared with RIPA buffer and used for scintillation counting to determine cellular [^{3}H]-R1881 accumulation (dpm/well of 6-well plate). Results are expressed as the mean specific cellular [^{3}H]-R1881 uptake of three replicates (±SEM).

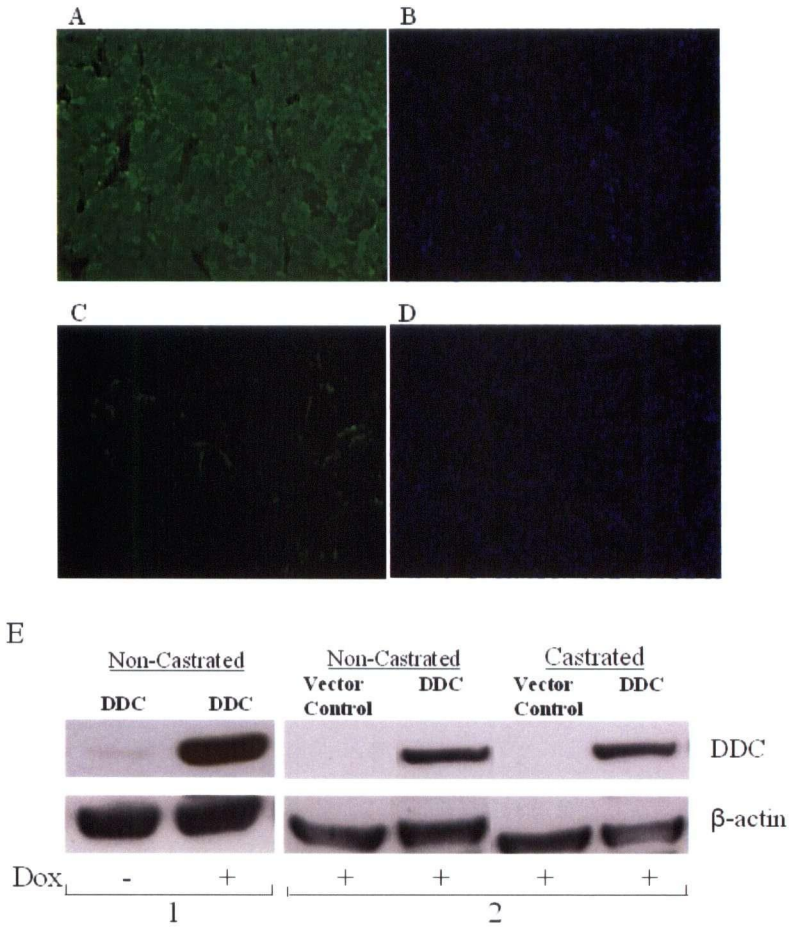

Figure 4.4 – Immunofluorescence and Western Blot Analysis of LNCaP-DDC and LNCaP-Vector Control Xenografts. A-D) Formalin-fixed/paraffin-embedded LNCaP-DDC and LNCaP-Vector control tumour tissues were mounted onto glass slides and stained with a rabbit anti-DDC antibody after antigen retrieval. Slides were incubated with rabbit biotinylated-secondary antibody, followed by fluorescein (green)-conjugated streptavidin treatment and counterstaining with hoechst nuclear stain (blue). Staining was visualized using fluorescent microscopy and Northern Eclipse imaging software. Results are shown for LNCaP-DDC (panels A/B) and LNCaP-Vector control (panels C/D) tumours treated with Dox for 53 days in non-castrated mice (original magnification × 200). E) Whole cell protein extracts were prepared in RIPA buffer from tumours at various time points and used for Western blot analysis (50μg protein/well) with an anti-DDC antibody (β-actin blotting was used for loading control). Results are shown for LNCaP-DDC and LNCaP-Vector control tumours treated with Dox for 53 days in non-castrated and castrated mice (group 2). The expression of DDC was also analyzed for LNCaP-DDC tumours treated with and without Dox for 32 days in non-castrated hosts (group 1; control study for determination of DDC expression leakiness).

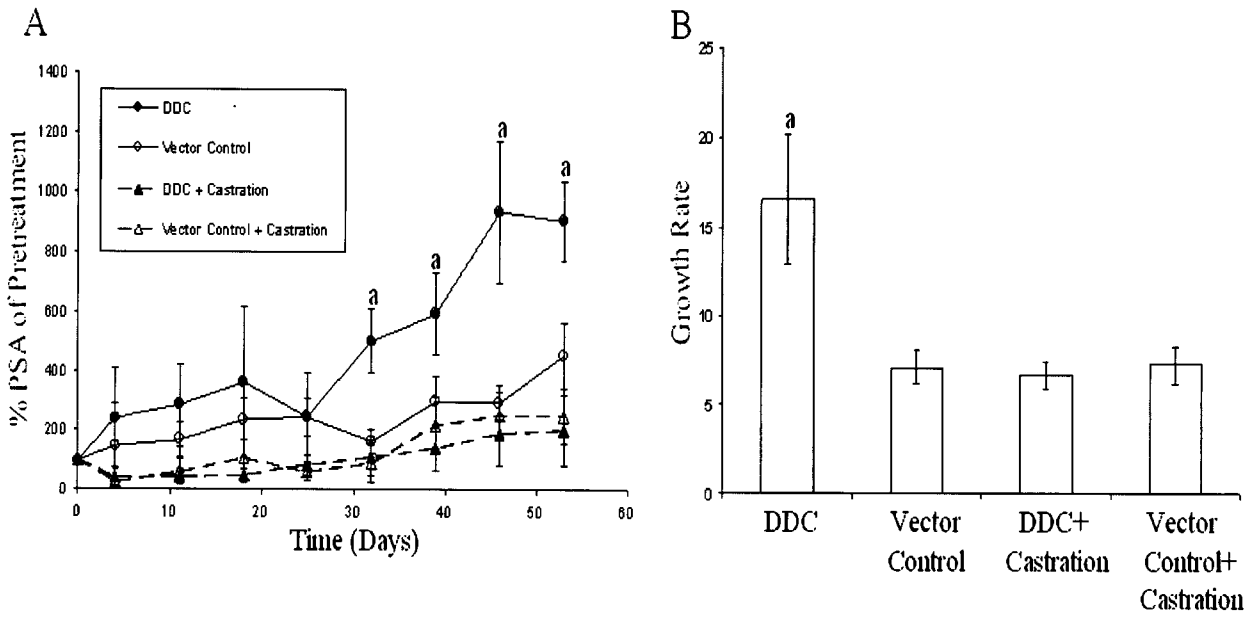

Figure 4.5 – Effect of Induced DDC Expression on Serum PSA and LNCaP Xenograft Tumour Growth. LNCaP-DDC and LNCaP-Vector control cells were inoculated subcutaneously into male nude mice with weekly monitoring of serum PSA and tumour volumes. Once serum PSA values reached 75–100 ng/mL, half of the mice in each tumour group were castrated and given Dox-treated water, while the remaining animals only received Dox-treated water. A) Serum PSA levels are expressed as percentage of pretreatment values and shown from the time of treatment initiation up to the end of the 53 day experiment. Dashed lines represent the castrated DDC (filled triangles) and vector control (open triangles) groups. Solid lines represent the non-castrated DDC (filled circles) and vector control (open circles) groups. Mean percentage PSA of pretreatment values from 3-6 mice (±SEM) are shown; $^{a}p < 0.05$ as compared to corresponding time points in the non-castrated vector control group. B) As done above for PSA, measured tumour volume values were normalized to pretreatment values (set at 100 %) to obtain the mean percentage tumour volume of pre-treatment (±SEM). The increase in tumour volume was divided by duration of the experiment (53 days) to determine growth rates for each group (linear regression slope analysis, GraphPad InStat); $^{a}p < 0.05$ as compared to non-castrated vector control, castrated DDC and castrated vector control groups.

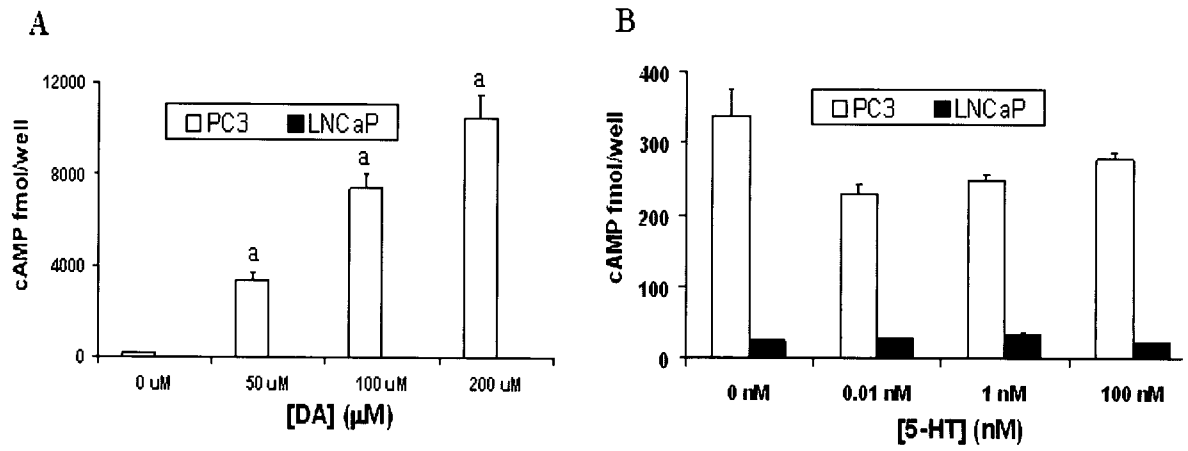

Figure 4.6 – Effect of DA and 5-HT on Induction of Intracellular cAMP Levels in PC3 and LNCaP Cells. A) DA and (B) 5-HT were used to treat PC3 and LNCaP cells in serum-free DMEM and RPMI media, respectively. Cells were stimulated with DA (15 minutes) or 5-HT (10 minutes) and cell lysates from triplicate wells (12-well plate) were used for cAMP analysis by ELISA. Results are expressed as the mean cAMP concentration (fmol/well) ±SEM; $^{a}p < 0.05$ as compared to control PC3 cells not treated with DA. Levels of cAMP were also normalized to mass (μg) of whole cell lysate protein (fmol/μg protein), which followed the same trend as above.

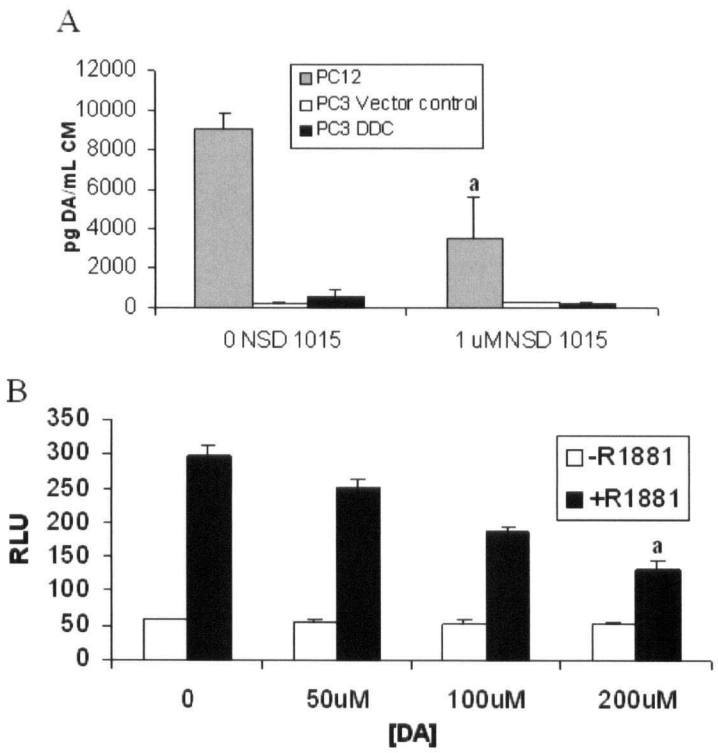

Figure 4.7 - DDC Coactivation of AR Transcription is Independent of DA Neurotransmitter. A) Dopamine ELISA; PC3 cells were transfected with AR expression plasmid (125 ng/well) and pDEST12.2-DDC (0.5 μg/well) or pDEST12.2 empty vector control (0.5 μg/well) to mimick previous transactivation assays (AR:DDC ratio of 1:4 versus 1:0). Cells were treated with 1 nM R1881 for 24 hours in the presence and absence of 1 μM NSD-1015 DDC enzymatic inhibitor. PC12 cells were not transfected or treated with R1881 but were incubated 24 hours with NSD-1015. Conditioned media (CM) was collected from PC3 and PC12 cells for DA ELISAs. Secreted DA levels (pg) were normalized to the assayed volume of CM (0.1 mL) and are expressed as the mean of triplicates (±SEM); $^{a}p < 0.05$ as compared to 0 NSD-1015 for PC12 cells. B) Dopamine transactivation assay; PC3 cells were transfected with AR expression plasmid, pRL-TK-renilla and pARR3-tk-Luc reporter as done above (**Figure 5.1A**), except DDC was not included. Cells were treated with or without 1 nM R1881 and increasing concentrations of pure DA for 24 hours before harvest and luciferase assay. RLU values were obtained by normalizing to total protein concentration and are presented as the mean of triplicates (±SEM); $^{a}p < 0.05$ as compared to +R1881/–DA control. This graph is representative of 3 independent trials.

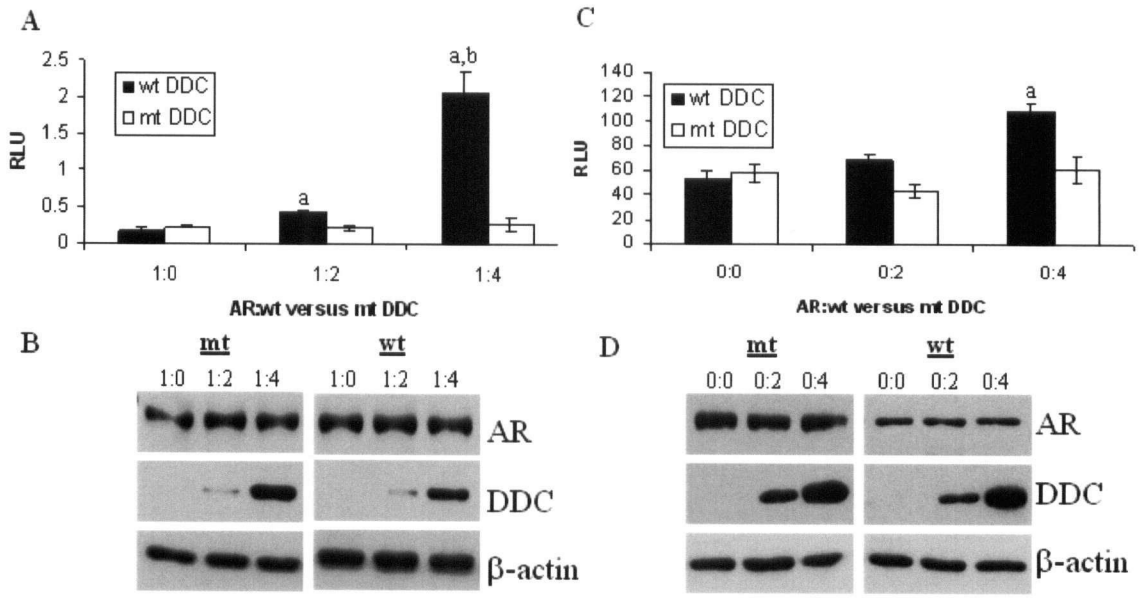

Figure 4.8 – Coactivation of AR Transactivation by DDC is Dependent on its Enzymatic Activity. A) PC3 cells were transfected with a constant amount of AR (250 ng/well), pARR3-tk-Luc (167 ng/well), pRL-TK-renilla (83 ng/well) and increasing amounts of pDEST12.2-DDC (wt) or pDEST12.2-mt-DDC (mt) vector. These amounts included 0, 0.5 or 1.0 μg/well, which correspond to the 1:0, 1:2 and 1:4 AR:DDC ratios, respectively. Total DNA was kept constant at 1.5 μg/well using pDEST12.2 empty vector control. Cells were treated with or without 1 nM R1881 for 24 hours prior to harvest and luciferase assay (results above only show +R1881 conditions for clarity; in the absence of R1881 AR activity was virtually undetectable). The renilla luciferase pRL-TK vector was used for normalization of transfection efficiency. RLU values are the mean of triplicates (±SEM); $^{a}p < 0.05$ as compared to +R1881 empty vector control (1:0 ratio) and $^{b}p < 0.05$ as compared to +R1881 1:2 AR:DDC ratio. Each graph is representative of 3 independent trials. C) LNCaP cells were transfected and assayed as for PC3 cells above, except AR was excluded (ratios of AR:DDC were 0:0, 0:2 and 0:4). Mean RLU values (±SEM) are presented; $^{a}p < 0.05$ as compared to +R1881 empty vector control (0:0 ratio). B) PC3 and (D) LNCaP cell transactivation assay protein lysates were used to determine AR and wt DDC versus mt DDC expression levels. A 10 μg aliquot of protein lysate from each triplicate assay was combined (30 μg total protein) and subjected to SDS-PAGE/Western blot analysis. Blots were probed with anti-AR, anti-DDC and anti-β-actin antibodies.

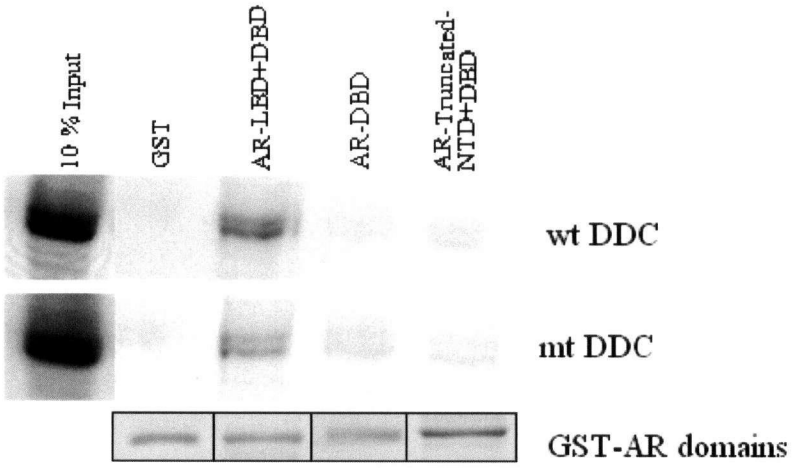

Figure 4.9 – Interaction of DDC with AR is Independent of its Enzymatic Activity. A) GST protein and GST-AR domain fusion proteins (Truncated-NTD+LBD, DBD and DBD+LBD) were coupled to glutathione-agarose beads at equimolar levels, as determined by Coomassie Blue staining (GST-AR domains). Wt DDC and mt DDC were expressed by *in vitro* transcription/translation and radiolabeled using [^{35}S]-methionine. After incubation of radiolabeled proteins and GST-AR domains, bound protein was eluted for SDS-PAGE and autoradiography analysis.

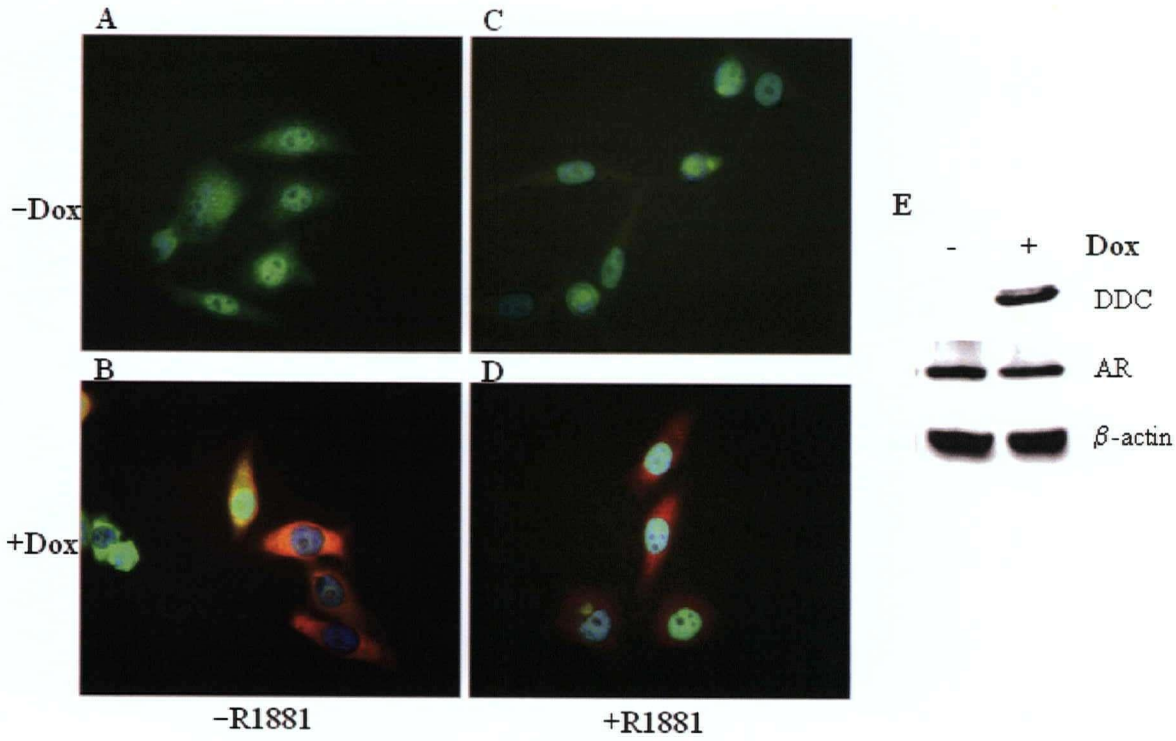

Figure 4.10 – Immunofluorescence and Western Blot Analysis of HeLa-AR/DDC Stable Cells. A-D) HeLa-AR/DDC cells were treated with or without Dox (1 μg/mL) and ±1 nM R1881 for 48 and 24 hours, respectively. Methanol-fixed cells were stained simultaneously using mouse anti-AR and rabbit anti-DDC antibodies followed by incubation with the corresponding species secondary antibody conjugated to fluorophores; green-fluorescein for AR and Texas-Red for DDC. Nuclei were stained with DAPI (blue) present in the mounting media. Positive staining was visualized using fluorescent microscopy and Northern Eclipse imaging software. All panels are AR/DDC/DAPI overlays (original magnification × 400). E) Whole cell extracts were prepared for HeLa-AR/DDC stable cells using RIPA buffer, after induction of DDC expression with Dox for 48 hours and 1nM R1881 treatment for 24 hours. Western blotting (25μg protein/well) was performed with anti-DDC, anti-AR and anti-β-actin antibodies.

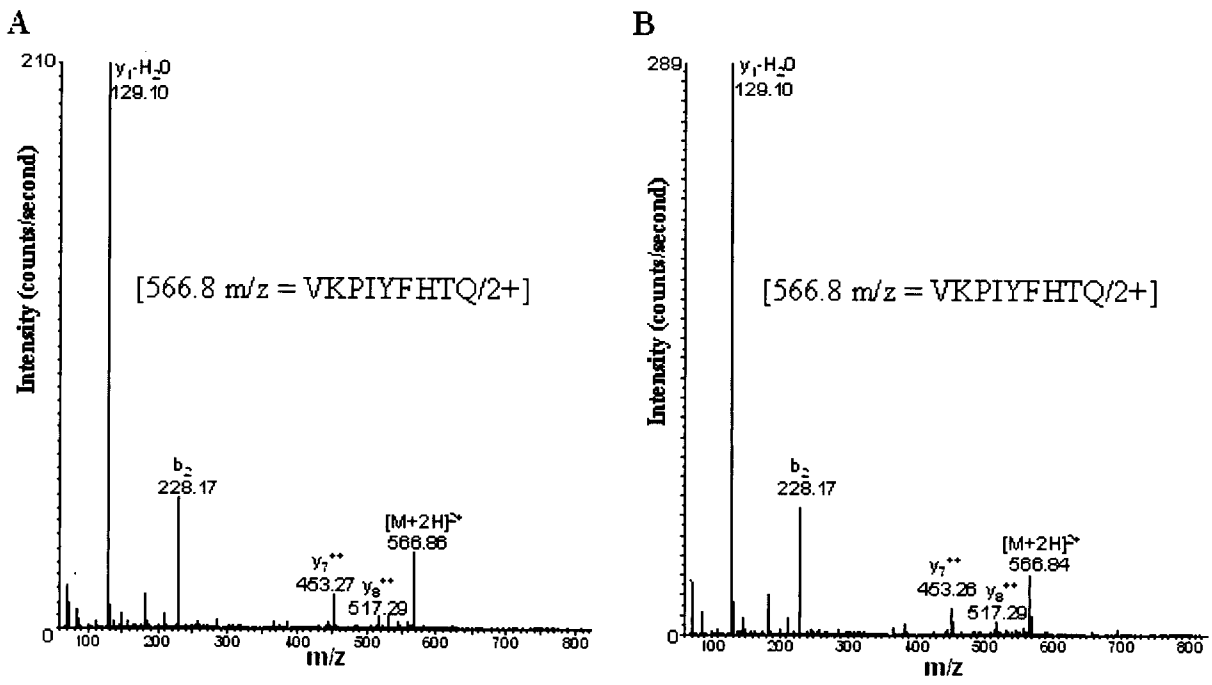

Figure 4.11 – Mass Spectrometric Identification of the AR C-terminal Peptide (Val911 – Gln919). HeLa-AR/DDC stable cells were grown in the absence (A) or presence (B) of Dox and FLAG-tagged AR was purified for trypsin digestion. Peptides were analyzed by LC-MS/MS with a Q-TOF mass spectrometer. The shown MS/MS spectra were acquired from a doubly charged $[M + 2H]^+$ peptide ion, at m/z 566.8, that was identified in the absence (–Dox) and presence (+Dox) of DDC overexpression. The sequence VKPIYFHTQ was unambiguously matched to amino acids 911-919 of human AR using MASCOT and the Swiss-Prot database.

4.5 Discussion

Several steroid receptor coactivators, including members of the SRC/p160 family, ARA54 and ART-27, have been shown to increase AR-regulated PSA expression (63-65). In addition to these, we have found that overexpression of DDC in prostate cancer cells also enhances AR transcription from the PSA promoter (**Figure 4.1A**). Activation of this promoter is dependent on the properties of the cell line examined, the presence of AR or possibly other steroid receptors, and the absence or presence of additional coregulators (66). Since *in vivo* PSA gene expression is regulated by AR (48), the 2.5-fold increase in receptor transactivation from the proximal PSA promoter is physiologically representative of the effects of DDC on AR activity. This also suggests that DDC may activate AR through a mechanism involving transcription factors important for regulation of the PSA gene.

The most effective means of treating advanced prostate cancer is by androgen ablation therapy, which results in temporary regression of prostate tumours but eventually leads to androgen-independent disease (67, 68). Although, androgen withdrawal treatment causes a 90 % reduction in the level of circulating androgens, low concentrations of this hormone from adrenal secretions still remain and may stimulate AR transcriptional activity (69, 70). Our observation that DDC sensitizes AR to low androgen levels in prostate cancer cells (**Figures 4.1B** and **4.1C**) suggests that during disease progression to androgen independence, DDC may allow for enhanced receptor activity and continued expression of AR-regulated genes involved in tumour growth under castration conditions. However, a minimum threshold androgen concentration of

0.1 nM R1881 was necessary for DDC to increase AR activity, suggesting that its coactivation function occurs *via* a ligand-dependent mechanism.

The sensitization of AR to low androgen concentrations should occur from a molecular mechanism that results in some increased efficiency of the receptor's ability to directly bind hormone. Using *in vitro* and cell line-based ligand-binding assays, we found an increase in the apparent affinity of AR for androgen by up to 2-fold and an elevation of 66-71 % in the maximum androgen-binding capacity (B_{max}) of the receptor, in the presence of DDC protein (**Figures 4.2** and **4.3**). Although with DDC overexpression, a higher apparent affinity for R1881 can contribute to the observed increase in B_{max} for AR in cells, the elevation in androgen-binding capacity must ultimately result from an increase in the number of cytosolic AR molecules that are capable of binding ligand. Overall, these finds provide a possible mechanism by which DDC enhances androgen-dependent AR activity.

AR mediates transcriptional activation predominantly through its N-terminal domain AF1 and C-terminal LBD AF2 activation functions (7, 71). The highly conserved AF2 hydrophobic surface in the LBD of AR is stabilized by androgen and required for recruitment of SRC/p160 coactivators (8). Binding of androgen to AR is thought to stabilize helix 12 of AF2 to complete the coactivator binding surface, allowing their recruitment and leading to enhanced AR transcription (72-74). The observed increase in apparent affinity of the AR-LBD for androgen in the presence of purified His-DDC protein (**Figure 4.2B**), suggests that DDC can actually modulate the mechanism by which ligand binds to the receptor. As this *in vitro* system only consists of AR-LBD, DDC and androgen, the increase in ligand-binding can be attributed to AR-LBD

molecules binding R1881 more efficiently, with a higher proportion binding androgen, when an interaction occurs between DDC and the AR-LBD (**Figure 4.2A**). The effect of DDC on AR ligand-binding may involve intramolecular changes to the conformation of the AR-LBD. This could lead to structural alterations of the 11 α-helices that form the hydrophobic pocket of steroid receptors involved in recognition of cognate ligand, as well as positioning of helix 12 near the extreme C-terminus of the AR-LBD to expose the surface required for coactivator binding (75, 76).

It has been reported that the recombinant bacterially-expressed AR-LBD used in our *in vitro* ligand binding assay exists in a stable conformation that is capable of binding androgen (77, 78). However, in the absence of DDC (**Figure 4.2B**), the need for excessive concentrations of R1881 (883 nM) to half-saturate the population of AR-LBD molecules reveals that chaperone components involved in the assembly of steroid receptor heterocomplexes are crucial for efficient ligand-binding. It has been proposed that steroid receptors acquire hormone-binding activity after their initial assembly with hsp70 and its co-chaperone hsp40, followed by association with hsp90 (79). The hsp90-associated chaperone machinery has been suggested to position helix 12 of the AR-LBD in a conformation that is optimal for hormone binding. The increase in AR-LBD androgen-binding efficiency in the presence of DDC protein (54 % control to 92 % in presence of His-DDC at 1000 nM R1881), suggests that the association of this coactivator with steroid receptor-chaperone complexes in the cellular environment may provide an additional means of modulating AR responsiveness to hormone.

As expected, cell line-based ligand binding assays indicated that the amount of androgen required to saturate wt AR in HeLa-AR cells, expressing necessary chaperone

machinery, is vastly lower than in the purified protein system, reaching a plateau at 1-10 nM R1881 (**Figure 4.3**). Overexpression of DDC in HeLa-AR cells confirmed that this coactivator can increase the apparent affinity of full-length AR for androgen in mammalian cells (**Figures 4.3A** and **4.3B**). In addition, with DDC overexpression, there is a significant increase in the total amount of bound-androgen, under saturation conditions (1-10 nM R1881), suggesting that a sub-population of AR molecules are not bound to ligand and remain localized to the cytoplasm even at high R1881 concentrations. These inactive AR molecules can be targeted by cytoplasmic coactivators, such as DDC, and by steroid receptor chaperone proteins. The interaction of DDC with AR can lead to a higher proportion of AR molecules being activated. Therefore, in contrast to the coactivation mechanism of classical steroid receptor coactivators, such as SRC/p160 family members and CBP/p300, which facilitate AR transcription by histone modifications, chromatin remodelling, and bridging of the receptor to components of basal transcriptional machinery, DDC can enhance AR transactivation by increasing ligand-binding affinity and capacity of the receptor in the cytoplasm, resulting in higher nuclear levels of AR.

Established coactivators of AR that can regulate receptor hormone-binding prior to nuclear translocation include ARA70 and the hsp90 chaperone protein (9, 18, 80). While hsp90 has been shown to maintain AR in a high affinity ligand-binding conformation, ARA70 has been suggested to change the conformation of cytosolic AR so that it binds and/or retains androgen more easily and also translocates to the nucleus at a faster rate (18). Moreover, ARA70 was shown to specifically retard the dissociation of steroid hormones, like estrogen (17 beta-estradiol), which is also known to enhance AR

activity, without affecting association of hormone with the receptor. Whether, DDC can similarly modify AR ligand-binding on/off rates in a time-dependent manner remains the subject of future studies (discussed in **Section 6.2** of **Chapter 6**). Since DDC is co-localized with AR only in the cytosol, one can speculate that the increased apparent affinity of AR for androgen, leading to elevation of AR maximum androgen-binding capacity (B_{max}) (**Figures 4.3A** and **4.3B**), is most likely associated with an increase in the on-rate of ligand-binding. Importantly, the lack of increased cellular androgen-uptake with DDC overexpression in HeLa-AR cells (**Figure 4.3C**), suggests that the increased B_{max} of AR is due to a direct effect of DDC on the receptor in the cytosol and not *via* production of its neurotransmitter products or other unknown pathways. Overall, our combination of *in vitro* purified protein and cell-based ligand-binding assays suggests that DDC can enhance AR transcriptional activity by increasing its androgen-binding capacity and apparent affinity for ligand, possibly through conformational changes in the ligand-binding domain of the receptor, prior to nuclear translocation.

The androgen-dependent enhancement of AR transactivation by DDC was also confirmed in animal studies. Increased PSA production with DDC overexpression was seen in LNCaP xenografts, demonstrating that DDC can act as an AR coactivator *in vivo* (**Figure 4.5A**). However, this effect was only observed under non-castration conditions suggesting that a minimal threshold concentration of androgens is required in order for DDC to enhance AR transactivation. Since mice have been reported not to produce adrenal precursor androgens (81), surgical castration may essentially eliminate circulating androgens (82), far below levels required by DDC to enhance AR activity. In contrast to removal of the testes in mice, human chemical castration/hormone-ablation therapy does

not completely remove circulating androgens and production of adrenal androgens in prostate cancer patients could account for continued androgen-dependent AR activation (69, 70). Hence, in patients undergoing androgen-withdrawal therapy, DDC may serve to activate AR utilizing limiting concentrations of hormone. In non-castrated mice, we also observed a significant increase in tumour growth rate with overexpression of DDC (**Figure 4.5B**), suggesting that its coactivation function may lead to sustained AR activity and androgen-dependent tumour growth.

Although enzymatic products of DDC (DA and 5-HT) are not involved in the indirect activation of AR in prostate cancer cells (**Figures 4.6** and **4.7**), mutational analysis of the PLP cofactor binding site (Lys303Ile) revealed that decarboxylation activity is necessary for enhancement of AR transcription (**Figures 4.8A** and **4.8C**). Replacing residue 303 of DDC with amino acids that are structurally distinct or even those that more closely resemble Lys results in dramatic loss of enzymatic activity, indicating that the shape and chemical properties of this residue are essential for catalysis (44). The lack of DA/5-HT involvement in the activation of AR and loss of coactivation function for enzymatically inactive mt DDC, suggest that this enzyme may be acting upon the receptor itself *via* the free α-COOH group of the AR terminal Gln919 residue or possibly upon another unknown amino acid substrate. However, due to the high level of specificity of PLP-dependent enzymes for α-carbons of free amino acids (83), it is unlikely that DDC can utilize proteins as substrate. Nevertheless, due to the necessity of DDC catalytic activity for AR coactivation and direct binding ability to the receptor, this possibility was tested using mass spectrometry analysis, which revealed that DDC does not modify the AR C-terminal G1n919 residue (**Figure 4.11**). The PLP cofactor of DDC

also plays an important role in the formation of the active homodimer enzyme (84). Hence, the inability of mt DDC to bind PLP can also prevent dimerization of DDC, which may be necessary for its enhancement of AR activity.

The Lys303Ile mt DDC bound to AR-domains (strongest with LBD) in the same manner as wt enzyme (**Figure 4.9**), suggesting that this amino acid mutation does not affect the receptor-coactivator association. A notable feature of DDC that may be important for its functional interaction with AR is the presence of an amphipathic alpha-helical LXXLL motif (amino acids 153-157), which has been shown to be important for docking onto the hydrophobic groove of steroid receptor ligand-binding domains (73, 76, 85, 86). Although it has been shown that among steroid receptors, the AR-LBD uniquely prefers binding to FXXLF motifs, one of which is located in the AR-NTD, others have also demonstrated that the AR AF2 binds a subset of SRC LXXLL motifs with high affinity to activate transcription (87-89). DDC also contains two additional FXXLL motifs (amino acids 251-255 and 284-288) utilized by nuclear receptor coregulators to bind receptor ligand-binding domains (90). We have previously found (**Chapter 3**) that the DDC central region containing the LXXLL motif and two FXXLL motifs, are not in involved in binding to AR *in vitro* (56). However, it is possible that in the cellular environment, DDC can use these motifs to dock onto the LBD of AR in the cytoplasm. Overall, the lack of a change in DDC binding to the AR-LBD after mutation of its PLP cofactor binding site suggests that this amino acid is not crucial for the direct association of DDC with AR (**Figure 4.9**). Therefore, mt DDC may still retain the conformation required to interact with AR, but is unable to use its enzymatic decarboxylation activity to exert coactivation function on the receptor.

In conclusion, this study has demonstrated that DDC can function as a coactivator of AR *in vitro* and *in vivo*. DDC sensitizes AR to limiting concentrations of androgen in prostate cancer cells possibly by increasing the apparent affinity of AR for ligand and through elevation of androgen-binding capacity. All our mechanistic studies suggest that enhancement of AR transactivation by DDC is an androgen-dependent process. Even though the enzymatic products of DDC are not involved in the indirect activation of AR, the decarboxylation activity of this enzyme is necessary for coactivation function. Future studies will further explore the mechanism by which DDC modulates AR ligand binding ability and the role of DDC enzymatic activity in regulation of receptor function (discussed in **Chapter 6**).

4.6 References

1. Chen CD, Welsbie DS, Tran C, Baek SH, Chen R, Vessella R, et al. Molecular determinants of resistance to antiandrogen therapy. Nat Med 2004;10(1):33-9.

2. Evans RM. The steroid and thyroid hormone receptor superfamily. Science 1988;240(4854):889-95.

3. Roy AK, Chatterjee B. Androgen action. Crit Rev Eukaryot Gene Expr 1995;5(2):157-76.

4. Tsai MJ, O'Malley BW. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 1994;63:451-86.

5. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, et al. The nuclear receptor superfamily: the second decade. Cell 1995;83(6):835-9.

6. Warnmark A, Treuter E, Wright AP, Gustafsson JA. Activation functions 1 and 2 of nuclear receptors: molecular strategies for transcriptional activation. Mol Endocrinol 2003;17(10):1901-9.

7. He B, Wilson EM. Electrostatic modulation in steroid receptor recruitment of LXXLL and FXXLF motifs. Mol Cell Biol 2003;23(6):2135-50.

8. He B, Gampe RT, Jr., Hnat AT, Faggart JL, Minges JT, French FS, et al. Probing the functional link between androgen receptor coactivator and ligand-binding sites in prostate cancer and androgen insensitivity. J Biol Chem 2006;281(10):6648-63.

9. Fang Y, Fliss AE, Robins DM, Caplan AJ. Hsp90 regulates androgen receptor hormone binding affinity in vivo. J Biol Chem 1996;271(45):28697-702.

10. van de Wijngaart DJ, van Royen ME, Hersmus R, Pike AC, Houtsmuller AB, Jenster G, et al. Novel FXXFF and FXXMF motifs in androgen receptor cofactors

mediate high affinity and specific interactions with the ligand-binding domain. J Biol Chem 2006;281(28):19407-16.

11. Heinlein CA, Chang C. Androgen receptor (AR) coregulators: an overview. Endocr Rev 2002;23(2):175-200.

12. Horwitz KB, Jackson TA, Bain DL, Richer JK, Takimoto GS, Tung L. Nuclear receptor coactivators and corepressors. Mol Endocrinol 1996;10(10):1167-77.

13. Sadovsky Y, Webb P, Lopez G, Baxter JD, Fitzpatrick PM, Gizang-Ginsberg E, et al. Transcriptional activators differ in their responses to overexpression of TATA-box-binding protein. Mol Cell Biol 1995;15(3):1554-63.

14. Baniahmad A, Ha I, Reinberg D, Tsai S, Tsai MJ, O'Malley BW. Interaction of human thyroid hormone receptor beta with transcription factor TFIIB may mediate target gene derepression and activation by thyroid hormone. Proc Natl Acad Sci U S A 1993;90(19):8832-6.

15. McEwan IJ, Gustafsson J. Interaction of the human androgen receptor transactivation function with the general transcription factor TFIIF. Proc Natl Acad Sci U S A 1997;94(16):8485-90.

16. Lee DK, Duan HO, Chang C. From androgen receptor to the general transcription factor TFIIH. Identification of cdk activating kinase (CAK) as an androgen receptor NH(2)-terminal associated coactivator. J Biol Chem 2000;275(13):9308-13.

17. Yeh S, Chang C. Cloning and characterization of a specific coactivator, ARA70, for the androgen receptor in human prostate cells. Proc Natl Acad Sci U S A 1996;93(11):5517-21.

18. Hu YC, Yeh S, Yeh SD, Sampson ER, Huang J, Li P, et al. Functional domain and motif analyses of androgen receptor coregulator ARA70 and its differential expression in prostate cancer. J Biol Chem 2004;279(32):33438-46.

19. Truica CI, Byers S, Gelmann EP. Beta-catenin affects androgen receptor transcriptional activity and ligand specificity. Cancer Res 2000;60(17):4709-13.

20. Lu ML, Schneider MC, Zheng Y, Zhang X, Richie JP. Caveolin-1 interacts with androgen receptor. A positive modulator of androgen receptor mediated transactivation. J Biol Chem 2001;276(16):13442-51.

21. Zhu MY, Juorio AV. Aromatic L-amino acid decarboxylase: biological characterization and functional role. Gen Pharmacol 1995;26(4):681-96.

22. Lee SP, Xie Z, Varghese G, Nguyen T, O'Dowd BF, George SR. Oligomerization of dopamine and serotonin receptors. Neuropsychopharmacology 2000;23(4 Suppl):S32-40.

23. Konradi C, Cole RL, Heckers S, Hyman SE. Amphetamine regulates gene expression in rat striatum via transcription factor CREB. J Neurosci 1994;14(9):5623-34.

24. Yan Z, Feng J, Fienberg AA, Greengard P. D(2) dopamine receptors induce mitogen-activated protein kinase and cAMP response element-binding protein phosphorylation in neurons. Proc Natl Acad Sci U S A 1999;96(20):11607-12.

25. Sidhu A, Niznik HB. Coupling of dopamine receptor subtypes to multiple and diverse G proteins. Int J Dev Neurosci 2000;18(7):669-77.

26. Zanassi P, Paolillo M, Feliciello A, Avvedimento EV, Gallo V, Schinelli S. cAMP-dependent protein kinase induces cAMP-response element-binding protein

phosphorylation via an intracellular calcium release/ERK-dependent pathway in striatal neurons. J Biol Chem 2001;276(15):11487-95.

27. Brami-Cherrier K, Valjent E, Garcia M, Pages C, Hipskind RA, Caboche J. Dopamine induces a PI3-kinase-independent activation of Akt in striatal neurons: a new route to cAMP response element-binding protein phosphorylation. J Neurosci 2002;22(20):8911-21.

28. Raymond JR, Mukhin YV, Gelasco A, Turner J, Collinsworth G, Gettys TW, et al. Multiplicity of mechanisms of serotonin receptor signal transduction. Pharmacol Ther 2001;92(2-3):179-212.

29. Ueda T, Mawji NR, Bruchovsky N, Sadar MD. Ligand-independent activation of the androgen receptor by interleukin-6 and the role of steroid receptor coactivator-1 in prostate cancer cells. J Biol Chem 2002;277(41):38087-94.

30. Culig Z, Hobisch A, Cronauer MV, Radmayr C, Trapman J, Hittmair A, et al. Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. Cancer Res 1994;54(20):5474-8.

31. Nazareth LV, Weigel NL. Activation of the human androgen receptor through a protein kinase A signaling pathway. J Biol Chem 1996;271(33):19900-7.

32. Sadar MD. Androgen-independent induction of prostate-specific antigen gene expression via cross-talk between the androgen receptor and protein kinase A signal transduction pathways. J Biol Chem 1999;274(12):7777-83.

33. Lee SO, Lou W, Hou M, Onate SA, Gao AC. Interleukin-4 enhances prostate-specific antigen expression by activation of the androgen receptor and Akt pathway. Oncogene 2003;22(39):7981-8.

34. Heinlein CA, Chang C. Androgen receptor in prostate cancer. Endocr Rev 2004;25(2):276-308.

35. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2002;2(7):489-501.

36. Smith CL, Conneely OM, O'Malley BW. Modulation of the ligand-independent activation of the human estrogen receptor by hormone and antihormone. Proc Natl Acad Sci U S A 1993;90(13):6120-4.

37. Mani SK, Allen JM, Clark JH, Blaustein JD, O'Malley BW. Convergent pathways for steroid hormone- and neurotransmitter-induced rat sexual behavior. Science 1994;265(5176):1246-9.

38. Apostolakis EM, Garai J, Fox C, Smith CL, Watson SJ, Clark JH, et al. Dopaminergic regulation of progesterone receptors: brain D5 dopamine receptors mediate induction of lordosis by D1-like agonists in rats. J Neurosci 1996;16(16):4823-34.

39. Power RF, Mani SK, Codina J, Conneely OM, O'Malley BW. Dopaminergic and ligand-independent activation of steroid hormone receptors. Science 1991;254(5038):1636-9.

40. Mani S. Ligand-independent activation of progestin receptors in sexual receptivity. Horm Behav 2001;40(2):183-90.

41. Burkhard P, Dominici P, Borri-Voltattorni C, Jansonius JN, Malashkevich VN. Structural insight into Parkinson's disease treatment from drug-inhibited DOPA decarboxylase. Nat Struct Biol 2001;8(11):963-7.

42. Poupon A, Jebai F, Labesse G, Gros F, Thibault J, Mornon JP, et al. Structure modelling and site-directed mutagenesis of the rat aromatic L-amino acid pyridoxal 5'-phosphate-dependent decarboxylase: a functional study. Proteins 1999;37(2):191-203.

43. Nishino J, Hayashi H, Ishii S, Kagamiyama H. An anomalous side reaction of the Lys303 mutant aromatic L-amino acid decarboxylase unravels the role of the residue in catalysis. J Biochem (Tokyo) 1997;121(3):604-11.

44. Ishii S, Mizuguchi H, Nishino J, Hayashi H, Kagamiyama H. Functionally important residues of aromatic L-amino acid decarboxylase probed by sequence alignment and site-directed mutagenesis. J Biochem (Tokyo) 1996;120(2):369-76.

45. Dominici P, Moore PS, Castellani S, Bertoldi M, Voltattorni CB. Mutation of cysteine 111 in Dopa decarboxylase leads to active site perturbation. Protein Sci 1997;6(9):2007-15.

46. Bertoldi M, Castellani S, Bori Voltattorni C. Mutation of residues in the coenzyme binding pocket of Dopa decarboxylase. Effects on catalytic properties. Eur J Biochem 2001;268(10):2975-81.

47. Ray MR, Wafa LA, Cheng H, Snoek R, Fazli L, Gleave M, et al. Cyclin G-associated kinase: a novel androgen receptor-interacting transcriptional coactivator that is overexpressed in hormone refractory prostate cancer. Int J Cancer 2006;118(5):1108-19.

48. Huang W, Shostak Y, Tarr P, Sawyers C, Carey M. Cooperative assembly of androgen receptor into a nucleoprotein complex that regulates the prostate-specific antigen enhancer. J Biol Chem 1999;274(36):25756-68.

49. Snoek R, Bruchovsky N, Kasper S, Matusik RJ, Gleave M, Sato N, et al. Differential transactivation by the androgen receptor in prostate cancer cells. Prostate 1998;36(4):256-63.

50. Schreiber E, Matthias P, Muller MM, Schaffner W. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res 1989;17(15):6419.

51. Yu D, Jia WW, Gleave ME, Nelson CC, Rennie PS. Prostate-tumor targeting of gene expression by lentiviral vectors containing elements of the probasin promoter. Prostate 2004;59(4):370-82.

52. Gleave M, Tolcher A, Miyake H, Nelson C, Brown B, Beraldi E, et al. Progression to androgen independence is delayed by adjuvant treatment with antisense Bcl-2 oligodeoxynucleotides after castration in the LNCaP prostate tumor model. Clin Cancer Res 1999;5(10):2891-8.

53. Koshimura K, Tanaka J, Murakami Y, Kato Y. Effects of dopamine and L-DOPA on survival of PC12 cells. J Neurosci Res 2000;62(1):112-9.

54. Sato N, Gleave ME, Bruchovsky N, Rennie PS, Beraldi E, Sullivan LD. A metastatic and androgen-sensitive human prostate cancer model using intraprostatic inoculation of LNCaP cells in SCID mice. Cancer Res 1997;57(8):1584-9.

55. Wilm M, Shevchenko A, Houthaeve T, Breit S, Schweigerer L, Fotsis T, et al. Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. Nature 1996;379(6564):466-9.

56. Wafa LA, Cheng H, Rao MA, Nelson CC, Cox M, Hirst M, et al. Isolation and identification of L-dopa decarboxylase as a protein that binds to and enhances transcriptional activity of the androgen receptor using the repressed transactivator yeast two-hybrid system. Biochem J 2003;375(Pt 2):373-83.

57. Abdul M, Anezinis PE, Logothetis CJ, Hoosein NM. Growth inhibition of human prostatic carcinoma cell lines by serotonin antagonists. Anticancer Res 1994;14(3A):1215-20.

58. Abdul M, Logothetis CJ, Hoosein NM. Growth-inhibitory effects of serotonin uptake inhibitors on human prostate carcinoma cell lines. J Urol 1995;154(1):247-50.

59. Dizeyi N, Bjartell A, Nilsson E, Hansson J, Gadaleanu V, Cross N, et al. Expression of serotonin receptors and role of serotonin in human prostate cancer tissue and cell lines. Prostate 2004;59(3):328-36.

60. Siddiqui EJ, Shabbir M, Mikhailidis DP, Thompson CS, Mumtaz FH. The role of serotonin (5-hydroxytryptamine1A and 1B) receptors in prostate cancer cell proliferation. J Urol 2006;176(4 Pt 1):1648-53.

61. Jongsma J, Oomen MH, Noordzij MA, Romijn JC, van Der Kwast TH, Schroder FH, et al. Androgen-independent growth is induced by neuropeptides in human prostate cancer cell lines. Prostate 2000;42(1):34-44.

62. Li XM, Juorio AV, Boulton AA. NSD-1015 alters the gene expression of aromatic L-amino acid decarboxylase in rat PC12 pheochromocytoma cells. Neurochem Res 1993;18(8):915-9.

63. He B, Lee LW, Minges JT, Wilson EM. Dependence of selective gene activation on the androgen receptor NH2- and COOH-terminal interaction. J Biol Chem 2002;277(28):25631-9.

64. Miyamoto H, Rahman M, Takatera H, Kang HY, Yeh S, Chang HC, et al. A dominant-negative mutant of androgen receptor coregulator ARA54 inhibits androgen receptor-mediated prostate cancer growth. J Biol Chem 2002;277(7):4609-17.

65. Taneja SS, Ha S, Swenson NK, Torra IP, Rome S, Walden PD, et al. ART-27, an androgen receptor coactivator regulated in prostate development and cancer. J Biol Chem 2004;279(14):13944-52.

66. Cleutjens KB, van der Korput HA, van Eekelen CC, van Rooij HC, Faber PW, Trapman J. An androgen response element in a far upstream enhancer region is essential for high, androgen-regulated activity of the prostate-specific antigen promoter. Mol Endocrinol 1997;11(2):148-61.

67. Taplin ME, Balk SP. Androgen receptor: a key molecule in the progression of prostate cancer to hormone independence. J Cell Biochem 2004;91(3):483-90.

68. Rennie PS, Nelson CC. Epigenetic mechanisms for progression of prostate cancer. Cancer Metastasis Rev 1998;17(4):401-9.

69. Belanger A, Brochu M, Cliche J. Levels of plasma steroid glucuronides in intact and castrated men with prostatic cancer. J Clin Endocrinol Metab 1986;62(5):812-5.

70. Mizokami A, Koh E, Fujita H, Maeda Y, Egawa M, Koshida K, et al. The adrenal androgen androstenediol is present in prostate cancer tissue after androgen deprivation therapy and activates mutated androgen receptor. Cancer Res 2004;64(2):765-71.

71. Simental JA, Sar M, Lane MV, French FS, Wilson EM. Transcriptional activation and nuclear targeting signals of the human androgen receptor. J Biol Chem 1991;266(1):510-8.

72. Darimont BD, Wagner RL, Apriletti JW, Stallcup MR, Kushner PJ, Baxter JD, et al. Structure and specificity of nuclear receptor-coactivator interactions. Genes Dev 1998;12(21):3343-56.

73. Feng W, Ribeiro RC, Wagner RL, Nguyen H, Apriletti JW, Fletterick RJ, et al. Hormone-dependent coactivator binding to a hydrophobic cleft on nuclear receptors. Science 1998;280(5370):1747-9.

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

75. Pike AC, Brzozowski AM, Hubbard RE, Bonn T, Thorsell AG, Engstrom O, et al. Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. Embo J 1999;18(17):4608-18.

76. Pike AC, Brzozowski AM, Hubbard RE. A structural biologist's view of the oestrogen receptor. J Steroid Biochem Mol Biol 2000;74(5):261-8.

77. Fang H, Tong W, Branham WS, Moland CL, Dial SL, Hong H, et al. Study of 202 natural, synthetic, and environmental chemicals for binding to the androgen receptor. Chem Res Toxicol 2003;16(10):1338-58.

78. Portigal CL, Cowell SP, Fedoruk MN, Butler CM, Rennie PS, Nelson CC. Polychlorinated biphenyls interfere with androgen-induced transcriptional activation and hormone binding. Toxicol Appl Pharmacol 2002;179(3):185-94.

79. Ratajczak T, Ward BK, Minchin RF. Immunophilin chaperones in steroid receptor signalling. Curr Top Med Chem 2003;3(12):1348-57.

80. Thin TH, Kim E, Yeh S, Sampson ER, Chen YT, Collins LL, et al. Mutations in the helix 3 region of the androgen receptor abrogate ARA70 promotion of 17beta-estradiol-induced androgen receptor transactivation. J Biol Chem 2002;277(39):36499-508.

81. Bair SR, Mellon SH. Deletion of the mouse P450c17 gene causes early embryonic lethality. Mol Cell Biol 2004;24(12):5383-90.

82. Wu JD, Haugk K, Woodke L, Nelson P, Coleman I, Plymate SR. Interaction of IGF signaling and the androgen receptor in prostate cancer progression. J Cell Biochem 2006;99(2):392-401.

83. John RA. Pyridoxal phosphate-dependent enzymes. Biochim Biophys Acta 1995;1248(2):81-96.

84. Jansonius JN. Structure, evolution and action of vitamin B6-dependent enzymes. Curr Opin Struct Biol 1998;8(6):759-69.

85. Heery DM, Kalkhoven E, Hoare S, Parker MG. A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature 1997;387(6634):733-6.

86. Greschik H, Moras D. Structure-activity relationship of nuclear receptor-ligand interactions. Curr Top Med Chem 2003;3(14):1573-99.

87. Dubbink HJ, Hersmus R, Verma CS, van der Korput HA, Berrevoets CA, van Tol J, et al. Distinct recognition modes of FXXLF and LXXLL motifs by the androgen receptor. Mol Endocrinol 2004;18(9):2132-50.

88. Steketee K, Berrevoets CA, Dubbink HJ, Doesburg P, Hersmus R, Brinkmann AO, et al. Amino acids 3-13 and amino acids in and flanking the 23FxxLF27 motif modulate the interaction between the N-terminal and ligand-binding domain of the androgen receptor. Eur J Biochem 2002;269(23):5780-91.

89. He B, Kemppainen JA, Wilson EM. FXXLF and WXXLF sequences mediate the NH2-terminal interaction with the ligand binding domain of the androgen receptor. J Biol Chem 2000;275(30):22986-94.

90. Huang N, vom Baur E, Garnier JM, Lerouge T, Vonesch JL, Lutz Y, et al. Two distinct nuclear receptor interaction domains in NSD1, a novel SET protein that exhibits characteristics of both corepressors and coactivators. Embo J 1998;17(12):3398-412.

CHAPTER 5. EXPRESSION PROFILE ANALYSIS OF L-DOPA DECARBOXYLASE AS A NEUROENDOCRINE MARKER IN NEO-ADJUVANT HORMONE TREATED VERSUS VARYING GLEASON GRADE PROSTATE TUMOURS

A version of this chapter has been published.

Wafa LA, Palmer J, Fazli L, Hurtado-Coll A, Bell R, Nelson CC, Gleave ME, Cox ME, Rennie PS. (2006). Comprehensive expression analysis of L-dopa decarboxylase and established neuroendocrine markers in neo-adjuvant hormone treated versus varying Gleason grade prostate tumours. Hum. Pathol. 38 (1): 161-170.

Dr. Michael Cox aided in the design of studies and provided critical comments for this work. Dr. Martin Gleave's laboratory constructed the tissue microarrays from prostate cancer patient specimens. Dr. Ladan Fazli (pathologist) performed the evaluation of scoring for stained tissue specimens. Dr. Hurtado-Coll carried out the digitized immunohistochemical scoring and Robert Bell aided with statistical analysis. Dr. Jodie Palmer performed the immunohistochemical staining for bombesin.

5.1 Introduction

Prostate cancer is the most commonly diagnosed non-skin cancer and a leading cause of cancer-death in North American men. Since prostate growth and development is dependent on androgens, hormone withdrawal therapy is frequently used to treat advanced prostate cancer (1). Although this is initially effective in controlling advanced disease, progression to an androgen independence (AI) is the usual outcome (discussed in **Section 1.2** of **Chapter 1**) (2). Growing evidence indicates that prostatic adenocarcinoma cells with neuroendocrine (NE) characteristics may play an important role in the mechanism of resistance to androgen ablation therapy and prostate cancer progression (3). The epithelial cell layer of the normal human prostate gland is made up

of specialized exocrine epithelial cells that secrete components into the seminal fluid, basal cells that replenish the supply of exocrine cells and occasional NE cells (discussed in **Section 1.1** of **Chapter 1**). These NE cells are thought to be involved in the regulation of cell growth, differentiation and exocrine secretions of the prostate through release of neurosecretory products. Although the origin of nascent prostatic NE cells appears to be from the neural crest, enrichment of NE cells in prostate tumours has been suggested to occur *via* NE differentiation of malignant epithelium (4). These cells have been suggested to promote growth of surrounding adenocarcinoma cells through secretion of mitogenic NE factors such as calcitonin, serotonin and bombesin, as well as production of the cytokine, interleukin 8 (5-7).

Immunohistochemical studies of the NE marker chromogranin A (CgA) have indicated that all prostate cancers contain NE cells (8) and about 10 % contain extensive multifocal NE features (3). However, the prognostic significance of NE differentiation remains controversial. Some studies suggest a significant correlation between NE differentiation and survival or pathologic stage, while others argue the lack of an association between Gleason grade and NE cell population density (9-12). Also, the exact nature of the relationship between hormone therapy and NE differentiation has not been clearly elucidated, as in several reports changes in NE cell population was not significantly affected by treatment (11, 13, 14).

A variety of mechanisms have also been proposed to account for continued or amplified AR signalling following androgen withdrawal therapy. These include gain of function AR mutations, amplification and overexpression of AR (15, 16), as well as ligand-independent (or ligand-reduced) activation of AR through the combination of cell

signalling pathways and altered expression of AR coregulator proteins (17, 18). Recent studies have demonstrated that the NE factor, bombesin (BB), can activate AR in the absence of and with low castrate levels of androgen in prostate cancer cells (19). Although the role of AR in epithelial-derived prostate cancer cells is well established, the expression status and role of AR in NE cells of prostate tumours are poorly defined. Some reports suggest the absence of AR in NE cells, while others observe a subpopulation of AR-positive tumour cells with a NE phenotype (20-22).

Clearly, several controversies concerning the contribution of NE differentiation in prostate cancer remains unanswered, necessitating the need for further systematic investigation of NE cells in disease progression. Questions such as the manner in which hormonal therapy affects NE differentiation status, the occurrence of correlations between Gleason grade and NE differentiation, and the status of AR expression in prostate cancer NE cells need to be answered. The current study directly addresses these issues using high throughput tissue microarray (TMA) methodology to monitor NE differentiation in patients receiving neo-adjuvant hormone therapy (NHT) prior to radical prostatectomy and in those with varying Gleason grade tumours. Due to the heterogeneity of NE cells, multiple NE markers were studied (CgA and BB), including a newly identified marker, L-dopa decarboxylase (DDC), which we have previously shown to directly bind to AR and enhance its transcriptional activity (**Chapters 3** and **4**) (23). Several cancers are characterized by extremely high levels of DDC activity and this enzyme has also been reported to be an excellent tumour marker for NE cell differentiation (24, 25). In order to determine whether DDC is also a NE marker for prostate cancer, we analyzed its expression profile in NHT-treated and varying Gleason

grade prostate tumour specimens. Because of the coactivation function of DDC on AR, a modified dual immunofluorescence protocol was also used to quantitatively determine the AR status of cells expressing DDC in prostate tumours.

5.2 Materials and Methods

Construction of Human Prostate Tissue Microarrays: NHT and Gleason Grade

Tissue microarrays (TMAs) were prepared using archival formalin-fixed and paraffin-embedded human prostate tumour specimens, as described by Kononen *et al.* (26). To minimize misrepresentation due to tumour heterogeneity, 3 (NHT TMA) or 4 cores (Gleason grade TMA), each 0.6 mm in diameter, were obtained from a total of 112 patients for the NHT TMA and 84 non-treated patients for the Gleason grade TMA. The NHT TMA was constructed from hormone naïve, NHT-treated and androgen-independent metastatic specimens (one set of 3 cores was obtained from a lung carcinoma patient). Naïve and NHT sample cores were obtained from radical prostatectomy specimens, while the AI tissues were isolated from rapid autopsy specimens of 13 men that succumbed to prostate cancer. Samples were segregated into 5 groups; non-treated (0 M, 21 patients), less than 3 months NHT (<3 M, 21 patients), 3 to 6 months NHT (3-6 M, 28 patients), greater than 6 months NHT (>6 M, 28 patients) and androgen-independent (AI, 13 patients) tumours. The donor paraffin blocks with corresponding H&E reference slides were analyzed by a pathologist to identify the most representative sections with cancer prior to core extraction. Three cores/specimen were arrayed for a total of 336 cores/NHT TMA.

Gleason grade TMAs were prepared from 84 non-treated patients, with 4 cores/patient, using the same procedure previously used for the NHT TMA. Benign prostatic hyperplasia (BPH) specimens were obtained from trans-urethral prostate resections (TURP) of patients with normal prostate specific antigen (PSA) and digital rectal exams. Cancerous specimens were from radical prostatectomies and TURP. H&E-stained slides for each block were reviewed by a pathologist to select areas with correct Gleason grades. Specimens were divided into one BPH and four Gleason groups. These include; benign BPH (7 cases), GL2 (14 cases), GL3 (21 cases), GL4 (21 cases) and GL5 (21 cases). Four cores/sample were arrayed for a total of 336 cores/Gleason grade TMA.

Immunohistochemical (IHC) Analysis

Mounted tissues on microarrays were de-paraffinized using xylene, re-hydrated with ethanol washes and permeabilized in a solution of 0.02 % triton-X 100. TMA sections were then steamed in citrate buffer (pH 6), cooled for 30 minutes, washed in PBS (3 minute washes) and incubated in 3 % hydrogen peroxide for 10 minutes to promote antigen retrieval. After blocking (3 % BSA in PBS) for 30 minutes, TMA slides were incubated at 4 °C overnight with the following antibodies; mouse monoclonal antibody against chromogranin A, CgA (clone 2H10, Chemicon Inc., used at 1:800), and rabbit polyclonal antibodies to L-dopa decarboxylase, DDC (Protos Biotech, used at 1:600), bombesin, BB (Immunostar, used at 1:2000) and the androgen receptor (Affinity BioReagents, used at 1:200). The slides were washed with PBS and developed using the LSAB+ kit detection system (Dako Corporation). Nova Red chromogen was applied and Hematoxylin counterstaining was performed (Vector Laboratories Inc.). Two TMA

slides were also used for negative controls, as described above, in which primary antibodies were replaced with the corresponding species normal IgG. Color reactions were not observed on negative control slides (data not shown).

Dual Immunofluorescence Analysis

Three adjacently sectioned NHT TMAs were prepared for primary antibody incubation, as described above for standard immunohistochemical staining. Subsequently, a modified version of conventional cytoimmunofluorescence, utilizing streptavidin-conjugated fluorophores and biotinylated-secondary antibodies (Vector Laboratories Inc.), was optimized to allow for dual labelling of human tissue on TMAs. The following 3 combinations were used; rabbit anti-DDC (Protos Biotech, 1:200 dilution) with mouse anti-CgA (Chemicon Inc., 1:300 dilution), rabbit anti-AR (Affinity BioReagents, 1:100 dilution) with mouse anti-CgA (Chemicon Inc., 1:300), and mouse anti-AR (Biogenex, 1:100 dilution) with rabbit anti-DDC (Protos Biotech, 1:200 dilution). In all cases the primary antibody incubations were carried out simultaneously at 4 °C overnight in a humidified chamber. After washing with PBS, slides were incubated with corresponding AR biotinylated-secondary antibodies (45 minutes). PBS washed samples were incubated with Texas Red-conjugated streptavidin (30 minutes). Slides were extensively washed with PBS and blocked sequentially with excess avidin followed by excess biotin to prevent possible binding of the biotinylated-secondary antibody for AR in the first step with streptavidin-conjugated fluorophore of the second step, used for detection of CgA and DDC. Slides were then washed with PBS and incubated with the appropriate biotinylated-secondary antibody for CgA and DDC

primary antibody detection, followed by fluorescein-conjugated streptavidin treatment. Specimens were counterstained for 10 minutes with Hoechst (Bisbenzimide) nuclear stain (Sigma, at 0.5 μg/mL). TMA slides were then visualized by fluorescent microscopy (Zeiss) with Northern Eclipse imaging software. All cells that showed DDC or CgA staining were visually counted and AR expression status was determined. The above protocol was also used for quantitation of DDC and CgA co-expression.

Evaluation of Staining

Immunohistochemically stained NHT and Gleason grade TMA slides were scanned using a BLISS scanner system from Bacus Laboratories Inc. (27). Digital images were evaluated independently by a pathologist (visual score) and using digitized IHC scoring (Image Pro Plus, IPP, by Media Cybernetics Inc.) at 200 × magnification. Visual staining of NE markers (CgA, DDC and BB) was based on the number of positive tumour cells (intensity of stain was high for all NE positive cells). Each core on the TMAs was classified using the following scoring system; 0 = no NE marker positive tumour cells, 1 = 1 - 5 NE marker positive tumour cells, score 2 = 6 - 10 NE marker positive tumour cells and score 3 = 11 or more NE marker positive tumour cells. Alternatively, visual AR scoring was based on stain intensity, which varied from no staining (score 0) to low (score 1), moderate (score 2) and high intensity staining (score 3). Digitized IPP scoring, ranging from 0 to 100, was based on positive staining area/core. Software was optimized to detect epithelial-derived adenocarcinoma cells for both NE markers and AR.

Statistical Evaluation

To determine significant differences between all comparison groups on NHT and Gleason grade TMAs, the Wilcoxon rank test was utilized since the distribution of scores was non-normal. This was the primary means of analysis when comparing patient mean, minimum, maximum and sum staining scores. Patient mean scores (±SEM) were calculated using scores from all patients, including those that stained negative for NE markers. Fisher's exact test was used to determine significant changes in the proportion of NE marker positive staining cores, independent of patient, and percentage of NE marker positive patients in each comparison group. All calculated p-values (R Statistical Environment, version R2.1.1, www.r-project.org) were two-sided and those less than 0.05 were considered statistically significant.

5.3 Results

DDC is a Marker of NE Differentiation in NHT-Treated Prostate Cancer Patients

To determine whether DDC is expressed in prostate tumour NE cells, tissue microarrays were constructed based on NHT treatment and stained with a polyclonal antibody to DDC (**Figure 5.1**). Sparse cytosolic DDC staining was observed in individual and clusters of adenocarcinoma cells, a characteristic pattern of expression for NE markers. DDC positive cells were present in normal and hormone treated patient cores (**Figures 5.1A** and **5.1C**). An adjacently sectioned NHT TMA was subsequently stained with a monoclonal antibody to CgA (**Figures 5.1B** and **5.1D**). The majority of NE positive cores contained cells that co-expressed both proteins in the cytoplasm, suggesting that DDC is a NE marker in prostate tumours. An exact measure of cellular

co-staining was then performed using dual immunofluorescence on the same NHT TMA (see below).

NE Differentiation in NHT Patients is Dependent on Duration of Therapy

To elucidate how hormone ablation therapy temporally influences the NE status of prostate tumours, TMAs constructed from specimens of untreated and NHT treated patients were used to comprehensively monitor the expression profile of multiple NE markers simultaneously. Adjacent NHT TMAs were stained with antibodies to DDC, CgA and AR. DDC and CgA staining was scored based on the number of NE marker positive tumour cells (**Figure 5.2**), whereas AR scoring was based on intensity of staining (data not shown). Clinicopathologic data (**Table 5.1**) for patients receiving NHT revealed that the median serum PSA levels and age for patients were not significantly different ($p > 0.05$). Also, two thirds of patients had a Gleason grade sum of 6 or 7 and a range of 4-9. Of 111 prostate cancer patients, 26 (23 %) had DDC positive adenocarcinoma cells and 30 (27 %) had CgA positive staining. All patients specimens stained positive for nuclear AR.

Visual and IPP software scoring methods revealed the same trend in DDC and CgA expression during treatment (**Table 5.2**). For DDC expression, the difference in visual patient mean score staining was significant ($p < 0.05$) between non-treated and > 6 months NHT-treated cancers, as well as with AI tumours, increasing ~ 3 and ~ 4 fold, respectively. IPP analysis showed the same significant differences with a ~ 3.5 and ~ 3 fold increase between untreated versus > 6 months NHT and AI cancer, respectively. Patient mean score did not change significantly with < 3 months NHT and was therefore

also significantly lower when compared to the > 6 months NHT and AI tumours. Patient mean score for CgA staining followed the same trend as that of DDC, showing significant differences between non-treated and < 3 months NHT versus > 6 months NHT and AI disease. Since TMAs were spotted with 3 cores/patient, we were also able to analyze patient minimum, maximum and sum total scores for each patient. The same significant differences as patient mean scores were found using Wilcoxon rank tests based on these criteria (data not shown). Staining differences for DDC and CgA between 3-6 months NHT and longer treatment or AI were statistically indistinguishable (**Table 5.2**). Fisher's exact test analysis using the proportion of positive cores, independent of patients, also confirmed the positive correlation between duration of NHT and extent of NE differentiation (data not shown). In addition, the proportion of NE marker positive patients, increased significantly from approximately 15 % to 40 % for the non-treated patients versus those treated with > 6 months NHT ($p < 0.05$).

The expression profile of a third NE marker, bombesin (BB), was also visually analyzed using NHT TMAs and showed a similar expression profile to CgA and DDC with respect to patient mean score and proportion of positive cores (**Table 5.2**). Although the population of DDC, CgA and BB positive cells increased significantly with NHT and in AI tumours, no such differences could be observed for expression of AR. All patient cores were AR positive and patient mean score staining remained constant between non-treated and AI tumours (**Table 5.2**). Overall, these results indicate that the duration of NHT dictates the extent of NE differentiation in prostate tumours.

NE Differentiation does not Correlate with Gleason Grade

Having observed evidence of increased NE differentiation with NHT, we next asked whether the expression of an established (CgA) and a novel (DDC) NE marker also change with tumour grade. Gleason grade TMA patients were not treated with NHT (**Table 5.3**). Two adjacently sectioned Gleason grade TMAs stained with antibodies to DDC and CgA were scored visually and using IPP software. Of 84 patients, 15 (18 %) contained DDC positive NE-phenotype adenocarcinoma cells and 20 (24 %) were CgA positive. Visual patient mean score staining was significantly different only between the highest GL5 grade and BPH, which showed no DDC or CgA immunoreactivity (**Table 5.4**). While we observed a general increase in NE marker staining with increasing tumour grade (GL2 to GL5), variances between samples rendered these differences statistically indistinguishable. IPP patient mean scores confirmed the same trends for DDC and CgA. Since four cores per patient were spotted on each TMA, patient minimum, maximum and sum total staining scores were also analyzed, revealing the same absence of significant differences as patient mean scores (data not shown). In addition, the proportion of DDC and CgA positive patients did not change with grade of tumour. Fisher's exact test using the proportion of NE marker positive cores confirmed the lack of a correlation between tumour grade and NE differentiation (data not shown). These data suggest that Gleason grade does not determine the extent of NE differentiation in prostate tumours.

Co-Expression of NE Markers and AR in Prostate Tumours

To address the question of whether prostate NE cells express the androgen receptor, dual immunofluorescence was carried using combinations of antibodies for

DDC, CgA and AR. Three adjacently sectioned NHT TMAs were co-stained for DDC/CgA, DDC/AR or CgA/AR. DDC and CgA were co-expressed exclusively in the cytoplasm (data not shown). Analysis of all DDC/CgA positive cores on the NHT TMA revealed that the vast majority (~ 84 %) of DDC positive cells also expressed CgA. The expression of AR in DDC and CgA positive cells was more variable (**Figure 5.3**, **Table 5.5**). Overall about 21 % of DDC and 30 % of CgA positive cells were clearly AR negative, while ~ 54 % of DDC and ~ 56 % of CgA expressing cells were positive for AR staining. Localization of the receptor in these AR positive NE adenocarcinoma cells was not always nuclear and included exclusively cytosolic or general cytosolic/nuclear staining. In cases where expression of AR was not obvious (due to uneven tissue sectioning), cells were designated as undefined. From the above observations we can conclude that AR is expressed in more than half of the DDC and CgA positive NE phenotype tumour cells.

Patient groupings, months (number of patients)	0 M (n = 21)	<3 M (n = 21)	3-6 M (n = 28)	>6 M (n = 28)	[a]AI (n = 13)
Median age in yrs (range)	60 (50-73)	60 (49-71)	61 (47-71)	63 (48-73)	N/A
Median PSA at pre-surgery, ng/dL (range)	6.0 (1.2-12)	7.1 (1.5-23)	8.0 (0.7-97)	8.2 (1.9-22)	N/A
Clinical stage (%)					
T1a, b, c	12 (57)	9 (43)	10 (35.7)	8 (28.6)	0
T2a, b	6 (29)	9 (43)	10 (35.7)	15 (53.6)	0
T3a, b	0	0	1 (3.6)	2 (7.1)	0
Undefined	3 (14)	3 (14)	7 (25)	3 (10.7)	0
Distant metastasis	0	0	0	0	13 (100)
Pathological stage (%)					
T1a, b, c	0	0	0	0	
T2a, b	16 (76)	14 (67)	19 (68)	19 (68)	
T3a, b	3 (14)	4 (19)	7 (25)	9 (32)	N/A
Undefined	2 (10)	3 (14)	2 (7)	0	
Distant metastasis	0	0	0	0	
Gleason grade sum (%)					
2-6	14 (66.7)	12 (57.1)	12 (42.9)	8 (28.5)	
7	5 (23.8)	6 (28.6)	10 (35.7)	7 (25)	N/A
> 7	1 (4.8)	1 (4.8)	2 (7.1)	12 (42.9)	
Undefined	1 (4.8)	2 (9.5)	4 (14.3)	1 (3.6)	

Table 5.1 - NHT Tissue Microarray Patient Profiles. The NHT TMA was constructed with hormone naïve (0 M), NHT-treated (<3 M to >6 M) and androgen-independent (AI) metastatic specimens from a total of 112 patients (one specimen was obtained from a lung carcinoma patient). Naïve and NHT sample cores were obtained from radical prostatectomy specimens and AI tissues were isolated from rapid autopsy specimens. Three cores from each specimen were arrayed for a total of 336 cores for the NHT TMA

[a]Clinicopathologic data not available for AI patients.

Marker	Treatment	Patient mean score ± SEM Visual	IPP
DDC	0 M (n = 21)	0.21 ± 0.11	3.41 ± 2.32
	<3 M (n = 21)	0.19 ± 0.13	3.68 ± 2.58
	3-6M (n = 28)	0.32 ± 0.15	5.75 ± 1.76
	>6M (n = 28)	0.71 ± 0.22[a, b]	12.57 ± 3.12[a, b]
	AI (n = 13)	0.93 ± 0.38[a, b]	10.81 ± 4.38[a, b]
CgA	0 M (n = 21)	0.19 ± 0.11	4.09 ± 2.04
	<3 M (n = 21)	0.33 ± 0.13	4.85 ± 2.99
	3-6M (n = 28)	0.57 ± 0.19	6.19 ± 2.51
	>6M (n = 28)	0.89 ± 0.23[a, b]	9.27 ± 2.02[a, b]
	AI (n = 13)	1.00 ± 0.39[a, b]	9.91 ± 3.64[a, b]
BB	0M (n = 21)	0.37 ± 0.08	
	<3 M (n = 21)	0.44 ± 0.08	
	3-6M (n = 28)	0.95 ± 0.13[a, b]	N/A
	>6M (n = 28)	1.18 ± 0.12[a, b]	
	AI (n = 13)	1.11 ± 0.18[a, b]	
AR	0 M (n = 21)	1.78 ± 0.13	68.5 ± 5.29
	AI (n = 13)	1.99 ± 0.06	64.5 ± 5.13

Table 5.2 - NE Marker and AR Expression with NHT. Patient mean score comparisons between treatment groups were performed using the Wilcoxon rank test; [a]$p < 0.05$ as compared to 0 M NHT and [b]$p < 0.05$ as compared to < 3 M NHT (patient minimum, maximum and sum scores were also analyzed and showed the same trend as patient mean scores).

Patient groupings (number of patients)	BPH (n = 7)	GL2 (n = 14)	GL3 (n = 21)	GL4 (n = 21)	GL5 (n = 21)
Median age in yrs (range)[a]	N/A	57 (46-64)	63 (46-70)	64 (54-75)	69 (56-89)
Median PSA at pre-surgery, ng/dL (range)[a]	N/A	6.5 (3.2-18)	6.0 (0.8-14)	6.1 (1.8-11)	29(12-47)
Clinical stage (%)					
T1a, b, c		9 (64)	12 (57)	9 (43)	0
T2a, b	N/A	0	4 (19)	5 (24)	0
T3a, b		0	0	1 (5)	0
Undefined		5 (36)	5(24)	6 (28)	21(100)
Pathological stage (%)					
T1a, b, c		0	0	0	0
T2a, b		13 (93)	18 (86)	17 (81)	6 (28.5)
T3a, b	N/A	0	3 (14)	3 (14)	4 (19)
TURP		0	0	0	9 (43)
Undefined		1(7)	0	1(5)	2 (9.5)

Table 5.3 - Gleason Grade Tissue Microarray Patient Profiles. Gleason grade TMAs were prepared from 84 non-treated patients. Benign prostatic hyperplasia (BPH) specimens were obtained from trans-urethral prostate resections (TURP) of patients with normal PSA and cancerous specimens were from radical prostatectomies and TURP. Four cores from each specimen were arrayed for a total of 336 cores for the Gleason grade TMA.

[a]Based on available values for known patients (overall 42 of 77 for age and 56 of 77 for PSA; information not available for BPH).

Marker	Patient Group	Patient mean score ± SEM Visual	IPP
DDC	BPH (n = 7)	0 ± 0	0 ± 0
	GL2 (n = 14)	0.07 ± 0.06	0.11 ± 0.11
	GL3 (n = 21)	0.08 ± 0.05	0.43 ± 0.31
	GL4 (n = 21)	0.11 ± 0.07	0.46 ± 0.31
	GL5 (n = 21)	0.45 ± 0.21[a]	1.92 ± 1.14[a]
CgA	BPH (n = 7)	0 ± 0	0 ± 0
	GL2 (n = 14)	0.14 ± 0.11	1.63 ± 1.16
	GL3 (n = 21)	0.11 ± 0.04	1.13 ± 0.44
	GL4 (n = 21)	0.14 ± 0.06	1.71 ± 0.69
	GL5 (n = 21)	0.46 ± 0.19[a]	4.11 ± 1.96[a]

Table 5.4 - NE Marker Expression with Gleason Grade. Patient mean score comparisons between groups were performed using the Wilcoxon rank test; [a]$p < 0.05$ as compared to BPH (patient minimum, maximum and sum scores were also analyzed and showed the same trend as patient mean scores).

	DDC Positive[a]	CgA Positive[a]
AR-negative (%)	21	30
AR-positive (%)	54	56
Nuclear (%)	34	37
Cytosolic (%)	10	13
Nuclear and cytosolic (%)	10	6
Undefined (%)	25	14

Table 5.5 - Prostate Adenocarcinoma Cells Co-Express AR and NE Markers. Three adjacently sectioned NHT TMAs were co-stained for DDC/CgA, DDC/AR or CgA/AR using dual immunofluorescence analysis. The expression status of AR was determined for all cells that showed DDC or CgA staining. Percentage of co-expression was calculated using all the NE marker positive cells on NHT tissue microarray.

[a]Approximately 84 % of DDC positive cells expressed CgA.

5.4 Figures

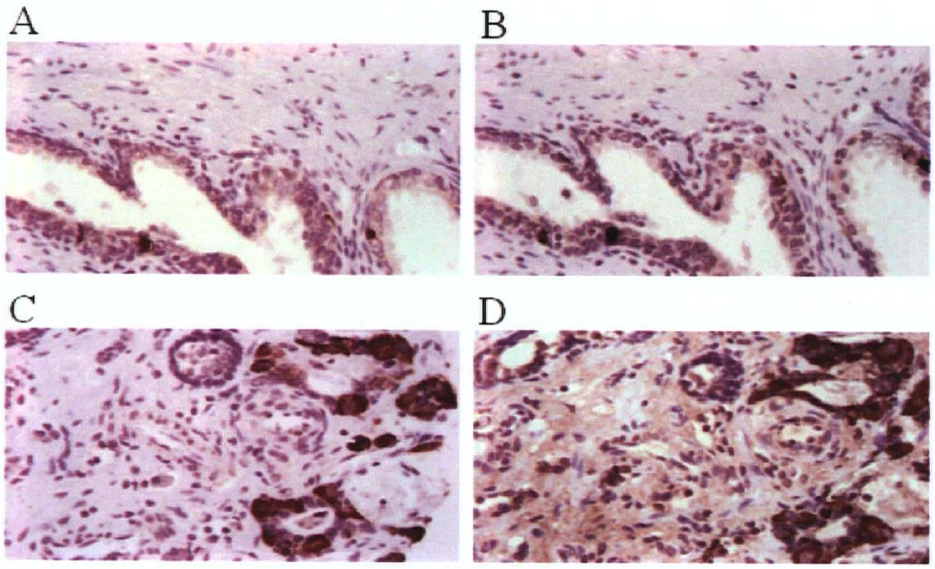

Figure 5.1 - DDC is Co-Expressed with CgA in NE Cells of the Prostate. Adjacently sectioned NHT tissue microarrays were prepared for standard immunohistochemical staining. A) and (B) are adjacent sections from a normal prostate core. C) and (D) are adjacent sections from the prostate core of a patient treated with NHT for 3-6 months. Sections (A) and (C) were stained with a polyclonal anti-DDC antibody. Sections (B) and (D) were stained with a monoclonal antibody to CgA for identification of NE cells (original magnification × 400).

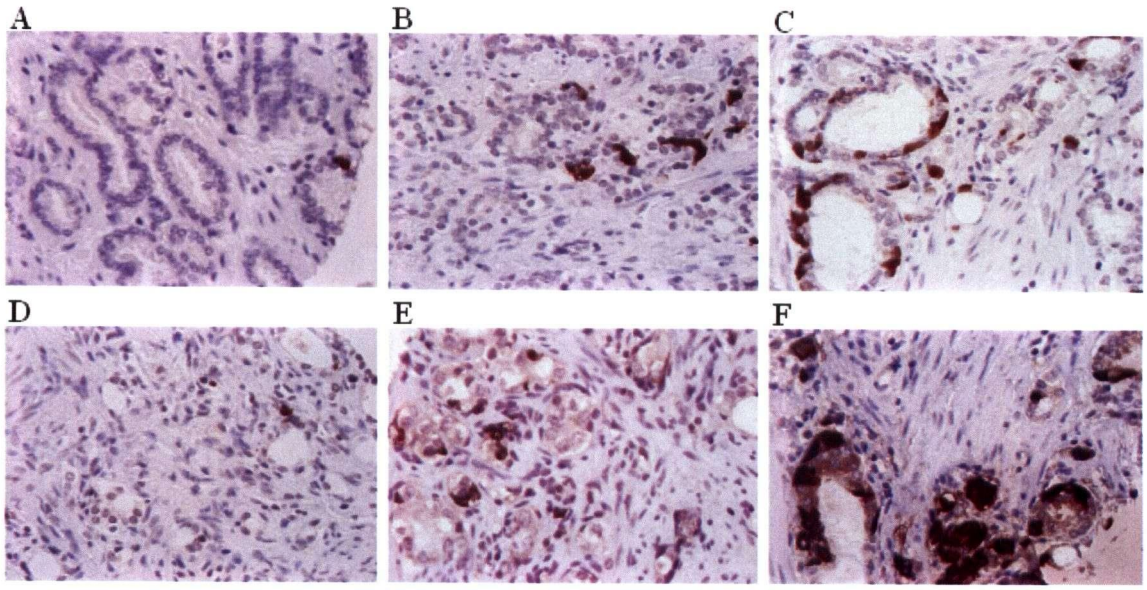

Figure 5.2 - Immunohistochemical Analysis of DDC and CgA Using the NHT Tissue Microarray. Sections (A), (B) and (C) represent DDC positive staining visual scores of 1, 2 and 3, respectively. Sections (D), (E) and (F) represent CgA positive staining visual scores of 1, 2 and 3, respectively. For both DDC and CgA, scoring was based on the number of NE marker positive adenocarcinoma cells; score 1 = 1 – 5 positive cells/core, score 2 = 6 – 10 positive cells/core, and score 3 = 11 or more positive cells/core (original magnification × 400).

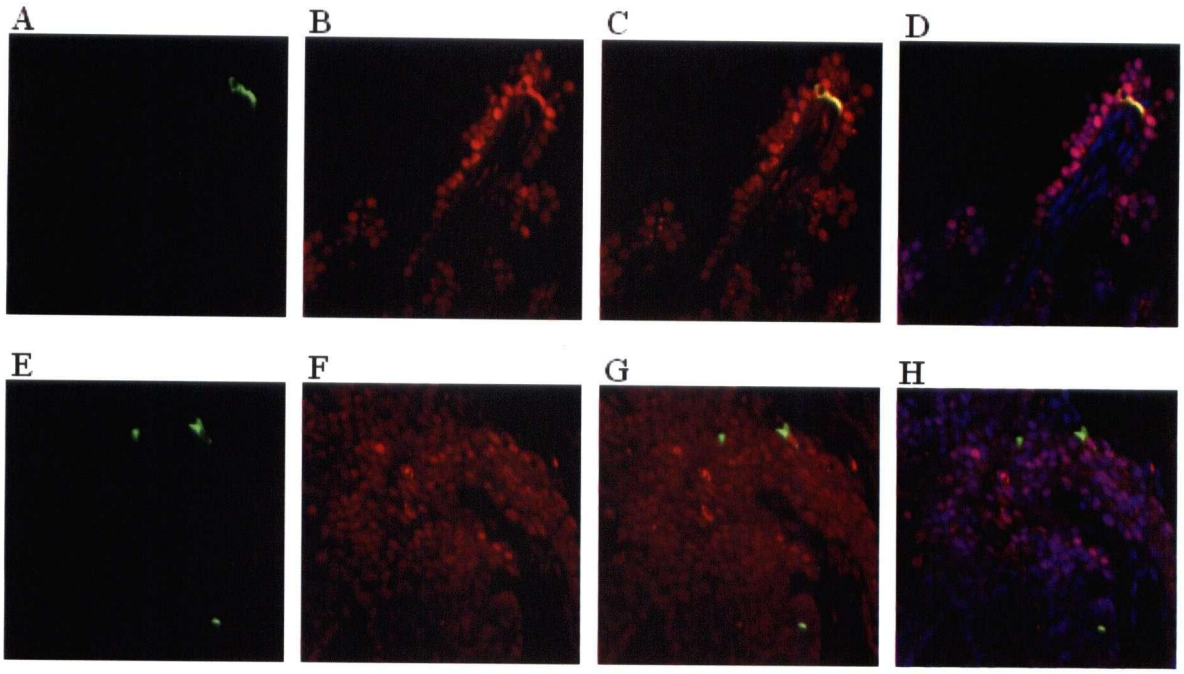

Figure 5.3 - DDC and CgA are Co-Expressed with AR in Prostate Adenocarcinoma Cells. NHT TMAs were stained with specific combinations of AR and DDC or CgA antibodies for dual immunofluorescence analysis. Sections (A), (B), (C) and (D) represent DDC, cytosolic AR, DDC/AR overlay and DDC/AR/hoechst nuclear stain overlay, respectively. Sections (E), (F), (G) and (H) represent CgA, nuclear AR, CgA/AR overlay and CgA/AR/hoechst nuclear stain overlay, respectively. Green = fluorescein, red = Texas-Red and blue = hoechst (original magnification × 400).

5.5 Discussion

A number of NE markers have been identified in prostate cancer, including CgA, synaptophysin, neuron-specific enolase, serotonin and BB, all of which have served as a means of identifying NE cells in human prostate tumours (28). In addition to these established markers, we found DDC to be a marker of NE differentiation in human prostate cancers treated with NHT (**Figure 5.1**). DDC is a well-characterized enzyme responsible for the synthesis of dopamine and serotonin in neural tissues and has also been identified as a NE marker in cancers such as neuroblastoma and lung carcinoma (24, 25). However, the study of DDC expression in prostate tumours has been limited to a single mouse model in which DDC enzymatic activity/expression was shown to increase in prostates of NE-cell-transformed transgenic mice and the co-expression of DDC with CgA was qualitatively identified in a small subset of prostate cancer specimens (29, 30). In the present study, we measured the extent of DDC co-expression with CgA in NHT treated human prostate TMAs using both immunohistochemical and dual immunofluorescence analysis. The high incidence (84 %) of DDC co-expression with CgA indicates that it is as reliable a NE marker as other known ones in neoplastic human prostate cancer cells that have been subjected to androgen ablation therapy. The presence of only DDC or CgA positive cells is not unexpected since diverse heterogeneity in the co-expression of multiple NE markers is common in prostate tumours, suggesting that there are actually several sub-populations of NE cells, each with their own distinct milieu of NE factors (21).

Although some studies have shown an increase in the population of CgA positive cells with NHT, there have also been contradictory reports that NE differentiation is not

affected by hormonal therapy (11, 13, 14, 31). The need for a systematic approach to determine any specific temporal associations between NE differentiation and hormonal therapy was addressed in the present study. We monitored NE differentiation with NHT that varied in duration from < 3 months up to 9 months (> 6 months group) and included prostate tumours that had progressed to AI. For all 3 NE markers analyzed (CgA, BB and DDC), the patient mean score was significantly elevated after 6 months of hormone-ablation and in AI tumours but not after short-term (< 3 months) NHT therapy, confirming that > 6 months of NHT is necessary for a significant increase in NE differentiation (**Table 5.2**). Also, an increase in the proportion of NE marker positive patients with > 6 months of treatment ($p < 0.05$) suggests that NHT actually results in more patients undergoing prostate tumour NE differentiation. Overall, these results indicate that the induction of NE differentiation is specifically dependent on duration of hormone therapy.

The lack of a correlation between NE differentiation and Gleason grade (**Table 5.4**) is in clear contrast to the substantial increase observed with > 6 months of NHT and in AI tumours. One possible explanation for the difference is that hormone ablation therapy may selectively induce apoptosis of androgen-responsive tumour cells resulting in an increased population density for NE cells (32). Alternatively, NHT may also force androgen-dependent adenocarcinoma cells to adopt other modes of survival. Increased acquisition of NE characteristics by this cell population upon hormone withdrawal then provides a source of mitogenic NE factors to enhance survival of the tumour. These NE factors can promote growth of prostate cancer cells and even modulate AR activity, independent of ligand or as in the case of BB, synergize with castrate levels of androgen

to activate AR (19, 33, 34). This hypothesis is supported by our observation of an increased population of BB positive cells with NHT (**Table 5.2**).

Since the Gleason grade TMA patients examined in this study were not subjected to NHT, an increase in NE cell population is not required for increased tumour aggressiveness. These patients possess sufficient androgen levels to allow for robust growth of AR-positive tumour cells. Gleason grade may still predict the capacity of tumours to undergo NE differentiation induced upon hormone ablation therapy. In our NHT TMA cohort, the majority of cases with elevated NE differentiation occurred in patients subjected to long term (> 6 months) NHT, which were diagnosed with high Gleason grade sums (> 7). However, in this cohort, patients with Gleason grade sums of > 7 were disproportionately divided into the longer (> 6 months) NHT group (12 of 28), with a lower proportion (1 of 21) segregated into the < 3 months and 0 NHT groups (**Table 5.1**). Due to this skewed categorization (based on clinical status) of NHT patients, the predictive potential of pre-treatment Gleason grade sum for the extent of NE differentiation induced upon hormonal therapy could not be determined.

Several studies have reported that AR is not expressed in prostate NE cells (20, 21), while others indicate the presence of intermediate cells expressing both CgA and exocrine markers, such as basal cell-specific cytokeratins, PSA and AR (22, 35, 36). Consistent with the latter studies (22, 35), we found a subpopulation of NE marker positive cells (~ 55 %) that expressed AR (**Figure 5.3** and **Table 5.5**). Co-expression of AR with NE markers supports the theory that increased NE differentiation seen in prostatic malignancies arises from trans-differentiation of conventional adenocarcinoma

cells into a NE phenotype, possibly in response to microenvironment changes of hormone levels and growth factors (31, 33).

More recently, use of small interfering RNA directed against AR (37), has demonstrated that the receptor actually represses NE trans-differentiation in prostate cancer cells. Hence, during androgen-deprivation therapy *in vivo*, the gradual loss of AR or its activity in transforming tumour cells may remove the repressive effect on trans-differentiation, allowing an increase in expression of NE markers such as DDC, CgA and BB. In AR expressing NE phenotype cells, it is possible that during trans-differentiation the receptor is still active and important in maintaining growth of the adenocarcinoma cell through utilization of residual amounts of androgen or ligand-independent mechanisms (17, 18). The observation that close to one-third (34 % DDC and 37 % CgA) of NE phenotype cells expressed AR exclusively in the nucleus suggests that it remains active. Additionally, during trans-differentiation increased expression of DDC, a protein we have previously shown to interact with and enhance AR transactivation (**Chapter 3**), could activate AR under androgen-limiting conditions and lead to sustained AR-dependent tumour cell growth (discussed in detail in **Chapter 6, Section 6.3**). NE cells that lack AR may be of neural crest origin or the end stage result of NE trans-differentiation where the receptor is completely lost and cells are sustained in an androgen-independent manner, secreting NE factors to maintain their own growth and that of other tumour cells.

In conclusion, the current study examined the controversial nature of NE differentiation in prostate cancer. We were able to demonstrate an association between NE differentiation and duration of NHT by analyzing the expression profile of DDC and

established NE markers (CgA and BB). However, NE differentiation and DDC expression were not significantly correlated with Gleason grade of prostate tumours. Dual immunofluorescence analysis allowed determination of AR status in NE cells and provided evidence for the NE trans-differentiation model in which AR activity may play a crucial role in maintaining growth of the developing NE cell. The role of DDC as a NE marker that is up-regulated with hormonal therapy of prostate tumours and the importance of its co-expression with AR in NE-phenotype adenocarcinoma cells is discussed in further detail in **Chapter 6**.

5.6 References

1. Albertsen PC, Hanley JA, Fine J. 20-year outcomes following conservative management of clinically localized prostate cancer. Jama 2005;293(17):2095-101.

2. Klotz L. Hormone therapy for patients with prostate carcinoma. Cancer 2000;88(12 Suppl):3009-14.

3. di Sant'Agnese PA, Cockett AT. Neuroendocrine differentiation in prostatic malignancy. Cancer 1996;78(2):357-61.

4. Abrahamsson PA. Neuroendocrine differentiation and hormone-refractory prostate cancer. Prostate Suppl 1996;6:3-8.

5. Theodorescu D, Broder SR, Boyd JC, Mills SE, Frierson HF, Jr. Cathepsin D and chromogranin A as predictors of long term disease specific survival after radical prostatectomy for localized carcinoma of the prostate. Cancer 1997;80(11):2109-19.

6. Weinstein MH, Partin AW, Veltri RW, Epstein JI. Neuroendocrine differentiation in prostate cancer: enhanced prediction of progression after radical prostatectomy. Hum Pathol 1996;27(7):683-7.

7. Huang J, Yao JL, Zhang L, Bourne PA, Quinn AM, di Sant'Agnese PA, et al. Differential expression of interleukin-8 and its receptors in the neuroendocrine and non-neuroendocrine compartments of prostate cancer. Am J Pathol 2005;166(6):1807-15.

8. Abrahamsson PA, Wadstrom LB, Alumets J, Falkmer S, Grimelius L. Peptide-hormone- and serotonin-immunoreactive cells in normal and hyperplastic prostate glands. Pathol Res Pract 1986;181(6):675-83.

9. Segawa N, Mori I, Utsunomiya H, Nakamura M, Nakamura Y, Shan L, et al. Prognostic significance of neuroendocrine differentiation, proliferation activity and androgen receptor expression in prostate cancer. Pathol Int 2001;51(6):452-9.

10. Pruneri G, Galli S, Rossi RS, Roncalli M, Coggi G, Ferrari A, et al. Chromogranin A and B and secretogranin II in prostatic adenocarcinomas: neuroendocrine expression in patients untreated and treated with androgen deprivation therapy. Prostate 1998;34(2):113-20.

11. Hirano D, Okada Y, Minei S, Takimoto Y, Nemoto N. Neuroendocrine differentiation in hormone refractory prostate cancer following androgen deprivation therapy. Eur Urol 2004;45(5):586-92; discussion 592.

12. Puccetti L, Supuran CT, Fasolo PP, Conti E, Sebastiani G, Lacquaniti S, et al. Skewing towards neuroendocrine phenotype in high grade or high stage androgen-responsive primary prostate cancer. Eur Urol 2005;48(2):215-21; Discussion 221-3.

13. Abrahamsson PA, Falkmer S, Falt K, Grimelius L. The course of neuroendocrine differentiation in prostatic carcinomas. An immunohistochemical study testing chromogranin A as an "endocrine marker". Pathol Res Pract 1989;185(3):373-80.

14. Kollermann J, Helpap B. Neuroendocrine differentiation and short-term neoadjuvant hormonal treatment of prostatic carcinoma with special regard to tumor regression. Eur Urol 2001;40(3):313-7.

15. Culig Z, Klocker H, Bartsch G, Steiner H, Hobisch A. Androgen receptors in prostate cancer. J Urol 2003;170(4 Pt 1):1363-9.

16. Ford OH, 3rd, Gregory CW, Kim D, Smitherman AB, Mohler JL. Androgen receptor gene amplification and protein expression in recurrent prostate cancer. J Urol 2003;170(5):1817-21.

17. Rennie PS, Nelson CC. Epigenetic mechanisms for progression of prostate cancer. Cancer Metastasis Rev 1998;17(4):401-9.

18. Ueda T, Mawji NR, Bruchovsky N, Sadar MD. Ligand-independent activation of the androgen receptor by interleukin-6 and the role of steroid receptor coactivator-1 in prostate cancer cells. J Biol Chem 2002;277(41):38087-94.

19. Dai J, Shen R, Sumitomo M, Stahl R, Navarro D, Gershengorn MC, et al. Synergistic activation of the androgen receptor by bombesin and low-dose androgen. Clin Cancer Res 2002;8(7):2399-405.

20. Bonkhoff H, Stein U, Remberger K. Androgen receptor status in endocrine-paracrine cell types of the normal, hyperplastic, and neoplastic human prostate. Virchows Arch A Pathol Anat Histopathol 1993;423(4):291-4.

21. Abrahamsson PA. Neuroendocrine cells in tumour growth of the prostate. Endocr Relat Cancer 1999;6(4):503-19.

22. Nakada SY, di Sant'Agnese PA, Moynes RA, Hiipakka RA, Liao S, Cockett AT, et al. The androgen receptor status of neuroendocrine cells in human benign and malignant prostatic tissue. Cancer Res 1993;53(9):1967-70.

23. Wafa LA, Cheng H, Rao MA, Nelson CC, Cox M, Hirst M, et al. Isolation and identification of L-dopa decarboxylase as a protein that binds to and enhances transcriptional activity of the androgen receptor using the repressed transactivator yeast two-hybrid system. Biochem J 2003;375(Pt 2):373-83.

24. Gazdar AF, Helman LJ, Israel MA, Russell EK, Linnoila RI, Mulshine JL, et al. Expression of neuroendocrine cell markers L-dopa decarboxylase, chromogranin A, and dense core granules in human tumors of endocrine and nonendocrine origin. Cancer Res 1988;48(14):4078-82.

25. Watanabe H, Imaizumi M, Ojika T, Abe T, Hida T, Kato K. Evaluation of biological characteristics of lung cancer by the human 28 kDa vitamin D-dependent calcium binding protein, calbindin-D28k. Jpn J Clin Oncol 1994;24(3):121-7.

26. Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 1998;4(7):844-7.

27. Ng TL, Gown AM, Barry TS, Cheang MC, Chan AK, Turbin DA, et al. Nuclear beta-catenin in mesenchymal tumors. Mod Pathol 2005;18(1):68-74.

28. Rodriguez R, Pozuelo JM, Martin R, Henriques-Gil N, Haro M, Arriazu R, et al. Presence of neuroendocrine cells during postnatal development in rat prostate: Immunohistochemical, molecular, and quantitative study. Prostate 2003;57(2):176-85.

29. Hu Y, Ippolito JE, Garabedian EM, Humphrey PA, Gordon JI. Molecular characterization of a metastatic neuroendocrine cell cancer arising in the prostates of transgenic mice. J Biol Chem 2002;277(46):44462-74.

30. Ippolito JE, Xu J, Jain S, Moulder K, Mennerick S, Crowley JR, et al. An integrated functional genomics and metabolomics approach for defining poor prognosis in human neuroendocrine cancers. Proc Natl Acad Sci U S A 2005;102(28):9901-6.

31. Jiborn T, Bjartell A, Abrahamsson PA. Neuroendocrine differentiation in prostatic carcinoma during hormonal treatment. Urology 1998;51(4):585-9.

32. Bonkhoff H, Fixemer T. [Neuroendocrine differentiation in prostate cancer: an unrecognized and therapy resistant phenotype]. Pathologe 2005;26(6):453-60.

33. Cox ME, Deeble PD, Lakhani S, Parsons SJ. Acquisition of neuroendocrine characteristics by prostate tumor cells is reversible: implications for prostate cancer progression. Cancer Res 1999;59(15):3821-30.

34. Larran J, Salido M, Aparicio J, Lopez A, de Palacio ML, Vilches J. In vitro characterization of bombesin and calcitonin on the proliferation of PC3, DU 145 and LNCaP cancer prostatic cell lines. Int J Dev Biol 1996;Suppl 1:275S-276S.

35. Roudier MP, True LD, Vessella RL, Higano CS. Metastatic conventional prostatic adenocarcinoma with diffuse chromogranin A and androgen receptor positivity. J Clin Pathol 2004;57(3):321-3.

36. Rumpold H, Heinrich E, Untergasser G, Hermann M, Pfister G, Plas E, et al. Neuroendocrine differentiation of human prostatic primary epithelial cells in vitro. Prostate 2002;53(2):101-8.

37. Wright ME, Tsai MJ, Aebersold R. Androgen receptor represses the neuroendocrine transdifferentiation process in prostate cancer cells. Mol Endocrinol 2003;17(9):1726-37.

CHAPTER 6. GENERAL DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

It is generally accepted that AR plays a crucial role in prostate cancer progression to androgen independence. Coregulator proteins that interact with AR can have an effect on the degree of receptor transactivation and may also allow for receptor-specific responses. Hence, the main hypothesis investigated in this study proposes that unique coregulators of AR have a role in the inappropriate activation of the receptor during progression of disease to AI. A wide number of AR-interacting proteins have already been identified as a consequence of their binding with DBD and LBD of the receptor (**Section 1.4** of **Chapter 1** and **Chapter 2**). However, due to the common amino acid sequences of these domains among steroid receptors, most of the reported AR-binding proteins have been found to also interact with other family members. In contrast, protein interactions with the variable NTD of steroid receptors, may allow identification of unique receptor protein partners. In order to identify AR-specific protein interactions, a yeast two-hybrid approach was employed using the AR-NTD as bait to screen a prostate cancer cell line cDNA library.

Due to the intrinsic transactivation activity of the AR N-terminal domain, the conventional yeast-two hybrid assay (GAL4 system) could not be used to identify AR-NTD-interacting proteins, as has been done previously for the DBD and LBD of AR. Instead, the repressed transactivator (RTA) yeast two-hybrid system, specifically designed for transactivator bait proteins, was utilized (**Chapter 2**). In addition to allowing identification of AR protein partners for the NTD, known to be a more important coregulator interaction site in AR versus other steroid receptors, another advantage of using the RTA system includes bypassing the need to fuse the screened

cDNA library with a heterologous DBD that binds onto the promoter of the reporter rather than the DBD fused AR-NTD. Although this approach has been previously used in conventional systems to also circumvent the problem of AR-NTD intrinsic transcriptional activity, fusion of library proteins to the DBD of GAL4 can affect its DNA binding ability, which ultimately introduces another level of variation during screening. Conversely, a disadvantage of the RTA system seems to be that it is not as stringent a yeast two-hybrid assay as the conventional GAL4 system. This is apparent from the large number of positive clones detected during screening (**Chapter 2**), which makes confirmation of interactions a more laborious process. Notably, the more established conventional GAL4 system can be used to identify protein interactions with deletion fragments of the AR N-terminal region that do not possess transactivation activity, but this will prevent detection of potentially important interactions that can be identified with the full-length functional AR-NTD.

Overall, use of the RTA yeast-two hybrid assay did allow for the identification of several AR-NTD-interacting proteins that were confirmable *in vitro* using GST-pulldown assays and in prostate cancer cells with co-immunoprecipitation tests (**Chapter 2**). These novel AR protein partners, which were proposed to exert coregulatory effects on the receptor, included DDC, GAK, eEF1A, $TAF_{II}250$, TRRAP/PAF400 and APC7. Of these, DDC, which was the most often detected clone from the RTA yeast two-hybrid assay, was studied in detail with respect to coregulation capacity and specificity for AR versus other steroid receptors (**Chapter 3**). The functional role of GAK, eEF1A, $TAF_{II}250$, TRRAP and APC7 as AR-interacting proteins will need to be assessed in the future (discussed below, **Section 6.1**). Although, DDC was initially identified as an AR-NTD-

interacting protein in the RTA system, this enzyme was found to interact more strongly with the AR-LBD (**Chapter 3**). Hence, it was of no surprise that DDC was able to robustly enhance both AR and GR transactivation in prostate cancer cells. However, DDC did exhibit a partially selective steroid receptor coactivation effect, since its enhancement of ERα transcriptional activity was almost negligible as compared to that of AR and GR. This observation may be explained by the fact that DDC is a cytosolic protein, which does not translocate to the nucleus, where ER is localized even in the absence of its cognate ligand (1). Alternatively, AR and GR become activated *via* binding ligand in the cytoplasm, prior to nuclear translocation.

Due to the strong AR coactivation function of DDC, this protein was studied further with respect to its mechanism of action on the receptor and expression profile in prostate cancer. DDC was shown to increase AR transcriptional activity *in vivo* (as measured by PSA production in LNCaP xenograft studies), sensitized AR activity to limiting concentrations of androgen and increased both the apparent affinity of the receptor for ligand, as well as its androgen-binding capacity (**Chapter 4**). Overall, these observations suggest that DDC is a physiologically relevant coactivator of AR that can enhance receptor activity under the androgen-deprived conditions often associated with AI prostate cancer. Moreover, the catalytic decarboxylation activity of DDC was found to be required for its enhancement of AR transactivation. However, the enzymatic products of DDC (dopamine and serotonin) were found to be not responsible for indirect activation of AR through their cell surface GPCR receptors, suggesting that DDC may act on the receptor itself or other unknown amino acid substrates. Expression profile analysis of DDC in prostate tumours revealed that this protein is a marker of NE

differentiation in prostate cancer (**Chapter 5**). NE cell density, and hence DDC expression, were shown to increase with NHT of prostate tumours in a time-dependent manner. Importantly, AR was found to be co-expressed with DDC in a subpopulation of NE-phenotype adenocarcinoma cells, in which DDC may enhance AR transactivation. Future studies that can be performed to further explore the mechanism of DDC coactivation function, and the implications of DDC as a NE marker that is up-regulated in hormone-treated prostate tumours, are discussed in **Sections 6.2** and **6.3**, respectively.

6.1 Coregulator Function of Novel AR-Binding Proteins

The identification of several previously unknown AR-interacting proteins using the RTA yeast two-hybrid system and the confirmation of these interactions *in vitro*, as well as in prostate cancer cell lines, can be used as a platform to further investigate several possible novel mechanisms by which AR activity may be regulated (**Chapter 2**). As discussed above, the role of DDC as an AR coregulator has been studied extensively (**Chapters 3, 4** and **5**) and can be examined further in the future (**Sections 6.2** and **6.3**). The other novel AR protein partners, including eEF1A, $TAF_{II}250$, TRRAP and APC7 need to be initially studied with respect to mapping the exact domain of interaction with AR and their effects on receptor transactivation or that of other steroid receptors. To accomplish this, full-length coding regions and truncations of these proteins will have to be cloned into appropriate expression vectors, which can be used for *in vitro* radiolabeling in GST-pulldowns and for transcriptional assays in prostate cancer cells. Both the overexpression of AR-interacting proteins with cloned constructs and the knock-down of endogenous protein expression, using small interference RNA (siRNA), can be

performed to ensure determination of a true coregulation effect. Although DDC was found to interact more strongly with the LBD than the NTD of AR (**Chapter 3**), it is possible that the full-length proteins of some other identified AR partners, may interact predominantly with the NTD of the receptor. This may also allow for AR-specific coregulation capacity. The coregulation effect of AR-binding proteins can then be confirmed *in vivo* with xenograft studies and their expression profiles can be examined in prostate cancer using tissue microarray methodology, as done previously for DDC (**Chapters 4** and **5**).

To determine how any newly identified AR coregulators modulate receptor transactivation, specific mechanistic studies will need to be performed based on the known functions of each protein. In the case of eEF1A, which has a well-established role as a protein translation elongation factor, its three isoforms, EF1A1, EF1A2 and the prostate cancer PTI-1 oncoprotein (2-4), can be cloned and tested individually for AR coregulation capacity. Mechanistic studies can then focus on the isoforms that have an impact on AR activity by exploiting the other known functions of eEF1A. This includes its ability to interact and co-translocate with zinc finger transcription factors to the nucleus in mammalian cells treated with the mitogen, EGF (5, 6). GFP-tagged AR and hcRed-tagged eEF1A fluorescent fusion proteins can be used to perform microscopy analysis. Nuclear translocation of AR and possibly eEF1A can be monitored simultaneously in prostate cancer cells in the absence and presence of R1881, combined with EGF treatment, which is known to activate AR (7). These experiments would determine whether EGF activation of AR involves its association with eEF1A. The interaction of eEF1A with filamentous actin (f-actin) has also been suggested to be

involved in the reorganization of the cytoskeleton (8, 9) and several AR coregulators (filamin, supervillin and gelsolin) are also f-actin binding proteins (10-13). Hence, it would be interesting to test the possible co-localization and interaction of eEF1A with these known AR coregulators using fluorescent microscopy and co-immunoprecipitation assays. These experiments would provide evidence for a role of eEF1A in cellular trafficking of AR along the cytoskeleton.

The Ser/Thr kinase, GAK, can be tested for its ability to use AR as a phosphorylation substrate by performing *in vitro* and *in vivo* kinase assays. For *in vitro* assays immunoprecipitated GAK from prostate cancer cells would be incubated with purified FLAG-tagged AR protein (**Chapter 4**) in the presence of [γ^{32}P]-ATP and phosphorylation would be assessed using phosphoimagery of an SDS-PAGE gel. For *in vivo* kinase assays, LNCaP cells, expressing endogenous AR and transfected with a GAK expression plasmid or control vector (GAK-specific siRNA as an alternative), would be cultured in the presence of [γ^{32}P]-ATP. Immunoprecipitated AR would then be analyzed by SDS-PAGE and autoradiography. Moreover, since GAK is known to interact with the hsp70 AR chaperone (14) and the classical AR coactivator CBP (15), co-immunoprecipitation assays can be performed using LNCaP cells to determine if GAK can also associate with these established AR binding proteins.

$TAF_{II}250$ possesses multiple enzymatic domains that include two Ser/Thr kinase domains, an acetylation domain, as well as ubiquitin activating domains (16). Hence, the mechanism by which $TAF_{II}250$ would modify AR transactivation may involve phosphorylation, acetylation or targeting of the receptor for ubiquitylation, all of which have been previously shown to regulate receptor activity (discussed in **Section 1.3** of

Chapter 1). Experiments to determine the role of $TAF_{II}250$ kinase activity in altering AR phosphorylation would be analogous to those performed for GAK (above). To test the possible acetylation of AR by $TAF_{II}250$, purified nuclei from prostate cancer cells would be used for immunoprecipitation of $TAF_{II}250$, which would then be incubated with FLAG-tagged AR protein (**Chapter 4**) in the presence of [^{14}C]-acetyl-Coenzyme A. This reaction mix would then be used for SDS-PAGE and autoradiography analysis. The possible role of the ubiquitin activating function of $TAF_{II}250$ in modulating AR activity or protein level can be examined by overexpressing (or knocking down by siRNA) $TAF_{II}250$ in LNCaP cells that have also been transfected with a V5-tagged ubiquitin expression construct. Cells would then be treated with the proteosomal inhibitor, MG132, and immunoprecipitated AR would be analyzed by Western blotting using an anti-V5 antibody to detect higher molecular weight ubiquitylated AR protein.

The transcriptional adapter protein, TRRAP, is known to associate with p/CAF and Tip60 (17, 18), both of which are coactivators of AR that increase receptor transactivation through their acetylation activities (19, 20). Thus, co-immunoprecipitation assays can be performed from purified LNCaP nuclei to determine if AR, TRRAP and Tip60 or p/CAF can co-exist as a multi-protein complex. This would indicate whether TRRAP can modulate AR activity by possibly recruiting known coactivators of the receptor. Finally, the APC7 subunit of the anaphase-promoting complex E3 ligase (21, 22) may regulate AR activity by targeting the receptor for degradation *via* the ubiquitin-proteosome pathway. AR does contain a D-box motif (^{386}RIKL^{389}) in its NTD, which is one of the target recognition sequences used by APC. Hence, in addition to testing the possible ubiquitylation of AR by APC as done above for

$TAF_{II}250$, mutational analysis (site-directed mutagenesis) of the AR D-box sequence may be used to confirm that APC can directly act on the receptor. Overall, the above mechanistic studies would provide solid evidence for the roles of several of the newly identified AR-interacting proteins in regulating AR activity.

6.2 Mechanism of DDC Coactivation Function

Although several mechanistic experiments have been performed in the current study to determine how DDC affects AR activity (**Chapter 4**), future studies will shed light on exactly how DDC affects the ligand binding ability of AR and why its enzymatic activity is required to enhance AR transactivation. To analyze the end-point effects of the observed increase in AR ligand binding affinity and maximum androgen binding capacity with DDC (**Figures 4.2** and **4.3**), ligand binding assays will be repeated in time course experiments. The effect of DDC on the rate of [^{3}H]-R1881 association and dissociation with AR can be determined by using a series of short (association; range 0 to 120 minutes) and longer (dissociation; range 0 to 6 hours) ligand incubation periods (23). For these experiments, cell-based ligand binding assays can be performed in the presence and absence of DDC, as done previously (**Chapter 4**). Importantly, ligand binding assays would be carried out with the mt DDC (Lys303Ile) that lacks coactivation function, which will determine if the catalytic activity of this enzyme is necessary for facilitation of AR ligand binding. The observed enhancement of AR ligand binding with DDC overexpression in the cytosol of HeLa-AR cells was also accompanied by an increase in the amount of androgen-bound nuclear receptor (**Figure 4.3**). This suggests that the effect of DDC on AR ligand binding ultimately results in more receptor

translocating into the nucleus. To confirm this hypothesis, cellular fractionation assays can be performed with or without DDC overexpression (±R1881) in HeLa-AR and prostate cancer cells, and the amount of nuclear AR can be quantitated by Western blot analysis. Moreover, GFP-tagged AR protein can be used in real-time fluorescent microscopy experiments to examine the movement of AR into the nucleus with DDC overexpression. These experiments would also be performed with mt DDC.

The observation that DDC requires its decarboxylation activity to enhance AR transactivation, independent of neurotransmitter products (dopamine and serotonin), and can directly interact with AR (**Chapter 4**), suggest the possibility that DDC may use the receptor as a substrate. Although in preliminary mass spectrometry studies, using purified FLAG-tagged AR from HeLa-AR cells (**Figure 4.11**), DDC was found not to decarboxylate the α-COOH group on the terminal Gln919 residue of the receptor, it may still be possible that DDC can use AR as a substrate in prostate cancer cells, such as PC3 cells, which show the greatest coactivation effect of DDC on the receptor (**Figures 4.1** and **4.8**). These cells may express other proteins or factors, absent in HeLa-AR cells, which may be required by DDC for its potential catalytic activity on AR. Hence, future studies can include the generation of PC3 cells that express FLAG-tagged AR constitutively and DDC under tetracycline regulation, as done previously for HeLa-AR cells (**Chapter 4, Section 4.2**). These cells can then be used for overexpression of DDC, purification of AR and subsequent LC-MS/MS analysis to identify any modifications of the receptor. Notably, rather than using AR as a direct substrate, the more likely scenario is that DDC decarboxylates other unknown amino acids into amines, which can then activate AR. The HeLa-AR/DDC stable cells generated in this study can still be used for

mass spectrometry analysis, to determine the indirect end-point effects of DDC on the status of established AR post-translational modifications. These include changes in phosphorylation, acetylation and sumoylation of the receptor, all of which have been shown to be involved in the regulation of AR transactivation (**Section 1.3** of **Chapter 1**). Site-directed mutagenesis can then be used to introduce changes to the AR amino acid sequence at any unique post-translationally modified site(s) and the impact of these changes on AR coactivation could be tested in transactivation assays.

The single LXXLL (amino acids 153-157) and two FXXLL motifs (amino acids 251-255 and 284-288) in the coding region of DDC maybe used to bind with the LBD of AR in cells (**Chapter 4**). To determine whether these motifs are functional, mutational analysis can be carried out prior to performing AR transcriptional assays with DDC overexpression. In addition, mammalian two-hybrid assays, where AR domains are fused to the GAL4-DBD, DDC is fused to the VP16 activation domain and a GAL4-regulated luciferase reporter is used to measure the strength of protein-protein interactions, can be used to determine if DDC motif mutations result in loss of binding to the receptor. Moreover, this assay can also be used to determine whether AR binds to the Lys303Ile mt DDC, despite its inability to coactivate receptor transactivation. Finally, to identify any other proteins that may be involved in the association of DDC with AR, a conventional yeast two-hybrid approach can be utilized, where DDC would be fused to GAL4-DBD and used as bait to screen a LNCaP cDNA library fused to the GAL4-AD. This could reveal the proteins related to subcellular structures or bridging proteins that may be involved in mechanistic pathways contributing to DDC's modulation of AR activity.

6.3 Role of DDC in Prostate Cancer

The coactivation function of DDC on AR may play an important role during prostate cancer progression. Elevated DDC expression in prostate cancer, as a result of increased NE differentiation, occurs in a specific time-dependent manner with neo-adjuvant hormonal therapy (**Chapter 5**). DDC is also co-expressed with AR in a subset of NE-phenotype adenocarcinoma cells. In addition, it has been previously shown that long-term androgen deprivation of AR-expressing LNCaP prostate cancer cells results in accumulation of NE characteristics *in vivo* (24), which can occur due to the active repression effect of AR on the NE-phenotype (25, 26). These studies combined with our observation that DDC activates AR transcription through an androgen-dependent mechanism, which involves sensitizing the receptor to limiting androgen concentrations and facilitating AR ligand-binding ability (**Chapter 4**), suggest that DDC may be important for the trans-differentiation process of AR-expressing luminal epithelial-derived adenocarcinoma cells into the NE-phenotype.

Encompassing the above observations, we propose a model to explain how the coactivation function of DDC on AR can influence NE trans-differentiation of prostate tumours during hormone ablation therapy (**Figure 6.1**). In the initial stages of disease, adenocarcinoma cells express AR, which is highly active and regulates androgen-dependent cell growth. Androgen withdrawal therapy temporarily reduces AR transcriptional activity, analogous to knocking down receptor function, which induces the NE-phenotype characterized by high-level expression of NE markers such as DDC. During NE trans-differentiation, under low androgen concentrations, intrinsic AR activity is minimal but DDC and other coactivators can interact with and sensitize the receptor to

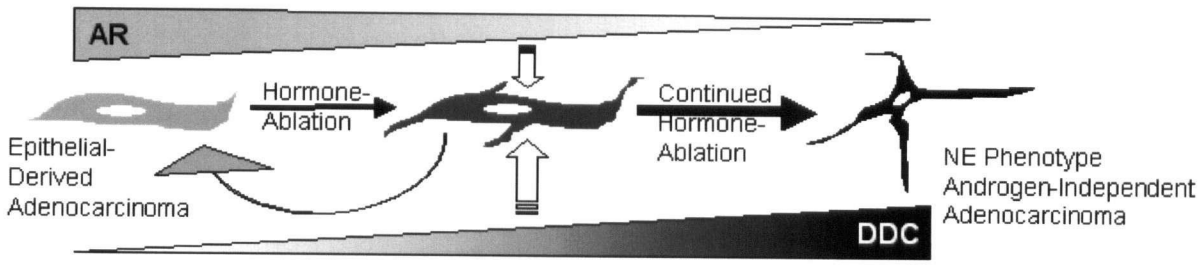

Figure 6.1 – Role of DDC-AR Coactivation in Neuroendocrine Trans-differentiation of Prostate Cancer Adenocarcinoma Cells. In the early stages of prostate cancer, AR exhibits robust activity and is susceptible to hormone ablation therapy. However, this treatment can induce the trans-differentiation of a population of luminal epithelial-derived adenocarcinoma cells into the NE-phenotype, which is characterized by increased expression of DDC and other NE markers. The resulting intermediate NE trans-differentiated cell can possess both luminal and NE characteristics, providing the environment in which DDC can coactivate AR. Prolonged hormonal therapy may lead to complete loss of AR activity/expression in some NE-phenotype cells and result in extremely high levels of DDC expression. These AR-independent NE cells can maintain their own growth through production of mitogenic factors and are resistant to hormone ablation treatment.

limiting levels of ligand to enhance its transactivation, leading back to more AR-dependent tumour cell growth. This process of trans-differentiation and de-differentiation may continue during the entire course of treatment as the patient succumbs to disease. Indeed, many studies have shown that AR is still expressed at high levels and is active, possibly *via* ligand-independent/reduced pathways, even in the most advanced stages of prostate cancer (27, 28).

It is also possible that sustained and extensive hormonal therapy may lead to total loss of AR expression and function, resulting in a completely NE cancer (**Figure 6.1**). Pure NE cell tumours of the prostate are rare but extremely aggressive (29, 30). At this stage adenocarcinoma cells are AR-independent and can maintain their own growth through secretion of mitogenic NE factors. Importantly, the enzymatic product of DDC, serotonin, has been well characterized as a NE-mitogenic factor produced by prostate tumours and increases with *in vitro* trans-differentiation of prostate cancer cells (31, 32). Hence, DDC may also drive NE trans-differentiation, independent of its coactivation function, by producing factors that contribute to the NE-phenotype. These enzymatic products can act in a paracrine or autocrine manner to promote growth of neighbouring adenocarcinoma cells or NE tumour cells, ultimately leading to further AR-independence. Overall, transition to the NE-phenotype may be a mechanism adopted by androgen-dependent carcinoma cells to allow their survival under androgen-deprived conditions.

Although a general consensus does not exist, one common mechanism for prostate cancer progression to AI has been suggested to include inappropriate continued activation of AR despite hormone ablation therapy (33, 34). Several mechanisms may explain hormone-refractory disease, including gain of function mutations for AR (28, 35,

36), amplification/overexpression of the receptor (27, 37, 38), and epigenetic mechanisms involving ligand-independent/reduced activation of AR *via* convergence of cell signalling pathways or altered activity/expression of receptor coregulators (33, 34, 39, 40) (discussed in **Section 1.5** of **Chapter 1**). Thus, in addition to hormonal therapy, new methods of inhibiting AR-mediated prostate cancer growth are required. Knocking down AR directly, using short hairpin RNA or antisense strategies, and targeting AR coactivators, using dominant negative approaches or mutagenesis of coactivator genes, have been used in the past (41-43). Notably, Cheng *et al.* have demonstrated that direct suppression of AR expression in the LNCaP xenograft model results in a dramatic inhibition of tumour growth and delay of progression to androgen independence (41). This suggests that tumour survival is dependent on AR and therefore targeting the receptor, with its associated coactivators, may serve as a useful therapeutic approach to treat prostate cancer. Since numerous coactivators have been shown to enhance AR activity and due to the existence of compensatory mechanisms (44), it is most likely necessary to simultaneously target many of these receptor-binding proteins in order to achieve maximal AR suppression.

The necessity of DDC decarboxylation activity for enhancement of AR transactivation (**Chapter 4**) provides an additional opportunity to target receptor function by simply utilizing existing clinically used enzymatic inhibitors (45). Moreover, targeting DDC, at two different stages of disease, may provide a unique means of repressing AR function and cancer progression in patients that exhibit extensive multifocal NE differentiation. First, DDC coactivation function can be targeted during the AR-dependent NE trans-differentiation phase, which may include early-stage up to AI

disease, depending on the patient, and secondly in rare cases of completely AR-independent disease. In the latter case, targeting DDC would not be due to its coactivation function on AR but because of its decarboxylation activity that can increase serotonin synthesis or other aromatic amino acid metabolic products, which may act as mitogenic factors. In addition, these mitogens may be important during the trans-differentiation process since some NE factors, such as bombesin, have been shown to indirectly activate AR under androgen-deprived conditions (46). The enzymatic inhibitor of DDC, carbidopa, has been recently used to treat mice bearing tumours from implanted prostate NE cancer cells, which were derived from a NE-cell-transformed transgenic mouse model. In combination with other drugs, carbidopa resulted in a 40 % reduction of tumour growth (45). It remains to be seen whether inhibition of DDC enzymatic activity is effective in reducing tumour progression in prostate cancer patients or for *in vivo* prostate tumour models that are not entirely NE-based but rather adopt a NE-phenotype with progression, as seen in actual disease. It is possible that patients diagnosed as having more extensive NE-differentiated tumours, which is correlated with development of more aggressive cancer, can be given more aggressive therapy that includes inhibitors targeting enzymatic activity of DDC.

6.4 Significance of Research

The AR plays a central role in progression of prostate cancer to androgen independence. Determining the factors that control AR-specific gene transcription and the degree of receptor activity are key to finding a means of delaying or preventing the emergence of the androgen-independent phenotype. The overall goal of the current study

was to identify novel proteins that interact with the unique AR-NTD and to determine their role in the regulation of receptor transactivation. In addition, the expression profiles of identified AR coregulator proteins were to be examined in prostate cancer progression. The results presented in this research project demonstrate the identification of several novel AR-interacting proteins, of which, DDC was studied further and shown to be a coactivator of AR *in vitro*, as well as *in vivo*. The coactivation effect of DDC was found to occur through an androgen-dependent mechanism and required its enzymatic activity. Moreover, DDC was found to be co-expressed with AR in NE-phenotype adenocarcinoma cells. The expression of DDC increased in hormone-treated prostate tumours and AI disease, suggesting that it may play an important role in prostate cancer progression through sustaining AR activation in an androgen-depleted environment. The clinical relevance of DDC as a coactivator of AR will be the subject of future studies, which may provide possible novel methods of treating prostate cancer.

6.5 References

1. Greene GL, Press MF. Structure and dynamics of the estrogen receptor. J Steroid Biochem 1986;24(1):1-7.

2. Thornton S, Anand N, Purcell D, Lee J. Not just for housekeeping: protein initiation and elongation factors in cell growth and tumorigenesis. J Mol Med 2003;81(9):536-48.

3. Shen R, Su ZZ, Olsson CA, Fisher PB. Identification of the human prostatic carcinoma oncogene PTI-1 by rapid expression cloning and differential RNA display. Proc Natl Acad Sci U S A 1995;92(15):6778-82.

4. Mansilla F, Hansen LL, Jakobsen H, Kjeldgaard NO, Clark BF, Knudsen CR. Deconstructing PTI-1: PTI-1 is a truncated, but not mutated, form of translation elongatin factor 1A1, eEF1A1. Biochim Biophys Acta 2005;1727(2):116-24.

5. Gangwani L, Mikrut M, Galcheva-Gargova Z, Davis RJ. Interaction of ZPR1 with translation elongation factor-1alpha in proliferating cells. J Cell Biol 1998;143(6):1471-84.

6. Galcheva-Gargova Z, Konstantinov KN, Wu IH, Klier FG, Barrett T, Davis RJ. Binding of zinc finger protein ZPR1 to the epidermal growth factor receptor. Science 1996;272(5269):1797-802.

7. Culig Z, Hobisch A, Cronauer MV, Radmayr C, Trapman J, Hittmair A, et al. Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. Cancer Res 1994;54(20):5474-8.

8. Edmonds BT, Wyckoff J, Yeung YG, Wang Y, Stanley ER, Jones J, et al. Elongation factor-1 alpha is an overexpressed actin binding protein in metastatic rat mammary adenocarcinoma. J Cell Sci 1996;109 (Pt 11):2705-14.

9. Ejiri S. Moonlighting functions of polypeptide elongation factor 1: from actin bundling to zinc finger protein R1-associated nuclear localization. Biosci Biotechnol Biochem 2002;66(1):1-21.

10. Ozanne DM, Brady ME, Cook S, Gaughan L, Neal DE, Robson CN. Androgen receptor nuclear translocation is facilitated by the f-actin cross-linking protein filamin. Mol Endocrinol 2000;14(10):1618-26.

11. Loy CJ, Sim KS, Yong EL. Filamin-A fragment localizes to the nucleus to regulate androgen receptor and coactivator functions. Proc Natl Acad Sci U S A 2003;100(8):4562-7.

12. Ting HJ, Yeh S, Nishimura K, Chang C. Supervillin associates with androgen receptor and modulates its transcriptional activity. Proc Natl Acad Sci U S A 2002;99(2):661-6.

13. Nishimura K, Ting HJ, Harada Y, Tokizane T, Nonomura N, Kang HY, et al. Modulation of androgen receptor transactivation by gelsolin: a newly identified androgen receptor coregulator. Cancer Res 2003;63(16):4888-94.

14. Lindquist S. The heat-shock response. Annu Rev Biochem 1986;55:1151-91.

15. Yin X, Warner DR, Roberts EA, Pisano MM, Greene RM. Identification of novel CBP interacting proteins in embryonic orofacial tissue. Biochem Biophys Res Commun 2005;329(3):1010-7.

16. Wassarman DA, Aoyagi N, Pile LA, Schlag EM. TAF250 is required for multiple developmental events in Drosophila. Proc Natl Acad Sci U S A 2000;97(3):1154-9.

17. Vassilev A, Yamauchi J, Kotani T, Prives C, Avantaggiati ML, Qin J, et al. The 400 kDa subunit of the PCAF histone acetylase complex belongs to the ATM superfamily. Mol Cell 1998;2(6):869-75.

18. Frank SR, Parisi T, Taubert S, Fernandez P, Fuchs M, Chan HM, et al. MYC recruits the TIP60 histone acetyltransferase complex to chromatin. EMBO Rep 2003;4(6):575-80.

19. Reutens AT, Fu M, Wang C, Albanese C, McPhaul MJ, Sun Z, et al. Cyclin D1 binds the androgen receptor and regulates hormone-dependent signaling in a p300/CBP-associated factor (P/CAF)-dependent manner. Mol Endocrinol 2001;15(5):797-811.

20. Gaughan L, Logan IR, Cook S, Neal DE, Robson CN. Tip60 and histone deacetylase 1 regulate androgen receptor activity through changes to the acetylation status of the receptor. J Biol Chem 2002;277(29):25904-13.

21. Peters JM. The anaphase-promoting complex: proteolysis in mitosis and beyond. Mol Cell 2002;9(5):931-43.

22. Harper JW, Burton JL, Solomon MJ. The anaphase-promoting complex: it's not just for mitosis any more. Genes Dev 2002;16(17):2179-206.

23. Thin TH, Kim E, Yeh S, Sampson ER, Chen YT, Collins LL, et al. Mutations in the helix 3 region of the androgen receptor abrogate ARA70 promotion of 17beta-estradiol-induced androgen receptor transactivation. J Biol Chem 2002;277(39):36499-508.

24. Burchardt T, Burchardt M, Chen MW, Cao Y, de la Taille A, Shabsigh A, et al. Transdifferentiation of prostate cancer cells to a neuroendocrine cell phenotype in vitro and in vivo. J Urol 1999;162(5):1800-5.

25. Wright ME, Tsai MJ, Aebersold R. Androgen receptor represses the neuroendocrine transdifferentiation process in prostate cancer cells. Mol Endocrinol 2003;17(9):1726-37.

26. Xie S, Lin HK, Ni J, Yang L, Wang L, di Sant'Agnese PA, et al. Regulation of interleukin-6-mediated PI3K activation and neuroendocrine differentiation by androgen signaling in prostate cancer LNCaP cells. Prostate 2004;60(1):61-7.

27. Ford OH, 3rd, Gregory CW, Kim D, Smitherman AB, Mohler JL. Androgen receptor gene amplification and protein expression in recurrent prostate cancer. J Urol 2003;170(5):1817-21.

28. Culig Z, Klocker H, Bartsch G, Steiner H, Hobisch A. Androgen receptors in prostate cancer. J Urol 2003;170(4 Pt 1):1363-9.

29. Oesterling JE, Hauzeur CG, Farrow GM. Small cell anaplastic carcinoma of the prostate: a clinical, pathological and immunohistological study of 27 patients. J Urol 1992;147(3 Pt 2):804-7.

30. Sarma DP, Weilbaecher TG. Small-cell carcinoma of prostate. Urology 1989;33(4):332-5.

31. Abrahamsson PA, Wadstrom LB, Alumets J, Falkmer S, Grimelius L. Peptide-hormone- and serotonin-immunoreactive cells in normal and hyperplastic prostate glands. Pathol Res Pract 1986;181(6):675-83.

32. Cox ME, Deeble PD, Lakhani S, Parsons SJ. Acquisition of neuroendocrine characteristics by prostate tumor cells is reversible: implications for prostate cancer progression. Cancer Res 1999;59(15):3821-30.

33. Taplin ME, Balk SP. Androgen receptor: a key molecule in the progression of prostate cancer to hormone independence. J Cell Biochem 2004;91(3):483-90.

34. Rennie PS, Nelson CC. Epigenetic mechanisms for progression of prostate cancer. Cancer Metastasis Rev 1998;17(4):401-9.

35. Marcelli M, Ittmann M, Mariani S, Sutherland R, Nigam R, Murthy L, et al. Androgen receptor mutations in prostate cancer. Cancer Res 2000;60(4):944-9.

36. Shi XB, Ma AH, Xia L, Kung HJ, de Vere White RW. Functional analysis of 44 mutant androgen receptors from human prostate cancer. Cancer Res 2002;62(5):1496-502.

37. Linja MJ, Savinainen KJ, Saramaki OR, Tammela TL, Vessella RL, Visakorpi T. Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. Cancer Res 2001;61(9):3550-5.

38. Visakorpi T, Hyytinen E, Koivisto P, Tanner M, Keinanen R, Palmberg C, et al. In vivo amplification of the androgen receptor gene and progression of human prostate cancer. Nat Genet 1995;9(4):401-6.

39. Ueda T, Mawji NR, Bruchovsky N, Sadar MD. Ligand-independent activation of the androgen receptor by interleukin-6 and the role of steroid receptor coactivator-1 in prostate cancer cells. J Biol Chem 2002;277(41):38087-94.

40. Sadar MD. Androgen-independent induction of prostate-specific antigen gene expression via cross-talk between the androgen receptor and protein kinase A signal transduction pathways. J Biol Chem 1999;274(12):7777-83.

41. Cheng H, Snoek R, Ghaidi F, Cox ME, Rennie PS. Short hairpin RNA knockdown of the androgen receptor attenuates ligand-independent activation and delays tumor progression. Cancer Res 2006;66(21):10613-20.

42. Miyamoto H, Rahman M, Takatera H, Kang HY, Yeh S, Chang HC, et al. A dominant-negative mutant of androgen receptor coregulator ARA54 inhibits androgen receptor-mediated prostate cancer growth. J Biol Chem 2002;277(7):4609-17.

43. Xu J, Qiu Y, DeMayo FJ, Tsai SY, Tsai MJ, O'Malley BW. Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene. Science 1998;279(5358):1922-5.

44. Taneja SS, Ha S, Swenson NK, Torra IP, Rome S, Walden PD, et al. ART-27, an androgen receptor coactivator regulated in prostate development and cancer. J Biol Chem 2004;279(14):13944-52.

45. Ippolito JE, Merritt ME, Backhed F, Moulder KL, Mennerick S, Manchester JK, et al. Linkage between cellular communications, energy utilization, and proliferation in metastatic neuroendocrine cancers. Proc Natl Acad Sci U S A 2006;103(33):12505-10.

46. Desai SJ, Ma AH, Tepper CG, Chen HW, Kung HJ. Inappropriate activation of the androgen receptor by nonsteroids: involvement of the Src kinase pathway and its therapeutic implications. Cancer Res 2006;66(21):10449-59.