博士論文

論文題目　　To purify and identify the novel androgen receptor cofactors in prostate cancer cells
(前立腺癌における新規アンドロゲン受容体転写共役因子群の精製及び機能解析)

氏　　名 陳 淑婷

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論文の内容の要旨

応用生命工学　専　攻
平成 23 年度博士課程　入学
氏　　名 陳 淑婷
指導教員名 白髭克彦

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To purify and identify the novel androgen receptor cofactors in prostate cancer cells
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Chapter 1 Introduction

The male sex hormone androgens are critical for expression of male phenotype including development and maintenance of secondary male characteristics. The physiological effects are outputted via diverse genes expression that are mainly mediated by its cognate receptor, androgen receptor (AR) which is a ligand-inducible transcription factor. Due to the extensive physiological role of AR, abnormal AR signaling is implicated in a wide variety of disorders including prostate cancer.

AR signaling start from the circulating androgens directly penetrate across cell membrane of target cells and bind to the inactive cytoplasmic AR. The androgen-bound AR undergoes conformational change, dimerization and translocates from cytosol to the nucleus where it binds to specific DNA sequences known as androgen-responsive elements (AREs) and recruits a series of coregulators (also termed cofactors) that exhibit distinct functions to regulate a vast number of target genes transcription.

More than 150 AR coregulators have been identified through basic molecular and biochemical approaches. Recent studies revealed the crucial role of chromatin architecture in gene regulation and also emerged many of these AR coregulators mediate transcription through building appropriate chromatin environment. On the other hand,

it has implicated that the posttranslational modifications of AR or AR complex components have a critical role for quantitative /qualitative regulation of AR. However, the mechanism by which these coregulators regulate AR posttranslational modifications and affect target genes expression is ill-defined.

In addition, given the advance of global genome-wide mapping technology, the function of AR coregulators as well as their specific target genes have been analyzed and brought new insights into AR transcriptional regulation.

The aim of these study is to reveal a novel AR transcriptional regulation mechanism by identifying AR coregulators. For this purpose I adopted biochemical purification of the AR-associated proteins, and investigated the AR quantitative /qualitative regulation as well as the global genome-wide transcript profiling by which these coregulators.

Chapter 2 Identification of novel AR transcriptional coregulators in prostate cancer cells

Identification of the AR-associated proteins using antibody affinity purification

To purify the AR-associated proteins in prostate cancer cells, I performed the anti-AR antibody affinity protein purification. The endogenous AR-associated proteins were subjected to LC-MS/MS analysis. In addition to the known AR coregulator PRMT5, several proteins were identified as AR coregulator candidates. Among these candidates, Ubiquitin Specific Protease 7 (USP7) was focused because of its high score identified by LC-MS/MS.

Ubiquitination is a reversible posttranslational modification that provides a tag either marking the labeled protein for degradation or modulates its function. Ubiquitination can be reversed by deubiquitinating enzymes (DUBs) such as USP7 through removing ubiquitin from target proteins. It is known that ligand-dependent ubiquitination of AR plays an important role in AR transcriptional regulation, including act as a scaffold for coactivators recruitment or transcriptional activation-coupled proteasomal degradation. However, the precise mechanism by which these ubiquitin network mediate AR transcriptional activity is poorly understood. To investigate the role of USP7 in AR transcriptional regulation may be helpful to clear the intricate ubiquitin network in AR signaling.

USP7 is a novel AR-associated protein in prostate cancer cells

To validate the interaction between USP7 and AR, co-immunoprecipitation was performed in potent androgen DHT-treated prostate cancer cells LNCaP and 22Rv1. The

result confirmed the LC-MS/MS identification and revealed that the interaction of USP7 and AR was DHT-dependently enhanced. To investigate whether USP7 modulates the ubiquitination of AR, *in vivo* ubiquitination assay was carried out. As a result, overexpression of USP7 attenuated ubiquitinated AR produced by DHT, suggesting USP7 may participate in AR transcriptional activity regulation through control of AR ubiquitination status.

Chapter 3 The elucidation of USP7 functional role in AR transcriptional regulation

USP7 associates with AR on AREs upon rapid DHT stimulation

Previous studies indicated that USP7 mainly localizes in nucleus, hence the investigation was focused on chromatin region. The time course recruitment of USP7 to AREs was assessed using ChIP-qPCR. The recruitment of USP7 to AREs increased upon 1 hr DHT treatment and declined rapidly, suggesting USP7 was involved in the early stage of AR transcriptional activation. In addition, the interaction of USP7 and AR on AREs was confirmed using re-ChIP. This result exhibited that the association and disassociation of USP7 and AR on AREs upon rapid DHT stimulation.

USP7 regulates AR transcriptional activity and specificity

Given the gene specific regulation manner of AR coregulators, I profiled the global effect of USP7 knockdown on androgen-dependent genes transcript using RNA-seq analysis.

Approximately 45% of androgen-dependent genes expression were affected by USP7 knockdown, indicating the essential role of USP7 in AR signaling. In addition, USP7 functioned as a coactivator in DHT up-regulated genes, whereas it acted as a corepressor in DHT down-regulated genes. This result suggested that USP7 is able to mediate AR transcriptional activity and specificity.

USP7 regulates liganded AR protein stability and chromatin recruitment

To investigate the mechanism by which USP7 regulates AR transcriptional activity, I observed the effect of USP7 knockdown on AR chromatin recruitment. The result indicated that AR binding to AREs was diminished by USP7 knockdown. Since USP7 attenuated ligand-induced ubiquitinated AR, it was expected that USP7 facilitates AR chromatin binding through receptor stabilization. To corroborate this speculation, I

observed the effect of USP7 overexpression on liganded AR protein degradation rates. The result revealed that the liganded AR protein half-life was prolonged by USP7.

Chapter 4 Conclusion and Discussion

To decipher the new AR transcriptional regulation mechanism, the endogenous AR-associated proteins in prostate cancer cells were affinity purified, and the DUBs USP7 was identified as a novel AR coregulator.

USP7 facilitates AR binding to AREs and activates gene transcription through attenuating ligand-induced ubiquitinated AR and receptor stabilization. By strictly controlling the dynamic USP7 association and disassociation, the AR transcriptional activity can be precisely fine-tuned.

In addition, the global profiling of USP7 knockdown effect on androgen-dependent gene transcripts supposed the gene specific regulation manner of USP7. Given that AR-mediated gene repression versus activation via distinct mechanisms, suggesting USP7 may regulate AR target genes expression through multiple pathways.

To conclude, this study biochemically identified USP7 as a novel AR coregulator which is required for mediation of AR transcriptional activity and specificity, thereby broadens the view of ubiquitin network in gene regulation.

Abbreviation

ADPC; androgen dependent prostate cancer
AF-1; activation function-1
AF-2; activation function-2
AR; androgen receptor
AREs; androgen response elements
CCS; charcoal-stripped serum
ChIP-seq; chromatin immunoprecipitation sequencing
DHT; dihydrotestosterone
DNMT1; DNA methyltransferase
DUBs; deubiquitinating enzyme
ER; estrogen receptor
FSH; follicle-stimulating hormone
FoxA1; Forkhead box A1
GR; glucocorticoid receptor
GnRH; gonadotropin-releasing hormone
HAT; histone acetyltransferase
HDAC; histone deacetylase
HMT; histone methyltransferase
HRPC; hormone refractory prostate cancer
LBD; ligand binding domain
LH; luteinizing hormone
MDM2; murine double minute 2
PR; prosgesterone receptor
PRC1; polycomb repressive complex1
PRMT5; histone-arginine N-methyltransferase
RNA-seq; RNA sequencing
USP7; ubiquitin specific peptidase 7
Ub; ubiquitin
nAREs; negative androgen response elements

Chapter 1

Introduction

Overview

The male sex hormone androgens play critical role in development and maintenance of male phenotype via the ligand-inducible nuclear receptor, androgen receptor (AR) to modulate a vast number of target genes expression. To study the mechanism of AR transcriptional regulation is necessary for understanding the physiological and pathological androgen actions.

The AR transcriptional regulation is mediated by collaborative protein complexes including coregulators and specific transcription factors. Recent years the mechanisms of these AR transcriptional coregulators rebuild chromatin environment for gene transcription are well demonstrated. However, how these coregulators mediate AR post-translational modifications and manipulate AR quantitative/ qualitative regulation are ill-defined. In addition, recent studies of mapping AR genome wide binding sites using ChIP-chip and ChIP-seq revealed the complexity of these collaborative proteins in androgen-dependent genes expression and broaden the view of AR transcriptional regulation.

In this chapter, the physiological and pathological AR actions especially in prostate cancer are introduced. And the molecular mechanism of AR transcriptional regulation including the composition of AR transcriptional collaborative proteins as well as the recent studies of genome-wide AR landscape mapping in prostate cancer model cell lines are overviewed. Based on these research, the study aim of the thesis is emerged.

The physiological function of androgens

1. Androgen types and synthesis

Androgens, a kind of steroid hormone are synthesis in a series of steroidogenesis pathway in tesis Leydig cells. The biosynthesis of androgens in testis are under strict regulation via the hypothalamic-pituitary-gonadal (HPG) axis.

Secretion of Gonadotropin-releasing hormone (GnRH) occurs in a pulsatile fashion from hypothalamus, and is transported to the gonadotrophs of the anterior pituitary gland that controls gonadotropin: follicle-stimulating hormone (FSH) and luteinizing hormone (LH) synthesis and secretion. In turn, LH stimulates testicular androgens synthesis and FSH promotes spermatogenesis, the release systemic androgen concentrations then feedback inhibit the signaling of LH, FSH and GnRH. The adrenal glands also secret androgens although it is too low to have significant physiological effect in male whereas in female the adrenal glands are major source of androgens (Figure 1-1 A).

The major circulating androgens are testosterone, in 5α-reductase expressed target tissues such as skin and prostate, testosterone is converted to more potent metabolite (dihydrotestosterone) DHT before bind to receptor. In addition to testosterone and DHT, testis also secrets Dehydroepiandrosterone (DHEA), is primary precursor of estrogens[1,2,3].

2. The physiological function of androgens

The male sex hormone androgens are critical for expression of male phenotype including sexual differentiation, development and maintenance of secondary male characteristics and spermatogenesis.

The sexual phenotype formation starts from embryo stage. During embryo stage, androgens play essential role in sexual differentiation, male development is induced in the presence of testosterone and DHT, in absent of these hormones female differentiation occurs. Another important stage of sexual phenotype development is puberty. During puberty, the increased production androgens act on wide range of target tissues including brain, skin, muscle, bone, adipocyte and sexual organs to develop secondary male characteristics and spermatogenesis. Spermatogenesis is a process that spermatogonia or germinal cells different into spermatozoon (commonly called sperm cells) that is essential for preserving the number of chromosomes in the offspring and sexual reproduction[1,2,3].

The biological function of androgen receptor (AR)

1. The physiological function of AR

The varied physiology function of androgens are exerted via androgen receptor (AR), a 110-kD ligand-inducible nuclear receptor that expression of target genes[4,5] (Figure 1-2). Androgen receptor is expressed in wide range of tissues especially in reproductive system such as testis and prostate gland. Studies from AR-deficient or AR knockout (ARKO) male mice models revealed the central role of AR in the androgen-regulated reproductive development. These genetic mouse models displayed reproductive tract and gonadal development, as well as sexual behavior impaired phenotypes, consistent with the known aberrant AR target gene expression[6].

Dysregulation of AR signaling perturbs normal reproductive development and results in a wide range of pathological condition such as androgen-insensitive syndrome, spinal bulbar muscular atrophy and prostate cancer[1,2,5,6].

2. AR and prostate cancer

The prostate gland is responsible for producing the fluid portion of semen and help for controlling the flow of urine. While the prostate functions is critically dependent on AR signaling, the hyperactive AR signaling modulates the expression of genes associated with cell cycle regulation, survival and growth that cause prostate carcinogenesis[6,7,8,9,10].

It is well accepted that AR is essential for androgen dependent prostate cancer (ADPC) and hormone refractory prostate cancer (HRPC) progression, however, the exact role of AR in prostate cancer initiation, progression and develop into hormone refractory prostate cancer (HRPC) remain elusive.

Somatic AR mutation is supposed to be the major mechanism by which prostate cancer cells acquire the ability to survive and proliferate in the absence of androgens. In addition, recent studies have identified several AR splice variants that lack LBD domain, resulting in constitutively activated AR and may promote the progression of HRPC. Other hyperactive AR signaling mechanisms include AR gene amplification, alteration in AR coregulators expression and tumor microenvironment effects[7-12].

3. The gene and structure of AR

AR is a member of the nuclear steroid receptor superfamily, member ligand-inducible transcription factors that mediate the expression of target genes in response to ligands specific to each receptor, including steroids, vitamin, thyroid hormone and xenobiotic agents[4,5]. The AR gene containing eight exons is located on the long arm of the X-

chromosome at Xq11-12. The eight exons encode AR distinct functional motifs: the N-terminal domain (NTD encoded by exon 1), DNA-binding domain (DBD encoded by exon2 and 3), a hinge region (H encoded by 5' portion of exon4), and a ligand-binding domain (LBD encoded by the remainder of exon4 to exon8) (Figure 1-1 B).

A hinge region separates the DBD and the LBD, which contains a nuclear localization signal to facilitate ligand-dependent nuclear translocation of receptor. PEST (proline-, glutamate-, serine-, and threonine-rich) sequence, a degradation motif is also found in hinge region. The DBD contains two zinc finger motifs are contributed to DNA binding site recognition. The LBD is posted in C terminus and is composed of twelve α-helices forming ligand-binding pocket. X-ray crystallographic analysis reveals that ligand binding induces LBD conformational change and generates a coregulators interaction surface. Contrary to the highly conserved DBD and LBD, the NTD is the least conserved region of AR contains polyglutamine and polyglycine repeats that result in variation of AR length.

Like most nuclear receptors, AR possesses two transactivation domain, AF-1 and AF-2 located in NTD and CTD respectively. The AF-1 and AF-2 domain function as docking sites for transcriptional coregulators recruitment and AF-1 activity is ligand independent whereas AF-2 activity shows ligand dependent. Although the relative transactivation activity of the AF-1 and the AF-2 are variable and depend on the cellular environment and expression of coregulators, the AF-1 and AF-2 are required for optimal AR transactivation[4,5,6].

The molecular mechanism of AR transcriptional regulation

In normal physiological condition, upon ligand binding the cytoplasmic AR undergoes conformation change results in dissociation of chaperones and reveals the NLS signal. The ligand-bound AR translocates to the nucleus where its binds to target gene regulatory regions containing DNA motif: 5'-AGAACANNNTGTTCT-3', known as androgen response elements (AREs) and associates with distinct functions of coregulators forming a large multi-protein transcriptional complex to cooperate with gene regulation[4,5,6,13,14] (Figure 1-2).

Apart from general transcription factors, the composition of AR transcriptional complex that recruited in regulatory regions of target gene can be divided into two classes: coregulators and specific transcription factors that bind to their consensus binding elements[13,14,15].

1. Coregulators (Cofactors)

In general, coregulators do not bind DNA by themselves and according to their ability to enhance or reduce AR transcriptional activity, coregulators can be classified into coactivators and corepressors. By virtue of assessing the functions by which these coregulators, a huge strides in the understanding of AR transcriptional regulation have been achieved. It is known that a large proportion of coregulators possess enzymatic activities and chromatin remodeling properties.

Enzymatic properties coregulators

Histone-modifying enzymes compose one major group of AR coregulators, they are able to modulate the acetylation, phosphorylation, methylation, ubiquitination and SUMOylation status of the local histone environment that are associated with transcription activation or repression. The p300/CBP and p160/SRC HATs are the best –characterized AR coactivators, and their expression level are correlated with androgen-dependent prostate cancer. Even AR itself and components of the general transcriptional machinery as well as cooperating coregulators can be the substrates.

Although these coregulators rebuild chromatin environment for transcriptional regulation is well demonstrated, the AR posttranslational modifications by these coregulators contribute to AR quantitative /qualitative regulation are ill-defined[16,17].

Chromatin remodeling coregulators

The gene transcriptional activation is associated with a series of reorganization of nucleosome position as well as increased accessibility of DNA for interaction with transcriptional machinery and integrated regulatory factors. These processes are able to

be accomplished by chromatin remodeling complexes. The SWI/SNF complex contains DNA-dependent ATPase subunit necessary for chromatin modification is one of the best characterized of the chromatin remodeling complexes. Activation of androgen target genes requires the assembly of the SWI/SNF complexes and is involved in promotion of prostate cancer cell proliferation[13,14].

2. Specific transcription factors

A special class of transcription factor refer to pioneer factors are able to associate with compacted chromatin directly and modulate chromatin accessibility, thereby facilitate the binding of additional transcription factors. Recently, they have been implicated in hormone-dependent cancer including prostate cancer[18,19].

Recent studies of genome-wide AR binding sites in different stages of prostate cancer model cell lines have identified hundreds of androgen-regulated genes and suggested that the AR transcriptional program may change as the cancer progress from hormone dependent to hormone independent by virtue of the pioneer factor FoxA1.

It is proposed that the development of ADPC into HRPC is accompanied the transition of cell-specific epigenetic marks distribution such as H3K4me1/me2. The cell-specific distribution of H3K4me1/me2 can recruit FoxA1, which in turn restricts the AR binding sites and transcription program. Hence, AR can drive distinct transcription program to promote ADPC and HRPC cell proliferation[18,19,20]. (Figure 1-3). Interestingly, the silencing of FoxA1 in prostate cancer cells results in reprogramming of AR binding site, suggesting the dual role of FoxA1 in AR transcriptional regulation[21,22]. In addition to FoxA1, other pioneer factor, GATA2, OCT1 and ETS are also reported to regulate AR binding[23,24].

These studies not only alter and expand the view of these collaborative proteins in AR transcriptional regulation but also impress the complexity of prostate cancer. However, other DNA binding proteins that might be involved in modulation of AR chromatin binding remain to be identified.[18-23]

AR transcriptional regulation model

The nuclear receptor such as ERα, PR and GR undergo hormone-induced receptor transactivation and couple receptor proteasomal degradation. The nuclear receptor cyclic nature is supposed as a mechanism by which cells to fine-tuning transcription activation in response to rapid changes in hormone concentration.[25] Unlike these nuclear receptors, AR is stabilized in the presence of androgens, hence cells may apply other mechanism to cope with the androgen response. Recent studies have proposed two speculations of AR transcriptional regulation model to explain the AR actions.

1. Long-term sustained manner

Androgen have been reported to stabilize AR protein, suggesting the different mechanism of androgen-induced AR transactivation compare to other nuclear receptors[26,27]. A study from Myles Brown's group demonstrated that AR regulates gene transcription is a long term process. They investigated the time course recruitment of AR and coregulators such as p300 and SRC1 as well as RNA pol II to PSA gene AREs in LNCaP cells using ChIP. The result revealed that the AR transcriptional complexes accumulated on AREs continuously over 16 h and gradually declined following longer treatment. This scenario also coincided with the long-term PSA mRNA expression pattern. They also supposed that the constant elevation of AR protein level by androgen is the potential for secondary events of transcription[28,29].

2. Cyclic manner

Contradict to long-term sustained manner, other studies demonstrated that the association and dissociation of AR and its coregulators is in alternative cyclic manner. It is assumed that nuclear receptors regulate transcription in cyclic manner that start from ligand binding and end in ubiquitin/proteasome-mediated degradation. As shown for ERα, transcription initiation is a highly dynamic process of transcriptional complex assembly that is coupled chromatin modification and remodeling. After transition to elongation, the ERα-transcriptional complex dissociate from regulatory elements through ubiquitin target receptor to degradation and allow a new round of transcription complex assembly and initiation[30-34].

The controversy of AR transcriptional regulation model may partly due to the intricate ubiquitin network in androgen signaling. Next section will simply introduce the ubiquitin modification system and overview the functional role of ubiquitination in AR transcriptional regulation.

The ubiquitin system in AR transcriptional regulation

1. Ubiquitin system

Ubiquitination, the covalently conjugated of 76-amino-acid polypeptide ubiquitin (Ub) to target protein, is a reversible posttranslational modification that provides as a tag either marking the labeled protein for degradation or modulates its biological function.

In general, ubiquitination is controlled by three step reaction initialized by E1-mediated activation of Ub, followed by conjugation to E2 enzyme and substrate targeting by E3 Ub ligase. This process can be reversed by specific deubiquitinating enzymes (DUBs) to remove Ub from substrates or cleave the Ub linkage[35] (Figure 1-4 A).

Ub can be conjugated to target proteins either as monomer or as Ub chains that vary in length and linkage type. Ub chains can form through seven lysine (Lys) residues on its surface (Lys 6, Lys 11, Lys27, Lys 29, Lys33, Lys 48 and Lys 63). Different Ub linkages result in various conformations of Ub chains and create varied molecular signals. Whereas the role of Lys 48-linked chains in proteasome degradation and Lys 63-linked chains in cell signaling is well characterized, the exact function of the remaining six atypical Ub chains are poorly defined[35,36,37].

These vital biological functions of ubiquitination can be reversed by deubiquitination. Deubiquitination is mediated via DUBs which can be subdivided into five structurally distinct families, ubiquitin C-terminal hydrolases(UCHs), ubiquitin-specific protease (USPs), ovarian tumor proteases (OTUs), Josephins and JAMM/MPN+. The UCH, USP, OTU and Josephin families are cysteine protease, whereas the JAMM/MPN+ family members are zinc metalloproteases (Figure 1-4 B). DUBs can display specificity for both substrates and particular Ub chains, the USP DUBs are substrate- rather than Ub chain linkage-specific whereas JAMM family DUBs are Lys63-specific[38,39].

2. The role of ubiquitination in AR transcriptional regulation

It has become clear that androgen activates AR is accompanied AR ubiquitination, suggesting this modification make a contribution to AR transcriptional activation.

Androgen-induced AR ubiquitination is accomplished by a sophisticated ubiquitin network[40,41]. Several E3 ligase has identified as AR coregulators that mediate AR transcriptional activity. AR is ubiquitinated by MDM2 subsequent to Akt phosphorylation which leads to AR degradation, hence reduces steady-state level of AR and attenuates AR transcriptional activity[42]. More recently, ChIP assay have shown that androgen-induced general transcription factor TFIIH phosphorylates AR and thereby recruits the E3 ligase: MDM2 and CHIP to AREs resulting AR polyubiquitination and

target receptor degradation and turnover[40,43,44]. Contrast to MDM2 and CHIP target receptor degradation, RNF6 generates Lys 6- and Lys 27-linked Ub chains that stabilize receptor and act as scaffold for coactivators such as ARA54 recruitment consequently modulate AR transcriptional activity and specificity [41,45] (Figure 1-5).

Although less understood, deubiquitination is also involved in this ubiquitin network. Previous studies has shown Tumor susceptibility gene 101 (TSG101), an E2-like enzyme deprived of ubiquitin conjugase activity, transiently locks AR in monoubiquitinated state by preventing polyubiquitination, thus extends AR-mediated transcription[46]. In addition, the DUBs USP26 have been proposed to counteract hormone-induced AR ubiquitination by MDM2 and prolong AR transcriptional activity[47].

Even though the androgen-mediated intricate AR ubiquitination that contribute to distinct cell signals are essential for AR transcriptional regulation. The mechanism of manipulating this ubiquitin network including E3 ligase and DUBs and fine-tuning AR transcriptional activity is remain elusive.

Study purpose

The male sex hormone androgens are critical for expression and maintenance of male phenotype. The extensive physiological function of androgens are mainly exerted by the ligand-inducible nuclear receptor, AR to mediate a vast number of target gene expression. Hence, aberrant AR signaling is implicated in a wide range of disease including prostate cancer. To understand the comprehensive molecular mechanism of AR transcriptional regulation is essential for elucidating the physiological and pathological role of androgens.

Studies gained from prostate cancer model cell lines revealed that the AR-mediated transcription is a highly regulated and complex process. Prior to ligand stimulation, the pioneer factors are recruited to the cell-specific distribution epigenetic marks and establish the cell-specific AR binding patterns. Upon androgen stimulation or other signaling that activate AR, AR binds to AREs that created by pioneer factors and recruits coregulators forming a huge complex to activate transcription.

The composition of these AR transcriptional complexes existed on AREs are temporal, quantitative variation and gene specific. They rebuild the chromatin environment and modulate AR or components of these complexes post-translational modifications to affect gene transcription. Hence, to unravel the composition of these AR transcriptional complexes and investigate how they manipulate AR quantitative /qualitative regulation as well as mediate target gene expression is an efficient strategy to understand the AR transcriptional regulation.

The aim of this study is to reveal a novel AR transcriptional regulation mechanism by identifying AR coregulators. For this purpose I biochemical purification of the AR-associated proteins in prostate cancer cells, and investigate the mechanism by which these coregulators regulate AR quantitative /qualitative regulation as well as identify their specific target gene using the genome-wide transcript profiling method. Through this integrate investigation, the comprehensive AR transcriptional regulation by these coregulators that identified can be deciphered (Figure 1-6).

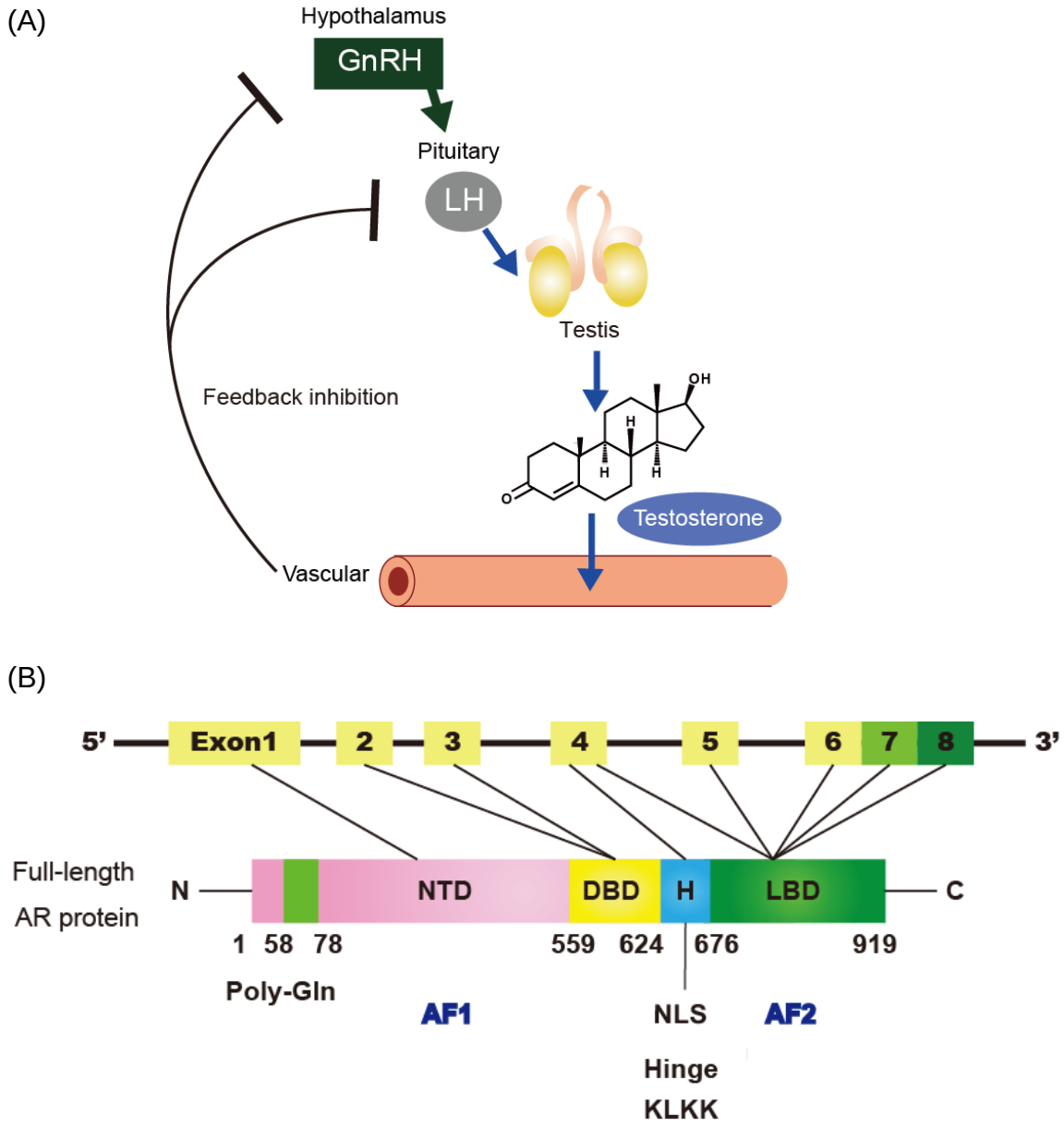


Figure 1-1 The androgens biosynthesis pathway and the structure domain of AR

(A) The biosynthesis of androgens is via hypothalamic-pituitary-gonadal (HPG) axis. The synthesized testosterone then feedback inhibit GnRH and LH secretion, making the system comes full circle. FSH is not shown in this diagram. (B) AR is a member of the nuclear steroid receptor superfamily. The AR protein comprises distinct functional domains and are encoded by eight exons.

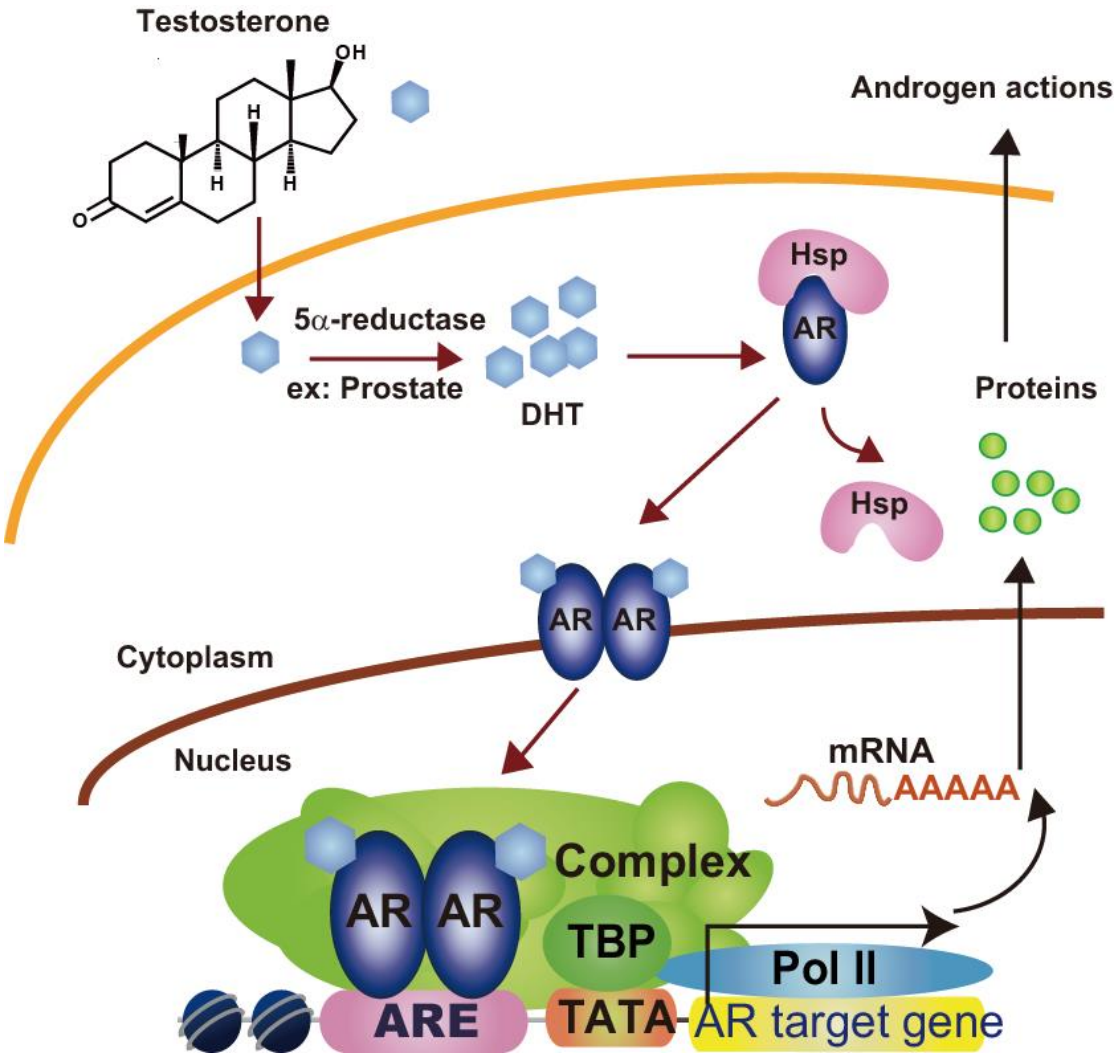


Figure 1-2 The androgen actions are exerted via androgen receptor (AR)

The circulating testosterone directly penetrates across cell membrane of target cells, in 5α-reductase express tissues such as prostate glands, testosterone is converted to DHT by 5α-reductase before binding to AR. The liganded AR dissociates with Hsp proteins and translocates to nucleus where it binds to AREs and recruits a series of coregulators that exhibit distinct functions to regulate a vast number of target gene transcription and output into androgen actions.

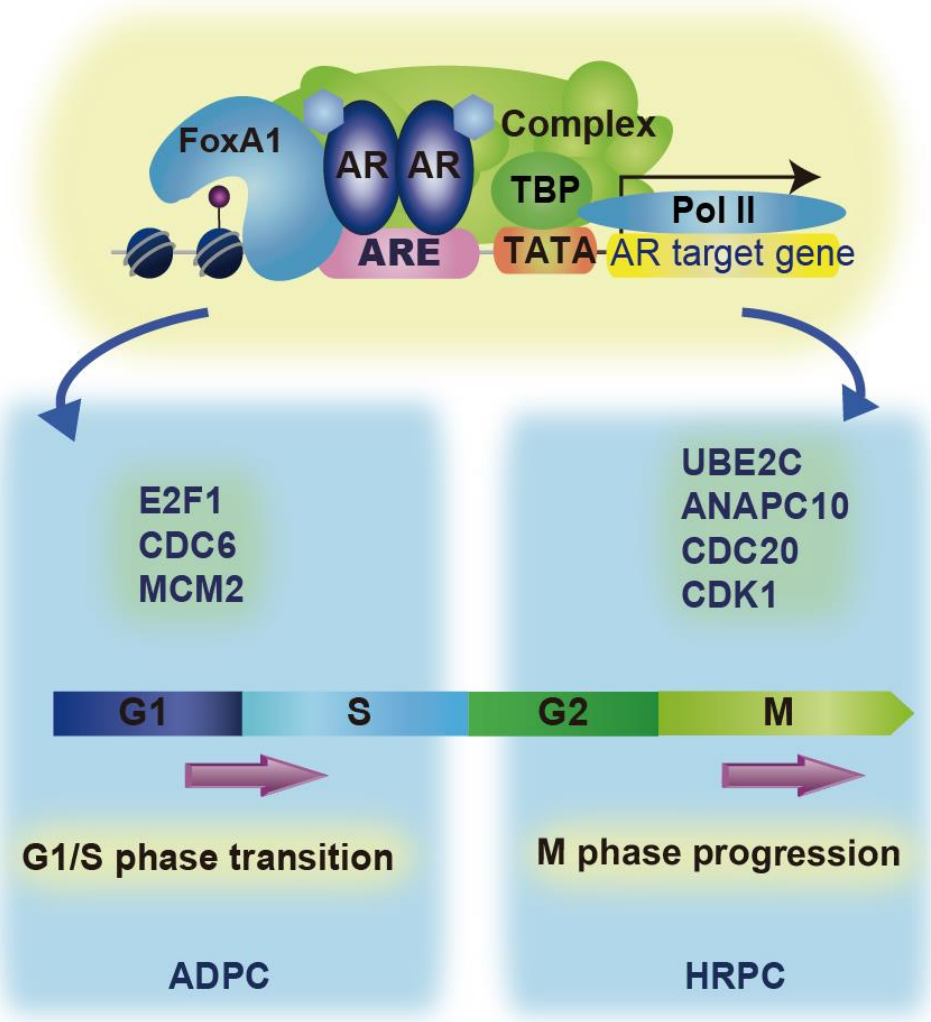
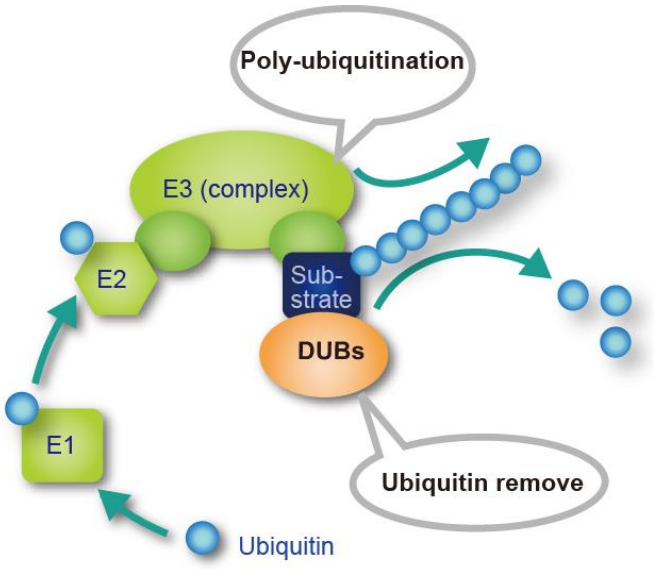


Figure 1-3 AR regulates cell-specific transcription program to promote prostate cancer cell proliferation

It is supposed that AR can drive distinct transcription program in ADPC and HRPC cells. In ADPC, AR promotes cell proliferation by expression of G1/S phase transition genes. Contrast to this, HRPC cell proliferation is mediated by expression of M phase cell cycle genes.

(A)



(B)

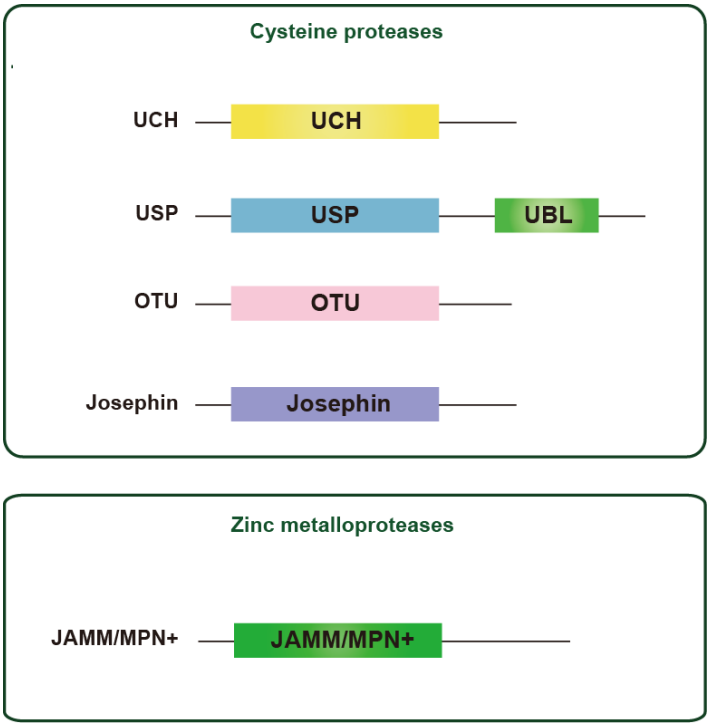


Figure 1-4 The ubiquitination chain formation and hydrolysis

(A) The ubiquitination is mediated by three step enzymatic cascades, and the ubiquitin chain can be reversed by DUBs. (B) The DUBs can be subdivided into five structurally distinct families according to their catalytic domains. The UCH, USP, OTU and Josephin families are cysteine proteases, whereas the JAMM/MPN+ family members are zinc metalloproteases

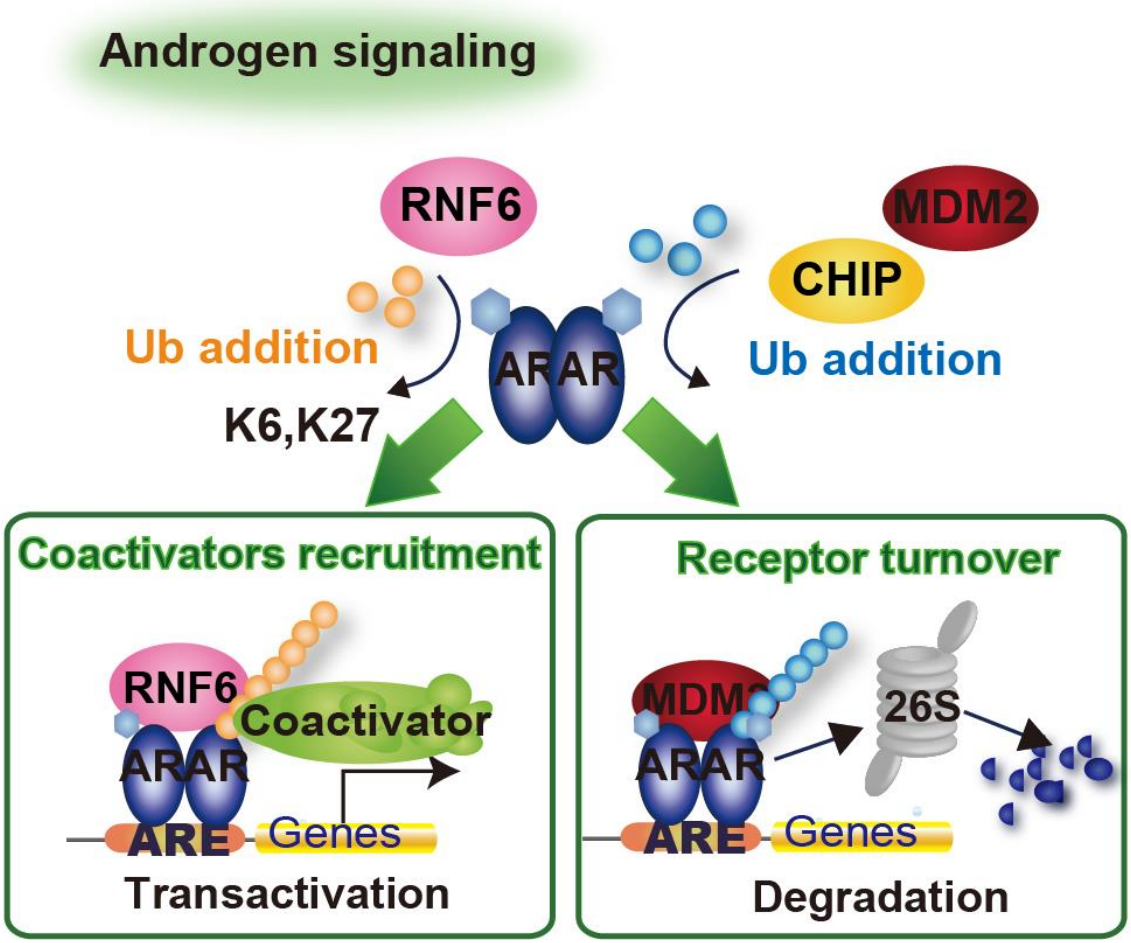


Figure 1-5 The role of ubiquitination in AR transcriptional activity regulation

The androgen-regulated AR ubiquitination including MDM2 and CHIP generate Ub chain that target receptor degradation as well as RNF6 generates K-6 and K-27 Ub chain that stabilize receptor and coactivators recruitment.

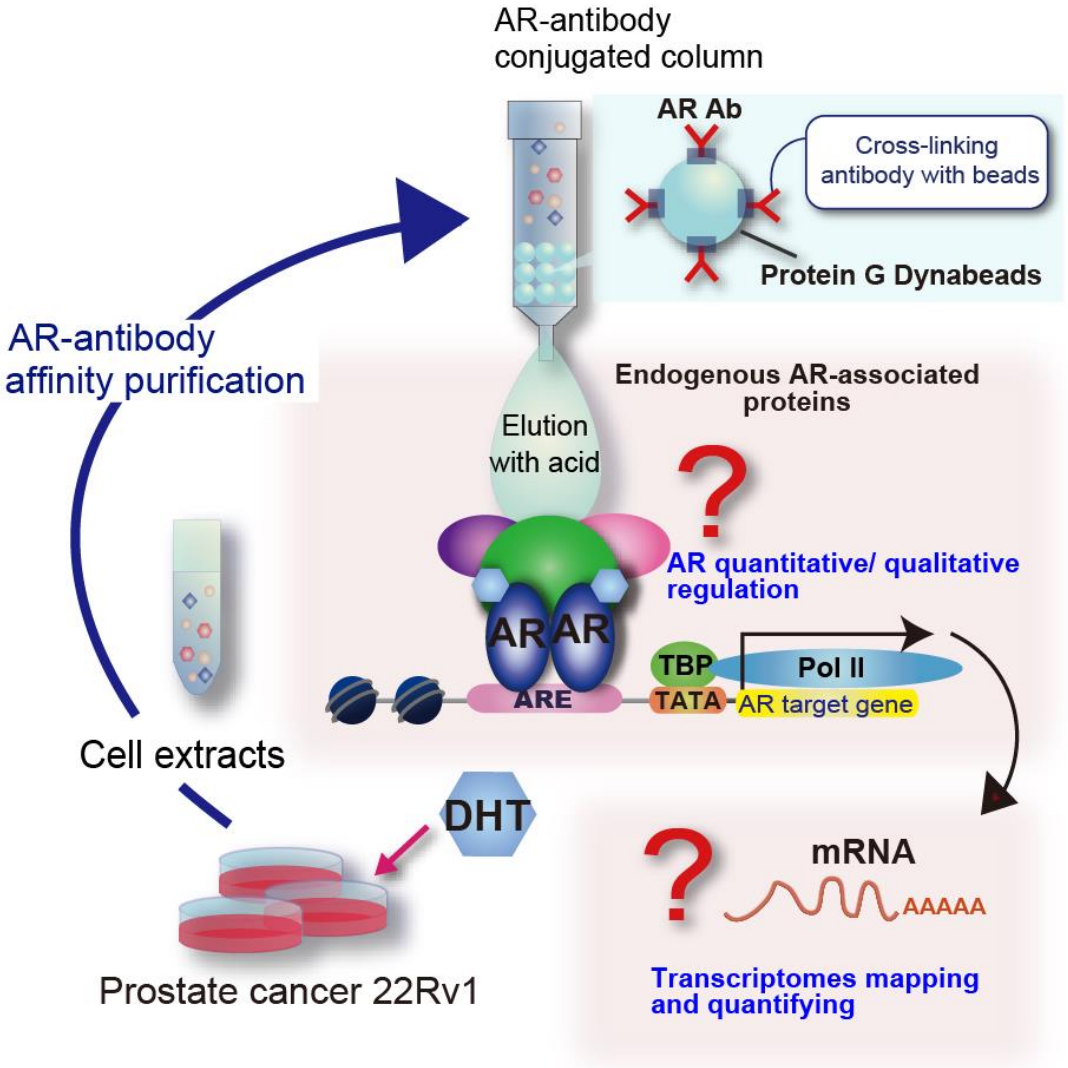


Figure 1-6 Study purpose

The aim of this study is to reveal a novel AR transcriptional regulation mechanism by identifying AR coregulators. For this purpose the endogenous AR-associated proteins in prostate cancer cells are purified using antibody affinity purification. The interested coregulator candidates are focused. And the AR quantitative/qualitative regulation as well as the cell transcriptome profiling that modulate by these coregulators are investigated.

Chapter 2

Identification of AR transcriptional coregulators in prostate cancer cells

Overview

Unraveling the composition of AR transcriptional complexes and investigate their functional role in AR quantitative /qualitative regulation is an efficient strategy to revel novel AR transcriptional regulation mechanism and understand the physiological and pathological AR action.

To identify the endogenous AR-associated proteins in prostate cancer cells, the anti-AR antibody affinity purification was performed. Among these AR coregulator candidates, the potential candidates that might modulate AR post-translational modifications were focused and further investigated their function in AR quantitative /qualitative regulation.

Through the biochemical method to identify the endogenous AR-associated proteins in prostate cancer cells and further examine their functional role in AR transcriptional regulation, I hope to discover a novel AR- target gene regulation mechanism.

Material and method

1. Material

1. Plasmid

Flag-USP7

USP7 cDNA fragments were generated by polymerase chain reaction (PCR) and were subcloned into flag-pcIneo vector (Invitrogen, Grand Island, NY) at the Xho I and SmaI sites. The forward and reverse primers were
5′-CGGGATCCATGGCGGCGACTACTG-3′and
5′-CGGATATCTTCTGAAAAGGGGGGAAAC -3′, respectively.

HA-MDM2

Human MDM2 cDNA fragments were generated by polymerase chain reaction (PCR) and were subcloned into HA-pcIneo vector (Invitrogen, Grand Island, NY) at the XhoI and SmaI sites. The forward and reverse primers were
5′- GGAACTCGAGATGGTGAGGAGCAGGCAAAT -3′and
5′- GGAACCCGGGCTAGGGGAAATAAGTTAGCACAATC -3′, respectively.

wt-AR

The wt-AR plasmid were kindly provided by Dr. Suzuki.
These vectors sequence were confirmed by DNA sequencing.

2. Antibody

① Immunoblotting

Primary antibodies

AR (N20): Santa Cruz Biotechnology (sc-816) 1: 3000 dilution
USP7: Abcam (ab4080) 1: 3000 dilution
MDM2 (SPM14): Santa Cruz Biotechnology (sc-965) 1:1000 dilution
Ubiquitin(P4D1): Santa Cruz Biotechnology(sc-8017)1:1000 dilution
Flag-M2: Sigma-Aldrich (F3165) 1:5000 dilution
α-Tubulin: Sigma-Aldrich (T6074) 1: 5000 dilution
β-Actin: Abcam (ab8224) 1: 5000 dilution

Secondary antibodies

Rabbit: Rabbit IgG HRP (Jackson Immuno research)
Mouse: Mouse IgG HRP (Jackson Immuno research)

② Immunoprecipitation

AR (441): Santa Cruz Biotechnology (sc-7305) 1: 1000 dilution
USP7: Bethyl Laboratory. (A300-033A) 1: 1000 dilution
Flag-M2 agarose: Sigma-Aldrich

3. Reagent

① Androgen: 5α-dihydrotestosterone (DHT) ; Sigma-Aldrich, was dissolved in ethanol to make 10^{-3}, 10^{-5} M for stock and diluted to 10^{-8} M prior to use.

② Protease inhibiton: MG132 ; PEPTIDE (3175-v) was dissolved in DMSO to make 10 mM for stock.

2. Method

1. Cell culture

LNCaP and 22Rv1 cells were grown at 37 ℃ under 5% CO2 in 10-cm plastic dishes containing 10 ml of RPMI 1640 media with 10% fetal bovine serum, 0.2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin. HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.2 mM L-glutamine, 100 U/ml penicillin, 100 ug/ml streptomycin. Before DHT treatment, cells were incubated in phenol-red free medium with 5% charcoal-stripped FBS (CCS) for 3 days.

2. Anti-AR antibody affinity protein purification

① Preparation of anti-AR antibody-conjugated column

Material

- BC100 buffer: 20 mM HEPES pH7.6, 10% glycerol, 100 mM KCl, 0.2 mM EDTA
- 0.2 M triethanolamine pH8.2
- DMP: dimethylpimelimidate (PIERCE)
- 0.1 M glycine-HCl pH 2.0
- 50 mM Tris pH 8.0
- Protein G dynabeads

Procedure

Coupling antibody with dynabeads protein G

100 μg AR and mouse IgG antibodies were diluted to final concentration 0.1 μg/μl using BC100, respectively. The dynabeads protein G slurry 200 ul was washed twice using BC100 prior to couple with antibody dilution, mixed well and rotated overnight at 4 ℃.

Crosslinking

The beads after coupling was washed using BC100, followed by 0.2 M triethanolamine (pH 8.2) washed. DMP is unstable in aqueous solution, should be prepared immediately prior use. Hence, to crosslinking Ab to beads, fresh prepared 20 mM DPM/ triethanolamine solution was added and rotated at room temperature (RT) for 1 h. 50 mM Tris-HCl (pH7.5) was added and rotated at RT for 15 min to stop crosslinking reaction and washed using BC100 three times. To remove the free antibodies that were not crosslinking to beads, 0.1 M glycine-HCl (pH 2.0) was added

and removed immediately. 50 mM Tris-HCl (pH 8.0) was used for neutralization.

After washed by BC100 three times, 400 ul of BC100 was added. For storage, added 0.01% NaN3 to beads-Ab solution and stored at 4 ℃.

Validation of crosslinking efficiency

To check the crosslinking efficiency, sampled beads before and after crosslinking. Meanwhile, to check the efficiency of antibody couple with beads, sampled the diluted Ab solution before coupling and the supernatant after coupling. The SDS loading buffer was added to these samples and boiled at 95 ℃ for 5min. After centrifuged, the supernatant were subjected to SDS-PAGE and visualized using silver staining. Before crosslinking, the IgG heavy chain (55 kD) and IgG light chain (25 kD) bands are exhibited in silver staining. After crosslinking, the two bands are unable to be observed. According to this, the crosslinking efficiency can be evaluated.

② Extraction of cell lysate

Material

• TNE buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40

Procedure

1×10^8 cells 22Rv1 cells maintained in 10 cm dish (10 cm dish, 40 plates) were treated with 10 nM DHT for 6 h and harvested using TNE buffer (add protease inhibitor cocktail (Roche), 1 mM Na_3VO_4, 2 mM NaF, 1 mM PMSF prior to use). The lysate were filtered through 0.22 um filter and the protein concentrations were measured using BCA protein assay.

③ Purification of AR-associated proteins using anti-AR antibody-conjugated column

Material

• 0.05%TFA (Trifluoroacetic acid)

• TCA (Trichloroacetic acid)

• Acetone (Keep at 4 ℃ prior to use)

Procedure

Immunoprecipitation

The cell lysate from② were divided to two part and contacted with AR and IgG antibody-conjugated beads gained from①, mixed well and rotated at 4 ℃ for overnight. The supernatant was discarded and beads were washed several times using TNE buffer. To elute the proteins that associated with beads, added 0.05% TFA and incubated at RT for 15 min. The eluate was transferred to new tube, and repeated this procedure again. The twice eluate were combined and subjected to TCA precipitation.

TCA precipitation

For TCA precipitation, the 1 volume of 100% TCA was added to 9 volumes of protein sample, vortex well and incubated at 4 ℃ for 30 min. After centrifuged, discarded supernatant and washed the pellet using cold acetone, repeated twice. Dried pellet until acetone totally evaporated. For silver staining and immunoblotting, added SDS loading buffer, boiled at 95 ℃ for 5 min. If needed, add Tris-HCl (pH 8.8) to neutralize the solution. The sample were visualized by silver staining and excised for LC-MS/MS identification.

Silver staining

The SDS loading buffer mixture sample were subjected to SDS-PAGE electrophoresis using 2-15% gradient gel: MULTIGEL II Mini 2/15 13W (Cosmo Bio, DCB-414855). Proteins that separated in the gel was visualized by silver staining. Silver staining was carried out using SilverQuest™ Silver Staining Kit (Invitrogen, LC6070) according to the manufacturer's instructions.

④ Gel digestion for LC-MS-MS Analysis

Material

(All prepare prior to use)

- Reduction buffer: 10 mM DTT in 100 mM NH_4HCO_3
- Alkylation buffer: 55 mM iodoacetamide in 100 mM NH_4HCO_3
- Trypsin buffer: 3-25 ng/ml Trypsin (Trypsin Gold-Mass Spec Grade, Promega) in 50 mM NH_4HCO_3
- Extraction buffer: 5% formic acid in 50% acetonitrile

Procedure

The gels from silver staining were placed on a clean glass and rinsed with MQ. The bands of interest were excised and the same molecular weight site of IgG gel were also excised for negative control respectively. The gel slices were destained in 50% acetonitrile in 100 mM NH_4HCO_3, lyophilized and rehydrated in 50 ul of 10 mM DTT in 100 mM NH_4HCO_3. After incubated at 56 ℃ for 1 h, the DTT supernatant was removed and 50 ul of 55 mM iodoacetamide solution was added and incubated in dark for 45 min. The supernatant was discarded and the gel slices were lyophilized and digested with trypsin at 37 ℃ for overnight. The tryptic peptides were extracted from gel slices using 5% formic acid in 50% acetonitrile. This solution was lyophilized and the peptides reconstituted in 0.1% formic acid prior to LC-MS/MS analysis.

3. Transfection of cells with plasmids

Transient transfection of cells with plasmids was performed with X-tremeGENE HP DNA Transfection Reagent (Roche) according to the manufacturer's instructions but with slight modifications.

4. Immunoblotting and Immunoprecipitation

For immunoblotting, cell lysates were collected using TNE lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, cOmplete protease inhibitor cocktail (Roche), 1 mM Na_3VO_4, 2 mM NaF, 1 mM PMSF and 10 mM N-ethylmaleimide NEM). For immunoblotting analyses, cell lysates were separated on 7-15% sodium dodecyl sulfate-polyacrylamide (SDS) gel, and then proteins in the SDS gels were transferred to a polyvinylidene difluoride membrane. The membrane were blocked with 3% skim milk in PBST and incubated with primary antibodies overnight at 4 ℃. After wash with PBST three times, the membrane with primary antibodies was incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at RT. Immunoreactive signals were detected with Luminata Western HRP Substrates (Millipore).

For immunoprecipitation, antibodies were added to lysate and incubated at 4 ℃ for 16 h. The immunocomplexes were collected using protein G dynabeads and the beads were washed with TNE buffer five times and visualized by immunoblotting.

For detecting ubiquitinated proteins, immunoprecipitation was performed under denaturing condition. The lysate were added with SDS to a concentration 1% , and then heated at 95 ℃ for 10 min. The lysates were diluted using TNE buffer to reduced SDS concentration and incubated at 4 ℃ for 30 min before antibody was added.

Result

1. Identification of the AR-associated proteins using antibody affinity purification

To identify the novel AR coregulators, the endogenous AR-associated proteins in 22Rv1 cells were purified using AR antibody-conjugated column. The HRPC 22Rv1 cells are derived from human prostatic carcinoma xenograft CWR22R, and widely utilized for HRPC study[47]. It is known that 22Rv1 cells not only express exon3 duplication (code for zinc finger) full-length AR protein but also exhibit 77-kD AR variants. This AR variants lack CTD domain (including ligand binding domain) of AR and are suggested to exert critical function in HRPC progression[49].

The cell lysate from DHT-treated 22Rv1 were incubated with AR antibody-conjugated column, the proteins that associated with antibody were eluated using acids and subjected to TCA precipitation subsequently (Figure 2-1 A). The protein in the extracts were visualized by silver staining, and the specific bands were excised and subjected to LC-MS/MS identification (Figure 2-1 B). Several proteins were identified as AR coregulator candidates including the known AR coregulator histone-arginine N-methyltransferase (PRMT5)

Among these candidates, the molecular weight 126-kD protein band was identified as ubiquitin specific peptidase 7 (USP7), which FDR<1%, peptides number＞10, score on the list top1 showing the high confidence.

USP7 belongs to USP family DUBs that possesses a wide range of substrates including the tumor suppressor protein P53 and its regulator murine double minute (MDM2) as well as the transcription factors FOXO4[50,51,52](Figure 2-2). By virtue of the deubiquitinase activity, USP7 is able to enhance the target protein stability or alter subcellular localization. Additionally, USP7 was reported overexpression in prostate cancer cells[53]. It is supposed that aberrant expression of coregulators contribute to hyperactive AR signaling and associate with prostate cancer progression, suggesting USP7 may exert important role in AR transcriptional regulation.

2. USP7 is involved in androgen-regulated AR ubiquitination

Although several E3 ligases have been identified as AR coregulators, the understanding of DUBs in AR transcriptional regulation is few and far between. Thus, USP7 was focused and further investigated its functional role in androgen-dependent gene expression.

The interaction between USP7 and AR was validated in AR and USP7 overexpression

293T cells using immunoprecipitation (Figure 2-3 A). The endogenous interaction was also confirmed in prostate cancer cell lines LNCaP and 22Rv1 upon DHT treatment (Figure 2-3 B). These results indicated that the interaction between USP7 and AR was androgen-dependent enhanced, implying USP7 is involved in androgen actions.

It was proposed that the ubiquitinated AR generated by androgen is important for AR transcriptional activity. The DUBs USP7 might participate in the ubiquitin network and regulate AR transcriptional activity. To corroborate this speculation, the time course of AR ubiquitination upon DHT stimulation in LNCaP cells was examined. Consistent with previous report, the result demonstrated that the ubiquitinated AR was accumulated upon androgens treatment time course (Figure 2-4 A). In addition, the ubiquitinated AR generated by DHT was abolished by overexpression of USP7. And the AR E3 ligase, MDM2 was used as positive control of this *in vivo* ubiquitination assay (Figure 2-4 B). Whether USP7 is an AR deubiquitinase need to be further investigated by *in vitro* ubiquitination assay.

The result demonstrated that USP7 is a novel AR-associated protein and is involved in AR ubiquitination regulation.

Summary

To revel a novel AR transcriptional regulation mechanism by unraveling the composition of AR transcriptional complexes in prostate cancer cells. The anti-AR antibody affinity purification was applied, and several AR coregulator candidates were identified.

USP7 was focused for further investigation due to its highly identified score and the elusive role of DUBs in AR transcriptional regulation. The endogenous interaction between USP7 and AR was validated, confirming the confidence and reliable of the identification result.

Given that the androgen-produced ubiquitinated AR implicates in AR transactivation, the effect of USP7 on AR ubiquitination was investigated. The result indicated that USP7 abolished androgen-generated ubiquitinated AR, while whether AR is a substrate of USP7 need to be further verified.

It is supposed that the ubiquitinated AR implicated in coactivators recruitment and transactivation coupled-proteasomal degradation. As a result, USP7 deubiquitinates AR may oppose these outcomes of AR ubiquitination, and is involved in AR transcriptional regulation.

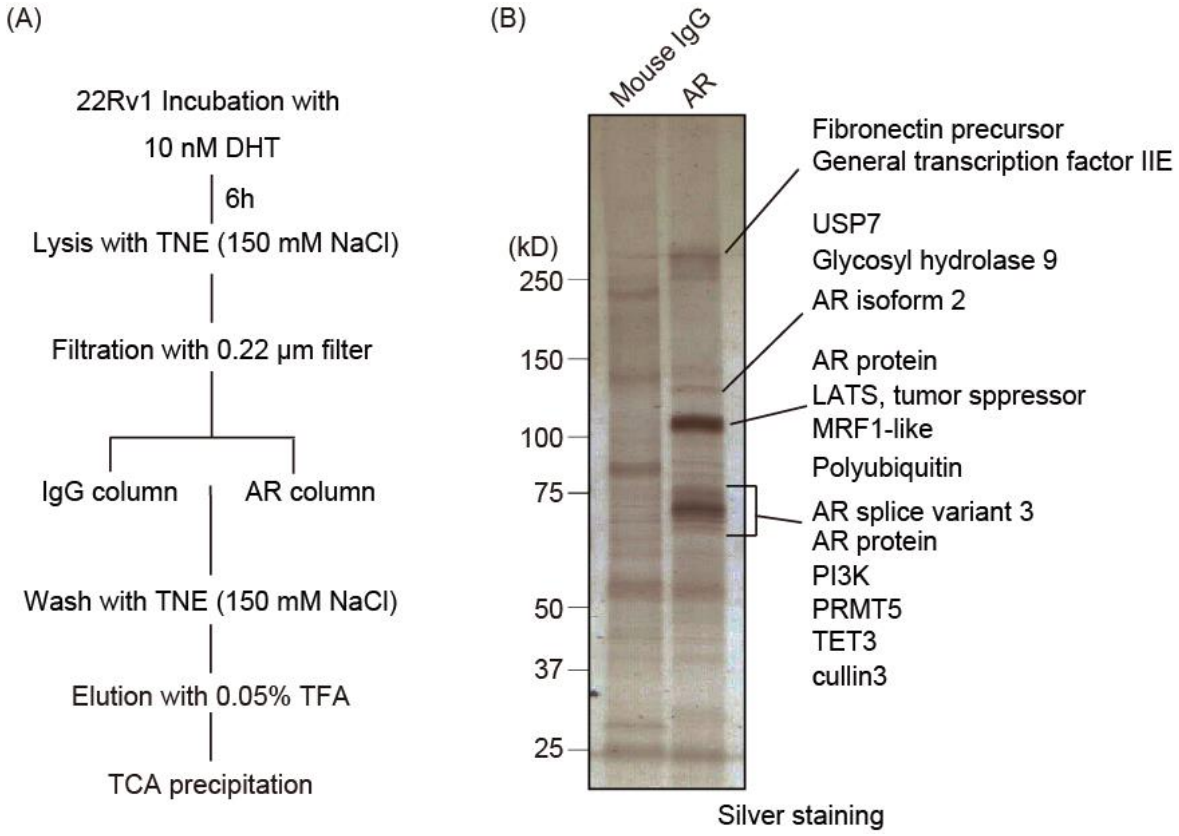
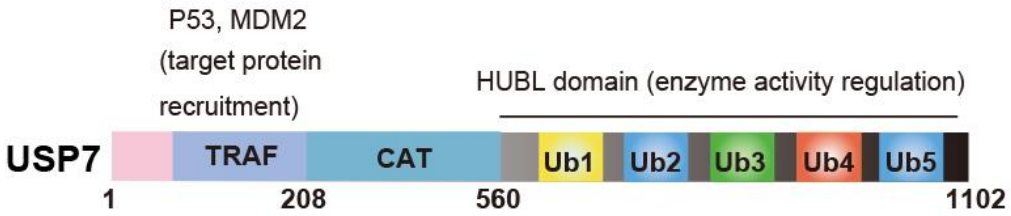


Figure 2-1 The identification of AR-associated proteins in prostate cancer cells using antibody affinity purification

(A) The scheme of antibody affinity protein purification (B) The sample from scheme (A) were visualized through silver staining. These specific bands were excised and subjected to LC-MS/MS identification subsequently.

(A)



(B)

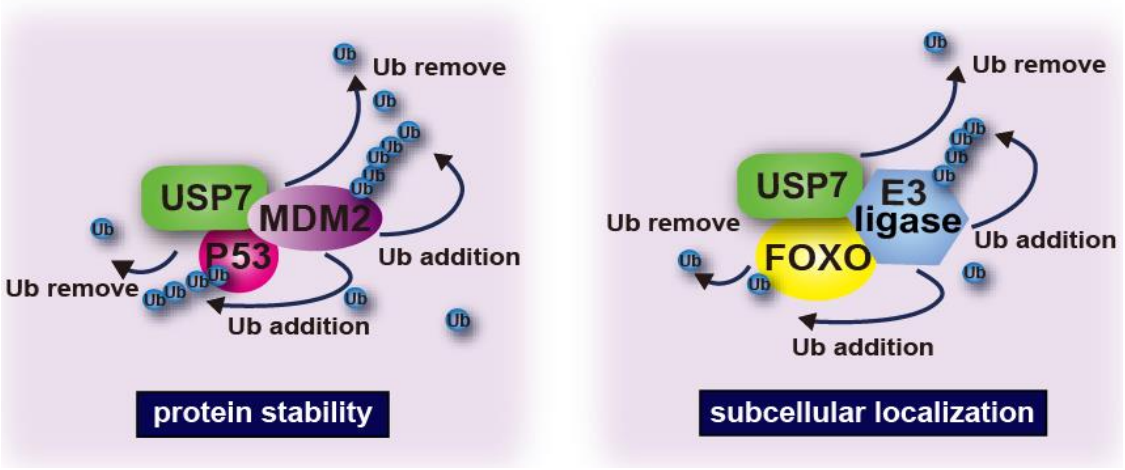


Figure 2-2 USP7 is a deubiquitinating enzyme (DUBs)

(A) The structure domain of USP7. USP7 belongs to USP family DUBs, and comprises TRAF domain that contributes substrates interaction and catalytic domain as well as five UBL domain. (B) The regulator role of USP7 in P53 and FOXO transcription factor. The DUBs USP7 is involved in P53 protein stability and FOXO subcellular location mediation through removing ubiquitin from substrates.

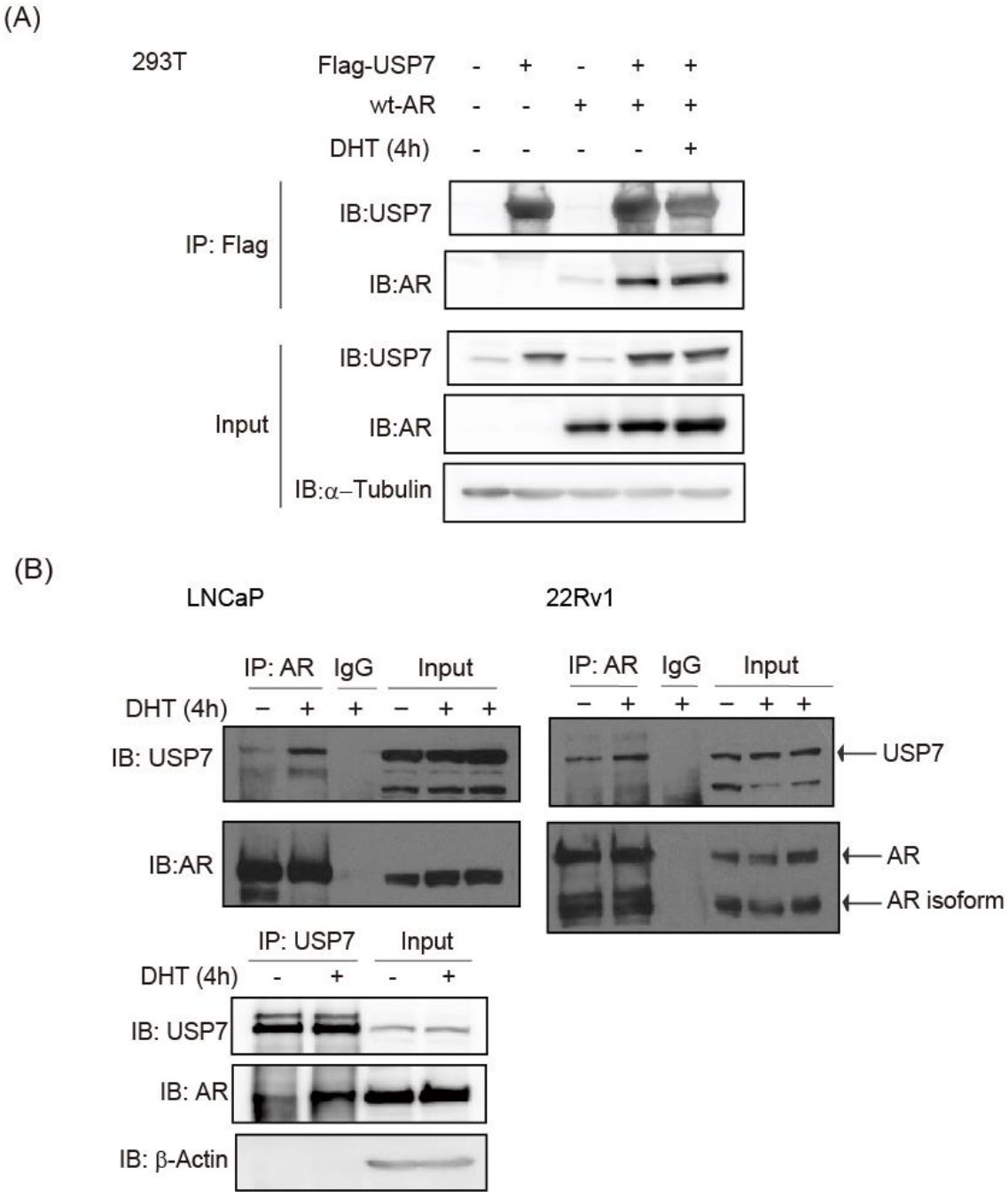


Figure 2-3 Validation of the interaction between AR and USP7

(A) 293T cells were transfected with Flag-USP7 and wt-AR expression vectors for 48 h and followed by 10 nM DHT incubation for 4 h before cells were harvested. Immunoprecipitation assay was adopted using anti-Flag M2 agarose. (B) The endogenous interaction of USP7 and AR was DHT-dependent enhanced in LNCaP and 22Rv1 cells by reciprocal coimmunoprecipitation assays using antibodies as indicated.

(A) (B)

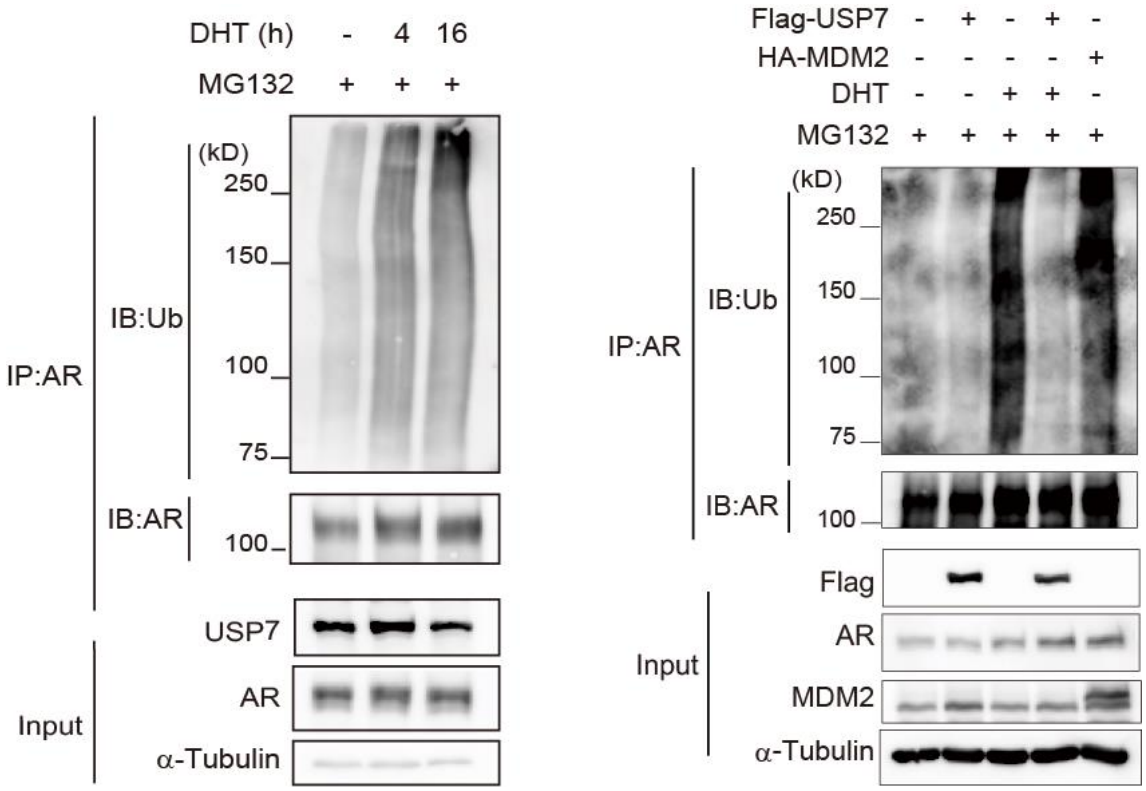


Figure 2-4 The DHT-generated polyubiquitinated AR is abolished by USP7

(A) DHT induced AR polyubiquitination in LNCaP cells. (B) USP7 attenuated ubiquitinated AR generated by DHT. LNCaP cells that treated with 10 nM DHT as indicated time periods (A) or transfected with plasmid as indicated (B) were harvested and subjected to immunoprecipitation using AR antibody under denature condition. The AR E3 ligase MDM2 was utilized as positive control in (B)

Chapter 3

The elucidation of USP7 functional role in AR transcriptional regulation

Overview

To reveal a novel AR transcriptional regulation mechanism, the endogenous AR-associated proteins were biochemically purified and the DUBs USP7 was identified as a novel AR-associated protein that is involved in androgen-regulated AR ubiquitination.

In this chapter the mechanism by which USP7 modulates AR quantitative/ qualitative regulation and affects target gene expression were further investigated. Given that USP7 is indicated mostly localized in nucleus, the study of USP7 in AR transcriptional regulation was focused on chromatin region. The dynamic USP7 recruitment to AREs was observed using ChIP-qPCR.

In addition, to decipher the exhaustive regulation profile by which USP7 in androgen-dependent gene expression, the RNA-seq analysis was adopted in USP7 knockdown LNCaP cells. Finally, the mechanism of USP7 modulates quantitative/ qualitative AR was clarified.

Through the integrated analysis of genome-wide transcripts profiling and the AR quantitative/qualitative regulation, the comprehensive mechanism of AR transcriptional regulation by USP7 was emerged.

Material and method

1. Material

1. Plasmid

ARE-Tk-Luciferase reporter

The ARE-Tk-Luc reporter plasmid was kindly provided by Dr. Suzuki.

2. siRNA

USP7 siRNA

158

GAGCUUUGUCGAGUGUUGCUCGAUA

UAUCGAGCAACACUCGACAAAGCUC

159

GGGAUGGCAAAUGGUGUAAAUUUGA

UCAAAUUUACACCAUUUGCCAUCCC

160

CCGGGACCUGUUAGAAGAAUGUAAA

UUUACAUUCUUCUAACAGGUCCCGG

3. Antibody

① Chromatin immunoprecipitation

AR (441): Santa Cruz Biotechnology (sc-7305) 1: 1000 dilution

USP7: Bethyl Laboratory. (A300-033A) 1: 1000 dilution

4. Reagent

① USP7 inhibitor : P22077 ; TOCRIS (4485) was dissolved in DMSO to make 20 mM for stock

② Cycloheximide: Sigma was dissolved in DMSO to make 50 mg/ml for stock

2. Method

1. Chromatin immunoprecipitation and quantitative PCR

LNCaP were crosslinked with 1% formaldehyde for 10 min, quenched with 125 mM glycine and then sonicated. Solube chromatin was immunoprecipitated using AR, USP7 and control antibodies that crosslinked with protein A dynabeads (Dynal) overnight at 4 ℃. After this, beads were wash several times and eluted with elution buffer (50 mM Tris-HCl, 10 mM EDTA and 1% SDS) for 20 min at 65℃. The eluates were incubated at 65 ℃ overnight to reverse crosslinks and then treated with RNaseA and followed by proteinase K. The samples were purified using PCR purification kit (Qiagen). Quantitative PCR was performed using KAPA SYBR Fast qPCR kit (Kapa Biosystems)

on an ABI 7500 PCR system (Applied Biosystems).

As for Re ChIP, the first-round antibody-crosslinked beads was added to chromatin extracts and incubated overnight at 4 ℃. The bounded protein complexes were eluted by 10 mM DTT at 37 ℃ for 30 min, and the elution was then diluted ten times and subsequently reimmunoprecipitated by second-round or control IgG antibodies overnight at 4 ℃. The results were presented as fold-enrichment over control ChIP or as the percentage of input chromatin that was precipitated. Primers used in this study were listed :

FKBP5

5′- CTTCACGCCTGTGTTGCTTTTA-3′and

5′-AGGGTGCAGGACGTTCCA-3′

PDE9A (B41)

5′- GCCTCCCCCGTGCAG-3′and

5′-TGCAAGGCACGTCTCAATTC -3′

PSA (AREIII)

5′- TGGGACAACTTGCAAACCTG-3′and

5′- CCAGAGTAGGTCTGTTTTCAATCCA -3′

TMPRSS2 (AREV)

5′- TGGTCCTGGATGATAAAAAAAGTTT-3′and

5′- GACATACGCCCCACAACAGA-3′

FKBP5-Ctril

5′- CTCTTTCAACTGAGGCCTGG-3′and

5′- CTCCCAAAGTGCTGGGATTA-3′

PSA-Ctril

5′- TAGCTGGGACTACAGGCACC-3′and

5′- TAATCCCAGCACTTTGGGAG-3′

2. RNA isolation and RT-PCR

Total RNA was isolated using Trizol (Invitrogen) and Nucleospin RNA II (Macherey-Nagel) following the manufacturer's instruction. Synthesis of complementary DNA was performed using ReverTra qPCR RT Master Mix (TOYOBO). cDNA was amplified with KAPA SYBR fast qPCR kit (Kapa Biosystem) on an ABI 7500 real time PCR system (Applied Biosystem). Primer sequences are listed:

BMF

5′-GAGGTACAGATTGCCCGAAA-3′

5′-TTCAAAGCAAGGTTGTGCAG-3′

CDC6

5′-ACCTATGCAACACTCCCCATT-3′

5′-TGGCTAGTTCTCTTTTGCTAGGA-3′

CLDN8

5′-CGGCTGGAATCATCTTCATCA-3′

5′-TTGGCAACCCAGCTCACAG-3′

E2F1

5′-TCCAAGAACCACATCCAGTG-3′

5′-CTGGGTCAACCCCTCAAG-3′

FKBP5

5′-GCGGAGAGTGACGGAGTC-3′

5′-TGGGGCTTTCTTCATTGTTC-3′

HES6

5′-AGCCCCTGGTGGAGAAGA -3′

5′-CAGCACTTCGGCGTTCTC -3′

NRDG1

5′-GTGGAGAAAGGGGAGACCAT-3′

5′-ACAGCGTGACGTGAACAGAG-3′

PDE9A

5′-GATCCCAATGTTTGAAACAGTGAC-3′

5′-TCCCAAAGTGGCTGCAGC-3′

PSA

5′-TGTGTGCTGGACGCTGGA-3′

5′-CACTGCCCCATGACGTGAT-3′

SLC44A1

5′-TTTCCTGCTATGCCAAGTTTGC -3′

5′-CCAGAATCGTTAAGATCCACACA -3′

SLC45A3

5′-GGCCCGGAGACACTATGAT -3′

5′-CCATGACCAGAGAGAAGACCA -3′

TMPRSS2

5′-GGACAGTGTGCACCTCAAAGAC-3′

5′-TCCCACGAGGAAGGTCCC-3′

UTG2B15

5′-GTG TTG GGAATA TTATGACTACAGTAA C -3′

5′-GGGTATGTT AAATAGTTCAGCCAGT -3′

UTG2B17

5′-TGA CTT TTGGTTTCAAGC ATA -3′

5′-TTCCATTTCCTTAGGCAAGGG -3′

3. RNA-Seq analysis.

After transfection with USP7 siRNA for 72 h, LNCaP cells were exposed to 10 nM DHT or vehicle for 16 h and harvested for RNA-seq analysis. Total RNA was isolated using Trizol (Invitrogen) and Nucleospin RNA II (Macherey-Nagel) following the manufacturer's instruction. One microgram of mRNA was used for library preparation using Illumina TruSeq RNA sample Preparation v2 kit (TruSeq) according to the manufacturer's instructions, followed by sequencing on the Illumina HiSeq2500 platform. The data graphic were made by R. (http://www.R-project.org.) Gene Ontology (GO) analysis was performed using the web tool Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/)

4. Cell proliferation assay

LNCaP cells maintained in 10 cm dish were transfected with USP7 siRNA for 24 h before cells were seeded in 12 well plate at a density of 6×10^4 cells/well, and incubated in RPMI 1640 (phenol-red free medium) containing 5 % charcoal stripped FBS (As 0 h). The cell number was counted using Neubauer Chamber at 48 h and 96 h.

5. Luciferase assay

The reporter assay is performed using Dual-luciferase Reporter assay system (Promega). The cells maintained in 10 cm dish were seed in 12 well plate for 18 h and co-transfected with ARE-tk-containing luciferase reporter (100 ng/well) and wt-AR (20 ng/well) and flag-USP7 (50 ng/well) expression vectors for 24 h. The cell were harvested and subjected to luciferase assay following the manufacturer's instruction.

Result

1. USP7 associates with AR on AREs upon rapid DHT stimulation

Given that USP7 is proposed mainly localized in nucleus, the investigation was focused on chromatin region. To observe the time course of USP7 recruitment to AREs, the chromatin immunoprecipitation with qPCR assay was performed in DHT-treated LNCaP cells. Considering the long-term sustained model, supposing that AR recruitment to AREs parallels with coactivators such as p300 and SRC1 over 16 h. The observation of the AR and USP7 recruitment to the well characterized AR-target genes: PSA, PDE9A and FKBP5 gene AREs was at DHT stimulation 0, 1, 4 and 16 h.

The recruitment of AR to AREs were gradually increased after DHT treatment and the accumulation was maintained until 16 h although with decline (Figure 3-1 A). Contrast to AR, the recruitment of USP7 reached the summit upon DHT treatment 1 h or may be earlier and declined rapidly, suggesting the short duration of USP7 associated with AR on chromatin (Figure 3-1 B).

To further confirm the association of USP7 and AR on AREs, Re ChIP assay was adopted in DHT-stimulated for 1 h LNCaP cells. As shown in Figure 3-2, the association of USP7 and AR on FKBP5 and PSA gene AREs was enhanced upon DHT stimulation.

Together, these results exhibited that USP7 and AR form complex on AREs upon short-term DHT stimulation and suggested that USP7 may participate in AR transcriptional activation early stage.

2. The recruitment of USP7 to AREs is AR dependent

Since USP7 associated with AR on AREs upon DHT treatment, to examine the binding of USP7 to AREs is dependent on AR or not, the pure AR antagonist bicalutamide was used. Bicalutamide inhibits receptor activation and chromatin recruitment by preventing the binding of endogenous androgens to AR.

LNCaP cells were pretreated with bicalutamide and followed by DHT stimulation, the DNA bound USP7 and AR was examined by ChIP-qPCR assay. The inhibition effect of bicalutamide on AR chromatin recruitment was confirmed in Figure 3-3. Parallel with AR, the DHT-enhanced recruitment of USP7 to PSA, PDE9A and FKBP5 gene AREs were diminished (Figure 3-4). This result indicated the AR-dependently recruitment of USP7 to AREs.

3. USP7 is involved in androgen-dependent gene transcription

The previous data revealed that USP7 and AR form complex on AREs is in ligand

dependent manner, suggesting USP7 may cooperate with AR to regulate target gene transcription.

To corroborate this speculation, the effect of USP7 overexpression on ARE-containing luciferase reporter activity was investigated. The result revealed that the DHT-induced ARE-containing reporter activity was inhibited by overexpression of USP7 in 293T and 22Rv1 cells (Figure 3-5 A). Consistent with this result, USP7 knockdown activated the ARE-containing reporter activity (Figure 3-5 B), showing the corepressor role of USP7 in AR-dependent gene transcription.

To further examine the role of USP7 in androgen-dependent genes transcription, I observed the effect of P22077, a recently identified small-molecule USP7 inhibitor on DHT-stimulated the known androgen inducible genes mRNA expression in LNCaP cells[53]. The time course of several AR-target genes mRNA expression stimulated by 10 nM DHT in LNCaP cells was validated, and 8 h was chosen for the subsequent analysis (Figure 3-6). Contradict to the data that USP7 trans-repressed ARE-reporter activity, P22077 dose dependently inhibited the DHT-stimulated expression of PSA and FKBP5 mRNA (Figure 3-7).

Due to the reporter assay cannot reliably reflect the AREs chromatin environment within cells, the controversial effect of USP7 on ARE-reporter activity and androgen-inducible gene mRNA expression suggested the epigenetic enzymes network and the cross-talk between the AREs chromatin specific marks are critical for USP7 function.

Together, the contradictive results revealed that USP7 is involved in androgen-dependent gene transcription and may mediate AR transcriptional regulation through multiple pathways.

4. USP7 specificity regulates a subset of androgen-dependent genes

Recent evidences supported that many AR coregulators function in gene-specific manner, such as RNF6 specificity affects a subset of AR target gene[41]. Additionally, the contradictive effect of USP7 on ARE-reporter activity and DHT-induced gene mRNA expression suggested that USP7 might exert diverse functions on AR-target gene regulation. Hence, to gain the comprehensive profile of USP7 in AR transcriptional regulation, and identify the USP7-dependent genes, the effect of USP7 knockdown on LNCaP cells transcriptome profiling was analyzed using RNA-sequencing (RNA-seq).

Three different USP7 specific siRNA oligonucleotides were used to knock down USP7 in LNCaP prostate cancer cells and the protein expression was examined by immunoblotting (Figure 3-8 A). The effect of the three USP7 siRNA on PSA mRNA expression was confirmed by qPCR. Consistent with the effect of USP7 inhibitor P22077,

DHT-stimulated PSA mRNA expression was attenuated in three USP7 siRNA transfected cells (Figure 3-8 B). The oligonucleotide 160 was used for subsequent RNA-seq analysis.

The expression of USP7 in LNCaP prostate cancer cells was knockdown by siRNA and treated with 10 nM DHT for 16 h in hormone starvation condition before RNA extraction. The isolated RNA were subjected to RNA-seq to prolife the transcriptome (Figure 3-9 A) and protein expression including USP7 and PSA were examined using immunoblotting (Figure 3-9 B).

Total 11460 transcripts were mapped and quantified by RNA-seq and their quantitative result were visualized using heatmap (Figure 3-10). Knockdown of USP7 resulted in profound global gene transcripts variations in DHT-treated cells. Androgen-response genes in LNCaP cells are mainly regulated by AR, suggesting the importance of USP7 in AR transcriptional regulation. In addition, several know AR target genes were filtered and visualized using heatmap. Among DHT-activated genes, knockdown of USP7 had significant effect on a subset of gene expression, including FAM111B, KLK2, KLK3, FKBP5, E2F1, MCM2, 4, 6, 7 and CDC6. Meanwhile, USP7 knockdown appeared to have little or no effect on another subset of AR target genes including TMPRSS2, SLC45A3, PMEPA1 and CLDN8. On the other hand, among DHT-repressed genes, knockdown of USP7 derepressed BMF, CXCR7 and TRPC4 but was without significant effect on UTG2B15 and UTG2B17 (Figure 3-11). The result revealed that USP7 specifically regulates a subset of androgen-dependent genes.

To identify the USP7-regulated AR target gene, the gene exhibited at least 1.5 fold alteration after DHT stimulation (relative to vehicle) were filtered as androgen-dependent genes. According to this, 332 genes were defined as DHT up-regulated and 189 genes were defined as DHT down-regulated, the others (10938) were define as stable. Meanwhile, the gene exhibited greater than 1.5 fold alteration by USP7 knockdown (relative to mock) were filtered as USP7-regulated (Figure 3-12). The overlapping genes between USP7-regulated and androgen-dependent were represented using Venn diagram (Figure 3-13).

In the absence of DHT, total 705 genes were differently expressed by USP7 knockdown including 399 down-regulated and 306 up-regulated, revealing the wide range of biological effects by which USP7. Among the USP7-regulated genes (including down- and up- regulated), approximately 30% overlapped with androgen-dependent (including down- and up- regulated), suggesting these overlapping genes were regulated independent of AR signaling (Figure 3-13 A).

To further observe the USP7 knockdown effect on androgen up-regulated and down-

regulated gene expression under androgen signaling, respectively. Approximately 54% (179 genes) of DHT up-regulated genes were decreased by USP7 knockdown, revealing the coactivator role of USP7. Contrast to this, knockdown of USP7 derepressed 28% (54 genes) of DHT down-regulated genes (Figure 3-13 B), implying the corepressor role of USP7.

Given that the mechanism of AR mediated-gene activation is distinct from repression, supposing USP7 is involved in AR transcriptional regulation via multiple pathways. Although the repression function by which USP7 need to be further verified, the data of USP7 trans-repressed the ARE-containing reporter activity supported the corepressor role of USP7 (Figure 3-5). This result revealed the requirement of USP7 in androgen-dependent gene regulation and indicated that USP7 is a gene specific AR coregulator.

To further confirm the importance of USP7 in androgen-dependent gene expression, I compared the effect of USP7 knockdown on DHT-stimulated gene expression. Knockdown of USP7 significantly abolished the DHT induction fold among androgen-dependent gene (down-regulated and up-regulated) but not stable, revealing the essential role of USP7 in androgen-dependent gene regulation (Figure 3-14).

The result of RNA-seq analysis was validated by qPCR to examine several androgen-activated genes including PSA, FKBP5 and PDE9A (Figure 3-15, 3-16), as well as androgen-repressed genes such as BMF and SLC44A1 (Figure 3-17).

The RNA-seq analysis of transcriptome profiling shown that USP7 affected a subset of androgen-dependent gene expression, to assess the biological function of USP7 in androgen signaling, the overlapping genes between androgen-dependent and USP7-regulated were subjected to gene ontology (GO) analysis using DAVID.

The analysis of the 179 overlapping gene between androgen up-regulated and USP7-regulated gene exhibited significantly enriched within several biological process including DNA replication and cell cycle regulation (Figure 3-18) revealing the essential role of USP7 in androgen signaling.

Together, these data exhibited the essential role of USP7 in AR transcriptional regulation, and is important for a specific subset of androgen-dependent gene expression that are highly associated with several androgen-mediated biological process such as DNA replication.

5. USP7 facilitates AR chromatin recruitment

Although the transcriptome profiling indicated the gene specific regulation manner of USP7, given that the androgen-activated gene expression mechanism is well established, the mechanism of USP7 mediates androgen-activated gene expression was investigated.

The previous data shown that inhibition and knockdown of USP7 abolished a subset of androgen inducible gene mRNA expression including FKBP5, PSA and PDE9A. To examine whether this gene expression inhibition results from affecting AR chromatin binding. The effect of USP7 inhibitor P22077 on AR recruitment to FKBP5 gene AREs was observed. Consistent with the mRNA expression, the recruitment of AR to FKBP5 gene AREs was reduced (Figure 3-19 A). In addition, USP7 knockdown also abolished the binding of AR to FKBP5, PSA and PDE9A gene AREs (Figure 3-19 B), supposing USP7 mediates AR transcriptional activity by facilitating AR chromatin recruitment.

6. USP7 regulates liganded AR protein stability

The data of USP7 overexpression attenuated ubiquitinated AR that generated by DHT, supposing USP7 may be involved in receptor stabilization thereby facilitates AR chromatin recruitment. To investigate whether USP7 mediates AR protein stability, the effect of USP7 overexpression on wt-AR protein degradation rates in the absence or presence of DHT was observed. The protein synthesis inhibitor cycloheximide (CHX) was added in 293T cells, and the wt-AR protein degradation rates were visualized using immunoblotting(Figure 3-20).

The result revealed that overexpression of USP7 was without obvious effect on AR protein stability in the absence of DHT. Upon DHT stimulation, the liganded AR protein half-life was significantly prolonged in USP7 overexpression cells, suggesting USP7 is involved in liganded AR protein stability regulation.

7. USP7 is required for prostate cancer LNCaP cell proliferation

It is well accepted that the transcriptional output of AR contribute to prostate cancer cell proliferation. Given the essential role of USP7 in androgen-dependent gene expression, suggesting USP7 may regulate prostate cancer cell proliferation. To assess the biological function of USP7 in androgen-stimulated prostate cancer cell growth, the cell proliferation assay was performed in USP7 knockdown LNCaP cells.

The result displayed that DHT-stimulated cell growth was severely diminished by USP7 knockdown (Figure 3-21). In addition, knockdown of USP7 also attenuated the cell growth in the absence of DHT, suggesting USP7 can regulate prostate cancer cell proliferation through AR-independent pathway. Consider to the RNA-seq analysis of USP7 knockdown affected wide range of gene expression independent of AR, implying USP7 can mediate prostate cancer cell proliferation through AR-dependent and -independent signaling.

Summary

In this chapter, the functional role of USP7 in AR transcriptional regulation was investigated. Upon DHT stimulation, USP7 was recruited to AREs and cooperated with AR to regulate gene transcription. The result of transcriptome profiling in LNCaP cells revealed the essential role of USP7 in androgen-dependent gene expression, approximately 54% of androgen up-regulated gene were affected by USP7 knockdown. Among the androgen inducible genes, USP7 may mediate gene expression via regulation of chromatin receptor ubiquitin-proteasomal degradation and facilitate AR chromatin binding.

In addition, among androgen down-regulated gene, 28% were derepressed by USP7 knockdown, although the exact role of USP7 in androgen down-regulated gene need to be further verified. The result suggested the gene specific regulation manner of USP7. This study not only exhibits the comprehensive role of USP7 in AR transcriptional regulation but also broadens the perspective of ubiquitin network in AR actions.

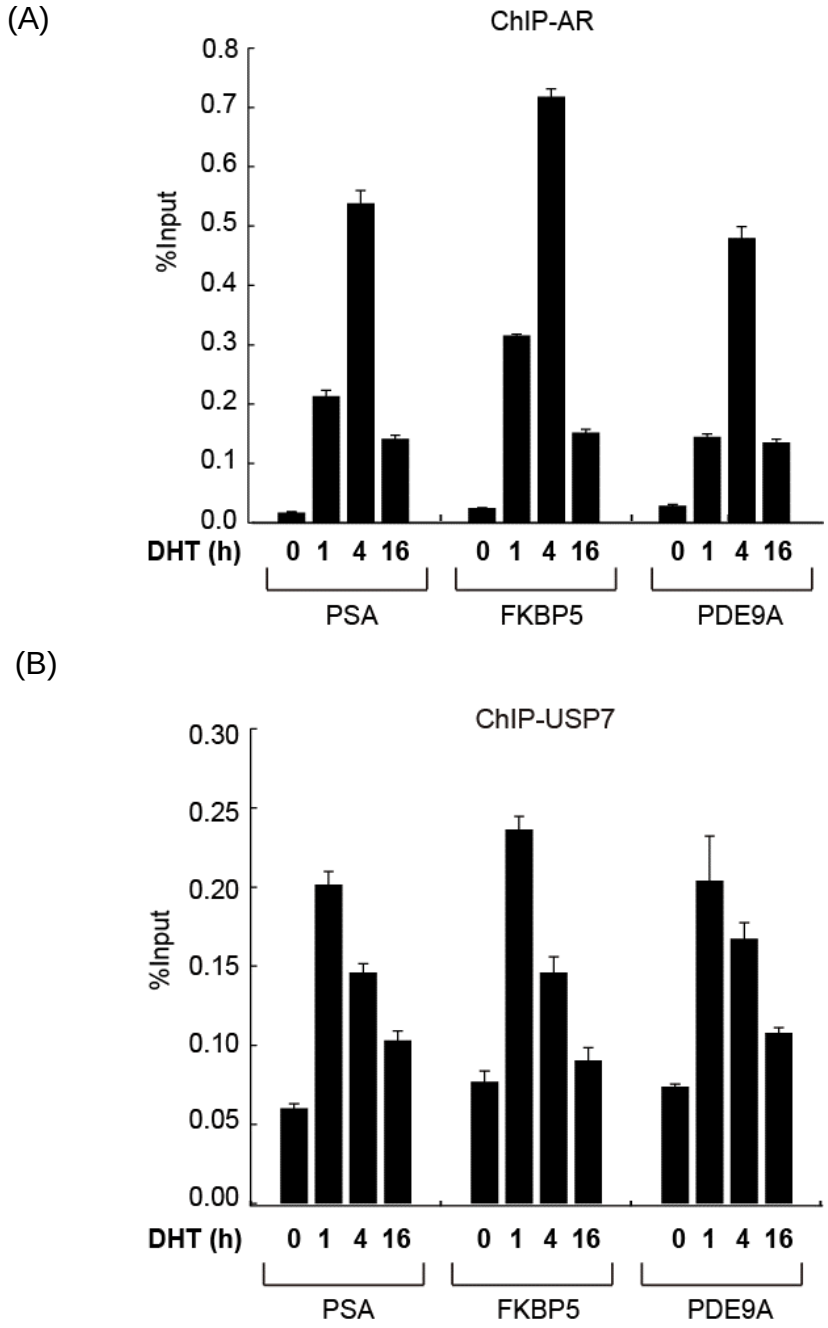


Figure 3-1 The time course recruitment of AR and USP7 to PSA, FKBP5 and PDE9A gene AREs

LNCaP cells were treated with 10 nM DHT for 0, 1, 4, 16 h and the DNA bound to AR (A) and USP7 (B) were measured by ChIP-qPCR.

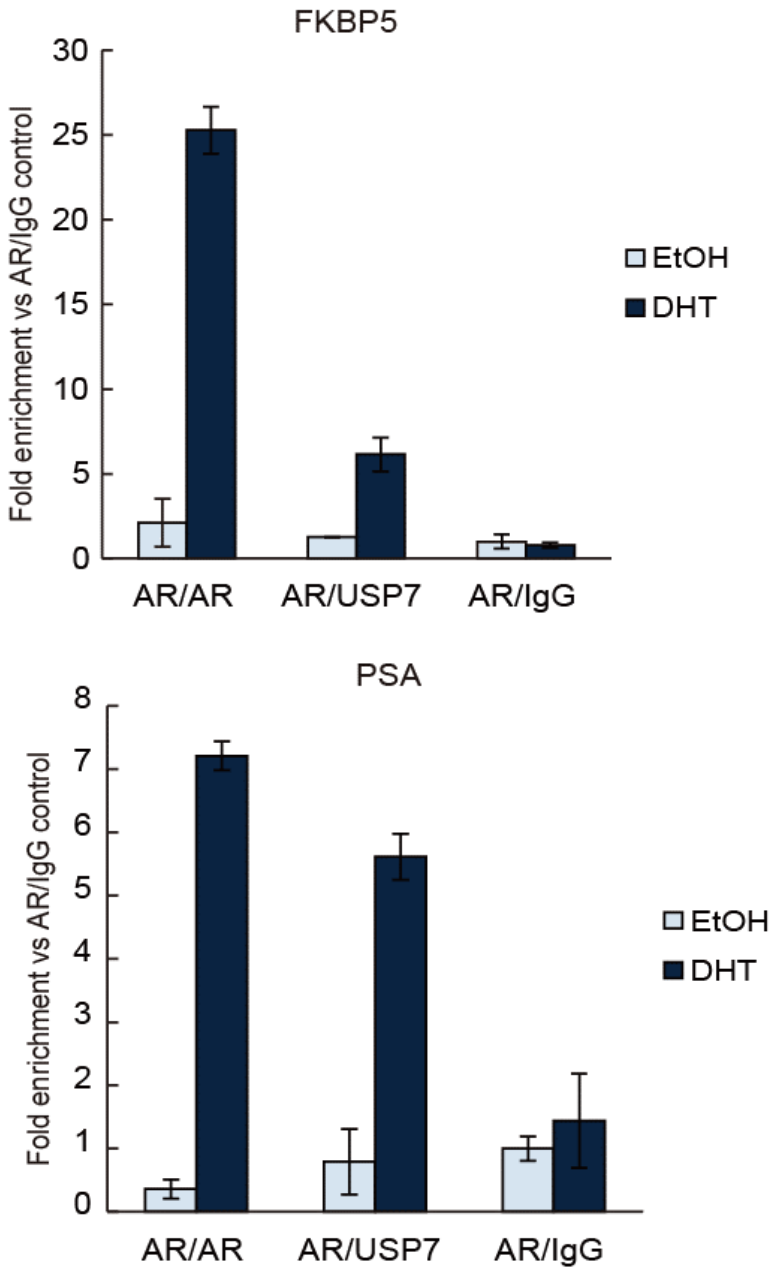


Figure 3-2 USP7 associates with AR on FKBP5 and PSA gene AREs is DHT-dependent enhanced

The Re ChIP assay was performed in DHT-treated 1 h LNCaP cells using anti-AR antibody and followed with second-round antibodies as indicated.

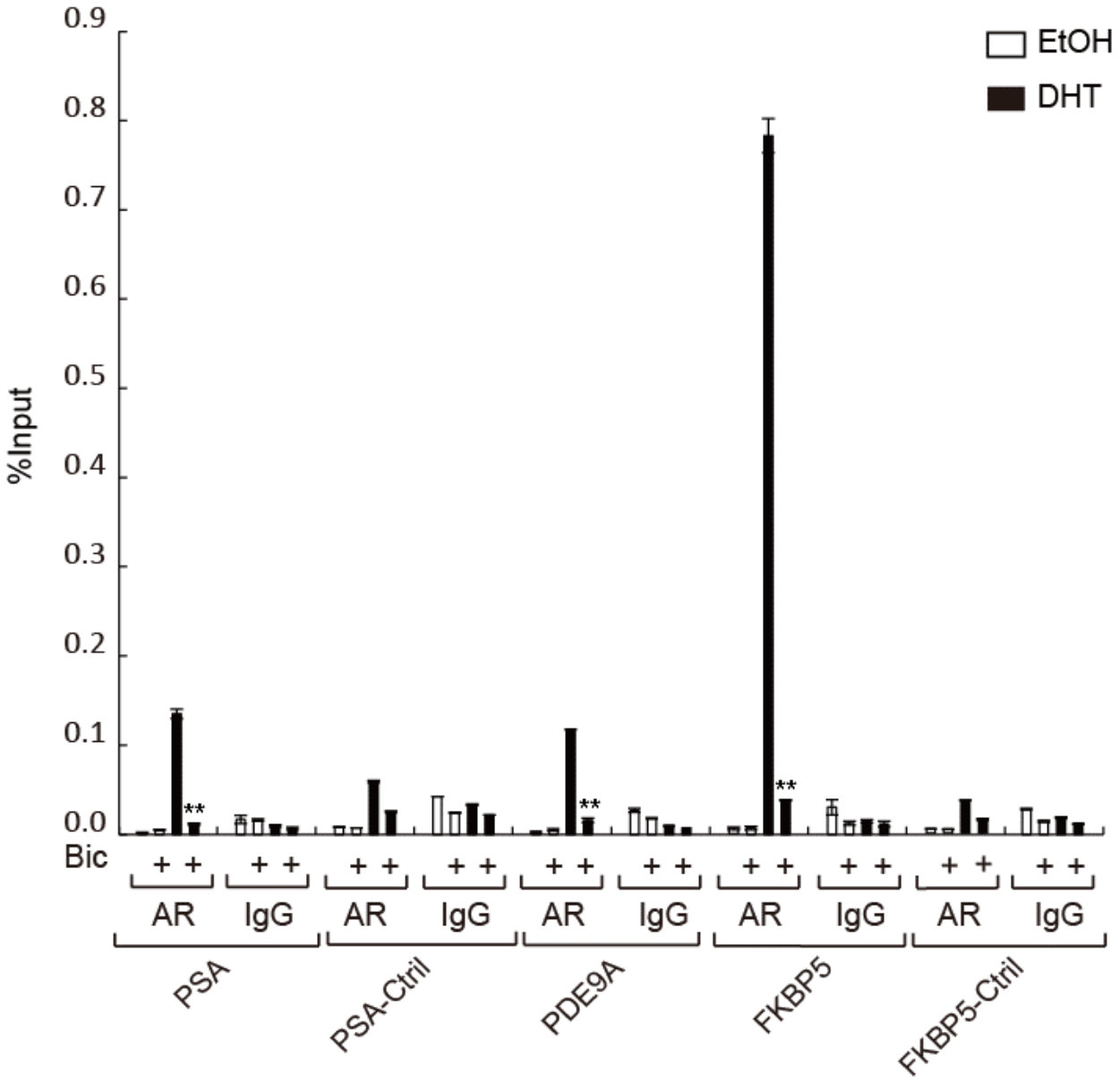


Figure 3-3 The recruitment of AR to AREs is attenuated by AR antagonist in LNCaP cells

LNCaP cells were pretreated with bicalutamide (Bic) 10 μM for 2 h and then treated with EtOH or DHT for 4 h. The ChIP-qPCR was performed using anti-AR antibody to observe the AR recruitment to PSA, PDE9A and FKBP5 gene. The random DNA sequence near the PSA and FKBP5 AREs were used as negative control.(**, $p<0.01$, two-tailed t-test)

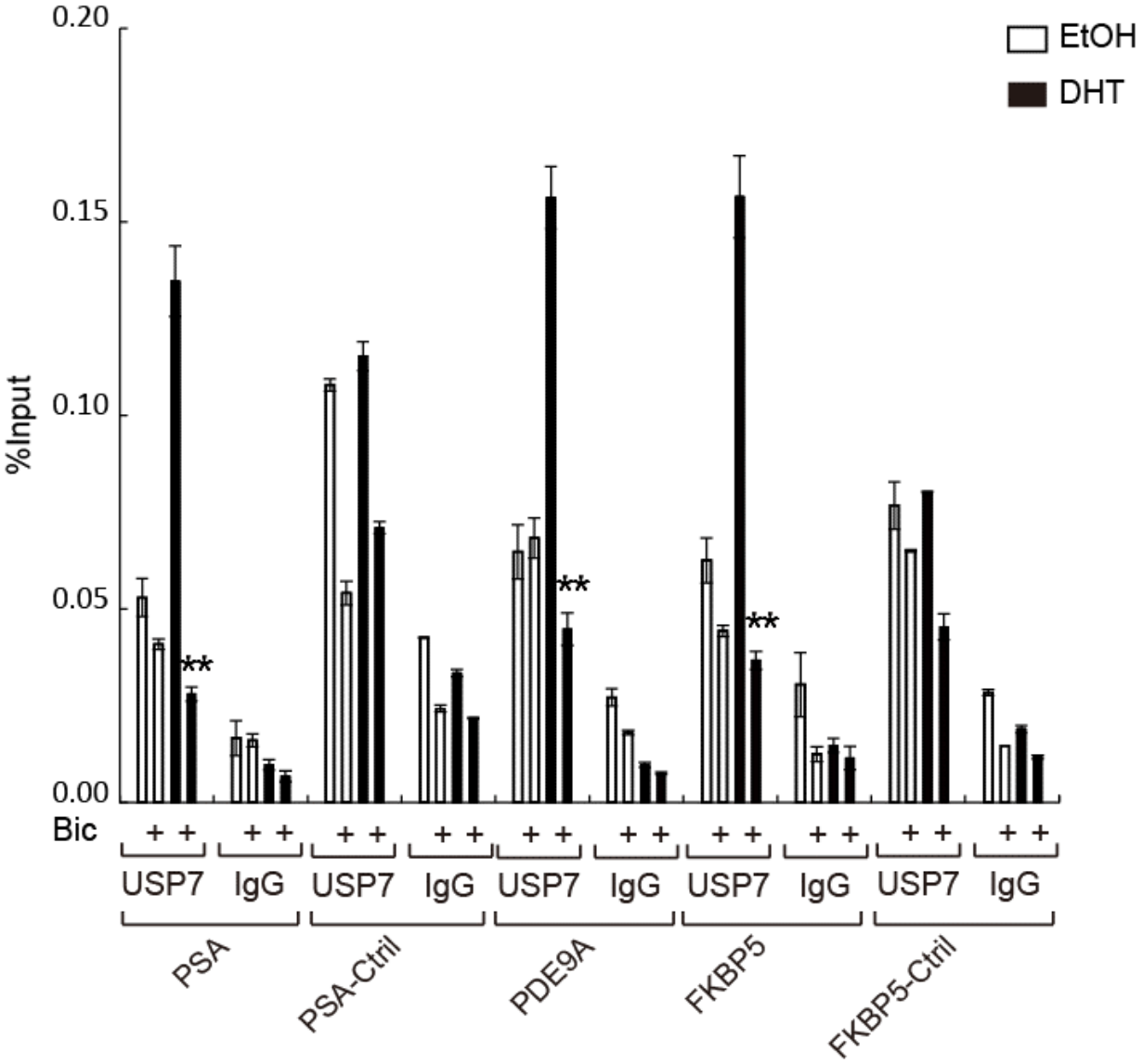


Figure 3-4 The recruitment of USP7 to AREs is attenuated by AR antagonist in LNCaP cells

LNCaP cells were pretreated with bicalutamide (Bic) 10 μM for 2 h and then treated with EtOH or DHT for 4 h. The ChIP-qPCR was performed using anti-USP7 antibody to observe the USP7 recruitment to PSA, PDE9A and FKBP5 gene. (**, $P<0.01$, two-tailed t-test)

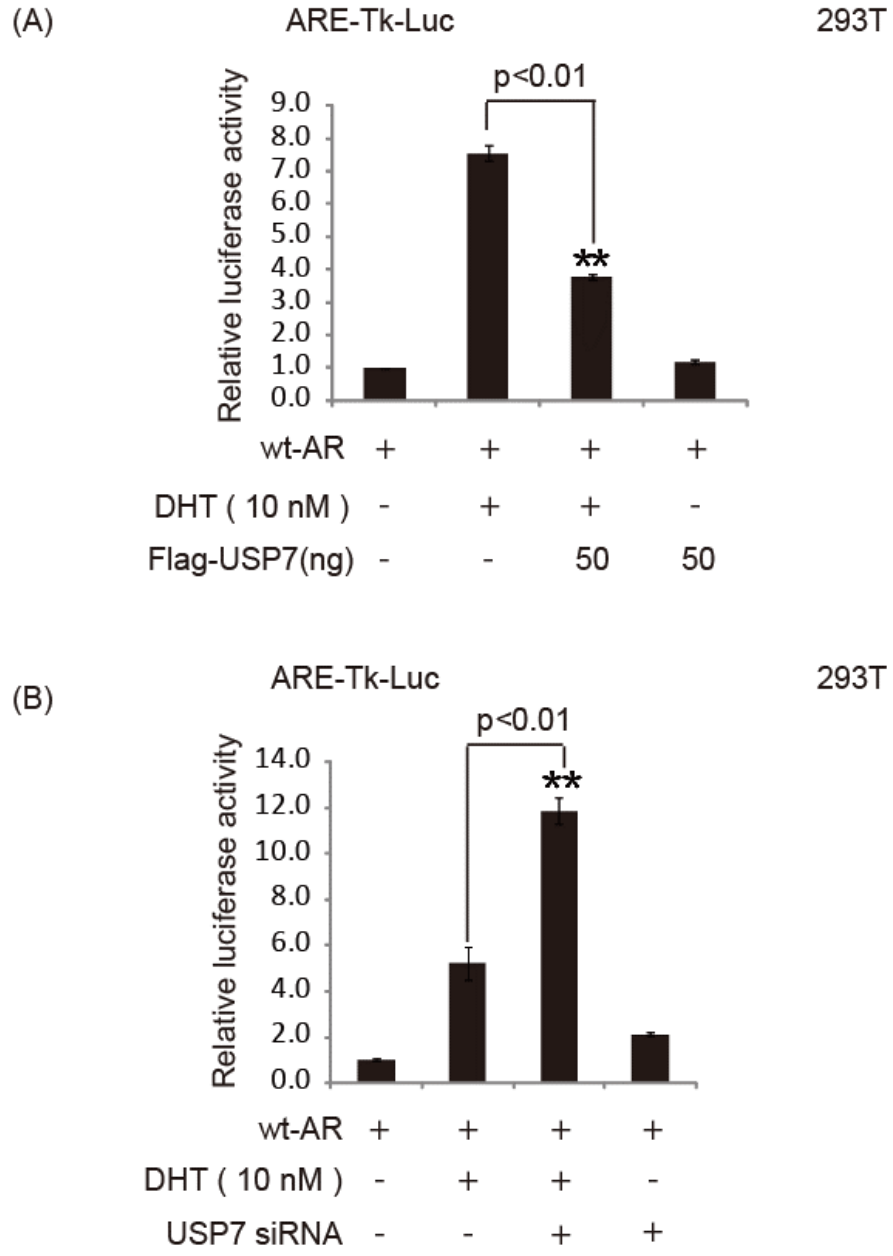


Figure 3-5 USP7 squelches AR-dependent transcription from an ARE-regulated Luciferase reporter

(A) 293T cells were transfected with plasmids as indicated for 24 h and followed by 18 h DHT treatment. The cell lysate were subjected to luciferase assay. (B) 293T cells were transfected USP7 siRNA for 72 h and followed by plasmids as indicated transfection for 24 h and subsequently treated with 10 nM DHT for 18 h before cell harvested. The cell lysate were subjected to luciferase assay. Data is shown relative to wt- AR transfected alone whose value was set at 1. **, $p<0.01$, two-tailed t-test)

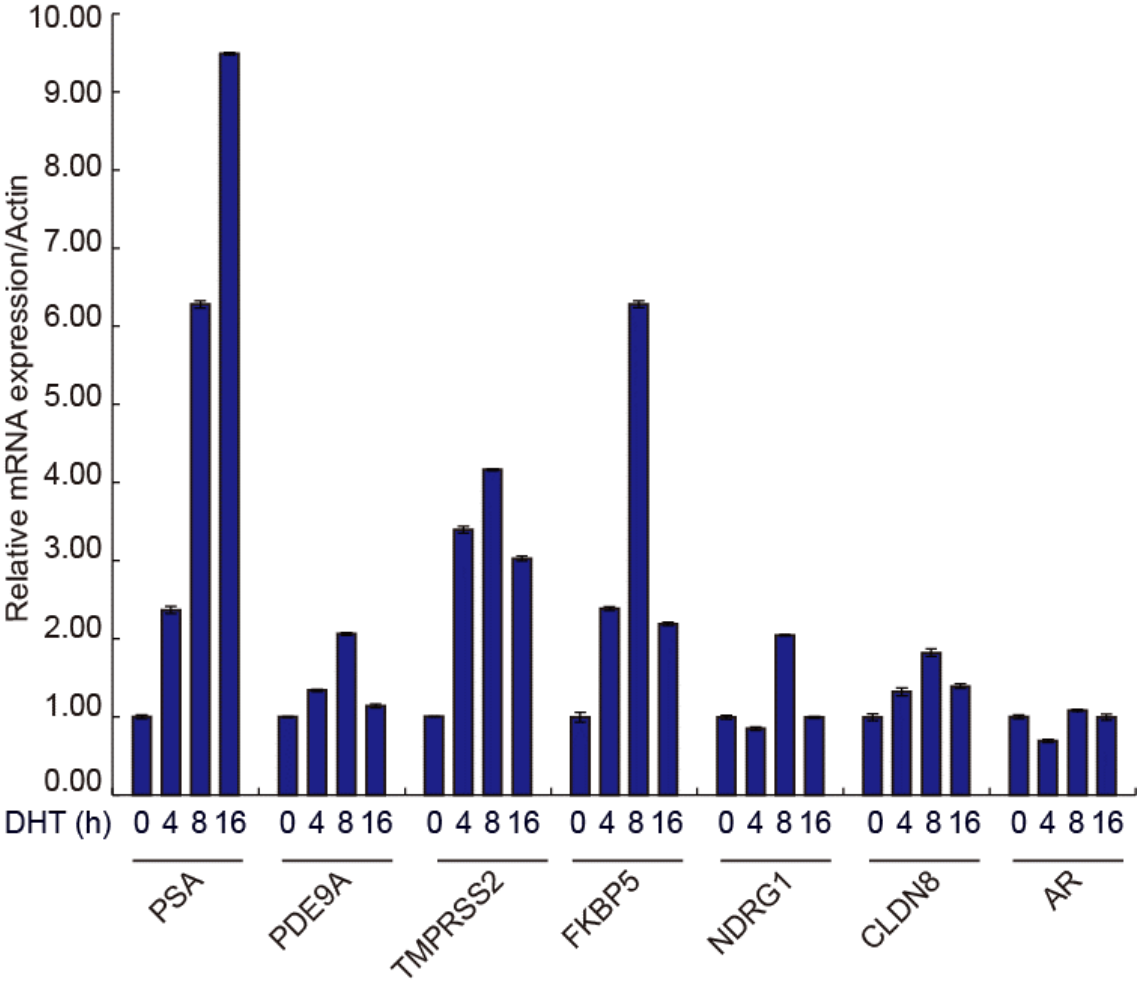


Figure 3-6 Validation the time course of AR-target genes mRNA expression stimulated by 10 nM DHT in LNCaP cells

LNCaP cells were maintained in phenol-red free medium with 5% CCS for 72 h and treated with 10 nM DHT for indicated time periods. The cells were harvested and the isolated RNA were measured using qPCR.

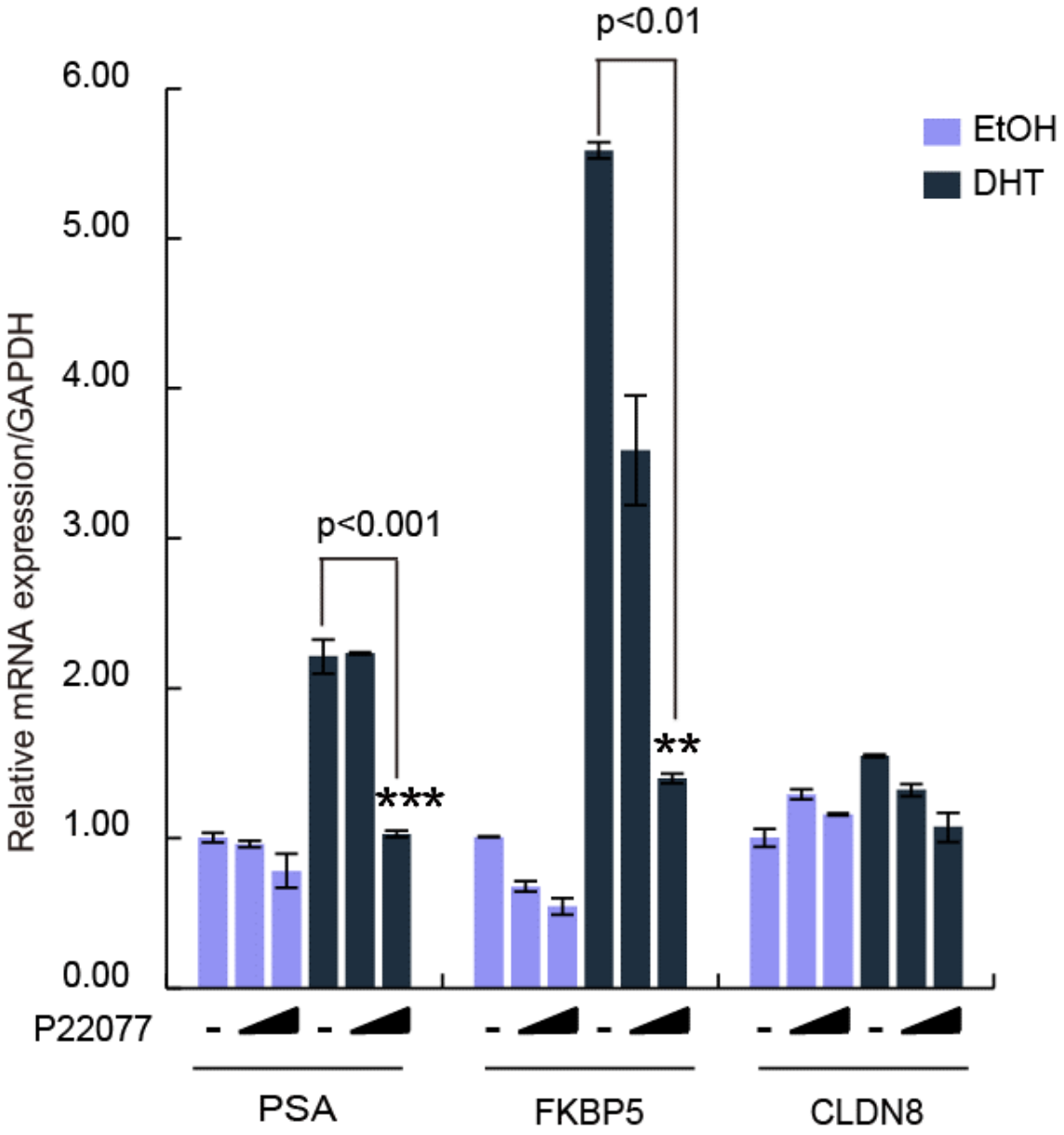


Figure 3-7 Effect of USP7 inhibitor P22077 on DHT-activated AR target genes mRNA expression

The mRNA expression level of AR-target genes PSA, FKBP5 and CLDN8 in LNCaP cells were determined by qPCR and normalized against expression level of the GAPDH gene in the absence or presence of DHT for 8 h upon pretreated with USP7 inhibitor P22077 with 10 uM or 20 uM for 30 min, respectively. (**, $p<0.01$, ***, $p<0.001$, two-tailed t-test)

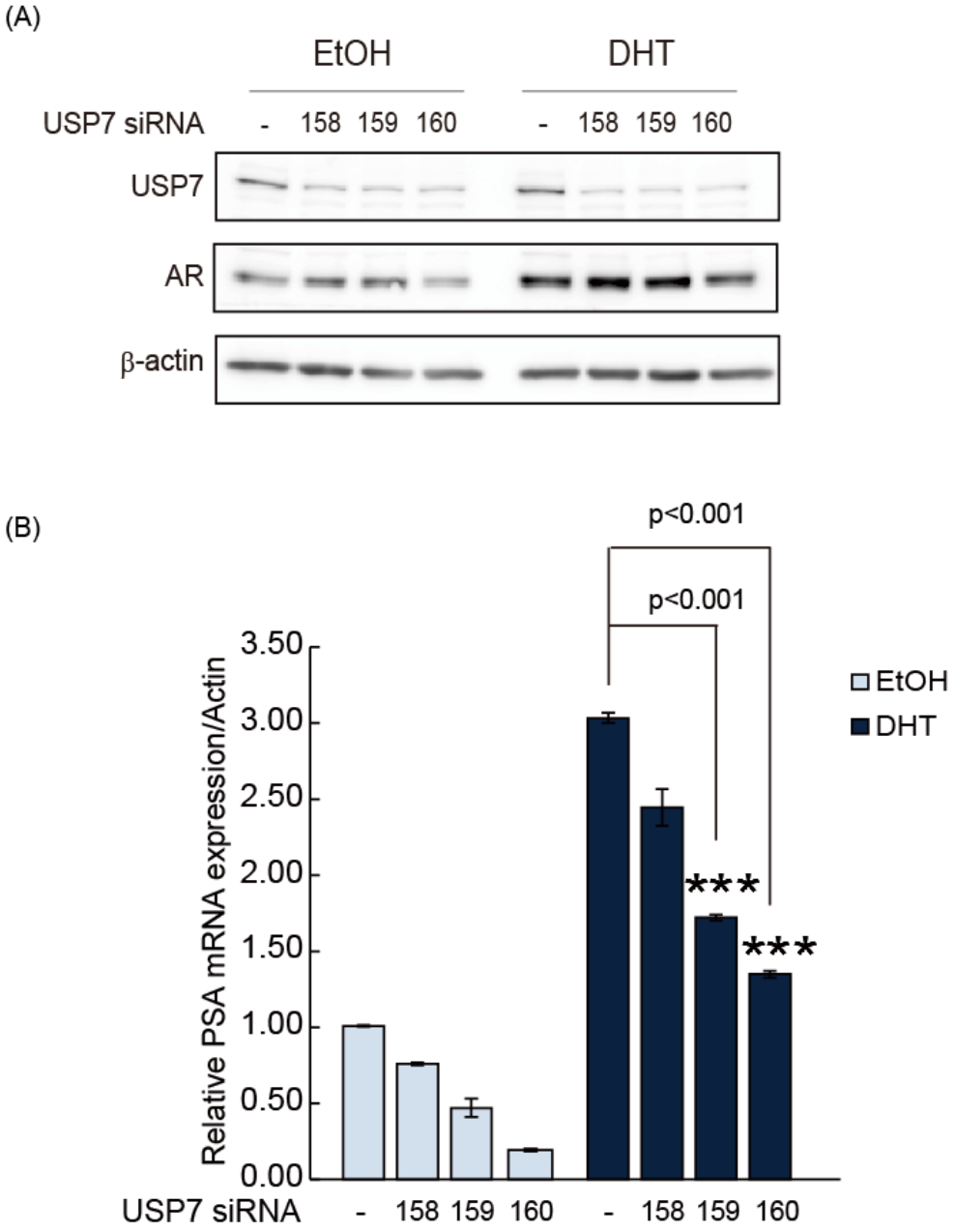


Figure 3-8 Validation of the three USP7 siRNA oligonucleotides

(A) Immunoblotting confirming the knockdown of USP7 protein in LNCaP cells transfected with three specific USP7 siRNA. (B) Validation of three USP7 siRNA effect on PSA mRNA expression using qPCR. ***, P<0.001, two-tailed t-test.

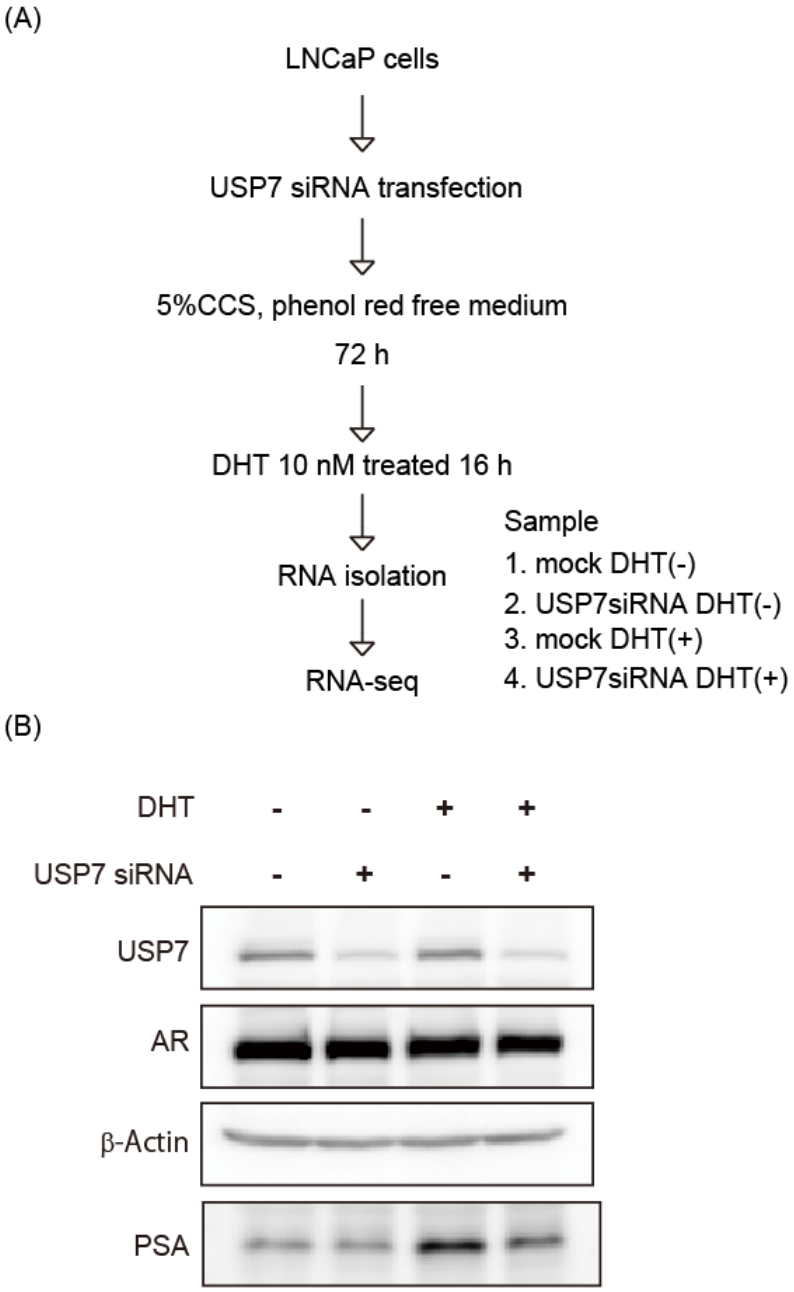


Figure 3-9 (A) The scheme of sample preparation for RNA-seq analysis (B) Validation of protein expression

(A) LNCaP cells were transfected with USP7 siRNA for 72 h and incubated in 10 nM DHT medium for 16 h. RNA was isolated and subjected to RNA-seq analysis. (B) The protein expression in sample (A) was examined using immunoblotting.

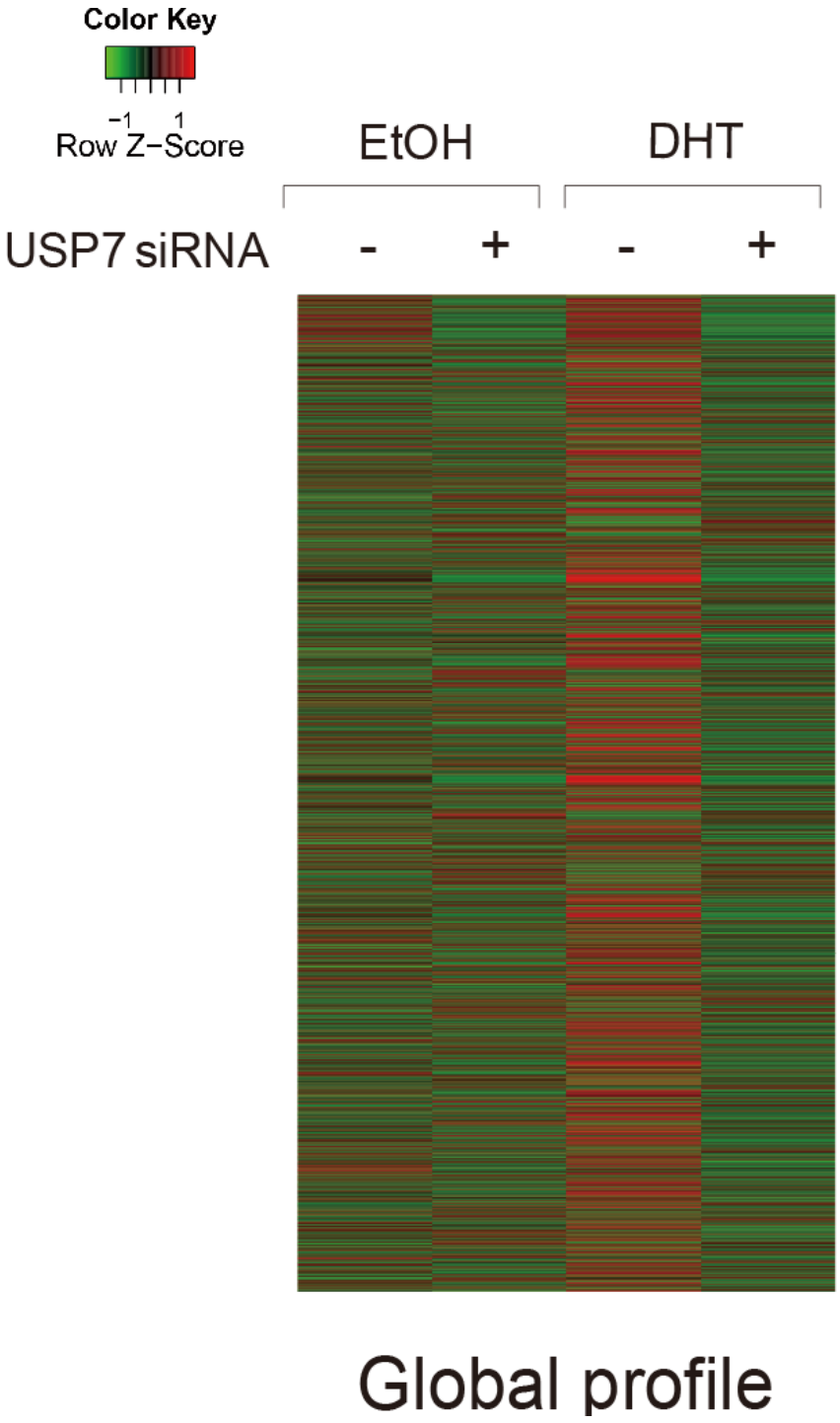


Figure 3-10 Heatmap representation of global genes expression profile by RNA-seq analysis

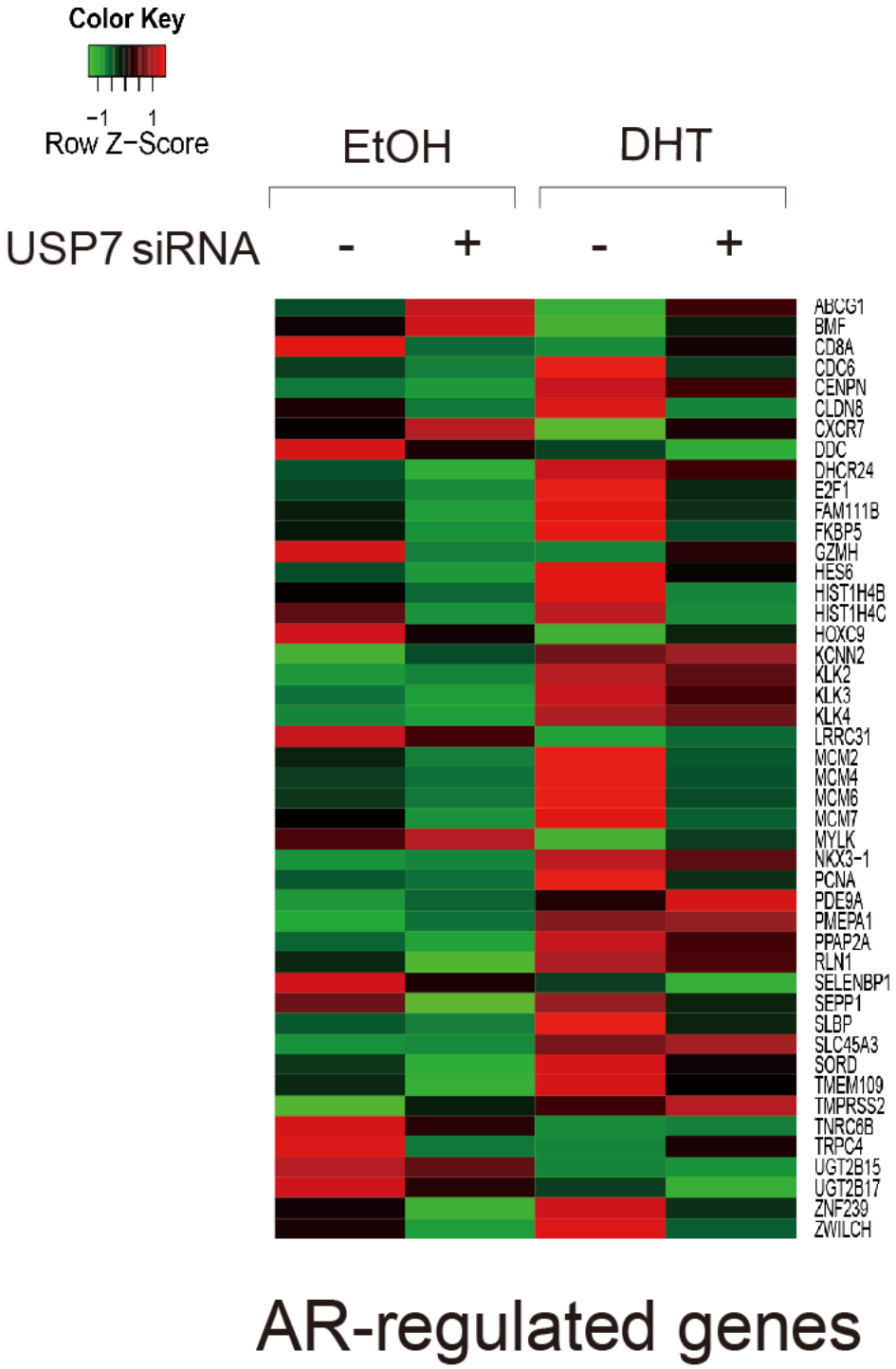


Figure 3-11 Heatmap representation of androgen-dependent genes expression profile by RNA-seq analysis

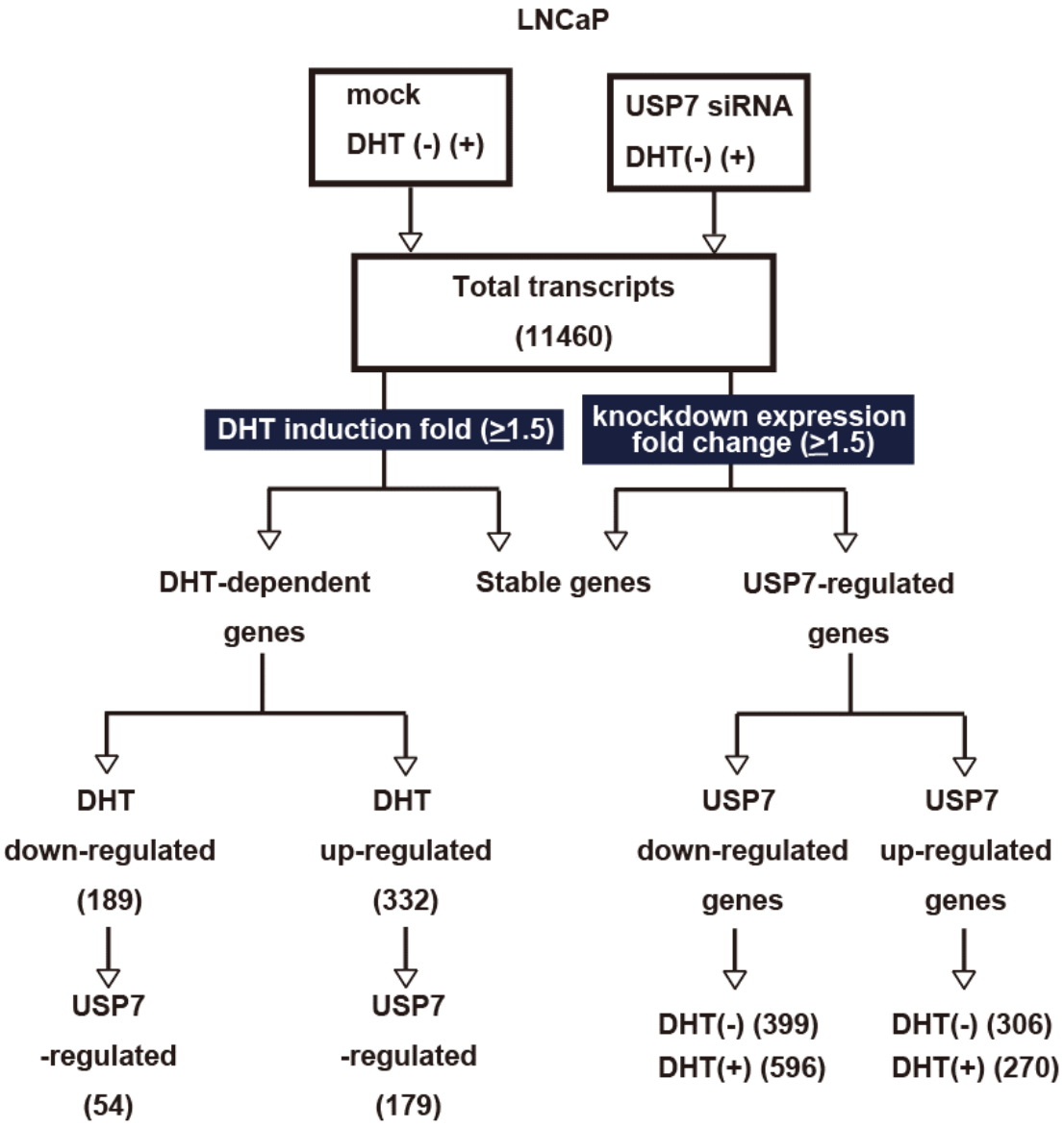


Figure 3-12 Workflow of androgen-dependent genes and USP7-regulated genes identification

To identify the USP7-dependent AR target genes, the genes exhibited at least 1.5 fold expression alteration after DHT stimulation were filtered as androgen-dependent genes. Meanwhile, the genes exhibited greater than 1.5 fold expression alteration by USP7 knockdown were filtered as USP7-regulated genes. The number in the brackets are gene number.

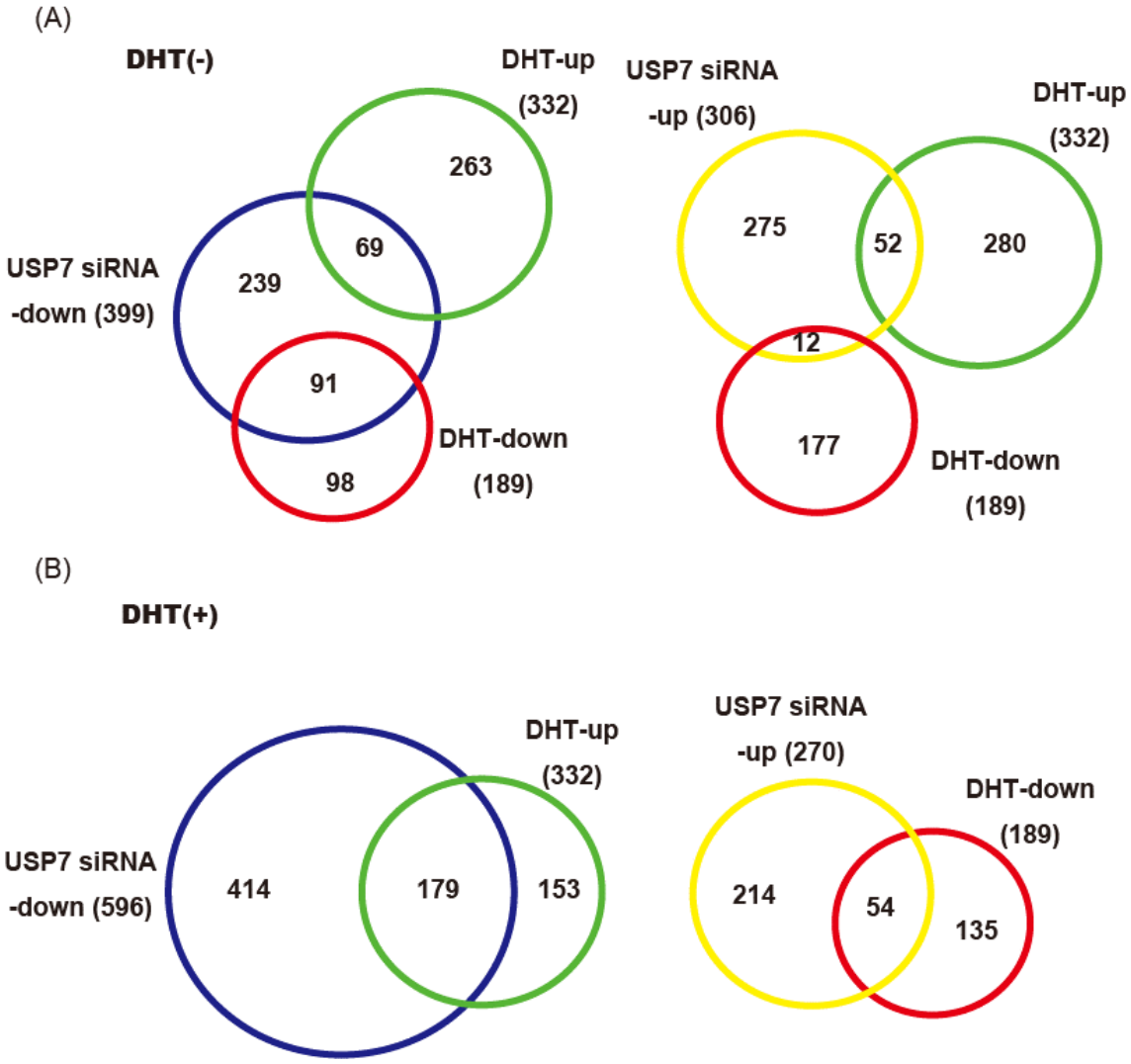


Figure 3-13 The overlap between USP7-regulated and androgen-dependent genes

(A-B) Venn diagram showing the overlap between USP7-regulated genes and androgen-dependent genes in the absence (A) or in the presence (B) of DHT. The number in the brackets are gene number. USP7 siRNA-down: genes that expression attenuated by USP7 knockdown, USP7 siRNA-up: genes that expression increased by USP7 knockdown

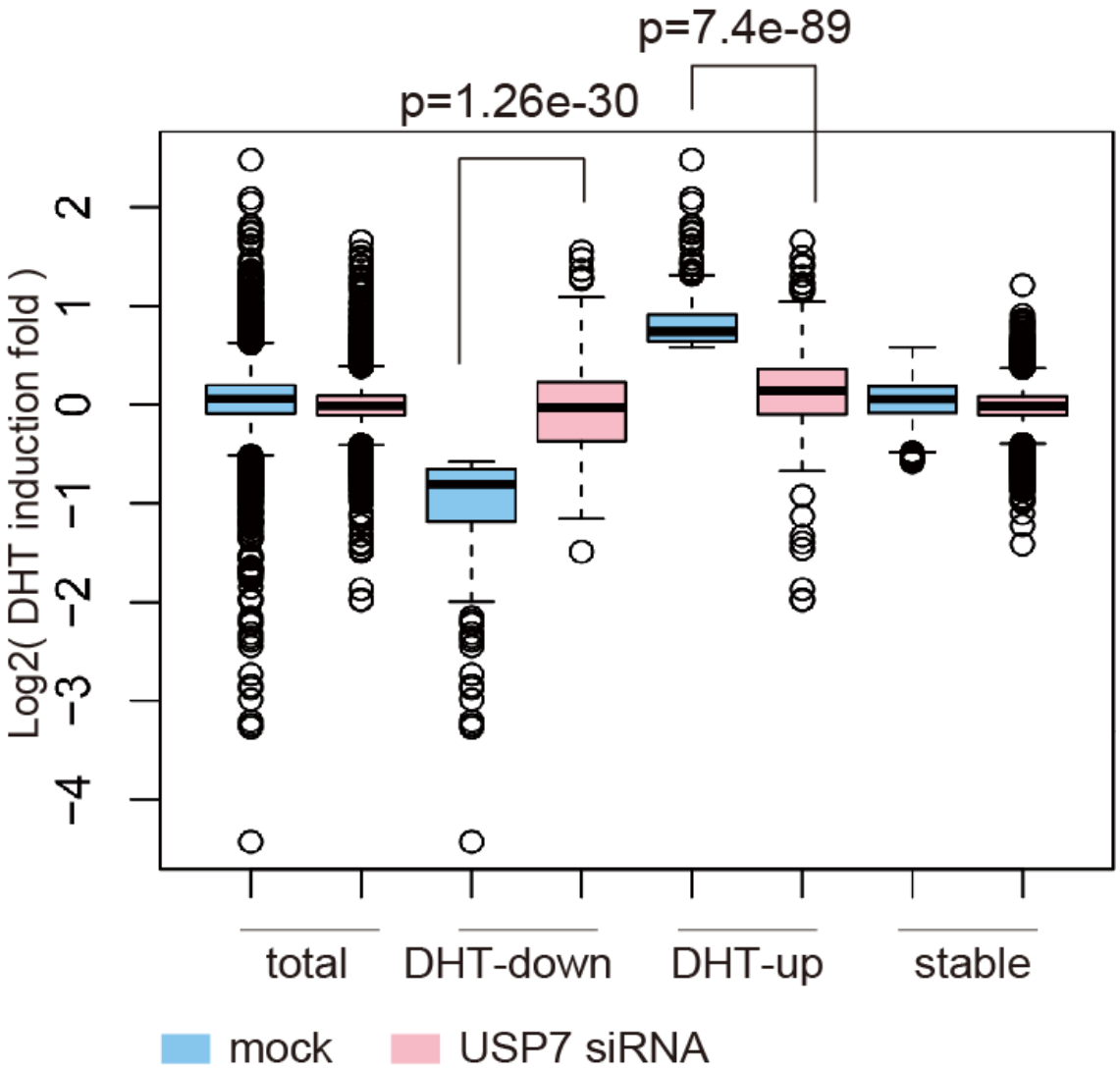


Figure 3-14 Boxplot showing the DHT induction fold change in mock and USP7 knockdown cells

Among the androgen-dependent genes, the DHT induction fold change is significantly affected by USP7 knockdown. P value are calculated using two-tailed t-test.

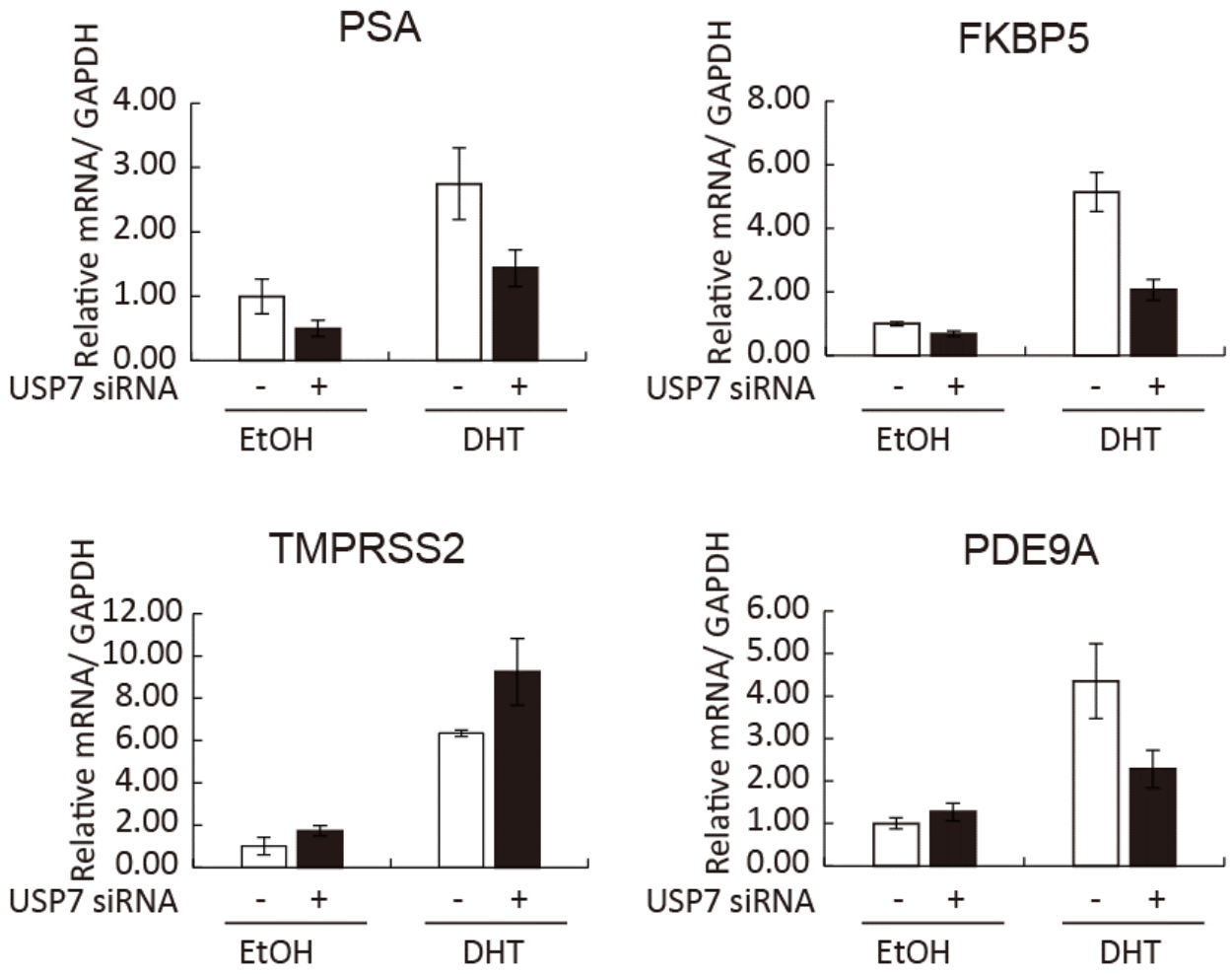


Figure 3-15 Validation of the RNA-seq result-1: androgen-activated genes

The mRNA expression level of androgen up-regulated genes in USP7 knockdown LNCaP cells were determined by qPCR and normalized against expression level of the GAPDH gene in the absence or presence of DHT for 8 h.

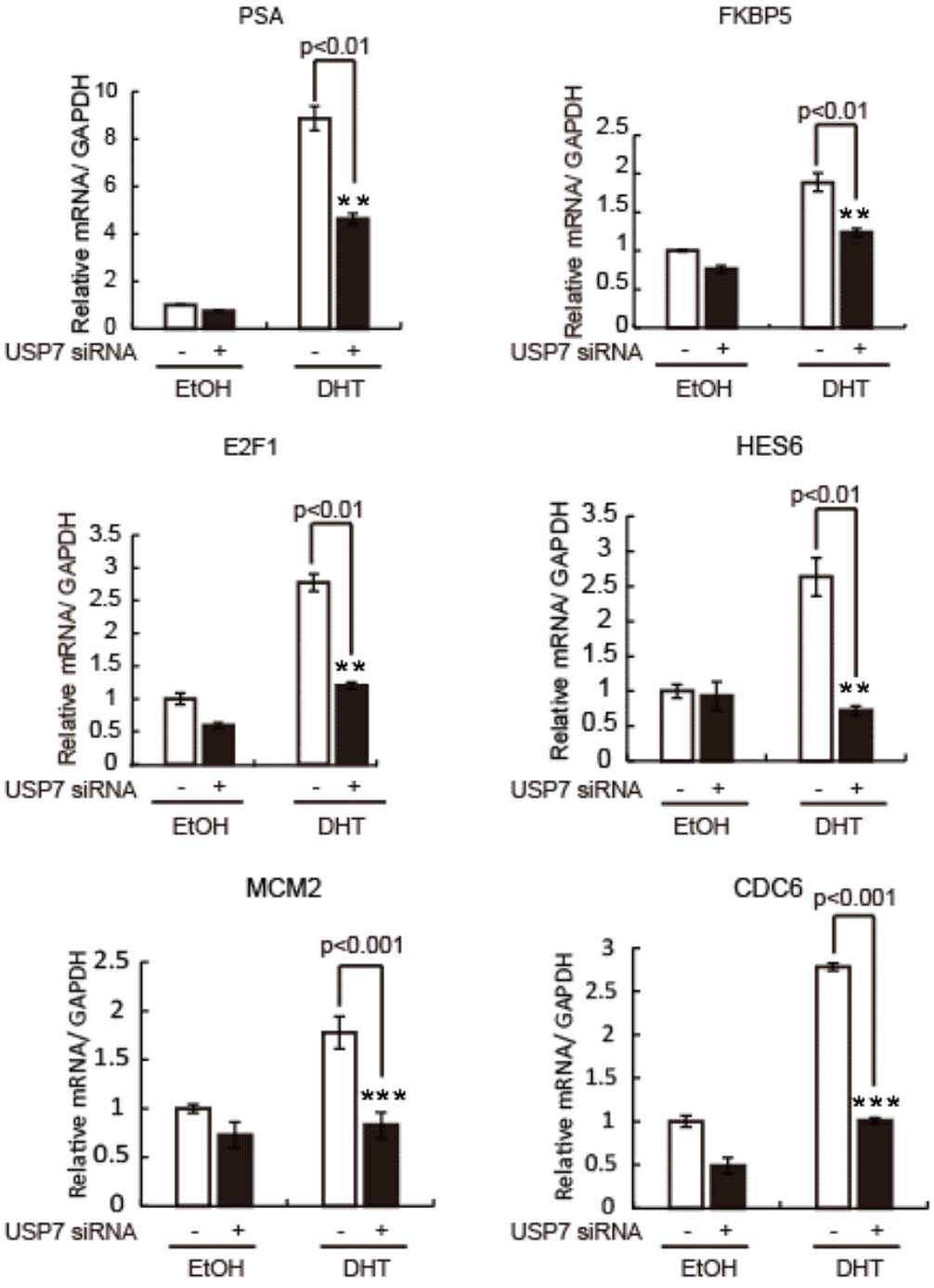


Figure 3-16 Validation of the RNA-seq result-1: androgen-activated genes

The mRNA expression level of androgen up-regulated genes in USP7 knockdown LNCaP cells were determined by qPCR and normalized against expression level of the GAPDH gene in the absence or presence of DHT for 16 h. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, two-tailed t-test.

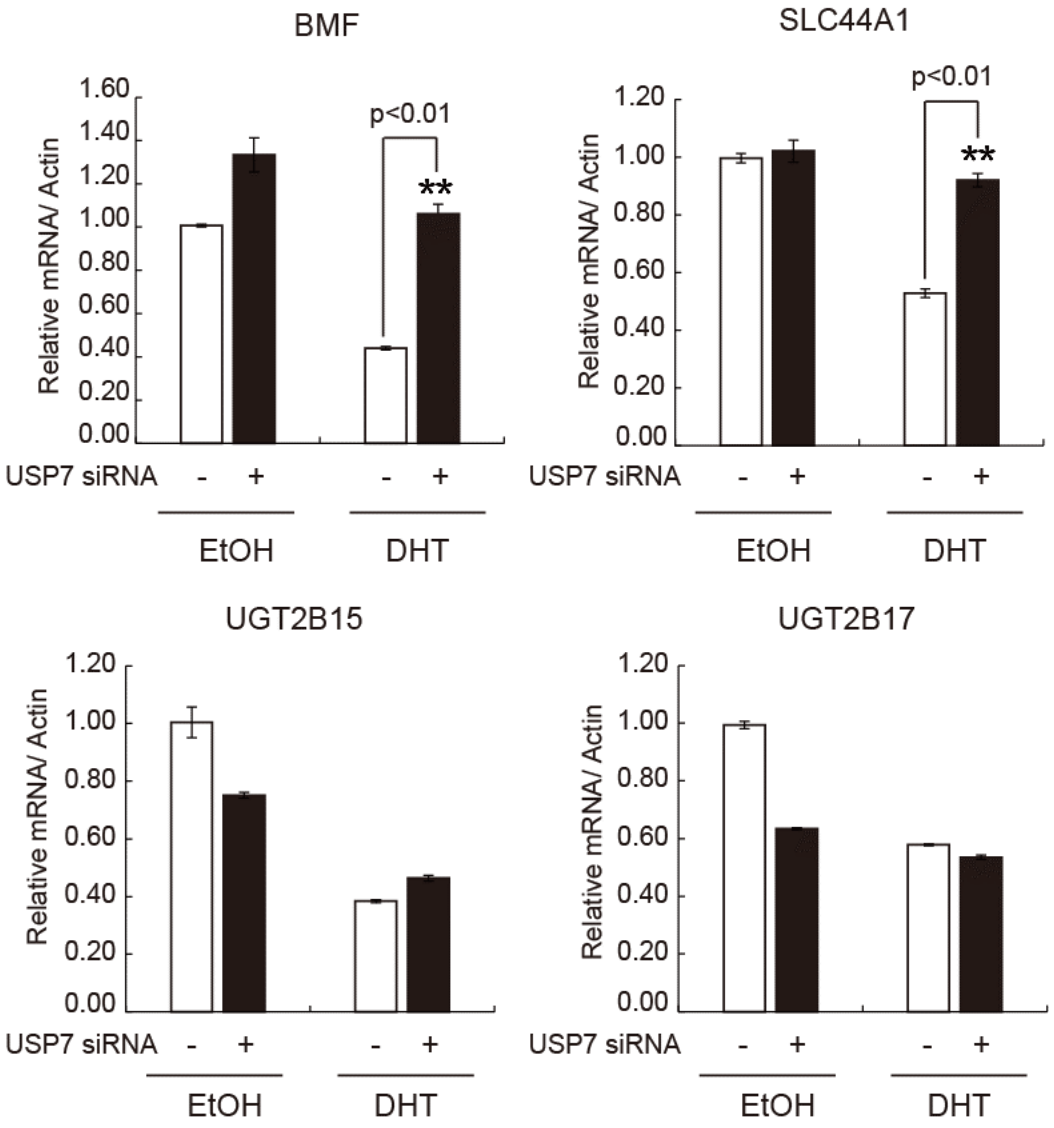


Figure 3-17 Validation of RNA-seq result-2: androgen-repressed genes

The mRNA expression level of androgen down-regulated genes in USP7 knockdown LNCaP cells were determined by qPCR and normalized against expression level of the Actin gene in the absence or presence of DHT for 16 h.**, $p<0.01$, two-tailed t-test.

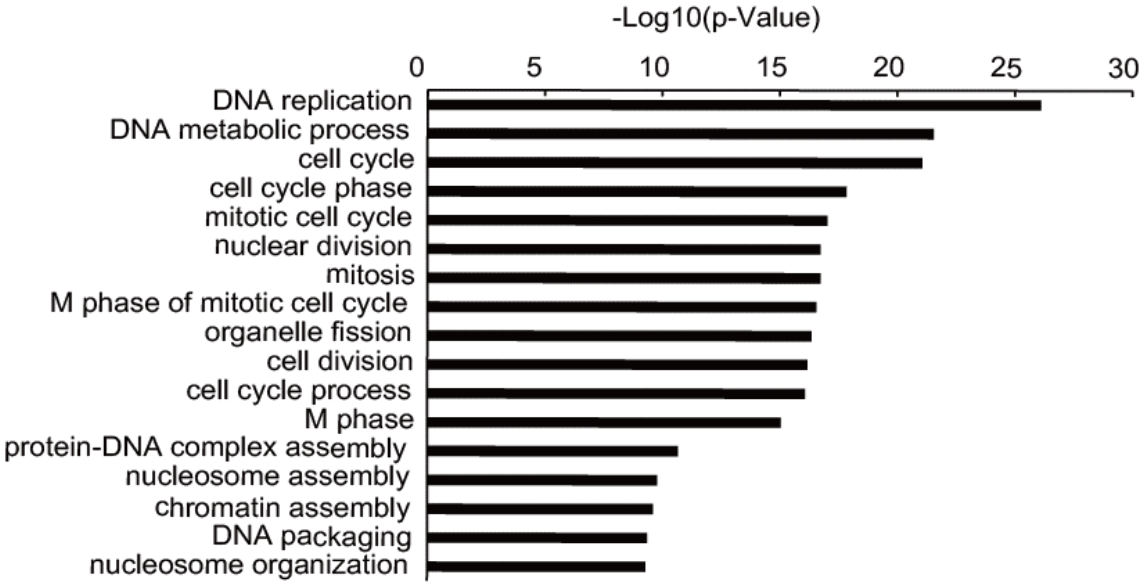


Figure 3-18 Functional annotation suggested that USP7 is involved in androgen-regulated biological function

Functional annotation of the 179 overlapping genes between DHT up-regulated and USP7 siRNA down-regulated genes using DAVID. Top overrepresented gene categories ($p<0.05$) from gene ontology (GO) biology process are shown.

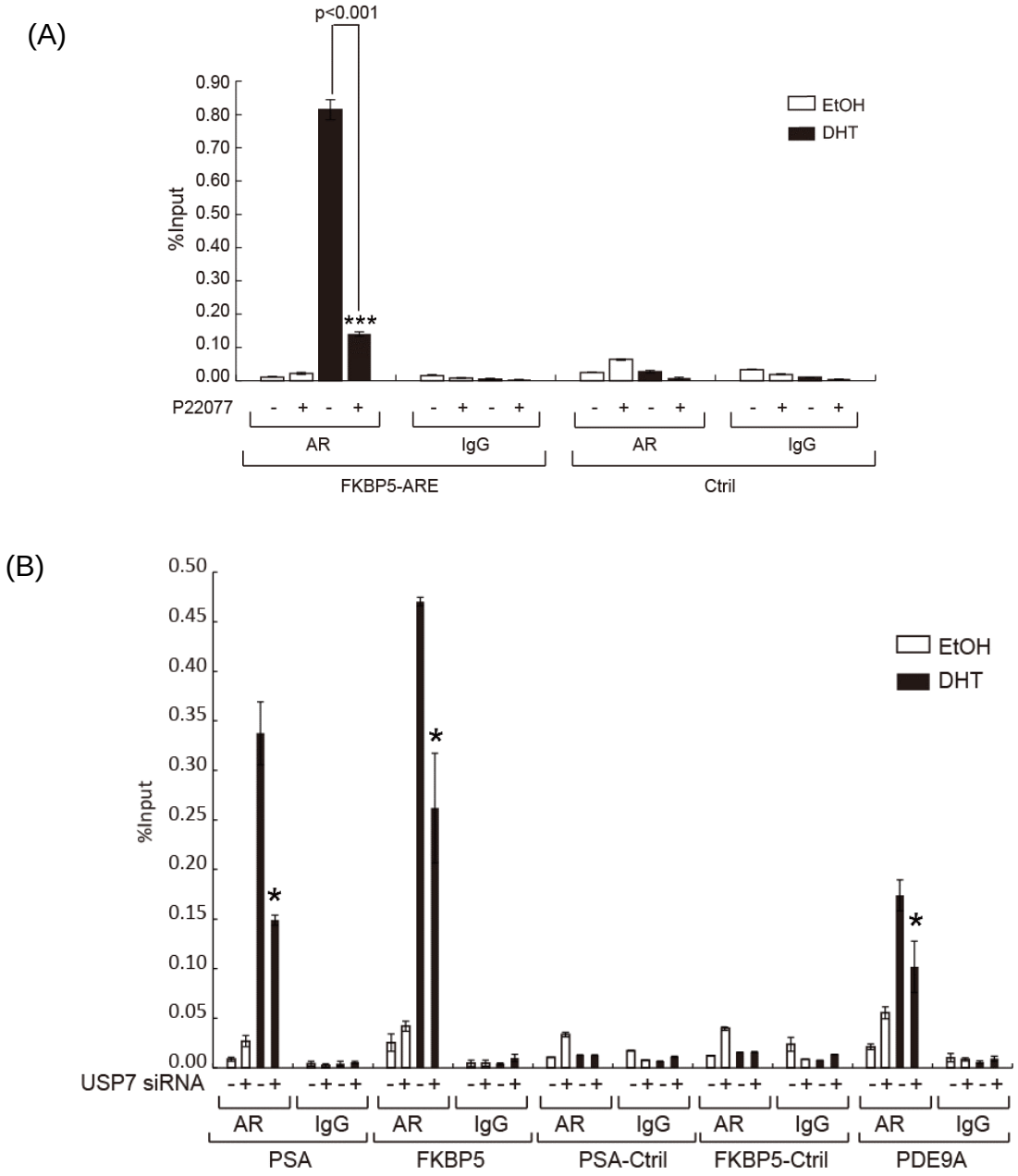


Figure 3-19 USP7 facilitates AR chromatin recruitment

(A) USP7 inhibitor P22077 inhibited AR recruitment to FKBP5 AREs. LNCaP cells were pretreated with P22077 (20 μM) for 30 min and then treated with 10 nM DHT or EtOH for 4 h. The ChIP-qPCR was performed using anti-AR antibody. (B) The recruitment of AR to AREs were diminished by USP7 knockdown. LNCaP cells were transfected with USP7 siRNA for 72 h and then treated with EtOH or 10 nM DHT for 1 h. The ChIP-qPCR was performed using anti-AR antibody. *, $p<0.05$, ***, $p<0.001$, two-tailed t-test.

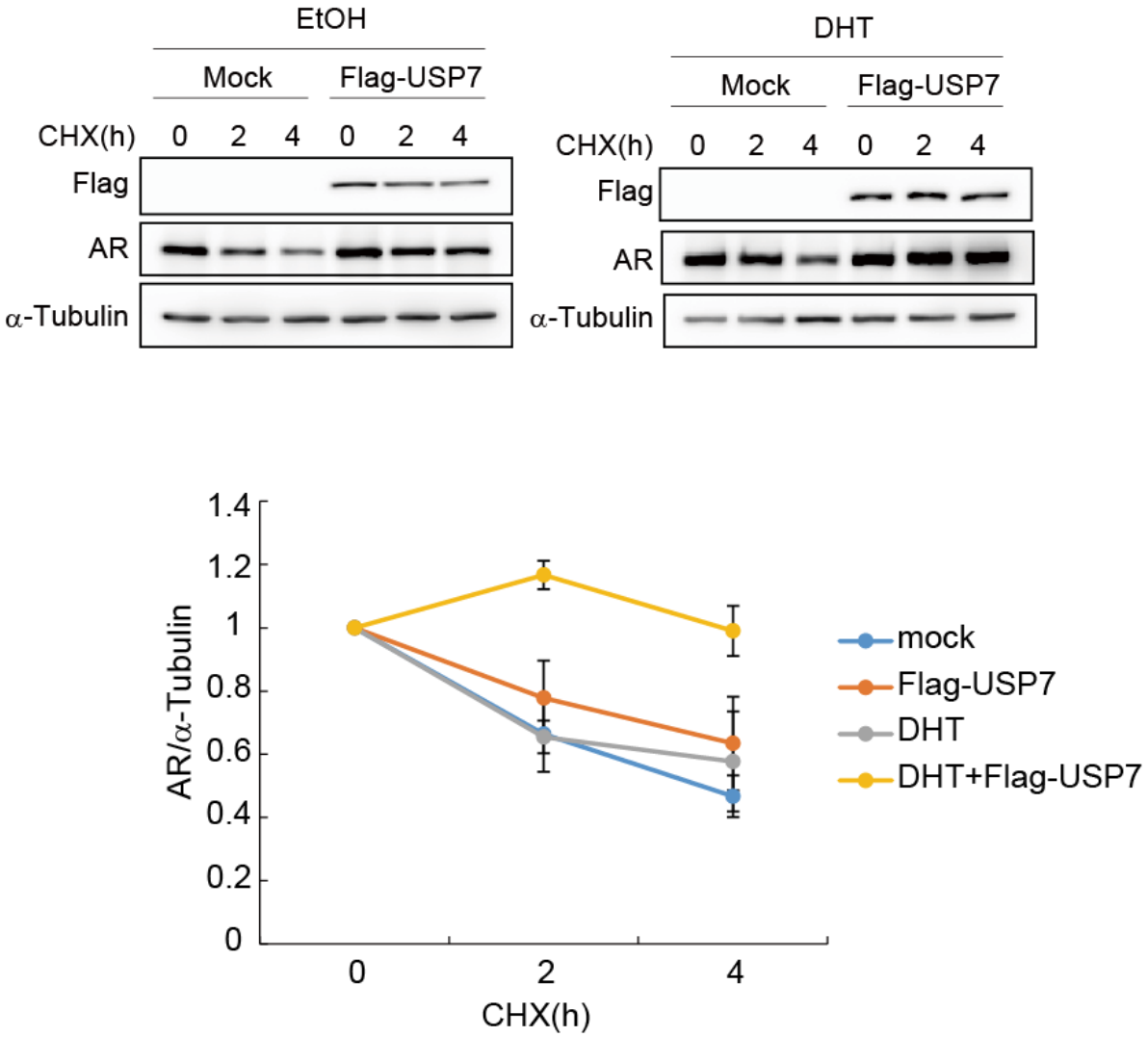


Figure 3-20 USP7 is involved in AR protein stability regulation

293T cells were transfected with AR and Flag-USP7 expression vectors for 24 h and added 10 nM DHT for 2 h followed by 150 μg/ml cycloheximide (CHX) time course treatment before cell harvested. Quantification of AR levels were carried out using densitometry by normalizing AR to α-Tubulin expression (bottom panel). Values were set relative to AR expression at time 0. The mean ± SD values of three independent experiment.

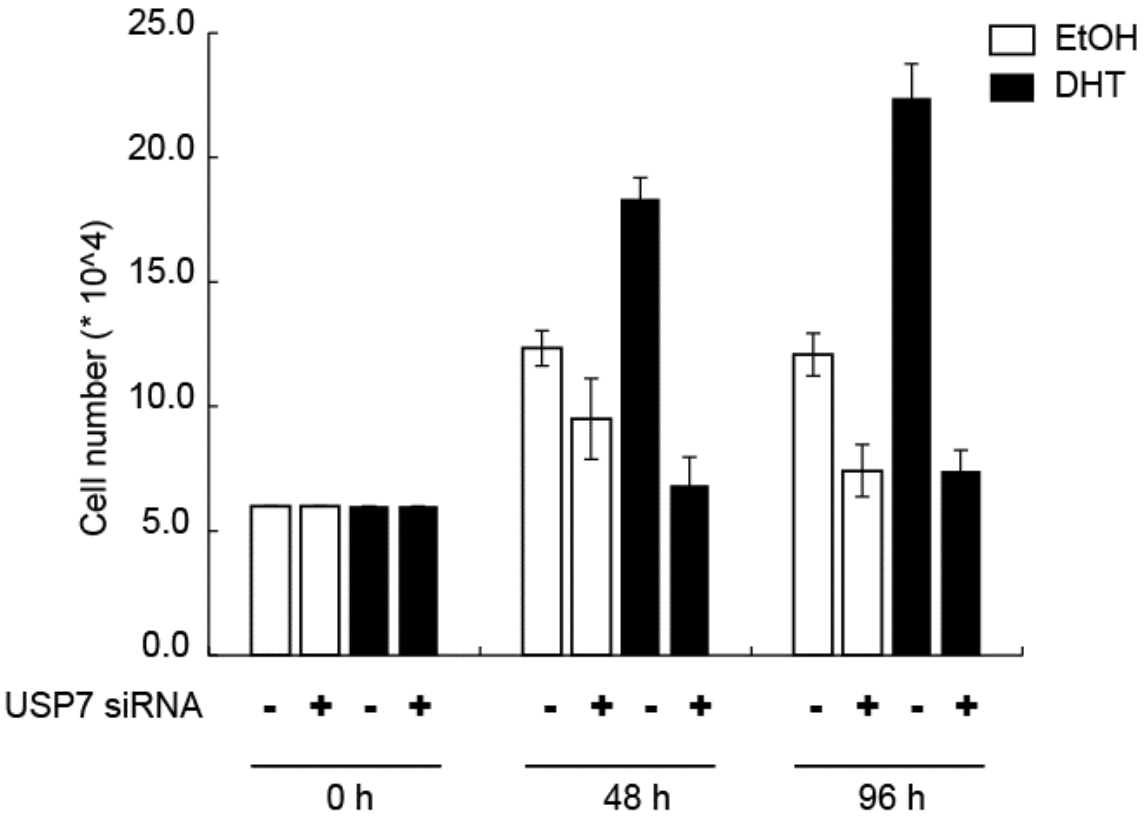


Figure 3-21 USP7 is required in androgen-stimulated LNCaP prostate cancer cell proliferation

LNCaP cells were transfected with USP7 siRNA for 24 h, and subsequently seeded in 12 well plate. The cells were incubated in hormone starvation condition in the absence or presence of 10 nM DHT, and the cell number of each well were counted at indicated time.

Chapter 4

Conclusion and Discussion

Conclusion

This study was endeavored to reveal a novel AR transcriptional regulation mechanism by identifying the composition of AR transcriptional protein complexes. For this purpose, the AR-associated proteins were affinity purified and the high score candidate USP7 was focused. Through investigating the functional role of USP7 in AR transcriptional regulation, a new insight of ubiquitin network was brought into in AR transcriptional regulation.

1. The proposal mechanism of USP7 regulates AR transcriptional activity

From this study, the molecular mechanism of USP7 mediates androgen-regulated genes especially activated genes expression can be proposed:

USP7 is associated with AR on AREs upon rapid DHT stimulation, where USP7 acts as guard to protect AR from ubiquitin/ proteasomal degradation thereby facilitates a subset of target genes expression that associate with several androgen-mediated biological processes. While USP7 deubiquitinates AR directly or indirectly need to be clarified (Figure 4-1).

2. The gene specific regulation manner of USP7 in androgen-dependent genes

To profile the USP7 knockdown effect on genome-wide androgen-dependent gene transcripts, revealing the essential role of USP7 in androgen-dependent genes regulation and the gene specific regulation manner by which USP7. While USP7 acts as a coactivator is much more impressive than corepressor, and the exact function of USP7 in androgen-repressed genes need to be verified. The data of USP7 trans-repressed the ARE-containing reporter activity supported the corepressor role of USP7. Given that AR mediates gene activation and repression via distinct mechanisms, implying USP7 might be involved in multiple levels of AR transcriptional regulation. While this speculation remain to be corroborated, this analysis impressed the importance of ubiquitin network in AR transcriptional activity and specificity regulation.

Discussion

1. The role of USP7 in ubiquitin network-regulated AR transcriptional activity

Hormone regulated ubiquitination of AR results in intricate effects on AR transcriptional activity, and vice versa deubiquitination of AR by USP7 also brings many possible consequences in AR transcriptional regulation.

The result of liganded AR protein half-life was prolonged by overexpression of USP7 suggested that USP7 is involved in hormone-regulated receptor ubiquitination and proteasomal degradation. The chromatin ubiquitin-proteasome system is supposed to allow proper progression through rounds of transcription and appropriate assembly of the necessary protein complexes as well as modulates the activation status of transcription factors and coregulators. On the other hand, RNF6 generates Lys 6- and Lys 27-linked Ub chains that stabilize AR receptor and act as scaffold for coactivators such as ARA54 recruitment, thereby regulates AR transcriptional activity and specificity. Hence, the DUBs activity of USP7 is possible to oppose these E3 ligase actions and blocks gene transcription.

This situation can be solved through mediation of the short-term USP7 recruitment to chromatin. By strictly controlling these ubiquitin network enzymes dynamic association and disassociation sequence and duration, the AR transcriptional activity can be precisely regulated. Similar mechanism was also described in the studies of TSG101 and USP26 in AR transcriptional activity regulation. TSG101 is proposed to maintain AR in monoubiquitination state by preventing AR polyubiquitination and stimulating AR transcriptional activity. Another DUBs USP26 is proposed to reverse MDM2-generated ubiquitination chains, and extend transcriptional activation.

It was reported that the interaction between E3 ligase and DUBs allow fine-tuning of the ubiquitination status of common substrates[38]. The well know case is the E3 ligase MDM2 and its substrate P53 are relevant USP7 substrates. USP7 can both remove ubiquitin from MDM2 and its substrate P53, thereby both stabilizes P53 and MDM2. Through the coupling of positive and negative ubiquitin regulation, the cell P53 protein level are precisely and strictly controlled. This working model may be utilized to fine-tuning of the AR ubiquitination status and affect AR quantitative /qualitative regulation. The well characterized AR E3 ligase MDM2 and RNF6 may cooperate with USP7 to regulated AR transcriptional activity.

Although the dynamic interplay between USP7 and other ubiquitin network enzymes remain to be clarified, this study not only impress the importance of DUBs in AR

transcriptional activity and specificity regulation but also broaden the view of ubiquitin network in gene regulation.

2. The proposal mechanism of USP7 mediated androgen-dependent gene repression

The result of globally profile the USP7 knockdown effect on genome-wide transcript not only revealed the coactivator role of USP7 in up-regulated genes but also showing 28% of DHT down-regulated genes were derepressed by USP7 knockdown and proposing the USP7 corepressor role.

The mechanism of AR-mediated gene repression versus activation is fundamental different. Although the mechanism of AR activates gene transcription is well established, the mechanisms by which AR-mediated gene repression are ill-defined. Several mechanisms of AR-repressed gene have been proposed including inhibition of other transcription factors/ activators and AR non-genomic actions as well as the direct binding of AR to negative AREs (nAREs)[55].

While the DNA consensus sequence specific for negative regulation by androgens has not yet been described. The binding of AR to nAREs is supposed resulting in displacement of RNA Polymerase II and formation of corepressor complexes which contribute to a more condensed chromatin conformation thereby represses gene. The data of USP7 squelches DHT-stimulated ARE-luciferase reporter activity suggested that USP7 regulates androgen-repressed gene through mediating the binding of AR to DNA/ nAREs.

It is supposed that enhancers are specified by particular chromatin marks by which cells establish patterns of gene expression. On the other hand, USP7 was reported to modulate histone H2A ubiquitinase Polycomb repressive complex 1 (PRC1) and DNA methyltransferase 1 (DNMT1)-suppressed gene expression[56,57]. Even USP7 itself was supposed to function as a histone H2B deubiquitinase and repress gene in insect cells[58].

As a result, USP7 might cooperate with the enhancer specific epigenetic enzymes network to modulate the enhancer epigenetic environment thereby destine gene activation or repression.

Perspective

USP7 is a potential therapeutic target in prostate cancer

The aberrant AR signaling mediate the initiation and progression of ADPC as well as the development of HRPC. To clarify the mechanism by which manipulation the AR transcriptional activity in ADPC and HRPC is important for prostate cancer therapy.

Several hyperactive AR signaling mechanism have been proposed, including AR receptor point mutation, AR gene amplification and aberrant expression of coregulators. Recent studies have shown that altered expression of AR coregulators such as SRC and p300 in prostate cancer may modify AR transcriptional activity and can be utilized as prostate cancer diagnosis mark.

Here, the DUBs USP7 was biochemically identified as a novel AR coregulator in prostate cancer, and is required for AR transcriptional activity and specificity. This study implicated the importance of USP7 in prostate cancer progression. Indeed, previous study has observed the overexpression of USP7 in human prostate cancer, and the high levels USP7 is involved in the aberrant PTEN nuclear exclusion regulation as well as directly correlated with tumor aggressiveness[52].

Taken together, these data suggested that USP7 can manipulate AR-dependent and -independent signaling to promote prostate cancer progression. Recent years, many USP7 specific small molecular inhibitors have been discovered for cancer target therapy such as colon cancer[59,60]. This study exhibits that USP7 mediates AR transcriptional activity and specificity as well as involved in AR-dependent and –independent cell proliferation. As a result, USP7 may also be as a potential target for prostate cancer therapy.

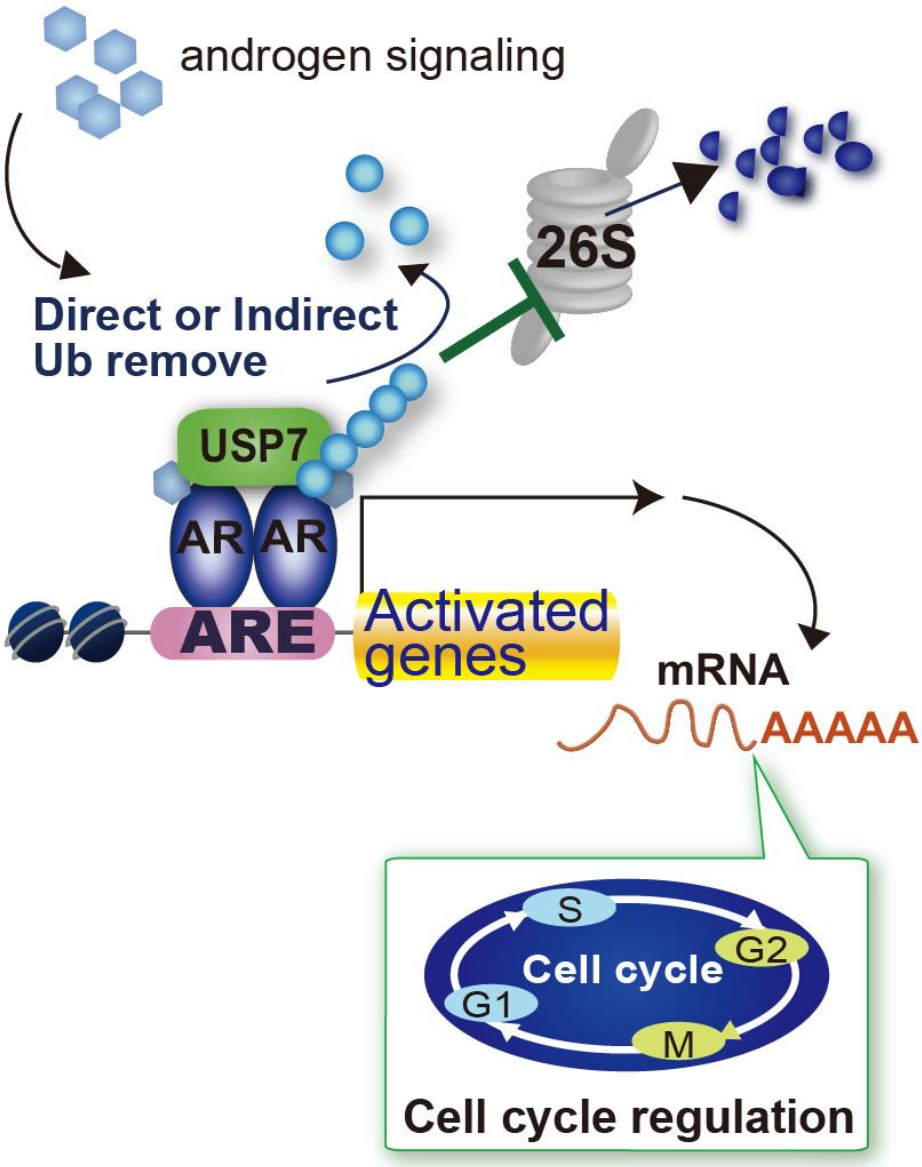


Figure 4-1 Conclusion-Model of USP7 regulates AR transcriptional activity

Upon androgen stimulation, liganded AR, USP7 and other coregulators are recruited to AREs. USP7 protects AR from ubiquitin-targeted proteasomal degradation and activates a subset of androgen-dependent gene expression that highly associate with androgen-mediated biological processes such as cell cycle regulation.

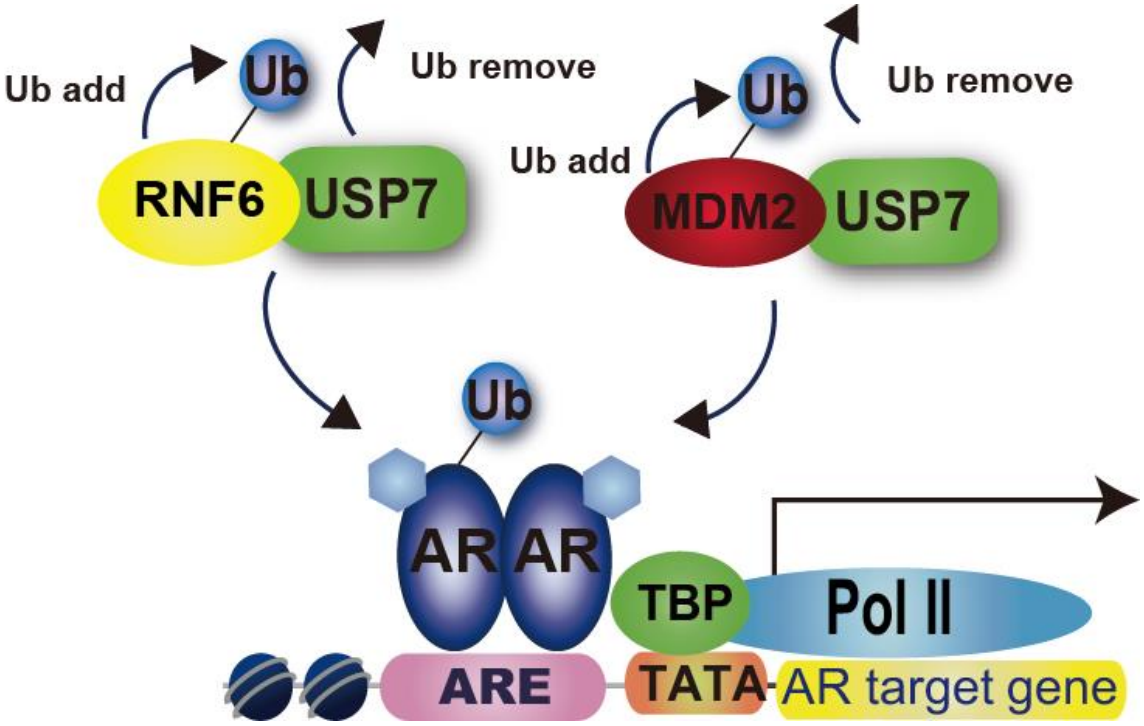


Figure 4-2 Discussion-The proposed working model of USP7 and AR E3 ligase to fine-tuning AR quantitative /qualitative regulation.

The DUBs USP7 and E3 ligase such as RNF6 and MDM2 may cooperate to fine-tuning AR ubiquitination status. Through the coupling of positive and negative ubiquitin regulation, the AR protein level and transcriptional activity are able to be precisely and strictly controlled.

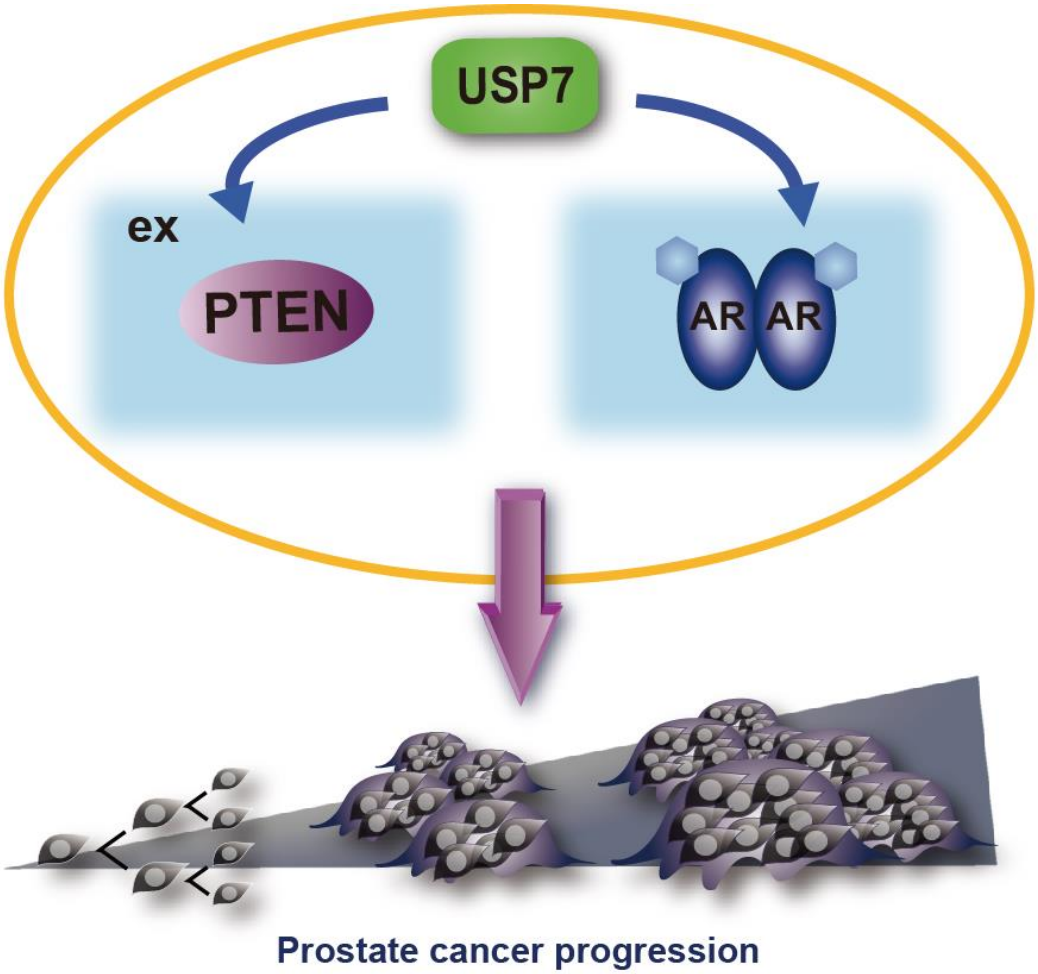


Figure 4-3 Perspective-USP7 mediates prostate cancer cell progression via AR-dependent and AR-independent signaling

The overexpression USP7 in prostate cancer is involved in hyperactive AR signaling and aberrant PTEN nuclear exclusion, thereby promote prostate cancer cell proliferation and progression. Consequently, USP7 might be a potential target for prostate cancer therapy.

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Shuting Chen

The University of Tokyo