THE ANDROGEN RECEPTOR CENTRIC TRANSCRIPTIONAL NETWORK IN PROSTATE CANCER

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NATIONAL UNIVERSITY OF SINGAPORE

2012

THE ANDROGEN RECEPTOR CENTRIC TRANSCRIPTIONAL NETWORK IN PROSTATE CANCER

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B.Sc. (Hons.), NUS

A THESIS SUBMITTED

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

NUS GRADUATE SCHOOL FOR INTEGRATIVE SCIENCES AND ENGINEERING

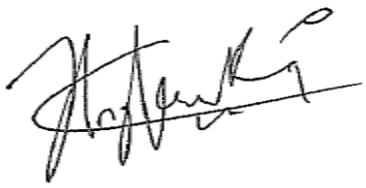
NATIONAL UNIVERSITY OF SINGAPORE

2012

DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.



Chng Kern Rei

01 March 2013

ACKNOWLEDGEMENTS

My Ph.D Supervisor, Dr Edwin Cheung, has been a dedicated mentor throughout the four years of my Ph.D study. He has been a constant source of encouragement and guidance. I would like to take this opportunity to express my profound gratitude to him. I would like to give my thanks to my TAC members, Dr Patrick Tan and Dr Neil Clarke who have given insightful suggestions and opinions.

I sincerely thank all my colleagues who have worked with me for the past four years and have provided their kind help and advice to me selflessly. Immense thanks go to the co-partners who have made important contributions to my Ph.D project: Mr Chang Cheng Wei, Ms Tan Si Kee, Ms Hong Shu Zhen, Mr Yang Chong and Mr Noel Sng. I also wish to give acknowledgements to Dr Tan Peck Yean for insightful discussions; Mr Lim Seong Soo, Dr Valere for guidance in the FISH experiments and the GTB sequencing group in the Genome Institute of Singapore for their technical assistance in second generation sequencing technology.

I convey my deepest appreciations to the Genome Institute of Singapore for hosting my Ph.D research and to A-STAR for providing my scholarship and the fundings for my Ph.D study.

Last, but not least, I am deeply indebted to my loving family members for their unwavering support and understanding.

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SUMMARY

A dysregulated Androgen Receptor (AR) transcriptional network is one of the main drivers behind prostate cancer initiation and development. Indeed, AR has always been a key target in prostate cancer therapeutics. A thorough understanding of the AR transcriptional network would shed valuable insights to prostate cancer etiology and contribute immensely to the development of new prostate cancer therapies. To function, AR has to interact and collaborate with a plethora of other transcription factors. It is the interplay between AR and its co-factors that ultimately define the output of the AR-centric transcriptional program. Consequently, aberrant expression of AR co-factors would contribute to a deregulated androgen receptor transcriptional circuitry that favors prostate cancer progression.

Prostate cancer was shown frequently to harbor recurrent gene fusions that led to over-expression of the transcription factor, ERG. The potential transcription crosstalk between AR and ERG is of exceptional interest as it represents a prostate cancer-specific collaboration that is suitable for therapeutic intervention. Herein, we sought to gain a deeper understanding on the AR and ERG transcriptional network in prostate cancer cells. By generating and analyzing a time-course Chromatin Immunoprecipitation-Sequencing (ChIP-Seq) of AR and ERG, we provided valuable insights into the temporal and spatial aspects of genome-wide AR/ERG cistromic profiles. Coupled with siRNA knockdown experiments, we showed that ERG could function as a transcriptional co-repressor of AR.

Apart from ERG, several transcriptional co-repressors such as histone deacetylases (HDACs) and the polycomb repressor, EZH2, which are implicated for cancer progression, are also commonly over-expressed in prostate cancers. Interestingly, several studies have reported a correlation between the expression of HDACs, EZH2 and ERG in prostate cancers. To reveal insights into the possible interplay between AR, ERG and these co-repressors, we proceed on to generate extensive cistromic profiles of these factors prior and after androgen stimulation. We observed that these co-repressors, like ERG, were also recruited to AR enhancers upon androgen treatment. In addition, we found that while substantial overlaps are present between the genome-wide occupancy profiles of ERG, each distinct HDAC members and EZH2, they are not indistinguishable. This implies a distinct role for each respective co-repressor.

Importantly, we assigned a functional role for the co-repressors in facilitating metastasis. Our results showed that ERG, HDACs and EZH2 transcriptionally suppressed the induction level of androgen induced cytoskeletal proteins that inhibit metastasis and maintain the epithelial phenotype in prostate cancer cells. Implicitly, VCL was validated as one such cytoskeleton protein.

Taken together, our data suggested that, through their repressive effects, ERG, HDACs and EZH2 could co-operate in this AR centric transcriptional network to attain optimal androgen signaling for cancer progression. This finding highlighted a formerly unappreciated auxiliary role of these co-repressors in regulating androgen signaling in prostate cancers.

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LIST OF SYMBOLS

ADT	Androgen deprivation therapy
AR	Androgen receptor
ARBS	AR binding site
ARE	Androgen response element
ATCC	American type culture collection
CDFBS	Charcoal dextran treated FBS
cDNA	Complementary DNA
ChIA-pet	Chromatin Interaction Analysis by Paired-End Tag
ChIP	Chromatin immunoprecipitation
ChIP-chip	ChIP on chip
ChIP-pet	ChIP paired end tag
ChIP-Seq	ChIP-Sequencing
Co-IP	Co-immunoprecipitation
cRNA	Complementary RNA
DAB2IP	Disabled homolog 2-interacting protein

DBD	DNA-binding domain
DHT	5α-dihydrotestosterone
DNA	Deoxyribonucleic acid
DSG	Disuccinimidyl Glutarate
Eed	Embryonic ectoderm development
EMT	Epithelial mesenchymal transition
ER	Estrogen Receptor
ERBS	Estrogen receptor Binding sites
ERG	v-ets Erythroblastosis virus E26 homologue (avian)
ETOH	Ethanol
ETS	Erythroblastosis virus E26 homologue
ETV	ETS translocation variant
EWS	Ewing sarcoma breakpoint region 1
EZH2	Enhancer of zeste homolog 2
FBS	Fetal bovine serum
FDR	False discovery rate
FISH	Fluoresence in-situ Hybridization
FITC	Fluorescein isothiocyanate
FKBP5	FK506 binding protein
FLI1	Friend leukemia virus integration 1
FoxA1	Forkhead Box A1
GTF	General transcription factors
HAT	Histone acetyltransferase

HDAC	Histone deacetylase
HSP	Heat shock protein
IgG	Immunoglobulin G
JAK2	Janus kinase 2
Krt	Keratins
LBD	Ligand Binding Domain
LEF-1	Lymphoid enhancer binding factor 1
MCM	Molecular Concepts Map
Mdm2	p53 E3 ubiquitin protein ligase homolog
Med1/TRAP220	Mediator complex subunit 1
MET	Mesenchymal to Epithelial Transition
MMP	Matrix metalloproteinase
NGS	Next generation sequencing
NKX3-1	NK3 homeobox 1
NTD	N terminal transactivation domain
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PLA1A	Phospholipase A1
PolII	RNA polymerase II
PRC2	Polycomb repressor complex 2

PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homolog
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SELEX	Systematic evolution of ligands by exponential enrichment
siCtrl	Control siRNA
Slit2	Slit homolog 2
siRNA	Small interfering RNA
Suz12	Suppressor of zeste 12 homolog
TBS	Tris-buffered saline
TEL	ETS translocation variant 6
TMPRSS2	Transmembrane protease, serine 2
TSS	Transcription start site
UBE2C	Ubiquitin-conjugating enzyme E 2C
VCL	Vinculin

CHAPTER 1: INTRODUCTION

1.1 Prostate Cancer Basics

Prostate cancer is one of the most frequent cancers among the male population. According to current available statistics (Howlader et al., 2011). 1 in 6 American males are expected to be diagnosed with prostate cancer within their lifetime. Apart from hereditary factors, certain dietary and environmental factors were also shown to be correlated with prostate cancer incidence (Carter et al., 1990). Given the high prevalence of prostate cancers, intense research efforts have been and are still being invested to understand and to combat the disease.

These efforts have resulted in significant progress for prostate cancer treatment. The seminal discovery by Charles Huggin that demonstrate the necessity of androgens (Male steroid hormones) in prostate cancer progression has led to the development of Androgen Deprivation Therapy (ADT) (Huggins, 1967; Huggins and Hodges, 2002). Clinically, most hormone naïve prostate cancers were shown to regress in response to ADT. However, recurrence is common with the disease progressing into aggressive, metastatic and castrate-resistant form through a variety of mechanisms (Feldman and Feldman, 2001). Median survival rate for such cases is only 1-2 years (Lassi and Dawson, 2009).

1.2 Androgens in Prostate Cells

Past research has provided strong evidence that demonstrate the role of androgens in fueling prostate cancer growth and development (Huggins, 1967; Huggins and Hodges, 2002). Physiologically, androgens are responsible for promoting male characteristics, which include but not limited to, regulating prostate gland development, maintenance and function (Cunha et al., 1987; Mooradian et al., 1987). There exist several types of androgens. The principal androgen in males is testosterone (T), which is usually metabolized and converted into its more potent counterpart, 5α-dihydrotestoterone (DHT) by the enzyme 5α-reductase (Russell and Wilson, 1994). Androgens as the cognate ligands of AR, typically exert their influence on cell biology through activating AR signaling.

1.3 A Brief Description of AR

AR is a member of the nuclear hormone receptor superfamily. Structurally, AR is comprised of several distinct functional domains, namely, a N-terminal domain (NTD) containing 2 transcriptional activation units (AF-1 and AF-5), a DNA binding domain (DBD) where 2 four-cysteine zinc-binding domains are located, a ligand binding domain (LBD) harboring another transcriptional activation unit AF-2 and a hinge region connecting LBD and DBD (Fig. 1.1) (Brinkmann et al., 1989; Chang et al., 1988; Jenster et al., 1995; Koochekpour, 2010). The functional domains of AR are consistent with the characteristics of a ligand dependent transcription factor. Being the predominant receptor

for androgens, AR is the main mediator for the genomic actions of androgens. The general simplified pathway for AR activation through androgen stimulation is as follows: After activation by androgens binding to its LBD, AR dissociates from prebound heatshock proteins (HSP), translocates into the nucleus and dimerizes. Within the nucleus, AR is recruited to the DNA via its DBD which structurally recognize the consensus DNA sequence AGAACANNNTGTTCT, the canonical motif for Androgen Response Elements (AREs) to mediate transcriptional regulation of the targeted gene (Fig. 1.2) (Heinlein and Chang, 2004; Saraon et al., 2011).

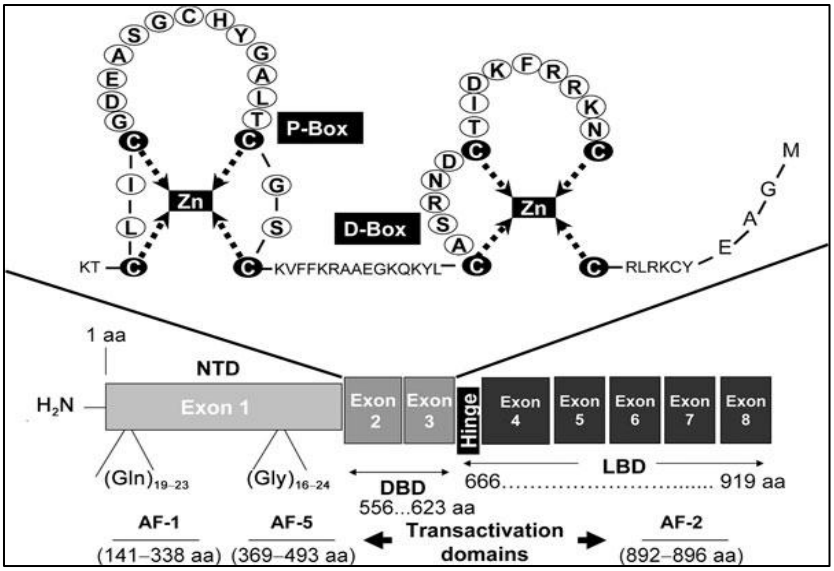


Figure 1.1 An Illustration on the Different Functional Domains of AR (Koochekpour, 2010). Structurally, AR comprise of a N-terminal domain, a DNA binding domain and a ligand binding domain.

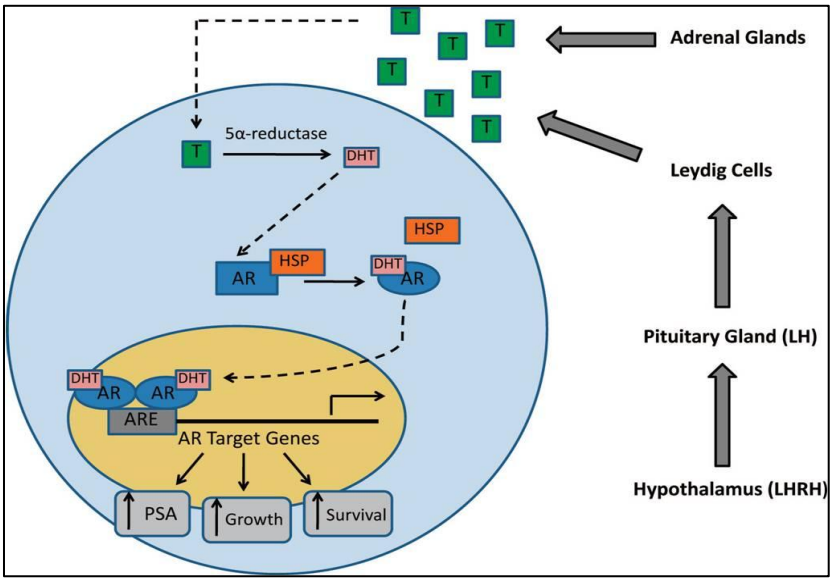


Figure 1.2 A Simplified Schematic on Androgen Signaling through AR (Saraon et al., 2011). AR is activated by its ligand, DHT, gets translocated into the nucleus and recruited to the chromatin to mediate transcription.

1.4 AR in Prostate Cancers

The AR signaling pathway is known to be critical in prostate cancer biology. Studies have revealed that the AR transcriptional program is responsible for regulating genes that are responsible for driving proliferation, survival and differentiation in prostate cancer cells (Buchanan et al., 2001; Debes and Tindall, 2002; Heinlein and Chang, 2004; Schiewer et al., 2012; Shen and Abate-Shen, 2010). Being the signaling core of this transcriptional program, the transcription activity of AR is crucial to the final output of the pathway and hence prostate cancer progression (Buchanan et al., 2001; Debes and Tindall, 2002; Heinlein and Chang, 2004; Schiewer et al., 2012; Shen and Abate-Shen, 2010). To take advantage of this relationship, a panel of AR direct transcriptional targets, including PSA, has been utilized as the main biomarkers for monitoring prostate cancer progression (Makarov et al., 2009). Furthermore, modulation of AR, the chief therapeutic target in prostate cancer treatment, has shown to be effective in the treatment of hormone naïve prostate cancers (Crawford et al., 1989). Although most prostate cancers eventually turn androgen independent (Feldman and Feldman, 2001), these advanced malignancies were shown to be still reliant on the AR signaling pathway for maintenance and continual progression (Chen et al., 2004; Zhang et al., 2003). Since castrate-resistant prostate cancers, while exhibiting resistant to anti-androgens, are still dependent on AR for survival, enhanced therapeutic targeting of AR in these forms of cancer should still be effective. This led to the development of second generation anti-androgens which have a higher affinity to AR in comparison to the currently used anti-androgens (Tran et al., 2009). Within expectations, these compounds have exhibited great potential in the

treatment of "androgen independent" prostate cancers (Tran et al., 2009). Second-site AR antagonists that target AR allosterically were also demonstrated to block AR action and block proliferation in castrate-resistant prostate cancer cells (Joseph et al., 2009). These further lend support to the feasibility of targeting AR as a therapeutic option in androgen independent cancers. Hence, it is imperative that we elucidate and understand the mechanisms underlying the transcriptional actions of AR so as to develop better therapeutic targeting strategies for treating castrate-resistant prostate cancers.

1.5 The Transcriptional Complex of AR

As a transcription factor, AR does not function alone. Gene transcription regulation via AR is a well-regulated process involving the participation of a diversity of other transcriptional factors. The functionality of AR-mediated transcription depends on a series of coordinated events which involve chromatin remodeling, epigenetic modifications, chromosomal looping and polymerase tracking (Wang et al., 2005). To gain understanding on the AR transcriptional machinery and its workings, previous efforts were focused on an individual gene level. The AR activated target gene, PSA, is one of the candidates most extensively studied model (Cleutjens et al., 1996; Shang et al., 2002; Wang et al., 2005) for insights into AR-mediated transcription. Transcriptional regulation of the PSA gene is governed by the co-operative actions of its AR bound proximal promoter and a distal enhancer (Shang et al., 2002; Wang et al., 2005). The proximal promoter of the PSA gene harbors 2 AREs (Cleutjens et al., 1996) while its

enhancer (4.2kb upstream of TSS) harbors 1 ARE (Cleutjens et al., 1997). Apart from AR, other co-operative transcription factors including histone acetylases (HATs), histone demethylases, mediator complexes, and polymerases are also recruited to the PSA enhancer and promoter (Louie et al., 2003; Metzger et al., 2005; Shang et al., 2002; Wang et al., 2005; Wang et al., 2002; Yamane et al., 2006), resulting in the formation of an AR transcriptional activator complex (Fig. 1.3). This AR transcriptional complex is responsible for initiating the required chromatin remodeling and epigenetic modifications that ensure chromatin competency for transcriptional regulation. A multitude of AR transcriptional collaborators are known to be recruited via the AF-1 and AF-2 domains of AR. For instance, co-activators such as P160 are recruited to the chromatin via association with the intra-molecular interaction between AF-1 and AF-2 of AR (Alen et al., 1999; He et al., 1999). Upon their engagement with the AR, some of these collaborators in turn mediate the assembly of other transcription factors, ultimately forming the AR transcriptional complex. The TRAP220/Med1 co-activator upon recruitment to AR serves to append the whole mediator complex to the chromatin for the direct recruitment of other general transcription factors (GTFs) and pol II (Lewis and Reinberg, 2003; Wang et al., 2005; Wang et al., 2002). Co-operation between the PSA enhancer and promoter is achieved through chromatin looping and sharing a core AR transcriptional complex (Shang et al., 2002; Wang et al., 2005). RNA polymerase II which is strongly recruited to the PSA enhancer then tracks to the promoter to enhance PSA gene transcription (Wang et al., 2005). Although much is already shown about the AR activator transcriptional complex regulating PSA transcription, the exact nature and dynamics of the complex is still unclear. Furthermore, apart from PSA, the AR

transcriptional regulatory machineries of hundreds of other AR target genes remain understudied.

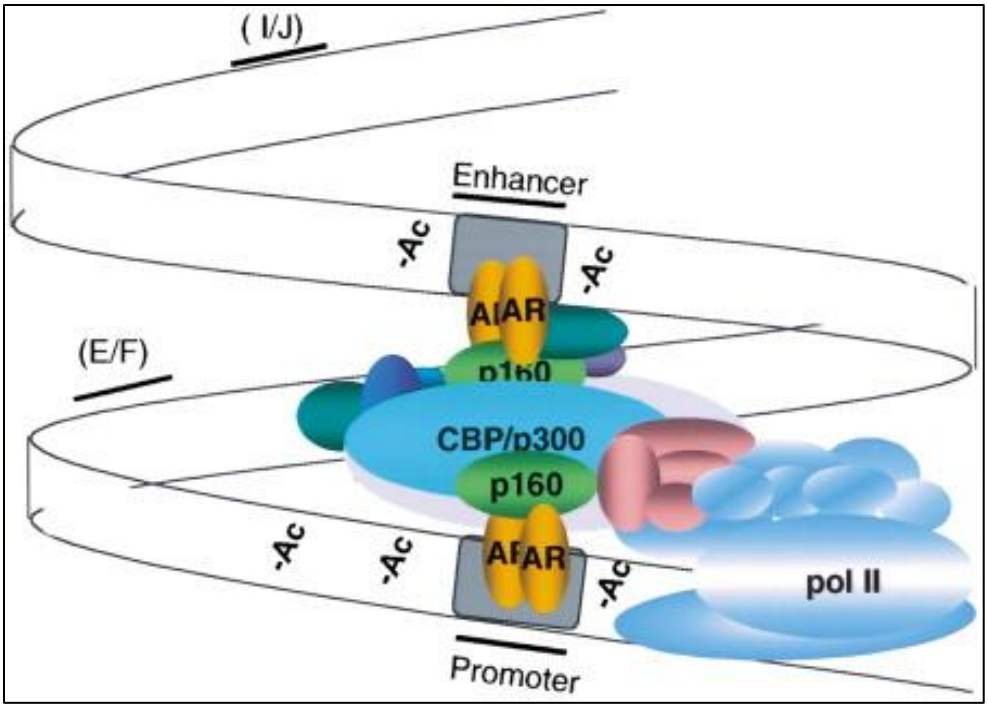


Figure 1.3 A Model of the AR Transcriptional Complex at the Enhancer and Promoter of PSA after Androgen Stimulation (Shang et al., 2002). Upon DHT stimulation, AR gets recruited to the cis-regulatory elements and initiates the recruitment of transcriptional cofactors such as p300 and p160. Finally, RNA polymerase II is also recruited for gene transcription.

1.6 Techniques for Genome-Wide Analysis of AR Binding Sites (ARBS) in Prostate Cancer Cells

The development of molecular biology has brought about unprecedented insights into cellular biology. Experimental research utilizing molecular biology techniques has elucidated much detail on the workings of cellular processes such as cell metabolism, cell signaling, transcription, translation, protein degradation and transportation. As discussed earlier, much progress has also been made on the field of AR transcriptional regulation. However, past studies were largely based on a single or a few genes (Cleutjens et al., 1997; Cleutjens et al., 1996; Shang et al., 2002; Wang et al., 2005) that provided limited information on the attributes of AR-mediated transcription at large. From gene profiling studies, it is known that hundreds of genes are actually regulated by androgen/AR (Holzbeierlein et al., 2004; Kazmin et al., 2006; Tan et al., 2012) in prostate cancer cells. In addition, time course gene expression profiling of androgen regulated genes revealed high differential expression kinetics among these genes (Tan et al., 2012). This suggests that the androgen regulated genes are likely to be regulated by a set of AR bound cis-regulatory sites that could possibly alter with the duration of androgen signaling. However, until recently, unlike AR induced gene expression profiles, AR cis-regulatory elements were comparatively understudied and there was no analysis of AR occupied cis-regulatory elements on a large scale. A large reason for this was the lack of a high throughput technology to identify ARBS in prostate cancer cells. Recently, the advent of experimental techniques such as Chromatin Immunoprecipitation coupled with

microarray (ChIP-chip) and Chromatin Immunoprecipitation coupled with massively parallel DNA sequencing (ChIP-seq) has provided the impetus for such studies.

1.6.1 ChIP-chip VS ChIP-seq

ChIP-chip and ChIP-seq are both high throughput methods for identifying and interrogating protein-DNA interactions *in-vivo*. They are both high throughput extensions of the Chromatin Immunoprecipitation (ChIP) technique. For both techniques, ChIP is first performed via crosslinking the interaction between the protein and DNA. Sonication is then performed to shear the chromatin into short pieces (~500bp). Immunoprecipitation to pull down the desired protein bound DNA fragment is then performed using specific antibodies against the protein of interest. For ChIP-chip, the pulled down DNA is then amplified and then hybridized on a tiling array for detection (Fig. 1.4), while ChIP-Seq involved the sequencing of the pulled down DNA and subsequent mapping back to the reference genome (Fig. 1.5).

The ChIP-chip technique was first utilized to establish ARBS *in-vivo* (Bolton et al., 2007; Massie et al., 2007; Wang et al., 2007). Although these studies have shed light on the rough landscape of the AR transcriptional regulatory network in prostate cancer cells, the shortfalls of the ChIP-chip technology is apparent. For instance, the resolution of the identified binding sites in ChIP-chip technology is low (i.e. few kb). Apart from that, the ChIP-chip technique is unable to interrogate repetitive regions of the genome and requires quite a huge amount of starting DNA material. Furthermore, ChIP-chip experiments are only possible depending on the availability of tiling arrays (which can be

costly if customization is required). In contrast, the ChIP-Seq emerged as an attractive alternative and offers several advantages over the ChIP-chip technology (Park, 2009) (Table 1). Consequently, recent studies have adopted ChIP-Seq as the preferred method for studying genome-wide ARBS (Tan et al., 2012; Wang et al., 2011; Yu et al., 2010b). Nevertheless, these 2 techniques have successfully advanced this research field, enabling a more detailed characterization of the AR cistrome (A genome-wide map of AR occupied cis-regulatory elements).

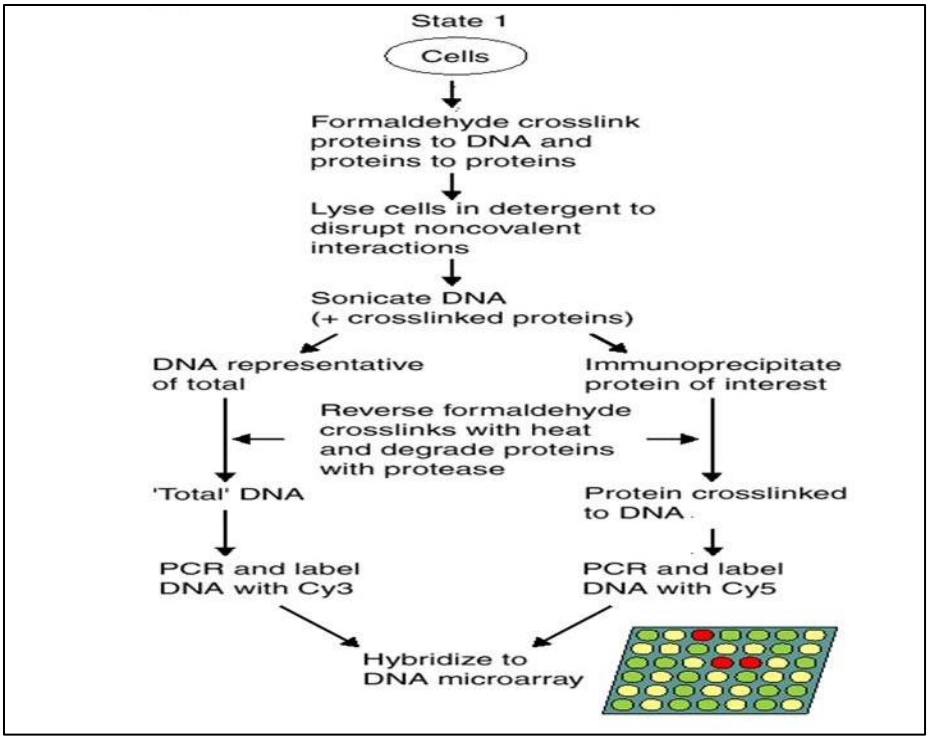


Figure 1.4 The Experimental Flow of ChIP-chip Technology (Pugh and Gilmour, 2001)

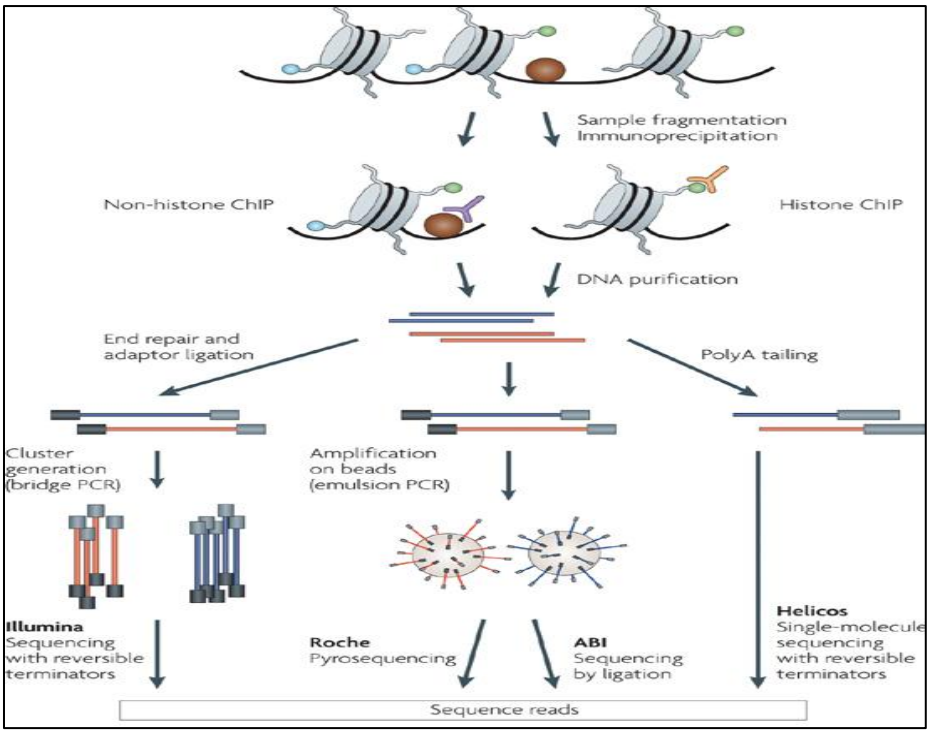


Figure 1.5 The Experimental Flow of ChIP-seq Technology (Park, 2009)

	ChIP–chip	ChIP–seq
Maximum resolution	Array-specific, generally 30–100 bp	Single nucleotide
Coverage	Limited by sequences on the array; repetitive regions are usually masked out	Limited only by alignability of reads to the genome; increases with read length; many repetitive regions can be covered
Cost	US$400–800 per array (1–6 million probes); multiple arrays may be needed for large genomes	Currently US$1,000–2,000 per lane (using the Illumina Genome Analyzer); 6–15 million reads before alignment
Source of platform noise	Cross-hybridization between probes and nonspecific targets	Some GC bias can be present
Experimental design	Single- or double-channel, depending on the platform	Single channel
Cost-effective cases	Profiling of selected regions; when a large fraction of the genome is enriched for the modification or protein of interest (broad binding)	Large genomes; when a small fraction of the genome is enriched for the modification or protein of interest (sharp binding)
Required amount of ChIP DNA	High (a few micrograms)	Low (10–50 ng)
Dynamic range	Lower detection limit; saturation at high signal	Not limited
Amplification	More required	Less required; single-molecule sequencing without amplification is available
Multiplexing	Not possible	Possible

Table 1 Comparison between the ChIP-chip and ChIP-seq methodology (Park, 2009)

1.6.2 The prospect of Next generation Sequencing (NGS) Technologies in Prostate Cancer Genomic Research

The advent of the NGS and its potential applications is likely to revolutionize genomic studies. NGS technology has endowed researchers the capability to examine in unprecedented details, the genomic profiles of different biological systems with unparalleled speed and ease. With its sheer technological power and potential, NGS technology has undoubtedly positioned itself as an indispensable driving force in future genomic research. In fact, most of the recent breakthroughs seen in the field are a result of NGS technology application. Correspondingly, future genomic studies on the AR transcriptional network in prostate cancers would likely hinge heavily on the further development of NGS technology.

1.7 Analyzing the AR Cistrome in Prostate Cancer Cells

Since AR functions primarily as a transcription factor in response to androgens, the genomic locations and characteristics of ARBS will directly affect the role of AR in regulating transcription. An understanding of the genomic features and distribution patterns of the AR cistrome in prostate cancer cells will contribute to the comprehension of the AR transcriptional regulatory mechanisms and the identities of the target genes it regulate. For instance, direct target genes of AR could be possibly identified from the locations of ARBS.

1.7.1 Location Analysis of ARBS in Prostate Cancer Cells

Initially, as with other transcription factors such as the promoter-bound E2F transcription factor family (Xu et al., 2007), it was generally assumed that genome-wide AR chromatin occupancy studies would allow easy identification of primary AR target genes responsible for the diverse downstream cascades of androgen signaling. However, AR was found generally to regulate transcription through occupying distal enhancers far away from the transcriptional start sites of regulated genes (Tan et al., 2012; Wang et al., 2007; Yu et al., 2010b). Consequently, this poses a major challenge in the determination of targets genes that are directly regulated by specific AR cis-regulatory elements. Moreover, in comparison to several hundreds of androgen regulated genes, thousands of ARBS were detected across the genome (Tan et al., 2012; Wang et al., 2009b; Yu et al., 2010b). This further adds to the complexity of the problem in associating binding sites with regulated genes whereby a single gene could be regulated by multiple enhancers (through extensive chromatin loopings). There is also the possibility of the presence of large numbers of AR enhancers that are non-functional under this situation which may only be transcriptionally activated under specific signaling conditions. Interestingly, the recruitment to distal enhancers might be a recurring feature for nuclear hormone receptor mediated transcriptional regulation (Carroll et al., 2006; Lefterova et al., 2008).

1.7.2 The Androgen Response Elements and other Motifs in ARBS

Like other DNA binding transcription factors, the AR DNA binding domain is mainly responsible for determining its DNA binding specificity and affinity. To determine the

DNA motifs to which AR binds (termed the Androgen Response Elements (AREs), earlier studies had performed DNAse footprinting and electrophoretic mobility shift assays (Cleutjens et al., 1997; De Vos et al., 1991) with cloned androgen-responsive enhancers near androgen regulated genes. Through these efforts, AR was found to bind to imperfect inverted repeats with a three base pair spacer bearing similarities to the sequence 5′-AGAACANNNTGTTCT-3′. However, this identification approach was tedious and low throughput in nature for the discovery of possible ARE sequences. Subsequently, a PCR-based SELEX (Systematic Evolution of Ligands by Exponential Enrichment) approach was utilized (Nelson et al., 1999; Roche et al., 1992; Zhou et al., 1997) to meet this challenge. Not only do these studies confirm AR's high binding affinity to sequences similar to 5′-AGAACANNNTGTTCT-3′, they also demonstrated that AR exhibit specific binding preferences to direct repeats and to certain nucleotides at the flanking or spacer region of AREs that differ from other Class I steroid nuclear hormone receptors (GR, PR and MR) (Nelson et al., 1999; Zhou et al., 1997). Even though these studies have extended our understanding on the binding specificity of AR, they only provided information on the *in-vitro* binding characteristics of AR. The *in-vivo* features of ARBS are likely to be influenced by the presence of other collaborative transcription factors and the chromatin status of the binding region. Unsurprisingly, by utilizing Chromatin Immunopreciptiation (ChIP) assays to interrogate AR occupancy *in-vivo*, a significant proportion of perfect AREs present in the genome were found to be devoid of AR binding in the LNCaP prostate cancer cells (Horie-Inoue et al., 2006). Nevertheless, this result confirms the disparity between *in-vitro* and *in-vivo* binding features of AR. Since ChIP assays could be used to identify *in-vivo* ARBS, the

application of a high throughput ChIP-based approach would enable the determination of the AR cistrome. To this end, methodologies such as ChIP on chip (ChIP-chip) (Iyer et al., 2001), ChIP paired-end tags (Wei et al., 2006) (ChIP-PET) and ChIP sequencing (Johnson et al., 2007) (ChIP-Seq) were developed. The application of these high throughput AR ChIP assays in prostate cancer cells has provided a large number of novel bona-fide ARBS for analysis. Although the canonical ARE consensus motif was observed to be enriched in the ARBS identified by several different studies (Bolton et al., 2007; Massie et al., 2007; Wang et al., 2007; Yu et al., 2010b), a substantial portion of the ARBS were reported to be devoid of canonical AREs. Through a chromosome wide AR ChIP-chip experiment in LNCaP cells (Wang et al., 2007), it was reported that only 10% of the 90 ARBS found on chromosome 21 and 22 harbor the canonical AR consensus motif. Interestingly, 68% of the 90 ARBS were described to contain non-canonical AREs. These non-canonical AREs are either in the form of isolated half AREs or half AREs arranged in a head-to-head, tail-to-tail or direct repeat manner with a varying spacer length between zero to eight nucleotides. Similarly, the other study (Massie et al., 2007) that performed AR ChIP on chip using promoter tiling arrays reported a relative small proportion of ARBS (~26.8%) harboring canonical AREs with the majority of the ARBS (~57.2%) having only half ARE motifs. In contrast, canonical form of AREs were found in majority of ARBS (~69%) identified in HPr-1AR cells through AR ChIP on chip assay that utilize customized tiling arrays interrogating ~104kb genomic regions centered on the transcription start sites of 548 candidate hormone responsive genes (Bolton et al., 2007). Analysis of our AR ChIP-Seq data in LNCaP cells (Tan et al., 2012) revealed canonical AREs in 44% and half AREs in 19% of the ARBS.

However, we were not able to detect enrichment of other previously reported forms (apart from ARE direct repeats of three base pair spacer) of non-canonical AREs (Wang et al., 2007). Indeed, a recent paper provided data that cast doubts on the functionality of the non-canonical AREs (Denayer et al., 2010). Despite substantial differences between these high throughput ChIP-based studies, these results largely confirmed that *in-vivo*, AR is not recruited exclusively to rigid canonical ARE motifs determined *in-vitro*. The surprisingly low occurrence of AREs in ARBS has provided the impetus for investigating the presence of other enriched motifs that could aid AR recruitment indirectly. Indeed, through motif enrichment analysis, motifs of several transcription factors were found to be overrepresented in ARBS and subsequently validated as transcriptional collaborators of AR in several studies (Massie et al., 2007; Tan et al., 2012; Wang et al., 2007; Yu et al., 2010b).

1.7.3 AR Cistrome in Advanced Prostate Cancers

As mentioned earlier, studies have demonstrated that AR activation remains critical to the survival and growth of castrate-resistant prostate cancers (Hara et al., 2003; Zhang et al., 2003). However, the exact role of AR in advanced prostate cancers is still largely unknown. Recent advances in AR cistromic studies have provided insights on the alterations to the AR transcriptional program accompanying prostate cancer progression. For instance, Wang and his colleagues provided evidence for a distinct AR transcriptional program in androgen independent prostate cancers (Wang et al., 2009b). Specifically, they found that relative to hormone dependent prostate cancers, AR display distinct

chromatin occupancy preferences to the cis-regulatory elements of a substantial number of cell cycle and M-phase genes in castrate-resistant prostate cancers. An example is the Ubiquitin-conjugating enzyme E 2C (UBE2C). Consequently, AR exclusively regulates the expression of these genes to promote proliferation in androgen independent prostate cancer cells but not in its androgen dependent counterpart. Remarkably, this study has provided novel insights to the AR transcriptional network in prostate cancers by demonstrating the capability of AR in mediating different transcriptional programs with prostate cancer progression. A more recent study has identified a possible mechanism that could regulate the transcription plasticity of AR (Wang et al., 2011).

1.8 Transcriptional Collaborators of AR

Transcription co-factors are able to exert profound influence on the AR transcriptional output through the regulation of AR's transcription activity. In prostate cancers, transcriptional co-regulatory factors of AR are commonly expressed aberrantly. Consequently, the AR transcriptional network is altered to one that promotes oncogenesis. Indeed, studies have suggested that altered expression of AR co-activators could contribute to castrate-resistant prostate cancers (Devlin and Mudryj, 2009). In another testament to the importance of these transcription collaborators, a recent study had identified the presence of mutated AR co-factors that could deregulate AR signaling in prostate cancers (Taylor et al., 2010). Given mounting evidence that point to the emergence of a transformed AR transcriptional circuitry essential for prostate cancer progression, it would be of therapeutic interest to identify and understand the other

different AR transcription collaborators that could possibly contribute towards the reshaping of AR cistrome and its transcriptional program. Next, I will give a discussion on two major AR transcriptional collaborating factors in prostate cancer cells.

1.8.1 Forkhead Box Protein A1

Recent studies have demonstrated the presence of transcription factors already preloaded to potential AR-enhancers prior androgen stimulation and AR recruitment (Sahu et al., 2011; Wang et al., 2011; Wang et al., 2007). This particular class of transcription factors was termed as pioneering factors. Their presence at these cis-regulatory elements was usually shown to be necessary for subsequent recruitment of AR or other transcriptional co-factors (Sahu et al., 2011; Wang et al., 2011; Wang et al., 2007). Correspondingly, pioneering factors were generally implicated to facilitate and prime the recruitment of other transcription factors through chromatin remodeling. Although the exact mechanisms responsible for initiating chromatin remodeling is likely to be specific for the different pioneering factors and is still largely unclear, a variety of general mechanisms have been studied and put forward (Magnani et al., 2011). Pioneering factors were suggested to be able to modulate the nucleosomal structure and facilitate transcription factor recruitment by directly evicting the nucleosomes, modulating higher-order chromatin structure and initiating/maintaining epigenetic modifications that are associated with potential transcription factor binding sites (Magnani et al., 2011).

Forkhead box protein A1 (FoxA1) is one of the identified pioneering factors for AR-mediated transcription (Sahu et al., 2011; Wang et al., 2011). The role of FoxA1 as a

pioneering factor was first discovered in the estrogen receptor (ER) transcriptional network system in breast cancers (Carroll et al., 2005). Apart from ER, FoxA1 was also shown to function as a pioneering factor of AR in prostate cancers (Lupien et al., 2008). In concordance with its purported role as a major pioneering factor of both estrogen receptor (ER) and AR, the cistromes of FoxA1 were revealed to overlap significantly with that of ER, in breast cancers and AR, in prostate cancers (Lupien et al., 2008). Intriguingly, even though FoxA1 binding was largely independent to estrogen or androgen stimulation, the cistrome of FoxA1 identified in breast and prostate cancers was significantly different (Lupien et al., 2008). The large number of lineage-specific FoxA1 binding sites found was functionally responsible for the determination of tissue-specific transcription programs of the nuclear receptors in breast and prostate cancers through pioneering differential tissue-specific AR and ER cistromic profiles (Lupien et al., 2008). The mechanisms underlying FoxA1 specific recruitment to the chromatin were also investigated. FoxA1 binding sites were found to be enriched for H3K4me1 and H3K4me2 histone marks (Lupien et al., 2008; Sahu et al., 2011; Serandour et al., 2011). Importantly, the removal of these marks through LSD1 overexpression was shown to abrogate FoxA1 binding (Lupien et al., 2008). In contrast, silencing of FoxA1 did not affect the H3K4 methylation levels but reduced DNAse I sensitivity at FoxA1 binding sites (Lupien et al., 2008). This data supports a model in which FoxA1 is recruited to H3k4me1/me2 sites to initiate chromatin opening and remodeling, priming the region for subsequent AR/ER recruitment.

Given the general consensus on FoxA1's role as a critical pioneering factor for AR, it would be important to determine its regulation of the AR transcriptional network in

prostate cancer cells. To address this, two recent studies (Sahu et al., 2011; Wang et al., 2011) separately performed in-depth analysis of the AR cistromes before and after FoxA1 depletion in prostate cancer cells. Even though the experimental conditions between the two studies were somewhat different, similar observations were derived (Sahu et al., 2011; Wang et al., 2011). The analyses defined three classes of ARBS that were differentially affected by FOXA1 depletion: The gained ARBS, the lost ARBS and the unchanged ARBS. Through coupling the AR cistromic maps with microarray profiling experiments, it was revealed that the genes regulated by the three different classes of ARBS had unique biological functions (Sahu et al., 2011; Wang et al., 2011), providing a mechanism in which FoxA1 determines prostate cancer progression. Intriguingly, even though the two studies both demonstrated similar capabilities of FoxA1 in reprogramming the AR transcriptional network, the two studies claimed contrasting phenotypical and functional effects exerted by FoxA1 in prostate cancer progression. In the first study (Wang et al., 2011), decreased levels of FoxA1 were associated with castrate resistant, poor prognostic prostate tumours. Moreover, depletion of FoxA1 enhanced S-phase cell entry of LNCaP prostate cancers under reduced androgen conditions. On the contrary, the second study (Sahu et al., 2011) associate low FoxA1 levels with good patient prognosis. While these opposing results may seem contradicting, they might be pointing to a dual role of FoxA1 on cancer progression under different subtypes or stages of prostate cancer. However, further experimentations would definitely be necessary to substantiate this claim.

1.8.2 The ETS Transcription Factor: ERG

The ETS family encompass a class of transcription factors that have a highly conserved DNA binding domain termed the ETS domain (Karim et al., 1990). The ETS domain is a winged helix-turn-helix structure that binds to DNA with the purine-rich core sequence GGAA (Karim et al., 1990; Kodandapani et al., 1996; Liang et al., 1994). The ETS transcription factors were known to play important roles in regulating a wide diversity of cellular and developmental processes including cell proliferation, cell differentiation, cellular senescence, haematopoiesis, angiogenesis and apoptosis (Ohtani et al., 2001; Sevilla et al., 1999; Sharrocks, 2001; Taylor et al., 1997; Treisman, 1994). Since all of the ETS family members recognized the same core DNA sequence as a result of their highly conserved ETS domain, the action specificity of each ETS transcription factor are generally specified by interacting with specific co-regulatory protein partners and/or by post translation modifications such as phosphorylation and ubiquitination during activation of cellular signaling (Chakrabarti et al., 2000; Li et al., 2000; Sharrocks, 2001; Wasylyk et al., 1998). The function of ETS transcription factors as activators or repressors was also dependent on cellular context, regulated by their protein interaction partners and linked to activation of specific signal transduction pathways (Sharrocks, 2001; Sharrocks et al., 1997). Interestingly, several ETS transcription factors were found to be highly associated with cancers through gene fusion with another protein. Examples include the EWS-ERG, EWS-FLI1 gene fusion in Ewing's sarcoma (Giovannini et al., 1994) and the TEL-JAK2 in leukemia (Lacronique et al., 1997; Peeters et al., 1997).

Of high relevance to prostate cancers, a recent landmark paper reported the discovery of recurrent ETS fusion genes (Tomlins et al., 2005). Strikingly, it was reported that almost

50% of prostate cancers from PSA screened cohorts (Kumar-Sinha et al., 2008) actually harbor recurrent ETS gene fusions. Out of all the different types of ETS gene fusion, the most commonly occurring variant is the TMPRSS2-ERG fusion gene (Kumar-Sinha et al., 2008). This fusion involves the promoter of the androgen regulated gene, TMPRSS2 to fuse to the promoter of ERG gene, rendering the expressing of ERG androgen dependent (Fig. 1.6). Other than ERG, ETS family members such as ETV1, ETV4 and ETV5 are also found to be involved in gene fusions in prostate cancers albeit at a substantially lower frequency (Kumar-Sinha et al., 2008). Given the widespread prevalence of ERG gene fusion in prostate cancers, intense research efforts have been directed at elucidating its function in prostate cancer progression. Consequently, ERG was established as a key player in prostate oncogenesis in multiple studies (Carver et al., 2009; King et al., 2009; Tomlins et al., 2008; Zong et al., 2009). Specifically, ERG was shown to synergize to PTEN loss and PI3K pathway activation to promote prostate cancer progression to invasive adenocarcinoma (Carver et al., 2009; King et al., 2009). In addition, increased ERG expression in the prostate cells was linked to the activation of the plasminogen activation and the matrix metalloproteinase (MMP) pathways through direct transcriptional upregulation of pathway components (Fig. 1.6) (Tomlins et al., 2008). Interestingly, ERG was also shown to co-operate with AR to induce the formation of poorly differentiated and invasive prostate carcinomas in mice (Zong et al., 2009). This posits a potential AR and ERG crosstalk that might be crucial for prostate cancer development and progression. Given that both AR and ERG are transcriptional factors, it is tempting to speculate a transcriptional collaboration (Fig. 1.6).

Indeed, the ETS binding motif was first found to be over-enriched at AR-bound promoter regions identified in an AR ChIP-on-chip study on prostate cancer cells (Massie et al., 2007). Through further ETS-1 ChIP experiments, the same study went on to show androgen-induced recruitment of ETS-1 to several AR-bound promoters (Massie et al., 2007). This was the first study to demonstrate the potential of ETS members as collaborative factors of AR in regulating transcription. Subsequently, both ETV1 (Shin et al., 2009) and ERG (Sun et al., 2008) were also revealed to regulate AR signaling through functioning as AR transcriptional co-factors. Intriguingly, while ETS-1 and ETV1 were implicated as transcriptional activators of AR (Feed Forward Regulation) (Massie et al., 2007; Shin et al., 2009), ERG was recently shown by others to exert repressive (Feedback Regulation) influence on AR dependent transcription (Sun et al., 2008; Yu et al., 2010b). As this finding suggests a different role for each unique ETS gene fusion in prostate oncogenesis, this finding implies a need to further stratify ETS fusion positive prostate cancers into more specific subgroups for diagnosis and treatment.

To understand the transcriptional network of AR and ERG, Yu and colleagues (Yu et al., 2010b) have generated genome-wide cistromic profiles of AR and ERG in ERG fusion positive VCaP prostate cancer cells. They observed substantial overlap between the cistromes of AR and ERG and hence provide evidence for a genome-wide transcriptional collaboration between the two factors. ERG was shown to occupy the AR enhancers as well as the promoter of AR target genes. Importantly, ERG was found to suppress androgen induced differentiation markers and consequently implicated as a suppressor of AR-mediated epithelial differentiation (Yu et al., 2010b). Although this study (Yu et al., 2010b) has provided substantial insights on the transcriptional collaboration between AR

and ERG, several important questions on the cross-talk remains unanswered. Some of these unresolved questions were highlighted by Chen and Sawyers (Chen and Sawyers, 2010). For instance, the model AR target genes that were shown to be repressed by ERG (Yu et al., 2010b) were only epithelial differentiation markers with no known functional role in oncogenesis. Consequently, it is important to identify the ERG repressed AR target genes that are responsible for cancer progression. Also, the mechanics of this particular transcriptional collaboration under androgen signaling remains vague as the analysed ERG cistrome was not under an androgen stimulated condition.

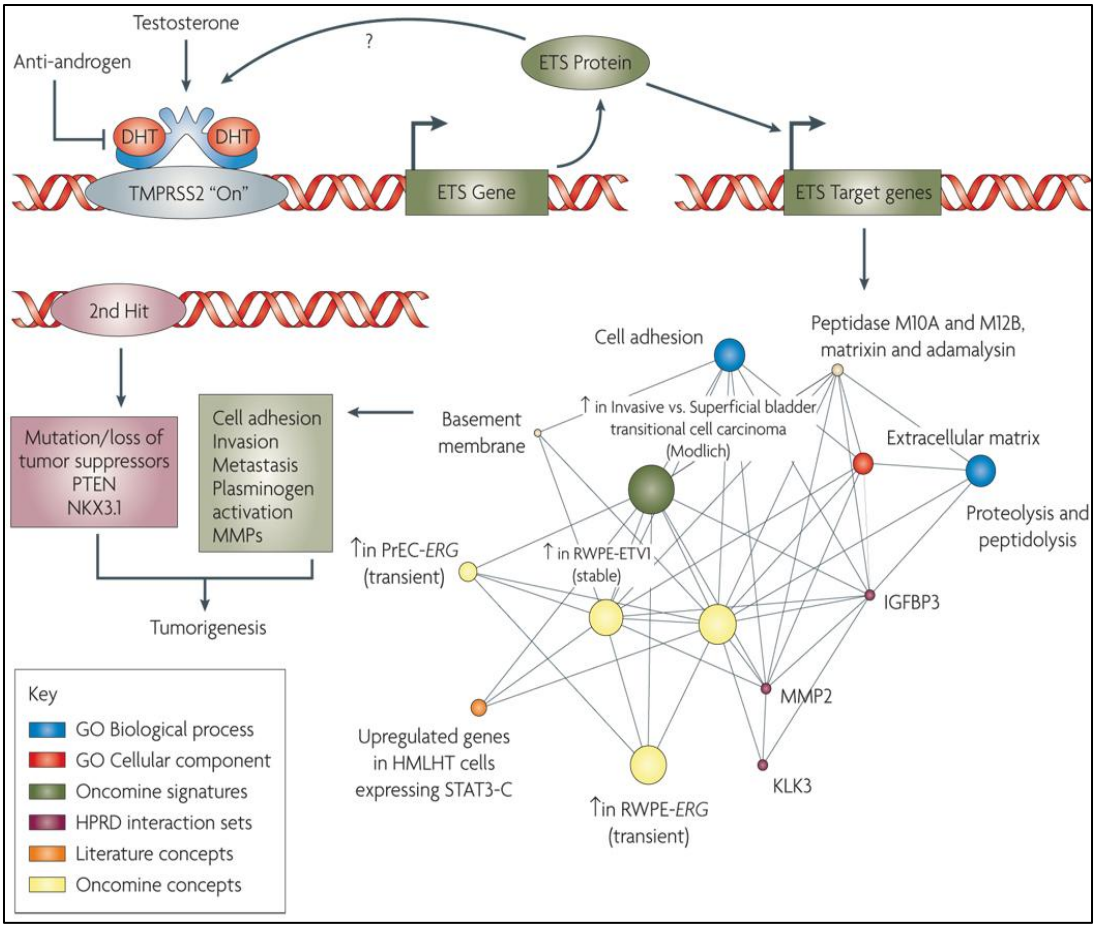


Figure 1.6 The Defined Role of ETS proteins in Prostate Cancers (Kumar-Sinha et al., 2008)

1.9 Reduced Androgen Signaling in Advanced Metastatic Prostate Cancers

A partial attenuation of androgen signaling was suggested to be a common feature of metastatic and advanced prostate cancers (Yu et al., 2010b). This was a surprising finding as AR is commonly known to enhance prostate cancer development and maintenance through driving proliferation and survival (Heinlein and Chang, 2004). In normal prostate development, apart from driving proliferation, AR is also known to promote differentiation of prostate cells (Kelly and Yin, 2008). Likewise, AR is implicated to drive a pro-differentiation transcription program in prostate cancer cells (Sun et al., 2008; Yu et al., 2010b). Consequently, this part of AR activity is inhibitory to the aggressive stem cell-like dedifferentiated state that is usually exhibited by metastatic prostate cancers (Yu et al., 2007b). To subvert this, in ERG-fusion positive prostate cancers, ERG functioned to directly mediate partial suppression of androgen signaling for prostate cancer advancement (Sun et al., 2008; Yu et al., 2010b). Apart from ERG, multiple transcriptional co-repressors implicated for cancer progression, are also commonly over-expressed in prostate cancers. These co-repressors were generally thought to exert their oncogenic influence through constitutive repression of tumor suppressor genes. However, their role in androgen signaling is still relatively unclear. It would thus be interesting to investigate their function, if any, in the AR transcriptional network and determine if they act in a similar way as ERG. In this study, we decide to focus our investigation on the HDACs (specifically HDAC1, HDAC2 and HDAC3) and EZH2 co-repressors as a result of their known importance in prostate cancer development and their relationship with AR and ERG.

1.10 Histone Deacetylases in Prostate Cancers

HDACs are a class of enzymes that catalyze the removal of acetyl groups from either transcription factors or the tails of histones to regulate the transcription of target genes (de Ruijter et al., 2003; Glozak and Seto, 2007). Deacetylation of the histones results in chromatin compaction, thereby restricting the recruitment of transcriptional machinery (RNA polymerases and other GTFs) (Fig. 1.7). Consequently, the transcriptional activity of the target gene is reduced or abolished (de Ruijter et al., 2003; Glozak and Seto, 2007). Apart from histones, deacetylation of transcription factors by HDACs could cause a change in protein conformation, which in turn alters the activity of the said transcription factor (Fig. 1.7) (Glozak and Seto, 2007). On the other hand, HATs act in counter to the activity of HDACs, catalyzing the acetylation of histones leading to the enhancement of transcriptional activity. Therefore, the regulation of net activity between HDACs and HATs could serve as a rheostat to the transcriptional output of the target genes (Fig. 1.7) (Glozak and Seto, 2007).

Essentially, HDACs are categorized into different classes according to their sequence similarity, domain organization and homology to yeast counterparts (Table 2) (Abbas and Gupta, 2008; de Ruijter et al., 2003; Dokmanovic et al., 2007; Gregoretti et al., 2004). HDACs usually are involved in multi-subunit repressive complex recruited by specific transcriptional co-repressors. For instance, HDACs could be recruited by nuclear receptor co-repressors such as N-CoR and SMRT to form part of the repressor complex for gene suppression (Abbas and Gupta, 2008).

Out of all the HDACs, the class I members, HDAC1, HDAC2 and HDAC3, were shown to be frequently over-expressed and to promote metastasis in prostate cancers (Wang et al., 2009a; Weichert et al., 2008). Other than being suggested to be involved in DNA-methylated repression of tumor suppressors that inhibit cellular proliferation and survival (Patra et al., 2001), HDACs are also known to impact AR-mediated transcription in prostate cancers (Abbas and Gupta, 2008; Gaughan et al., 2002; Gaughan et al., 2005; Shang et al., 2002). In fact, AR and HDAC1 were demonstrated to form a complex at the PSA promoter (Gaughan et al., 2002; Gaughan et al., 2005). AR was demonstrated to be directly de-acetylated by HDAC1, culminating into the downregulation of AR activity (Gaughan et al., 2002; Gaughan et al., 2005). A de-acetylated AR was shown to be de-stabilized through Mdm2-mediated ubiquitylation (Gaughan et al., 2005). In another study, HDAC1 and HDAC2 were shown to be only recruited to the PSA promoter to form the corepressor complex after bicalutamide (androgen antagonist) stimulation (Shang et al., 2002). Given the importance of HDACs and AR in prostate cancers, it was suprising that, to date, we found no published study on the genome-wide cross-talk between HDACs and AR.

It was interesting for us to note that high levels of HDAC1 coupled with a low HDAC1 target gene profile expression are key features of TMPRSS2-ERG fusion positive prostate cancers (Gupta et al., 2010; Iljin et al., 2006). Furthermore, ERG fusion positive prostate cancer cells were shown to be particularly sensitive to HDAC inhibitor treatment (Bjorkman et al., 2008). Accordingly, ERG fusion positive prostate cancers were postulated to adopt epigenetic reprogramming through HDAC1 as an oncogenicity driving mechanism (Bjorkman et al., 2008). This particular feature of ERG positive

prostate cancer is suggestive of a possible transcriptional collaboration between HDACs and ERG.

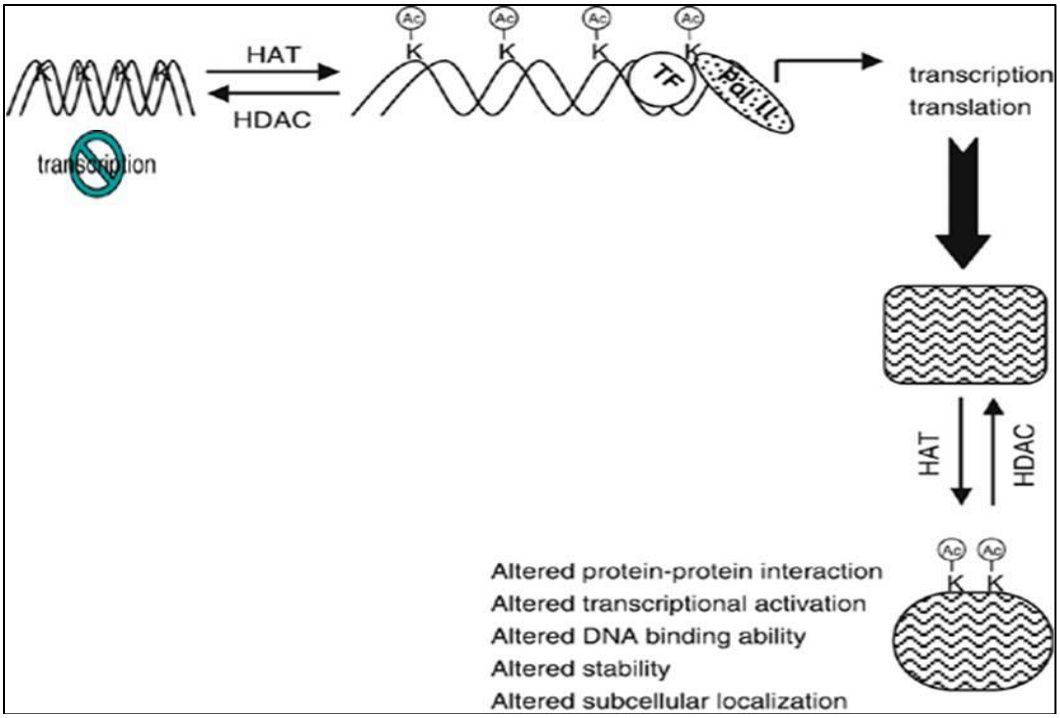


Figure 1.7 Involvement of HDACs in Transcriptional Regulation (Glozak and Seto, 2007)

HDAC classification depending on sequence identity and domain organization

Histone Classes	Members	Localization	Target Proteins (partial list)
Class I	HDAC1	Nucleus	AR, p53, E2F-1, SHP, STAT3, MyoD
	HDAC2	Nucleus	STAT3, Bcl6, glucocorticoid receptor GATA-1, STAT-3,SHP, RelA, YY-1
	HDAC3	Nucleus	
	HDAC8	Nucleus	-
Class II	HDAC4	Nucleus/Cytoplasm	GCMa, GATA-1, HP-1
	HDAC5	Nucleus/Cytoplasm	GCMa, Smad7, HP-1
	HDAC6	Mostly Cytoplasm	HSP70, Smad7, SHP, α-tubulin
	HDAC7A	Nucleus/Cytoplasm	PLAG1, PLAG2
	HDAC9	Nucleus/Cytoplasm	-
	HDAC10	Mostly Cytoplasm	-
Class III	SIRT1	Nucleus	NF-κB, p53, FOXO
	SIRT2	Cytoplasm	α-tubulin, H4
	SIRT3	Nucleus/Mitochondria	Acetyl-CoA synthetases
	SIRT4	Mitochondria	Glutamate dehydrogenate
	SIRT5	Mitochondria	-
	SIRT6	Nucleus	DNA polymerase B
	SIRT7	Nucleus	RNA polymerase 1
Class IV	HDAC11	Nucleus/Cytoplasm	-

Table 2 Different Classes of HDACs (Abbas and Gupta, 2008)

1.11 The Methyltransferase Polycomb Protein EZH2 in Prostate Cancers

The enhancer of zeste homolog 2 (EZH2), is a member of the polycomb group family that catalyzes the trimethylation of histone H3K27 at the promoter of genes for targeted transcriptional repression (Fig. 1.8) (Cao and Zhang, 2004). EZH2 was first discovered as part of a multiprotein polycomb complex that mediates the silencing of HOX genes for proper embryonic development in *Drosophila* (Fig. 1.8) (Franke et al., 1992). EZH2 was postulated to be an important regulator of development as its deletion in mice resulted in early lethality during early stage of development (O'Carroll et al., 2001). In embryonic stem cells, EZH2 usually functions in complex with Eed and Suz12, forming the polycomb repressor complex 2 (PRC2) to silence the expression of genes that are involved in differentiation and in the regulation of specific developmental lineages (Fig. 1.8) (Boyer et al., 2006; Lee et al., 2006b). EZH2 was also shown to be involved in X-chromosome inactivation (Fig. 1.8) (Plath et al., 2003). In cancers, the oncogenic role of EZH2 in cancer progression has been well established in a diversity of cancers including breast, prostate and lymphoma (Kleer et al., 2003; Neff et al., 2012; Simon and Lange, 2008; Varambally et al., 2002). Aberrantly high expression of EZH2 was demonstrated to inhibit differentiation, drive proliferation, metastasis and angiogenesis in cancers (Bryant et al., 2007; Chang et al., 2011; Li et al., 2010; Lu et al., 2010; Min et al., 2010; Richter et al., 2009; Simon and Lange, 2008). Accordingly, elevated levels of EZH2 were commonly associated with advanced cancers exhibiting poor prognostic outcomes (Simon and Lange, 2008) (Yu et al., 2007b). In particular, EZH2 were found to be commonly overexpressed in invasive and castrate-resistant prostate cancers (Varambally

et al., 2002). The oncogenic capability of EZH2 has been strongly linked with its methyltransferase activity as the presence of a functional SET domain is vital for EZH2 mediated repression of its target genes in prostate cancers (Varambally et al., 2002). In support, EZH2 has been shown to function as a repressor of tumor suppressors (E-cadherin and Slit2) in prostate cancer cells through trimethylation of h3k27 at their promoters (Fig. 1.8) (Cao et al., 2008; Yu et al., 2010a). The suppression of these genes has led to increased invasive capabilities of the prostate cancer cells (Cao et al., 2008; Yu et al., 2010a). The epigenetic silencing of DAB2IP expression by EZH2 was also demonstrated to enhance prostate cancer invasiveness through activating RAS and NFKB (Min et al., 2010). In addition, EZH2 was also implicated to play a role in maintaining an aggressive stem-cell-like state in prostate cancer cells by triggering a cellular de-differentiation program through epigenetic silencing (Yu et al., 2010b; Yu et al., 2007b). Recent data suggests that EZH2 might play a role in AR-mediated repression of target genes (Zhao et al., 2012). However, no co-localization between EZH2 and AR on specific cis-regulatory elements was shown in the study to establish direct transcriptional collaboration between the two factors (Zhao et al., 2012). A genome-wide colocalization between AR and EZH2 that would clarify the generality of the transcriptional cross-talk would be beneficial for further insights. The results from Zhao et al. (Zhao et al., 2012) suggest the presence of an EZH2 and AR cross-talk in AR-mediated transcriptional repression but since the analysis is only limited to a few genes, it may not be representative of the exact nature of the cross-talk. Recent findings in the field have shown that the cross talk between AR and the transcriptional repressor, ERG, could promote prostate cancer progression through regulated suppression of AR signaling (Yu

et al., 2010b). Being a transcriptional repressor itself, EZH2 has the potential to facilitate prostate cancer development in a similar fashion as ERG, perhaps even through collaboration with ERG. Moreover, the expression levels of ERG and EZH2 were shown to be correlated in prostate cancers (Yu et al., 2010b). Accordingly, we think that further studies would be necessary for dissecting the possible cross-talk between AR, ERG and EZH2.

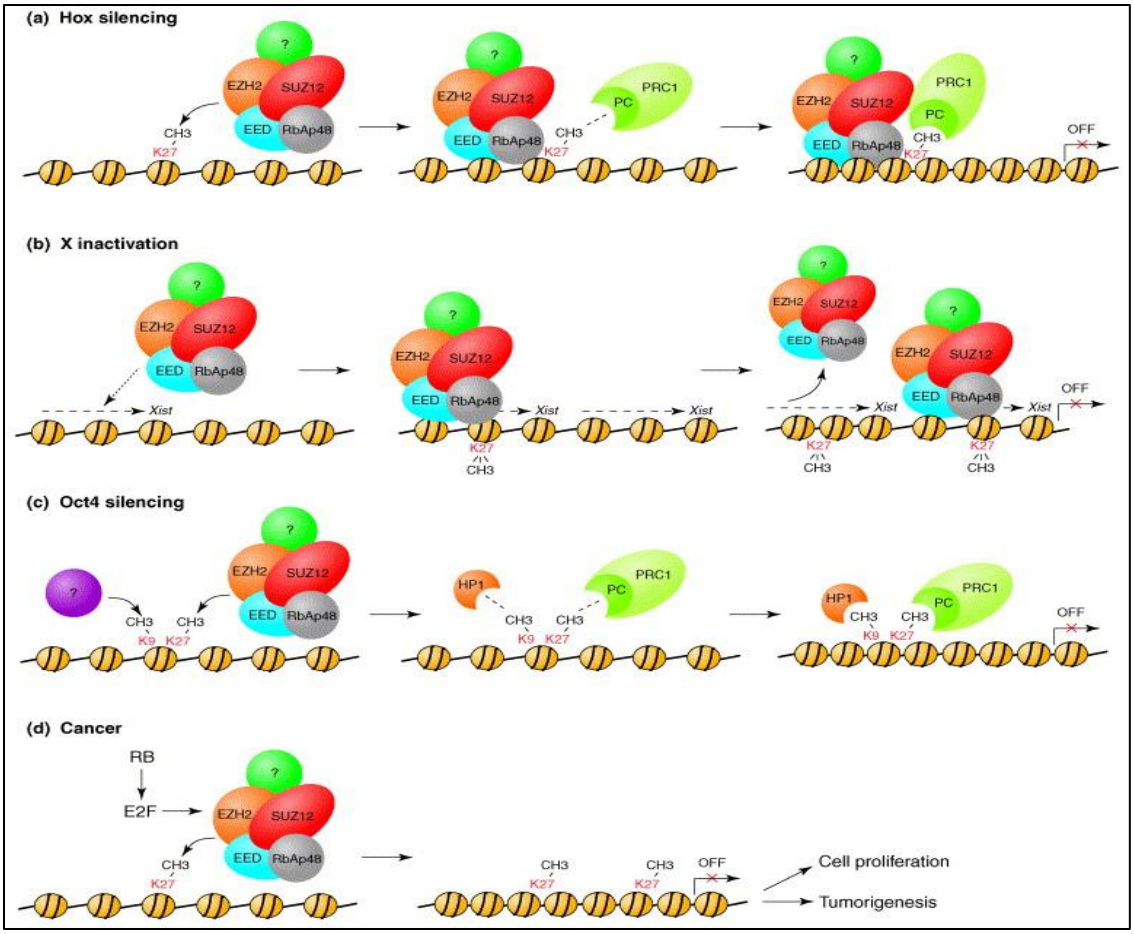


Figure 1.8 Examples of the Different Mechanisms of EZH2-mediated Transcriptional Repression (Cao and Zhang, 2004)

1.12 Aims of Study

As discussed above, various pieces of evidence have strongly linked the process of prostate cancer development to an alteration of the AR transcriptional output. The AR transcriptional output is a direct manifestation of the workings of the AR transcriptional circuitry. The AR transcriptional network exhibits high plasticity (Wang et al., 2009b). As such, prostate cancers are able to adapt, progress and recur through recalibration of the AR transcriptional circuitry. This could be accomplished via manipulating the expression levels of AR (Chen et al., 2004) and/or its transcriptional collaborators (Sahu et al., 2011; Wang et al., 2011). Hence, a thorough understanding of the cross-talk between AR and its collaborators could be useful to the development of new therapeutics for prostate cancers.

In this study, we aim to investigate the transcriptional cross-talk between AR and the recurrent fusion gene, ERG. During the course of our work, other studies have provided evidence that ERG functions as an AR transcriptional collaborator that contributes to the development of prostate cancers by suppressing AR signaling (Sun et al., 2008; Yu et al., 2010b). These independent studies, which are largely in agreement with our findings, have elucidated much insight about the AR and ERG crosstalk. However, these studies did not address the underlying mechanisms and functional consequences of the ERG-mediated repression of AR signaling. Furthermore, as mentioned earlier in this thesis, apart from ERG, several other transcriptional repressors (HDACs and EZH2) were also shown to be over-expressed and had their expression levels correlated with ERG in prostate cancers. However, their potential involvement with the AR and ERG

transcriptional cross-talk remained unstudied. Utilizing genomic technologies and a variety of molecular biology techniques, we sought to characterize and analyze the AR and ERG centric co-repressor transcriptional network and its role in prostate cancer progression.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell Culture

LNCaP and VCaP, human prostate cancer cell lines were obtained from American Type Culture Collection (ATCC). Both are androgen dependent prostate cancer cell lines expressing functional AR protein. VCaP cells were maintained under 5% CO_2 in DMEM solution supplemented with 10% fetal bovine serum (FBS), sodium pyruvate, sodium bicarbonate, and penicillin/streptomycin. LNCaP cells were also maintained under 5% CO_2, but in RPMI medium 1640 supplemented with 10% FBS, sodium pyruvate, gentamycin and penicillin/streptomycin. Unless otherwise stated, VCaP cells were usually grown for 24 hrs prior to DHT (Tokyo Chemical Industry) stimulation, in phenol red free DMEM solution supplemented with 10% charcoal-dextran stripped fetal bovine serum (CDFBS), sodium pyruvate, sodium bicarbonate, and penicillin/streptomycin. LNCaP cells were usually cultured in phenol red free RPMI supplemented with 5% CDFBS, sodium pyruvate, gentamycin, and penicillin/streptomycin for 72 hrs prior to stimulation. For maintanence, the cells were subcultured once a confluency of 80% is reached (~5-7days).

2.2 Fluoresence in-situ Hybridization (FISH)

VCaP/LNCaP cells were treated with colcemide (10ug/ml) for 2 hrs prior harvesting. After which, the interphase and metaphase cells were prepared for FISH analysis by

fixation and standard hypotonic treatment. The cells were then treated with pepsin (100mg/ml) (Sigma) and HCl (0.01 mol/L) at 37°C (5 min) before fixation in 1% formaldehyde (Sigma) (10 min) and dehydration with a series of ethanol concentrations. Fosmid probes were obtained from the BACPAC Resource Center (BPRC, CHORI, Oakland, California, USA), and grown in accordance to the manufacturer's protocols. DNA extraction was performed using Nucleobond PC500 (Macherey-Nagel) kit. Utilizing an Enzo Nick Translation DNA labeling system, the extracted DNA was labeled with either biotin-16-dUTP (Roche) or digoxigenin-11-dUTP (Roche). An estimated 20ng of probe, together with 10ug of Cot1-DNA, was used per hybridization. The slides and probes mixes were codenatured at 75°C prior overnight hybridization at 37°C. Post hybridization washes were performed at 45°C and blocking was performed with blocking reagent (Roche). The slide was then incubated with avidin-conjugated fluorescein isothiocyanate (FITC) (Roche) and anti-Digoxygenin-Rhodamine (Roche). To enable visualization, the nuclei were stained by DAPI counterstain (Vector Laboratories). The fluorescence images were captured with a cooled charge-coupled device (CCD) camera attached to a Nikon fluorescence microscope using a 60X objective. The fosmid probe sequences utilized are shown in Appendix I.

2.3 Chromatin Immunoprecipitation (ChIP)

After starving, VCaP cells (24hrs) and LNCaP cells (72 hrs) were treated with 100nM DHT/EtOH for the indicated durations. Following that, the cells were fixed with 1% formaldehyde (Sigma-Aldrich, Missouri) prior collection. The harvested cell pellets were

then lysed with SDS lysis buffer (with Proteinase Inhibitor) and sonicated so as to shear the genomic DNA into lengths of 500-1000 bp. 3.33% (by volume) of the sheared chromatin was aliquoted and kept as input in -80^{o}C fridge. The rest of the sheared chromatin was then precleared with normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz) and sepharose beads A (Zymed, San Francisco) for at least 2 hours in 4^{o}C. After which, overnight immunoprecipitation was performed by incubating the precleared chromatin with the desired antibodies and sepharose beads A (Zymed, San Francisco). On the following morning, the beads were consecutively washed to remove non-specific bindings. After washing, the captured DNA-protein complex was eluted from the sepharose beads. De-crosslinking of the DNA from the DNA-protein complex was done at 65^{o}C overnight. The de-crosslinked DNA was then purified with QIAquick spin PCR purification kit (Qiagen, California) and q-PCR quantified using the KAPA SYBR FAST qPCR kit with specific primers. DNA detection is performed with the ABI 7900HT Fast Real-Time PCR System. Primer Express (Applied Biosystems, California) was utilized for primer design (Appendix II). For HDAC1, HDAC2, HDAC3, and EZH2 ChIP, a double cross-linking strategy was utilized for the stabilization of protein-protein interactions. This would increase the pull-down efficiency of the DNA that are indirectly bound to these transcription factors. Specifically, cells were first fixed with 2 mM DSG (Pierce) for 45 minutes prior 1% formaldehyde fixation. Antibodies that were used for ChIP analysis are anti-AR (sc-815x), anti-ERG (sc-353), anti-HDAC3 (sc-11417) from Santa Cruz Biotechnology, anti-HDAC1 (ab7028-50), anti-HDAC2 (ab7029-50) from Abcam, and anti-EZH2 (39639/39901) from Active Motif.

2.4 ChIP-Sequencing

Altogether, 5ng of immunoprecipitated DNA from VCaP cells for each respective transcription factor was quantified using Pico-green ds DNA assay kit (Invitrogen) and used for ChIP-Seq library construction. The ChIP-Seq DNA sample Prep Kit (Illumina) was utilized for library preparation with some minor modifications. The ChIP DNA was made to undergo end-repair followed by adapter ligation. Platinum Pfx DNA polymerase (Invitrogen) was then used to amplify the ChIP-DNA. Electrophoresis of the amplified DNA products on a 2% agarose gel was subsequently performed. Detection of the amplified DNA products was done by staining the gel with SYBR® Green I Nucleic Acid Gel Stain (Invitrogen). DNA products of size 200-300bp were extracted by gel excision and purified. Confirmation of the size and quality of the extracted purified ChIP DNA was done using bioanalyzer prior sequencing on the Solexa platform. The sequencing depth for each library was at least 10million tags. ChIP-Seq read tags were aligned to the reference human genome (UCSC, hg18). The binding peaks were determined with CCAT (Xu et al., 2010) using input reads as control. To determine that the antibodies used for ChIP assays do recognize their intended target, validation was done with western blot assays using VCaP cells that were treated with siRNAs targeting the different specific transcription factors respectively (Fig. 2.1).

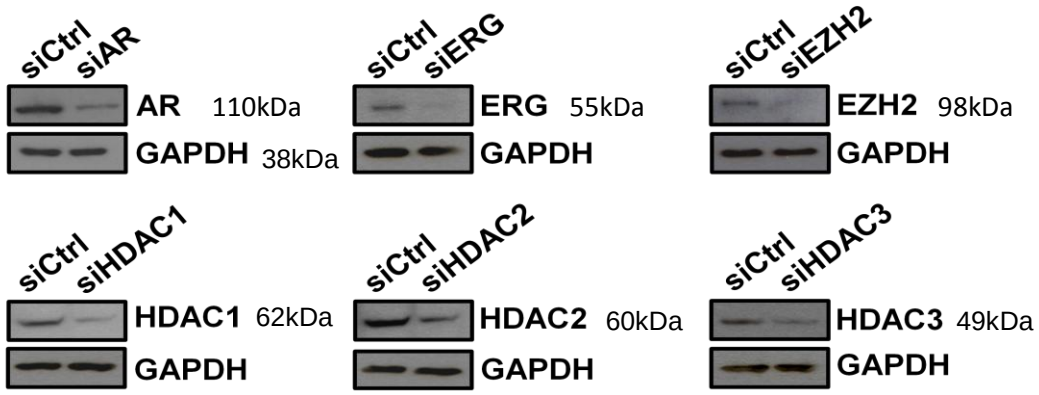


Figure 2.1 Validation of ChIP antibodies

Western blot analysis using VCaP cells treated with different siRNAs targeting different specific transcription factors. Probing was performed with respective matching ChIP antibody as indicated.

2.5 Western Blot Analysis

VCaP/LNCaP cell pellets were harvested and lysed by vortexing in Triton X-100 at 4^{o}C for 30mins. To determine the amount of protein for loading, protein quantification was done using Pierce BCA Protein Assay kit. The protein was denatured by boiling at 99^{o}C in 4X SDS loading buffer (with mercaptoethanol) for 5 minutes prior separation by SDS polyacrylamide gel electrophoresis. After electrophoresis, the separated proteins were transferred to a nitrocellulose membrane, blocked at 37^{o}C for 30mins with 5% milk and then incubated with adequate concentration of desired antibodies (primary and secondary). Protein bands were then detected using a chemiluminescent approach using ECL Plus (Amersham). The antibodies used for western blot analysis include anti-AR (sc-816), anti-ERG (sc-354), anti-Vinculin (sc-25336) from Santa Cruz, anti-AR (AR441) from Labvision, anti-HDAC1 (#05-100), anti-HDAC2 (#05-814) from Millipore, anti-HDAC3 (#3949) and anti-EZH2 (#3147) from Cell Signaling Technology.

2.6 Co-Immunoprecipitation

VCaP cells growing in full serum conditions were trypsinized and lysed to obtain whole cell lysate. A small aliquot of the whole cell lysate was first stored at -80°C. This aliquot was to be used as input during western blot analysis of the co-ip samples. After which, the rest of the cell lysate was pre-cleared with Protein A/G-Agarose beads (Roche Applied Science) for 4 hrs at 4^{o}C. Post pre-clearing, the lysate was then incubated overnight with 5 µg of anti-AR (sc-815x), anti-ERG (sc-353) or anti-rabbit IgG as

required at 4°C. Protein A/G-Agarose Roche beads were added into the mixture on the following day and then further incubated at 4°C for another 1.5 hrs. After incubation, the agarose beads were washed with TBS for a total of four times so as to remove non-specific binding. Finally, the beads were boiled for 5 minutes at 99°C prior being eluted with SDS loading buffer for western blot analysis.

2.7 Short Interfering RNAs (siRNAs)

Unless stated otherwise, a double knockdown approach was utilized for siRNA knockdown studies. VCaP cells seeded in a six well plate for 24 hrs were transfected with the selected siRNA at a concentration of 100 nM/transfection using 4ul of Lipofectamine RNAi Max (Invitrogen) twice with a 24 hours interval being observed between each transfection. For experiments requiring EtOH/DHT stimulation, the cells were starved, 24hrs after the second transfection, in media containing CDFBS for another 24hrs. The siRNAs used in the study were siAR (ON-TARGETplus SMARTpool L-003400-00), siERG (SiGENOME D-003886-01), siHDAC1 (ON-TARGETplus SMARTpool L-003493-00), siHDAC2 (ON-TARGETplus SMARTpool L-003495-00), siHDAC3 (ON-TARGETplus SMARTpool L-003496-00), siEZH2 (ON-TARGETplus SMARTpool L-004218-00) from Dharmacon, and siVCL synthesized from 1stBase. The siVCL sequence is rCrUrGrGrCrUrUrGrCrArGrArUrCrCrArArArUrUrU. The control siRNA for the siAR, siERG, siHDAC1/2/3 and siEZH2 experiments was from Dharmacon (D-001206-13), while the control siRNA for the siVCL experiments was from 1stBase (rUrUrCrUrCrCrGrArArCrGrUrGrUrCrArCrGrUdTdT).

2.8 Gene Expression Analysis

Pre-treated VCaP/LNCaP cells were harvested and their total RNA collected in TRI-reagent (Sigma). Purification of RNA was done with PureLink^{TM} RNA Mini Kit (Invitrogen) in accordance with the manufacturer's protocol. Reverse transcription of RNA to cDNA was carried out using M-MLV reverse transcriptase (Promega). cDNA levels for specific genes were measured by quantitative PCR using the KAPA SYBR FAST qPCR kit and detected with the ABI 7900HT Fast Real-Time PCR System. All gene expression levels were normalized to GAPDH prior comparison. The primers for cDNA quantification were designed with Primer Express software (Applied Biosystems) and are listed in APPENDIX III.

2.9 Microarray Expression Profiling

In accordance with the manufacturer's instructions, the purified total RNA from three independent biological replicates of VCaP cells, pre-treated with varying conditions (as stated), were converted to cRNA using the Illumina® TotalPrepTM-96 RNA Amplification Kit (Ambion). This was followed by hybridization of the synthesized cRNA onto Sentrix® HumanRef-8 v3 Expression BeadChip Kit (Illumina). The BeadChips were then scanned with the BeadArray Reader to obtain the image data that would be subsequently processed using GenomeStudio. Finally, the GeneSpring GX 11.0 software was utilized for analysis of the gene expression data.

2.10 Matrigel Invasion Assay

Invasion assay was performed with the HTS FluoroBlok Cell Culture Inserts (8.0 nm pore size) (BD). 750 μl of media (with 20% FBS) was added into each well of a 24-well plate before the inserts were placed individually into each well. Each insert was first pre-coated with 80 μl of the pre-diluted (250 μg/ml) Matrigel Basement Matrix (BD). Following which, the pre-knockdown cells (4 X 10^5 siRNA-treated VCaP cells/2 X 10^5 siRNA-treated LNCaP cells) that were already resuspended in 200 μl media (with 0.5% FBS) were then seeded into each well respectively. The cells were then allowed to grow in the incubator for another 48 hrs prior harvested for assaying. During harvesting, the cells that invaded through the basement membrane and attached to the bottom of the inserts were fixed with 3.7% formaldehyde for 30 minutes. After fixation, the cells were then stained under dark conditions with 25 μg/ml propidium iodide (PI) for 60 minutes. The was then used to scan, detect and count any cells that passed through the base membrane of the inserts. Ten different fields were taken for each insert for cell count averaging. Each condition was assayed in technical triplicates (three inserts for one condition) for biological triplicates.

2.11 BrdU Assay for measuring Cell Proliferation

BrdU assay was performed using the FITC BrdU Flow Kit (BD Pharmingen) in accordance with the manufacturer's protocol. siRNA transfected VCaP cells growing in full serum were treated with BrdU (final conc. of 10uM) for another 48hrs, 24hrs after

the second transfection, before harvested and fixed. The fixed cells were then permeabilized, re-fixed and incubated with DNAse for the cells to expose the incorporated BrdU. The exposed BrdU are then recognized and bound by the fluorescent anti-BrdU antibodies. Prior flow cytometry analysis, the cells were resuspended in 7-AAD to stain for total DNA content for cell cycle analysis. The percentage number of cells in S phase was reported for 10000 cells.

2.12 PI FACs Analysis for measuring Cell Survival

The siRNA treated VCaP cells growing in full serum were harvested 48hrs after the second transfection, washed with PBS and subsequently fixed with 70% cold ethanol for 45mins at 4^{o}C. The fixed cells were then incubated at room temperature with RNAse (100ug/ml) for 5 mins. Following which, the cells were stained with 50ug/ml of propidium iodide for another hour in the dark. The percentage number of cells in sub G1 phase was then detected through flow cytometry analysis of the genomic DNA content of 10000 cells using the CellQuest Analysis software.

2.13 Motif Discovery Analysis

To find enriched DNA motifs around the ChIP binding peaks, the bioinformatic tools, MEME (Bailey et al., 2009) and CentDist (Zhang et al., 2011) were utilized. The MEME program was used for the discovery of enriched DNA motifs in a de novo manner while

the CentDist program was used for finding the enrichment of known DNA motifs that are available in the TRANSFAC database (Wingender et al., 1996).

2.14 Generation of Heatmap Binding Signals

To generate the plot, ERG and ARBS that were within 500 bp of each other were clustered together. The AR and ERG libraries were re-sampled to 10 million tag reads and subsequently plotted out as intensity signals around a region of -/+2 kb centralized at the respective AR/ERG ChIP-Seq peak or AR/ERG clusters. Re-sampling was done to ensure a fair comparison of tag intensities between libraries. Individual binding region was then sorted according to their binding signal intensity at their respective categories (AR only, ERG only and AR/ERG overlap) for easy visualization.

2.15 Conservation Analysis for Binding Peaks

The conservation scores for the alignment of 27 vertebrate genomes with the Human genome (PhastCons28way) were downloaded from the UCSC Genome Browser database. The sequence conservation score of every nucleotide in a 2000 bp window centered on the defined ChIP-Seq peak/cluster were plotted and compared.

2.16 Survival Curve Analysis

Prostate cancer patient survival data and the gene expression profiles of their tumor samples were obtained from the MSKCC dataset available in this study (Taylor et al., 2010). Patients were separated into two categories based on the expression levels of VCL in their tumor samples. Those expressing VCL levels higher than the median VCL expression level of all patients (in the dataset) were classified as high VCL expressing while the rest of the patients were classified as low VCL expressing. Survival association with VCL expression status (high/low) was computed using Cox-Proportional Hazards model implementation that is available in the R-library under ‘‘survival’’. Kaplan-Meier survival analysis was utilized for the analysis of the clinical outcome.

2.17 Oncomine Concept Map and Gene Ontology Analysis

The Oncomine Molecular Concept Map analysis was utilized for associating ERG-bound (5 kb from TSS) androgen induced genes (>2 fold) that were classified in this study with the defined prostate cancer gene signatures that were derived and deposited in the Oncomine database. Different nodes represent different sets of gene signatures and the size of the node proportional to the number of genes in that gene signature. The criteria for significant associations (represented as edges) between nodes is defined as Odds Ratio > = 2 and p-value < 1e-4.

2.18 Data deposition

The generated raw ChIP-Seq and gene expression profiling data in this study have been deposited at the NCBI GEO repository under accession number GSE28951.

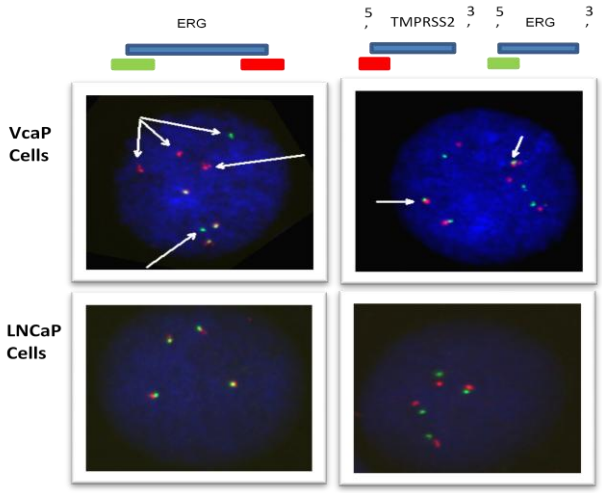
CHAPTER 3: RESULTS

3.1 Confirmation of VCaP Cells as TMPRSS2-ERG Fusion Positive

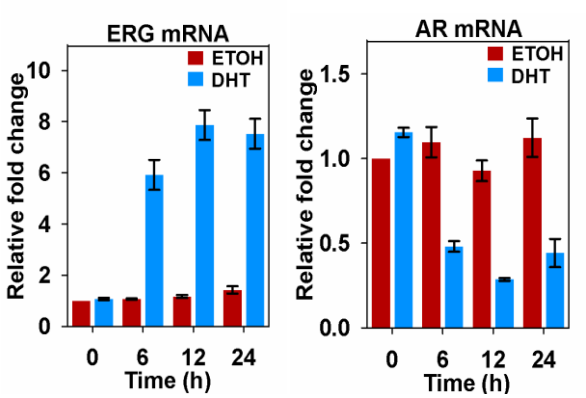
To study the AR and ERG transcriptional cross-talk, an ERG-fusion positive prostate cancer cell line would be necessary as a model system. The VCaP prostate cancer cell line was previously established as TMPRSS2-ERG fusion positive, resulting in ERG expression being induced by androgen stimulation (Tomlins et al., 2005). The FISH technique was utilized to validate the TMPRSS2-ERG fusion in VCaP cells (Fig. 3.1A). Indeed, through FISH, using probes that hybridize to the upstream and downstream region of the ERG gene loci separately, structural rearrangement was detected in the ERG gene locus of VCaP cells (some of the red and green probes were far apart) but not of LNCaP cells (all the red and green probes were co-localized). In addition, the presence of TMPRSS2-ERG fusion in VCaP cells (some of the red and green probes were co-localised), but not in LNCaP cells (all of the red and green probes were far apart), was confirmed using probes that target the TMPRSS2 loci and the ERG loci respectively (Fig. 3.1A). Furthermore, a time course profiling of both mRNA and proteins levels of ERG expression post DHT stimulation (Fig. 3.1B-C) indeed corroborates with previous published results that the ERG gene expression becomes transcriptionally induced by androgens as a result of the TMPRSS2-ERG fusion (Tomlins et al., 2005). As shown in Fig. 3.1B-C, DHT stimulation resulted in the repression of AR mRNA expression across time (Fig. 3.1B) with the protein level remaining relatively constant albeit exhibiting a slight decrease after prolonged DHT exposure (Fig. 3.1C). In contrast, the mRNA and

protein levels of ERG were both upregulated in response to androgen stimulation (Fig. 3.1B-C). The prompt increment in ERG mRNA expression (6 hours) suggests a rapid initiation of AR mediated transcription upon androgen treatment. The observed difference in the androgen induced expression kinetics between ERG mRNA and proteins levels (peaking at 12 hrs for RNA and 24 hrs for protein) (Fig. 3.1B-C) is likely to be due to a lag time between transcription and translation. So far, the experiments have shown that VCaP prostate cancer cells could serve as a model experimental cell system for the purpose of our study.

A



B



C

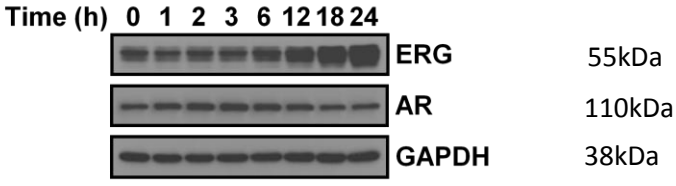


Figure 3.1 Androgen Regulated Expression of TMPRSS2-ERG Fusion Gene in VCaP cells

(A) FISH validation of the presence of TMPRSS2-ERG fusion gene in VCaP cells. LNCaP cells were utilized as the negative control. Expression profiling analysis of AR and ERG (B) mRNA and (C) protein levels in VCaP cells after treatment with 10nM DHT at the various indicated time points. Error bars represent S.E.M of at least 3 independent repeats.

3.2 Binding Kinetic Analysis of AR and ERG to the Chromatin post Androgen Stimulation

As AR-mediated transcription is likely to precede any increase in ERG protein levels post androgen stimulation, we sought to study the AR and ERG transcriptional cross-talk as a temporal event with regards to androgen signaling (Fig 3.2). The androgen induced recruitment of AR and ERG to the chromatin in VCaP cells was traced via a time-course AR/ERG ChIP at various durations (0hrs, 2hrs and 18hrs) post DHT stimulation. The 0 hours time point is a representation of the unstimulated cell state. The 2 hours time point provides information on the transcriptional events arising during early androgen signaling while the 18 hours time point depicts late androgen signaling transcriptional events occurring after the increase in ERG protein expression. Post androgen stimulation, we observed strong early (2 hours) AR recruitment to the enhancer of the model AR target gene, PSA. This observation of strong early AR recruitment at the 2 hours time point is in concordance with our earlier conclusion that AR-mediated transcription could occur rapidly after DHT treatment. At the late phase of androgen signaling (18 hours), AR occupancy at the PSA enhancer persisted but was significantly reduced (Fig. 3.2). In comparison, ERG was also recruited to the PSA enhancer 2 hours post stimulation but unlike AR, whose binding was reduced at late phase of androgen signaling (18 hours), the recruitment of ERG remained strong (Fig. 3.2). Taken together, this set of results provides some evidence that AR and ERG could probably be co-localized on the chromatin together upon androgen stimulation but with different and distinct binding kinetics. Apart from the AR target gene, PSA, we also attempted to study the loading of ERG onto regulatory element unoccupied by AR. To this end, we performed an ERG

time course chip on the regulatory element of the ERG target gene, PLA1A. PLA1A was first established as a direct target of ERG and had its ERG-bound regulatory element identified in a previous study (Tomlins et al., 2008). Importantly, no significant AR occupancy was detected by our AR ChIP at this regulatory element (Fig. 3.2). We also observed a rise in ERG binding at this site during the late phase of androgen signaling (18 hours) (Fig 3.2), possibly as a consequence of the increased ERG protein expression. As there was no significant increase in ERG protein levels at the early phase of androgen signaling (2 hours) (Fig. 3.1C), the recruitment of ERG to the PSA enhancer is likely to be a direct effect of AR binding.

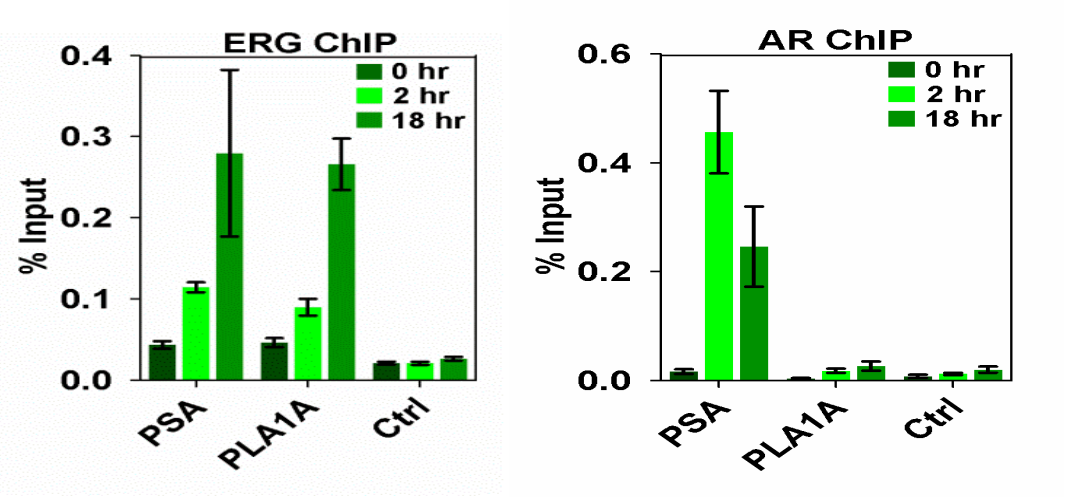


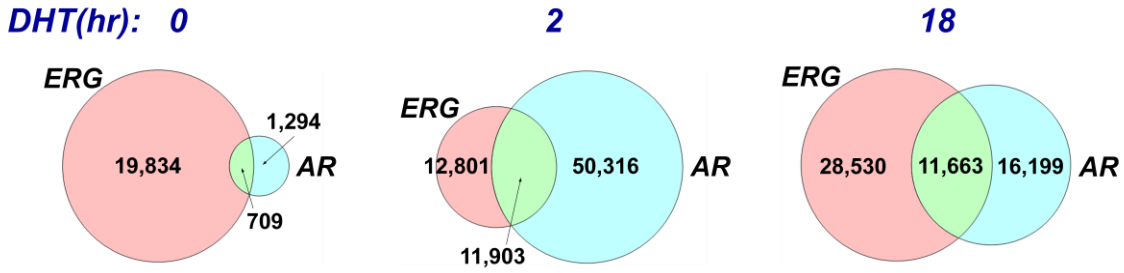
Figure 3.2 Kinetic Analysis of AR and ERG binding to Chromatin

Hormone depleted VCaP cells were treated with 100nM DHT for different durations (0, 2 or 18 hours) respectively prior formaldehyde cross-linking. The cross-linked chromatin was immunoprecipitated with antibody against either AR or ERG before quantifying with qPCR for selected binding sites. %input refers to % with respect to the total chromatin used for immuno-precipitation. Control (Ctrl) refers to a randomly selected genomic location. Error bars represent S.E.M of at least 3 independent repeats.

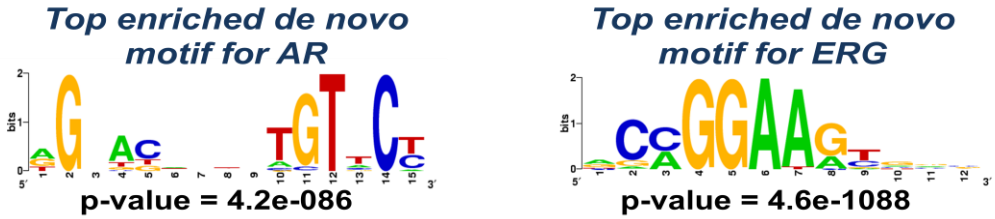
3.3 Generation of the AR and ERG Cistromes using ChIP-Seq

To extend our earlier observations on the spatial and temporal characteristics of AR and ERG chromatin occupancy to a genome-wide scale in prostate cancer cells, several AR and ERG ChIP-Seq libraries at various time points post androgen stimulation (0, 2, 18 hours) were generated (Table 3). Generally, substantial overlaps between AR and ERG cistromes, indicating the existence of a genome-wide transcriptional collaboration was observed (Fig. 3.3A). Interestingly, we noted that substantial overlap between AR and ERG cistrome was only observed after androgen stimulation as a result of a drastic increase in ARBS (Fig. 3.3A) after androgen stimulation. As expected, de novo motif analysis of the identified VCaP AR and ERG binding sites using MEME (Bailey et al., 2009) reveals the presence of motifs that are strikingly similar to the canonical ARE and ETS-like sequence respectively (Fig. 3.3B). We also compared our work to a recently generated genome-wide map of AR and ERG in VCaP cells that was published in the course of our study (Yu et al., 2010b). Although the experimental conditions adopted in that study were different from ours, the cistromic maps generated in both studies still overlap significantly with each other (Fig. 3.3C).

A)



B)



C)

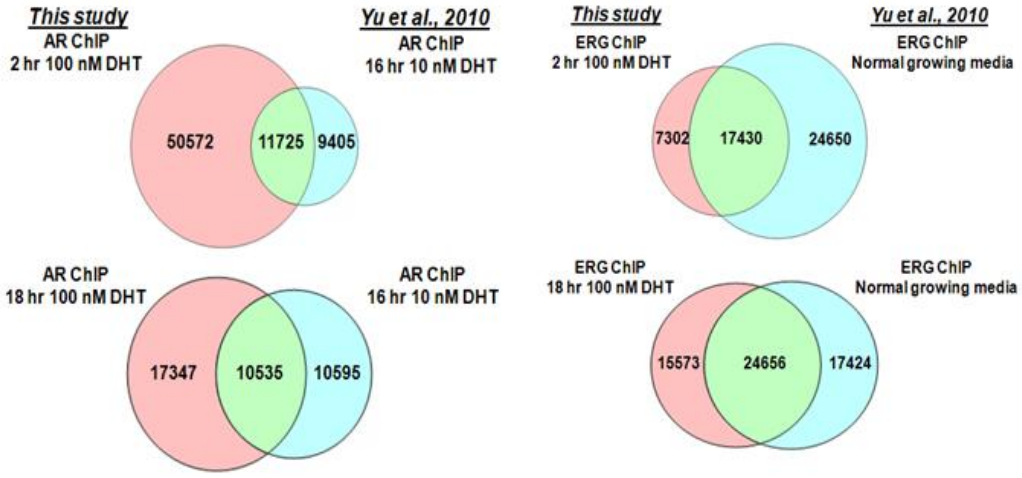


Figure 3.3 The AR and ERG Cistromes in VCaP Cells

A) Venn diagrams illustration of the overlap between AR and ERG cistromes in VCaP cells treated with 100 nM DHT for different durations (0, 2, 18 hrs). B) Weblogos of the most enriched motif found in AR and ERG binding sites respectively using the *de novo* motif discovery software, MEME (Bailey et al., 2009). C) Venn diagram illustration of the comparison between the AR and ERG cistromes in VCaP cells derived from this study and that of Yu et al., 2010b.

Library Name	FDR=0.05	FDR=0.1	FDR=0.2	Sequencing Depth (reads)
ERG ChIP 0hr DHT	20545	23823	27474	13946853
ERG ChIP 2hr DHT	24732	28579	34120	13075571
ERG ChIP 18hr DHT	40229	41908	47725	15254728
AR ChIP 0hr DHT	2004	2481	3137	12670989
AR ChIP 2hr DHT	62297	68689	74834	15590194
AR ChIP 18hr DHT	27882	33185	33958	11885706

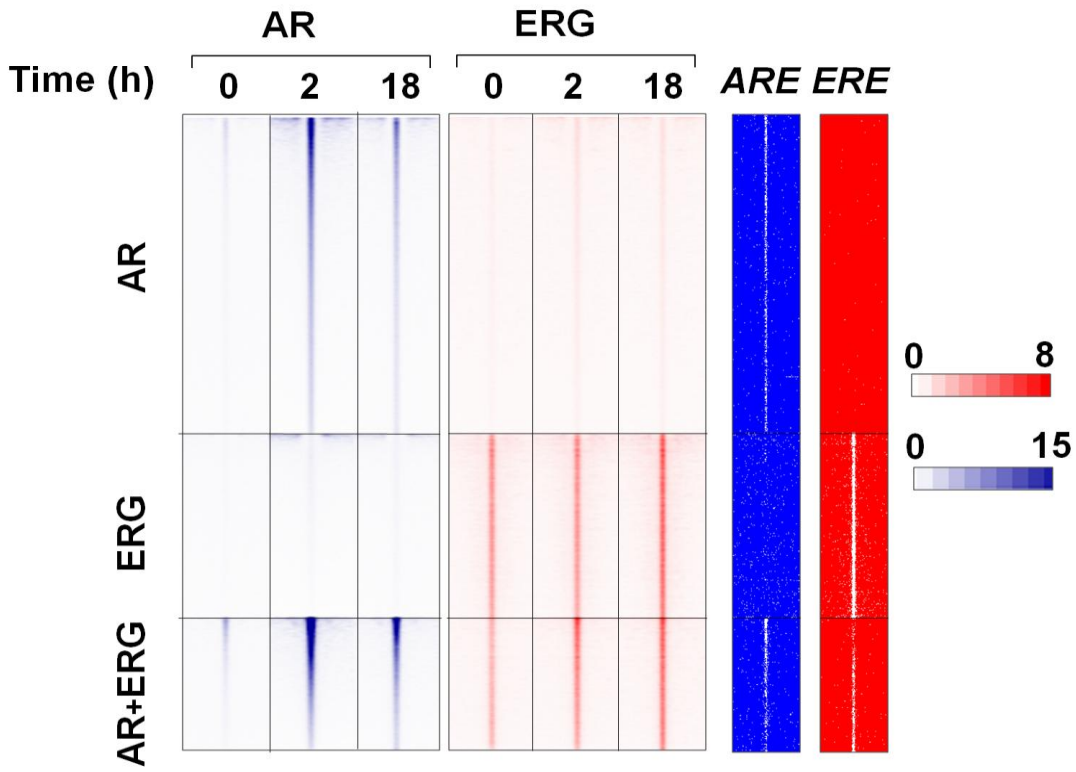
Table 3 Sequencing depth and peak numbers (under several FDR) of the different AR and ERG ChIP-Seq libraries

3.4 Binding Kinetic Cistromic Profiles of AR and ERG under Different Phases of Androgen Signaling

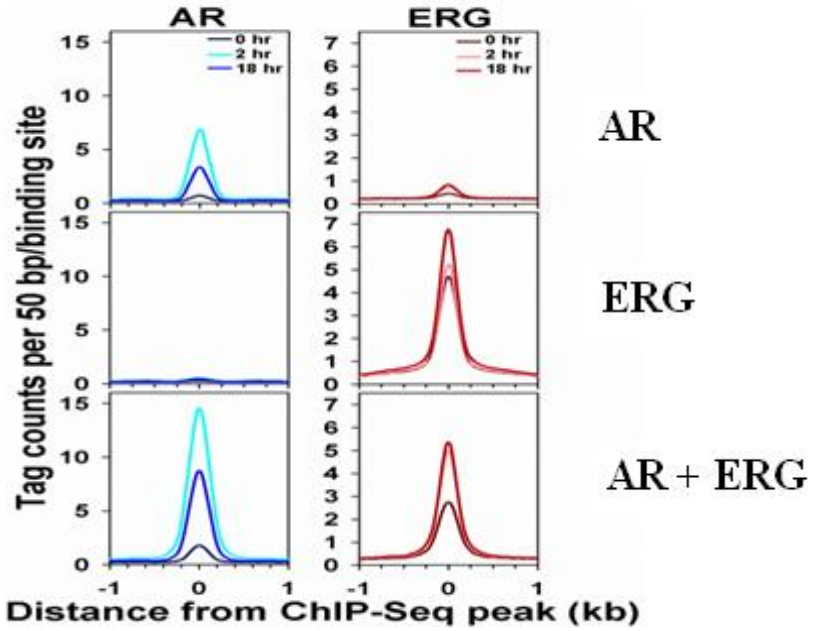
For a visual representation of the AR and ERG binding events, we plotted the binding profiles of AR and ERG centered on their binding clusters in a heatmap format (Figure 3.4A). From the plot, we were able to make several interesting observations pertaining to the AR/ERG chromatin occupancy profiles in response to androgen signaling. Similar to our previous observation derived from individual loci (Fig. 3.2), the androgen-induced global kinetic binding profiles of AR and ERG were clearly distinct (Fig. 3.4A-B). Globally, there was minimal AR binding in the genome prior to any stimuli (Fig. 3.4A-B). After a short period (2 hours) of DHT stimulation, AR was already strongly recruited to AR unique and AR+ERG co-localized sites across the genome. In concordance with the trend at PSA enhancer, there was a global reduction in AR occupancy at the late phase of androgen signaling (18 hours), an indication that the rate of AR recruitment is being outpaced by the rate of AR dissociation (Fig. 3.4A-B). Unexpectedly, for ERG, in contrast to AR, there was already a substantial amount of ERG preloaded at both ERG unique and AR+ERG co-localized binding sites prior to androgen induction (Fig. 3.4A-B). We observed that while the binding of ERG at AR+ERG co-localized binding sites was generally enhanced after 2 hours of DHT stimulation, ERG binding at ERG unique binding sites remained mostly constant. This suggests the possibility that AR recruitment might be enhancing ERG loading at shared binding sites (Fig. 3.4A-B). ERG occupancy at the ERG unique sites eventually increased in the late phase of androgen signaling (18 hrs) (Fig. 3.4A-B). This could be result of the presence of an increased level of ERG protein expression at that phase of androgen signaling. Interestingly, AR and ERG

consensus motifs were found strongly associated with the respective corresponding AR/ERG binding sites (Fig. 3.4A-B), suggesting that the presence of binding motifs is likely one of the major determinants of AR and ERG chromatin co-occupancy. We also made the observation that while ARBS harboring ERG are generally stronger AR binders than those that do not, ERG binding sites (ERGBS) that harbor AR did not display higher ERG tag intensities compared to their counterparts (Fig. 3.4C). This suggests that ERG tends to occupy stronger ARBS than weak ones (Fig. 3.4C (Left)) while AR exhibit no preference to stronger ERGBS (Fig. 3.4C(Right)).

A)


AR
ERG
Time (h)
0
2
18
0
2
18
ARE ERE
AR
ERG
AR+ERG
0
8
0
15

B)


AR
ERG
0 hr
2 hr
18 hr
Tag counts per 50 bp/binding site
Distance from ChIP-Seq peak (kb)
AR
ERG
AR + ERG

C)

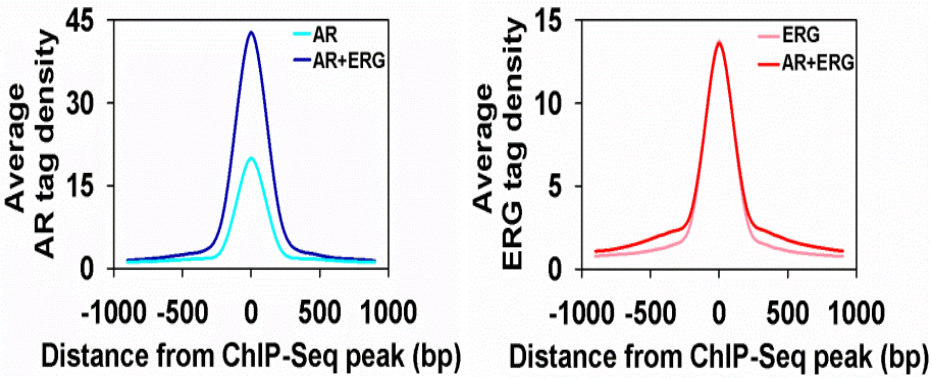


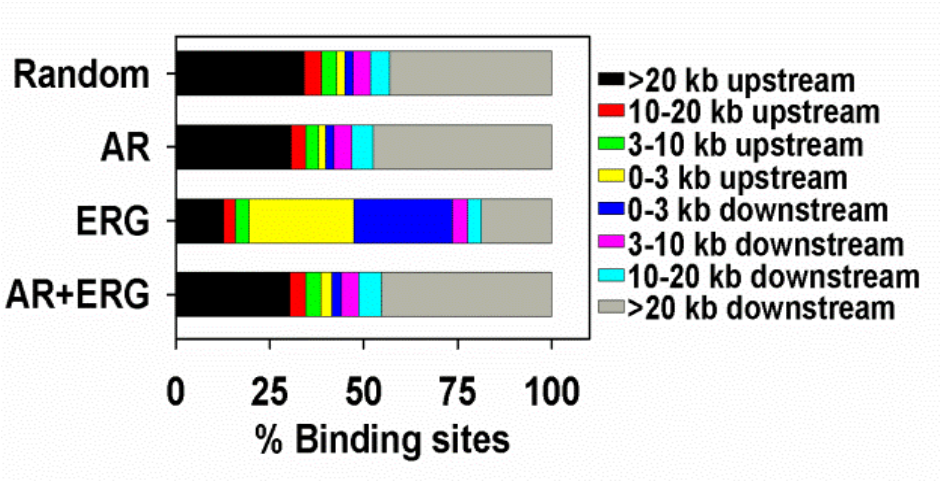
Figure 3.4 Kinetic binding profiles of the AR and ERG cistromes under androgen signaling

A) Heatmap display of sorted ChIP-Seq signals of AR and ERG chromatin occupancy events in VCaP cells. Signals are plotted in reference to the center of AR/ERG ChIP-Seq cluster peak (-/+2 kb). Corresponding occurrence of predicted ARE and ETS binding motif at the respective cluster peak are depicted in heatmap on the right. B) Plots showing the average AR and ERG ChIP-Seq tag counts at the different subsets of the AR and ERG binding cluster after 0, 2, and 18 hrs of androgen stimulation. C) A comparison of the average tag intensity of (Left) AR at AR unique and AR+ERG co-occupied binding sites (Right) ERG at ERG unique and ERG+AR co-occupied binding sites (2hr after DHT stimulation).

3.5 Genomic Distribution and Sequence Conservation Analysis of AR and ERG Binding Sites

We proceeded to examine the genome-wide distribution of AR/ERG binding sites. From our AR ChIP-Seq data, we noted that ARBS are mostly located at distal enhancers far away from the transcriptional start sites (TSS) of genes rather than at proximal promoters (Fig. 3.5A). This is consistent to what was previously reported about ARBS at chromosomal level (Wang et al., 2007). In comparision, ERGBS were found substantially at both promoter proximal and distal locations. For AR and ERG co-localized sites, they were also located at sites distal to the TSS, a distribution similar to AR unique binding sites (Fig. 3.5A). From evolutionary sequence conservation analysis, we found the peaks of AR and ERG binding sites to be generally conserved as they display higher conservation score relative to their flanking regions (background) (Fig. 3.5B). ERG unique binding sites were noted to exhibit the highest conservation score relative to AR unique and AR+ERG co-occupied binding sites (Fig. 3.5B). This is probably as a result of a large proportion of ERGBS being localized to the generally highly conserved TSS of genes. On the other hand, AR+ERG overlapping binding sites were generally more conserved when compared to AR unique binding sites (Fig. 3.5B), suggesting that these overlapping sites might be of a higher functional importance.

A)



B)

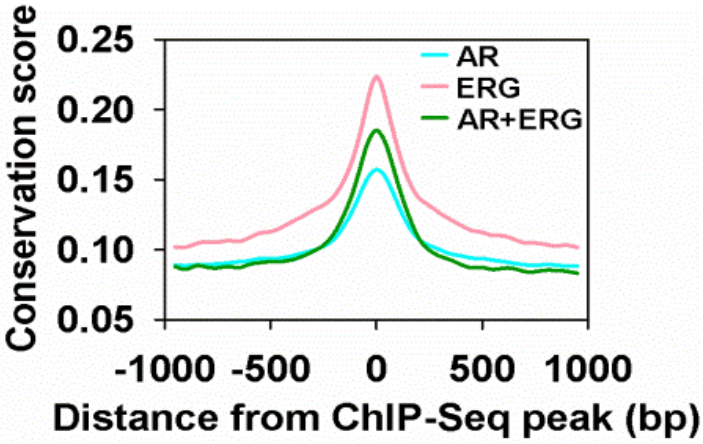


Figure 3.5 Genomic Distribution and Sequence Conservation Analysis

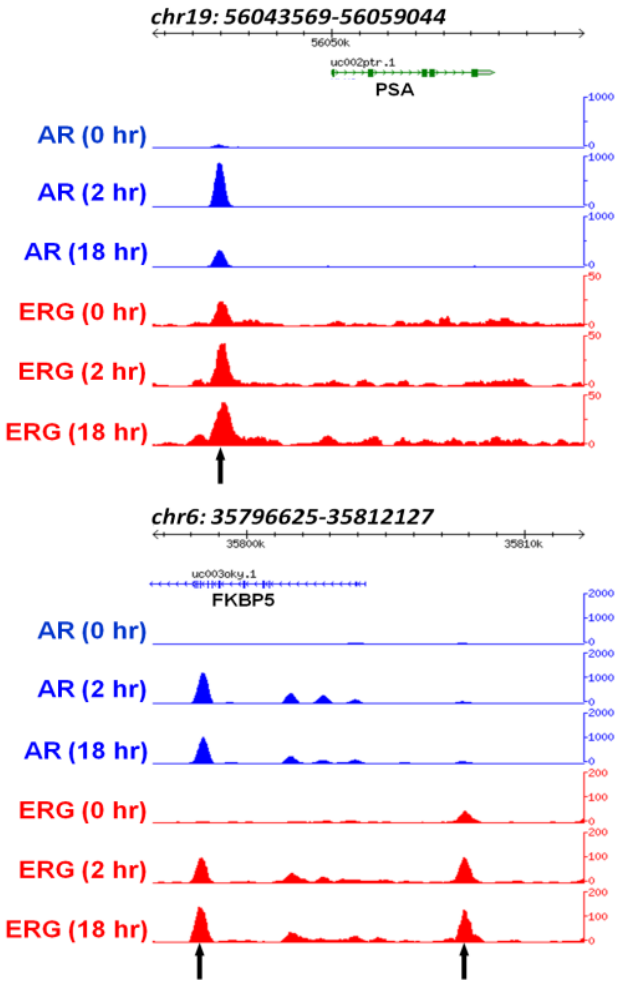
A) Genome-wide distribution of AR and ERG occupied sites with respect to the transcription start sites (TSSs) of RefSeq genes. (B) Conservation sequence analysis of AR and ERG binding sites.

3.6 The Transcriptional Collaborative Nature of AR and ERG

3.6.1 Interplay between ERG and AR

Basing on the substantial global co-occupancy of AR and ERG at cis-regulatory elements, we postulate the existence of a genome-wide transcriptional crosstalk between AR and ERG. Strikingly, we observed strong co-localization of AR and ERG at important regulatory elements of AR model target genes such as PSA and FKBP5 (Fig. 3.6A). As a further support for the direct collaboration between AR and ERG, an endogenous interaction between AR and ERG was detected through immunoprecipitation (Fig. 3.6B). Interestingly, apart from occupying AR-bound enhancers, ERG occupancy was also enriched at the promoters of AR target genes identified from our time course microarray experiments (Fig. 3.6C and Fig. 3.6D). Interestingly, we observed that the ERG binding at these gene promoters are generally not influenced by androgen stimulation (Data not shown). These findings point to an extensive interplay between ERG and androgen signaling.

A)


chr19: 56043569-56059044
56050k
uc002ptr.1
PSA
AR (0 hr)
AR (2 hr)
AR (18 hr)
ERG (0 hr)
ERG (2 hr)
ERG (18 hr)
chr6: 35796625-35812127
35800k
35810k
uc003oky.1
FKBP5
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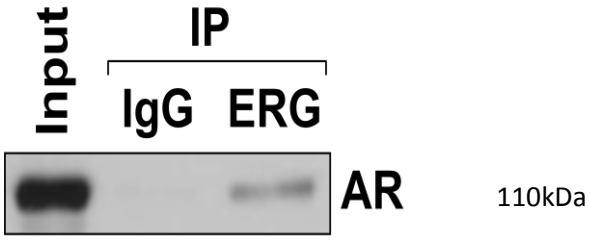
AR (2 hr)
AR (18 hr)
ERG (0 hr)
ERG (2 hr)
ERG (18 hr)

B)


Input
IP
IgG
ERG
AR
110kDa

C) **D)**

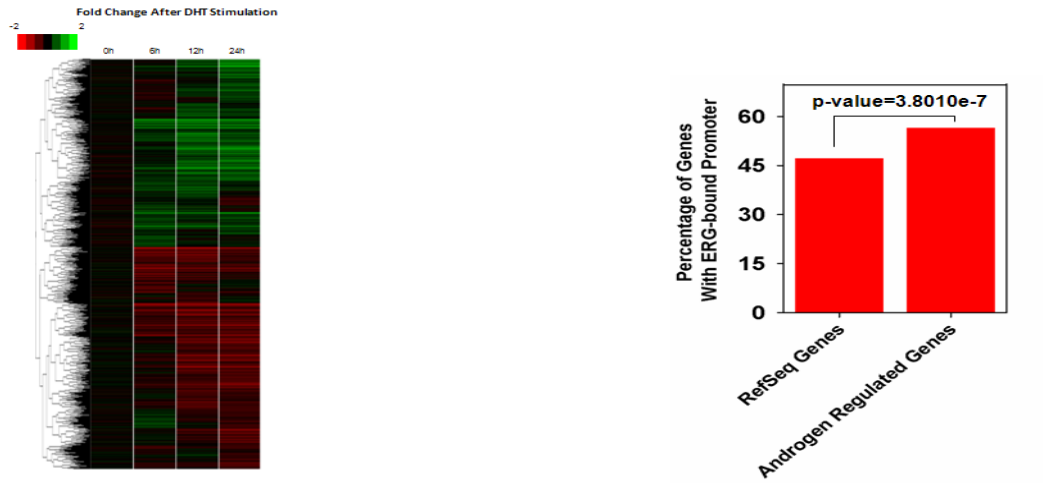


Figure 3.6 AR and ERG Cross-Talk

A) Snapshots of AR and ERG binding sites at two of the model AR target genes: (Top) PSA and (Bottom) FKBP5. The black arrows indicate the position of the co-localized AR and ERG binding sites. B) Western blot analsysis depicting endogenous interactions between AR and ERG in VCaP cells. C) Androgen deprived VCaP cells treated with 10nM DHT/vehicle (EtOH) for the specified durations were harvested for total RNA. Gene profiling was subsequently performed. Fold change represents ratio of gene expression under DHT stimulation to their respective expression under vehicle (EtOH) stimulation. 3 independent repeats were performed. D) Bar graphs depicting the percentage of genes with an ERG occupied promoter. Androgen regulated genes are defined as genes regulated (> 2 fold) by our androgen induced time course profiling data.

3.6.2 Androgen Induced Transcriptional Programs Regulated by Distinct Subsets of AR Cistrome

To examine the androgen-mediated transcriptional programs regulated by AR unique and AR+ERG binding sites respectively, we performed a gene ontology analysis using Ingenuity systems Pathway Analysis (IPA) on identified androgen-regulated genes associated with AR unique or AR+ERG binding sites. We observed that while AR+ERG binding sites were more associated with genes responsible for cellular movement, growth and proliferation as well as cell cycle and morphology, AR unique binding sites were more associated with genes related to cell death (Fig. 3.7). Taken together, our results so far indicate that AR and ERG binding across the genome shares a large overlap but yet distinct. This strongly points to a potential collaboration between the two factors.

Androgen-regulated genes associated with AR+ERG overlapping binding sites

Category	p-value
Cellular Movement	3.71E-07
Cellular Growth and Proliferation	6.16E-07
Cell Cycle	2.09E-06
Cellular Development	1.1E-05
Carbohydrate Metabolism	1.76E-05
Cell Morphology	1.78E-05

Androgen-regulated genes associated with AR unique binding sites

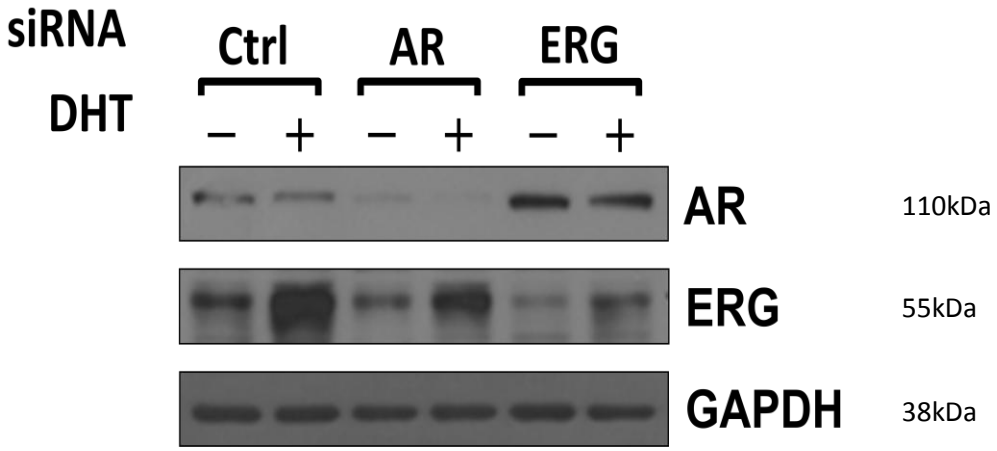
Category	p-value
Cell Death	2.89E-07
Cellular Growth and Proliferation	2.13E-05
Cellular Movement	2.16E-05

Figure 3.7 IPA analysis of genes associated with AR unique or AR+ERG overlapping binding sites

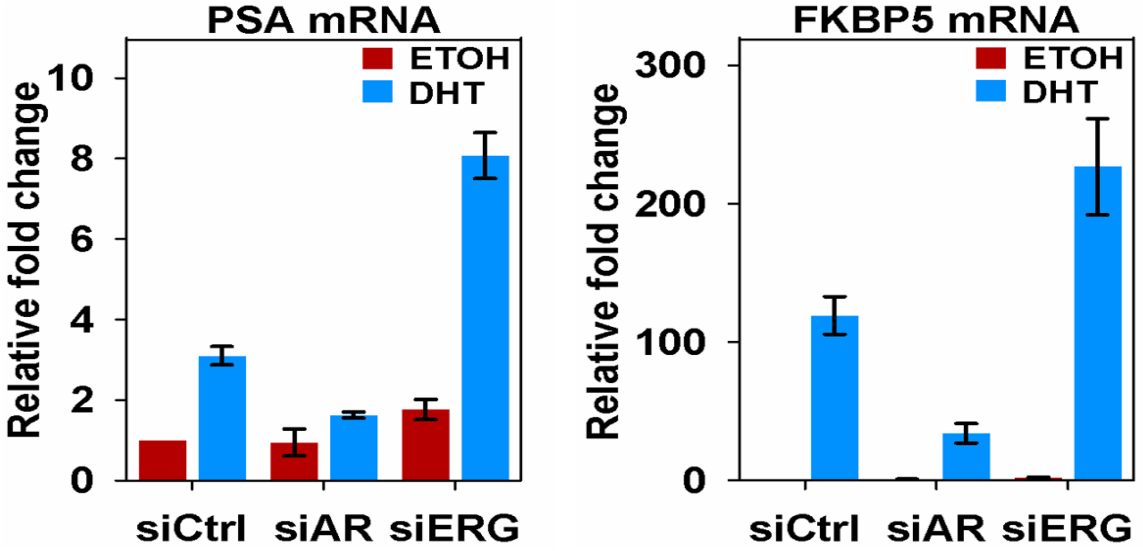
3.6.3 Microarray Profiling of Androgen Regulated Genes after ERG Depletion

To investigate the nature of this cross-talk, we assessed the effect of siRNA-mediated ERG silencing on androgen regulated AR target genes. Intriguingly, we found that ERG depletion in VCaP cells (Fig. 3.8A) culminated into a further induction of androgen upregulated model target genes such as PSA and FKBP5 (Fig. 3.8B). This result suggests that ERG could function to repress androgen signalling. To assess if the extent of the repressive effect of ERG on androgen signalling, we went further on to perform a microarray profiling of androgen regulated genes after ERG knockdown in VCaP cells (Fig. 3.8C). We noted that ERG silencing affects both androgen up and down regulated genes. Expectedly, we found that ERG knockdown indeed led to the enhancement of the expression levels of a substantial set of androgen-upregulated genes (393). This implies that attenuation of AR signaling is probably one of the major transcriptional processes mediated by ERG in ERG-fusion positive prostate cancer cells.

A)


siRNA
DHT
Ctrl
AR
ERG
– + – + – +
AR
110kDa
ERG
55kDa
GAPDH
38kDa

B)


PSA mRNA
ETOH
DHT
Relative fold change
0
2
4
6
8
10
siCtrl
siAR
siERG
FKBP5 mRNA
ETOH
DHT
Relative fold change
0
100
200
300
siCtrl
siAR
siERG

C)

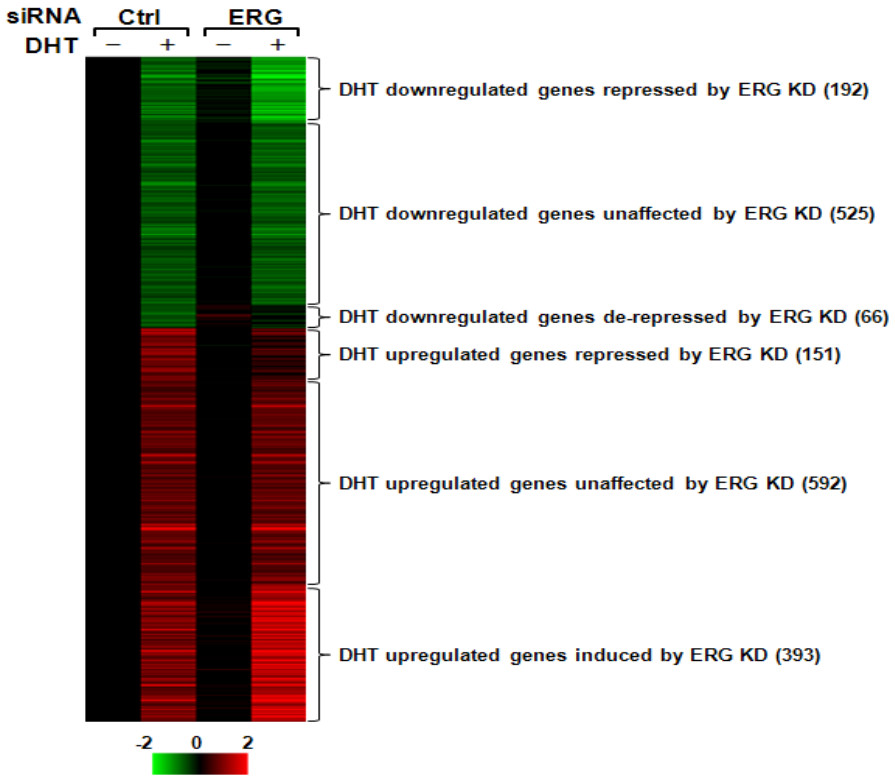


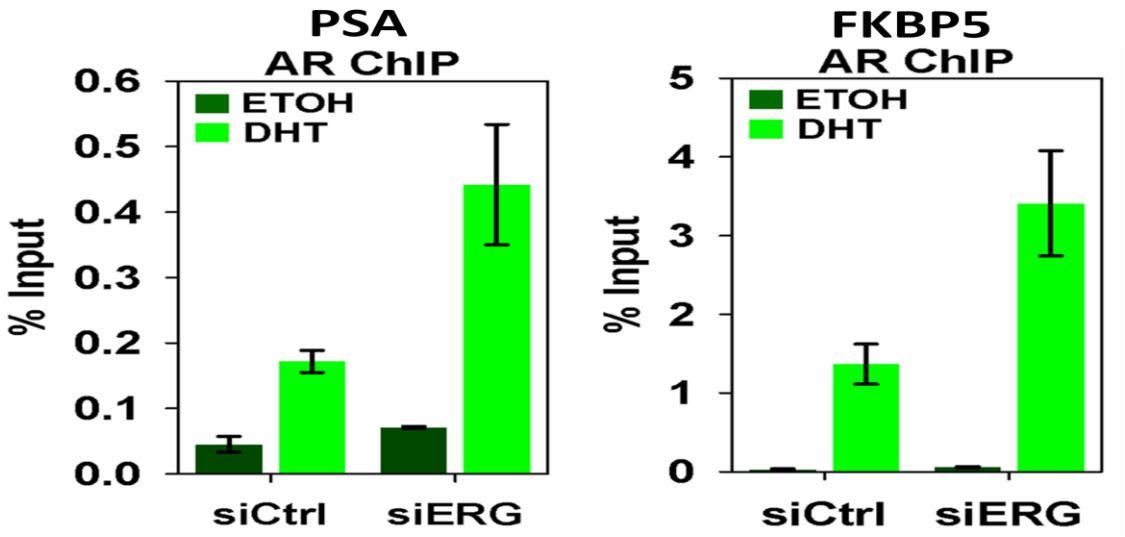
Figure 3.8 Effect of ERG silencing on androgen induced gene transcription

A) AR and ERG expression levels in androgen deprived VCaP cells that were transfected with control siRNA or siRNA against AR/ERG prior stimulation with EtOH/10 nM DHT for 18 hrs. GAPDH was utilized as loading control. B) Androgen deprived VCaP cells were first transfected with control siRNA or siRNA targeting AR/ERG. After 8 hrs of EtOH/ 10 nM DHT stimulation, cells were then harvested for total RNA. The total RNA was converted to cDNA for quantification with qPCR. GAPDH was utilized as a control for internal normalization. Error bars represent S.E.M of at least 3 independent experiments. C) Heatmap representation of gene profiling data. VCaP cells that were transfected with control siRNA or siRNA targeting ERG were subsequently deprived of androgens prior stimulation with ETOH/10 nM DHT for 8 hrs. Cells were then harvested for total RNA and utilized for microarray analysis. Genes that exhibited at least 1.5 fold alteration after DHT stimulation (Ctrl+) (relative to vehicle (Ctrl-)) were filtered as androgen responsive genes. Genes that displayed at least 1.2 fold change after ERG depletion (ERG+) (relative to control siRNA treated with DHT (Ctrl+)) were regarded as affected by ERG knockdown. The numbers of genes in the different groups after ERG knockdown are shown in parenthesis. Data shown is from 3 independent biological repeats.

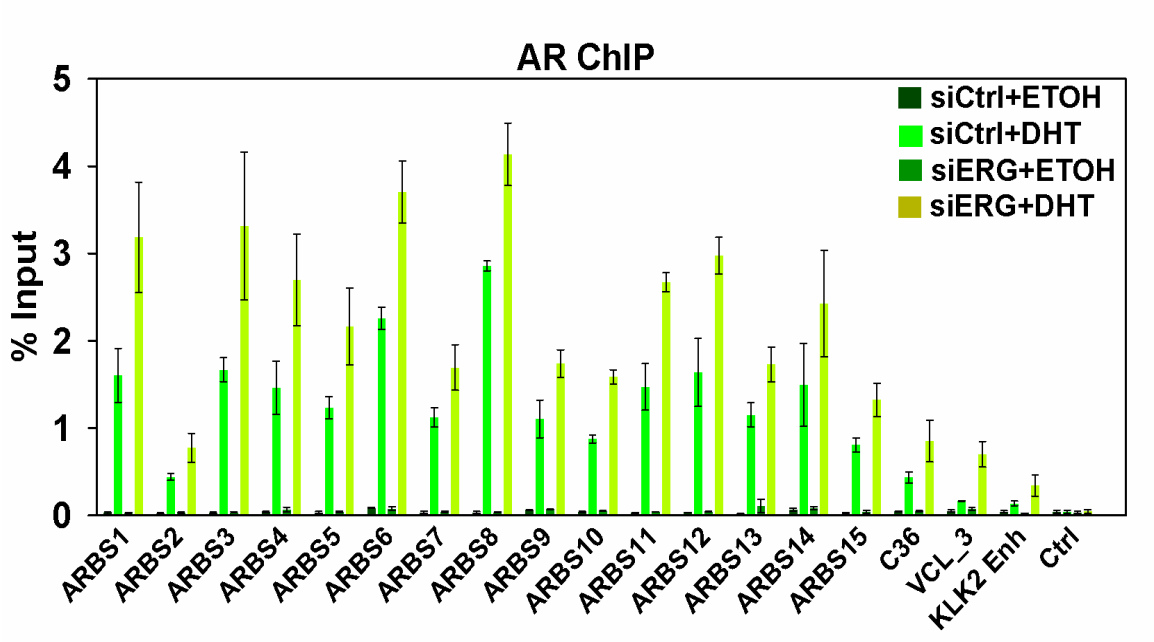
3.6.4 ERG Depletion Enhanced AR Recruitment to the Chromatin

We proceeded to examine the possible mechanisms underlying of ERG-mediated attenuation of androgen-dependent transcription. We postulated that ERG knockdown could enhance AR binding and in turn induce higher AR-target gene expression. To test if ERG suppresses AR recruitment to the chromatin, we performed AR ChIP in VCaP cells after ERG depletion. Depletion of ERG resulted in a significant rise in AR binding at multiple tested AR+ERG occupied binding sites including those ARBS that are associated with PSA and FKBP5 (Fig. 3.9A and 3.9B). To assess the effect of ERG depletion on genome-wide AR binding, we examined the AR ChIP-Seq dataset recently generated by another group (Yu et al., 2010b) in ERG knockdown VCaP cells. Their data suggest that ERG silencing could result in a gain of new AR binding sites (Fig. 3.9C). Taken together, our results and those of another (Yu et al., 2010b) suggest that the repressive influence of ERG on AR induced transcription could be attributed, at least in part, to the reduction of AR binding to its cis-regulatory elements, possibly as a result of ERG occupancy at the same cis-regulatory elements.

A)


PSA
AR ChIP
ETOH
DHT
% Input
0.6
0.5
0.4
0.3
0.2
0.1
0.0
siCtrl
siERG
FKBP5
AR ChIP
ETOH
DHT
% Input
5
4
3
2
1
0
siCtrl
siERG

B)


AR ChIP
siCtrl+ETOH
siCtrl+DHT
siERG+ETOH
siERG+DHT
% Input
5
4
3
2
1
0
ARBS1
ARBS2
ARBS3
ARBS4
ARBS5
ARBS6
ARBS7
ARBS8
ARBS9
ARBS10
ARBS11
ARBS12
ARBS13
ARBS14
ARBS15
C36
VCL_3
KLK2 Enh
Ctrl

C)

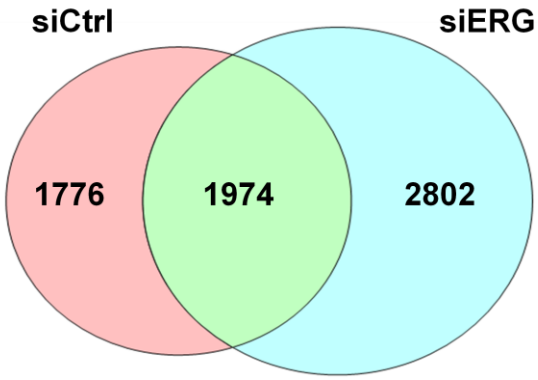


Figure 3.9 ERG Depletion Induce AR Recruitment to the Chromatin

A) and B) VCaP cells transfected with control siRNA or ERG targeting siRNA were deprived of androgens for 24 hours prior a 2h stimulation with EtOH/100 nM DHT. ChIP assays were performed using antibodies against AR. The immunoprecipitated DNA was quantified with qPCR. Error bars represent S.E.M of at least 3 independent biological repeats. C) The overlap of AR cistromes in VCaP cells treated with control siRNA or ERG targeting siRNA under normal full serum condition. The ChIP-Seq datasets were retrived from GSE14097 deposited in the NCBI GEO repository.

3.7 Involvement of HDACs and EZH2 in AR and ERG Transcriptional Cross-talk.

3.7.1 Overexpression of HDACs and EZH2 in Prostate Cancer

As discussed in the introduction, several transcriptional co-repressors (the histone deacetylases (HDAC1, HDAC2, and HDAC3) and the methyltransferase EZH2) were known to be widely overexpressed in prostate cancers (Fig. 3.10). Although these co-repressors were shown to play important roles in the progression of prostate cancers (Min et al., 2010; Wang et al., 2009a; Yu et al., 2010a; Yu et al., 2007b), their relationship with AR and ERG cross-talk is virtually unknown. Interestingly, the expression of these co-repressors was recently demonstrated to exhibit a positive correlation to ERG levels (Iljin et al., 2006; Yu et al., 2010b) in clinical prostate cancer samples. Furthermore, ERG-fusion positive prostate cancer cells were shown to exhibit enhanced sensitivity to HDAC inhibitors (Bjorkman et al., 2008). Basing on these observations, we postulate a potential direct collaboration between these co-repressors and ERG in suppressing AR transcriptional activity.

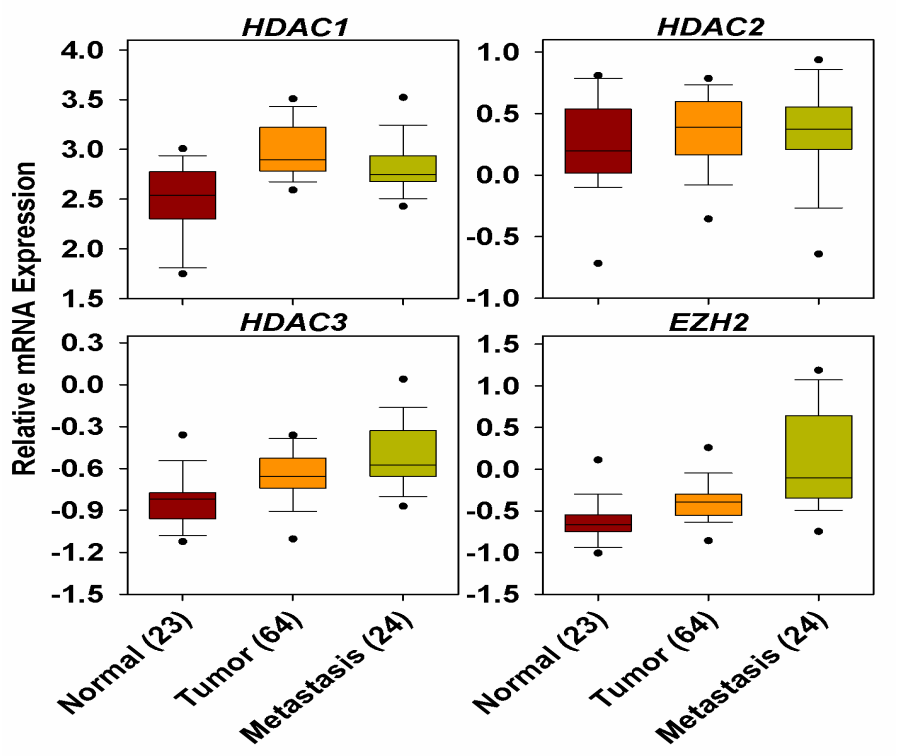


Figure 3.10 Overexpression of HDACs and EZH2 in Prostate Cancer

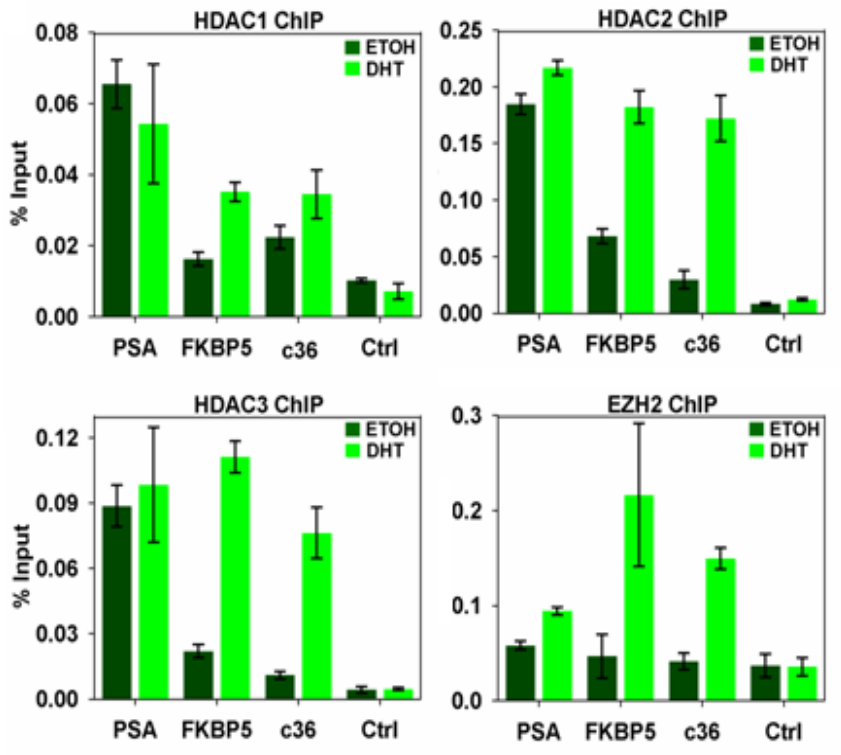
Boxplots of the relative mRNA expression levels of the transcriptional co-repressors HDAC1, HDAC2, HDAC3 and EZH2, in clinical prostate samples from the Yu's (Yu et al., 2004) study. The data were retrieved from the Oncomine database.

3.7.2 Chromatin Occupancy of HDACs and EZH2 at ARBS

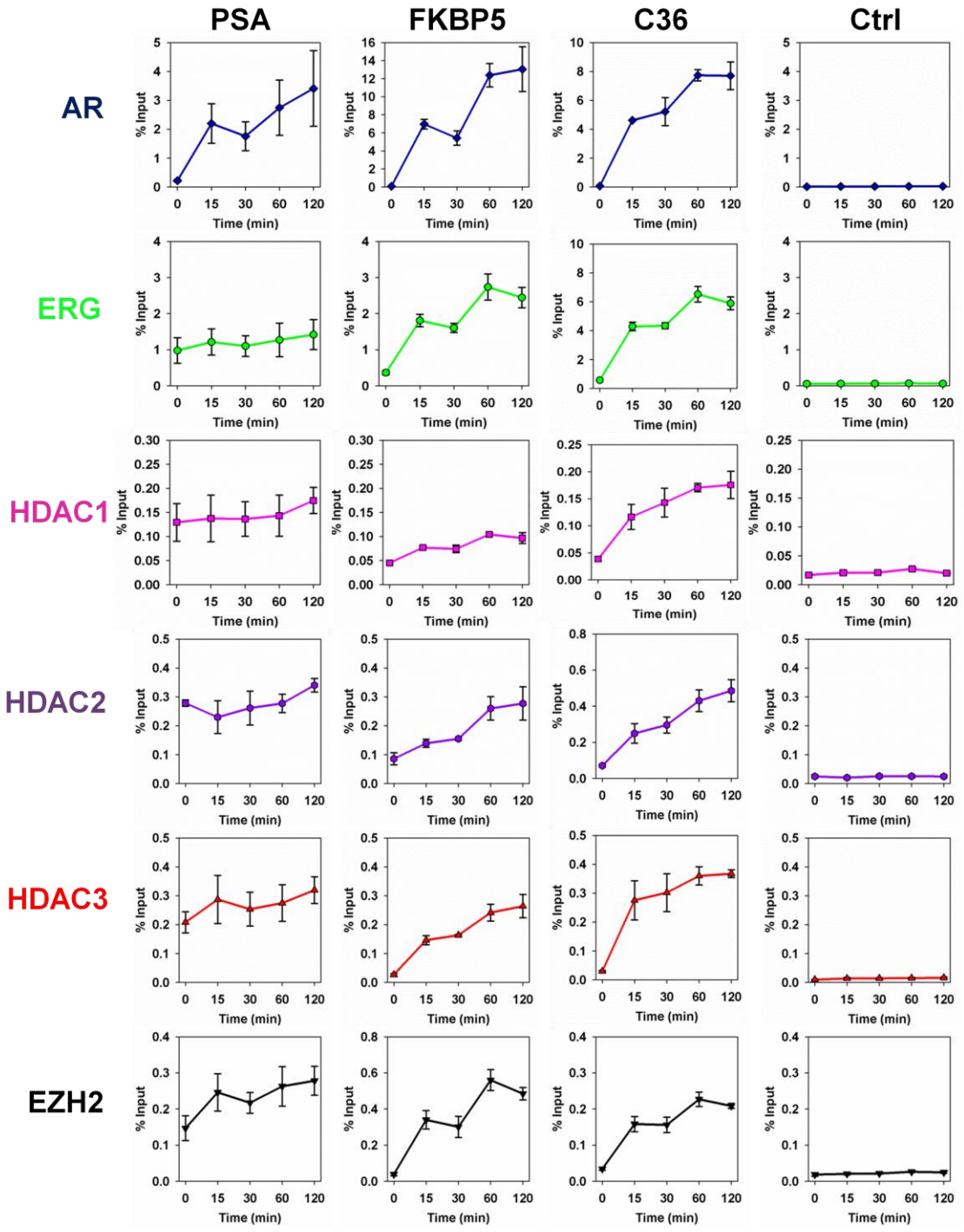
Both HDACs and EZH2 are transcriptional factors that are usually recruited to the chromatin and subsequently exert their repressive influence via modifying the histone tails. Hence, to validate our hypothesis that these co-repressors directly participate in the ERG-mediated suppression of AR transcriptional activity, we first tested their recruitment to AR+ERG binding sites by performing ChIP assays for these factors in VCaP cells before and after androgen stimulation. In support of our postulation of a transcriptional collaboration, we found that HDAC1, HDAC2, HDAC3, and EZH2 were all recruited to several AR + ERG co-localized binding sites including those that were associated with PSA and FKBP5 (Fig. 3.11A). Interestingly, we observed that androgen actually stimulate the recruitment of these corepressors in most cases, suggesting that this transcriptional co-operation is under the regulation of androgen signaling (Fig. 3.11A). Next, we proceeded to investigate the binding kinetics of HDACs and EZH2 with reference to AR and ERG recruitment to the chromatin, we peformed time-course ChIP assays at a series of timepoints upon androgen stimulation. We observed that similar to AR, HDAC1-3 and EZH2 were recruited to the several tested AR + ERG co-localized binding sites shortly (as early as 15 mins) after androgen stimulation (Fig. 3.11B). Furthermore, the binding profiles of HDAC1-3 and EZH2 overlapped substantially to that of AR and ERG, suggesting a link between the recruitment of AR and ERG with that of HDAC1-3 and EZH2 (Fig. 3.11B). To further establish the transcriptional co-operation between AR and ERG with HDAC1-3 and EZH2, we performed co-immunoprecipitation to probe for any endogenous physical interactions that would provide further evidence for a direct collaboration between these transcription factors. Indeed, we were able to detect

physical endogenous interactions between HDAC1, HDAC2 and EZH2 with both AR and ERG (Fig. 3.11C). However, intriguingly, we were unable to detect any interaction between HDAC3 with either AR or ERG from our co-immunoprecipitation assays. We reckoned that this might be a result of a much weaker and/or more transient interaction between HDAC3 with AR or ERG. Integrated together, our results suggest that HDACs and EZH2 are likely to collaborate with AR and ERG to regulate androgen-dependent transcription.

A)


HDAC1 ChIP
HDAC2 ChIP
HDAC3 ChIP
EZH2 ChIP
ETOH
DHT
% Input
PSA
FKBP5
c36
Ctrl
0.08
0.06
0.04
0.02
0.00
0.25
0.20
0.15
0.10
0.05
0.12
0.09
0.03
0.3
0.2
0.1
0.0

B)


PSA
FKBP5
C36
Ctrl
AR
ERG
HDAC1
HDAC2
HDAC3
EZH2
% Input
Time (min)
0
15
30
60
120

C)

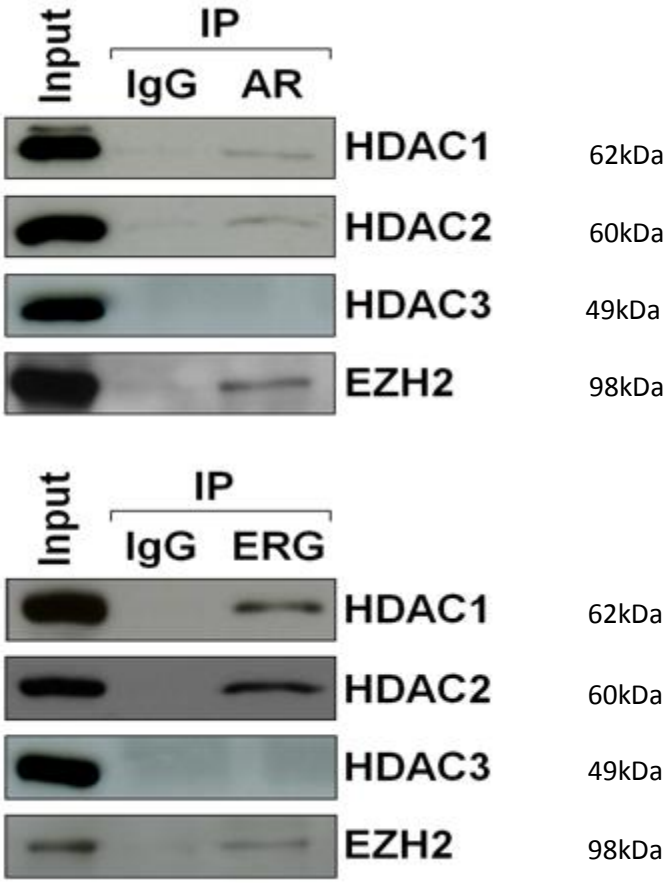


Figure 3.11 Physical Interaction and chromatin co-occupancyof HDACs and EZH2 with AR and ERG

A) VCaP cells were starved in androgen-free media for 24 h prior treatment with EtOH/100 nM DHT for 2 h. Subsequently, the cells were first double crosslinked with DSG followed by formaldehyde fixation. Immunoprecipitation was carried out with antibodies against HDAC1, HDAC2, HDAC3, or EZH2. qPCR quantification was performed for specific binding sites. Error bars are indicative of S.E.M for at least 3 independent experiments. B) Time course ChIP assays for the indicated DHT stimulated timings for AR, ERG, HDAC1-3 and EZH2 in VCaP cells were performed as described in (A). C) Western Blot analysis showing the results of the AR/ERG co-immunoprecipitation assays that were performed for probing endogenous interactions between AR and ERG with HDAC1, HDAC2, HDAC3, and EZH2 in VCaP cells.

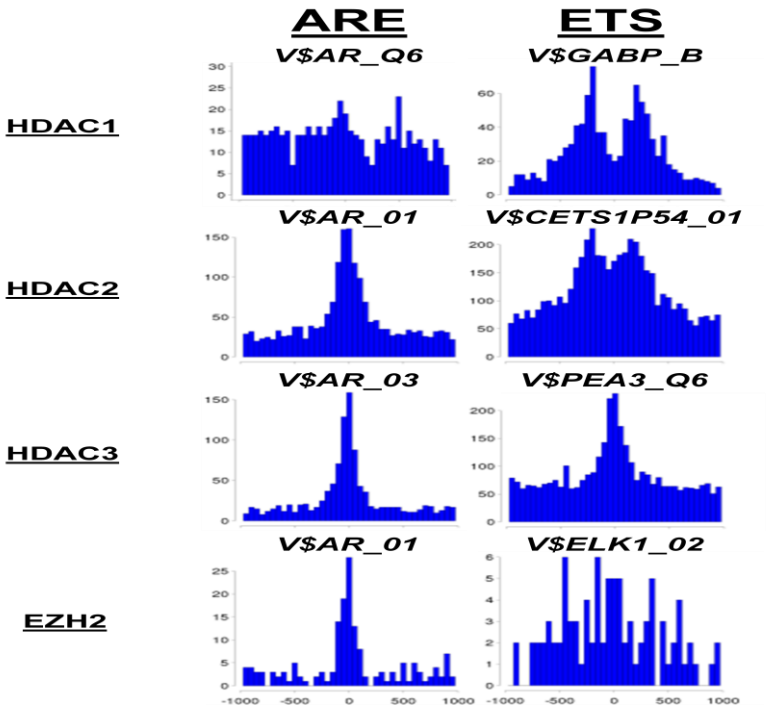
3.8 Cistromic Analysis of HDACs and EZH2 in VCaP Cells

3.8.1 Motif and Location Analysis of HDACs and EZH2 Cistromes

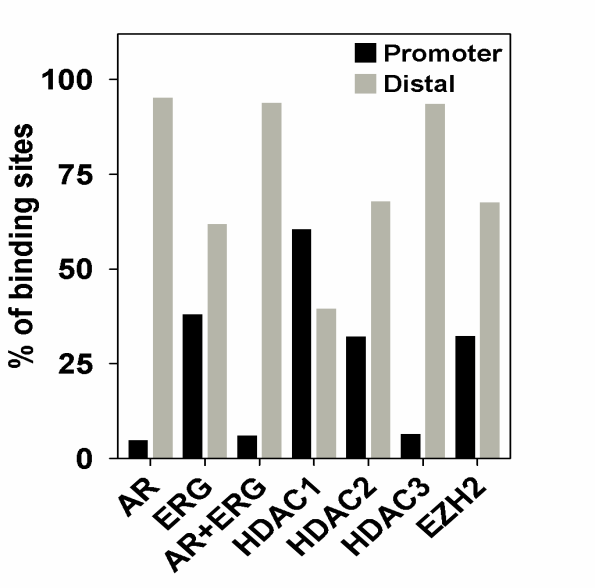
To further establish the extent of transcriptional collaboration between AR, ERG and the corepressors, HDACs and EZH2 on androgen signaling, we went on to generate the cistromes of these factors in VCaP cells prior and after (2 hrs) DHT stimulation (Table 4). The 2 hrs time-point was chosen as it corresponds to the largest overlap in AR and ERG co-localized binding from our earlier studies (Fig. 3.3A). Using the in-house generated bioinformatic tool, CENTDIST (Zhang et al., 2011), which detects motifs enriched in a set of DNA sequence, we found good center of distribution scores for both AR and ERG binding motifs at HDAC2, HDAC3, and EZH2 binding sites. This means these transcription factors are generally recruited to the DNA at a position that is near the AR and ERG binding motifs, suggesting a potential indirect recruitment via tethering to AR and ERG (Fig. 3.12A). Accordingly, since only ERG motifs were found enriched for HDAC1 binding peaks, this might be an indication that the recruitment of HDAC1 is occurring mainly through ERG. Next, we examined the location genomic distribution of the corepressors. HDAC1 was found to have a high binding preference to promoters, while HDAC2 and HDAC3 were mostly found at distal enhancers (Fig. 3.12B). From past studies, it was generally assumed that EZH2 was mainly recruited to the promoter regions to regulate transcription (Ku et al., 2008; Margueron et al., 2008; Yu et al., 2007a), but data from our EZH2 cistromic analysis surprisingly revealed that EZH2 is actually found substantially at distal enhancers in prostate cancer cells during androgen signaling (Fig. 3.12B). This was an intriguing finding that suggests a novel

transcriptional role for EZH2. To validate the authenticity of our generated cistromic maps and the co-localization of the co-repressors (HDACs1-3 and EZH2) with AR and ERG, we performed ChIP-qPCR validation of all these co-repressors for multiple AR+ERG co-localized binding sites (Fig 3.12C).

A)


ARE
ETS
HDAC1
HDAC2
HDAC3
EZH2
V$AR_Q6
V$GABP_B
V$AR_01
V$CETS1P54_01
V$AR_03
V$PEA3_Q6
V$AR_01
V$ELK1_02

B)


Promoter
Distal
% of binding sites
100
75
50
25
0
AR
ERG
AR+ERG
HDAC1
HDAC2
HDAC3
EZH2

C)

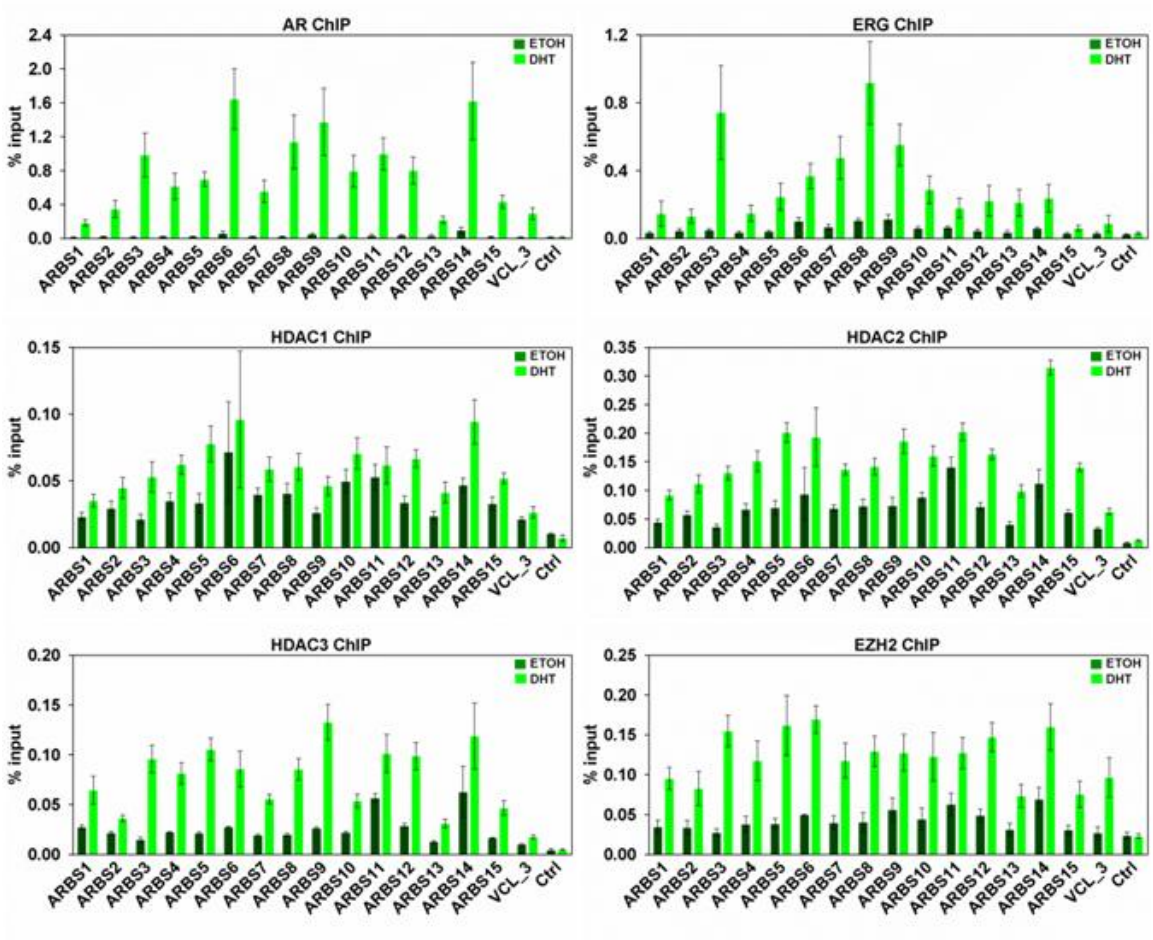


Figure 3.12 Motif and Location Analysis of HDACs and EZH2 Binding Sites

A) The binding sites of each respective co-repressor were input into the CentDist program. The generated average tag count distribution of Androgen Response Elements (AREs) and ETS binding motif (ETS) relative to the peaks of the respective co-repressor binding sites is displayed in graphical form. B) Bar chart showing the proportion of binding sites located at the promoter proximal (-/+3 kb from TSS) or distal regions for AR, ERG, HDAC1, HDAC2, HDAC3, and EZH2 respectively. C) VCaP cells were depleted of androgens and treated with either ETOH/100 nM DHT for 2 hrs. Chromatin Immunoprecipitation was carried out as described in Fig 3.11A with antibodies against AR, ERG, HDAC1, HDAC2, HDAC3, or EZH2. Immunoprecipitated DNA was quantified with qPCR for the specified AR+ERG co-occupied sites. Error bars represent S.E.M of at least 3 independent experiments.

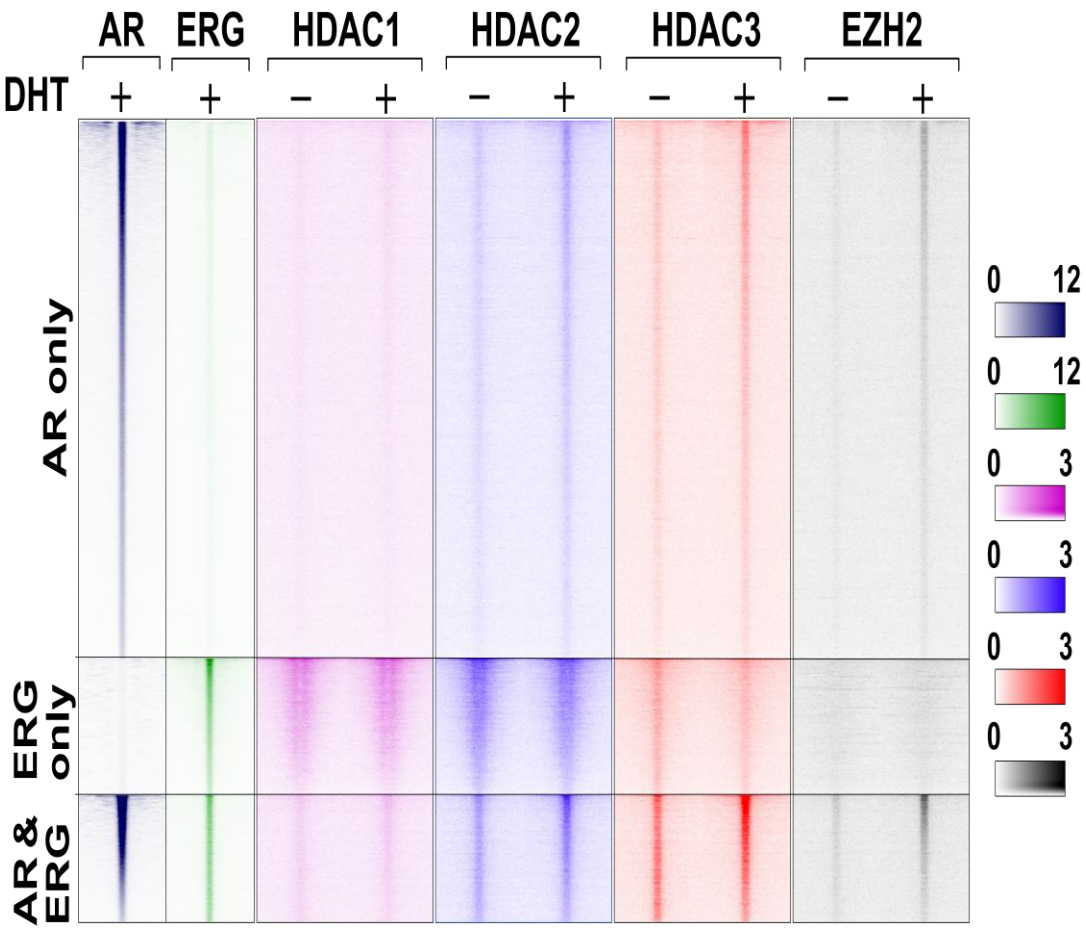
Library Name	FDR=0.05	FDR=0.1	FDR=0.2	Sequencing Depth (reads)
HDAC1 ChIP EtOH	0	0	1925	24944881
HDAC1 ChIP DHT	0	614	3434	25133481
HDAC2 ChIP EtOH	11288	16581	24309	14508414
HDAC2 ChIP DHT	10500	19813	25669	19626271
HDAC3 ChIP EtOH	0	1785	3667	23135520
HDAC3 ChIP DHT	1664	3225	5311	23350682
EZH2 ChIP EtOH	516	1336	1677	25434932
EZH2 ChIP DHT	815	1559	2794	24458897

Table 4 Sequencing depth and peak numbers (under several FDR) of HDAC1-3 and EZH2 ChIP-Seq libraries

3.8.2 Characterization and Analysis of the AR-Centric Co-repressor Regulatory Transcriptional Network in ERG-fusion Positive VCaP Cells

In concordance with our hypothesis that ERG, HDAC1-3 and EZH2 are widely involved in the direct regulation of androgen induced transcription, our cistromic studies of AR, ERG and the co-repressors revealed an intricate AR-centric transcription network in which the co-repressors are integrated into the network via occupation of different subsets of the AR and ERG cistromes with varying binding kinetics. In addition, the integration of these co-repressors is strongly enhanced on androgen signaling, suggestive of a feedback mechanism. We noted distinct combinations of corepressor recruitment in relation to AR and ERG binding sites. Both AR+ERG co-localized and AR unique binding sites are largely occupied by HDAC2, HDAC3, and EZH2 but the strength of the recruitment to AR unique sites was of a much weaker degree (Figs. 3.13A and 3.13B). In comparison, at ERG unique binding sites, we only observed HDAC1 and 2, but not EZH2 occupancy (Figs. 3.13A and 3.13B). Interestingly, we found that recruitments of HDAC2, HDAC3 and EZH2 to ARBS sites were enhanced upon androgen stimulation while no changes in HDAC1 binding were observed at the same binding sites. The strongest (average ChIP-Seq tag count) recruitment of these factors was observed at AR+ERG co-occupied sites (Figs. 3.13A and 3.13B).

A)


AR
ERG
HDAC1
HDAC2
HDAC3
EZH2
DHT
+
+
−
+
−
+
−
+
−
+
AR only
ERG only
AR & ERG
0 12
0 12
0 3
0 3
0 3
0 3

B)

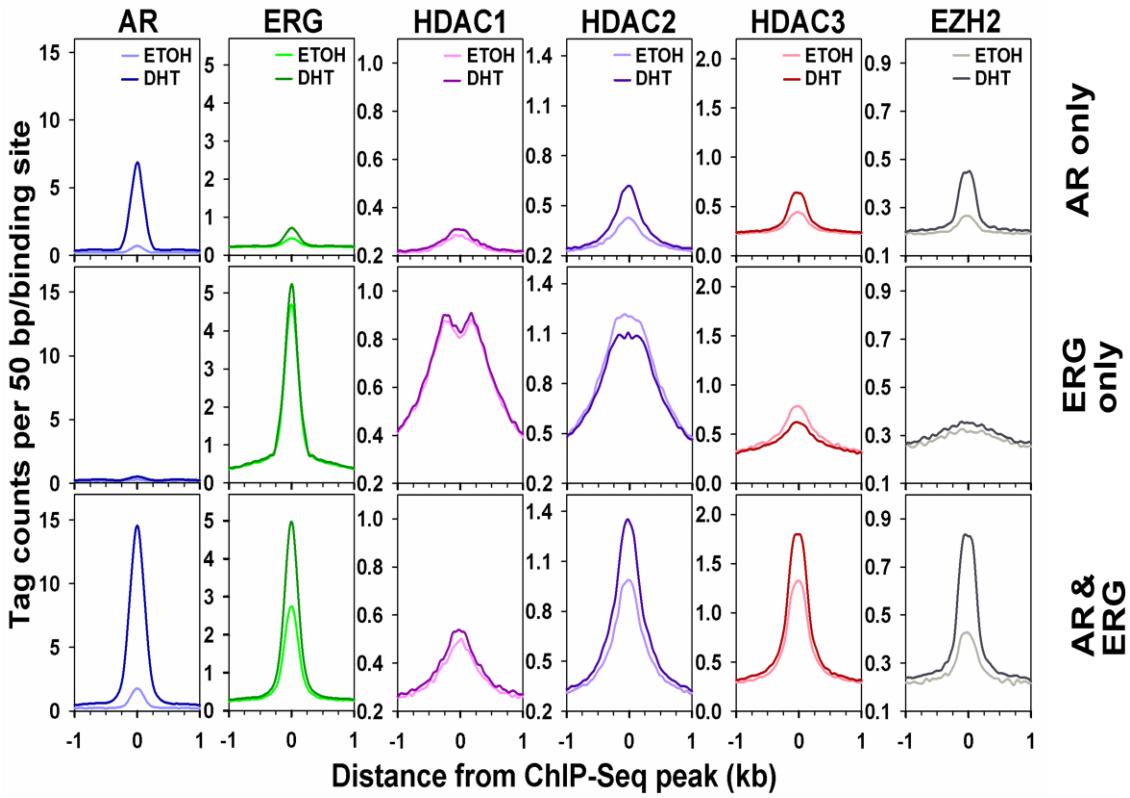


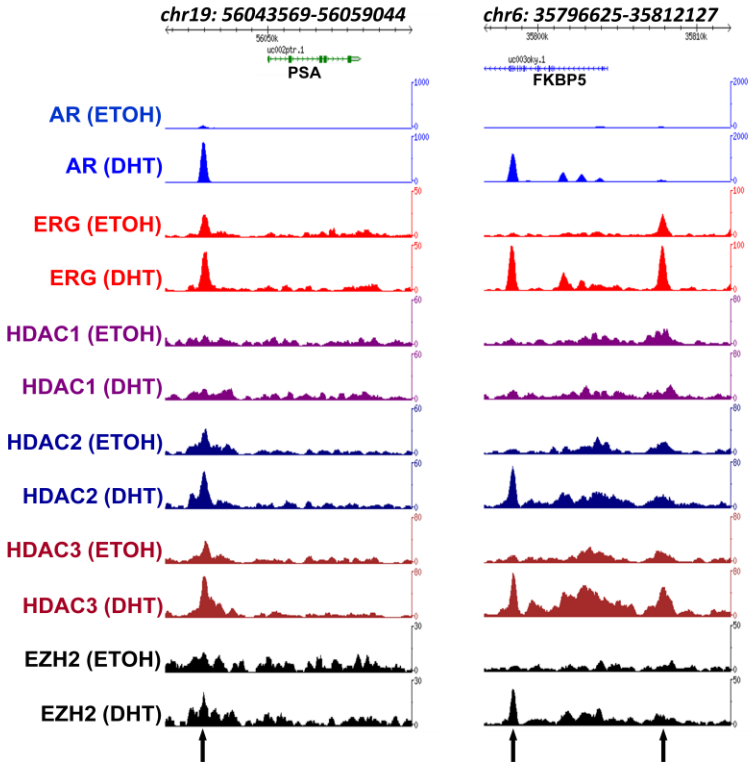
Figure 3.13 Characterization of the HDAC1-3 and EZH2 Cistrome in relation to AR and/or ERG Binding sites on Androgen Signalling

A) Heatmap representation of sorted normalised ChIP-Seq tag count of AR, ERG, HDAC1, HDAC2, HDAC3 and EZH2 binding, centralized on the center of AR and/or ERG ChIP-Seq peak (-/+2 kb) in VCaP cells. (B) Graphical display of the average ChIP-Seq tag count intensities of AR, ERG, HDAC1, HDAC2, HDAC3, and EZH2 at different subsets of the AR and ERG cistrome prior and after 2 hrs of androgen stimulation.

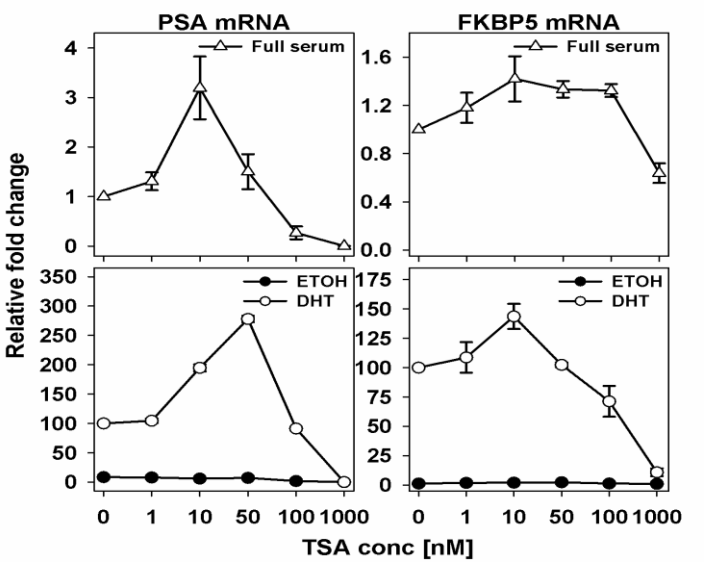
3.9 Attenuation of Androgen Induced Transcription by HDACs and EZH2 in ERG-Fusion Positive VCaP Cells

So far, our observation of the genome-wide recruitment of HDACs and EZH2 to AR+ERG co-localized binding sites across the prostate cancer genome strongly suggests the participation of these co-repressors in ERG-mediated attenuation of androgen-dependent transcription. Consistent with this observation, we found co-localization of HDACs and EZH2 at AR+ERG co-occupied transcriptional regulatory elements of PSA and FKBP5, model androgen induced direct target genes that are repressed by ERG (Fig. 3.14A). To determine the role HDACs and EZH2 in androgen-dependent transcription, we utilized specific small molecule inhibitors that block the activity of these co-repressors and examined its effects on the androgen-induced transcript levels of PSA and FKBP5. Specifically, TSA and DZNep were used to inhibit the activities of HDACs and EZH2 respectively. Interestingly, androgen-induced transcript exhibited a biphasic transcriptional response to TSA treatment: at low concentrations, TSA enhanced PSA and FKBP5 transcript levels but was repressive at high concentrations. This suggests a dual (activation and repression) role for HDACs in regulating AR transcriptional activity (Fig. 3.14B). As for DZNep, we observed enhancement in the expression of both PSA and FKBP5 after treatment, consistent with a role for EZH2 in repressing AR transcriptional activity (Fig. 3.14C).

A)


chr19: 56043569-56059044
chr6: 35796625-35812127
PSA
FKBP5
AR (ETOH)
AR (DHT)
ERG (ETOH)
ERG (DHT)
HDAC1 (ETOH)
HDAC1 (DHT)
HDAC2 (ETOH)
HDAC2 (DHT)
HDAC3 (ETOH)
HDAC3 (DHT)
EZH2 (ETOH)
EZH2 (DHT)

B)


PSA mRNA
FKBP5 mRNA
Full serum
ETOH
DHT
Relative fold change
TSA conc [nM]
0
1
10
50
100
1000

C)

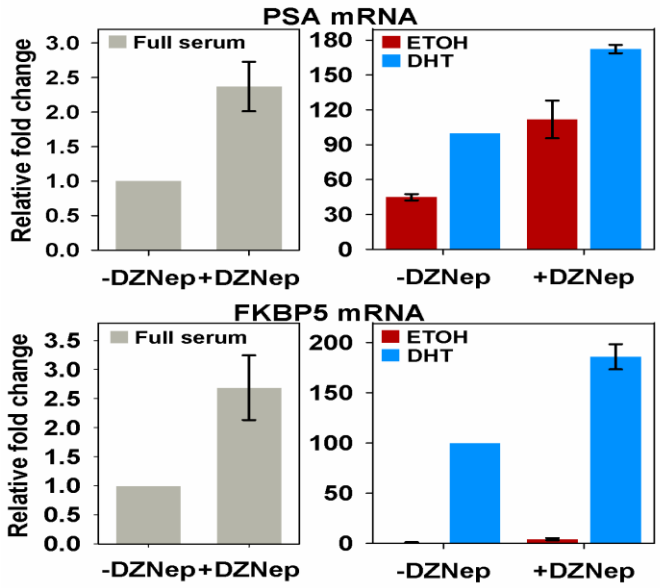


Figure 3.14 Co-recruitment of HDACs, and EZH2 to AR+ERG occupied sites repressed AR-dependent transcription

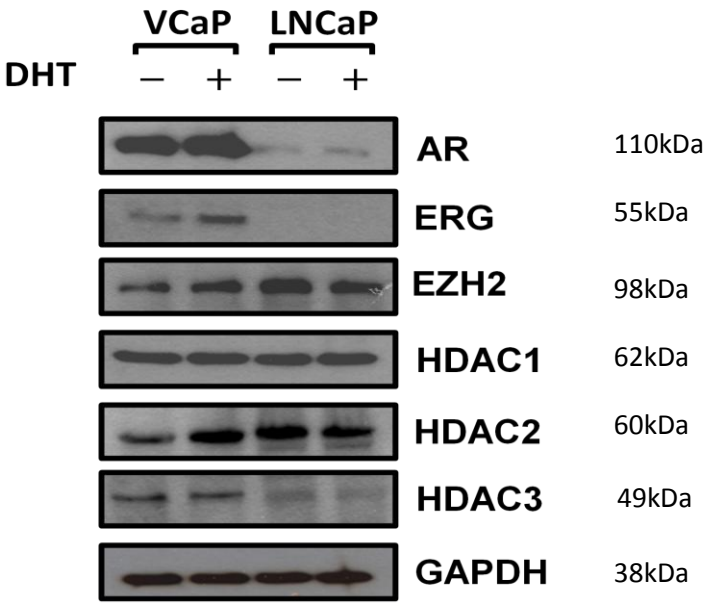
(A) Snapshots of HDAC1, HDAC2, HDAC3, and EZH2 at AR+ERG occupied regulatory elements of model AR target genes, PSA and FKBP5. (B) VCaP cells grown in full serum (top) or starving medium were co-treated with vehicle/10 nM DHT (bottom) and varying concentrations of TSA for 24 hours. After which, total RNA from the cells were extracted and converted to cDNA. The transcript levels were quantified by qPCR using GAPDH as an internal normalization control. Error bars represent S.E.M of at least 3 independent experiments. (C) VCaP cells grown in full serum (left) or starving medium treated with vehicle/10 nM DHT for 8 hours (right) were first subjected to vehicle/3 μM DZNep stimulation for 24 (left) or 48 (right) hrs. Total RNA was harvested and processed as described in Fig. 3.14B. Error bars represent S.E.M of at least 3 independent experiments.

3.10 Roles of HDACs and EZH2 on Androgen Induced Transcription in ERG-Fusion Negative LNCaP Cells

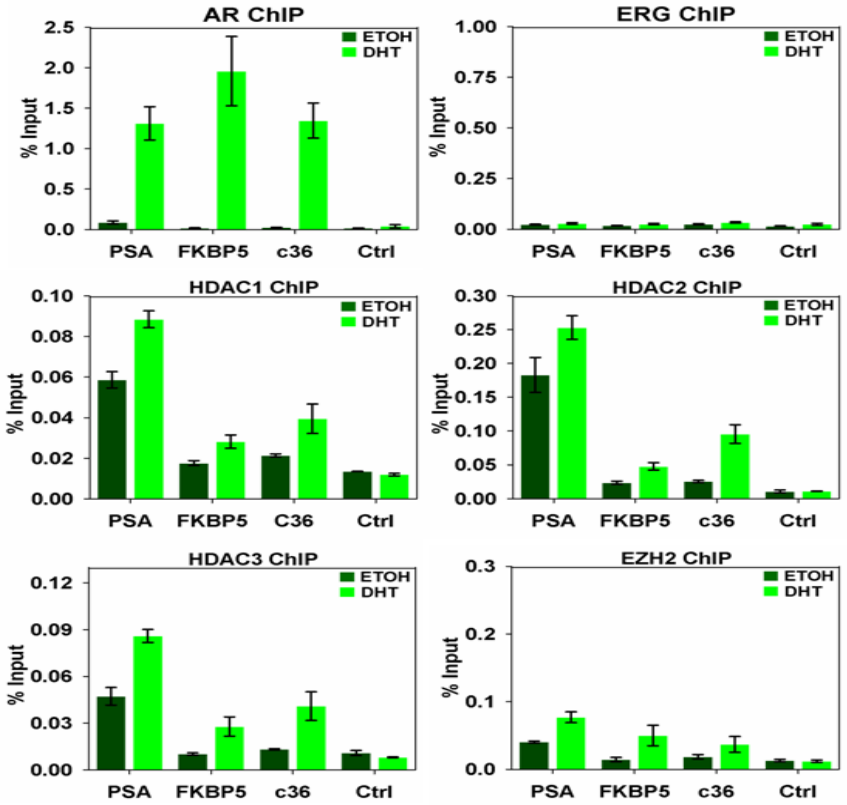
Thus far, we have only investigated the role of HDACs and ERG in AR-mediated transcription using the ERG overexpressing VCaP prostate cancer cells. Since there has been no evidence suggesting that the overexpression of HDACs and EZH2 in prostate cancer is exclusively limited to ERG-fusion positive subtypes, we questioned if these co-repressors played similar roles in ERG-fusion negative prostate cancer with little or no ERG expression. To address this question, we examined the effects of HDACs and EZH2 on AR-dependent transcription in LNCaP cells (an AR-positive but ERG negative prostate cancer cell line). Earlier, we observed that unlike VCaP cells, LNCaP cells do not harbor the TMPRSS2-ERG fusion (Fig. 3.1A). We further demonstrated that there is negligible ERG expression in LNCaP relative to VCaP (Fig. 3.15A). In addition, we compared the expression levels of AR, HDAC1-3 and EZH2 between the two cell lines. From our results, we noted that apart from not expressing ERG, the expression levels of AR and HDAC3 was also significantly lower in LNCaP as compared to VCaP (Fig. 3.15A). The extremely high level of AR in VCaP was within our expectations as AR is known to be highly amplified in VCaP cells (Liu et al., 2008). On the other hand, the expression levels of EZH2, HDAC1 and HDAC2 was similar in both LNCaP and VCaP cells (Fig. 3.15A). We then tested the recruitment of these transcription factors via ChIP assays. Interestingly, we found that in LNCaP cells, HDACs and EZH2 were also recruited after androgen stimulation, similar to the ARBS that were earlier tested in VCaP cells (Fig. 3.11A) but their binding affinities, were in most instances, lower (with respect to % input) than VCaP cells (Fig. 3.15B). Within expectations, while AR was

significantly recruited to the ARBS tested, negligible ERG recruitment was detected (Fig. 3.15B). Next, we assessed the role of HDACs and EZH2 on AR-mediated transcription in LNCaP cells. LNCaP cells were treated with small molecule inhibitors of HDACs or EZH2 (TSA or DZNep). We found that there was a much weaker (compared to LNCaP) or no response in androgen upregulation of model AR target genes such as PSA and FKBP5 after treatment with DZNep and TSA, respectively (Fig. 3.15C). Taken together, while our results are suggestive towards the conclusion for a more pronounced role of HDACs and EZH2 in the repression of AR activity under an ERG-fusion positive prostate cancer system such as VCaP cells, further experiments are required for establishing the nature of the corepressor complex in the absence of ERG.

A)


VCaP
LNCaP
DHT
– + – +
AR
110kDa
ERG
55kDa
EZH2
98kDa
HDAC1
62kDa
HDAC2
60kDa
HDAC3
49kDa
GAPDH
38kDa

B)


AR ChIP
ERG ChIP
HDAC1 ChIP
HDAC2 ChIP
HDAC3 ChIP
EZH2 ChIP
ETOH
DHT
% Input
PSA
FKBP5
c36
C36
Ctrl

C)

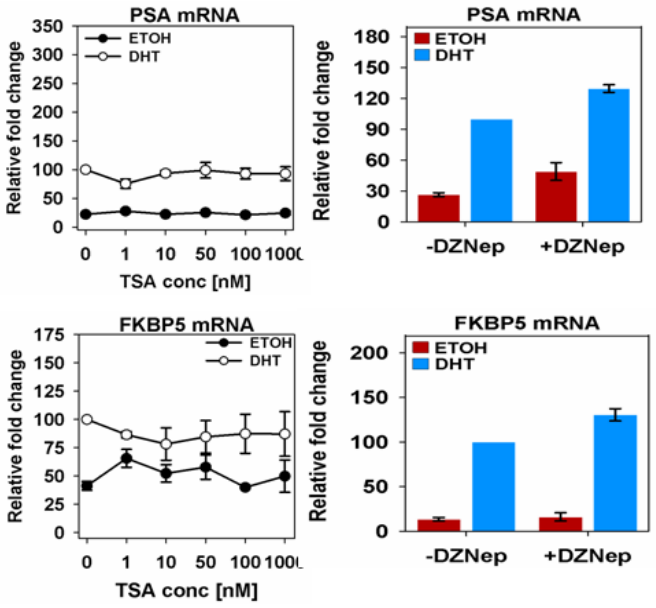


Figure 3.15 Role of HDACs and EZH2 on AR-mediated Transcription in ERG-Fusion Negative Prostate Cancer Cells

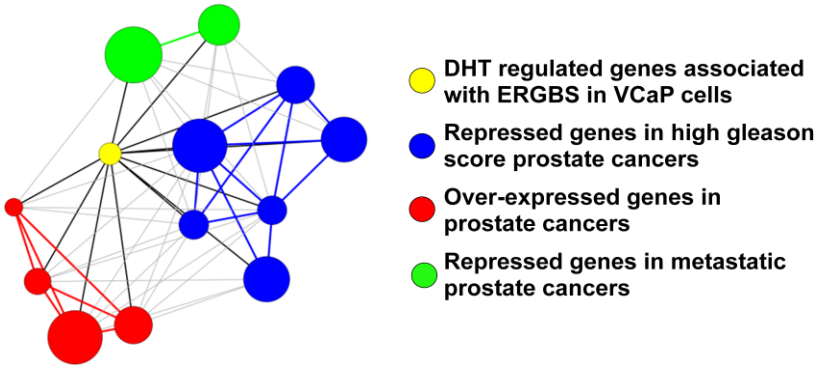
A) Western Blot analysis probing for AR, ERG, HDAC1-3 and EZH2 in androgen deprived LNCaP and VCaP cells after 2 hrs of 100nM DHT/EtOH stimulation. B) Androgen-depleted LNCaP cells were treated for 2 hrs with ETOH/100 nM DHT. Chromatin was immunoprecipitated with specific antibodies against AR, ERG, HDAC1, HDAC2, HDAC3, or EZH2 using the same procedures as those for VCaP cells (Fig 3.11A). Immunoprecipitated DNA was quantified through qPCR. Error bars represent S.E.M of at least 3 independent experiments. C) (Left panels) Hormone depleted LNCaP cells co-treated with vehicle or 10 nM DHT were subjected to varying concentrations of TSA for 24 hrs as for VCaP cells in Fig 3.14B (bottom). (Right panels) Hormone depleted LNCaP cells were pre-treated with vehicle or 3μM DZNeP for 48 hrs and then subjected to with or without 10 nM DHT for 8 hrs as for VCaP cells in Fig 3.14C (right). Total RNA from the treated cells were then harvested and converted to cDNA before quantifying for gene expression levels. GAPDH was used as an internal normalization control. Error bars represent S.E.M of at least 3 independent experiments.

3.11 The Role of ERG in AR-Directed Prostate Cancer Progression

ERG was suggested to inhibit differentiation, expedite (Epithelial Mesenchymal Transition) EMT and promote metastasis in prostate cancer cells through direct transcriptional upregulation of genes such as PLA1A, PLAT, PLAU, and EZH2 (Tomlins et al., 2008; Yu et al., 2010b). Apart from being a transcriptional activator, our findings indicate that ERG also functions as a repressor of AR-dependent transcription, possibly by working together with corepressors such as HDACs and EZH2. However, it is still unclear as to whether if ERG can promote prostate cancer progression through the direct suppression of AR-mediated transcription. To assess the clinical importance of ERG inhibition on AR-dependent transcription in prostate cancer development, we performed an Oncomine molecular concept map (MCM) analysis with androgen-upregulated genes that are associated with ERGBS using data of clinical prostate samples deposited in the Oncomine database. From the analysis, we observed ERG bound androgen induced genes to be associated with several concepts related to prostate cancer, especially with those concepts depicting genes that are over-expressed in primary cancer (compared to normal prostate) but repressed in advanced and metastatic prostate cancer (compared to primary prostate cancer) (Fig. 3.16A and Table 5). This result implied the occurrence of reduced androgen signaling in aggressive forms of prostate cancer during prostate cancer progression. Interestingly, this finding was corroborated by a recent study that was published in the course of this work (Yu et al., 2010b) by another group using their own defined target gene signature but with a similar methodology. To further support the validity of this claim, we performed an additional analysis on the clinical prostate cancer dataset of a very extensive clinical study (Taylor et al., 2010) and also observed similar

findings (Fig. 3.16B). Again, the same set of defined androgen induced genes was found to exhibit significantly higher expression levels in primary prostate tumors (compared to normal prostate) albeit lower expression levels in metastatic prostate tumors (compared to primary tumors) (Fig. 3.16B).

A)



B)

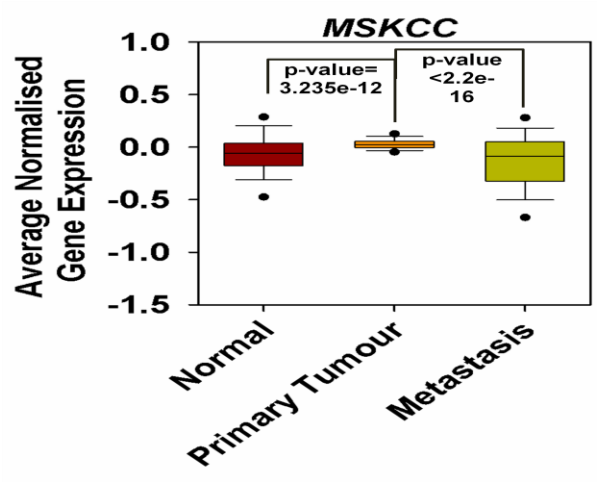


Figure 3.16 Expression Profiles of ERG associated Androgen Induced Gene Set in Clinical Prostate Samples

A) A network display of clinical prostate cancer gene signatures that have a significant correlation with the ERG-associated androgen upregulated gene set defined in this study. The Oncomine Molecular Concept Map (MCM) analysis was utilized to compare defined ERG-associated (5 kb from TSS) androgen induced genes (>2 fold) against clinical prostate cancer gene signatures depositied in the Oncomine database. Criteria for establishing significant associations between node is defined as OD≥2; p-value < 1e-4. B) Boxplot displays of the average normalised expression for each individual gene in the defined androgen induced gene signature under respective category of clinical prostate sample. ERG-associated androgen induced gene signature were identified as described in Fig. 3.16A. Using the normalized MSKCC prostate cancer clinical dataset (Taylor et al., 2010), the average expression of each gene in the androgen induced gene signature for each sample type was assessed and plotted**.** Normalization is done by log transformation of the expression of each probe and then subtracting by probe median. The mean of the expression of each probe in the respective category is then plotted out in box plot form.

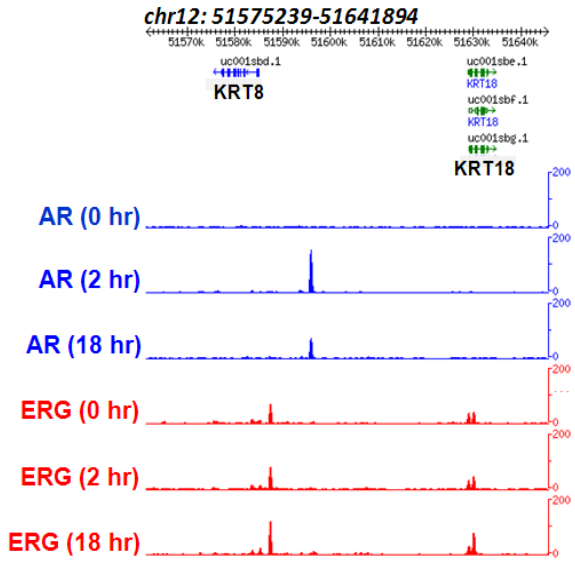
Concept Name	P-value	Odds Ratio
Cancer Type: Prostate Cancer - Top 10% Over-expressed (Bittner Multi-cancer)	1.17E-32	5.1
Prostate Adenocarcinoma - Advanced Gleason Score - Top 10% Under-expressed (Vanaja Prostate)	2.22E-22	4
Prostate Carcinoma vs. Normal - Top 10% Over-expressed (Welsh Prostate)	8.57E-22	5.3
Prostate Carcinoma Epithelia - Advanced Gleason Score - Top 5% Under-expressed (Tomlins Prostate)	1.89E-19	5.9
R1881 Treatment + Vector Only Transfection Control - LNCaP Cell Line - Top 10% Over-expressed (Chen CellLine)	1.02E-13	3.3
Benign Prostatic Hyperplasia Tissue Subtype - Epithelia - Top 10% Over-expressed (Tomlins Prostate)	3.49E-13	3.6
Cancer Type: Prostate Cancer - Top 10% Over-expressed (Ramaswamy Multi-cancer)	1.25E-11	3.3
Prostate Carcinoma vs. Normal - Top 5% Over-expressed (Singh Prostate)	1.89E-10	4.3
Prostate Carcinoma - Advanced Gleason Score - Top 10% Under-expressed (Yu Prostate)	1.92E-10	3.3
Amsacrine Sensitive - Multi-cancer Cell Line - Top 10% Under-expressed (Compendia CellLine)	2.32E-09	2.7
Prostate Adenocarcinoma - Smoker - Top 10% Under-expressed (Wallace Prostate)	2.32E-09	2.7
Prostate Carcinoma Primary Cell Culture - Recurrence at 1 Year - Top 10% Under-expressed (Nanni Prostate)	6.32E-09	2.6
Cancer Type: Prostate Cancer - Top 10% Over-expressed (Su Multi-cancer)	1.77E-08	2.9
Cancer Type: Prostate Cancer - Top 10% Over-expressed (Ramaswamy Multi-cancer 2)	8.08E-08	2.6
Prostate Adenocarcinoma vs. Normal - Top 10% Over-expressed (Vanaja Prostate)	1.55E-07	2.3
Prostate Cancer - Metastasis - Top 10% Under-expressed (Varambally Prostate)	4.72E-07	2.2
Benign Prostatic Hyperplasia Epithelia vs. Normal - Top 10% Over-expressed (Tomlins Prostate)	7.77E-07	2.5
Prostate Carcinoma - Advanced Gleason Score - Top 5% Under-expressed (Lapointe Prostate)	1.01E-06	2.9
Prostate Carcinoma Primary Cell Culture - Advanced Gleason Score - Top 10% Under-expressed (Nanni Prostate)	3.67E-06	2.2
Prostate Carcinoma vs. Normal - Top 1% Over-expressed (Varambally Prostate)	9.31E-06	4.8
Prostate Adenocarcinoma - Advanced Gleason Score - Top 10% Under-expressed (Wallace Prostate)	1.82E-05	2.1
Topotecan Sensitive - Cell Line - Top 5% Under-expressed (Gyorffy CellLine)	2.39E-05	2.5
Prostate Carcinoma - Recurrence at 5 Years - Top 10% Over-expressed (Holzbeierlein Prostate)	2.58E-05	2.8
Prostate Cancer - Metastasis - Top 10% Under-expressed (Lapointe Prostate)	3.67E-05	2.1
Acinar Prostate Adenocarcinoma - Smoker - Top 5% Under-expressed (Bittner Prostate)	3.77E-05	2.3

Table 5 List of Oncomine concepts significantly associated with the defined ERG-targeted androgen-induced gene signature

3.12 ERG-mediated Attenuation of Androgen Induced Epithelial Cytoskeletal Proteins that are associated with an Epithelial Phenotype.

Results derived from our analysis on available clinical data (Fig. 3.16A and Fig. 3.16B) are suggestive towards the conclusion that regulated repression of a significant portion of ERG-associated androgen induced genes facilitate the progression of prostate cancers to the advanced and metastatic forms. As discussed in the introduction, although ERG repressed androgen induced targets like PSA and FKPB5 are markers of epithelial differentiation; they have no known functional role in driving metastasis and cancer progression. Interestingly, a detailed examination of the ERG-associated androgen induced genes elucidated previously reported mediators of Mesenchymal to Epithelial Transition (MET) (a process that reduces metastasis) in breast cancer. Examples include KRT8 and KRT18 (Buhler and Schaller, 2005; Tomaskovic-Crook et al., 2009) (Fig. 3.17A). qPCR confirmed the androgen induced upregulation in the expression of these keratin genes in VCaP cells and the further enhancement post ERG silencing (Fig. 3.17B).

A)



B)

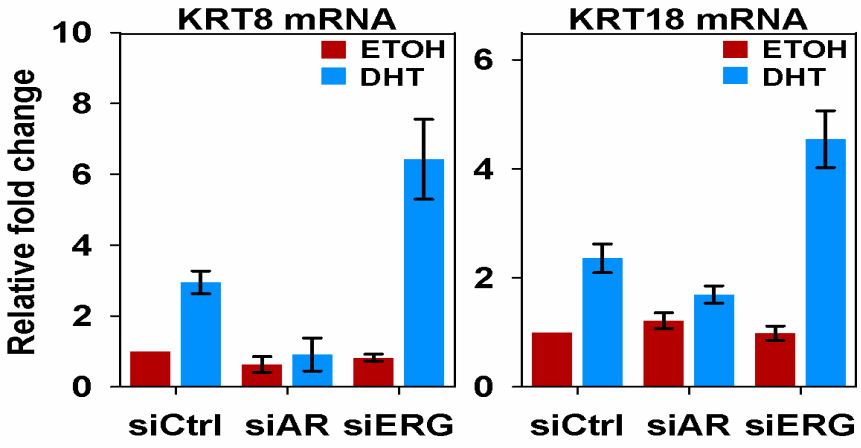


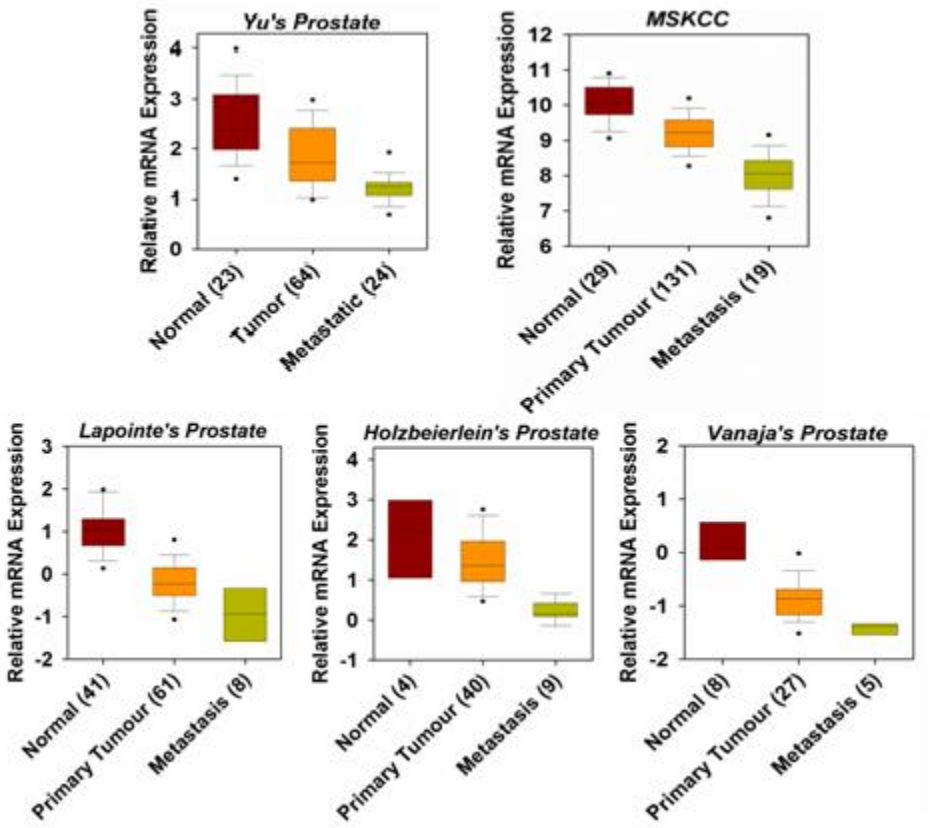
Figure 3.17 Transcription Regulation of Keratin Genes by AR and ERG

A) ChIP-Seq dervived snapshot of AR and ERG binding events near KRT8 and KRT18 gene locus. B) VCaP cells were processed, RNA collected and converted to cDNA as described in Fig. 3.8B. Gene expressions were quantified by qPCR using specific primers. GAPDH was used as an internal normalization control. Error bars represent S.E.M of at least 3 independent experiments.

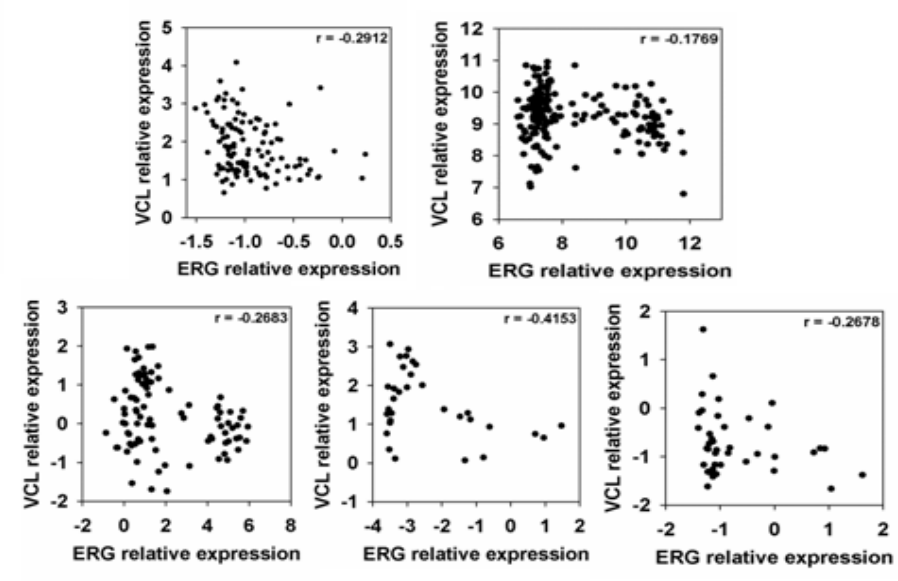
3.13 VCL, a Tumor Suppressor in Prostate Cancer

Within our defined gene set (Fig. 3.16A), we sought to identify novel AR target genes that are suppressed and facilitate metastasis in prostate cancer. From literature review, Vinculin (VCL) was identified as a potential candidate. VCL is a membrane cytoskeletal protein required for regulating focal adhesion turnover. This is a process important for proper cell movement (Saunders et al., 2006). Importantly, a previous study identified an interaction between VCL and the MET mediator, E-Cadherin. This interaction was found to be critical for mechanosensing enhancement (le Duc et al., 2010). We then turned to clinical data deposited in the Oncomine data for evidence that supported a role of VCL as a suppressor of cancer progression. In line with our expectations, we found the mRNA expression of VCL was low in primary prostate cancers and even lower in advanced metastatic counterparts. This was a trend that was supported by several clinical studies (Fig. 3.18A). Furthermore, we observed a negative correlation relationship between the mRNA levels of ERG and VCL (Fig 3.18B). Survival analysis using data from the Taylor *et al* clinical study (Taylor et al., 2010) also showed that patients with low expression of VCL have a significantly lower recurrence free survival (Fig. 3.18C), indicative of VCL's postulated role as a tumor suppressor. Taken together, these clinical data were in concordance with our postulation of VCL as a potential novel AR target gene that is suppressed by ERG in prostate cancer to facilitate cancer progression and metastasis.

A)


Yu's Prostate
Relative mRNA Expression
Normal (23)
Tumor (64)
Metastatic (24)
MSKCC
Relative mRNA Expression
Normal (29)
Primary Tumour (131)
Metastasis (19)
Lapointe's Prostate
Relative mRNA Expression
Normal (41)
Primary Tumour (61)
Metastasis (8)
Holzbeierlein's Prostate
Relative mRNA Expression
Normal (4)
Primary Tumour (40)
Metastasis (9)
Vanaja's Prostate
Relative mRNA Expression
Normal (8)
Primary Tumour (27)
Metastasis (5)

B)


r = -0.2912
VCL relative expression
ERG relative expression
r = -0.1769
VCL relative expression
ERG relative expression
r = -0.2683
VCL relative expression
ERG relative expression
r = -0.4153
VCL relative expression
ERG relative expression
r = -0.2678
VCL relative expression
ERG relative expression

C)

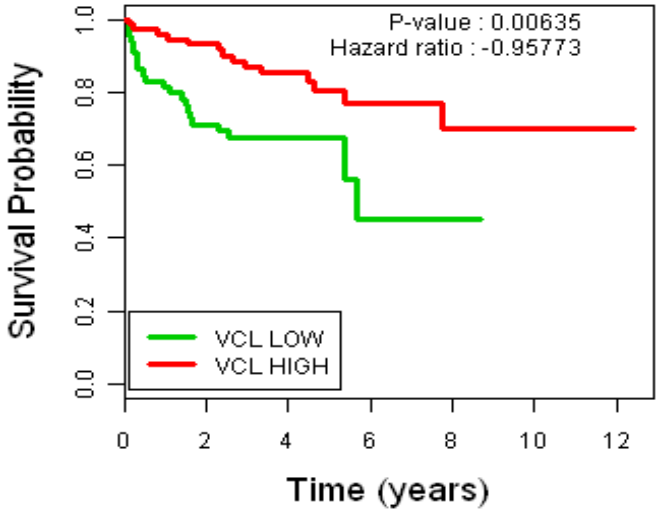


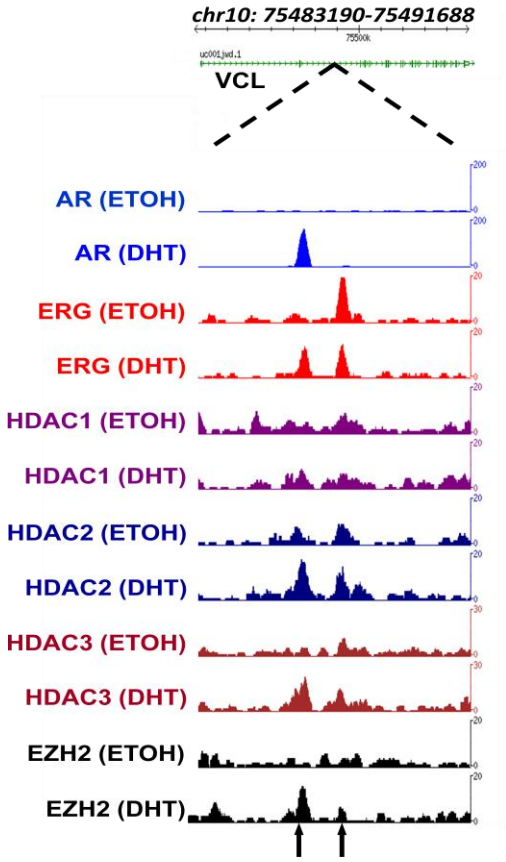
Figure 3.18 Expression Levels of Vinculin in Clinical Prostate Cancer Studies

A) Boxplots showing relative mRNA expression of VCL in clinical prostate samples from the MSKCC study and studies deposited in the Oncomine database. B) Scatterplots showing the correlation between the relative mRNA expression of VCL and the corresponding ERG mRNA expression in clinical prostate samples from the same studies in Fig. 3.18A. C) Kaplan-Meier survival curve showing the risk of biochemical relapse in prostate cancer patients expressing high (red line) or low (green line) VCL levels. Data from the analysis was retrieved from the MSKCC prostate cancer dataset (Taylor et al., 2010).

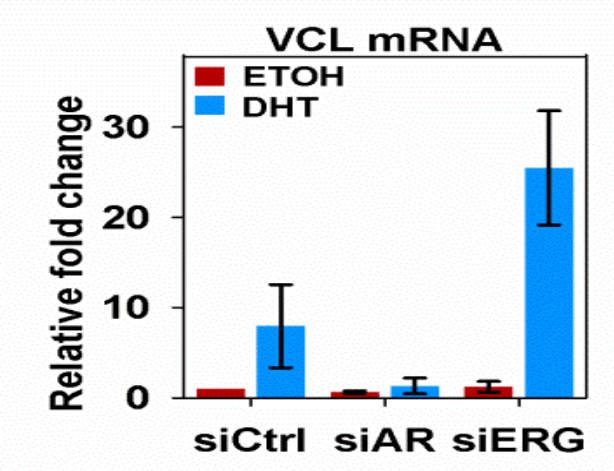
3.14 VCL, an Androgen Induced Gene that is Suppressed by ERG, HDACs and EZH2 in VCaP Cells

To provide evidence that inhibition of VCL directly links ERG and AR with prostate cancer progression, we first showed that VCL is a direct target of AR and ERG. As shown in our Chip-Seq data (Fig. 3.19A), AR and ERG are recruited to an intronic region of VCL. Interestingly, HDACs and EZH2 were also recruited to the same site (Fig. 3.19B). Furthermore, while androgens stimulate the expression of VCL, silencing of ERG via siRNA and the inhibition of HDACs/EZH2 by small molecules all led to enhanced VCL expression (Fig. 3.19B, Fig 3.19C and Fig 3.19D).

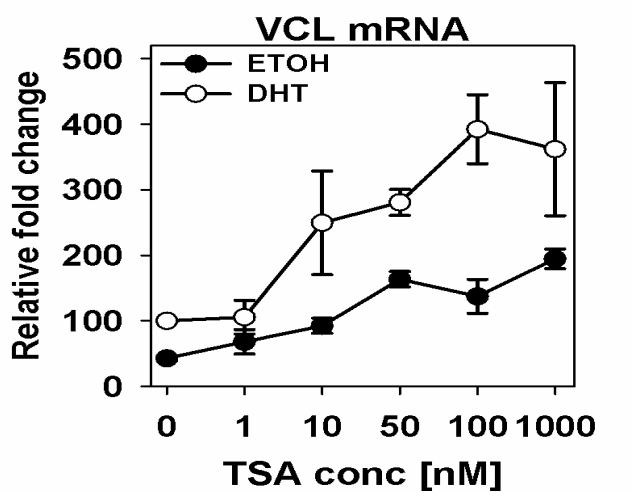
A)


chr10: 75483190-75491688
VCL
AR (ETOH)
AR (DHT)
ERG (ETOH)
ERG (DHT)
HDAC1 (ETOH)
HDAC1 (DHT)
HDAC2 (ETOH)
HDAC2 (DHT)
HDAC3 (ETOH)
HDAC3 (DHT)
EZH2 (ETOH)
EZH2 (DHT)

B)


VCL mRNA
ETOH
DHT
Relative fold change
30
20
10
0
siCtrl
siAR
siERG

C)



D)

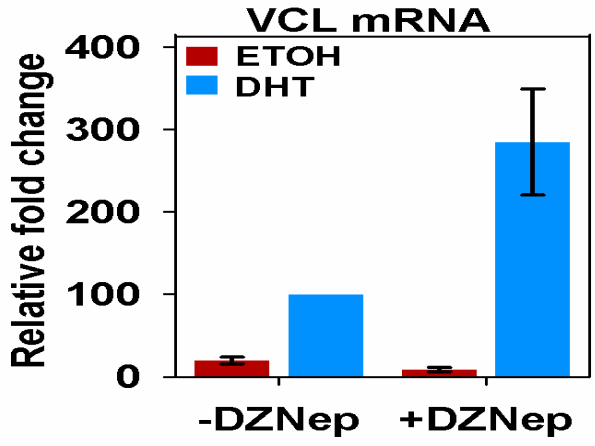


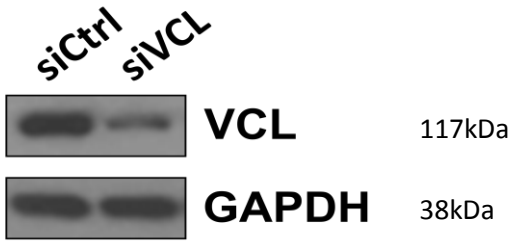
Figure 3.19 Suppression of Androgen Induced Upregulation of VCL by ERG, HDACs and EZH2

A) Snapshot showing the co-localization of AR, ERG, HDACs 1-3 and EZH2 at a potential cis-regulatory element of VCL. B) The consequences of AR and ERG siRNA-mediated depleteion on VCL expression in VCaP cells were assessed using methods as described in Fig. 3.8B. C) The consequences of TSA-mediated inhibition of HDACs on VCL expression in VCaP cells were assessed as described in Fig. 3.14B (bottom). D) The consequences of DZNep-mediated inhibition of EZH2 on VCL expression in VCaP cells were assessed as described in Fig. 3.14C (right).

3.15 Silencing of VCL Led to Increased Prostate Cancer Cell invasiveness

Thus far, we have only provided correlative evidence that would support VCL role as a tumor suppressor in prostate cancer. It would be important to demonstrate the functional phenotypic effect of VCL suppression on prostate cancer progression. To address this question, we investigated the effect of VCL depletion on prostate cancer metastasis through performing invasion assays using VCaP cells with or without VCL depletion. In concordance with our postulated tumor suppressor role for VCL, our results showed that silencing of VCL (Fig 3.20A) culminated in an increase in the matrigel invasiveness of VCaP cells (Fig. 3.20B). Through performing PI FACs and BrdU assays, we confirmed that the increase in matrigel invasion capability of VCaP cells post VCL depletion was not due to decreased cell death (Fig. 3.20C) or increased cell proliferation (Fig. 3.20D). To provide further support for the generality of VCL as a suppressor of cell invasion in prostate cancers, we also assessed the effect of VCL depletion on cell invasion in another prostate cancer cell line, LNCaP (Fig. 3.20E). Similar to VCaP cells, we also observed an increase in matrigel invasiveness of LNCaP cells after VCL depletion (Fig 3.20F). Overall, our results support the postulation that ERG, HDACs and EZH2 facilitate prostate cancer cell invasion and metastasis, in part, by suppressing AR-mediated upregulation of VCL.

A)



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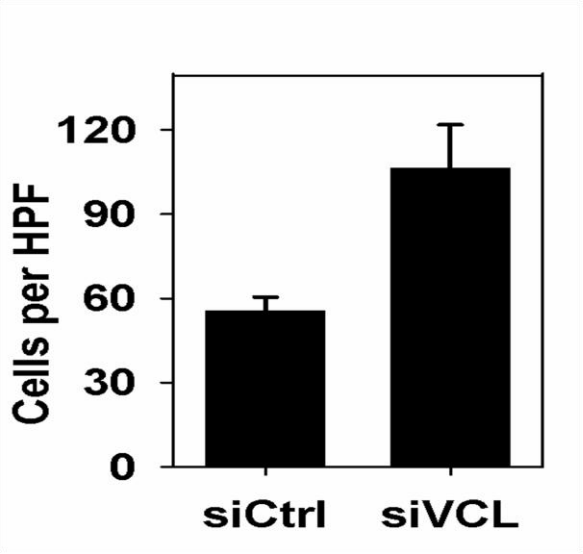
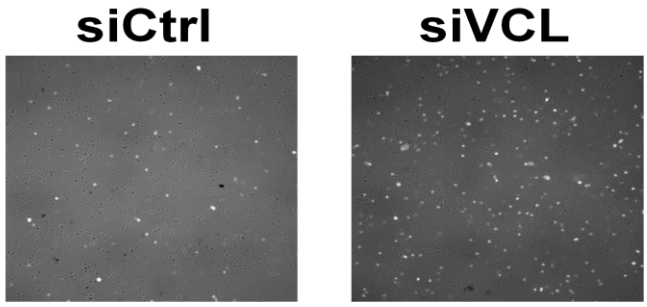
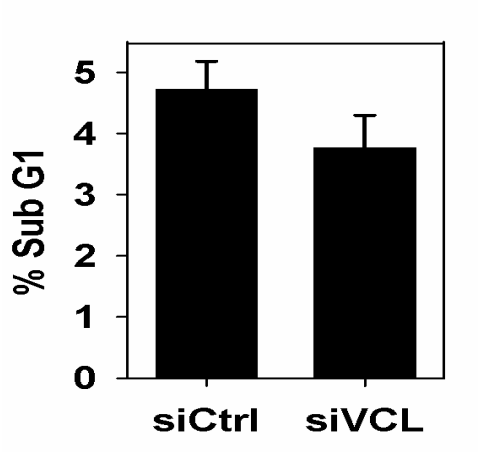


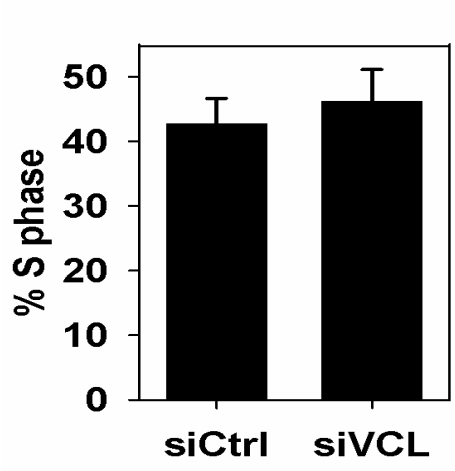


Taken using 20X Objective Lens

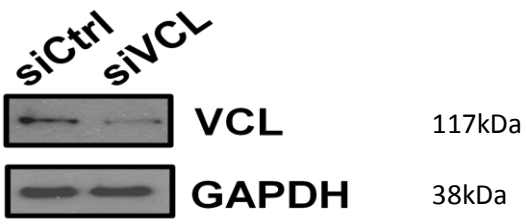
C)


% Sub G1
5
4
3
2
1
0
siCtrl
siVCL

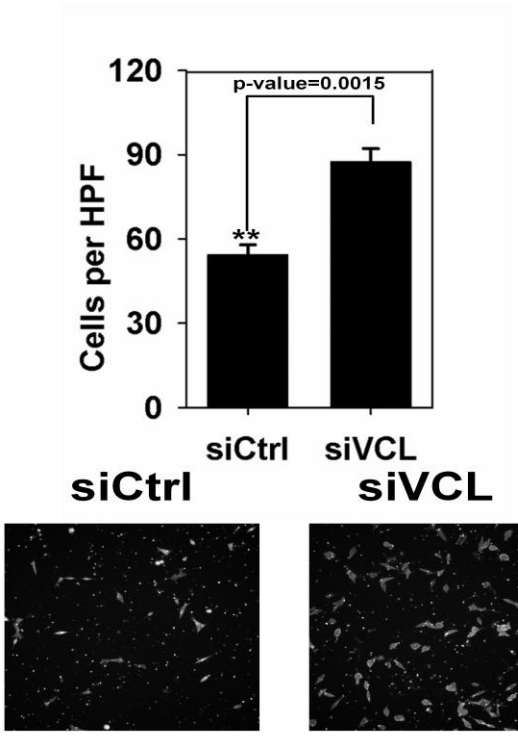
D)


% S phase
50
40
30
20
10
0
siCtrl
siVCL

E)


siCtrl
siVCL
VCL
GAPDH
117kDa
38kDa

F)



Taken using 20X Objective Lens

Figure 3.20 VCL as a Suppressor of Invasion in Prostate Cancer Cells

A) Western blot analysis of VCL expression in VCaP cells grown under normal full serum conditions and treated with control siRNA or siRNA against VCL. GAPDH was utilized as the loading control. B) VCaP cells, treated with control siRNA or VCL targeting siRNA, were used for Matrigel invasion assay. Bar charts showing the average number of cells that have passed through the transwell per high power field (HPF) (Top). Error bars represent S.E.M of at least 3 biological replicates. Representative high HPF images of siRNA treated VCaP cells that have passed through the transwell in a matrigel invasion assay (Bottom). C) Bar chart showing the percentage of VCaP cells in Sub G1 phase after treatment with control siRNA or siRNA against VCL as assessed by PI FACs analysis. D) Bar chart showing the percentage of VCaP cells in S phase after treatment with control siRNA or siRNA against VCL as assessed by BrdU assay analysis. E) Western blot analysis of VCL expression in LNCaP cells grown under normal full serum conditions and treated with control siRNA or siRNA against VCL using GAPDH as the loading control. F) LNCaP cells, treated with control siRNA or VCL targeting siRNA, were used for Matrigel invasion assay. Bar charts showing the average number of cells that have passed through the transwell per high power field (HPF) (Top). Error bars represent S.E.M of at least 3 biological replicates. Representative high HPF images of siRNA treated LNCaP cells that have passed through the transwell in a matrigel invasion assay (Bottom).

Chapter 4: Discussion

Previous research has established a close and intricate link between the output of AR transcriptional network and prostate cancer development (Debes and Tindall, 2002; Heinlein and Chang, 2004). Consequently, manipulation of the AR transcriptional activity as a therapeutic strategy against prostate cancer harbors the potential to be highly effective (Huggins, 1967; Huggins and Hodges, 2002). To facilitate the development of this form of therapeutics, a clear understanding of the transcriptional mechanisms underlying the AR transcriptional network is highly desired.

AR-mediated transcription is a complex process encompassing a series of highly coordinated steps. The recruitment of the receptor, collaborative factors, coactivators, and corepressors had to occur in a temporal and spatial manner to ensure optimal transcriptional output. Furthermore, the transcriptional activity of AR at the different gene loci had to be independently regulated so as to achieve a precise transcriptional output driving the desired cellular phenotype.. From our understanding of the field, we noted that most of the studies to date have focused on the function of transcriptional coactivators such as SRCs and p300 in the activation of AR-mediated transcription. Although these studies have provided much knowledge into the workings of the AR-mediated transcription, it is unfortunate that this does not provide a comprehensive view of the whole AR transcriptional network as the net transcriptional output is determined by a coordinated crosstalk between AR with both co-activators and co-repressors. Consequently, co-repressors are likely to also play a major role in the regulation of transcriptional output of the AR transcriptional network. For instance, the widely

overexpressed TMPRSS2-ERG was surprisingly shown to function as a repressor of AR-mediated transcription (Sun et al., 2008; Yu et al., 2010b). Other than ERG, multiple transcriptional co-repressors (HDACs and EZH2) were also known to be overexpressed in prostate cancers (Varambally et al., 2002; Weichert et al., 2008). However, our understanding on the extent of androgen signaling attenuation by transcriptional co-repressors at the genomic level and the resulting functional consequences on prostate cancer progression remains limited.

We attempted to address this question with a genomic approach. Utilizing ChIP-Seq, we mapped out the genome-wide binding profiles of AR. ERG, HDAC1, HDAC2, HDAC3 and EZH2 in prostate cancer cells prior and post androgen stimulation. Briefly, we uncover an AR-centric transcriptional network consisting of the transcriptional repressors ERG, HDACs and EZH2. Our data suggest that the integration of transcriptional co-repressors in AR transcriptional network provide the mechanism for regulated suppression of androgen signaling.

Similar to a recent study (Yu et al., 2010b), which was published during the course of our work, we found widespread co-localization of AR and ERG after 2 hrs of DHT stimulation. This is indicative of a genome-wide transcriptional collaboration between the two factors in prostate cancer. Even though both our study and that of Yu et al. (Yu et al., 2010b) showed substantial overlap between the cistromes of AR and ERG, our study provided several novel insights on the AR and ERG cross-talk. In contrast to the Yu et al. study (Yu et al., 2010b) which examine AR cistrome only after a prolonged duration of

androgen treatment and ERG cistrome under full serum condition, we have provided a comprehensive profile of AR and ERG cistrome in prostate cancer cells at both short and long time intervals after androgen stimulation and even under conditions of androgen deprivation. In addition, we treated the prostate cancer cells with a saturating concentration of DHT (100nM) as opposed to the other study (10nM). Consequently, we are likely to discover novel functional AR binding sites that are only occupied under androgen-saturated conditions as was shown by a recent study (Cai et al., 2011). Interestingly, our data showed that a substantial number of ERG unique and AR+ERG co-localized binding sites had prebound ERG prior to androgen treatment, suggestive of a pioneering role for ERG. This was an unexpected finding as the expression of ERG was shown to arise from a fusion event, resulting in it being induced upon androgen stimulation (Tomlins et al., 2005). Unlike the conventional pioneering factors, our time-course ChIP-Seq data showed that the recruitment of ERG to AR+ERG co-localized binding sites can be further enhanced with short-term DHT stimulation even though some ERG is prebound prior stimulation. In contrast, the increment of ERG at ERG unique sites is mostly only observed after an increase in ERG protein levels on prolonged androgen stimulation. Apart from providing evidence for AR's role as a facilitator of ERG recruitment to ARBS, this result also demonstrate ERG as an unique nuclear receptor transcriptional repressor different from other counterparts such as NKX3-1 and LEF-1, which exert their repressive function via competition with the Estrogen Receptor (ER) for binding to the ERBS (Holmes et al., 2008). Intriguingly, we also noticed that the promoters of androgen regulated genes were also frequently occupied by ERG in addition to the AR-bound enhancers. However, unlike ERG binding at AR-bound enhancers, their

occupancy at the promoters of androgen regulated genes was usually unresponsive to androgen stimulation. Further studies are required to address the functional relevance of ERG binding at the promoter region of androgen regulated genes in the attenuation of androgen signaling.

The expression levels of HDACs and EZH2 were shown by recent studies to be positively correlated to that of ERG in prostate cancers (Gupta et al., 2010; Iljin et al., 2006; Yu et al., 2010b). Interestingly, HDACs1-3 and EZH2 are also transcriptional repressors that were observed to be ovexpressed in prostate cancers (Varambally et al., 2002; Weichert et al., 2008) and associated with prostate oncogenesis (Min et al., 2010; Wang et al., 2009a; Yu et al., 2010a; Yu et al., 2007b). It is thus tempting to speculate a transcriptional collaboration between ERG, HDACs and EZH2 in the partial attenuation of AR transcriptional activity. Indeed, analysis of our generated ChIP-Seq data elucidated a complex and intricate transcriptional network between AR, ERG with other widely over-expressed transcriptional corepressor proteins (HDAC1, HDAC2, HDAC3, and EZH2) in prostate cancer cells. Interestingly, our study revealed a general increased in the occupancy of AR, ERG, HDAC2, HDAC3, and EZH2 to shared elements of the transcriptional network during androgen signaling, suggestive of a tightly regulated mechanism that is incorporated in the AR transcriptional program for feedback during androgen signaling. Whereas previous studies have demonstrated the recruitment of HDACs to ARBS, this was under the context of antiandrogen (casodex) stimulation (Shang et al., 2002) and not under androgen signaling. Even more unexpected was our finding on androgen-induced EZH2 recruitment to a substantial of ARBS enhancer sites,

an observation that suggests a role for EZH2 in transcriptional regulation, unique from its commonly perceived role as the catalytic enzyme for histone methylation at the promoters of repressed genes (Ku et al., 2008; Margueron et al., 2008; Yu et al., 2007a).

Even though our study revealed some insights on the integrated transcriptional co-repressor network of AR, ERG, HDACs and EZH2, our understanding of this network is still incomplete. For instance, the exact mechanism of co-operative action by ERG, HDACs and EZH2 in AR transcriptional attenuation remains largely unknown. Furthermore, we showed the involvement of HDACs and EZH2 in ERG-mediated suppression of androgen signaling in ERG-positive VCaP prostate cancer cells, their specificity to the type of regulatory elements remains unresolved. While we have shown a mild attenuation effect on AR transcriptional activity by HDACs and EZH2 in ERG-fusion negative LNCaP cells, we acknowledge the need for a more comprehensive study to establish the role of HDACs and EZH2 in AR-mediated transcription in ERG-null prostate cancers.

Although our work and others (Sun et al., 2008; Yu et al., 2010b) highlighted the role of ERG in attenuating AR induced differentiation markers including PSA and FKBP5, there were no major known direct functional roles for these genes in prostate cancer progression (Chen and Sawyers, 2010). An attenuated level of androgen signaling was suggested to be a common feature of metastatic and advanced prostate cancers (Yu et al., 2010b). Importantly, studies have shown that a low AR content is responsible for epithelial mesenchymal transition (EMT) and invasion in prostate cancer cells (Zhu and

Kyprianou, 2010). Strikingly, ERG is also implicated to disrupt the cortical cytoskeletal network (Schulz et al., 2010), promote EMT and invasion in prostate cancers by upregulating matrix metalloproteinases (MMPs) (Tomlins et al., 2008), ZEB1/2 (Leshem et al., 2011) and through FZD4 (Gupta et al., 2010). So the question begets: Could ERG mediated suppression of AR signaling be a possible avenue for EMT in prostate cancers? In the EMT process, epithelial cytoskeletal and cell adhesion proteins including keratins and E-Cadherins are substituted with their mesenchymal counterparts such as Vimentin and N-Cadherins (Lee et al., 2006a). Composition alteration of the cell adhesion and cytoskeleton molecules will culminate into weaker cell adhesion and cell-cell cohesion. Consequently, this leads to enhanced cancer cell motility and invasiveness (Lee et al., 2006a). Indeed, we observed that ERG could directly repress AR-mediated upregulation of epithelial cytoskeletal protein markers KRT8 and KRT18. Apart from epithelial cytokeratins, the androgen induced expression of the cytoskeletal protein Vinculin (VCL), was also repressed directly by ERG, together with HDACs1-3 and EZH2. VCL is a cytoskeletal protein responsible for regulating focal adhesion turnover (Saunders et al., 2006) and hence cell motility. VCL was recently implicated to potentiate the mechanosensing function of E-cadherin (le Duc et al., 2010), an important adhesion molecule that suppressed metastasis in prostate cancers (Cao et al., 2008). In addition, VCL was also shown to stabilize E-cadherin expression at cell surface (Peng et al., 2010). These results highlight the importance of VCL to the optimal functionality of E-cadherin in regulating cell adhesion and cell motility. Interestingly, we showed that depletion of VCL also led to an increase in the invasive capability of prostate cancer cells. This suggests VCL role as an important androgen induced target that is repressed by ERG,

HDACs and EZH2 for prostate cancer progression. Analysis on clinical data also revealed an association between low VCL levels and advanced metastatic prostate cancers. On top of that, low VCL level was shown to be a predictor of poor prognosis in prostate cancer patients. Intriguingly, however, there was also recent data that showed non-conformity to the role of VCL as a general tumor suppressor in prostate cancers. For instance, enhanced proliferative capability was attributed to the amplification of the VCL gene in a subset of prostate cancers (Ruiz et al., 2011), as exemplified by the AR negative PC3 prostate cancer cell line model. On the hand, proliferation rate of the androgen sensitive 22Rv1 (Ruiz et al., 2011) and VCaP (in this study) prostate cancer cell line was unaffected by VCL depletion. Consequently, these results suggest a possible dual role of VCL in prostate cancer progression that is likely to sensitive to the levels of VCL under a specific cellular context. Again, further studies would be necessary to establish this postulation.

At first sight, the notion deriving from our data that regulated partial suppression of androgen signaling drives prostate cancer progression seem to be contradictory to the generally accepted consensus that increased AR activity is associated with both naïve and castrate-resistant prostate cancer development. Certainly, the importance of high AR activity in prostate cancer progression is undisputable. The role of high AR activity in promoting prostate cancer cell survival and proliferation is well established (Liao et al., 2005; Schiewer et al., 2012). An active and functioning AR was also widely recognized as driver for continued prostate cancer progression, crucial for the development, growth and survival of androgen independent prostate cancers (Chen et al., 2004; Wang et al.,

2009b). For instance, with AR amplification, prostate cancer cells could become highly sensitive to basal levels of androgens, ensuring adequate AR transcriptional activation for continued prostate cancer cell proliferation and survival in patients that undergo androgen deprivation therapy (Chen et al., 2004). We are in full agreement with these accepted insights that AR activity is essential for prostate cancer progression. This is highlighted in our recently published paper that showed high AR activity being crucial for hormone naïve prostate cancer cell survival (Tan et al., 2012). However, we feel that our current data in this paper is not in opposition to these commonly accepted consensuses but offers an additional dimension to prostate cancer progression. We conclude that while an enhanced level of AR transcriptional output may drive cancer cell survival and proliferation, aberrant hyperactivation of the whole AR transcriptional program might be detrimental for prostate cancer progression to metastatic forms. This might be the explanation for studies that have observed a slowing of prostate cancer progression occurring under high doses of DHT treatment (Hofman et al., 2001; Tsihlias et al., 2000). In support, defined sets of androgen upregulated genes in our study and that of Yu et al. (Yu et al., 2010b) were both shown to be associated with overexpressed genes in prostate primary tumours (in comparison to normal prostate) and underexpressed genes in advanced and metastatic prostate cancers (in comparison to primary prostate tumors). This implies some forms of reduced androgen signaling in advanced and aggressive prostate tumours when compared to primary prostate tumours. Hyperactivation of AR might drive transcription programs associated with epithelial differentiation and thus inhibit metastasis. Indeed, a low level of AR content was shown to be necessary for an EMT phenotype in prostate cancers (Zhu and Kyprianou, 2010). Consequently,

regulation of androgen signaling in prostate cancer would be crucial for ensuring an equilibrium that allows for cancer cell proliferation, survival, as well as metastasis. To achieve this equilibrium level of androgen signaling, we speculate that multiple feedback mechanisms are likely to be incorporated in the AR transcriptional network. In fact, AR was recently shown to mediate its own transcriptional repression under high levels of androgens in prostate cancer cells (Cai et al., 2011). We speculate that this might be one of the possible feedback mechanisms for modulating androgen signaling repression. Additionally, we think that the integration of ERG and transcriptional co-repressors into the AR transcription network, discovered in this study, constitutes another level of feedback mechanism for maintaining an optimal androgen signaling output. Taken together, the results from this study are consistent with a dual opposing role of AR in driving proliferation and survival but inhibiting metastasis in prostate cancer and so to modulate the AR transcriptional output ideal for metastasis, a highly integrated transcriptional network of AR with ERG, HDACs and EZH2, functions to limit AR-driven epithelial differentiation and to facilitate EMT via regulated suppression of AR signaling (Fig. 4.1).

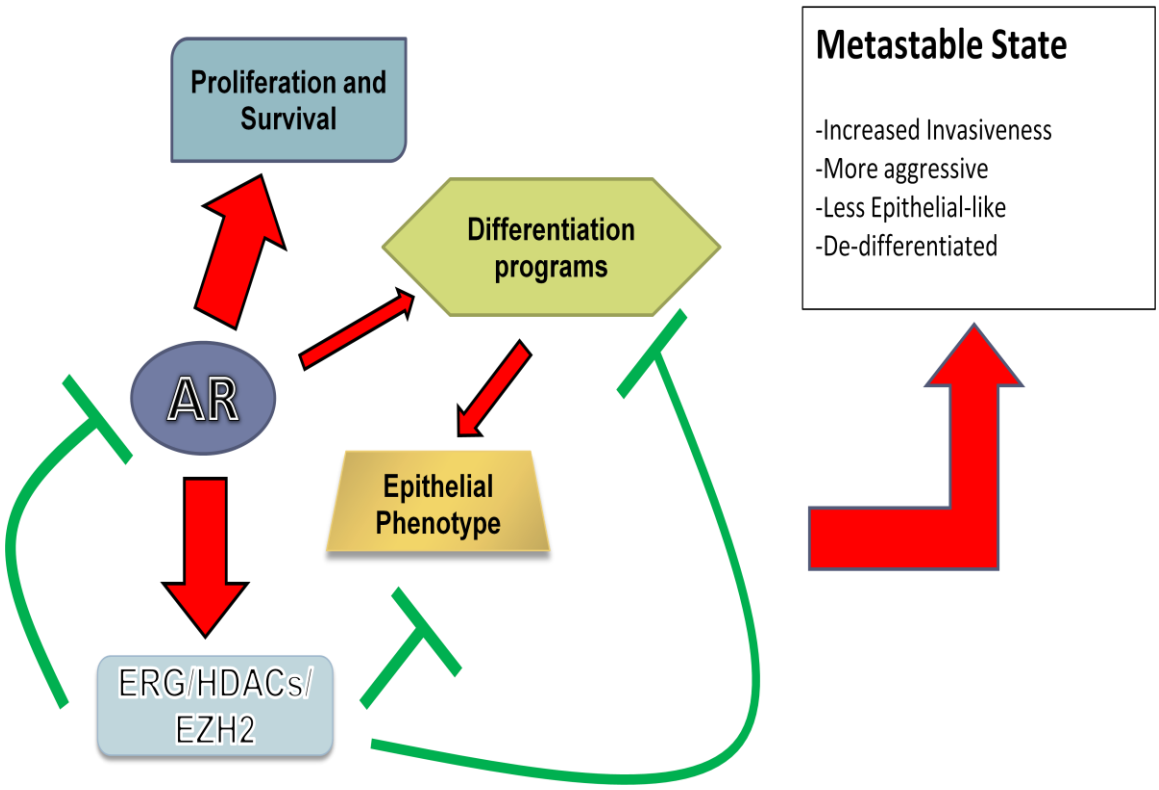


Figure 4.1 A Working Model for Prostate Cancer Development and Progression

Chapter 5: Future Directions

Thus far, our work had shed several interesting and novel insights on the AR-centric transcriptional network regulating prostate cancer progression, especially in relation to the fusion gene transcription factor, ERG, and the oncogenic transcription co-repressors, HDACs1-3 and EZH2. However, these findings are still vastly inadequate for a thorough understanding of AR, ERG, HDACs1-3 and EZH2 cross-talk. Future efforts should be directed to several areas.

5.1 Determining the Transcriptional Mechanisms and the Specificity Underlying the AR-ERG-HDACs-EZH2 transcriptional Cross-Talk

Notwithstanding the fact that our data have provided some evidence for the involvement of ERG, HDACs and EZH2 for regulated suppression of androgen signaling, the mechanistic details and the specificity of their actions is still very much unclear.

Although HDACs/EZH2 binding to ARBS was established in our study to be somewhat androgen responsive, understanding of the factors regulating their recruitment remained incomplete. For instance, it is not known whether HDACs/EZH2 is recruited to the chromatin in response to AR and/or ERG chromatin occupancy. AR/ERG Knock-down HDACs/EZH2 ChIP experiments could be performed to better address this query. However, it will be a technically challenging feat if AR/ERG silencing leads to changes in the expression of HDACs/EZH2. Even though our current data have established a chromatin co-localization and a direct endogenous interaction between HDACs/EZH2 with AR and ERG, the functionality of these factors as possible multi-protein complexes

ought to be resolved with further evidence from protein complex purifications and re-ChIP experiments.

To exploit this crosstalk as a possible therapeutic target, the transcriptional mechanisms undertaken by these co-factors in attenuating AR-mediated transcription ought to be thoroughly addressed. For example, it would be important to determine the exact location of the endogenous interactions between the transcription factors and the essentiality of the interactions on ERG/HDACs/EZH2 suppression of AR-mediated transcription. It would also be interesting to assess if the influence of HDACs/EZH2 recruitment on the acetylation and/or methylation status of histones and/or other transcriptional collaborators. This is especially so for EZH2 as a recent study had proposed a SET-independent function of EZH2 in regulating transcription (Lee et al., 2011) .

5.2 Unraveling the 3 Dimensional Transcriptional Interactome of the AR-ERG Cross-Talk

The genome is not a 2-dimensional system. Data in this study have revealed a complex network of multiple promoters and enhancers. The enhancers are found to be interspersed throughout the genome with no obvious patterns relative to the regulated genes. To function, the enhancers had to communicate with each other and with specific promoters though the spatial nuclear space via chromatin looping. Consequently, analysis of the 3 dimensional transcriptional interactome would be essential for comprehending the AR-ERG crosstalk. This kind of information would be helpful to the validation and

characterization of the potential communication between AR enhancers with ERG-bound/unbound promoters.

Recently, AR-mediated chromosomal looping was attributed as a facilitator for the formation of certain chromosomal rearrangements in prostate cancers. Specifically, studies have shown that TMPRSS2-ERG gene fusions could be made to form de-novo in TMPRSS2-ERG fusion negative LNCaP cells that were stimulated by androgens and exposed to genotoxic stress, facilitated by AR-mediated chromatin interactions between regions of the TMPRSS2 loci and the ERG loci (Lin et al., 2009; Mani et al., 2009). Of high interest, research on clinical prostate cancer samples has suggested chromosomal rearrangements to be associated with ETS-fusion positive status (Pflueger et al., 2011) and AR and ERG binding sites to be enriched with chromosomal DNA breakpoints (Berger et al., 2011). These findings imply the involvement AR and ERG in mediating chromosomal rearrangements through chromosomal looping.

Analysis of the 3 dimensional transcriptional interactome would be crucial to understanding the role of AR and ERG in mediating chromosomal interactions. Newly developed genomic technology such as ChIA-PET (Fullwood et al., 2009) and Hi-C (Lieberman-Aiden et al., 2009) would be most apt for these studies. However, to date, no such studies have been carried out in prostate cancer cells. To address this gap of knowledge, we have actually generated an AR ChIA-PET library in VCaP cells and in the process of doing so for an ERG ChIA-PET. We believe that the analysis of these maps will provide unprecedented information that could help resolve the role of AR and ERG in mediating chromosomal interactions.

5.3 Delving Deeper into the Downstream Functional Consequences of the AR-ERG-HDACs-EZH2 Transcriptional Crosstalk

In this study, we have tried to address the potential therapeutic implications of the AR-ERG-HDACs-EZH2 transcriptional crosstalk through investigating the downstream functional consequences of this transcriptional network. From this, we could also establish potential therapeutic targets and/or biomarkers targeting this cross-talk. In that regard, we have identified VCL as a novel AR-induced downstream functional target that is suppressed by ERG, HDACs and EZH2 to facilitate prostate cancer metastasis. This is a substantial advancement compared to past studies which only identified differentiation markers with no known functional influence in prostate cancer progression (Chen and Sawyers, 2010). However, this is still insufficient. Our analysis had revealed the AR-ERG-HDACs-EZH2 crosstalk as an extensive genome-wide transcription network. Consequently, it would be almost a certainty that VCL is not the only gene regulated by this crosstalk. Future efforts should be aimed at elucidating the whole regulated transcriptome and identifying other regulated genes that exhibit functional relevance in driving prostate cancer progression.

5.4 Bringing Clinical Relevance onto the AR-ERG-HDACs-EZH2 Transcriptional Cross-Talk

Since our main goal is to formulate clinical therapies for prostate cancer, it is an eventuality that we need to validate our findings under clinical settings. ChIP-Seq assays of AR, ERG, HDACs and EZH2 should also be carried out in a panel of patients' prostate

tumor samples to validate the existence of the transcriptional network clinically. Although this has already been done in prostate tumors for AR and ERG (Yu et al., 2010b), we believe that it will be a technical challenge with transcriptional co-repressors such as HDACs and EZH2 which usually does not bind to DNA directly. Transcriptome profiling of the prostate tumor samples should also be carried out as complementation to the elucidated cistromic profiles. These data should be integrated and correlated to the clinical parameters such as cancer stage, subtype and prognosis to derive possible clinical significance. Immunostaining for the presence of the downstream targets (i.e. VCL) of the crosstalk and evaluating their correlation with clinical parameters (i.e. cancer subtype) of the prostate tumors would establish much support and validity for the clinical relevance of the downstream targets in driving prostate cancer progression.

Chapter 6: Conclusion Remarks

A comprehensive understanding of the AR transcriptional network in prostate cancers would provide valuable information on prostate cancer initiation, development and maintenance, critical to the advancement of prostate cancer diagnostics and therapeutics. In addition, research into the AR transcriptional network could yield insights on nuclear receptor biology and possibly provide the basis for understanding hormone response and transcriptional regulatory mechanisms in general. Research so far has allowed us to progress from the primitive understanding of AR as a transcription factor that recruits polII to up-regulate transcription. Currently, we are beginning to understand that the AR transcriptional network is not a rigid, two-dimensional system that could produce only an all-or-none output. Rather, the AR transcriptional circuitry is a system that exhibits high plasticity and complexity, both spatially and temporally, with the degree of the output determined through specific interactions with a diversity of transcriptional co-factors, both co-activators and co-repressors. With the inherent plasticity, only then the AR network is able to evolve and adapt under different cellular environments and context to meet the needs of prostate cancer progression. To be able to formulate strategies to target effectively this "plasticity" therapeutically, it would be important for us to comprehend the intricate complexities of the transcriptional cross-talks between AR and other important transcription collaborators. For instance, such knowledge would also be useful in establishing predictive models for the AR transcriptional output during therapy. In this work, we have provided some preliminary insights into the cross-talk of several transcription factors that are commonly overexpressed in prostate cancers, namely, ERG,

HDACs1-3 and EZH2, with AR. Although our findings here is not fully comprehensive, we believed that our study herein has provided a strong basis and a good resource for future work that aim to further delineate the regulatory role of ERG, HDACs1-3 and EZH2 in the AR transcriptional network for prostate cancer advancement.

Appendix I

List of Fosmid Probes

(Mani et al., 2009)

For Detecting Gene Rearrangement within the ERG locus

1st Probe for ERG loci: RP11-476D17

2nd Probe for ERG loci: RP11-95I21

For Detecting Fusion between TMPRSS2 and ERG

Probe for ERG loci: RP11-476D17

Probe for TMPRSS2 loci: RP11-35C4

Appendix II

List of qPCR Primers

ChIP-qPCR Primers	Sequence
PLA1A_ChIP_F	AGTGGGAGAGGTGCAGGAAA
PLA1A_ChIP_R	TGAAACACACTGTCCCTCTTTGA
FKBP5_ChIP_F	CTTCACGCCTGTGTGCTTTTAT
FKBP5_ChIP_R	AGGGTGCAGGACGTTCCA
PSA_ChIP_F	TGGGACAACTTGCAAACCTG
PSA_ChIP_R	CCAGAGTAGGTCTGTTTTCAA
c36_ChIP_F	AACAGGCATTATTGTCTTTGAAAAAG
c36_ChIP_R	TCTCATTCTGTGGCTGTGTACTCTCT
CTRL1_ChIP_F (AR/ERG ChIP)	CCTGGAGGGCTTGGAGAT
CTRL1_ChIP_R (AR/ERG ChIP)	ATCCTACGGCTGGCTGTGA
CTRL2_ChIP_F (HDACs/EZH2 ChIP)	GTTTTCCATCTTTTCCAGTTGTCTATAA
CTRL2_ChIP_R (HDACs/EZH2 ChIP)	CATATGGCCTGTGAAGCTTTCA
1_F:	AAGCGTAGGAAACAGCCAGTCT
1_R:	GGTCACAGCAGTGGCCTATTTAC
2_F:	TGTTGAGCAGCCGGAAGAG
2_R:	GGGAGTCCTACCATCTCCTCACT
3_F:	GACCTGGTCGTTTGGATGGA
3_R:	CTCTCTGCCTTTCCTCTCGAATAT
4_F:	CCTTTGGAGTCCTGTCTGTTCTC
4_R:	TGGGAAGTGGTTGGAACACA
5_F:	CGCCGCATCCTTGCA
5_R:	CCCTCGTTTTCAGAGCCAACT
6_F:	TGTGCCTCCTGCTGTGATGT
6_R:	TTTGGCAAGAACACCACAGAAG
7_F:	GGCACAGGAAAAAGCAGTAGTGT
7_R:	AGTGGCACGGGAGAAGTAGGT
8_F:	GTTTTCCTTTCCTGAGATATCATGTG
8_R:	TGTCCCCACGTGTTTTCAAA
9_F:	CTGAGATAAAGAGGAAATGTCTGGAA
9_R:	GCACGGAGCACAAGCATTG
10_F:	ACATGGGAACGAAGTGTCTTCA
10_R:	GCTATTGTGCCTGGGCTGAT
11_F:	CCCTTGTCCTCTGGACTTCTAAGT
11_R:	ACGGGTATTTCAGAGATTGTTTCTG
12_F:	CTGTCTGCCAGGATCTCTGTGT
12_R:	GCTGCTGATGTGCCAGTGAT
13_F:	TGTGCCACTGCATGTGTTCTT
13_R:	CAGGGAAAACCAACAGAGTTAGGA
14_F:	CTACGATGACAACAAATCTCAACTGA
14_R:	TTTGCCTGTGTTGATTGTTCTGT
15_F:	GGACAGCAGGAGGCACAGA
15_R:	TTCCAGATGCCTGCACTTTG
vcl3_F:	CAGGGTTGGAACAGCATGTATTAA
vcl3_R:	CAAGTATGCAGCACCAACTCACA
klk2_F	GTTGAAAGCAGACCTACTCTGGA
klk2_R	CTGGACCATCTTTTCAAGCAT

Appendix III

List of cDNA Primers

cDNA RT Primers	Sequence
ERG_Forward	CGCAGAGTTATCGTGCCAGCAGAT
ERG_Reverse	CCATATTCTTTCACCGCCCACTCC
AR_Forward	GTGTCACTATGGAGCTCTCACATGT
AR_Reverse	GTTTCCCTTCAGCGGCTCTT
PSA_Forward	TGTGTGCTGGACGCTGGA
PSA_Reverse	CACTGCCCCATGACGTGAT
FKBP5_Forward	GGCTGGCAGTCTCCCTAAAA
FKBP5_Reverse	ATCAAGGAGCTCAATCTCAAAAAAG
KRT8_Forward	CAGGCAGCTATATGAAGAGGAGATC
KRT8_Reverse	ATGGACAGCACCACAGATGTG
KRT18_Forward	GCGAGGACTTTAATCTTGGTGATG
KRT18_Reverse	TGGTCTTTTGGATGGTTTGCA
VCL_Forward	CCTCGTCCGGGTTGGAA
VCL_Reverse	TAAATGCTGGTGGCATATCTCTCT
GAPDH_Forward	GGCCTCCAAGGAGTAAGACC
GAPDH_Reverse	AGGGGAGATTCAGTGTGGTG

My Publications During The Course Of PhD And On Which This Thesis Was Derived From

1) **Chng, K.R.**, Chang, C.W., Tan, S.K., Yang, C., Hong, S.Z., Sng, N.Y., and Cheung, E. (2012). A transcriptional repressor co-regulatory network governing androgen response in prostate cancers. EMBO J 31, 2810-2823.

2) **Kern Rei Chng**, Shin Chet Chuah and Edwin Cheung. 2012. Genomics of Prostate Cancer. In Stem Cells and Human Diseases. Edited by Rakesh Srivastava and Sharmila Shankar. Springer Netherlands. 175-196

3) **Chng, K.R.** and Cheung, E. (2012). Sequencing the Transcriptional Network of Androgen Receptor in Prostate Cancer. Cancer Lett. (Accepted)

4) Tan, P.Y., Chang, C.W., **Chng, K.R.**, Wansa, K.D., Sung, W.K., and Cheung, E. (2012). Integration of Regulatory Networks by NKX3-1 Promotes Androgen-Dependent Prostate Cancer Survival. Mol Cell Biol 32, 399-414.

5) *Tan, S. K., *Lin, Z. H., Chang, C. W., Varang, V., **Chng, K. R.**, Yong, E. L., Sung, W. K., and Cheung, E. (2011) "AP-2γ Regulates Oestrogen Receptor-Mediated Long-Range Chromatin Interaction and Gene Transcription" The EMBO Journal 30 (13), 2569-81. *Equal contributions

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