Investigating the role of a somatically acquired H3K27ac gain in aggressive prostate cancer

M.Sc. thesis presented by

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ABSTRACT

Prostate cancer is a complex malignancy that affects millions of men worldwide. Several epigenetic mechanisms critical for chromatin remodeling and accessibility are crucially altered in prostate cancer progression. As such, epigenetic alterations represent a driving mechanism of advanced prostate cancer. The epigenetic changes underlying prostate carcinogenesis include the silencing of tumor suppressor genes by promoter hypermethylation, deregulation of histone modulating enzymes, and global DNA hypomethylation. Understanding how epigenetic deregulation contributes to prostate cancer onset and progression may improve risk stratification and treatment selection for prostate cancer patients. In addition, a somatically acquired enhancer has been recently described being a noncoding driver of advanced prostate cancer. This finding emphasizes the emerging role of regulatory elements as noncoding oncogenic drivers in prostate cancer. Thus, our goal was to identify and characterize somatically acquired regulatory elements implicated in aggressive prostate cancer. Here, by analyzing publicly available epigenetic data generated in aggressive prostate tumors and indolent specimens, we identify a somatically acquired regulatory element, demarcated with H3K27ac, in the *ANKRD30A* gene in aggressive prostate cancers. We show that *ANKRD30A* is significantly more expressed in a subset of prostate cancers compared to benign prostatic hyperplasia. We also observe that *ANKRD30A* expression is associated with accelerated disease recurrence. Consistently with a role in metastatic progression, somatic H3K27ac gain at *ANKRD30A* locus is identified in ~36% of AR^+ castration-resistant prostate cancer (mCRPC) patient derived xenografts (PDXs) models established from prostate cancer metastases and correlates with transcript expression. Moreover, we reveal that *ANKRD30A* transcript expression is amplified in LAPC4 and 22Rv1 prostate cancer cell lines. We defined the H3K27ac profile at the *ANKRD30A* locus in multiple prostate cancer cell lines. *In vitro*, we show that inducible expression of *ANKRD30A* increases metastatic prostate cancer cell proliferation. Collectively, our observations suggest an *ANKRD30A*-associated regulatory element epigenetically activated in aggressive prostate cancers and this finding might help stratify men who would benefit from more aggressive treatment modalities.

RÉSUMÉ

Le cancer de la prostate est une affection très complexe qui affecte des millions d'hommes dans le monde. Plusieurs mécanismes épigénétiques qui jouent un rôle clé dans le remodelage de la chromatine et l'accessibilité à l'ADN sont considérablement dérégulés lors de l'initiation et la progression du cancer de la prostate. Ces changements épigénétiques comprennent la perte de fonction de gènes suppresseurs de tumeurs par l'hyperméthylation de promoteurs, la dérégulation des enzymes de modulation des histones et l'hypométhylation globale de l'ADN. Comprendre comment la dérégulation épigénétique contribue à l'apparition et à la progression du cancer de la prostate peut améliorer la stratification du risque et la sélection du traitement pour les patients atteints d'un cancer de la prostate. De plus, un amplificateur somatiquement acquis a été récemment décrit comme un facteur non codant du cancer avancé de la prostate. Cette découverte souligne le rôle émergent des éléments régulateurs en tant qu'oncogènes non codants dans le cancer de la prostate. L'objectif de notre étude était d'identifier et de caractériser des éléments régulateurs acquis somatiquement impliqués dans le cancer agressif de la prostate. En analysant des données épigénétiques provenant de tumeurs agressives de la prostate et de spécimens indolents, nous avons identifié un élément régulateur somatiquement acquis, marqué par H3K27ac, au niveau du gène *ANKRD30A* dans les cancers de la prostate agressifs. Nous montrons que *ANKRD30A* est significativement plus exprimé dans le cancer de la prostate par rapport à l'hyperplasie bénigne de la prostate. Nous observons également que l'expression d'*ANKRD30A* est associée à une récurrence accélérée de la maladie. Conformément à son rôle dans la progression métastatique, le gain somatique de H3K27ac au locus *ANKRD30A* est observé dans environ 36 % des modèles AR^+ de xénogreffes dérivées de métastases de patients atteints d'un cancer de la prostate résistant à la castration. Ce gain est aussi corrélé avec l'expression du transcrit. De plus, nous révélons que l'expression du gène *ANKRD30A* est amplifiée dans les lignées cellulaires de cancer de la prostate LAPC4 et 22Rv1. Nous définissons le profil H3K27ac au locus *ANKRD30A* dans plusieurs lignées cellulaires du cancer de la prostate. *In vitro*, nous montrons que l'expression d'*ANKRD30A* augmente la prolifération des PC-3, soit des cellules du cancer de la prostate métastatique. Collectivement, nos observations suggèrent un élément régulateur associé à *ANKRD30A* activé épigénétiquement dans des cancers de la prostate agressifs et ceci pourrait aider à stratifier les hommes qui bénéficieraient de traitements plus agressifs.

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LIST OF ABBREVIATIONS

ADN	acide désoxyribonucléique
AJCC	American joint committee on cancer
AKT	ak strain transforming
ANKDR30A	ankyrin repeat domain 30A
AR	androgen receptor
ARE	androgen receptor element
ATAC-seq	Assay for Transposase-Accessible Chromatin using sequencing
ATAD2	ATPase family AAA domain containing 2
ATCC	American type culture collection
BCR	biochemical recurrence
BET	bromodomain and extraterminal
BMPR1B	bone morphogenetic protein receptor type 1B
BPE	bovine pituitary extract
BPH	benign prostatic hyperplasia
BRD4	bromodomain-containing protein 4
CHD1	chromodomain helicase DNA binding protein 1
ChIP-seq	chromatin immunoprecipitation using sequencing
CIHR	Canadian institute of health research
CO_2	carbon dioxyde
CPRC	castration resistant prostate cancer
CRE	cis regulatory element
CRISPR	clustered regularly interspaced short palindromic repeats
DHT	dihydrotestosterone
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
DNMT1	DNA methyltransferase 1
DNMT3A	DNA methyltransferase 3A
DNMT3B	DNA methyltransferase 3B

DRE	digital rectal exam
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EMT	epithelial to mesenchymal transition
EP300	E1A Binding Protein P300
ERG	ETS-related gene
ERSPC	European randomized study of screening for prostate cancer
ETS	E26 transformation-specific
ETV1	ETS Variant Transcription Factor 1
ETV4	ETS Variant Transcription Factor 4
EV	empty vector
EZH2	Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit
FBS	fetal bovine serum
FDA	food and drug administration
FOXA1	forkhead box a1
FPKM	fragments per kilobase of transcript per million
G418	geneticin
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GATA2	GATA-binding factor 2
GATK	genome analysis toolkit
GEO	European Genome-phenome Archive
GOI	gene of interest
GSTP1	glutathione S-transferase pi gene
H2A	histone H2A
H2B	Histone H2B
H3	Histone H3
H3K27	histone H3 at lysine 27
H3K18	histone H3 at lysine 18
H3K4	histone H3 at lysine 4

H3K9	histone H3 at lysine 9
H3T11	histone H3 at threonine 11
H4	histone H4
H4K20	histone H4 lysine 20
H4R3	histone H4 arginine 3
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDAC2	histone deacetylase 2
HDM	histone demethylase
HMT	histone methyltransferase
HOX	homeobox
HOXB13	homeobox B13
HS	high sensitivity
IP	immunoprecipitation
KAT3B	lysine acetyltransferases p300
KAT5	lysine acetyltransferases 5
KAT8	lysine acetyltransferases 8
KLK3	lallikrein related peptidase 3
KRAB	Krüppel-associated box
LSD1	lysine-specific demethylase 1A
NAD	nicotinamide adenine dinucleotide
NEB	new england biolabs
NKX3-1	NK3 homeobox 1
PBS	phoshate buffered saline
PCR	polymerase chain reaction
PDX	patient-derived xenograft
PIN	prostatic intraepithelial neoplasia
PKN1	proteine kinase 1
PLCO	prostate, lung, colorectal, ovarian
PRC1	protein regulator of cytokinesis 1
PRC2	protein regulator of cytokinesis 2

PRMT5	protein arginine methyltransferase 5
PSA	prostate serum antigen
PTEN	phosphate nd tensin homolog
P_{TRE3G}	*TRE3G* promoter
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute Medium
RT	reverse transcriptase
SDS	sodium dodecyl sulfate
SMYD3	SET and MYND domain containing 3
SNP	single nucleotide polymorphism
SPOP	speckle type BTB/POZ protein
TAF1	TATA-box binding protein associated factor 1
TAL1	TAL BHLH transcription factor 1
TBST	tris-buffered saline and polysorbate 20
TCGA	the cancer genome atlas
TET	ten-eleven translocation
TET1	ten-eleven translocation 1
TET2	ten-eleven translocation 2
TET3	ten-eleven translocation 3
TF	transcriptional factor
TGF	transforming growth factor
TMA	tissue microarrays
TP63	tumor protein p63
TRIM24	tripartite motif containing 24
TRUS	transrectal ultrasound
TSS	transcriptional start site
UBE2C	ubiquitin conjugating enzyme E2 C
VEL	variant enhancer loci
WDR5	WD repeat domain 5
WHSC1	Wolf-Hirschhorn syndrome candidate 1

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CONTRIBUTION OF AUTHORS

This thesis follows a manuscript-based structure. I wrote the first, third and fourth chapters under the direction of David P. Labbé.

The presented work in the second chapter is the result of the collaboration between the Labbé laboratory (Janie Larocque, M.Sc. candidate, Nadia Boufaied, research associate, and David P. Labbé, principal investigator) and the Bailey laboratory (Swneke Bailey, principal investigator). This manuscript was primarily written by me and proofed by David P. Labbé. Co-authors wrote the methodology for the sections that they contributed to. Reanalysis of the Stelloo *et al*. ChIP-seq dataset was conducted by Swneke Bailey. Analysis of ChIP-seq of LuCaP patient-derived xenografts was performed by Nadia Boufaied using data provided by Eva Corey. RNA isolation for sequencing, ChIP-qPCR, cloning, and proliferation assays were designed, optimized, performed, and analyzed by me with the help of Nadia Boufaied and David P. Labbé. Validation datasets were selected by Nadia Boufaied and David P. Labbé and analyzed by Nadia Boufaied. Western blotting and quantitative polymerase chain reaction (qPCR) were conducted and analyzed by me.

Figures 1-1, 1-2 and 1-3 were adapted by me from the literature. Figures 1-4 and 2-1 were prepared by Swneke Bailey. Figures 2-2, 2-3, 2-4, 2-5 and Supplementary Data 1-B were prepared by Nadia Boufaied. Figures 2-6, 2-7, S1, S2, S3, S4, S5 and Supplementary Data 1-A were prepared by me.

Chapter 1: Research Background

1.1 The prostate

The prostate is part of the reproductive and urinary systems of a male. As shown in **Figure 1-1**, it sits anterior to the rectum and surrounds the urethra just at the base of the bladder. Enclosed by a fibrous capsule, the prostate is made up of 20 to 30 compound tubuloalveolar glands embedded in a stroma of circular smooth muscle fibers and collagenous tissue [1]. The prostate is usually the size of a walnut in young men but can change over time and grow larger in older men. This process, referred as benign hyperplastic hyperplasia (BPH), involves benign overgrowth of the glands surrounding the prostatic urethra. As the tissue increases in size, it compresses the urethra and can produce symptoms of bladder outlet obstruction [2]. The prostate is primarily a reproductive organ. In conjunction with the seminal vesicles, the prostate produces the fluid that supports the sperm. During ejaculation, prostatic smooth muscle contracts, squeezing this fluid into the prostatic urethra via several ducts. The prostatic secretion plays a role in activating the sperm and accounts for up to one-third of the semen volume. It is a milky, slightly acidic fluid that contains citrate (a nutrient source), several enzymes, and prostate-specific antigen (PSA) [1].

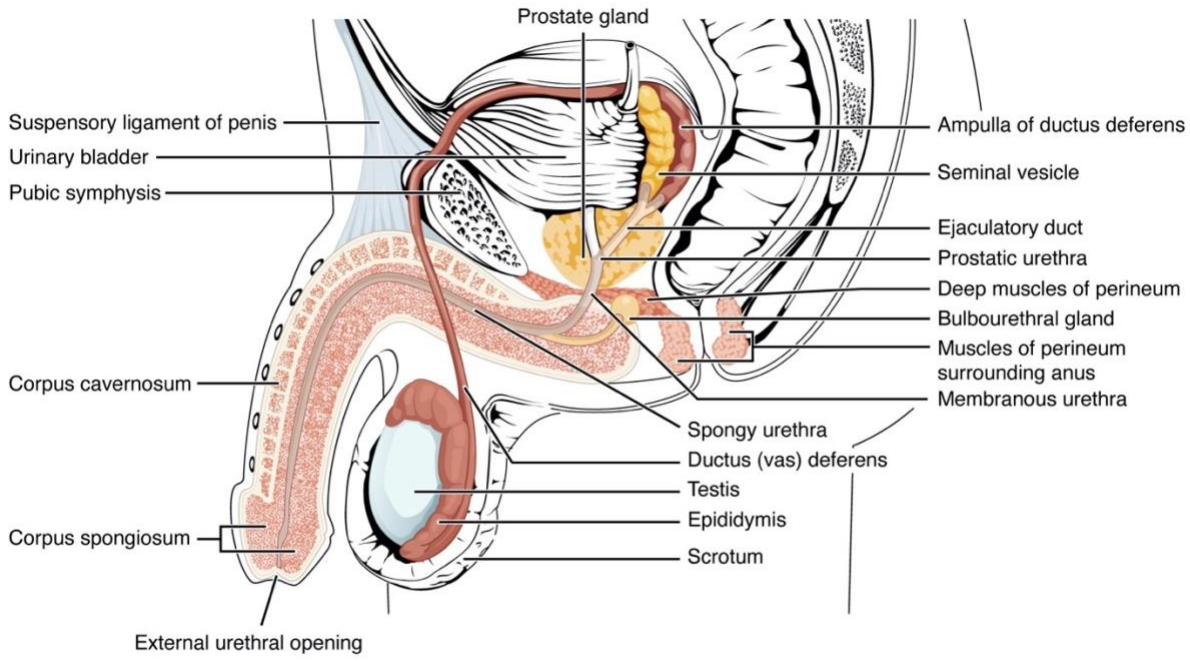

Figure 1- 1. Male reproductive system. Lateral view of the structures of the male reproductive system. Adapted from Figure 27.2, Anatomy and Physiology. Textbook content produced by

The prostate is the male organ most commonly afflicted with either benign or malignant neoplasms. Benign overgrowth of the gland and prostate cancer are very common, especially in in older men. McNeal *et al.* (1988) defined a concept of zonal anatomy of the prostate. They described three distinct zones of the glandular prostate: the peripheral zone, the central zone, and the transition zone. These anatomic zones vary in size and have distinct ductal systems that are differentially afflicted with neoplastic processes [3]. The peripheral zone is close to the rectal wall and accounts for 70% of the volume of the young adult prostate. During a digital rectal examination (DRE), the physician assesses the posterior aspect of the peripheral zone, which is important because 60-70% of carcinomas of the prostate originate in the peripheral zone. The central zone is the area that surrounds the ejaculatory ducts and accounts for 25% of the volume of the prostate. Only 5-10% of prostate cancers begin in the central zone, but they are believed to be more aggressive and more likely to invade the seminal vesicles. The transition zone encircles the urethra. It is small in young adults; accounting for 5% of the volume of the prostate, but it grows throughout life. This enlargement of the prostatic gland that occurs with aging is called benign prostatic hyperplasia (BPH) and can cause urinary problems. Roughly 10-20% of prostate cancers originate in the transition zone [3, 4].

1.2 Prostate cancer

1.2.1 Epidemiology

Prostate cancer is a complex malignancy of the prostate that affects millions of men worldwide, mainly in developed countries [5]. Other than skin-cancers, prostate cancer is now recognized as the most prevalent type of cancer in men. It ranks as the third leading cause of cancer death among Canadian men. In 2021, the Canadian Cancer Society estimated that 24,000 new cases of prostate cancer would be diagnosed, accounting for about 20% of all new male cancer cases, and that 4,500 men would die of the disease. Currently, 1 in 8 Canadian men will develop prostate cancer during their lifetime, and 1 in 29 will die from the disease [6]. In Canada, between 1979 and 1990, the incidence of prostate cancer increased steadily every year at an average rate of about 3% [7]. Since then, incidence rates have changed greatly. They increased rapidly from 1990 to 1993 (12.7%),

declined steeply from 1993 to 1995 (8.4%), increased again steadily between 1995 and 2007, then declined sharply from 2007 to 2017 (4.4%) [6]. The incidence rate peaked in 1993 at 140.5 incident cases per 100,000 men and again in 2001 at 132.4 per 100,000 men [8, 9]. These peaks largely reflect the increase in early detection of prostate cancer following the introduction of the prostate-specific antigen (PSA) blood test. The first peak, in 1993, follows the introduction of PSA as a screening tool, and the second peak, in 2001, is best explained by the publicity around diagnosis of early prostate cancer using serial PSA testing [9]. The trends in mortality have a different pattern. In fact, mortality has declined more in later years than the incidence. In Canada, the age-standardized mortality rates of prostate cancer increased gradually, from 1977 to 1993, then decreased significantly ever since 1995. The mortality rate peaked in 1995 at 45.1 deaths per 100,000 men but is estimated by the Canadian Cancer Society to be 22.7 per 100 000 for 2021, a 50% mortality decline in 26 years despite a significant increase in lifetime expectancy of men during the same period [8, 9]. This trend is likely due to the earlier diagnosis and improved treatment of prostate cancer. Unfortunately, it comes at the expense of an increased incidence of men being diagnosed with prostate cancer of whom a significant proportion are at little risk of dying of their cancer [10]. Indeed, an increasing number of men are diagnosed with prostate cancer due to increasing life expectancy and the increased use of PSA screening. However, most prostate cancers have an indolent course and men with prostate cancer are more likely to die from other conditions, such as heart disease, rather than from their cancer [11]. Thus, many prostate cancers are indolent and inconsequential to the patient while others are aggressive and lethal if detected too late or left untreated. This broad spectrum of biological activity can make decision making for individual patients difficult and highlights the critical need for distinguishing indolent from aggressive prostate cancers, which will be discussed in further detail later.

1.2.2 Risk factors

1.2.2.1 Age

Prostate cancer is a disease of older men. Age is a well-known risk factor for prostate cancer. Although premalignant lesions can be detected in men in their 30s and 40s, prostate cancer rarely occurs in men younger than 40 years, and its incidence increases with age [9, 12] . The rate of prostate cancer diagnosis in Canada is approximately 100 per 100,000 men aged 50 to 54, 500 per

100,000 men aged 60 to 64 and greater than 700 per 100,000 men over the age of 80. In fact, the incidence of prostate cancer increases faster with age than that of any other major cancer [9].

1.2.2.2 Family history

Since the first report of familial aggregation of prostate cancer in 1956, several epidemiologic studies have shown that the risk of developing prostate cancer in a man with one affected first-degree relative is 2-3 times higher compared with a man with no family history of prostate cancer. If a man has a positive family history of prostate cancer, the relative risk increases according to the number of affected family members, their degree of relatedness, and the age at which they were affected [13, 14]. It has also been noted that men whose fathers or brothers had prostate cancer are typically diagnosed 6 to 7 years earlier than men without a similar history. [15]. Men whose brother had prostate cancer before the age of 60 have a 25% chance of developing the disease, compared with 8% for men with no family history prostate cancer. The risk is slightly lower at 20% for men whose father had prostate cancer before 60 years. Furthermore, men who have 3 or more relatives with prostate cancer have a 35% to 45% risk of developing prostate cancer [16].

1.2.2.3 Genetics

Prostate cancer is a genetically heterogenous disease. More than 40% of prostate cancer cases diagnosed in men before the age of 55 years may be due to heredity. Dominantly inherited susceptibility genes with either high penetrance or low penetrance are likely to be involved [16]. These genetic factors are also predicted to cause 5% to 10% of cases of prostate cancer [14]. Genome-wide scans have identified five single nucleotide polymorphisms (SNPs) associated with prostate cancer in various loci, especially at 8q24, 17q12 and 12q24. The cumulative effect of these genetic variants was shown to increase prostate cancer risk [17, 18]. In addition, specific susceptibility genes to prostate cancer include mutations of *BRAC2* and *HOXB13*, which are commonly observed in patients with early onset disease [19].

Although non-hereditary, epigenetic alterations also influence prostate cancer related genes. Epigenetic silencing of *GSTP1* through methylation occurs in approximately 70% of prostatic intraepithelial neoplasia (PIN), which is a neoplastic growth of epithelial cells in benign prostatic

acini or ducts, and virtually in all cases of prostate cancer, making it the most common epigenetic alteration in this malignancy [20, 21].

1.2.2.4 Ethnicity

African-American men have the highest reported incidence of prostate cancer in the world [22]. Although African Americans have experienced a greater decline in mortality than white men since the early 1990s, their death rates remain more than 2.4 times higher than whites and they are more likely to be diagnosed at an advanced stage [22, 23]. It seems likely that the source of the disparity is multifactorial [24]. Many biological, environmental, and social hypotheses have been advanced to explain these differences. African American men have a higher intake of dietary fat than Caucasians and Asians, and this could contribute to their higher risk [25]. Access to proper health care and other socioeconomic factors may place African Americans at a higher risk for poorer disease outcomes than white men but does not explain fully the higher incidence rate [26].

1.2.2.5 Diet

In a review of 33 published case-control studies that examined the association between prostate cancer and dietary fat, eight studies reported that increased dietary fat intake is significantly associated with prostate cancer risk [22]. Others have shown that the risk of prostate cancer progression to an advanced stage is greater in men with a high-saturated fat diet [23]. Moreover, Labbé *et al.* demonstrated that saturated fat intake in a murine prostate cancer model contributes to prostate cancer lethality [24]. Although the molecular underpinnings of the association between obesity and prostate cancer incidence remain elusive, obesity has been consistently associated with an increased risk of prostate cancer aggressiveness and mortality [25]. In addition, studies have found several micronutrients that appear to reduce the risk of prostate cancer, including soy (isoflavones), green tea and tomatoes (lycopene) [26, 27]. Initial report of a large intervention study from SELECT (the Selenium and Vitamin E Cancer Prevention Trial), showed that selenium and α-tocopherol (vitamin E) supplements, taken either alone or together for an average of five years, did not prevent prostate cancer but increased non significantly prostate cancer risk. However, extended follow-up of participants showed that dietary supplementation with vitamin E significantly increased the risk of prostate cancer in healthy men. This demonstrates the potential for seemingly healthy substances, such as vitamins, to cause harm, and underscores the need for

consumers to be skeptical of health claims for unregulated over-the-counter products in the absence of strong evidence from clinical trials [28]. In fact, the World Cancer Research Fund advise against taking supplements to prevent prostate cancer [29]. Collectively, this data highlights the emerging role of diet as an extrinsic risk factor that can increase or diminish the risk of disease progression.

1.2.3 Diagnosis

Much interest has focused on the search for effective measures to detect prostate cancer in its early and most easily manageable stages. The diagnosis of prostate cancer may involve several clinical scenarios. The disease can be detected on microscopic inspection of prostate tissue removed for the management of presumed BPH. Prostate cancer can also be diagnosed after digital rectal examination (DRE) by palpating the prostate through the anterior rectal wall of a patient. The diagnosis of prostate cancer can also be made by checking the PSA (prostate-specific antigen) levels. This simple blood test has been a major factor in the early detection of adenocarcinoma of the prostate [2]. PSA is a normal component of blood at levels below 2.5 ng/ml, but it is also a tumor marker that usually follows the clinical course of prostate cancer [1]. Typically, the diagnosis is based on an abnormal PSA blood test result, which usually leads to a transrectal ultrasound (TRUS) and biopsy of the prostate. Since most patients are now diagnosed at an early stage, there is little need for further staging tests. However, if there is concern for a more advanced cancer, then the patient can be evaluated with a bone scan or CT scan of the abdomen and pelvis [2].

Since the adoption of its widespread use, the PSA blood test has resulted in significant increases in the number of men who are diagnosed at both a younger age and at an early stage of the disease. Initial reports from two large, randomized trials assessing the effect of PSA screening on prostate cancer mortality yielded conflicting results [30, 31]. In the U.S. Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial, Andriole *et al*. reported no mortality benefit from combined screening with PSA testing and digital rectal examination (DRE) during a median follow-up of 7 years [30]. In contrast, the European Randomized Study of Screening for Prostate Cancer (ERSPC) trial reported a 20% relative reduction in the risk of death from prostate cancer at a median follow-up of 9 years for men who underwent PSA screening without DRE. The PLCO

trial has been criticized for its relatively short follow-up given prostate cancer's long natural history, and for the fact that more than half the men in the control arm had a PSA test done outside of the trial. This translates to an absolute reduction of about 7 prostate cancer deaths per 10,000 men screened. However, PSA-based screening was also associated with a high risk of overdiagnosis. According to ERSPC investigators, 1410 men would need to be screened and 48 additional cases of prostate cancer would need to be treated to prevent one death from prostate cancer [31]. In 2011, the U.S. Preventive Services Task Force recommended that healthy men should stop having routine PSA screening, arguing that the test showed little or no benefit for men with no symptoms of the disease and that it was needlessly exposing hundreds of thousands of men to common complications of surgical or radiation treatment, such as impotence and urinary incompetence, when their cancer was too slow growing to be fatal any time soon. In fact, PSA levels rise due to many factors, such as benign growth of the prostate, infection, and growth of a cancer. Thus, the PSA test does not really reveal that a man has prostate cancer, only that he might have prostate cancer [1]. This test should then be used in conjunction with other measures of disease detection for initial screening, such a DRE and ultrasonography [32].

Although most patients are diagnosed before becoming symptomatic, many have voiding symptoms because of BPH, which is common in the age group that is also at risk for prostate cancer. In the rare cases that a patient has symptoms from his cancer (hematuria, weight loss, malaise, anorexia, and back pain), it is often too late for a cure because the symptoms usually indicate an advanced stage of the disease [2].

1.2.4 Pathology

There are two main growth-related disorders of the prostate: benign prostatic hyperplasia (BPH), and prostate cancer. BPH is a very common disorder that affects both the epithelial and mesenchymal components of the prostate. An estimated 80% of men older than 60 years of age experience BPH. It is important to recognize that BPH and prostate cancer are not related entities. They are related only by their close anatomic site of origin and high incidence in older men. No study has conclusively demonstrated that BPH predisposes to the development of prostate cancer [33]. The clinical presentation of prostate cancer can range from localized indolent (prostate-confined) to a rapidly progressing lethal metastatic disease. In its initial stages, when confined to

the prostatic capsule, prostate cancer is generally curable by surgical intervention or radiation therapy. However, if not detected at an early stage, or in more aggressive forms of the disease, prostate cancer can progress to advance stages characterized by local invasion of the seminal vesicles, followed by metastasis primarily to the bone. This transition to metastatic disease is often followed by a shift from androgen dependence to androgen independence, which is provoked by androgen deprivation therapy. The disease is then considered castration-resistant and incurable [34]. More than 95% of prostate cancers are adenocarcinomas with abnormal proliferation of prostatic glandular structures [35].

1.2.4.1 Pathogenesis

Like other cancers, prostate cancer is caused by an accumulation of genetic alterations that drives cells to malignant growth. Although prostate cancer is a disease of older men, histologic evidence can be found in the prostates of healthy men in their 20s to 40s, but the diagnosis is typically made three to four decades later, which suggests that the development of the disease is a multistep process resulting from a variety of genetic and epigenetic alterations [35].

The key genetic alterations that occur during prostate carcinogenesis and progression are shown in **Figure 2**. Although it is a matter of some debate, PIN has been widely accepted as the main precursor lesion to prostate cancer. PIN refers to the proliferation of atypical or dysplastic prostate glandular epithelial cells within the confines of prostatic ducts and acini [36]. It precedes the development of cancer by 10 years or more. Prostate cancer progression generally follows an increasing burden of genomic aberrations. Early genomic events in tumor evolution include loss of *NKX3-1* (an AR-regulated gene involved in normal differentiation of prostate epithelium) and fusions of *TMPRSS2* with *ERG* (*ETS*-related gene), which are later followed by additional lesions that mediate progression. *TMPRSS2-ERG* is the most common gene fusion in localized prostate cancer and involves the 5′ untranslated region of the androgen-regulated *TMPRSS2* gene fused to the exon 4 of ERG (ETS-related gene), which leads to the overexpression of oncogenic transcription factors [37]. Furthermore, loss of *TP53* and *PTEN* as well as *MYC* amplification are frequent events prior to metastasis that appear to contribute to metastatic spread. In advanced disease, AR alterations (*i.e.*, *AR* amplification, *AR* activating mutations, constitutively active *AR*

variants) that promote resistance to androgen deprivation have been found to arise after metastases have developed [35, 38].

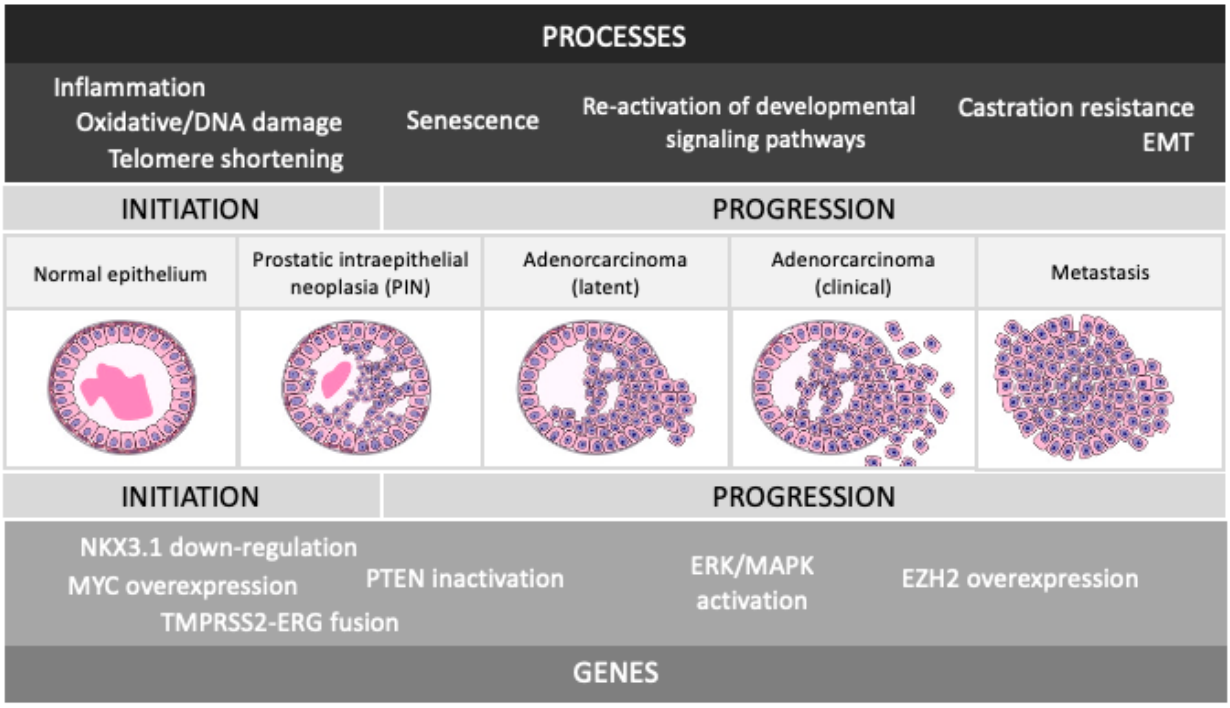

Figure 1- 2. Overview of prostate cancer pathogenesis. Stages of progression are shown along with key molecular processes and genetic alterations during each stage. Adapted from Shen et al. (2010) with the permission of the author.

1.2.5 Tumor grading and staging

The Gleason system is the grading system for prostate cancer. It describes the architecture and structure of tumor cells under a microscope and relies on the results of the prostate biopsy. Tumor cells usually fall into five distinct patterns as they change from normal cells to cancerous cells [39]. Cells of the more aggressive or poorly differentiated prostate cancers have more indistinct cell borders, larger nuclei, and loss of gland formation [40]. In the Gleason system, cells are graded on scale of 1 to 5. A grade of 1 corresponds with low-grade disease (variable-sized glands that infiltrate through normal stroma and normal glands), grades 2-3 correspond with intermediate grade disease (incompletely formed glands with variable amounts of fusion and more infiltrative growth pattern), and grades 4-5 correspond with high-grade disease (single infiltrating cells with no gland formation) [39, 40]. The pathologist looking at the biopsy sample assigns a primary grade

to the predominant pattern in the tumor and a secondary grade to the second most observed pattern. The Gleason score is obtained by adding the primary and secondary grades together. Theoretically, Gleason scores range from 2 to 10, but pathologists rarely assign scores of 2 to 5, and most Gleason scores assigned range from 6 to 10. Thus, a tumor with a Gleason score of 6 (3 + 3) is the lowest grade [39]. If the cancer has only one pattern present, then both the primary and secondary grade are reported as the same grade (*e.g.*, 4 + 4). The primary Gleason pattern is the most important determinant of biologic risk to differentiate between intermediate- and high-grade tumors. For example, among Gleason score 7 tumors, those assigned 4 + 3 are more aggressive than those read as 3 + 4 [40].

The most widely employed staging system for prostate cancer is the American Joint Committee on Cancer (AJCC) TNM staging system. It uses results of the digital rectal exam (DRE) and transrectal ultrasound (TRUS) to consider the size of the primary tumor (T stage), if the cancer has spread to nearby lymph **nodes (N** category) and if the cancer has **metastasized** to other parts of the body (**M** category). There are four stages to prostate cancer. In stage 1, the tumor is microscopic and intracapsular. Stage 2 involves a palpable tumor on rectal examination that is confined to the prostate. Stage 3 has a tumor that extended beyond the capsule of the prostate. In stage 4, the tumor has metastasized to distant organs (*e.g.*, bones, brain). T1 and T2 tumors account for most tumors diagnosed in contemporary practice. Tumor staging in prostate cancer is a relatively weak prognostic factor, compared with risk factors such as Gleason score and PSA levels, partly due to the subjectivity of DRE and TRUS interpretation [2, 40].

1.2.6 Treatments

Management of prostate cancer depends on several factors, including the stage of the tumor, as well as the age and health of the patient. The optimal form of therapy for all stages of prostate cancer remains a subject of great debate. For men with localized disease, treatment ranges from aggressive therapy to observation called “watchful waiting” or active surveillance. During active surveillance, patients who have been diagnosed with prostate cancer do not receive treatment but are closely monitored and begin treatment only when indicated by screening changes. Usually this includes regular PSA blood tests and DRE. Watchful waiting is a less invasive type of monitoring that requires fewer tests and relies more on the patient’s symptoms to decide if treatment is needed.

Because prostate cancer is often slow growing, many physicians recommend watchful waiting or active surveillance [41]. They may be an option for older men with asymptomatic stage 1 prostate cancer, while younger men may be candidates for the more aggressive approach, which includes surgery to remove the prostate and surrounding tissue (radical prostatectomy) and radiation therapy [2]. When indicated, prostate cancer can be treated surgically, alone or in conjunction with radiation. Radical prostatectomy has been shown to be superior to watchful waiting in reducing localized prostate cancer in a randomized trial performed in Sweden. In this largely unscreened population, the reduction in disease-specific mortality because of radical prostatectomy was greatest among patients younger than 65 years [42]. In some cases, lymph nodes in the pelvis are also removed. As for radiation therapy, this treatment uses high-energy rays or particles to destroy prostate cancer cells. It is another standard treatment option for localized prostate cancer, and it provides favorable clinical outcomes over a 12-year period [43].

Prostate cancers are typically sensitive to androgens (*e.g.,* testosterone) and may be temporarily controlled with androgen ablation that reduces testosterone level in the serum of a patient. Deprivation of androgens is achieved by surgical (removal of the testis) or chemical (drug) castration. Many advanced prostate cancers initially respond to androgen deprivation therapy [2], but over time the disease is no longer hormonally responsive, often spreads to bones, and is then considered castration resistant and incurable [34]. Because there is no cure for men with castration resistant prostate cancer, palliative measures are required, such as spot radiation treatment of painful areas of bone metastasis and analgesics. However, other treatments are slowly becoming more available and effective. Cryosurgery, high-intensity focused ultrasound, and proton beam therapy are now in clinical trials [1]. There also have been advances in chemotherapy and immunotherapy for the treatment of advanced prostate cancer. For example, the biologic agent sipuleucel-T has been shown to prolong life of patients with CRPC. This therapy is based on autologous cellular immunotherapy to induce the patient's immune system to attack the prostate cancer cells [44]. A great deal more research is being conducted to identify vaccines and other therapies to prevent and cure this highly prevalent cancer.

Although potentially curative, primary treatments for localized prostate cancer can have side effects that can affect the quality of life among prostate cancer patients. More men are seeking

treatment and thus, more are living with the possible short- and long-term side effects of such treatments. Incontinence and impotence are by far the most common side effects of prostate cancer treatments. Approximately 70% to 90% of men who undergo radical prostatectomy will experience impotence. Bowel dysfunction is very common after radiation therapy [2, 45]. Osteoporosis can also occur in males with prostate cancer who are being treated with androgen-suppressing drugs [46]. Given the issues arising from the effect of various treatments and their potential complications, it is important that patients have access to preoperative and postoperative counseling that contain comprehensive information about side effects and their impact on quality of life [47].

1.2.7 Major clinical challenges

1.2.7.1 Castration-resistant prostate cancer

Prostate cancer cells, like normal prostate cells, require androgens and their interaction with the androgen receptor (AR) to grow and survive. Therefore, the perturbation of the AR pathway is the mechanistic rationale for the use of androgen suppression to treat prostate cancer. Deprivation of androgens is typically achieved by surgical or chemical castration. In prostate cancer patients, androgen deprivation therapy initially reduces tumor burden and circulating PSA to low or undetectable levels. However, the development of a castration-resistant prostate cancer is inevitable. This form of prostate cancer is lethal, and patients no longer respond to androgen deprivation therapy. The mechanisms of castration resistance remain unclear [48]. Thus, a second major clinical challenge that could be significantly impacted by basic research in prostate tumor biology is the investigation of pathways of castration resistance, which could lead to the identification of new therapeutic approaches.

1.2.7.2 Distinguishing indolent from aggressive disease

At present, there are several major clinical challenges associated with prostate cancer diagnosis and treatment. The advent of PSA screening has led to a vast increase in the diagnosis of clinically localized low-grade carcinomas, many of which are relatively indolent forms of the disease that may not require treatment. Thus, the main drawback of this increase in prostate cancer detection is the overtreatment of indolent disease. For instance, patients with prostate cancer rarely relapse after primary therapy, and very likely can be managed and monitored with active surveillance.

However, a small fraction of these low-grade tumors will progress rapidly and require further treatments. Consequently, a major clinical challenge is the current inability to accurately distinguish indolent from aggressive tumors in prostate cancer patients. This could be addressed by better understanding the molecular basis of cancer initiation and identifying improved biomarkers that distinguish between indolent and aggressive forms of prostate cancer. The ability to identify patients with more aggressive disease would help prevent overtreatment of those with low-risk tumors [35].

1.3 Transcriptional regulation in prostate cancer

Prostate cancer development involves mutations or disruption of expression of key transcription factors that are critical for normal prostate homeostasis [49]. Basal transcription factors (e.g., TFIIA, TFIIB) along with RNA polymerase II make up the most basic assembly of proteins necessary for the transcription of protein-coding genes. Basal transcription factors bind to the promoter region of genes, such as a TATA-box element or initiator sequence [50]. On the other hand, regulatory transcription factors are proteins that bind to specific DNA-regulatory sequences located farther from the promoter, including enhancers and silencers, to control chromatin and modulate levels of transcription. In combination with basal transcription factors, regulatory transcription factors can form complex systems that increase or decrease gene transcription and protein synthesis, subsequently altering cellular function [51]. The role of enhancers in prostate cancer will be discussed later in this chapter. Here, we focus on the transcription factors that are important in the maintenance of normal prostatic identity (NKX3-1, p63, AR) and transcription factors that are deregulated in primary prostate cancer (ETS family members, c-MYC). We also review the factors involved in the transcriptional reprogramming after androgen-deprivation therapy and those implicated in initiating and maintaining CRPC.

1.3.1 Transcriptional maintenance of normal prostate identity

1.3.1.1 NKX3-1

The *NKX3-1* homeobox gene is expressed early during the development of the prostatic epithelium [52]. *Nkx3-1* is primarily expressed in secretory luminal cells of the adult prostate as well as in a small subset of basal cells and plays a critical role in maintaining tissue identity and differentiation of the prostatic epithelium [52, 53]. In the prostate, loss of *Nkx3-1* leads to dysplasia and PIN,

which often precedes the onset of prostate cancer [13], but it is not sufficient to initiate disease progression. In addition, *NKX3-1* is involved with *MYC* and *AR* in networks defining prostate cell fate. Indeed, *NKX3-1* and the *AR* are directly intertwined in a feedforward loop, and *NKX3-1* and *MYC* cross-regulate shared target genes in prostate tumorigenesis [54, 55]. *MYC* is the most frequently amplified gene in primary prostate cancer, while *AR* is the gene most amplified in metastatic disease [56]. Thus, loss of *NKX3-1* is a tumor-initiating event that disrupts normal prostate epithelial differentiation.

1.3.1.2 p63

The p63 transcription factor is a marker of prostate basal cells and is required for the normal epithelial development of the prostate [57]. The *TP63* gene encodes five variants of the p63 protein: TA, ΔN, α, β, and γ [58]. Loss of the ΔNp63 isoform, but not TAp63, prevents the development of the prostate [59, 60]. As such, ΔNp63-expressing cells are believed to be prostatic stem cells that are required for the generation of ΔNp63-negative prostate luminal cells. Moreover, prostate adenocarcinoma lacks basal cells, so p63 can be used to distinguish clinically benign tumors from prostate cancer tumors [61]. However, p63 is observed in a rare subset of prostate cancer tumors, which represent a molecularly distinct subclass of prostate cancer. Although these tumors maintain a basal-like phenotype, they still express typical prostate luminal-type proteins, such as NKX3-1 [62]. Aberrant expression of p63 is also associated with increased prostate cancer-specific mortality [63]. Therefore, p63 is critical for prostate epithelial development and homeostasis, as well as for prostate cancer diagnosis and prognosis.

1.3.1.3 AR

The ligand-inducible transcription factor, AR, is essential in maintaining the integrity of the prostate epithelium. In the prostate, androgen deprivation through castration induces apoptosis of the prostatic epithelium and reduces gland size [49]. This phenotype results from an intricate interaction between stromal and epithelial cells. Stromal AR is central to the maintenance of normal prostate identity. It is highly expressed in the prostate and binds to androgens to maintain the integrity of prostatic epithelial cells. Thus, androgen deprivation alters paracrine signaling of stromal cells, which leads to disruption of the homeostasis between the stroma and epithelium and apoptosis of epithelial cells [64]. Epithelial AR is also necessary for normal prostate maturation as

it maintains epithelial differentiation and suppresses cell proliferation [65]. Overall, AR activation by androgens sustains a transcriptional program that is essential for the maintenance of normal prostate identity. Deprivation of androgens by castration severely suppresses loading of AR onto AR response elements (AREs), but this effect can be rescued with treatment with testosterone that restores an AR-dependent transcriptional program [65]. The AR dependency of prostatic tissues has been exploited for the treatment of prostate cancer over 80 years ago. At present, androgen deprivation therapy remains the treatment of choice for prostate cancer [49].

1.3.2 Prostate cancer transcriptional reprogramming

1.3.2.1 ETS-mediated transcription

In primary prostate cancer, the most common genomic alteration is the gene fusion of the 5' untranslated region of *TMPRSS2* to transcription factors of the ETS family, such as *ERG*, *ETV1*, *ETV4*, and *FLI1*. Notably, androgens regulate the 5' untranslated region of *TMPRSS2*, so its fusions with *ETS* genes result in the deregulation of the *ETS* transcriptional program by AR [49]. The *TMPRSS2-ERG* gene fusion, which is found in up to 46% of primary prostate cancers, is the most frequent chromosomal rearrangement involving ETS family members [66]. In mice, overexpression of *ETS* genes under the control of an androgen-dependent promoter can lead to PIN or focal prostatic hyperplasia or even result in no appreciable phenotype [49]. Moreover, obesity in men harboring the *TMPRSS2-ERG* gene fusion is associated with poorer prostate cancer prognosis [67]. Taken together, these findings suggest that as with the loss of *NKX3-1*, *TMPRSS2-ETS*-related gene fusion is an early, if not an initiating event, in tumor evolution with an impact that is highly context-dependent [68]. Additionally, ERG overexpression in a mouse model of prostate cancer driven by *Pten* loss accelerates disease progression and leads to more invasive and poorly differentiated carcinoma and reduced survival [69]. This shows that deregulation of *ETS*-mediated transcription in the prostate by *TMPRSS2-ERG* gene fusion co-occurs with *PTEN* loss as the disease progresses [70].

1.3.2.2 MYC

The MYC proto-oncogene is a key driver of prostate cancer tumorigenesis and progression [71]. MYC is overexpressed at early stages of the disease, but it is also observed in 37% of metastatic prostate cancer patients [72] and associated with poor survival [73]. In primary prostate cancer,

MYC overexpression is associated with a gain of chromosome 8q and focal amplification of 8q24.21 [56]. Combined with *Pten* loss, *MYC* overexpression robustly recapitulates primary disease in murine prostate [74]. At the metabolomic level, *MYC* overexpression induces global rewiring of cell metabolism that supports cancer cell survival and growth [75]. Overexpression of *MYC* also impacts the transcriptional program of the prostate. It is intertwined with many genetic alterations and signaling defects that are found in prostate cancer. Notably, protein levels of MYC are increased by *TMPRSS2-ERG* gene fusion [76], which subsequently drives the loss of Nkx3-1 in murine prostatic epithelial cells [77] and affects the expression of Nkx3-1 and MYC coregulated target genes [55]. Additionally, deregulation of MYC protein levels drives disease progression to metastasis in a murine prostate cancer model with loss of *Pten/Trp53* [78]. Thus, MYC overexpression, alone or in combination with other genetic alterations, is central to prostate cancer initiation and progression to a metastatic disease [49].

1.3.3 Transcriptional rewiring of castration-resistant prostate cancer

1.3.3.1 AR in transition to androgen-independent AR signaling

The majority of prostate cancers are sensitive to androgens and their progression often depends on the transcriptional activity of AR. In the 1940s, the surgical removal of testes in men was found to strikingly relieve symptoms of prostate cancer and reduce tumor burden [79]. Since then, androgen deprivation therapies targeting AR activity has been the standard of treatment for advanced prostate cancer that recurs after prostatectomy or radiotherapy. Typically, this therapy combines chemical castration through the chronic use of gonadotropin-releasing hormone agonists or antagonists, which improves symptoms, decreases tumor burden, and prolong patient survival [80]. Unfortunately, androgen deprivation therapy rarely cures the cancer itself. Over time, it exerts an evolutionary pressure on the AR signaling activity of the tumor and prostate cancer almost always recurs and progresses to deadly CRPC that usually continues to rely on the AR for its progression [49]. The underlying mechanisms of therapeutic resistance to androgen ablation include mutations in AR, amplification of AR gene or enhancer, AR variants, coactivator overexpression, and *de novo* androgen synthesis in tumors [81]. One of the most frequent of these mechanisms is the amplification of AR. It is observed in more than half of metastatic prostate tumors and dramatically reduces the threshold of androgen-mediated binding of AR to the chromatin [56]. Another important mechanism of resistance to therapies is increased expression of AR variants that lack

the ligand-binding domain. These variants possess constitutive AR activity and can be generated through either alternative splicing or AR genomic structural rearrangements [49].

In CRPC, AR signaling is partly reactivated and restores AR-mediated secretion of PSA, further expanding the repertoire of AR-targeted genes [80]. Changes in levels of androgens can impact the activation of enhancer and suppressor elements which might explain the partial restoration of AR activity following hormonal therapy [82]. For instance, the expression of AR is controlled by a regulatory element in the AR gene that acts as a suppressor at high-androgen levels or as an enhancer at low-androgen levels [83]. Interestingly, a somatically acquired enhancer of AR was recently discovered to be a noncoding driver in advanced prostate cancer. This enhancer located upstream of the androgen locus is frequently activated and amplified by histone acetylation which leads to progression of metastatic CRPC [84]. Restored AR activity also drives the upregulation of specific genes, such as UBE2C, that is not observed in androgen-dependent cells [85]. Therapy with AR antagonists selectively disrupts weaker AR-binding sites, and thus reprograms the AR cistrome [86]. Moreover, AR binds to two different motifs based on whether the ligand is an agonist, such as dihydrotestosterone (DHT), or an antagonist (e.g., enzalutamide). This results in distinct transcriptional outcomes [87]. Collectively, these findings show that AR transcriptional reprogramming plays a prominent role at every stage of prostate cancer, from an androgen-sensitive to a castration-resistant disease.

1.3.3.2 FOXA1

In the prostate, FOXA1 is a pioneer factor that is involved in the establishment of the AR transcriptional program. Transcription factors can generally only bind to open and accessible regions of chromatin to regulate gene expression. In contrast, pioneer factors are a subgroup of transcription factors that bind to closed regions of chromatin to induce rearrangements of nucleosomes and recruit other transcription factors to establish gene-expression programs [88]. FOXA1 plays a critical role in controlling prostate cell maturation. Indeed, loss of FOXA1 in prostate tissues results in an epithelium with basal-like cells and no differentiated or mature luminal cells [89]. The AR cistrome is directly reprogrammed by FOXA1 expression. FOXA1 recruits the AR to low-affinity half androgen regulatory elements. Therefore, higher levels of FOXA1 allow for opening of chromatin regions and binding of liganded AR to many half androgen

regulatory elements, whereas lower levels of FOXA1 lead to binding of unliganded AR to androgen regulatory elements, which reprograms the AR transcriptional network [90]. Additionally, in prostate cancer tissues, AR-binding sites colocalize with the binding motifs of FOXA1 and with the transcription factor HOXB13. When overexpressed, both are sufficient to trigger reprogramming of the normal AR cistrome to a tumor-related AR cistrome. This suggests that both transcription factors are essential for prostate cancer progression and survival [91]. In primary prostate cancer, FOXA1 mutations are frequently detected. They repress AR activity and increase prostate cancer growth. Finally, point mutations in FOXA1 are associated with focal genomic instability in localized prostate cancer, which likely explains why tumors harboring FOXA1 mutations represent a distinct molecular subtype of prostate cancer, and why they are enriched in castration-resistant and metastatic disease [92].

1.4 Epigenetics

1.4.1 Introduction to epigenetic regulation

The term, “epigenetics,” was first used in 1942 by Conrad Waddington to refer to the dynamic interactions between the genome and the environment that are involved in cell differentiation and development in higher organisms. He defined epigenetics as heritable alterations in gene expression that are not due to changes in DNA sequence [93]. Waddington articulated his model in the complete absence of any potential epigenetic mechanisms.

Epigenetic modifications are reversible changes in gene expression that result from modifications of the chromatin structure and DNA accessibility without alteration of the cell’s DNA sequence [94]. They regulate patterns of gene expression via three mechanisms (i) DNA methylation, principally, but not exclusively at CpG sites, often in clusters within the human genome (ii) the status of chromatin (condensed, relaxed) which is determined by the modification status of the histones (acetylation, methylation) bound at specific sites and (iii) the expression of non-coding RNA, such as microRNAs (miRNA), which can control the expression of multiple genes by recognition of target sites in mature mRNA, resulting in their destruction by ribonuclease complexes [95].

1.4.2 Epigenetics in cancer

Somatic mutations that alter DNA sequences appear to be a fundamental and universal feature of cancer, and cancer is in this sense a genetic disease. Although the complete sequence of the 3 billion base pairs that make up the human genome has been analysed and mapped thousands of times [96, 97], identifying genomic alterations that contribute to cancer remains a major challenge. Initially, cancer was thought to be solely a consequence of genetic changes in key tumor-suppressor genes and oncogenes that regulate cell proliferation, DNA repair, cell differentiation, and other homeostatic functions. However, recent advances in the field of epigenetics have shown that human cancer cells harbor global epigenetic abnormalities, in addition to numerous genetic alterations. We now know that cancer cells have a specific epigenome. Although the mechanisms behind the establishment, maintenance, and plasticity of this epigenome are not fully understood, it is clear that alterations in the epigenetic programming are nearly universal in human cancers, including prostate cancer [98].

1.4.3 Epigenetic alterations in prostate cancer

Prostate cancer is a complex and heterogeneous disease that arises from both genetic and epigenetic alterations. Such epigenetic alterations are part of the causal chain that allow prostate cancer cells to evade the rules governing normal cells, which enables invasion, survival, and self-renewal [98]. For instance, silencing of tumor suppressor genes by promoter specific hypermethylation, aberrant expression of histone modulating proteins, and global DNA hypomethylation, contribute not only to prostate cancer onset but also to its progression to advanced and castration-resistant prostate cancer (**Figure 3**) [99].

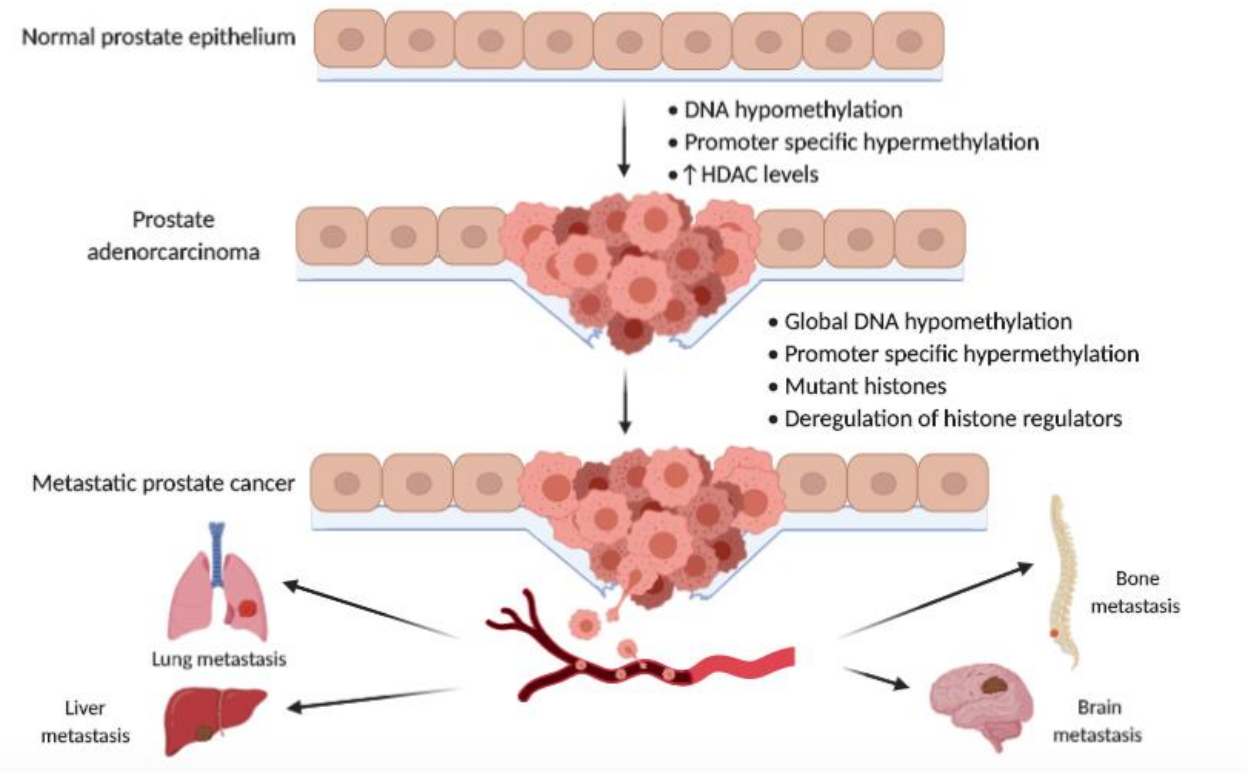

Figure 1- 3. Epigenetic mechanisms in prostate cancer initiation and progression. Key epigenetic alterations occurring during disease progression are shown. Adapted from Graça et al. (2016) under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/). Created with BioRender.com. The figure was reproduced for educational and non-publishing uses.

1.4.3.1 DNA methylation

DNA methylation is the most studied epigenetic event in prostate cancer and has been examined throughout carcinogenesis and disease progression. DNA methylation is performed by DNA methyltransferases (DNMTs) consisting of DNMT1, DNMT3A, and DNMT3B, that use S-adenosyl methionine as donor to transfer a methyl group onto the C5 position of the cytosine to form 5-methylcytosine within CpG dinucleotides, which are often found in large clusters called CpG islands [100]. Methylated cytosine can be converted into 5-hydroxymethylcytosine (5hmC) by TET proteins, including TET1, TET2, and TET3, and 5hmC can be further oxidized to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) [101]. DNA methylation is one of several epigenetic mechanisms that normal cells use to control gene expression and gene silencing. Aberrant DNA methylation, such as hypermethylation within promoter regions of tumor

suppressor genes or global hypomethylation, contributes to cancer through silencing of tumor suppressor genes and genome instability, respectively [102].

DNMT expression and activity are elevated in prostate tumor models and in androgen-resistant prostate cancer cell lines [103, 104]. DNMT1 has also been shown to act as a tumor suppressor gene in mice with early stage prostate cancer and as an oncogene in mice with late stage prostate cancer, particularly in the metastasis process through regulation of the epithelial to mesenchymal transition (EMT) and cancer stem cell programs [105]. Recent studies uncovered that DNMTs are involved in transcriptional activation through their interaction with TET proteins [106]. Both TET1 and TET2 were shown to play a tumor-suppressive role in prostate cancer through regulation of cell proliferation, migration, and invasion [107, 108]. Furthermore, the expression of several genes (*i.e., GSTP1* and *HOX* family members) is frequently downregulated in prostate cancer due to promoter hypermethylation [100]. In CRPC, changes in DNA methylation of AR target genes are also observed [109]. This led to the proposal of panels of DNA methylation-based biomarkers for the diagnosis of prostate cancer [110]. A stratification of prostate cancer subtypes based on DNA methylation patterns has been proposed [111], but the clinical usefulness is still unclear.

The classic demethylating agents, azacitidine and decitabine, have been evaluated *in vivo* in prostate cancer xenografts and showed some efficacy in many studies [112]. A novel decitabine formulation, based on a refined drug delivery system, was shown to prevent tumor growth in two different prostate cancer xenografts [113]. One other inhibitor of DNA methylation is the DNA hydroxymethylase ten–eleven translocation 1 which can revert cytosine methylation and is described as tumor suppressor. Its expression is often reduced in prostate cancer tissues and associated with decreased survival [112].

1.4.3.2 Histone modifications

DNA is packaged into complex quaternary structures in chromatin via interactions with proteins and RNA. The primary level of DNA organization is composed of nucleosome subunits in which DNA is wrapped around a histone octamer containing dimers of the four histone subunits: H2A, H2B, H3, and H4. These histone subunits have long tails that protrude outward from the core nucleosome structure. It is at these tails that occurs numerous posttranslational modifications,

including methylation, acetylation, phosphorylation, and several others, establish an "epigenetic code" that controls the surrounding chromatin state. Repressive, active, or poised (bivalent) chromatin states are associated with specific combinations of modifications on the histone tails [114]. Histone modifications are controlled by specific "writers," "erasers," and "readers". The writers of histone methylation are the histone methyltransferases (HMTs) whereas the writers of histone acetylation are histone acetyltransferases (HATs). The "erasers" are the histone demethylases (HDMs) and histone deacetylases (HDACs). The "readers" of histone marks include proteins with specific protein folds, including bromodomains (histone acetylation readers), chromodomains, and several others. Together, they help constitute chromatin remodeling and epigenetic reprogramming [98].

1.4.3.2.1 Histone acetylation

Histone acetylation is among the most studied of the histone modifications. It is uniformly associated with open or active chromatin [98].The balanced activity of cellular histone acetyltransferases (HATs) and histone deacetylases (HDACs) control global and local histone acetylation. In line with increased HDAC activity, local reductions of acetylated histone H3 and H4 are observed in prostate cancer [115]. On the other hand, high levels of global H3K18 acetylation are associated with higher risk of prostate cancer recurrence [116]. Taken together, this suggests a deregulation of HATs and HDACs in prostate cancer.

Elevated histone H3 acetylation is found in the vicinity of AR binding regions which impacts androgen target gene expression [117]. In CPRC, chromatin opening through local hyper-acetylation of super-enhancer regions contribute to reduced androgen dependency [118]. There is an emerging role of such super-enhancers as drivers of the expression of oncogenes. Recently, super-enhancers and the enrichment of the acetyl mark binder bromodomain-containing protein 4 (BRD4) at genetic risk loci have been reported in prostate cancer [119]. Moreover, the AR was shown to interact with many cofactors possessing HAT activity, which regulates the local histone acetylation status and downstream expression of androgen target genes [120]. Notably, EP300/KAT3B is a cofactor that cooperates with GATA2 to open chromatin at AR-targeted enhancers and facilitate gene expression in prostate cancer [121]. The HAT activity of EP300/KAT3B increases histone acetylation, mostly at H3K18, which alters prostate cancer cell

proliferation [122]. Levels of EP300/KAT3B are decreased by androgens but stimulated after androgen ablation [123]. Other cofactors that control the activity of androgen target genes include MYST1/KAT8 and TIP60/KAT5. Knockdown of MYST1/KAT8 reduces prostate cancer cell proliferation and TIP60/KAT5 is up-regulated in prostate cancer [112]. Presently, only few selective and potent HAT inhibitors are available and their impact on prostate cancer has been described in some studies. EP300 inhibitors (C646, NK13650A, NK13650B) were shown to block AR signaling and induce apoptosis, but only at high doses [124]. The TIP60 inhibitor NU9056 also exhibits anti-proliferative effects of prostate cancer cells [125].

In parallel with site-specific reduction of histone acetylation marks, HDAC levels are elevated in prostate cancer, especially in high-grade tumors [126]. HDACs are essential players in androgen-driven gene expression by changing local histone acetylation [127]. Zinc-dependent HDAC inhibitors include pan-HDAC inhibitors (e.g., panobinostat, belinostat) and more selective inhibitors, such as entinostat and mocetinostat [112]. In castration-resistant models, panobinostat reduces tumor growth [128]. Interestingly, the effect of HDAC inhibitors is stronger in models harboring the *ERG* gene fusion, which is detected in about 50% of prostate tumors [129]. Moreover, the NAD^+-dependent HDAC, sirtuin 1, was observed to directly repress AR activity via local reduction of histone acetylation [130].

Concerning bromodomain proteins, they are readers of histone acetylation marks and translate epigenetic modifications into a transcriptional response. The bromodomain and extra-terminal protein (BET) subgroup is the most studied because of highly potent and selective BET inhibitors [131]. The functional impact of BET proteins in prostate cancer, most notably BRD4, has been reported in several studies. In CRPC, BRD4 is known to interact with ERG to control the expression of common target genes. Examples of BET bromodomain inhibitors include JQ1, OTX015/MK-8628, I-BET762 and ABVV-075. Two bromodomain inhibitors (JQ1 and I-BET762) were shown to reduce tumor growth by partially preventing the interaction between BRD4 and ERG [132]. Other inhibitors reduced tumor growth in castration-resistant prostate cancer through reduced expression and binding of either full-length AR or splice variant [112]. This implies various mechanisms by which BET bromodomain inhibitors reduce prostate tumor growth. Also, their impact is explained in part by their disruption of transcriptional networks

following the targeting of enhancers/super-enhancers that are required for proliferation and cellular identity [133]. Several other bromodomain proteins, such as ATAD2, TRIM24 and TAF1, have been linked to prostate cancer. For instance, ATAD2 is cofactor of AR that is upregulated in a subset of prostate tumors [134]. As for TRIM24, it is stabilized by SPOP mutations that are often detected in recurring prostate cancer and was shown to interact with AR [135]. Finally, levels of TAF1 enhances the transcriptional activity of AR by affecting AR ubiquitination, implying a role of TAF1 in prostate cancer progression [136]. The ongoing efforts to identify better, highly potent, and selective compounds that inhibit individual HATs and HDACs will be of great help to further elucidate the individual roles of these enzymes in prostate cancer.

1.4.3.2.2 Histone methylation

Histone methylation can be associated with either open or closed chromatin, depending on which amino acid on the histone tail is methylated and the number of methyl groups [137]. Dynamic changes in methylation patterns of histones, mainly lysine methylation, is implicated in prostate cancer progression. For example, androgen ablation stimulates H3K4 dimethylation which correlates with disease recurrence [138]. In contrast, diminished H3K4 monomethylation, as well as H3K9 di- and trimethylation are found in prostate tumors. Also, H4K20 methylation is much reduced in CRPC [139]. Interestingly, histone H4K20 hypomethylation at the promoter regions of MYC regulated genes is increased in a murine prostate cancer model following saturated fat intake which contributes to prostate cancer lethality [30]. Much less in known about arginine methylation but H4R3 dimethylation is positively linked with prostate cancer recurrence [115].

The AR cooperates with several factors, such as proteins of the Polycomb group (PcG) complexes, to govern histone methylation. EZH2, an essential component of the complex PRC2, maintains the repressive histone mark H3K27me3, and is often overexpressed in prostate cancer and has been demonstrated to promote disease progression and castration resistance [140]. Inhibition of EZH2 with compounds such as DZNeP and GSK126 alone or in combination with other drugs, decreases prostate tumor size and proliferation [141]. A member of PRC1, BMI1, also plays a role in basal prostate stem cell maintenance, marks a distinct population of castration-resistant luminal progenitor cells, and is associated with prostate cancer initiation and progression [142].

Elevated levels of histone methyltransferase SMYD3, which methylates specific lysine residues in histones H3 and H4, is predictive of prostate cancer aggressiveness, thereby gene silencing of *SMYD3* reduces tumor growth [112]. Another histone lysine methyltransferase, WHSC1, has been shown to be stabilized by AKT, leading to promotion of prostate cancer metastasis [102]. Further, the arginine methyltransferase PRMT5 has an oncogenic function in prostate tumors. Apart from histones, it also methylates the AR and regulated its activity on downstream target genes [112].

Concerning histone demethylases (HDMs), they also play a role in AR signaling and prostate cancer. For example, cooperative demethylation of H3K9 by JMJD2C and LSD1 promotes AR activity [143]. LSD1 also directly represses *AR* gene expression via H3K4 demethylation in the second intron of AR. NCL1 is a recently reported inhibitor of LSD1. This inhibitor reduces tumor growth in CRPC. Combination of JMJD2C with JMJD2B also controls AR transcriptional activity as well as AR stability [112].

Chromodomain proteins are readers of histone methylation marks. For instance, CHD1 is a reader of H3K4 di- and trimethylation marks that is frequently mutated in *ETS* fusion-negative late-stage prostate cancer, and its inactivation promotes prostate cancer aggressiveness [92]. Interestingly, loss of *CHD1* dramatically reduces proliferation and survival in PTEN-deficient prostate cancer [144].

1.4.3.2.3 Histone phosphorylation

Up to now, few data was published on the impact of histone phosphorylation on AR signaling and prostate cancer. Phosphorylation of H3T11 by the phosphotransferase PKN1 leads to androgen-mediated recruitment of WDR5 to AR target genes. Importantly, inhibition of PKN1 with Ro318220 reduces androgen target gene expression and loss of *WDR5* decreases prostate cancer cell proliferation [112].

1.4.3.3 Non-coding RNAs

Changes in non-coding RNA profiles are also associated with prostate cancer progression and therapy response. For instance, the microRNA miR-141 is elevated in prostate cancer and correlates with the serum PSA levels. Many other microRNAs were found to be up-regulated (e.g.,

miR-20a, miR-21, miR-195 and miR-375) or down-regulated (e.g., miR-34a, miR-143/145, miR-205 and miR-488) in prostate cancer [145]. Some microRNAs including miR-34a, miR-34c and miR-130b, directly control AR activity by targeting its 3′-untranslated region. It was also shown that miR-130b increases invasion and therapy resistance prostate cancer patients [146]. On the other hand, several microRNAs that are involved in prostate cancer cell proliferation are regulated by AR expression [145]. Further, a recent study revealed a novel long non-coding RNA, *LINC00844*, that is central in the androgen transcriptional network and the development and progression of prostate cancer [147]. A few clinical trials are currently ongoing to evaluate circulating non-coding RNAs as potential biomarkers for prostate cancer to help identify risks early on or to predict therapy response [112].

1.5 *Cis* regulatory elements in cancer

It is clear now that genetic and epigenetic alterations dysregulate oncogenic and tumor-suppressor signaling pathways associated with tumorigenesis and the development of cancer. With the advent of high-throughput and relatively inexpensive whole-genome sequencing technology, the focus of cancer research has started to shift towards identifying alterations in non-coding *cis*-regulatory elements of the cancer genome. Non-coding *cis* gene regulatory elements have an emerging role in cancer. They play an important role in gene regulation with mutations and epigenetic changes in these elements that can result in abnormal expression of target genes. The rate of mutation in non-coding DNA is almost twice as high as the rate in coding DNA, possibly due to lack of selective pressure and different DNA repair mechanisms [148]. Thus, identifying driver non-coding mutations amongst the vast background of passenger mutations is difficult. Further, it is challenging to determine how a given mutation-harbouring region regulates expression, and which genes are affected [149].

Non-coding *cis* gene regulatory elements include promoters, enhancers, super-enhancers, insulators, and silencers. Recently, *TERT* promoter mutations in melanoma, and recurrent mutations that create a super-enhancer regulating *TAL1* expression in leukemia have been discovered. This has sparked significant interest in the search for more somatic *cis*-regulatory alterations driving cancer development [148].

1.5.1 Enhancers in prostate cancer

In prostate cancer, somatic alterations of *cis* non-coding regulatory elements mostly reside in enhancers and super-enhancers [150]. Enhancers and super-enhancers are regulatory DNA sequences that define the regulation of genes. Enhancers function as binding platforms. They contain binding sites for RNA polymerase, TFs, and co-activators. For instance, enhancers closely interact with promoters, over either short or long distances to activate target genes [151] Importantly, accumulated evidence reveals that enhancers are marked by epigenetic modifications, such as mono-methylation at H3 lysine 4 (H3K4me1) and acetylation at H3 lysine 27 (H3K27ac) [152]. Super-enhancers are defined as a grouping of enhancers with aberrant high levels of transcription factor binding [153].

Enhancer mutations lead to activation of gene expression through three major mechanisms: copy number gain of enhancer regions, structural rearrangements or "enhancer hijacking" of a target gene and somatically acquired mutations that create or alter transcription factor binding sites [84]. Enhancer malfunction is a key process that drives the aberrant regulation of oncogenes in cancer. In prostate cancer, growth-related genes and oncogenes commonly acquire enhancers or super-enhancers, which drives uncontrolled proliferation by locking the growth regulatory network to an "on" state. For example, the *MYC* oncogene, which drives the growth of various cancers, acquires large super-enhancers that are tumor specific and absent from normal cells [154]. Because the cancer phenotype relies on the abnormal transcription programs driven by enhancers, this leads to dependency of some prostate cancers on enhancer and super-enhancer-related transcription programs [152]. Notably, in prostate cancer, the acquisition of BRD4-associated super-enhancers leads to elevated expression of key oncogenic genes, including *TMPRSS2-ETS*, *KLK3*, and *BMPR1B*, especially CRPC [152]. Also, the formation of an extended enhancer through chromosomal rearrangements of TMPRSS2 and ERG promotes ERG overexpression leading to subsequent overexpression of ERG target genes that drive the development of prostate cancer [155]. Importantly, Takeda *et al.* recently discovered that an enhancer upstream of the androgen receptor locus is activated and amplified in advanced prostate cancer. The amplification of this enhancer also drives metastatic CRPC [84]. These findings emphasize that enhancers are crucial regulators within prostate cancer.

1.6 Research objectives

1.6.1 Rationale

Advances in our understanding of prostate cancer biology have reshaped clinical practice for the detection, diagnosis, and management of this disease. The advent of active surveillance has changed the management of indolent prostate cancer, while chemotherapy and hormonal therapy have improved the outcomes of patients with advanced and aggressive disease [156]. However, prostate cancer remains the most common malignancy affecting men. An increasing number of men are diagnosed with prostate cancer due to increasing life expectancy and use of PSA screening. Most prostate cancers are indolent and inconsequential to the patient while a minority are aggressive and lethal if detected too late or not treated [11]. Thus, the risk of over-diagnosing and over-treating many prostate cancer cases is real. This highlights the critical need to accurately stratify indolent prostate cancers from aggressive prostate cancers.

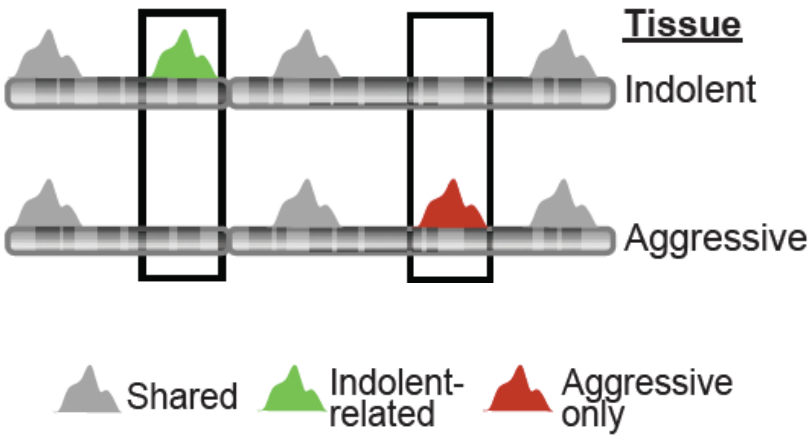

Figure 1- 4. Research strategy. Schematic representation of our research strategy to determine gene regulatory elements and the epigenetic landscape associated with aggressive prostate cancer. Aggressive only peaks are in red, indolent-related peaks are in green, shared peaks are in grey [157].

1.6.2 Hypothesis

We hypothesize that epigenetic reprogramming, through alterations of key epigenetic factors and gene regulatory elements, underlies prostate cancer aggressiveness.

1.6.3 Objectives

Given the emerging role of epigenetic configuration and the need for risk stratification of prostate cancer, the objectives of this project are to 1) identify and 2) characterize somatically acquired regulatory elements associated with aggressive prostate cancer.

1.6.4 Research design and methods

To identify somatically acquired regulatory elements that might be drivers of aggressive prostate cancer, we reanalyzed the publicly available epigenomic dataset from Stelloo *et al.* [111]. We confirmed correlative evidence of the identified regulatory element with prostate cancer using publicly available transcriptomic datasets. To establish a causative link between the identified regulatory element and prostate cancer progression, we selected the best prostate cancer cell line based on the H3K27ac profile (ChIP-seq) and transcript expression (RNA-seq). *In vitro*, we established a Tet-On-3G system in PC-3 to induce the candidate gene associated with H3K27ac gain in aggressive disease and investigate its role in prostate cancer cell proliferation.

1.6.5 Contributions to the advancement of knowledge

This project explores the knowledge gap in the literature regarding the role of a somatically acquired H3K27ac gain at the *ANKRD30A* locus in prostate cancer biology.

Chapter 2: Investigating the role of a somatically acquired H3K27ac gain in aggressive prostate cancer

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2.1 Abstract

Several epigenetic mechanisms are altered in prostate cancer progression and become drivers of advanced prostate cancer. Understanding how epigenetic deregulation contributes to the development and progression of prostate cancer may improve risk stratification and treatment selection for prostate cancer patients. In addition, a somatically acquired enhancer has been recently described being a noncoding driver of advanced prostate cancer. This finding emphasizes the emerging role of regulatory elements as noncoding oncogenic drivers in prostate cancer. As such, we aimed to identify and characterize somatically acquired regulatory elements, defined by H3K27 acetylation (H3K27ac), in metastatic prostate cancer. In this study, we identified a somatically acquired regulatory element associated with the *ANKRD30A* locus in aggressive prostate cancer by using publicly available epigenetic data. Further, we showed that *ANKRD30A* expression is increased in a subset of prostate cancers and associated with accelerated disease recurrence. Consistent with a role in metastatic progression, somatic H3K27ac gain at *ANKRD30A* locus was identified in ~36% of AR^+ castration-resistant prostate cancer (mCRPC) patient derived xenografts (PDXs) models established from prostate cancer metastases which also correlated with transcript expression. *In vitro*, we revealed that *ANKRD30A* is aberrantly expressed in LAPC4 and 22Rv1. We also determined the status of H3K27ac at the *ANKRD30A* locus in PC-3, LAPC4, LNCaP, 22Rv1 and RWPE-1. Finally, we showed that inducible expression of *ANKRD30A* increases the proliferation of PC-3, a metastatic prostate cancer cell line. Altogether, our data suggest that activation of *ANKRD30A* might contribute to the development of aggressive prostate cancer. This epigenetic event could potentially be used as an epigenetic biomarker to identify patients with aggressive prostate cancer.

2.2 Introduction

Prostate cancer is a major societal burden. It is the second most common cancer in men worldwide, with over 1 million newly diagnosed cases and a quarter of a million deaths each year [1]. Mortality has declined more in later years than the incidence. This improvement is likely due to the earlier diagnosis and improved treatment of prostate cancer [2]. Unfortunately, it comes at the expense of an increased incidence of men being diagnosed with prostate cancer of whom a significant proportion are at little risk of having a life-threatening disease during their lifetime. In fact, most cases of prostate carcinoma are relatively indolent and require no treatment, such that the majority

of men diagnosed with prostate cancer will instead die of other causes [2]. Nonetheless, a small fraction of these tumors will progress rapidly and result in prostate cancer-specific lethality if detected too late or left untreated. Consequently, distinguishing the rare and aggressive forms of prostate cancer from the majority of indolent cancers is a major clinical challenge [3].

Prostate cancer is a complex and heterogeneous disease that arises from both genetic and epigenetic alterations. Several epigenetic aberrations, such as silencing of tumor suppressor genes by promoter hypermethylation, deregulation of histone modulating enzymes, and DNA hypomethylation contribute not only to prostate cancer onset, but also to its progression to advanced and castration-resistant cancer [4]. Moreover, a recent study revealed that a somatically acquired enhancer of the androgen receptor is a noncoding driver in advanced prostate cancer. This enhancer located upstream of the androgen locus is frequently activated and amplified by histone H3 acetylation on lysine 27 (H3K27ac) and drives progression of metastatic castration-resistant prostate cancer. These findings emphasize the emerging role of epigenetic alterations and the modulation of regulatory elements in prostate cancer progression [5]. Thus, our goal was to identify and characterize epigenetic events implicated in aggressive prostate cancer. Overall, we revealed a somatically acquired active regulatory element at the *ANKRD30A* locus in aggressive prostate cancer. We showed that *ANKRD30A* is more expressed in prostate cancer compared to benign prostatic hyperplasia. Moreover, we find that *ANKRD30A* expression is associated with accelerated disease recurrence and correlates with H3K27ac status in mCRPC PDXs. We determine H3K27 acetylation status at the *ANKRD30A* locus in four prostate cancer cell lines and one normal prostate cell line. Finally, inducible overexpression of *ANKRD30A* in a metastatic prostate cancer cell line increased cellular proliferation. Altogether, we reveal an *ANKRD30A*-associated regulatory element that is epigenetically activated in aggressive prostate cancers, providing a basis for prostate cancer risk stratification. This underscores the importance of epigenomic profiling in prostate cancer to functionally characterize regulatory elements that might be driving the progression of prostate cancer to a metastatic and lethal disease.

2.3 Materials and Methods

Bioinformatics:

Data source and description. Publicly available prostate cancer transcriptomic and epigenomic datasets used in this study were downloaded from the European Genome-phenome Archive under de following accession code: GSE80609 (ChIP-seq), GSE80609 (RNA-seq). Firstly, in the Stelloo *et al*. cohort, 49 samples were selected from patients who developed a relapse within ~5 years after diagnosis and matched on age, Gleason score, PSA level, and T-stage with 50 samples from patients with non-relapsed disease within ~10 years after diagnosis. All samples included were primary prostate cancer specimens. Two samples had no follow-up data available [6]. Secondly, the Yun *et al*. cohort consisted of 45 prostate tissue samples (8 BPHs, 16 localized prostate cancers, 9 advanced prostate cancers, and 12 CRPCs) from 41 patients. Four patients provided both advanced and CRPC samples at different time points. All samples in this cohort were collected from the primary cancer site in the prostate [7]. Thirdly, TCGA-PRAD readcount matrix, methylation levels and samples clinicopathological information were downloaded from The Cancer Genome Atlas (TCGA) database (http://tcga-data.nci.nih.gov/tcga/) [8] using Bioconductor package TCGAbiolinks [9]. The TCGA level 3 cohort had 52 normal prostate samples and 502 cancer samples. One metastasis sample was excluded from the analysis.

Somatic gain analysis. Using the Stelloo *et al*. dataset, H3K27ac ChIP-seq reads were aligned to hg19 with BWA [10] and significantly enriched regions (peaks) were called with MACS [11]. The union of all H3K27Ac peaks and reads counts across patient samples were determined using BEDTools [12]. Somatically acquired regulatory elements differentially activated between aggressive and indolent prostate cancer groups were identified using EdgeR [13].

Survival analysis. To conduct survival analysis on *ANKRD30A* gene expression, TCGA RNA-seq dataset was transformed using the variance-stabilizing transformation implemented in the Deseq2 package [14]. For the methylation status survival analysis, the mean methylation levels of eleven probes at the *ANKRD30A* locus were calculated from the TCGA dataset. Patients were divided into two groups either high expression and low expression or high methylation and low methylation by optimal cutpoint calculated by surv_cutpoint function of survminer

survminer_0.4.6 package [15]. Differences between groups in patient's recurrence-free survival was estimated by Kaplan–Meier survival analysis and log-rank tests using R package survival_3.1-12 [16]. Survival curves were generated using survminer_0.4.6 9 package [15].

Cell lines and cell culture: The prostate cancer cell lines, PC-3 (CRL-1435, ATCC), PC-3M, and PC-3M LN4 (kindly provided by Dr. Mario Chevrette, McGill University), which were isolated from bone metastasis, were cultured in Roswell Park Memorial Institute Medium 1640 (RPMI-1640; 350-015-CL, Wisent). Similarly, lymph node metastasis derived LNCaP (CRL-1740, ATCC), LAPC4 (kindly provided by Dr. Jacques Lapointe, McGill University), and 22Rv1 (CRL-2505, ATCC) were maintained in RPMI medium. The C4-2 (CRL-3314, ATCC) cell line constitute a subline grown from LNCaP. C4-2 cells were cultured in Dulbecco's Modified Eagle Medium plus F-12 Medium (DMEM/F-12 (4:1); 301-075-CL, Wisent) supplemented with custom-made insulin, triiodothyronine, apo-transferrin, d–Biotin (301-076-EL, Wisent). DU-145 cells (HTB-81, ATCC), which are derived from a brain metastasis of prostate cancer, were maintained in Dulbecco's Modified Eagle Medium (DMEM; 319-015-CL, Wisent). The three media, RPMI, DMEM and DMEM/F-12, were supplemented with heat-inactivated (56℃ for 30 minutes) 10% fetal bovine serum (FBS; 12483020, Life Technologies) and 1% HyClone penicillin/streptomycin (SV30010, Fisher Scientific). The RWPE-1 (kindly provided by Dr. Jacques Lapointe, McGill University) and RWPE-2 cell lines (CRL-11610, ATCC), which are prostate epithelial cells established from normal human prostate, were cultured in Keratinocyte Serum Free Medium (17005042, Invitrogen) supplemented with bovine pituitary extract (BPE) and human recombinant epidermal growth factor (rEGF). Primary prostate epithelial cells, PrEc (CC-2555, Lonza), were cultured in Prostate Epithelial Cell Growth Medium BulletKit (CC-3166, Lonza). The complete growth media for this cell line was PrEBM Basal Medium (CC-3165, Lonza) and PrEGM SingleQuots Supplements (CC-41-77, Lonza) containing the following growth factors: L-Glutamine, Extract P, Epinephrine, rh TGF-a, Hydrocortisone hemisuccinate, rh Insulin and Apo-transferrin. Penicillin and streptomycin were added to the complete media. All cell lines were kept in culture at 37°C and 5% CO_2.

RNA-sequencing and data analysis: RNA from 300,000 cells was extracted using the miRNeasy Micro Kit (217084, QIAGEN). DNase digestion was performed during RNA purification using

the RNase-Free DNase Set (79254, Qiagen). Isolated RNA was quantified with NanoDrop® spectrophotometer (ND-1000) and quality assessed with the Qubit dsDNA HS (High Sensitivity) Kit (Q32854, Thermo Fisher Scientific). The mRNA stranded libraries were amplified and sequenced by Génome Québec. RNA-seq alignment and analysis was performed using GenPipes "RNA-seq" pipeline [17]. Raw reads were trimmed and filtered for quality using Trimmomatic [18] and aligned to the human reference genome (GRCh38) using STAR [19]. Mapped reads are further refined using GATK [20, 21] and Picard suite [22]. The readcounts were estimated using HT-seq Count [23].

ChIP-qPCR: Cells in 150 mm plates were fixed with 1% formaldehyde (PI28906, Thermo Fisher Scientific) for 20 minutes and quenched with glycin for 5 minutes. After washings, cells were collected at 500 x g for 5 minutes. Cell pellets containing 7 million cells were flash-frozen and stored at -80 °C. Next, cell pellets were lysed for 1 hour in 500 µl sarkosyl buffer containing 0.25% sarkosyl (L5125, Sigma), 1 mM DTT, RIPA 0.3 buffer, 1x protease inhibitors (04693132001, Roche), 5 mM NaButyrate (19-137, Sigma). Chromatin of 1 million cells was sheared in the Bioruptor Pico (Diagenode) device using a 30 second on/30 second off, 15 cycles regimen. For the shearing assessment, an aliquot of sheared chromatin was quickly de-crosslinked with 500 U/mL RNAse (18021014, Thermo Fisher Scientific) for 30 min at 37 °C and proteinase K (P6556-10MG, Sigma) for 30 min at 55 °C. DNA was then purified using the MinElute Purification Kit (28004, Qiagen) and quantified using the Qubit dsDNA HS (High Sensitivity) Kit (Q32854, Thermo Fisher Scientific). Purified de-crosslinked DNA was analyzed for fragment size on a 2% E-Gel (A42135, Thermo Fisher Scientific) with the E-Gel Power Snap Electrophoresis System (G8300, Thermo Fisher Scientific). Chromatin with fragments of 100-350 base pairs long was used for further testing. Sheared chromatin with a concentration of 370 ng was magnetically immunoprecipitated with 1 µg of the polyclonal anti-H3K27ac antibody (C15410196, Abcam) in combination with 1 µg of the negative IgG control antibody (C01011009, Diagenode) using the ChIPmentation Kit for Histones (C01011009, Diagenode) on the IP-Star Compact Automated System (Diagenode) device, according to the manufacturer's instructions. Subsequently, stripping and end repair of immunoprecipitated and input DNA were performed according to the ChIPmentation Kit guidelines. Quantitative PCR was performed for recovery analysis with the positive control ChIP-seq grade GAPDH-TSS primer pair (human) supplied with the

ChIPmentation Kit for Histones that specifically amplifies a genomic region containing the *GAPDH* promoter. Quantitative PCR was also performed for H3K27ac enrichment analysis at the *ANKRD30A* locus using a custom primer pair designed to target a specific region of *ANKRD30A* enriched for H3K27ac in aggressive prostate cancer clinical samples **(Table S2-1)**.

Generation of Tet-On 3G inducible expression systems: The Tet-On 3G inducible systems protocol had three major steps:

Cloning ANKRD30A insert into pTRE3G-BI-mCherry vector

The ANKRD30A-pCMV6-Entry (SC305713, Origene) plasmid was digested with NotI and BglI at 37 °C overnight. In parallel, the pTRE3G-BI-mCherry (631333, Clontech) was digested with BamHI-HF and NotlI at 37 °C overnight. The 5'-ends of digested *pTRE3G-BI-mCherry* (3564 bp) were dephosphorylated using Antarctic Phosphatase (M0289S, NEB) at 37 °C for 30 minutes. The reaction was stopped by heat inactivation at 80 °C for 5 minutes. Digested *ANKRD30A* (4664 bp) and digested-blunted *pTRE3G-BI-mCherry* were purified from a 1% agarose gel using the Monarch DNA Gel Extraction Kit (T1020S, NEB). Purified *ANKRD30A* (insert DNA) and *pTREG3G-BI-mCherry* (vector DNA) were ligated using T4 DNA Ligase (M0202S, NEB). Recombinant DNA was transformed into Stbl3 chemically competent *E. coli* strain (C737303, Thermo Fisher Scientific). Transformed colonies were amplified and plasmid DNA was screened by restriction digest and sequenced by Génome Québec.

Pilot testing Tet-based induction of ANKRD30A. 5µg of customized response *pTRE3G-BI-mCherry-ANKRD30A* plasmid was co-transfected with 1µg of regulator *pCMV-Tet3G* (631335, Clontech) plasmid into 22Rv1 cells in a 6-well plate using Xfect transfection reagents (631317, Clontech). The empty plasmid *pTRE3G-BI-mCherry* was used as a control. After 24 hours, the transfected cells were treated with doxycycline (1 µg/ml) (S4163, Selleck Chemicals) for 48 hours to co-induce mCherry and *ANKRD30A* expressions. mCherry expression was assessed by an IncuCyte S3 (Sartorius).

Creating and screening Tet3G and ANKRD30A expressing double stable cell lines. PC-3 cells were co-transfected with 2.5 µg of the *pCMV-Tet3G* plasmid and 2.5 µg of the *pTRE3G-BI-mCherry-ANKRD30A* construct in 6-well plates using Lipofectamine 3000 (Invitrogen). After 48

hours, the transfected cells were treated with G418 (450-130-QL, Wisent) at the selection concentration that is optimal for the PC-3 cell line (200 µg/ml). The medium was replaced with fresh complete medium plus G418 every four days. After ~2-3 weeks of drug selection, cells were cultured in a maintenance concentration of G418 (200 µg/ml). For screening, we performed luciferase reporter assays. In 6-well plates, 4 µg of *pTRE3G-Luc* (631333, Clontech) plasmid was transfected along with 1 µg of the *pCMV-Renilla* plasmid driving the expression of the *Renilla* luciferase gene used as an internal control. After 4 hours, the transfected cells were treated with doxycycline (2 µg/ml). Luciferase activity was measured 24 hours after transfection with the Dual-Luciferase Reporter Assay kit (E2940, Promega). Double-stable pool of cells with high fold induction was selected for propagation and further testing. Double-stable cells were induced by doxycycline (2 µg/ml) for 48 hours to co-induce *mCherry* and *ANKRD30A* expressions, sorted for *mCherry* positive populations, and processed for downstream analyses such as qRT-PCR and proliferation assays.

Quantitative RT-PCR: RNA was extracted using QIAzol Lysis Reagent (79306, Qiagen). Isolated RNA was then quantified with the NanoDrop spectrophotometer (ND-1000) and reverse-transcribed into cDNA with the iScript Reverse Transcription Supermix (1708841, BioRad) or the LunaScript RT SuperMix Kit (E3010L, NEB) using 1,000 ng of RNA. Quantitative-PCR was performed using iQ SYBR Green Supermix (1708882, BioRad) or the Luna Universal qPCR Master Mix (M3003X, NEB) on the CFX Connect Real-Time System (BioRad). *GAPDH* was used as control. Custom primer sequences are listed in **Table S2-1**.

Proliferation assay: Double-stable Tet-inducible PC-3 expressing *Tet3G* and *ANKRD30A* or *EV* were seeded at a density of 5,000 cells per well in 96-well plates. To induce the expression of *ANKRD30A*, cells were treated with doxycycline (2 µg/ml) at day 0. The plate was then placed in the IncuCyte S3 (Sartorius) and images of whole wells were acquired every 8 hours for 5 days. Images were analyzed using the Basic Analyzer software (included with IncuCyte system) and data was shown as confluency (%) over time.

Immunoblotting: Cells were lysed using RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% TRITON-X). Equal amounts of protein were loaded on 8% Tris-glycine SDS-

polyacrylamide gel and transferred to nitrocellulose blotting membranes (1704271, Bio-Rad). Following 1 hour blocking in 5% non-fat milk in TBST-T, membranes were incubated with rabbit polyclonal ANKRD30A (1:1000, Aviva Biosystems, OALA07447) or NY-BR-1 Polyclonal Antibody (Invitrogen, PA5-101251) overnight at 4°C. Following washing, the membranes were incubated with goat anti-rabbit (1:10000, Jackson ImmunoResearch, 111-035-003) for 1 hour and developed using Clarity Western ECL Substrate detection reagent (BioRad, 1705061). Images were obtained using ChemiDoc Imaging System. The same protocol was used for the loading control, GAPDH antibody (Cell Signaling, 21187).

Statistics

Statistical analyses of bioinformatics data were performed in R version 3.6.2 (2019-12-12). Statistical analysis to compare two groups were performed using student's *t*-test or Wilcox test. Two-way ANOVA with Sidak's multiple comparison tests were performed for experiments involving multiple group comparisons.

2.4 Results

A somatic H3K27 acetylation mark is gained at the *ANKRD30A* locus in aggressive prostate cancers

Distinguishing indolent from aggressive disease is a major clinical challenge in prostate cancer management and decision-making [24]. Also, epigenetic reprogramming is an emerging mechanism driving prostate cancer initiation and progression. Thus, to identify somatic epigenetic events in aggressive prostate cancer, we analyzed a publicly available ChIP-seq dataset comparing the H3K27ac profile of 45 indolent prostate cancer tumors with 45 aggressive prostate tumors obtained following radical prostatectomy. The aggressive prostate tumors were collected from patients who developed biochemical recurrence (BCR) within 5 years whereas indolent tumors were collected from patients with no BCR within 10 years after diagnosis [6]. Our analysis identified 150,693 H3K27ac peaks in all prostate cancer cases and revealed multiple metastatic variant enhancer loci (Met-VELs) at specific genes and regulatory elements (**Figure 2-1A**). Notably, one of the top gained Met-VEL that we identified was linked to the *ANKRD30A* gene locus and was particularly enriched in aggressive tumors (**Figure 2-1B**; log2[fold change (FC)]=1.22, P=2.22x10^{-6}, FDR=0.02). This peak (chr10:37457090-37457725) is 636 bp in length

and localized between exon 19 and 20. Using publicly available ChIP-seq and ATAC-seq data, we visualized the peaks at the *ANKRD30A* locus on IGV. It appears that our H3K27ac peak of interest, which is detected in aggressive prostate tissues, has no overlap with other peaks from 22Rv1, LNCaP and PC-3 cell lines. This suggests a somatically acquired regulatory element at the *ANKRD30A* locus in aggressive prostate tumors.

ANKRD30A expression is increased in prostate cancers and is associated with disease recurrence

ANKRD30A (ankyrin repeat domain 30A) is regarded as a putative transcription factor [25]. It is uniquely expressed in the mammary epithelium and associated with breast cancer progression [26]. Although, mRNA has been detected in the testis and some prostate cancer tumors [27], expression levels of *ANKRD30A* in prostate cancer remain largely elusive. Thus, following the analysis of the ChIP-seq dataset, we analyzed two publicly available transcriptomic datasets [7, 8] to compare the expression of *ANKRD30A* in different prostate tissues, including benign prostatic hyperplasia, localized prostate cancer, advanced prostate cancer and CRPC. We found that *ANKRD30A* is significantly increased in early-stage prostate cancer, advanced disease and CRPC compared to benign prostatic hyperplasia (**Figure 2-2A**). In prostate cancer, biochemical recurrence (BCR) is defined as the rise in PSA levels following primary treatment (e.g., prostatectomy and/or radiation therapy). It is widely used for reporting the clinical outcomes of the disease. Therefore, to investigate the potential role of *ANKRD30A* expression in prostate cancer progression, we compared BCR-free survival between prostate cancer patients expressing either high or low *ANKDR30A.* We observed that high *ANKRD30A* expression was associated with a shorter time to BCR (**Figure 2-2B**), which indicates that *ANKRD30A* expression is associated with prostate cancer progression.

Promoter of *ANKRD30A* is globally hypomethylated in prostate tumors

Given the critical role of DNA methylation in prostate cancer progression, we also compared the methylation pattern at the promoter of *ANKRD30A* by tissue types. We found that the promoter region of *ANKRD30A* was generally hypomethylated in prostate tumors compared to benign tissues (**Figure 2-3A**). Promoter hypomethylation is generally associated with gene activation [28]. Although not significant, we also observed a trend toward a poorer outcome in prostate cancer

patients with low methylation at the promoter region of *ANKRD30A* (**Figure 2-3B**). The genomes of cancer cells are globally hypomethylated when compared to normal cells, except for hypermethylation of promoters of genes that are silenced in cancer (*e.g.*, tumor suppressors) [29]. Taken with the previous observation that *ANKRD30A* expression is increased in localized and advanced prostate, the above results indicate that hypomethylation at the promoter of *ANKRD30A* is associated with an increased gene expression and trends with disease recurrence.

Somatic H3K27ac gain in *ANKRD30A* gene is associated with transcript expression in LuCaP patient-derived xenografts

The LuCaP patient-derived xenografts (PDXs) are metastatic, androgen receptor positive (AR^+) and castration-resistant. They reflect the molecular heterogeneity of advanced prostate cancer and serve as models to study mechanisms underlying treatment response and resistance [30]. Comparison of 14 LuCaP prostate cancer PDXs identified H3K27ac gain at the *ANKRD30A* locus in a subset of models (**Figure 2-4A**). This somatically acquired H3K27ac gain was observed in approximately 36% of LuCaP PDXs and correlated with transcript expression (**Figure 2-4B**). These results support an association between this H3K27ac gain and *ANKRD30A* expression in prostate cancer.

***ANKRD30A* is aberrantly expressed in LAPC4 and 22Rv1 prostate cancer cell lines**

Next, we leveraged RNA-seq data from multiple prostate cancer and normal prostatic cell lines to define the levels of *ANKRD30A* expression *in vitro*. We showed that *ANKRD30A*, which is a known breast cancer antigen and normally restricted to the mammary epithelium [25, 27], was mostly expressed in LAPC4 and to a lesser extent, in 22Rv1. No *ANKRD30A* expression was detected in other prostate cancer cell lines (C4-2, LNCaP, PC-3, PC-3M, PC-3M LN4, DU-145) and normal prostate cell lines (PrEC, RWPE-1, RWPE-2) (**Figure 2-5**). Interestingly, the LAPC4 cell line, which expressed the greatest levels of *ANKRD30A*, is sensitive to androgens. In contrast, the other cell lines with no *ANKRD30A* expression (PC-3, PC-3M, PC-3M LN4, DU-145, C4-2) are androgen-insensitive and castration-resistant. However, LNCaP cells are androgen-sensitive but didn't express *ANKRD30A* and 22rv1, a CRPC cell line, expressed intermediate levels of *ANKRD30A*, suggesting that *ANKRD30A* activity might be independent from AR-dependent signaling.

Defining the H3K27 acetylation status at the *ANKRD30A* locus in prostate cancer cell lines

Next, we defined the status of H3K27 acetylation at the *ANKRD30A* locus across four metastatic prostate cancer cell lines (PC-3, LNCaP, LAPC4, 22Rv1) and one normal prostatic cell line (RWPE-1) using ChIP-qPCR. First, we optimized the shearing settings for each cell line (e.g., cell number, number of sonication cycles) and analyzed the fragment size. We obtained chromatin fragments between 100-350 bp in size **(Figure S2-1A)**. After immunoprecipitation, we calculated the recovery of immunoprecipitated DNA by qPCR in all cell lines using the positive control GAPDH-TSS that specifically amplifies a genomic region containing the *GAPDH* promoter. The percentage of DNA recovery was of 2.4%, 1.2%, 2.8%, 2.0% and 3.4% for the RWPE-1, LAPC4, 22Rv1, LNCaP and PC-3, respectively **(Figure 2-6A)**. Following recovery analysis, we analyzed H3K27ac enrichment at the *ANKRD30A* locus by targeting specifically a region positive for *ANKRD30A*-associated H3K27ac in prostate cancer tissues **(Figure S2-1B)**. The H3K27ac mark was highly enriched in LAPC4 (fold change = 3120), RWPE-1 (fold change = 584) and 22Rv1 (fold change = 113), but also modestly enriched in LNCaP (fold change = 36) and PC-3 (fold-change = 38) (**Figure 2-6B**). This indicates that LAPC4 is the prostate cancer cell line with the strongest H3K27ac gain at the *ANKRD30A* locus.

***ANKRD30A* increases proliferation of metastatic prostate cancer cells**

To investigate the role of *ANKRD30A* in disease progression, we overexpressed the gene in prostate cancer cells using a Tet-On 3G inducible expression system. To establish a Tet-On 3G system that allows inducible *ANKRD30A* expression in the presence of Dox, we first cloned *ANKRD30A* into a TET-inducible vector (*pTRE3G-BI-mCherry*) to create a system in which the expression of *ANKRD30A* and mCherry will be co-induced by doxycycline (**Figure S2-2A**). We screened by restriction digestion the recombinant DNA for the correct insert (**Figure S2-2B**) and verified the sequence integrity of the cloned fragment by Sanger sequencing. Then, we tested our *pTRE3G* construct for functionality. We transiently co-transfected our customized response plasmid (*pTRE3G-BI-mCherry-ANKRD30A*) together with the regulator plasmid *pCMV-Tet3G* into HEK293FT and 22Rv1 and tested for *ANKRD30A* induction in the presence of doxycycline. After a Dox induction of 24-48 hours, cells transfected with our construct expressed higher levels of *mCherry* and *ANKRD30A* compared to non-induced transfected cells (**Figure S2-3A, B**). Next, we generated a drug-resistant double-stable *Tet3G* and *ANKRD30A*-expressing PC-3 cells.

Functional screening of double-stable PC-3 confirmed that cells expressed the Tet-On 3G transactivator and exhibited high levels of induction from the promoter P_{TRE3G} (**Figure S2-3C**). Further, we screened for a stable population of cells capable of high induction by sorting cells for *mCherry* and we validated the induction of *ANKRD30A* in double-stable PC-3 by quantitative PCR (**Figure 2-7A, B**). Finally, we observed that induction of *ANKRD30A* with Dox increased the proliferation of PC-3 cells (**Figure 2-7C)**, suggesting that *ANKRD30A* might play a role in prostate cancer progression by promoting cell proliferation.

2.5 Discussion

Advances in our understanding of prostate cancer detection, diagnosis, and management have changed clinical practice and reshaped guideline recommendations. The advent of active surveillance has changed the management of low-risk disease, while chemotherapy and hormonal therapy have improved the outcomes of patients with advanced disease [31]. However, prostate cancer remains the most common malignancy affecting men and the risk of over-diagnosing and overtreating many prostate cancer cases is real [24]. This poses many question marks and highlights our current inability to accurately stratify indolent prostate cancers from aggressive prostate cancers.

Epigenetic reprogramming of DNA *cis*-regulatory elements (CREs) is an emerging mechanism in prostate cancer initiation and progression. Consistently, we identified a differentially enriched H3K27ac site that predicts a regulatory element at the *ANKRD30A* locus in aggressive prostate tumors (**Figure 2-1**). CREs are modulated by epigenetic events (*e.g.*, histone modifications) that control chromatin accessibility. CREs include promoters, enhancers, and silencers. Active enhancers are typically marked by H3K27ac and associated with active transcription [32]. In particular, enhancer malfunction is a key process that drives the aberrant regulation of oncogenes in cancer and uncontrolled proliferation by locking the growth regulatory network to an 'ON' state. During normal development and homeostasis, enhancers are found close to many genes that specify cell fate [33]. In contrast, during tumorigenesis, they commonly form *de novo* near growth-related genes and oncogenes. For example, the *MYC* oncogene, which drives the growth of various cancers, acquires large super-enhancers that are tumor specific and absent from normal cells [34]. Moreover, the AR gene acquires an enhancer upstream of its locus which drives advanced prostate

cancer and desensitizes cells to hormonal therapy [5]. Here, we predicted the activation of an enhancer element at the *ANKRD30A* locus in aggressive prostate tumors (**Figure 2-1, 4**). Although *ANKRD30A* is not considered an oncogene, it is defined as a breast cancer antigen and regarded as a putative transcription factor [25]. It is composed of 37 exons and is localized to chromosome 10. *ANKRD30A* has an open reading frame of about 4.5 kb and encodes a peptide of 158 kDa. While it is mostly restricted to the mammary epithelium, altered expression levels of *ANKRD30A* are associated with breast cancer progression [26]. While mRNA was previously detected in testis and some prostate cancer samples by PCR [27], the role of *ANKRD30A* in prostate cancer remains unexplored. In this study, we determined *ANKRD30A* expression levels across multiple prostate cancer cell lines (**Figure 2-5**). *ANKRD30A* mRNA was mostly detected in LAPC4 and 22Rv1, so these cell lines will be used in future experiments to test the functional role of *ANKRD30A* expression in prostate cancer. Of note, we used transcriptomic data and implicitly assumed that changes in mRNA expression corresponded to changes in protein levels. Yet genome-wide correlations between mRNA and protein expression levels appear to be poor. This creates concern for inferences from only mRNA expression data [35]. We attempted to quantify ANKRD30A protein levels by western blot in multiple prostate cancer cell lines to support our RNA-seq data. Up to date, only a few commercial antibodies targeting ANKRD30A are available. We tested two antibodies for appropriate protein recognition in prostate cancer cells lines. However, immunoblotting of both antibodies showed lack of protein recognition and various nonspecific bands, so these antibodies were considered unreliable for analysis (**Figure S2-4**).

Tumor cells grow rapidly and often have defects in DNA repair mechanisms and in the regulation of the cell cycle. As a result, they typically carry a large mutational load. Somatic non-coding mutations are common in sporadic tumors. They commonly give rise to a greatly increased transcription rate of the affected target genes, providing tumor cells with a strong growth advantage. Somatic mutations can alter enhancer activity in *cis* by increasing the enhancer copy number to amplify its output. Many of the copy number variations affect key oncogenes, but some involve only the non-coding genome [32]. Although enhancers can be activated by direct mutations that function in *cis*, a more common mechanism of oncogenic enhancer activation involves *trans*-acting epigenetic mechanisms [32]. One mechanism that can contribute to epigenetic dysregulation of enhancer activity involves changes in the activity of histone-modifying

enzymes, such as histone acetyltransferases and histone deacetylases (HDACs). Histone modifications modulate the accessibility of chromatin and transcriptional activity [36]. They can also contribute to the onset and progression of prostate cancer [37]. Recent technical advances significantly increased our understanding of the genome-wide epigenetic regulation of gene expression in prostate cancer. H3K27ac HiChIP in prostate cell lines recently identified risk genes for prostate cancer susceptibility, which provide potential insights into causal mechanisms [38]. Interestingly, with H3K27ac ChIP-seq data from 14 LuCaP prostate cancer PDXs, we illustrated that *ANKRD30A* expression correlated with the enrichment of the somatic H3K27ac mark (**Figure 2-4)**. High *ANKRD30A* expression was also associated with poor clinical outcomes (**Figure 2-2**). Moreover, we revealed that the H3K27ac mark was enriched in LAPC4 and 22Rv1 prostate cancer cell lines (**Figure 2-6**). This suggests an *ANKRD30A*-associated enhancer activated epigenetically in a subset of prostate cancers that might predict disease outcomes. Again, this transcriptomic data implicitly assumes that changes in mRNA expression is indicative of changes in protein expression. In this regard, patient tissue microarrays (TMA) should be used to investigate changes in protein levels to directly infer a correlation between ANKRD30A and patient clinical outcomes. It is not presently clear if H3K27ac enrichment at the *ANKRD30A* locus contributes directly to prostate cancer tumorigenesis. ChIP-sequencing should be performed in the future to better profile the H3K27ac mark in prostate cancer cell lines. However, given the important role of histone modifications in regulating CREs activity, it is likely that the somatic H3K27ac gain at the *ANKRD30A* locus contributes to cancer development in a subset of aggressive tumors by altering the activity of an enhancer element.

Similarly to cancer-associated alterations in histone modifications, changes in the activity of DNA methylation via methyltransferases also reshape the activity of CREs [32]. Prostate cancer cells often display global hypomethylation and specific hypermethylation at promoter regions of tumor suppressor genes compared with normal cell [29]. Interestingly, we observed hypomethylation of the *ANKRD30A* promoter in prostate cancer that was associated with poorer clinical outcomes (**Figure 2-3**). Considering that promoter hypomethylation is associated with gene activation [28], the above results indicate that hypomethylation at the promoter of *ANKRD30A* correlates with higher gene expression in prostate tumors as well as disease recurrence. Thus, the promoter methylation status of *ANKRD30A* and/or a somatic H3K27ac gain regulates *ANKRD30A*

expression that may contribute to cancer development in a subset of aggressive tumors. We plan on using CRISPR-dCas9 systems to modulate the activity of the regulatory element at *ANKRD30A* in cancer cell lines to test its functional role in regulating *ANKRD30A* itself and perhaps nearby genes.

Finally, we observed that overexpression of *ANKRD30A* in PC-3 increases cell proliferation (**Figure 2-7)**. Additional *in vitro* assays (e.g., migration, invasion assays) should be performed in the future to better characterize the functional role of *ANKRD30A* in prostate cancer progression as we provided correlative evidence between *ANKRD30A* expression and prostate cancer aggressiveness (**Figure 2-3**). An important future direction is the functional validation our *ANKRD30A*-associated regulatory element using epigenetic perturbation experiments. With the recent discovery of the CRISPR–dCas9 perturbation systems, it is now possible to identify and test the functional relevance of regulatory elements observed in tumor cells. Several laboratories are now using CRISPR–dCas9 to repress or activate cancer-associated CREs in cancer cell lines to test their functional role in regulating nearby genes [32]. In the future, our Tet-3G-expressing stable cell lines will be used for inducible CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa) to regulate and functionally validate the identified regulatory element at the *ANKRD30A* locus. These technical approaches will further help expose the epigenetic events and regulatory elements that might be driving the progression of prostate cancer to a metastatic and lethal disease.

2.6 Conclusion

In summary, we identified a somatically acquired regulatory element at the *ANKRD30A* locus in aggressive prostate cancer which supports the emerging role of epigenetic alterations as highly recurrent drivers of prostate cancer. We showed that *ANKRD30A* was aberrantly expressed in a subset of prostate cancers and associated with accelerated disease recurrence. We observed that the promoter of *ANKRD30A* was globally hypomethylated. Gain of H3K27ac at the *ANKRD30A* locus was also identified in ~36% of LuCaP patient derived xenografts and correlated with transcript expression. We defined H3K27ac status at the *ANKRD30A* locus in prostate cancer cells. Finally, we also revealed that overexpression of *ANKRD30A* increases prostate cancer cell proliferation. Collectively, our observations underscore the importance of epigenomic profiling in

prostate cancer and the value of genome editing to functionally characterize gene regulatory elements. Further studies are needed to elucidate the role of our identified regulatory element in prostate cancer. It is critical to modulate and functionally validate the *ANKRD30A*-associated regulatory element with CRISPR perturbation systems. If confirmed as a prostate cancer risk locus, testing for the activity of this regulatory element could be used to identify prostate cancer patients who would benefit from more aggressive therapy regimens.

2.7 Disclosure of potential conflicts of interests

The authors report no potential conflicts of interest.

2.8 Acknowledgments

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2.9 Figures

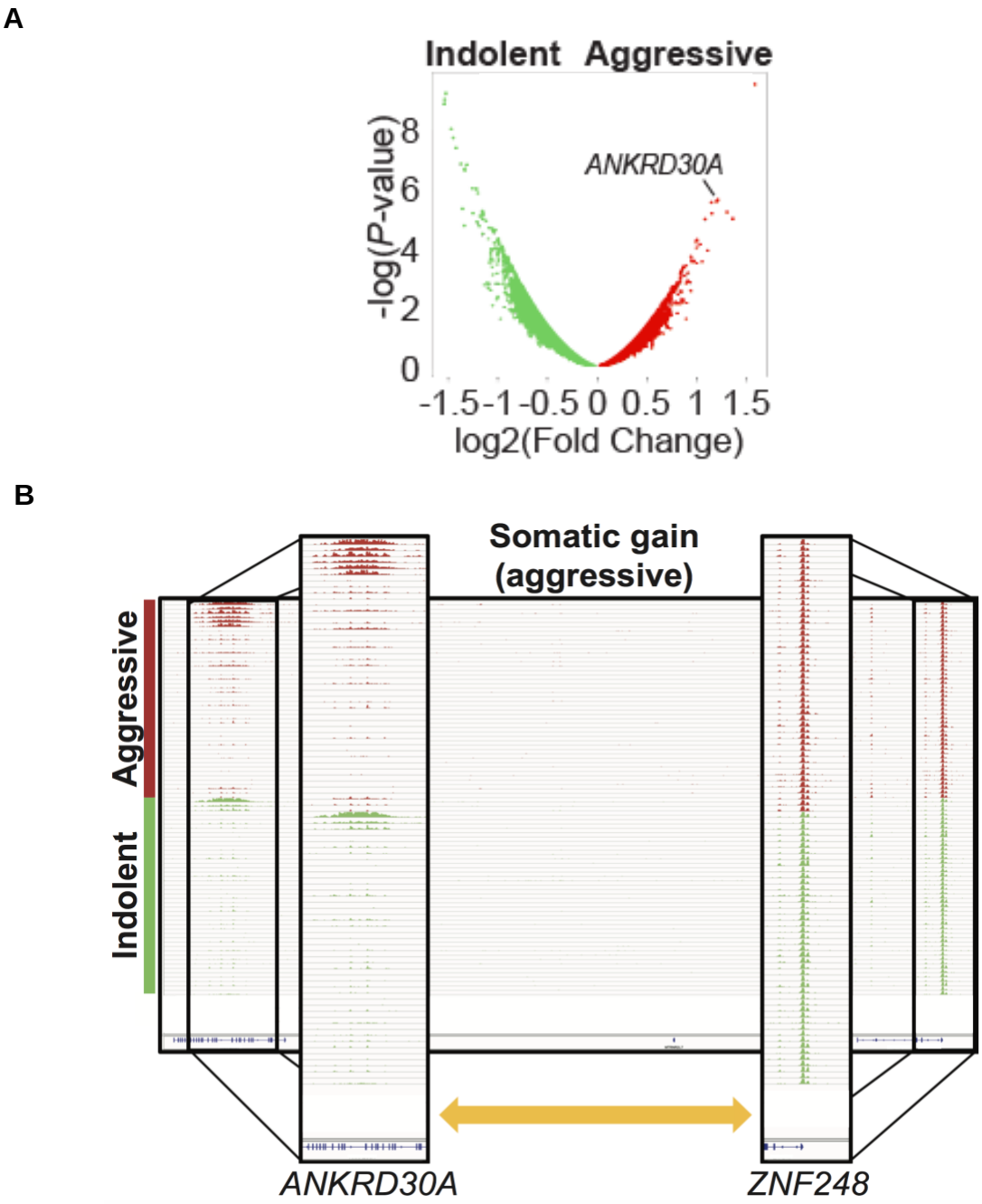

Figure 2-1.The H3K27 acetylation mark is enriched within aggressive tumors at the *ANKRD30A* locus.A. Volcano plot of the association results of gained/lost somatic regulatory elements between indolent and aggressive tumors. **B.** Analysis of a ChIP-seq dataset comparing the H3K27ac mark in 45 indolent (green) and 45 aggressive (red) prostate cancer cases (log2[fold change] =1.22, P=2.22x10-6) [6].

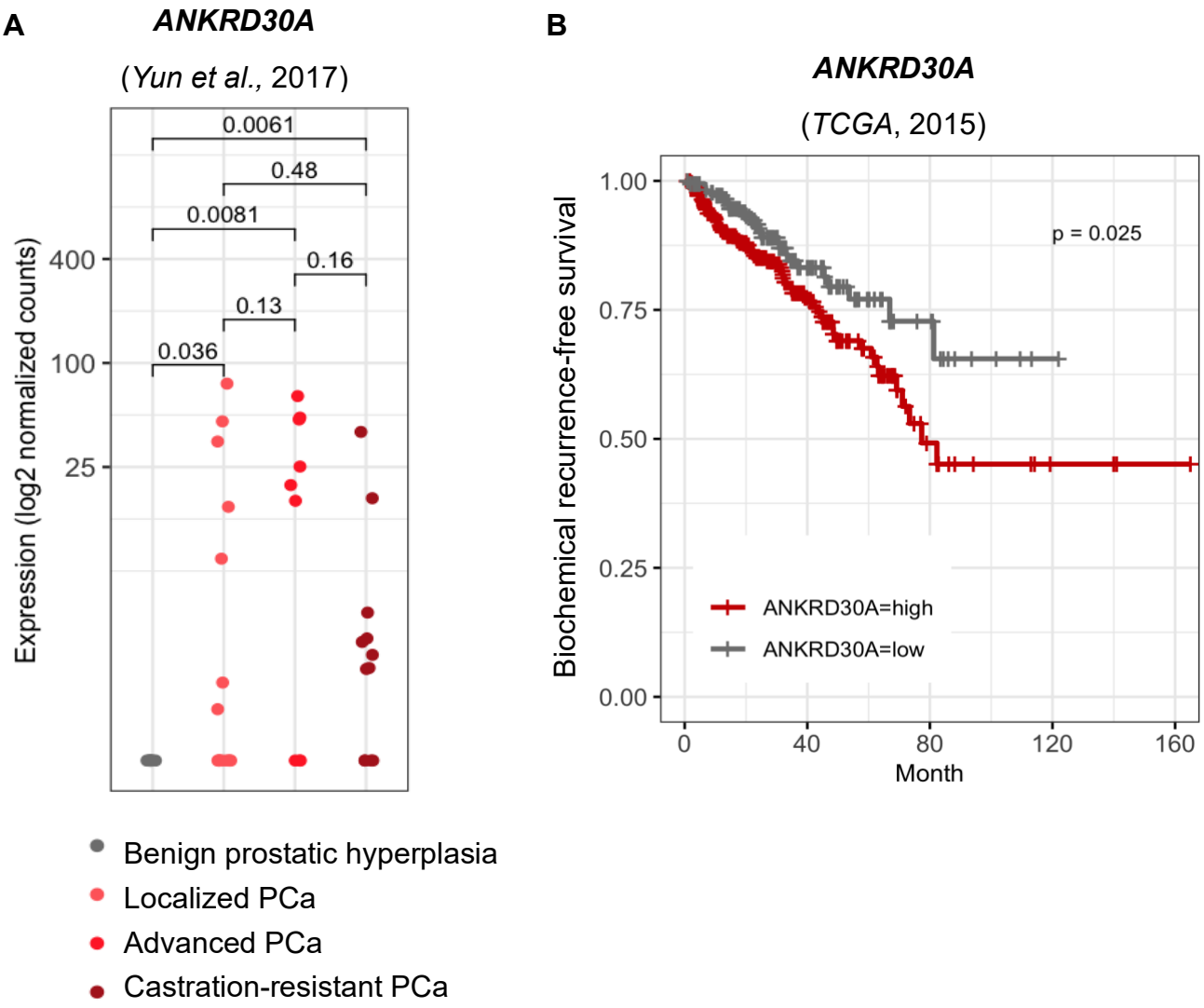

Figure 2-2. *ANKRD30A* is increased in early-stage and advanced prostate cancer and is associated with disease recurrence. A. Dot plot showing *ANKRD30A* expression (log2 normalized counts) in different prostate tissues (benign prostatic hyperplasia n=8, localized PCa n=16, advanced PCa n=9 and castration resistant PCa n=12 from the Yun *et al.* dataset (GSE80609) [7]. Two-tailed Student's *t*-test was applied. **B.** Kaplan-Meier plot showing biochemical recurrence (BCR)-free survival for high and low *ANKDR30A* expression in 91 recurred patients and 397 disease-free patients from the TCGA-PRAD (provisional) cohort [8] using the maximally selected rank statistic.

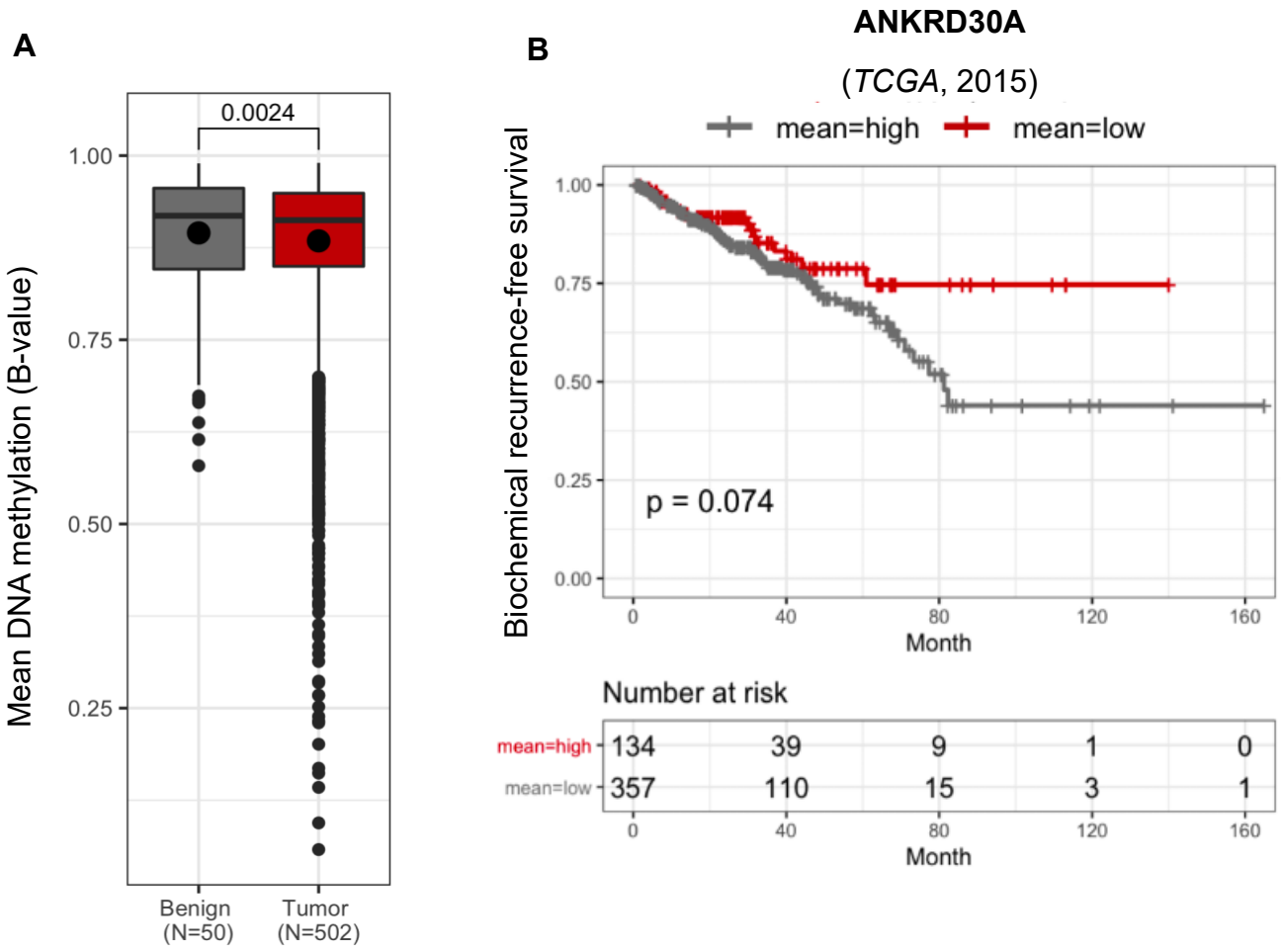

Figure 2-3. A. Promoter region of *ANKRD30A* is globally hypomethylated in prostate cancer and trends towards accelerated disease recurrence. Box plot comparing mean DNA methylation (B-values) at the *ANKRD30A* promoter region between 50 benign tissues (grey) and 502 tumor tissues (red) from the TCGA-PRAD (provisional) cohort (Cell, 2015). Student's t-test was applied. **B.** Kaplan-Meier showing biochemical recurrence-free survival relative to the mean methylation value at *ANKDR30A* in 488 patients from the TCGA-PRAD (provisional) cohort (Cell, 2015) using the maximally selected rank statistic. Low methylation is in grey and high methylation is in red.

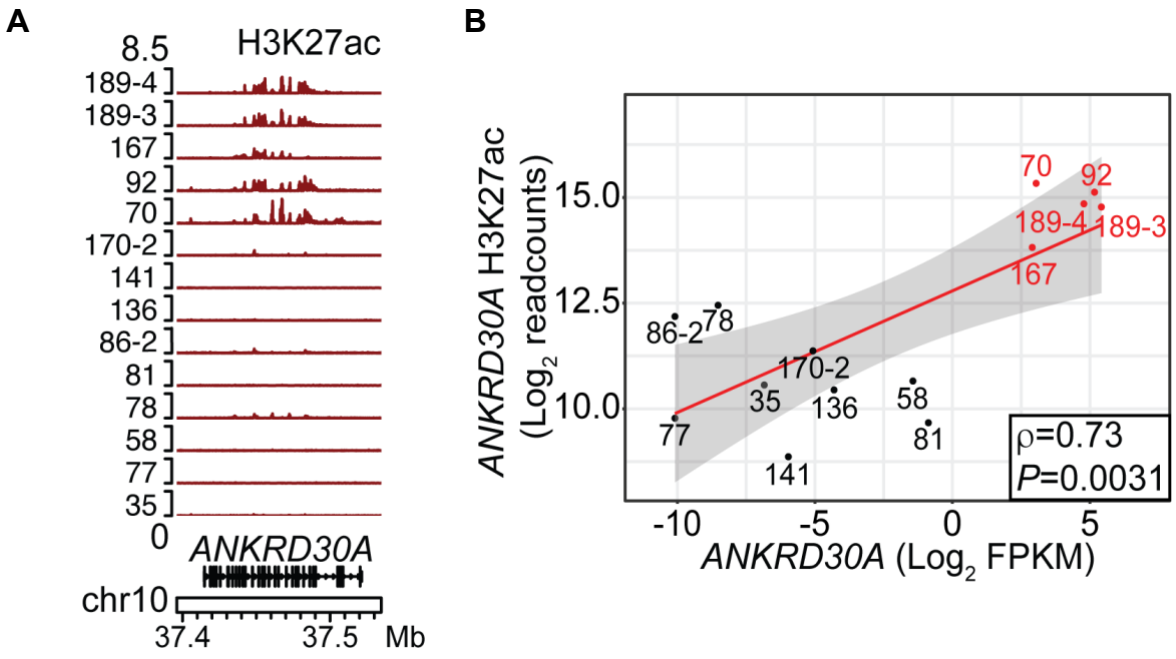

Figure 2-4. Somatic H3K27 acetylation gain at *ANKRD30A* correlates with transcript expression in LuCaP PDXs. A. ChIP-seq analysis comparing the H3K27ac mark at the *ANKRD30A* locus of 14 LuCaP prostate cancer patient-derived xenografts. **B.** *ANKRD30A* expression (log2 FPKM) relative to *ANKRD30A* H3K27ac (log2 readcounts) in 14 LuCaP prostate cancer patient-derived xenografts (p-value=0.0031).

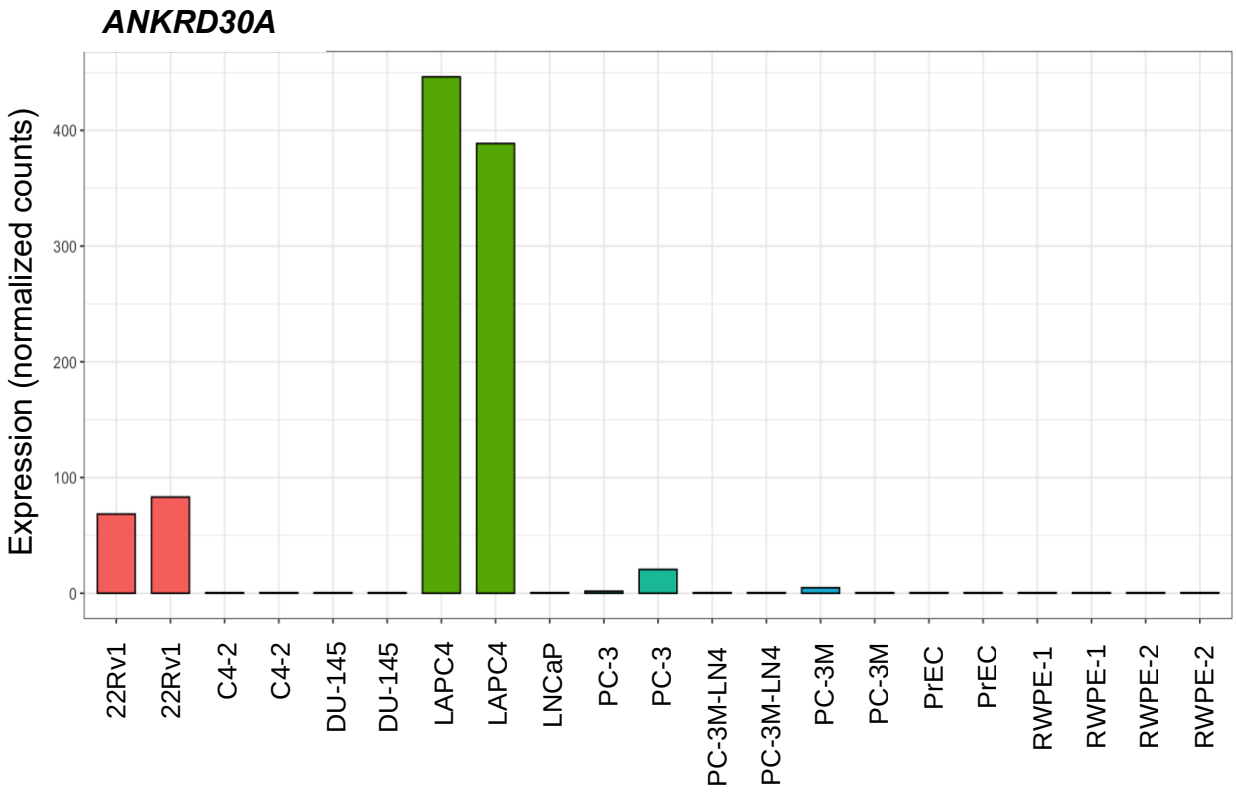

Figure 2-5. *ANKRD30A* is aberrantly expressed in LAPC4 and 22Rv1 prostate cancer cell lines. RNA-seq analysis showing *ANKRD30A* expression (normalized counts) in different human prostate cancer and normal prostatic cell lines (n=2 per cell line, LNCaP n=1).

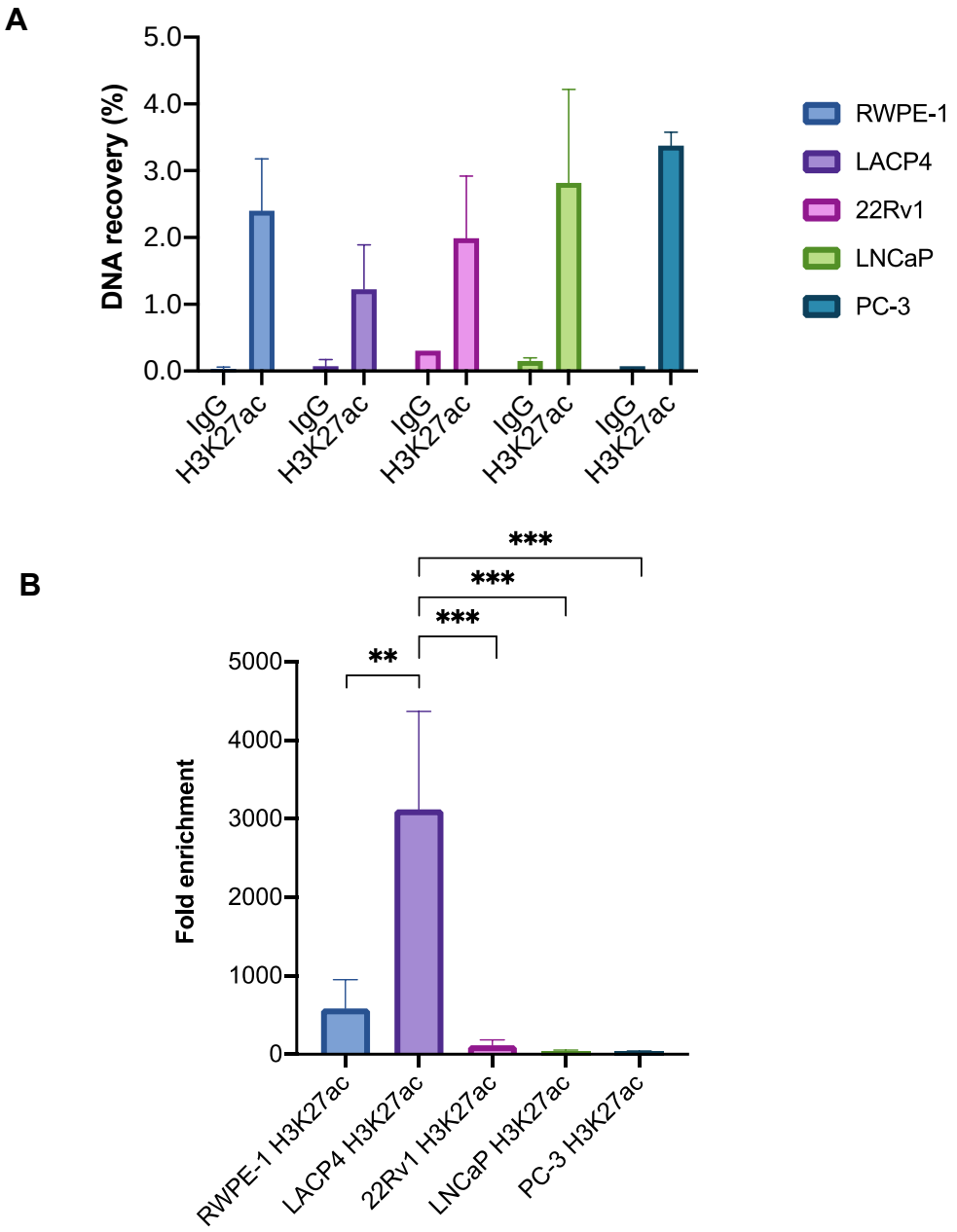

Figure 2-6. Status of H3K27 acetylation at the *ANKRD30A* locus in prostate cancer cell lines. **A.** DNA recovery analysis of ChIP for H3K27ac in RWPE-1, LAPC4, 22Rv1, LNCaP and PC-3 cell lines. The relative amount of immunoprecipitated DNA compared to Input DNA for the control region GAPDH TSS (% of recovery) was calculated using the following formula: % recovery = 2^[(Ctinput-log2(5))-Ctsample]x100 where log2(5) accounts for the input dilution (5x). Immunoprecipitated IgG is the negative control. Error bars indicate SEM (n=2). **B.** ChIP-qPCR for the histone mark H3K27ac showing *ANKRD30A* in RWPE-1, LAPC4, 22Rv1, LNCaP and PC-3 cell lines. Fold enrichment was calculated relative to IgG. Error bars indicate SEM (n=3). $^{**}P < 0.01$, $^{***}P < 0.001$. One-way ANOVA with Tukey's multiple comparisons test were applied.

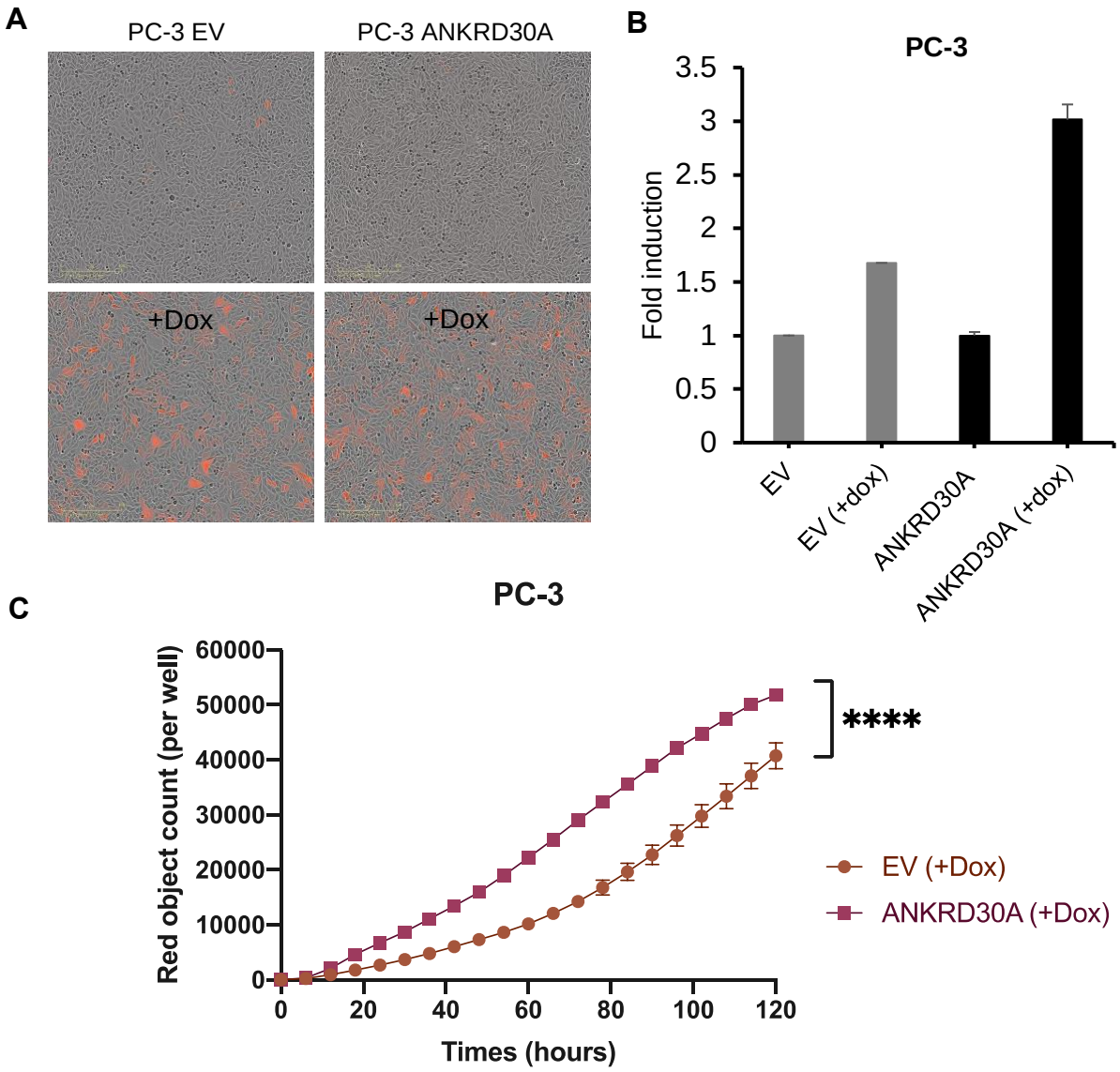

Figure 2-7. Inducible expression of ANKRD30A increases cell growth. A. Double-stable PC-3 cultured in the presence of Dox in a 6-well plate. PC-3 expressing the empty vector (EV) was used as control. Cells that were induced with Dox (2 ug/mL) expressed mCherry and are shown in red. **B.** qPCR analysis showing induction of *ANKRD30A* mRNA expression with Dox in double-stable PC-3. Gene expression was normalized to *GAPDH*, and fold induction was calculated relative to no Dox conditions. **C.** Proliferation of double stable PC-3 in the presence of Dox. Cells were seeded at 5,000 cells/well in a 96-well plate. Dox (2 ug/mL) was added at day 0. Cells that were induced with Dox expressed mCherry and were counted (red object count). Experiment was repeated 3 times with at least 6 technical replicates (experimental replicates provided as **Supplementary Data 1**). Error bars indicate SEM. **** $P < 0.01$. Two-way ANOVA with Sidak's multiple comparisons test were applied.

2.10 Supplementary figures

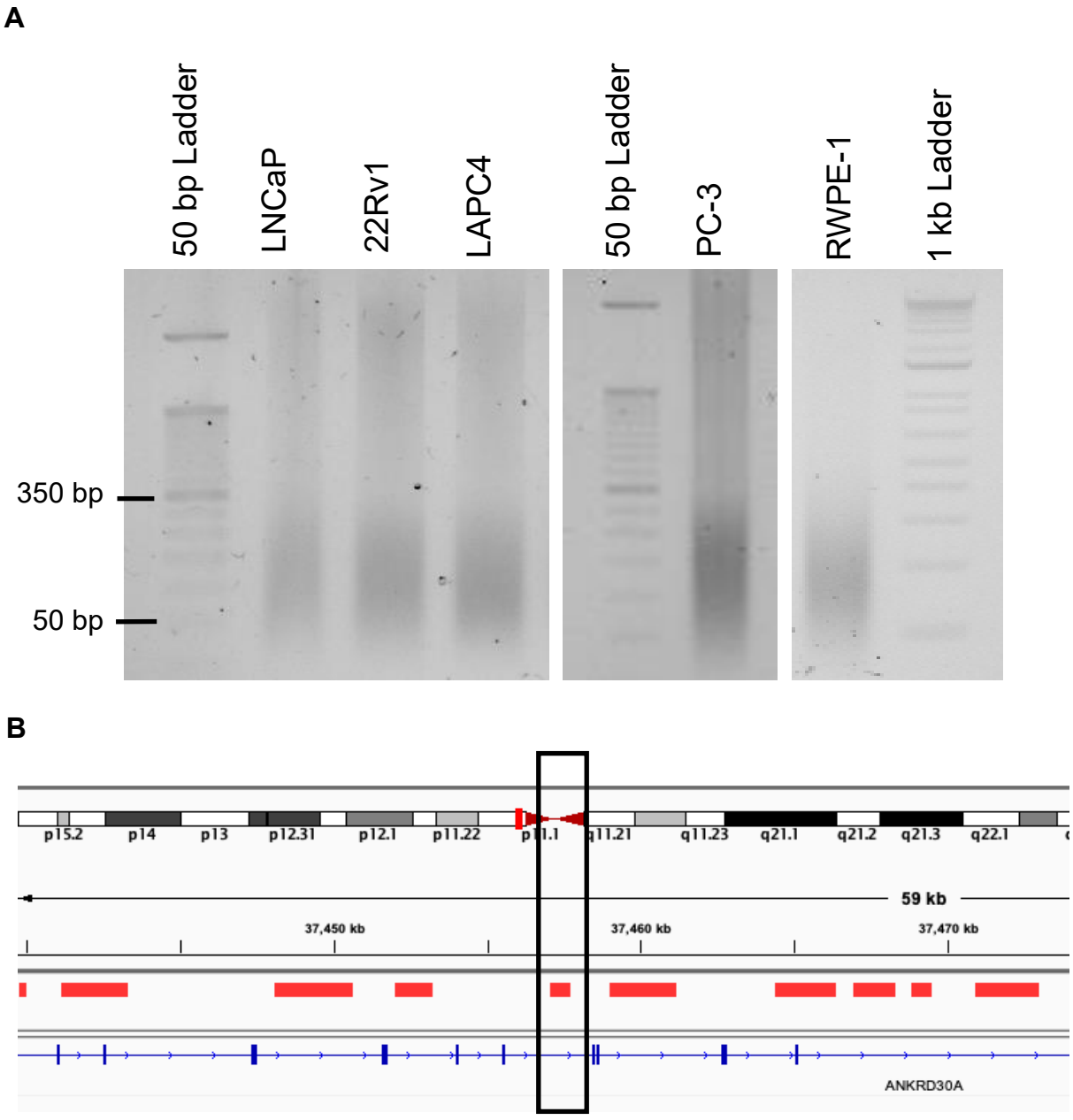

Figure S2-1. Shearing assessment of prostate cancer cell lines. A. Approximately 300 ng of de-crosslinked DNA was loaded in each well. Fragment size was analyzed on a 2% agarose E-gel. **B.** Map view of *ANKRD30A*-associated H3K27ac peaks in prostate cancer tissues. Target H3K27ac peak at chr10:37457090-37457725 used for ChIP-qPCR is highlighted in black.

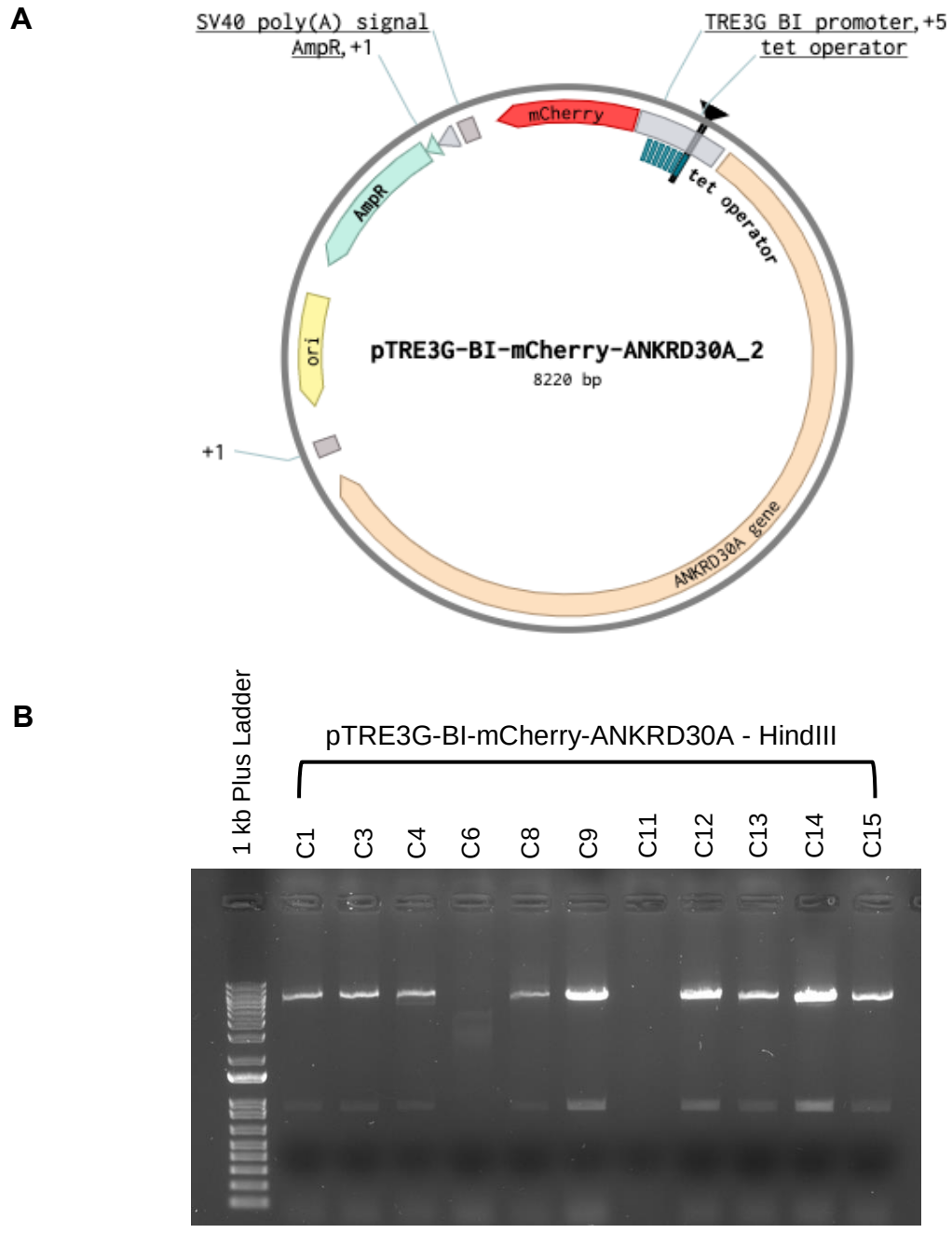

Figure S2-2. Cloning of *pTRE3G-BI-mCherry-ANKRD30A*.A. Map of pTRE3G-BI-mCherry-ANKRD30A. Plasmid was designed with Benchling. **B.** Digestion with HindIII of transformed clones with *pTRE3G-BI-mCherry-ANKRD30A*. Clones 1, 3, 4, 8, 9, 12,13, 14 and 15 expressed the plasmid.

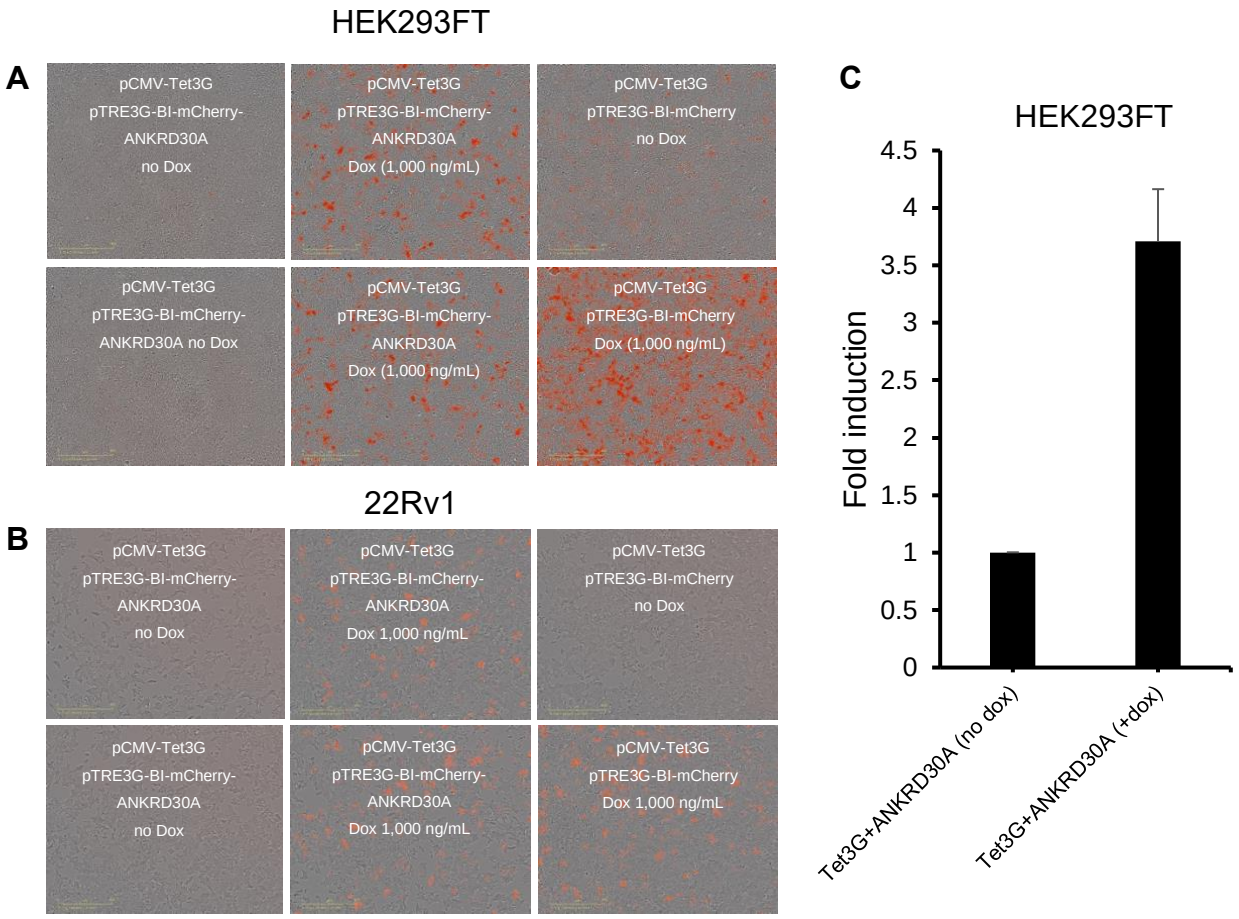

Figure S2-3. Pilot testing Tet-based induction of *pTRE3G-BI-mCherry-ANKRD30A* construct. A-B. The regulator plasmid pCMV-Tet3G and customized response plasmid *pTRE3G-BI-mCherry-ANKRD30A* were co-transfected into HEK293FT and 22Rv1 in a 6-well plate. The empty vector (EV) plasmid *pTRE3G-BI-mCherry* was used as control. Cells that were induced with Dox (1,000 ng/mL) expressed mCherry and are shown in red. **C.** After 24 hours, cell pellets were harvested and induced expression levels to uninduced expression levels was compared using qPCR for *ANKRD30A*. Gene expression was normalized to *GAPDH*, and fold induction was calculated relative to no Dox condition. Error bars indicate SEM (n=2).

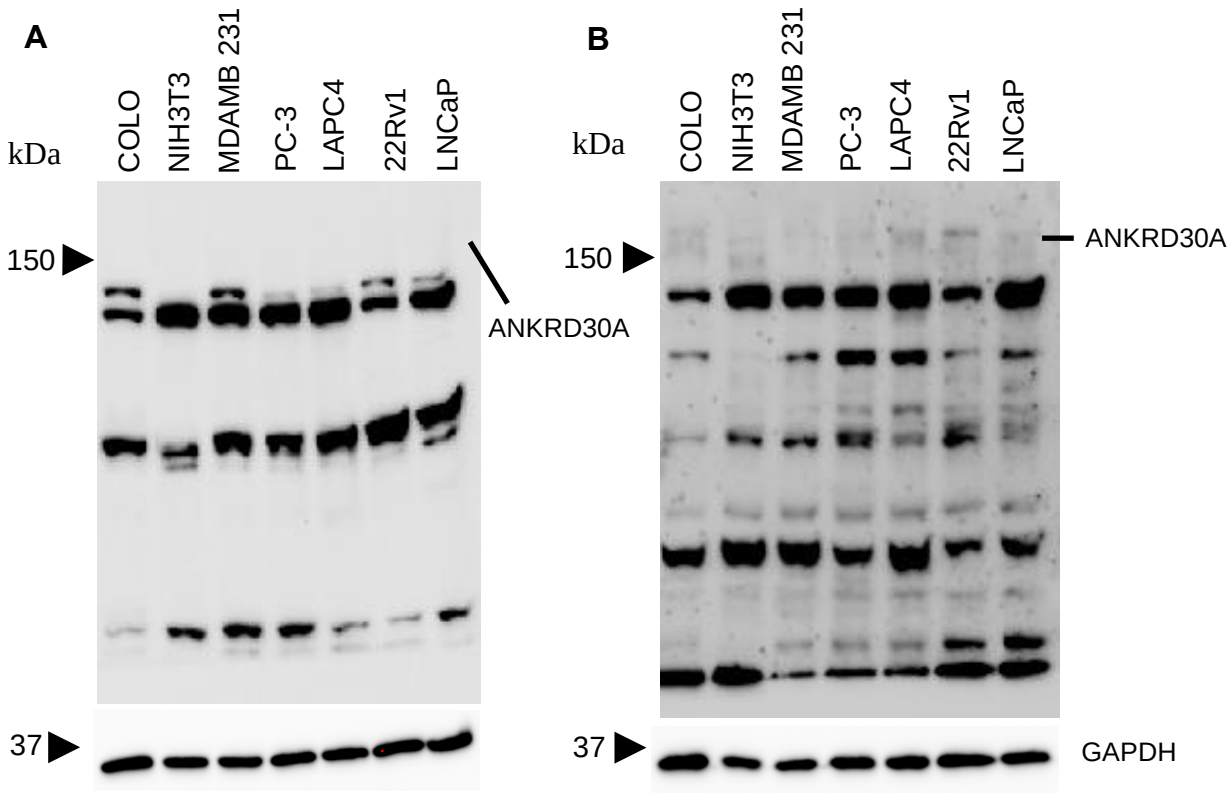

Figure S2-4. Testing two ANKRD30A antibodies. Protein was collected from COLO (human colon cancer cell line), NIH3T3 (mouse embryonic fibroblast cell line), MDAMB231 (human breast cancer cell line), and PC-3, LAPC4, 22Rv1 and LNCaP (prostate cancer cell lines). Western blot of proteins was probed for ANKRD30A (**A**) with Aviva Biosystems antibody and (**B**) with Invitrogen antibody. GAPDH was used as a loading control. The expected molecular weight of ANKRD30A is 158 kDa. COLO, NIH3T3 and MDAMB231 were used as positive controls for ANKRD30A protein expression according to manufacturer's product sheets.

2.11 Supplementary table

Target gene	Forward sequence	Reverse sequence
ANKRD30A (ChIP-qPCR)	gggtgcctgtagtcccagctac	ggctcaccacaagctccg
ANKRD30A (qPCR)	tggttttctgaaggctccctgca	aggcagatggcttctcgggagg
GAPDH (qPCR)	ctttcccgcctctcagcctttg	agatggggaattggagccggag

Table S2-1. List of customized primers used for ChIP-qPCR and qPCR.

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Chapter 3: General discussion

3.1 Summary of findings

In this study, we identified an aberrantly activated regulatory element at the *ANKRD30A* locus in aggressive prostate cancer. Investigation of a transcriptomic dataset confirmed that the *ANKRD30A* gene is aberrantly expressed in a subset of prostate cancers. Interestingly, high *ANKRD30A* expression was associated with a faster time to BCR in a validation cohort. We also observed that the promoter of *ANKRD30A* was globally hypomethylated. Gain of H3K27ac at the *ANKRD30A* locus was also identified in ~36% of LuCaP patient derived xenografts of mCRPC and correlated with transcript expression. *In vitro*, we revealed that *ANKRD30A* is amplified in LAPC4 and 22Rv1. We also defined the status of H3K27 acetylation at the *ANKRD30A* locus in multiple prostate cancer cell lines (PC-3, LAPC4, LNCaP, 22Rv1) and a normal prostatic cell line (RWPE-1). Finally, we observed that overexpression of *ANKRD30A* increased proliferation of a metastatic prostate cancer cell line, but the functional role of *ANKRD30A* in prostate cancer progression requires further characterization, which will be discussed in this chapter. Taken together, our data suggest that activation of *ANKRD30A* might contribute to the development of aggressive prostate cancer and could potentially be used as an epigenetic biomarker to identify patients with aggressive prostate cancer.

3.2 Somatically acquired regulatory elements in aggressive prostate cancer

Prostate cancer development is initiated by the rewiring of the normal prostate transcriptional network with deregulated expression or mutation of key transcription factors [49]. Importantly, the binding of these transcription factors to DNA *cis*-regulatory elements (CREs) is regulated by epigenetic events, including histone modifications, that control chromatin accessibility. CREs include promoters, enhancers, and silencers. Acetylation of lysine 27 on histone H3, or H3K27ac, is associated with active transcription and aberrant H3K27ac enrichment at enhancer elements is a hallmark of cancer [158, 159]. Therefore, CREs and epigenetic changes are intertwined, and both are important drivers of prostate cancer initiation and progression [49, 112]. For example, a somatically acquired AR enhancer, marked by H3K27ac, was recently identified as a noncoding driver in advanced prostate cancer [84]. Epigenomic reprogramming is an emerging mechanism in prostate cancer development and progression, but only two studies have focused on the

identification of CREs, demarcated by H3K27ac, in prostate tumors [111, 155]. Moreover, the majority of prostate cancers are indolent and inconsequential to the patient while a minority are aggressive and lethal if detected too late or left untreated. This can make decision-making for individual patients difficult and highlights the critical need for more robust risk stratification strategies [160]. Thus, in this study, we analyzed the Stelloo *et al.* epigenomic dataset with a goal to identify somatic epigenetic events associated with CREs in aggressive prostate cancer. In selecting high-confidence H3K27ac peaks that were reliably identified in multiple samples, we identified differences in epigenetic marks between aggressive and indolent prostate cancer cases and defined the *ANKRD30A* locus as a genomic region of interest. Previously, Stelloo *et al.* revealed distinct prostate cancer subtypes with signature clusters, but they were unable to identify somatic epigenetic events in aggressive prostate cancer [111]. In our study, the epigenetic event identified is an enriched H3K27ac region at the *ANKRD30A* locus in aggressive prostate tumors (**Figure 2-1**). This indicates a regulatory element at the *ANKRD30A* locus. The nucleosomes that flank the nucleosome-free regions of active enhancers are typically marked by the histone modification H3 lysine 27 acetylation (H3K27ac) [150]. Thus, we also predict the activation of an enhancer element at the *ANKRD30A* locus in aggressive prostate tumors (**Figure 2-1, 4**). Enhancer deregulation is a key process in cancer predisposition. Activation of putative enhancer elements via H3K27ac can drive the aberrant expression of oncogenes in cancer by locking the growth regulatory network to an 'ON' state, thereby driving uncontrolled proliferation. During normal development and homeostasis, enhancers are found close to many genes that specify cell fate [153]. In tumorigenesis, enhancers commonly form *de novo* near growth-related genes and oncogenes [154]. For example, the AR gene acquires an enhancer upstream of its locus which drives advanced prostate cancer [84].

A common mechanism of oncogenic enhancer activation involves epigenetic events that function in *trans* [150]. One such epigenetic event involves histone modifications via changes in the activity of histone-modifying enzymes, such as histone acetyltransferases and histone deacetylases (HDACs). These histone modifications impact the accessibility of chromatin and transcriptional activity [161], and also contribute to prostate cancer initiation and progression [162]. In line with the role of histone modifications in prostate cancer progression, we supported our previous findings using H3K27ac ChIP-seq data from 14 LuCaP prostate cancer PDXs, which also

illustrated that *ANKRD30A* expression and H3K27ac show comparable profiles (**Figure 2-4**). Furthermore, we confirmed with ChIP-qPCR that H3K27ac is highly enriched in LAPC4, prostate cancer cell line. This further suggests an *ANKRD30A*-associated regulatory element that is epigenetically activated in a subset of prostate cancers. The mechanism activating the *ANKRD30A*-associated regulatory element remains unknown. Although we showed that differences in the level of H3K27ac enrichment are associated strongly with *ANKRD30A* transcript expression changes (**Figure 2-4**), we have yet to investigate the potential effect of H3K27ac on transcription factor binding sites prediction and *ANKRD30A* RNA abundance changes. Performing ChIP-seq and CRISPR-dCas9-VPR / CRISPR-dCas9-KRAB on normal and prostate cancer cell lines will enable to explore the regulatory mechanisms associated with modified H3K27ac at *ANKRD30A* gene expression and the functional role of H3K27ac in *ANKRD30A* expression, respectively. For now, we can hypothesize that the *ANKRD30A* region marked by H3K27ac is enriched for a transcription factor binding motif of a transcription factor that is involved in cell proliferation and survival.

Furthermore, we demonstrated *ANKRD30A* is significantly increased in early-stage prostate cancer, advanced disease and castration-resistant prostate cancer compared to benign prostatic hyperplasia. However, *ANKRD30A* was not differentially expressed between early and late-stage prostate cancer (**Figure 2-2A**). Thus, we can speculate that the acetylation of H3K27 at the *ANKRD30A* locus is occurring early during cancer progression and in a subset of prostate cancer cases. According to our RNA-seq data, the three normal prostate cancer cell lines (PrEC, RWPE-1, RWPE-2) don't overexpress *ANKRD30A* transcripts. Thus, it is unlikely that *ANKRD30A* levels in normal prostatic cells could indicate a predisposition factor to prostate cancer. However, RNA profiling of additional normal prostatic cell lines and normal prostate tissues is needed to further substantiate our findings. Interestingly, while the H3K27ac mark at *ANKRD30A* was highly enriched in LAPC4 (fold change = 3120), it was also enriched in RWPE-1 (fold change = 584), indicating that metastatic prostate cancer cell lines and normal prostate cancer cell lines can have acetylated H3K27 at this specific region (**Figure 2-6B**). This highlights the need to explore the role of H3K27ac in *ANKRD30A* expression.

It is not presently clear if H3K27ac enrichment at the *ANKRD30A* locus contributes directly to prostate cancer tumorigenesis. However, given the important role of histone modifications in

CREs regulation and transcriptional activation, it is likely that enrichment of H3K27ac at the *ANKRD30A* locus contributes to prostate cancer development in a subset of tumors by altering the activity of an enhancer element via epigenetic reprogramming. To follow with the transcriptional profiling of multiple prostate cancer cell lines that was already performed by our laboratory, we will also perform matched H3K27ac (ChIP-seq) and open chromatin (ATAC-seq) profiling. We intend to use the profile of RWPE-1, a normal prostatic cell line, as background to identify tumor-specific elements associated with prostate cancer aggressivity.

Similarly to cancer-associated alterations in histone modifications, changes in the activity of DNA methylation via methyltransferases also reshape the activity of CRE-associated genes [150]. Global hypomethylation is frequently observed in prostate cancer cells. Tumor suppressor genes also often display promoter specific hypermethylation compared to normal cells [163]. Interestingly, we observed global hypomethylation of the *ANKRD30A* promoter in prostate cancer that was associated with poorer clinical outcomes (**Figure 2-3**). Considering that promoter hypomethylation is associated with gene activation [164], our results indicate that hypomethylation at the promoter of *ANKRD30A* correlates with higher gene expression in prostate tumors as well as disease recurrence. Thus, promoter hypomethylation of *ANKRD30A* and somatic H3K27ac gain at the *ANKRD30A* locus converge to regulate its expression and possibly contribute to prostate cancer progression in a subset of aggressive tumors. We will further use CRISPR-dCas9 systems to repress and activate the predicted regulatory element at *ANKRD30A* locus in cancer cell lines to test its functional role in regulating *ANKRD30A* itself and perhaps nearby genes.

3.3 Investigating the role of *ANKRD30A* in prostate cancer

The role *ANKRD30A* gene is not well defined. So far, most of the work on *ANKRD30A* has focused on breast cancer because it was initially defined as a breast cancer antigen. This gene is localized to chromosome 10 and consists of 37 exons. It has an open reading frame of about 4.5 kb and encodes a peptide of 158 kDa. In normal tissues, *ANKRD30A* expression through promoter hypermethylation is restricted to placenta, brain, breast, testis, and sperm, with no expression in other tissues including the prostate [165, 166]. Altered expression levels of *ANKRD30A* are associated with breast cancer progression [167], but the role of *ANKRD30A* in prostate cancer

remains largely unexplored. In our study, analysis of a prostate transcriptomic dataset revealed that *ANKRD30A* expression is silenced in BPH tissues but is significantly expressed in a subset of prostate cancer samples (**Figure 2-2A**). Kaplan-Meier curves further revealed that high *ANKRD30A* expression correlated with shorter time to BCR (**Figure 2-2B**), suggesting that *ANKRD30A* is linked to an aggressive subtype of primary prostate cancer and associated with disease recurrence. Interestingly, *ANKRD30A* is regarded as a putative transcription factor; it encodes an uncharacterized DNA-binding transcription domain previously associated with metastatic breast cancer progression [168-170]. In accordance with a role in metastatic progression, somatic H3K27ac gain at *ANKRD30A* locus was identified in approximately 36% of AR-positive mCRPC patient derived xenografts models established from prostate cancer metastases (LuCaP series [171]) (**Figure 2-4A)**. As previously mentioned, this somatic gain also correlated with *ANKRD30A* transcript expression (**Figure 2-4B)**. Further, using RNA-seq we observed an amplification of the *ANKRD30A* gene in LAPC4 and 22Rv1 prostate cancer cell lines (**Figure 2-5**). LAPC4 cells, which expressed the most *ANKRD30A*, are sensitive to androgens, but the other cell lines not expressing or lesser amount of *ANKRD30A* (PC-3, PC-3M, PC-3M LN4, DU-145, C4-2) are androgen-insensitive and castration-resistant. Interestingly, LNCaP cells are androgen-sensitive but didn't express *ANKRD30A* while the CRPC 22Rv1 expressed moderate levels. This suggests that *ANKRD30A* expression might be independent of AR transcriptional signaling. Furthermore, LAPC4 and 22Rv1 will be used in future experiments to test the functional role of *ANKRD30A* expression in prostate cancer. Indeed, to presume that *ANKRD30A* itself is the target of H3K27ac-mediated activation, more studies are needed.

Of note, we used transcriptomic data and implicitly assumed that changes in mRNA expression is indicative of changes in protein expression. Yet, mRNA and protein expression levels are not always correlated. Therefore, this is problematic for inferences from only mRNA expression data [172]. We attempted to quantify ANKRD30A protein levels by western blot in multiple prostate cancer cell lines to corroborate our RNA-seq data. Up to date, only a few commercial antibodies targeting ANKRD30A are available. We tested two antibodies for appropriate protein recognition in prostate cancer cells lines. However, immunoblotting of both antibodies showed lack of protein recognition and provided various nonspecific bands. Both were regarded as unreliable for analysis (**Figure S2-5).**

Finally, we observed that overexpression of *ANKRD30A* increased PC-3 proliferation, a metastatic prostate cancer cell line (**Figure 2-7)**. According to RNA-seq, this cell line expresses little to no *ANKRD30A* (**Figure 2-5**), thereby it was selected for the generation of a Tet-inducible system for *ANKRD30A* overexpression. Of note, we worked polyclonal populations of stable cells, rather than selecting for single stable clones, because there was poor cell viability after clonal dilution and no fold induction of *ANKRD30A* expression in selected drug-resistant double-stable cell clones. Using polyclonal instead of monoclonal cell populations could have affected the consistency of induction due to the possible outgrowth of poorly inducing clones as the cells were passaged. In fact, in double-stable cells, we observed higher basal expression from P_{TRE3G} than expected. Generally, because transiently transfected cells contain more copies of the TRE-containing plasmid than do stable cell lines, fold induction (ratio of maximal to basal GOI expression) levels are almost always lower in transient assays than in properly selected stable and double-stable clonal cell lines. However, fold induction of *ANKRD30A* was slightly lower in our double-stable cells compared to transiently transfected cells **(Figure 2-7B, S2-4C)**.

Collectively, we hypothesize that activation of *ANKRD30A* supports the development of aggressive and metastatic prostate cancer through reactivation of developmental epigenomic programs during disease progression, but the overall effect of *ANKRD30A* overexpression on prostate cancer remains to be explored. Although testing for cell proliferation is an excellent approach to study cancer-associated features, additional *in vitro* assays (*e.g.*, migration, invasion assays) should be performed in the future to extrapolate robustly to prostate cancer progression. Currently, we are generating a Tet-On 3G system in *ANKRD30A*-expressing cell lines (22Rv1, LAPC4) and a cell line that express no *ANKRD30A* (LNCaP). Moreover, the gene expression pattern of *ANKRD30A* remains largely unexplored. There are six proteins that were shown to interact with ANKRD30A: ANKRD30B, LRRC31, RAB3IP, SCLT1, SPAG5, and UBC [173]. An interesting future analysis would be to identify genes that are co-expressed with *ANKRD30A* to determine its interaction with other genes and signaling pathways. For example, we could specifically investigate the expression pattern of AR along with the expression of *ANKRD30A*.

3.4 Overdiagnosis and overtreatment of prostate cancer

There is a major clinical challenge associated with overdiagnosis and overtreatment of prostate cancer under the current use of clinical practices. Although prostate cancer is estimated to claim the lives of 4,500 Canadians in 2021, most of the 24,000 men that will be diagnosed with prostate cancer this year in Canada will likely have indolent disease that may never become life-threatening even without primary therapy [6]. Importantly, it is estimated that about 5 to 48 men would need to be diagnosed and potentially treated with radical therapy for primary prostate cancer to prevent one death from prostate cancer [98]. This means that the majority of men with prostate cancer live many years after diagnosis and may never suffer from the disease. However, the treatment of prostate cancer with surgical, radiation, or hormonal therapy involves important short-term and long-term side-effects. For instance, radical prostatectomy in men with localized prostate cancer can result in long-term urinary incontinence and impotence additionally to immediate postoperative complications. Moreover, radiation therapy of primary disease can lead to urinary, gastrointestinal, and sexual complications. Since most men are diagnosed with indolent localized prostate cancer, they are unfortunately at risk for complications following radical prostatectomy or radiation therapy when such treatments are not necessary for the management of their cancer [174]. This highlights the urgent need to better understand prostate cancer initiation and progression.

In addition to the screening of asymptomatic men for prostate cancer, PSA is also widely used in all the main phases of prostate cancer detection, such as active surveillance, monitoring response to therapy and risk stratification for recurrence. However, PSA as a biomarker for prostate cancer is controversial because it has limited specificity and leads to overdiagnosis which in turn results in overtreatment [175]. Several new biomarkers supplementing the role of PSA are now available for men with prostate cancer (e.g., *PCA3*, *TMPRSS2-ERG*, methylated *GSTP1*). The combined measurement of these biomarkers results in enhanced specificity for prostate cancer diagnosis [176]. Emerging biomarkers predictive of aggressiveness and outcomes in patients with newly diagnosed disease include for example concurrent TOP2A and EZH2 mRNA and protein levels. Both are biomarkers for the early detection of a subgroup of aggressive prostate cancer [177]. Because these biomarkers only identify subsets of prostate cancers, the identification of novel

biomarkers is needed to better stratify indolent from aggressive prostate cancer to predict outcomes and guide therapies [98].

3.5 Targeted epigenetic profiling to improve prostate cancer stratification

Advances in molecular biology have led to detection of multiple DNA-based biomarkers in cancer. DNA-based biomarkers include genetic alterations, such as mutations and genomic rearrangements, that can be very specific for cancer [98]. However, such genetic alterations can be highly heterogeneous in prostate cancer. One of the most frequent genetic alteration in prostate cancer is the *TMPRSS2-ERG* gene fusion which occurs in about 50% of cases [56]. On the other hand, multiple consistent and recurrent epigenetic alterations are more frequently observed in many prostate cancers. For example, dozens of alterations in DNA methylation occur in 70% of prostate cancer tissues but not in normal prostate tissues. Many studies have reported that DNA methylation alterations associated with Gleason grade, with metastasis, and with disease recurrence after therapy for primary prostate cancer may serve as effective biomarkers [178-180]. In addition, global histone modification patterns detected in prostate cancer tissues were also shown to predict disease recurrence and may be useful for risk stratification of patients with prostate cancer [181]. Thus, such epigenetic alterations may be used as prostate cancer epigenetic biomarkers alone, or in combination with other genetic alterations to improve current clinical decision-making [98].

As mentioned previously, the current management of prostate cancer is challenged by our inability to stratify indolent from aggressive disease which leads to systemic prostate cancer overdiagnosis and overtreatment [160]. Thus, the identification of epigenetic biomarkers should be guided by comparative epigenomics of indolent versus aggressive tumors. In line with this, in our study, we used epigenomic data comparing indolent with aggressive prostate cancer cases and revealed a somatically acquired epigenetic event associated with aggressive prostate cancer. The H3K27ac mark was enriched at the *ANKRD30A* gene in aggressive prostate cancers but not in indolent tumors (**Figure 2-1B)**. This epigenetic event enriched in aggressive tumors indicates previously undescribed molecular heterogeneity in prostate cancer and could be used for disease classification and risk stratification. Also, the global promoter hypomethylation of the *ANKRD30A* gene in tumor tissues suggests a potential epigenetic event specific to prostate cancer that might also predict

disease recurrence (**Figure 2-3A, B**). Taken together, these epigenetic events may be valuable as prostate cancer epigenetic markers. If these epigenetic alterations are validated in more studies, they could help in risk stratification of prostate cancer and monitoring of indolent disease during active surveillance. Our understanding of the key epigenetic events driving prostate cancer initiation and progression is improving. We can expect that more epigenetic alterations in prostate cancer will be used as biomarkers to inform prostate cancer management decisions. Novel epigenetic biomarkers may be impactful in multiple clinical contexts of prostate cancer, such as the management of risk stratification at the time of diagnosis and active surveillance, especially when the diagnosis involves localized prostate cancer. Thus, development of effective epigenetic biomarkers to help guide clinical decision-making would be of tremendous value [98].

3.6 Preclinical efficacy of inhibitors of epigenetic targets

Our advancing knowledge of the epigenetic mechanisms in prostate cancer is fueling the development of novel therapeutic strategies that can target epigenetic alterations. Given the highly frequent and recurrent epigenetic alterations driving prostate cancer initiation and progression (as discussed in **Chapter 1**), it would be logical to presume that targeting these epigenetic events may be beneficial for treating prostate cancer.

Currently, available DNMT inhibitors include the nucleoside analog drugs decitabine and azacitidine, as well as a new decitabine prodrug called guadecitabine [98]. Decitabine and azacitidine are Food and Drug Administration (FDA) approved for treatment of myelodysplastic syndrome [182], but they have not been effective as single agents for prostate cancer. This may be due partly because of poor bioavailability, metabolism, or other pharmacological properties conferring a primary resistance to these inhibitors. For instance, nucleoside metabolizing enzymes may confer intrinsic primary resistance of cancer cells to DNMT inhibitors. One nucleoside metabolizing enzyme, cytidine deaminase, is known to inactivate decitabine and azacytidine [183]. Novel nucleoside analog DNMT inhibitors that are resistant to the effects of cytidine deaminase are in development. They include guadecitabine and ASTX-727, which is the combination of a cytidine deaminase inhibitor with decitabine. Other combinations of drugs that can overcome such resistance mechanisms are currently under investigation [184]. Moreover, the low proliferation rates in many prostate cancers (except in very late-stage disease) may also be a factor of resistance

because nucleoside analog DNMT inhibitors can only inhibit DNA methyltransferase enzymes if cells are replicating their DNA. Therefore, non-nucleoside DNMT inhibitors that can modulate the DNA methylation machinery may be more helpful in treating prostate cancers with low proliferation rates. Such non-nucleoside DNMT are under investigation in preclinical studies with some limited success [98].

Some HDAC inhibitors, including vorinostat and panabinostat, are FDA approved for treatment of advanced primary cutaneous T-cell lymphomas and multiple myeloma, respectively [185, 186]. However, like DNMT inhibitors, HDAC inhibitors have not been effective as single agents in prostate cancer. Nonetheless, a clinical trial reported that combination of the DNMT inhibitor azacitidine and the HDAC inhibitor entinostat has some efficacy in treating advanced non-small-cell lung cancer. Interestingly, most patients who received subsequent therapies, including chemotherapy, targeted therapies, and immunotherapy, had more pronounced responses to these therapies than would have been expected [187]. This suggests that epigenetic drugs, even if only modestly effective as a stand-alone treatment, might sensitize cancers to other therapeutic agents. Multiple mechanistic studies have shown that epigenetic drugs, including DNMT inhibitors and HDAC inhibitors, can modulate the immunogenicity and immune response of cancer cells, resulting in an increased response to immunotherapies. These mechanisms are also being tested in many cancer types, including prostate cancer [98].

Several new classes of drugs targeting multiple “writers”, “erasers”, and “readers” of epigenetic marks are in development, but are mostly still in early stages [98]. One new class of epigenetic drugs are EZH2 inhibitors (e.g., tazemetostat, DZNep, CPI-1205). The histone methyltransferase EZH2 is frequently upregulated in patients with more aggressive prostate cancer and there is emerging evidence that it is a therapeutic target for prevention of prostate cancer [188]. In metastatic CRPC, CPI-1205 is being tested in combination with androgen deprivation therapy [189]. Recently, the inhibition of EZH2 by tazemostostat or DZNep was shown to enhance prostate cancer response to PD-1 checkpoint blockade [190]. There are also multiple efforts to target the BET-bromodomain “readers” of histone acetylation marks, such as BRD4 [191]. Given the importance of BRD4 in the regulation of MYC and AR, both of which are key players in prostate cancer initiation and progression, inhibitors of BRD4 (e.g., JQ-1) and several others, are being

investigated for prostate cancer therapy in preclinical and clinical settings. There is also significant interest in testing drugs targeting HATs which are predicted to have similar effects to targeting BRD4, because they would antagonize the "writing" of histone acetylation marks that would then be "read" by BRD4. Whether these agents will have utility in prostate cancer is not yet known [98].

Finally, our results suggest that enrichment of the H3K27ac mark at the *ANKRD30A* gene and promoter hypomethylation of *ANKRD30A* are epigenetic events in aggressive prostate cancer (**Figure 2-1, Figure 2-3**). The pattern of H3K27ac and gene promoter hypomethylation are distinctly different between aggressive and indolent prostate cancers. Both promoter hypomethylation and the H3K27ac mark are associated with the activation of gene transcription. Although correlational, our data suggests that *ANKRD30A* is epigenetically activated in aggressive prostate cancer. If we can show a functional role of the H3K27ac gain at the *ANKRD30A* locus in prostate cancer progression, targeting this epigenetic alteration using drugs with favorable therapeutic index and clinical utility might be an interesting focus of research in the future.

Chapter 4: General conclusions and future directions

4.1 General conclusion

Distinguishing indolent from aggressive prostate cancer cases is a major clinical challenge in disease management and decision-making [160]. Advances in our understanding of epigenetic mechanisms also highlights epigenetic alterations as drivers of prostate cancer initiation and progression. Thus, our main goal was to identify and investigate the role of somatic epigenetic events in aggressive prostate cancer using epigenomic profiling.

Importantly, we discovered a somatically acquired regulatory element in the *ANKRD30A* gene in a subset of aggressive prostate cancers that was associated with disease biochemical recurrence. We investigated cancer-associated phenotypes that are related to tumor aggressivity, such as proliferation, and reported that *ANKRD30A* expression confers increased proliferative potential.

These results also support an *ANKRD30A*-associated regulatory element as a potential biomarker for prostate cancer progression and raise the exciting possibility for targeted epigenetic profiling for risk stratification and improved management of prostate cancer.

4.2 Future directions

An important future direction of our study is to establish a causative link between *ANKRD30A* expression and disease progression. In the future, we will continue to investigate the necessity of *ANKRD30A* to initiate or maintain the aggressivity of prostate cancer with additional *in vitro* assays (*e.g.*, migration, invasion assays). We will modulate *ANKRD30A* expression with gene knockout in relevant prostate cancer cell lines and evaluate the impact on transcriptional (RNA-seq) and epigenetic (H3K27ac; ChIP-seq) reprogramming. Currently, we are generating Tet-On 3G systems in *ANKRD30A*-expressing cell lines (22Rv1, LAPC4) and non-expressing cell lines (LNCaP, PC-3). These Tet-3G-expressing stable cell lines will be used to either disrupt (CRISPRi) or activate (CRISPRa) the *ANKRD30A*-associated regulatory element using the inducible CRISPR/dCas9-KRAB and CRISPR/dCas9-VPR systems, respectively. This will enable the validation of our findings through customized single-guide RNAs (sgRNA) targeting the candidate regulatory element. We plan on using customized sgRNAs that target various regions of

ANKRD30A, including our candidate regulatory element, to modulate the activity of this regulatory element in prostate cancer cell lines. To design these single-guide RNAs, we used coordinates of four *ANKRD30A*-associated H3K27ac peaks. Currently, we are generating the CRISPR-dCas9 system with either the LSD1/KRAB or the p300/VP64 systems in addition to designing these single-guide RNAs. Importantly, since *ANKRD30A* encodes an uncharacterized transcription factor, we will define the *ANKRD30A* cistrome via ChIP-seq. Moreover, assays of chromatin accessibility (ATAC-seq), which determine how "open" a region is, will also be used to further profile the epigenomic landscape of prostate cancer cell lines at the *ANKRD30A* locus. Finally, we will characterize the role of *ANKRD30A* on *in vivo* orthotopic tumor growth and metastasis. Altogether, these approaches will help elucidate the role of the *ANKRD30A*-associated element in driving the progression of prostate cancer to a metastatic and lethal disease. This will provide a much-needed tool to identify men who would benefit from aggressive treatments. In summary, *in vitro* and *in vivo* should be conducted to elucidate the overall effect of the *ANKRD30A*-associated regulatory element, and *ANKRD30A per se*, in prostate cancer development and progression.

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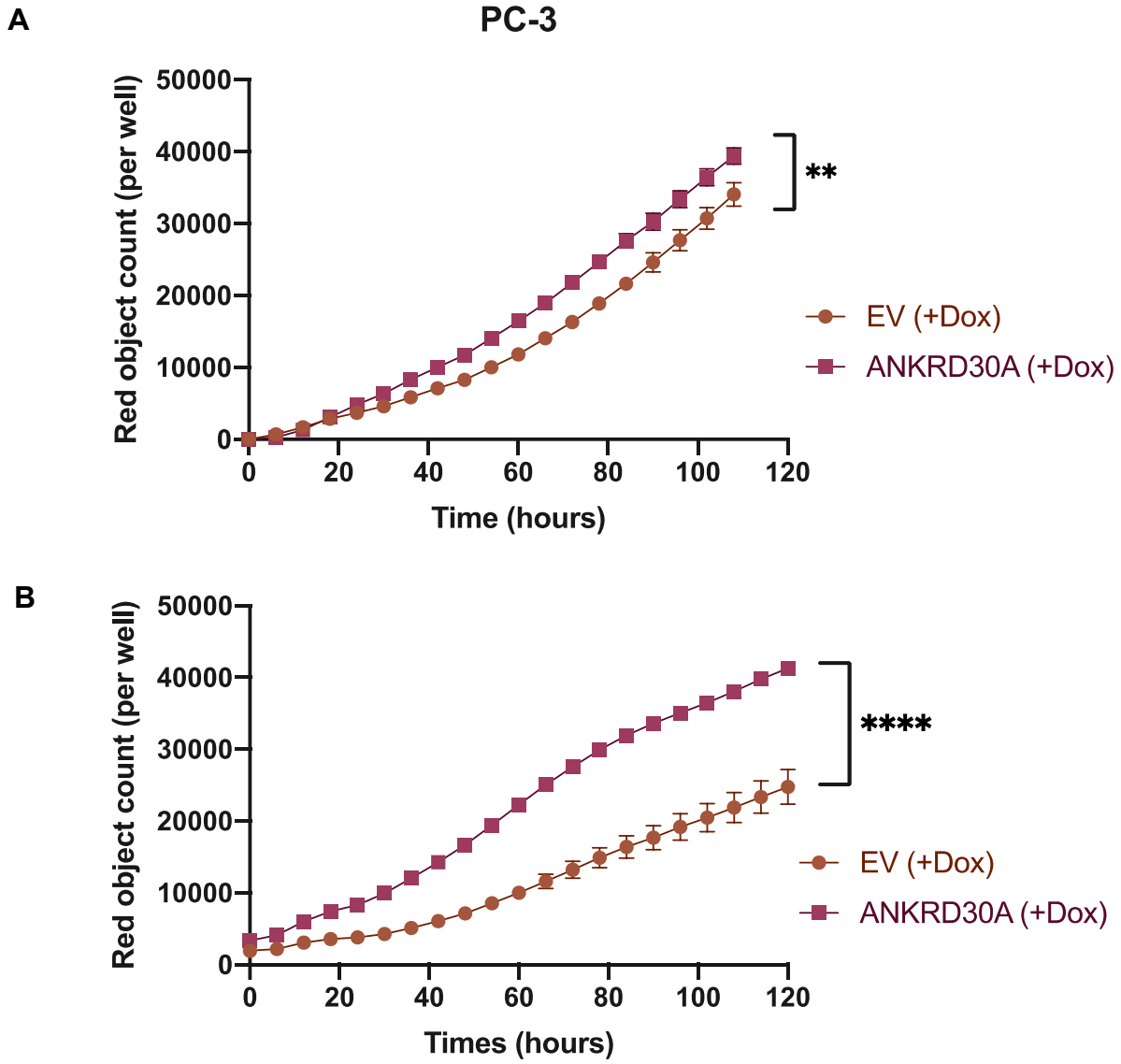
Appendix
Supplementary data for Chapter 2

This section provides the reader with additional information regarding the data presented in **Chapter 2**. Since some of the figures are representative of individual experiments (done with at least six technical replicates), additional figures from repeat of these experiments are shown here.



Supplementary Data 1. Other replicates for **Figure 2-7C.** Proliferation of double stable PC-3 in the presence of Dox. Cells were seeded at 5,000 cells/well in a 96-well plate. **A.** Dox (2 ug/mL) was added at day 0 (n=6). **B.** Cells were cultures for a few days in Dox (2 ug/mL) prior to seeding (n=16). Cells that were induced with Dox expressed mCherry and were counted (red object count). Error bars indicate SEM. ** $P < 0.01$, **** $P < 0.0001$. Two-way ANOVA with Sidak's multiple comparisons test were applied.