Mechanism of action of ID4 as a tumor suppressor

Shravan Kumar Komaragiri
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MECHANISM OF ACTION OF ID4 AS A TUMOR SUPPRESSOR

A DISSERTATION
SUBMITTED TO THE FACULTY OF CLARK ATLANTA UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

BY
SHRAVAN KUMAR KOMARAGIRI

DEPARTMENT OF BIOLOGICAL SCIENCES

ATLANTA, GEORGIA

DECEMBER 2016
ABSTRACT

BIOLOGICAL SCIENCES

KOMARAGIRI, SHRAVAN K. M.S. CLAFLIN UNIVERSITY, 2009

MECHANISM OF ACTION OF ID4 AS A TUMOR SUPPRESSOR

Committee Chair: Jaideep Chaudhary, Ph.D.

Dissertation dated December 2016

Initial studies demonstrated that Inhibitor of DNA binding/differentiation protein 4 (Id4) acts as a tumor suppressor in prostate cancer (PCa). To further confirm and investigate the mechanism by which ID4 acts as a tumor suppressor, herein we concentrated on two different approaches. In the first approach we investigated ID4 role as a tumor suppressor by regulating AKT/PI3K pathway. In the second approach, we examined the role of Id4 in blocking the tumorogenic properties of metastatic PC3 cells. Phosphoinositide 3-kinase/Protein kinase B (PI3/AKT) pathway regulates multiple biological processes leading to cell survival, proliferation and growth in cancerous cells. We performed immunohistochemistry (IHC) to determine AKT, pAKT and PTEN protein expression in Id4 knockout mouse prostates and PCa cell lines. IHC on Id4 knockout mouse prostates demonstrated a significant decrease in PTEN expression as compared to normal mouse prostates. Consistent with decrease PTEN, the expression of pAKT expression increased. Similar pattern was observed in PCa cell lines: DU145 cells lacking Id4 had low PTEN as compared to Id4 over-expressing DU145 cells. In addition,
Chromatin immunoprecipitation (ChIP) analysis also demonstrated an increase in the binding of acetylated p53 on PTEN promoter in the presence of Id4.

The second approach demonstrated the tumor suppressor function of Id4 in highly tumorogenic and metastatic PC3 cell line. Interestingly, this study demonstrated that overexpression of Id4 in PC3 cells results in decreased tumorogenicity in part through increased expression of Androgen receptor (AR) and its target genes Cyclin dependent inhibitor 1 (p21) and FK506-binding protein 51 (FKBP51). Apoptosis, migration and cell proliferation decreased in the Id4 overexpressed PC3 cells. Mice injected with PC3 + Id4 cells showed decreased tumor size and volume. IHC studies on tumor xenografts demonstrated increased levels of AR, Ki67, and p21 in Id4 overexpressed xenograft. Collectively, our data indicate that ID4 acts as tumor suppressor by regulating the levels of PTEN by prompting the binding of acetylated p53 onto PTEN promoter which eventually results in inhibiting P13K/AKT pathway. Furthermore, Id4 not only increases the expression of AR in PC3 cells but also regulates the factors responsible for AR tumor suppressor activity.
ACKNOWLEDGEMENTS

With God everything is possible. First, I would like to thank God for giving me the wisdom to put you in the center of things I do. I would like to thank my mentor and doctoral advisor, Dr. Jaideep Chaudhary, for his kindness, dedication, invaluable support and advice during my matriculation at Clark Atlanta University. I would also like to thank my committee members: Drs. Cimona Hinton, Valerie Marah, Joann Powell, and Shailesh Singh, for their accolades, affirmations, and criticisms required along this journey. I would like to thank all the former/current members of Dr. Chaudhary’s lab and graduate students in Department of Biological Sciences. Finally, I would like to thank my beloved mother and father for their unconditional love, sacrifice and support. I would like to thank my wife, brother, sister and my family for their endless love, support and great encouragement at all times throughout my studies. I would also like to thank the financial, academic and technical support of the Clark Atlanta University, the chair and the staff of the Department of Biological Sciences and Center for Cancer Research and Therapeutic Development. This work was funded by NIH/NCRR/RCMI grant #G12RR03062 and NIH grant #R01CA128914 and the Department of Biological Sciences for supporting my doctoral thesis.
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<table>
<thead>
<tr>
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AP-2</td>
<td>Activating Protein 2</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen Receptor</td>
</tr>
<tr>
<td>BCS</td>
<td>Bovine Calf Serum</td>
</tr>
<tr>
<td>Bp</td>
<td>Base Pair</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign Prostatic Hyperplasia</td>
</tr>
<tr>
<td>BCRP</td>
<td>Breast Cancer Resistance Proteins</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast Cancer 1</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CBF1</td>
<td>Centromere Binding Factor 1</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle Threshold</td>
</tr>
<tr>
<td>DAPI</td>
<td>4'-6-Diamidino-2-Phenyllindole</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Dinucleotide Triphosphate</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>Egr1-1</td>
<td>Early Growth Response</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-Regulated</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FKBP51</td>
<td>FK506-Binding Protein</td>
</tr>
<tr>
<td>HLH</td>
<td>Helix Loop Helix</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
</tr>
<tr>
<td>ID</td>
<td>Inhibitor of DNA Binding</td>
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<tr>
<td>ERK</td>
<td>Extracellular Signal-Regulated Kinases</td>
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<tr>
<td>EZH2</td>
<td>Enhancer of Zeta Homolog 2</td>
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<tr>
<td>H3K27Me3</td>
<td>Tri Methylation of Histone 3 at Lysine 27</td>
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<td>JHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>MDM2</td>
<td>Mouse Double Minute 2 Homolog</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>MS-PCR</td>
<td>Methylation Specific-Polymerase Chain Reaction</td>
</tr>
<tr>
<td>NF-Kb</td>
<td>Nuclear Factor KappaB</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-Kinase</td>
</tr>
<tr>
<td>PIN</td>
<td>Prostatic-Intraepithelial Neoplasia</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>Peroxisome Proliferator-Activated Receptor Gamma</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate Specific Antigen</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and Tensin Homolog</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Difluoride</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse Transcriptase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription Polymerase Chain</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
</tbody>
</table>
PART I

ROLE OF ID4 IN PTEN/AKT PATHWAY
CHAPTER I
INTRODUCTION

The helix-loop-helix (HLH) family of transcription factors comprises >200 members, which have been identified in organisms from yeast to man.\textsuperscript{1-2} The basic helix-loop-helix (bHLH) family of transcription factors are key players in many developmental processes,\textsuperscript{3} such as differentiation of various cell types, such as in B and T-lymphocytes, muscle lineages, pancreatic B cells, neurons and osteoblasts.\textsuperscript{4-5} Most (bHLH) proteins that form a large super family of transcription factors that regulate tissue-specific transcription,\textsuperscript{6} positively regulate sets of genes during cell fate determination and cell differentiation. Members of this family have two highly conserved domains. The carboxyl terminal end of bHLH contains the HLH domain involved in homo or heterodimerization with other bHLH proteins that regulate transcription and the amino terminal end which contains the basic domain facilitates binding to DNA containing the canonical ‘E box’ recognition sequence, CANNTG and ‘N box’ sequence, CACNAG\textsuperscript{2} or Ets recognition sequence, GGAA/T present in the promoter regions of regulated proteins.\textsuperscript{4} Thus bHLH proteins form a large super family of transcription factors that regulate tissue-specific transcription.\textsuperscript{6} The bHLH hetero-homo dimer plays an important role in many physiological processes including cellular differentiation, apoptosis, cell cycle arrest and regulate critical developmental processes.\textsuperscript{7} A distinct subfamily of HLH proteins, the Inhibitor of differentiation (ID) proteins, lack these basic DNA-binding
region and instead function solely by dimerization with other transcriptional regulators, principally those of the bHLH type and such ID-bHLH heterodimers are unable to bind to DNA, and hence ID proteins act as dominant negative regulators of bHLH proteins.\textsuperscript{8-9} Ids are known to interact with other bHLH proteins such as E2A, E2-2, or HEB, and also with factors such as RB (retinoblastoma) family members that have a role in cell cycle.\textsuperscript{4}

In highly proliferating cells, ID proteins are typically expressed while there expression levels decrease in differentiated cells. Ids are known to be positive regulators of cell growth and negative regulators of cell differentiation.\textsuperscript{10} ID proteins play key roles in the regulation of lineage commitment, cell fate decisions,\textsuperscript{11} and also have a role in cell signaling, DNA damage control, and apoptosis. They also act in the timing of differentiation during neurogenesis, lymphopoiesis and neovascularisation (angiogenesis) and are essential for embryogenesis and cell cycle progression.\textsuperscript{11} ID proteins that consists of ID1, ID2, ID3 and ID4 are dominant negative transcriptional regulators of basic Helix Loop Helix (bHLH) transcription factors that lack the basic DNA binding domain but have intact HLH domain that is conserved in all ID proteins.\textsuperscript{12} Out of four ID proteins, ID4 appears to act primarily as a tumor suppressor in most cancers as opposed to ID1, ID2 and ID4, which in most cases acts as tumor-promoters or supporting oncogenes.\textsuperscript{13-14} Previous studies from our lab, demonstrated that Id4 acts as a tumor suppressor in PCa via regulating not only various tumor suppressors and tumor promoters but also through various regulatory pathways such as PTEN/AKT, p53 and AR pathways.\textsuperscript{15-20} Id4 has been shown to act differently depending on the tumor type as shown in Table 1.
Table 1: Id4 Modulation in Cancer

<table>
<thead>
<tr>
<th>Kind of Modulation</th>
<th>Kind of Analysis</th>
<th>Tumor Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Downregulated</td>
<td>mRNA</td>
<td>Prostate</td>
</tr>
<tr>
<td>Down Regulated in Low Grade Cancer vs</td>
<td>Protein</td>
<td>Prostate</td>
</tr>
<tr>
<td>Hyperplasia Upregulated in High Grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade Cancer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypermethylation</td>
<td>Promoter, DNA</td>
<td>Leukemia</td>
</tr>
<tr>
<td>Hypermethylation</td>
<td>Promoter, DNA</td>
<td>Lymphoma</td>
</tr>
<tr>
<td>Downregulated</td>
<td>Protein</td>
<td>Breast</td>
</tr>
<tr>
<td>Hypermethylation</td>
<td>Promoter, DNA</td>
<td>Breast</td>
</tr>
</tbody>
</table>

Study between Id4 and PTEN expression suggests that there is some cross-talk between AKT pathway and Id4. In this study loss of Id4 in mouse prostates resulted in decreased expression of PTEN leading to increased pAKT expression levels.\textsuperscript{18} Previous studies demonstrated, binding of p53 to PTEN promoter can enhance the regulation of PTEN and thus can mediate cell cycle arrest and apoptosis by promoting stabilization, acetylation and tetramerization of p53.\textsuperscript{21-22} Id4 that regulates PTEN activity and expression, and vice-versa pAKT expression levels, might play a role when PTEN inactivation increases the expression and activity levels of Mouse double minute 2 (MDM2) a known p53 repressor by PI3K/AKT dependent pathway.\textsuperscript{23} In the presence of PTEN, p53 gets upregulated through translational mechanisms mediated by mTORC1.\textsuperscript{24} Previously we have shown that Id4 has a role in p53 pathway where Id4 restores p53 transcriptional activity.\textsuperscript{15} HLH domain is shown to be conserved in all ID proteins that is involved in DNA binding and transcriptional activity of ID proteins.\textsuperscript{25} Domain structure
of HLH protein family has HLH domain, and ld4 which belongs to HLH family lack the basic domain, which is shown in Figure 1. Serine residue at position 5 in ld2 is the one to get phosphorylated, that inturn makes ld2 to act as a tumor promoter and vice-versa. ld4 which has the same HLH domain and serine 5, might also act in similar fashion as ld2 when it gets phosphorylated. To study this, we investigated, whether the association between PTEN and ld4 in PCa, has any effect on AKT pathway.

![Diagram](image)

**Figure 1. Domain structure of HLH family.** A represents HLH domain that also contains basic amino group needed to bind to DNA. B represents HLH domain that lack basic amino group, which is seen in ID proteins.
CHAPTER II
LITERATURE REVIEW

2.1 Cancer Epigenetics and Genetic Instability

Disruption of regulatory circuits which maintain cellular homeostasis leads to development of cancer. The hallmarks of cancer include, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis. Genes can be inactivated as a result of deletions, mutations or epigenetic mechanisms and also can be deregulated by amplifications or chromosomal translocations.

There is often more than one genetic aberration present in many cancers. Cancer cells acquire somatic mutations as part of normal development which differ from any germline mutations. Those mutations which give proliferative and/or survival responses to cells are acquired as part of an on-going process during development. These somatic mutations are categorised as ‘drivers’ or ‘passengers’. ‘Drivers’ are defined as those mutations which give the cells a survival advantage. ‘Passenger’ mutations are those mutations which allow the ancestry of the cell to be mapped but are not beneficial for the cells survival. According to the classical mechanism, genomic instability (deletions or translocations) due to inactivating mutations in tumor suppressor genes or gain of function mutations in oncogenes resulting in uncontrolled growth is considered as a tumor initiating event.
More recently, loss of function (expression) through epigenetic changes due to altered pattern of DNA methylation and histone modification, is gaining significant attention as an additional pathway involved in cancer initiation and progression.\textsuperscript{30-32} These epigenetic changes could predispose the inherited genome of subsequent generations to mutagenic/genotoxic alterations leading to the development of sporadic PCa.\textsuperscript{33} Thus, combined genetic and epigenetic changes are now considered as cancer initiating events.

2.2. Structure / Function of Prostate

The prostate gland which weighs approximately 40 grams, is a walnut sized gland in the male reproductive system just below the bladder that supplies fluid for the sperm during ejaculation.\textsuperscript{34} A newborn male’s prostate is very small about the size of a grain of wheat. At the onset of puberty it begins to grow dramatically until around the age of twenty and fairly constant until the forties, then due to dihydrotestosterone (DHT) and estrogens the prostate begins to bulk up. It is divided into 2 lobes and contains smooth muscle cells capable of contracting to expel the prostatic fluid.\textsuperscript{35} The structure and the normal vs cancer of prostate is shown in Figure 2.

\textbf{Figure 2. The adult prostate and surrounding structures.}\ The base of the prostate is located at the bladder neck. The urethra bisects the prostate. (B) Illustration showing, two examples of PCa: the top, which grew up next to the urethra causing its compression and to the bottom showing it comes out of urethra causing urinary problems.
2.3 Molecular Biology and Histology of PCa

Anatomically prostate is composed of the central, transition, peripheral zones, and a region of the anterior fibromuscular stroma, which is the most anterior prostatic structure. Posterior to the stromal region are the paired central zones. Interior to the central zones are the transition zones which are located on either side of urethra. The transition zones represent the smallest zone in the normal prostate. And lastly the peripheral zone is the largest region of the normal adult prostate, which is located on the posterior side of the prostate. Benign prostatic hyperplasia (BPH), a nonmalignant overgrowth that is fairly common among aging men, occurs mainly in the transition zone, whereas prostate carcinoma arises primarily in the peripheral zone.  

Histologically, prostatic glands are composed of different cell types. The luminal epithelial cells which are androgen-dependent, produce prostatic secretions. Luminal epithelial cells are characterized by expression of androgen receptor and cytokeratins 8 and 18. Basal cells that form a continuous layer between the luminal cells and basement membrane are most abundant cells with cytokeratins 4, 14 and p63 as markers. The third cell type is neuroendocrine cells which are androgen independent and are dispersed throughout the basal layer. Stroma that surrounds the epithelial glands is composed of smooth muscle fibers, fibroblastic, neuronal, vascular and lymphatic cell types, and can be identified using alpha-actin and vimentin as markers.

2.4 PCa

PCa also known as adenocarcinoma develops mostly from gland cells that make prostate fluid which is added to the semen. Other types of cancers such as sarcomas,
small cell carcinomas and transitional cell carcinomas which are rare can also start in the prostate gland. Most PCa’s grow slowly, and don’t cause any health problems in men who have them.\textsuperscript{41}

2.5 PCa Incidence

PCa is the second leading cause of cancer deaths in men.\textsuperscript{42} In the year 2013 there are an estimated 238,590 new cases of PCa and predicted deaths are almost 29,750 in the United States. Of all the cancer deaths, PCa deaths account for approximately 5.1 percent with 15.3 percent of men will be diagnosed with PCa at some point during their lifetime.\textsuperscript{43} African American men are twice more likely to have PCa incidence compared to that of Caucasians and Asian men and have nearly a two-fold higher mortality rate than Caucasian men.\textsuperscript{44} Most PCa related deaths are due to spread of malignant cells to tissues other than prostate, known as metastasis.\textsuperscript{45}

2.6 Risk Factors

A risk factor separates patients without known disease into those at increased or decreased likelihood for disease and for men who develops aggressive cancer there is a great need for such factors. Risk factors can be classified as endogenous or exogenous. Endogenous risk factors for prostate include family history, hormones, race, aging and oxidative stress. Diet, environmental agents, occupation and other factors such as smoking, energy intake, sexual activity, marital status, vasectomy, social factors, physical activity and anthropometry come under exogenous risk factors,\textsuperscript{46} for PCa. Any study of risk of PCa depends on five unique characteristics that contribute to its unpredictable and paradoxical clinical behavior. First, PCa is a slow growing, with a doubling time of 3-4
years. Secondly PCa is remarkably age related that rarely appears before 40 years of age and typically identified in men around 70 years of age.\textsuperscript{46} A Spanish study on Mediterranean men stated 33\% of men in their 8\textsuperscript{th} decade had evidence of PCa at necropsy and died with the disease, but not from it.\textsuperscript{47} Third, PCa usually is multifocal, so that most men with PCa die not just because of it but because of other cancers or disease conditions. Fourth, PCa and PIN are heterogenous in their morphology and genotype, virtually the entire genome participates in prostatic carcinogenesis.\textsuperscript{46}

### 2.7 Progression

The development of prostatic tumor in men is generally slow, taking up to 4 to 10 years to develop a 0.4 inch-size tumor.\textsuperscript{48} Multiple molecular events have been associated with the development of PCa. An early event in PCa progression is the loss of a region of chromosome 8p, which occurs in approximately 80\% of tumors.\textsuperscript{49} Another step in PCa progression occurs with the loss of chromosome 10q and the tumor suppressor PTEN (Phosphatase and Tensin deleted on chromosome 10), which accounts for 50\% - 80\% of prostate tumors, thus supporting the role of PTEN in PCa progression.\textsuperscript{50} A third common event in PCa progression that results in carcinoma is the loss of chromosome 13q which includes Retinoblastoma tumor suppressor gene, that occurs in 50\% of prostate tumors.\textsuperscript{51} Progression of PCa is also based on androgen-based pathways that continue to have a clinically significant role in the progression of castrate-resistant PCa.\textsuperscript{52} Mostly all men eventually develop progressive disease following androgen deprivation therapy (ADT), leading to castrate-resistant PCa.\textsuperscript{53-54} ADT might also be because of adrenal gland and testis that produce several enzymes involved in the synthesis of testosterone and
dihydrotestosterone that are highly expressed in tumor tissue in addition to androgen production.  

2.8 Treatment

The therapeutic approaches such as radical prostatectomy and radiotherapy are considered curative for localized disease, yet no treatments for metastatic PCa is available that can significantly increases patient survival. PCa treatment is primarily by surgery and/or radiotherapy due to the intimate organ localization. A prostatectomy usually leads to an excellent prognosis with low risk of death from PCa after surgery. However, deregulated production and secretion of growth factors by stromal cells within the PCa microenvironment, as well as mutations in androgen signaling pathway components and further physiological modifications, including angiogenesis, local migration, invasion, intravasation, circulation, and extravasation of the tumor, potentially lead to systemic recurrence of the cancer, including the appearance of focal tumor in advanced stage. Maximal therapeutic efficacy in the treatment of castration-resistant PCa will require novel agents or drugs that act by inhibition of the enzymes responsible for androgen production, as well as agents that inhibit the androgen receptor that target intracrine steroidogenic pathways within the prostate tumor microenvironment.

2.9 Tumor Promoters in PCa

Development of any neoplasm reflects a progressive and cumulative alteration in various genes. Oncogenes function in a dominant mode, whereas tumour suppressor genes (TSGs) are recessive. If the protein is over-active and contributes to prostate carcinogenesis, or metastasis, it could be termed a prostate-specific 'oncogene.' Of the
oncogenes, the RAS gene family and neuroblastoma (N) RAS), have been most widely studied in PCa.\textsuperscript{65} C-ERB-B2 oncogene, as a tumor marker or therapeutic target remains unclear in PCa. The MYC family of oncogenes, in particular C-MYC involved in differentiation has high level of mRNA in proliferating cells of PCa.\textsuperscript{66} The polycomb group protein, ‘enhancer of zeste homologue 2’, is activated in advanced PCa that causes generalized reduction in transcription by increasing histone deacetylation via histone deacetylase-2.\textsuperscript{67} Members of the RAF oncogene family that encode serine/threonine kinases, which activate the mitogen-activated Mitogen-activated protein kinases (MAPK) or Extracellular signal-regulated (ERK) kinases through direct phosphorylation are the other kind of genes that are also expressed in PCa.\textsuperscript{68}

2.10 Tumor Suppressors in PCa

PCa progression occurs due to the inactivation, deletion or mutation of tumor suppressor genes such as PTEN, p53, retinoblastoma (RB), which are notable tumor suppressors.\textsuperscript{69-73} RB was the first identified gene product to suppress cell division by preventing cells in G1 phase from entering S phase,\textsuperscript{74} Protein p53, named after the 53-kDa gene product, is a nuclear phosphoprotein that can produce growth arrest at the G1-S checkpoint, allowing DNA repair,\textsuperscript{75} is frequently mutated in PCa.\textsuperscript{76} PTEN which is the second most deleted gene after p53 has a protein phosphatases activity that can act as either positive or negative regulator during signal transduction or cell transformation and acts as a marker for metastatic progression.\textsuperscript{71,77}
2.11 Tumor Suppressor: PTEN

PTEN is an enzyme that is found in almost every tissue in the body. As a tumor suppressor, its primary role is to stop cells from dividing. Inactivation of PTEN results in uncontrolled proliferation of cells leading to the growth of tumors. PTEN plays a major role in the development of the nervous system and has been implicated in diseases such as autism and brain development. Germline PTEN mutations are primarily found in 80% of patients with the autosomal dominant Cowden disease (CS), and 60% of individuals with Bannayan-Riley-Ruvalcaba syndrome (BRRS) another autosomal dominant hamartoma syndrome. However, PCa has not been found associated with Cowden syndrome and germline PTEN loss, perhaps providing credence to the understanding that loss of PTEN is a late event in PCa.

PTEN also known as MMAC1 (mutated in multiple advanced cancers 1) and TEP1 (TGF- beta regulated and epithelial cell enriched phosphatase 1), is mutated in multiple advanced cancers and was identified in 1997 when cloned and mapped to 10q23, a region undergoing frequent somatic deletion in tumors. Genome wide linkage analysis and loss of heterozygosity (LOH) studies have also identified 10q23.3 as one of the potential PCa (PC) susceptibility loci, which is often deleted in PCa.

PTEN is also deleted or inactivated in many tumors such as renal, melanoma, prostate, breast, lung, bladder and thyroid. Both monoallelic and biallelic deletions of PTEN occur in PCa cell lines and non-cultured human prostate tumor specimens. Decreased PTEN expression correlates with increased tumor grade, advanced disease stage and poor prognosis. Human PTEN promoter is GC rich and lacks TATA box with
several transcriptional start sites that contain binding sites for transcription factors such as Specificity protein 1 (SP-1), Tumor protein p53 (p53), Centromere binding factor 1 (CBF-1), Snail family transcriptional factor 1 (Snail1), Proto-oncogene Jun in humans (C-Jun), Early growth factor (Egr-1) and Activating protein 2 (AP-2) whereas Nuclear factor kappaB (NF-kB) and Peroxisome proliferator-activated receptor gamma (PPAR-\gamma) regulate PTEN expression.\textsuperscript{88} Mutation in PTEN gene can lead to protein formation but that PTEN is not active as it cannot dephosphorylate pAKT to AKT.\textsuperscript{89} PTEN can be regulated at different levels, transcriptionally, translationally post-translationally and also at the protein level. Figure 3 shows PTEN can be regulated by different genes transcriptionally.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{pten_regulation}
\caption{Regulation of PTEN transcription. Numerous genes regulate PTEN transcription both positively (EGR-1, PPARγ, Myc, and p53) and negatively (NFκB, c-Jun, HES, and TGFβ signaling). NOTCH1 may be able to activate or repress PTEN transcription depending on the cellular context. Recently, miR-21 was identified as the first microRNA to regulate the expression of PTEN.}
\end{figure}

\subsection*{2.12 PTEN Structure}

PTEN is a 47 kDa (403 aa) protein with nine exons. The PTEN amino acid sequence resembles dual specific protein phosphatases and tensin, a chicken cytoskeletal
C2 domain that mediates membrane attachment of cell signaling proteins. Other three functional domains of PTEN, include short phosphatidylinositol-4,5-bisphosphate (PIP2) binding domain on the N terminus, PEST sequences and a PDZ interaction motif on the C-terminal tail that regulate protein stability and binding to PDZ domain containing proteins, are thought to be central to its biological functions. The phosphatase domain, directly responsible for removing a phosphate group from the lipid PIP3, is evolutionarily conserved. Almost 40% of the cancer associated mutations in PTEN are found in the phosphatase domain. Tumorigenic mutations also occur on C-terminal C2 domain and tail sequence, highlighting the role of C terminus in maintaining PTEN protein stability. PTEN loss in PCa is commonly from somatic mutations generated through copy number loss, rather than point mutation. With the absence of PTEN there is reduced survival in PCa patients.

2.13 PTEN Localization (in the Nucleus)

PTEN contains dual nuclear localization signal like sequences, regulate cell cycle progression and genomic integrity in the nucleus, by inducing G1 cell cycle arrest in part by reducing cyclin D1 levels through its phosphatase activity, or through MAPK signaling. There is a marked reduction in nuclear PTEN in rapidly cycling cancer cells in comparison to resting or differentiated cells. Nuclear export of PTEN is inhibited by oxidative stress, a process dependent on phosphorylation of Ser380. p53 transcriptional activity and stability can be regulated by nuclear PTEN, independent of its phosphatase activity, leading to p53 mediated G1 growth arrest and cell death. Nuclear PTEN itself is sufficient to reduce human prostate xenograft growth in-vivo in a p53 dependent
Nuclear expression of PTEN can reduce nuclear levels of pAKT, but whether this is a PI3K dependent or independent mechanism is not known. Through its phosphatase independent mechanism, nuclear PTEN increases the activity of RAD5, a protein involved in double strand break repair, and also increases E3 ligase activity of APC/C with its activator CDH1, that is involved in degrading oncogenic proteins PLK1 and Aurora kinases.

2.14 PTEN and Regulation of the PI3K/AKT/mTOR Pathway-PCa

PI3K/AKT pathway which is active in human cancers, controls cell growth, migration, differentiation and survival at cellular level. The major substrate of PTEN is phosphatidylinositol (3,4,5)-triphosphate (PIP-3), a lipid second messenger molecule generated by the action of PI3Ks. PTEN by its lipid phosphatase activity removes phosphate from the D3 position of PIP3, thus antagonizing the action of PI3K, and thereby stops cell division. In the absence of PTEN, PIP3 accumulates at plasma membrane. This results in the recruitment and activation of kinases such as phosphoinoside-dependent kinase1 (PDK1) and AKT that are involved in cell growth and survival via their pleckstrin homology (PH) domains. AKT (isoforms 1,2, 3) which gets phosphorylated at Thr308 by PDK1, and Ser473 by mammalian target of rapamycin complex2 (mTOR), drives cell survival, proliferation, growth, angiogenesis, and metabolism by phosphorylating downstream signaling proteins which include inhibitory phosphorylation of GSK3 beta, FOXO, BAD, p21, p27, and PGC1, and activating phosphorylation of mTOR1, IKK beta, MDM2, ENTPD5, SREBP1C, AS160 and SKP2. AKT promotes cell cycle progression and proliferation by inhibiting
and SKP2.\textsuperscript{110-111} AKT promotes cell cycle progression and proliferation by inhibiting p21, p27 and alleviating GSK3-beta induced cyclin D1 degradation,\textsuperscript{111-112} and can also evade apoptosis by phosphorylating pro-apoptotic protein BAD.\textsuperscript{113} mTOR signaling also triggers a negative feedback loop that inhibits PI3K/AKT pathway through phosphorylation that degrades insulin receptor substrate 1 by S6K.\textsuperscript{114} Conversely inhibiting mTOR1 results in activation of PI3K/AKT pathway through RAS/MAPK pathway.\textsuperscript{115} Figure 4 describes mechanism of PTEN function can in the AKT pathway.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{AKT pathway: The solid arrows mark the directional change of proteins (up- or down-regulation). Doted arrows mark hypothesized change in protein expression/activity. CDC42, cell division cycle 42; G3BP1, GTPase-activating protein SH3-domain-binding protein 1; GD1-2, Rho GDP dissociation inhibitor 2; LIMK, LIM domain kinase; MAPK, mitogen-activated protein kinase; MCM7, minichromosome maintenance protein 7; MSH2, MutS homolog 2; NDRG1, N-myc downstream regulated gene 1; p21, cyclin-dependent kinase inhibitor 1A; PCNA, proliferating cell nuclear antigen; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; Rb, retinoblastoma protein; RhoA, Ras homolog gene family member A.}
\end{figure}

In both heritable and sporadic cancers, when PTEN is absent or dysfunctional, Akt phosphorylation and activity are significantly increased \textit{in vitro} and \textit{in vivo}.\textsuperscript{116} PTEN inhibits phosphorylation of Akt, which, in turn, stimulates AR phosphorylation and
domain/Hinge domain and inhibits AR nuclear translocation and AR-mediated transcriptional activity in PCa cells.\textsuperscript{118}

### 2.15 PI3K/AKT/mTOR- Independent Functions of PTEN

The PTEN C2 domain that lacks phosphatase activity can interact directly with p53 to enhance p53 mediated cell cycle arrest and apoptosis by promoting stabilization, acetylation and tetramerization of p53.\textsuperscript{21-22} And also the same domain can regulate cell motility.\textsuperscript{119} PTEN inactivation increases the expression and activity levels of MDM2 a known p53 repressor by PI3K/AKT dependent pathway,\textsuperscript{23} but when PTEN is present it upregulates p53 through translational mechanisms mediated by mTORC1.\textsuperscript{120} Conversely, p53 can also regulate PTEN at the transcriptional level,\textsuperscript{121} and in PTEN null mouse model, deletion of p53 accelerated PTEN null PCa by reducing cellular senescence.\textsuperscript{122} PTEN also regulates NKK3.1, a tumor suppressor whose function is lost as an early event in PCa initiation.\textsuperscript{123}

### 2.16 Function of PTEN

Lipid phosphatase activity of PTEN is important to its tumor suppressor role. PTEN as a dual specific phosphatase with activity towards acidic substrates, removes phosphates from both lipids and proteins and thus acts as a multifunctional protein.\textsuperscript{84} Reconstitution of PTEN expression to certain PTEN null cells results in an increase in the population of cells at G1 phase of the cell cycle,\textsuperscript{124} but in other PTEN null cells it resulted in the induction of apoptosis suggesting the evidence of lipid phosphatase activity of PTEN,\textsuperscript{125} that is associated with tumor suppression. PTEN phosphatase activity can be inhibited by direct binding of DJ1.\textsuperscript{126} PTEN is also capable of dephosphorylating
phosphorylated serine, threonine and tyrosine residues on peptide substrates in vitro,\textsuperscript{127} as well as protein substrates such as FAK,\textsuperscript{128} CREB,\textsuperscript{129} Elf2,\textsuperscript{130} and SRC,\textsuperscript{131} in vivo, thereby inhibiting cell survival, proliferation and migration.

\textbf{2.17 Transcriptional Role of PTEN}

PTEN is one of the most frequently mutated genes in cancer after p53, with inactivating mutations found in many solid tumor types.\textsuperscript{132} The PTEN gene has been shown to be inactivated through somatic and germline mutations as well as transcriptionally suppressed through epigenetic mechanisms,\textsuperscript{133} and often lost in late-stage human cancers, especially those of the brain, prostate and endometrium.\textsuperscript{134}

Epigenetic inactivation of PTEN expression has been described in cancer xenografts, where loss of PTEN protein is a result of promoter methylation.\textsuperscript{50} Transcriptionally, TGF-beta regulates PTEN expression depending on Ras/MAPK pathway. In late stage aggressive diseases, when Ras/MAPK pathway is active, TGF-beta suppresses PTEN through Smad4 independent pathway,\textsuperscript{135} and also through c-Jun.\textsuperscript{136} In the case when Ras/MAPK pathway is blocked, TGF-beta induces PTEN expression through Smad dependent pathway.\textsuperscript{137} Mitogen activated protein kinase kinase 4 (MKK-4) inhibits PTEN transcription by activating Nf-kB, a transcriptional suppressor of PTEN.\textsuperscript{138}

Epithelial to mesenchymal transition (EMT) transcription factor SNAIL, that negatively regulates PTEN expression competes with p53 to bind to PTEN promoter and leads to activation of PTEN transcription during p53 mediated apoptosis.\textsuperscript{139-140} PTEN transcription is also regulated by transcription factors such as PPAR-gamma,\textsuperscript{141} EGR1,\textsuperscript{142}
BMJ,\textsuperscript{143} whereas activated NOTCH1 both positively and negatively regulates PTEN through MYC and CBF1.\textsuperscript{144-145}

### 2.18 Post-Transcriptional Role of PTEN

Post-transcriptionally, PTEN mRNA at miRNA loci is regulated by both miR-22 and miR-106B-25 cluster, that are overexpressed in human PCa.\textsuperscript{146} PTENP1 the PTEN pseudogene which shares vast homology with PTEN mRNA acts as a decoy for PTEN targeting miRNAs and therefore sequester and inhibit negative regulatory effects of miRNAs on PTEN mRNA, and thus PTENP1 influence PTEN expression through a coding independent function.\textsuperscript{147-148}

### 2.19 Post-Translational Role of PTEN

Post-translationally, in inactive state PTEN is phosphorylated at various serine and threonine residues on its C-terminal tail, that increases PTEN stability.\textsuperscript{149} This modification reduces its plasma membrane localization,\textsuperscript{150} and its ability to form complex with PDZ domain-containing proteins,\textsuperscript{151} and as a result there is a decrease in its PIP3 lipid phosphatase activity.\textsuperscript{152} Whereas when PTEN is dephosphorylated at C-terminal tail, it opens its phosphatase domain and interacts with binding partners, that makes PTEN more unstable,\textsuperscript{153} and this open state is more prone to ubiquitin mediated proteosomal degradation at Lys13 and Lys289 that is mediated by NEDD4-1, E3 ligase.\textsuperscript{101,154} Phosphorylation of different amino acids in C-terminal tail of PTEN has different effects and functions that are phosphorylated by different mechanisms. Ser370 can be phosphorylated by tyrosine protein kinase SRC, and protein kinase CK2,\textsuperscript{155} while Thr366 by GSK3-beta.\textsuperscript{156} Phosphorylation at Ser229 and Thr321 targets PTEN to plasma
membrane by protein kinase ROCK,\textsuperscript{157-159} whereas phosphorylation at Tyr336 by RAK can lead to its tumor suppressor activity.\textsuperscript{160}

2.20 Helix Loop Helix (HLH) and Basic HLH (bHLH) Proteins

The helix-loop-helix (HLH) family of transcription factors comprises >200 members, which have been identified in organisms from yeast to man.\textsuperscript{1-2} The basic helix-loop-helix (HLH) family of transcription factors are key players in many developmental processes,\textsuperscript{3} such as differentiation in various cell types, such as in B and T-lymphocytes, muscle lineages, pancreatic B cells, neurons and osteoblasts.\textsuperscript{4-5} Most basic helix-loop-helix (bHLH) proteins positively regulate sets of genes during cell fate determination and cell differentiation form a large super family of transcription factors that regulate tissue-specific transcription.\textsuperscript{6} Members of this family have two highly conserved domains. The carboxyl terminal end of bHLH contains the helix loop helix (HLH) domain involved in forming a homo or hetero dimerization with other bHLH proteins that regulates transcriptional regulation and the amino terminal end contains the basic domain that binds to DNA sequences called E-box,\textsuperscript{19} that plays a very important role in many physiological processes including cellular differentiation, apoptosis, cell cycle arrest and regulate critical developmental processes.\textsuperscript{7}

2.21. Inhibitor of Differentiation/Inhibitor of DNA Binding (ID) Proteins

Id proteins are typically expressed in highly proliferating cells and their expression levels decrease in differentiated cells. Ids are known to be positive regulators of cell growth and negative regulators of cell differentiation.\textsuperscript{10} ID proteins play key roles in the regulation of lineage commitment, cell fate decisions,\textsuperscript{11} and also have a role in cell
signaling, DNA damage control, and apoptosis. They also act in the timing of differentiation during neurogenesis, lymphopoiesis and neovascularisation (angiogenesis) and are essential for embryogenesis and cell cycle progression.\textsuperscript{11}

HLH proteins that positively regulate sets of genes during cell fate determination and cell differentiation, possess a region high in basic amino acids adjacent to HLH domain, that facilitates binding to DNA containing the canonical ‘E box’ recognition sequence, CANNTG and ‘N box’ sequence, CACNAG,\textsuperscript{2} or Ets recognition sequence, GGAA/T present in the promoter regions of regulated proteins,\textsuperscript{4} and thus form a large super family of transcription factors that regulate tissue-specific transcription.\textsuperscript{6} However, a distinct subfamily of HLH proteins, the ID proteins, lack these basic DNA-binding region and instead function solely by dimerization with other transcriptional regulators, principally those of the bHLH type and such ID-bHLH heterodimers are unable to bind to DNA, and hence ID proteins act as dominant negative regulators of bHLH proteins.\textsuperscript{8, 9} Ids are known to interact with other bHLH proteins such as E2A, E2-2, or HEB, and also with factors such as RB (retinoblastoma) family members that have a role in cell cycle.\textsuperscript{4} The Id family consists of four isoforms, Id1, Id2, Id3, and Id4,\textsuperscript{4} that act as negative regulators of basic bHLH transcription factors, by inhibiting the binding of ID-bHLH complex to DNA. Structurally, the core HLH domain between Id and bHLH proteins is highly conserved that allows efficient Id-bHLH dimerization. However, the Id-bHLH dimer is transcriptionally inactive due to the lack of DNA-binding basic domain in Id proteins\textsuperscript{161} (see Figure 5). The interference of Id proteins with the key regulatory bHLH proteins is therefore an important interaction for proliferation and differentiation.
Figure 5. bHLH transcription factors: Part A, represents Basic helix loop helix domain of bHLH transcription factors containing DNA-binding domain. Part B, represents bHLH proteins that bind to DNA. Id proteins lacking DNA binding domain, binds to E-proteins, but the complex cannot bind to DNA.

In man, the Id1, Id2, Id3, and Id4 genes map to same chromosome at positions 20q11, 2p25, 1p36, and 6p21.3-22, where all of them share considerable homology to one another and seem to have derived from a common ancestral gene.

Widespread expression of Id1, Id2 and Id3 genes was demonstrated throughout from early gestation to birth during mouse development, with considerable overlap of Id1 and Id3, with distinct Id4 expression limited to nervous system. Deletion of Id1 gene in mice, failed to generate phenotype, and Id2 null mice possessed defects in immunity due to lack of lymph nodes, while mice null for Id3 had defects in B cell proliferation and humoral immunity. Mice null for both Id1 and Id3 possessed embryonic lethality with aberrant neuronal differentiation and angiogenesis. Id1, Id2 and Id3 which are identified as mitogen responsive, are further implicated in cell cycle progression when antisense Id constructs are introduced into serum stimulated NIH3T3 cells. Id1 had shown to inhibit, E-protein and ETS mediated activation of the CDK inhibitor p16/INK4a. Id2 and Id3 have been shown to get phosphorylated in late G1 stage of cell cycle by cyclin dependent kinase 2 (CDK2), and Id2 by interacting via
its HLH domain with retinoblastoma protein (pRb), p107 and p130, reverses cellular growth inhibition.\textsuperscript{3} Id proteins induce apoptosis in accordance with their expression. Expression of Id1 in dense mammary epithelial cell cultures,\textsuperscript{173} Id3 in B-lymphocytes,\textsuperscript{174} and Id4 in astrocyte-derived cell line,\textsuperscript{175} induces apoptosis.

In general, the expression of Id proteins is high in proliferating cells as Id proteins (Id1-Id3) promote cell proliferation,\textsuperscript{10,176} and expression is down regulated during differentiation.\textsuperscript{177} Id1, Id2, and Id3 are upregulated are in pancreatic cancers,\textsuperscript{178} colorectal cancers,\textsuperscript{179} astrocytic tumors,\textsuperscript{180} and squamous cell carcinomas of head and neck.\textsuperscript{181} Id4 expression is seen to decrease in many cancers,\textsuperscript{182} due to promoter hypermethylation,\textsuperscript{183} except in testicular seminomas where all Id genes are upregulated.\textsuperscript{184} As key regulators of cell cycle and differentiation, Id proteins have shown a vast regulatory function across diverse cellular functions including cell cycle, apoptosis, senescence and tumorigenesis in cancer.\textsuperscript{185}

ID2 and ID3 contain an N terminal region targeted by Cyclin A and Cyclin E cdk2 complexes for phosphorylation, and that region is encoded by a Salmonella typhimurium virulence gene regulator (SPVR) site of which a serine residue is phosphorylated, the SPVR region is also present on ID4 but absent in ID1.\textsuperscript{172} Phosphorylation of ID2 inhibits the binding to E proteins, whereas the phosphorylation of ID3 changes the specificity of the binding to either E12 homodimers or E12-MyoD heterodimers in B-cells.\textsuperscript{171} The inhibition of the phosphorylation of either ID2 or ID3 also affects their ability to promote S phase entry and therefore affects the proliferative potential of the ID2 and ID3.\textsuperscript{186} The half-life of the ID proteins varies between 15
minutes to 1 hour depending on cell type and cellular localization. The degradation of the ID proteins has been linked to the ubiquitin proteasome system. This system relies on an ubiquitin activating enzyme, E1, an ubiquitin carrier protein, E2, and an ubiquitin ligase, E3. The addition of multiple molecules of ubiquitin then targets the protein for degradation via the proteasome. This process of degradation is apparent with ID1, ID2 and ID3, and also ID4. However, ID4 that is ubiquitinated, is dependent on E1 enzyme for degradation.

2.22 Inhibitor of DNA Binding 4 (Id4) Protein

Id4 mRNA and protein, which are primarily expressed in the nervous system, progressively decrease as the precursor cells proliferate in vitro and in vivo and do so more quickly when the cells are cultured at 33°C compared with at 37°C. The progressive decrease in Id4 transcription may be part of the cell-intrinsic timer that helps determine when oligodendrocyte precursor cells withdraw from the cell cycle and differentiate. The human ID4 has two coding exons and produces a protein predicted to be approximately 17kDa. In mice, Id4 RNA is expressed predominately in developing and mature neuronal tissue, bladder and bone marrow that differs from the expression patterns of Id1, Id2 and Id3, while low levels of Id4 RNA (with all the other ID transcripts) is seen in in vitro embryonic stem cell line cultures, but this disappeared as the cells differentiated into blast colonies.

Id4 expression in PCa is associated with an increased risk of metastasis. A recent study by Sharma et al. found Id4 promoter methylation in PCa. Another study from our lab shows, ectopic expression of Id4 results in cell cycle arrest.
methylation and loss of expression has also been documented in colorectal adenocarcinomas.\textsuperscript{193} Id4 expression has been linked to breast tumourigenesis and Id4 protein down-regulates \textit{BRCA1} expression,\textsuperscript{194} and its expression is inversely correlated with estrogen receptor expression in breast cancer malignancies.\textsuperscript{195} Id4 promoter methylation which is evident in breast cancer demonstrates its role as a potential tumour suppressor and oncogene in a context dependent manner.\textsuperscript{196}

The sub-cellular localisation of the Id proteins is considered nuclear, but differs with cell type, differentiation stage and post translational modification.\textsuperscript{197} In normal rat breast tissue Id4 sub-cellular localisation changes with the differentiation stage of the epithelia, with more nuclear staining in proliferating epithelia and in all other stages it is either mostly cytoplasmic or very weakly expressed.\textsuperscript{198} In PCa cell lines, it shows that Id4 protein has a different effect on the cell cycle to the other Id proteins. Id4, when ectopically over-expressed in PCa cell lines, induced S phase arrest and apoptosis and this was attributed to Id4 up-regulating the expression of \textit{E2A} which in turn increased the levels of \textit{p21} and \textit{p27} expression. Id4 has also been demonstrated to increase \textit{cyclin e} levels in \textit{cdkn2a} -/- astrocytes, which drives their proliferation.\textsuperscript{199} In neural precursors, Id4 protein is required for the G1 to S phase transition in the cell cycle.\textsuperscript{200}

Id4 plays an important role in nervous system and in particularly in oligodendrocyte differentiation by directly binding to OLIG1 and OLIG2 proteins responsible for in neural progenitor cells,\textsuperscript{201-202} and acts as a tumor suppressor in breast cancer.\textsuperscript{203} The specific nature of Id4 protein expression in cancer has not been clarified, however studies support that Id4 has both pro-tumor and anti-tumor activity. Epigenetic
silencing of Id4 in leukemia, breast, colorectal, mouse and human chronic lymphocytic leukemia (CLL), and gastric cancer tend to support its antitumor activity. It is highly expressed in bladder and rat mammary gland carcinomas suggesting that it may have pro-tumor activity. All knockout Id4 mice studies revealed that Id4 plays an essential role in neural stem cell proliferation and differentiation, and normal brain development.

The t(6;14)(p22;q32) translocation was initially identified using fluorescence 'in situ' hybridization (FISH) and inverse PCR in one case of adult B cell precursor acute lymphoblastic leukaemia (BCP-ALL) and showed ID4 RNA expression, and also overexpression of ID4 protein by immunohistochemical staining in four other cases of BCP-ALL which harboured other genetic abnormalities such as a t(8;14) translocation, a P53 mutation, 46XY12p- and hyperdiploidy.

The involvement of ID4 in an immunoglobulin heavy chain locus (IGH) translocation was identified in 13 patients with BCP-ALL. The t (6;14) (p22;q32) was cloned from 3 cases using long distance inverse PCR (LDI-PCR) and showed the translocation of IGHJ into the centromeric 3’ portion of the ID4 gene resulting in the over expression of ID4 RNA in BCP-ALL. The current literature and preliminary gene expression experiments therefore suggest that ID4 may be a tumor suppressor or an oncogene in a context dependent manner.
2.23 Id4 Expression in Normal Prostate

Oncomine database suggested that Id4 is highly expressed in the luminal epithelial cells of the adult normal prostate and its expression decreases in PCa (Figure 6) and that was also seen in mouse prostate (Figure 7). To further elucidate this phenomenon, a tissue microarray (TMA) also showed that Id4 is highly expressed in normal prostate whereas its expression decreases with increase in cancer progression (Figure 8). Majority of studies have suggested that Id4 acts a potential tumor suppressor in many other cancers such as breast, leukemia, glial neoplasia, gastric cancer, pancreatic cancer, colorectal adenocarcinoma, and malignant lymphoma.

Figure 6. Oncomine database: Shows that Id4 expression is down regulated in PCa.

Figure 7. Expression of Id4 in normal prostate: In normal mouse prostate Id4 is highly expressed.
Figure 8. PCa tissue microarrays. They were used to investigate Id4 expression. Id4 was highly expressed in normal prostate (A) 200X and (B) 400X as seen by intense brown staining in the nuclei. Overall, Id4 expression decreased with increasing grade of PCa (C) grade I (200X), (D) grade I (400X), (E) Grade II (200X), (F) grade II (400X), (G) Grade III (200X), and (H) Grade III (400X). The sections are also representative of scores used to quantify staining intensity: A and B - score 3; C and D - score 2; E - score 0. Id4 is mostly nuclear as seen by intense nuclear staining (brown, indicated by red arrow in D). At higher stages a clear large nucleus with no apparent brown staining is observed (yellow arrow in D and F). The sections were counterstained with hematoxylin that is reflected in the blue nuclei observed primarily in PCa sections with undetectable Id4 expression. The 400X images in panels B, D, F, and H are corresponding images of boxed regions shown in panels A, C, E, and G (2009). The inset in panel G is the 4009 image of the region showing high Id4 expression in normal prostate adjacent to cancer (stage III).

2.24 Id4 is Epigenetically Silenced in Cancer

Studies performed in other cancers such as breast, colorectal and lymphoma have showed that Id4 is epigenetically silenced due to promoter hypermethylation. EZH2 is a transcriptional repressor that plays a vital role in maintaining the homeostatic balance between gene repression and expression.\textsuperscript{213} In PCa EZH2 is involved in epigenetic silencing of many tumor suppressor genes such as DAB2 interacting protein (DAB2IP),\textsuperscript{214} Adrenoceptor Beta 2 (ADRB2),\textsuperscript{215} E-Cadherin (CDH1),\textsuperscript{216} and KLF2.\textsuperscript{217}
2.26 Id4 Acts as a Tumor Suppressor

The specific nature of Id4 protein expression in cancer has not been clarified, however studies support that Id4 has both pro-tumor and anti-tumor activity. Epigenetic silencing of Id4 in leukemia, breast, colorectal, mouse and human chronic lymphocytic leukemia (CLL), and gastric cancer tend to support its antitumor activity.

PCa by promoting apoptosis through caspase 3/7, cytochrome c and p53 dependent pathways. Also, we have shown that Id4 promotes senescence by promoting E2F1 expression. Id4 has also been shown to attenuate cell proliferation by increasing the expression of cyclin dependent kinase inhibitors p16 and p27 expression and inhibits migration by down-regulating MMP2 expression. Nkx3.1, a marker of epithelial differentiation that regulates rate of proliferating luminal epithelial cells in prostate, is downregulated in LNCaP – Id4 cells, (Id4 was silenced with gene specific
shRNA) whereas it is upregulated in DU145+Id4 cells (Id4 is ectopically expressed). PTEN that acts as a negative regulator of AKT is upregulated in DU145+Id4 cells.\textsuperscript{18} Sox9 whose expression is observed in early stages of prostate hyperplasia and is a part of prostate development that gets reactivated in prostate neoplasia is upregulated in LNCaP-Id4 cell and vice versa in DU145+Id4 cells.\textsuperscript{223-224} Our studies from lab show that Id4 overexpression in DU145 cells promoted apoptosis and senescence (Figures 11 and 12). Overall these studies show that in PCa cell models, in which Id4 was either silenced or over-expressed, Id4 acts as a tumor suppressor.\textsuperscript{18}

![Table Image]

**Figure: 11. Id4 induces apoptosis in DU145 cells.** DU145 + Id4 cells have almost three times more apoptotic cells than DU145 cells, along with more 50% more dead cells.

![Image](Cells_B-gal Staining.png)

**Figure: 12. Id4 promotes senescence in the DU145 cells.** Cells (DU145 and DU145+Id4) cells were stained with SA-b-galactosidase. The blue nuclei due to SA-b-galactosidase staining were counted in 15 randomly-selected fields and are expressed as mean±SEM (panel F). The flattened nuclei with intense blue staining were classified as cells with advanced senescence and smaller light blue nuclei were counted as cells with moderate senescence. **p<0.001, t-test performed for columns “a” and “b”**
2.27 PTEN/AKT Pathway and Id4

Study between Id4 and PTEN expression, suggest that there is some cross-talk between AKT pathway and Id4.\textsuperscript{18} Recent study by Sharma et al suggested that loss of Id4 in mouse prostates resulted in decrease in expression levels of PTEN and vice-versa. pAKT expression levels increased in the absence of Id4 suggesting its role in the AKT pathway. In Id2 and Id3 the serines in Salmonella typhimurium virulence gene regulator (SPVR) site are being phosphorylated.\textsuperscript{26} Id2 acts as a growth suppressor in myoblasts when one of its serine at position 5 in SPVR region gets dephosphorylated.\textsuperscript{26} Id4 also possess similar sequence containing the serine’s at the same positions as seen in Id2. So these findings led us to investigate whether Id4 functions in the similar fashion as Id2. From previous study,\textsuperscript{18} and from preliminary studies (data shown below) we know that Id4 plays a role in regulating PTEN and pAKT expressions.

p53 binding to PTEN promoter can enhance the regulation of PTEN by Id4 and thus can mediate cell cycle arrest and apoptosis by promoting stabilization, acetylation and tetramerization of p53.\textsuperscript{21-22} Id4 that regulates PTEN activity and expression, might play a role when PTEN inactivation increases the expression and activity levels of MDM2 a known p53 repressor by PI3K/AKT dependent pathway\textsuperscript{23}, but when PTEN is present it upregulates p53 through translational mechanisms mediated by mTORC1. All this studies along with some bioinformatics studies made us to investigate the role of PTEN/AKT pathway in regulating Id4 phosphorylation and vice-versa the role of Id4 in regulating PTEN/AKT pathway.
2.28 Id4 Regulates PTEN/AKT Pathway

IHC studies on prostates from 6-8 weeks old Id4-/- mice were analyzed. Data suggested the loss or decreased PTEN expression in Id4-/- prostates as compared to wild type. In Id4 -/-, expression levels of pAKT increased compared to wild type with no significant change in AKT levels. Id4 -/- leads to PIN like lessons suggesting Id4s role in PCa initiation and progression as shown in Figure 13. These results lead us to investigate AKT, pAKT and PTEN mRNA and protein levels in PCa cell in context of Id4 expression.

**Figure 13. IHC data on PTEN, AKT and phospho-AKT (p-AKT).** Expression in wild type (Id4+/+) and Id4 knockout (Id4-/-) mice. A: Pten was expressed at high level in the normal prostate both in the nucleus and cytoplasm of Id4+/+ prostate. B and C: Pten expression was significantly reduced or undetectable (black arrowheads) in the Id4-/- prostate ducts. Note the hyperplastic regions in Panel B. Occasionally, few Pten positive cells were observed that were primarily localized to epithelial cells near the basement membrane (inset in Panel B). Pten expression was observed in the urethra (asterisk, Panel C) but not in prostatic ducts. The inset in Panels A and B are enlarged boxed regions in corresponding panels. Panels D-F: Lobe specific expression of phospho-Akt in Id4-/- mice. Increased phospho-Akt was observed in dorsal prostate (Panel D) but not in ventral (E and inset)) and lateral (F) prostate. Phosphorylation of Akt correlated with total Akt expression (G-I) in Id4-/- prostate. Akt expression was undetectable in lateral and ventral prostate (G) but was detectable in dorsal prostate (H and I) from Id4-/- mice. Panels J-L: Total Akt expression in wild type mice prostate. Akt expression was highly variable within the glandular epithelium (J). Regions of undetectable to high Akt expression were juxtaposed (K and L). Similar expression profile (low to high) of phospho-Akt was observed in wild type prostates (Panels M-O). The cells staining positive for phospho-Akt were counted in tubules that also stained positive for Akt. The ratio of pAkt/Akt positive cells is shown in Panel L. (**: P < 0.001). Representative images are shown. The scale bar is 100 um.
In comparison with DU145 cell line, in DU145+ld4 (ld4 ectopically expressed), pAKT levels decreased and AKT levels remained the same as seen in Figures 14, 15, and 16. PTEN levels increased, in DU145+ld4 as compared to DU145 cells, suggesting that these results are similar to those obtained in ld4 -/- mice prostates.

**Figure 14. PTEN mRNA data.** Showing increase in PTEN expression in the presence of ld4 in RT PCR. There is increase in PTEN mRNA in DU145+ld4 compared to DU145, with no change in LNCaP and LNCaP-ld4.

**Figure 15. PTEN protein expression.** Data showing increase in PTEN expression in the presence of ld4 in DU145. GAPDH was used as a loading control.

**Figure 16. Expression levels of phospho AKT and AKT.** LNCaP- ld4 cells show more expression of pAKT when compared to LNCaP cells. DU145 + ld4 cells have less expression pAKT when compared to DU145 cells. Total AKT remained similar in comparative cell lines. GAPDH was used as a loading control.
Till now, no studies have been performed to address the mechanism by which Id4 act as a tumor suppressor (Prostate) or tumor promoter (Glioblastoma). All Ids have conserved HLH domain with variables C and N terminals as shown in Figure 17. Id2 which belongs to the same family as Id4 is known to act as tumor promoter when it is phosphorylated at serine 5 of SPVR region and as tumor promoter when it is phosphoablated.

![Figure 17](image)

**Figure 17. ID protein sequences.** Conserved HLH domain in all Id proteins shown in Red box. Yellow box is the serine at position 5 that is phosphorylated in Id2 and present in Id4.

Analysis of data from different Bioinformatics tools like NET PhosK 1.0 Server (Figure 18) predicted the possible phosphorylation sites in Id4. This results along with the role Id4 role in regulating phospho-AKT via PTEN prompted us investigate the role of PTEN/AKT pathway in Id4 phosphorylation. Thus these findings led us to propose a model (Figure 19) whereby, Id4 could regulate PTEN/AKT pathway via phosphorylation. Collectively, the initial data prompted us to investigate the role of Id4 in regulating PTEN and AKT pathway, and whether this study can answer the Id4 form of action in PCa is through phosphorylation or not.
**NetPhosK 1.0 Server - prediction results**

Technical University of Denmark

Method: NetPhosK without ESS filtering:

Query: gi_661546_gb_AAA73923.1

<table>
<thead>
<tr>
<th>Site</th>
<th>Kinase</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-5</td>
<td>cdk5</td>
<td>0.61</td>
</tr>
<tr>
<td>S-10</td>
<td>PKC</td>
<td>0.58</td>
</tr>
<tr>
<td>S-16</td>
<td>cdc2</td>
<td>0.53</td>
</tr>
<tr>
<td>S-34</td>
<td>cdc2</td>
<td>0.50</td>
</tr>
<tr>
<td>S-73</td>
<td>PKC</td>
<td>0.55</td>
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<tr>
<td>T-11</td>
<td>PKC</td>
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<tr>
<td>S-59</td>
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<tr>
<td>T-145</td>
<td>cdc2</td>
<td>0.50</td>
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</table>

Highest Score: 0.82 PKC at position 133

**Figure 18. NetPhosK 1.0 Server.** Showing Id4 Predicted phosphorylation sites with highest score at Serine5 and Serine10.

**Figure 19. Predicted Id4 phosphorylation model:** This graphical representation shows possible phosphorylation of Id4. In the presence of PTEN, pAKT is converted back to AKT leading to dephosphorylation of Id4, that now acts as a tumor suppressor as seen in PCa. In the absence of PTEN, AKT gets phosphorylated and as result it leads to Id4 phosphorylation, that now acts as a tumor promoter as seen in Glioblastoma. Now Id4 that is in dephosphorylated form inturn upregulates PTEN vice-versa, thus forming a positive feedback loop.
3.1 ID4 Overexpression and Silencing in PCa Cell Lines

The PCa cell lines LNCaP, DU145 and PC3 were purchased from ATCC and cultured as per ATCC recommendations. Human Id4 was over-expressed in DU145 cells as previously described.\(^{19}\) ID4 was stably silenced in LNCaP cells using gene specific shRNA retroviral vectors (Open Biosystems #RHS1764-97196818, -97186620 and 9193923 in pSM2c, termed as Id4shRNA A, B and C respectively). The cells transfected with non-silencing shRNA (RHS1707) was used as control cell line. Transfections and selection of transfectants (puromycin) were performed as suggested by the supplier. Successful ID4 gene silencing was confirmed by qRT-PCR, Western blot analysis, and ICC.

The pCMV + ID4 vector and pCMV vector alone was transfected in sub-confluent (60%) PC3 cells grown in six well plates using TransIT-prostate transfection reagent cocktail (10 µL TransIT prostate reagent, 5 µL prostate boost reagent (Mirus Bio) and 2 µg pCMV-Id4 DNA in 200 µL of serum free media) for around 5 to 6 hours. The culture media which was without serum and antibiotics was changed. Once after an overnight that is after 12 hours incubation with the cocktail. Forty-eight hours after transfection, the cells with incorporated pCMV + ID4 were selected by incubation in the fresh media containing antibiotic named G418 with about 15 to 20 days with changing
fresh media containing 350 µg/mL G418 (Invitrogen) for one week with media change every 2 days. Following this selection cycle, the transfected cells were passaged once in F12-BCS-Antibody and then re-exposed to F12-BCS-Antibody with 350 µg of G418/ml for an additional week (second G418 selection). This approach ensured the survival of only transfected cells. Simultaneous experiments were also performed in which cells were transfected with no DNA (control, parental) or with pCMV DNA (transfection control). The G418 selection procedure described above resulted in no surviving PC3 parental cells. The cells were grown to confluence (80%), trypsinized (0.25% v/v trypsin and 0.03% w/v EDTA in calcium- and magnesium free phosphate buffered saline), counted, and plated at a 1:2 dilution in new 100-mm plates.

3.2 Reverse Transcriptase

RNA (2 µg or 4µg) was reverse transcribed in a final volume of 25 µl as per standard protocols\textsuperscript{18,225} (RT-Mix: 1.25 mM each of dNTP's; 250 ng oligo dT (Promega, Madison, WI), 10 mM dithiothreitol, and 200 U MMLV reverse transcriptase (Invitrogen) in the MMLV first-strand synthesis buffer (Invitrogen)). RNA was denatured for 10 min at 65°C, and then cooled on ice for 2 minutes before addition of RT mix and enzyme. The reverse transcriptase reaction was carried out at 42°C for 1 h and 95°C for 5 minutes. The reverse transcribed RNA were stored at -20°C until further analysis.

3.3 Quantitative Real Time PCR (qRT-PCR)

qRT-PCR was performed as described previously using gene specific primers (Table 2) on RNA purified from cell lines\textsuperscript{226}.
Table 2. qRT-PCR and ChIP Primers Used in the Study

<table>
<thead>
<tr>
<th>PCR primers</th>
<th>Forward (5')</th>
<th>Reverse (5')</th>
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<td>Id4</td>
<td>CCCTCCCTCTCTAGTGCTCC</td>
<td>GTGAACAAGCAGGCGCA</td>
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<tr>
<td>GAPDH</td>
<td>GAAGGTGAAGGTCGGAGTC</td>
<td>GAAGATGGTGATGGGATTC</td>
</tr>
<tr>
<td>AR</td>
<td>GAAGCCATTGAGCCAGGTGT</td>
<td>TCGTCCACGTGTAAGTGCG</td>
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<td>p21</td>
<td>GCCATTAGGCGATGCACAG</td>
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</tr>
<tr>
<td>FKBP51</td>
<td>TTTCCCTCGAATGCAACTCTC</td>
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<table>
<thead>
<tr>
<th>CHiP Primers</th>
<th>Forward (5')</th>
<th>Reverse (5')</th>
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<tr>
<td>BAX</td>
<td>GGAGCC1CTTTCTCAGTTTG</td>
<td>CAATCGGAGTGTAACCACATC</td>
</tr>
<tr>
<td>PUMA</td>
<td>CATGTACACATTAGTACACCTTGCC</td>
<td>TCTCAGATCCAGGCTTTGCTACTGTC</td>
</tr>
</tbody>
</table>

3.4 Protein Extraction

Total cellular proteins were prepared from cultured PCa cell lines using M-PER (Thermo Scientific). Protein samples were quantitated using the BCA protein assay protocol from Bio-Rad. A standard curve was determined using BSA and sample absorbance read at 750 nm. Samples were concentrated in 30 µg/µL volume and then mixed 1:1 with 2X laemmli sample Buffer.

3.5 Western Blot Analysis

30 µg of total protein was size fractionated on 4-20% SDS-polyacrylamide gel and subsequently blotted onto a nitrocellulose membrane (Whatman). The blotted nitrocellulose membrane was subjected to western blot analysis using respective protein specific antibodies (Table 3).
Table 3. Antibodies for Western Blotting, Co-IP, IHC, ICC, and ChiP Analysis

<table>
<thead>
<tr>
<th>Protein</th>
<th>Company Antibodies</th>
<th>Dilutions WB/ICC</th>
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</thead>
<tbody>
<tr>
<td>ID4</td>
<td>Aviva</td>
<td>1:1200, 1:200</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Cell Signaling</td>
<td>1:1000, 1:200</td>
</tr>
<tr>
<td>PUMA</td>
<td>Rockland Immunochemical</td>
<td>1:1000</td>
</tr>
<tr>
<td>BAX</td>
<td>Cell Signaling</td>
<td>1:1000</td>
</tr>
<tr>
<td>PTEN</td>
<td>Abcam</td>
<td>1:1000, 1:150</td>
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<tr>
<td>p21</td>
<td>Cell Signaling</td>
<td>1:1000</td>
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<td>p53</td>
<td>Cell Signaling</td>
<td>1:1000, 1:200</td>
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<tr>
<td>E-Cad</td>
<td>Novus Biologicals</td>
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<td>AR</td>
<td>Santa Cruz</td>
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<td>EGF1</td>
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<td>KI67</td>
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<tr>
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<td>Global Acetylated lysine</td>
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<td>K320</td>
<td>Millipore</td>
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<td>K373</td>
<td>Abcam</td>
<td>1:1000</td>
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<td>Goat anti-rabbit</td>
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<tr>
<td>Secondary Antibody</td>
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<td></td>
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<td>PDH</td>
<td>Cell Signaling</td>
<td>1:250</td>
</tr>
<tr>
<td>ICC secondary antibodies</td>
<td>Cell Signaling</td>
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<tr>
<td>DyLight 594 goat anti-mouse (red)</td>
<td>Thermoscientific</td>
<td>1:200</td>
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<tr>
<td>DyLight 488 goat anti-rabbit (green)</td>
<td>Thermoscientific</td>
<td>1:200</td>
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<tr>
<td>DyLight 594 goat anti-rabbit (red)</td>
<td>Thermoscientific</td>
<td>1:200</td>
</tr>
<tr>
<td>DyLight 488 goat anti-mouse (green)</td>
<td>Thermoscientific</td>
<td>1:200</td>
</tr>
</tbody>
</table>

After washing with 1x PBS, 0.5% Tween 20, the membranes were incubated with horseradish peroxidase (HRP) coupled secondary antibody against rabbit IgG and visualized using the Super Signal West Dura Extended Duration Substrate (Thermo Scientific) on Fuji Film LAS-3000 Imager.
3.6 Cell Proliferation Assay

PC3-CMV and PC3+1D4 cells were plated in a U shaped 96 well plate at a density of 5 x 10^3 cells/well. After the cells attached, cell proliferation analyses were performed using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. PC3-CMV and PC3+1D4 cells were seeded in 96-multi well plates at a density of 5 x 10^5 cells/well without serum overnight. Cells were then cultured for 72 hrs. MTT assay was performed using CellTiter 96 Non-Radioactive Cell Proliferation Assay kit (Promega) following manufacturer’s instructions.

3.7 Statistical Analysis (Proliferation)

Data was analyzed by SPSS 13.0 statistics software. Experimental data is presented as means ± SEM. A p-value of <0.05 was considered statistically significant.

3.8 Apoptosis Assay

Apoptosis was quantitated using Propidium Iodide and Alexa Fluor 488 conjugated Annexin V (Molecular Probes) and dual-sensor MitoCasp (Cell Technology) as described previously.\textsuperscript{227}

3.9 Statistical Analysis (Apoptosis)

Quantitative real time data was analyzed using the delta delta Ct method. The ChIP data was analyzed using % chromatin (1%) as input (Life Technologies). Within group Student’s t-test was used for evaluating the statistical differences between groups.
3.10 AKT Kinase Assay

Akt kinase assay was performed using a nonradioactive IP-kinase assay kit (Cell Signaling, Beverly, USA) as directed by the manufacturer. Briefly, immunoprecipitated Akt was incubated with cold ATP and GSK-3 fusion protein, an AKT substrate, followed by detection of phosphorylated GSK-3 by immunoblotting with a specific antiphospho-GSK-3α/β (Ser21/9) antibody, in DU145 and DU145 + Id4 cells.

3.11 Migration Assay

In vitro cell migration assay was conducted using 24-well transwell inserts (8mm, BD Biosciences, Palo Alto, California). Cells were harvested and centrifuged at 1500 rpm for 5 minutes at room temperature. The pellets were suspended into F12 supplemented with 0.2% BSA. Aliquots of 100µL cell suspension (3x10^4 cells/insert) were added to each insert. Chemo attractant solutions were made by diluting EGF (10 ng/mL) into F12 supplemented with 0.2% BSA and then cells were allowed to migrate through a porous membrane coated with rat tail collagen (50 mg/mL) at 37°C for 5 hours. F-12 containing 0.2% BSA served as the control medium. Cells inside the transwell inserts were removed by cotton swabs. The cleaned inserts were fixed in 4% paraformaldehyde (pH 7.5). Cells on the outside of the transwell insert membranes were stained using DAPI (3 µg/mL). The images were captured in five random areas using Axiovert 200M, Carl Zeiss (Göttingen, Germany) microscope. Stained nuclei were counted using image analysis software (ZEN 2012; Carl Zeiss). Results were expressed as migration index defined as: the average number of cells per field for test substance/the average number of cells per field for the medium control.
3.12 Tunel Assay

The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assay was used to detect fragmented DNA as marker for apoptosis in FFPE tissue sections using TACS 2 TdT-DAB apoptosis detection kit (Trevigen). The slides were counterstained in hematoxylin and mounted with Immuno-mount (Thermo Scientific).

3.13 Luciferase Reporter Gene Assay

PC3-CMV and PC3+1D4 cells were plated in a 96-well plate at a density of 2.5×10^4 cells/well. After the cells attached, they were transiently transfected by mixing either mutated androgen response element (ARR3-luciferase) or AR response element driven luciferase reporter plasmid (PSA-luciferase) with pGL4.74 plasmid (hRluc/TK: Renilla luciferase, Promega) DNA in a 9:1 ratio with FuGENE HD transfection reagent (Promega) in a final volume of 100 µL of RPMI 1640 medium and incubated for 15 minutes at room temperature. The transfection mix was then added to the cells followed by addition of R1881 (10 nM) or vehicle after 4 hours. After a total of 24 hours, the cells were assayed for firefly and renilla luciferase activities using the Dual-Glo Luciferase reporter assay system (Promega) in LUMIstar OPTIMA (MHG Labtech). The results were normalized for the internal renilla luciferase control. Both of the luciferase plasmids were provided as a generous gift from Dr Amina Zoubeidi (University of British Columbia, Canada).
3.14 Immunohistochemistry (IHC)

Slides were processed through standard protocols. Following antigen retrieval (autoclave in 0.01 M sodium citrate buffer pH 6.0 at 121°C/20 psi for 30 min), the peroxidase activity was blocked in 3% H2O2 and nonspecific binding sites blocked in 10% Goat serum. The blocked sections were incubated overnight at 4°C with respective protein specific primary antibodies followed by incubation with secondary antibody for 1 hour. The slides were stained with DAB for 2 min, counterstained with haematoxylin and mounted with Immuno-mount (Thermo Scientific), examined and photo-micrographs taken using the Zeiss microscope with an AxioVision version 4.8 imaging system. All the antibodies were mono-reactive, that is a single reactive band was observed in western blot using total cell lysate from PCa cell lines LNCaP, DU1545 and PC3.

3.15 DNA Methylation Analysis

PTEN promoter methylation was analyzed using methylation-specific PCR (MSP) as described previously. The MSP region amplified in context of the PTEN genome in this study has been previously investigated and well characterized in hepatocarcinoma. Briefly, Genomic DNA from cell lines was isolated using DNeasy kit (Qiagen) and from laser captured sections using ZR Genomic DNA tissue MicroPrep Kit (Zymo Research). Approximately 1µg of DNA was sodium bisulfite-modified using EZ DNA methylation Kit (Zymo Research) and subjected to MSP as described previously. Polymerase chain reactions were performed in a 25µL reaction using GoTaq Green master mix (12.5ul, Promega) with 500pm each of the 5’ and 3’ primers (Table 2). Temperature conditions for PCR were as follows: 40 cycles of 94°C for 30 sec, 58°C for 45 sec and 72°C for 30
sec, followed by 1 cycle at 72°C for 10 min. PCR products were separated on 1.5% agarose gels and visualized using GelDoc XR+ (BioRad).

3.16 Bisulfite Sequencing

Direct bisulfite sequencing of the PCR product was performed as reported earlier using ID4 specific primers as listed in Table 2 and analyzed using ABI sequencer.

3.17 siRNA Transient Transfections

Cells were seeded in 6 well plates at 1.25×10⁵ cells/well in 5% bovine calf serum (VWR) and allowed to attach overnight. The following day, cells were rinsed with serum free media immediately prior to transfection. Transient transfection reagent was used to transfect p14ARF siRNA and p14ARF siRNA (Catlog # AM51331) control in DU145 cells according to manufacturer's protocol from Ambion Life Technologies and the cells were then cultured for additional 48-72 hr. Subsequently, the transiently transfected cells were harvested for RNA/protein or cross linked with formaldehyde for Chromatin immuno-precipitation.

3.18 Chromatin Immunoprecipitation (ChIP) Assay

Formalin-fixed paraffin-embedded (FFPE) samples were used for ChIP based analysis for enrichment of p53, Acetyl p53, H3K27Me3 and H3Ac on PTEN promoter. For this analysis, the regions showing >75% cancerous regions or more than >80% normal/ benign regions were dissected using Leica LMD6500 and captured in micro centrifuge tubes. Genomic DNA was isolated from these sections by the method of Fanelli et al., except that tissue samples were de-paraffinized with xylene instead of
histolemon. The chromatin extracted from tissue samples was sheared (Covaris S220), subjected to immuno-precipitation with either p53, Acetyl p53, RNA POL II, H3Acetylation or IgG antibodies (see Table 3), reverse cross linked and subjected to quantitative ChIP- PCR (qChIP).

Chromatin immuno-precipitation in cell lines was performed using the ChIP assay kit (Millipore, Billerica, MD) as per manufacturer’s instructions. The chromatin (total DNA) extracted from cells was sheared (Covaris S220), subjected to immuno-precipitation with respective antibodies (see above), reverse cross linked and subjected to quantitative ChIP- PCR in Bio-Rad CFX.

3.19 Statistical Analysis (ChIP)

Quantitative real time data was analyzed using the ΔΔCt method. The ChIP data was analyzed against non-immune IgG used as a negative control. Within group Student’s t-test was used for evaluating the statistical differences between groups.

3.20 Immuno-Cytochemistry

Cells were grown on glass chamber slides up to 75% confluency. The slides were then washed with PBS (3x) and fixed in ice cold methanol for 10 min at room temperature and stored at -20°C until further use. Before use, the slides were equilibrated at room temperature, washed with PBS (5 min x 3), blocked with 1%BSA in PBST for 30 min at room temp and incubated overnight (4°C) with primary antibody (1% BSA in PBST). The slides were then washed in PBS and incubated with secondary antibody with fluorochrome conjugated to DyLight in 1% BSA for 1 hr at room temp in dark. The
slides were subsequently washed again and stained in DAPI for 1 min and mounted with glycerol. Images were acquired by Zeiss fluorescence microscope through Axiovision software.

3.21 Site-Directed Mutagenesis

In-vitro site-directed mutagenesis was performed by using cloned Pfu DNA polymerase (Agilent technologies) on bacterial expression plasmids. The PCR product was first digested overnight with Dpn-I (Promega) and then used to transform JM101 super-competent cells (Agilent). All the mutants were verified by DNA sequencing (Applied Biosciences). Mutations in the SPVR region at serine 5 of Id4 were introduced by PCR using complementary primers incorporating specific site mutations. The mutation of the serine with alanine serves as a phospho- deficient and mutation of serine with glutamic acid serve as a phosphor- mimic. Followed by transfection of the plasmid into cell lines to further determine the effect of mutagenesis at serine 5 of Id4 that explains whether Id4 is phosphorylated or not.
CHAPTER IV
RESULTS

4.1. Id4 Regulates PTEN Expression/Function

Previous studies from both our lab and from other labs showed that Id4 acts as a tumor suppressor in PCa by promoting apoptosis through caspase 3/7, cytochrome c and p53 dependent pathways. But the underlying mechanism of how Id4 regulates p53 dependent pathways is not known. Studies showing the positive feedback loop between PTEN and p53 lead us to investigate one of the mechanisms of how p53 pathway can be regulated by PTEN in the presence of Id4. PTEN presence blocks the PI3-AKT pathway which controls fundamental cellular processes such as cell survival and cell cycle. In my preliminary data I have shown that Id4 increases the expression levels of PTEN and also I have shown that decreases pAKT expression levels with no change in total AKT levels. I have seen both at gene, protein and also with IHC data. These lead us to investigate the mechanism by which Id4 regulates PTEN expression/function and whether transcriptional regulation of PTEN is (acetyl) p53 dependent. Id4 acts as a tumor promoter in glioblastoma, but tumor suppressor in prostate, gastric, colon and leukemia. Functional difference of Id4 in different cancers might be because of phosphorylated or unphosphorylated forms as seen in the case of Id2. Recent evidences based on the Bioinformatics data on Id4 suggested possible phosphorylation sites that might get phosphorylated. PTEN by converting PIP3 to PIP2, (dephosphorylates)
pAKT to AKT), might function in dephosphorylating Id4 which leads to tumor suppression that we see in PCa and increase in dephosphorylated Id4 increases PTEN levels which serves as a feedback loop. Overall these observations lead us to investigate phosphorylation of serines at position 5 on Id4 protein.

Based on preliminary results demonstrating that Id4 dependent regulation of (IHC data PTEN mRNA and protein levels) of PTEN, pAKT and AKT made us to investigate our first aim to determine the mechanism by which Id4 regulates PTEN expression/function.

4.1.1 Methylation and Acetylation of PTEN Promoter

At the epigenetic level, methylation and acetylation of PTEN were studied in the presence or absence of Id4. We observed that there was partial methylation of PTEN promoter in DU145+Id4 cell line to no methylation in DU145. These results prompted us to check histone acetylation status of PTEN promoter in the presence of Id4. We were able to identify H3K27 acetylation marks near the PTEN promoter region using bioinformatics approaches. Experimentally we did not see significant difference in acetylation of PTEN in DU145 and DU145+ID4 cell lines (Figure 20).

**Figure 20. PTEN promoter methylation and acetylation**: a. Showing Partial methylation of the PTEN Promoter in DU145. b. Showing no significant difference comparatively in Acetylation status in DU145.
4.1.2 Id4 and p53 Acetylation

PTEN regulation occurs at the transcriptional level also. Many proteins regulate PTEN transcription negatively (sal-like protein 4 (SALL4), SNAIL, inhibitor of DNA binding 1 (ID1), BMI1, c-JUN, ecotropic virus integration site 1 protein (EVI1) and MYC) and positively (peroxisome proliferator-activated receptor-γ (PPARγ), early growth-response protein 1 (EGR1), p53 and C-repeat binding factor (CBF1)). Human genomic PTEN locus contains a p53 binding element directly upstream of the PTEN gene, which is necessary for inducible transactivation of PTEN by p53. These studies along with data from our lab demonstrating that Id4 promotes p53 transcriptional activity through CBP/p300 dependent acetylation, led us to investigate on whether transcriptional regulation of PTEN by Id4 is p53 dependent. IHC data on Id4 -/- also confirms that acetylated p53 expression is decreased in Id4 -/- mice as compared to wild type and the expression of p21 a known p53 target is also decreased in Id4 -/- mice prostates as shown in Figure 21.
Figure 21. IHC on Id4-/- with p53, Acetyl p53 and p21. The results suggested that acetylated p53 expression is decreased in Id4-/- mice as compared to wild type along with p21 a known p53 target, with no change in total p53. Acetyl p53 is the active form of p53 that is involved in tumor suppressor role of p53.

4.1.3. Binding of Acetyl p53 on PTEN Promoter

These results prompted us to investigate whether PTEN expression is also regulated by acetylated p53 in the prostate. ChIP analysis on the PTEN promoter showed significant increase in binding of acetylated p53 on PTEN promoter in DU145 + Id4 cell line compared to DU145 cell line as shown in Figure 22. Overall these results suggested Id4 dependent increase in binding of acetylated p53 on PTEN promoter and Id4 regulates PTEN expression/ function at the transcriptional level via p53 pathway.
Figure 22. ChIP Assay: Increase in the binding of Acetylated p53 to PTEN Promoter. Enrichment of RNA Pol II, p53, and Ac-p53 on PTEN promoter in prostate cancer cell lines DU145 and DU45 + ld4 cells. The data is expressed (mean+SEM, n=3 in triplicate) as % of input. The statistical significance between enrichment (indicated by letters "*** " corresponding to Pol II and Ac-p53 respectively) is based on comparison with DU145 cells (*: P<0.001).

4.2. Role of PTEN-pAKT Axis in Regulating ld4 Phosphorylation

Id2 acts as a tumor suppressor in dephosphorylated form and tumor promoter in phosphorylated form in different cancers where serine 5 of SPVR region gets phosphorylated.26 With this observation along with using one of the Bioinformatic tool, NetPhosK 1.0 Server (see Figure 15), we were able to focus on possible serines in ld4 that might get phosphorylated based on this score. Serines 5 and 10 possessed highest score but we initially focused on serine 5 based on the previous study.26 This observation along with the observation that Id2 study as previously discussed, made us to study aim 2, that is whether PTEN-pAKT axis regulates ld4 activity by phosphorylation and vice-versa ld4 regulate AKT pathway by dephosphorylating pAKT to AKT via regulating PTEN.
4.2.1 Kinase Assay: Id4 Regulates PTEN/Akt Axis

Phosphorylation of proteins is a posttranslational modification that affects intracellular signaling. Functional regulation by phosphorylation has been well documented for many transcription factors. For example, tumor suppressor Rb gene that is composed of two sets -p110Rb, an un- or under-phosphorylated species, and pp112-114Rb, a group of overtly phosphorylated proteins is wholly unphosphorylated in G1, and gets phosphorylated at the beginning of S phase and remained phosphorylated through S phase and G2 phase of the cell cycle. 233-234 2% of eukaryotic genes code for protein kinases that are involved in catalyzing phosphorylation events. 235 Conserved amino acid sequence SPVR, in Id proteins is a phosphorylation target by cyclin E-cdk2 and cyclin A cdk2 kinases. 26 Kinase Assay was performed to study the role of Id4 in phosphorylation of AKT as initial studies, show Id4 regulates pAKT. Kinase assay determines AKT kinase activity in the cell that is measured in terms of Glycogen synthase kinase 3 (GSK-3) phosphorylation. Results from Figure 23 indicated that kinase levels in terms of GSK phosphorylation decreased in the presence of Id4 in DU145 suggesting the antitumor role of Id4. Kinase assay not only determines Id4 role in PTEN/AKT pathway but also can solve whether in this process Id4 gets phosphorylated or unphosphorylated as seen in Id2.

![Figure 23. Kinase Assay. Showing pAKT activity (in terms of pGSK levels) is decreased in the presence of Id4, thus indicating the role of Id4 in phosphorylation that can be antitumoric. GAPDH is used as loading control.](image-url)
4.2.2 Treatment with PI3K Inhibitor Lead to Id4 Expression in DU145 Cells

In the initial studies we observed Id4 upregulates and vice-versa downregulates pAKT, speculating the role of Id4 in PTEN/AKT pathway in DU145 cells. Id4 also reduced the levels of phospho-GSK as seen above. To further confirm the role of Id4 in PTEN/AKT axis we treated DU145 cells with PI3K inhibitor (LY294002) that inhibits PIP2 phosphorylation to PIP3 which further blocks AKT phosphorylation. Treatment with PI3K inhibitor increased PTEN expression in DU145 cells as noticed in initial studies. Thus this study further confirms that Id4 induces PTEN in DU145 cells that, in turn, dephosphorylates AKT. Expression of Id4 after treatment with LY294002, as seen in Figure 24, indicates that Id4 may be is subjected to phosphorylation even though there is no change in kinase levels before and after treatment with LY294002. Further, Kinase levels are determined in terms of phospho-GSK as seen in Figure 25 after treatment with LY294002. Id4 by regulating PTEN, can maintain AKT phosphorylation status and by doing so may acquire one of the phosphorylation forms. To clarify this, we performed IP with Id4, followed by western blot with global phosphoserine antibody. Possible phosphorylation of Id4 was seen in DU145+Id4, PC3 and MDA-MB-231, breast cancer cell line used as loading control for Id4 (Figure 26). To further confirm the phosphorylation, future experiments are discussed in next section.
Figure 24. Treatment with LY294002. Western Blot showing the expression levels of Id4 after treatment with LY294002 in DU145 cell line, with a comparative increase in PTEN after treatment with LY294002. GAPDH is used as a loading control.

Figure 25. Kinase Assay showing pAKT activity. In terms of Psk, levels remained almost same in DU145 after treatment with PI3K inhibitor. GAPDH is used as a loading control.

Figure 26. Id4 Phosphorylation -IP with ID4 & Western blot with phospho serine. Phosphorylation was observed in DU145+Id4, PC3 and MDA-MB-231 (Breast cancer cell line that expresses Id4 used as a control).
CHAPTER V
DISCUSSION AND CONCLUSION

Phosphoinositide 3-kinase (PI3K)-AKT pathway modulates cell growth, metabolism, differentiation, migration, survival, and proliferation of a wide variety of cell types.\textsuperscript{236} Misregulation of this signaling pathway can cause several disorders, including leukemia and/or autoimmunity or alternatively immune deficiencies.\textsuperscript{237} Since the discovery of PTEN and its role as a phosphatase, the PIP3-Akt pathway assumes significance as a major cancer pathway.\textsuperscript{238} PTEN remains one of the main factors in modulating proteins such as AKT and p53 that are altered in cancer.\textsuperscript{90, 239} PTEN, the main negative regulator of PI3K-Akt pathway functions as regulator of cell polarity and also as a switch between cell proliferation and migration, various types of cancer.\textsuperscript{238}

Unlike p53, a tumor suppressor which is acutely and rapidly upregulated in response to oncogenic stress, PTEN expression is constitutive and essential at all times.\textsuperscript{240} PTEN is shown to be regulated by transforming growth factor β (TGFβ).\textsuperscript{241} PTEN is upregulated transcriptionally by factors such as peroxisome proliferation-activated receptor λ (PPARλ),\textsuperscript{141} and the early growth regulated transcription factor-1 (EGR1).\textsuperscript{142} There is a P53 putative binding element in the promoter sequence of \textit{PTEN},\textsuperscript{231} that suggests a p53 mediated cellular survival mechanism that functions through the activation of PTEN transcription.
In Id4 -/- studies, we observed focal hyperplastic regions resembling PIN like lesions. Many of the genes associated with PCa and their respective knockout/transgenic phenotypes are also recapitulated in the Id4-/- model that support the role of Id4 in PCa. Apart from loss of Nkx3.1 as discussed in our recent study, a decrease in PTEN specifically in the prostate, sustained androgen receptor expression, increased Myc and Sox9 also promote early stages prostatic intraepithelial neoplasia. Our results suggest that the above noted genes and their regulated pathways are downstream of Id4. However, in spite of these complex alterations, we did not observe a significantly greater number of pre-neoplastic lesions in Id4-/- prostate suggesting the possibility of mechanisms/pathways that restrains the formation of significant pre-cancerous lesions and PCa. One of these pathways could involve AKT, a kinase on which many of these pathways converge. AKT 1 and AKT 2 deficiency is sufficient to reduce the incidence of tumors in Pten (+/-) mice, and Myc also cooperates with Akt1 in promoting prostate tumorigenesis. Thus loss of AKT could be a key mechanism that negatively regulates the formation of PIN like lesions given the remarkable pro-neoplastic gene signature in Id4-/- mice. Loss of AKT 1 also leads to increased apoptosis and general growth retardation that affect the size of organs. We speculate that the smaller genital tract and prostate in Id4-/- could be in part due to decreased AKT expression.

In this study, we provide significant evidence demonstrating the role of Id4 in PI3K - AKT pathway. At the core of the pathway is the expression of PTEN, which is positively regulated by Id4. Upregulation of PTEN in the presence of Id4 led to a decrease in levels of pAKT. p53 acetylation by Id4 and its subsequent binding to the
PTEN promoter appears to the underlying regulatory mechanism. We predict ld4 plays an important role in modulation of PTEN activity in PCa cells. If this theory with evidence works, it would suggest that ld4 might be used for the first time as physiological agent to upregulate PTEN which will in turn regulate AKT pathway, thus regulating cell proliferation and cell division in cancerous cells. Additionally ld4 by modulating PTEN and regulating AKT pathway may in turn get phosphorylated or dephosphorylated that can address one of the mechanism by which ld4 acts as a tumor suppressor in PCa. Although it has been reported that ld4 exerts tumor suppressive activities in several cancer models as presented in literature herein, it is possible that ld4 may act as tumor suppressor in a PTEN- p53-independent manner by regulating AKT pathway. However in tumors with PTEN loss, both mTORC1 and other molecules upstream in the pathway including PI3K and AKT PI3/AKT should be inhibited. If we find that ld4 act as tumor suppressor in the absence of PTEN, we will continue to investigate the functional role of ld4 and its effects on the apoptotic and/or senescence pathway in the cellular environment.
PART II

ID4 PROMOTES AR EXPRESSION AND BLOCKS TUMORIGENICITY
OF PC3 PROSTATE CANCER CELLS
CHAPTER VI
INTRODUCTION

Deregulation of tumor suppressor genes is associated with tumorigenesis and the development of cancer. In PCa, ID4 is epigenetically silenced and acts as a tumor suppressor. In normal prostate epithelial cells, ID4 collaborates with androgen receptor (AR) and p53 to exert its tumor suppressor activity.\textsuperscript{15,17,20} Previous studies have shown that ID4 promotes tumor suppressive function of AR whereas loss of ID4 results in tumor promoter activity of AR.\textsuperscript{17–19} Ectopic ID4 expression in DU145 cells attenuates proliferation and promotes AR expression suggesting that ID4 promotes AR expression. In this study, we examined the effect of ectopic expression of ID4 on highly malignant PCa cell PC3. Stable overexpression of ID4 in PC3 cells leads to increased apoptosis and decreased cell proliferation and migration. In addition, in vivo studies showed a decrease in tumor size and volume of ID4 overexpressing PC3 cells, in nude mice. At the molecular level, these changes were associated with increased androgen receptor (AR), p21, and AR dependent FKBP51 expression. At the mechanistic level, ID4 may regulate the expression or function of AR through specific but yet unknown AR co-regulators that may determine the final outcome of AR function.
CHAPTER VII
LITERATURE REVIEW

7.1 AR in Prostate

The prenatal development of the prostate is dependent on androgen, particularly DHT. The fetal testis produces testosterone, which is reduced by 5α-reductase enzyme that is present in the urogenital sinus before and during prostate development. After the development of the prostate, androgens continue to function in promoting the survival of the secretory epithelia, the primary cell type thought to be transformed in prostate adenocarcinoma. In the normal prostate, the rate of cell death is 1–2% per day, which is balanced by a 1–2% rate of proliferation. The reduction of serum and prostatic DHT levels by castration results in a loss of 70% of the prostate secretory epithelial cells due to apoptosis in adult male rats, but the basal epithelia and stromal cell populations are relatively unaffected. AR is also expressed in the prostatic stroma, although castration results in the loss of stromal AR expression. The prostatic stroma therefore has the capacity to respond to androgen, but androgen is not required for its survival.

In humans, the proliferation occurs predominantly in the transitional zone of the prostate, the region that is primarily affected in benign prostatic hypertrophy but is seldom the initial site of prostate carcinoma formation. Although individual cases of PCa have been reported in anabolic steroid users, epidemiological studies have failed to establish a link between elevated serum testosterone, DHT, or adrenal androgens and PCa
risk, suggesting that elevated testicular and adrenal androgens alone do not significantly promote prostate carcinogenesis. Although serum androgens alone may not promote prostate carcinogenesis, androgen action and the functional status of AR are important mediators of PCa progression. Low serum testosterone levels in men with newly diagnosed and untreated PCa have been found to correlate with higher AR expression, increased capillary vessel density within the tumor, and higher Gleason score. AR expression is observed in primary PCa and can be detected throughout progression in both hormone sensitive and hormone refractory cancers. Epigenetic silencing of AR expression by methylation may occur and has been observed in 8% of primary PCas. Another possibility for the loss of AR expression in some tumor cells is a decrease in AR protein stability that reduces the AR protein level to one difficult to detect immunohistologically. AR is also degraded by ubiquitin targeting to the proteasome. Ubiquitination of AR is promoted by AKT kinase-mediated phosphorylation of the receptor, suggesting that cells with increased AKT activation may have a reduced AR protein level.

7.2 AR as Tumor Suppressor and Tumor Promoter

AR may act both as a tumor suppressor and a proliferator in the prostate. Over-expression of AR in PC3 cells results in decreased invasion in, in vivo mouse models whereas mice lacking the prostate epithelial AR (PEARKO) have increased apoptosis in epithelial luminal cells and increased proliferation in epithelial basal cells resulting in the expansion of CK5/CK8-positive intermediate cells. The PERKO mice
developed larger and more invasive metastatic tumors in lymph nodes and died earlier than wild-type littermates.²⁴⁶

AR activity and function is regulated by many co-factors and chaperones.²⁴⁸ In this context, ID4 appears to be one of the key regulators of AR function. Results suggest that in the presence of ID4, AR functions as a tumor suppressor whereas loss of ID4 promotes AR to act as a tumor promoter.¹⁷,¹⁹-²⁰ Identifying new or complex interactions between AR co-/regulators could provide some insight into possible mechanisms by which AR undergoes transition between a tumor suppressor vs. tumor promoter. Here, we report that ID4 acts as a regulator of AR by not only inducing AR expression but promoting its tumor suppressor activity, leading to induction of apoptosis and inhibition of cell migration and growth, in more metastatic PC3 cells.

7.3 AR Regulated Transcriptional Factor: FKBPS1

FKBPs which belong to the family of immunophilins, exert important nuclear functions, mediated by histone chaperone activity, interaction with transcription factors, and modifications of chromatin structure and also play a role in cancer pathogenesis.²⁴⁹ FKBPs, such as FKBPS1, FKBPS25, FKBPS38, FKBPS12, and FKBPS52 indirectly associate with DNA and affect gene expression through interaction with transcription factors or by histone modification.²⁵⁰ In PCa, FKBPS1 is part of a super chaperone complex that includes androgen receptor (AR) and androgen and plays an active role in cell proliferation in both the physiologic conditions of cell growth and differentiation, and in pre-neoplastic and neoplastic diseases and its deregulation leads to resistance to cell death.²⁴⁹,²⁵¹ Studies show that deregulated FKBPS1 as a prime factor in the etiology of
androgen-dependent PCa.\textsuperscript{252} In androgen-dependent tumor cell lines, FKBP51 hyperexpression increased androgen receptor transcriptional activity in the presence and absence of androgens; whereas, knockdown of FKBP51 dramatically decreased androgen dependent gene transcription and proliferation.\textsuperscript{253} On the other hand, it has been shown that FKBP51 is a more reliable marker than PSA because it is induced by AR more rapidly and more strongly than does PSA.\textsuperscript{254} On the basis of these studies, FKBP51 serves not only as a useful target for innovative therapies that block the androgen-receptor signaling axis in PCa but also as a AR regulated transcriptional factor.

7.4 ID4 and AR

In PCa cell lines it shows that Id4 protein has a different effect on the cell cycle to the other Id proteins. Id4, when ectopically over-expressed in PCa cell lines, induced S phase arrest and apoptosis and this was attributed to Id4 up-regulating the expression of E2A which in turn increased the levels of p21 and p27 expression. Id4 has also been demonstrated to increase cyclin e levels in cdkn2a \textsuperscript{-/-} astrocytes, which drives their proliferation.\textsuperscript{199} And in neural precursors, Id4 protein is required for the G1 to S phase transition in the cell cycle.\textsuperscript{200} All knockout Id4 mice studies revealed that Id4 plays an essential role in neural stem cell proliferation and differentiation,\textsuperscript{200} and normal brain development.\textsuperscript{211} ID4 acts as a tumor suppressor in PCa, and its loss, frequently observed in PCa, promotes CRPC through constitutive AR activation.\textsuperscript{17} Id4 is regulated by androgens in cells that respond to androgen stimulation such as testicular sertoli cells and prostate epithelial cells.\textsuperscript{225} Id4 also restores androgen receptor expression and activity in the androgen receptor negative PCa cell line DU145.\textsuperscript{19}
ID4, a dominant negative helix-loop-helix transcriptional regulator is highly expressed in normal epithelial cells of the prostate. In PCa, ID4 expression is progressively lost with increasing stage of the disease due to promoter hypermethylation. We have previously reported that knockdown of Inhibitor of differentiation-4 (ID4) in PCa LNCaP cells, promoted tumorigenicity with a gene expression signature that resembles that of constitutively activated AR in castrated mice. Conversely, ectopic ID4 expression induced re-expression of AR that led to decreased proliferation and increased apoptosis in otherwise androgen receptor negative PCa cell line DU145. Previous study from our lab shown in Figures 27 and 28, determined that in DU145 cells, ID4 functions as a tumor suppressor and when overexpressed in DU145 cells, it led to expression of AR and other genes such as p21, p27 that are needed for AR to function as a tumor suppressor protein. To further elucidate the role of ID4 in PCa, we utilized more metastatic and tumorigenic PC3 cells, which do not express AR and have very low levels of ID4, due to promoter methylation.

![Figure 27. Androgen receptor expression analysis](image)

**Figure 27. Androgen receptor expression analysis.** A. (Upper Panel) Expression of androgen receptor (AR) and E2A (E12/E47) bHLH transcription factor by RT-PCR. The gain of androgen receptor expression in DU145-Id4 cells as compared to DU145 cells at the transcript and protein level is evident. As controls, the parental, mock transfected DU145 cells (AR -ve) and LNCaP (AR +ve) cell lines were used. The expression of beta-actin was used as loading and RT-PCR control. B. Real time PCR analysis, performed on the same batch of reverse transcribed RNA used in panel A confirms the RT-PCR data. The fold change in AR expression is normalized to beta-actin.
Figure 28. Gene expression changes in DU145+ld4 cell lines. (A): RT-PCR and semi-quantitative expression levels of cyclin dependent kinase inhibitors p27 and p21 in DU145, DU145-ld4 and PrEC (normal prostate epithelial) cells. (B) Semi-quantitative analysis of RT-PCR results shown in (A). (C): Real time analysis of ld4, p53 and E-cadherin gene expression in DU145, DU145-CMV and DU145-ld4 cell lines. The real time data is normalized to the constitutively expressed gene beta-actin.
CHAPTER VIII
RESULTS

8.1 Generation of ID4 Expressing PCa Cell Line

Stably transfected PC3 with pCMV+ID4 vector expressed nearly 2.5 fold higher ID4 expression (PC3+ID4) cells as compared to the control vector (PC3+CMV) transfected cells. The ID4 expression in control vector transfected cells was negligible and was comparable to that in parental PC3 cells. Expression of ID4 was measured by quantitative PCR (Figure 29A) and western blotting (Figure 29B).

8.2 Effect of ID4 on Morphology, Cell Proliferation and Migration

A change in morphology in PC3+ID4 cells was observed (Figure 29C, Left panel). PC3+ID4 cells had an "epithelial like" morphology that was associated with increased cell-cell adhesion as compared to a mesenchymal morphology of the PC3-CMV cells (Figure 29C, Right panel). At the molecular level, the transition towards "epithelial" morphology and increased cell adhesion of PC3+ID4 cells could be due an increase in E-cadherin expression (Figure 29D and 29E). Overexpression of ID4 also attenuated proliferation of the PC3 cells. The PC3+ID4 cells had a 2 fold decrease in proliferation (Figure 29F), as compared to control cells. KI-67, a cellular marker for proliferation present during all active phases of cell cycle, was also reduced in PC3+ID4 cells compared to control cells (Figure 29G). Next, we investigated the effect on

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apoptosis in these cells by flow cytometry. The rate of apoptosis in PC3+ID4 cells was significantly higher as compared to PC3-CMV control cells (Figure 29H). Western blot analysis showed an increase in BAX expression, a marker associated with apoptosis in PC3+ID4 cells, compared to PC3-CMV cells (Figure 29I). The Cyclin dependent kinase inhibitor p21, a well-known inhibitor of cyclin-dependent kinases acts as a key factor for the regulation of cell growth is upregulated by AR in PCa cell lines. Consistent with this observation, Western blot analysis showed stable ID4 expression in PC3 cells resulted in upregulation of p21 levels (Figure 29I), compared to PC3 control cells.

Figure 29. Stable overexpression of ID4 with pCMV-1D4 in PC3 cells. Expression of ID4 was evaluated with real time PCR (A) and Western Blot (B). C: Morphology of the PC3-CMV and PC3+ID4 cells in culture. D and E: Analysis of E-cadherin by Western blot and immuno-cytochemistry. F: Proliferation rate of PC3-CMV and PC3+ID4 cells expressed as absorption at 570 nm (means ± SEM, n = 3; ***, P < .001). G: Ki-67 expression by Western Blot. H: Percent of cells undergoing apoptosis by flow cytometry (***: P < 0.001). I: Western blot analysis of BAX, and p21. GAPDH was used as loading control.
PC3+ID4 cells showed significantly decreased migration compared to the PC3-CMV control cells (Figure 30A and 30B) in a transwell migration assay. Collectively, these results demonstrate that ID4 expression decreases proliferation and increases apoptosis via BAX upregulation.

Figure 30. ID4 regulates migration. A. ID4 significantly inhibited migration in PC3+ID4 cells compared to PC3-CMV and PC3+EGF cells (positive control). Each bar represents Mean ± SEM (n = 3). (** p < 0.001 compared to PC3-CMV). B: Representative images of PC3+EGF, PC3 CMV and PC3+ID4 cells after migration of cells through Transwell (×10).

8.3 ID4 Promotes AR Expression

Real time PCR demonstrated that PC3+ID4 cells have 4 fold greater AR expression compared to PC3-CMV cells (Figure 31A). Western blot analysis further demonstrated greater AR protein expression in PC3+ID4 cells compared to PC3-CMV cells (Figure 31C). Immuno-cytochemistry further confirmed increased expression of
androgen receptor in PC3+1D4 cells (Figure 31D). The AR expression in PC3+1D4 cells was localized primarily to the nucleus (Figure 31D2) with an increase in expression and nuclear localization after treatment with 10nM R1881 (Figure 31D4). In contrast, AR was not expressed in PC3-CMV cells treated with vehicle alone but was expressed only after R1881 treatment (31D3).

 FKBP51, a well-established AR regulated gene was used to assess the transcriptional activity of AR. ID4 induced FKBP51 expression at both mRNA and protein levels in PC3+1D4 cells (Figure 31B and 31C). Androgen-dependent transcription
of the FKBP51 is conferred through a non-canonical androgen response element (ARE) element within an intron. Chromatin immuno-precipitation analysis using androgen receptor antibody revealed that binding to FKBP51 promoter is significantly increased (P < 0.001) in PC3+1D4 cells compared to PC3 control cells (Figure 31E). These results suggest that ID4 promotes binding of AR to FKBP51 promoter.

In order to further assess the transcriptional activity of AR, the activity of luciferase driven by the PSA promoter was performed. The relative PSA luciferase activity increased significantly in PC3+1D4 cells as compared to PC3-CMV cells (Figure 31F), which is consistent with the increased expression of AR in these cell lines. The mutant ARR3 luciferase plasmid (mt-ARR3 RE) used as a negative control, did not result in significant luciferase activity. (Firefly PSA is normalized to Renilla empty vector).

8.4 ID4 Results in Decreased Tumor Growth in Vivo

The influence of ID4 on tumor formation by PC3 was examined by measuring the size and weight of the tumors. PC3-CMV control cell tumors in nude mice were observed within 1 week of injection (Figure 32A and 32B). In contrast, PC3+1D4 cells formed tumors after a latency period of approximately 3 weeks, which led to a significant decrease in tumor growth. At the end of the experiments (6 weeks), the tumors were excised and volume and weights were measured (Figure 32C and 32D). The PC3+1D4 cells formed smaller tumors compared to PC3 control cells. Collectively, these results indicate that overexpression of ID4 decreases tumor growth of PC3 in nude mice.
Figure 32. Stable overexpression of ID4 in PC3 cells suppresses tumor growth in vivo. Male and nude mice were injected with PC3-CMV and PC3+ID4 cells and evaluated for tumor volumes and tumor weights. A: Representative xenograft images with numbers of mice with similar tumor (n) are shown. B: Volumes of the tumors were measured weekly (expressed as mm$^3$, means±SEM, n = 3/group, *, p < .05, and **, p < .01, and ***p<0.01 between PC3-CMV and PC3+ID4). The mice were sacrificed at 6 weeks. C, D: Relative volumes and weights (means±SEM, n = 3) of the tumors after excision (***, P < .001, between PC3-CMV and PC3+ID4 tumors). E: TUNEL assay demonstrated increased apoptosis in in PC3+ID4 mice xenograft (Brown staining) as compared to the PC3-CMV cells. The TUNEL positive cells were counted in five fields (at 400×) on three different tissue samples.

Xenografts derived from PC3+ID4 cells showed significantly more apoptotic cells compared to xenografts from PC3-CMV cells, confirming a role for ID4 in promoting apoptosis. The average number of TUNEL positive cells in PC3+ID4 xenograft tissue is significantly higher compared to PC3 control xenograft tissue (Figure 32E).
CHAPTER IX
DISCUSSION AND CONCLUSION

The role of ID4 in cancer has not been clarified; however, studies support both a pro-tumor and anti-tumor activity of ID4. ID4 promoter methylation demonstrates its role as a potential tumor suppressor and oncogene in a context dependent manner. We have shown that ID4 expression is decreased due to promoter methylation in PCa. Epigenetic silencing of ID4 promoter tend to support its tumor suppressor role in PCa.

In PCa cell lines, ID4 promotes cell cycle arrest and apoptosis, by up-regulating the expression of p21 and p27. In the present study also, ID4 decreases migration and cell proliferation of PC3 cells, which is associated with an increase in p21, a regulator of cell cycle progression at G1 and S phase and with a significant decrease in Ki67, a marker of proliferation. PC3+ID4 cells showed increased apoptosis and decreased cell migration compared to control cells. Most importantly we show an up-regulation of a E-cadherin a well-established tumor suppressor and inhibitor of epithelial to mesenchymal transition, by ID4. ID4 may directly regulate E-cadherin expression by neutralizing basic helix loop helix E2A proteins that negatively regulate E-cadherin gene expression through E-Box response elements. Alternatively, increased AR expression in PC3+ID4 cells may itself promote e-cadherin expression.

Collectively, these results demonstrated that ID4 expression induces a change in cell morphology AR plays a tumor suppressor role in normal cells and functions as tumor
protein promoter in PCa cells. Various studies have shown that AR mRNA and protein expression are low but detectable in PC-3 cells. Studies with treatment of PC3, DU145, and LAPC4 cells with R1881 and DHT showed increased AR levels only in PC3 cells, with no effect in DU145 and a negative effect in LAPC4 cells suggesting that PC3 retained the necessary cofactors to engage AR as a tumor suppressor. Addition of functional AR in PC3 cells resulted in decreased invasion in bone lesion assay and in mouse models. Induction of AR expression and activity in PC3 cells indeed results in growth suppression, which is probably mediated by co-regulators that enable AR’s normal tumor suppressor function.

Our results demonstrate that ID4 may activate the tumor suppressor activity of AR by inducing expression of target genes P21 and FKBP51, and by decreasing expression of Ki67, in PC3 cells. At the mechanistic level, ID4 may regulate the expression or function of specific but yet unknown AR co-regulators that may determine the final outcome of AR function.

In conclusion, ID4 may restore AR expression and activity in PC3 cells, which may contribute to the tumor suppressor function of ID4. Therefore, AR-dependent and -independent pathways may contribute to the tumor suppressor function of ID4. Identification of the factors that mediate these effects in these PCa cells will improve our understanding of mechanisms that contribute to tumor invasion and therapeutic resistance associated with androgen deprivation therapy.
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