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ID4 as a tumor suppressor: mechanism of action of ID4 in prostate cancer

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ABSTRACT

BIOLOGICAL SCIENCES

EVANS, ASHLEY L       B.S., South Carolina State University, 2006

ID4 AS A TUMOR SUPPRESSOR: MECHANISM OF ACTION
OF ID4 IN PROSTATE CANCER

Committee Chair: Dr. Jaideep Chaudhary, Ph.D.

Dissertation dated July 2013

Id proteins are members of basic helix-loop-helix family. However, Id proteins lack the basic binding domain, which prevents DNA binding, and thereby regulates transcription. There are four members in the Id protein family termed Id1-4. In prostate cancer the expression of Id1 and Id3 is high, whereas member Id4 expression is low. Decreased expression of Id4 is due to promoter hypermethylation in prostate cancer as well as many other cancers. This observation led us to hypothesize that Id4 acts as a tumor suppressor in prostate cancer. Furthermore, evidence suggests ectopic Id4 expression in metastatic prostate cancer cell line DU145 induced cell cycle arrest, apoptosis, and senescence. In this study, we expanded on these earlier studies to demonstrate that gain of Id4 attenuates cancer phenotype whereas loss of Id4 promotes cancer phenotype in prostate cancer cell lines DU145 and LNCaP, respectively. Upon over-expression of Id4 in DU145 cells (DU145+Id4), there was an increase in apoptosis, due to decreased mitochondrial membrane potential (MMP) and increased expression of...
pro-apoptotic markers (PUMA, BAX, and p21). Inversely, silencing of Id4 in LNCaP cells (LNCaP-Id4) led to decreased apoptosis due to an intact mitochondrial membrane and decrease in the expression of pro-apoptotic markers (PUMA, BAX, and p21). Since BAX, PUMA, and p21 are direct transcriptional targets of p53, these results therefore prompted us to investigate the effect of Id4 on expression and activity of p53. LNCaP cells express wild-type p53. DU145 cells harbor mutant p53 (P223L and V274F), which lies within the DNA binding domain and abrogates p53 transcriptional activity. DU145 cells also express high levels of p53, due extended half-life. Surprisingly, there was decreased expression of p53 in DU145+Id4 cells associated with nuclear localization indicating enhanced transcriptional activity. We investigated p53 DNA binding and transcriptional activity. We determined that mutant p53 in DU145+Id4 cells was transcriptionally active evident by increased luciferase activity and binding of p53 to the promoters of its targets. In LNCaP-Id4, p53 expression was decreased which resulted in decreased p53 transcriptional activity and decreased DNA binding ability. The data suggested that Id4 can restore mutant p53 activity, which is a significant observation. Our results also suggest that Id4 promotes the assembly of a macromolecular complex involving CBP/p300 that results in acetylation of p53 at K373, a critical post-translational modification required for its biological activity. Loss of Id4 in LNCaP cells also abrogated wild type p53 DNA binding and transcriptional activity with concomitant loss of CBP/p300 requirement and decreased acetylation. In conclusion, we demonstrated that loss of Id4 promotes cancer phenotype in LNCaP cells. We also demonstrated that the tumor suppressor activity of Id4 is in part through regulation of CBP/p300 dependent acetylation and function of p53.
ID4 AS A TUMOR SUPPRESSOR: MECHANISM OF ACTION OF ID4 IN
PROSTATE CANCER

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

BY
ASHLEY LECOLE EVANS

DEPARTMENT OF BIOLOGICAL SCIENCES

ATLANTA, GEORGIA
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LIST OF ABBREVIATIONS

ANOVA ............................................................. Analysis of Variance
AR ................................................................. Androgen Receptor
BCS ............................................................... Bovine Calf Serum
bp ................................................................. base pair
BPH ............................................................... Benign Prostatic Hyperplasia
BCRP ............................................................ Breast Cancer Resistance Proteins
BMP .............................................................. Bone Morphogenetic protein
BRCA1 ........................................................... Breast Cancer 1
BSA .............................................................. Bovine Serum Albumin
cDNA ............................................................. Complementary Deoxyribonucleic Acid
Ct ................................................................. Cycle threshold
DAPI ............................................................. 4′-6-Diamidino-2-phenylindole
DNA ............................................................. Deoxyribonucleic Acid
dNTP ............................................................. Dinucleotide Triphosphate
ECL ............................................................... Enhanced Chemiluminescence
EGF ............................................................. Epidermal Growth Factor
ER ............................................................... Estrogen Receptor
FBS ............................................................. Fetal Bovine Serum
HLH ................................................................. Helix Loop Helix
ICC ............................................................. Immunocytochemistry
ID ............................................................... Inhibitor of Differentiation
MAPK ........................................................... Mitogen Activated Protein Kinases
MDM2 ............................................................. Murine double minute 2
MMP .............................................................. Matrix Metalloproteinase
MMP .............................................................. Mitochondrial Membrane Potential
mRNA ............................................................. Messenger Ribonucleic Acid
MS-PCR ......................................................... Methylation Specific-Polymerase Chain Reaction
PBS .............................................................. Phosphate Buffered Saline
PCR .............................................................. Polymerase Chain Reaction
PIN .............................................................. Prostatic-Intraepithelial Neoplasia
PSA .............................................................. Prostate Specific Antigen
PVDF ............................................................ Polyvinylidene Difluoride
RT ................................................................. Reverse Transcriptase
RT-PCR ........................................................ Reverse Transcription Polymerase Chain
RNA ............................................................. Ribonucleic Acid
RPMI ............................................................. Roswell Park Memorial Institute
CHAPTER 1
INTRODUCTION

Id proteins have been implicated in the progression of many malignant cancers (1) that include adenocarcinoma, neural tumors, squamous cell carcinoma, melanoma, sarcoma, leukemia, breast cancer, lymphocytes, and prostate cancer (2-7). In general, Id1, Id2, and Id3 are highly expressed in cancer. Id1, Id2, and Id3 are expressed at low levels in the normal prostate epithelium and benign prostate hyperplasia (8), whereas the expression of Id4 is relatively high (9). In prostate cancer tissues, there were increased Id1, Id2, and Id3 levels (8) whereas Id4 is decreased (9). Id4 expression has been found to be decreased due to promoter hypermethylation in prostate cancer. However, the exact function of Id proteins in prostate cancer is not known. Id proteins have the ability to affect multiple molecular pathways. Id proteins are capable of regulating the expression of a large number of genes through specific bHLH and non-bHLH interactions that in turn regulate many cellular processes such as cell cycle progression (10, 11), cell fate (12-14), invasiveness (15, 16), and apoptosis (12, 17). Id proteins homo or hetero dimerize with other bHLH proteins in the HLH domains. However, Id proteins lack the basic DNA binding domain and cannot bind to DNA (18) (Figure 1). All Id proteins interact with bHLH, TCF3 (19, 20), but their interaction with non-bHLH proteins appears in large part to be isoform dependent- Id1: CASK, ELK1, GATA4, caveolin; Id2: ELK1,
3 and 4, CDK2, PAX2, 5 and 8, Rb and related pocket proteins, Id3: ELK1 and 4, ADD1 ((19, 20) and public databases). Specific non-bHLH interaction partners for Id4 are currently not known.

Many of the factors that contribute to prostate cancer progression have been associated with Id1. Id1 has been implicated in the transition from androgen dependent prostate cancer to androgen independent prostate cancer (21), which is a hallmark of cancer progression. Id1 is also involved in VEGF stimulation of angiogenesis (22) and proliferation mediated by EGFR (21) as well as by inactivation of cyclin dependent kinases (23). Over expression of Id1 in LNCaP cells resulted increased proliferation, and increased invasiveness thorough increased MMP-2. (24, 25). Silencing of Id1 sensitized prostate cancer cells to chemotherapeutic drug Taxol. Also in DU145 and PC3 cells, which are androgen independent cell lines, the levels of Id1, Id2, and Id3 are significantly higher than normal prostate epithelial cells (26). However, Id4 has an inverse expression pattern as compared to other Ids in prostate cancer. Id4 is the most recently discovered member of the Id family and is the focus of this study. Oncomine database provides evidence that in more advanced stages of prostate cancer Id4 was found to be decreased. In metastatic prostate cancer cell line DU145, there is decreased expression of Id4 due to promoter hypermethylation (26). Collectively these studies suggest that Id4 acts as a tumor suppressor. Previously we published data to support the role of Id4 as a tumor suppressor, in which we studied cellular processes such as cell cycle, proliferation, senescence, and apoptosis. Upon over-expression of Id4 into DU145 cells (which have very little to no Id4 expression due to promoter methylation), there is a marked decrease
in proliferation due to increased cyclin dependent kinases p21 and p27 expression (26). Cyclin dependent kinase inhibitors are involved in proliferation and cell cycle arrest. The induction of p21 has been linked to G1 arrest, making it a key component of G1 checkpoint response. P27 has been found to attenuate proliferation in prostate cancer cells. Id4 also induces senescence and apoptosis in DU145 cells (26). Collectively the data suggested that Id4 acts as a tumor suppressor in prostate cancer. To further investigate the anti-tumor properties of Id4, we silenced Id4 in LNCaP cells (express Id4) to determine whether loss of Id4 facilitates cancer phenotype. We hypothesize that loss of Id4 will promote tumorigenesis in prostate cancer cells. We investigated processes associated with cancer phenotype which included proliferation, apoptosis, anchorage independent growth, and tumor growth \textit{in vivo}.

Tumor suppressor p53 is one of the most commonly inactivated genes in human cancer (27, 28). The inactivation of p53 occurs for many reasons, such as lesions that prevent p53 activation, mutations within the gene itself or mutations in downstream effectors that affect p53’s function (29). Some mutations in p53 usually fall within the DNA binding domain and renders p53 transcriptionally inactive (30). Transcriptional inactivation of p53 hinders the activation of p53 transcriptional targets that may induce cell cycle arrest or apoptosis. Deregulated apoptosis is a common event in cancer progression. Interestingly, DU145 cells harbor mutant p53 that lacks transcriptional activity (31). The p53 mutants (P223L and V274F) in DU145 cells are rare but within the DNA binding domain (DBD 94-292) known to abrogate p53 transcriptional activity due to structural de-stabilization and/or DNA interactions (32, 33). The 274F in DU145 cells
is next to 273H, a DNA contact and one of the most highly mutated amino acid (33).

Both these amino acids (274F and 273H) are within the conserved region of p53 beta strand S10 whereas 223L lies in the outer loop (30). Some but not all p53 mutations maintain transactivation potential for some promoters (e.g. CDKN1A) but not others (e.g. BAX, PUMA and Pig3) (34). The mutant p53 in DU145 also lacks the ability to transactivate CDKN1A (37).

While there is overwhelming evidence that Id4 acts as tumor suppressor in prostate cancer, its mechanism of action is not known. We hypothesize Id4 functions as a tumors suppressor by inducing apoptosis in a p53 dependent manner in prostate cancer cells. The aim of this study is to investigate the molecular mechanism by which Id4 acts as a tumor suppressor in prostate cancer. To investigate this hypothesis following aims were designed:

1. To identify Id4 regulated molecular pathways involved in tumor suppression.
2. Determine the mechanism of Id4 regulated apoptotic pathways in prostate cancer cells.
2.1 Prostate Development

The growth and development of the prostate begins at the fetal stages and concludes at sexual maturity (35). The prostate develops from the urogenital sinus (UGS), which is part of the cloaca. Urogenital sinus is a midline structure with epithelial layer surrounded by a mesodermally derived mesenchymal layer and is found at the neck of the developing bladder (36). The UGS is found in both male and female humans at about 7 weeks of gestation (35). Prostatic morphogenesis occurs once the UGS is distinguishable at 10-12 weeks. The adult human prostate has a compact morphology and is about the size of a walnut (Figure 2A). The adult prostate is organized into three zones, a central zone, a transition zone, and a peripheral zone (Figure 2B) (37). The paired central zone is posterior to the stromal region. Interior to the central zone is the transition zones which is located on either side of the urethra. The transition zone represents the smallest zone in the normal prostate (38). The peripheral zone is the largest region of the normal adult prostate, which is located on the posterior side of the prostate. The significance of this architecture is based upon the relationship of these zones to prostatic disease. 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 (39).

Mature prostatic ducts consist of three major cell types (Figure 3B), luminal secretory epithelial cells, basal epithelial cells and stromal smooth muscle cells (40, 41). The cell types can be distinguished from one another by differentiation markers. The expression markers for the luminal cells are androgen receptor and cytokeratins 8 and 18. Markers for basal epithelial cells are p63, cytokeratins 4 and 14. There are also less common cell types that include neuroendocrine cells and rare basal epithelial cells that have unique marker expression profiles and are candidates for epithelial stem cells (41).

The stromal layer is mostly composed of smooth muscle, but also contains fibroblasts, neuronal, lymphatic, and vascular cell types (40). Stromal markers include smooth muscle alpha-actin and vimentin. Neuroendocrine cells are a minor population of cells in the normal prostate, are androgen-independent, and express chromogranin A, serotonin, and neuropeptides (36).

2.1.1 Androgens and Androgen Receptor

Leydig cells, a minor population of non-epithelial cells in the testis, produce androgens. Androgens have an important role in male sexual differentiation, sexual maturation, and the maintenance of spermatogenesis (42-44). Androgens also have an essential role in reproductive organ development, and mediate physiological response in many other tissues and organs which include the skin, skeletal muscle, cardiac muscle, liver, kidney, central nervous system and the hematopoietic system (42, 45). Androgens mainly target the prostate gland and modulates its response through androgen receptor
Androgen action functions through an axis involving the testicular synthesis of testosterone, its transport to target tissues, and the conversion by 5-reductase to the more active metabolite dihydrotestosterone (DHT). Testosterone and DHT exert their biological effects through binding to AR and inducing AR transcriptional activity. After the development of the prostate gland, androgens continue to promote survival of the secretory epithelial cells, the primary cell type involved in the malignant transformation to prostate adenocarcinoma. AR belongs to the nuclear receptor superfamily and is a ligand-inducible transcription factor that regulates tissue-specific expression of target genes. It is uniformly expressed in most human organs; however there is an absence of expression in the spleen and bone marrow (46). The AR gene consists of eight exons and encodes four functional domains which are similar to other nuclear receptor superfamily members. These domains include a conserved DNA-binding domain, hinge region, a COOH-terminal ligand-binding domain (LBD), and less conserved NH2-terminal domain (47).

2.2 Prostate Cancer Incidence

Prostate cancer is the second most common cancer among men in the United States, only behind non-melanoma skin cancer. It is also the leading cause of cancer death among men of all races (48). Statistics suggest that black men are more likely to die of prostate cancer than any other group. The incidence of prostate cancer dramatically increases in later stages of life and affects men at a ratio of 1:9 over the age of 65 (49) (50).
2.3 Prostate Cancer Progression

In the normal prostate the balance between low rates of proliferation and low levels of apoptosis maintain homeostasis. In prostate cancer there is a disturbance of this balanced state (51). Prostatic intraepithelial neoplasia (PIN) has been coined the precursor of prostate cancer. PIN is low to high grade lesions that are localized in the prostate ducts (52, 53). In PIN and early invasive carcinomas there is a seven to ten fold increase in proliferation. PIN eventually leads to invasive carcinoma (Figure 3A), characterized by loss of basal lamina, over proliferation of basal and luminal cells, and full expression of markers associated with invasive carcinoma such as MMP 2 (matrix metalloproteinase-2) (54). Invasive carcinoma may progress to a more aggressive phenotype which allows local invasion to the seminal vesicles (54) (55). The transition from androgen dependent to androgen independent may result in metastatic prostate cancer (54) (55). Metastatic prostate cancers also display an approximately 60% decrease in the rate of apoptosis (51) and migration to other body parts (54, 55).

Prostate cancer can be detected through elevated serum prostate serum antigen (PSA) levels. The PSA test has been widely used to screen and help in early detection of prostate cancer. PSA levels are detected at a range of 2.5-10 ng/mL in prostate cancer samples, results confirmed by histology of biopsies. Although PSA detection is very sensitive, it is an imperfect system for detecting prostate cancers (56). Previously, prostate cancer was detected by clinical symptoms or digital rectal examination (DRE). This method of detection resulted in the discovery of advanced tumors that had stretched beyond the organ capsule or whether metastasis had occurred (56).
2.3.1 Androgens, AR, and prostate cancer

In 1941, Huggins and Hodges reported that the normal prostate and early stages of prostate cancer were strongly dependent on androgens (57). The activity of AR and AR modulators is an essential aspect of prostate cancer. Approximately 80-90% of prostate cancers are sensitive to androgens at its initial diagnosis (58). After Huggins and Hodges discovery, treatments of prostate cancer focused on androgen ablation by castration or chemical castration. Androgen deprivation usually results in favorable clinical response and dramatic tumor regression. Androgen depletion is an essential part of many prostate cancer treatments, despite the frequent reoccurrence of prostate cancer post androgen ablation therapy. However, this does not mean that the cancer is fully gone, after continuous treatments, the treatment can stop working and an androgen independent cancer may re-develop and spread throughout the body. Casodex (bicalutamide) is a pharmaceutical drug, and is commonly used as an anti-androgen drug to treat recurrent prostate cancer. Casodex prevents androgen binding to AR by blocking its binding sites and acting as a competitive inhibitor of AR. Androgen dependent cells are growth inhibited by Casodex; however androgen independent cells are refractory to this type of treatment. AR is expressed throughout prostate cancer progression and persists in the majority of patients with hormone refractory prostate cancer. The mechanisms involving progression of prostate cancer have been extensively studied and are seemingly involved in prostate carcinoma development. However, frequent amplification of AR gene is observed in prostate carcinomas growing under low levels of androgens resulting in increased sensitivity towards minimal levels of androgens (59).
AR coactivators are also responsible for mediating effects of AR on chromatin structure and transcriptional initiation (60). Low serum testosterone levels of men newly diagnosed with prostate cancer have shown a correlation with increased AR expression, increased capillary density within the tumor, and higher Gleason score (61). Increased AR expression also correlates with decreased recurrence free survival and disease progression.

AR can be transactivated by coactivators also referred to as coregulators. It has been suggested that differing levels in the expression of coregulators may be involved in the development and progression of prostate cancer (60). Coactivators are coregulators that activate AR and corepressors repress AR. Examples of coactivators include ARA70 (Androgen receptor associated coregulator 70), SRC1 (alias NCOA1 nuclear receptor coactivator 1), CBP (alias CREBBP CREB binding protein (Rubinstein-Taybi syndrome)), p300 (alias EP300 E1A binding protein p300) (62), and 5 putative corepressors include NCoR1 (nuclear receptor co-repressor 1), AES (amino-terminal enhancer of split), cyclin D1 (alias CCND1), prohibitin (PHB), and PAK6 (p21(CDKN1A)-activated kinase 6) (63).

In the absence of androgens, androgen receptor is located in the cytoplasm in a complex with HSP90. Upon infusion of androgens the globular C-terminal domain of the receptor accepts the ligand and structural changes occur that result in the dissociation of AR from HSP90 or other heat shock proteins. Then, nuclear AR binds to AREs, and recruits various cofactors. HSP90 forms a stable heterocomplex with AR and has been shown to translocate AR to the nucleus.
Androgen receptor also has regulatory abilities for other prostate cancer associated proteins. Three known AR transcriptional targets are PSA, NKX3.1, and ETV1. Prostate specific antigen is considered to be the most sensitive biochemical marker for evaluating prostatic disease and response to therapy. PSA is a known glycoprotein that is a member of the kallikrein family of serine proteases (64). In the normal prostate PSA is secreted into the glandular ducts, it is there that it degrades proteins produced in seminal vesicles to prevent coagulation of the semen. During prostate cancer progression, PSA levels increase as a result of aberration of the normal prostate ductal structure by neoplastic epithelial cells. This allows PSA to be actively secreted into the extracellular space and enter the circulation. AR is the primary regulator of PSA expression, by binding to three androgen response element containing enhancer elements located within the PSA promoter region (64). E-twenty six (Ets) transcription factors, epithelium-specific Ets factor 2 (ESE2), and prostate-derived Ets factor (PDEF) can also induce the transcription of a PSA reporter gene in AR negative cell line CV-1.

NKX3.1 is also a transcriptional target of AR and required for prostate tumor progression. Androgen receptor (AR) positively regulates NKX3.1 expression, whereas NKX3.1 negatively modulates AR transcription and consequently the AR-associated signaling events (65). Ets Variant Gene 1 (ETV1) is a novel androgen-regulated gene. Studies demonstrate that ETV1 mRNA and protein are up-regulated in response to ligand-activated AR in androgen-dependent cells, but there is no detectable ETV1 expression in normal prostate cells (66). The ETV1 promoter is induced by androgens and recruits AR, as a feedback loop (67).
2.3.2 Genetic disposition in prostate cancer progression

Literature suggests that genetic disposition may play a role in prostate cancer progression since about 5-10% of prostate cancer cases are considered to be hereditary (68, 69). Polymorphisms are DNA sequence variation in genes that are associated with hormone response, DNA repair, cell protection, and nucleotide metabolism. Polymorphisms have been implicated in prostate cancer progression. Gene amplifications as a result of chromosomal alterations have been observed in many cancers. The frequency of structural chromosomal alterations have been found to be increased in advanced stages of prostate cancer. Specific chromosomal regions have been identified as having a role in chromosomal alterations, these regions include 5q, 6q and 7p (70).

Another facet of prostate cancer progression is the inactivation of tumor suppressor genes such as p53, retinoblastoma (RB), and Phosphatase and tensin homolog (PTEN), which are the most notable tumor suppressors and are inactivated, silenced or mutated in cancers (71) (72). Chromosomal alterations and point mutations are responsible for functional inactivation of p53 in prostate cancer (73). Point mutations have also been reported for PTEN that result in transcriptional reduction of expression (72). The Rb gene is located at 13q14, which is a chromosomal region frequently deleted in prostate carcinoma suggesting that tissue specific tumor suppressor genes play key roles in prostate carcinoma (74).
2.4 Id Proteins

The inhibitor of differentiation (Id) proteins belong to a class of helix loop helix (HLH) proteins involved in the development of many cell types. Id proteins were first characterized in the early 1990s (75). There are four members of the Id family, Id1, Id2, Id3 and Id4. The most studied Id proteins are Id1 and Id3. Id4 is the most recently discovered family member and the least understood. The Id protein have a conserved HLH domain, that allows for homo or hetero dimerization with other proteins that have a HLH domain. However, Id proteins lack a basic region (DNA binding domain) and cannot bind DNA. Through protein-protein interactions Id proteins regulate transcription (Figure 1) (18). Id proteins have divergent structures and functions, but they do share sequence homology in the HLH domain. Id1 and Id3 share the highest sequence similarity (Figure 4). There are seven classes of HLH proteins that are categorized by their tissue distribution, dimerization and tissue binding abilities (1) (Table 4). The class I HLH protein are referred to as basic HLH proteins and E proteins contain a motif of positively charged amino acids adjacent to the HLH domain and bind to the E box sequence CANNTG. These proteins possess the ability to homodimerize or heterodimerize with their HLH domains; however their binding specificity is limited to E boxes. The class II proteins can also form homodimers and heterodimerize with the class I proteins (18, 76). The class IV proteins are known to interact with the class III proteins. The class V proteins (consists of Id1, Id2, Id3 and Id4) lack the basic DNA binding domain and have the ability to negatively regulate class I and II proteins (18). The size of Ids range from 119 amino acids (Id3) to 199 residues (Id4) (14). Id proteins have
different expression patterns which suggests an isoform specific role for individual Id proteins (77). Id protein expression is high in developing tissue and proliferating cells but absent in terminally differentiated tissues such as adult tissues (78) (77). Id proteins hetero dimerize with several classes of bHLH proteins as well as non bHLH proteins. The interaction repertoire of Id proteins also involves several non-bHLH proteins. All Id proteins interact with bHLH TCF3 (19, 20), but their interaction with non-bHLH proteins appears in large part to be isoform dependent. Specific non-bHLH interaction partners for Id4 are currently not known. Thus Id proteins are capable of regulating the expression of a large number of genes through specific bHLH and non-bHLH interactions that in turn regulates many cellular processes such as cell growth, differentiation, and apoptosis (12).

2.4.1 Id Proteins in Development

Id proteins are expressed in many organs and tissues during development (79) and the expression of Id1, Id2, and Id3 are overlapping, whereas Id4 has a unique pattern (80). It has been determined that many organs and cell types require Id proteins for normal development, which is when cells rapidly proliferate and differentiate in various living systems. Id1 and Id3 are essential components for neurogenesis (81), angiogenesis (82), and tumor vascularization (81), which provides evidence of the role of Id proteins during development and in cancer. Id1/Id3 knockout mice have severe brain deficiencies (81) and angiogenesis and invasion were severely inhibited in knockout mice (83). As for overexpression studies, constitutive expression of Id1 impaired mouse B cell development (76) (84). Also, overexpression of Id2 has been determined to lead to inhibition of T-cell development (85). Id proteins may also act as antagonists to other
HLH transcription factor proteins during development (76). Inhibitory effects of Id proteins on E proteins blocks the activation of E protein target genes, which results in the loss of tissue specific gene expression and a disruption of differentiation program in many cell types (86). In lymphocyte development, E2A is required for normal lymphocyte development and function, when Id2 binds E2A, its function is blocked which results in defective lymphocyte development. Based on that study it was determined that Id2 expression is an essential factor in lymphatic organ development (87). The retroviral overexpression of Id4 induces the disappearance of ectoderm overlaying the neural tube which causes these cells to be converted into neural crest cells (88). From these studies, we can conclude that Id4 has essential functions in neuronal cells and mammalian embryos. Id2 and Id4 show similar expression patterns in migrating post-mitotic neurons (89). Id4 is selectively expressed in the nervous system during development suggesting a unique role of Id4 compared to other Id proteins during development. Id4 is expressed in the cerebellum and olfactory bulb, while Id1 and Id3 are not expressed in the brain of adult animals (82). In Id4 -/- mice, the brains are severely transformed and the hypothalamus is disorganized and reduced in size (76). In Id4 -/- mice, oligodendrocyte production is also extremely reduced in the brain of these mice (90). Id4 is an essential regulator of neural stem cell proliferation and fate determination (91). Id4 expression is unique during embryogenesis as compared to otherIds (80). During embryogenesis, Id1, Id2, and Id3 were expressed in multiple tissues, whereas Id4 was only detected in neuronal tissues and the ventral portion of the
epithelium of the developing stomach (92). Collectively while all Id proteins have a role in development, their roles may not be the same.

2.4.2 Id Proteins in Cancer

Id proteins have been recognized as molecules coordinating inhibition of differentiation with a host of cellular functions that include proliferation, cell cycle progression, migration, invasiveness, cell fate, and angiogenesis. Id proteins have the ability to affect multiple molecular pathways. (93, 94). More tumor promoting abilities exist with Id1 and Id3. Ids affect the invasiveness of cells. Id1 over-expression in SCp2 mammary epithelial cells caused increased invasiveness (7). Id1 has also been implicated in promoting migration in mammary epithelial cells due to increased production of MMP2 (95). Studies have shown that Id proteins, more specifically Id1, have a role in regulating senescence and immortalization. Id1 was found to have a role in promoting the immortalization of primary keratinocytes (96), esophageal epithelial cells (97), and prostate epithelial cells (98). In breast cancer, elevation of Id1 correlates with a more aggressive subset of breast cancer and cervical cancer (99). Id2 also has role in regulating cell processes. In neuroblastoma, elevation of Id2 is able to inactivate the Rb pathway, which promotes the cell cycle (10). Over-expression of Id2 was found to determine cell fate specification in the ectoderm by suppressing the epidermal lineage (88). Id3 has been shown to be highly expressed in various cancers including prostate cancer, pancreatic cancer, and colorectal cancer, while Id3 expression is decreased in ovarian cancer (100).
2.4.3 Id Proteins in Cell Cycle and Apoptosis

Id proteins play an important role in G1 -S-phase transition in the cell cycle (Figure 5). Id1 and Id2 proteins are required for cell cycle progression through the G1 phase and levels of these proteins decline as cells enter senescence in fibroblasts (101). The retinoblastoma (Rb) protein represents a crucial protein in cell cycle regulation and the disruption of Rb is one hallmark of cancer (102). In U2OS cells, which have functional Rb and p53, Id2 over expression results in increased proliferation. However, this is not the case in SAOS-2 cells, which lack functional Rb, indicating that Id2 protein exerts its effect on the cell cycle in an Rb dependent manner (10). Furthermore, the loss of Id2 expression rescues Rb-/- embryos and delays tumorigenesis of pituitary tumors in Rb+/- mice (103). Id2 is therefore an effector of cell cycle regulation and proliferation via Rb regulation. Evidence suggests that Id4 may also have a role in cell cycle regulation in neural precursors, being that it is required for the G1 to S phase transition (104). Id4 has also been demonstrated to increase cyclin E levels in cdkn2a +/- astrocytes, which drives their proliferation (17). Id proteins are regulated by mitogenic factors, which causes an up-regulation in expression and propelling of the cell cycle. Literature suggests a role for Id proteins in apoptosis. Id1 is highly expressed in advanced prostate cancer cells that are resistant to apoptosis, and down regulation may result in increased sensitization to prostate cancer treatments (105). Inhibition of Id2 led to apoptosis in some prostate cancer cells (LNCaP and PC3) and not others (DU145) (106).
2.4.4 Id proteins in prostate cancer

Id1, Id2, and Id3 are expressed at low levels in the normal prostate epithelium and benign prostate hyperplasia (8), whereas the expression of Id4 is relatively high (9). In prostate cancer tissues, there were increased Id1, Id2, and Id3 levels (8) whereas Id4 was decreased (9). Id4 expression has been found to be decreased due to promoter hypermethylation in prostate cancer. However, the exact function of Id proteins in prostate cancer is not known. Id proteins are capable of regulating many pathways via specific bHLH and non-bHLH interactions. Many of the factors that contribute to prostate cancer progression have been associated with Id1. Expression of Id1 has been associated with poor survival of prostate cancer patients, which suggests a role of Id1 in prostate cancer progression (105). Id1 has been implicated in the transition from androgen dependent prostate cancer to androgen independent prostate cancer (21), which a hallmark of cancer progression. Over expression of Id1 in LNCaP cells resulted in androgen independent proliferation, which suggests the regulation of AR expression and activity by Id1 (24). Id1 is also involved in VEGF stimulation of angiogenesis (22) and proliferation mediated by EGFR (21) as well as by inactivation of cyclin dependent kinase inhibitors (23). Silencing of Id1 sensitized prostate cancer cells to chemotherapeutic drug Taxol. Also in DU145 and PC3 cells, which are androgen independent cell lines, the levels of Id1, Id2, and Id3 are significantly higher than normal prostate epithelial cells (26). Inactivation of Id1 by small interfering RNA attenuated cell proliferation and promotes apoptosis and senescence in PC3 cells (107). In vivo studies in which PC3 cells with silenced Id1, developed smaller tumors as compared PC3
control. Decreased tumor formation of PC3-Id1 was possibly due to decreased proliferation marker PCNA and invasion marker MMP2 (107).

2.5 Id4

2.5.1 Id4 in development

Id4 is the most recently discovered member of the Id family and the focus of the current studies. Id4 plays a critical role during development. Several studies provide evidence that Id4 plays a major role in the development of the nervous system, more specifically oligodendrocyte development (97). Id4 is expressed in oligodendrocyte precursor cells and may have a role in controlling the timing of differentiation of oligodendrocytes (108). Recently, Id4 was found to directly interact with bHLH, OLIG1 and OLIG2 in neural progenitor cells. Id4 inhibits oligodendrocyte development by blocking the transport of Olig1 from the cytoplasm to the nucleus (109). Knock-out studies revealed that Id4 is required for normal brain size. Knock-out studies also suggested Id4 regulates neural stem cells proliferation and differentiation, specifically Id4 regulates lateral expansion of proliferative zone in the developing cortex and hippocampus (104).

Dong et al. found that Id4 null mice exhibit impaired mammary development such as ductal elongation, side-branching, and possibly alveologenesis (110). Id4 also stimulates the proliferation of mammary epithelium during puberty, pregnancy as well as cell proliferation induced by estrogen and/or progesterone. Data provided evidence that Id4 promotes mammary gland development by suppressing p38MAPK activity. Id4 was
identified as a distinguishing marker of spermatogonial stem cells in the mammalian germline and plays an important role in the regulation of self-renewal (110).

### 2.5.2 Id4 as tumor promoter vs. tumor suppressor

Localization studies of Id4 during cancer progression suggest an inverse relationship to Id1, Id2 and Id3 in many different cancers such as prostate cancer, colorectal adenocarcinoma, and thyroid cancer (26, 80, 111-114). Therefore, Id4 expression is unique as compared to Id1, Id2, and Id3. In many cancers, Id4 is epigenetically silenced due to promoter hypermethylation, which leads to reduced levels (115). The reduced levels of Id4 in these cancers may indicate its role as a tumor suppressor. However, there are also lines of evidence that suggests Id4 may act as a tumor promoter in some cases of cancer (115). Id4 has been implicated in many malignancies and may function as both a tumor suppressor and/or an oncogene in a context dependent manner.

### 2.5.3 Id4 as a tumor promoter

There are some cases in which Id4 acts as a tumor promoter in ovarian, breast, and bladder cancer. In ovarian cancer, nanoparticle delivery of siRNA sequence for Id4 in ovarian tumor-bearing mice suppressed growth of established tumors while significantly improving survival. They also found that Id4 down-regulates BRCA1 in ovarian and breast cancers (116). In breast cancer, Park et al. found that knockdown of Id4 expression suppressed the properties of cancer stem cells (CSCs) including sphere-forming ability and side population phenotype. Based on this data, Id4 may be a
therapeutic target for the treatment of advanced breast cancer. Id4 is also a potential oncogene in a small subset of bladder cancers (117).

2.5.4 Id4 as a tumor suppressor

Chan et al. found that Id4 is hypermethylated in gastric adenocarcinomas. Id4 was determined to be a potential tumor suppressor gene since epigenetic inactivation has a role in the colorectal cancer progression (118). Also in colorectal adenocarcinoma, cdc42 induced methylation of CpG island of the Id4 promoter, which resulted in decreased Id4 expression. In 60% of colorectal cancer samples, Cdc42 was found to be over-expressed and this expression was associated with Id4 silencing (119). Id4 gene methylation status was examined in patients with acute myeloid leukemia (AML) and 84.8% (39/46) AML patients were Id4 gene was methylated while the 10 iron deficiency anemia (IDA) patients with completely non-methylated Id4. They did determine that the degree of Id4 gene methylation changes in AML patients with different subtypes and stages, which suggests that Id4 gene methylation may be an early molecular event in the progression of AML (120). Zhao et al. found increased occurrence of acute leukemia in AL-CR patients, which are patients with methylated Id4 gene (121). Castro et al. examined multiplexed methylation profiles of tumor suppressor genes including Id4 and clinical outcomes in lung cancer and Id4 was frequently methylated in lung cancer cell lines (122). In breast cancer, Id4 promoter methylation was found to be 68.9% (117/170) in breast cancer samples. The focus of this paper was to investigate the impact of Id4 promoter methylation on breast cancer progression. Treatments to demethylate breast cancer cells lines were associated with Id4 re-expression (100). Another study found that
Id4 is constitutively expressed in normal human mammary epithelium but is suppressed in ER-positive breast carcinomas and preneoplastic lesions; however in ER-negative carcinomas Id4 is expressed. This identifies a role for Id4 as a tumor suppressor in human breast cancer and suggests expression is regulated by estrogen (123). Collectively, in breast cancer tissues Id4 is a potential tumor suppressor gene that undergoes epigenetic silencing during carcinogenesis which may lead to an increased risk for tumor relapse.

In Burkitt’s lymphoma, Id4 gene was also found to be methylated. Qu et al. used arsenic trioxide (ATO) in Raji cells to demethylate Id4 and recover Id4 mRNA expression. Id4 gene hypermethylation may be the cause of malignant proliferation of Raji cells (124). Id4 was also found to be inactivated by DNA methylation in malignant lymphoma and may function as a tumor suppressor gene (125). Id4 has been identified as a potential tumor suppressor gene in many cancers; however the mechanism of action of Id4 has not been investigated.

2.5.5 Id4 in prostate cancer

There is convincing evidence that Id4 acts as tumor suppressor in prostate cancer, data suggests that over-expression of Id4 induces apoptosis, senescence, and cell cycle arrest in metastatic prostate cancer cell line DU145 (26). Carey et al. provided evidence that in the presence of ectopic Id4 in prostate cancer cell lines, an S phase arrest and apoptosis occurs, which resulted in increased the levels of p21 and p27 expression (26). Sharma et al. also showed that Id4 is hypermethylated in prostate cancer tissues as compared to normal controls, which suggests tumor suppressive functions for Id4 (9). Vinarskaja et al. found that Id4 expression is significantly decreased in prostate cancer,
and hypermethylation was detected by MS-PCR and pyrosequencing (126). This study used 47 carcinoma and 13 benign prostatic tissues. The results from this study, directly supports that role of Id4 as a tumor suppressor in prostate cancer (126).

2.5.6 Summary

Several lines of evidence have been mentioned that suggests a unique role of Id4 as compared to other Ids (Id1, Id2, and Id3) in developmental biology as well as cancer biology. There are more instances of how Id4 promoter methylation in cancer results in little to no expression of Id4, which may suggest its role as a tumor suppressor. However, the mechanisms by which Id4 may act as a tumor suppressor or tumor promoter are far from understood.

2.6 Tumor suppressor protein p53

p53 was first identified as a transformation-related protein in 1979 (127). p53 was identified as a cellular protein which binds tightly to the simian virus 40 large T antigen and accumulates in the nuclei of cancer cells (128, 129). Initially p53 was thought to have oncogenic properties due to its overexpression in mice and in tumor cells (130). About ten years later, it was discovered that mutant p53 (missense mutant form) was originally coined as wild-type p53, so the oncogenic properties (131) observed actually resulted from a p53 mutation (132, 133). The first knockout studies of wild type p53 provided clear evidence that p53 is indeed a tumor suppressor (134). Many studies thereafter provided more lines of evidence of p53 as a tumor suppressor. p53 was found to be involved in cell-cycle regulation, induction of apoptosis, development, differentiation, gene amplification, DNA recombination, chromosomal segregation, and
cellular senescence (135). Mutations or loss of p53 has been observed in more 50% of all human cancer cases. p53 is a member of a unique family of proteins that also includes p63 (136) and p73 (137). While this family is structurally and functionally related, p53 has evolved more than the others. p53 is able to prevent tumor development, whereas p63 and p73 have distinct roles in normal development biology (138).

Human p53 has molecular weight of 53 kD and is encoded by a 20-Kb gene containing 11 exons and 10 introns (139). P53 is located on chromosome 17p. Wildtype p53 consists of 393 amino acids and consists of several domains: transactivation domain, proline-rich region, DNA-binding domain, tetramerization domain, and regulatory domain (Figure 6A) (29, 140, 141). The N-terminal domain is required for transactivation activity and this domain interacts with various transcription factors including murine double minute 2 (MDM2) and acetyltransferases (142, 143). The central core, which contains the DNA binding domain, is required for sequence specific DNA binding (144). The C-terminal domain includes tetramerization and regulatory domains and functions as a negative regulatory domain (29) and has been implicated in induction of cell death (145). When the C-terminus and core DNA binding domain are disrupted by posttranslational modifications, such as phosphorylation or acetylation, the DNA binding domain becomes active and thereby induces an enhanced transcriptional activity (146). The enhanced transcriptional activity is the activation of p53 and targets that induce cell cycle arrest and apoptosis. Structure-based studies have revealed the most p53 mutations found in cancers are missense mutations that are located in the...
central DNA-binding domain. 80% of p53 mutation studies are centered around mutations that lie within the residues between 126-306 (Figure 6B) (30).

p53 has an important role in maintaining the balance of proliferation and genome integrity following genotoxic stress (29, 147). There are several types of genotoxic stress that include DNA damage, heat shock, hypoxia, and oncogene over-expression, following stress, wildtype p53 is activated and triggers biological responses to combat that stress through DNA repair or apoptosis mechanisms (147, 148). This activated p53, likely causes an increase in the expression of p53 as well as other changes through posttranslational modifications, which results in activation of p53-target genes (149). In the case of DNA damage, ATM protein kinase is activated and then activates Chk2 kinase (150). ATM and ChK2 then both phosphorylate p53 at distinct sites which can lead to p53-dependent cell cycle arrest and apoptosis (151, 152). p53 target genes activate downstream pathways that have a role in cell-cycle checkpoints, cell survival, apoptosis, and senescence (153). Many different approaches have been used to identify targets of p53, resulting in hundreds of physiologically p53 responsive genes being reported (Figure 6). The genes identified are involved in cell cycle (p21 and GADD45), DNA repair (GADD45, p48, p53R2, APE1), apoptosis (PUMA, BAX, BCL-2 family), and senescence genes (p21 and p66) (154). p53 is also involved in the repression of gene expression; the genes that may be repressed are bcl-2, bcl-X, B1, MAP4, and survivin. Some of these genes are negative regulators of apoptosis (29, 155).
2.6.1 p53 and cell cycle

p53 plays a role in the regulation of the cell cycle, by inducing a cell cycle arrest. Cell cycle arrest allows the cell time to repair DNA damage before entering the critical stages of DNA synthesis and mitosis. p53 can use nucleotide excision repair and base excision repair mechanisms before the arrested cells are released back into the proliferating pool (156). One of the most well-known and studied p53 target genes is cyclin dependent kinase inhibitor, p21. P21 is a known cell cycle regulator, which is a primary mediator of p53-dependent G1 cell cycle arrest following DNA damage. p21 is upregulated by p53 in response to stress and DNA damage (157-159), therefore blocks cyclin E/CDK-mediated phosphorylation of Rb and release of E2F, which functions to induces the expression of gene involved in S phase entry (145). Increased expression of GADD45 results in p53-driven G2 cell cycle arrest (160, 161). This occurs through the binding of GADD45 to CDC2, which prevents cyclin B/CDC2 complex formation and inhibits kinase activity (160).

2.6.2 p53 and apoptosis

One of the tumor suppressive functions of p53 is to monitor cellular stress and to induce apoptosis as necessary. In situations of cell stress and damage p53 can initiate apoptosis and eliminate damaged cells (153). Apoptotic genes that regulate apoptosis includes BAX, DR5/KILLER, DRSL, Fas/CD95, PIG3, Puma, Noxa, PIDD, PERP, Apaf-1, Scotin, p53AIP1, and many others. The p53 apoptotic targets are divided into two groups, extrinsic pathway (Fas/CD95, DR5/KILLER, DR4) and intrinsic pathway (Bcl-2 family pro-apoptotic and anti-apoptotic members) (154).
When the extrinsic pathway (also referred to as death receptor pathway) is activated, p53 initiates apoptosis through activation of the death receptors located on the plasma membrane (162). P53 signals the inhibition of the production of IAPs (inhibitor of apoptosis proteins), which controls apoptosis (162). Alternatively, the intrinsic pathway is the main pathway activated in p53 dependent apoptosis, whereas extrinsic pathway is used to supplement the apoptotic response (163). The intrinsic pathway is the mitochondrial pathway, and is activated when cells undergo stress. This pathway is regulated by the Bcl-2 family proteins (164, 165). Bcl-family consists of both pro-apoptotic members (Bax, Bak, Bcl-xl, BH3-only members Bid, Bad, Puma, and Noxa) and anti-apoptotic members (Bcl-2 and Bcl-Xl). When the intrinsic pathway is activated, the pro-apoptotic proteins such Bax, Bid, Puma, Noxa, and p53αIPl localize to the mitochondria which causes loss of the mitochondrial membrane potential and cytochrome c release. Next the grouping of the apoptosome complex with Apaf-1 triggers the activation of caspases through caspase 9, which executes apoptosis. (166-168). Loss of Bax is responsible for nearly 50% of accelerated tumor growth in brain tumors (169, 170). Bax is also required for p53-dependent apoptosis in colorectal cancer cells, but is dispensable in apoptosis of thymocytes and intestinal epithelial cells (154). In some instances cell cycle arrest can protect cells from apoptosis. There is a crucial balance between Puma and p21 which determines if cell undergo cell cycle arrest or apoptosis in colorectal cancer cells, if p21 is disrupted cells die through apoptosis, however if Puma is disrupted, apoptosis is prevented (171, 172).
2.6.3 Regulation of p53

In normal conditions within the cell, p53 is maintained at very low levels and in the inactive latent form (148). In normal growing cells, the half-life of p53 is restricted to minutes, however cellular stress or DNA-damage can prolong it to hours (173). Increased expression of p53 protein is usually a result of lengthening of its half-life. Expression of p53 and its activities are dependent upon the cell’s needs and extracellular stimuli. Many proteins can regulate p53 levels and activity, such as HPV16 E6, WT-1, E1B/E4, SV40 T-antigen, MDM2, JNK, Pirh2, and PARP-1 (154).

2.6.4 Protein stability

The stability of p53 can be increased by binding of SV40 T antigen, WT1 or E1B/E4, whereas MDM2 or E6 association results in degradation of p53 (174). MDM2 is a well-known p53 regulator that inhibits p53 activity by blocking its transcriptional activity, by facilitating its nuclear export and degradation (175, 176). The E3 ubiquitin-ligase activity that MDM2 possess can regulate ubiquitylation and proteasome-dependent degradation of p53 (176). The MDM2 gene is also under the control of p53 and can be transcriptionally regulated by p53 following cellular stress (177). The p53 protein is targeted by MDM2 for degradation, however mt p53 is not targeted by MDM2 while results in high levels in cancer cells (154).

2.6.5 Phosphorylation of p53

In response to stress, posttranslational modifications can also affect p53 activity and stability (178). These posttranslational modifications include phosphorylation, acetylation, ADP-ribosylation, ubiquitination, sumoylation, neddylation, and cytoplasmic
sequestration, and usually occur in the N- and C-terminal regions of p53 \((154)\). The most commonly studied modifications are acetylation and phosphorylation because of their enhancement of p53 transcriptional activity. These modifications result in p53 stabilization and nuclear accumulation which allows p53 to interact with sequence-specific sites of its target genes \((179, 180)\). While phosphorylation is the most commonly reported protein modification in mammalian cells, it seems that acetylation may actually be more crucial than phosphorylation \((154, 181)\). p53 phosphorylation results in stabilization and increased DNA-binding, twenty serine and threonine sites have been identified as phosphorylated on p53. Phosphorylation at these sites is mostly induced by DNA damage, but a few sites are repressed by genotoxic stress \((181)\). P53 can be phosphorylated by protein kinases (ATM/ATR) \((152)\).

### 2.6.6 Acetylation of p53

Acetylation may also have an important role in stabilization \((182)\) and transcriptional activation of p53 \((183)\). Many types of cell stress may increase acetylated p53 in various cell types. Acetylation occurs on lysine residues, and commonly acetylated p53 residues include Lys305, Lys320, Lys372, Lys373, Lys381, Lys382 and Lys386. The aforementioned residues are located in the regulatory domains adjacent to tetramerization domain \((154)\). P53 can also be acetylated by two histone acetyltransferases, CBP/300 and PCAF. CBP/300 acetylates the C terminus of p53 at Lys305, Lys372, Lys373, Lys381, and Lys382, however PCAF acetylates Lys320 \((184-186)\). It is believed that the recruitment of coactivators stabilizes p53 and facilitates DNA binding, which enhances the transactivation activity of p53 in cells, whereas deacetylation has an
opposite effect and suppresses activity (141). The acetylation of p53 can promote the sequence-specific DNA binding activity, which may be due to an acetylation induced conformational changes in p53 (185, 187, 188).

Acetylation and phosphorylation can work together to promote p53 activity, in response to UV radiation, the p53 N terminus is first phosphorylated at Ser37 and Ser33, and then the phosphorylated p53 activates CBP/300 and PCAF to induce p53 acetylation at Lys373/Lys382 and Lys320 (189, 190). In some instances phosphorylation may be required in order for acetylation to occur (191). Overall, p53 modulation is a very complex process and p53 may be activated or regulated in different ways including posttranslational modifications.

2.6.7 p53 mutations

P53 is one of the most commonly inactivated genes in human cancers (27, 28). More than 18,000 mutations have been found in the p53 gene in different types of cancers. Studies suggest that about mutations of p53 occur in all tumor types and at varying rates of 10% (in hematopoietic malignancies) to close to 100% (in high-grade serous carcinoma of the ovary), which leads to p53 loss of function (154). In lung cancer, p53 mutations occur in 70% of tumors, 60% in colon, head and neck, ovary, and bladder cancer, and 45% in stomach cancer (154). In 50% of human tumors in which p53 is mutated, this loss of function is due to mutations in regulators of p53, MDM2 or E6 of HPV, or deletion of p14arf (192). While wt p53 is a tumor suppressor, mt p53 harbors oncogenic properties. For example, patients with Li-Fraumeni syndrome, who have an inherited p53 germline mutation in one of the p53 alleles, are higher risk for developing
cancer during their lifetime (154). As well as loss of wt p53 allele led to tumors of the brain, breast, connective tissue, hematological system, and adrenal gland (134). Loss of wt p53 also leads to tumor formation. Mice with deficient wt p53 are more susceptible to spontaneous tumor development (134). p53 protein mutations can result in eradication of protein function and loss of function may be related to tumor progression and genetic instability (154). Collectively, mutations in p53 gene aids in the progression of cancer.

2.6.8 p53 Missense mutations

While most tumor suppressor genes, such as RB, APC, or BRCA1 are inactivated during cancer progression by deletions or truncating mechanisms, p53 usually undergoes missense mutations, which means a single nucleotide is substituted by another (193). When this happens a full-length protein containing only a single amino acid substitution is produced. Mutations of p53 have diverse locations however the majority of mutations result in loss of p53 DNA binding capabilities. This affects p53’s ability to bind DNA in a sequence-specific manner and thus activate transcriptional of p53 target genes (194). P53 mutations are found in all coding exons of the gene, with a strong preference to the exon 4-9, which encodes the DNA-binding domain of the protein. Of mutations in the DNA-binding domain, 30% fall within 6 “hotspot” residues which are frequently mutated in all cancers. These residues are R175, G425, R248, R249, R273, and R282), and may exist due to susceptibility of particular codons to alterations (30).

2.6.9 P53 Dominant negative manner and Gain of Function

Many p53 mutants are able to promote tumor development not only through loss of wild-type function but also other mechanisms (195). For example, when there is
heterozygous mutation, which means there are wild-type and mutant alleles, mutant p53 can antagonize the wild-type p53 functions and render it inactive in a dominant negative manner. The inactivation of wt p53 by mt p53 in a dominant negative manner relies on the fact that transcriptional activity of wt p53 is dependent upon p53 as a tetramer and somehow mt p53 may interfere with the structure (131, 196, 197). p53 mutations are commonly followed by loss of heterozygosity during cancer progression (198). Lines of evidence support the idea that many mutant p53 isoforms may exert oncogenic properties by a gain-of-function mechanism, referring to the oncogenic properties acquired by mt p53. Collectively, DN and GOF effects have a major role in the selection of missense mutations in p53 during tumor progression (135, 199). Data provides evidence that p53 may be mutated in early and late stages of tumorigenesis. However, the genetic aberrations determine the tumor progression and aggressiveness of the disease rather than the stage that the mutations occur.

2.6.10 Therapeutic Applications of p53

Inactivation of tumor suppressor p53 has a major role in tumorigenesis so it of interest to determine mechanisms that may activate p53. One of the commonly studied negative regulators of p53 is MDM2. It is believed that inhibiting the E3 activity of MDM2 may provide a strategy to kill tumor cells by restoring p53 activity (200). As a result, many drug development studies are focused on p53-MDM2 interaction, one being nutlins. Nutlins are small molecule inhibitors that are able to inhibit p53-MDM2 interaction in vitro and in vivo. Treatment with this molecule induces expression of p53 and its target genes which induces apoptosis (201, 202). Another inhibitor of p53-
MDM2 interaction, benzodiazepinedione, was found to induce p53 and its target, decrease proliferation of tumors cell with wt p53 (203). Antisense oligodeoxynucleotides targeted against MDM2 and p21 were also found be a potential therapeutic strategy because it sensitized tumor cells to antineoplastic agents (204). Since p53 has a known role in apoptosis, other therapies include treatments that induce apoptosis though p53 targets. Surprisingly, p53 apoptotic targets are rarely mutated in human cancers. Therefore, p53 apoptotic targets Bax, Puma, p53AIP1, Noxa, and others could be used as targets for gene therapy (205). Introduction of wt p53 into tumors is also a cancer therapy, expression of wt p53 was found to induce rapid loss of cell viability with characteristics of apoptosis (154). There are also therapies to reactivate p53 function from mt p53, since mt p53 is not able to perform wt p53 functions due to protein folding. Peptide and small molecule development have been investigated to combat protein folding. These molecules stabilize the structure of p53 which leads to restored p53 DNA binding abilities, transcription, and apoptotic functions (154). Synthetic peptides have been derived from the C-terminus of p53 (206), CBD3 (207), and PRIMA-1 (208). CBD3 is known to stabilize the structure of p53, binds mt p53, and induce the refolding of two hot spot mutations (His273 and His175) in cancer cells (207, 209). PRIMA-1 inhibits the growth of tumor cells by inducing apoptosis in transcription-dependent manner through the conformational changes of p53 mutants that promotes p53 DNA binding (208). A small synthetic molecule, CP-31398 is able to restore wt p53 function to mutants, possibly through the intrinsic pathway (210, 211). While there are clues of
mechanistic insight of these molecules, the exact mechanism by which these molecules regulate the misfolded mt p53 to restore functionality is not known.

2.6.11 p53 and Id4

In the literature there are correlations between Id4 and p53 expression, which suggest that there is some interaction between p53 and Id4. Coradini et al found that in breast cancer tissues with missense mutations in p53 there is decreased Id4 expression, whereas in severe mutations (loss of p53 expression through deletions) Id4 expression is surprisingly high (212). While these findings were unexpected, its suggests an interesting relationship between p53 mutations, mammary cell dedifferentiation, and the concomitant acquisition of stem-like properties. Fontemaggi et al. determined altered p53 protein is prevalent in a class of triple-negative breast cancers. Further analysis of Id4 and p53 interaction in breast cancer cell lines suggested that transcriptional activation of Id4 promoter is exerted by a complex mutant p53/E2F1/p300 axis. In this study, mutant p53 regulation of Id4 expression was specific to mutation and R273H exerted the highest regulatory function over Id4 expression, which may explain rarity in mutant p53 regulation of Id4 in prostate cancer. They concluded that Id4 displays tumor suppressor functions in ER+ breast tumors, while it is frequently inactivated by promoter hypemethylation, whereas in tumors that express mutant p53 and are ER-, Id4 displays oncogenic activities (213). There has been an association between mutant p53 and Id4 in breast cancer, however this association has not been investigated in other cancers.
2.6.12 Summary

In summary, p53 is characterized as a tumor suppressor due to its role in apoptosis, cell cycle arrest, DNA damage, senescence, differentiation, and DNA repair. P53 is at the heart of many stress response pathways and regulator of many genes that modulate various cellular processes such as DNA damage control, cell cycle arrest, senescence, and apoptosis. Mutations of p53 can provide an environment for genetic instability of tumor cells, which can result in accelerating tumor progression. There has also been a correlation between Id4 and mutant p53 in breast cancer, which suggests an interaction between the two proteins.
3.1 Id4 over-expression and silencing in prostate cancer cell lines

The prostate cancer 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 (26). 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.

3.2 RNA extraction

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) as described previously [43]. The final RNA pellet was re-suspended in diethylpyrocarbonate (DEPC)-treated H2O at a concentration of 1 mg/ml and stored at -80°C until analysis.
3.3 **Reverse Transcriptase**

RNA (2 μg) was reverse transcribed in a final volume of 25 μl as per standard protocols (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)). The RNA was denatured for 10 min at 65°C, and then cooled on ice 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. Samples were stored at -20°C until analysis.

3.4 **Quantitative Real Time PCR (qRT-PCR)**

qRT-PCR was performed as described previously using gene specific primers (Table 1) on RNA purified from cell lines (8).

3.5 **Protein Extraction**

Total cellular proteins were prepared from cultured prostate cancer cell lines using M-PER (Thermo Scientific) (26). Protein samples were quantitated using the The Bio-Rad DC Protein Assay according to manufacturer protocol. A standard curve was determined using BSA and sample absorbance read at 750 nm. Samples were concentrated in 30 μg/ul volume and then mixed 1:1 with 2X Sample Buffer.

3.6 **Western Blot Analysis**

30 μg of total protein was size fractionated on 4-20% SDS-polyacrylamide gel (5% for CBP/p300 western blotting) and subsequently blotted onto a nitrocellulose membrane (Whatman). The blotted nitrocellulose membrane was subjected to western blot analysis using respective protein specific antibodies (Table 2). 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.

3.7 Proliferation Assay

Cell proliferation analyses were performed using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. LNCaP+NS and LNCaP-Id4 cells were seeded in 96-multi well plates at a density of $5 \times 10^5$ 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.

3.8 Scratch Wound Assay

Migratory properties of LNCaP+NS and LNCaP-Id4 cells were measured using a scratch wound assay. Cells were plated in 6-well plates ($5 \times 10^5$ cells/well) in RPMI with 5 % FBS and cultured overnight. Before treatment, wells were scratched down the middle with a 200 µl pipette tip. Culture media were replaced with RPMI containing 5 % FBS. Cells were allowed to migrate across the scratch for 48 h. Images of the scratch area were recorded at three random spots at 0 and 48 h. The migrating cells were counted using a standard size field for each image.

3.9 Migration Assay

LNCaP+NS and LNCaP-Id4 cells were used for in vitro cell migration assay. Assay was performed using 24-well trans-well inserts (8 µm). Briefly, cells were washed
once with RPMI and harvested from cell culture dishes by EDTA-trypsin into 50 ml conical tubes. The cells were centrifuged at 500 × g for 10 minutes at room temperature; the pellets were re-suspended into RPMI supplemented with 0.2% bovine serum albumin at a cell density of 3 × 10⁵ cells/ml. Outsides of the trans-well insert membrane were coated with 50 μl of rat tail collagen (50 μg/ml) overnight at 4°C. Following day, aliquots of rat tail collagen (50 μl) were added into the trans-well inserts to coat the inside of the membranes. Insert were kept at room temperature for 1.5 h before being washed thoroughly with 3 ml of RPMI. Chemoattractant solutions were made in RPMI supplemented with 0.2% bovine serum albumin. Control and chemoattractant solutions (400 μl) were added into different wells of a 24-well plate. 100 μl aliquots of cell suspension were loaded into trans-well inserts that were and placed into the 24-well plate. The trans-well insert-loaded plate was placed in a cell culture incubator for 5 h. At the end of the incubation. The cleaned inserts were fixed in 300 μl of 4% paraformaldehyde (pH 7.5) at room temperature for 20 minutes. Cells on the outside of the trans-well insert membrane were stained using HEMA 3 staining kit (Fisher Scientific, Inc.). Then, stained cells were counted in four non-overlapping fields using low-power with a light microscope, and the average number of cells reflected the cell migration status in each trans-well insert. Results were indicated by migration index, which is defined as the average number of cells per field for test substance / the average number of cells per field for the medium control. (214).
3.10 Soft agar assay

Prepared 0.8% base agar layer, followed by 0.7% top agarose solution in 10cm plates. LNCaP+NS and LNCaP-Id4 cells (~20,000 cells) were harvested, and resuspended in the top agar. Cells were then aliquoted appropriately on top of base agar layer (pre-warmed to 37°C). Then incubated the plates for 21 days and fed cells 2 times a week. Colony growth was monitored and at the end of incubation colony was quantitated and charted.

3.11 Mouse model studies

Male nude mice that are 6-8 weeks old were obtained upon IACUC approval from Charles River Laboratories International Inc (Wilmington, MA). These mice were kept in conventional pathogen-free housing at Mercer University and were used for study in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals. LNCaP+NS and LNCaP-Id4 cells were cultured until growth reached a concentration of 3 x 10^6 million cells. Then cells were trypsinized, rinsed with PBS, and resuspended in serum-free media. Cells were then mixed with a 1:1 dilution of matrixgel (BD biosciences) and kept on ice until further use. Mice were injected subcutaneously, the left flank of nude mice with LNCaP+NS cells and the right flank with LNCaP-Id4 cells. Then tumor growth was observed over a period of 12 weeks. Weight, tumor growth dimensions were monitored several times weekly. Weight measured in grams, tumor size measured by weight x height.
3.12 Statistical Analysis

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

3.13 Co-Immunoprecipitation

To detect the protein-protein interactions, co-immunoprecipitation was performed using protein A coupled to magnetic beads (Protein A Mag beads, GenScript) as per manufacturer’s instructions. Briefly, protein specific IgG (anti-p53 or -Id4, Table S1) was first immobilized to Protein A Mag Beads by incubating overnight at 4°C. To minimize the co-elution of IgG following immuno-precipitation, the immobilized IgG on protein A Mag beads was cross-linked in the presence of 20mM dimethyl pimelic acid dihydrochloride (DMP) in 0.2M triethanolamine, pH8.2, washed twice in Tris (50mM Tris pH7.5) and PBS followed by final re-suspension and storage in PBS. The cross-linked protein specific IgG-protein A-Mag beads were incubated overnight (4C) with freshly extracted total cellular proteins (500 µg/ml). The complex was then eluted with 0.1 M Glycine (pH 2-3) after appropriate washing with PBS and neutralized by adding neutralization buffer (1 M Tris, pH 8.5) per 100 µl of elution buffer.

3.14 Chromatin Immuno-precipitation (ChIP) Assay

Chromatin immuno-precipitation was performed using the ChIP assay kit (Millipore, Billerica, MD) according to the manufacturer’s instructions. The chromatin (total DNA) extracted from cells was sheared (Covaris S220), subjected to immuno-precipitation with p53, mouse/ rabbit IgG or RNA pol II antibodies (Table S1), reverse
cross linked and subjected to qRT-PCR in Bio-Rad CFX. The previously published ChIP primer sets spanning the consensus p53 response element sites in the promoters of BAX (215), p21 (215), PUMA (216) and MDM2 (215) were used (Table SII).

3.15 Electrophoretic Mobility Shift Assay (EMSA)

The nuclear proteins from respective cell lines were prepared using the nuclear extraction kit from Affymetrix (AY2002) according to the manufacturer’s instructions. 1μl of nuclear proteins were used in an EMSA reaction using Biotin end labeled p53 double stranded oligonucleotide (Affymerix, AY1032, p53(1) EMSA kit containing the p53 response element (215)). The nuclear proteins and labeled oligonucleotide or excess unlabeled oligonucleotide were incubated for 20 min at room temperature, separated on 5% non-denaturing polyacrylamide gel and transferred onto nitrocellulose membrane and detected following manufacturer’s instructions. The EMSA using LNCaP+NS cells with wild type p53 and p53 null PC3 was used as positive and negative controls, respectively.

3.16 p53 Activity Assay

p53 DNA binding activity and quantitation on nuclear extracts was performed by capturing p53 with double stranded oligonucleotides containing a p53 consensus binding site immobilized in a 96 well format (TF-Detect p53 Assay, Genecopoeia) followed by detection with p53 specific antibody in a sandwich ELISA based format according to manufacturer’s instructions (essentially a quantitative super-shift assay).

3.17 Transient Transfections and Reporter Gene Assay

Cells were cultured in 96-well plates to 70-80% confluency and transiently transfected by mixing either PG13-luc (containing 13 copies wt p53 binding sites (217),
Addgene) or MG15-luc (containing 15 mutant p53 binding sites (217), Addgene) with pGL4.74 plasmid (hRLuc/TK: Renilla luciferase, Promega) DNA in a 10:1 ratio with FuGENE HD transfection reagent (Promega) in a final volume of 100 ul of Opti-MEM and incubated for 15 min at room temperature. The transfection mix was then added to the cells. After 24 h, 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.

**3.18 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 (5min x3), blocked with 1% BSA in PBST for 30 min at room temp and incubated overnight (4°C) with primary antibody (1% BSA in PBST, Table SI). The slides were then washed in PBS and incubated with secondary antibody with fluorochrome conjugated to DyLight (Table SI) in 1% BSA for 1hr at room temp in dark. The slides were subsequently washed again and stained in DAPI (1 μg/ml) for 1 min and mounted with glycerol. Images were acquired by Zeiss fluorescence microscopy through Axiovision software.

**3.19 Apoptosis Assay and mitochondrial membrane potential (MMP)**

Apoptosis and MMP was quantitated using Propidium Iodide and Alexa Fluor 488 conjugated Annexin V (Molecular Probes) and dual-sensor MitoCasp (Cell Technology) as described previously (218)
3.20 Statistical Analysis:

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.

3.21 Cell Treatments

2x10^5 cells were seeded in a tissue grade six well plate and allowed to attach overnight. The following day 500nM of Doxorubicin was added to the media and incubated for 24hrs for apoptosis assay. 10^3 cells were plated in a 96 cell culture plate and allowed to attach overnight. The following day 500nM of Doxorubicin was added to the plates and cells were incubated for 24, 48 and 72 hours to measure proliferation.
CHAPTER 4
RESULTS

4.1 Loss of Id4 promotes tumorigenecity of prostate cancer cells

4.1.1 Expression of Id4 in prostate cancer cell lines

Id4 is undetectable in DU145 cells due to promoter hyper-methylation (9). In contrast, Id4 was expressed in LNCaP cells due to promoter hypo-methylation. These two cell lines were used to either over-express (DU145+Id4) or silence (LNCaP-Id4) Id4. Two different retroviral shRNA vectors (vectors A and C) were used to silence Id4 (Figure 8) in LNCaP cells. The shRNA mediated stable knockdown of Id4 in LNCaP using vector A (LNCaP-Id4), Id4 over-expressing DU145 cells (DU145+Id4, Figure 8C) and their respective vectors only transfected cells were used for all subsequent experiments. A non-silencing control vector was also transfected into LNCaP cells (LNCaP+NS). Expression of Id4 was measured by q-PCR, western blotting, and ICC.

4.1.2 Effect of Loss of Id4 on cell apoptosis and proliferation

Upon Id4 silencing, there is a marked decrease in apoptosis as compared to LNCaP+NS control (Figure 9A). The rate of apoptosis was measured using Annexin V staining and analyzed by FACs analysis. Apoptotic cells decreased from 47\% in LNCaP+NS to 10\% in LNCaP-Id4. Proliferation rates almost doubled after Id4 silencing.
as shown by MTT assay (Figure (9B). This data suggests loss of Id4 promotes increased proliferation and suppresses apoptosis.

4.1.3 Effect of loss of Id4 on cell migration

To investigate the effect of loss of Id4 on migratory abilities of LNCaP cells, which do not typically migrate, we employed the scratch wound assay (Figure 10A). We plated 5 x 10^5 cells in a six well plate and then used a 20μL tip to make an even wound in the control samples as well as LNCaP-Id4 cells. The wound was allowed to heal over a 48 h period. After 48 h, we measured wound healing, and we determined that the wound was more healed in LNCaP-Id4 cells. Data indicated that loss of Id4 increased migratory abilities of cells. However, it is difficult to quantitate the scratch wound assay so migration was measured using a more quantitative manner. We used the Transwell migration assay to measure the number of migratory cells (Figure 10B). After the 5 hours of incubation, the inserts were then stained and counted under an inverted microscope. We counted twice the amount of migratory cells in LNCaP-Id4 as compared to LNCaP+NS controls. Collectively, the data provides evidence that loss of Id4 promote migration in LNCaP cells.

4.1.4 Gene expression changes following loss of Id4

We investigated proliferation marker cyclin dependent kinase inhibitor p27. The expression of p27 was detected by q-PCR (Figure 11C). The expression of p27 is decreased by 9-fold upon loss of Id4, as compared to LNCaP+NS controls. Thus the increase in proliferation may be due deregulation of p27. The decrease in apoptosis could be due to the decrease in p53 and its downstream targets, which will be discussed
later. We also observed increased migratory capabilities after silencing of Id4 in LNCaP cells. So then, expression of markers involved in migration and invasion, (MMP2, MMP13, and Id1) were investigated. We found a four fold increase in MMP2 expression (Figure 11B) and MMP13 by a little more than two fold induction (Figure 11B). We also noted 14 fold increase in Id1 expression in LNCaP-Id4 cells as compared to the controls (Figure 11A). So the increased migratory capabilities of LNCaP-Id4 may be a result of increased expression of MMPs and Id1.

4.1.5 Effect of loss of Id4 on tumor growth

Next, we wanted to determine the effect of loss of Id4 on tumor growth. First, we used an in vitro technique, the clongenic assay, to determine if LNCaP-Id4 would form larger and more colonies as compared to the parental LNCaP+NS (Figure 12A). We plated 50,000 cells in the agar filled plates and incubated them for 14 days. After 14 days, we observed not only more colony growth, but also larger colonies (2.5 fold increase), which would indicate that loss of Id4 promotes tumor growth in LNCaP cells. Since the LNCaP-Id4 cells were able to form colonies, we decided to proceed with in vivo studies in nude mice. We injected 6 mice with LNCaP+NS cells on the left flank and LNCaP-Id4 cells on the right flank. After the mice were injected tumor growth was monitored. After about three weeks, we noticed tumor growth on the LNCaP-Id4 flank (Figure 12B). We monitored tumor growth and mice weight weekly throughout the growth period (Figure 12C). After six weeks, the mice were sacrificed and the tumors excised (Figure 12E). The tumor weight and volume of LNCaP-Id4 cells (weight 0.62, tumor size 15mm x 10mm) were significantly higher than the LNCaP+NS control (weight
0.02g, tumor size 4mm x 2mm), which indicates loss of Id4 promotes tumor growth in nude mice. Not only were the weight (Figure 12 D) and volume (Figure 12F) significantly increased, but the LNCaP-Id4 tumors grew at a faster rate than LNCaP+NS tumors (Figure 12G).

4.2 Id4 dependent acetylation restores mutant-p53 transcriptional activity

4.2.1 Id4 Promotes Apoptosis

A significant increase in apoptotic cells was observed in DU145+Id4 (64±3.3%, P<0.001, Figure 13A) cells as compared DU145 cells (24±5.1%, Figure 13A) whereas number of cells undergoing apoptosis decreased in LNCaP-Id4 (10.5±2.3%) as compared to LNCaP (47.2±6.5%) cells (Figure 13A). Apoptosis in DU145+Id4 cells was accompanied by increased fraction of cells with lower mitochondrial membrane potential (MMP, 21.3±4.65%, Figure 13B) whereas decreased apoptosis in LNCaP-Id4 cells was associated with increased fraction of cell with high MMP (1.5±0.09%) as compared to DU145 (0.7±0.08%) and LNCaP+NS (14.3±2.60%) respectively (Figure 13B). Thus Id4 promotes apoptosis through changes in MMP that eventually promotes cytochrome c release from the mitochondria (219).

Increased BAX expression and/or PUMA dependent dissociation of BAX from Bcl-2 promotes translocation of BAX to mitochondria resulting in decreased mitochondrial membrane potential (220). The expression of pro-apoptotic BAX and PUMA increased in DU145+Id4 cells whereas a corresponding decrease in BAX and PUMA was observed in LNCaP-Id4 cells at the protein (Figure 13C) and transcript (Fig. 13D) level as compared to DU145 and LNCaP+NS cells respectively (Figures. 13C and
These results clearly implicated the role of Id4 in promoting apoptosis through increased expression of BAX and PUMA. Apoptotic stimuli induce BAX activation, characterized by translocation and multimerization on the mitochondrial membrane surface resulting in exposure of an amino terminal epitope recognized by the confirmation specific monoclonal antibody BAX 6A7 (221). Co-localization of BAX (BAX 6A7 antibody) with mitochondrial PDH (pyruvate dehydrogenase) demonstrated that BAX undergoes conformational change and translocates to the mitochondria in DU145+Id4 and LNCaP+NS cells (Figure 13E) but not in DU145 and LNCaP-Id4 cells possibly due to undetectable levels of BAX (Figure 13C).

Both BAX and PUMA are also transcriptional targets of the tumor suppressor protein p53 (222). As expected, decreased apoptosis in part due to loss of BAX and PUMA expression in LNCaP-Id4 cells was associated with low p53 expression as compared to LNCaP+NS cells (Figure 14A). A similar relationship between Id4 and p53 expression was not observed in DU145 cells. Unlike wt-p53 in LNCaP+NS cells, the DU145 cells harbor mut-p53. The two mutations (P223L and V274F) are within the DNA binding domain that results in a transcriptionally inactive form of p53 (32). Mut-p53 protein generally accumulates at high levels due to loss of regulatory mechanisms (223) as seen in DU145 cells (Figures 14A and 14B, 12 fold higher as compared to LNCaP+N cells). Surprisingly, we observed decreased levels of mut-p53 in DU145+Id4 cells at the transcript (Figure 14B, 2 fold compared with LNCaP cells normalized to 1) and protein level (Figure 14A). These results are significant especially in context of increased expression of BAX and PUMA in DU145+Id4 cells in spite of low mut-p53.
expression. We reasoned that one of the mechanisms by which mut-p53 could up-regulate BAX/PUMA expression could be through gain of transcriptional activity by mut-p53 in DU145+Id4 cells. Immuno-cytochemical localization of p53 also revealed that mut-p53 is localized to the nucleus and cytoplasm in DU145 (Figure 14 C, DU145, arrows) cells but is primarily nuclear in DU145+Id4 cells (Figure 14 C, DU145+Id4, arrows). Previous studies have also shown a predominant cytoplasmic staining of mutant p53 in prostate cancer whereas wt- p53 is primarily nuclear (224).

We also investigated the expression of CDKN1A (p21) which is also a well characterized p53 target gene (225). Increase in the expression of p21 (Figures 13C and 13D, 9 fold as compared to DU145), in addition to PUMA and BAX further consolidated our observations that mut-p53 in DU145+Id4 cells may have gained transcriptional activity through site specific DNA binding in the respective promoter elements.

4.2.2 Id4 restores mutant p53 DNA binding and transcriptional activity

An EMSA with canonical p53 DNA response element was used to determine the DNA binding ability of wt- (LNCaP+NS) and mut-p53 (DU145). LNCaP+NS cells with wt-p53 resulted in a gel shift (Fig. 15A) whereas, a gel shift of lower intensity was observed in LNCaP-Id4 as compared to LNCaP+NS cells possibly due to lower expression of wt-p53 (Figures 15A and B). A distinct gel shift was observed in the presence of DU145+Id4 nuclear extracts but no gel shift was observed with DU145 nuclear extracts suggesting that mut-p53 in the absence of Id4 lacks DNA binding activity. Increased p53 DNA binding activity using p53 response element immobilized on a 96 well plate followed by detection with p53 specific antibody was also observed in
LNCaP+NS and DU145+Id4 that was significantly higher as compared to LNCaP-Id4 and DU145 cells respectively (Figure 15B). In a functional transcriptional assay using a p53 response element (wt-p53RE) luciferase reporter plasmid, the relative p53 luciferase activity decreased significantly in LNCaP-Id4 cells as compared to LNCaP+NS cells (normalized to 1, Figure 15C), which is consistent with the expression of p53 in these cell lines. Surprisingly, mut-p53 in DU145+Id4 cells demonstrated high luciferase activity as compared to DU145 (normalized to 1, wt-p53RE). The mutant p53 luciferase plasmid (mt-p53RE) used as a negative control, as expected, did not result in luciferase activity. In context of using LNCaP+NS as a positive control, our results strongly suggested that mut-p53 gains DNA binding and transcriptional activity in the presence of Id4 that is in part independent of its expression level. Silencing of p53 through siRNA was used to further clarify the role of mutant p53 in DU145. However, siRNA based p53 silencing led to massive apoptosis in DU145 (226).

Real time quantitative PCR analysis on Chromatin immuno-precipitated (ChIP) with p53 antibody demonstrated the binding of wt-p53 to its respective response elements on BAX (Figure 15D), p21 (Figure 15E) and PUMA (Figure 15F) promoters in LNCaP cells. The decreased p53 expression in LNCaP-Id4 correlated with decreased binding to its respective promoter elements on BAX, p21 and PUMA promoters (P<0.001) (Figures 15D-F). As anticipated, in DU145 no significant binding of mutant p53 was observed on p21, PUMA and BAX promoters (Figures 15D-F). However, in DU145+Id4 cells, a significant increase in the binding of mut-p53 as compared to DU145 was observed on BAX, p21 and PUMA promoters (Figures 15D-F).
Incidentally, p53 regulates MDM2, (an E3 ubiquitin ligase involved in p53 protein degradation) expression in a highly complex manner. In this study we focused on investigating whether MDM2 expression is regulated in a p53 dependent manner at the promoter level, rather than on interaction between wt- and mut-p53 with MDM2 at the protein level. Unpredictably, MDM2 protein expression was higher in LNCaP-Id4 (2.4±0.32 fold, Figure 16A) cells as compared to LNCaP+NS cells (Figure 16A and semi quantitation in lower panel) in spite of lower p53 expression (Figure 16A and B). The expression in DU145 cells (2.6±0.11 fold) was comparable to LNCaP-Id4 cells (Figure 16A). However, MDM2 expression was lower in DU145+Id4 (1.2±0.13) cells as compared to DU145 but was comparable to LNCaP+NS cells (normalized to 1). MDM2 expression is regulated by a p53 response element located within the P2 promoter in intron 1 (Figure 16B) (227). The alternative, P1 promoter, upstream of exon1 is generally considered p53 independent (228). Both P1 and P2 transcript however are translated from the common start site in exon 2. Abundance of P1 and P2 transcripts was then performed to understand whether MDM2 expression is regulated in a p53 dependent (P2) or independent (P1) manner. The results suggested that MDM2 expression in LNCaP+NS cells is primarily due to transcription from the P2 promoter in part due to the binding of p53 (Figure 16D), whereas in LNCaP-Id4 cells, MDM2 expression is a result of activation from the P1 promoter (Figure 16C). In DU145 cells, the P1 promoter was active as compared to P2, but in DU145+Id4 cells, the p53 dependent (Figure 16D) P2 promoter was transcriptionally active (Figure 16C). These results suggested that the regulation of MDM2 expression is highly complex and that in cells lacking Id4 (LNCaP-
Id4 and DU145), the P1 promoter is transcriptionally active whereas in cells with Id4
(LNCaP+NS and DU145+Id4) the p53 dependent P2 promoter is active (Figure 16D).

4.2.3 Id4 Recruits CBP/p300 to promote p53 acetylation

Acetylation, independent of phosphorylation status promotes p53 stabilization and
transcriptional activity but de-stabilizes its interaction with MDM2 (229). Recent studies
have also shown that acetylation of some mutant forms of p53 can restore the DNA
binding activity (230). These studies led us to explore whether Id4 promotes acetylation
of mut-p53 in DU145+Id4 cells. The total p53 protein was first immuno-precipitated and
then immuno-blotted with acetylated lysine antibody. Increased global p53 lysine
acetylation was observed in DU145+Id4 and LNCaP+NS cells as compared to LNCaP-
Id4 and DU145 cells. In p53, K320 is acetylated by PCAF and promotes p53-mediated
activation of cell cycle arrest genes such as p21 (231). In contrast, acetylation of K373
leads to hyper-phosphorylation of p53 NH2-terminal residues and enhances the
interaction with promoters for which p53 possesses low DNA binding affinity, such as
those contained in pro-apoptotic genes, BAX and PUMA (231). The results shown in
Figure 17A demonstrated a significant increase in K373 acetylation in DU145+Id4 cells
whereas no significant change was observed between LNCaP+NS and LNCaP-Id4 cells.
The K320 expression was also significantly higher in DU145+Id4 and LNCaP+NS cells
as compared to DU145 and LNCaP-Id4 cells. These results provided evidence that Id4 is
involved in promoting acetylation of specific residues in wt- and mut-p53 that promotes
its binding to respective response elements. The increased K320 acetylation in
DU145+Id4 cells clearly is consistent with the study by Parez et al (230) in which the
authors demonstrated acetylation at this specific residue restores mutant p53 biological activity. We were however intrigued with a significant increase in the expression of acetylated K373 in DU145+Id4 cells. Acetylation at K373 is CBP/P300 dependent (232). We hypothesized that if CBP/P300 is involved in K373 acetylation than it could co-precipitate with p53. Results demonstrated that indeed mutant p53 is physically associated with CBP/P300 at significantly higher levels than mut-p53 from DU145 cells alone (Figure 17A). These results led us to propose a model whereby, Id4 could recruit or promote the assembly of CBP/P300 and p53. Immuno-precipitation with Id4 and blotting with p53 demonstrated the presence of p53 in this complex in DU145+Id4 and LNCaP+NS cells but not in DU145 and LNCaP-Id4 cells suggesting that Id4 directly associates with p53. These results consolidated our hypothesis that Id4 promotes the recruitment of CBP/p300 on p53 to promote acetylation and restore its biological activity.

4.3 Id4 sensitizes prostate cancer cells to antitumor drug Doxorubicin

4.3.1 Id4 sensitizes prostate cancer cell DU145 to doxorubicin treatments by apoptosis and proliferation

DU145 and DU145+Id4 cells were treated with 500 nM of Doxorubicin for 24 hours. After 24 hours apoptosis was measured, there was an increase in apoptosis in both DU145 and DU145+Id4 cells (Figure 18A); however the observation that Id4 alone can induce apoptosis at a rate similar to that of antitumor drug, Doxorubicin is an intriguing observation. There were also significantly more dead cells in DU145+Id4 as compared to DU145 cells, 30% to 22%, respectively. This data suggested that Id4 sensitizes cells to doxorubicin treatment.
4.3.2 Id4 may induce sensitization via MYC regulation

To investigate the mechanisms by which Id4 may sensitize DU145 cells to chemotherapeutic agent Doxorubicin, we wanted to investigate genes known to sensitize cells to doxorubicin treatment. There were two genes that were of interest p53 and MYC. Since we have provided evidence that Id4 restores mutant p53 function in an acetylation dependent manner, this may be the mechanism by which Id4 sensitizes prostate cancer cells to therapeutic treatment. However, there may be an alternate pathway involved, MYC regulation. So we first investigated the expression of MYC in prostate cancer cells, DU145 and DU145+Id4, we observed a decrease in expression MYC upon over-expression of Id4 (Figures 19 A and B). Downstream of MYC lies MDM2, which also a well-known tumor promoter. In DU145+Id4 cell, MDM2 expression is decreased as well. Therefore Id4 down regulates MDM2 possibly via MYC regulation (Figure 18C). Then we wanted to investigate whether MYC is binding to its downstream target MDM2, so we used a ChIP assay (Figure 19D). We immunoprecipitated with MYC and measuring binding to the MDM2 promoter. We determined that MYC is binding to the MDM2 promoter in DU145 cells, however there is decreased binding in DU145+Id4 possibly as result of decreased expression of MYC in DU145+Id4 cells. Collectively, the data provides evidence that Id4 down regulated MYC expression in DU145+Id4 cells, as a result MDM2 expression is down regulated due to decreased MYC expression.
CHAPTER 5

DISCUSSION

5.1 Loss of Id4 promotes tumorigenecity of prostate cancer cells

Id4 has been characterized as a tumor suppressor in many cancers, due to its promoter methylation in cancer tissues. Previous studies from Carey et al, provided evidence in prostate cancer that ectopic Id4 induced apoptosis in prostate cancer cell lines DU145, while attenuating the cell cycle and proliferation (26). Since LNCaP cells express Id4, it was determined to be an ideal model in which to silence Id4. Upon silencing of Id4, there was decreased apoptosis, which may be contributed to decreased p53, BAX, and PUMA expression. Upon silencing of Id4, there was an increase in proliferation of the LNCaP-Id4 cells. Studies have shown that there is inhibition of p27kip1 expression in prostate cancer, which results a proliferative phenotype (233-235). So the increased proliferation of LNCaP-Id4 cells may be due to decreased p27kip1 expression. Increased migration also occurred in LNCaP-Id4 cells which may be a result of increased Id1, MMP2, and MMP9.

Id1 is strongly associated with prostate cancer. Id1 has been found to promote migration and invasiveness of cancer cells (16, 106). During invasion, the extracellular matrix can be degraded by MMPs, which results in their increased expression (236). Aalinkeel et al. found that upon overexpression of MMP-9 in LNCaP cells, there was fairly common
increased invasion. While there was no overexpression of MMP-9 in these studies, loss of Id4 did increase the expression of MMP-9 which may support the increased migration and invasion that was observed (237). Not only did loss of Id4 increase migratory and invasive abilities of LNCaP-Id4 cells, but also resulted increased tumor formation in nude mice. LNCaP-Id4 tumors were not only larger than the controls (LNCaP+Id4) but also formed at a faster rate which may also be due to increased Id1 levels. Id1 expression has been associated with tumor growth in prostate cancer through activation of VEGF (22).

5.2 Id4 dependent acetylation restores mutant p53 transcriptional activity

In this study, we provide evidence that Id4 regulates p53 at two different levels: transcriptional regulation of wt-p53 in LNCaP cells and restoring the biological activity of mutant p53 in DU145 cells. Although the transcriptional regulation of wt- p53 by Id4 is clearly evident in our studies, we focused on investigating the mechanism by which Id4 restores the biological activity of mutant p53, clearly an area of high interest given that mutant p53 is observed in one third of prostate cancers (238) and more than 50% of all cancers (239).

The core domain of p53 is inherently unstable. Point mutations in this domain promote instability and unfolding, leading to decreased or completely abrogated transcriptional activity (240). Both alleles of p53 in DU145 cells carry mutations resulting in the expression of mutant p53 (p223L and V274F) (32). Although none of these mutations represent the known hotspot mutations but together these mutants maintain a predominantly denatured confirmation, are accumulated in large amounts in cells and lacks transactivation function in DU145 cells and when over-expressed in p53
null PC3 cells (24). Hence the mutants in DU145 cells are excellent models to understand the mechanisms involved in promoting its function in context of Id4 which is epigenetically silenced in these cells.

In our studies, we clearly show that mutant p53 expression is high in DU145 cells but lacks transactivation potential and DNA binding activity as compared to LNCaP cells with wt-p53. Studies have also shown that virtually all tumor derived p53 mutants are unable to activate BAX transcription but some retain the ability to activate p21 transcription (34). However, our results clearly suggest the p53 mutations in DU145 are incapable of transactivating not only p21 but BAX as well due to lack of promoter binding.

Multiple lines of evidence support the gain of transactivation potential of mutant p53 in DU145 cell over-expressing Id4: First, mutant p53 in DU145+Id4 cells promotes p53 dependent luciferase reporter activity, second, mutant p53 gains DNA binding activity as demonstrated by EMSA and direct DNA binding followed by detection with p53 specific antibody and thirdly, site specific binding to the respective p53 binding sites on BAX, PUMA, p21 and MDM2 P2 promoters, that is also consistent with their corresponding increase in expression and apoptosis. The decrease in the expression of mutant p53 in DU145+Id4 cells as compared to DU145 could also suggest that mutant p53 responds to the regulatory network required to maintain its normal physiological (compared to LNCaP cells) levels. Although we did not follow up on the mechanisms involved in the regulation of p53 expression, post-translation modifications within p53 (discussed below) can promote its function at multiple levels by attenuating its interaction
with MDM2, recruitment to p53 responsive promoters and favoring nuclear retention as observed in DU145+Id4 cells.

Acetylation at lysine residues has emerged as a critical post-translational modification of p53 for its function in vivo such as growth arrest, DNA binding, stability and co-activator recruitment ((229, 231) and reviewed in (242)). The global de-acetylation of p53 and specifically at K320 and K373 in LNCaP-Id4 cells provide strong evidence that acetylation is a major modification required to maintain p53 activity. Previous studies have demonstrated that certain post-translational modifications such as PCAF dependent acetylation of K320 can restore mutant p53 activity (G245A and R175H) (230). Our results on mutant p53 acetylation, global and K320/373 in DU145+Id4 are particularly novel and provide direct evidence that mutant p53 activity can be restored by acetylation. The increased K320 acetylation of DU145 p53 mutants is most likely also mediated by PCAF but we did not directly investigate this mechanism. However, a significant observation made in this study was co-elution CBP/P300 with wt-(LNCaP) and mutant p53 (DU145+Id4) and increased K373 acetylation in an Id4 dependent manner. Moreover, co-elution of Id4 as part of this complex with p53 antibody and co-elution of p53 with Id4 antibody suggest that Id4 can recruit CBP/P300 on wt- and mutant p53 to promote acetylation. Alternatively, CBP/p300 could recruit Id4 to promote large macromolecular assembly on p53 that could result in its acetylation and increased biological activity. Thus certain p53 mutations with some degree of conformational flexibility, upon co-factor recruitment such as Id4 and CBP/p300 could gain biological activity that is similar to wt-p53.
Acetylation at specific lysine residues can also promote specific p53 functional modifications: acetylation at K320 by PCAF results in increased cytoplasmic levels whereas CBP/P300 dependent acetylation of K370/372/373 leads to increased nuclear retention of p53 (231, 232). In contrast, MDM2, a negative regulator of p53, actively suppresses p300/CBP-mediated p53 acetylation in vivo and in vitro (243). Although we did not investigate phosphorylation of wt- or mut-p53, but K373 acetylation mimic p53Q373 undergoes hyper-phosphorylation and interacts more strongly with low affinity pro-apoptotic promoters such as BAX. In contrast, the p53Q320 interacts efficiently with the high-affinity p21 promoter (231). The ChIP data demonstrating high p53 binding on p21 promoter in DU145+Id4 cells with increased p53 K320 acetylation may suggest increased phosphorylation that correlates well and further supports acetylation dependent increase in mutant p53 activity.

As such low MDM2 levels observed in DU145+Id4 cells as compared to DU145 could be one of the mechanism by which mutant p53 could gain its trans-activation potential together with increased acetylation. MDM2 binds to the N-terminal end of p53 to inhibit its trans-activation function and consequently, disruption of p53-MDM2 interaction promotes p53 to act as transcription factor (244). Studies in DU145 and LNCaP cells using nutlin, a disruptor of p53-MDM2 interaction suggested that blocking MDM2 interaction or decreasing its cellular levels may be sufficient to promote wt- p53 activity (LNCaP cells) but is not sufficient for promoting mutant p53 transcriptional activity in DU145 cells (31). Acetylation also destabilizes p53-MDM2 interaction and
enables p53 mediated response including recruitment to respective promoters and apoptosis (225).

Collectively, we provide evidence that mutant p53 in DU145 cells retains the ability to undergo acetylation in the presence of Id4. Id4, a transcriptional regulator, may promote the p53 acetylation by recruiting CBP/p300 and/or PCAF independent of p53 mutations. Acetylated p53 in turn acquires transcriptional activity through structural changes that could possibly involve destabilization of p53-MDM2 interaction, and subsequent recruitment to p53 responsive promoters and apoptosis. The global acetylation promoted by Id4 suggests that additional lysines such as K120 and K164, known to promote binding to specific promoters such as PUMA could also be involved, but remains to be investigated. Whether Id4 promotes the activity of p53 mutants found only in DU145 cells or it has the ability to promote transactivation potential of other well-known p53 hot-spot mutants is an obvious next step that needs to be investigated. Nevertheless, the acetylation mechanism is nearly universal in nature and suggests that Id4 could promote the biological activity of other mutants, however whether such mutants retains sufficient structural flexibility following acetylation remains to be determined.

5.3 Id4 sensitizes DU145 cells to Doxorubicin treatment

Id4 sensitized prostate cancer cell lines DU145 to Doxorubicin (anti-cancer drug) via MYC regulation. There is a correlation between decreased MYC expression and increased sensitivity to doxorubicin. Abaza et al. determined that decreased MYC expression (antisense oligonucleotides) sensitized colorectal cancer cells to
chemotherapeutic drugs (245). Combination studies of doxorubicin and MYC antisense resulted in increased therapeutic effects in melanoma (246). Also a MYC inhibitor, 10058-F4 was found to inhibit proliferation and enhance chemosensitivity in human hepatocellular carcinoma cells (247). As a consequence of decreased MYC expression, there is also a decreased in tumor promoter MDM2. Slack et al. determined that MDM2 is a direct transcriptional target of MDM2 (248). Here we provide evidence that MYC binding to MDM2 promoter in DU145 cells, however there is decreased binding in DU145+Id4 cells, which may be due to decreased expression. Collectively these studies provide evidence that decreased MYC, regardless of mode of down regulation, may result in chemosensitization of cancer cells. So we conclude through Id4 regulation of MYC and/or reactivation of mutant-p53, DU145 cells are sensitized to Doxorubicin treatment, which is very important observation.
CHAPTER 6
CONCLUSION

In many cancers including prostate cancer Id4 gene is hypermethylated which suppresses expression of Id4. Previous studies found that upon overexpression of Id4 in metastatic prostate cancer cell line DU145, in which Id4 is methylated there was an induction of senescence and apoptosis. Despite the fact that three genes primarily involved in senescence, RB, p53, and p16, are mutated in this cell line. P53 is also involved in apoptosis. That led us to investigate the mechanism by which Id4 may induce apoptosis in DU145 cells. After evaluating the expression of known and well-studied pro-apoptotic markers, PUMA, BAX, and p21, we determined that Id4 up regulated the expression of each target. One common factor of these pro-apoptotic markers is that p53 can regulate their expression. So therefore it was imperative to investigate p53 expression and function, since p53 is mutated in DU145 cells there is an accumulation of p53 protein due to an extended half-life. In DU145+Id4 cells there is decreased expression of p53 and increased binding of p53 to the promoters of its targets (BAX, p21, and PUMA). This data suggested that Id4 restores mutant p53 function in DU145 cells. We determined that Id4 is promoting wild-type p53 function through acetylation of mutant p53. We also determined that Id4 is recruiting CBP/p300 to acetylate p53. This is a very important observation, because in many cases small molecules or other drugs
are needed for mutant p53 restoration. We also determined that loss of Id4 promotes tumorigenicity of prostate cancer cells. LNCaP cells express Id4, so we decided to silence Id4 in these cells to see if it would promote cancer phenotype. Upon silencing of Id4 in LNCaP cells, there was a decrease in apoptosis, but an increase in proliferation and migration. Loss of Id4 also resulted in tumor formation in nude mice; the tumors were not only larger but formed at a faster rate than the LNCaP+NS controls. This data further supports our hypothesis that Id4 acts as a tumor suppressor in prostate cancer. We also determined that Id4 sensitizes prostate cancer cells to antitumor drug, Doxorubicin. While this data is intriguing, it is of interest to determine if this phenomenon is mutation specific or cancer specific or if Id4 may be able to restore mutant p53 in other systems. It is also very important to understand exactly how Id4 may regulate tumor suppression pathways as (Figure 20) as well as translational modifications such as acetylation.
### APPENDIX

**Table 1.** qRT-PCR and ChIP primers used in the study.

<table>
<thead>
<tr>
<th>PCR primers</th>
<th>Forward (5')</th>
<th>Reverse (5')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Id4</td>
<td>CCCTCCCTCTCTAGTGCTCC</td>
<td>GTGAACAAGCAGGGCGCA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GAAGGTGAAGGTCGGAGTC</td>
<td>GAAGATGGTGATGGGATTTC</td>
</tr>
<tr>
<td>PUMA</td>
<td>CTGTATCTCTGACGCCTTGC</td>
<td>ACGGGCGACTCTAAGTGCT</td>
</tr>
<tr>
<td>BAX</td>
<td>CAG AGG CGG GGT TTC ATC</td>
<td>AGC TTC TTG GTG GAC GCA T</td>
</tr>
<tr>
<td>p21</td>
<td>GCCATTAGCGCATCACAG</td>
<td>TCGGTTCACAGGTGTTTCTG</td>
</tr>
<tr>
<td>p53</td>
<td>GCTCGACGCTAGGATCTGAC</td>
<td>GCTTTCCACGACGGTGAC</td>
</tr>
<tr>
<td>MDM2 P1 Promoter</td>
<td>TTTTCAGCGCCAGGACGCACCG</td>
<td>GGGTCTCTTGGTCCG</td>
</tr>
<tr>
<td>MDM2 P2 Promoter</td>
<td>CTTTTTTCTCTGCTGATCCAGGC</td>
<td>CAGGGTCTCTTGGTCCGAAGCTG</td>
</tr>
</tbody>
</table>

**CHIP Primers spanning the p53 response elements**

<table>
<thead>
<tr>
<th></th>
<th>Forward (5')</th>
<th>Reverse (5')</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDM2</td>
<td>GGTTGACCTCAGCTTTTCTCTT</td>
<td>GCTATTTAAACCATGCAATTTC</td>
</tr>
<tr>
<td>p21 Chip</td>
<td>GTGGCTCTGATTGGCTTTTCTG</td>
<td>TCCTTGGGCTGCTGTTTTCCAG</td>
</tr>
<tr>
<td>BAX chip</td>
<td>TAATCCCAGCGCTTTGGAAG</td>
<td>GCTGAGACGGGGTTATCTC</td>
</tr>
<tr>
<td>PUMA chip</td>
<td>GCGAGACTGTGGCCTTTG</td>
<td>CGTTCCAGGGGTCACAAAGT</td>
</tr>
</tbody>
</table>
## APPENDIX

**Table 2. Antibodies used for Immunoblots and immunocytochemistry, and ChIP**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Company Antibodies</th>
<th>Dilutions WB/ICC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Id4</td>
<td>Aviva</td>
<td>1:1200, 1:200</td>
</tr>
<tr>
<td>Id4</td>
<td>BioCheck</td>
<td>1:1000</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Cell Signalling</td>
<td>1:1000, 1:200</td>
</tr>
<tr>
<td>PUMA</td>
<td>Rockland Immunocchemical</td>
<td>1:1000</td>
</tr>
<tr>
<td>BAX</td>
<td>Cell Signalling</td>
<td>1:1000</td>
</tr>
<tr>
<td>BAX(6A7)</td>
<td>Abcam</td>
<td>1:1000, 1:150</td>
</tr>
<tr>
<td>p21</td>
<td>Cell Signalling</td>
<td>1:1000</td>
</tr>
<tr>
<td>p53</td>
<td>Cell Signalling</td>
<td>1:1000, 1:200</td>
</tr>
<tr>
<td>MDM2</td>
<td>Novus Biologicals</td>
<td>1:1000</td>
</tr>
<tr>
<td>MYC</td>
<td>Cell Signalling</td>
<td>1:1000</td>
</tr>
<tr>
<td>RNA Pol II</td>
<td>Millipore</td>
<td></td>
</tr>
<tr>
<td>Global Acetylated lysine</td>
<td>Cell Signalling</td>
<td>1:1000</td>
</tr>
<tr>
<td>K320</td>
<td>Millipore</td>
<td>1:1000</td>
</tr>
<tr>
<td>K373</td>
<td>Abcam</td>
<td>1:1000</td>
</tr>
<tr>
<td>goat anti-rabbit Secondary Antibody</td>
<td>Millipore</td>
<td>1:10000</td>
</tr>
<tr>
<td>PDH</td>
<td>Cell Signalling</td>
<td>1:250</td>
</tr>
<tr>
<td>ICC secondary antibodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DyLight 594 goat anti-mouse (red)</td>
<td>Thermoscientific</td>
<td>1:200</td>
</tr>
<tr>
<td>DyLight 488 goat anti-rabbit (green)</td>
<td>Thermoscientific</td>
<td>1:200</td>
</tr>
<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>
**APPENDIX**

Table 3. Id4 and p53 status in cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Id4 status</th>
<th>p53 status</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP</td>
<td>+</td>
<td>wildtype</td>
</tr>
<tr>
<td>LNCaP-Id4</td>
<td>- (silenced)</td>
<td>wildtype</td>
</tr>
<tr>
<td>DU145</td>
<td>- (promoter methylation)</td>
<td>mutated</td>
</tr>
<tr>
<td>DU145+Id4</td>
<td>+++ (over-expressed)</td>
<td>mutated</td>
</tr>
<tr>
<td>PC3</td>
<td>++</td>
<td>null</td>
</tr>
</tbody>
</table>
APPENDIX

Table 4: The classes of the HLH proteins with their specific domains and tissues distribution adapted from (18)

<table>
<thead>
<tr>
<th>Class</th>
<th>Examples of Proteins</th>
<th>Specific Domain</th>
<th>Tissue Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>E2A(E12/E47), TCF12, TCF4</td>
<td>Basic DNA binding domain</td>
<td>Ubiquitously expressed</td>
</tr>
<tr>
<td>II</td>
<td>MyoD, Neuro D, ABF-1</td>
<td>Basic DNA binding domain</td>
<td>More tissue specific expression</td>
</tr>
<tr>
<td>III</td>
<td>MYC family of transcription factors</td>
<td>Basic DNA binding domain, leucine zipper frame</td>
<td>Ubiquitously expressed</td>
</tr>
<tr>
<td>IV</td>
<td>Mad, Max, Mixi, c-Myc</td>
<td>Basic DNA binding domain</td>
<td>Varied tissue specific expression</td>
</tr>
<tr>
<td>V</td>
<td>Id1, Id2, Id3, and Id4</td>
<td>No Basic DNA binding domain</td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>Hairy (Drosophilia)</td>
<td>Basic DNA binding domain and a proline in the DNA binding domain with PAS domain</td>
<td></td>
</tr>
<tr>
<td>VII</td>
<td>Aromatic hydrocarbon receptor</td>
<td>Basic DNA binding domain with PAS domain</td>
<td></td>
</tr>
</tbody>
</table>
Table 4. Modulation of Id4 in cancer adapted from (115) Id4 was found to be hypermethylated in a variety of malignancies such as leukemia, prostate cancer, and breast cancer. Hypermethylation of Id4 leads to decreased expression of Id4.

<table>
<thead>
<tr>
<th>Kind of Modulation</th>
<th>Kind of analysis</th>
<th>Tumor type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear localization in cancer vs. cytoplasmic localization in spermatogonia</td>
<td>protein</td>
<td>Seminoma</td>
</tr>
<tr>
<td>Upregulation association to amplification at 6p22.3</td>
<td>mRNA</td>
<td>Bladder</td>
</tr>
<tr>
<td>Hypermethylation</td>
<td>promoter DNA</td>
<td>Gastric adenocarcinoma</td>
</tr>
<tr>
<td>Hypermethylation</td>
<td>promoter DNA</td>
<td>Colorectal carcinoma</td>
</tr>
<tr>
<td>Downregulation</td>
<td>protein</td>
<td>Colorectal adenocarcinoma</td>
</tr>
<tr>
<td>Hypermethylation</td>
<td>promoter DNA</td>
<td>Prostate</td>
</tr>
<tr>
<td>Downregulated in low grade cancer vs. hyperplasia upregulated in high grade vs low grade</td>
<td>protein</td>
<td>Prostate</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>Upregulated</td>
<td>protein</td>
<td>Breast (rat)</td>
</tr>
<tr>
<td>Hypermethylation</td>
<td>promoter DNA</td>
<td>Breast</td>
</tr>
<tr>
<td>Upregulated in basal-like cancer vs non-basal cancer</td>
<td>mRNA</td>
<td>Breast</td>
</tr>
<tr>
<td>Hypermethylation</td>
<td>promoter DNA</td>
<td>Breast</td>
</tr>
<tr>
<td>Upregulated in p53-expression cancer</td>
<td>protein</td>
<td>Breast</td>
</tr>
<tr>
<td>Upregulated in cancer vs normal brain</td>
<td>mRNA</td>
<td>Glioblastoma multifome (GBM)</td>
</tr>
<tr>
<td>Upregulated in cancer vs adjacent normal tissue</td>
<td>protein</td>
<td>Small cell lung cancer</td>
</tr>
<tr>
<td>Hypermethylation</td>
<td>promoter DNA</td>
<td>Cholangiocarcinoma</td>
</tr>
</tbody>
</table>
## APPENDIX

### Figure 1

<table>
<thead>
<tr>
<th></th>
<th>E-proteins</th>
<th>Id proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AD1</td>
<td>Iα1</td>
</tr>
<tr>
<td>TA</td>
<td></td>
<td>Iα2</td>
</tr>
<tr>
<td>αβ</td>
<td></td>
<td>Iα3</td>
</tr>
<tr>
<td>βδ</td>
<td></td>
<td>Iα4</td>
</tr>
</tbody>
</table>

**Figure 1b**

- **E-proteins**
  - E box
  - Target gene

- **Id proteins**
  - E box
  - Target gene

*Nature Reviews | Immunology*
Figure 1. Bhlh family of transcription factors: Id proteins. (A) Basic helix loop helix (bHLH) transcription factors regulate the differentiation programs of multiple cell lineages. This protein family shares a common sequence domain, which is where these proteins bind. (B) However Id protein family lacks the basic binding domain which prevents DNA binding. Id proteins are transcriptional regulators in many developmental processes. Id proteins drive differentiation. Figure taken from (249)
Figure 2

A

This shows the prostate and nearby organs

B

This shows the inside of the prostate, urethra, rectum, and bladder
APPENDIX

Figure 2. The adult prostate and surrounding structures. (A) The base of the prostate is located at the bladder neck. The urethra bisects the prostate. Image taken from http://www.cancer.gov/cancertopics/. (B) The three histologically distinct zones are shown: the central zone, the transitional zone, and the peripheral zone. Figure taken from Prostate Histology April 13, 2012 (2003-2012) Pathology Outlines.com, Inc.
Figure 3

A

NORMAL PROSTATE DUCT

Lumen

Stroma

Basal lamina

Tight junctions

Luminal epithelial cells

Secretory epithelial cells

Basal epithelial cells

Neuroendocrine cell

B

PIN

SECRETORY EPITHELIAL CELLS

TIGHT JUNCTIONS

BASAL EPITHELIAL CELLS

NEUROENDOCRINE CELL

INVASIVE CARCINOMA

METASTASIS
Figure 3. Progression of prostate cancer. (A) Schematic representation of cancer progression. Normal prostate duct to metastasis of the prostate. The structure of the normal prostate is maintained by its androgen dependence. However the onset of PIN leads to disorganization of prostate cells and eventually leads to invasive carcinoma and then metastasis to other body sites. (B) Schematic of metastatic cascade and depiction of the cell types within a human prostatic duct. There are three types of cells in the normal prostate, secretory luminal epithelial cells, and neuroendocrine cells, and basal epithelial cells. Image taken from (250)
Figure 4

Figure 4. Id sequence and protein structure comparison. Id proteins shared a conserved HLH domain sequence.
Figure 5. Model for Id gene function in cell cycle progression. The role of Id proteins in the cell cycle. Id proteins are key regulators of the GI phase of the cell cycle and regulate RB, a crucial cell cycle gene. Figure taken from (251)
Figure 6

A

Functional domain of p53

Activation domain: Sequence-specific DNA binding domain

Tetramerization domain 393

B

core domain: sequence specific DNA binding

cOOH-terminal domain: tetramerization
Figure 6. **P53 gene and cancer derived p53 mutations.** (A) The full length protein of p53 is shown schematically and four domains are defined: activation domain, DNA binding domain, tetramerization domain, and regulatory domain. (B) Commonly mutated regions of p53 protein. The relative number of times and placement of a particular point mutation is depicted as a histogram above the schematic. The majority of tumor-derived mutations are found in the core/DNA binding domain. This figure was adapted from (30).
Figure 7. p53 pathway, depicting p53 regulatory network. P53 can be regulated by MDM2 and p14ARF. P53 can also regulate p21, MDM2, Bax, and Gadd45, which eventually leads to cell-cycle arrest and apoptosis. This figure was taken from (252).
Figure 8

A

![Bar graph showing Relative Expression/GAPDH](chart.png)

B

![Images of Id4/DAPI](images.png)

C

![Images of LNCaP and DU145 with Id4/DAPI](images.png)
Figure 8 Stable knockdown of Id4 by retroviral shRNA in LNCaP cells (retroviral vectors A and C) and stable over-expression of hId4 in Du145 cells. (A) Real time quantitative polymerase chain reaction for Id4 expression in LNCaP (NS, non-specific) following transfection with Id4shRNA vectors A and C and non-silencing shRNA (NS) (***: P<0.001). (B) Western blot analysis of Id4 expression in LNCaP cells with non-specific shRNA (NS) and Id4 specific shRNA (-Id4, vector A). (C) Immuno-cytochemical analysis of stable knockdown of Id4 expression in LNCaP cells (LNCaP-Id4, vector A) as compared to cells with non-specific shRNA (LNCaP+NS). The red staining indicates Id4 expression (DyLight 594). Id4 expression in DU145 cells stably transfected with Id4 expression vector (DU145+Id4) as compared to DU145 cells transfected with empty vector (DU145+NS). The green staining represents Id4 (DyLight 488). DAPI was used to stain the nuclei (blue) in both LNCaP and DU145 cells. Representative images are shown.
APPENDIX

Figure 9

**A**

### Apoptosis Data

<table>
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<tr>
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<th>Live cells</th>
<th>Apoptotic cells</th>
<th>Dead cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP+NS</td>
<td>50%</td>
<td>47%</td>
<td>4%</td>
</tr>
<tr>
<td>LNCaP-Id4</td>
<td>83%</td>
<td>10.5%</td>
<td>6.5%</td>
</tr>
</tbody>
</table>

**B**

### Proliferation Assay

![Graph showing proliferation assay results](image)
Figure 9. Loss of Id4 attenuates apoptosis and promotes proliferation. Apoptosis was measured using Annexin V staining. Proliferation was measured using an MTT assay. (A) In cell line LNCaP-Id4, apoptosis is decreased by \( \sim 37\% \). (B) There is an increase in the proliferation of LNCaP-Id4 cells by fold change of 2. Data is statistically significant. Each bar represents Mean \( \pm \) SD from a representative experiment. (P< 0.05).
Figure 10

A

B

LNCaP+NS

LNCaP+Ida

0 hr

48 hr

Transwell Migration Assay

# of cells

LNCaP+NS

LNCaP+Ida

***
APPENDIX

Figure 10. Loss of Id4 promotes migration. Effects of loss of Id4 on cell migration in prostate cancer cell line LNCaP. Representative images of (A) Scratch Wound Assay (B) LNCaP and LNCaP-Id4 cells after migration of cells through transwell. Cells were visualized under 10X objectives. LNCaP+NS was used as a control. The number of migratory cells doubles upon loss of Id4. Each bar represents Mean SEM (n=3). * Significantly different (P < 0.05) compared to untreated controls.
Figure 11. Gene expression changes following loss of Id4. The expression of cyclin dependent kinase p27 was decreased upon loss of Id4. P27 has been implicated in cell proliferation (11C). The expression of migration markers Id1 (11A) and MMPs 2 and 13 were increased in LNCaP-Id4 cells as compared to LNCaP+NS. The increase in migration markers may provide the mechanism by which loss of Id4 promotes migration of prostate cancer cells. The mean+SEM of three experiments in triplicate are shown. The delta delta Ct (normalized to GAPDH and LNCaP normalized to 1) is shown (*: P<0.001).
Figure 12

A

B

C

D

E

F

G

APPENDIX
APPENDIX

Figure 12. Loss of Id4 promotes anchorage independent growth and tumor growth in vivo. Loss of Id4 increases anchorage-independent cell growth. The colonies were grown for 21 days and then the images were captured using a microscope (10X) (A). After quantitation of the colonies, there were 3 times as many colonies in LNCaP-Id4 as there were in the control LNCaP-Id4 (B). (C) Images of the tumor growth were captured weekly to visual tumor size. (D) After the extraction of the tumor, the tumor size was determined to be LNCaP+NS Weight: 0.02g Tumor dimensions: 4mm x 2mm and LNCaP-Id4 Weight: 0.62g Tumor dimensions 15mm x10mm, which a significant increase in tumor size. (E) and (F) Graph represent tumor weight and volume which increased 5 and 6 times greater than the LNCaP+NS control, respectively. (G) Representation of the rate and size of tumor growth over a period of six weeks, shows not only increased tumor size in LNCaP-Id4 but also increased rate of tumor growth. Data is expressed of six different mice mean+SEM, *: P<0.05).
Figure 13

A. Western Blot

B. q-Real Time PCR

C. ICC: BAX localization with PDH

E
Figure 13. Id4 promotes apoptosis by regulating mitochondrial membrane potential and the expression of pro-apoptotic genes. A: percent cells undergoing apoptosis was determined by propidium iodide and Annexin V staining followed by flow cytometry. Significant increase in apoptosis (***: P<0.001) was observed in DU145 cells over-expressing Id4 (D+Id4) when compared with DU145 cells alone (D). A significant decrease in apoptosis was observed in LNCaP cells that lacked Id4 (L-Id4) as compared to LNCaP cells (L, ***: P<0.001). B. Percent cells with high mitochondrial membrane potential (Gated, FL2>100 fluorescence units). In the presence of Id4 (D+Id4 and L), the mitochondrial membrane potential decreased as compared to the corresponding cells that lack Id4 (D and L-Id4). (***: P<0.001 – L vs. L-Id4 and D vs. D+Id4). C. Western blot analysis of p21, BAX, confirmation specific BAX (BAX6A7) and PUMA in D, D+Id4, L and L-Id4 cells. GAPDH was used as loading control. Representative western blots of three different experiments are shown. D. Real time quantitative analysis of p21, BAX and PUMA expression in D, D+Id4, L and L-Id4 cells. The mean+SEM of three experiments in triplicate are shown. The delta delta Ct (normalized to GAPDH) between D and D+Id4 (D normalized to 1, designated as “a”) and between L and L-Id4 (L normalized to 1, designated as “b”) is shown (*: P<0.001). E. Immuno-cytochemical analysis demonstrating co-localization of confirmation specific BAX (using BAX 6A7...
antibody) with mitochondrial pyruvate dehydrogenase (PDH). Blue: DAPI, red: PDH, green: BAX 6A7 and yellow: co-localization of BAX and PDH (observed only in LNCaP and DU145+Id4 panels. Representative images from three different experiments are shown.
Figure 14

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Figure 14. **Id4 regulates p53 expression and cellular localization.** Analysis of p53 protein (A) and transcript (B) expression in L, L-Id4, D and D+Id4 cells. The western blot analysis shown in panel A is the representative of three different experiments. The p53 real time quantitative expression is mean±SEM of triplicates. The data normalized to GAPDH and represented as fold change with p53 expression in LNCaP used as positive control set to 1 (□ □ □ Ct). (*:P<0.001 as compared to LNCaP). C. Immuno-cytochemical localization of p53 in L, L-Id4, D and D+Id4 cells. Nuclear and cytoplasmic (253) expression of p53 is clearly evident in L, L-Id4 and D cells. Whereas p53 is primarily nuclear in D+Id4 cells (253). Red: p53, Blue: DAPI. Representative images are shown.
APPENDIX

Figure 15

A

EU  L  L-id4  D  D+ld4  P

B

p53 Activity (OD450nm)

L  L-id4  D  D+id4

***

C

Relative Luciferase Act.

wt-p53RE  mt-p53RE

L  L-id4  D  D+id4

*

D

% Input

L  L-id4  D  D+ld4

E

F

BD  BD  D+ld4

BD  D+ld4

a  a*

b  b*

0.00  0.01  0.02  0.03

0.00  0.05  0.10  0.15  0.20  0.25
Figure 15. Id4 promotes DNA binding and transcriptional activity of wild type and mutant p53. A. EMSA with p53 consensus DNA binding response element with nuclear extracts from LNCaP (L), LNCaP-Id4 (L-Id4), DU145 (D), DU145+Id4 (D+Id4) and PC3 cells. Nuclear extracts from PC3 cells, null for p53 and LNCaP cells with wild type p53 were used as negative and positive controls respectively. Excess unlabeled (EU) p53 response element was used to monitor non-specific binding. B. Quantitative p53 DNA binding in a sandwich ELISA based system. P53 was captured by double stranded oligonucleotide with p53 response element immobilized on a 96 well plate. The captured p53 was detected using p53 antibody by measuring the intensity at 450nm using HRP coupled secondary antibody. C. The p53 transcriptional activity as determined by transiently transfecting cell lines as indicated above with p53 response element driven luciferase reported plasmid (wt-p53RE). The data is normalized to Renilla luciferase. The mutant p53 luciferase reporter plasmid was used as a negative control (mt-p53RE). The p53-luciferase reporter activity in LNCaP-Id4 (L-Id4) was normalized to LNCaP (L) and that of DU145+Id4 (D+Id4) with DU145 (D). The data from 3 different experiments in triplicate is expressed as mean+SEM (*: P<0.001). D, E and F. Chromatin immuno-precipitation assay demonstrating the binding of p53 to its respective response element in the BAX (D), p21 (E) and PUMA (F) promoters. The data is expressed as percent input
APPENDIX

is mean+SEM of three experiments in triplicate (a: between L and L-id4 and b: between D and D-id4, *: P<0.001, BD: Below Detection).
Figure 16

A

<table>
<thead>
<tr>
<th></th>
<th>L</th>
<th>L-I4</th>
<th>D</th>
<th>D+I4</th>
</tr>
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<tbody>
<tr>
<td>MDM2</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>GAPDH</td>
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</table>

B

Exon 1a → p53 RE → Exon 1b → Exon 2

P1

P2

C

D

% Input

Fold Change

P1

P2

L L-I4 D D+I4

P1

P2

L L-I4 D D+I4

% Input

Fold Change
APPENDIX

Figure 16. Expression of MDM2 and its transcriptional regulation. A. MDM2 immuno blot in cells with (L and D+Id4) and without Id4 (L-Id4 and D). GAPDH was used as loading control. Representative data from three different experiments is shown. The bottom panel is semi-quantitative analysis of fold change in MDM2 expression relative to LNCaP (L) and normalized to GAPDH (mean+SEM, *: P<0.001, compared to L). B. Schematic of MDM2 promoter organization. MDM2 is transcribed from two independent promoters P1 and P2 but both the transcripts are translated from a common start site in exon2. P1 promoter is p53 independent whereas P2 promoter is p53 dependent due to a p53 response element in intron 1 (p53RE). Specific primers were used to determine the transcript abundance of P1 (p53 independent) and P2 (p53 dependent) transcripts. C. P1 and P2 transcript abundance with Real time quantitative PCR analysis in cell lines expressed as fold change from three different experiments in triplicate (mean+SEM). The expression is first normalized to GAPDH and then to P1 transcript in L and D cells set to 1 (comparison between L and L-Id4 and between D and D+Id4, a: P<0.001 as compared to P1 transcript b: P<0.001 compared to P2 transcript). D. Chromatin immuno-precipitation assay demonstrating the binding of p53 to its respective response element in the MDEM2 P2 promoter (intron 1). Data is expressed as mean+SEM of three different experiments performed in triplicate (mean+SEM, *: P<0.001).
Figure 17. Acetylation of p53 and interaction with CBP/p300 and Id4. A. p53, immunoprecipitated from cell lines was blotted with antibodies against acetylated lysine (global), p53 acetylated at either K373 (Ac-373) or K320 (Ac-320) and CBP/p300. B. The total protein lysate from cell lines as indicated was immunoprecipitated (IP) with either p53 or Id4 antibody. The immunoprecipitated lysate was then immuno-blotted with p53 antibody (IB:p53).

Representative data is shown.
APPENDIX

Figure 18

<table>
<thead>
<tr>
<th></th>
<th>% Live</th>
<th>% Apoptotic</th>
<th>% Dead</th>
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<tr>
<td>DU145 control</td>
<td>95.9</td>
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<td>0.8</td>
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<tr>
<td>DU145+ 500nM Dox</td>
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<td>44.1</td>
<td>19.5</td>
</tr>
<tr>
<td>DU145+Id4 control</td>
<td>40.2</td>
<td>31.0</td>
<td>28.8</td>
</tr>
<tr>
<td>DU145+Id4 +500nM Dox</td>
<td>22.2</td>
<td>44.1</td>
<td>33.7</td>
</tr>
</tbody>
</table>
Figure 18. Id4 sensitizes prostate cancer cell to doxorubicin treatment. (A) and increase in apoptosis (16B). Proliferation was measured at 0, 24H, 48H, and 72H and apoptosis was measured after 24H. Cells were treated with 500nM of dox.
Figure 19. Id4 regulation of MYC and MDM2. Id4 down-regulated Myc expression in DU145+Id4 cells (17A). MDM2 is a direct target of MYC and its expression is decreased in DU145+Id4 probably due to decreased levels of MYC (17B). There is decreased binding of MYC in DU145+Id4 possibly due to decreased level of MYC (17C). DU145 was calculated as 1.
Figure 20

P21 degradation, Mdm2

p53 independent functions

Mdm2

myc

p53

Bax

Apoptosis

PUMA

Apoptosis

p21

Cell cycle
Figure 20. **Id4 pathway involved in tumor suppression.** Id4 may regulate multiple tumor suppression pathways, but we have provided evidence that Id4 regulates MYC which can in turn regulate many unique pathways. We also provided concise evidence that Id4 protein directly binding to p53 protein which can then activate cell cycle and apoptosis pathways.
REFERENCES


with poor differentiation and unfavorable prognosis, *Clin Cancer Res* 10, 7475-7483.


