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ID4 Acts as a Tumor Suppressor via p53: Mechanistic Insight

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ABSTRACT

DEPARTMENT OF BIOLOGICAL SCIENCES

MORTON, DERRICK J. B.S. EASTERN KENTUCKY UNIVERSITY, 2009

ID4 ACTS AS A TUMOR SUPPRESSOR VIA P53:

MECHANISTIC INSIGHT

Committee Chair: Jaideep Chaudhary, Ph.D.

Dissertation dated May 2016

Overexpression of tumor-derived mutant p53 is a common event in tumorigenesis, suggesting an advantageous selective pressure in cancer initiation and progression. Given that p53 is found to be mutated in 50% of all human cancers, restoration of mutant p53 to its wild type biological function has been a widely sought after avenue for cancer therapy. Most research efforts have largely focused on restoration of mutant p53 by artificial means given that p53 has some degree of conformational flexibility allowing for introduction of short peptides and artificial compounds. Recently, theoretical modeling and studies focused on restoration of mutant p53 by physiological means has raised the question of whether there are effective therapies worth exploring that focus on global physiological mechanisms of restoration of p53. Herein, we provide computational analysis of the thermodynamic stabilities of both wild-type and mutant p53
core domains by studying their respective minimum potential energies. Also, it is widely accepted that wild type p53 is modulated by various acetyl transferases as well as deactylases, but whether this mechanism of p53 modulation can be exploited for physiological restoration of mutant p53 remains under intense investigation. Using prostate cancer cell lines representative of varying stages of aggressiveness as a model, we show that ID4 dependent acetylation promotes mutant p53 DNA-binding capabilities to its wild type consensus sequence, thus regulating p53-dependent target genes leading to subsequent cell cycle arrest and apoptosis. Specifically, we identify that ID4 promotes acetylation of K373 and to a lesser extent K320, in turn regulating p53-dependent biological activities. Together, our data provides computational analysis of the core domain of certain mutant forms of p53 and a molecular understanding of ID4 dependent acetylation that suggests a strategy of enhancing p53 acetylation at sites K373 and K320, critical sites of post translational modification of p53, that may serve as a viable mechanism of physiological restoration of mutant p53 to its wild type biological function.
ID4 ACTS AS A TUMOR SUPPRESSOR VIA P53:
MECHANISTIC INSIGHT

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

BY
DERRICK J. MORTON, JR.

DEPARTMENT OF BIOLOGICAL SCIENCES

ATLANTA, GEORGIA
MAY 2016
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<table>
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<th>Full Form</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen Receptor</td>
</tr>
<tr>
<td>BCS</td>
<td>Bovine Calf Serum</td>
</tr>
<tr>
<td>bp</td>
<td>Base Pair</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign Prostatic Hyperplasia</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenetic protein</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast Cancer 1</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immuno precipitation</td>
</tr>
<tr>
<td>CRPC</td>
<td>Castration Resistant Prostate Cancer</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′-6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Dinucleotide Triphosphate</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
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</tbody>
</table>
EGF ................................................................. Epidermal Growth Factor

EMSA .................................................................... Electrophoretic Mobility Shift Assay

ER ............................................................................ Estrogen Receptor

FBS ....................................................................... Fetal Bovine Serum

HLH ...................................................................... Helix Loop Helix

ICC ........................................................................ Immunocytochemistry

ID .......................................................................... Inhibitor of Differentiation

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

PCa ....................................................................... Prostate Cancer

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 I
INTRODUCTION

The presence of p53 missense mutations is an overwhelming characteristic and likely critical step in the oncogenic process, usually as a result of somatic mutations, which occur in approximately half of all human cancers and constitute a cornerstone in tumorigenesis. As reviewed in Oren et al. in principle, emergence of a p53 mutation within a cell might have three, not mutually exclusive, types of outcomes. First, such mutation is expected to abrogate the tumor suppressor function of the affected TP53 allele, reducing the overall capacity of the cell to mount a proper p53 response; if both alleles eventually become mutated, or if the remaining allele is lost, such cells will be totally deprived of anticancer protection by p53. Second, many common mutant p53 isoforms can exert dominant–negative effects over coexpressed wild type p53, largely by forming mixed tetramers that are incapable of DNA binding and transactivation. Hence, even if one wild-type allele is retained, the cell may be rendered practically devoid of wild type p53 function through such mechanism, particularly if the mutant protein is expressed in excess over its wild type counterpart. Third, and most relevant, the emergent mutant p53 protein possess activities of its own, often not present in the original wild type p53 protein, which can actively contribute to various aspects of tumor progression. These new abilities attributed to mutant p53 are commonly referred to as gain-of-function. Structural studies of mutant forms of p53 found in human tumors
have mainly been classified into two main categories: type I mutations, which affect amino residues directly involved in the DNA interaction (R248H, V274F, and R273H), and class II mutations involving residues responsible for the stabilization of the three-dimensional structure of p53. Structural mutants, includes the majority of p53 proteins found in human tumors, such as R175H, P223L, R249H, and R282H mutants, all of which destabilize p53 conformation and the p53 DNA-binding.

Numerous studies of mutant p53 have been designed to explore restoration of DNA-binding capability artificially by site-specific phosphorylation and amino acid substitution primarily in the c-terminus of p53 to resemble wild type p53. Theoretical modeling of p53 has also been central to understanding the DNA-binding capabilities of mutant p53, as well as conformation changes induced by protein-protein interactions, as well as a number of artificial compounds have been identified that can reactivate mutant p53 by directly stabilizing the interaction with DNA and/or by preventing misfolding or aggregation. Similarly, the structure of wild-type p53 itself naturally comprises unfolded regions and displays high tendency to aggregation. Several of these reactivating agents include: CP-31398, ellipticine, MIRA-1, RITA, and PRIMA-1. Lastly, reactivating molecules represented by short peptides introduced in the c-terminal domain of p53, when introduced into tumor cells harboring p53 mutants, lead to induction of p53-regulated genes and apoptosis. In this case, however, the mechanisms of activation are less clear, which provides a physiological basis of exploration into this phenomenon.
Moreover, interestingly, studies have shown that the p53 mutations are indeed not all functionally equivalent.⁴ It’s widely been reported that mutations in the DNA-contacting residues of p53 have a less dramatic effect on the folding of the p53 protein than the structural mutants.¹⁴ On the other hand, mutant p53 can engage in protein–protein interactions with a growing number of transcription factors, often being recruited to binding sites of those factors on chromatin, and modulate their transcriptional output both positively and negatively.¹⁵ In fact, very early studies showed that certain p53 mutants retain the ability to activate transcription of genes known to be regulated by wild-type p53 and to induce cell cycle arrest or apoptosis in some cell types but not in others providing a physiologically relevant basis for study of mutant p53 restoration to wild type biological activity by non-artificial means.

Our laboratory as well as other groups have shown that in the case of wild type and mutant forms of p53, the interaction with acetylases and acetylation of p53 itself is indispensible in regulation of DNA binding capabilities and transcription activation potential, as well as the ability of p53 to trigger cell cycle arrest or apoptosis.⁷a,¹⁶ In a previous study done by our laboratory we provide evidence that ID4, a transcriptional regulator could recruit CBP/p300 to promote large macromolecular assembly on p53 that could result in its acetylation and increased biological activity. Here, we demonstrate that ID4, a transcriptional regulator can promote p53-dependent induced cellular death and cell cycle arrest in prostate cancer cells by specifically modifying the acetylation pattern of p53, which increases its transcriptional activity and promotes the expression of pro-apoptotic and cell cycle regulator genes. Furthermore, using acetylation mimics of p53,
we identify lysines 373 and 320 as critical acetylation sites that aid in p53 biological activities. Together, the results presented herein suggest that ID4 dependent p53 acetylation can be used as model to revert mutant p53 to wild type biological activity.

ID proteins (ID1, ID2, ID3 and ID4) are dominant negative transcriptional regulators of basic Helix Loop Helix (bHLH) transcription factors that lack the basic DNA binding domain but have intact HLH domain. Thus, ID proteins can interact with bHLH proteins but the heterodimer fails to bind DNA and activate E-Box dependent transcription of target genes. ID proteins regulate the function of various ubiquitously expressed and tissue specific bHLH transcription factors as well as many non-bHLH proteins with different affinities in complex transcriptional networks. Herein, we focus on the current understanding of ID4 in development and disease.

The de-regulation of ID proteins contributes to developmental defects and neoplastic progression. Based on significant sequence homology of ID protein sub-types within the HLH domain, a degree of functional redundancy is expected at least in their interaction with bHLH transcription factors. However, recent studies suggest that each of the ID sub-type also participates/regulates unique biological activities which is evident from non-compensatory phenotypes in ID specific knockout models and preferred protein interactions, both bHLH and non-bHLH. In this context Inhibitor of differentiation/DNA-binding 4 (ID4) has emerged as an outlier in terms of expression and function. Evidence from animal models indicates that phenotypic changes and molecular pathways regulated by ID1, ID2 and ID3, in general are not similar to those regulated by ID4. The opposing function of ID4 versus IDs 1, 2, and 3 also suggest that the core function of ID proteins as
dominant negative bHLH transcriptional regulators may be just a fraction of their overall function. The majority of ID sub-type specific function could involve unknown and perhaps yet undefined interactions with sequence specific bHLH or non-bHLH proteins resulting in non-overlapping biological endpoints.\textsuperscript{20}

As key regulators of cell cycle and differentiation, ID proteins have shown a vast regulatory function across diverse cellular functions, including cell cycle, apoptosis, senescence and cancer.\textsuperscript{20}

ID4 appears to act primarily as a tumor suppressor in most cancers as opposed to ID1, ID2 and ID4, which in most cases acts as tumor-promoters or supporting oncogenes.\textsuperscript{17,21} In a small subset of cancers, ID4 also acts as a tumor promoter (Figure 1). Beyond correlative studies showing ID4 expression in tumors, the underlying molecular mechanism remains largely unexplored.

\textbf{Figure 1.} ID4 expression in cancers. A: ID4 expression profile in various cancers from the Oncomine database. The blue and red boxes represent cancers in which ID4 expression is decreased or increased respectively as compared to normal counterparts. B: The ID4 expression in various cancers from literature mining. The green lines point to cancers in which ID4 is down-regulated whereas red lines point to cancers where ID4 is up-regulated.
In general, the expression of ID proteins (ID1-ID3) is high in proliferating cells.\textsuperscript{19c, 19d} Their expression is down regulated during differentiation.\textsuperscript{22} Consistent with these observations the expression of ID1, ID2 and ID3 proteins is increasingly observed in many cancers and, in most cases, associated with aggressiveness of the disease including poor prognosis, metastasis, and angiogenesis.\textsuperscript{21} In contrast, the expression of ID4 decreases in many cancers.\textsuperscript{23} Of all the four ID proteins, the underlying mechanism of ID1, ID2 and, to a lesser extent, ID3 in cancer is relatively well known.\textsuperscript{24} ID1, ID2 and ID3 have been shown to negatively regulate the expression of Cyclin D1, p16 and p21, but promote the expression of matrix metalloproteinase (MMPs)\textsuperscript{24-25} and thus, promote aggressiveness of disease. On the contrary, large numbers of studies have reported reduced ID4 expression due to promoter hyper-methylation during cancer progression to a more malignant phenotype.\textsuperscript{26}

ID4 is required for normal prostate morphogenesis where it is specifically expressed in the luminal epithelial cells.\textsuperscript{26b} Prostates from ID4\textsuperscript{-/-} mice exhibit decreased branching morphogenesis and often display prostatic intra-epithelial neoplastic lesions (PIN).\textsuperscript{27} Loss of ID4 expression is also frequently observed in prostate cancer, suggesting its essential role as a tumor suppressor.\textsuperscript{28} Knockdown of ID4 in LNCaP prostate cancer cell lines results in aggressive growth, increased cell survival and acquisition of castration resistance phenotype usually associated with advanced disease.\textsuperscript{29} In addition to prostate cancer, decreased ID4 expression is also observed in leukemia,\textsuperscript{30} AML,\textsuperscript{31} CLL,\textsuperscript{32} ALL,\textsuperscript{33} glial neoplasia,\textsuperscript{34} squamous cell carcinoma,\textsuperscript{35} gastric cancer,\textsuperscript{36} pancreatic cancer,\textsuperscript{37} colorectal adenocarcinoma,\textsuperscript{37-38} malignant lymphoma,\textsuperscript{39} cholangiocarcinoma,\textsuperscript{31c} Barrett's
esophagus and esophageal adenocarcinoma\textsuperscript{40} and lung cancer.\textsuperscript{41} Thus, epigenetic inactivation of ID4 due to promoter methylation appears to be the key mechanism in many cancers. ID4 is also overexpressed in a small subset of cancers, which will be discussed herein.

Previous studies have explored possible correlations between ID4 and p53. A negative correlation was observed between mutant p53 and ID4 expression in meta-analysis of clinical studies.\textsuperscript{42} Similarly, in the TCGA dataset (Breast Invasive Carcinoma, TCGA Provisional), p53 mutations were observed in 32\% of cases (306 cases out of 956) whereas ID4 was altered in only 3\% cases. Interestingly, ID4 expression was altered in only 6\% of cases in which p53 was mutated. Hence a significant correlation was not observed between p53 mutations and ID4 expression (Figure 2). However, previous studies from our laboratory in prostate cancer found that ID4 was modulating wild type and mutant p53 suggesting a positive role for ID4 and p53 cross-talk.\textsuperscript{43}

**Figure 2.** Relation between p53 mutations and ID4 expression in breast cancer obtained from TCGA provisional dataset (cBIO Portal). A total of 956 samples were analyzed of which 306 (32\% demonstrated p53 mutations (green) and 3\% demonstrated ID4 alterations (red bars: up-regulated, blue bars: down-regulated)). Only 20 samples with p53 mutations showed alterations in ID4 expression. The figure only shows the samples with p53 mutations and ID4 expression. The grey bars represent samples with no change in either p53 (mutations/ expression) or ID4 (expression) (majority of these samples not shown). The expression is based on microarray analysis with Z-score of more than 2.0.
The molecular mechanisms by which mutant p53 may function have been reviewed extensively. These pathways include alterations in the DNA-binding ability of mutant p53, interaction of mutant p53 with other proteins, including transcription factors or proteins not directly related to the regulation of gene expression. It is clear that the effects of mutant p53 are strongly context dependent, and interactions that promote activity in some circumstances may be inhibitory in others.

Previous studies from our laboratory have demonstrated that ID4 promotes transcriptional activity of wild type p53 in a CBP/p300 dependent acetylation at K320 and K373 residues. Interestingly, ID4 also restores the wild type transcriptional activity of mutant p53 in DU145 cells through a similar mechanism. The restoration of mutant p53 transcriptional activity in DU145 is evident that ectopic ID4 promotes apoptosis, attenuates cell cycle and promotes senescence in these cells in spite of mutations in Rb and p16. Moreover, electrophoretic shift assays (EMSA) and chromatin immunoprecipitation (ChIP) analysis demonstrated that ID4 promotes binding of mutant p53 to the consensus wild type p53 DNA response element and enrichment to p53 binding sites on p21, BAX and PUMA promoters. Co-elution of ID4 as part of the p53-CBP/p300 complex with p53 antibody and co-elution of p53 with ID4 antibody suggest that ID4 can recruit CBP/P300 on wild type and mutant p53 to promote acetylation. Whether ID4 can also restore the wild type transcriptional activity of various p53 hot spot mutants remains to be established.

While there is overwhelming evidence that demonstrates that there is ID4 and p53 cross-talk in prostate cancer, the underlying mechanism remain unexplored. In this study,
we hypothesized that ID4 acts as tumor suppressor by regulating p53 expression and/or function. To investigate this hypothesis the following aims were proposed:

1. Determine whether ID4 regulates p53 expression and/or stability.
2. Investigate how ID4 interacts and regulates p53 transcriptional activity.
CHAPTER II
REVIEW OF LITERATURE

2.1 Prostate and Prostate Cancer

The human prostate glands are paired, branched, secretory structures that bud from the urogenital sinus (UGS) in a testosterone-dependent process. The prostate develops from 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. The UGS is found in both male and female humans at about 7 weeks of gestation. 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 3A). The adult prostate is organized into three zones, a central zone, a transition zone, and a peripheral zone (Figure 3B). 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. 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), an overgrowth that is common
among aging men, occurs mainly in the transition zone, whereas prostate carcinoma arises primarily in the peripheral zone.\textsuperscript{50}

**Figure 3.** 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.

Mature prostatic ducts consist of three major cell types luminal secretory epithelial cells, basal epithelial cells and stromal smooth muscle cells.\textsuperscript{51} Differentiation markers can distinguish these cell types from one another. 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.\textsuperscript{51b} The stromal layer is mostly composed of smooth muscle, but also contains fibroblasts, neuronal, lymphatic, and vascular cell types.\textsuperscript{51a} 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.\textsuperscript{46}

Prostate cancer (PCa) is the most prevalent solid tumor and a leading cause of cancer-related deaths among males in the United States, with an estimated 238,590 and 233,000 new cases diagnosed in 2013 and 2014 respectively, and 29,720 deaths in 2013.\textsuperscript{52} In 2015, it is estimated that 220,800 new cases will be diagnosed with 27,540 estimated deaths according to the American Cancer Society. Due to technical improvement in screening and diagnostics, early surgical resection and introduction of novel anti-neoplasms agents, the incidence of PCa has been declining approximately 2.4% annually from 2002 to 2011. The mortality of PCa has dropped 3.3% each year over the last decade, with elevated overall survival rate from 66\% in the 1970s to 99.6\% currently.\textsuperscript{52b, 53}

Most prostate cancers are adenocarcinomas, which arise from glandular epithelial cells. Prostate cancer rarely begins from the tissues surrounding the gland.\textsuperscript{54} Prostate intraepithelial neoplasia (PIN) is defined by “neoplastic growth of epithelial cells within pre-existing benign prostatic acini or ducts.”\textsuperscript{55} PIN is widely accepted as a precursor to prostate cancer.\textsuperscript{56} Prostate carcinoma is usually observed near the surface of the glands, which can be diagnosed during a digital rectal examination.\textsuperscript{57} As the tumors grow, the prostate expands around urethra and cause urinary problems with time it starts to invade surrounding organs such as seminal vesicle, neck of the bladder, lymph nodes and later prostate cancer can spread to bones, spine and pelvis. Metastasis often occurs in the lungs, liver and adrenal glands.\textsuperscript{58}
American cancer society has identified five risk factors, which may increase the likelihood that a person develops prostate cancer: family history, age, race, nationality and diet. It has been found that African Americans have higher rates of prostate cancer incidence compared to that of Caucasians and Asian men. The risk of prostate cancer increases with age: one out of 6 American men during their life time is diagnosed with the disease. The therapeutic approaches such as radical prostatectomy and radiotherapy are considered curative for localized disease, yet no treatments for metastatic prostate cancer are available that significantly increases patient survival. Epidemiological studies have shown environmental factors such as diet is also a factor that increases the risk of prostate cancer.

2.1.1 Androgens and Androgen Receptor (AR)

AR is a nuclear hormone receptor consisting of eight exons, which encode four functional domains: the NH2-terminal domain (NTD), DNA-binding domain (DBD), the hinge region and ligand-binding domain (LBD). The NTD accounts for majority of AR transcriptional activities, and the LBD binds androgens and transfers AR to the nucleus. The DBD, composed of two zinc fingers, is critical to DNA recognition and binding; whereas the hinge domain regulates the translocation of AR into the nucleus. PCa cells depend on androgens for growth and survival via the AR axis, and the roles of AR in maintenance of prostate tissue lineage, as well as in PCa initiation and development, are the basis for the effectiveness of androgen depletion therapy (ADT). Since Huggins and Hodges first demonstrated that PCa was hormone responsive and castration could be used as an effective therapy for patients in 1941, efforts focusing on ablating AR
signaling have never been halted.\textsuperscript{65} Unfortunately, although surgical or chemical castration is highly effective in shrinking tumor burden decreasing serum PSA levels, and improving survival rate during initial treatment, PCa recurs after a median duration of response for 12 - 24 months and gradually develops into castration resistant prostate cancer (CRPC).\textsuperscript{53b}

Many studies have suggested that CRPC cells express mutated AR, which exhibited enhancement in both gene expression level and functional sensitivity. In clinical settings, AR amplification, promiscuity, and splice variant isoforms were more frequently observed in PCa previously treated with ADT, as compared to primary PCa without any treatment.\textsuperscript{62, 66} Therefore, it is generally believed that most CRPC cases are not truly hormone refractory, in which AR transcription is aberrantly reactivated despite low serum level of androgen after castration.\textsuperscript{62, 64} Conversely, mutations involving the AR gene were found in up to around 44\% of CRPC cases.\textsuperscript{67} Moreover, hundreds of types of mutations in the AR have been identified, though 90\% are non-sense and missense mutations.\textsuperscript{68} AR mutations mainly occur in the LBD and NTD, with only 7\% mutations in the DBD and 2\% in the hinge region. Mutations in the LBD, which were demonstrated to increase the sensitivity and decrease the specificity of the ligand binding, are clinically significant.\textsuperscript{53b, 63a, 69}

\subsection{Molecular Mechanisms of Prostate Cancer}

Since Huggins and Hodges’ initial discovery, research efforts to elucidate the molecular basis of prostate cancer has been at the forefront of cancer research. A number of mouse models (PTEN/-/- \textsuperscript{70}, Myc hi/ lo \textsuperscript{71}, Nkx3.1/-/- \textsuperscript{72}, ID4/-/- \textsuperscript{27} and reviewed in \textsuperscript{73})
have been developed that recapitulate several aspects of human prostate cancer initiation and progression. Most studies have centrally focused toward the role of androgen receptor (AR) in prostate cancer. As aforementioned AR, a member of the nuclear receptor superfamily, is required for normal prostate function and is involved in cytodifferentiation of the prostate epithelial cells. In a majority of prostate cancer cases, AR function contributes to the survival and proliferation of cancer cells in primary disease and in most cases the presence of AR continues to be indispensable after progression to hormone independent disease. However, the prostate epithelial specific AR knockout mouse also develops prostate cancer, suggesting that AR function is not absolutely required for disease development. Also, unlike other cancers (most notably breast and colorectal) where a familial mutation in a single gene leads to a higher risk of developing the respective cancers, no such major risk allele has been identified for prostate cancer to date. The heterogeneity of prostate cancer suggests that there could be multiple initiating events leading to inactivation of tumor suppressors and/or activation of tumor promoters/oncogenes that could at some point of disease progression cross-talk with AR. These early or late events may promote the transition of androgen receptor from a tumor suppressor to an oncogene.

2.1.3 Genetic Disposition in Prostate Cancer Progression

Genetic disposition has been shown to play a pivotal role in prostate cancer progression since studies have shown a significant amount of prostate cancer cases are considered to be hereditary. Polymorphisms have been implicated in prostate cancer progression and have been central to studies in an effort to link genetics to disease.
Polymorphisms are DNA sequence variation in genes that are associated with hormone response, DNA repair, cell protection, and nucleotide metabolism. 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.\textsuperscript{80}

The heterogeneity of prostate cancer has led to the investigation of inactivation of critical 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.\textsuperscript{81,82} Chromosomal alterations and point mutations are responsible for functional inactivation of p53 in prostate cancer.\textsuperscript{83} Point mutations have also been reported for PTEN that result in transcriptional reduction of expression.\textsuperscript{82} 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.\textsuperscript{84}

2.2 Tumor Suppressor p53 “Guardian of the Genome”

The tumor suppressor protein p53 remains one of the most intensively studied genes in cancer biology and therapy.\textsuperscript{85} It was regarded as an oncogene for 10 years after it was discovered more than twenty years ago.\textsuperscript{86} Subsequent studies revealed that p53 (encoded by the human gene TP53), in fact was a tumor suppressor, which binds to a specific DNA sequence and transactivates target genes leading to cell cycle arrest and/or apoptosis.\textsuperscript{85} p53 is a stress response protein that functions primarily as a tetrameric
transcription factor, which regulates a large number of genes in response to a variety of cellular insults, including oncogene activation and DNA damage. These signals activate p53 primarily through post-translational modifications that result in augmented p53 protein level and transactivation activity.

2.2.1 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. 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, 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. Increased expression of GADD45 results in p53-driven G2 cell cycle arrest. This occurs through the binding of GADD45 to CDC2, which prevents cyclin B/CDC2 complex formation and inhibits kinase activity.

2.2.2 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. 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).\textsuperscript{93}

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.\textsuperscript{94} p53 signals the inhibition of the production of IAPs (inhibitor of apoptosis proteins), which controls apoptosis.\textsuperscript{94} Alternatively, the intrinsic pathway is the main pathway activated in p53 dependent apoptosis, whereas extrinsic pathway is used to supplement the apoptotic response.\textsuperscript{95} The intrinsic pathway is the mitochondrial pathway, and is activated when cells undergo stress. This pathway is regulated by the Bcl-2 family proteins.\textsuperscript{96} 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 p53aIP1 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.\textsuperscript{97} Loss of Bax is responsible for nearly 50\% of accelerated tumor growth in brain tumors.\textsuperscript{98} BAX is also required for p53-dependent apoptosis in colorectal cancer cells, but is dispensable in apoptosis of thymocytes and intestinal epithelial cells.\textsuperscript{93} 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 \(^9\) (Figure 4).

**Figure 4.** 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. \(^1\)

### 2.2.3 Acetylation of p53

Acetylation has many important effects on p53. It increases p53 protein stability, binding to low affinity promoters, association with other proteins, antiviral activities, and is required for its checkpoint responses to DNA damage and activated oncogenes.\(^2\) Six acetyltransferases have been identified that modify p53 at lysines predominantly in the C-terminus or its central DNA binding domain. Acetylation of p53 directly affects its transcriptional activity by opening up its normally closed conformation or by altering its binding to certain response elements in gene targets (Figure 5). In general, these modifications are mediated by two different groupings of acetyltransferases, p300/CBP/PCAF or Tip60/MOF/MOZ. There appears to be significant redundancy in
sites of p53 acetylation since loss of one or more sites, including all seven C-terminal lysines in mouse p53, can be largely compensated for by acetylation of remaining lysines. However, combined loss of eight major acetylation sites in human p53 (8KR mutant altered at K120, 164, 370, 372, 373, 381, 382 and 386) renders p53 transcriptionally inert and prevents its induction of cell cycle arrest and/or apoptosis. Conversely, in many cell types the inhibition of histone deacetylases (HDACs) that remove acetyl groups from p53 (i.e., HDAC1 and SIRT1) causes increased p53 acetylation and p53-dependent activation of apoptosis and senescence. Together, these findings suggested that acetylation is an essential regulator of the anti-cancer functions of p53.

Figure 5. The acetylation of p53 stimulates its sequence-specific DNA-binding activity. Acetylation of lysine residues in C-terminal region results in neutralization of positive charges and leads to the disruption of interactions between the C-terminal domain and the core domain. DNA-binding domain adopts an active conformation and binds to target DNA.
2.3 p53 and Cancer

2.3.1 p53 Mutations

p53 function has been found to be compromised in tumor cells, usually as a result of somatic mutations, which occur in approximately half of all human cancers and constitute a cornerstone in tumorigenesis. In principle, emergence of a p53 mutation within a cell might have three, not mutually exclusive, types of outcomes. First, such mutation is expected to abrogate the tumor suppressor function of the affected TP53 allele, reducing the overall capacity of the cell to mount a proper p53 response; if both alleles eventually become mutated, or if the remaining allele is lost, such cells will be totally deprived of anticancer protection by p53. Second, many common mutant p53 isoforms can exert dominant–negative effects over coexpressed wild type p53, largely by forming mixed tetramers that are incapable of DNA binding and transactivation. Hence, even if one wild-type allele is retained, the cell may be rendered practically devoid of wild type p53 function through such mechanism, particularly if the mutant protein is expressed in excess over its wild type counterpart. Third, and most relevant, the emergent mutant p53 protein possess activities of its own, often not present in the original wild type p53 protein, which can actively contribute to various aspects of tumor progression. These new abilities attributed to mutant p53 are commonly referred to as gain-of-function (GOF).
2.3.2 Gain-of-Function Mutant p53

The concept of mutp53 GOF was formally introduced in the early 1990s, when it was shown that mutp53 isoforms of both human and mouse origin, but not wild type p53, could transform p53-null cells and give them the ability to form colonies in soft agar in vitro and tumors in mice. Moreover, the most convincing support for the gain-of-function hypothesis came from mice engineered to harbor some of the most frequently occurring tumor-associated p53 mutations. In comparison to heterozygous or null (p53+/− or p53−/−) mice, animals with one mutant allele show a different and broader tumor spectrum — with the appearance of more carcinomas and sarcomas, in addition to lymphomas. Interestingly, these mutant p53 driven cancers play a pivotal role in tumor progression and also aided in genomic instability. A whole host of oncogenic functions of mutant p53 has been characterized in cell culture models, including: an ability to promote invasion, migration, angiogenesis, stem cell expansion, survival, proliferation and tissue remodeling. These evidences has aided in the theory the mutant p53 has a wide range of response and acts in several different pathways to effect tumor progression.

2.4 Mechanisms of Mutant p53 Function

There is much discussion of the molecular mechanism by which mutant p53 may function, notably there have been numerous characterizations of pathways mutant p53 may employ. These molecular mechanisms as laid out by Muller et al., 2013, consist of alterations in the DNA-binding ability of mutant p53, changes in the interaction of mutant
p53 with other proteins, including other transcription factors, or proteins not directly related to the regulation of gene expression.⁴

**2.4.1 Mutant p53 Binds to DNA to Alter Gene Expression**

Although, two thirds of missense mutations in the DBD, including all hotspot mutants, abrogate the ability of p53 to activate target genes, modulation of gene transcription by mutant p53 is well documented as an important gain-of-function mechanism.¹⁰⁵ Tumor derived mutant p53 proteins have often been seen to retain the N-terminal transcriptional transactivation domains and most mutant p53 function has been attributed to direct or indirect abilities to regulate gene expression.¹⁰⁶ Most amino acid substitutions occur within the DNA-binding domain of the majority of tumor-derived p53 mutations may change, rather than completely abolish, sequence-specific DNA binding. Mutant p53 has been shown to have DNA binding ability, though most cases show that in mutant form, p53 is unable to bind its wild-type response element. Mutant p53 has been shown to bind to other parts of DNA such as the nuclear matrix, providing an alternate mechanism to regulate gene expression.⁴

**2.4.2 Mutant p53 Binds to Transcription Factors**

Another well established mechanism for gene specific transcriptional regulation is the interaction of mutant p53 with sequence-specific transcription factors, which result in either augmentation or attenuation of their activity. Besides p63 and p73, which are well characterized transcription factors that interact with mutant p53, SP1 and ETS1 also interact with wild-type p53.¹⁰⁷ Interestingly, the effects of mutant and wild-type p53 are antagonistic, suggesting that additional but distinct co-factors are recruited by mutant p53
and wild-type p53. The best-described transcriptional functions of mutant p53 relate to its ability to interact with other transcription factors and modulate the expression of their target genes. In some cases, mutant p53 increases the activity of the transcription factor partner, with further complexity added by the role of cellular stimuli, transcriptional cofactors and other proteins.

2.4.3 Mutant p53 Interacts with Proteins to Change their Function

The interaction of mutant p53 with transcription factors can also be inhibitory. Probably the best understood of these involve these interactions. For example, studies have previously shown that the anti-proliferative effect of TGF-β is facilitated by the presence of wtp53, elucidating that these two pivotal signaling pathways are necessary in suppression of early stages of tumor progression. Moreover, current investigation of cross-talk and mutant p53 have shown a fascinating correlation as well. Intriguingly, mutant p53 can repress the expression of TGF-β receptor type II, thus attenuating TGF-β-mediated signaling. Also, in response to TGF-β treatment, SMAD2 promotes the complex between mutant p53 and p63, leading to the inhibition of p63-driven gene expression.

Although there has been much focus on the role of mutant p53 in affecting transcriptional programs, mutant p53 also binds and modulates the function of proteins that are not directly involved in transcription. For example, by interacting with MRE11, a DNA nuclease required for DNA repair, mutant p53 prevents the MRE11–RAD50–NSB1 complex from phosphorylating ATM, leading to impaired homologous recombination. An interesting example of a protein whose function is enhanced by mutant p53
binding is topoisomerase 1 (Top1), which maintains topology of DNA. Whereas wild-type p53 both promotes and counteracts Top1 function, mutant p53 has specifically lost the negative regulation of Top1, resulting in hyper-recombination and genomic instability.\textsuperscript{114}

A major hallmark of cancer progression is a gradual increase in genome instability, manifested all the way from higher mutation rates to gross aberrations in chromosome number and structure. A link between mutant p53 and increased genomic instability was clearly demonstrated by showing that human mutant p53 can disrupt normal spindle checkpoint control, leading to accumulation of cells with polyploidy genomes.\textsuperscript{115}

\textbf{2.4.4 Stability of Mutant p53}

A major phenomenon of mutant p53’s ability to endow the cellular environment with a whole host of new abilities lies within its increased stability as compared to its wild type counterpart, which is quickly degraded. So, how does mutant p53 gain its newfound stability? The degradation of wild-type p53 is achieved through a multitude of mechanisms, involving several E3 ubiquitin ligases that target p53 for ubiquitination and subsequent proteasomal degradation.\textsuperscript{116} Of those, the best studied and probably the most important driver of p53 degradation is MDM2, an E3 ligase and also plays a role in repressing p53 mRNA translation. Escape from MDM2-mediated degradation is therefore an appealing mechanism for mutant p53 stabilization,\textsuperscript{117} however; mutant p53 is still susceptible to MDM2-mediated degradation.\textsuperscript{117b, 118}

The simplest explanation for mutant p53 stabilization draws on the fact that
cancer-associated mutp53 isoforms lack the ability to transactivate wild type p53 target genes. The MDM2 gene is a classical positive transcriptional target of wild type p53, and this drives a negative feedback loop that helps maintain wild type p53 levels very low in unstressed cells. However, mutant p53 fails to transactivate the MDM2 gene. Hence, MDM2 protein levels are likely to be rather low in cells that express only mutant p53.  

2.4.5 Animal Models for Mutant p53 GOF  

Increasingly animal models are playing a larger role in demonstrating mutant p53 GOF capabilities and giving insight to the biological significance of these newfound abilities. Several studies have also shown that several mutants often found in various cancers known, often referred to as hotspot mutants were also shown to enhance tumor formation in p53-null fibroblasts and also in human p53-null osteosarcoma cells 104, 119, establishing that mutant p53 indeed has GOF.

2.4.6 Mutant p53: Different in the Same Way?  

Similarly, most studies indicate that cancer-associated mutant p53 has gain-of-function activities that aid the invasiveness and metastatic properties of the cell. The gain-of-function activities of mutant p53 in cancer models, poses the question are all p53 mutations created equal and why are there so many? For example, Gurova et al.120 tested this paradigm when they used p53 mutants found in prostate cancer cell line, DU145 that has two different p53 mutations P223L and V274F. They used a p53-null cell line (PC3) to study both mutants individually and when coexpressed. Gurova et al.120 found that these mutants are different from wild-type p53 protein in at least some of their properties. Although p53–274Phe has more features of a “real” cancer-derived mutant (maintains the
denatured conformation of the DNA-binding domain, lacks transactivation ability) than the p53–223Leu one (wild-type conformation, transactivation ability), they both showed some properties that are unusual for tumor-derived mutants, and they more closely resemble wild-type p53 (can be stabilized by DNA damage, cause growth suppression in some cell types, do not possess dominant-negative activity). However, these relatively “weak” mutants create p53 protein with new structural and functional properties when coexpressed in one cell. Whereas when both mutants were coexpressed they were seen to serve a similar function as in DU145 cells.\textsuperscript{120}

Interestingly, studies have shown that the p53 mutations are indeed not all functionally equivalent.\textsuperscript{4} It is widely been reported that mutations in the DNA-contacting residues of p53 have a less dramatic effect on the folding of the p53 protein than the structural mutants.\textsuperscript{14} The native and denatured forms of p53 can be differentiated using conformation-specific antibodies, suggesting that the structural switch could reveal or obscure epitopes for binding to different partner proteins.\textsuperscript{5} On the other hand, mutant p53 can engage in protein–protein interactions with a growing number of transcription factors, often being recruited to binding sites of those factors on chromatin, and modulate their transcriptional output both positively and negatively.\textsuperscript{15} Interaction studies of mutant p53 with p63 or p73 \textsuperscript{107-108, 121} have shown that the structural mutants bind p63 or p73 with a much higher affinity than the contact mutants, although both groups of mutations have the ability of inhibiting p63 and/or p73 to promote invasion and metastasis or to prevent apoptosis.\textsuperscript{112, 121-122}
2.5 Therapeutic Applications of p53

Since p53 mutation, as well as wild-type p53 impairment is widely observed in human cancers, restoration of p53 function in tumors has been pursued as a promising strategy for cancer therapy.\textsuperscript{123} Also, it is also important to know whether tumors with different types of p53 mutations require other therapeutic approaches. The presence of a mutant form of the tumor suppressor gene p53 has the potential to disrupt the apoptosis pathway and cell cycle arrest after DNA damage, which results in increased radiation resistance and cell survival.\textsuperscript{4} Promising studies using wild-type p53 adenoviral or retroviral gene transfer in tumors are ongoing. Overexpression of p53 has been shown to be sufficient to induce apoptosis or sensitize the tumor cells to radiotherapy or chemotherapy in most cancer cells with reduced efficacy in tumor cells containing wild-type p53.\textsuperscript{124} However, because gain-of-function mutants exert additional oncogenic functions, wild-type p53 gene transfer might not work in these tumors, and the malignant phenotype may be maintained. p53 antisense therapy\textsuperscript{125} that blocks the function of the p53 mutant might be more useful for tumors with gain-of-function mutants. Other promising therapies that might interfere with the different properties of the gain-of-function mutants are structure-based rescue therapies.\textsuperscript{126} Since, mutant p53 proteins are highly expressed in many cancers, making them extremely attractive targets for therapy. Current strategies have focused on destabilization or inactivation of mutant p53, or reactivation of wild-type function in the mutant p53 protein. The latter strategy is difficult
to achieve, but has gained great traction recently with current research using mouse models showing that the activation of wild-type p53 is established tumors can lead to efficient tumor regression.\textsuperscript{4}

2.6 ID4-p53 Cross-talk

The molecular mechanism by which mutant p53 may function have been reviewed extensively.\textsuperscript{4} These pathways include alterations in the DNA-binding ability of mutant p53, interaction of mutant p53 with other proteins, including transcription factors or proteins not directly related to the regulation of gene expression. It is clear that the effects of mutant p53 are strongly context dependent, and interactions that promote activity in some circumstances may be inhibitory in others.

Fontemaggi and colleagues have extensively characterized the molecular interaction between ID4 and mutant p53 in promoting neo-angiogenesis in breast cancer.\textsuperscript{127} In breast cancer cell lines ID4 is a transcriptional target of gain-of-function p53 mutants R175H and R280K in SKBr3 and MDA-MB-231 but not in MCF7 breast cancer cells expressing wild type p53. The protein complex involving mutant p53–E2F1-p65 assembles on specific regions of the ID4 promoter and positively controls ID4 expression.\textsuperscript{127} The study further demonstrated that mutant p53 R273H had the highest regulatory control over ID4 expression.\textsuperscript{127} Overexpression of ID4 in SKBr3 cells, stabilized the mRNA of pro-angiogenic factors IL8 and GRO-\textsuperscript{3,4}which resulted in the increased secretion of the respective proteins. ID4 expression was also increased in response to Adriamycin and Cisplatin treatments on mutant p53/ID4 expressing cell lines. Studies
involving the knockdown of mutant p53 abrogated this response, indicating that in response to DNA damage mutant p53 in complex with E2F1 and p300 bind to the CDE element closest to the transcriptional start site, on the ID4 promoter, to achieve “mutant p53 associated” chemo-resistance. Thus, direct interaction between mutant p53 and E2F1 on the ID4 promoter supports the oncogenic functions of mutant p53 in at least breast cancer.

ID4 functions as a tumor suppressor in ER+ve breast tumors where it is frequently inactivated by promoter hypermethylation; however, ID4 displays oncogenic activity in ER-ve breast cancer cells SKBr3 and MDA-MB-237 that express mutant p53. These results suggest that ER may have a role in regulating ID4 dependent expression of mutant p53. The corresponding estrogen receptor levels in the meta-analyses discussed above were not reported but could determine the outcome of the ID4-mutant p53 cross-talk. These conflicting results between cell lines and clinical studies suggest that the ID4 regulatory mechanism could be controlled by a complex mechanism that still remains to be investigated.

2.7 Molecular Dynamic Studies of p53

More than 80% of missense mutations in p53 found in human cancers are located in the core DNA domain. Many of these mutations disrupt DNA binding directly or reduce the folding or stability of the core domain suggesting that DNA binding is critical to p53 function. Damaged cells harboring p53 mutants that abrogate wild-type p53 functionality will begin and continue uncontrolled cellular division and often equip the cell with a host of new abilities (gain-of-function) aiding in adverse conditions in the
The concept of mutant p53 gain of function was formally introduced in the early 1990s, when it was shown that mutant p53 isoforms of both human and mouse origin, but not wild-type p53, could transform p53-null cells and give them the ability to form colonies in soft agar in vitro and tumors in mice. Moreover, the most convincing support for the gain-of-function hypothesis came from mice engineered to harbor some of the most frequently occurring tumor-associated p53 mutations.

The structure of the p53 core domain, commonly associated p53 mutations and their complex with DNA are currently under intense investigation. However, studies of the core domain of wild-type p53 have revealed the details of protein folding, DNA recognition, and given some insight into potential mechanisms of some cancer-associated mutations. Some of these missense mutations are temperature sensitive e.g. the P223L and V274F in DU145 prostate cancer cells that retain transactivation functions at lower temperatures (32C), while other mutations result in complete loss of activity (gain of function/ loss of transactivation). Extensive structural studies suggest that the temperature sensitive mutants and/or other mutants promote near wild type structure of p53 under certain conditions. Protein-protein interaction between wild type and mutant p53 core domains in higher-order p53 complexes has also been a subject of experimental and modeling studies. Based on these results, we hypothesized that theoretical modeling of p53 and its mutants could provide a parameter that can link the type of mutation with p53 structure and possibly function. These structural simulation parameters of various known p53 mutations could be used to establish an association between cancer prognosis, response to treatment and more importantly develop strategies to recover the
structure of mutant p53 to simulate as a wild type protein. Studies have suggested that common p53 cancer mutants exhibit a variety of distinct local structural changes while the overall structural scaffold remains largely preserved. Thus, a focus on the structural changes associated with the local core DNA binding domain may be more informative in terms associating the severity of mutants with function.

Modeling studies via computational methods, including molecular docking, molecular dynamics (MD) simulation and binding free energy prediction are usually applied to supplement the experimental studies in order to understand the mechanism at an atomic level or to improve the efficiency of molecular design or discovery. Studies including theoretical modeling of p53, and the p53 conformation changes induced by protein-protein and protein-ligand interactions, including peptide and small molecular inhibitors have significantly expanded our current understanding of the dynamic nature of p53 structure. While there have been several studies discussing the dynamic simulations of p53 in recent years, the dynamics and flexibility of mutation induced conformational changes of p53 have not been completely characterized or understood. Additionally, the dynamics characteristic of other domains of p53 has not been fully studied. In this study, we aim to understand via computational analysis of p53 structural models the energy differences of wild-type and mutant forms of p53. Moreover, this study will aid in providing better understanding of how disparate energy, temperature sensitivity, and missense mutations differences amongst different forms of mutant p53 compared to wild-type affect the disease state of cancer.
The molecular dynamics study presented herein was inspired by many experimental observations that focused on the restoration of mutant p53 due to the intrinsic flexibility of its domain structure. Studies have shown that certain p53 mutants can be rescued via temperature (temperature sensitive mutants P223L and V274F), and artificial means such as small molecules and short peptides (reviewed in 135). Aforementioned studies in our laboratory revealed that mutant p53 could be restored to function as wild-type via physiological means through acetylation of lysine residues (K373). Thermodynamic studies revealed that the R248Q mutant is stable at sub-physiological low temperatures. The R248Q stability was less than that of the wild-type protein by ~2 kcal/mol. The mutant structure also retained a two-stage unfolding transition, similar to the wild-type protein 137, which indicated well-defined structures at low temperature. PRIMA-1 (p53-reactivation and induction of massive apoptosis-1, APR-017, 2,2-bis(hydroxymethyl)-1-azabicyclo [2,2,2]octan -3-one)138 and its methylated form, RRIMA-1MET (APR-246)139 was shown to restore sequence-specific DNA binding region of R273H and R175H by forming adducts with thiols in mutant p53 and activating several p53 target genes and promoting apoptosis in human cancer cells.12 Thus, thermodynamic stability calculated exclusively through global energy minimization simulations could provide an insight into the structural flexibility of mutant p53. Our long-term goal is to use these simulations to discover mutants that could be 1) sensitive to restoration of mutant p53 biological activity and 2) severity of the mutant that can subsequently be used to predict prognosis.
2.8 Conclusion

In conclusion, 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.

2.9 ID4 Function, Sequence and Structural Properties

The origins of ID proteins can be traced back to the D. melanogaster extramacrochaetae (emc) gene (reviewed in 21b). As orthologs of emc, the four ID proteins are paralogs that appeared through gene duplication in vertebrates.

ID4 only shares the core HLH domain but divergent N- and C-terminal domains compared with other ID proteins (Figure 6). Therefore, ID4 can be considered as a remote homologue of ID1, 2 and 3. ID4 is also the longest protein within the ID family with 161 residues (Figure 6). The sequence divergence of ID4 at the N-terminal (Alanine rich) and C-terminal (Proline rich) (Figure 6) could allow distinct ID4 functions as opposed to the other ID protein family members.

Figure 6. The red lines indicate the alanine and proline rich regions at the N- and C-terminal of ID4. The conserved helix–loop–helix region and the amino acids that are unique to ID4 HLH domain is indicated (asterisk).
The poly-alanine rich N-terminal tract in ID4 appears to be rapidly evolving. Similar to the poly-alanine tracts of HOXA13 and class III POU transcription factors that appeared only in mammals, the poly-alanine tract in ID4 also first appeared in primitive mammals such as opossum (marsupials) but is absent in lower vertebrates such as alligators, xenopus, zebra fish and birds (Gallus, Finch). Thus, the poly-alanine tract in ID4 appeared independently in paralogs, hence conserved for functional reasons. The poly-alanine tract in ID4 is consistent with similar tracts which are preferentially found in many transcription factors and lies outside, usually towards the N-terminal of the functional domains (such as N-terminal to HLH domain in ID4). This tract generally acts as a flexible spacer element located between the functional domain of a protein and therefore essential to protein conformation, protein–protein interactions and/or DNA binding. The length of the poly-alanine tract in ID4 (n=10) is also within threshold for normal function whereas an increase in the length beyond this threshold results in human diseases. In fact, structural studies suggest that none of the N- and C- terminal fragments of any of the ID proteins adopts a helical formation, except the N-terminal 27-64 fragment of ID4, a motif that is dictated by the Alanine residues 39 and 48 (Figure 5). Thus the alanine rich N-terminal domain could be a functionally important domain in ID4. In this context, the unique function of ID4 suggests the potential for positive selective pressure for developing and maintaining the divergent ID4 locus during evolution.
The over-representation of proline within the C-terminal end may also impart additional structural and functional features unique to ID4. Proline is a strong promoter of intrinsic disorder\textsuperscript{144} and thus likely supports the internal disorder at the ID4 C-terminal domain. Indeed, the overall percent disorder in ID4 is highest among all the ID proteins: ID4-70.2%, ID2-59.7%, ID3-35.29% and ID1-28.6%. The low complexity proline rich region in ID4 suggest that ID4 like other internally disordered proteins also lacks a defined 3-D structure at the C-terminal that favors protein-protein interactions by presenting a larger interaction surface allowing multiple binding partners.\textsuperscript{145} Thus, structural divergence through acquisition of new functionally relevant domains in ID4 suggests a potential novel role in development and differentiation as compared to IDs 1, 2 and 3.

2.10 The Role of ID4 During Development and Differentiation

ID proteins are expressed by essentially all cell lineages at some point during embryonic development. Consensus suggests that ID expression is highest in undifferentiated, proliferating populations that is subsequently down-regulated as primitive cells exit from cell cycle and terminally differentiate.\textsuperscript{19c}

By in situ hybridization on mouse development post-gastrulation, ID1, 2 and 3 expression is observed in multiple tissues whereas the expression of ID4 is non-overlapping and restricted to neuronal tissues and in the ventral portion of the epithelium in developing stomach.\textsuperscript{146} In adult human tissues, ID4 expression is observed in brain, thyroid, testis and pancreas.\textsuperscript{147} ID4 is also required for normal mammary\textsuperscript{148} and prostate gland development.\textsuperscript{27}
ID4 is highly expressed in osteoblasts, adipocytes, prostate epithelial cells, neurons, testicular Sertoli cells and during differentiation in glial cells supporting its role as a pro-differentiation factor. ID4 is also expressed in germ cells at various stages of development: spermatogonial stem cells, spermatocytes, pachytene spermatocytes and spermatids. ID4 is also required to maintain spermatogonial stem cell renewal.

Unexpectedly, overexpression of ID4 in oligodendrocyte progenitors cells (OPC) prevents differentiation, associated with a decrease in the endogenous expression of all myelin genes. Conversely, OPCs lacking ID4 display precocious differentiation and increased apoptosis suggesting that ID4 is required for the development of oligodendrocytes. A progressive decline in ID4 transcription is also a part of the intracellular timer that helps determine when oligodendrocyte precursor cells withdraw from the cell cycle and differentiate. This unique phenotype supports the role of ID4 as inhibitor of differentiation, the classical function of ID proteins.

Immuno-cytochemical studies have shown that ID4 is localized to the nucleus in OPCs and spermatids, but remains cytoplasmic in spermatocytes.

Collectively, the studies suggest that ID4 can act as pro- or anti-differentiation factor in a cell specific manner. The integration of various cellular events such as response to specific ligands and possibly cell specific interacting proteins could eventually determine whether ID4 regulates proliferation and/or differentiation and cellular localization.
2.11 ID4 Knockout Mouse Models

2.11.1 Global ID4 Knockout Model

Two different global ID4 knockout (ID4-/-) mouse models exist. In the first ID4-/- model (strain 129X1/SvJ, developed by Mark Israel’s group (ID4<sup>tm1Mais</sup> in MGI database), exons 1 and 2 were replaced by GFP and neomycin-resistance genes via homologous recombination in JM-1 ES line. The second ID4-/- model (strain 129P2/OlaHsd) was developed by Fred Sablitzky’s group (ID4<sup>tm1Fsky</sup> in MGI database). In this model the sequence encoding HLH domain and most of the C-terminus of the gene was replaced lacZ-neo cassette. The cassette was inserted in-frame, allowing the expression of a fusion gene encoding the N-terminal 65 amino acids of ID4 fused to beta-galactosidase.

Both the ID4-/- mouse models displayed essentially similar phenotypes in the brain (decreased brain size, abnormal fat cell morphology) and mammary gland. The ID4<sup>tm1Fsky</sup> model appears to have a more severe phenotype as a result of associated embryonic lethality (50% die in uteri or neonatally). The surviving homozygous mutant mice lose weight rapidly probably due to a defect in abnormal adipose tissue development and osteoporosis with only 20% surviving through adulthood. A similar phenotype in the ID4<sup>tm1Mais</sup> model was not reported.

2.11.2 ID4 and Neuronal Development

During development, depletion of ID4 in mice also revealed its essential role in normal brain development and function where it is required for: neural stem cell proliferation and differentiation, lateral expansion of the proliferative zone in the
developing cortex and hippocampus and proliferation in the ventricular zones.\(^{151, 158}\) In the absence of ID4, neural precursor cells proliferate more slowly than their wild type counterpart highlighting the essential regulatory role of ID4 during neural stem cell proliferation and fate determination. Though not investigated, but the cognitive abilities of ID4\(-/-\) mice could provide some interesting clinical correlates.

2.11.3 ID4 and Mammary Gland Morphogenesis

In mouse mammary gland, \textit{ID4} is expressed in cap cells, basal cells and in a subset of luminal epithelial cells where it promotes ductal elongation and branching morphogenesis.\(^{148}\) Targeted ID4 deletion impairs ductal expansion and branching morphogenesis as well as cell proliferation induced by estrogen and/or progesterone. ID4 also maintained the survival of normal mammary cells as well as cultured mammary tumor cells. These normal and tumorigenic functions of ID4 were primarily achieved by suppressing p38MAPK activity.\(^ {148}\)

2.11.4 ID4 and Spermatogenesis

In mice lacking ID4 expression, quantitatively normal spermatogenesis was found to be impaired due to progressive loss of the undifferentiated spermatogonial population during adulthood. ID4 expression was observed in spermatogonial stem cells (SSCs) in the mouse germline where it was shown to regulate self-renewal. Specifically, ID4 is expressed by a sub-population of type A single spermatogonia in mouse male germ line.\(^ {154}\) GDNF, a key growth factor driving self-renewal ID4 expression was found to up-regulate ID4 expression in isolated SSC-enriched fractions.\(^ {154}\)
2.11.5 ID4 and Osteoporosis

A drastic reduction in osteoblast differentiation with a corresponding increase in differentiation toward adipocytes was observed in ID4-/− mice. ID4 promotes osteoblast differentiation by releasing Hes1 from Hes1-Hey2 complexes. Subsequently, Hes1 increases the stability and transcriptional activity of Runx2, a key molecule of osteoblast differentiation, which results in an enhanced osteoblast-specific gene expression.149

2.11.6 ID4 and Prostate

A large cohort of studies demonstrating a strong association between loss of ID4 expression in many cancers suggest that ID4-/− mouse could present with multiple cancers at some point in the lifetime. Till date, the cancer phenotype has been well established only in the prostate of ID4-/− mice. ID4 is highly expressed in wild type mouse prostate as well as normal human prostate. We have shown that ID4 is a key regulator in the normal development of various androgen receptor organs of the male genital tract in general and specifically of the prostate. Prostate from ID4-/− mice have smaller size, decreased branching morphogenesis and decreased differentiated luminal cells as determined by almost complete loss of NKX3.1 expression, a marker of differentiated luminal epithelial cells.27 A similar defect in branching morphogenesis in the developing mammary gland of ID4-/−148 therefore appears to be a consistent mechanism. The presence of PIN lesions, the earliest stage of prostate cancer27 in 6 weeks old ID4-/− mouse prostate suggested that loss of ID4 may be an initiating event in prostate cancer development. ID4 had no effect on androgen receptor expression as well as its translocation to the nucleus. Interestingly, the expression of androgen dependent
genes such as the homeobox NKX3.1 decreased, that was in part due loss of AR binding to its respective response element. In contrast, the expression of other AR target genes such as probasin and Myc increased suggesting that ID4 may differentially regulate AR binding to its corresponding binding site. Furthermore, the expression Pten a known tumor suppressor is also decreased in ID4-/- mice.27

Clearly, tissue specific ID4 knockouts need to be developed in order to fully understand its role in development, differentiation and disease. The existing global ID4 knockouts should also be thoroughly analyzed since some of the effects could be subtle and may not result in easily identifiable large scale developmental phenotypes such as those observed in brain and reproductive tracts.

2.12 ID4 and Cancer

ID4 appears to act primarily as a tumor suppressor in most cancers as opposed to ID1, ID2 and ID3, which in most cases acts as tumor-promoters or supporting oncogenes.17 In a small subset of cancers, ID4 also acts as a tumor promoter (Figure 1). Beyond correlative studies showing ID4 expression in tumors, the underlying molecular mechanism remains largely unexplored.

2.12.1 ID4 as a Tumor Promoter

The role of ID4 as a tumor promoter is based on the evidence (experimental and meta-analysis) that increased ID4 expression is observed in a small subset of cancers (Figure 1).
2.12.2 Breast Cancer

A clear picture relating ID4 expression with breast cancer has not emerged. Epigenetic silencing of ID4 is observed in Columnar Cell lesions (CCL) and Ductal Carcinoma In situ (DCIS)/invasive carcinoma and majority of sporadic breast cancers. ID4 expression is also absent in ER positive atypical ductal hyperplasia, ductal carcinoma in situ and invasive carcinomas but present in normal ER negative mammary epithelial cells suggesting that ER could negatively regulate ID4. Increased ID4 expression is observed in basal cell-like breast cancer, triple negative breast cancer (TNBC) (including 4T1 mouse mammary cancer cell line) but not in non-TNBC. Functional studies demonstrated that ID4 down-regulates BRCA1 and allows for anchorage-independent growth of breast (SKBr3) and ovarian (PA-1) cancer cells which are ER-ve. However, another study demonstrated that BRCA1 up-regulates ID4 that correlates with increased BRCA1 and ID4 expression in normal breast and ovarian tissues. In meta-analysis, no significant association was found between ID4 and breast cancer.

The ID4 gene expression profile in The Cancer Genome Atlas (TCGA) dataset is consistent with results discussed above. ID4 is highly expressed in the normal breast tissue and in Basal cell like cancers (ER-ve). Interestingly, ID4 expression was found to be lower in Luminal B type ER+ve cancers (Figure 7).
Figure 7. ID4 expression profile from The Cancer Genome Atlas database. The respective cancers (A; breast cancer, B: GBM and C: prostate cancer), the gene expression analysis platform and number of samples (N) used for analysis are indicated above each panel. The cluster is represented by decreased (green), increased (red) or no change (black) in ID4 expression. The bars toward the right of each cluster represent sample type and sub-type. The PAM50 array represents major molecular subtypes in breast cancer. The estrogen receptor status (ER) is indicated by + (present) and − (absent). ANP: adjacent normal prostate, PCA: prostate cancer.

2.12.3 Other Cancers

ID4 expression is observed in Ovarian Cancer, only 32% of high grade ovarian cancer. ID4 is also highly expressed in Glioblastoma. In GBM, high ID4 expression was also observed in TCGA data sets, specifically in classical, neuronal and pro-neural types (Figure 7).
2.12.4 ID4 DNA Alterations

ID4, located in a 4 Mb region of the chromosome 6p22.3, is consistently amplified in bladder cancers with a positive outcome.\textsuperscript{169} On the contrary, genomic loss of ID4 is observed in Hodgkin’s Lymphoma.\textsuperscript{170}

Cytogenetic abnormalities resulting from chromosomal 6 translocations are also very common in hematologic malignancies. ID4 was demonstrated in one case of B-cell lineage acute lymphoblastic leukemia (ALL) with t(6;14) (p22;q32)\textsuperscript{171} indicating that ID4 may act as an oncogene in some human leukemia cases through its ability to sequester specific B-cell transcription factors. In general, ID4 expression is down-regulated in ALL\textsuperscript{33}, hence the translocation observed in one clinical case could be an isolated incidence in the backdrop of other genomic alterations.

2.12.5 ID4 as a Tumor Suppressor

Epigenetic silencing of ID4 in many cancers has prompted investigators to classify it as a tumor suppressor.\textsuperscript{30, 31c, 41, 161, 170, 172} (Figure 4) ID4 is epigenetically silenced in: Leukemia,\textsuperscript{30} AML,\textsuperscript{173} CLL,\textsuperscript{32} ALL,\textsuperscript{33} glioblastoma,\textsuperscript{34} squamous cell carcinoma,\textsuperscript{35} gastric cancer,\textsuperscript{174} pancreatic cancer,\textsuperscript{175} colorectal,\textsuperscript{38} lymphoma,\textsuperscript{39} cholangiocarcinoma,\textsuperscript{31c} esophageal,\textsuperscript{40} and lung cancer\textsuperscript{41}. Meta-analysis in Oncomine database\textsuperscript{176} also suggested that ID4 expression is decreased in majority of cancers (Figure 7).

2.12.6 ID4 and Prostate Cancer

Previous studies from our laboratory strongly support the role of ID4 as a tumor suppressor in prostate cancer (PCA).\textsuperscript{172a, 177} Immuno-histochemical analysis of normal and malignant human prostate tissue microarray shows that ID4 is down regulated
specifically in prostate cancer but not in normal adjacent prostate glands\cite{177} (see also TCGA dataset in Figure 1 and PRAD in Figure 7). Decrease in ID4 expression is stage dependent with majority of high grade tumor samples showing negligible ID4 expression while high ID4 expression is observed in the normal prostate tissue.\cite{177} We have shown that ID4 is expressed in PCa cell line LNCaP (low tumorigenic, androgen sensitive\cite{43}), low in PC3, but not in C81 (androgen insensitive and more tumorigenic LNCaP derivative\cite{178}), and DU145 cells.\cite{177} Furthermore, similar to other cancers, the decrease in ID4 expression in prostate cancer tissue and cell lines was attributed to promoter hypermethylation.\cite{172a, 177} Our study was further validated by Vinarskaja et al., who confirmed that ID4 promoter hypermethylation and down-regulation during prostate cancer progression.\cite{28}

Silencing of ID4 in LNCaP prostate cancer cells (LNCaP(-)ID4) results in a castration resistant phenotype, partly due to gain of de novo steroidogenesis.\cite{29} The LNCaP(-)ID4 cells also form tumors in castrated mice as compared to LNCaP cells.\cite{29} These results suggested that ID4 is required to maintain the tumor suppressive function of AR whereas as loss of ID4 results in tumor promoter activity of AR. These results could explain the ID4-/- mice prostate phenotype where AR is expressed at the levels similar to wild type mice but associated with PIN lesions.\cite{27} At the mechanistic level, ID4 may regulate the expression or function of AR co-regulators that may determine the final outcome of AR function.
2.12.7 ID4 in Prognosis and Survival

Inactivation of ID4 is also associated with poor differentiation and unfavorable prognosis in colorectal carcinoma.\textsuperscript{172c} In breast cancer loss of ID4\textsuperscript{179} is associated with recurrence free survival, increased tumor relapse.\textsuperscript{172b} AML patients with myelodysplastic syndrome (MDS) exhibited a significantly higher frequency of ID4 methylation with shorter survival.\textsuperscript{180}

In chronic myeloid leukemia, methylation of the ID4 promoter increases as the disease progressed from ‘chronic phase’ to ‘accelerated phase’ to ‘blast crises’.\textsuperscript{179} In a separate study ID4 was found methylated in 86\% of acute leukemia patients and 100\% in leukemia-relapse patients.\textsuperscript{31b} ID4 expression was reduced by promoter methylation in 95\% of B-cell lymphoma samples and 100\% of follicular lymphoma samples.

2.12.8 ID4 and Angiogenesis

Glioblastoma derived tumor cell expressing elevated levels of ID4 produce enlarged xenografts in immunosuppressed mice that were better vascularized than corresponding control tumors suggesting a novel pro-angiogenic function to ID4 (mediated by Matrix GLA protein (MGP)).\textsuperscript{181} In glial neoplasms down regulation of ID4 is associated with inhibition of angiogenesis.\textsuperscript{34} In breast cancer cells increased ID4 expression may also promote angiogenesis through stabilization of GRO-a and IL-8 mRNA\textsuperscript{127}).
2.12.9 ID4 and Chemo-resistance

High ID4 expression was shown to be responsible for chemo resistance to anticancer drugs in glioma stem cells (GSCs) by enhancing the expression of SOX2 by suppressing microRNA-9. SOX2 is a non-bHLH transcription factor known to play key role in development and maintenance of GSCs and is repressed by microRNA-9. The resulting ID4 mediated increased expression of SOX2 further induced ABC transporters such as ABCC3 and ABCC6 through direct transcriptional regulation resulting in chemoresistance of GSCs.\textsuperscript{182}

It is evident from the above studies that ID4 expression and association with various disease modalities even in the same cancer types such as breast and glioblastoma is often conflicting. The analytical tools such as specific ID4 antibodies, RT-PCR strategy (given that ID4 gene is highly CG rich) and specific CpG islands in the promoter itself needs to be reevaluated and validated. This is apparent from a prostate cancer study which demonstrated a positive correlation between ID4 overexpression with the Gleason score and metastatic progression.\textsuperscript{183} Use of highly specific ID4 antibodies, TCGA prostate adenocarcinoma (Figure 1) and Oncomine databases (Figure 7) clearly presented an inverse correlation between ID4 expression and prostate cancer. Most of the ID4 promoter methylation studies on cancers where ID4 is silenced have focused on the proximal promoter (surrounding the transcriptional start site) hence comparable and confirms epigenetic inactivation. Cancer heterogeneity may also contribute equally to inconsistent results. It is likely that ID4 expression is limited to a sub-set of cancers,
particularly in context of heterogeneity observed in many cancers. Decreased angiogenesis with better prognosis and increased survival in a subset of glioblastoma with low ID4 \(^{25b, 34}\) whereas high ID4 particularly in cells which are in close proximity to the vasculature\(^{181, 184}\) clearly supports an association between ID4 expression and cancer subtypes. A strong relationship between ID4 expression and cancer subtypes, particularly in GBM and breast is also evident from TCGA datasets (Figure 1). A clear discrimination between cancer subtypes (Breast and Glioblastoma), apparent from TCGA datasets (Figure 7) suggests that ID4 expression is a strong predictor of associated disease modalities.

The contrasting expression of ID4 in various cancers (and sub-types) is suggestive of a complex molecular mechanism which could involve tissue/ stage/ sub-type specific interaction proteins, post-transcriptional modifications and regulatory mechanisms.

### 2.13 Regulation of ID4 Gene Expression

#### 2.13.1 Transcriptional Regulation

At least two functional elements located downstream of the TATA box have been identified in the ID4 promoter. The first, a positive regulatory element contains a consensus E-Box that binds the bHLH leucine zipper upstream stimulatory factor (USF). The second, a negative regulatory element is a GA motif recognized by Sp1 and Sp3 transcription factors.\(^{185}\)

Interestingly, the predicted ER response element\(^{186}\) could result in the down-regulation of ID4 expression in ER positive breast cancer cells treated with estradiol.\(^{161}\)
Increased ID4 expression in TNBC\textsuperscript{164a} therefore could in part be due to loss of ER. In sporadic breast tumors, ID4 is also inversely correlated with ER mRNA expression.\textsuperscript{160} In several tumor specimens, ID4 mRNA expression was lowest in samples expressing high levels of ER mRNA whereas treatment of ER positive breast cancer cells with estradiol resulted in decreased expression of ID4\textsuperscript{165} (also see Figure 7A).

Our studies demonstrated that ID4 is regulated by the androgens in normal prostate epithelial cells and less metastatic prostate cancer cell line LNCaP.\textsuperscript{172a} Ectopic ID4 expression also induced re-expression of androgen receptor and downstream transcriptional target PSA in otherwise androgen receptor negative prostate cancer cell line DU145.\textsuperscript{172a}

AR-mediated prostate tumorigenesis in mice expressing mutant androgen receptor (E231G) demonstrated negligible ID4 expression together with other signature genes to predict biochemical relapse in androgen receptor dependent prostate cancer.\textsuperscript{187} Taken together, these studies suggest that ID4 exists in positive feedback loop with AR but in a negative feedback loop with ER. However, direct evidence that ER or AR binds directly to ID4 promoter is lacking.

Other studies suggest that the tumor promoting role of ID4 in breast cancer can be linked to the ability of mutated p53 to regulate ID4 at the promoter level through specific binding sites mediated by E2F1 and p65.\textsuperscript{127} ID4 is also the downstream target of BMP4 in neural progenitor cells and apparently mediates the inhibitory effects on oligodendroglial differentiation\textsuperscript{188} whereas ID4 dependent osteoblast differentiation is independent of BMP4\textsuperscript{149}, suggesting cell type specific regulation (Figure 2).
2.13.2 Epigenetic Regulation

Gene silencing through promoter hypermethylation is the most widely accepted mechanism involved in the regulation of ID4 expression. ID4 promoter is CpG rich that spans the proximal promoter through entire exon 1 thus making this region highly attractive for epigenetic modifications. Several studies have not only extensively characterized the down regulation of ID4 expression through promoter hypermethylation in many cancers and cell lines, but have also linked ID4 expression to clinic-pathological variables, such as stage, tumor grade, age, cancer recurrence and poor prognosis such as in lung, bladder, breast and colorectal cancer as discussed above. Demethylation of the breast cancer cell lines (BT20, MCF7 and T47D) by 5-Aza-2’-Deoxycytidine (AZA) and Trichostatin A and prostate cancer cell line DU145 by AZA resulted in clear re-expression of ID4 further supporting the hypermethylation as the primary mechanism involved in ID4 down-regulation in cancers.

Studies in colorectal carcinoma demonstrated that ID4 promoter is regulated by Cdc42, a small GTPase of the Rho family. High Cdc42 expression in many cancers including colorectal carcinoma, may suggest a role of cdc24 in ID4 promoter methylation. How a protein involved in promoting cell cycle regulates epigenetic re-programming of the ID4 promoter remains to be addressed.

PRMT5, a type II protein arginine methyl-transferase which catalyzes mono- and di-methylation of arginine residues, is generally considered as an oncogene that promotes cell cycle. PRMT5 was shown to occupy the CpG rich islands in ID4 promoter resulting in its promoter hypermethylation during glial cell and oligodendrocyte differentiation.
In cancer cells, high PRMT5 expression may tend to support its role in epigenetic silencing of ID4. However, in prostate cancer cells PRMT5 expression is primarily cytoplasmic and promotes growth. In contrast, PRMT5 is nuclear in benign prostate epithelial cells where it inhibits growth.\(^{190}\) Thus PRMT5 localization (predominantly cytoplasmic) in prostate cancer does not correlate with its role in ID4 methylation or association with CpG islands, which as one would expect to be in the nucleus. The transcription factor YY1 was also shown to inhibit ID4 transcription by recruiting histone deacetylase-1 to its promoter\(^ {191}\) suggesting that histone acetylation may also play a role in regulating ID4 expression.

EZH2 (enhancer of Zeste 2), part of the Polycomb repressor complex 2 (PRC2) is involved in epigenetic re-programming in both normal and disease states including cancer. EZH2 is specifically involved in covalent modification of histone tails, specifically tri-methylation (me3) of lysine 27 (K27) on histone 3 (H3) (H3K27me3), a repressive mark found on many gene promoters that are silenced.\(^ {192}\) EZH2, as part of the PRC2 complex also recruits DNMTs (DNA methyl transferases) that in turn promotes DNA methylation at CpG islands (CGI) thus connecting the two key epigenetic repression systems.\(^ {193}\) Increased EZH2 expression is also observed in many cancers\(^ {194}\) including prostate cancer.\(^ {195}\) We recently demonstrated that ID4 is an EZH2 target gene in prostate cancer. Assembly of PRC2 complex initiated by increased recruitment of EZH2 on ID4 promoter increases the repressive H3K27me3 histone modification and recruits DNMT1/ 3a resulting in ID4 promoter hyper-methylation in DU145 prostate cancer cell lines and prostate cancer tissue. Demethylation of the breast cancer (BT20,
MCF7 and T47D\textsuperscript{172b} and prostate cancer (DU145)\textsuperscript{172a} resulted in clear re-expression of ID4 by 5-Aza-2’-Deoxycytidine (AZA) a DNMT1 inhibitor and HDAC inhibitor Trichostatin A.

Epigenetic inactivation in many but not all cancers suggests that ID4 could be highly regulated in a tissue specific manner. Basal transcriptional machinery consisting of a subset of bHLH (USF1) transcription factors together with the assembly of tissue specific positive (AR, BMP4) or negative (ER) regulatory complexes could determine optimal ID4 transcription. The binding of Sp1 to GC rich regions, which are targets of hyper-methylation, could be a rate-limiting step in the assembly of such transcription factor complexes on ID4 promoter that could eventually determine ID4 transcriptional output.

2.13.3 Regulation of ID4 Expression by MicroRNA

Recent studies have demonstrated that ID4 expression is also regulated by miRNAs. ID4 is repressed by miR342 (breast cancer cell line MDA-MB-231\textsuperscript{196}, miR335 (breast cancer cell line MCF7\textsuperscript{197} and senescence associated (SA) miR485-5p (fibroblast cell lines).\textsuperscript{198}

2.14 Mechanism of Action of ID4

Protein interaction studies have demonstrated that ID4 sequesters OLIG1 and OLIG2, members of class B bHLH proteins, which plays key roles in early oligodendrocyte specification and regulates oligodendrocyte differentiation.\textsuperscript{188} ID4 also promotes osteoblast differentiation by interacting with the bHLH protein Hes1.\textsuperscript{149}
Recently, ID4 was also shown to interact with another bHLH transcription factor, Twist 1 in GBM. The sequestration of Twist-1 a putative tumor promoter also led to decreased MMP2 mediated invasion of glioblastoma derived cells.

ID4 was found to down-regulate the expression of breast cancer susceptibility gene BRCA1 suggesting a negative feed-back loop between BRCA1 and ID4. Surprisingly, BRCA1 induced the expression of ID4 in a study by Welcsh et al.

In ID4-/- mice, a decrease in ductal expansion and branching morphogenesis was attributed to activation of p38MAPK. Thus, ID4 may regulate major stress activated pathways through down-regulation of p38MAPK.

A novel but un-expected mechanism of action of ID4 has recently emerged. In a ChIP study, ID4 was found to be part of the transcriptional complex that could potentially regulate ERa and Foxa1. The lack of ID4 DNA binding domain in ID4 suggests that it most likely regulates transcription as part of a larger protein complex.

As opposed to the protein interaction network of ID1 and ID2, the regulatory network of ID4 remains to be fully explored. Given the inverse association between ID1 and ID4 in cancer, the ID4 regulatory network is expected to be unique and non-overlapping. The major questions that need to be addressed are whether ID4 negatively regulates ID1 dependent pathways. If this is true then obviously the HLH independent mechanisms should be explored and/or contributions of the non-HLH domains towards selective binding with bHLH and non-bHLH proteins between ID4 and other three ID proteins. Moreover, the degree of overlapping functions between a subset of ID proteins such as ID2 and ID4 which both bind Rb should be closely examined to evaluate
structure/ function relationship. Recent data supporting the indirect interaction of ID4 with DNA as part of the larger transcriptional complex is also interesting and opens an entirely novel dimension to the function of ID4.

2.15 The role of ID4 in Cell Cycle and Proliferation

Ectopic expression of ID4 blocks cell cycle and inhibits proliferation that is associated with increased expression of p21, and p27 in prostate cancer cell line DU145. The ID4 dependent cell cycle arrest appears to be primarily at S-Phase in DU145 and PC3 prostate cancer cell lines and not at G1 as would be expected from increased p21 and p27 expression. The block is S-phase appears to be due to decrease in the expression of E2F1 that is required for transition through S-phase. Loss of ID4 also promotes progression into S-phase in neuronal early cortical progenitor cells and LNCaP prostate cancer cell lines. Epigenetic knockdown of ID4 through promoter hypermethylation in colon carcinoma by Cdc42, a member of Rho GTPases family may be required for cell cycle progression. Nevertheless, these results suggest that unlike ID1, ID2 and ID3, the expression of ID4 is not associated with progression of cell cycle and points towards the role of ID4 in promoting differentiation in specific cell types since both these processes are largely mutually exclusive.

ID4 can also drive the malignant transformation of astrocytes via de-regulation of cell cycle and differentiation through the up-regulation of cyclin E and activation of Jagged-Notch1 signaling but only in primary murine Ink4a/Arf/-/- astrocytes. Increased ID4 expression and deletion of the INK4a-ARF locus is also found in majority of human malignant gliomas. These results suggest that the Ink4a/ Arf could be an
upstream regulator of ID4 in a subset of gliomas. Whether ID4 itself drives the expression of Ink4a/Arf or vice versa to block cell cycle could be an interesting avenue to investigate.

2.16 ID4 in Therapeutics

Overwhelming evidence suggests that ID4 is epigenetically silenced in many cancers. Hence therapeutic strategies which alter the cancer epigenome via inhibition of DNMT1 such as by AZA\textsuperscript{201} are viable strategies. Alternatively, mechanisms involved in regulating ID4 levels at the transcriptional level such as those involving ER and AR could be exploited to elevate ID4 levels in breast and prostate cancer. A study recently demonstrated that treatment cells by arsenic trioxide (ATO) reversed the hyper-methylation of ID4 promoter and decreased proliferation of Raji cells.\textsuperscript{202} The specificity of this treatment on ID4 promoter however remains to be investigated.

A tumor-penetrating nano-complex (TPN) comprising of siRNA complexed with a tandem tumor-penetrating and membrane-trans-locating peptide, which enables the specific delivery of siRNA deep into the tumor parenchyma was employed in vivo to evaluate the role of ID4 as an oncogene\textsuperscript{167} in tumors, where ID4 expression is elevated such as those of the ovary. Treatment of ovarian tumor-bearing mice with ID4-specific TPN suppressed growth of established tumors and significantly improved survival.

2.17 Conclusion

It is evident that ID4 shows divergence from the other ID family members during development, differentiation and diseases such as cancer. The mouse knockout models
have clearly established the essential role of ID4 in development that is not compensated by other ID proteins. Overall, the role of ID4 in cancer appears to be that of a tumor suppressor that is largely based on its epigenetic silencing in majority of cancers. The expression of ID4 in limited cancer types (GBM) is associated with favorable prognosis further supporting the role of ID4 as a putative tumor suppressor. How ID4 acts as a tumor suppressor is an open question and should be the major focus of future studies. The other major question that remains to be addressed is the mechanism by which ID4 acts as a tumor promoter in limited cancer sub-types such as TNBC, basal cell carcinoma of the breast and ovarian cancer. Interaction of ID4 with bHLH proteins such as OLIG and TWIST and cross-talk with BRCA1, p38MAPK and Notch signaling pathway may provide a significant insight into the mechanism of action of ID4 that needs to be further explored. The lack of a DNA-binding domain and unique sequence of ID4 (Alanine and Proline rich domains that support protein interactions) also suggest that ID4 may act as a major hub in protein–protein interaction networks, possibly as a putative co-chaperone. These interactions may help unravel the complex biology of ID4 in development and cancer. The strong anti-cancer effect of ID4 in prostate cancer suggests that mechanisms to up-regulate ID4 expression in cancers is a therapeutic strategy and needs to be further explored.
CHAPTER III
MATERIALS AND METHODS

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.\(^{172a}\) 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 TRlZol (Invitrogen, Carlsbad, CA) as described previously. 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 anal
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.203

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>GTGAACAGCAACGAGGGAAGCA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GAAGGTTGAAGTGGATGGTGC</td>
<td>GAAGATGGTGATGAGGATTTTC</td>
</tr>
<tr>
<td>PUMA</td>
<td>CTGTATCTGTGACCTTTTGC</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>GCCATTAGCAGTGACATCACAG</td>
<td>TGCCTTCACAGTGTTTGTG</td>
</tr>
<tr>
<td>p53</td>
<td>GCTCGACGCTAGATTCTGAC</td>
<td>GCCTTCCACGACGGTGAC</td>
</tr>
</tbody>
</table>

CHiP Primers spanning the p53 response elements

| p21 Chip | GTGGGCTCTGATTTGCTTTTCTG | TCCTTGGGCTGCCCTGTTTTCAG |
| BAX chip  | TAATCCACAGCGCTTTGGAAG | GCTGAGACGAGGGTTATCTC |
| PUMA chip | GCCGAGACTGTGGCCTTGTG | CGTCCACGGTCCACAAAGT |
3.5 Protein Extraction

Total cellular proteins were prepared from cultured prostate cancer cell lines using M-PER (Thermo Scientific). 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.

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 Signaling</td>
<td>1:1000, 1:200</td>
</tr>
<tr>
<td>PUMA</td>
<td>Rockland Immunocel</td>
<td>1:1000</td>
</tr>
<tr>
<td>BAX</td>
<td>Cell Signaling</td>
<td>1:1000</td>
</tr>
<tr>
<td>BAX6A7</td>
<td>Abcam</td>
<td>1:1000, 1:150</td>
</tr>
</tbody>
</table>
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 x 10^5 cells/ well without serum overnight. Cells were then cultured for 72 hrs. MTT assay was performed using CellTiter 96 Non-Radioactive Cell Proliferation Assay kit (Promega) following manufacturer’s instructions.

3.8 Statistical Analysis (Proliferation)

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.
3.9 Co-Immunoprecipitation (ChIP)

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 3) 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 pimelimidate 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.

3.10 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 EMSA kit containing the p53 response element). 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.

3.11 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).

3.12 Transient Transfections and Luciferase 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, Addgene) or MG15-luc (containing 15 mutant p53 binding sites, 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.

3.13 Apoptosis Assay

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

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

3.15 Mutagenesis of p53

Mutagenesis of p53, the retroviral p53 vector previously described was manipulated via mutation induced by site-directed mutagenesis mimicking DU145 cells that harbor mutant p53 (P223L and V274F). Also, hotspot mutations of p53 found any many cancers (R273H and R175H) purchased from Addgene, Inc, pCMV-Neo-Bam p53 R175H was a gift from Bert Vogelstein (Addgene plasmid #16436) as well as pCMV-Neo-Bam p53 R273H(Addgene plasmid # 16439).

3.16 Immunohistochemistry (IHC)

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

The cells were cultured in six well plates with respective media. The cells at 60-70% confluency were stained for senescence associated-β-galactosidase (SA-βgal) staining kit (Cell signaling) as per manufacturer’s instructions. At least 15 representative fields were randomly selected for the quantitation of the percentage of SA-βgal positive cells. The images were captured in both phase contrast and bright field to better visualize cellular details.

3.18 The structures of Wild Type and Mutant p53

The wild type p53 core domain (residues 94-269) structure 1TSR\(^5\) was obtained from the Protein Data Bank (PDB), a widely used portal for biological macromolecular structures\(^{210}\). The 1TSR represents the X-ray crystal structure of a wild type core domain p53 -DNA complex at 2.2 Å resolution with a crystallographic R factor of 20.5\%.\(^5\) The DNA binding domain of p53 consists of a beta sandwich that serves as a scaffold for two large loops and a loop-sheet-helix motif. The two loops, which are held together in part by a tetrahedral coordinated zinc atom, and the loop-sheet-helix motif form the DNA binding surface of p53. We therefore used 1TSR as a framework to study p53 mutations that fall within the DNA binding domain. The base 1TSR peptide sequence was used to introduce prototype p53 mutations: R175H, R273H, P223L, and V274F. The widespread R175H and R273H were used as hot spot gain of function p53 mutations while P223L, and V274F were used a weak temperature sensitive mutants found in DU145 prostate cancer cell lines.
3.19 Molecular Dynamics Simulation

In collaboration with the physics department at Clark Atlanta University, we performed molecular dynamic simulations of 1TSR (wild type) and core domain mutants using the CHARMM (Chemistry at HARvard Macromolecular Mechanics), an all-atom empirical energy function force field. The CHARMM force field treats interactions of amino acids with bonded and non-bonded terms. It is worth noting that in order to properly characterize the relevant interactions, it is necessary to include explicit solvent. For this reason, our MD calculation results were rectified with simulations with explicit water. Computational simulations largely utilize the atomic description of biomolecules. However, many studies have shown that molecular systems may have diverse conformations due to large numbers of degrees of freedom around chemical bonds, leading to a plethora of local minima conformations. Biological function is associated when the structure which is in the proximity of a global energy minimum. In an effort to optimize the system, we used simulated annealing with repeated search cycles with various initial conformations using NAMD (NAnoscale Molecular Dynamics) and subsequent visualization in VMD (Visual Molecular Dynamics). NAMD was developed by the Theoretical and Computational Biophysics Group in the Beckman Institute for Advanced Science and Technology at the University of Illinois at Urbana-Champaign. NAMD is a parallel molecular dynamics platform (on Charm++) for high performance simulation of large biomolecules systems. Shown in supplementary figures 2a-2h are the optimized conformations of aforementioned mutant forms of p53. Simulated annealing
serves as an optimization technique that readily converges energy of biomolecules after 20 to 30 cycles.

The potential energy of core domain 1TSR and mutants was calculated in a given conformation as a sum of individual energy terms of bonded and non-bonded interactions. The following assumptions were made to address the effect of mutation in a confirmation: 1) Changing a residue in a wild type affects overall bonding, 2) Non-bonded terms such as electrostatic, van der Waals and polarization are independent of mass, and 3) Bonded terms such as energy of stretch, bending and torsion depend on the molar mass of residues. A bond was considered as a spring with its own equilibrium length \( r_0 \) (in our assumption the center of mass of a residue is \( r_0 \)). The energy that is required to stretch or compress a bond between two atoms or to bend a bond from its equilibrium angle depends on the constant of the spring constant. Therefore, the overall energy of bending and stretching depends on molar mass while a mutation is considered similar to changing the spring in a harmonic motion. Periodic Fourier function was used to represent the energy of torsions between particles based on molecular dynamics. The interaction mode \( v_n \) was defined as \( v_n \propto \frac{1}{2m} \), where \( m \) is the molar mass of the residue. Therefore the changes in energy (\( \beta \)) can be considered as the effect of molar mass of residue (\( m \)) harmonious to changes in energy of stretch, bending, and torsion in a protein by: \( \beta \propto m \)

We also assumed at the minimum that mutating a residue in a protein may have an effect on at least two near-neighbor residues. The molar mass was therefore...
extracted from the PDB file. Specifically, for the residues involved, the effective mass is calculated as $\sum_{n=1}^{3} \frac{1}{m_n}$, where $m_n$ is the molar mass of the $n$th residue. As such we can define the relative change of the molar mass as:

$$\mu = \frac{\sum_{n=1}^{3} \frac{1}{m_{n_{mutated}}}}{\sum_{n=1}^{3} \frac{1}{m_{n_{wild\ type}}}}$$

where the nominator and denominator denote the reciprocal effective mass for the mutated residue and wild type, respectively.

Finally, we consider the effective mass of a 3-sequence segment as

$$1/m = \sum_{n=1}^{3} \frac{1}{m_{n}}$$

where the central segment can be either a mutated or wild type residue. As such, we can characterize the relative change of the effective mass as

$$\mu = m_{\text{mutated}}/m_{\text{wild type}}.$$ 

Thus, $\mu$ is dimensionless and characterizes the correlation between the mutated protein and the wild type protein.
CHAPTER IV
RESULTS

4.1 ID4 Regulates Wild-type and Mutant p53

4.1.1 Expression of ID4 in Prostate Cancer cell Lines

The cellular and molecular alterations of epithelial cells in the prostate and their microenvironments during cancer development are complicated, and it is difficult to use a single cell line to study these changes and also to study the pathophysiological effects mediated by p53. Cell lines that will be utilized herein are derived from different Prostate Cancer (PCa) hosts and have their own characteristics that may be representative of different stages of PCa. LNCaP cells, isolated from a human PCa lymph node metastasis\textsuperscript{215}, are positive for ID4 and have wild-type p53; DU145 cells, derived from a brain metastasis of human PCa\textsuperscript{216}, harbors a mutant form of p53 and ID4 has been reported to be epigenetically silenced; PC3 cells isolated from a human PCa bone metastasis with high malignancy\textsuperscript{58}, is p53-null and has endogenous levels of ID4.

ID4 is undetectable in DU145 cells due to promoter hyper-methylation.\textsuperscript{217} In contrast, ID4 was expressed in LNCaP cells due to promoter hypo-methylation. These two cell lines were used to either overexpress (DU145+ID4) or silence (LNCaP-ID4)
ID4. Lastly, we wanted to use the highly metastatic cell line, PC3 to investigate possible ID4-p53 cross-talk. Since, PC3 cells are null for p53 and express ID4, we overexpressed wild-type p53 as well as mutant p53 via site-directed mutagenesis mimicking DU145 p53 mutations (P223L and V274F) and p53-hotspot mutations (R273H and R175H) (Figure 8B,C) a gift from Bert Vogelstein’s group (Table III) to study possible cross-talk of ID4 and p53 mediated pathways. In the same manner as our LNCAP-ID4 cellular model (Figure 9), we stably silenced ID4 in PC3 cells as well created and PC3+p53 (wild-type and mutant) overexpression model (+/-) ID4 (Figure 9). Herein, our p53 overexpression (P223L and V274F: DU145 cells) (R175H and R273H: hotspot p53 mutants) model system will be referred to as wild type and mutant p53 to represent both DU145 and hotspot p53 mutants as aforementioned.

**Table 3.** PC3 Wild type and Mutant p53 Overexpression Model in the Presence and Absence of ID4

<table>
<thead>
<tr>
<th>PC3+NS</th>
<th>PC3+ID4shRNA</th>
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<tbody>
<tr>
<td>wtp53</td>
<td>wtp53</td>
</tr>
<tr>
<td>mutp53PL</td>
<td>mutp53PL</td>
</tr>
<tr>
<td>mutp53VF</td>
<td>mutp53VF</td>
</tr>
<tr>
<td>mutp53VPL</td>
<td>mutp53VPL</td>
</tr>
<tr>
<td>mutp53R175H</td>
<td>mutp53R175H</td>
</tr>
<tr>
<td>mutp53R273H</td>
<td>mutp53R273H</td>
</tr>
</tbody>
</table>

**Figure 8.** (A) Schematic view of p53 domain with mutations in DBD domain (R175H,P223L,R273H,V274F). (B) Confirmation of mutant p53 (P223L) and (V274F) by sequencing
Two different retroviral shRNA vectors (vectors A and C) were used to silence ID4 (Figure 9) in LNCaP and PC3 cells. The experiments reported here were performed on stable knockdown of ID4 in LNCaP and PC3 cells using vector A (LNCaP-ID4) (PC3-ID4). p53 (wild type and mutant) over-expression in PC3 cells (Figure 9) and their respective vectors were used for all subsequent experiments. A non-silencing control vector was also transfected into LNCaP and PC3 cells (LNCaP+NS) (PC3+NS). Expression of ID4 was measured by quantitative PCR and western blotting (Figure 9).

**Figure 9.** Stable knockdown of ID4 by retroviral shRNA in LNCaP and PC3 cells (retroviral vectors A and C). (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. (D) Western Blot analysis of ID4 expression with non-specific shRNA (NS) and ID4 specific shRNA (-ID4, vector A) and p53 expression (DU145 specific p53 mutants: P223L and V274F) in PC3 cells via site-directed mutagenesis. (E) Western blot analysis of ID4 expression with non-specific shRNA (NS) and ID4 specific shRNA (-ID4, vector A) and p53 expression (Hotspot p53 mutants: R175H and R273H).

4.2 ID4 Effects on Proliferation and Gene Profile upon Silencing of ID4 in LNCaP Cells

Upon ID4 silencing we investigated proliferation in LNCaP cells via a MTT assay, which measures the rate of cell proliferation. We observed that the rate of proliferation nearly doubled after ID4 silencing (Figure 10A). This data suggests loss of ID4 promotes increased proliferation and suggests that in the absence of ID4 cellular apoptosis could be adversely affected as well.

After observing the effect of ID4 on proliferation, we investigated proliferation marker cylin dependent kinase inhibitor p27, which could provide insight into the molecular mechanism by which ID4 may effect proliferation. The expression of p27 was detected by q-PCR (Figure 10B). 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. Also, this phenomenon could have an adverse effect on apoptosis, possibly due to p53 transactivation capability, which will be discussed later.
Loss of ID4 promotes proliferation. (A) There is an increase in the proliferation of LNCaP-Id4 cells by fold change of 2. Data is statistically significant. Each bar represents Mean ± SD from a representative experiment. (P< 0.05). (B) The mRNA expression of cyclin dependent kinase p27 was decreased upon loss of ID4. p27 has been implicated in cell proliferation.

4.3 ID4 Effects on p53-mediated Apoptosis

A significant increase in apoptotic cells was observed in DU145+ID4 (64±3.3%, P<0.001, Figure 11A) cells as compared DU145 cells (24±5.1%, Figure 11A) whereas a number of cells undergoing apoptosis decreased in LNCaP-Id4 (10.5±2.3%) as compared to LNCaP (47.2±6.5%) cells (Figure 11A). Also, we observed that p53 promoted basal apoptosis in an ID4 dependent manner in PC3 cells where both wild type and mutant p53 had been overexpressed (Figure 11B). We observed a marked increased in apoptosis in PC3+wtp53 (42.2±3.2%) as compared to control pcDNA (9.9±1.3%) as well observing significant apoptosis in mutant forms of p53 (PL-34.6±6.2%, VF-32.0±4.0%, VFPL-21.6±3.0%) overexpression as compared to wtp53. As expected, we observed that mutant
p53 overexpression did not induce apoptosis at the rate of wild-type p53 given the mutant status of p53 (Figure 11B). Conversely, we observed the opposite effect took place upon ID4 silencing. We found that ID4 silencing reduced the rate of apoptosis amongst both wild type p53 (27.5, 6.2%) and mutant p53 (PL-6.7, 3.0%, VF-7.7, 5.6%, VFPL-13.5, 6.2%) overexpression models compared to controls NS (14.2, 2.9%) and PC3+ID4shRNA (10.2, 2.2%) (Figure 11B). We also investigated the rate of apoptosis when p53 hotspot mutations (R175H and R273H) were overexpressed (Figure 11C). We observed that both hotspot mutants did induce apoptosis (R175H-20.6+7.6%, R273H-15.0, 4.6%) compared to wtp53 (36.4, 5.9%) and negative control pcDNA (10, 2.9%) (Figure 11C). As expected the rate of apoptosis with mutant p53 was significantly lower as compared to that observed with wild type p53. However, significant rates of apoptosis with mutant p53 were observed as compared to the negative control pcDNA (Figure 11C). Thus, the results indicate that p53 promotes apoptosis in an ID4 dependent manner in PC3 cells. We also observed that upon ID4 silencing in PC3 cells we observed a similar pattern of apoptosis as compared to basal levels of apoptosis in PC3 cells with endogenous ID4 as previously shown.218 We observed an increase in apoptosis in wild-type p53 (27.5, 8.6%) compared to controls NS (9.8, 2.1%) and PC3+ID4shRNA (8.7, 3.0%), but there was a significant decrease of apoptosis compared to the PC3+wtp53 (36.4, 5.9%) with endogenous ID4. We also observed a significant decrease in apoptosis compared to wtp53+ID4shRNA in p53 hotspot mutants upon silencing of ID4 as well (R175H-13.6, 4.3%, R273H-16.2, 5.7%), however there was not a significant decrease
in apoptosis with regard to the hotspot p53 mutant R273H compared to overexpression with endogenous ID4 likely due to R273H a classified DNA contact mutant.

Figure 11. ID4 promotes p53-mediated apoptosis. (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) A significant increase in apoptosis (***: P<0.001) was observed in PC3+pcDNA versus wtp53 and p53 mutants (p53 mutants: P223L and V274F) “a”: significant difference between wtp53 versus mutant p53. A significant decrease in apoptosis was observed in PC3 cells that lacked ID4 (PC3-ID4) compared to PC3 cells with wtp53 and p53 mutants. (C) A significant increase in apoptosis (Hotspots: R175H and R273H) (***: P<0.001) was observed in PC3+pcDNA versus wtp53 and p53 mutants. A significant decrease in apoptosis was observed in PC3 cells that lacked ID4 (PC3-ID4) compared to PC3 cells.
4.3.1 ID4 Effects on p53 Mediated Apoptosis Gene Profile upon ID4 Silencing in LNCaP and PC3 Cells as well as Overexpression of ID4 in DU145 Cells

After observing the effect of ID4 on apoptosis, we investigated known target genes of p53 to elucidate the molecular mechanism by which ID4 may promote apoptosis via p53. The target genes studied were p21, PUMA, and BAX. BAX expression and/or PUMA dependent dissociation of BAX from Bcl-2 promotes translocation of BAX to mitochondria resulting in decreased mitochondrial membrane potential. 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 transcript (Figure 12A) and protein (Figure 12B) level as compared to DU145 and LNCaP+NS cells respectively (Figures 12A,B). These results suggest 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. 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 12B) but not in DU145 and LNCaP-ID4 cells possibly due to undetectable levels of BAX (Figure 12B).

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 as previously shown.\textsuperscript{218} 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 mutant p53. The two mutations (P223L and V274F) are within the DNA binding domain that results in a transcriptionally inactive form of p53.\textsuperscript{221} Mutant p53 protein generally accumulates at high levels due to loss of regulatory mechanisms as seen in DU145 cells.\textsuperscript{222}

We also investigated the expression of CDKN1A (p21), which is also a well characterized p53 target gene.\textsuperscript{223} Increase in the expression of p21, (9 fold as compared to DU145) (Figure 12A), in addition to PUMA and BAX further consolidated our observations that mutant p53 in DU145+ID4 cells may have gained transcriptional activity through site specific DNA binding in the respective promoter elements.

To further elucidate the molecular mechanism by which ID4 promoted apoptosis in LNCaP and DU145 cells, p53 target genes: p21, PUMA, and BAX were also used to investigate ID4 mediated apoptosis when wild-type and mutant p53 were overexpressed in PC3 cells. Investigation of p53 in PC3 cells, which have endogenous ID4 served as the model to examine the transactivation potential of p53 as shown when ID4 was overexpressed in DU145 cells (Figure 12C).

As aforementioned study of CDKN1A (p21), which is a well characterized p53 target gene\textsuperscript{223} was investigated. Increase in the expression of p21, in addition to PUMA and BAX (Figures 12C, compared to PC3) further consolidated our observations that wild-type p53 as well as over-expressed mutant p53 in PC3 cells may have gained transcriptional activity in the presence of ID4. Moreover we observed an increase in
downstream targets of p53 when hotspot mutants: R175H and R273H that were transfected into PC3 cells (Figure 12D). This finding was critical to this study as this suggested ID4 has an effect on well-established p53 mutations, suggesting that ID4 may play a role in an array of different p53 mutants. Conversely, we observed the opposite effect upon silencing of ID4 in PC3 cells (DU145-p53 mutants and hotspot mutants). The expression of p21, PUMA, and BAX were down-regulated compared to PC3 cells with endogenous ID4 (Figure 12D).

Figure 12. Gene expression profile following ID4 silencing and wild type and mutant p53 overexpression in PC3 cells. (A) 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). (B) 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. (C,D) Representative western blots of three different experiments are shown. Western blot analysis of p21, BAX, and PUMA in PC3 cells transfected with wild type and mutant p53 (C) Transfection of p53 mutations: P223L and V274F (D) Transfection of hotspot p53 mutations: R175H and R273H.
4.3.2 ID4 Promotes p53-mediated UV-Induced Apoptosis

p53 plays a crucial role in mediating DNA damage responses.\textsuperscript{224} UV treatment was utilized to activate the DNA-damage pathway mediated by p53. The PC3 cellular (wild-type and mutant p53) (+/-) ID4 model was used to investigate the DNA damage pathway via UV radiation treatments at 40 J/m\textsuperscript{2}. Similarly to the previous study, where we examined apoptosis at the basal level in PC3 cells with p53 overexpression (wild-type and mutant) (+/-) ID4, we sought to investigate apoptosis in the same manner after UV-radiation. After UV-radiation we observed a significant increase in apoptotic cells in PC3 cells with overexpressed wild type p53 (42.2, 6.2\%) and mutant p53 (PL- 34.6, 11.2\%, VF- 32.0, 4.6\%, VFPL-21.6, 8.9\%) compared to control pcDNA (9.9, 2.2\%) (Figure 13A). Upon ID4 silencing in PC3 cells we observed a reduced rate of apoptosis compared to PC3 cell lines with endogenous ID4. When ID4 was silenced the rate of apoptosis in wild-type p53 was (39.7, 8.9\%) and mutant p53 (PL- 22.3, 8.6\%, VF- 23.1, 7.6\%, VFPL-18.6, 6.9\%) (Figure 13B). Thus, this data suggests that p53 (wild-type and mutant) promoted apoptosis after UV-radiation treatment in an ID4 dependent manner in PC3 cells.

The results observed in the UV induced apoptotic study led us to investigate the molecular mechanism by which ID4 promotes apoptosis. Subsequently, cellular protein was extracted and investigated for the expression of proliferation marker cyclin dependent kinase inhibitor p21 and apoptotic markers PUMA, and BAX in PC3 cells harboring wild-type p53 and p53 mutants (+/-) ID4 via western blot analysis to understand the underlying molecular mechanism. The expression of p21, PUMA, and
BAX were decreased upon loss of ID4, as compared to PC3 cells harboring wild type and mutant p53 in the presence of ID4 (Figure 13C). Thus, the decrease in cell cycle regulator (p21) and apoptotic markers (PUMA and BAX) upon silencing of ID4 could suggest that ID4 plays a critical role in p53 transactivation and the ability of p53 to effect both apoptosis and senescence.

**Figure 13.** ID4 promotes UV-induced p53-mediated apoptosis. (A) A significant increase in apoptosis (****: P<0.001) was observed in PC3+p53 and PC3+mutp53 (p53 mutants: P223L and V274F) compared with PC3 cells alone when treated with UV-radiation. (B) A significant decrease in apoptosis was observed in PC3 cells that lacked ID4 (PC3-ID4) compared PC3 cells (PC3, ***: P<0.001) when treated with UV-radiation. (C) Western blot analysis of p21, BAX, and PUMA in PC3 cells transfected with wild type and mutant p53. GAPDH was used as loading control. Representative western blots of three different experiments are shown.
4.4 ID4 Promotes p53-mediated Senescence

Microscopic examination of PC3 + wtp53 cells revealed visibly enlarged morphology and accumulation of cytoplasmic aggregates as compared to mutant forms of p53 overexpressed in PC3 cells (Figure 14A). Several studies have described the accumulation of similar cytoplasmic aggregates such as lipofuscin vesicles, a molecular byproduct associated with replicative senescence and cellular aging. Lipofuscin molecules are an “aging pigment” and the byproduct of incomplete lysosomal degradation of damaged mitochondria. These observations lead us to investigate the induction of senescence in PC3+p53 (wild-type and mutant) in the presence and absence of ID4. A decrease in senescence associated betagalactosidase (SA-βgal) staining upon ID4 silencing suggested that loss of ID4 expression in PC3 cells upon overexpression of p53 (wild-type and mutant p53) down-regulated senescence at a higher frequency than transfected PC3 cells with endogenous ID4 (Figure 14A). The number of cells with strong (High) and those with light (Low-Moderate) blue staining were quantitated. The results summarized in (Figure 14 B) demonstrated a significant increase in the number of cells with SA-βgal staining in PC3 with endogenous ID4 (wild-type and mutant p53) (wt-p53- 81, 2.3%, Moderate and 17, 0.9% High) (PL-29, 4.0%, Low, 66, 8.0%, High) (VF-65, 0.9%, Low, 25, 4.0%, Moderate, 8, 0.07%, High) (VFPL- 79,6.3%, None, 20, 1.0%, Low) as compared to silencing of ID4 (wt-p53- 44, 1.3% , Low, 60, 15%, Moderate) (PL-8,2.3%, None, 78, 13%, Low, 12, 2.5%, Moderate) (VF- 4, 0.9%, None, 88, 10.2%, Low, 10, 3.6%, Moderate) (VFPL-85, 6.3%, None, 15, 7.8%, Low) (Figure 14C). Furthermore,
none of the PC3 (wild-type and mutant p53)–ID4 cells demonstrated High SA-βgal staining. Senescence in normal cells is associated with a flattened, enlarged morphology with >2 fold increase in cellular diameter compared to non-senescent cells. Over 60% of PC3 (wild-type and mutant p53) cells stained positive for the SA-βgal and ~20% of those cells appeared to have flattened morphology (Figure 14A) suggesting cyto-architectural changes in PC3 (wild-type and mutant p53) cells.

Several tumor suppressor genes are capable of inducing senescence through inhibition of the cell cycle by inducing a G1 arrest via either p21 [34], p27 [35] or p16 and subsequently inhibiting the CDK2 dependent phosphorylation of the RB protein. However, PC3 cells have a highly deregulated cell cycle with a frame shift deletion mutation in the p53 gene, the key regulators of cell cycle and senescent pathways [36].

Our results demonstrate an increase in senescence in an ID4 dependent manner in PC3 (wild-type and mutant p53) cells. These results prompted us to investigate the underlying molecular mechanisms such as the influence of ID4 on regulatory mechanisms of p53 (MDM2, p14ARF); p53 DNA-binding and transcriptional activation; and a mechanism (acetylation) by which ID4 may influence mutant p53 to regain wild-type biological activity.
Figure 14. ID4 promotes p53-mediated senescence. (A) ID4 promotes senescence in PC3 cells with overexpressed wild type and mutant p53. The cells (PC3+p53 and mutant p53) cells were stained with SA-b-galactosidase. (B, C) The blue nuclei due to SA-bgalactosidase staining were counted in 15 randomly selected fields and expressed as mean±SEM. The flattened nuclei with intense blue staining were classified as cells with High senescence and smaller light blue nuclei were counted as cells with low-moderate senescence.

4.5 ID4 Regulates p53 Regulatory Mechanisms: p14ARF-MDM2 Interaction

In the PC3+p53 (wild-type and mutant) (+/-) ID4 cellular model total MDM2 protein was first immuno-precipitated and then immuno-blotted with p14ARF antibody from all cell lines. Increased p14ARF was observed in PC3 cells with stably overexpressed wild type and mutant p53 as compared to PC3 cells transfected in the same manner with ID4shRNA (Figure 15A). p14ARF serves as a hyper-proliferative sensor for
the cell and directly interacts with MDM2 to regulate p53 biological activity.\textsuperscript{117a}

Moreover, when we immuno-blotted for p53 we saw decreased expression as compared to PC3 cells where ID4shRNA was used. This result suggested that ID4 promoted the sequestering of MDM2 via p14ARF allowing p53 activation. In contrast, immuno-blot for p53 in the absence of ID4 (ID4shRNA). An increase in p53 expression was observed suggesting that MDM2 was still bound to p53. These results suggested that p53 may be targeted for degradation by its E3 ubiquitin ligase MDM2 and hence not functional (Figure 15). These results provided evidence that ID4 promotes interaction between MDM2 and p14ARF one of the most common regulatory mechanisms of p53.\textsuperscript{117a}

![Figure 15. ID4 regulates p53 via p14ARF and MDM2 interaction.](Image)

4.6 ID4 Restores Mutant p53 DNA Binding and Transcriptional Activity

An electrophoretic shift assay (EMSA) with canonical p53 DNA response element was used to determine the DNA binding ability of wild-type (LNCaP+NS) and mutant p53 (DU145). LNCaP+NS cells with wild-type p53 resulted in a gel shift (Figure 16A) whereas, a gel shift of lower intensity was observed in LNCaP-ID4 as compared to
LNCaP+NS cells possibly due to lower expression of wild-type p53 (Figures 16A). 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 mutant 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 16B). 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 16C), which is consistent with the expression of p53 in these cell lines. Surprisingly, mutant 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.

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 16D), p21 (Figure 16E) and PUMA (Figure 16F) 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 16 E-F). As anticipated, in DU145 no significant binding of mutant p53 was observed on p21, PUMA and BAX promoters (Figures 16E-F). However, in DU145+ID4 cells, a significant increase in the binding of mutant p53 as compared to DU145 was observed on BAX, p21 and PUMA promoters (Figures 16E-F).

These observations lead us to investigate whether wild-type and mutant p53 in PC3 cells would also have the ability to regain DNA binding and transcriptional activity in an ID4 dependent manner. We used a p53 DNA binding activity assay that utilized nuclear extracts from our PC3 system. We found increased p53 DNA binding activity using p53 response element immobilized on a 96 well plate followed by detection with p53 specific antibody in the PC3+p53 (wild-type and mutant p53) in the presence of ID4 as compared the ID4 silencing model in PC3 cells where we observed a significant decrease in p53-DNA binding. (Figure 17A). In a functional transcriptional assay using a p53 response element (wt-p53RE) luciferase reporter plasmid, the relative p53 luciferase activity decreased significantly in PC3+p53 (wild-type and mutant) upon ID4 silencing in cells as compared to PC3+p53 (wild-type and mutant) cells with endogenous ID4 (normalized to 1, Figure 17B,C). Surprisingly, mutant p53 (DU145 p53 mutant P223L and V274F) as well as hot spot mutants (R175H and R273H) overexpressed in cells demonstrated moderately high luciferase activity as compared to wild-type p53 overexpressed in PC3 cells (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 (Figure 17D,E). In context of using PC3+wtp53 as a positive control, our results strongly suggested that mutant p53 gains DNA binding and transcriptional activity in the presence of ID4.

Figure 16. ID4 promotes DNA binding and transcriptional activity of wild type and mutant p53 in LNCaP and DU145 cells. 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 reporter 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 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 17. ID4 promotes DNA binding and transcriptional activity of wild type and mutant p53 in PC3 cells. (A) 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 in PC3 cells with overexpressed wild type and mutant p53 (+/-) ID4. (B-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. (D-E) The mutant p53 luciferase reporter plasmid was used as a negative control (mt-p53RE). The p53-luciferase reporter activity in PC3+wtp53 and mutant p53 was normalized to PC3 and compared to PC3+wtp53 and mutant p53 (-ID4) and PC3-ID4.
4.7 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. Recent studies have also shown that acetylation of some mutant forms of p53 can restore the DNA binding activity. These studies led us to explore whether ID4 promotes acetylation of mutant 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. 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. 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 wild-type and mutant 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. 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. We hypothesized that if CBP/p300 is involved in K373 acetylation than it could co-precipitate with p53. Our results demonstrated that indeed mutant p53 is physically associated with CBP/P300 at significantly higher levels than mutant p53 from DU145 cells alone (Figure 18A). 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 (Figure 18A). These results consolidated our hypothesis that ID4 promotes the recruitment of CBP/p300 on p53 to promote acetylation and restore its biology.

Moreover, we also investigated the acetylation profile used in the previous study via a histological examination of prostate xenografts using (+/-) ID4 model system. LNCaP+NS, LNCaP -ID4, DU145, and DU145+ID4 cells were subcutaneously injected into the flanks of non-castrated nude male mice. Increased expression of Global lysine acetylation, acetylated lysine 320 and 373 in xenografts of LNCaP +NS and DU145+ID4 were observed (Figure 18B). Tumors obtained from LNCaP-ID4 and DU145 xenografts saw significant decrease in acetylation markers (lysine 320 and 373) in the absence of ID4 (Figure 17B) Also, we observed modulation of total p53 in an ID4 dependent manner in prostate xenografts (Figure 18B). Upon ID4 silencing we observed decrease in total p53 expression as compared to LNCaP+NS and DU145 xenograft tissue (Figure 18B). To compliment this study we performed a TUNEL assay to examine apoptosis, a marker of
cellular death. The degree of apoptosis by TUNEL assay in the xenografts was used to understand the mechanism of tumor regression in response to various treatments.

Representative examples of apoptosis at the conclusion of the experiments are shown in Figure 18C. The apoptotic index (no. of apoptotic nuclei/ total number of nuclei counted in 5 random fields) clearly suggested a significant increase in apoptosis in xenografts in LNCaP and DU145+ID4 as compared to LNCaP-ID4 and DU145. The increase in the number of apoptotic nuclei (dark brown) in LNCaP and DU145+ID4 groups as compared to LNCaP-ID4 and DU145, control is clearly visible in the images. We observed an increase in TUNEL in ID4 positive xenografts and compared to ID4 negative xenografts where we observed decrease in TUNEL staining indicating less apoptosis (Figure 18C). This result was consistent with our findings in our in vitro studies and correlated well with our acetylation profile (+/-) ID4.

To further elucidate the mechanism of activation of mutant p53 to wild-type function, we investigated whether ID4 could promote a similar acetylation profile in the highly metastatic cell line PC3 as shown in LNCaP+NS and DU145+ID4 xenografts through immunohistological studies. 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. The results shown in Figure 18D demonstrated a significant increase in global p53 acetylated lysine and K373 acetylation in PC3+p53 (wild type and mutant) cells whereas there was a significant decrease observed in both global p53 lysine acetylation and K373 acetylation (Figure 18D). We also used an acetyl-mimic model to
study the effects of p53 acetylation and deacetylation (acetyl-mimics: K320Q/R and K373Q/R) via the study of site-specific residues critical to the regulation of p53’s activity (Figure 19A). By overexpressing a collection of p53-R175H acetylation-mimic mutants in PC3 cells, we show that specific acetylation at K373, and to a lesser extent at K320, are sufficient for inducing p53 target gene dependent transactivation apoptosis and restoration of DNA-binding capabilities. Interestingly, we show that the inhibitory effect of deacetylation/non-acetylation (373R and 320R) has on p53 induced apoptosis and DNA-binding capability seemingly disrupting the global transcriptional program of p53 by decreasing these activities (Figures 19B-C). Moreover, we find that ID4 was able to preferentially modulate apoptosis and DNA-binding capabilities at higher rate via acetyl-mimic K373Q as compared to K320Q suggesting the K373 is most critical for ID4 dependent p53 acetylation. Remarkably, our study reveals that a single lysine, K373 and/or K320, in the c-terminal region of p53 can be acetylated to provide mutant forms of p53 DNA-binding and apoptotic capabilities. We however did observe the K320R and K373R (deacetylation mimics) mutants showed some relative luciferase activity as well as apoptosis, although not significant compared to K320Q and K373Q, respectively. Interestingly, Liu et al. reported activation of downstream target a p21-thymidine kinase construct was modest (15%) with deacetylation mimics; this result might have been due to the presence of two flanking lysines (K319 and K321), one of which might be an optional binding site for PCAF³, which could provide justification of similar apoptotic activity and luciferase activity we observed as well with both K320 and K373. Flanking lysines may be sufficient for acetylation of mutant p53 by acetyl-transferases. Thus, these results...
further consolidate our hypothesis that ID4 acts as a tumor suppressor via p53 by promoting acetylation of mutant p53 restoration to its wild-type biological activity.

Figure 18. Acetylation of p53 and interaction with CBP/p300 and ID4. (A) p53, immuno-precipitated from cell lines LNCaP, LNCaP-ID4, DU145, and DU145+ID4 was blotted with antibodies against acetylated lysine (global), p53 acetylated at either K373 (Ac-373) or K320 (Ac-320), ID4, and CBP/p300. (B) Representative images of xenografts from non-castrated nude male mice. Brown immunostaining of acetylated lysine (global), K373 (Ac-373), K320 (Ac-K320), and p53. (C) Representative images of xenografts from castrated SCID male mice, brown immunostaining of TUNEL indicating apoptosis. (D) p53, immuno-precipitated from cell lines PC3+wtp53 and mutant p53 was blotted with antibodies against acetylated lysine (global), p53 acetylated at either K373 (Ac-373).
Figure 19. ID4 promotes p53-R175H transcriptional activity and apoptosis. (A) p53-R175H Acetyl-mimic and deacetylation mimics constructs. (B) 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. (D-E) The mutant p53 luciferase reporter plasmid was used as a negative control (mt-p53RE). The p53-luciferase reporter activity in PC3+mimics was normalized to PC3+NS and compared to PC3+mimics (-ID4) and PC3+shID4. (C) A significant increase in apoptosis (***: P<0.001) was observed in PC3+320Q+373Q and PC3+320R+373Q compared with PC3+320R+373R and PC3+320Q+373R. A significant decrease in apoptosis was observed in PC3 cells that lacked ID4 (PC3-ID4) compared PC3 cells (PC3, ***: P<0.001).
4.8 Molecular Dynamics: Studies of Mutations in p53

The calculated energies based on the simulated annealing of the wild type and mutated core domain of p53 is shown in Table 4 (and Figure 20A). The total calculated energies ($E$) and normalized calculated energies per atom ($E_a$) were lower for wild type p53 (-19244 and -2.123 kcal/mol, respectively) as compared to the normalized energies per atom of hot spot mutants R175H and R273H (-2.0 kcal/mol), suggesting that the wild type p53 core domain was more thermodynamically stable as compared to the hot spot mutants. In fact the $E_a$ of the two temperature sensitive mutants P223L and V274F were essentially similar to the $E_a$ of wild type suggesting similar thermodynamic stabilities. The same data (Table 4) plotted with wild-type set as a reference point clearly shows the magnitude of energy differences of the various core domain mutants (Figure 20B). The $E_a$ between the wild type and R175H and R273H have more difference in minimized energy to the wild type and with temperature sensitive mutants.

Figure 20. (A) Difference in calculated energy between mutated proteins and the wild type based on results of simulated annealing optimization. (B) Arginine mutation, R248Q, compared with previous simulations.
The stability of wild type p53 core domain is 6.0 kcal (1 kcal = 4.18 kJ)/mol at
25°C and 9.8 kcal/mol at 10°C based on denaturation curves measured by differential
scanning calorimetry/spectroscopy\(^{136}\) suggested that the p53 core domain is of moderate
thermodynamic stability. Based on the above method the calculated equilibrium
denaturation of p53 core domain at 10°C was -2.97 kCal mol\(^{-1}\) M\(^{-1}\).\(^{136}\) The calculated
equilibrium denaturation at 10°C of p53 mutants R175H, C242S, R248Q, R249S AND
R273H WAS -2.59, -2.68, -2.91, -3.09 and -3.11 kCal mol\(^{-1}\) M\(^{-1}\) respectively.\(^{136}\) Except
R249S and R273H the equilibrium denaturation at 10°C of p53 mutants was lower than
the wild type, possibly because these mutants appear to part of the DNA binding domain.
In contrast, the simulated normalized (per atom) calculated energy difference in our study
at least for R273H is less than the respective wild type p53 core domain (Table 4 and
Figure 20). Functionally the R273H is also a gain of function mutant found in majority of
cancers.

As compared to the temperature sensitive mutants, the effect of mutating an
arginine is noticeable; changing an arginine to a histidine in two different positions shows
the similarity of impact on minimized energy. Therefore, to understand whether the
energy differences were specifically due to mutating arginine to histidine, we simulated
the minimum energy calculations using another arginine mutation, R248Q. R248Q is
also a common mutation in many cancer types. Table V shows the minimized energy \((E_a)\)
of R248Q. The energy difference between R248Q and wild type in context of R273H
and R175H, shown in Figure 20B suggests that arginine residue places wild type p53 in
lower energy with respect to the mutants. It is therefore not surprising that majority of
mutations involve the arginine residue that alters the minimum energy of p53. Thus, irrespective of whether arginine mutations are structural (R175H) or functional (R273H), targeting an arginine residue appears to play a very significant role in thermodynamic stability of the p53 core domain.

We hypothesized that replacing arginine, the third heaviest residue in terms of molar mass, by a lighter residue may decrease thermodynamic stability. To study the effect of mutating a residue on the overall energy, we focused on linking the specific mutation to the overall bonded terms in energy. As stated in the methods section, we used a dimensionless quantity, to establish a correlation between the mutated and the wild type protein. Table VI summarizes of results for p53 core domain mutants, which are also shown in Figure 21.

![Figure 21. Approximation (μ) factor in correlation between residue molar masses and potential energies for mutated protein](image-url)
Table 6. Calculation of $\mathcal{P}$, a Dimensionless Approximation Entity for the Nearest Neighbors of the Mutant Residue

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</tbody>
</table>

A comparison between approximation in Figure 21 and simulation in Figure 20A indicates that two different methods place mutated proteins in a similar context as compared to the wild type. Since $\mathcal{P}$ was a calculated approximation of mutants and its 2 immediate neighbors with an approximation similar to that achieved by simulations in CHARRM, we therefore used a similar approximation for additional core domain p53 mutants found in various cancers (Table VII). The results indicate that changes in potential energy depend not only on the molar mass of the target residue, but also to at least two other residues near the target residue. If two different mutations label the same residue position (e.g. R248Q & R248W), replacement by the lighter residue (Q) in molar mass, sets protein at the higher potential energy. Moreover, when the target and
replacement residues are the same (R175H & R273H), in the different positions, then changing the potential energy will be determined by the molar mass of near-neighbor residues. Mutations may present similar results (R248W & R282W) in our approximation if two near-neighbor residues are very similar or the same in molar masses. Every mutation with μ > 1 in our results referred to a higher frequency in human cancer in p53. The rate of mutation in p53 in cancer references compares to μ factor (Table 8). Our approximation therefore matches to p53 mutations in cancer adequately.  

Table 7. Results for More Mutations in p53 Based on our Approximation

<table>
<thead>
<tr>
<th>Mutated protein</th>
<th>μ</th>
<th>3 sequence residues in the wild type</th>
<th>3 sequence residues in the mute protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>R248Q</td>
<td>1.0578</td>
<td>ASN-ARG-ARG</td>
<td>ASN-GLN-ARG</td>
</tr>
<tr>
<td>R248W</td>
<td>0.9556</td>
<td>ASN-ARG-ARG</td>
<td>ASN-TRP-ARG</td>
</tr>
<tr>
<td>G245S</td>
<td>0.8858</td>
<td>GLY-GLY-MET</td>
<td>GLY-SER-MET</td>
</tr>
<tr>
<td>R273C</td>
<td>1.1101</td>
<td>VAL-ARG-VAL</td>
<td>VAL-CYS-VAL</td>
</tr>
<tr>
<td>R282W</td>
<td>0.9555</td>
<td>ASP-ARG-ARG</td>
<td>ASP-TRP-ARG</td>
</tr>
<tr>
<td>R282S</td>
<td>1.1981</td>
<td>ASN-ARG-ARG</td>
<td>ASN-SER-ARG</td>
</tr>
<tr>
<td>G245D</td>
<td>0.8253</td>
<td>GLY-GLY-MET</td>
<td>GLY-ASP-MET</td>
</tr>
<tr>
<td>D281G</td>
<td>1.3058</td>
<td>ARG-ASP-ARG</td>
<td>ARG-GLY-ARG</td>
</tr>
<tr>
<td>R240K</td>
<td>1.0413</td>
<td>GLY-ARG-ASP</td>
<td>GLY-LYS-ASP</td>
</tr>
<tr>
<td>R174Y</td>
<td>0.9889</td>
<td>VAL-ARG-ARG</td>
<td>VAL-TYR-ARG</td>
</tr>
<tr>
<td>V143A</td>
<td>1.1169</td>
<td>PRO-VAL-GLN</td>
<td>PRO-ALA-GLN</td>
</tr>
<tr>
<td>L194F</td>
<td>0.9276</td>
<td>HIS-LEU-ILE</td>
<td>HIS-PHE-ILE</td>
</tr>
</tbody>
</table>
Table 8. The Overall Frequency of Mutations in Human Cancer and $\mu$ Factor (Approximation). The minimum percentage of mutation in biology references matches to our prediction (underlined mutated protein)

<table>
<thead>
<tr>
<th>Mutated protein</th>
<th>$\mu$ factor</th>
<th>Percentage of mutation frequency in biology</th>
</tr>
</thead>
<tbody>
<tr>
<td>R248Q</td>
<td>1.0578</td>
<td>3.50%</td>
</tr>
<tr>
<td>R248W</td>
<td>0.9556</td>
<td>2.80%</td>
</tr>
<tr>
<td>G245S</td>
<td>0.8858</td>
<td>2.80%</td>
</tr>
<tr>
<td>R273C</td>
<td>1.1101</td>
<td>2.70%</td>
</tr>
<tr>
<td>R282W</td>
<td>0.9555</td>
<td>2.40%</td>
</tr>
<tr>
<td>G245D</td>
<td>0.8258</td>
<td>0.68%</td>
</tr>
<tr>
<td>R273H</td>
<td>1.0303</td>
<td>3.10%</td>
</tr>
<tr>
<td>R175H</td>
<td>1.0379</td>
<td>4.50%</td>
</tr>
</tbody>
</table>

4.8.1 Solvation

All the energy calculations described above are for minimized protein structure in the vacuum as an approximation and reduce noise. Since the proteins rarely exist in a dehydrated/vacuum space, we therefore calculated the energies of the minimized structure of wild type and four mutated p53 core domains solvated in a box of water as large as the dimensions of each protein individually (block of water) (Figure 22A-E). The calculated energy in the vacuum and solvation for the wild type and mutant proteins was compared (Table 9). The results suggested that there is no significant change in calculated the energy in the vacuum as compared to the block of water.
Figure 22. Wild-Type protein (A), mutated protein V274F (B), mutated protein R273H (C), mutated protein R175H (D) and mutated protein R175H (E) in a box of water individually. Each protein sets in a box of water as large as protein dimensions.

Table 9. The Calculated Energy of Solvation and the Vacuum for the Wild-type and Four Mutated Proteins

$E_{(0)}$ = Energy of protein in the vacuum.  
$E_{(2)}$ = Energy of protein in the box of water.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Na (protein)</th>
<th>Na (water)</th>
<th>$E_{(0)}$ kcal/mol</th>
<th>$E_{(2)}$ kcal/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>W-T</td>
<td>9063</td>
<td>33498</td>
<td>-17434.8</td>
<td>-17434.0</td>
</tr>
<tr>
<td>R175H</td>
<td>9042</td>
<td>33234</td>
<td>-16653.0</td>
<td>-16652.2</td>
</tr>
<tr>
<td>R273H</td>
<td>9042</td>
<td>32367</td>
<td>-16706.9</td>
<td>-16714.8</td>
</tr>
<tr>
<td>V274F</td>
<td>9075</td>
<td>33303</td>
<td>-17373.8</td>
<td>-17373.0</td>
</tr>
<tr>
<td>P223L</td>
<td>9078</td>
<td>34200</td>
<td>-17400.2</td>
<td>-17401.5</td>
</tr>
</tbody>
</table>
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. Since LNCaP cells express ID4, it was determined to be an ideal model in which to silence ID4. Upon silencing of ID4, there was a decrease in apoptosis via increase in 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. So the increased proliferation of LNCaP-ID4 cells may be due to decreased p27kip1 expression.

5.2 ID4 Regulates Wild-type and Mut-p53 Biological Activity

The molecular mechanism by which wild type and mutant p53 may function has been reviewed extensively. These mechanisms include: p53 DNA-binding capabilities and transactivation of p53-dependent downstream targets. Mutant p53 function has been characterized by alterations in the DNA-binding ability of mutant p53, interaction of mutant p53 with other proteins, including transcription factors or proteins not directly
related to the regulation of gene expression.\textsuperscript{4} It is clear from our study herein that the effects of mutant p53 are strongly context dependent in regard to ID4. We provide evidence that ID4 regulates p53 regulatory mechanisms in PC3 cells where we have overexpressed wild type and mutant p53. MDM2 and p14ARF, key players in p53 regulation of p53 are both modulated in the presence of ID4. Upon silencing of ID4 we observe increased co-elution of p53 as part of the MDM2-p14ARF-p53 complex, suggesting that MDM2 an E3 ubitiquin ligase for p53 is tightly bound to p53 and possibly targeting p53 for degradation. In this study we study both DU145 mutants (P223L and V274F) and hotspots mutants (R175H and R273H) DNA binding capabilities as well as transactivation potential. When wild type and mutant forms of p53 were overexpressed in PC3 cells we observed increased regulation of p53 downstream targets (p21, BAX, and PUMA) and upon ID4 silencing we observed decreased expression of all studied downstream targets of p53 in both DU145 mutant forms of p53 as well as hotspot mutants. We found that when wild type and mutant forms of p53 in the presence of ID4 were overexpressed in PC3 cells we increase in p53-mediated apoptosis and senescence, suggesting that ID4 is playing a role in the biological activity of both wild type and mutant forms of p53.

5.3 ID4 Dependent Acetylation Restores Mutant p53 Transcriptional Activity

In the case of DU145 specific mutants (P223L and V274F) and hotspot mutants (R175H and R273H) we further establish that ID4 dependent acetylation is necessary for certain contact and structural mutant forms of p53 to gain sequence specific DNA-binding and subsequent transactivation of downstream targets of p53. Increased apoptosis
and senescence, p53-dependent processes observed in DU145 cells in the presence of ID4 further supports the role of ID4 in restoring the biological activity of mutant p53. Based upon our earlier observation, we speculate that ID4 dependent regulation of p53 is mediated via acetyltransferases such as CBP/p300 or by additional posttranslational modifications that occur in an acetylation dependent manner. It has been well established that several C-terminal lysines (evolutionarily conserved across species) of p53 (K370, K372, K373, K381, K382) are acetylated by CBP/p300. Acetylation of residues found in the C-terminal of p53 promotes an open conformation by inhibiting the ability of its C-terminus to bind and occlude the DNA binding domain, thereby enhancing p53 transcriptional activity. Inhibition of the C-terminal binding and occluding DNA via acetylation poses acetylation as a global mechanism of restoration of mutant p53.

Studies have shown that acetylation of p53 by CBP/p300 is generally considered activating, but that is not always the case. The biological consequences of p53 acetylation by CBP/p300 may depend significantly on cellular context, such as cell type and/or transformation status (i.e., primary, immortalized or tumor-derived). For example, p300-mediated acetylation of p53 in human cancer cell lines has been shown to be essential for p21 promoter transactivation and cell cycle arrest. Yet analyses of primary mouse embryo fibroblasts (MEFs) lacking CBP/p300 revealed those factors are not required for p53-mediated upregulation of p21 and MDM2 following DNA damage, although it is notable that the magnitude of their induction was reduced.

Cell type differences also influence the role of CBP/p300 in apoptosis. Most studies agree with early findings that CBP/p300 is required for p53 acetylation and p53-
dependent apoptosis. Numerous cell types have been examined in the analyses, including primary MEFs and various human cancer lines. In contrast, loss of CBP/p300-mediated p53 acetylation in HCT116 colorectal cells resulted in increased expression of PUMA and apoptosis following DNA damage, suggesting CBP/p300 expression normally suppresses p53-dependent apoptosis in those cells. Intriguingly, CBP/p300 may act similarly in neurons. It was recently shown that p53 acetylation at K381 and K382 in neuronal cells specifically inhibits p53 binding to the PUMA promoter, preventing PUMA expression and DNA damage-induced cell death.

In our studies we used an acetyl-mimic model to study the effects of p53 acetylation and deacetylation (acetyl-mimics: K320Q/R and K373Q/R) by studying site-specific residues critical to the regulation of p53’s activity. By overexpressing a collection of p53-R175H acetylation-mimic mutants in PC3 cells, we show that specific acetylation at K373, and to a lesser extent at K320, are sufficient for inducing p53 target gene dependent transactivation apoptosis and restoration of DNA-binding capabilities. Interestingly, we show that the inhibitory effect of deacetylation/non-acetylation (373R and 320R) has on p53 induced apoptosis and DNA-binding capability seemingly disrupting the global transcriptional program of p53 by decreasing these activities. Moreover, we find that ID4 may be preferentially modulating apoptosis and DNA-binding capabilities at higher rate via acetyl-mimic K373Q as compared to K320Q suggesting the K373 is most critical for ID4 dependent p53 acetylation. Although not significant we did observe K320R and K373R (deacetylation mimics) relative luciferase activity as well as apoptosis as compared to K320Q and K373Q acetyl-mimics.
Interestingly, Liu et al.\textsuperscript{122a} found similar results when they reported activation of downstream target a p21-thymidine kinase construct was modest (15\%) with deacytlation mimics; this result might have been due to the presence of two flanking lysines (K319 and K321), one of which might be an optional binding site for PCAF, which could also provide an explanation of apoptotic activity and luciferase activity observed in our model with both K320R and K373R deacetylation mimics.

It is also clear from our study herein that the effect of ID4 dependent acetylation of p53 has an affect on other known p53-mediated events in the cell. We provide evidence that ID4 regulates target genes of p53: BAX, PUMA, and p21, which are critical to p53-dependent cell cycle arrest and apoptotic event and modulation of MDM2 and p14ARF, key players in p53 regulation.

As discussed the main E3 ubiquitin ligase and negative regulator of p53 is MDM2, MDM2 is itself a transcriptional target of p53 that functions in a critical negative feedback loop to restrict p53 levels in non-stressed cells.\textsuperscript{207} Importantly, ubiquitin ligases negatively regulate the acetylation of p53 through several different mechanisms. First, MDM2 ubiquitylates the acetyltransferases CBP/p300, PCAF, and Tip60, which targets those proteins for degradation at the proteasome, thereby reducing their expression and ability to acetylate p53.\textsuperscript{231} Other studies have shown that C-terminal lysines of p53 (K370, K372, K373, K381, K382) are competitively targeted for either acetylation or ubiquitylation as well.\textsuperscript{231}

Additional crosstalk between ubiquitylation and acetylation occurs because some p53 acetyltransferases, CBP/p300 and PCAF, are dual-capability enzymes with ubiquitin
ligase functions. Specifically, CBP/p300 can function as an E4 ubiquitin ligase for p53 and cooperate with MDM2 to promote p53 polyubiquitylation.\textsuperscript{236} Also, interestingly it has been reported that changes in p53 phosphorylation can either facilitate or inhibit the binding of specific cofactors of p53, which can then block or promote acetylation. For example, N-terminal phosphorylation of p53 at S15, T18, S20 and/or S37 blocks MDM2 association, with multisite phosphorylation more effectively than isolated phosphorylation events.\textsuperscript{231} Subsequent stabilization of p53 accompanied by reduced modification of lysines with competing ubiquitin fosters increased acetylation. Additionally, N-terminal p53 phosphorylation enhances binding to its acetyltransferases.

The impact of other p53 post-translational modifications, such as methylation, sumoylation, and neddylation, on p53 acetylation and functions is generally not well defined. Methylation has been shown to counteract, cooperate with or not affect p53 acetylation and activation depending on the site of modification and the study. p53 is methylated at K370 by Smyd2 (Set/MYND Domain-2), K372 by Set 7/9 (Su(var)3-9 and “Enhancer of zeste” protein 7/9), and K382 by Set8 (Su(var)3-9 and “Enhancer of zeste” protein 8).\textsuperscript{231} Methylation of K370 and K382 inhibits p53 DNA binding and transcriptional activity, and K382 methylation was shown to impair K382 acetylation.\textsuperscript{237}

There are currently not many investigators exploring p53 sumoylation and its impact on p53 acetylation. In fact, the functional consequences of sumoylation on p53 activity in general remains unclear. Some early reports indicated that sumoylation promotes p53 recruitment into PML nuclear bodies and transcriptional activation.\textsuperscript{231} Neddylation of p53 has not been thoroughly studied, but still poorly understood. It can
occur on K370, K372, and K373 by MDM2 and on K320 and K321 by FBXO11 (F-box protein 11), and in each case it is associated with inhibition of p53-mediated transcription. The effect of p53 neddylation on its acetylation remains to be determined but it is predicted to interfere since both modifications occur on the same lysines.

The acetylation mechanism is nearly universal in nature and is suggestive from our studies that ID4 could promote acetylation of other known mutants as well; however, it is understood that sufficient structural flexibility of mutant forms of p53 are essential for recruitment of ID4 and macromolecular assembly of co-factor to p53, which could promote a similar acetylation pattern as shown in our studies (Figure 23). A profile of other known p53 hotspot mutants and study of their inherent structural flexibility in context of ID4 dependent acetylation is an area of great interest, which could provide a universal method of restoration of mutant p53 to that of wild type p53 function.

**Figure 23.** ID4-p53 biological network. A representative image of ID4-p53 cross-talk. Green arrows are representative of up-regulation, whereas blue represents physical interaction.
Studies including theoretical modeling of p53, and the p53 conformation changes induced by protein-protein and protein-ligand interactions, including peptide and small molecular inhibitors have significantly expanded our current understanding of the dynamic nature of p53 structure.\textsuperscript{133-134} While there have been several studies discussing the dynamic simulations of p53 in recent years, the dynamics and flexibility of mutation induced conformational changes of p53 have not been completely characterized or understood.

5.4 Molecular Dynamic Simulation of Mut-p53 is Predictive and Correlative

The current study evidenced herein confirmed that our method of molecular dynamic simulation of mutant forms of p53 can be predictive and correlative of the rate of mutation in human cancer. Consequently, further investigations may be required before these findings can be generalized and applicable to diverse p53 mutants. Nonetheless, the R175H and R273H mutations are highly relevant to human cancers as well as many other hotspot mutations studied herein. A typical search of the IARC database establishes the prevalence of R175H and R273H as a somatic mutation in numerous different tumors and as a germline mutation in families with Li Fraumeni syndromes.\textsuperscript{132e}

The less frequently occurring p53 mutations (P223L and V274F used in this study) have been shown to essentially act as wild type at lower temperatures suggesting intrinsic flexibility. It is therefore not surprising that the calculated energies of these two mutants are similar to the wild type p53. Thus taken together our studies reveal that p53’s intrinsic flexibility can yield promising therapeutic avenues for restoration of mutant p53.
biological processes. This study via computational analysis aims to provide an understanding of p53 core domain structure and how manipulation of missense mutations found in p53 via simulation can be predictive of the rate of frequency of p53 mutations found in human cancer, thus giving insight to what therapeutic avenues could be most suitable for p53 restoration.
CHAPTER VI

CONCLUSION

Even though p53 is one the best-studied genes as the result of 30 years extensive research, our comprehension and appreciation of its complexity in regulating many crucial biological processes are far from complete. It is indisputable that p53 suppresses tumorigenesis via the canonical pathway of inducing apoptosis and/or senescence and non-canonical pathways, some of which are still emerging.\textsuperscript{239} Conversely, p53 dysfunction-induced tumorigenesis is mediated by loss of cell cycle arrest and apoptosis, as well as by compromising host immune surveillance.\textsuperscript{116, 240}

Since many p53 mutants are associated with a conformational change that hinders its DNA binding and transactivation capacity, it is rationalized that small molecules that revert the mutant p53 to its wild-type configuration will restore p53 function. Indeed, based on crystallographic structural and computational analyses, PRIMA-1 (p53 reactivation and induction of massive apoptosis-1) and MIRA-3 (mutant p53 reactivation and induction of rapid apoptosis \textit{in vivo}) were developed to convert mutant p53 and restore p53 function leading to effective activation of downstream apoptosis-inducing targets, such as caspase-2, PUMA, and BAX.\textsuperscript{138, 241}
As in these previous studies, given the unique and the inherent conformational flexibility of p53 we also used computational analyses to study wild type and mutant p53 that could eventually help to develop strategies to revert mutant p53 to its wild type configuration. The study presented herein also provided evidence that molecular dynamic simulation of mutant forms of p53 can be predictive and correlative of the rate of mutation in human cancers. In addition to our computational analysis of the core domain of wild type and mutant p53, we also investigated a novel physiological mechanism of restoration of mutant p53 via an hlh transcriptional regulator ID4.

In many cancers, ID4 has been found to be hyper-methylated suppressing expression and function of ID4. Previous studies form our laboratory found that upon overexpression of ID4 in prostate cancer cell line DU145, in which ID4 is methylated there was an induction of senescence and apoptosis. Two genes primarily involved in senescence and apoptosis (RB and p16) are found to be non-functional in DU145 cells, whereas p53 is found to be mutated in this cell line,\textsuperscript{44} however when ID4 was overexpressed recovery of apoptosis and senescence was observed. The overlap in biological function of ID4 and p53 led us to investigate possible connections between both genes. From previous studies, we found that ID4 regulates the activity of wild type and mutant p53 via the assembly of a macromolecular complex involving CBP/p300 that resulted in acetylation of p53 at K373, a critical post-translational modification required for its biological activity.\textsuperscript{43} Furthermore, the study herein also found that ID4 could regulate wild type and mutant p53 when overexpressed in a highly metastatic cell line PC3, which complimented our prior investigation of ID4-p53 cross-talk in LNCaP and
DU145 cells. Moreover, we observed that ID4 had a similar effect on hotspot p53-mutants, most frequently found in cancers. Taken together, our present study finds that ID4 plays a pivotal role in modulation of acetylation and transactivation of p53 in context of both DU145 specific p53 mutants and hotspot mutants overexpressed in PC3 cells providing clear evidence that ID4 may serve as a physiological agent to restore p53 wild-type function in a non-cell line/non-mutation specific manner given ID4 mediated mutant p53 restoration to wild type biological function in the PC3 cell line. While this data is intriguing, it is of great interest to determine if this phenomenon of p53 restoration via ID4 is effective in other organ systems as well as with a variety of other p53 mutations. Continued computational analysis along with further studies elucidating the mechanism of action will help to unravel the complex biological processes of ID4 and p53 in cancer. The strong anti-cancer effect of ID4 in prostate cancer and cross-talk with p53 will provide a strong framework of study in other systems, which eventually could serve as an attractive therapeutic approach.
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