Theses and Dissertations--Toxicology and Cancer Biology

Toxicology and Cancer Biology

2015

CROSS-TALK BETWEEN THE TUMOR SUPPRESSORS PAR-4 AND P53

Tripti Shrestha Bhattarai
University of Kentucky, tsbhat2@uky.edu

CROSS-TALK BETWEEN THE

TUMOR SUPPRESSORS PAR-4 AND P53

_____

Dissertation

_____

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in
The Graduate School in the University of Kentucky

By

Tripti Shrestha Bhattarai

Director: Dr. Vivek Rangnekar, Professor, Department of Radiation Medicine

University of Kentucky

Lexington, Kentucky

2015

ABSTRACT OF DISSERTATION

CROSS-TALK BETWEEN THE TUMOR SUPPRESSORS

PAR-4 AND P53

This work describes the fascinating interplay between two tumor suppressors Prostate apoptosis response-4 (Par-4) and p53. The guardian of the genome, p53, is frequently mutated in human cancers, and may contribute to therapeutic resistance. However, p53 is intact and functional in normal tissues, and we observed that specific activation of p53 in normal fibroblasts could induce apoptosis selectively in p53-deficient cancer cells. This paracrine apoptotic effect was executed by Par-4 secreted in response to p53 activation. Accordingly, activation of p53 in wild-type mice, but not in $p53^{-/-}$ or $Par-4^{-/-}$ mice, caused systemic elevation of Par-4 that induced apoptosis of p53-deficient tumor cells. Mechanistically, p53 induced Par-4 secretion by suppressing the expression of UACA (Uveal Auto-antigen with Coiled-coil domains and Ankyrin repeats), a binding partner of Par-4, and thereby releasing Par-4 from sequestration by UACA. Thus, normal cells can be empowered by p53 activation to induce Par-4 secretion for inhibition of therapy-resistant tumors.

Conversely, our studies have also revealed a definite role for Par-4 in regulating p53 expression. The pro-apoptotic tumor suppressor Par-4 is lost, down-regulated, inactivated or mutated in a number of cancers. Loss of Par-4 is associated with therapeutic resistance and poor disease prognosis, yet the mechanism for resistance is not clearly understood. Using genetically matched cells, we show that Par-4 expression is required for stabilization and function of the tumor suppressor p53, which constitutes the hub of signaling networks controlling important cellular and organismal phenotypes. In particular, the expression of p53 protein and its stabilization in response to genotoxic stress were remarkably attenuated in response to Par-4 loss. Accordingly, Par-4-null or -knockdown cells demonstrated increased resistance to apoptosis induced by genotoxic stress. Par-4 loss resulted in elevated Mdm2 activity, which is known to cause p53 degradation. Our findings suggest that Par-4 stabilizes p53 by inhibiting Akt-mediated phosphorylation of Mdm2 that is known to prevent

translocation of Mdm2 into the nucleus for p53 ubiquitination and degradation. These studies identify a novel regulatory relationship between two tumor suppressors and may provide a better understanding of therapeutic resistance in tumors with p53 wild type status.

Tripti Shrestha Bhattarai
Student's Signature

5/3/2015
Date

CROSS-TALK BETWEEN THE TUMOR SUPPRESSORS

PAR-4 AND P53

By

Tripti Shrestha Bhattarai

Vivek Rangnekar, PhD_____
Director of Dissertation

Isabel Mellon, PhD_____
Director of Graduate Studies

5/3/2015___
Date

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER ONE: INTRODUCTION

In the United States, cancer is ranked second only to heart disease in terms of the total number of deaths; and, in adults belonging to the age group 40-79 years, it is the leading cause of death (Siegel et al., 2014). According to the report prepared by the International Agency for Research in Cancer (IARC), cancer is the leading cause of mortality worldwide- an estimated 8.2 million deaths was caused by cancer alone in 2012. Furthermore, cancer cases are projected to increase by 75% globally, and reach close to 25 million over the next two decades (World Cancer Report 2014). The global burden of cancer continues to increase, and represents one of the most formidable public health problems.

Extensive research in the field of cancer biology over the past few decades has made it possible to appreciate the remarkable diversity and intricate complexities found within this disease. It is now widely accepted that tumorigenesis involves a multi-step process leading to an accumulation of genetic aberrations. Many adult human tumors reflect 4-7 rate-limiting, stochastic mutation events. Often such changes reflect a dominant gain of function in oncogenes, or a recessive loss of function in the case of tumor suppressors, which lead to defects in cellular regulatory processes that enable them to override cell cycle control checkpoints, and escape apoptosis, thus tipping the balance in favor of uncontrolled growth and proliferation. According to the model projected by Hanahan and Weinberg,

individual tumors will sustain as many mutation events, in as many genes, as are necessary to acquire the six traits that today are famously known as the "hallmarks of cancer". These six hallmarks are: a) self-sufficiency in growth signals; b) insensitivity to growth-inhibitory cues; c) evasion of programmed cell death (apoptosis); d) limitless replicative potential; e) sustained angiogenesis; and f) tissue invasion and metastasis (Hanahan and Weinberg, 2000). In addition, in recent times, the role of a supportive microenvironment in tumorigenesis is being increasingly appreciated. Earlier perceptions of a tumor as an insular mass of transformed cells has given way to the concept of tumor as an assemblage of distinct, specialized cell types and sub-types, which collectively constitute a complex tumor microenvironment in which the dynamic interaction between the neoplastic cells (which forms the tumor parenchyma) and the mesenchymal compartment (which forms the tumor-associated stroma) is believed to be increasingly important for tumor initiation, progression and metastasis (Hanahan and Weinberg, 2011).

TUMOR SUPPRESSOR GENES

Knudson's "two-hit theory" following his historical analysis of familial and non-familial retinoblastomas, the phenomenon of loss of heterozygosity (LOH), and his mutational analysis of the Retinoblastoma gene (Rb) thereafter, laid out the framework for the classical paradigm for tumor suppressor genes (Friend et al., 1986; Knudson, 1971). Tumor suppressor genes have been traditionally characterized as 'recessive' genes, and bi-allelic inactivation of a tumor suppressor is considered to be essential for tumorigenesis. Thus, germline

mutation in a tumor suppressor gene can be cause of familial cancer syndrome that shows an autosomal dominant pattern of inheritance. In addition, the same tumor suppressor gene is supposed to be inactivated in a number of sporadic cancers.

A functional classification categorizes tumor suppressors as a) gatekeepers; or b) caretakers; or c) landscapers (Kinzler and Vogelstein, 1996, 1998). Gatekeeper genes include all genes that have distinct function in cell cycle regulation and in suppressing proliferation, promoting differentiation, and inducing apoptotic cell death. Examples include *APC, Rb1, PTEN, Tp53, Par-4*. Loss of function of a gatekeeper tumor suppressor is 'rate-limiting' for either tumor initiation, or progression, or metastasis in multi-stage tumorigenesis. "Caretaker" tumor suppressors function by playing pivotal roles in maintaining genomic integrity and preventing aberrations such as aneuploidy and microsatellite instability. Examples of this class include DNA repair genes such as *MSH2, MLH1, BRCA1, ATM* etc. Disruption of a caretaker gene would lead to increased genomic instability and higher mutation rates, thus raising the probability of a gatekeeper being inactivated. "Landscaper" genes function by regulating the extracellular matrix, or the expression of cell surface markers, or by modulating the action of secreted proteins/growth factors, thus controlling the tumor microenvironment (Kinzler and Vogelstein, 1996, 1998; Macleod, 2000).

For the purpose of this dissertation, we will focus on apoptosis (or lack thereof) in cancer, and on the tumor suppressor genes that target apoptotic pathways as a means to cause tumor suppression and achieve tissue homeostasis.

APOPTOSIS

Apoptosis is a highly organized complex process of programmed cell death that utilizes the cellular machinery of gene transcription and translation in order to get rid of damaged or superfluous cells. Apoptosis is characterized by distinct morphological features such as chromatin condensation, DNA fragmentation, membrane blebbing, preservation of organelle structure, and containment of cellular contents within membrane-bound apoptotic bodies (Kerr et al., 1972).

Apoptotic cell death in vertebrates usually occurs via one of the two- extrinsic or intrinsic- pathways, both of which ultimately utilize the aspartate-specific cysteine protease (caspase)-mediated proteolytic cascade. Extrinsic (death receptor-initiated) pathway is triggered by the interaction of extracellular signals with cell surface death receptors of the CD95 (Apo-1 or Fas)/TRAIL/TNF receptor 1 family, or Death Receptors 3, 4 and 5 (DR3, DR4, DR5), which often leads to receptor clustering and recruitment of the adaptor protein Fas-activated Death Domain (FADD) to the intracellular effector domain of the death receptor. FADD proteins in turn recruit initiator procaspase 8 to form a death inducing signaling complex (DISC).

The intrinsic (or mitochondrial) apoptotic pathway is regulated by the Bcl-2 family of proteins, which promote or inhibit the apoptotic process by governing the activation/inactivation of mitochondrial permeability transition pore (MPTP). Based on functional relevance, this family is sub-divided into two groups:

a) Anti-apoptotic group includes Bcl-2, Bcl-xL, Mcl-1, A-1, and Bcl-W-molecules that are essential for cell survival.

b) Pro-apoptotic group is represented by proteins such as Bax and Bak, which, among others, are essential for mitochondrial outer membrane permeability (MOMP). Other pro-apoptotic members of this family, namely, PUMA, Noxa, Bim, Bad and Bmf act by sequestering Bcl-2 and Bcl-xL, and thereby inhibiting the action of these anti-apoptotic molecules.

In response to apoptotic cues such as cytotoxic stresses or developmental signals, Bax and Bak, the pro-apoptotic members of Bcl-2 family, can homo- or hetero-dimerize with other pro-apoptotic partners of the Bcl2 family to form pores on the outer mitochondrial membrane, and thereby trigger the release of apoptogenic molecules (such as cytochrome c, Smac/DIABLO) from the mitochondria into the cytosol, leading to the formation of a procaspase-activating adaptor protein called apoptotic protease activating factor-1 (Apaf1). Apaf1 then recruits and activates procaspase 9. Activation of both caspases 8 and 9 unleashes the caspase cascade by activating downstream executioner caspases (caspase-3, -6, and -7) to ultimately result in apoptosis of the cell by causing the release of the endonuclease caspase-activated DNAse (CAD) from its inhibitor iCAD, and by proteolytic cleavage of key cellular enzymes and proteins (Delbridge et al., 2012; Strasser et al., 2000).

Apoptosis and cancer

As early as 1972, Kerr *et. al.* speculated that apoptosis, a highly regulated process of "cell deletion", was crucial to maintaining normal tissue homeostasis. In addition, they theorized that this "cell loss factor" or apoptosis played a vital role in counter-balancing the rapid rate of mitosis in neoplastic growth, and the resulting equation between these two processes determined the rate of tumor enlargement. They also proposed that apoptosis may be involved in tumor regression following therapeutic intervention (Kerr et al., 1972). Subsequent studies revealed a high rate of apoptosis in spontaneously regressing tumors, as well as in tumors treated with cytotoxic anti-cancer agents (Kerr et al., 1994).

Cloning, characterization, and recognition of *Bcl-2* proto-oncogene (activated by chromosomal translocation) in B-cell follicular lymphomas consolidated the proposition that, apart from "transforming" or "immortalizing" hyper-proliferative phenotype that oncogenes were heretofore attributed with, oncogenes like *Bcl-2* could promote tumor growth by blocking programmed cell death or apoptosis (Lowe and Lin, 2000). Likewise, many human cancers demonstrate up- or down-modulation in the expression and/or functions of key molecules involved in the apoptotic process at multiple layers of regulation, thus promoting incongruous cell survival, and contributing to oncogenesis. Overexpression of Bcl-2 is observed in non-Hodgkin's lymphomas, small cell lung cancers, chronic lymphocytic leukemia, neuroblastoma and prostate cancer, and this up-regulation is also associated with a chemo-resistant phenotype (Reed, 1999; Yip and Reed, 2008). Bcl-xL is also reported to be over-expressed in multiple myeloma,

Kaposi's sarcoma, colorectal adenocarcinoma, hepatocellular carcinoma, and pancreatic cancer (Plati et al., 2011). On the other hand, loss or inactivation of the pro-apoptotic members of the Bcl-2 family leads to defects in the apoptotic pathway, which, in conjunction with an oncogenic mutation, is potentially tumorigenic. For example, homozygous deletion of *Bim* was found in approximately 20% of mantle cell lymphomas; and accordingly, Bim inactivation accelerated Eμ-myc-driven lymphomagenesis in mice. Similarly, loss of PUMA in mice also results in accelerated lymphoma development in Eμ-myc transgenic mice, and PUMA expression is also lost from nearly 40% of Burkitt's lymphoma. In addition, loss of pro-apoptotic Noxa, Bax or Bmf have been shown to promote lymphoma development in experimental mouse models, but alterations in these genes in human cancers have not yet been reported (Delbridge et al., 2012). This shows that deregulation of apoptosis might not exert a potent transforming influence; however, it predisposes cells to survive in inappropriate circumstances, and sustain other mutation(s) that can then lead to oncogenic transformation.

Dysregulation of the extrinsic or receptor-mediated apoptotic signaling pathway is also frequently observed in various cancers. Mutations in Fas or Fas ligand is known to promote lympho-proliferation, and therefore constitute a predisposition to hematopoietic malignancy in both mice and humans (Drappa et al., 1996; O' Reilly et al., 2009; Rieux-Laucat et al., 1995). In addition, many human cancers, for example, melanoma, hepatocellular carcinoma, non-small cell lung carcinoma, endometrial, colon and prostate cancers show elevated expression of

caspase-inhibitory proteins known as c-FLIP (FLICE Inhibiting Proteins), which are recruited to the DISC at the cell membrane via their Death Effector Domains (DED), and act as Caspase-8 decoys to inhibit its activation, thereby blocking death receptor-mediated apoptosis (Plati et al., 2011).

Another facet of caspase regulation involves the IAP (Inhibitors of Apoptosis) family of proteins, composed of eight members, and encoded by genes *BIRC1* to *BIRC-8*. Among these, cellular IAP1 (c-IAP1) *(BIRC2)*, c-IAP2 *(BIRC3)*, XIAP *(BIRC4)*, and ML-IAP *(BIRC7)* are directly involved in apoptosis regulation whereas the other members modulate cell survival by other means, for instance, by cell cycle regulation or inflammation. These IAP molecules inhibit the apoptotic process either by directly binding to the caspases and inhibiting their activity or by virtue of their ubiquitin ligase activity (de Almagro and Vucic, 2012). Over-expression of IAP proteins across a variety of cancers, for example, colon cancer, breast cancer, melanomas, clear-cell renal carcinomas etc (de Almagro and Vucic, 2012) further emphasize the fact that survival advantage acquired by apoptosis inhibition provides an impetus to the tumorigenic process. Mutations in caspases are not frequently encountered in cancers. Nevertheless, disruption of Apaf-1 is associated with Noonan syndrome; caspase-10 mutations contribute to autoimmune lympho-proliferative syndrome type II; and frameshift mutations in caspase-5 have been observed in gastrointestinal, endometrial, and in hereditary non-polyposis colorectal cancers (Lowe and Lin, 2000).

The process of apoptosis has evolved as a means to eliminate altered or superfluous cells that show tumorigenic potential or otherwise threaten tissue

homeostasis. Conversely, transformed/neoplastic cells employ various cellular strategies to suppress protective apoptotic response and develop significantly increased tolerance to apoptotic stimuli such as growth factor depletion, hypoxia, DNA damage, telomere malfunction, activation of mitogenic oncogenes, and treatment with radiation or chemotherapy in order to acquire one of the hallmarks of cancer- evasion of apoptosis. Some tumor suppressor genes, such as *Tp53* and *Par-4*, play an integral role in this process of apoptosis, which constitutes a critical aspect to tumor inhibition mediated by these tumor suppressors. We will elaborate a little bit about these tumor suppressors, p53 and Par-4, whose cross-talk constitutes the framework of this dissertation.

TUMOR SUPPRESSOR P53

The tumor suppressor p53 appears to transcend borders of classification that Kinzler and Vogelstein (Kinzler and Vogelstein, 1996) used to define tumor suppressors, since p53 can be considered both as a 'gatekeeper' for its role in cell cycle control and apoptosis; and a 'caretaker' in its role as the 'guardian of the genome'. However, it is interesting to note that p53, the product of the gene *Tp53*, was discovered in 1979 as a 53kD protein bound by the large T-antigen oncoprotein of the sarcoma-associated virus SV40 in transformed cells and tumors, and was postulated to be an oncogene product linked to viral transformation process (Lane and Crawford, 1979; Linzer and Levine, 1979). Other observations regarding p53 around the same time seemed to support this hypothesis. Ten years later, cloning of wild-type p53 cDNA by Finlay *et. al.*, and their seminal observation that wild-type p53 prevented transformation of cell

cultures by oncogenes began the evolution of p53 as a potential tumor suppressor (Finlay et al., 1989).

Tumor suppression by p53

Functional studies on *Tp53* performed in the decade of 1980's led to the notion that p53 behaved as a proto-oncogene. It was not only found complexed with viral oncoproteins, but was observed to be over-expressed in tumor-derived and transformed cell lines. Moreover, isolation of several cDNA clones showed that p53 had the potential to transform cells in combination with the *Ras* oncogene (Levine et al., 2004). However, the evidence for its supposed oncogenic potential was later realized to be erroneously collected from mutant p53 cDNA clones. Finlay *et. al.* demonstrated that the wild-type p53 cDNA actually blocked the transformation process driven by the oncogenes E1A plus Ras, as well as those initiated by mutant p53 plus Ras. They also observed that cells that were transformed in culture selected for mutated p53, and that mutant p53 not only lost its tumor suppressive ability, but could act in a dominant negative fashion to inactivate wild-type p53, thus making an unambiguous case for the tumor suppressive effects of wild-type p53 (Finlay et al., 1989). Indeed, mutations in both alleles of *Tp53* were identified in mouse tumors and in cultured human cell lines; and in human colorectal cancers, wild-type *Tp53* alleles were frequently observed to be lost by mutations and/or deletions: all of these being definite indications of a candidate tumor suppressor (Lane and Levine, 2010).

The ensuing upsurge of interest in p53 research continued to bring forth more evidence in support of the tumor suppressive actions of p53. By 1992, p53 was

clearly established as a check-point protein involved in cell cycle arrest and in maintaining genomic integrity following DNA damage (Lowe and Lin, 2000). Studies with knock-out mouse models have demonstrated that mice deficient in p53, in spite of being developmentally normal, spontaneously develop cancer (particularly lymphomas and sarcomas) with high penetrance at a very young age (Donehower et al., 1992; Jacks et al., 1994). P53 knock-out mice are also more susceptible to oncogene- or carcinogen-induced tumors (Donehower and Lozano, 2009). Additionally, transgenic mice engineered to over-express p53 were shown to be significantly resistant to cancer, thus emphasizing the tumor-protective effects of p53 (Garcia-Cao et al., 2002).

Substantive evidence from human cancers and cancer-derived cell lines has also served to conclusively establish p53 as a bona-fide tumor suppressor. Germline *Tp53* mutations are known to cause a rare type of cancer predisposition called the Li-Fraumeni syndrome. Li-Fraumeni patients with an inherited mutant allele of Tp53 show an increased susceptibility to early-onset cancers of diverse types (Levine and Oren, 2009). Approximately 50% of all sporadic human tumors show mutations in both alleles of *Tp53* gene, and more than 25,000 *Tp53* mutations have been identified so far, making it the most frequently mutated gene in all human cancers. Moreover, both germline and somatic mutations at the *Tp53* locus are usually followed by loss of heterozygosity (LOH) during tumor progression, suggesting that a selective force inactivates wild-type p53 in the process (Brosh and Rotter, 2009). Furthermore, p53 mutations are often associated with advanced tumor stage and poor patient prognosis (Wallace-

Brodeur and Lowe, 1999). Approximately 75% of all p53 mutations are missense substitutions. Mutant p53 tends to be more stable than wild-type p53 (hence the aberrant over-expression observed in tumors). Most common p53 mutants are known to exert tumor-promoting effects by dominant negative inactivation of endogenous wild-type p53, in addition to oncogenic gain of function activities. It is now known that abrogation of wild-type p53 transcriptional activity is the key factor in the selection of such missense mutations. Not surprisingly, majority of p53 missense mutations occurs in the DNA binding domain (DBD), thus impacting its transactivation capacity which is crucial for its tumor suppressive functions (Brosh and Rotter, 2009; Petitjean et al., 2007). The structure of p53; and its central role in eliciting a transcriptional program that can control critical cellular processes (such as cell cycle arrest, cellular senescence and apoptosis) that bring about tumor suppression are briefly discussed below.

Structure

P53 protein, a stress-induced transcription factor, is biologically active as a homo-tetramer comprising 4 x 393 amino acid residues; each monomer consisting of 393 amino acids, and representing a highly modular structure of intricate complexity. P53 contains folded DNA-binding and tetramerization domains flanked by intrinsically disordered regions at both amino (N)- and carboxy (C)-termini. N-terminus of p53 comprises the transcription-activation domain (TAD) (residues 1–62), which is essential for p53 transcriptional activity. The p53 TAD connects target gene recognition to its expression by directly binding to the transcriptional co-activators p300/CBP and components of the

basic transcriptional machinery. It can be further divided into sub-domains TAD1 and TAD2, which is followed by a proline rich domain (PRD) (residues 63-94) (Joerger and Fersht, 2010). TAD2 is shown to be essential for transcriptional activation of a number of target apoptotic genes. In addition, the PRD is considered necessary for apoptosis and growth suppression mediated by p53 (Harms and Chen, 2005).

Amino acid residues (94-292) in the p53 protein confer DNA-binding potential, and therefore, constitute its "core" domain. This core domain is responsible for recognizing and binding to its target duplex DNA in a sequence-specific manner, and is also involved in co-repressor binding. The p53 binding-site sequence consensus is discussed in more detail in forthcoming sections below. Nuclear entry of p53 is regulated by a nuclear localization signaling domain (residues 316-324). Moreover, residues 325-355 are involved in p53 tetramerization, which is essential for its activity *in vivo*. Residues 356-393 of p53 form the basic C-terminal domain (CTD) which performs important regulatory functions, and is involved in down-modulation of DNA-binding activity of p53 core domain (Harms and Chen, 2005; Joerger and Fersht, 2010).

P53 also interacts with a large number of protein partners, and a majority of its interactions are mediated through its N-terminal TAD, although a good number of partners also interact with the C-terminal domain, and only a few bind to the core domain (Joerger and Fersht, 2010). In addition, within the N-terminal and C-terminal domains of p53 reside a multitude of amino acid residues subject to an

array of post-translational modifications that can modulate p53 activation, stabilization, as well as binding to its partners.

Transcriptional regulation by p53

More than three decades of research in the field of p53 has revealed that a broad range of cellular processes are regulated by p53, and continues, to this day, to ascribe new roles for p53 in the regulation of metabolism, autophagy, fecundity, and various aspects of differentiation and development. The best characterized function of p53 is that of a pivotal signaling node that translates diverse upstream stress signals into downstream responses including, but not limited to, cell cycle arrest, senescence, and apoptosis. As a transcriptional factor, p53 regulates the expression of several critical molecules necessary to co-ordinate these multiple aforementioned processes that produce anti-proliferative and anti-transformation outcomes. Thus transcriptional regulation of its target genes by p53 is integral to its role as a tumor suppressor.

Extensive research in the p53 field over the years has identified an estimated several hundreds of RNA-polymerase II (RNAP II)-transcribed genes that are directly, and thousands others that are indirectly, regulated by p53 (Beckerman and Prives, 2010). Although p53 mRNA is constitutively expressed, basal levels of p53 protein are kept extremely low in unstressed cells. A plethora of cellular stresses, including but not limited to, genotoxic stresses such as radiation, telomere erosion, nucleotide depletion; hypoxia; heat/cold shock; nutritional starvation; mitochondrial biogenesis stress; oncogenic activation etc, can stabilize and activate p53. Stabilization of p53 expression levels primarily occurs

through events that disrupt its interaction with its negative regulator Mdm2 protein, which mediates the proteasomal degradation of p53.

Once stabilized, p53 translocates into the nucleus and binds, as a tetramer, specific DNA sequences that are designated p53 response elements (REs) (McLure and Lee, 1998). The consensus p53-binding DNA sequence consists of two copies of the 10-base pair motif RRRCWWGYYY (n=0-13) RRRCWWGYYY (where R is Adenine or Guanine, W is a purine base, and Y is a pyrimidine base) with or without a spacer region (n), which can be up to 13 bases long. C4 and G7 in each motif are considered critical for effective binding (el-Deiry et al., 1992; Funk et al., 1992). However, an analysis of functional p53 target genes suggests that mismatches in one or more bases in the consensus sequence do not result in loss of binding. Non-canonical sites, such as those found in *pig3* (TGYCC)$_n$ response microsatellite element; the triplet pairs of the pentameric element at the *aqp3* locus; or the "head-to-tail" configuration of the *Mdr1* site show that p53 REs can accommodate moderate alterations in the consensus sequence (Beckerman and Prives, 2010).

P53 REs are usually found to cluster in the non-coding areas of its target gene, and are most commonly found in the promoter at varying distances upstream (e.g., *p21, noxa*) of the transcription start site (TSS), although some might be located closer (within 300 base pairs) of the TSS (e.g. *Hdm2, Pcna*). However, these REs have also been found at diverse regions within the target gene locus; for instance, within early intronic sequences (e.g. *Puma, Pig3*), and also within exons (e.g. *miR-34a*). Once bound to its RE, p53 can then recruit diverse

transcriptional co-regulators, such as histone modifying enzymes, chromatin remodeling complexes, and components of the general transcriptional machinery and the pre-initiation complex (PIC) to modulate RNAP II activity at the target gene locus.

Although majority of research has focused on p53 as a transcriptional activator, p53 can also cause repression of some of its target genes. It is yet not clear what distinguishes an RE from being a transcriptional activator-site versus a transcriptional-repressor site. Nonetheless, p53 is considered to cause gene repression via the following three generally accepted mechanisms (Riley et al., 2008):

i.   Steric interference, wherein p53 binding to its RE overlaps the binding site of another (more powerful) trans-activating agent (e.g. *Afp, Bcl-2*).

ii. P53 squelching of transcriptional activators (e.g. *cyclin B1, TERT*).

iii. P53-mediated recruitment of transcriptional co-repressors, such as histone deacetylases (HDACs) (*MAP4, Hsp90AB1*) (Riley et al., 2008).

Diverse cofactors that interact with, or are recruited to specific RE's by p53, can post-translationally modify histones, other cofactors, or p53 itself. The precise mechanisms whereby p53 targets a select group of genes regulating a specific pathway in response to distinct stresses are poorly understood. However, it is now thought that combinations of cofactors and post-translational modifications (e.g. phosphorylation, acetylation, sumoylation, neddylation etc) may serve as distinct "barcodes" to enable the selection of specific promoters. P53 thus

regulates a number of genes directly involved in the control of the following cellular processes that are considered to be the mechanisms for tumor suppression by p53: most notably cell cycle arrest, senescence, and apoptosis. P53-mediated transactivation of $p21^{Waf1/Cip1}$ is considered important for G1/S arrest; and repression of *Cdc25c*, in addition to activation of 14-3-3σ can initiate G2/M arrest (Zilfou and Lowe, 2009). P53 and its downstream effectors can initiate a program of cellular senescence leading to a terminal state for the replication of cells. Many studies have emphasized the importance of senescence in the inhibition of tumor progression, and have identified a key role for p53 and p53-regulated genes $p21^{Waf1/Cip1}$ and Plasminogen Activator Inhibitor-1 (*PAI- 1*) in this process (Vousden and Prives, 2009).

P53 and apoptosis

Incidentally, p53 was the first tumor suppressor to be linked to apoptosis, and this aspect of p53 function constitutes one of the most extensively researched areas in the field. Endogenous p53 has been shown to be essential for radiation-induced apoptosis in mouse thymocytes (Lowe et al., 1993b). Marked reduction in apoptosis correlates with the occurrence of some p53 mutations in transgenic mice, and with clonal progression in Wilm's tumor (Lowe and Lin, 2000). Studies using p53 knock-out mice have demonstrated that p53 plays a critical role in apoptosis induced by a range of stimuli, including DNA damaging anti-cancer agents, radiation, hypoxia, and mitogenic oncogenes. On the other hand, mice expressing a p53 mutant protein that lacks the ability to induce cell cycle arrest, but retains apoptotic potential are efficiently protected from spontaneous tumor

development (Toledo et al., 2006). P53 regulates the promoters of numerous genes involved in apoptosis. Upon activation, p53 transactivates various pro-apoptotic effectors of both intrinsic and extrinsic apoptotic pathways, including Noxa, PUMA, Bax, PIG3, p53AIP1, Perp, CD95 and DR5 (Zilfou and Lowe, 2009). In addition, disruption of several p53 effectors, such as, Bax, Apaf-1 and Caspase-9 can promote oncogenic transformation and tumor development in mouse models (Lowe and Lin, 2000).

Apart from these transcription-dependent actions, p53-mediated apoptosis also involves its transcription-independent functions. Specifically, in response to some cell death signals, such as IR, p53 rapidly localizes to the mitochondria, where it can induce mitochondrial outer membrane permeabilization (MOMP), thus effecting the release of pro-apoptotic factors from the mitochondrial inter-membrane space. Additionally, p53 is known to bind Bcl-2, Bcl-xL, and Bak at the mitochondria, acting either as a direct activator of the pro-apoptotic Bax and/or Bak, or as a derepressor. P53-regulated apoptosis effector molecule PUMA appears to be the link between transcription-dependent and -independent mechanisms of p53-mediated apoptosis. PUMA, which is directly up-regulated by p53 in response to cellular stress, translocates into the mitochondria where it interact with Bcl-xL, thus releasing p53 to activate Bax (Zilfou and Lowe, 2009).

Many more p53 target genes have been implicated in apoptosis than in other effector functions, suggesting that its control of the apoptotic response is complex. P53 simultaneously targets multiple death circuit pathways to co-ordinate an apoptotic response, and hence, no single p53-regulated molecule

can account for all of its pro-apoptotic activity. This fact is exemplified by PUMA, which is considered to be essential for apoptosis induction by p53, and is the only p53 target gene whose loss produces apoptotic defect similar to that caused by p53 loss in irradiated T lymphocytes. Yet, PUMA-null mice do not exhibit the overt tumor-prone phenotype like their p53-null counterparts do, implying that multiple effector functions of p53, rather than just disruption of apoptosis, must be simultaneously inactivated to initiate tumorigenesis and that the end result of p53 activation is context-dependent. Apoptosis can be integrated into a larger p53 tumor suppressor network controlled by different signals, environmental contexts, and cell type (Fridman and Lowe, 2003; Zilfou and Lowe, 2009).

Non-autonomous tumor suppression by p53

Although most of the extensive research on p53 has concentrated on the cell autonomous role of p53 in tumor suppression, emerging evidence also points towards non-cell autonomous tumor suppressive effects of p53. Many studies have explored p53 status in the tumor mesenchyme, particularly in the supporting fibroblasts that constitute the supporting stroma, and concluded that p53 can inhibit tumorigenesis and tumor progression in a non-cell autonomous manner. High frequencies of somatic mutations in Tp53 have been reported in breast neoplastic epithelium and stroma (Kurose et al., 2002). There is also evidence that tumor latency is reduced in p53-null mice relative to wild-type mice, indicating that the presence of wild-type p53 in the host exerts an inhibitory effect on tumor progression. In addition, reconstitution of tumors with either wild-type or p53$^{-/-}$ MEFs in mice showed accelerated tumor growth in the presence of p53-null

fibroblasts, even though the composition differed only in p53 status of fibroblasts, the neoplastic component being identical (Kiaris et al., 2005). This reinforces the idea that p53 loss produces a supportive stroma that is more conducive to tumor growth. Moreover, tumor progress imposes a strong selective pressure for loss of p53 function in cancer-associated fibroblasts (CAFs), and stromal cells frequently show LOH at the Tp53 locus, similar to those found in primary tumors (Hill et al., 2005; Kiaris et al., 2005; Kurose et al., 2002).

P53 and cancer therapy

Because of its control of multiple arms in the apoptotic process, the p53 pathway can influence tumor responsiveness to therapeutic agents. Numerous cell culture and in vivo studies have demonstrated that tumors that are wild-type for p53 undergo p53-dependent apoptosis when treated with a wide variety of therapeutic agents, such as IR, Adriamycin, 5-Fluorouracil (5-FU), Etoposide, Vincristine (Dudley et al., 2008; Levine, 1997; Lowe et al., 1994; Lowe et al., 1993a; Mirzayans et al., 2013), and p53 loss/inactivation leads to resistance to apoptosis of tumor cells in response to such agents. Additionally, cytogenetic changes in tumor stroma including p53 mutations can compromise p53 function, and modulate response to chemotherapy. Tumor-associated stromal cells have been reported to show diminished p53 accumulation and decreased sensitivity to the effects of p53-activating agents such as Etoposide and Vincristine (Dudley et al., 2008) .

In view of such far-reaching tumor suppressive effects of p53, much scientific effort has been channelized to reinstate p53 pathway in tumors with the help of

gene therapy or using strategies to reactivate p53 in tumor cells, in order to restore responsiveness to therapy and improve patient prognosis (Vousden and Prives, 2009). Although such efforts have met with mixed results, exploring new avenues in p53 restoration/reactivation does appear to promise therapeutic benefit. One such approach to reactivate wild-type p53 is by inhibiting its interaction with Mdm2, thereby increasing its half-life and leading to p53 accumulation within cells.

MURINE DOUBLE MINUTE 2 HOMOLOG (MDM2)

The expression of the tumor suppressor protein p53 is maintained at low levels within normal unstressed cells (despite constitutive expression of its mRNA); this tight regulation of its expression is largely attributable to Mdm2 protein. Originally identified as a gene amplified in double minute chromosomes in mouse fibroblasts, it is now best characterized as the negative regulator of p53 protein. Multiple mechanisms by which Mdm2 regulates p53 expression and function have been identified. First, Mdm2 strongly binds p53 at its N-terminal TAD1 sub-domain and inhibits transcriptional activation function of p53 (Momand et al., 1992). Second, Mdm2 is a RING-finger ubiquitin ligase with high specificity for p53. It targets p53 for ubiquitination and thereby mediates its degradation by the 26S proteasome (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997). Third, Mdm2 contains a signal sequence that is similar to the nuclear export signal of many viral proteins. It can, therefore, bind to p53 and promote its nuclear export, hence making p53 inaccessible to targeted DNA for transcriptional regulation (Chene, 2003). Furthermore, Mdm2 can also suppress

p53 protein translation either by binding directly to p53 mRNA, or by mediating degradation of the ribosomal protein RPL26 which has been shown to have an obligate role for p53 translation in response to genotoxic stress (Manfredi, 2010). On the other hand, Mdm2 gene promoter contains p53 REs, and is transcriptionally activated by p53. Mdm2 and p53 are therefore part of an auto-regulatory feedback loop.

Human Mdm2 is a protein consisting of 491 amino acids in which four distinct regions have been defined. At the N-terminus is the binding site for p53. Central part of the protein contains an acidic region and a zinc-finger domain, whereas the C-terminus contains a RING finger domain. The middle region of Mdm2 is known to interact with a number of proteins, and the C-terminus RING finger is also a binding site for a closely related partner, Mdmx (Manfredi, 2010).

The importance of p53-Mdm2 interaction was demonstrated with homozygous deletion of the *Mdm2* gene in mice. Their embryos die very early during gestation (blastocyst stage), but this phenotype could be rescued with additional deletion of the *Trp53* gene. This clearly indicates that, during development, regulation of p53 apoptotic activity by Mdm2 is essential (Jones et al., 1995; Montes de Oca Luna et al., 1995). It has also been shown that Mdm2 over-expression blocks p53-mediated cell cycle arrest and apoptosis (Chen et al., 1996). Many studies have described over-expression of Mdm2 in different kinds of tumors. Increased levels of Mdm2 can thus confer growth advantage and contribute to tumorigenesis by inactivating the p53 pathway in cancers that do possess wild-type p53. Over-expression of Mdm2 protein can be achieved by amplification of its gene; single

nucleotide polymorphism at position 309 (SNP309) in its gene promoter; enhanced transcription; or increased translation (Zhao Y, 2013).

Analysis of over 3000 tumors revealed that Mdm2 gene is amplified in ~7% of these tissues; the highest frequency of amplification being observed in soft tumor tissues, osteosarcomas, and esophageal carcinomas (Chene, 2003). Mdm2 amplification and p53 mutations were found to be mutually exclusive. Over-expression of Mdm2 in human tumors correlates with poor clinical prognosis and poor treatment response to current therapeutics (Zhao Y, 2013). Animal experiments have revealed that targeted expression of Mdm2 in the mouse leads to increased tumorigenesis. Likewise, in mouse models of cancer in which p53 inactivation is known to play a role, evidence suggests that this can also occur through enhanced expression of Mdm2 (Manfredi, 2010).

A variety of stress signals disrupt the p53-Mdm2 interaction, thus leading to p53 accumulation/activation, and setting into motion downstream p53 effector pathway(s). Disruption of p53-Mdm2 interaction can be achieved by two means (Zilfou and Lowe, 2009):

a) By post-translational modifications (mainly phosphorylation) of multiple residues in the NH2-terminus of p53 protein by various kinases including ATM, ATR, DNA-PK, Chk1, Chk2 in response to DNA damage from IR or chemotherapeutic agents. Such post-translational events inhibit the ability of Mdm2 to ubiquitinate and degrade p53, thereby stabilizing its expression.

b) P53-Mdm2 interaction is also antagonized by negative regulation of Mdm2 mediated by p14ARF (p19ARF in mouse) in response to oncogenic challenges, resulting in p53 protein stabilization. Notably, p14^{ARF} binds to Mdm2 at a site distinct from p53-Mdm2 interface, and therefore is not a competitive inhibitor (Eischen et al., 1999; Palmero et al., 1998).

Recent evidence also suggests that mere disruption of p53-Mdm2 interaction using chemical inhibitors, even in the absence of phosphorylation on key p53 residues, is sufficient for p53 to act as a transcriptional regulator (Thompson et al., 2004; Vassilev et al., 2004). Likewise, ablation of Mdm2 levels using RNAi approaches resulted in p53-dependent transcriptional activity in the absence of either phosphorylation or acetylation on specific relevant sites (Giono and Manfredi, 2007).

Like p53, Mdm2 protein is subject to regulation by post-translational modifications such as phosphorylation and acetylation. Various phosphorylation sites on Mdm2 have been identified, and based on the site and the kinase involved, these modifications can inhibit or enhance Mdm2 activity. ATM and c-Abl kinases inhibit Mdm2 activity by inducing phosphorylation of Serine 395 (S395) and Tyrosine 394 respectively. CBP/p300-mediated acetylation of Mdm2 also exerts an inhibitory effect on its activity (Kruse and Gu, 2009). Such modifications lead to p53 accumulation and activation of p53-regulated pathways. Conversely, phosphorylation of Mdm2 at positions S166 and S186 by the cell survival kinase Akt/PKB not only enhances its E3 ligase activity but also promotes its nuclear entry, and facilitates the export of p53 from the nucleus to

the cytoplasm, leading to increased degradation of p53 and significant inhibition of p53-mediated apoptosis (Limesand et al., 2006; Mayo and Donner, 2001).

PROSTATE APOPTOSIS RESPONSE- 4 (PAR-4)

Identification of Par-4:

The prostate apoptosis response-4 (*Par-4*) gene was first identified by differential hybridization technique as an immediate early apoptotic gene induced in response to elevated intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in androgen-independent rat prostate cancer cell line, AT-3, treated with Ionomycin. Par-4 expression was also observed in apoptotic androgen-dependent rat ventral prostate following castration. Furthermore, *Par-4* gene induction was not observed in liver, kidneys or other androgen receptor containing organs which do not undergo apoptosis upon castration. This observation, combined with the fact that Par-4 expression occurs only downstream to apoptotic signaling, and not in other processes such as growth stimulation, necrosis, oxidative stress or growth arrest established *Par-4* as an apoptosis-specific gene (Sells et al., 1994).

Human Par-4, which was found to share significant sequence homology with its rat counterpart, was subsequently identified in yeast-two hybrid studies as a partner of the tumor suppressor Wilm's tumor1 (WT1) (Johnstone et al., 1996) and atypical Protein Kinase C (aPKC) (Diaz-Meco et al., 1996). Ubiquitous Par-4 expression has also been observed in nearly all tissues of mice, horses, pigs, and cows. In addition, Par-4 is also known to be expressed in other vertebrates such as fish, birds, and *Xenopus* (El-Guendy and Rangnekar, 2003). Constitutive

expression of Par-4 is seen in virtually all tissues of ectodermal, mesodermal, and endodermal origin (Boghaert et al., 1997).

Following the discovery of Par-4 and its pro-apoptotic functions, the majority of studies revolved around its role as an intracellular protein. However, a seminal study by Burikhanov et al. showed that Par-4 is secreted by almost all types of cells, and is available in systemic circulation. Secretion of Par-4 was shown to occur independently of apoptosis, and through the classical secretory pathway involving the endoplasmic reticulum (ER) and the golgi network. Secreted Par-4 is known to retain its pro-apoptotic potential and is able to selectively target cancer cells when applied extracellularly (Burikhanov et al., 2009). Research over the years has established Par-4 as a cancer cell-selective pro-apoptotic protein, and a bona fide tumor suppressor.

Structure

Human *Par-4* gene has been mapped to the minus strand of chromosome 12q21 (Johnstone et al., 1998). The gene comprises seven exons and six introns, and a total of 99.06 kb of DNA (Zhao and Rangnekar, 2008). Par-4 is known to be evolutionarily conserved among all vertebrates (Boghaert et al., 1997). The product of the *Par-4* gene is an approximately 38 kDa protein consisting of 342 amino acids in humans; 332 amino acids in rats; and 333 amino acids in mice. Rat and mouse Par-4 proteins exhibit a 93% amino acid homology, and rat and human Par-4 proteins possess about 75% identical and 84% functionally similar amino acids. Moreover, the protein domains that are presumably of high

functional importance are 100% conserved across these three species (El-Guendy and Rangnekar, 2003).

Some of the conserved functional domains of Par-4 are: a) two putative nuclear localization sequences (NLS) designated NLS1 (amino acid residues 20-25) and NLS2 (a bipartite sequence comprising residues 137-153) in the N-terminal region, b) a nuclear export sequence (NES) in the C-terminus, and c) a leucine-zipper domain spanning amino acids 290-332 in the C-terminal region (El-Guendy and Rangnekar, 2003). Both NLS1 and NLS2 are conserved across the human, mouse and rat species. However, studies using deletion mutants of Par-4 have shown that only NLS2 is required for translocation of Par-4 into the nucleus and subsequent induction of apoptosis. The leucine zipper domain at the C-terminus of Par-4 is not required for its apoptotic function. Considering the role of this domain in various protein-protein interactions, the leucine zipper appears more important for the regulation of Par-4 function by partner proteins such as WT-1, PKCζ, Akt1 etc (El-Guendy et al., 2003). Par-4 also contains in its sequence several consensus sites for post-translational modifications. Phosphorylation of Par-4 by protein kinase A- I (PKA- I) at the Threonine 155 (T155) residue is known to activate Par-4, and this step is required for Par-4 function (Gurumurthy et al., 2005). Conversely, phosphorylation of Serine 249 (S249) by the Akt1/PKB Serine/threonine (S/T) kinase has been shown to inactivate Par-4 by subjecting it to 14-3-3-mediated sequestration in the cytoplasm (Goswami et al., 2005). Par-4 also possesses a putative ATP/GTP-binding domain, and several consensus sites for phosphorylation by kinases

such as protein kinase C (PKC) and casein kinase 2 (CK2), although their functional importance remains to be investigated (Hebbar et al., 2012).

Selective for Apoptosis in Cancer Cells (SAC) Domain

Analysis of several mutants resulting from serial deletion of the full-length Par-4 protein from both the amino and carboxy termini led to the identification of a unique core domain, spanning amino acids 137-195, that is sufficient to carry out the apoptotic function of Par-4. It was observed that this 58-amino acid fragment of Par-4, when over-expressed, induces apoptosis specifically in cancer cells only, and therefore is called the 'selective for apoptosis of cancer cells' (SAC) domain. The SAC domain is 100% conserved among mammals (including rodents, humans and chimpanzee), chicken, frog and zebra fish. This segment contains the NLS2, which facilitates its nuclear translocation, and the PKA phosphorylation site, T155, which is responsible for its activation, but excludes the C-terminal leucine zipper domain. It is interesting to note that the SAC domain shows nuclear localization, and is capable of inducing apoptosis not only in Par-4 sensitive cancer cells, such as PC3, DU145, LNCaP/IL-6, but also in the cells that show primarily cytoplasmic localization of full-length Par-4, and are resistant to its apoptotic actions, such as LNCaP, MCF-7 etc. However, neither Par-4 nor the SAC domain causes apoptosis in normal cells (El-Guendy et al., 2003).

Par-4 is a tumor suppressor

Evidence from numerous studies across a variety of human cancers, as well as experiments in knock-out and transgenic mouse models, has validated Par-4 as

a bona fide tumor suppressor. Pancreatic and gastric cancers frequently show a deletion or instability in chromosome 12q21, the region where Par-4 is located (Kimura et al., 1998; Schneider et al., 2003). It is also possible that aberrant expression of this region contributes to Wilm's tumorigenesis (Johnstone et al., 1998). Par-4 is observed to be down-regulated in over 70% of renal cell carcinoma (Cook et al., 1999); in neuroblastoma (Kogel et al., 2001); endometrial cancer (Moreno-Bueno et al., 2007); pancreatic cancer (Ahmed et al., 2008); breast cancer (Mendez-Lopez et al., 2010; Zapata-Benavides et al., 2009); cholangiocarcinoma (Franchitto et al., 2010); and in high-grade glioblastoma (Liu et al., 2014b). In prostate cancer, Par-4 is not down-regulated or mutated, but inactivated by Akt-mediated phosphorylation and subsequent sequestration in the cytoplasm by chaperone protein 14-3-3 (Goswami et al., 2005).

In about 32% of the endometrial carcinomas, diminished Par-4 expression was observed to be due to promoter hypermethylation. A strong correlation was also reported between low Par-4 levels and microsatellite instability in these tumors. In addition, Par-4 promoter hypermethylation and silencing could also be detected in endometrial cancer cell lines SKUT1B and AN3CA. A single base mutation in exon 3 of the Par-4 gene, converting an Arginine residue (CGA) to a Stop codon (TGA) thus resulting in a truncated protein, has also been identified in endometrial cancer (Moreno-Bueno et al., 2007). In the case of breast cancer, emerging data have implicated Par-4 down-regulation as a prognostic factor for poor disease outcome and decreased overall survival (Mendez-Lopez et al., 2010; Nagai et al., 2010). A recent study also identified low Par-4 expression as

an independent predictor of reduced recurrence-free survival, and demonstrated that down-regulation of Par-4 is necessary and sufficient to promote breast cancer recurrence (Alvarez et al., 2013).

K-Ras mutations are the most frequent mutations in pancreatic cancer, and Par-4 is reported to be significantly down-regulated in pancreatic cancers harboring K-Ras mutations. Tumors with such K-ras mutations tend to be highly aggressive and therapy-resistant, whereas tumors with higher Par-4 levels have better prognosis (Ahmed et al., 2008). Oncogenic Ras is known to down-regulate Par-4 in a variety of cells via the MEK-ERK pathway, and this is considered to be an important step towards Ras-induced transformation. Restoration of Par-4 levels either by the MEK inhibition or by stable expression of ectopic Par-4 abrogates cellular transformation (Barradas et al., 1999; Pruitt et al., 2005; Qiu et al., 1999). Furthermore, in Ras-transformed epithelial cells, long-term decrease in Par-4 expression has been demonstrated to be achieved by MEK-dependent hypermethylation of the *Par-4* promoter (Pruitt et al., 2005). There is also evidence that if Par-4 is reinstated in these cells by transient transfection, it will selectively sensitize the cells expressing oncogenic Ras, but not those that lack oncogenic Ras, to apoptosis; and this action of Par-4 is achieved by inhibition of NF-κB-mediated transcriptional activation (Nalca et al., 1999). The tumor-suppressor function of Par-4 has also been demonstrated in hematopoietic stem cells where it antagonizes the activation of oncogenic Ras, and disrupts BCR-Abl signaling to produce anti-transformation outcomes (Kukoc-Zivojnov et al., 2004).

Animal studies have demonstrated that Par-4 knockout mice are prone to spontaneous development of tumors in various tissues, e.g. lungs, liver, urinary bladder and endometrium, and also exhibit prostatic intraepithelial neoplasia (PIN). The study also shows that Par-4 knockout mice are more susceptible to chemical- or hormone-induced endometrial and prostatic lesions. Animals homozygous for Par-4 knockout also have a significantly shorter life span compared to heterozygous knockout or wild-type animals (Garcia-Cao et al., 2005).

In contrast, transgenic mice over-expressing full-length Par-4 or the SAC domain of Par-4 show remarkable protection against the growth of spontaneous or oncogene-induced autochthonous tumors. Interestingly, when SAC transgene was co-expressed with oncogenic SV40 antigens in mice, normal or benign prostatic epithelial cells continued to express SAC, whereas all prostate intra-epithelial neoplasia (PIN) and adenocarcinoma lesions showed down-regulation or elimination of the SAC transgene, indicating that loss of SAC is a pre-requisite for progression into malignant disease (Zhao et al., 2007). A similar observation was recently made when Alvarez *et. al.* noted that endogenous, and even ectopically expressed Par-4 was lost from recurrent breast tumors in their study with mouse models (Alvarez et al., 2013). In addition, studies with transgenic mouse models indicate that mice that express systemic Par-4/SAC protein are resistant to the growth of non-autochthonous tumors. Importantly, transplantation of bone marrow from cancer-resistant transgenic mice to cancer-susceptible wild-type mice conferred cancer resistance, further confirming the potent anti-tumor

activity displayed by secretory Par-4/SAC. Moreover, intravenous injection of recombinant Par-4 or SAC protein could effectively inhibit lung metastasis of Lewis Lung Carcinoma (LLC 1) cells (Zhao et al., 2011). Overall, these results reiterate the cancer cell-specific apoptotic action and tumor-suppressive role of Par-4.

Par-4 and apoptosis

The pro-apoptotic action of Par-4 is indicated by its increased expression in actively apoptosing cells, e.g. the granulosa cells of atretic ovarian follicles; interdigitating web cells of the mouse embryo; involuting tadpole tail; and dying neurons during neuronal development, wherein apoptosis induced in the neurons serves to maintain the number of neurons in the nervous system (El-Guendy and Rangnekar, 2003). The fact that the prostatic ductal cells do not normally express Par-4, but when subjected to testosterone ablation by castration, these apoptosing cells do express Par-4 further underscores the pro-apoptotic function of Par-4 (Boghaert et al., 1997).

Studies conducted in cell culture models show that over-expression of Par-4 is sufficient to directly induce apoptosis in most cancer cells, and this apoptotic action of Par-4 can override cell-protective mechanisms, such as presence of Bcl-2, Bcl-xL or absence of wild-type p53 or PTEN function. In contrast, over-expression of Par-4 does not induce apoptosis in normal or immortalized but non-transformed cells, but only serves to sensitize such "resistant" cells to additional apoptotic signal(s), such as increased intracellular Calcium (Ca^{2+}), growth factor withdrawal, Tumor Necrosis Factor- α (TNF-α), or ultraviolet (UV),

X-ray and gamma radiation (Chakraborty et al., 2001; El-Guendy et al., 2003; Nalca et al., 1999). Similar to Par-4, SAC domain causes apoptosis in a wide variety of cancer cells while leaving the normal cells unharmed (El-Guendy et al., 2003).

The cancer cell-specific apoptotic effect of Par-4 is evident in vivo as well. A single injection of adenoviral construct of Par-4 into solid subcutaneous tumors produced in mice by implanting PC-3 cells resulted in a drastic reduction in tumor volume within 3 weeks. This outcome was largely due to an increase in apoptosis caused by Par-4 (Chakraborty et al., 2001). Similarly, in tumors generated by xenotransplanted A375-C6 melanoma cells in SCID mice, over-expression of Par-4 correlated with decreased tumor development, and an increase in apoptosis (Lucas et al., 2001). The Par-4/SAC transgenic mice are physiologically and developmentally normal when scored in terms of fertility, gender ratio, viability, body weight and aging characteristics, but show remarkable resistance to the growth of spontaneous as well as induced tumors, which further underscores the fact that Par-4 induces apoptosis specifically in cancer cells, while normal cells are largely unaffected (Zhao et al., 2007).

A number of studies in cell culture models have described the role of Par-4 in sensitizing different cell types to the apoptotic effects of various stimuli, such as growth factor withdrawal, TNF- α, UV, X-ray and gamma radiation (Chakraborty et al., 2001; Chendil et al., 2002; El-Guendy and Rangnekar, 2003; Nalca et al., 1999). Par-4 is also considered to be an essential down-stream regulator of the apoptotic actions of a wide variety of chemical/physical therapeutic agents such

as Vincristine, TNF-related apoptosis inducing ligand (TRAIL), Adriamycin, and Ionizing Radiation (IR) (Gurumurthy et al., 2005; Ranganathan and Rangnekar, 2005). Diminished expression of Par-4 has often been linked with reduced sensitivity to apoptosis in response to treatment with therapeutic agents. Par-4 is down-regulated in many cancers, and reduced levels or loss of Par-4 has been shown to confer increased resistance of cancer cells to chemotherapeutic agents such as Adriamycin, Etoposide, Vincristine, Cisplatin and 5-Fluorouracil (5-FU) (Affar el et al., 2006; Alvarez et al., 2013; Tan et al., 2014; Wang et al., 2010). In a recent study, extensive analysis of patient datasets revealed that breast tumors with the poorest response to neo-adjuvant chemotherapy (represented by the greatest amount of residual cancer burden) exhibited substantially lower expression of Par-4 prior to therapy than tumors that showed a better response to chemotherapy. Using clinically relevant mouse models, the same study showed that breast cancer cells with reduced Par-4 expression could survive apoptosis caused by oncogene inhibition and by treatment with chemotherapeutic agents, and that this therapy-resistant population later on emerges as recurrent tumor (Alvarez et al., 2013).

Mechanisms of apoptosis by Par-4

The apoptotic effect due to Par-4 involves either an activation of the cellular apoptotic machinery or inhibition of the cellular pro-survival mechanisms. Par-4 induces apoptosis in cancer cells by enabling the translocation of Fas and Fas ligand (FasL) to the plasma membrane. Fas receptor belongs to the TNF-receptor (TNFR) family of type-I transmembrane proteins that possess a

conserved intracellular C-terminal 'death domain'. Activation of Fas receptor, for example by binding to FasL, triggers trimerization of the receptor, and recruits the adapter protein FADD, thereby initiating the formation of DISC and unleashing the caspase cascade. Therefore, in these cells, over-expression of Par-4 is a sufficient signal for cell death (Chakraborty et al., 2001).

In parallel, Par-4 also translocates into the nucleus and inhibits NFκB-mediated cell survival mechanisms; and this inhibition constitutes one of the essential mechanisms of Par-4-induced apoptosis (Nalca et al., 1999). NFκB regulates a number of pro-survival genes, including but not limited to, cell-protective genes such as those of the Bcl-2 family, e.g. Bcl-xL, Al/Bfl1; and anti-apoptotic genes such as X-linked inhibitor of apoptosis (XIAP) that can protect the cell from TNF-induced apoptosis (Barkett and Gilmore, 1999). Activation of Par-4 by PKA-dependent phosphorylation at T155 is considered to be critical for both Fas/FasL translocation and NF-κB inhibition (Gurumurthy et al., 2005). In other studies, Par-4 has been shown to function in the cytoplasm, wherein it represses NFκB-dependent gene transcription by inhibiting the TNFα-induced nuclear translocation of p65 (Rel A) subunit by blocking the aPKC- or IKKβ-mediated phosphorylation of IκB, and this mechanism occurs without disrupting DNA-binding capability of the NFκB complex (Diaz-Meco et al., 1999). A novel mechanism for Par-4-induced apoptosis in breast cancer was recently described. In response to oncogene inhibition or treatment with chemotherapeutic agents, Par-4 was observed to induce ZIP-kinase-dependent phosphorylation of Myosin

Light Chain 2 (MLC2), resulting in multinucleation, and ultimately apoptosis due to cytokinesis failure (Alvarez et al., 2013).

Apoptotic action of extracellular or secreted Par-4 is known to be mediated by Glucose Regulated Protein 78 (GRP78), which acts as a receptor for Par-4 at the cell surface. Par-4 is known to bind the N-terminal region of GRP78 via its SAC domain, and this ligation results in the initiation of a robust feedback loop which involves the trafficking of more GRP78 to cell surface in a Par-4 dependent manner. The binding of Par-4 to GRP78 results in elevated ER stress and, therefore, activation of the ER stress response protein PERK (PKR-like ER kinase), which in turn leads to the activation of caspase-8 and caspase-3 through a FADD dependent mechanism. The cancer-specific activity of secreted Par-4 is due to differential expression of GRP78 in cancer and normal cells. GRP78 is found almost exclusively on the surface of cancer cells, and this accounts for the susceptibility of cancer cells to the apoptotic action of secreted Par-4 (Burikhanov et al., 2009). Intracellularly, cancer cell specificity of Par-4 may also be due to enhanced PKA-mediated phosphorylation of T155 residue in Par-4/SAC domain as a result of elevated expression and/or activity of the enzyme PKA1 in cancer cells and primary tumors compared with normal cells (Gurumurthy et al., 2005).

Project objectives

Research from our lab has shown that the tumor suppressor Par-4 is secreted, and induces apoptosis specifically in cancer cells. Such cancer cell selectivity exhibited by extracellular Par-4 is a much desired characteristic which can be harnessed and exploited to conceptualize novel effective therapeutic strategies.

The aim of this dissertation project work is to identify means to enhance Par-4 secretion and elevate its systemic levels so as to eliminate remote tumor cells (chapter 2). Secondly, this dissertation work also investigates the mechanism(s) related to apoptosis resistance engendered by Par-4 loss or down-regulation using cells derived from Par-4$^{+/+}$ (wild-type) or Par-4-null background (chapter 3).

Chapter 2: Par-4 mediates paracrine apoptotic effect of p53

1. To identify molecular regulator(s) of Par-4 secretion.

2. To identify mechanism by which Par-4 secretion is regulated by the said molecular regulator.

3. To determine if small molecules can be used to modulate Par-4 secretion and cause apoptosis of cancer cells in a paracrine manner.

Chapter 3:

1. To determine the effect of Par-4 loss on apoptosis caused by different agents using primary fibroblasts differing only in Par-4 status.

2. To investigate the mechanisms pertaining to apoptosis resistance engendered by Par-4 loss.

Figure 1.1: Intrinsic and extrinsic pathways apoptotic pathways
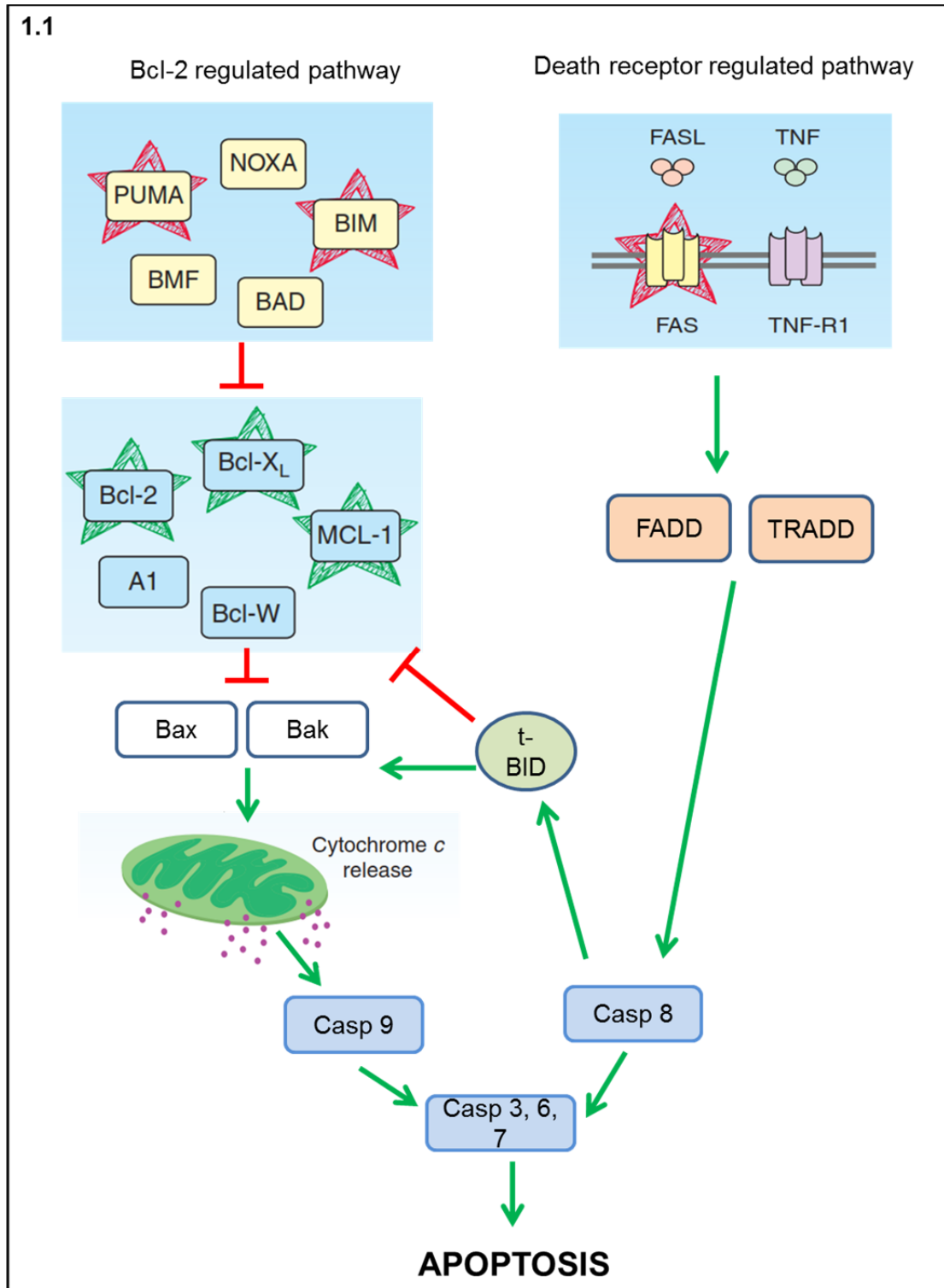
Figure 1.1: Intrinsic and extrinsic pathways apoptotic pathways. Apoptosis can be initiated by activation of two distinct, albeit ultimately converging, pathways, the "Bcl-2-regulated pathway" (left; also known as the "stress" or "mitochondrial" or "intrinsic" pathway) and the "death receptor pathway", also known as the "extrinsic pathway" (right). Protein–protein interactions between members of the Bcl-2 family govern activation of the Bcl-2-regulated pathway, whereas binding of their cognate ligands activates the death receptors (e.g., FAS, TNF-R1). Intrinsic pathway leads to the activation of Caspase 9, whereas extrinsic pathway activates Caspase 8. Cellular demolition is performed by the effector caspases 3, 6, and 7 that act downstream of both pathways.

Figure adapted from Delbridge et. al. Cold Spring Harb Perspect Biol. 2012 Nov 1;4(11).

Figure 1.2: Domain structure of p53
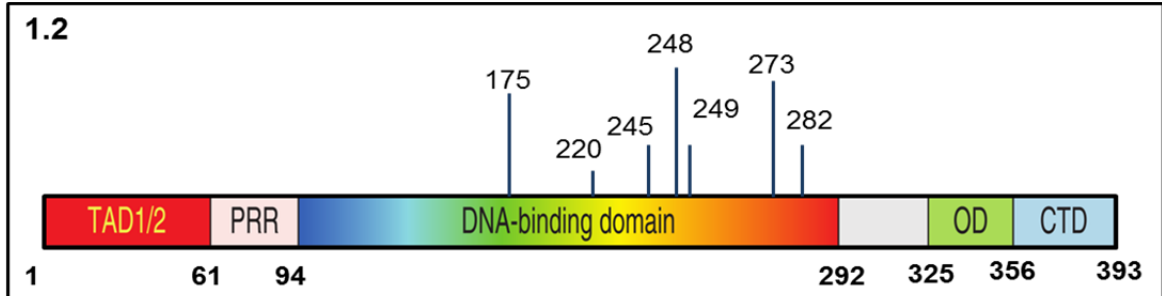
Figure 1.2: Domain structure of p53. p53 contains a natively unfolded amino-terminal transactivation domain (TAD), which can be further subdivided into the subdomains TAD1 and TAD2, followed by a proline-rich region (PRR). The structured DNA-binding and tetramerization domains (OD) are connected through a flexible linker region. Similarly to the TAD region, the regulatory domain at the extreme carboxyl terminus (CTD) is also intrinsically disordered. The vertical bars indicate "hot-spots" for relative missense-mutation frequency in human cancer.

Figure adapted from Joerger and Fersht, Cold Spring Harb Perspect Biol. 2010 Jun;2(6).

Figure 1.3: P53 effector pathways in mediating tumor suppression
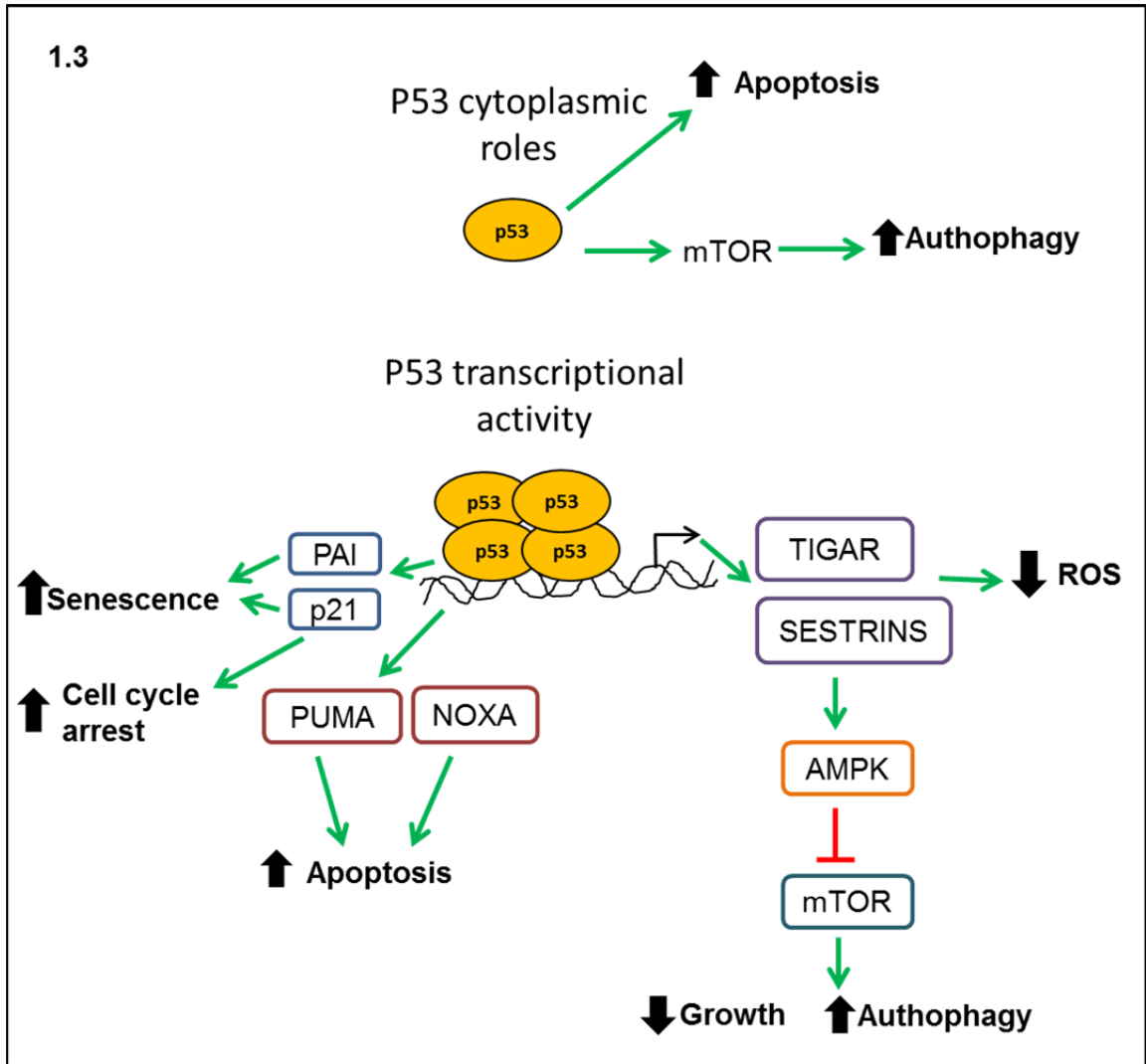
Figure 1.3: P53 effector pathways in mediating tumor suppression. The p53 effector functions of cell cycle arrest, senescence, apoptosis, autophagy, and metabolism regulation are co-ordinated by a number of p53 target genes. These genes are turned on depending on the type and extent of stimulus received; cell type; and general physiological context. These effector functions of p53 are critical in p53-mediated tumor suppression.

Figure 1.4: Domain structure of human Par-4
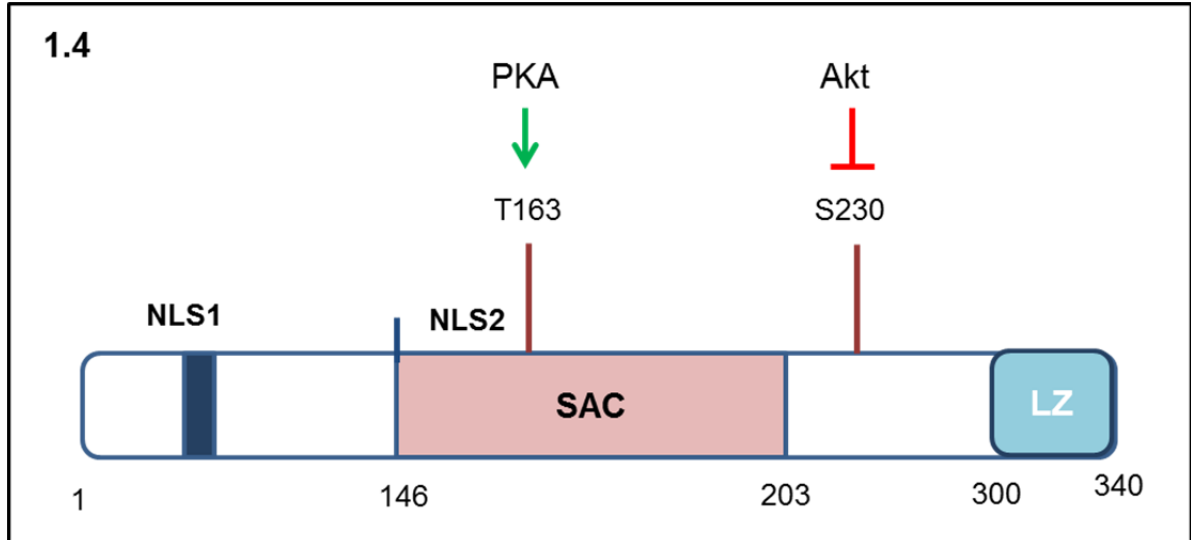
Figure 1.4: Domain structure of human Par-4. The best characterized conserved domains of Par-4, namely, the N-terminal Nuclear Localization Sequences 1 and 2 (NLS1 and NLS2), Selective for Apoptosis of Cancer Cells (SAC), and the C-terminal Leucine Zipper (LZ) domains are illustrated. Activation by PKA-mediated phosphorylation at T163 is essential for Par-4 function. Conversely, Par-4 is inactivated by Akt-mediated phosphorylation at S230.

Figure 1.5: Apoptosis by intracellular and extracellular Par-4
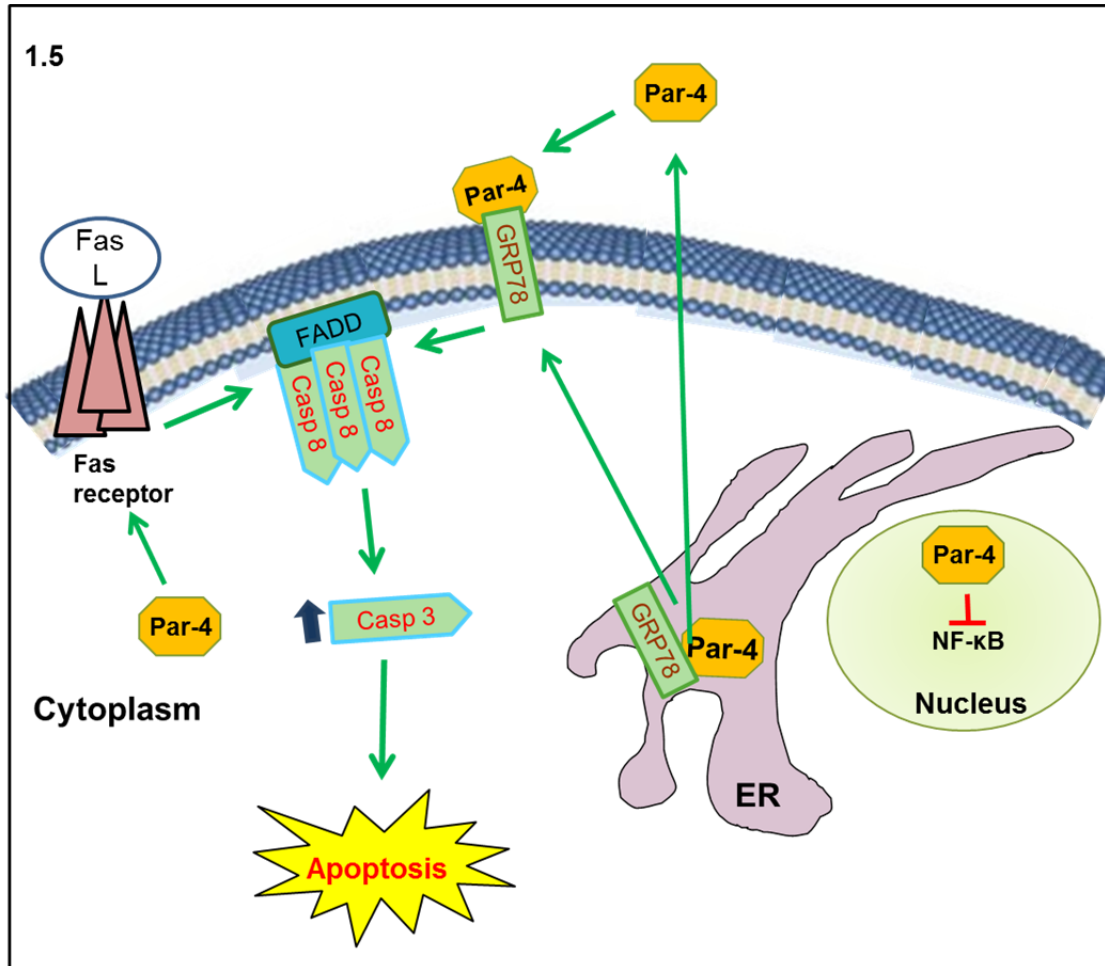
Figure 1.5: Apoptosis by intracellular and extracellular Par-4. Apoptosis induction by Par-4 involves membrane translocation of death receptors of the CD95 family (Fas/FasL). Ligation of Fas with FasL recruits FADD, an adapter protein with cytoplasmic death domain, which in turn serves to recruit procaspase 8 molecules and initiate the proteolytic caspase cascade. Par-4-mediated apoptosis also involved inhibition of NF-κB-mediated pro-survival pathways. Extracellular Par-4 interacts with its receptor GRP78 and drives membrane recruitment of FADD, thus initiating the extrinsic pathway for apoptosis.

CHAPTER TWO: PAR-4 MEDIATES PARACRINE APOPTOTIC EFFECT OF P53

INTRODUCTION

Lung cancer is the leading cause of cancer deaths in both men and women in the United States (Siegel et al., 2014). Activation of the Ras oncogene and loss of tumor suppressor p53 are the two most commonly occurring alterations in lung cancer (Wang et al., 2006). An intact p53 signaling pathway is believed to be essential for growth arrest and cell death caused by diverse chemotherapeutic agents and ionizing radiation. Conversely, loss of p53 function in the tumors may confer therapeutic resistance, ultimately leading to death of the patients (Chen et al., 2010; Levine, 1997; Sekido, 2001). P53 knock-out or p53-mutant mice develop spontaneous, as well as inducible tumors (Donehower et al., 1995; Donehower et al., 1992; Jacks et al., 1994) while p53 transgenic mice exhibit tumor-free survival with an increased life span (Garcia-Cao et al., 2002; Matheu et al., 2007). As up- or down-regulation of transcriptional target genes involved in cell cycle progression or apoptosis plays an important role in the tumor suppressor function of p53, it is not surprising that p53 is mutated within its DNA binding domain in a large number of human cancers (Petitjean et al., 2007).

Given the role of p53- as the "guardian of the genome"- in controlling diverse cellular processes that contribute towards preventing oncogenic transformation, it is perhaps not surprising that a major part of p53 research has focused on its cell-autonomous functions. However, in the recent years, with increasing interest in the role of tumor microenvironment, we can also find a growing body of evidence suggesting a non cell-autonomous role for p53, whereby p53 function in

the stromal compartment can be instrumental in tumor inhibition. Kiaris *et. al.* observed reduced latency in tumor development in p53-null mice relative to wild-type mice, indicating that p53 activity in the host may exert an inhibitory effect on cancer progression (Kiaris et al., 2005). Several studies have revealed that tumor-associated stromal cells also frequently undergo genetic alterations in the *Tp53* locus manifested by somatic mutations and loss of heterozygosity (LOH), similar to those observed in primary tumors (Kurose et al., 2002; Moinfar et al., 2000; Paterson et al., 2003; Wernert et al., 2001). Moreover, tumor progression imposes a strong selective pressure for loss of p53 function in cancer-associated fibroblasts (Addadi et al., 2010; Hill et al., 2005).

These observations support the view that p53 function in both normal and cancer compartments of the tumor is important for suppression of tumor progression. However, the precise mechanism by which p53 loss in normal or stromal cells may contribute to tumor progression is not well understood. Because normal cells in the body of the patient except in the tumor micro-environment are expected to express wild type p53, we determined whether activation of p53 function in normal cells causes paracrine growth inhibitory effects in cancer cells. Specifically, we asked whether p53 activation in normal cells induces the secretion of pro-apoptotic proteins systemically and whether such proteins act to inhibit the growth of p53-deficient tumor cells in a paracrine manner. Our findings indicate that activation of p53 in normal cells promotes the secretion of Prostate apoptosis response-4 (Par-4), a pro-apoptotic tumor suppressor protein

(Burikhanov et al., 2009). Importantly, secreted Par-4 mediates a paracrine growth-inhibitory effect by inducing apoptosis of p53-deficient cancer cells.

MATERIALS AND METHODS

Cells, plasmids, and chemical reagents

Lung cancer cells H1299, HOP92, prostate cancer cells PC-3, and primary lung fibroblast cells HEL were from ATCC, MD. IKKβ$^{+/+}$ and IKKβ$^{-/-}$ MEFs were previously described (Burikhanov et al., 2013). Par-4$^{+/+}$ and Par-4$^{-/-}$ MEFs were derived from wild type and Par-4-null C57/B6 mice (Figure 2.5). P53$^{+/+}$ and p53$^{-/-}$ MEFs in the 3rd passage were from Tyler Jacks (MIT, MA). Nutlin-3a was from Cayman Chemicals, and PS-1145, z-VAD-fmk and BFA were from Sigma Chemicals. UACA constructs were described previously (Burikhanov et al., 2013). The p53 constructs were from Wafik El-Deiry (Penn State University, PA). Immortalized Balb/c fibroblasts (10)1, with no endogenous p53, and (10.1)Val5 cell line derived from 10(1) cells by stable transfection with the temperature-sensitive p53 allele encoding Valine at 135aa, were from Arnold Levine (Institute for Advanced Study, NJ). The p53 mutant in (10.1)Val5 cells exhibits wild type conformation at 32 °C, judged by p21/WAF1 induction, and mutant conformation at 37-39 °C (Harvey and Levine, 1991; Wu and Levine, 1994).

Generation of Par-4/Pawr knock out mice

Par-4 knock out (KO) mice were generated using the C57/B6 mouse background. As Exon 2 contains the initiating ATG codon, it was flanked by loxP

sites. The selection marker (Neomycin, Neo) was flanked by FRT sites and introduced into intron 2. Constitutive knock out allele was generated after *in vivo* Cre-mediated recombination that resulted in deletion of Exon 2 and loss of Par-4 function, owing to removal of the initiating ATG codon. Par-4$^{+/-}$ mice were intercrossed to generate Par-4$^{-/-}$ mice. Par-4 null mice grow normally and develop spontaneous tumors in diverse tissues as previously reported (Moreno-Bueno et al., 2007). MEFs isolated from the Par-4$^{-/-}$ mice and corresponding Par-4$^{+/+}$ littermate control mice were studied for Par-4 secretion (Figure 2.5).

Co-immunoprecipitation and Western blot analysis

Protein extracted from cell lysates was filtered, pre-cleared with 25 µl (bed volume) of protein G-Sepharose beads and immunoprecipitated with 1 µg of respective antibodies. The eluted proteins were resolved by SDS-PAGE, and subjected to Western blot analysis as described (Goswami et al., 2008).

Antibodies and siRNA duplexes

The polyclonal antibody for UACA was from Bethyl Laboratories. Par-4 (R332), Col1A1 (H-197), pan-cytokeratin (C11), p53 (FL-393), p21/WAF1 (F5), and rabbit IgG antibodies were from Santa Cruz Biotechnology, Inc. The mouse monoclonal antibody for p53 (1C12 mAb), cleaved caspase 3 antibodies were from Cell Signaling, and that for β-actin was from Sigma Chemical Corp. The control siRNA and pools of siRNA for UACA were from Dharmacon and SantaCruz Biotechnology, Inc.

Quantitative Real Time-PCR analysis

Approximately 1×10^6 cells were infected with GFP control or GFP-tagged p53-adenovirus for 24 hours, and total RNA was extracted using Trizol reagent. Quantitative Real Time-PCR (qRT-PCR) was performed using SuperScript First-Strand Synthesis System (Invitrogen) using oligo (dT) primers for RT. Brilliant II SYBR Green Master Mix (Agilent Technologies) was used for subsequent qPCR. Primers used for qPCR were as follows:

<u>18S RNA</u>

Forward primer: 5'-cgc cc tag agg tga aat tct-3'

Reverse primer: 5'-cga acc tcc gac ttt cgt tct-3'

<u>Human UACA</u>

Forward primer: 5'-ggc gga gaa cga taa gtt gac taa-3'

Reverse primer: 5'-cat gtt tct cgg gag cta caa ac-3'

Reverse transcriptase-PCR analysis

For the reverse-transcriptase PCR experiment with co-transfection, approximately 1.5×10^6 IKKβ$^{-/-}$ cells were co-transfected with either vector, wild-type(wt) UACA, or mutant(m) UACA construct with or without p53 plasmid for 72 hours, and total RNA was extracted using RNeasy mini kit (Qiagen). Reverse transcription was performed to synthesize cDNA with random hexamers using SuperScript III First-Strand Synthesis System (Invitrogen), and was followed by PCR. PCR products were then subjected to DNA gel electrophoresis. Primers used were as follows:

18S RNA

Forward primer: 5'- gta acc cgt tga acc cca tt -3'

Reverse primer: 5'- cca tcc aat cgg tag tag cg -3'

pCB6$^+$ human UACA wild type or mutant constructs

Forward primer: 5'-aag agc ctc aag tcc cgc c -3'

Reverse primer: 5'- ccc ccc ttt ctg ctg ctt-3'

ChIP analysis

ChIP assays for endogenous p53 binding to the consensus binding site in UACA were performed by treating 2x10^6 HEL cells with either DMSO vehicle or with Nutlin-3a for 24 h, and 1x10^6 cells were used per ChIP reaction in each treatment group. Alternatively, for ChIP analysis with ectopic p53 expression, 1x10^6 cells infected with either GFP control or GFP-tagged p53-adenovirus were used per ChIP assay.ChIP analysis was performed using the ChIP kit from Millipore according to the instructions provided by the manufacturer. Briefly, the proteins and DNA were cross-linked with 1% formaldehyde, lysed, and the DNA was sheared into 200-800 bp fragments. Proteins linked to the DNA were immunoprecipitated with appropriate antibodies (using rabbit IgG or no antibody as control). Subsequently, immune complexes were eluted from the beads, protein-DNA crosslinks were reversed, and DNA was isolated after phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation. DNA fragments were amplified by PCR using the following primers.

UACA:

Forward primer 5'-gtc tac tcc ttg cgc gct gg-3'

Reverse primer 5'-gcg gcg cca gac gac-3'.

p21:

Forward primer 5'-ctg gac tgg gca ctc ttg tc- 3'

Reverse primer 5'-ctc cta cca tcc cct tcc tc- 3'

GAPDH:

Forward primer 5' – atg gtt gcc act ggg gat ct- 3'

Reverse primer 5' – tgc caa agc cta ggg gaa ga- 3'

Apoptosis assays

Apoptotic nuclei were identified by immunocytochemical (ICC) analysis for active caspase-3, or by 4, 6-diamidino-2-phenylindole (DAPI) staining. A total of three independent experiments were performed; and approximately 500 cells were scored in each experiment for apoptosis under a fluorescent microscope.

Animal experiments

Whole-blood samples and various tissues were collected from mice, 24 h after injection via the intra-peritoneal (i.p.) route with Nutlin-3a plus PS-1145 (10mg/kg and 5mg/kg body weight, respectively) or corn oil (100 µl) as vehicle control. Serum was separated from the blood samples and used for testing. All animal procedures were performed with IACUC approval.

Statistical analysis

All experiments were performed in triplicate to verify the reproducibility of the findings. Statistical analyses were carried out with Statistical Analysis System

software (SAS Institute, Cary, NC) and P values were calculated using the Student t test unless otherwise mentioned.

RESULTS

Normal fibroblasts secrete Par-4 protein in a p53-dependent manner.

To examine whether p53 activation in normal cells exhibits paracrine effects in cancer cells, we used co-cultures of mouse embryonic fibroblasts (MEFs) from p53$^{+/+}$ or p53$^{-/-}$ mice with p53-null H1299 and PC-3 cells and p53-mutant HOP92 cells. The cell cultures were treated with Nutlin-3a, a specific activator of p53 (Vassilev et al., 2004). Nutlin-3a induced apoptosis in p53-deficient cancer cells that were co-cultured with p53$^{+/+}$ MEFs but not with p53$^{-/-}$ MEFs (Figure 2.1A, left panel). As expected, the p53-deficient cancer cells and the MEFs were resistant to apoptosis by Nutlin-3a when cultured individually (Figure 2.1A, right panel). Because p53 may function by partial inhibition of NF-κB activity (Dey et al., 2007), we combined Nutlin-3a with PS-1145, a small molecule that specifically inhibits IKKβ (Hideshima et al., 2002). By itself, PS-1145 does not induce apoptosis of normal or lung cancer cells [Figure 1A, right panel; and (Burikhanov et al., 2013)]. However, treatment of the co-cultures with PS-1145 induced apoptosis in cancer cells, and the combination of Nutlin-3a plus PS-1145 highly augmented that effect (Figure 2.1A).

To determine whether extracellular factors secreted by the MEFs in response to Nutlin-3a and/or PS-1145 treatment contributed to apoptosis of the cancer cells, the MEFs were treated with these small molecules, and conditioned medium

(CM) was transferred to p53-deficient lung cancer cells or normal lung cells. The CM from p53$^{+/+}$ MEFs but not the CM from p53$^{-/-}$ MEFs treated with Nutlin-3a or PS-1145 produced apoptosis of H1299 cells. Moreover, the CM from p53$^{+/+}$ MEFs treated with a combination of Nutlin-3a and PS1145 exhibited an additive apoptotic effect in H1299 cells. By contrast, the CM from MEFs treated with Nutlin-3a and/or PS-1145 did not induce apoptosis in wild-type *TP53* primary human lung fibroblasts HEL cells (Figure 2.1B, left panel). Similarly, CM from HEL cells treated with Nutlin-3a plus PS-1145 induced apoptosis of H1299 and HOP92 cells, but not in HEL cells (Figure 2.1B, right panel). These findings indicated that Nutlin-3a and PS-1145 may regulate the secretion of cancer-selective pro-apoptotic factor(s) in a p53-dependent manner.

We then examined the CM for secreted proteins, especially TRAIL, maspin, IGFBP3, and Par-4, which are known to act extracellularly and induce cancer-specific apoptosis. The CM from p53$^{+/+}$ MEFs treated with Nutlin-3a or PS-1145 showed elevated levels of Par-4 protein, and combination of Nutlin-3a plus PS-1145 additively increased the secretion of Par-4 (Figure 2.1C). None of the other proteins showed elevated secretion with Nutlin-3a plus PS-1145 (data not shown). By contrast, p53$^{-/-}$ MEFs accumulated Par-4 protein in the lysate, but failed to secrete it in response to these treatments (Figure 2.1C). However, secretion of Collagen (Col1A1) was unaffected by the treatments in p53$^{+/+}$ or p53$^{-/-}$ MEFs, implying that p53$^{-/-}$ MEFs were not generally deficient in protein secretion (Figure 2.1C, lower panel). Moreover, pre-treatment of p53$^{+/+}$ cells with pan-caspase inhibitor z-VAD-fmk did not diminish Nutlin-3a plus PS-1145-

inducible secretion of Par-4 protein, indicating that Par-4 secretion was not a post-apoptosis event (Figure 2.1D).

Importantly, the Par-4 antibody but not the PTEN control antibody inhibited apoptotic activity in the CM (Figure 2.1E, left panel), implying that secreted Par-4 mediates the paracrine apoptotic action of p53. Consistent with these observations, the CM from Par-4$^{+/+}$ MEFs but not Par-4$^{-/-}$ MEFs treated with Nutlin-3a plus PS-1145 induced apoptosis of p53-deficient cancer cells (Figure 2.1E, middle and right panels). Moreover, doxorubicin, which is known to activate p53, induced Par-4 secretion from Par-4$^{+/+}$ but not Par-4$^{-/-}$ MEFs, and the CM collected from Par-4$^{+/+}$ but not Par-4$^{-/-}$ MEFs induced apoptosis of p53-deficient cancer cells (Figure 2.1F). Together, these findings suggested that p53 regulates the secretion of Par-4 protein, which selectively induces apoptosis in cancer cells.

p53 induces systemic expression of Par-4 in normal mice.

We examined whether p53 regulates the secretion of Par-4 *in vivo*. The serum of untreated C57/B6 p53$^{+/+}$ mice contained higher levels of Par-4 relative to p53$^{-/-}$ mice (Figure 2.2A, left panel). When p53$^{+/+}$, p53$^{-/-}$, and Par-4$^{-/-}$ mice were injected with Nutlin-3a plus PS-1145 or vehicle control, and serum samples were collected for analysis of Par-4, we noted that relative to vehicle control, Nutlin-3a plus PS-1145 caused a ~5-fold increase in serum levels of Par-4 protein in p53$^{+/+}$ mice. By contrast, Nutlin-3a plus PS-1145 failed to elevate systemic levels of Par-4 in p53$^{-/-}$ mice or Par-4$^{-/-}$ mice (Figure 2.2A, right panel), implying that p53 function was essential for up-regulation of Par-4 secretion in mice. Importantly,

the serum from Nutlin-3a plus PS-1145 treated p53$^{+/+}$ mice, but not p53$^{-/-}$ mice or

Par-4$^{-/-}$ mice, induced *ex vivo* apoptosis of cancer cell cultures but not normal cell

cultures (Figure 2.2B, upper panel). By contrast, the serum from vehicle-treated

mice failed to induce apoptosis over background levels in normal or cancer cells

(Figure 2.2B, lower panel). Moreover, pre-incubation of the serum samples

collected from Nutlin-3a plus PS-1145 treated p53$^{+/+}$ mice with the Par-4 antibody

but not the PTEN control antibody, significantly inhibited the ability of the serum

to induce apoptosis of cancer cell cultures (Figure 2.2C). Together, these

findings suggest that p53 activation in normal mice induces adequate levels of

systemic Par-4 protein that is functionally effective in inducing apoptosis of

cancer cells.

P53 down-regulates the expression of UACA to induce Par-4 secretion.

As Par-4 does not contain a p53 consensus binding site in its DNA, Par-4

secretion is likely regulated by another downstream target of p53. To identify the

primary gene target of p53, we used three criteria: (1) it should encode a protein

that binds to Par-4, (2) it should be regulated by p53, and (3) it should be induced

by NF-κB activity. We recently identified UACA (uveal autoantigen with coiled-coil

domains and ankyrin repeats) as a strong binding partner of Par-4 (Burikhanov et

al., 2013). UACA was co-immunoprecipitated from normal MEF and HEL

fibroblast with Par-4 antibody, and Par-4 was reciprocally co-immunoprecipitated

with UACA antibody (Figure 2.3 A). A comparison of p53$^{+/+}$ and p53$^{-/-}$ MEFs

indicated that UACA was lower in p53$^{+/+}$ normal cells and mouse tissues relative

to p53$^{-/-}$ cells and mouse tissues. Nutlin-3a suppressed the expression of UACA

in p53$^{+/+}$ but not p53$^{-/-}$ MEFs (Figure 2.3B), implying suppression of UACA expression by p53. Our previous studies (Burikhanov et al., 2013) have shown that UACA is regulated by NF-κB and, as expected, UACA expression was inhibited by PS-1145 (Figure 2.3B).

UACA levels in p53$^{+/+}$ and p53$^{-/-}$ MEFs correlated inversely with the levels of Par-4 secreted in the CM. Restoration of p53 activity in p53$^{-/-}$ MEFs by p53-adenoviral infection or at 32°C in 10(1)/Val5 fibroblasts, which contain a temperature-sensitive mutant of p53 (Harvey and Levine, 1991; Wu and Levine, 1994), suppressed the expression of UACA and induced secretion of Par-4 (Figure 2.3C). Moreover, knock-down of UACA using several different mouse and human siRNA duplex pools, resulted in elevated Par-4 levels in the CM of p53$^{+/+}$ MEFs and HEL cells (Figure 2.3D). By contrast, knock down of UACA in p53$^{-/-}$ MEFs did not elevate the secretion of Par-4 (Figure 2.3D), indicating that p53 function was necessary to regulate the secretion of Par-4. Consistently, ectopic expression of UACA inhibited the secretion of Par-4 (Figure 2.3E). UACA binding to Par-4 was essential to prevent Par-4 secretion, and accordingly, mutant UACA (631-1413aa), which does not bind to Par-4 (Burikhanov et al. 2013), was unable to prevent Par-4 secretion (Figure 2.3F).

UACA and p53 reciprocally regulate Par-4 secretion via the classical pathway.

Previous studies have indicated that Par-4 is secreted via the classical ER-Golgi pathway (Burikhanov et al., 2009), as well as via the exosomal pathway (Wang et al., 2012). On the other hand, p53 has been shown to promote secretion of

proteins via the exosomal pathway (Yu et al., 2006; Yu et al., 2009). To determine whether p53 activation leads to Par-4 secretion via the classical pathway, we used brefeldin A (BFA), which blocks the ER-Golgi pathway. BFA inhibited the secretion of Par-4 by p53 restoration at $32^{o}C$ in 10(1)/Val5 fibroblasts (Figure 2.3G, left panel). As both Nutlin-3a and PS-1145 induced the secretion of Par-4 in a p53-dependent manner, we investigated whether Par-4 secretion following Nutlin-3a plus PS-1145 treatment occurred via the classical pathway. Nutlin-3a plus PS-1145-inducible secretion of Par-4 from p53$^{+/+}$ MEFs was about 60% inhibited by BFA (Figure 2.3G, middle panel). These findings imply that p53 regulates the secretion of Par-4 largely by the classical pathway. Moreover, Par-4 secretion following UACA knock down in p53$^{+/+}$ MEFs was inhibited by BFA (Figure 2.3G, right panel). Collectively, these findings indicate that UACA inhibition by siRNA, Nutlin-3a plus PS-1145, or p53 activation induces Par-4 secretion via the classical pathway.

UACA is a direct target of p53.

A potential p53-binding site (el-Deiry et al., 1992) is present in the DNA corresponding to exon 1 within the coding region of UACA (Figure 2.4A). P53 suppresses the expression of UACA RNA, as judged by qRT-PCR (Figure 2.4B). In order to determine direct binding of p53 to its consensus binding site in UACA, we performed chromatin immuno-precipitation (ChIP) studies in p53-deficient cells that were infected with a GFP-p53 adenoviral expression construct. Our results showed that ectopically expressed p53 construct was bound to the putative p53 consensus element present in exon 1 within the *UACA* gene (Figure

2.4C). We next corroborated this finding with endogenous p53. HEL cells treated with Nutlin-3a, but not with vehicle, showed chromatin immuno-precipitation of endogenous p53 bound to the p53-consensus motif in UACA (Figure 2.4D). Consistently, Nutlin-3a treatment, which activates endogenous p53, suppressed the expression of UACA in HEL cells (Figure 2.4E).

To determine whether p53 regulated UACA expression via its binding motif in an NF-κB activity-independent manner, we performed our experiments in IKKβ$^{-/-}$ MEFs, which lack NF-κB activity (Burikhanov et al., 2013; Li et al., 1999). Nutlin-3a treatment of IKKβ$^{-/-}$ MEFs resulted in suppression of UACA expression (Figure 2.4E). Moreover, ectopic p53 suppressed the expression of UACA containing the p53-binding motif, but did not suppress the expression of mutant-UACA, which contained mutations in the p53-binding sequence in IKKβ$^{-/-}$ MEFs at both protein (Figure 2.4F, upper right panel), and mRNA levels (Figure 2.4F, lower panel). Together, these findings suggest that p53 suppressed the expression of UACA via its binding motif in an NF-κB-independent manner. Thus, UACA is a relevant target of p53, and p53 may regulate Par-4 secretion by down-regulation of UACA.

DISCUSSION

The tumor suppressor p53 is known to suppress tumor growth by intracellular activation of growth arrest and apoptotic cell death pathways. However, p53 is mutated in over 50% of cancers, and mutant forms of p53 may render cancer cells resistant to both chemotherapy and radiation therapy. Lung cancer, the leading cause of deaths in the US, is often associated with inactivating mutations

in p53. We determined whether wild type p53 function in normal cells could be effectively propelled to target lung cancer cells. The present study revealed that p53 activation in normal cells induces paracrine apoptosis of p53-deficient lung cancer and prostate cancer cells. This action of p53 is mediated by the tumor suppressor protein Par-4. The paracrine effect of p53 activation on Par-4 secretion was bolstered by co-parallel inhibition of NF-κB activity in normal cells. We noted that UACA binds to Par-4 and prevents it from being secreted, and inhibition of UACA by p53 activation and/or inhibition of NF-κB activity, or several different siRNAs, results in elevated secretion of Par-4. Importantly, Par-4 secreted by cells following co-parallel activation of p53 and inhibition of NF-κB activity caused apoptosis in p53-deficient lung cancer cells but not normal cells. The physiological relevance of the findings was confirmed by studies indicating that normal cells in mice can be triggered to secrete pro-apoptotic Par-4 activity in circulation in a p53-dependent manner. The elevated levels of Par-4 in serum induced *ex vivo* apoptosis in tumor cells but not normal cells. Because our previous studies indicated a good correlation between inhibition of lung tumors in immuno-competent mice and induction of *ex vivo* apoptosis by Par-4 in the serum of these mice [Zhao et al, 2011], the activation of p53 to trigger pro-apoptotic Par-4 protein secretion and elevate its systemic levels may be an effective strategy to induce apoptosis of cancer cells that metastasize through the circulatory route. Collectively, our findings suggest that secreted Par-4 mediates the paracrine apoptotic effects of p53, and that secretagogues of Par-4

may therefore empower normal cells to execute paracrine tumor growth inhibition.

Our findings indicate that Par-4 secretion following p53 activation occurs via the classical BFA-sensitive pathway. Secretion of Par-4 via this pathway is dependent on down-modulation of UACA, a novel functional target of p53. Chromatin immunoprecipitation experiments confirmed that p53 directly binds to its consensus motif in UACA. Importantly, p53 suppressed the expression of UACA in an NF-κB-independent manner. As UACA is a principal binding partner that sequesters Par-4 in normal cells, UACA inhibition by p53 is necessary to release Par-4 for secretion via the classical pathway. However, normal cells lacking p53 are deficient in Par-4 secretion despite UACA down-regulation, implying that p53 function is critical for Par-4 secretion and is suggestive of an additional role for p53 in triggering Par4 secretion. Several integral components of the classical ER-Golgi-transport vesicle/membrane fusion pathway are currently being investigated as targets for p53-dependent Par-4 secretion. Given that previous studies have indicated that p53 regulates protein secretion via the exosomal pathway, we tested whether Par-4 secretion is also regulated via the exosomes. Our findings indicate that of the total Par-4 secreted by cells, <5% is secreted via the exosomal pathway and the large majority is secreted by the classical pathway (unpublished data).

In summary, our findings suggest that the tumor suppressor p53 regulates the secretion of the pro-apoptotic, tumor suppressor Par-4 via the classical pathway by suppressing UACA, and that Par-4 executes the paracrine apoptotic effects of

p53. As systemic Par-4 inhibits the growth of lung tumors [Zhao et al, 2011], Par-4 secretagogues can be exploited to activate p53 and unleash the power of normal cells in the tumor micro- and macro-environment to elevate systemic Par-4 and suppress tumor cell survival.

Figure 2.1: P53 activation in normal cells produces paracrine apoptosis in p53-deficient cancer cells

C

		v	N	P	N+P	v	N	P	N+P
CM	Par-4								
	fold change:	1	2	2	4.5	<0.1	<0.1	<0.1	<0.1
	albumin								

lysate	p53								
	Par-4								
	actin								

		v	N	P	N+P	v	N	P	N+P
CM	Col1A1								
	albumin								

p53$^{+/+}$ p53$^{-/-}$

D

		v		N+P	
	zVAD	−	+	−	+
CM	Par-4				
	COL1A1				
	albumin				
lysate	Par-4				
	actin				
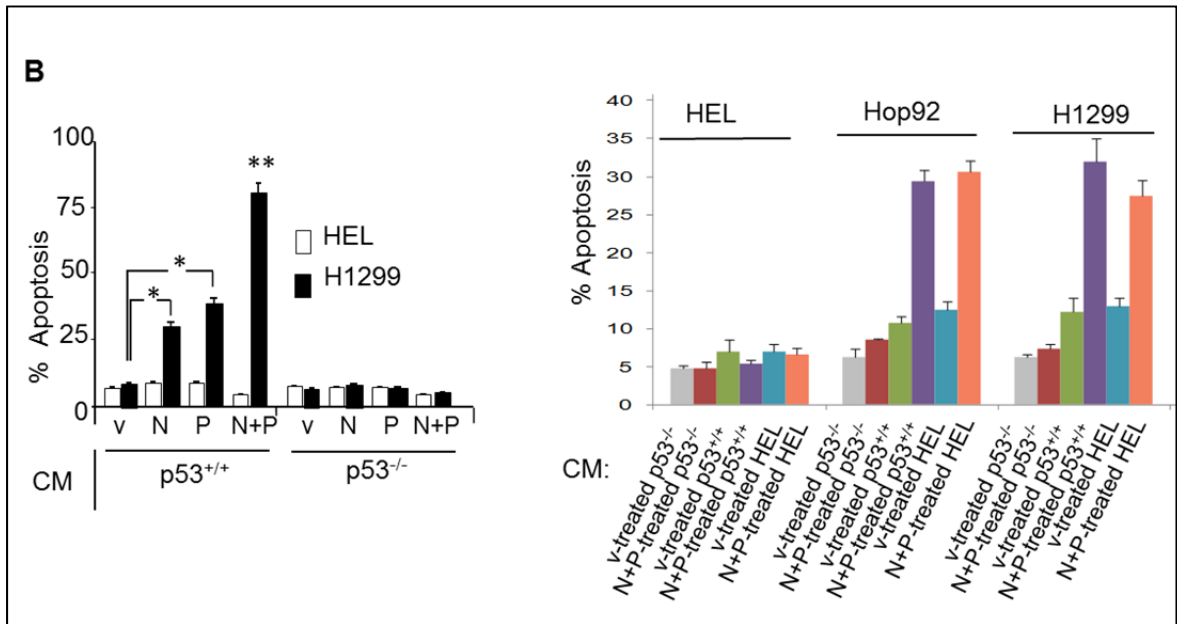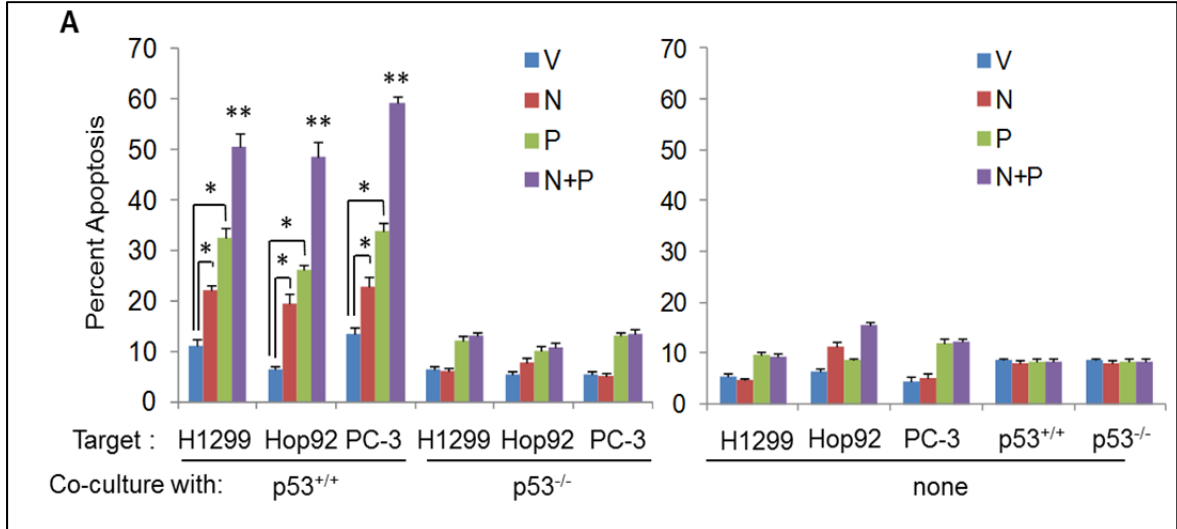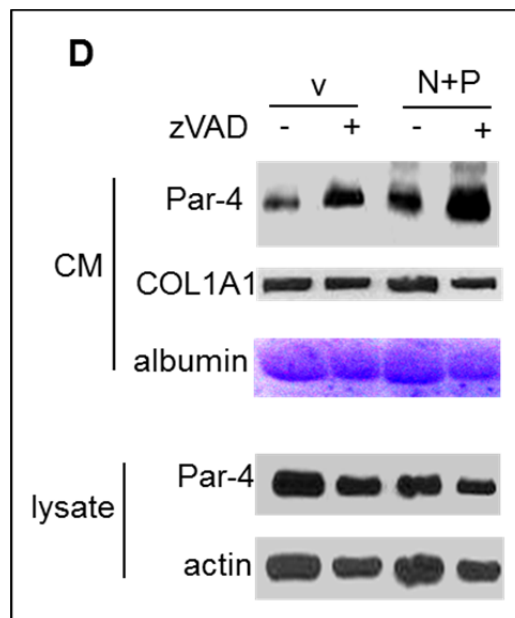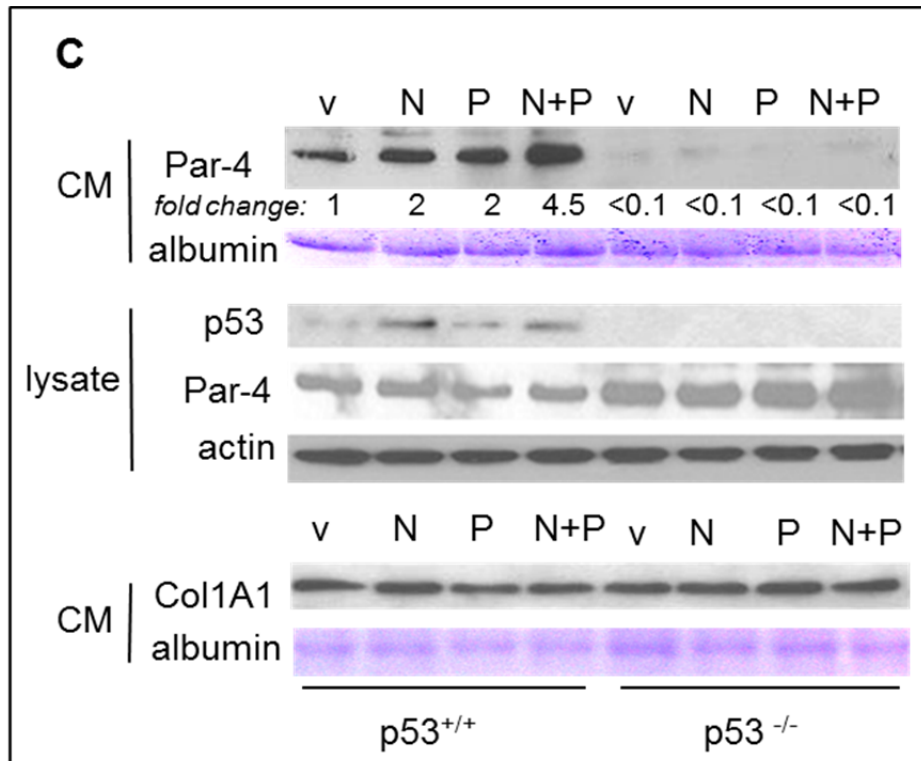
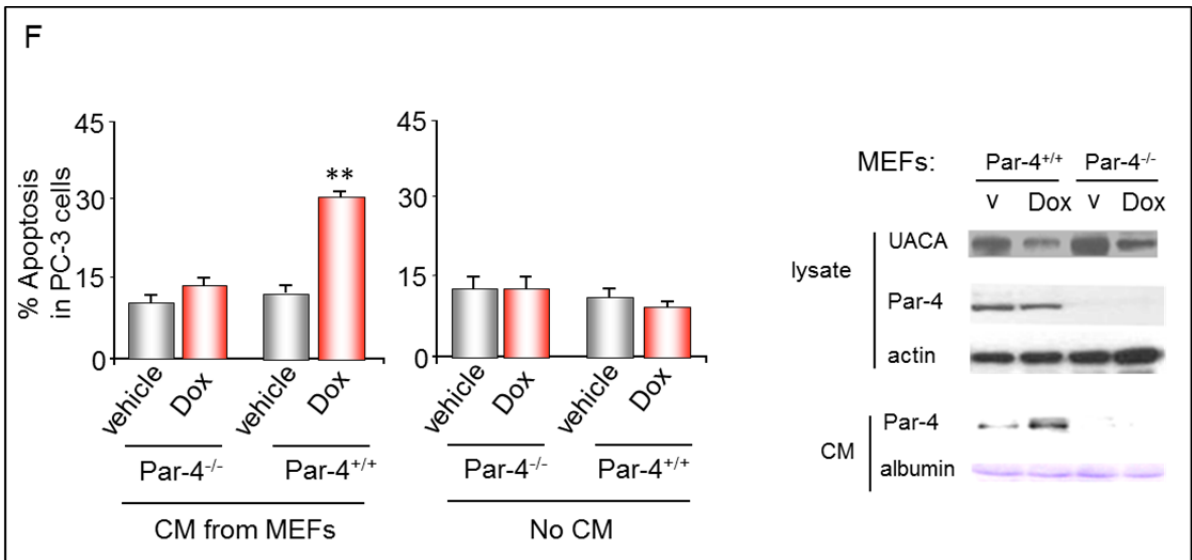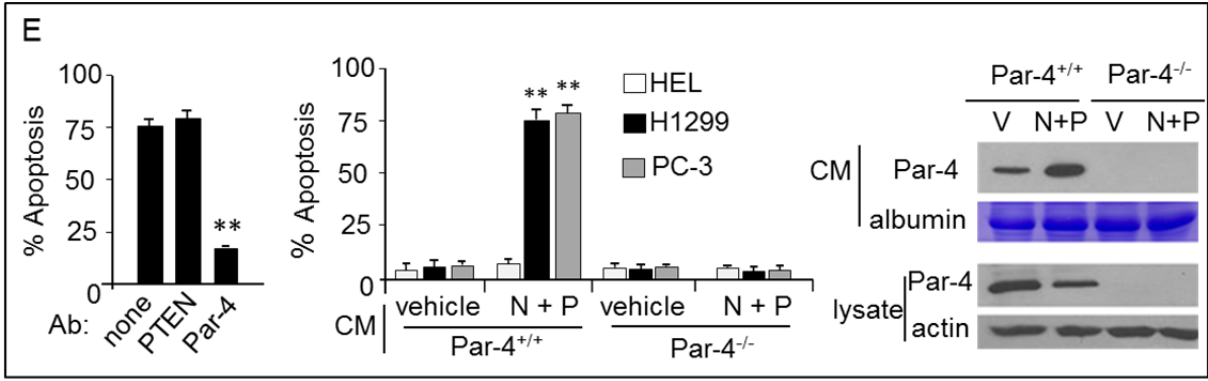**Figure 2.1: P53 activation in normal cells produces paracrine apoptosis in p53-deficient cancer cells**

(A) **Activation of p53 in MEFs induces apoptosis of co-cultured p53-deficient cancer cells**. Co-cultures of p53$^{+/+}$ or p53$^{-/-}$ MEFs with the indicated cancer cells, were treated with vehicle (v), Nutlin-3a (N, 10 µM) and/or PS-1145 (P, 10 µM) for 24 h. The cells were subjected to ICC for cytokeratins to detect epithelial cancer cells and for active caspase-3 to determine apoptotic cells. Performed by Dr. Ravshan Burikhanov.

(B) **Apoptosis of cancer cells by CM obtained from p53-activated MEFs**. P53$^{+/+}$ or p53$^{-/-}$ MEFs were treated with vehicle (v), Nutlin-3a (N, 10 µM) and/or PS-1145 (P, 10 µM) for 24 h, and their CM was transferred to normal (HEL) or p53-deficient cancer (H1299) cells. Apoptotic cells were scored after 24 h by ICC for active caspase-3 (left panel). Similarly, p53$^{+/+}$ or P53$^{-/-}$ MEFs and HEL cells were treated with vehicle (v), Nutlin-3a (N, 10 µM) and/or PS-1145 (P, 10 µM) for 24 h and the CM was transferred to various normal (HEL) and p53-deficient cancer (H1299, HOP-92) cell lines. After 24 h, the cells were scored for apoptosis (right panel).

(C) **Co-parallel activation of p53 and inhibition of NF-κB activity additively increases Par-4 secretion in fibroblasts**. MEFs were treated with vehicle (v), Nutlin-3a (N) and/or PS-1145 (P) for 24 h, and their CM, as well as whole-cell lysates were subjected to Western blot analysis for Par-4. β-actin was used to normalize loading of lysate. Coomassie blue-stained albumin in the serum was used to normalize loading of CM. Par-4 secretion, but not Col1A1 secretion, was dependent on the p53 status of the cells. Fold change in secreted Par-4 is shown.

(D) **Par-4 secretion in response to p53 activation does not require apoptosis of the normal cells**. P53$^{+/+}$ or P53$^{-/-}$ MEFs were treated with z-VAD-fmk (2 µM) and with Nutlin-3a (N, 10 µM) plus PS-1145 (P, 10 µM) or vehicle (v) for 24 h. The CM was examined for Par-4 levels by Western blot analysis.

(E) **Par-4 secretion is critical for the paracrine effect resulting from simultaneous p53-activation and NF-κB-inhibition**. The CM from p53$^{+/+}$ cells treated with Nutlin-3a + PS-1145 was incubated with polyclonal antibody for Par-4 or PTEN control, and then added to H1299 cells. Apoptosis of H1299 cells was scored after 24 h by ICC for active caspase 3 (left panel). Par-4$^{+/+}$ and Par-4$^{-/-}$ MEFs were treated with Nutlin-3a + PS-1145 (N+P) or vehicle, then the CM was applied to the indicated cell lines, and apoptotic cells were scored by ICC for active caspase-3 (middle panel). Expression of Par-4 in CM was verified on Western blots (right panel). Performed by Dr. Ravshan Burikhanov.

(F) Doxorubicin induces paracrine apoptosis of cancer cells by Par-4 secretion. PC-3 cells were treated with 100 nM doxorubicin (Dox) or vehicle for 24 h and scored for apoptosis by ICC for active caspase-3. Note 100 nM Dox does not directly induce apoptosis in PC-3 cells (middle panel). Par-4$^{+/+}$ or Par-4$^{-/-}$ MEFs were treated with 100 nM doxorubicin (Dox) or vehicle for 24 h and the CM from these cells was transferred to PC-3 cells; after 24 h the PC-3 cells were scored for apoptosis (left panel). Elevation of Par-4 in the CM of doxorubicin (Dox)- or vehicle (v)-treated Par-4$^{+/+}$ or Par-4$^{-/-}$ MEFs was confirmed by Western blot analysis (right panel). Right panel performed by Dr. Ravshan Burikhanov.

(A, B) Asterisk (*) indicates statistical significance (P < 0.001) by the Student *t* test. (**) indicates that N+P is significantly (P < 0.001) more effective than individual treatments among different cell lines based on two-way ANOVA followed by Bonferroni correction.

(E, F) (**) indicates that the indicated treatments are significantly (P < 0.001) more effective than their respective control groups based on two-way ANOVA followed by Bonferroni correction.

Figure 2.2: Activation of p53 in mice induces systemic expression of Par-4 pro-apoptotic activity

C

HOP92 cells   KP cells   LLC1 cells

serum from
mice injected
with:

vehicle   N+P
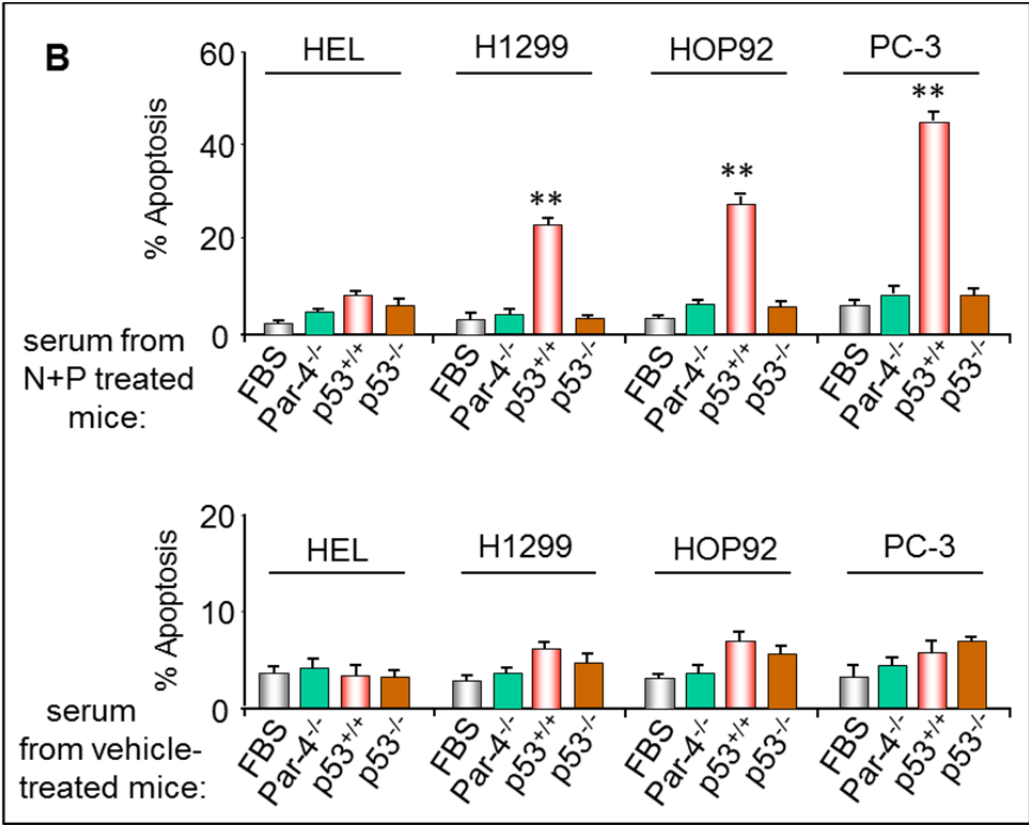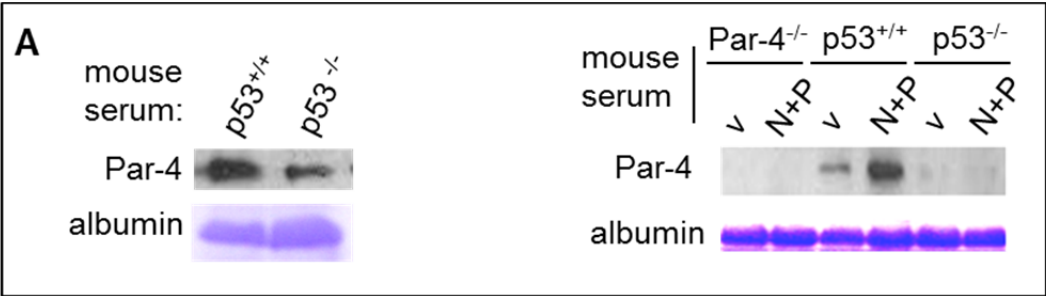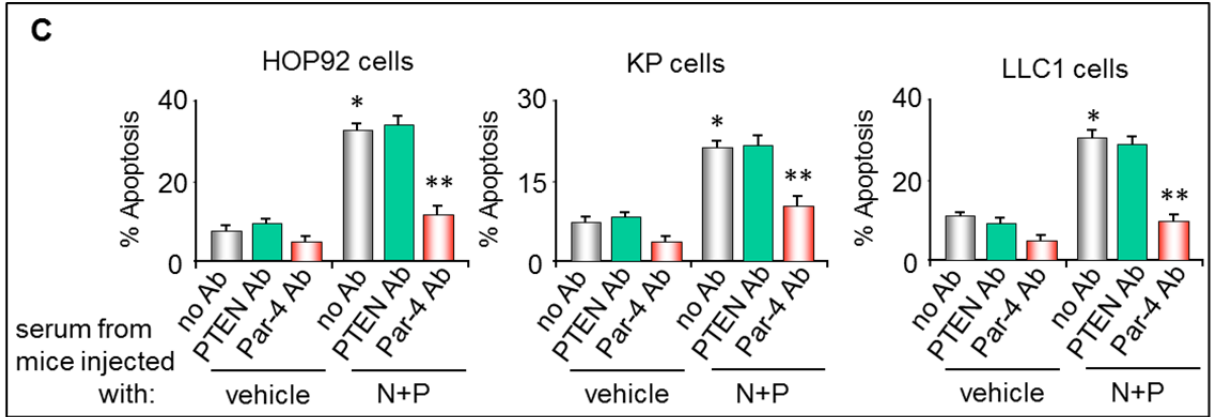
Figure 2.2: Activation of p53 in mice induces systemic expression of Par-4 pro-apoptotic activity.

(A) P53 function is essential for induction of systemic Par-4 in mice. Serum samples from p53$^{+/+}$ mice (4 µl per lane) and p53$^{-/-}$ mice (8 µl per lane) (left panel); and also from p53$^{+/+}$, p53$^{-/-}$, and Par-4$^{-/-}$ mice (right panel) injected via i.p. route with either vehicle (v) or with Nutlin-3a + PS-1145 (N+P) were examined for Par-4 expression by Western blot analysis using serum albumin to normalize loading. Data are representative of 4 mice per treatment. Performed by Dr. Ravshan Burikhanov.

(B) Serum from Nutlin-3a plus PS-1145-treated p53$^{+/+}$ mice induces *ex vivo* apoptosis of cancer cells. Serum samples collected from p53$^{+/+}$, p53$^{-/-}$, and Par-4$^{-/-}$ mice injected i.p. with either vehicle (lower panel) or with Nutlin-3a + PS-1145 (N+P) (upper panel) was applied at a final concentration of 10% to the indicated normal or cancer cell lines. The cells were scored for apoptosis after 24 h. Fetal bovine serum (FBS, 10%) was used as an additional control. Performed by Dr. Ravshan Burikhanov.

(C) Secreted Par-4 is essential for the paracrine effect of p53 activation. Serum samples collected from p53$^{+/+}$ mice treated with vehicle or Nutlin-3a + PS-1145 (N+P) were pre-incubated with Par-4 antibody, control PTEN antibody or no antibody (Ab), and then applied on lung cancer cells. After 24 hours of treatment, the cells were scored for apoptosis. Performed by Dr. Ravshan Burikhanov.

(B) Asterisk (**) indicates there is a significant ($P < 0.001$) increase in apoptosis of cancer cells with serum obtained from p53$^{+/+}$ mice treated with N+P, based on two-way ANOVA followed by Tukey's test.

(C) Asterisk (*) indicates statistically significant ($P < 0.001$) increase in apoptosis of cancer cells with serum obtained from wild-type mice treated with N+P, and (**) indicates significant ($P < 0.001$) reduction in this effect when Par-4 antibody is used. The statistical analysis was performed using two-way ANOVA followed by Tukey's test.

Figure 2.3: P53 stimulates Par-4 secretion by suppressing the expression of UACA

F

CM
- Par-4
- Col1A1
- albumin

Lysate
- RFP antibody
- Par-4
- actin

RFP
UACA 1-630-RFP
UACA 631-1413-RFP
UACA-RFP

UACA-RFP
1 — UACA — 1413aa — RFP

UACA 1-630aa-RFP
1 — 630aa — RFP

UACA 631-1413aa-RFP
631 — 1413aa — RFP

(1-225aa) **RFP**

p53-binding site

G

10(1)Val5/32°C

CM
- Par-4
- Col1A1
- albumin

lysate
- UACA
- Par-4
- actin

v   BFA

v
v+BFA
N+P+v
N+P+BFA

CM
- Par-4
- Col1A1
- albumin

lysate
- UACA
- Par-4
- actin

siRNA: control   UACA

v BFA   v BFA

CM
- Par-4
- Col1A1
- albumin

lysate
- UACA
- Par-4
- actin
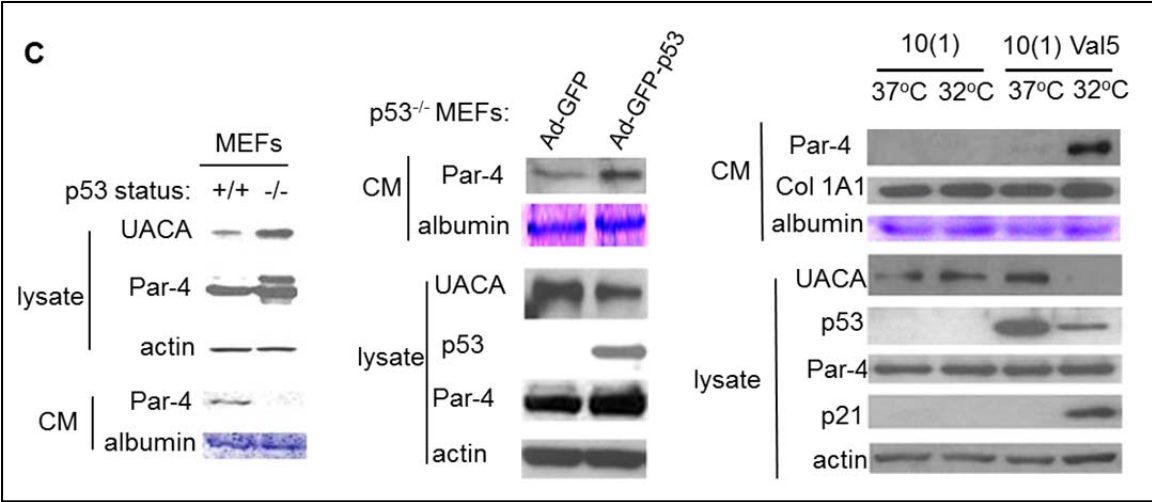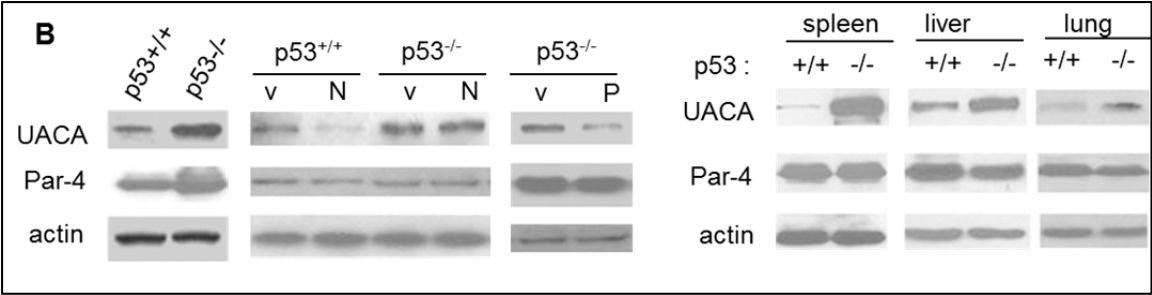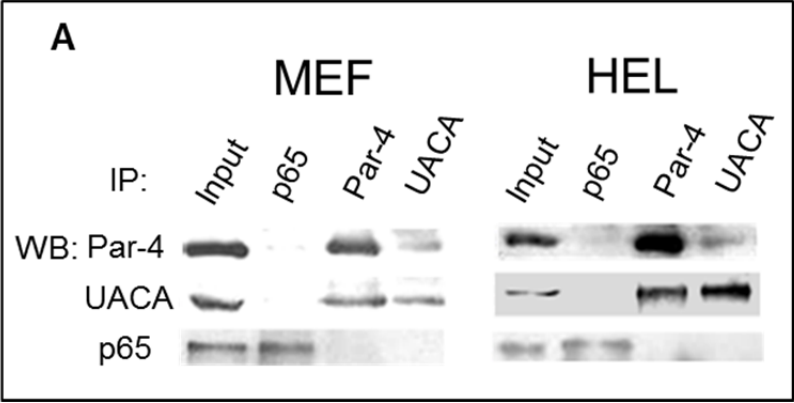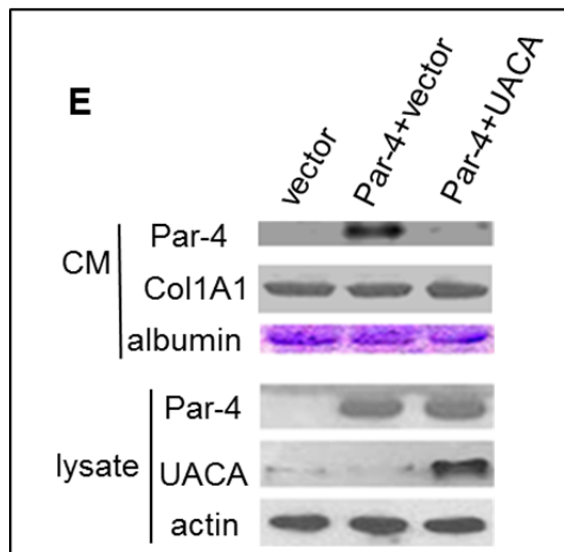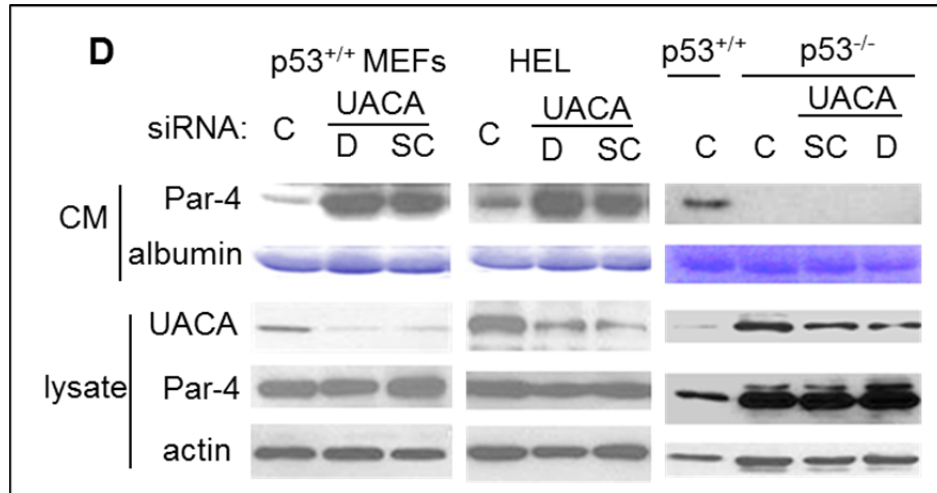
Figure 2.3: P53 stimulates Par-4 secretion by suppressing the expression of UACA.

(A) **UACA binds to Par-4 in fibroblasts**. Whole-cell lysates of human lung fibroblasts HEL and p53$^{+/+}$ MEFs were subjected to co-immunoprecipitation with Par-4, UACA, or p65/NF-κB (control) antibody. The immunoprecipitates and i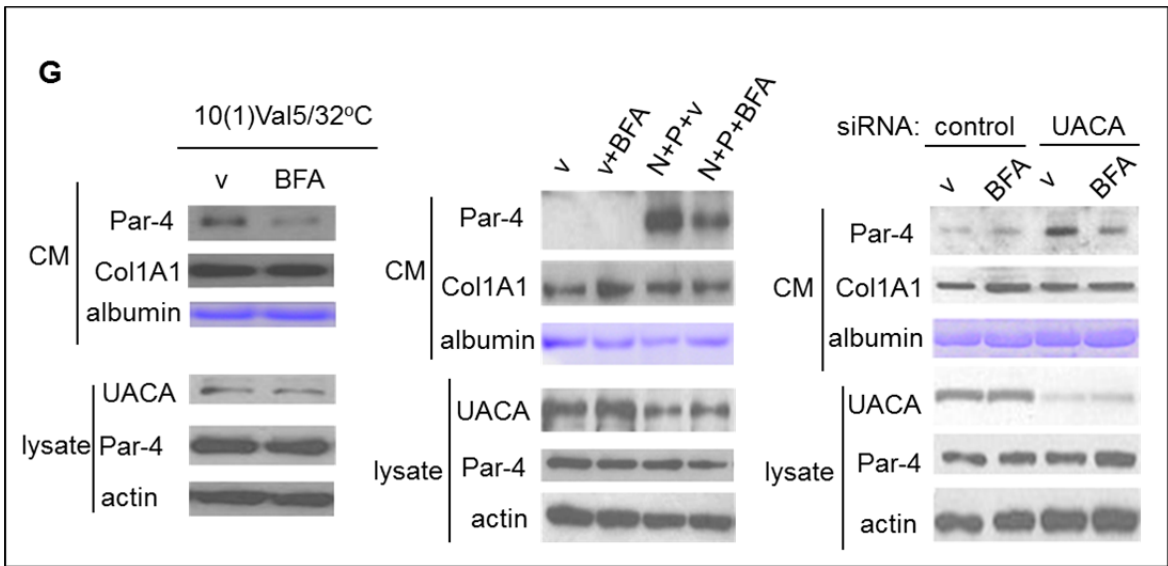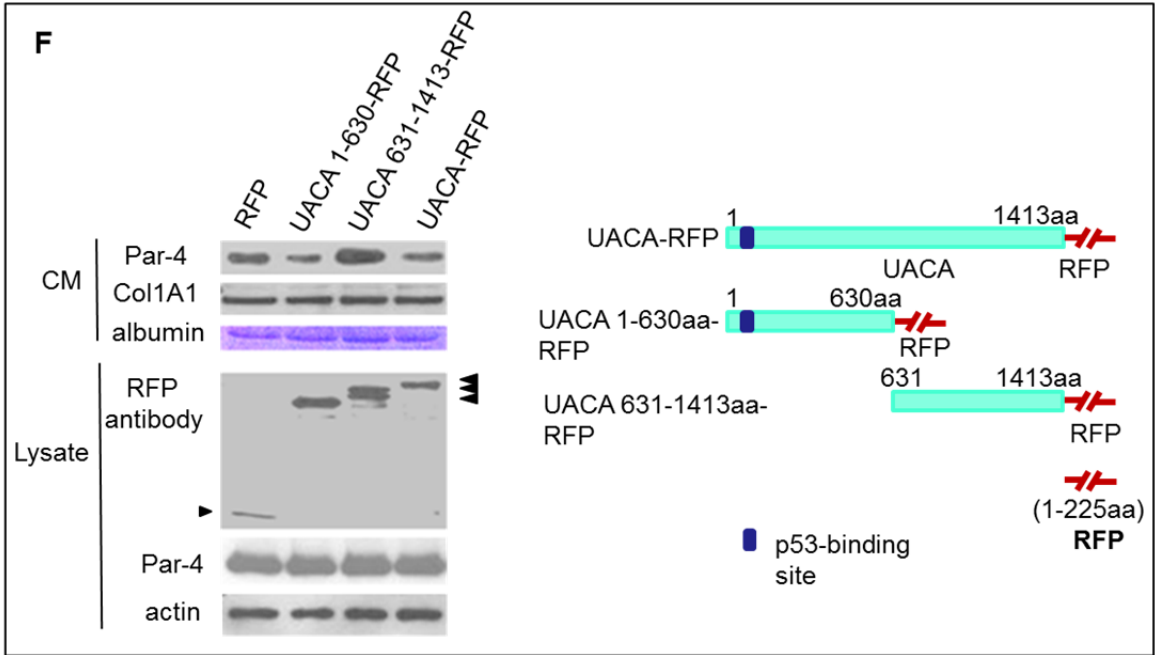nput lysates were examined by Western blot analysis to anallyze binding interaction between Par-4 and UACA in fibroblasts.

(B) **P53 down-regulates UACA**. Whole-cell lysates from p53$^{+/+}$ and p53$^{-/-}$ MEFs that were either untreated or treated with vehicle (v), Nutlin-3a (N, 10μM), or PS-1145 (P, 10 μM) for 24 hours (left 3 panels) ; or whole-tissue lysates of highly vascular organs obtained from p53$^{+/+}$ and p53$^{-/-}$ mice (right 3 panels), were examined for UACA by Western blot analysis. Right panel performed by Dr. Ravshan Burikhanov.

(C) **Restoration of p53 activity inhibits UACA expression and promotes Par-4 secretion.** CM or whole-cell lysates from p53$^{+/+}$ and p53$^{-/-}$ MEFs were subjected to Western blot analysis for UACA or Par-4 expression. Results indicate an inverse relation between UACA expression and Par-4 secretion (left panel). P53$^{-/-}$ MEFs were infected with GFP-tagged p53- or GFP-producing adenoviral constructs (middle panel). Also, the mouse fibroblasts 10(1), which do not express any p53, and 10(1) derived Val5 cells, which are engineered to stably over-express wild-type p53 at 32 °C or mutant p53 at 37 °C, were grown at 37°C or shifted to 32°C to activate p53 (right panel). Expression of the indicated proteins in the CM or whole-cell lysate was examined by Western blot analysis. Middle panel performed by Dr. Ravshan Burikhanov.

(D) **UACA inhibits Par-4 secretion.** UACA expression was knocked down in mouse (p53$^{+/+}$ MEF) and human (HEL) cells with distinct siRNA pools from two different sources- Dharmacon (D), and SantaCruz Biotechnology, Inc. (SC), and the CM, as well as the whole cell lysates, were subjected to Western blot analysis. C, control (scrambled) siRNA. Performed by Dr. Ravshan Burikhanov.

(E) **Ectopic expression of UACA inhibits the secretion of Par-4.** MEFs from Par-4$^{-/-}$ mice were co-transfected with Par-4 expression construct plus vector control, Par-4 and UACA expression construct (1:1 ratio), or with vector control. Transfected populations were selected, confirmed for stable expression of Par-4 and UACA using whole-cell lysates, and then secretion of Par-4 in the CM was examined by Western blot analysis. Performed by Dr. Ravshan Burikhanov.

(F) **UACA binding to Par-4 is essential in order to prevent Par-4 secretion.** Par-4$^{+/+}$ MEFs were transfected with expression constructs for Red

Fluorescent Protein (RFP; vector), RFP-tagged UACA, or RFP-tagged UACA deletion mutants, 1-630 (which binds to Par-4) or 631-1413 (which does not bind to Par-4) (Burikhanov et al., 2013). Transfected populations were selected, confirmed for stable expression of UACA or UACA-mutants using whole-cell lysates, and secretion of Par-4 in their CM was determined by Western blot analysis. Diagrammatical representations of full-length UACA and its deletion mutants are shown (right panel). Performed by Dr. Ravshan Burikhanov.

(G) **P53 activation and UACA inhibition promotes secretion by a BFA-sensitive pathway.** 10(1)/Val5 fibroblasts grown at 32°C were treated with BFA (1 μg/ml) or vehicle (v) for 3 h (left panel). UACA expression was knocked down in MEFs (p53$^{+/+}$) with Nutlin-3a plus PS-1145 (N+P; 10 μM each) or vehicle (v) control (middle panel), or with an siRNA pool (from Dharmacon) (right panel), and then the cells were further placed in the presence of BFA or vehicle (v) for 3 h. The CM, as well as the whole-cell lysates, were subjected to Western blot analysis. Right panel performed by Dr. Ravshan Burikhanov.

Figure 2.4: P53 directly binds to UACA and inhibits its expression

B

C

F

UACA
p53
actin

Wild type mouse fibroblasts

UACA
p53
Par-4
actin

IKKβ⁻/⁻ mouse fibroblasts

UACA
132 bp

18S
151 bp

IKKβ⁻/⁻ mouse fibroblasts

77
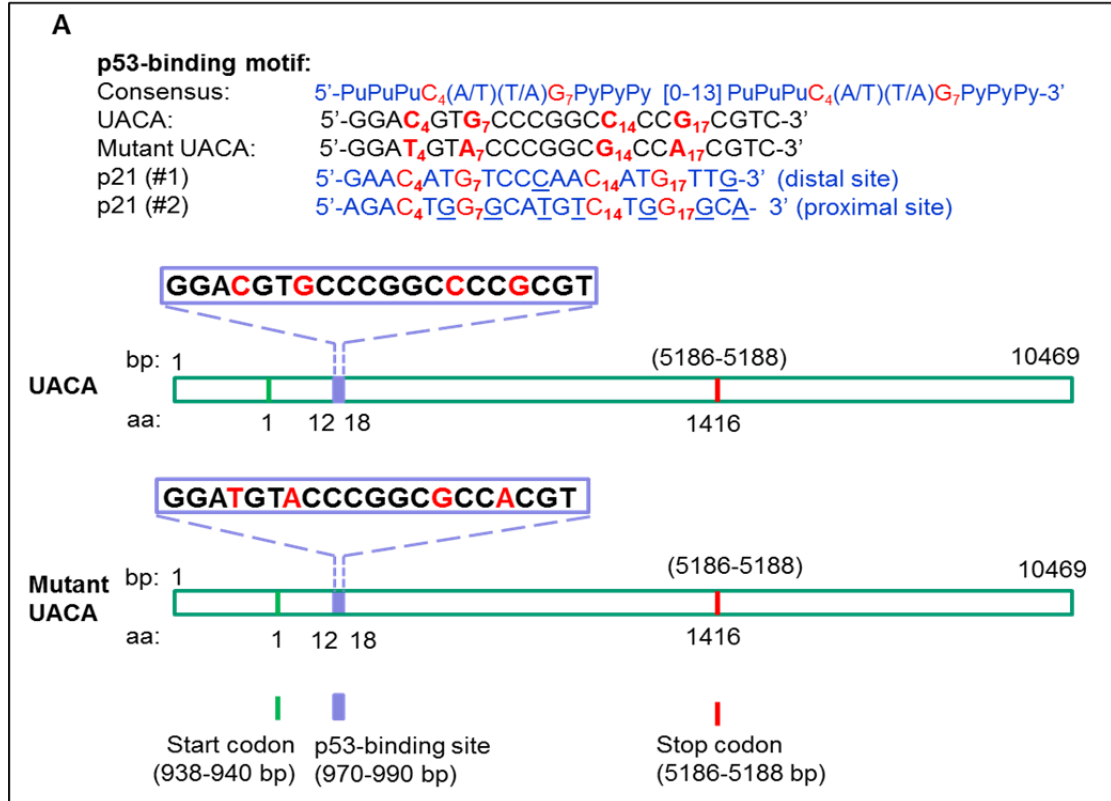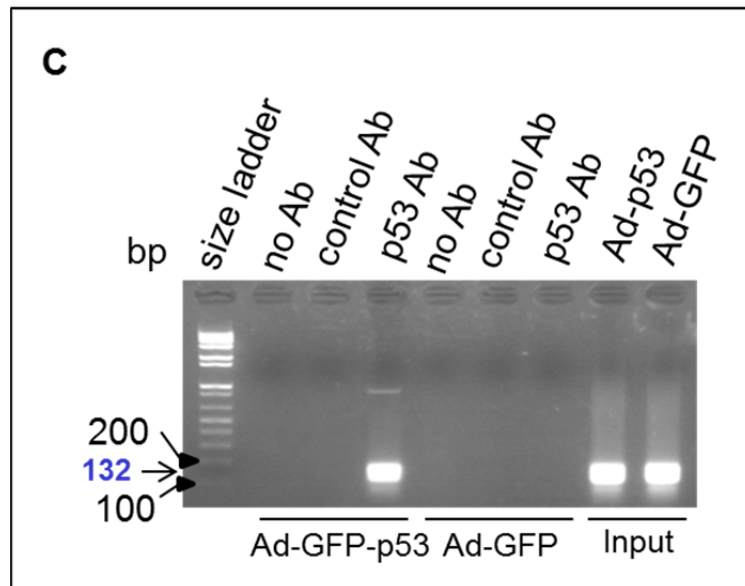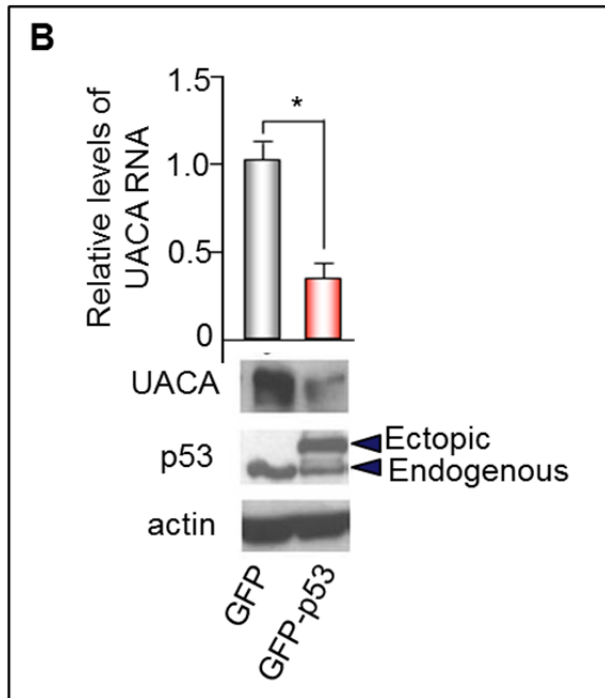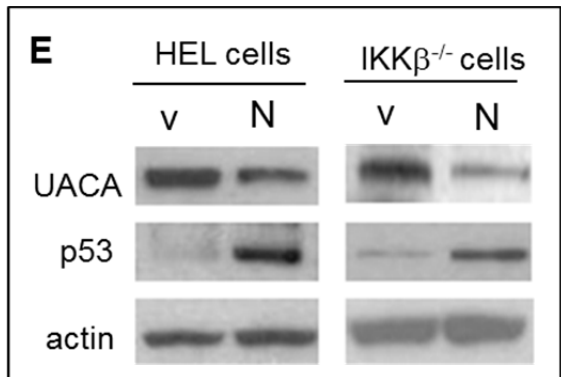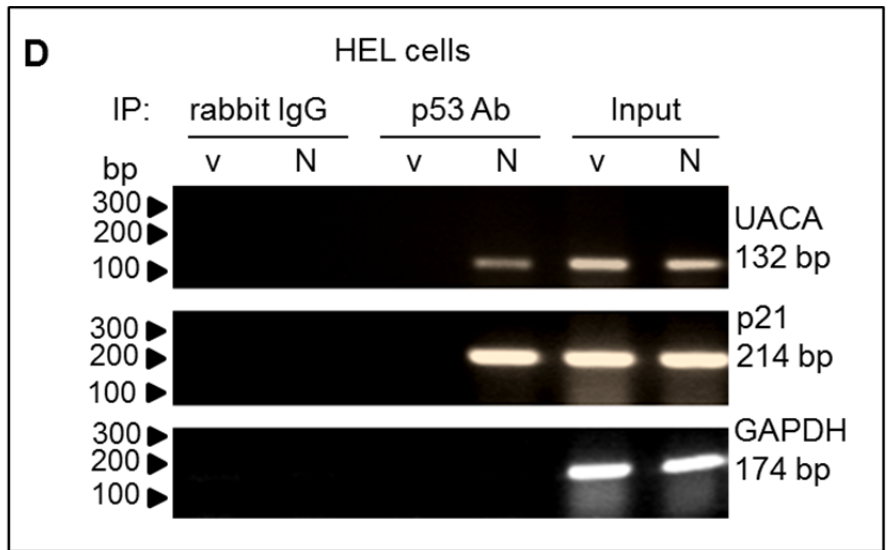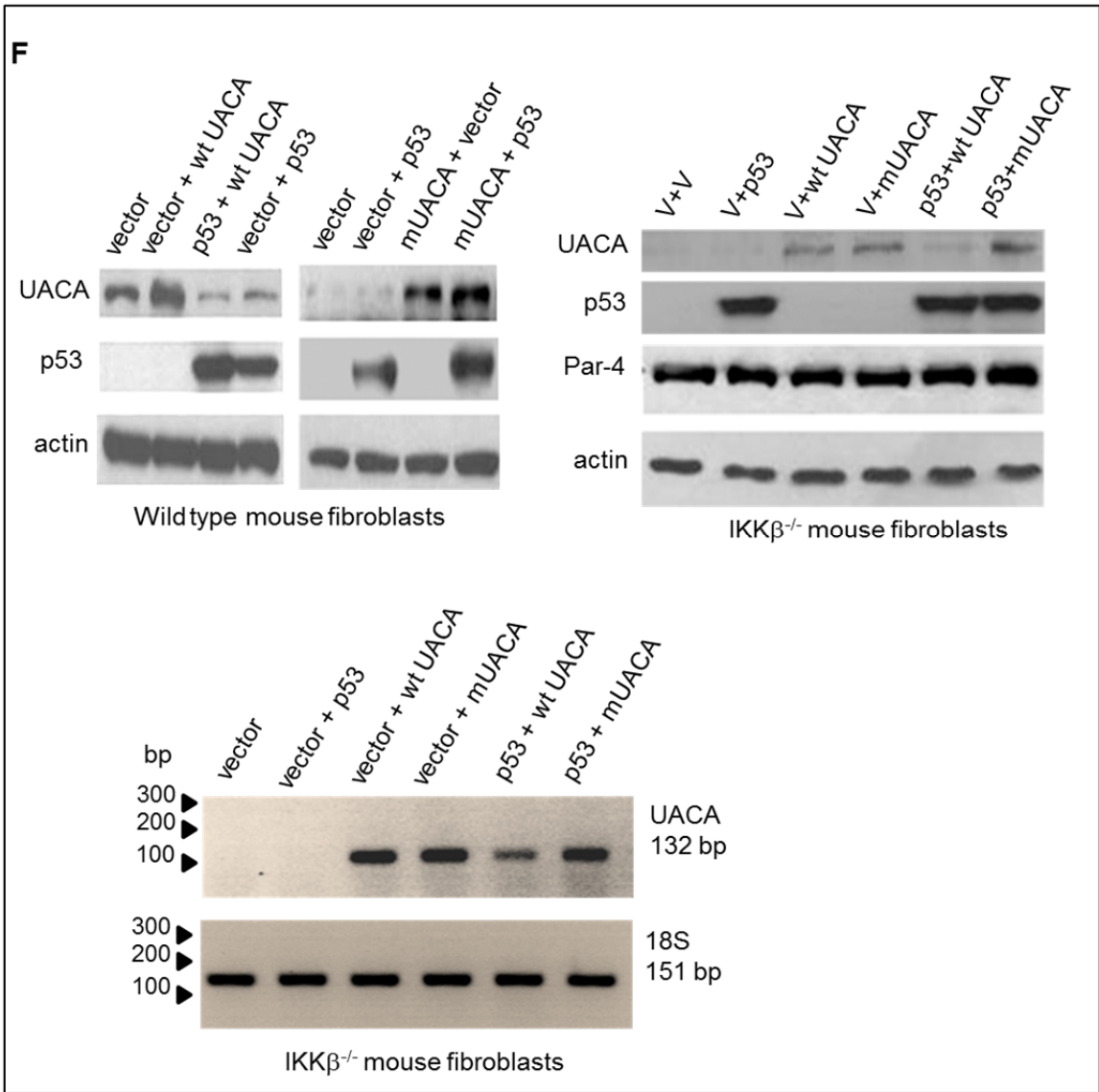
Figure 2.4: P53 directly binds to UACA and inhibits its expression.

(A)   **Comparison of the p53 response elements in UACA and p21, and location of wild type p53 binding motif and mutations introduced in UACA.** The p53 binding site consensus DNA motif is shown along with the p53-binding motifs in UACA and p21. The indicated mutations were introduced at C4, G7, C14 and G17 into the p53-binding motif of UACA. P21 has two p53-binding motifs (#1 and #2). The base pairs in the p53-binding motif of p21 that deviate from the p53 consensus binding motif are underlined. Mutant-UACA with the altered p53-binding site was generated by PCR using mutant-UACA primers and wild type UACA DNA as template. Fidelity of the constructs was confirmed by Retrogen Inc., CA.

(B)   **P53 inhibits the expression of UACA RNA.** Cells were infected with either control GFP-producing adenovirus or GFP-tagged p53-producing adenovirus for 24 h. Total RNA was isolated from each of the samples and subjected to qRT-PCR (upper panel) or subjected to Western blot analysis (lower panel). Performed by Dr. Shirley Qiu.

(C)   **P53 binds to its consensus binding motif in *UACA*.** A putative p53 binding motif (5'-GGAC_4GTG_7CCCGGCC_{14}CCG_{17}CGTC-3'; where C4 and G7 have been previously shown to be critical) is present in the coding region of UACA. P53-deficient cells were infected with GFP-tagged p53- or GFP-producing adenovirus and subjected to ChIP analysis with p53 antibody (Ab) or control rabbit Ab, and immunoprecipitated DNA fragments were analyzed by PCR with primers flanking the p53 binding site.

(D)   **Endogenous p53 binds to its consensus binding motif in *UACA*.** HEL cells were treated with Nutlin-3a (N) or vehicle (v) for 24 h and subjected to ChIP analysis with p53 antibody (Ab) or control rabbit IgG Ab, and immunoprecipitated DNA fragments were analyzed by PCR with primers flanking the p53-binding site in UACA. Primers flanking the p53-binding motif #1 in p21 (Figure 2.4A) or for GAPDH (which does not contain a p53-binding site), were used as positive or negative control, respectively. Input samples for each set of primers are shown.

(E)   **Nutlin-3a causes inhibition of endogenous UACA expression in an NF-κB independent manner**. IKKβ$^{-/-}$ MEFs or IKKβ$^{+/+}$ MEFs and HEL cells, as controls, were treated with Nutlin-3a or vehicle for 24 h, and whole-cell lysates were subjected to Western blot analysis for UACA, p53 or actin.

(F)   **P53 regulates UACA expression via its binding motif in an NF-κB activity-independent manner.** IKKβ$^{-/-}$ MEFs or IKKβ$^{+/+}$ MEFs, as

controls, were co-transfected with the indicated expression constructs. Whole-cell lysates were subjected to Western blot analysis for UACA, p53 or actin (top panels) or RNA was prepared from the cells and subjected to reverse transcriptase PCR for UACA and 18S rRNA (bottom panel). Wild type (wt) UACA contained wild type p53-binding motif 5'-GGAC_4GTG_7CCCGGCC_{14}C CG_{17}CGTC-3', and mutant (m) UACA contained mutations 5'-GGAT_4GTA_7 CCCGGCG_{14}CCA_{17}CGTC-3' in the p53 binding motifs.

Top right panel performed by Dr. Ravshan Burikhanov, and bottom panel performed by Nikhil Hebbar.

(B) Asterisk (*) indicates statistical significance (P< 0.001) by the Student *t* test.

Fig 2.5: Generation of PAWR knock-out mice
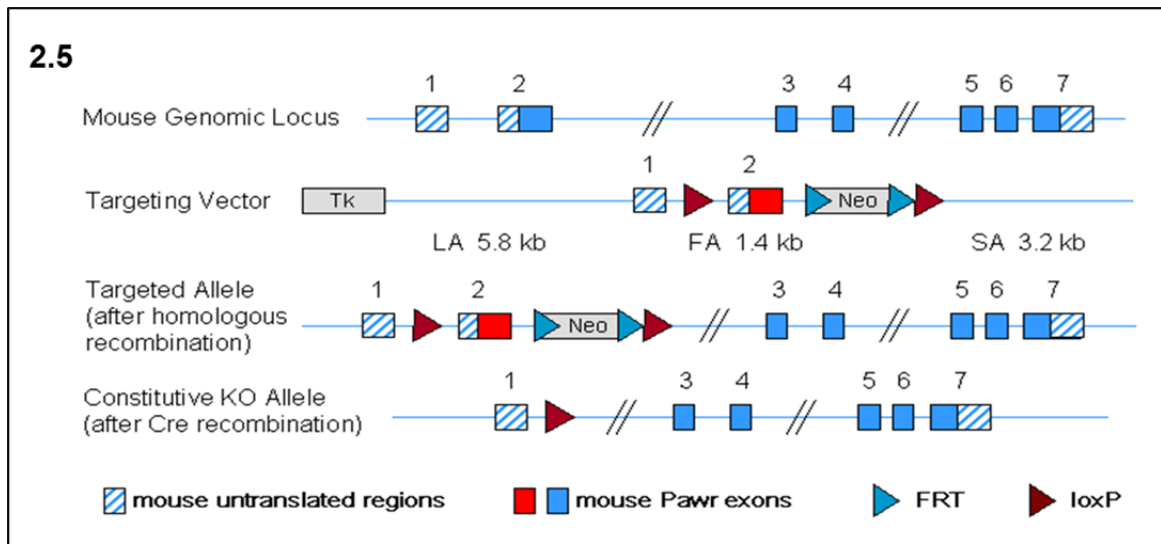
Fig 2.5: Generation of PAWR knock-out mice: Diagrammatical illustration of the targeting cassette used in the generation of PAWR knock-out mice, as described in Materials and Methods section.

CHAPTER 3: PAR-4 REGULATES P53 STABILITY

INTRODUCTION

Since the discovery of Par-4 in 1994, research in this field has primarily revolved around the apoptotic function of Par-4. The apoptotic nature of Par-4 is exemplified by its elevated expression in cells undergoing active apoptosis, for example, granulosa cells of atretic ovarian follicles, interdigitating web cells of the mouse embryo, involuting tadpole tails, and dying neurons during neuronal development (El-Guendy and Rangnekar, 2003). Par-4 is reported to be an essential downstream regulator of the apoptotic action of various physical and chemical agents, such as TRAIL, Vincristine, Adriamycin, and Ionizing Radiation (IR) (Gurumurthy et al., 2005; Ranganathan and Rangnekar, 2005). In addition, several studies in cell culture models have emphasized the role of Par-4 in sensitizing different cell types to a wide array of apoptotic stimuli, namely, growth factor withdrawal; tumor necrosis factor-α (TNF- α); ultraviolet (UV), X-ray and gamma radiation (Chakraborty et al., 2001; Chendil et al., 2002; El-Guendy and Rangnekar, 2003; Nalca et al., 1999); as well as to chemotherapeutic agents such as Docetaxel (Pereira et al., 2013) and 5-Fluorouracil (5-FU) (Wang et al., 2010). Induction of cancer cell-specific apoptosis by Par-4 in involves concomitant activation of the pro-death pathway mediated by Fas/Fas ligand (Fas/FasL) and inhibition of pro-survival pathway mediated by Nuclear Factor-kappa B (NF-κB) (Chakraborty et al., 2001). In addition, some reports have cited Par-4-mediated repression of the *Bcl-2* gene as a factor contributing to apoptosis-sensitizing action of Par-4 (Chendil et al., 2002; Wang et al., 2010).

A predominant observation across a broad spectrum of human cancer cells denotes that cells that have down-modulated or lost the expression of Par-4 exhibit increased resistance to apoptosis due to therapeutic agents such as Etoposide, Vincristine, Adriamycin, and Cisplatin (Affar el et al., 2006; Alvarez et al., 2013; Tan et al., 2014). Low Par-4 expression was also identified as the reason for diminished sensitivity of oncogene-driven breast cancer cells to apoptosis following oncogene inhibition (Alvarez et al., 2013). Furthermore, inactivation of Par-4 by Akt-mediated phosphorylation and subsequent cytoplasmic retention was also noted to inhibit apoptosis in prostate cancer (Goswami et al., 2005). Consistent with these observations, reduced Par-4 expression is known to correlate with decreased overall survival in pancreatic (Ahmed et al., 2008) and breast cancers (Mendez-Lopez et al., 2010; Nagai et al., 2010), and glioblastomas (Liu et al., 2014b), as well as reduced recurrence-free survival in breast cancers (Alvarez et al., 2013).

Despite such glaring instances which place Par-4 right at the center of apoptosis evasion, therapy resistance, and poor disease prognosis, not enough is understood about the mechanism(s) that contribute to the apoptotic pathway mediated by Par-4 in response to various physical and chemical stimuli. High resistance to apoptosis shown by Par-4-null cancer cells is reminiscent of therapeutic resistance conferred by p53 loss (or mutation/inactivation) to UV (Barley et al., 1998; Mirzayans et al., 2013), IR (Lowe et al., 1993b; Mirzayans et al., 2013), and to chemotherapeutic agents such as Adriamycin, 5-FU (Lowe et al., 1993a), as well as to Etoposide and Vincristine (Dudley et al., 2008). All of

such studies have been performed in cancer cells, which by dint of genomic instability, might harbor many more genetic changes than just Par-4 loss or down-regulation. One study (Affar el et al., 2006) has utilized primary cells, but the MEFs used do not represent true Par-4 loss because they express a leucine zipper deletion mutant of Par-4, which still contains the potent SAC domain. In this study, for the first time, we study the functional relevance of Par-4 loss using MEFs prepared from Par-4 knock-out embryos. We demonstrate that loss of Par-4 results in a significant reduction in p53 expression, as well as function. In addition, our findings indicate that attenuation of p53 stabilization renders the Par-4-null cells resistant to apoptosis in response to genotoxic stresses such as treatment with 5-FU or IR. Mechanistically, Par-4 appears to stabilize p53 by preventing Akt-mediated phosphorylation of Mdm2, thereby inhibiting stabilization and nuclear translocation of Mdm2.

MATERIALS AND METHODS

Cell lines and generation of Par-4$^{+/+}$ and Par-4$^{-/-}$ MEFs

A549 and HOP92 lung cancer cells were from ATCC; and were cultured in RPMI+10% FBS. Cells expressing constitutively active Akt were kindly provided by Dr. Morris Birnbaum (University of Pennsylvania).

Par-4$^{-/-}$ and Par-4$^{+/+}$ MEFs were generated from C57BL/6 Par-4 knock-out mice and from their wild-type littermates (Burikhanov et al., 2014a), and were grown in DMEM supplemented with 10% Fetal Bovine Serum (FBS). Briefly, 14.5 days old embryos were isolated from female uteri; heads were severed and used for genotyping. The embryos were cleaned to remove unwanted tissues; each

embryo was placed in a separate petri dish, and aseptically minced into fine fragments with a clean razor blade. The resulting pulp was transferred into a tube containing trypsin, pipetted to disintegrate tissue clumps, and incubated for approximately 10 minutes. This process was repeated until a satisfactory cell suspension was obtained. The cell suspension was then transferred to a tube containing pre-warmed media, centrifuged, and plated in DMEM supplemented with 15% FBS, non-essential amino acids (NEAA), glutamine, and sodium pyruvate.

Generation of stable cell lines

pLKO.1 lentiviral vectors expressing control short hairpin (sh)RNA or shRNAs targeting Par-4 were gift from Dr. Zhang. pLKO.1 lentiviruses were packaged by co-transfecting the pMD2.G (VSVG) envelope plasmid and Gag, Pol expressing psPAX2 packaging plasmid (both from Addgene) into HEK-293T cells. Cells were cultured for 48 hours after transfection and the lentiviral particles collected from the supernatants were used to transduce A549 and HOP92 cell with or without concentration using Lenti-X Concentrator (Clontech). Cells were subsequently selected with puromycin and gene silencing efficiency was analyzed by immunoblotting for the respective proteins.

Plasmid constructs and reagents

Luciferase construct containing wild-type p53 consensus binding sites (PG13) was purchased from Addgene. Par-4 shRNA construct in pLKO.1 vector, and Adenoviral p53 constructs were kindly provided by Dr. Zhang and Dr. Wafik el-Deiry (Penn State University), respectively. Par-4 adenoviral constructs have

been described previously (Chakraborty et al., 2001). Nutlin-3a was purchased from Cayman Chemicals. Cycloheximide and 5-FU were from Sigma.

Western blotting and antibodies

Cell lysates or tissue extracts were subjected to gel electrophoresis by SDS-PAGE followed by western blotting with appropriate antibodies. Par-4 (R-332), Akt-1 (B-1), Egr-1 and p53 (DO-1) were from Santa Cruz Biotechnology. Mouse specific p53 antibody (mAb, 1C12), cleaved caspase-3 (D175), phospho-Akt (S-473), and phospho-Mdm2 (S166) antibodies were from Cell Signaling Technology. Mdm2 antibody (Ab3) was purchased from Calbiochem, and β-actin antibody from Sigma.

Reverse transcriptase-Polymerase Chain Reaction (RT-PCR) analysis

Total RNA was extracted from lung tissues harvested from Par-4+/+ and Par-4-/- mice and preserved in RNAlater solution (Qiagen) RNeasy Plus Mini kit (Qiagen). Reverse transcription was performed to synthesize cDNA with random hexamers using SuperScript III First-Strand Synthesis System (Invitrogen), followed by PCR. PCR products were then subjected to agarose gel electrophoresis. The primers used for p53 and 18S are listed below.

Mouse p53 RNA

Forward primer: 5'- tat gtg cac gta ctc tcc tc -3'

Reverse primer: 5'- tgc tgt gac ttc ttg tag atg -3'

18S RNA

Forward primer: 5'- gta acc cgt tga acc cca tt -3'

Reverse primer: 5'- cca tcc aat cgg tag tag cg -3'

Luciferase assay

Cells were co-transfected with the PG-13 wild-type p53-luc or empty pGL3-luc reporter plasmid together with β-galactosidase for normalization. 24 hours after transfection, cells were washed, lysed, and the cell extracts were mixed with luciferase substrate Steadylite plus from PerkinElmer. Luminescence was assayed using TopCount plate reader.

Apoptosis assays

Apoptotic nuclei were identified by immunocytochemical (ICC) analysis for active caspase-3, and/or by 4, 6-diamidino-2-phenylindole (DAPI) staining. A total of three independent experiments were performed; and approximately 500 cells were scored in each experiment for apoptosis under a fluorescent microscope.

Statistical Analysis

All experiments were performed in triplicate to verify reproducibility. Two-way ANOVA (with appropriate post-hoc test) and Student's t-test were used to calculate p-values.

RESULTS

Par-4 is necessary for apoptosis induction by 5-FU and IR

In order to assess the cellular role of Par-4 in apoptosis, we used genetically matched Par-4$^{+/+}$ or Par-4$^{-/-}$ mouse embryonic fibroblasts (MEFs) and investigated their response to genotoxic stresses exerted by treatment with 5-Fluorouracil (5-FU) or Ionizing Radiation (IR). We noted that, after 24 hours of treatment, Par-4$^{+/+}$ MEFs underwent a gradual dose-dependent increase in

apoptosis with 5-FU and IR treatment, whereas Par-4^{-/-} MEFs remained significantly resistant to these agents (Fig. 3.1A and 3.1B).

Par-4 is required for stress-induced p53 stabilization, transcriptional activity, and apoptosis

It is widely known that wild-type p53 undergoes rapid activation and stabilization in response to treatment with 5-FU and IR, and induces p53-dependent apoptosis in the event of intractable cellular damage (Bunz et al., 1999; Lowe et al., 1993a; Mirzayans et al., 2013). Since both Par-4^{+/+} and Par-4^{-/-} MEFs possess wild-type p53, treatment with 5-FU and IR was therefore expected to elicit a normal p53 stabilization response in both the MEFs. Hence, we decided to probe for p53 accumulation in response to these treatments in the wild-type and Par-4-null MEFs. Par-4^{+/+} MEFs, as expected, exhibited p53 accumulation when treated with 5-FU (10µM) or IR in a time- and dose-dependent manner. However, we were most intrigued to notice little or no induction of p53 protein expression in Par-4^{-/-} MEFs after such treatments (Fig. 3.2A and 3.2B). In order to determine that Par-4-null cells were not inherently defective in regulating the expression of stress-inducible genes, we tested for the expression of Early growth response-1 gene product (Egr-1) in 5-FU treated samples. Our results showed a steady increase in Egr-1 protein expression 30 minutes after 5-FU treatment in both Par-4^{+/+} and Par-4^{-/-} MEFs. In addition, we observed similar temporal sequence of Egr-1 induction and degradation in both Par-4^{+/+} and Par-4^{-/-} cells. Interestingly, p53, which is known to be transcriptionally regulated by Egr-1, was not appreciably induced in Par-4^{-/-} cells in spite of robust Egr-1 induction

(Fig. 3.2A). Next, we assessed p53 function in both the MEFs using PG13-luc, a luciferase reporter construct containing 13 copies of the wild-type p53-binding consensus sequence. When we co-transfected the PG13-luc construct along with β-galactosidase (for normalization), our results indicated that lower expression of p53 protein in Par-4$^{-/-}$ cells also translated to a corresponding reduction in p53 transcriptional activity (Fig 3.2C).

These results implied that Par-4$^{-/-}$ MEFs were resistant to 5-FU- or IR-induced apoptosis because of impaired p53 activation/stabilization and function. We sought to investigate if the apoptosis resistance exhibited by Par-4$^{-/-}$ MEFs could entirely be ascribed to attenuated p53 expression. For this purpose, Par-4$^{-/-}$ MEFs were infected with either GFP (control), or GFP-p53, or GFP-Par-4 adenovirus constructs, and after confirming the expression of these constructs, these cells were treated with either vehicle control, or with different doses of 5-FU for 24 hours, after which they were analyzed for apoptosis. As seen in Fig. 3.2D, re-introducing Par-4 into Par-4-null system restored the apoptotic potential of 5-FU in these cells. Replenishing p53 levels in these cells showed an increase in apoptosis; however, this effect did not match the extent of apoptosis produced by Par-4 over-expression, and thus represented only a partial recovery of 5-FU-induced apoptosis. Therefore, Par-4-induced apoptosis in response to 5-FU appears to be only partially mediated by p53, and also involves p53-independent pathways.

Par-4 is required for p53 protein stability under basal conditions

Based on our observation that p53 stabilization in response to genotoxic stresses was so severely compromised in the absence of Par-4, we decided to assess if there was any difference in basal expression of p53 in various tissues harvested from Par-4$^{+/+}$ and Par-4$^{-/-}$ animals, as well as in MEFs derived from these animals. As is evident in Fig. 3.3A, Par-4$^{-/-}$ tissues and MEFs showed markedly reduced expression of p53 in comparison to their Par-4$^{+/+}$ counterparts; the difference being more pronounced in tissues than in MEFs. Next, we sought to verify that our results reflected a direct role of Par-4 in p53 stability. We re-introduced Par-4 into Par-4-null MEFs by adenoviral infection, and assessed p53 expression 24 hours post infection. Interestingly, with increasing dosage of Par-4 re-expression in Par-4$^{-/-}$ cells, p53 expression also showed gradual stabilization (Fig. 3.3B).

Moreover, we wanted to ascertain that our observation was not limited to Par-4 knock-out animals and MEFs produced from them, but was a global effect of Par-4 loss. To test this, Par-4 expression was knocked-down in A549 and HOP92 lung cancer cells by transfection of lentiviral shRNA particles, and the cells stably expressing the shRNA were selected with the help of Puromycin. Knock-down of Par-4 expression in A549 cells, which possess wild-type p53, resulted in lowering of p53 expression, similar to our results in Par-4$^{-/-}$ background; whereas in HOP92 cells, which possess mutant p53, Par-4 knock-down had no effect (Fig. 3.3C). We could draw two major inferences from this result: a) Par-4 does not regulate the p53 promoter, because if it did, Par-4 knock-down should have

resulted in reduced expression of mutant p53 in HOP92 cells also. b) Par-4 is required for p53 protein stability. As a confirmatory step, we performed reverse transcription-polymerase chain reaction (RT-PCR) on RNA derived from lungs of Par-4$^{+/+}$ and Par-4$^{-/-}$ mice, and as depicted in Fig. 3.3D, p53 mRNA in these samples did not exhibit any difference in expression. These experiments clearly implicated a role for Par-4 in p53 stabilization, and hence we decided to investigate p53 protein stability in cells with or without Par-4.

Par-4 loss results in elevated Mdm2 activity and reduced p53 stability

We examined p53 protein expression in isogenic pairs of cell lines differing only in their expression of Par-4 (with Par-4 levels either intact, or entirely absent or knocked down) after treatment with Cycloheximide (CHX) (10µg/ml). CHX inhibits *de novo* protein synthesis, and hence would help us study the kinetics of p53 degradation, and therefore, its overall stability, in the presence or absence of Par-4. In Par-4-null MEFs, and in A549 cells in which Par-4 is knocked-down (A549 Par-4 sh), stability of p53 protein appeared to be significantly reduced in comparison to Par-4$^{+/+}$ MEFs (Fig. 3.4A) and to A549 cells with normal levels of Par-4 (A549 Con sh) (Fig. 3.4B), respectively. Moreover, as expected, the same treatment of HOP92 cells revealed highly stable form of mutant p53, high expression of which was maintained irrespective of CHX treatments or Par-4 status (Fig. 3.4C).

On the basis of these results, we hypothesized that p53 faced an accelerated rate of degradation, and therefore displayed less stability in the absence of Par-4,

which led us to evaluate Mdm2-mediated p53 degradation in the presence and absence of Par-4. Mdm2, an E3 ubiquitin ligase, is the best characterized negative regulator of p53 stability. We sought to investigate if changes in the expression and/or activity of Mdm2 could account for decreased stability of p53 in Par-4-null situation. To this end, we used Nutlin-3a to inhibit Mdm2-p53 interaction (Vassilev et al., 2004) in both Par-4$^{+/+}$ and Par-4$^{-/-}$ MEFs, the rationale being p53 expression should rise to comparable levels in both the MEFs if Mdm2 was involved. We treated these MEFs for 24 hours with Nutlin-3a, and further incubated them in the presence of 10µg/ml CHX, or vehicle control, after which cell lysates were harvested at different time points and analyzed by western blot. As seen in Fig. (3.4D), treatment with Nutlin-3a significantly stabilized p53 expression in both Par-4$^{+/+}$ and Par-4$^{-/-}$ MEFs, and the discrepancy in p53 levels that was observed without Mdm2 inhibition was lost. This result clearly pointed towards elevated Mdm2-mediated degradation of p53 due to Par-4 loss.

Several groups have reported that the cell survival kinase Akt negatively regulates p53 expression and function by phosphorylating Mdm2 at Serine-166 (S166) and Serine-186 (S186) - an event that not only enhances Mdm2 ubiquitin ligase activity, but also promotes its translocation into the nucleus, where it induces rapid ubiquitination, subsequent nuclear export and proteasomal degradation of p53, thus effectively inhibiting p53 function (Gottlieb et al., 2002; Mayo and Donner, 2001; Ogawara et al., 2002; Schmitz et al., 2006; Zhou et al., 2001). Previous research has revealed that loss of Par-4 results in elevation of Akt activity (Joshi et al., 2008). Therefore, we next performed serum stimulation

experiment with Par-4$^{+/+}$ and Par-4$^{-/-}$ in order to evaluate the levels and activity of Akt as well as Mdm2 in these cells. When Par-4$^{+/+}$ and Par-4$^{-/-}$ MEFs were made quiescent with serum-free media for four hours, and then stimulated by addition of serum, we found that Akt was activated to a greater degree in Par-4$^{-/-}$ cells than in Par-4$^{+/+}$ [reflected by increased Akt phosphorylation at Serine 473 (S473)]. Even more remarkable was the increase in levels of phospho-Mdm2 (S166) in Par-4$^{-/-}$ cells (Fig. 3.4E). Re-stimulation with serum elicits a cell survival program wherein activated Akt rapidly phosphorylates Mdm2 at Serine-166 and at Serine-186; these phosphorylation events augment Mdm2-mediated ubiquitination and degradation of p53. Our results suggest that the absence of Par-4 and consequent elevation of Akt activity promotes the activation of Akt-Mdm2-p53 axis, which leads to reduced expression and attenuated function of p53. These results were further validated using cells expressing constitutively active form of Akt. When compared to wild-type cells, cells with constitutively active Akt displayed, as expected, increased Akt phosphorylation at S473; very low levels of Par-4; enhanced Mdm2 (S166) phosphorylation; and significantly diminished expression of p53 (Figure 3.4F), which corroborates our findings with Par-4-null MEFs.

DISCUSSION

In this study, we have identified a novel role for the tumor suppressor Par-4 in regulating the expression and function of another tumor suppressor- p53. P53, a stress-induced transcription factor, constitutes a hub of signaling networks controlling critical cellular and organismal phenotypes. Many therapeutic agents

and genotoxic stresses exert their apoptotic effect by activating and stabilizing p53, which then sets into motion complex cellular machineries to orchestrate apoptotic cell death. Consequently, p53 loss or mutation, which occurs in more than half of human cancers, is known to confer resistance to apoptosis by various radio- and chemo-therapeutic agents, namely, UV, IR, 5-FU, Adriamycin, Etoposide, Vincristine etc (Lowe et al., 1993a). Interestingly, Par-4 loss or down-regulation is also deemed to confer chemo- or radio-resistance, as documented by a number of research studies conducted on cell culture systems, mouse models, as well as tumor specimens. This study, performed on MEFs obtained from either wild-type or Par-4 knock-out mice, also demonstrates that lack of Par-4 results in increased resistance to apoptosis by 5-FU and IR (both of which are known to activate p53) in spite of the presence of wild-type p53 in both cell types. Alvarez et al., 2013, have also observed that breast cancer cells in which Par-4 expression is down-regulated are resistant to apoptosis by p53-activating agents such as Adriamycin despite possessing wild-type p53. It is thus interesting to note how the presence of wild-type p53 appears to be ineffectual in causing apoptosis when Par-4 expression is lost or down-regulated. Our study hereby presents evidence that p53 expression and function is severely compromised by loss of Par-4; and therefore serves to shed light on the potential mechanism of therapy resistance engendered by Par-4 loss or down-regulation.

Our results show that p53 expression is reduced in Par-4$^{-/-}$ mice; in MEFs produced from Par-4$^{-/-}$ mice; as well as in A549 cells in which Par-4 expression level has been knocked-down, as compared to their Par-4$^{+/+}$ counterparts, which

indicates a definite role for Par-4 in regulating p53 expression. Furthermore, restoration of p53 expression in Par-4$^{-/-}$ cells with Par-4 re-introduction unequivocally corroborates this finding. The tight regulation of p53 protein level within cells is achieved not so much by changes in p53 transcription, but primarily by modulating Mdm2-mediated p53 degradation (Kruse and Gu, 2009; Oren, 1999). Indeed, several lines of evidence in our study indicate that regulation of p53 by Par-4 is at the level protein stabilization, as outlined below.

➢ Our RT-PCR data failed to reveal significant differences in p53 mRNA expression.

➢ Par-4 knock-down failed to impact p53 levels in HOP92 lung cancer cell line, which carries an arginine to leucine mutation at position 175 (175 R/L), and is known to possess relatively elevated basal levels of the mutant protein (O'Connor et al., 1997). Conversely, wild-type p53 levels in A549 lung cancer cells were effectively reduced by knock-down of Par- 4 expression. This result rules out the possibility of Par-4 regulating p53 promoter, because, if this effect were promoter-driven, expression levels of both the mutant and wild-type forms of p53 should have been impacted.

➢ In addition, our cycloheximide experiments clearly indicate that, when de novo protein translation is inhibited, Par-4 loss or down-regulation results in a significant drop in wild-type p53 protein stability, thereby suggesting the presence of a hyper-active p53 degradation mechanism in cells that lack Par-4.

Mdm2, an E3 ubiquitin ligase with a high specificity for p53, is the best characterized negative regulator of p53 expression and function. Mdm2 directly binds to p53, and tightly controls its basal levels via continuous ubiquitination and consequent degradation by the 26S proteasome (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997). Mdm2-mediated ubiquitination also promotes nuclear export of p53; and this exit from the nucleus and cytoplasmic relocation is essential for its proteasomal degradation (Boyd et al., 2000; Geyer et al., 2000). In addition, Mdm2 complexed with p53 is known to localize to regulatory regions of a number of p53 target genes and repress their transcriptional activation (Manfredi, 2010; Momand et al., 1992). Upon exposure to genotoxic or other cellular stresses, interaction between Mdm2 and p53 is disrupted, which alleviates p53 from Mdm2-mediated repression, leading to p53 stabilization and transactivation of specific p53 target genes (Manfredi, 2010). The small molecule Mdm2 antagonist Nutlin-3a, which binds to the p53-binding pocket in Mdm2 and thus disrupts Mdm2-p53 interaction, is shown to be sufficient to cause p53 accumulation and transcriptional activation of p53 target genes (Vassilev et al., 2004). Our experiment with Par-4$^{-/-}$ cells treated with Nutlin-3a and CHX, and the stabilization of p53 observed thereof, distinctly implicates Mdm2-mediated p53 degradation as a plausible cause for p53 protein instability in Par-4-null cells.

Excessive Mdm2-mediated degradation of p53 could reflect either augmented expression, or elevated activity of Mdm2 protein, or both. Myriad of post-

translational modifications on both p53 and Mdm2 have been described to influence the interaction between the two. Phosphorylation of Mdm2 by Akt/PKB serine/threonine kinase at two consensus sites- S166 and S186- is known to: a) enhance its E3 ubiquitin ligase activity; b) facilitate its translocation from the cytoplasm to the nucleus; c) lead to nuclear export of p53 and subsequent proteasomal degradation; and d) afford protection against p53-mediated apoptosis induced by genotoxic agents across a number of cellular and tumor paradigms (Gottlieb et al., 2002; Limesand et al., 2006; Mayo and Donner, 2001; Ogawara et al., 2002; Zhou et al., 2001). In this light, it is interesting to note that Par-4 has been shown to inhibit Akt activity (Joshi et al., 2008), and our serum re-stimulation results also indicate elevated Akt activity, as well as augmented levels of phospho-Mdm2 (S166), which invariably translates to lower p53 levels, in Par-4$^{-/-}$ cells in comparison to that in Par-4$^{+/+}$ cells. Taken collectively, our results suggest that loss of Par-4-mediated Akt inhibition, and consequent elevation in Mdm2 activity, is responsible for decreased p53 expression and transcriptional activity; impaired p53 stabilization in response to genotoxic stresses, such as treatment with 5-FU and IR; and increased threshold for apoptosis induction in response to 5-FU and IR observed in Par-4-null cells. This work, therefore, provides a novel mechanistic insight into how Par-4 loss or down-regulation could be associated with therapy resistance in tumors possessing wild-type p53.

Fig. 3.1: Par-4-null cells are resistant to apoptosis by 5-FU or IR
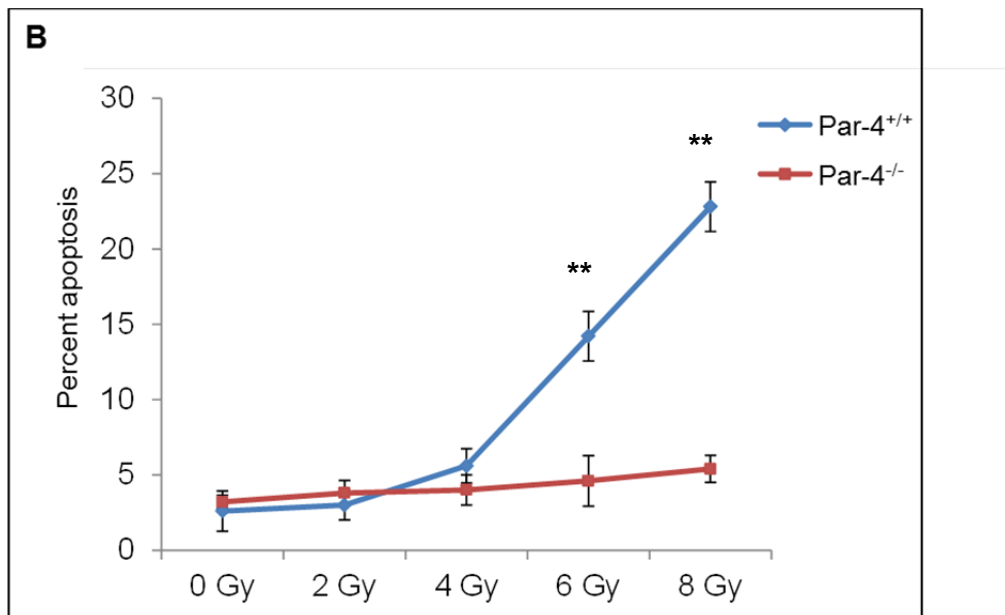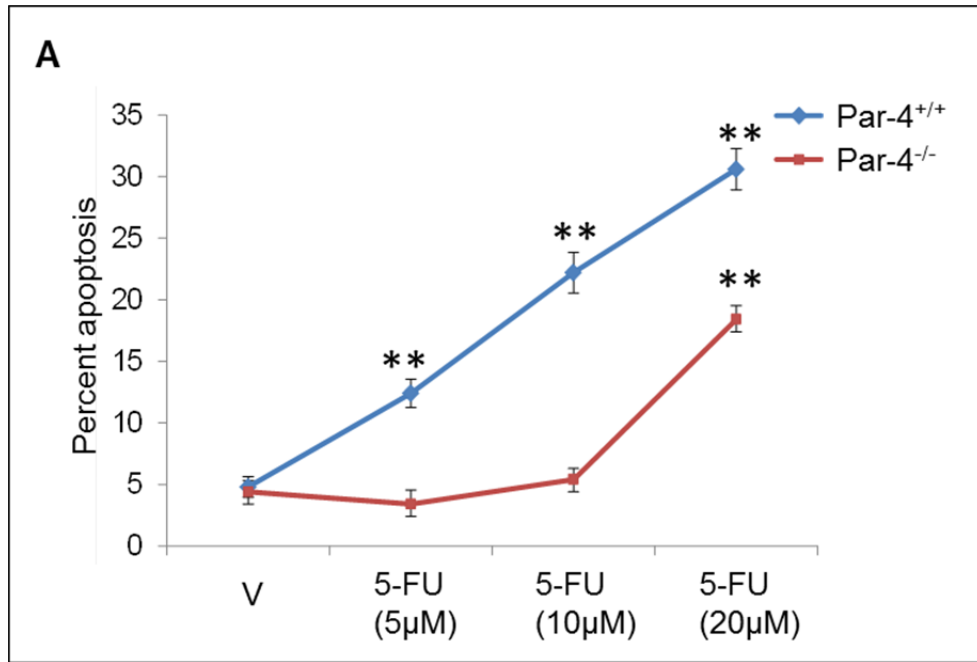
Figure 3.1: Par-4-null cells are resistant to apoptosis by 5-FU or IR

(A) **Par-4-null MEFs demonstrate increased resistance to 5-FU treatment.** Par-4$^{+/+}$ and Par-4$^{-/-}$ MEFs were treated with indicated doses of 5-FU for 24 hours, and the cells were scored for apoptosis by ICC for DAPI and active caspase-3 staining.

(B) **Par-4-null MEFs demonstrate increased resistance to IR**. Par-4$^{+/+}$ and Par-4$^{-/-}$ MEFs were exposed to 2, 4 or 6 Grays (Gy) of radiation for 24 hours, and apoptosis was assayed by ICC for DAPI and active caspase-3.

(A, B) Asterisk (**) indicates statistical significance (P < 0.001) based on two-way ANOVA followed by Bonferroni correction.

Fig. 3.2: Stress-inducible p53 stabilization and function is compromised in Par-4 loss
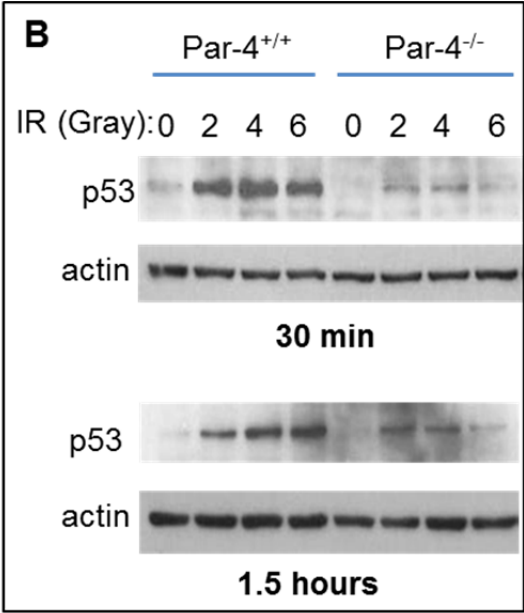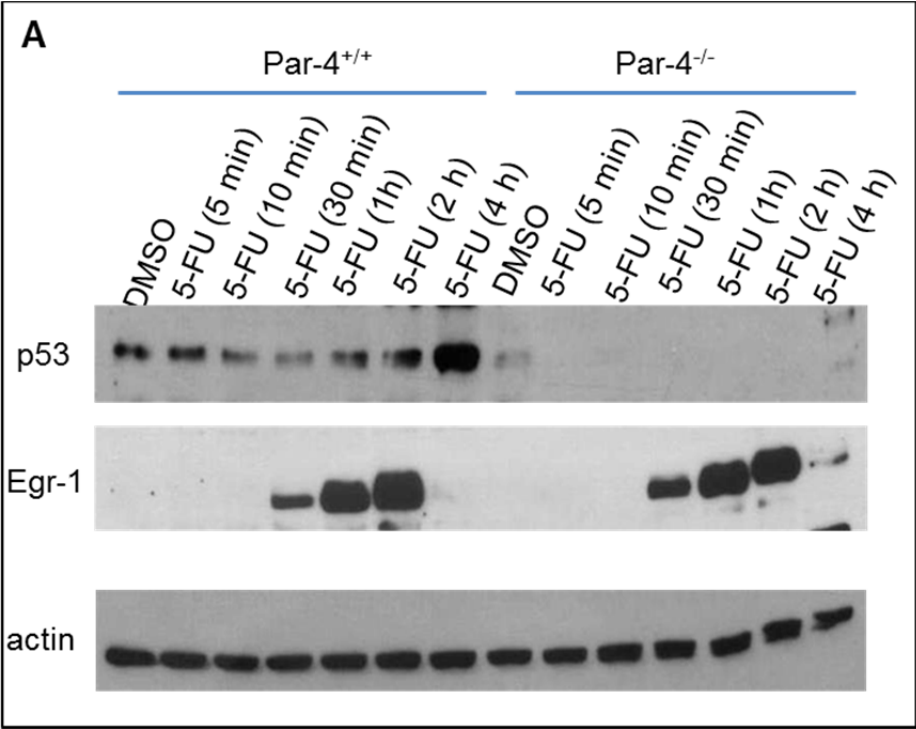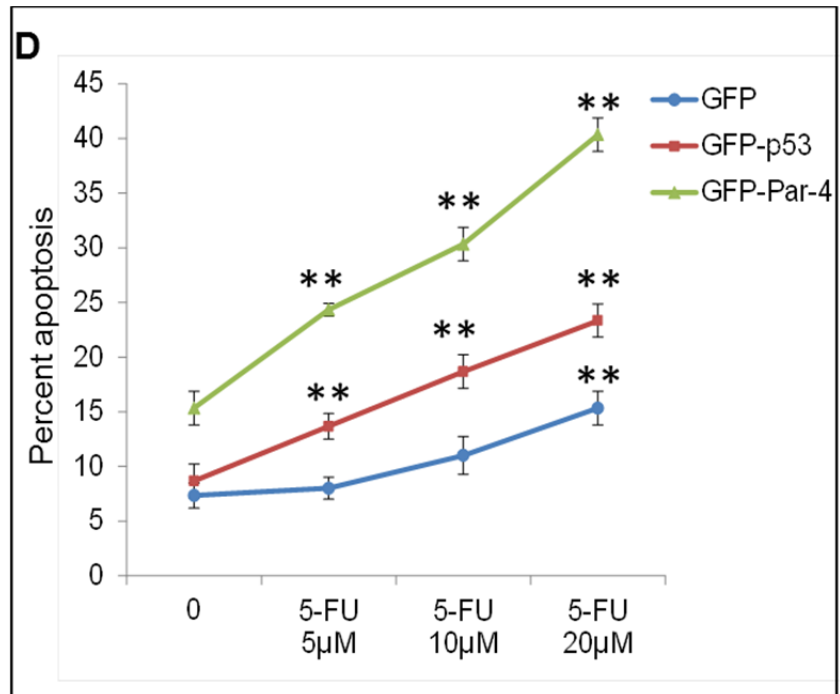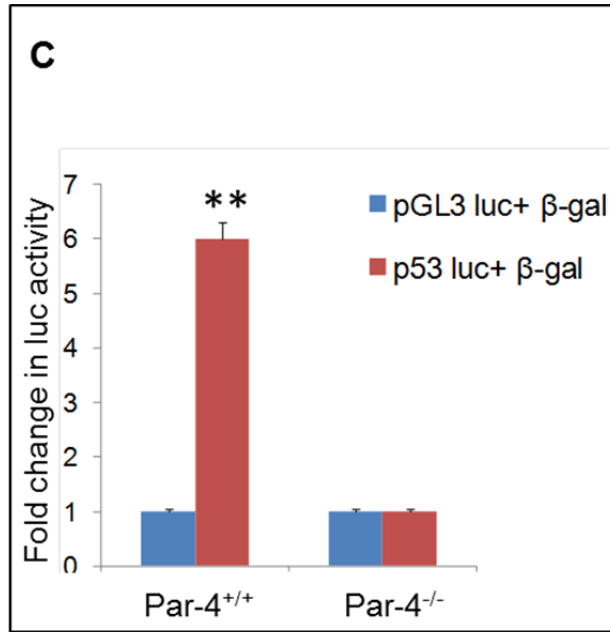
Fig. 3.2: Stress-inducible p53 stabilization and function is compromised in Par-4 loss

(A) **Par-4 is required for p53 accumulation after 5-FU treatment.** Par-4$^{+/+}$ and Par-4$^{-/-}$ MEFs were treated with either 10μM 5-FU or with vehicle control, DMSO. Cell lysates were harvested at the indicated time points and analyzed for p53 and Egr-1 expression by western blotting. Actin is used as loading control.

(B) **Par-4 is required for p53 accumulation after exposure to IR.** Par-4$^{+/+}$ and Par-4$^{-/-}$ MEFs were treated with 2, 4, and 6 Gy radiation, and the lysates were harvested 30 minutes or 90 minutes after exposure to IR. The whole cell lysates were subjected to western blotting and analyzed for p53 expression. Actin is used as loading control.

(C) **P53 transcriptional activity is attenuated in Par-4-null situation.** Par-4$^{+/+}$ and Par-4$^{-/-}$ MEFs were transfected with either empty luciferase construct (pGL3-luc) or with a luciferase construct driven by p53 response elements (p53-luc). 24 hours later, cell extracts were assayed for luciferase activity, which is expressed as fold induction over basal activity produced by empty luc vector pGL3-luc.

(D) **P53 replenishment in Par-4-null cells partially rescues the apoptotic potential of 5-FU.** Par-4$^{-/-}$ MEFs were infected with adenoviral constructs expressing either GFP, or GFP-p53 or GFP-Par-4 proteins. 18 hours after infection, cells were treated with the indicated doses of 5-FU for 24 hours, and assayed for apoptosis by DAPI positivity of pyknotic nuclei.

(C, D) Asterisk (**) indicates statistical significance ($P < 0.001$) based on two-way ANOVA.

Fig. 3.3: Par-4 is required for p53 protein stability under basal conditions

C

| | A549 | | HOP92 | |
| | Con sh | Par-4 sh | Con sh | Par-4 sh |

p53

actin

Par-4

D

Lung RNA

Ladder (bp)

Par-4$^{+/+}$ Par-4$^{-/-}$

200 —
100 —   p53

200 —
100 —   18S RNA
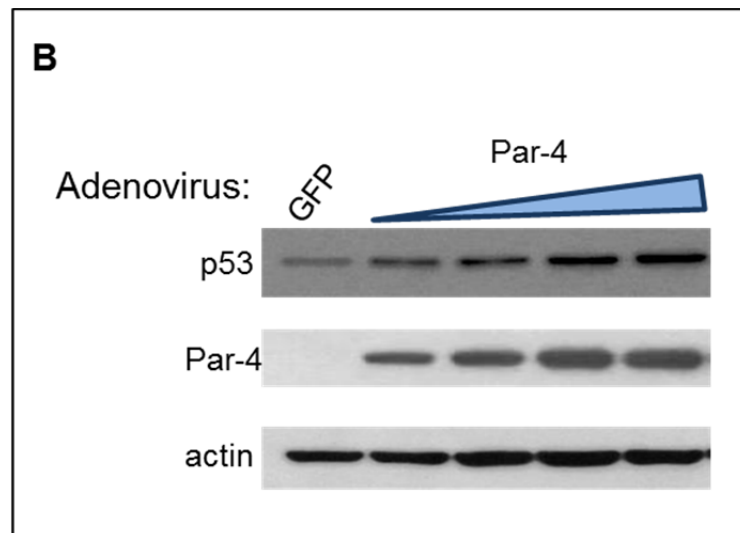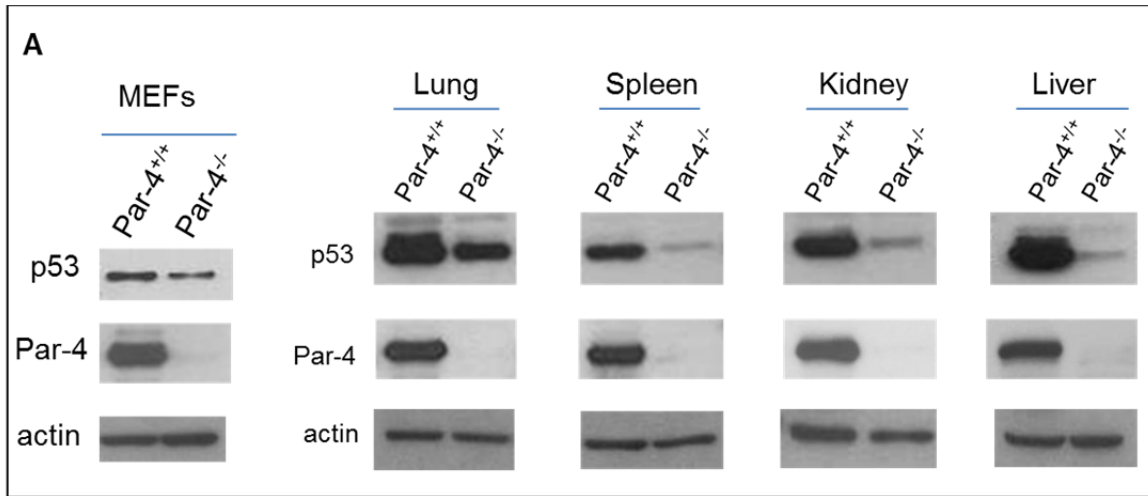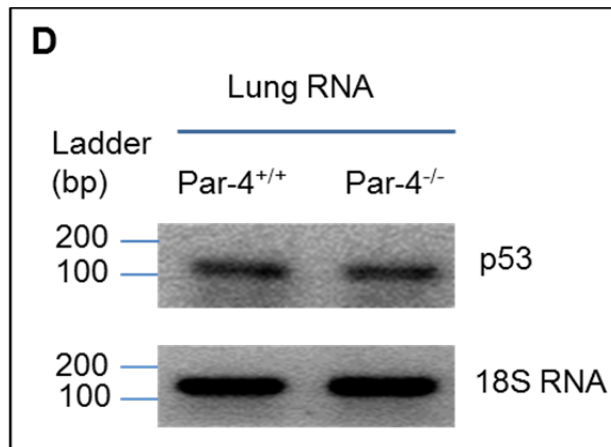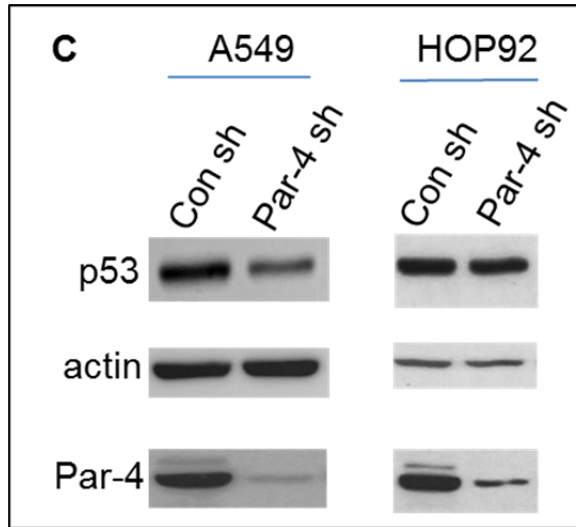
Fig. 3.3: Par-4 is required for p53 protein stability under basal conditions

(A) **Basal expression of p53 is reduced in Par-4-null situation**. Par-4$^{+/+}$ and Par-4$^{-/-}$ cell lysates, as well as protein extracts prepared from the indicated organs harvested from Par-4$^{+/+}$ and Par-4$^{-/-}$ mice were subjected to western blotting and probed for p53, Par-4 and actin.

(B) **Par-4 induces p53 protein stability.** Par-4$^{-/-}$ MEFs were transduced with Par-4 expressing adenoviral construct. Cell lysates were collected 24 hours after infection and analyzed by western blotting.

(C) **Par-4 is required for stability of wild-type, but not mutant, p53.** A549 and HOP92 lung cancer cells were infected with lentiviral particles containing either control shRNA (Con sh) or Par-4 shRNA (Par-4 sh) in order to silence Par-4 expression. Cells stably expressing the desired shRNA were selected by Puromycin treatment, and the lysates were analyzed for the expression of p53 by western blotting. A549 and HOP92 cells stably expressing either Control or Par-4 shRNA were prepared by Dr. Sunil Nooti.

(D) **Par-4 does not regulate p53 RNA levels.** Total RNA was extracted from lungs harvested Par-4$^{+/+}$ and Par-4$^{-/-}$ mice, and subjected to reverse transcriptase PCR (RT-PCR) for analysis of p53 mRNA levels. 18S RNA is used as control.

Fig. 3.4: Par-4 loss results in elevated Mdm2 activity

C

HOP92 Control sh

HOP92 Par-4 sh

HOP92

CHX

CHX

V   15m   30m   45m   60m   V   15m   30m 45m 60m

Con sh   Par-4 sh

p53

actin

Par-4

D

Par-4$^{+/+}$

Par-4$^{-/-}$

N+CHX

N+CHX

Ut   N+V   15m 30m   45m   60m   Ut   N+V   15m   30m   45m   60m

p53

actin
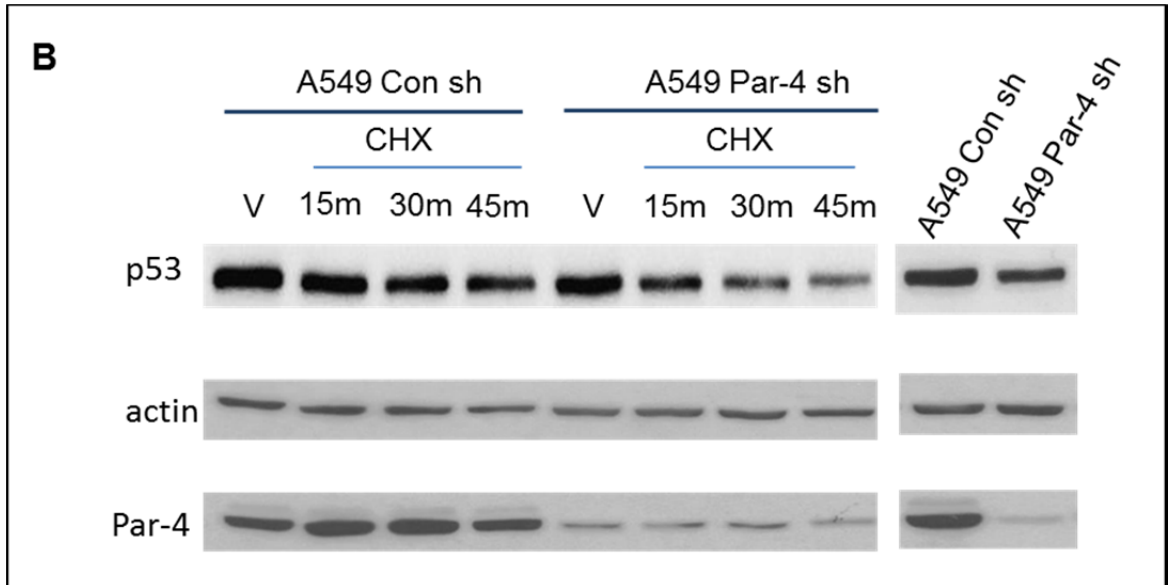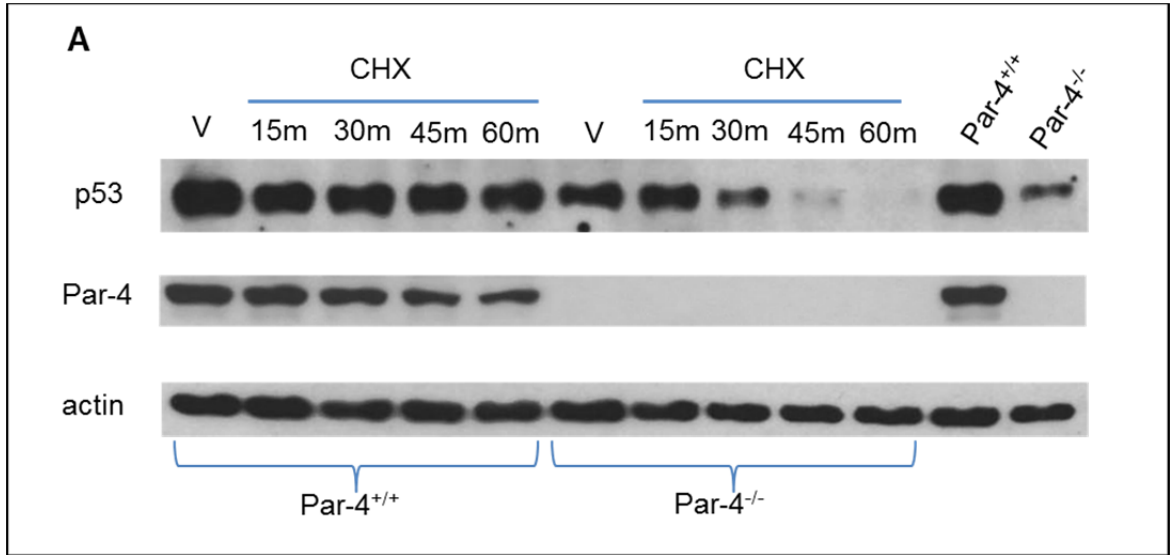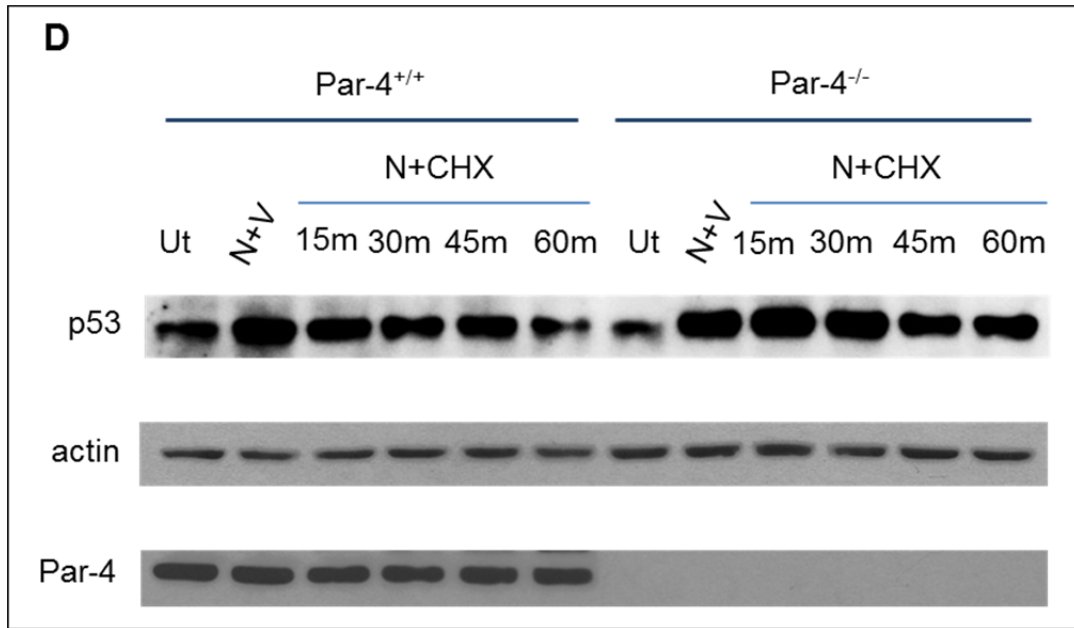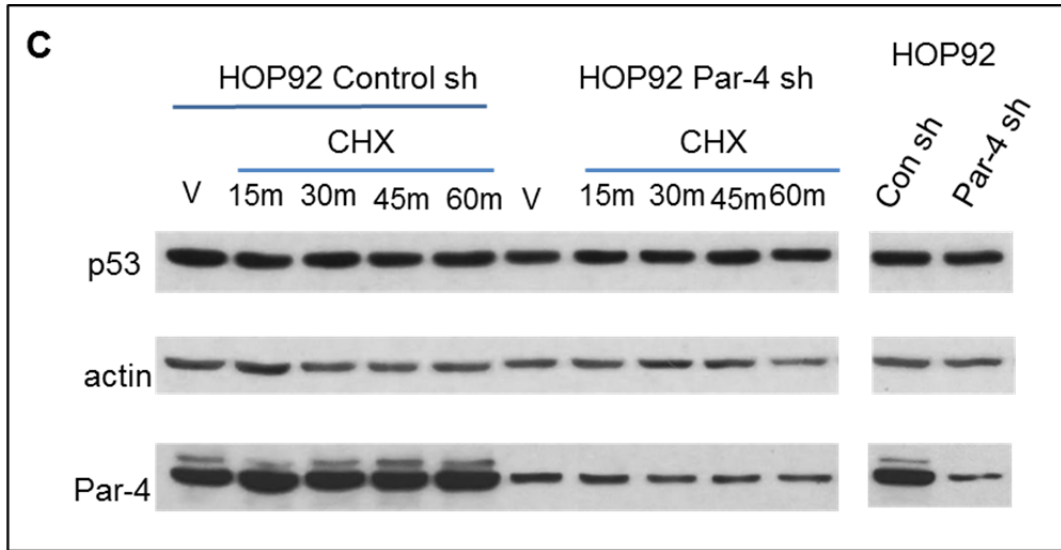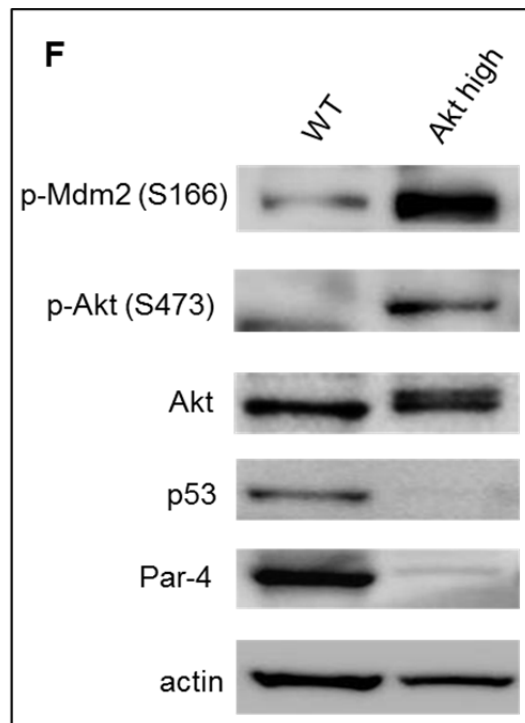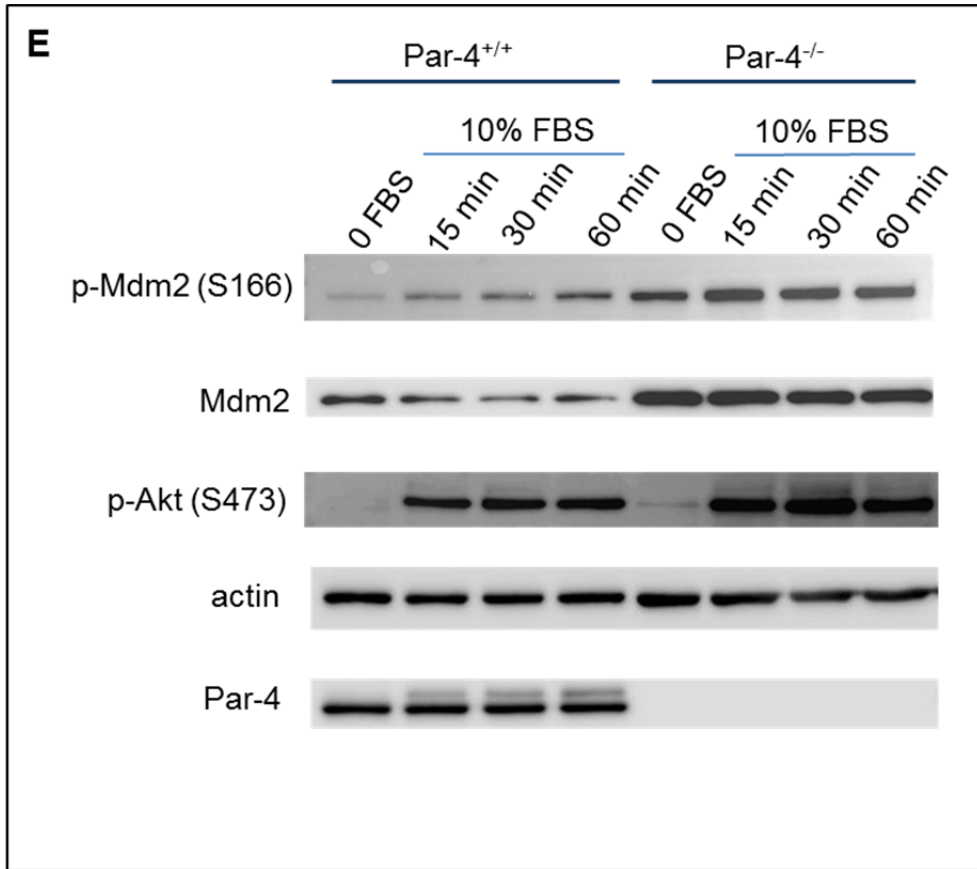
Par-4

Fig. 3.4: Par-4 loss results in elevated Mdm2 activity

(A) p53 protein stability is reduced in the absence of Par-4 in primary MEFs. Par-4$^{+/+}$ and Par-4$^{-/-}$ MEFs were treated with Cycloheximide (CHX), inhibitor of de novo protein synthesis, at the concentration of 10µg/ml. Cell lysates were harvested at the indicated time points and analyzed by western blot for the indicated proteins.

(B) P53 protein stability is reduced in the absence of Par-4 in lung cancer cells. A549 cells (possessing wild-type p53) expressing either control shRNA or shRNA for Par-4 were treated with 10µg/ml Cycloheximide or vehicle control; cell lysates were harvested at indicated time points and subjected to western blot to analyze p53 protein.

(C) Par-4 does not affect stability of mutant p53. HOP92 cells (possessing mutant p53) expressing either control shRNA or shRNA for Par-4 were treated with 10µg/ml Cycloheximide or vehicle control; cell lysates were harvested at indicated time points and subjected to western blot to analyze p53 protein.

(D) P53 stability caused by Par-4 loss is rescued by Mdm2 inhibition. Par-4$^{+/+}$ and Par-4$^{-/-}$ MEFs were pre-treated for 24 hours, and then treated with CHX for the indicated time points. Cell lysates were analyzed by western blotting.

(E) Par-4 inhibits Akt-mediated phosphorylation of Mdm2 at S166. Par-4$^{+/+}$ and Par-4$^{-/-}$ MEFs were serum starved for 4 hours, and re-stimulated with 10% serum for short time periods in order to activate Akt. Cell lysates were collected at the indicated time points, and analyzed for the indicated proteins and phospho-proteins by western blotting.

(F) Akt activity induces Mdm2 (S166) phosphorylation. Wild-type (WT) cells and cells expressing constitutively active form of Akt were lysed, harvested, and the lysates were subjected to western blotting and probed for the indicated proteins. Performed by Nikhil Hebbar.

CHAPTER FOUR: DISCUSSION AND FUTURE DIRECTIONS

Selectivity towards cancer cells is perhaps the most desirable, and yet, the most elusive trait sought after in cancer therapy. The tumor suppressor Par-4, because of its unique cancer cell-specific apoptotic action, presents considerable therapeutic promise. In addition, Par-4 is secreted, and can act both intracellularly (both in the cytoplasm and the nucleus) and extracellularly to induce apoptosis specifically in cancer cells. The tumor suppressor p53, rightfully titled "the guardian of the genome", holds the reins to a broad range of effector functions that contribute to its tumor suppressive effects. P53 transcriptionally controls and co-ordinates critical processes like cell cycle arrest, cellular senescence and apoptosis; and its emerging role in other important processes like autophagy and cellular metabolism is being increasingly chronicled. This dissertation work describes a fascinating cross-talk between these two tumor suppressors wherein they mutually regulate different facets of each other's functions. Par-4, which is secreted in response to p53 activation, executes the paracrine apoptotic effect of p53 in a cancer cell-selective manner (chapter 2). Par-4, on the other hand, plays a role in p53 stabilization; and this mechanism probably serves to explain the apoptosis resistance conferred by par-4 loss to genotoxic insults that normally induce p53-dependent apoptosis (chapter 3).

Reciprocal interactions between the tumor and its microenvironment, and the role of a supportive, reactive stroma in the development, progression and metastasis of tumors have been well documented. Lately, many studies have reported that

wild-type p53 in the stroma inhibits tumor growth, and that tumor progression selects for inactivation of p53 in the stromal compartment, particularly in cancer associated fibroblasts (CAFs) (Hill et al., 2005; Kiaris et al., 2005; Kurose et al., 2002). Most of these studies, however, have been largely descriptive, and the mechanism whereby wild-type p53 in the fibroblasts could exert inhibitory effect on tumor growth is yet to be addressed.

Our findings in chapter 2 reveal that Par-4, a tumor suppressor with potent apoptotic activity towards cancer cells, is secreted in response to p53 activation, and mediates the paracrine tumor suppressive effects of p53 (Figure 4.1). Since p53 function is frequently compromised in human cancers due to deletion or mutation of *Tp53* gene, it is obvious that p53 protein in such scenarios will not be amenable to activation by Mdm2 inhibitor Nutlin-3a and NF-кB inhibitor PS1145. Therefore, we circumvented that problem by empowering the normal cells (normal fibroblasts which possess wild-type p53) to induce Par-4 secretion. Our experiments with mice indicate that a single injection of Nutlin-3a+PS1145 builds up adequate Par-4 in the serum to cause *ex vivo* apoptosis of cancer cells. Like p53, other molecular regulators of Par-4 secretion can be identified, and then pharmacologically targeted to induce Par-4 secretion from normal cells. In fact, a recent publication identified one such target, vimentin, which can be inhibited by Arylquin 1, to enhance Par-4 secretion (Burikhanov et al., 2014b). Therefore, identification of such molecular targets opens up avenues for rational drug design and development of Par-4 'secretagogues' which promise immense therapeutic potential.

Our results suggest that Par-4 secretion in response to Nutlin-3a+PS1145 is induced by p53-mediated repression of UACA, a binding partner of Par-4. Binding of Par-4 by UACA results in Par-4 sequestration; and inhibition of UACA expression either by p53-mediated transcriptional repression or by NF-κB inhibition can have two consequences:

a) In cancer cells: Liberation of Par-4 from sequestration by UACA, and an increase in the expression of Par-4 receptor GRP78 on the surface of cancer cells, rendering them more sensitive to extracellular Par-4-mediated apoptosis (Burikhanov et al., 2013).

b) In cells with intact p53 pathway: Liberation of Par-4 from sequestration by UACA, and an increase in Par-4 secretion.

Our results with NF-κB inhibitors showing UACA down-regulation and enhanced Par-4 secretion from normal wild-type fibroblasts may have wider implications. Interestingly, UACA was identified as a protein up-regulated in inflammatory disease (panuveitis) (Yamada et al., 2001), and is positively regulated by NF-κB activity (Tran et al., 2010). By logical extension, then, we can hypothesize that drugs with anti-inflammatory properties (for example, NSAIDs) will be able to induce Par-4 secretion. Although NSAIDs are known to inhibit the enzymes cyclooxygenase-1 and/or -2 (COX-1, COX-2), some NSAIDs such as Aspirin, Sodium salicylate, Sulfasalazine, Sulindac (Yamamoto and Gaynor, 2001), and Ibuprofen (Greenspan et al., 2011) also inhibit NF-κB-mediated transcriptional activation.

Meta analyses of epidemiological data collected from different patient cohorts have discussed potential chemo-preventive effects associated with NSAID use against tumors at various sites, including prostate, breast, lung, brain, and melanoma (Liu et al., 2014a). In addition, these agents are also known to induce regression of adenomatous polyps of the colon and prevent development of colon cancer (Yamamoto and Gaynor, 2001). It will be interesting to examine if NF-κB inhibition by NSAIDs will result in elevated Par-4 secretion, and better still, to investigate if the chemo-preventive potential demonstrated by the use of these NSAIDs can be associated with the increase in Par-4 secretion. Data from Par-4/SAC transgenic mice indicate an increased resistance to cancer with elevated serum levels of Par-4 protein. Considering cancer cell-specific apoptotic nature and tumor suppressive function of Par-4, it is possible that there is a correlation between secreted Par-4 and cancer risk, which may constitute an important aspect in Par-4 research.

A particularly intriguing aspect of cross-talk between Par-4 and p53 is regulation of p53 stabilization by Par-4 (chapter 3). Using Par-4 wild-type and Par-4-null MEFs, we show that Par-4-null cells are significantly resistant to apoptosis by genotoxic agents such as IR and 5-FU, in spite of the fact that both sets of MEFs possess wild-type p53. It is well established that treatment with IR or 5-FU results in p53 stabilization/accumulation, and ultimately leads to p53-dependent apoptosis (Lowe et al., 1993a; Lowe et al., 1993b; Mirzayans et al., 2013). Even though apoptosis resistance conferred by Par-4 loss or down-regulation has been well studied, we show for the first time that p53 stress response is severely

attenuated in the absence of Par-4. Our results indicate that Par-4 loss leads to elevated Mdm2 activity, as judged by remarkable increase in Mdm2 (S166) phosphorylation, thereby resulting in reduced p53 stability (Figure 4.2). These results might have implications in therapy resistance shown by some cancers bearing wild-type p53.

Renal cell carcinoma (RCC) is one of the few types of cancers that retain wild-type p53. In fact, existing data suggests that approximately 86% of RCC contain wild-type p53. However, RCC is also characterized by increased co-expression of Mdm2; and such co-expression pattern is linked with poor disease prognosis (Polanski et al., 2014). Despite majority of RCC cases possessing wild-type p53, this disease exhibits exceptionally high resistance to radiation and chemotherapy (Cook et al., 1999). It is interesting to note that over 70% of human RCC show significant down-regulation of Par-4. Furthermore, replenishment of Par-4 protein into RCC cell lines established from two different RCC tumors restored therapeutic sensitivity of these cells to the apoptotic effects of Adriamycin and TNF- α (Cook et al., 1999). This result definitely indicates that apoptotic response of RCC cell lines is regained with Par-4 restoration. On the other hand, p53 stabilization using Mdm2 inhibitor Nutlin-3 is reported to induce growth arrest and senescence in RCC cells (Polanski et al., 2014). Was p53 activation/stabilization in response to Adriamycin compromised in RCC cells containing low Par-4? Does Par-4 down-regulation in RCC correlate with therapy resistance and poor disease outcome? Do Par-4 down-regulation and Mdm2 over-expression show

an inverse correlation? These not only constitute interesting research questions for the future, but might influence the choice of therapy used for RCC.

Dudley *et. al.* reported a similar phenomenon of impaired p53 function in tumor-associated stromal cells from A375 melanoma and PC3 prostate carcinoma xenografts and a spontaneous prostate tumor model TRAMP. They found that, upon treatment with p53-activating agents such as Etoposide and Vincristine, these tumor-associated stromal cells failed to accumulate p53, and showed increased resistance to growth inhibitory and apoptotic effects of these drugs, despite the fact that p53 sequence remained wild-type. Such lack of response demonstrated by tumor-associated stromal cells is reminiscent of that shown by Par-4-null fibroblasts. Therefore, it may be worthwhile investigating if Par-4 status is altered in cancer-associated stroma during tumor progression, and examining whether such alteration, if present, has a bearing on how the stroma responds to therapy.

It is known that the kind of effector function elicited by p53 is highly context dependent (Zilfou and Lowe, 2009). We now know that loss of Par-4 compromises p53 pathway and renders cells more resistant to apoptosis. However, whether other p53-regulated processes, such as cell cycle arrest, senescence, autophagy, and cellular metabolism also get similarly affected by Par-4 loss remains to be investigated. In addition, is reduced stability of p53 in the absence of Par-4 solely due to Akt-mediated activation of Mdm2? It is also likely that Par-4 can directly bind and modulate Mdm2 activity, given our

observation of such strong inhibition of p53 expression due to Par-4 loss. Direct regulation of Mdm2 expression/activity by Par-4 also needs to be examined.

In summary, the studies described in this dissertation elaborate how the mutual interplay between the two tumor suppressors p53 and Par-4 has helped:

- ➢ Conceptualize a novel strategy of empowering normal cells to combat remote cancer cells by releasing a cancer cell-specific killer protein.
- ➢ Elucidate the mechanism by which Par-4 loss may confer therapy resistance in cells that possess wild-type p53.

Figure 4.1: Par-4 mediates the paracrine apoptotic effect of p53
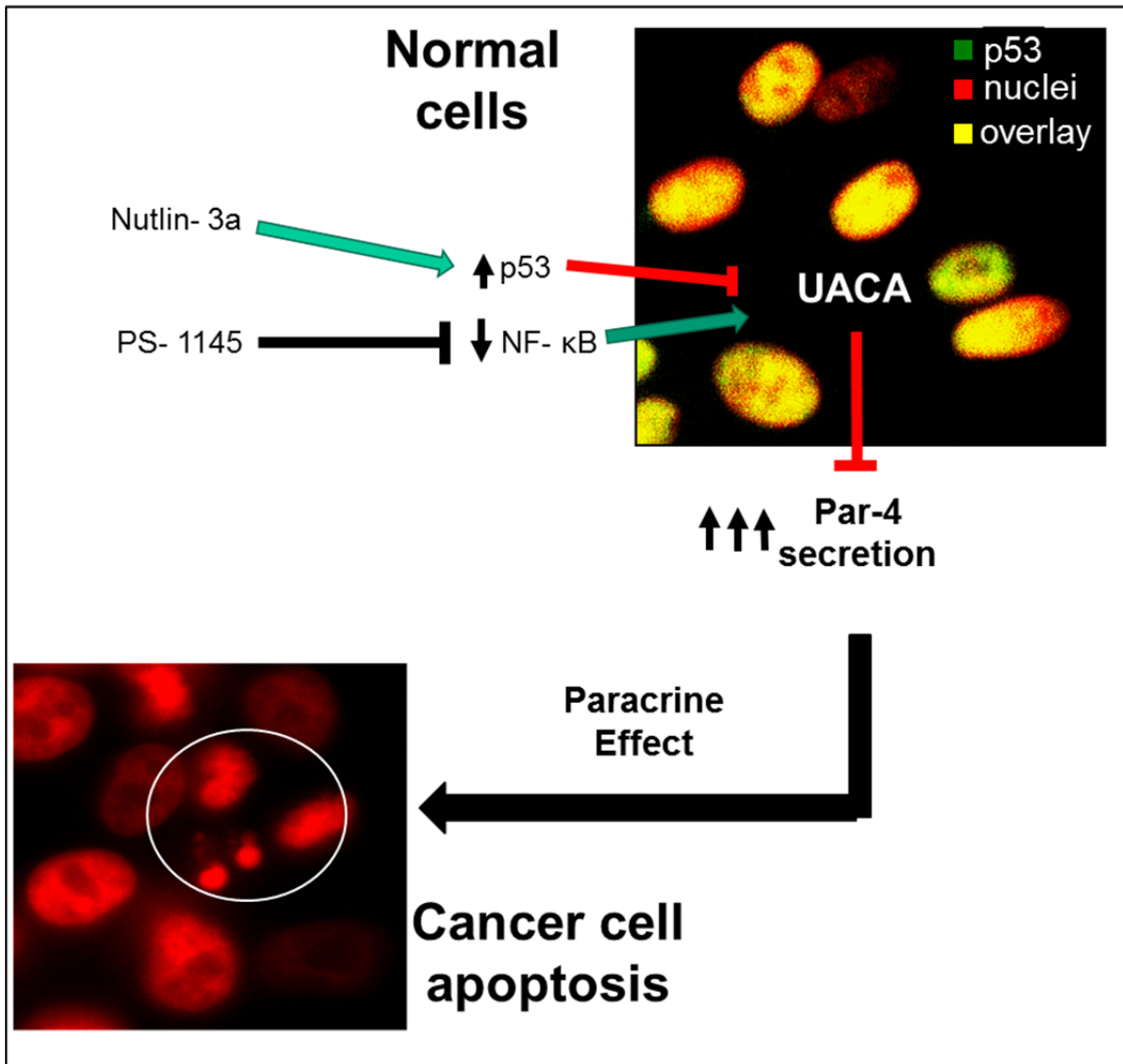
Fig 4.1: Par-4 mediates the paracrine apoptotic effect of p53. P53 activation by Nutlin-3a and NF-κB inhibition by PS-1145 results in robust p53 activation, thereby leading to synergistic increase in Par-4 secretion. Extracellular Par-4 then targets cancer cells in remote locations, thus executing the paracrine apoptotic effect of p53 in a cancer cell-specific manner.

Adapted and modified from Burikhanov et. al., Cell Rep. 2014 Jan 30;6(2):271-7.

Figure 4.2: Par-4 regulates p53 stability
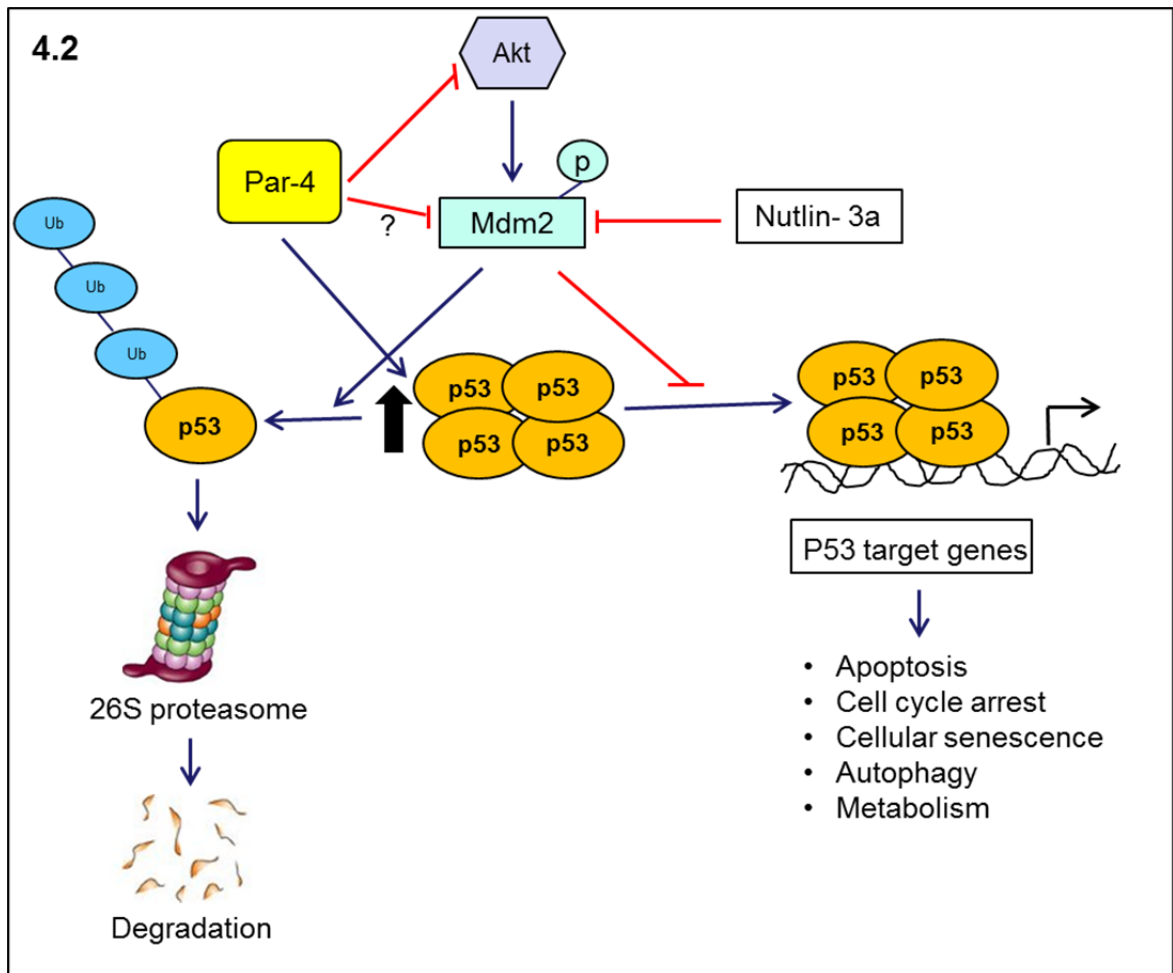
Figure 4.2: Par-4 regulates p53 stability. The E3 ligase activity is enhanced by Akt, which in turn is inhibited by Par-4. In the absence of Par-4, Akt-mediated phosphorylation of Mdm2 at S166 activates it, and results in an increased rate of p53 degradation, as well as inhibition of p53 transcriptional activity.

REFERENCES

(World Cancer Report 2014). World Cancer Report 2014 (International Agency for Research on Cancer, Lyon, France.).

Addadi, Y., Moskovits, N., Granot, D., Lozano, G., Carmi, Y., Apte, R.N., Neeman, M., and Oren, M. (2010). p53 status in stromal fibroblasts modulates tumor growth in an SDF1-dependent manner. Cancer research *70*, 9650-9658.

Affar el, B., Luke, M.P., Gay, F., Calvo, D., Sui, G., Weiss, R.S., Li, E., and Shi, Y. (2006). Targeted ablation of Par-4 reveals a cell type-specific susceptibility to apoptosis-inducing agents. Cancer research *66*, 3456-3462.

Ahmed, M.M., Sheldon, D., Fruitwala, M.A., Venkatasubbarao, K., Lee, E.Y., Gupta, S., Wood, C., Mohiuddin, M., and Strodel, W.E. (2008). Downregulation of PAR-4, a pro-apoptotic gene, in pancreatic tumors harboring K-ras mutation. International journal of cancer Journal international du cancer *122*, 63-70.

Alvarez, J.V., Pan, T.C., Ruth, J., Feng, Y., Zhou, A., Pant, D., Grimley, J.S., Wandless, T.J., Demichele, A., Investigators, I.S.T.*, et al.* (2013). Par-4 downregulation promotes breast cancer recurrence by preventing multinucleation following targeted therapy. Cancer cell *24*, 30-44.

Barkett, M., and Gilmore, T.D. (1999). Control of apoptosis by Rel/NF-kappaB transcription factors. Oncogene *18*, 6910-6924.

Barley, R.D., Enns, L., Paterson, M.C., and Mirzayans, R. (1998). Aberrant p21WAF1-dependent growth arrest as the possible mechanism of abnormal resistance to ultraviolet light cytotoxicity in Li-Fraumeni syndrome fibroblast strains heterozygous for TP53 mutations. Oncogene *17*, 533-543.

Barradas, M., Monjas, A., Diaz-Meco, M.T., Serrano, M., and Moscat, J. (1999). The downregulation of the pro-apoptotic protein Par-4 is critical for Ras-induced survival and tumor progression. The EMBO journal *18*, 6362-6369.

Beckerman, R., and Prives, C. (2010). Transcriptional regulation by p53. Cold Spring Harbor perspectives in biology *2*, a000935.

Boghaert, E.R., Sells, S.F., Walid, A.J., Malone, P., Williams, N.M., Weinstein, M.H., Strange, R., and Rangnekar, V.M. (1997). Immunohistochemical analysis of the proapoptotic protein Par-4 in normal rat tissues. Cell Growth Differ *8*, 881-890.

Boyd, S.D., Tsai, K.Y., and Jacks, T. (2000). An intact HDM2 RING-finger domain is required for nuclear exclusion of p53. Nature cell biology *2*, 563-568.

Brosh, R., and Rotter, V. (2009). When mutants gain new powers: news from the mutant p53 field. Nat Rev Cancer *9*, 701-713.

Bunz, F., Hwang, P.M., Torrance, C., Waldman, T., Zhang, Y., Dillehay, L., Williams, J., Lengauer, C., Kinzler, K.W., and Vogelstein, B. (1999). Disruption of p53 in human cancer cells alters the responses to therapeutic agents. The Journal of clinical investigation *104*, 263-269.

Burikhanov, R., Shrestha-Bhattarai, T., Hebbar, N., Qiu, S., Zhao, Y., Zambetti, G.P., and Rangnekar, V.M. (2014a). Paracrine apoptotic effect of p53 mediated by tumor suppressor Par-4. Cell reports *6*, 271-277.

Burikhanov, R., Shrestha-Bhattarai, T., Qiu, S., Shukla, N., Hebbar, N., Lele, S.M., Horbinski, C., and Rangnekar, V.M. (2013). Novel mechanism of apoptosis resistance in cancer mediated by extracellular PAR-4. Cancer research *73*, 1011-1019.

Burikhanov, R., Sviripa, V.M., Hebbar, N., Zhang, W., Layton, W.J., Hamza, A., Zhan, C.G., Watt, D.S., Liu, C., and Rangnekar, V.M. (2014b). Arylquins target vimentin to trigger Par-4 secretion for tumor cell apoptosis. Nature chemical biology *10*, 924-926.

Burikhanov, R., Zhao, Y., Goswami, A., Qiu, S., Schwarze, S.R., and Rangnekar, V.M. (2009). The tumor suppressor Par-4 activates an extrinsic pathway for apoptosis. Cell *138*, 377-388.

Chakraborty, M., Qiu, S.G., Vasudevan, K.M., and Rangnekar, V.M. (2001). Par-4 drives trafficking and activation of Fas and Fasl to induce prostate cancer cell apoptosis and tumor regression. Cancer research *61*, 7255-7263.

Chen, F., Wang, W., and El-Deiry, W.S. (2010). Current strategies to target p53 in cancer. Biochemical pharmacology *80*, 724-730.

Chen, J., Wu, X., Lin, J., and Levine, A.J. (1996). mdm-2 inhibits the G1 arrest and apoptosis functions of the p53 tumor suppressor protein. Molecular and cellular biology *16*, 2445-2452.

Chendil, D., Das, A., Dey, S., Mohiuddin, M., and Ahmed, M.M. (2002). Par-4, a pro-apoptotic gene, inhibits radiation-induced NF kappa B activity and Bcl-2 expression leading to induction of radiosensitivity in human prostate cancer cells PC-3. Cancer biology & therapy *1*, 152-160.

Chene, P. (2003). Inhibiting the p53-MDM2 interaction: an important target for cancer therapy. Nat Rev Cancer *3*, 102-109.

Cook, J., Krishnan, S., Ananth, S., Sells, S.F., Shi, Y., Walther, M.M., Linehan, W.M., Sukhatme, V.P., Weinstein, M.H., and Rangnekar, V.M. (1999). Decreased expression of the pro-apoptotic protein Par-4 in renal cell carcinoma. Oncogene *18*, 1205-1208.

de Almagro, M.C., and Vucic, D. (2012). The inhibitor of apoptosis (IAP) proteins are critical regulators of signaling pathways and targets for anti-cancer therapy. Experimental oncology *34*, 200-211.

Delbridge, A.R., Valente, L.J., and Strasser, A. (2012). The role of the apoptotic machinery in tumor suppression. Cold Spring Harbor perspectives in biology *4*.

Dey, A., Wong, E.T., Bist, P., Tergaonkar, V., and Lane, D.P. (2007). Nutlin-3 inhibits the NFkappaB pathway in a p53-dependent manner: implications in lung cancer therapy. Cell cycle *6*, 2178-2185.

Diaz-Meco, M.T., Lallena, M.J., Monjas, A., Frutos, S., and Moscat, J. (1999). Inactivation of the inhibitory kappaB protein kinase/nuclear factor kappaB pathway by Par-4 expression potentiates tumor necrosis factor alpha-induced apoptosis. The Journal of biological chemistry *274*, 19606-19612.

Diaz-Meco, M.T., Municio, M.M., Frutos, S., Sanchez, P., Lozano, J., Sanz, L., and Moscat, J. (1996). The product of par-4, a gene induced during apoptosis, interacts selectively with the atypical isoforms of protein kinase C. Cell *86*, 777-786.

Donehower, L.A., Godley, L.A., Aldaz, C.M., Pyle, R., Shi, Y.P., Pinkel, D., Gray, J., Bradley, A., Medina, D., and Varmus, H.E. (1995). Deficiency of p53 accelerates mammary tumorigenesis in Wnt-1 transgenic mice and promotes chromosomal instability. Genes & development *9*, 882-895.

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Jr., Butel, J.S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature *356*, 215-221.

Donehower, L.A., and Lozano, G. (2009). 20 years studying p53 functions in genetically engineered mice. Nat Rev Cancer *9*, 831-841.

Drappa, J., Vaishnaw, A.K., Sullivan, K.E., Chu, J.L., and Elkon, K.B. (1996). Fas gene mutations in the Canale-Smith syndrome, an inherited lymphoproliferative disorder associated with autoimmunity. The New England journal of medicine *335*, 1643-1649.

Dudley, A.C., Shih, S.C., Cliffe, A.R., Hida, K., and Klagsbrun, M. (2008). Attenuated p53 activation in tumour-associated stromal cells accompanies

decreased sensitivity to etoposide and vincristine. British journal of cancer *99*, 118-125.

Eischen, C.M., Weber, J.D., Roussel, M.F., Sherr, C.J., and Cleveland, J.L. (1999). Disruption of the ARF-Mdm2-p53 tumor suppressor pathway in Myc-induced lymphomagenesis. Genes & development *13*, 2658-2669.

el-Deiry, W.S., Kern, S.E., Pietenpol, J.A., Kinzler, K.W., and Vogelstein, B. (1992). Definition of a consensus binding site for p53. Nat Genet *1*, 45-49.

El-Guendy, N., and Rangnekar, V.M. (2003). Apoptosis by Par-4 in cancer and neurodegenerative diseases. Experimental cell research *283*, 51-66.

El-Guendy, N., Zhao, Y., Gurumurthy, S., Burikhanov, R., and Rangnekar, V.M. (2003). Identification of a unique core domain of par-4 sufficient for selective apoptosis induction in cancer cells. Molecular and cellular biology *23*, 5516-5525.

Finlay, C.A., Hinds, P.W., and Levine, A.J. (1989). The p53 proto-oncogene can act as a suppressor of transformation. Cell *57*, 1083-1093.

Franchitto, A., Torrice, A., Semeraro, R., Napoli, C., Nuzzo, G., Giuliante, F., Alpini, G., Carpino, G., Berloco, P.B., Izzo, L.*, et al.* (2010). Prostate apoptosis response-4 is expressed in normal cholangiocytes, is down-regulated in human cholangiocarcinoma, and promotes apoptosis of neoplastic cholangiocytes when induced pharmacologically. The American journal of pathology *177*, 1779-1790.

Fridman, J.S., and Lowe, S.W. (2003). Control of apoptosis by p53. Oncogene *22*, 9030-9040.

Friend, S.H., Bernards, R., Rogelj, S., Weinberg, R.A., Rapaport, J.M., Albert, D.M., and Dryja, T.P. (1986). A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature *323*, 643-646.

Funk, W.D., Pak, D.T., Karas, R.H., Wright, W.E., and Shay, J.W. (1992). A transcriptionally active DNA-binding site for human p53 protein complexes. Molecular and cellular biology *12*, 2866-2871.

Garcia-Cao, I., Duran, A., Collado, M., Carrascosa, M.J., Martin-Caballero, J., Flores, J.M., Diaz-Meco, M.T., Moscat, J., and Serrano, M. (2005). Tumour-suppression activity of the proapoptotic regulator Par4. EMBO reports *6*, 577-583.

Garcia-Cao, I., Garcia-Cao, M., Martin-Caballero, J., Criado, L.M., Klatt, P., Flores, J.M., Weill, J.C., Blasco, M.A., and Serrano, M. (2002). "Super p53" mice exhibit enhanced DNA damage response, are tumor resistant and age normally. The EMBO journal *21*, 6225-6235.

Geyer, R.K., Yu, Z.K., and Maki, C.G. (2000). The MDM2 RING-finger domain is required to promote p53 nuclear export. Nature cell biology *2*, 569-573.

Giono, L.E., and Manfredi, J.J. (2007). Mdm2 is required for inhibition of Cdk2 activity by p21, thereby contributing to p53-dependent cell cycle arrest. Molecular and cellular biology *27*, 4166-4178.

Goswami, A., Burikhanov, R., de Thonel, A., Fujita, N., Goswami, M., Zhao, Y., Eriksson, J.E., Tsuruo, T., and Rangnekar, V.M. (2005). Binding and phosphorylation of par-4 by akt is essential for cancer cell survival. Molecular cell *20*, 33-44.

Goswami, A., Qiu, S., Dexheimer, T.S., Ranganathan, P., Burikhanov, R., Pommier, Y., and Rangnekar, V.M. (2008). Par-4 binds to topoisomerase 1 and attenuates its DNA relaxation activity. Cancer research *68*, 6190-6198.

Gottlieb, T.M., Leal, J.F., Seger, R., Taya, Y., and Oren, M. (2002). Cross-talk between Akt, p53 and Mdm2: possible implications for the regulation of apoptosis. Oncogene *21*, 1299-1303.

Greenspan, E.J., Madigan, J.P., Boardman, L.A., and Rosenberg, D.W. (2011). Ibuprofen inhibits activation of nuclear {beta}-catenin in human colon adenomas and induces the phosphorylation of GSK-3{beta}. Cancer prevention research *4*, 161-171.

Gurumurthy, S., Goswami, A., Vasudevan, K.M., and Rangnekar, V.M. (2005). Phosphorylation of Par-4 by protein kinase A is critical for apoptosis. Molecular and cellular biology *25*, 1146-1161.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674.
Harms, K.L., and Chen, X. (2005). The C terminus of p53 family proteins is a cell fate determinant. Molecular and cellular biology *25*, 2014-2030.

Harvey, D.M., and Levine, A.J. (1991). p53 alteration is a common event in the spontaneous immortalization of primary BALB/c murine embryo fibroblasts. Genes & development *5*, 2375-2385.

Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997). Mdm2 promotes the rapid degradation of p53. Nature *387*, 296-299.

Hebbar, N., Wang, C., and Rangnekar, V.M. (2012). Mechanisms of apoptosis by the tumor suppressor Par-4. Journal of cellular physiology *227*, 3715-3721.

Hill, R., Song, Y., Cardiff, R.D., and Van Dyke, T. (2005). Selective evolution of stromal mesenchyme with p53 loss in response to epithelial tumorigenesis. Cell *123*, 1001-1011.

Honda, R., Tanaka, H., and Yasuda, H. (1997). Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS letters *420*, 25-27.

Jacks, T., Remington, L., Williams, B.O., Schmitt, E.M., Halachmi, S., Bronson, R.T., and Weinberg, R.A. (1994). Tumor spectrum analysis in p53-mutant mice. Current biology : CB *4*, 1-7.

Joerger, A.C., and Fersht, A.R. (2010). The tumor suppressor p53: from structures to drug discovery. Cold Spring Harbor perspectives in biology *2*, a000919.

Johnstone, R.W., See, R.H., Sells, S.F., Wang, J., Muthukkumar, S., Englert, C., Haber, D.A., Licht, J.D., Sugrue, S.P., Roberts, T.*, et al.* (1996). A novel repressor, par-4, modulates transcription and growth suppression functions of the Wilms' tumor suppressor WT1. Molecular and cellular biology *16*, 6945-6956.

Johnstone, R.W., Tommerup, N., Hansen, C., Vissing, H., and Shi, Y. (1998). Mapping of the human PAWR (par-4) gene to chromosome 12q21. Genomics *53*, 241-243.

Jones, S.N., Roe, A.E., Donehower, L.A., and Bradley, A. (1995). Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. Nature *378*, 206-208.

Joshi, J., Fernandez-Marcos, P.J., Galvez, A., Amanchy, R., Linares, J.F., Duran, A., Pathrose, P., Leitges, M., Canamero, M., Collado, M.*, et al.* (2008). Par-4 inhibits Akt and suppresses Ras-induced lung tumorigenesis. The EMBO journal *27*, 2181-2193.

Kerr, J.F., Winterford, C.M., and Harmon, B.V. (1994). Apoptosis. Its significance in cancer and cancer therapy. Cancer *73*, 2013-2026.

Kerr, J.F., Wyllie, A.H., and Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. British journal of cancer *26*, 239-257.

Kiaris, H., Chatzistamou, I., Trimis, G., Frangou-Plemmenou, M., Pafiti-Kondi, A., and Kalofoutis, A. (2005). Evidence for nonautonomous effect of p53 tumor suppressor in carcinogenesis. Cancer research *65*, 1627-1630.

Kimura, M., Furukawa, T., Abe, T., Yatsuoka, T., Youssef, E.M., Yokoyama, T., Ouyang, H., Ohnishi, Y., Sunamura, M., Kobari, M.*, et al.* (1998). Identification of

two common regions of allelic loss in chromosome arm 12q in human pancreatic cancer. Cancer research *58*, 2456-2460.

Kinzler, K.W., and Vogelstein, B. (1996). Lessons from hereditary colorectal cancer. Cell *87*, 159-170.

Kinzler, K.W., and Vogelstein, B. (1998). Landscaping the cancer terrain. Science *280*, 1036-1037.

Knudson, A.G., Jr. (1971). Mutation and cancer: statistical study of retinoblastoma. Proceedings of the National Academy of Sciences of the United States of America *68*, 820-823.

Kogel, D., Reimertz, C., Mech, P., Poppe, M., Fruhwald, M.C., Engemann, H., Scheidtmann, K.H., and Prehn, J.H. (2001). Dlk/ZIP kinase-induced apoptosis in human medulloblastoma cells: requirement of the mitochondrial apoptosis pathway. British journal of cancer *85*, 1801-1808.

Kruse, J.P., and Gu, W. (2009). Modes of p53 regulation. Cell *137*, 609-622.
Kubbutat, M.H., Jones, S.N., and Vousden, K.H. (1997). Regulation of p53 stability by Mdm2. Nature *387*, 299-303.

Kukoc-Zivojnov, N., Puccetti, E., Chow, K.U., Bergmann, M., Ruthardt, M., Hoelzer, D., Mitrou, P.S., Weidmann, E., and Boehrer, S. (2004). Prostate apoptosis response gene-4 (par-4) abrogates the survival function of p185(BCR-ABL) in hematopoietic cells. Experimental hematology *32*, 649-656.

Kurose, K., Gilley, K., Matsumoto, S., Watson, P.H., Zhou, X.P., and Eng, C. (2002). Frequent somatic mutations in PTEN and TP53 are mutually exclusive in the stroma of breast carcinomas. Nat Genet *32*, 355-357.

Lane, D., and Levine, A. (2010). p53 Research: the past thirty years and the next thirty years. Cold Spring Harbor perspectives in biology *2*, a000893.

Lane, D.P., and Crawford, L.V. (1979). T antigen is bound to a host protein in SV40-transformed cells. Nature *278*, 261-263.

Levine, A.J. (1997). p53, the cellular gatekeeper for growth and division. Cell *88*, 323-331.

Levine, A.J., Finlay, C.A., and Hinds, P.W. (2004). P53 is a tumor suppressor gene. Cell *116, Supplement 2*, S67-S70.

Levine, A.J., and Oren, M. (2009). The first 30 years of p53: growing ever more complex. Nat Rev Cancer *9*, 749-758.

Li, Z.W., Chu, W., Hu, Y., Delhase, M., Deerinck, T., Ellisman, M., Johnson, R., and Karin, M. (1999). The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. The Journal of experimental medicine *189*, 1839-1845.

Limesand, K.H., Schwertfeger, K.L., and Anderson, S.M. (2006). MDM2 is required for suppression of apoptosis by activated Akt1 in salivary acinar cells. Molecular and cellular biology *26*, 8840-8856.

Linzer, D.I., and Levine, A.J. (1979). Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell *17*, 43-52.

Liu, Y., Chen, J.Q., Xie, L., Wang, J., Li, T., He, Y., Gao, Y., Qin, X., and Li, S. (2014a). Effect of aspirin and other non-steroidal anti-inflammatory drugs on prostate cancer incidence and mortality: a systematic review and meta-analysis. BMC medicine *12*, 55.

Liu, Y., Gilbert, M.R., Kyprianou, N., Rangnekar, V.M., and Horbinski, C. (2014b). The tumor suppressor prostate apoptosis response-4 (Par-4) is regulated by mutant IDH1 and kills glioma stem cells. Acta neuropathologica *128*, 723-732.

Lowe, S.W., Bodis, S., McClatchey, A., Remington, L., Ruley, H.E., Fisher, D.E., Housman, D.E., and Jacks, T. (1994). p53 status and the efficacy of cancer therapy in vivo. Science *266*, 807-810.

Lowe, S.W., and Lin, A.W. (2000). Apoptosis in cancer. Carcinogenesis *21*, 485-495.

Lowe, S.W., Ruley, H.E., Jacks, T., and Housman, D.E. (1993a). p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell *74*, 957-967.

Lowe, S.W., Schmitt, E.M., Smith, S.W., Osborne, B.A., and Jacks, T. (1993b). p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature *362*, 847-849.

Lucas, T., Pratscher, B., Krishnan, S., Fink, D., Gunsberg, P., Wolschek, M., Wacheck, V., Muster, T., Romirer, I., Wolff, K.*, et al.* (2001). Differential expression levels of Par-4 in melanoma. Melanoma research *11*, 379-383.

Macleod, K. (2000). Tumor suppressor genes. Current opinion in genetics & development *10*, 81-93.

Manfredi, J.J. (2010). The Mdm2-p53 relationship evolves: Mdm2 swings both ways as an oncogene and a tumor suppressor. Genes & development *24*, 1580-1589.

Matheu, A., Maraver, A., Klatt, P., Flores, I., Garcia-Cao, I., Borras, C., Flores, J.M., Vina, J., Blasco, M.A., and Serrano, M. (2007). Delayed ageing through damage protection by the Arf/p53 pathway. Nature *448*, 375-379.

Mayo, L.D., and Donner, D.B. (2001). A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. Proceedings of the National Academy of Sciences of the United States of America *98*, 11598-11603.

McLure, K.G., and Lee, P.W. (1998). How p53 binds DNA as a tetramer. The EMBO journal *17*, 3342-3350.

Mendez-Lopez, L.F., Zapata-Benavides, P., Zavala-Pompa, A., Aguado-Barrera, M.E., Pacheco-Calleros, J., Rodriguez-Padilla, C., Cerda-Flores, R.M., Cortes-Gutierrez, E.I., and Davila-Rodriguez, M.I. (2010). Immunohistochemical analysis of prostate apoptosis response-4 (Par-4) in Mexican women with breast cancer: a preliminary study. Archives of medical research *41*, 261-268.

Mirzayans, R., Andrais, B., Scott, A., Wang, Y.W., and Murray, D. (2013). Ionizing radiation-induced responses in human cells with differing TP53 status. International journal of molecular sciences *14*, 22409-22435.

Moinfar, F., Man, Y.G., Arnould, L., Bratthauer, G.L., Ratschek, M., and Tavassoli, F.A. (2000). Concurrent and independent genetic alterations in the stromal and epithelial cells of mammary carcinoma: implications for tumorigenesis. Cancer research *60*, 2562-2566.

Moinfar, F., Man, Y.G., Arnould, L., Bratthauer, G.L., Ratschek, M., and Tavassoli, F.A. (2000). Concurrent and independent genetic alterations in the stromal and epithelial cells of mammary carcinoma: implications for tumorigenesis. Cancer research *60*, 2562-2566.

Momand, J., Zambetti, G.P., Olson, D.C., George, D., and Levine, A.J. (1992). The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell *69*, 1237-1245.

Montes de Oca Luna, R., Wagner, D.S., and Lozano, G. (1995). Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. Nature *378*, 203-206.

Moreno-Bueno, G., Fernandez-Marcos, P.J., Collado, M., Tendero, M.J., Rodriguez-Pinilla, S.M., Garcia-Cao, I., Hardisson, D., Diaz-Meco, M.T., Moscat, J., Serrano, M.*, et al.* (2007). Inactivation of the candidate tumor suppressor par-4 in endometrial cancer. Cancer research *67*, 1927-1934.

Nagai, M.A., Gerhard, R., Salaorni, S., Fregnani, J.H., Nonogaki, S., Netto, M.M., and Soares, F.A. (2010). Down-regulation of the candidate tumor suppressor gene PAR-4 is associated with poor prognosis in breast cancer. International journal of oncology *37*, 41-49.

Nalca, A., Qiu, S.G., El-Guendy, N., Krishnan, S., and Rangnekar, V.M. (1999). Oncogenic Ras sensitizes cells to apoptosis by Par-4. The Journal of biological chemistry *274*, 29976-29983.

O' Reilly, L.A., Tai, L., Lee, L., Kruse, E.A., Grabow, S., Fairlie, W.D., Haynes, N.M., Tarlinton, D.M., Zhang, J.G., Belz, G.T.*, et al.* (2009). Membrane-bound Fas ligand only is essential for Fas-induced apoptosis. Nature *461*, 659-663.

O'Connor, P.M., Jackman, J., Bae, I., Myers, T.G., Fan, S., Mutoh, M., Scudiero, D.A., Monks, A., Sausville, E.A., Weinstein, J.N.*, et al.* (1997). Characterization of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth-inhibitory potency of 123 anticancer agents. Cancer research *57*, 4285-4300.

Ogawara, Y., Kishishita, S., Obata, T., Isazawa, Y., Suzuki, T., Tanaka, K., Masuyama, N., and Gotoh, Y. (2002). Akt enhances Mdm2-mediated ubiquitination and degradation of p53. The Journal of biological chemistry *277*, 21843-21850.

Oren, M. (1999). Regulation of the p53 tumor suppressor protein. The Journal of biological chemistry *274*, 36031-36034.

Palmero, I., Pantoja, C., and Serrano, M. (1998). p19ARF links the tumour suppressor p53 to Ras. Nature *395*, 125-126.

Paterson, R.F., Ulbright, T.M., MacLennan, G.T., Zhang, S., Pan, C.X., Sweeney, C.J., Moore, C.R., Foster, R.S., Koch, M.O., Eble, J.N.*, et al.* (2003). Molecular genetic alterations in the laser-capture-microdissected stroma adjacent to bladder carcinoma. Cancer *98*, 1830-1836.

Pereira, M.C., de Bessa-Garcia, S.A., Burikhanov, R., Pavanelli, A.C., Antunes, L., Rangnekar, V.M., and Nagai, M.A. (2013). Prostate apoptosis response-4 is involved in the apoptosis response to docetaxel in MCF-7 breast cancer cells. International journal of oncology *43*, 531-538.

Petitjean, A., Mathe, E., Kato, S., Ishioka, C., Tavtigian, S.V., Hainaut, P., and Olivier, M. (2007). Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. Human mutation *28*, 622-629.

Plati, J., Bucur, O., and Khosravi-Far, R. (2011). Apoptotic cell signaling in cancer progression and therapy. Integrative biology : quantitative biosciences from nano to macro *3*, 279-296.

Polanski, R., Noon, A.P., Blaydes, J., Phillips, A., Rubbi, C.P., Parsons, K., Vlatkovic, N., and Boyd, M.T. (2014). Senescence induction in renal carcinoma cells by Nutlin-3: a potential therapeutic strategy based on MDM2 antagonism. Cancer letters *353*, 211-219.

Pruitt, K., Ulku, A.S., Frantz, K., Rojas, R.J., Muniz-Medina, V.M., Rangnekar, V.M., Der, C.J., and Shields, J.M. (2005). Ras-mediated loss of the pro-apoptotic response protein Par-4 is mediated by DNA hypermethylation through Raf-independent and Raf-dependent signaling cascades in epithelial cells. The Journal of biological chemistry *280*, 23363-23370.

Qiu, S.G., Krishnan, S., el-Guendy, N., and Rangnekar, V.M. (1999). Negative regulation of Par-4 by oncogenic Ras is essential for cellular transformation. Oncogene *18*, 7115-7123.

Ranganathan, P., and Rangnekar, V.M. (2005). Regulation of cancer cell survival by Par-4. Annals of the New York Academy of Sciences *1059*, 76-85.

Reed, J.C. (1999). Dysregulation of apoptosis in cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *17*, 2941-2953.

Rieux-Laucat, F., Le Deist, F., Hivroz, C., Roberts, I.A., Debatin, K.M., Fischer, A., and de Villartay, J.P. (1995). Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. Science *268*, 1347-1349.

Riley, T., Sontag, E., Chen, P., and Levine, A. (2008). Transcriptional control of human p53-regulated genes. Nature reviews Molecular cell biology *9*, 402-412.

Schmitz, K.J., Grabellus, F., Callies, R., Wohlschlaeger, J., Otterbach, F., Kimmig, R., Levkau, B., Schmid, K.W., and Baba, H.A. (2006). Relationship and prognostic significance of phospho-(serine 166)-murine double minute 2 and Akt activation in node-negative breast cancer with regard to p53 expression. Virchows Archiv : an international journal of pathology *448*, 16-23.

Schneider, B.G., Rha, S.Y., Chung, H.C., Bravo, J.C., Mera, R., Torres, J.C., Plaisance, K.T., Jr., Schlegel, R., McBride, C.M., Reveles, X.T.*, et al.* (2003). Regions of allelic imbalance in the distal portion of chromosome 12q in gastric cancer. Molecular pathology : MP *56*, 141-149.

Sekido, Y., Fong, K.M., and Minna, J.D. (2001). Molecular biology of lung cancer. In Cancer: principles and practice of oncology, H.S. De-Vita VT, and Rosenberg SA, ed. (Philadelphia: Lippincott), pp. 917-925.

Sells, S.F., Wood, D.P., Jr., Joshi-Barve, S.S., Muthukumar, S., Jacob, R.J., Crist, S.A., Humphreys, S., and Rangnekar, V.M. (1994). Commonality of the gene programs induced by effectors of apoptosis in androgen-dependent and -independent prostate cells. Cell Growth Differ *5*, 457-466.

Siegel, R., Ma, J., Zou, Z., and Jemal, A. (2014). Cancer statistics, 2014. CA: a cancer journal for clinicians *64*, 9-29.

Strasser, A., O'Connor, L., and Dixit, V.M. (2000). Apoptosis signaling. Annu Rev Biochem *69*, 217-245.

Tan, J., You, Y., Xu, T., Yu, P., Wu, D., Deng, H., Zhang, Y., and Bie, P. (2014). Par-4 downregulation confers cisplatin resistance in pancreatic cancer cells via PI3K/Akt pathway-dependent EMT. Toxicology letters *224*, 7-15.

Thompson, T., Tovar, C., Yang, H., Carvajal, D., Vu, B.T., Xu, Q., Wahl, G.M., Heimbrook, D.C., and Vassilev, L.T. (2004). Phosphorylation of p53 on key serines is dispensable for transcriptional activation and apoptosis. The Journal of biological chemistry *279*, 53015-53022.

Toledo, F., Krummel, K.A., Lee, C.J., Liu, C.W., Rodewald, L.W., Tang, M., and Wahl, G.M. (2006). A mouse p53 mutant lacking the proline-rich domain rescues Mdm4 deficiency and provides insight into the Mdm2-Mdm4-p53 regulatory network. Cancer cell *9*, 273-285.

Tran, N.H., Sakai, T., Kim, S.M., and Fukui, K. (2010). NF-kappaB regulates the expression of Nucling, a novel apoptosis regulator, with involvement of proteasome and caspase for its degradation. J Biochem *148*, 573-580.

Vassilev, L.T., Vu, B.T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukacs, C., Klein, C.*, et al.* (2004). In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. Science *303*, 844-848.

Vousden, K.H., and Prives, C. (2009). Blinded by the Light: The Growing Complexity of p53. Cell *137*, 413-431.

Wallace-Brodeur, R.R., and Lowe, S.W. (1999). Clinical implications of p53 mutations. Cellular and molecular life sciences : CMLS *55*, 64-75.

Wang, B.D., Kline, C.L., Pastor, D.M., Olson, T.L., Frank, B., Luu, T., Sharma, A.K., Robertson, G., Weirauch, M.T., Patierno, S.R.*, et al.* (2010). Prostate apoptosis response protein 4 sensitizes human colon cancer cells to

chemotherapeutic 5-FU through mediation of an NF kappaB and microRNA network. Molecular cancer *9*, 98.

Wang, G., Dinkins, M., He, Q., Zhu, G., Poirier, C., Campbell, A., Mayer-Proschel, M., and Bieberich, E. (2012). Astrocytes secrete exosomes enriched with proapoptotic ceramide and prostate apoptosis response 4 (PAR-4): potential mechanism of apoptosis induction in Alzheimer disease (AD). The Journal of biological chemistry *287*, 21384-21395.

Wang, Y., Zhang, Z., Lubet, R.A., and You, M. (2006). A mouse model for tumor progression of lung cancer in ras and p53 transgenic mice. Oncogene *25*, 1277-1280.

Wernert, N., Locherbach, C., Wellmann, A., Behrens, P., and Hugel, A. (2001). Presence of genetic alterations in microdissected stroma of human colon and breast cancers. Anticancer research *21*, 2259-2264.

Wu, X., and Levine, A.J. (1994). p53 and E2F-1 cooperate to mediate apoptosis. Proceedings of the National Academy of Sciences of the United States of America *91*, 3602-3606.

Yamada, K., Senju, S., Nakatsura, T., Murata, Y., Ishihara, M., Nakamura, S., Ohno, S., Negi, A., and Nishimura, Y. (2001). Identification of a novel autoantigen UACA in patients with panuveitis. Biochem Biophys Res Commun *280*, 1169-1176.

Yamamoto, Y., and Gaynor, R.B. (2001). Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. The Journal of clinical investigation *107*, 135-142.
Yip, K.W., and Reed, J.C. (2008). Bcl-2 family proteins and cancer. Oncogene *27*, 6398-6406.

Yu, X., Harris, S.L., and Levine, A.J. (2006). The regulation of exosome secretion: a novel function of the p53 protein. Cancer research *66*, 4795-4801.

Yu, X., Riley, T., and Levine, A.J. (2009). The regulation of the endosomal compartment by p53 the tumor suppressor gene. The FEBS journal *276*, 2201-2212.

Zapata-Benavides, P., Mendez-Vazquez, J.L., Gonzalez-Rocha, T.R., Zamora-Avila, D.E., Franco-Molina, M.A., Garza-Garza, R., and Rodriguez-Padilla, C. (2009). Expression of prostate apoptosis response (Par-4) is associated with progesterone receptor in breast cancer. Archives of medical research *40*, 595-599.

Zhao Y, B.D.a.W.S. (2013). Small Molecule Inhibitors of MDM2-p53 and MDMX-p53 Interactions as New Cancer Therapeutics. BioDiscovery *8*.

Zhao, Y., Burikhanov, R., Brandon, J., Qiu, S., Shelton, B.J., Spear, B., Bondada, S., Bryson, S., and Rangnekar, V.M. (2011). Systemic Par-4 inhibits non-autochthonous tumor growth. Cancer biology & therapy *12*, 152-157.

Zhao, Y., Burikhanov, R., Qiu, S., Lele, S.M., Jennings, C.D., Bondada, S., Spear, B., and Rangnekar, V.M. (2007). Cancer resistance in transgenic mice expressing the SAC module of Par-4. Cancer research *67*, 9276-9285.

Zhao, Y., and Rangnekar, V.M. (2008). Apoptosis and tumor resistance conferred by Par-4. Cancer biology & therapy *7*, 1867-1874.

Zhou, B.P., Liao, Y., Xia, W., Zou, Y., Spohn, B., and Hung, M.C. (2001). HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. Nature cell biology *3*, 973-982.

Zilfou, J.T., and Lowe, S.W. (2009). Tumor suppressive functions of p53. Cold Spring Harbor perspectives in biology *1*, a001883.

VITA

Name: Tripti Shrestha Bhattarai
Place of birth: Kathmandu, Nepal.

EDUCATION

2010-present   Ph.D. Fall 2010-present.
Graduate Center for Toxicology, University of Kentucky
Lexington, Kentucky.
Research project involves studying the "Cross-talk between
the tumor suppressors Par-4 and p53"
Mentor: Dr. Vivek Rangnekar

2010   Master's in Toxicology. Graduate Center for Toxicology,
University of Kentucky, Lexington, Kentucky.
Review Article: Cancer-selective apoptotic effects of
extracellular and intracellular Par-4. **Oncogene (2010) 29,
3873-3880.**
Mentor: Dr. Vivek Rangnekar

2004   B. Pharmacy (Hons.)
Jahangirnagar University, Savar, Dhaka, Bangladesh.
Research Project: Pharmacological and Toxicological
Effects of Mustakarista
Training: In-plant training performed at Renata Limited
(formerly Pfizer Laboratories), Dhaka, Bangladesh.
Mentor: Prof. Shahabuddin Kabir Choudhuri

PROFESSIONAL POSITION

November, 2004 – April, 2008   Instructor and Coordinator, Diploma in
Pharmacy program.
Nepal Institute of Health Sciences, Tusal,
Bouddha- 6, Kathmandu Nepal.

PUBLICATIONS

Cancer-Selective Apoptosis by Tumor Suppressor Par-4.
Hebbar, N., **Shrestha-Bhattarai T.**, and Rangnekar, V.M..
Adv Exp Med Biol. 2014;818:155-66.

Paracrine Apoptotic Effect of p53 Mediated by the Tumor Suppressor Par-4.
Burikhanov, R., **Shrestha-Bhattarai T.**, Hebbar, N., Qiu, S., Zhao, Y., Zambetti, G., and Rangnekar, V.M..
Cell Rep. 2014 Jan 30;6(2):271-7.

Par-4 prevents breast cancer recurrence.
Hebbar, N., **Shrestha-Bhattarai T.**, and Rangnekar, V.M..
Breast Cancer Res. 2013;15(5):314.

Par(-4)oxysm in breast cancer.
Shrestha-Bhattarai T, Hebbar N, and Rangnekar V.M..
Cancer Cell. 2013 Jul 8;24(1):3-5.

Novel mechanism of apoptosis resistance in cancer mediated by extracellular PAR-4.
Burikhanov, R., **Shrestha-Bhattarai T.**, Qiu, S., Shukla, N., Hebbar, N. K., Lele, S.M., Horbinsky, C., and Rangnekar, V.M..
Cancer Res. 2013 Jan 15;73(2):1011-9.

Cancer-selective apoptotic effects of extracellular and intracellular Par-4.
Shrestha-Bhattarai T. and Rangnekar, V.M..
Oncogene (2010) 29, 3873–3880.

Pharmacological and toxicological studies of an Ayurvedic formulation ("Louhasava") on the biological systems of rats and mice.
Ullah M.O., **Shrestha T**, Munira T.S., Choudhuri M.S..
Pakistan Journal of Biological Sciences. 2008 August; 11(16): 2013-17.

SCHOLARSHIPS & FELLOWSHIPS

Dissertation Year Fellowship 2013-2014 awarded by The Graduate School, University of Kentucky.

Kentucky Opportunity Fellowship 2012-2013 awarded by The Graduate School, University of Kentucky.

Kentucky Opportunity Fellowship 2011-2012 awarded by The Graduate School, University of Kentucky.

Fulbright Student Scholarship 2008-2010 granted by the United States Department of State to pursue a Master's degree in Toxicology.

Kentucky Graduate Scholarship (KGS) 2008-2010 granted by The Graduate School, University of Kentucky.

American Association of University Women (AAUW) International Fellowship 2008-2009 to study Master's in Toxicology.

Economic Relations Division (ERD) Scholarship provided by the Ministry of Planning, Government of the People's Republic of Bangladesh for academic excellence.

AWARDS & HONORS

University of Kentucky graduate student **Travel Award** to attend the AACR Cellular Heterogeneity in the Tumor Microenvironment conference, Feb 26 –Mar 1, 2014.

1st Prize in poster presentation, Graduate Student/Basic Sciences Division, 2013 Markey Cancer Center Research Day, University of Kentucky.

Abstract selected **as outstanding graduate student abstract invited for oral presentation** on Markey Cancer Center Research Day Symposium, 2012, University of Kentucky.

2nd Prize in poster presentation, Graduate Student/Basic Sciences Division, 2011 Markey Cancer Center Research Day, University of Kentucky.

Prime Minister's Gold Medal Award granted by the University Grants Commission (UGC), Bangladesh for scoring the highest grades in the Faculty of Biological Sciences at Jahangirnagar University.

Nepal Vidyabhushan from the Ministry of Education and Sports, Government of Nepal, for placing 1st Class 1st in B. Pharm. (Hons.).

CONFERENCES AND PRESENTATIONS

Cross-talk between the tumor suppressors Par-4 and p53.
Tripti Shrestha Bhattarai, James Sledziona, Sunil K. Nooti, and Vivek M. Rangnekar.
Markey Cancer Center Research Day, May 22, 2014, University of Kentucky.

Paracrine Apoptotic Effect of the Tumor Suppressor p53 is Mediated by Secreted Par-4.
Tripti Shrestha Bhattarai, Ravshan Burikhanov, Shirley Qiu, Yanming Zhao, and Vivek M. Rangnekar.
Markey Cancer Center Research Day, April 15, 2013, University of Kentucky.
Cellular Heterogeneity in the Tumor Microenvironment conference, American Association of Cancer Research (AACR), Feb 26-Mar 1, 2014.

The Tumor Suppressor p53 Induces Par-4 Secretion and Non-cell Autonomous Apoptosis.
Tripti Shrestha Bhattarai, Ravshan Burikhanov, Shirley Qiu, Yanming Zhao, and Vivek M. Rangnekar.
Invited for oral presentation at the Markey Cancer Center Research Day, May 16, 2012, University of Kentucky.

Golgin-160 Binds to the Tumor Suppressor Par-4 and Attenuates its Secretion.
Tripti Shrestha Bhattarai, Jaya Singh, Guofang Qiu, Carolyn E. Machamer, Vivek M. Rangnekar.
Markey Cancer Center Research Day, University of Kentucky on March 22, 2011.
AACR 102nd Annual Meeting, April 2-6 2011. Orange County Convention Center, Orlando,Florida.