Cloning, Expression and Characterization of Different Isoform of p53

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CERTIFICATE

The research work embodied in this thesis entitled **"Cloning, Expression and Characterization of Different Isoform of p53"** has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi, India. This work is original and has not been submitted so far, in part or in full for any other degree or diploma of any University.

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Dedicated to my Grandparents

Contents *Page No.*

Contents *Page No.*

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ABBREVIATIONS

β	Beta
α	Alpha
°C	Degree Celsius
ab	Antibody
bp	Base pair
dATP	Deoxyadenosine-5'-triphosphate
dGTP	Deoxyguanosine-5'-triphosphate
dCTP	Deoxycytidine-5'-triphosphate
dTTP	Deoxythymidine-5'-triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphates
EDTA	Ethylenediamine tetra-acetic acid
EtBr	Ethidium Bromide
EtOH	Ethanol
gm	Gram
hr	Hour
IPTG	Isopropyl-β-D-Thiogalactopyranoside
Kb	Kilobase
mM	Millimolar
mg	Milligram
Min	Minute
ml	Milliliter
nM	Nanomolar
ng	Nanogram
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease

rpm	Revolutions per minute
TE	Tris-EDTA
Tris	Tris (hydroxymethyl)-amino-methane
UV	Ultra violet
V	Volt
V/V	Volume/volume
W/V	Weight/volume
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-Galactopyaranoside
μ l	Microliter

CHAPTER 1

Introduction

1. Introduction

Overall development of an organism is dependant on the tight regulations of the key pathways and their components, and if there is any damage to a component of the pathway, the growth and development of the organism is affected. But nature has devised mechanisms, which check and guard the entire system through a set of key regulators in the pathway. Also if one pathway gets dis-regulated it cross-talks this to the other pathways and entire system gets de-stabilized. The p53 pathway is one of the very important pathways as it responses correctly to the damaging input signals like DNA damage, cellular stress, genotoxic stress, radiations like UV rays etc.

The p53 was discovered 26 years ago as a protein interacting with oncogenic T antigen from SV40 virus (Lane DP *et al.*, 1979). p53 is the most frequently mutated gene in human cancers as about half of the human cancers have mutation in p53 gene and loss of p53 activity is ubiquitous to all cancers as shown in **Tables 1** and **2** (Hofseth *et al.*, 2004). It is called as the cellular gate-keeper (Levine *et al.*, 1997) and tumor suppressor. The p53 protein responses to stress signals through post-translational modifications like phosphorylation, acetylation, methylation, ubiquitination, neddylation, sumolation etc. p53 protein is a sequence specific DNA binding transcription factor which is latent and less concentrated under normal conditions of growth and development. But it becomes activated and stabilized through above mentioned modifications in cells exposed to DNA damage and other types of stresses, and this leads to increase in the p53 protein levels and activities in the cell. Specific genes are activated and new products are accumulated which cause a variety of antiproliferative activities like cell cycle arrest, apoptosis and cellular senescence. Genes encoding DNA repair factors are also activated (Sax *et al.*, 2003). These responses prevent the replication of damaged DNA and the division of genetically altered cells. In this way p53 plays an essential role in maintaining the integrity of the genome (Lane *et al.*, 1992) and is known as the gatekeeper of the genome. p53 recognizes and binds to the p53-response elements in target genes and activates their transcription. It does so as a tetramer where a dimmer of two p53 protein subunits is first assembled on the response element and then two dimmers interact to give a transcriptionally active tetramer.

Two p53 related genes, p63 and p73 were discovered in 1997 and were shown to produce p63 and p73 proteins that are homologous and function similarly to p53. (Kaghad *et al.*, 1997; Yang *et al.*, 1998; Mondal *et al.*, 2005). The three proteins share a very good homology and are grouped together as a family. They all are able to bind to the p53 response elements (Mondal *et al.*, 2005). All the three members of the p53 family of transcription factors encode multiple splice variants called isoforms due to the usage of alternative promoter and alternative splicing (Kaghad *et al.* 1997; Yang *et al.* 1998 and Bourdon *et al.* 2005). p53 alone produces nine isoforms due to the usage of alternative promoter in intron 4 and alternative splicing in the intron 9. These isoforms are expressed in normal tissues in tissue-dependant fashion. The isoforms are p53 full length, p53 beta, p53 gamma, del133p53, del133p53 beta, del 133p53gamma, del40p53, del40p53 beta and del40p53 gamma. There is also another isoform of p53 called p53i9 produced due to the alternative splicing of intron 9 and deleted of the last 60 amino acids. But this isoform has never been reported to be expressed at the protein level.

The p53 protein has N-terminal transactivation, central DNA-binding and C-terminal oligomerization domains. N-terminal domain has two sub-domains, TA1 and TA2. The N-terminal deleted isoforms of p53 lack either first (TA1) or both (TA1 and TA2) transactivation domains. We hypothesize that these N-terminally deleted p53 isoforms may suppress p53-dependant transcription of target genes by repressing the activity of normal p53 protein either by inhibiting functional homotetramer formation on p53-response elements or by formation of transcriptionally inactive heterotetramer. Mutant and normal p53 proteins may form transcriptionally inactive heterotetramer because their oligomerization domains as well as DN-binding domains are intact. In the present study, we take three p53 isoforms; full length, del40-p53 and del133-p53. We first cloned them in baculovirus shuttle plasmids as flag-tagged genes and then transpose our genes into the baculoviral genome so that we can transfect insect cells with recombinat baculoviruses for recombinant protein expression and purification.

CHAPTER 2

Review of Literature

2. Review of Literature

p53 gene is a tumor suppressor gene which was discovered in 1979 (Lane *et al.*, 1979). In human, p53 gene is composed of 19.20 Kb, spanning over 11 exons and separated by 10 introns on chromosome 17p13.1 (GenBank Accession Number: NC-000017). The p53 gene was cloned from neoplastic rodent and human cells, and found to have weak oncogenic activity when expressed in rodent cells. In the late 1980,s, however, scientists discovered that they were studying missense mutants of Tp53 gene instead of the wild type gene. But these missense mutants of Tp53 gene proved to be the key to the understanding of the pathobiological activities of the p53. The fact that p53 is regarded as the tumor suppressor comes from the ability of the p53 to suppress the tumorigenic growth of cell lines (Symonds *et al.*, 1994). This was supported by the data where it has been shown that oncogenic human DNA viruses have evolved a mechanism to inactivate p53 functions (e.g., enhanced ubiquitin- dependant proteolysis of p53 by the E6 protein of human papilloma viruses (HPV) type 16 and 18 (Scheffner *et al.*, 1990). Also it has been found that certain missence mutations in p53 lead to oncogenic gain of functions (Sigal *et al.*, 2000). Mice deficient in TP53 are susceptible to spontaneous tumorigenesis (Donehower *et al.*, 1992) and germline TP53 mutations occur in individuals with the cancer prone Li- Fraumeni Syndrome (Malkin *et al.*, 1990; Srivastava *et al.*, 1990).

p53 is multifunctional with varied functions, many of which can be traced to its activity as a transcription factor. It was first shown to be involved in cell cycle regulation and apoptosis, and then in development, differentiation, gene amplification, DNA recombination, chromosomal segregation, cellular senescence and angiogenesis (Harris *et al.*, 1996; Oren *et al.*, 1999; Cho *et al.*, 1994). It is a well established fact that the wild type p53 facilitates DNA repair including nucleotide excision repair and base excision repair (Adimoolam *et al.*, 2003; Offer *et al.*, 1999; Zhou *et al.*, 2001) (Figure 1). It has been shown that chronic constitutive expression of p53 accelerates aging in mice (Tyner *et al.*, 2002). However mice age normally when transgenic p53 gene is under the control of its own promoter and not constitutively expressed (Garcia-Cola *et al.*, 2002). The most extensively studied p53 activation pathways are apoptosis and cell cycle arrest (**Figure 2** and **3**). Angiogenesis and DNA-repair pathways are also being studied thoroughly.

Random errors in DNA replication and environmental mutagens can produce mutations in p53 (Levine *et al.,* 1997) and 90% of mutations in p53 in human tumors are point mutations of six residues that affect its DNA binding domains (Vousden *et al.,* 2002). p53 acts as a cellular stress sensor that is activated in response to DNA damage, hypoxia, nucleotide depletion, aberrant growth signals, and chemotherapeutic drugs (Levine *et al.,* 1997), and then binds to specific target sequences to activate or repress expression of a relatively large number genes.

The p53 protein has a modular organization comprising an N-terminal transactivation domain, a central DNA binding domain and a C-terminal oligomerization domain (**Figure 4**). Transcriptionally active form of p53 is a homotetramer whose assemblage is governed by oligomerization domain. DNA–binding domain is responsible for the recognition and binding of p53 responsive elements in p53-regulated genes, while transactivation domain transactivates gene expression. Proper functioning of the p53 protein requires that all the domains must be intact because any mutation in any critical amino acid leads to the distortion of p53 structure and hence, function is impaired. Mutations in p53 in human cancers generally map in the DNA–binding domain and are mostly missense, but nonsense mutations also occur (Figure 5). Mutations in DNA binding domain either disrupt DNA–protein interactions or distort overall structure of the domain (Cho *et al.,* 1994; Friend *et al.,* 1994). Wild–type, but not tumor derived mutant, p53 binds to a double-stranded DNA consensus binding site containing two or more copies (consecutive or separated by one or two helical turns) of the ten base pair half-site 5'- PuPuPuC(A/T)(T/A)GPyPyPy -3', where Pu and Py represent purines and pyrimidines respectively (Kern *et al.,* 1991; el-Diery *et al.,* 1992; Funk *et al.,* 1992; Cho *et al.,* 1994; Wang *et al.,* 1993; Waterman *et al.,* 1995). Thus p53 binding site is represented by at least two half-sites where each half site is being formed by head-to-head quarter sites. Each p53 subunit in a homotetramer binds to each quarter site resulting in all four DNA quarter sites being occupied by p53 homotetramer. The p53 tetramer is a symmetric dimmer of dimmers in which all four subunits are geometrically equivalent (Clore *et al.,* 1994; Lee *et al.,* 1994; Jeffrey *et al.,* 1995; McLure *et al.,* 1998). Monomers in a dimmer bind to two consecutive quarter sites leading to one dimmer binding to one half site. One dimmer in a tetramer is sufficient to bind to a half site in the

S. NO.	Type of cancer.	Percentage p53 mutation (%).
1.	Lung cancer	70%
2.	Stomach cancer	45%
3.	Breast cancer	20%
4.	Colon cancer	60%
5.	Liver cancer	20%
6.	Prostate cancer	10-30%
7.	Head and neck cancer	60%
8.	Esophagus cancer	40%
9.	Leukemia	10%
10.	Lymphoma	30%
11.	Ovarian cancer	60%
12.	Cancer of Bladder	60%

Table 1. Percentage of p53 mutations in various human cancers. As can be inferred from the table, in human cancers p53 mutation/inactivation is a major cause of the disease. In some cancers although mutation in p53 is not usually found (like in Cervical cancers caused by human papilloma virus), p53 functions are abrogated due to various mechanisms like binding of E6 protein from human papilloma virus to p53 and hence, inactivating it. Hence, p53 mutations/inactivations are main responsible for cancer progression.

Codon (hotspot)	**Breast cancer**	**Colorectal cancer**	**Liver cancer**	**Lung cancer**	**Ovarian cancer**
Most frequent (%)	273 (6.7%) **G to A**	248 (13.8%) ***C to T***	249 (36.4%) ***G to T***	273 (8.9%) ***G to T***	273 (11.2%) ***G to A***
Second most frequent (%)	175 (6.0%) ***G to A***	175 (13.6%) ***G to A***	273 (4.1%) ***C to T***	248 (4.5%) ***G to T***	248 (5.6%) ***C to T***
Third most frequent (%)	248 (6.0%) ***G to A***	273 (9.5%) ***G to A***	166 (3.5%) ***T to A***	179 (4.2%) ***A to G***	282 (5.2%) ***C to T***
Fourth most frequent (%)	245 (3.1%) ***G to A***	245 (7.0%) ***G to A***	157 (1.8%) ***G to T***	245 (4.2%) ***G to T***	245 (4.8%) ***G to A***
Fifth most frequent (%)	249 (2.2%) ***G to T***	282 (5.0%) ***C to T***	159 (1.8%) ***G to C***	157 (3.5%) ***G to T***	175 (4%) 4% ***G to A***
Cumulative %age of five most frequent mutated codon sites and most frequent base pair change out of total gene.	24.0% ***G to A***	48.9% ***G to A***	47.6% ***G to T***	25.3% ***G to T***	30.8% ***G to A***

Table 2. The five most frequent codon sites (called hotspots) for p53 mutations with their most frequent base pair changes in breast, colorectal, liver, lung and ovarian cancers, and the percentage of p53 mutations occurring at these sites for each cancer. The most frequent base pair change at each hotspot codon in these cancers is shown in *italics*. As can be seen from codon numbers, these hotspot mutations occur in DNA- binding domain of p53 protein.

p53 response element but, concurrent interaction of the second dimmer with the second half site in the same response element drastically increases binding affinity (about 50 fold). This cooperative dimmer-dimmer interaction occurs independently of tetramerization and is a primary mechanism responsible for the stabilization of p53 DNA binding (McLure *et al.*, 1998).

2.1 The discovery of p53.

As stated earlier p53 was first identified in 1979 as a cellular protein that bound to the Semian Virus (SV40) large T-antigen and accumulated in the nuclei of cancer cells (Lane D.P. *et al.*, 1979). The discovery in 1979 of p53 protein was the culmination of two types of studies involving a virological approach and a serological approach.

2.1.1 Virological approach: - Studies of SV40-transformed cells showed that a 55 kDa protein was coprecipitated with the large T-antigen. This was due to the in vivo association of the two proteins (Lane *et al.*, 1979). It was also found that 54kDa protein was overexpressed in a wide variety of murine SV40 transformed cells, but also in un-infected embryonic carcinoma cells (Linzer and Levine 1979). The peptide map of this 54kDa protein was identical among different cell lines. It was then postulated that SV40 infection or transformation of mouse cells stimulates the synthesis or stability of a cellular 54kDa protein.

2.1.2 The serological approach:- It has been shown that humoral response of mice to some methylcholanthrene-induced tumor cell lines, such as MethA was directed toward the p53 protein (DeLeo *et al.*,1979). Later it was found that animals bearing several types of tumors elicited an immune response specific for p53 (Kress *et al.*, 1979). Antibodies against human p53 protein was described in 9% of breast cancer patient sera (Crowford *et al.*, 1982).

2.2. p53 as a tumor suppressor

2.2.1 Loss of p53 caused rapid tumor progression and p53 dependant apoptosis is required to suppress this tumor development:-

It has been shown that transgenic mice carrying a fragment of the large T antigen of the SV40 virus develop choroid plexus tumors through the inactivation of the Rb protein

although the large T antigen does not interfere with the p53 function (Symonds et al., 1994). In this system loss of Rb protein function leads to the de-repression of E2F (which is the oncogenic event) resulting in the oncogenic activation and entry into S-phase, which should cause p53-mediated apoptosis and elimination of the tumor cells. By crossing these transgenic mice into a p53 null mice backgrounds, it was observed that loss of p53 caused rapid tumor progression and that p53-dependant apoptosis was required to suppress this tumor development (Symonds *et al.*, 1994).

2.2.2 p53 in Friend murine erythroleukema:-

In these tumors induced by the Friend virus, the p53 gene found in the tumor cells is very often rearranged, leading to an absence of expression or the synthesis of a truncated or mutant protein (Mowat et al., 1985). The mutation often affects one of the conserved blocks of the protein (Munroe et al., 1988). In all cases studied, the second allele is either lost through loss of chromosome, or inactivated by deletion. In this tumor model, functional inactivation of the p53 gene seems to confer a selective growth advantage to erythroid cells during the development of Friend leukemia in vivo.

2.2.3 There are mutations in the various murine p53 cDNA clones:-

The finding that one murine p53 cDNA clone isolated from the F9 cell failed to cooperate with an activated Ha-Ras gene was another clue that the p53 cDNA clones differ from one another in their behavior (Finlay *et al.*, 1988). Examination of all murine p53 cDNA clones available revealed several codon changes which were primarily assumed to be due to polymorphism. However, comparison of these sequence differences with p53 from lower species indicated that some of them occur in highly conserved regions (Soussi *et al.*, 1990 and 1987), a feature which is not linked to polymorphism. Careful re-investigation of all sequences led to the conclusion that the F9 cDNA clone was a wild type, while most of the others used in transfection experiments contain point mutations that activate their transforming properties.

2.2.4 Wild type p53 has antiproliferative properties and does not cooperate with Ha-ras: -

A new set of experiments has shown that cotransfection of a plasmid encoding wild type p53 reduced the transformation potential of plasmids encoding mutant p53 and an activated Ha-ras gene (Eliyahu et al., 1989; Finlay et al., 1989). Furthermore, wild-type

p53 was shown to suppress transformation by a mixture of E1A or myc and an activated Ha-ras gene. These set of experiments showed that wild-type p53 is a suppressor of cell transformation in vitro.

2.2.5 p53 gene is mutated in a wide variety of human cancers: -

The expression of p53 in different human cancers or in tumor cell lines has long been under study by several different investigators. Genetic analyses of colorectal cancers reveal a very high rate of heterozygous loss of the short arm of chromosome 17, which carries the p53 gene (Vogelstein et al., 1988). PCR and sequencing analyses of the remaining p53 allele shows that it often contains a point mutation (Baker et al., 1989). Similar observations have been made in the case of lung cancer (Takahashi *et al.*, 1989). On the basis of these initial observations have come several hundred reports of alterations of the p53 gene in all types of human cancers (**Table 1**). In many cases these mutations are accompanied by a heterozygous loss of the short arm of the chromosome 17. These mutations in p53 gene in various human cancers are mainly transitions and transversions. In p53 gene, codon numbers 175, 248, 249, 273 and 282 are hotspots, which are frequently mutated in human cancers (**Table 2**). Most of the p53 inactivating mutations in human cancers are found in DNA-binding domain that account for more than 90% of them (**Figure 5**).

2.2.6 Germline mutations of the p53 gene are found in Li-Fraumeni patients:

Transgenic mice carrying a mutant p53 gene develop many types of cancers, with a high proportion of sarcomas (Lavigueur *et. al.*, 1989). This observation led to study patients with Li-Fraumeni syndrome. This syndrome represents a familial association of a broad spectrum of cancers including osteosarcomas, breast cancer, soft tissue sarcoma and leukemias, appearing at a very early age. Germline mutations in the p53 gene have been found in several families with this syndrome (Malkin *et al.*, 1990; Srivastava *et al.*, 1990). In all cases there is a strict correlation between transmission of the mutant allele and development of cancer.

2.2.7 Micro-injection of specific monoclonal antibody induces a growth arrest: -

The carboxyl-terminus of p53 has been shown to play an important role in controlling the specific DNA –binding functions. Wild-type p53 is found in a latent form that does not bind to the DNA. The specific DNA binding activity was shown to be activated by

various pathways: phosphorylation (Hupp *et al.*, 1992), small peptides which could mimic the carboxy-terminus of the p53 (Hupp *et al.*, 1995), short single stranded DNA (Jayaraman and Prives 1995), deletion of the last 30 amino acids (Hupp *et al.*, 1992) and the interaction with a cellular protein (Jayaraman *et al.*, 1997; Miller *et al.*, 1997). The p53 contains a basic region in C-terminus that represses DNA binding and hence p53-activity. When monoclonal antibody PAb421, directed to this region was introduced into the mouse cell line NIH 3T3; which was already co-transfected with the wild-type p53 and beta-galactosidase driven by p53 response element-containing promoter, suppression of growth occurred and this effect was shown to be due to the activation of p53 protein as expression of beta-galactosidase increased above basal levels (Hupp *et al.*, 1992 and 1995; Shaw *et al.*, 1996).

2.2.8 Wild type p53 as a tumor suppressor gene and mutant p53 as a dominant oncogene: -

Based on the above findings, it made it possible to define the p53 gene as a tumor suppressor gene. Yet unlike the Rb gene, which is the archetype of tumor suppressor genes, the p53 gene has some original features. In particular, more than 95% alterations in the p53 gene are point mutations that produce a mutant protein, which in all cases has lost its trans-activation activity. Nevertheless, the synthesis of these mutant proteins are harmful for the cell. In particular, it has been shown that some p53 mutants (depending on the site of mutation) exhibit a transdominant phenotype and are able to associate with wild type p53 to induce the formation of an inactive hetero-oligomer (Milner and Medcalf 1991). Moreover, co-transfection of mutant p53 with an activated ras gene showed that some p53 mutants have high, dominat oncogenic activity (Halevy et al., 1990). These observations led to the proposal that several classes of p53 mutants exist, according to the site of mutation and its phenotype which are as follows (Michalovitz et al., 1990): 1) null mutations with totally inactive p53 that do not directly intervene in transformation; 2) dominant negative mutations with a totally inactive p53 that is able to interfere with wild-type p53 protein expressed from the wild-type allele, and 3) positive dominant mutations where the normal functioning of TP53 protein is altered but in this case the mutant p53 acquires an oncogenic activity that is directly involved in transformation.

2.2.9 p53 and development: -

In human cancers, mutations in the p53 tumor suppressor gene are the most frequently observed genetic lesions. A null mutation was introduced into the p53 gene by homologous recombination in murine embryonic stem cells to investigate the role of the p53 gene in mammalian development and tumorigenesis. Mice homozygous for null allele appear normal but are prone to the spontaneous development of a variety of neoplasms by six months of age (Donehower *et al.*, 1992). Mice with disrupted germline p53 alleles have been shown to have enhanced susceptibility to spontaneous tumors of various types (Donehower *et al.*, 1995) but the viability of these animals indicates that p53 function is not essential for embryonic development. p53 tumor suppressor gene which facilitates DNA repair is embryo-protective . It has been shown that pregnant heterozygous p53-deficient mice when treated with environmental teratogen, benzo[a] pyrene exhibit between 2-4 fold higher embryo-toxicity than normal p53 controls (Nicol *et al.*, 1995). This gives first direct evidence that p53 gene is teratogen suppressor which protects the embryo from the DNA –damaging chemicals and developmental oxidative stresses.

2.3 Pathways toward p53 inactivation

Inactivation of the p53 gene is essentially due to missense and nonsense mutations or insertions/deletions of several nucleotides (**Figure 5**), which lead to either expression of a mutant protein (90% of cases) or absence of protein (10% of cases). In many cases, these mutations are associated with loss of the wild type allele of the p53 gene located on the short arm of the chromosome 17 (Srivastava *et al.*, 1990), which is why the p53 gene is said to behave like a classical tumor suppressor gene with a mutation in one allele and loss of heterozygosity (LOH) of second allele (Knudson's two hit model). This is justified by the observation that cancers with a high frequency of p53 gene mutations show high frequency of LOH of the short arm of chromosome 17.

Besides mutations in the p53 gene itself, other mechanisms also lead to the p53 functional inactivation as discussed as follows:

2.3.1 mdm2 alterations: -

The Mdm2 protein regulates the stability of the p53 protein by ubiquitination and transport towards the proteosome as shown in **Figure 6** (Haupt *et al.*, 1997). Mdm2 is an ubiquitin ligase. Abnormal accumulation of mdm2 protein is observed in many tumors, especially sarcomas. This accumulation can be due to amplification of the mdm2 gene, enhanced transcription of the gene or enhanced translation of mdm2 mRNA. Mdm2 protein is upregulated by p53 itself. On DNA damage stress, p53 is activated modifications and it binds to target genes one of which is MDM2 gene. Initially p53 protein is stabilized and accumulated through post-translational modifications after stress signal. From the period of p53 stabilization and accumulation till p53-dependant accumulation of Mdm2, p53 activates expression of its target genes and performs its tumor suppression functions. But when Mdm2 protein is accumulated, it starts targeting p53 to ubiquitin-proteasomal degradation. Hence, negative feedback loop is generated to control the activity of p53. Mdm2 protein has p53-binding domain that assists in p53-binding and subsequent proteasomal degradation. But when p53 is phosphorylated, Mdm2 protein cannot bind and hence p53 levels increase during stress (which phosphorylate p53) to performs its effector functions.

2.3.2 HPV infection (HPV): -

Human papilloma virus infection in some cancers leads to the p53 protein degradation. It has been shown to cause human cervical cancers and it has been also suggested that it may play a role in cancer of anus, vulva, vagina, penis and some cancers of oropharynx. In case of e.g., cervical cancers, E6 viral protein expressed by HPV specifically binds to the p53 protein and induces its degradation (Rapp *et al.*, 1998). This observation explains the rarity of p53 gene mutations in cervical cancers.

2.3.3 Cytoplasmic localization: -

In inflammatory breast cancers or neuroblastomas, molecular and immunohistochemical analyses demonstrate accumulation of wild type p53 protein in the cytoplasm of tumor cell, leading to the functional inactivation of the p53 protein (Alexander *et al.*, 1999). A protein named parc was isolated which sequesters the p53 protein in the cytoplasm of cells in the absence of any mutations in the p53 gene (Nikolaev *et al.*, 2003 a and b). An abnormal accumulation of this protein is observed in the neuroblastoma cells and could

therefore account for the functional inactivation of the p53 in this cancer. In such cases cytoplasmically sequestered p53 protein is mdm2-resistant.

2.3.4 hCHK2 mutations: -

hCHK2 is a kinase activated by ATM following irradiation. It is the human homologue of yeast Cds1 and Rad53 G2 checkpoint kinases and prevents cellular entry into mitosis after DNA damage. Heterozygous germline mutations in chk2 have been identified in some patients with Li-Fraumeni Syndrome (Bell DN *et al.*, 1999). Many studies have shown that hchk2 is necessary for phosphorylation and stabilization of p53 after genotoxic lesions. These studies are strongly supported with the description of hchk2 germline mutations in families with Li-Fraumeni syndrome not presenting any p53 mutations (Bell *et al.*, 1999).

2.4 The p53 Pathway

The p53 protein and its signal transduction pathway are composed of hundreds of genes and their products that respond to a wide variety of stress signals. These responses to stress include apoptosis, cellular senescence or cell cycle arrest. Also p53 regulated genes produce products that communicate these stress signals to the adjacent cells, prevent and repair damaged DNA and create feed back loops that enhance or attenuate p53 activity and communicate with other signal transduction pathways.

The p53 pathway has been conveniently divided up into five parts (Levine *et al.*, 2005): -

a). The input signals that trigger or induce the network into a functional state.

b). The upstream mediators that detect and interpret those signals that initiate the functional pathway and relay the inputs to the p53 protein or molecules that most immediately regulate the concentration and activity of the p53 protein.

c). The core set of proteins, including the p53 protein itself, which regulates p53 activity and function.

d). The downstream events which are composed of a set of genes and their proteins that are regulated by the p53 protein, most commonly by transcriptional activation but in some cases by protein-protein interactions.

e). The cellular outputs of these downstream events which include cell cycle arrest, cellular senescence or apoptosis and often result in extensive communication with other signal transduction pathways in the cell.

(2.4.1) The input signals that Trigger or induce the p53 pathway: -

The p53 protein and its signal transduction pathway are composed of a set of genes and their protein products that are designed to respond to a wide variety of intrinsic and extrinsic stress signals. These stress signals all impact upon the cellular homeostatic mechanisms that monitor and control the fidelity of DNA replication, chromosome segregation and cell division (Vogelstein *et al.,* 2000). Among the stresses that activate p53 protein is damage to the integrity of DNA in a cell. There are many physical and chemical causes to the DNA damage including gamma or UV irradiation, alkylation of bases, DNA cross-linking, depurination of DNA, alteration of the deoxyribose sugar moiety, reaction with oxidative free radicals and more. Each of these types of DNA damage is different and each is detected by a different set of proteins and then repaired by different enzymes or activities that reverse the damage in diverse ways. There are multiple DNA damage detection and repair systems in the cell but every type of DNA damage is reported to the p53 protein and its pathway (Giaccia *et al.,* 1998; Gudkov *et al.,* 2003 and Oren *et al.,* 2003). The duplication system of DNA often makes mistakes e.g., mismatch errors that are in the main edited and repaired but when lesions remain, the p53 protein too can detect them. In all of these cases the p53 pathway functions to respond to errors and eliminates those cells that make such mistakes. As cells divide, their telomeres become shorter and reach a critical length. This can result in the abnormal chromosomal translocations or abnormal recombination events. But the presence of these shorter telomeres in a cell is reported to the p53 protein resulting in the p53 response that in turn limits the abnormal chromosomal translocations. In addition to the DNA damage, several cellular deprivations activate the p53 response such as hypoxia, glucose starvation or the depletion of ribonucleoside triphosphate pool in the cell. When ribosome biogenesis is stopped or falls below a critical level the p53 protein is alerted and activated. Agents that damage the cell spindle and result in the faulty chromosome segregation activate p53. Both heat and cold shock as well as altered and denatured protein response in cells communicate with p53 by activating it. Nitric oxide, which is

associated with infections and inflammations activate the p53 protein and its response (Wang *et al.,*). All of the above processes will result in the loss of the fidelity of cellular duplication processes and as such their communication with the p53 pathway acts as a checkpoint to prevent abnormal clones of cells from arising. The overall process is very complex and regulated (**Figures 1** and **2**).

The mutational activation of some oncogenes also result in the sensitization of the p53 response and can often change the downstream output of the p53 pathway from cell cycle arrest to apoptosis (Lowe SW *et al.,* 1999). When mutational inactivation in some tumor suppressor genes such as retinoblastoma (Rb) protein (which activates the E2F-1), and adenomatous polyposis coli (APC) (which activates the beta-catinin-TCF4 transcription factor) or mutational activation of some oncogenes such as ras and myc occurs, p53 protein in the cell gets activated and p53 response occurs. Actually the activities of the transcription factors that result from mutations in these genes can transcribe the ARF gene and the ARF protein then binds to the MDM-2 protein and inhibits its activity as ubiquitin ligase. Here, a mutation or alteration in a protein that inappropriately signals a cell for the entry into the cell cycle is detected by the p53 check point and the cell is usually killed. This phenotype is mediated by the positive feed back loop in the p53 pathway that connects the p53 node to the other signal transduction pathways that regulate cell division (Lowe and Sherr, 2003).

The activation of the p53 protein in response to stresses is mediated and regulated by protein kinases, histone acetyle transferases, methylases, ubiquitin and sumo ligases. As the p53 protein is activated by these modifications, it can also be inactivated by phosphatases, histone deacetylases, ubiquitinases or even inhibitors of ubiquitin ligases. In addition, the activated p53 protein appears to interact with a number of proteins that are important for its transcriptional activity such as PML bodies (promyelocytic leukemia bodies) (Louria- Hayon *et al.,* 2003; Zu *et al.,* 2003) and the Werner helicase (Blander *et al.,* 2000).

(2.4.2) The upstream mediators of the p53 response: -

In response to input stress signals the p53 protein is activated. Activation can be define as an increase in the concentration of the p53 protein and an increased activity of p53 protein for the transcription of a set of genes that have a p53 DNA response element (Hoh

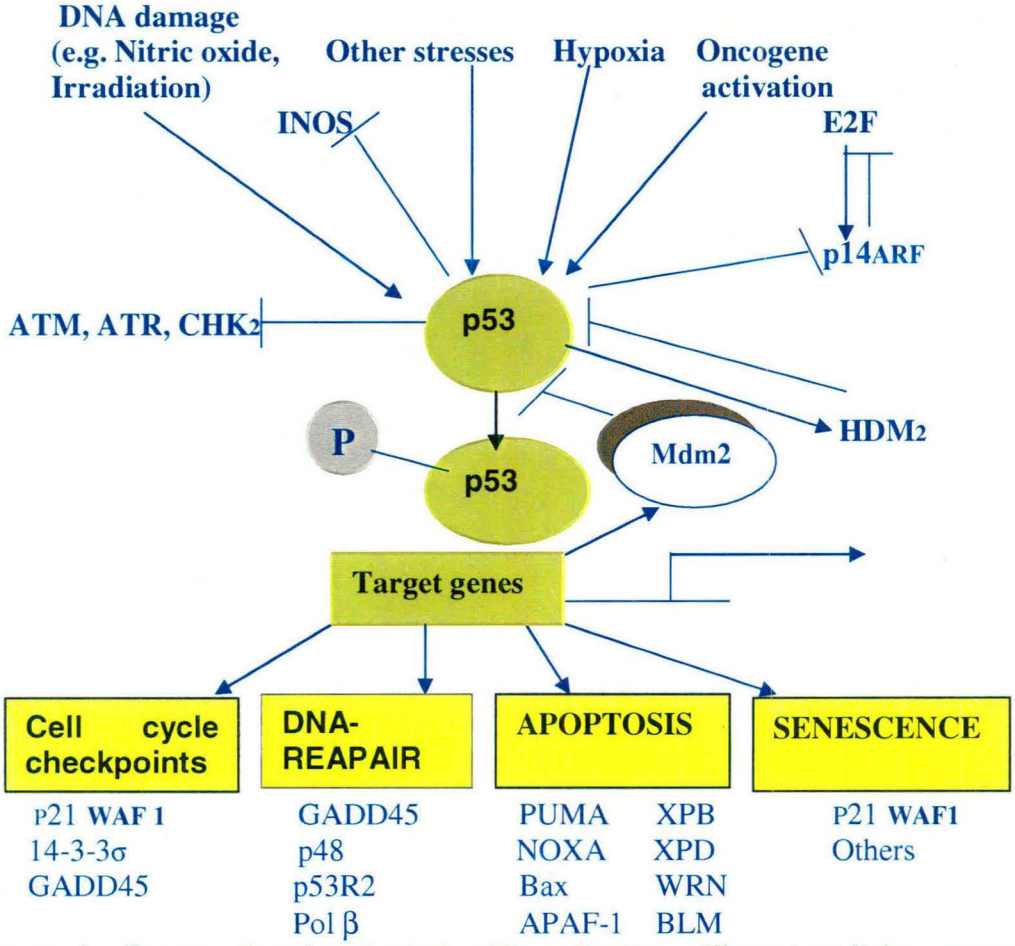

Figure 1. Stress signal-activated p53 activates different cellular processes through specific gene activations. Examples of upstream sensors of stress [e.g., the ATM (ataxia telangiectasia mutated) or ATR (ATM and Rad 3-related) kinase cascades] and negative feedback loops [e.g., inducible nitric oxide synthase (iNOS) upstream and HDM2 (human double minute 2) downstream of p53] are shown. Following activation, by post-translational modifications Including phosphorylation, methylation, sumolation and acetylation, p53 plays a key role in the protection of cells from further stress, through the activation of genes that play a role in cell-cycle check-points, apoptosis, DNA repair or cellular senescence. Examples of proteins that are encoded by these effector genes are listed. Abbreviation: APAF1, apoptotic protease -activating factor1; APE1, APEX nuclease (multifunctional DNA repair) 1; Bax, Bcl-2- associated X protein; BLM, bloom syndrome; CHK2, checkpoint 2 protein; GADD45, growth arrest and DNA damage inducible, alpha; p53R2, p53-inducible reductase small subunit homolog; polβ, DNA polymerase beta; PUMA, p53 up-regulated modulator of apoptosis; WRN, Werner syndrome; XPB, xeroderma pigmentosum group B; XPD, xeroderma pigmentosum group D.

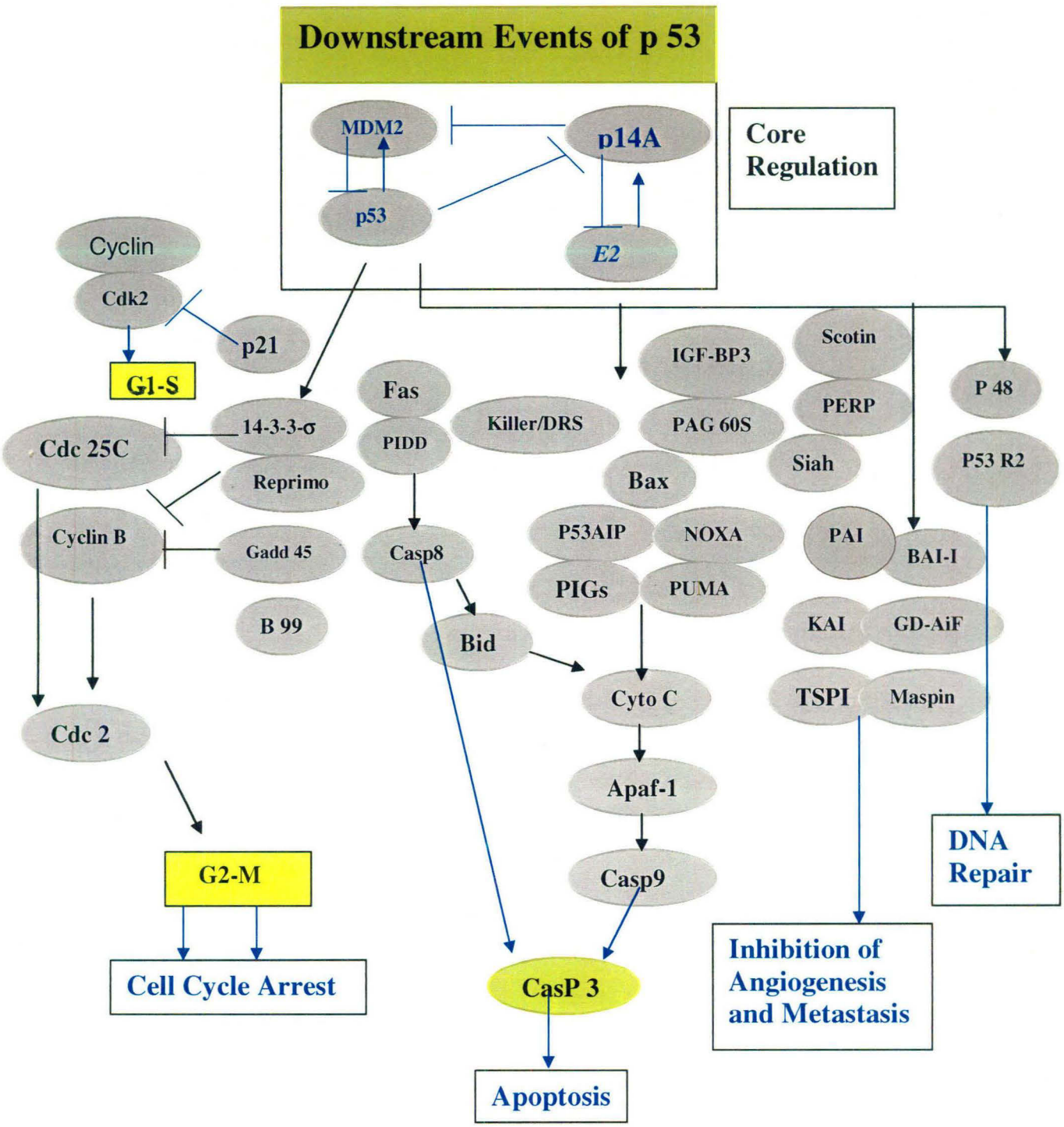

Figure 2. Downstream targets of the p53 transcription factor mediate its different biological outcomes. Different sets of p53-regulated genes control different functions to lead to either cell cycle arrest or apoptosis or DNA repair or inhibition of angiogenesis and metastasis.

et al., 2002) and are transcriptionally regulated by the p53 protein. The levels of the p53 protein are predominantly regulated by its proteolytic turn over. The p53 protein has a short half-life of 6-20 minutes in several cell types and the ubiquitin ligase that confers this short half-life is MDM2 protein. Recently two other ubiquitin ligases have been shown to act upon the p53 protein, CPO-1 and PIRH-2 (Leng *et al.*, 2003; Dornan *et al.*, 2004). These ubiquitin ligases target TP53 protein for proteasomal degradation (**Figure 6**). The main explanation for this redundancy can be had from the fact that after some DNA damage (gamma irradiation) the MDM-2 protein is auto-poly-ubiquitinated resulting in its degradation and an associated increase in the p53 levels and activity (Stommel *et al.*, 2004). Because of this fact p53 protein has a longer half-life after these DNA damages, but this observation is not seen in all DNA damages and stress signals. Given a large number of stress signals as an input to the p53 pathway, almost nothing is known about how these diverse inputs are communicated to the p53 protein. It is known that p53 and MDM-2 proteins are extensively modified after stress signals (**Figure 7**). The p53 protein is phosphorylated on a large number serine and threonine residues by many different protein kinases. It is acetylated by histone acetyl-transferases, methylated by methylases, ubiquitinated, summolated and nedylated (at epsilon amino groups of lysine at the carboxy terminus of the p53 protein) (Appela *et al.*, 200; Xirodimas *et al.*, 2004; Brooks *et al.*, 2003 and Xu *et al.*, 2003). In some cases it is clear that this is part of the process that mediates the response from DNA damage to the p53 protein. For example, gamma irradiation activates ATM kinase that after activation phosphorylates MDM-2 and p53 proteins (probably through chk kinases) as well as participates in the DNA repairing processes. Inspite of these clear observations, mutations in many p53 serine and threonine residues that block the phosphorylations at these residues still result in fairly normal p53 activation and function. Similarly, the use of Nutlin (a drug that blocks the p53-MDM-2 binding) activates p53 normally with no phosphorylation events (Vassilev *et al.*, 2004). Changes in the lysine residues in p53 protein, which are normally acetylated or modified by various peptides, to arginine residues that cannot be modified resulted in normal p53 responses in mice with these mutant proteins. These types of experiments indicate that the protein modifications of the p53 protein after a stress signal are not essential for the activation of the p53 protein (or are heavily backed up by the

other unknown processes not eliminated in these experiments). These phenomena question; what are the functions of the modifications of p53 and MDM-2 proteins after exposure to stress signals? Most believe that there is a functional role of these modifications. First of all the extent and nature of these protein modifications of p53 differ with different types of stress signals, thus forming the chemical codes that will inform p53 protein and the cell about the type of stress occurring. Second the p53 regulated transcriptional output is different after different stress signal, and these protein modifications could well dictate which p53-regulated genes are transcribed by the cell based on the specific protein modifications of the p53 protein.

(2.4.3) The core control of p53: -

Once activated, the p53 protein transcribes a number of genes. Among these is MDM-2 gene (and the COP-1 and PIRH-2 genes), which is the major negative regulator of p53 in the cell (Harris *et al.,* 2005). The transcriptional regulation of MDM-2 is a slow step (hours) but once the MDM-2 protein is produced it binds to the p53 (inhibiting its activity as a transcription factor) and ubiquitinates it enhancing its proteolytic degradation. This occurs as a rapid step. This auto regulatory loop produces an MDM-2-p53 oscillator composed of changed protein levels of p53 and MDM-2 out of phase with one another, in the cell (Lev Bar *et al.,* 2000 and Lahav *et al.,* 2004). When p53 levels in the cell increase it increases MDM-2 protein levels which in turn lower p53 levels, resulting in less MDM-2 levels. These oscillations are variable from cell to cell and they can last a long time and the number of such oscillations is roughly proportional to the input dose of stress signals (e.g., radiation) (Lahav *et al.,* 2004). COP-1 and PIRH-2 may act similarly but much focus has been put on MDM-2 study (**Figure 6**). Gene knockout of MDM-2 in mice is lethal early in development, just after implantation (Montes de Oca Luna *et al.,* 1995). This could be due to the hypoxia response of the p53 protein in the absence of MDM-2 protein, so that p53 activity is uncontrolled. Double knockout mice, with no MDM-2 and p53 genes, are viable proving that MDM-2 acts to block p53 mediated cell death early in development. This also demonstrated that PIRH-2 and COP-1 do not back up MDM-2, at least early in development.

The gene products that regulate MDM-2 and p53 levels and activities will need to be better characterized. The MDM-X (or MDM-4) gene was identified because it is related

to the MDM-2 gene in DNA and protein sequences. A knockout of MDM-X gene in mice is lethal and MDM-2 and p53 double knockout mice are viable (Parant *et al.*, 2001). Thus MDM-X is as important as MDM-2 in regulating p53 in vivo. Hence MDM-X can negatively regulate p53 directly and positively regulates MDM-2. After a stress signal, poly-ubiquitination of MDM-2 results in the degradation of MDM-2 and MDM-X. The activities that modify MDM-2 and MDM-X (cyclin G-PP2A, ARF and its activators, and HAUSP) act only on MDM-2 and remain to be explored in more detail. High levels of p53 protein appear to repress the transcription of ARF gene and the p53 knock-out mice (no p53 protein) has much higher levels of ARF mRNA and protein than wild type mice.

p53 and MDM-2 proteins shuttle between the nucleus and cytoplasm which undoubtedly change their activity and regulation (Tao *et al.*, 1999 and Boyd *et al.*, 2000). Just what factors control this cellular localization and therefore can impact upon p53 function remain to be explored. The AKT-1 kinase can phosphorylate MDM-2 protein increasing its activity and sending it into the nucleus of the cell (Mayo *et al.*, 2001 and Zhou *et al.*, 2001). Cyclin-G, a p53 regulated gene product, combines with the catalytic subunit of the PP2A phosphatase to remove a phosphate residue from the MDM-2 protein and increases its activity (Okamoto *et al.*, 2002). These two modifiers of the of the MDM-2 protein form feed back loops that connect the core p53 activity to the other signal transduction pathways as well as regulate the core functions of the network. Indeed there are at least ten negative or positive feedback loops that start with a p53 regulated gene product and result in increasing or decreasing the activity of p53 (Harris *et al.*, 2005). These feedback loops connect the p53 pathway to other signal transduction pathways. When a positive feedback loop occurs it results in the apoptosis and cell death but when negative one occurs it attenuates the p53 pathway.

One important thing to point out here is that there are two distinct transcriptional start sites for the p53 transcription and several alternative-splicing patterns of this mRNA. Altogether there are nine isoforms of the p53 protein that have been detected. So it is important to take the role of other p53 isoforms into account if p53 signaling is to be studied perfectly. Trans- activation domain truncated isoforms may e.g., suppress the activity of full-length p53 by interfering with the tetramer formation or activate or repress the p53 regulated genes. So the situation is complex. Recently these isoforms has been

shown to have a distinct and critical function in the development of the gut of the zebra fish (Chen *et al.*, 2005).

(2.4.4) The downstream events in the p53 pathway: -

Once the p53 protein is activated it gains the ability to bind to the p53 response elements in the p53-regulated genes and effects vital process like cell cycle arrest, apoptosis, DNA-repair, inhibition of angiogenesis and metastasis etc (Figure 2). The consensus DNA binding sequence is PuPuPuCWWGPyPyPy, where Pu is purine, C is cytosine, W is A or T, G is guanine and Py is pyrimidine. A p53 response element contains two of these 10 bp sequence, separated by a spacer of 0-20 bp, and the sequences are often located 5' to the gene or in the first or second intron of the gene regulated by p53 (el Deiry *et al.*, 1992). It is very challenging to identify all the novel p53 responsive sequences in the genome because degenerate sequences also function in a p53–dependant fashion. An algorithm has been described (Hoh *et al.*, 2002) that has successfully described novel p53 responsive genes (Feng *et al.*, a & b 2005). Different types of stress signals as inputs result in different genes being transcribed under p53 control (Zhao *et al.*, 2005). Also stress signals received by different cells or tissue types produce different transcriptional programmes regulated by the p53 protein. This has been implicated mainly due to the p53 protein modifications, protein–protein interactions as well as differences in DNA sequences of response elements. There appear to be some p53 regulated genes that are transcribed in response to many types of different stress signals and in all tissues responding to stress (p21, cyclin G, MDM-2, GADD45) and others are either stress specific or tissue specific (PTEN, TSC-2). What regulates these differences is not fully clear. The functions of the p53 response genes fall into several categories. A set of genes is clearly involved in cell cycle arrest (p21, 14-3-3 sigma, GADD45). A second set of p53-regulated genes is regulated in case of apoptotic pathway. These can be divided into the intrinsic and extrinsic apoptotic pathways. In the extrinsic pathway p53 regulates Fas production (a secreted protein), as well as killer/DR5, the trail receptor and a membrane protein. These proteins along with PIDD activate caspase 8 and BID to release cytochrome c that acts with APAF-1 (a p53 regulated gene) to activate caspase 9 and then they together lead to apoptosis. The intrinsic pathway is populated with many p53- regulated genes of which bax, noxa and puma may work in different cell types to

promote cytochrome c release. Several other p53-regulated genes have been implicated in the enhancement of apoptosis (Perp, Scotin, some PIGS, p53 AIP) but their mechanism of action remains to be elucidated. More recently some reports came which showed that p53 protein itself can move out of the nucleus and act upon the mitochondria or proteins in the mitochondria (BCL-2, BCL-XL) to promote cytochrome c release and apoptosis (Moll *et al.*, 2005; Chipuk *et al.*, 2004). The third out put of the p53 response is the senescence of cells that is as important as apoptosis or cell cycle arrest in mediating tumor suppression. Very little is known how p53 mediates senescence. As normal cells grow and divide in culture, with age their telomeres shorten and p53 levels increase. Mice with an overactive p53 protein, where the threshold for stress response is lowered, die prematurely and have compromised stem cell capabilities (senescence of stem cells) (Donehower *et al.*, 2002). These mice are more resistant to developing cancers initiated with carcinogens. These mice also die at younger age than wild-type mice with a normal p53 protein.

While cell cycle arrest, apoptosis and senescence are usually thought as the major out puts of the p53 pathway, other p53 responsive genes are beginning to define additional functions of the p53 pathway. The p48 protein and p53 R2 subunit of ribonucleotide reductase are p53 responsive genes that aid in DNA repair. The sestrins are a set of p53-regulated genes that counter the presence of reactive oxygen species in the cell (Budanov *et al.*, 2004). A second p53 regulated function is to communicate with cells in its environment that a cell has DNA damage or sense a stress signal. A number of p53 regulated or responsive genes produce secreted proteins. These proteins fall into several functional categories. The IGF-BP3 binds the IGF-1 hormone and prevents it from activating a growth response signal transduction pathway (Harris *et al.*, 2005; Buckbinder *et al.*, 1995). In this case p53 is negatively regulating cell growth, mitogen signaling and preventing cell division in adjacent cells after a stress signal. The p53 regulated and secreted gene products; PAI and maspin are protease inhibitors that result in the alterations to the extracellular matrix and cell surface (Kunz *et al.*, 1995; Zou *et al.*, 2000). Similarly the p53-regulated thrombospondin gene produces a secreted protein that alters the cellular matrix and is anti-angiogenic (Dameron *et al.*, 1994). Secreted proteins permit communication between cells. Recently the p53 regulated TSAP-6 gene was

shown to enhance the rate of exosome production from cells undergoing a p53 response to cells (Amzallag *et al.,* 2004). Exosomes can also communicate with other cells both in immune system and cells in its own vicinity. Finally a large number of p53 responsive genes (p21, WIP-1, SIAH-1, PTEN, TSC-2, IGF-BP-3, cyclin G, p73 delta N, mdm-2, COP-1, and PIRH-2) initiate positive and negative feedback loops with the p53 protein and the core gene products of the pathway (Harris *et al.,* 2005; Grob *et al.,* 2001). This results in the integration of a stress signal with other pathways in the cell that regulate cell growth, division or cell death.

(2.4.5) The cellular out puts of the downstream events: -

Broadly there are six different downstream outputs of the p53 pathway that fall into two distinct groups, primary and secondary responses. The three primary responses to stress input signal by the p53 pathway are; either cell cycle arrest, cellular senescence or apoptosis. The cell cycle arrest may be reversible or in other cases irreversible (which might be related to senescence). Cell cycle arrest by p53 response to a stress signal is not fully known. While p21 clearly plays some role in a G-1 arrest, it is not the whole event as has been shown by knockout experiments (Deng *et al.,* 1995; Brugarolas *et al.,* 1995). p53 blocks the re-initiation of a second S-phase in cells that can not enter cytokinesis because of treatments with spindle poisons (Vaziri *et al.,* 2003). Thus p53 prevents karyotypic instability in cells, which is a major functional output of the p53 pathway. There is very good evidence that the p53 protein and its functions regulate or monitor the number of centrosomes produced at each cell cycle. The p53 knockout mouse produces cells with abnormal number of centrosomes (Wang *et al.,* 1998). The way in which the p53 pathway brings about cellular senescence is completely unknown. The many p53-regulated genes play a role in promoting apoptosis. The transcriptional programme that initiates apoptosis triggers both the intrinsic and the extrinsic apoptotic pathways. The proposed p53 transcription independent pathway to apoptosis needs additional evidence. After stress signal input, p53 activates a pathway that ultimately leads to the release of cytochrome c from the mitochondria which with the help of Apaf-1 activates caspase 9 and caspase 3 to induce and promote apoptosis (**Figures 2** and **3**). Large number of p53-target genes have been proposed to mediate p53-dependent apoptosis (**Table 3**).

p53-Target gene	Function
ALDH4	Enzyme-proline degradation
APAF1	Activates procaspase 9
BAX	Pro-apoptotic BCL2 family member
BID	Pro-apoptotic BCL2 family member (BH3-only)
DcR1, DcR2	Decoy receptors for TRAIL
DR4	Death receptor for TRAIL
DR5/KILLER	Death receptor for TRAIL
FAS/APO-1	Death receptor
FDXR	Steroid biosynthesis
GML	GP1 anchored membrane protein
Hi95	Sestrin gene family/redox enzyme
IGF-BP3	IGF inhibitor, antimitogenic
IKIP	Unknown
mtCLIC	Chloride channel protein
NOXA	Pro-apoptotic BCL2 family member (BH3-only)
P2XM	Putative P2X family member
P53AIP1	Dissipates mitochondrial transmembrane potential
P53DINP1	Kinase co-factor for p53 Ser46 phosphorylation
PA26	Sestrin gene family/redox enzyme
PERP	Putative PMP-22/gas3 family member
PIDD	Death domain protein/caspase 2 activation
PIG3	Redox regulation
PIG8/ei24	Redox regulation
PRG3/AMID	Homology to AIF

p53-Target gene	Function
PTEN	Tumour suppressor/phosphatase
PUMA	Pro-apoptotic BCL2 family member (BH3-only)
SCN3B	Unknown
SCOTIN	Unknown
SIVA	Death domain protein
TP53TG3	Unknown
TRAF4	Adaptor protein for signal transduction
WIP1	Serine/threonine kinase

Adapted from Michalak et al., BBRC 331;(2005):786-798.

TABLE 3. p53 target genes proposed to mediate p53-dependent apoptosis.
Table shows the various p53-target genes proposed to play essential roles in p53-mediated apoptosis. Large numbers of these protein factors have been assigned specific roles, others are thought to play important roles and some factors have not been assigned any specific roles.

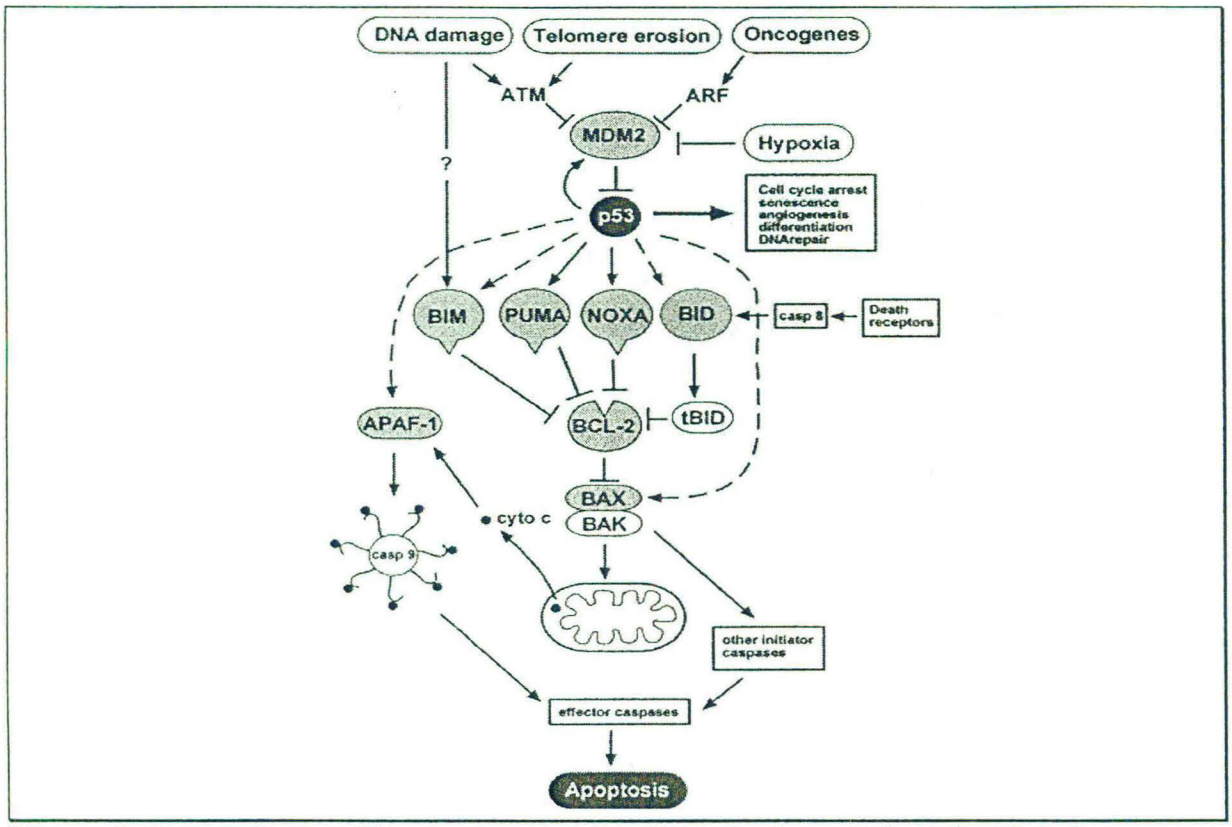

Figure 3. Apoptosis signalling in response to p53 activation. Activated p53 induces expression of a number of downstream target genes implicated in apoptosis, including members of the BCL-2 protein family. Pro-apoptotic BIM, PUMA, and NOXA can bind to the anti-apoptotic BCL-2 family members and thereby block their survival function. BAX and BAK are required for apoptosis signalling, but how BH3-only proteins induce their activity is presently not clear. It has been reported that BH3-only proteins can directly bind to BAX/BAK but this has not yet been demonstrated in cells expressing endogenous levels of these proteins and no measurements for affinity of BIM-BAX/BAK or PUMA-BAX/BAK interactions have been reported to date. BID expression has been reported to be increased by p53 and, following cleavage by caspase-8, tBID can promote apoptosis by translocating to mitochondria where it can bind to BCL-2-like proteins. Activation of BAX/BAK causes mitochondrial release of cytochrome *c*, which then causes APAF-1-mediated activation of caspase-9, which in turn leads to activation of the effector caspases, caspase-3, -6, and -7. Since loss of BH3-only proteins, combined loss of BAX/BAK or BCL-2 over-expression can promote survival of cells that are not protected from apoptosis by loss of APAF-1 or caspase-9, the BCL-2 protein family must regulate apoptosis also by a mechanism that is independent of the cytochrome *c*/APAF-1/caspase-9 'apoptosome.' Dashed lines indicate p53 target genes whose importance for p53-dependent apoptosis is debated.

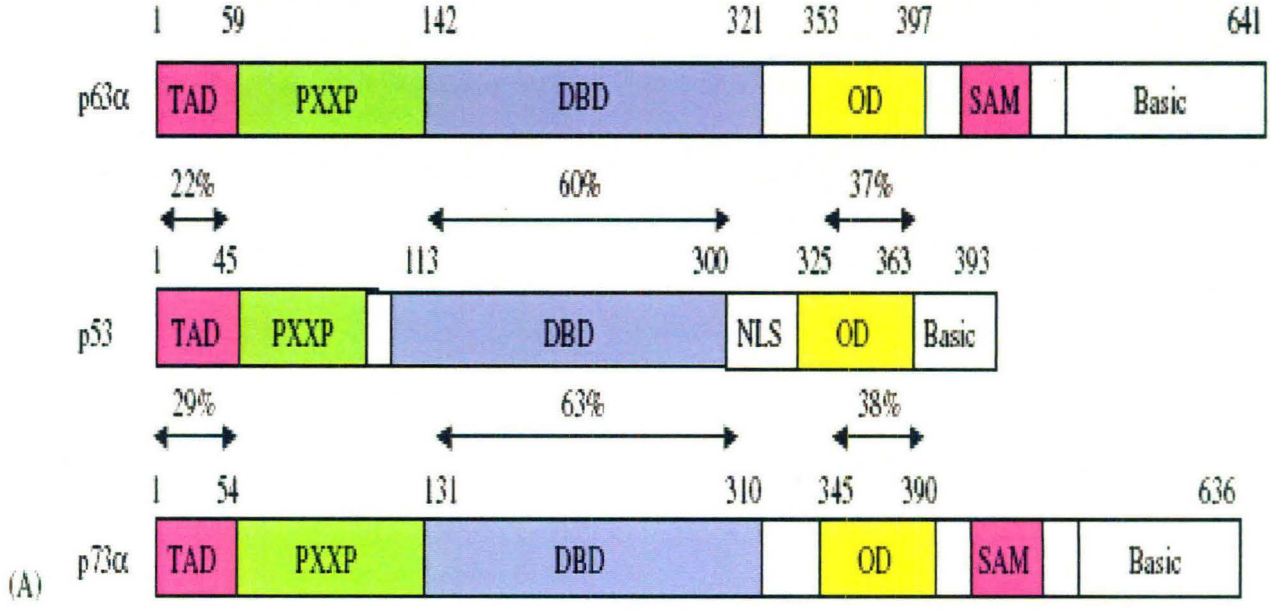

FIGURE 4. Comparative gene structures and functional organization of the p53 family members. The p53 family members have a modular organization of a typical transcription factor. For each gene, the transactivation (TAD), proline-rich (PXXP), DNA binding (DBD), oligomerization (OD), sterile a motif (SAM), and post SAM basic domains are represented. The N-terminal transactivation domain is responsible for transcription activation; Core DNA-binding domain mediates DNA-binding to p53-responsive elements and C-terminal oligomerization controls tetramerization. All the three domains must be intact to effect transcription on promoters by p53, p63 and p73 tetramers. The three members share a very good sequence homology in all the three domains as shown in figure.

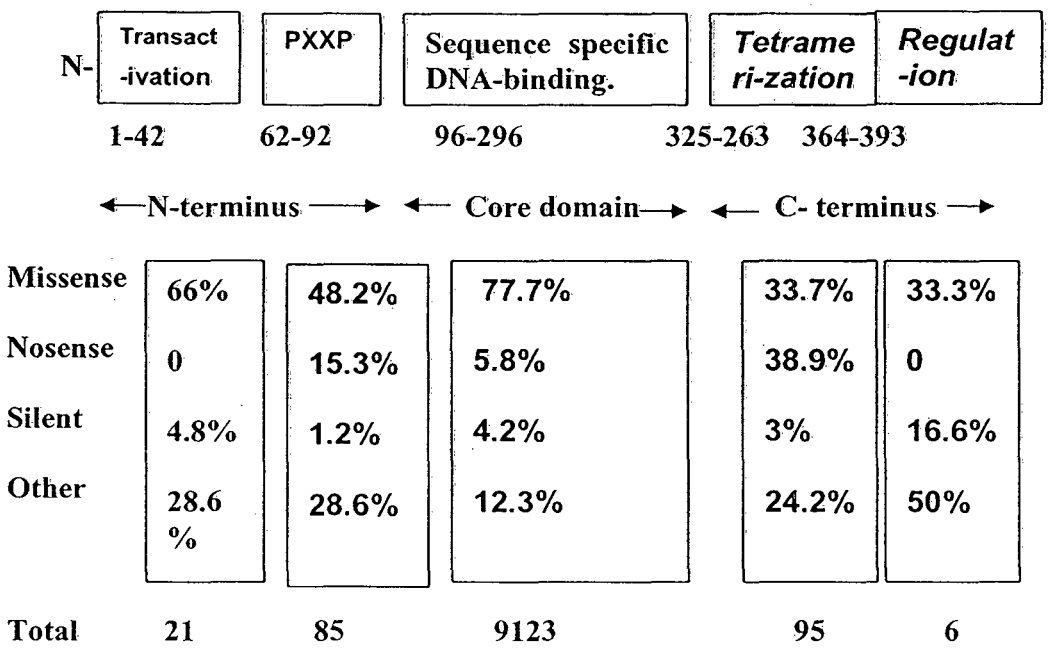

Figure 5. Organization of the human p53 gene and frequency of different mutations in its coding regions. TP53 is a 393 amino acids long protein with a typical structure of a transcription factor. Frequency of mutation in each region is shown as percent. It can be inferred from the values that DNA-binding domain harbors 90% of them alone. The results are based on the 10,000 mutations from the IARC database.

The secondary responses of the p53 pathway come from p53 regulated gene products that 1. either prevent DNA damage (sestrins) or aid in DNA repair, 2. mediate communication between the cells under stress and their neighbors, the extra cellular matrix shared by these cells or even other more distant cells in the body, and 3. create intracellular or extracellular p53 feedback loops that modulate p53 activity and the pathway and simultaneously communicate with the other pathways in the cell (Harris *et al.,* 2005). The soluble secreted p53 responsive proteins can change the extra-cellular matrix (maspin, PAI-1), block hormonal signaling (IGF-BP3) to another pathway, and alter angiogenesis in the vicinity of the alarmed cells (thrombospondin). In addition, p53 activation can enhance the rate of exosome production by the damaged cells by inducing the p53 regulated TSAP-6 gene (Amzallag *et al.,* 2004; Passer *et al.,* 2003). Exosomes can communicate with other cells by cell fusion and with the T-cells by presenting antigens to the immune system. The negative and positive feedback loops that are initiated by the p53 response to input stress signal serve two important functions, a). to modulate up or down the p53 activity; and b). to communicate with other signal transduction pathways in the cell. For example, after a commitment to apoptosis, a PTEN –mediated feedback loop modulates up p53 activity (a positive feedback) and shuts down the IGF- 1 and mTOR pathways (growth and cell division pathways). This activates other processes like autophagy that provides nutrients for adjacent cells and exosome production. Both autophagy and exosome production are functions of the lysosomal compartment and pathway. So p53 pathway seems to communicate with other pathways too.

2.5 p53 and genomic stability

Fibroblasts derived from Li-Fraumeni patients display an unusual capacity for immortalization in vitro, in contrast with the normal human fibroblasts, and even with fibroblasts from other cancer prone syndromes, which are rarely immortalized (Bischoff *et al.,* 1990). This growth characteristic was studied in detail by Bischoff *et al.,* 1990, who reported that it is accompanied by random chromosome loss and various chromosome anomalies. The in vitro immortalized cells when introduced into nude mice do not lead to tumorigenesis. Hence, in vitro immortalization and chromosome instability

are distinctive features of Li-Fraumeni derived fibroblasts. This chromosome instability explained why the Li-Fraumeni syndrome is linked to the germ-line mutation of the p53 gene. Yin *et al.* (1992) demonstrated that Li-Fraumeni fibroblasts, with chromosomal instability at low passage number, would show loss or mutation of the remaining wild type p53 allele and this loss was always accompanied with immortalization. This p53 LOH also resulted in loss of cell cycle arrest response. Also p53 +/- and p53-/- mouse embryonic fibroblasts displayed a high spontaneous conversion to aneuploidy in vitro which is not displayed by p53 +/+ genotypes (Livingstone *et al.*, 1992).

p53 is also involved in nucleotide excision repair (NER) (Wang *et al.*, 1995; Smith *et al.*, 1995; Ford *et al.*, 1995). This mode of repair is responsible for the removal of bulky adducts and UV photoproducts from DNA (usually called NER lesions) and comprises two pathways: transcription coupled- repair (TCR) responsible for the removal of lesions from the transcribed strand of active genes, and global genomic repair (GGR) that removes lesions from the rest of the chromatin (Freidberg *et al.*, 1995). p53 is involved in the repair of double stranded DNA breaks. In 1997, the groups of Lopez and Powell independently reported that inactivation of p53 or expression of mutant p53 increased the rate of spontaneous homologous recombination (Bertrand *et al.*, 1997; Mekeel *et al.*, 1997). This is highly significant because homologous recombination is one of the major pathways for the repair of double stranded DNA breaks. In recent years evidence has accumulated that indicates that p53 is also involved in the base excision repair (Offer *et al.*, 2001a and b; Zhou *et al.*, 2001). The mechanism for this is the p53 transcriptional-independent repair, where it has been suggested that p53 directly binds and stabilizes the base excision repair system.

2.6 Regulation of p53

In addition to the mutation, the tumor suppression function of p53 is likely to be inactivated by many other mechanisms in tumor cells. The best known mechanism to abrogate this function is to target the protein for proteasomal degradation. This is achieved largely by Mdm2, a p53-binding protein and the E3 ubiquitin liagase of p53 (Haupt *et al.*, 1997). Expression levels of p53 can be regulated at both transcriptional and translational stages; post-translational modifications take an important role in regulating

its stability (Appella *et al.*, 2001). It has been shown that signals derived from interferon-αβ treatments induce p53 expression at the transcriptional level (Takaoka *et al.*, 2003). Also interaction with the RNA-binding protein HuR leads to the increase in p53 expression as a result of enhanced rate of translation (Mazan-Mamczarz *et al., 2003;* Galban *et al.*, 2003).

Nevertheless, the majority of the stress signals increase stability of p53 protein. Under normal circumstances, Mdm2 binds to the p53 and targets it for proteasomal degradation. This process is enhanced by the transcription factor Yin Yang 1, called YY1 (Gronroos *et al., 2004; Sui* et al., 2004) or reversed by the de-ubiquitination enzyme HAUSP (Cummins *et al.*, 2004; Li *et al.*, 2004). Proteins such as p14-ARF can bind Mdm2 and inhibits its ubiquitin ligase activity E3 (**Fig.6**). Proteins Rb and ribosomal protein L11 bind to the acidic domain of Mdm2 and prevent it from targeting p53 to proteasomal degradation (Hsieh *et al.*, 199; Zhang *et al.*, 2003). Also 14-3-3-σ binds to C-terminus of p53 and increases its stability by preventing Mdm2-mediated ubiquitination (Yang *et al.*, 2003). By contrast promyelocytic leukemia (PML) protein can bind Mdm2 and sequeter it to the nucleolus (Bernardi *et al.*, 2004). Nucleoli are increasingly implicated in the regulation of Mdm2 –mediated p53 stability; proteins such as Mdm2, p14-ARF, Rb and L11 can be found in nucleoli, and the disruption of the nucleolus by microinjection of anti-upstream binding factor (UBF) causes stabilization of the p53 (Rubbi *et al.*, 2003). However the exact nature of such regulation is not known.

Like Mdm2, other ring-finger-containing proteins, such as Pirh2 and cop1 also possess ubiquitin E3 ligase activity and target p53 for degradation through protein-protein interaction (Leng *et al.*, 2003; Dornan *et al.*, 2004). They physically interact with p53 and mediate its ubiquitin-mediated degradation. Therefore together with Mdm2, they form a negative regulatory feedback-loop for p53 function. Human papilloma virus E6 protein also acts as an ubiquitin ligase and lead to ubiquitin-mediated p53 degradation (Scheffner *et al;* 1990).

p53 is heavily modified post-translationally as discussed earlier (**Figure 7**). Its is phosphorylated, acetylated, methylated, sumolated, neddylated and glycosylated (Chuikov *et al.*, 2004; Xerodimas *et al.*, 2004). Phosphorylation of Ser315 by aurora kinase increases p53 stability (Katayama *et. al.*, 2004), phosphorylation at Thr 55 by

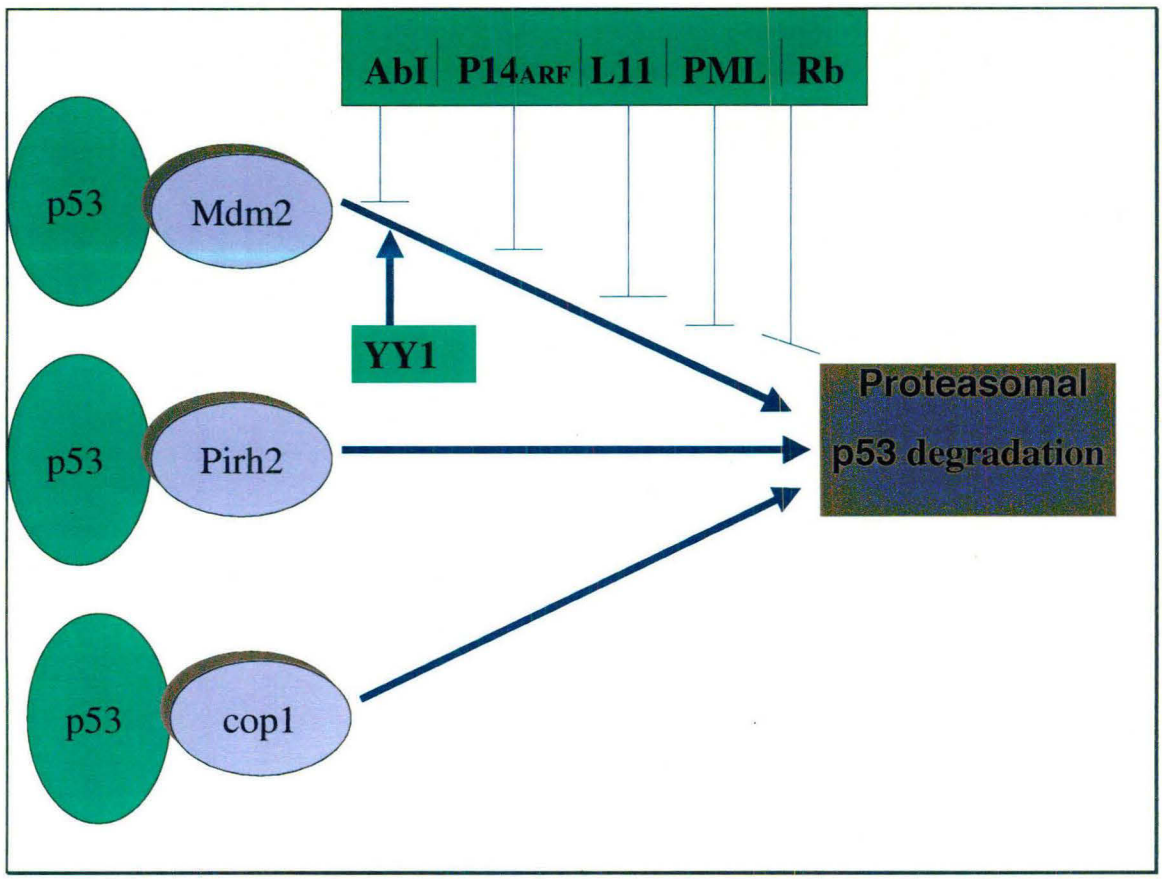

Figure 6. p53 degradation mediated by ubiquitin ligases. Three different E3 ubiquitin ligases; Mdm2, Pirh2 and Cop1 target non-phosphorylated-p53 for proteasomal degradation under normal growth conditions. This process in enhanced by Yang Yin 1 factor and inhibited by various inhibitory factors like Abl, P14ARF, L11, PML and Rb as shown in figure. All the three E3 ubiquitin ligases are ring-finger containing proteins.

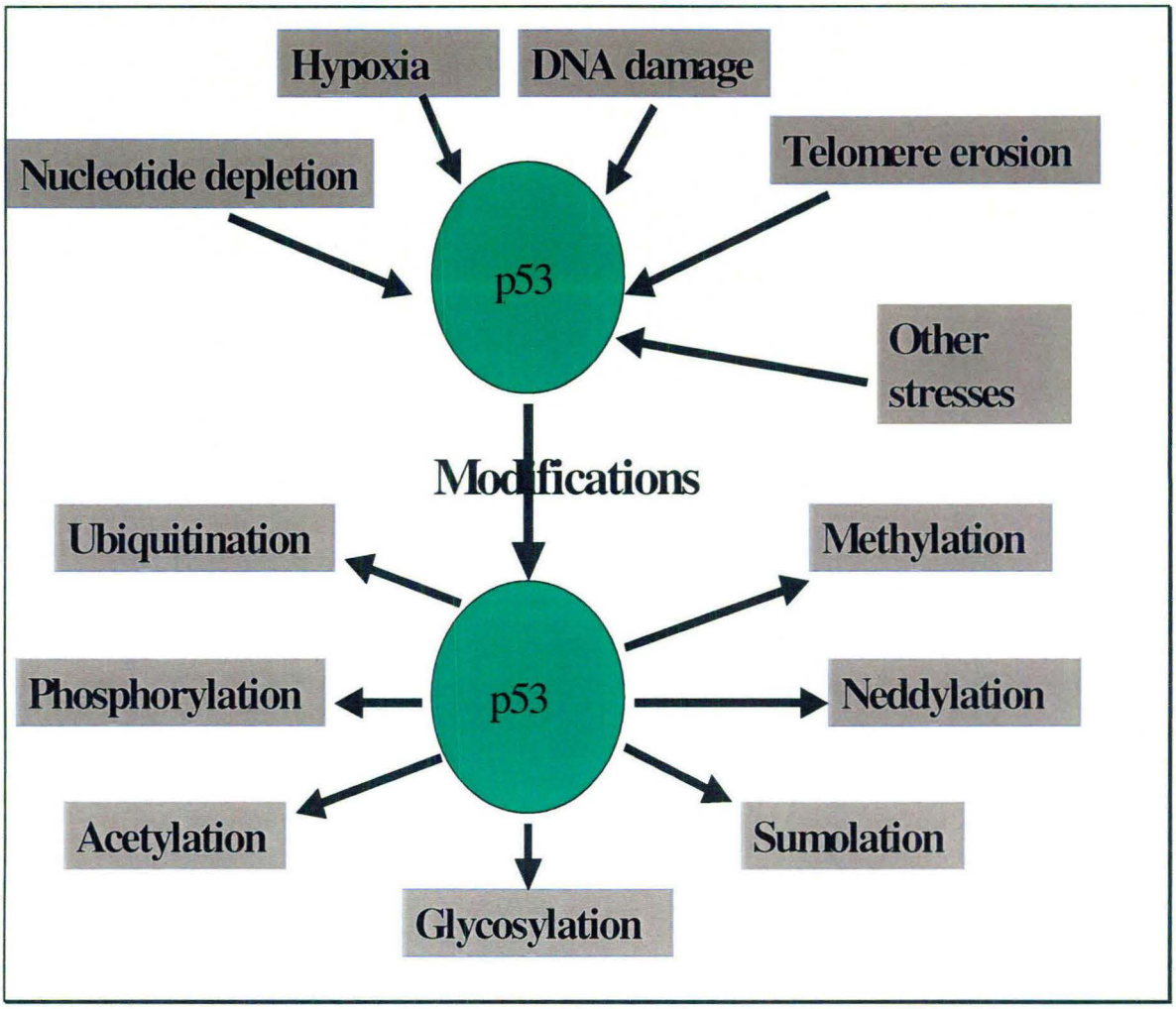

Figure 7. p53 activation by stress signals. During normal growth conditions, p53 is latent and highly unstable but becomes activated through post-translational modifications when the cell perceives stress signals. p53 is heavily modified by phosphorylation, acetylation, methylation, glycosylation, sumolation, neddylation and ubiquitination and after modifications, it becomes activated and performs its job as tumor suppressor to either lead to cell cycle arrest, apoptosis, cellular senescence or/and DNA repair.

TAF1 promotes p53 for degradation (Li *et al.*, 2004). Deacetylation of p53 by histone deacetylase (HDAC) through PML-RAR (PML-retinoic acid receptor) recruitment also leads to the degradation of the p53 (Insinga *et al.*, 2004). Methylation of p53 at Lys372 by Set9 methyltransferase stabilizes p53 and retains it in the nucleus, thereby increasing its transcription activity. By contrast, neddylation of p53 by NEDD8 inhibits its transcriptional activity (Xirodimas *et al.*, 2004). Specific modifications of p53 can change its fate by either targeting it for degradation or it may differentially bind to target promoters with different affinities to cause either cell cycle arrest or apoptosis (Vousden *et al.*, 2002). Two p53-target genes p53AIPI and p21 are activated at different time points after DNA damage. p53AIPI was shown to be induced in thymocytes 24 hours after DNA damage and requires phosphorylation of p53 at Ser46, and is proposed to be important for apoptosis (Oda *et al.*, 2000). Phosphorylation of p53 at Ser46 requires the p53-regulated nuclear protein p53DINPI that is reversed by another p53-regulated WIPI by inactivating p38MAPK (Okamura *et al.*, 2001; Tekekawa *et al.*, 2000). Another p53-target gene, PTEN, a tumor suppressor mutated in large number of human tumors and in three related human autosomal dominant disorders characterized by developmental defects and high tumor incidence (Cristofano *et al.*, 2000). PTEN stabilizes p53 and is required for p53-mediated apoptosis in immortalized MEF (Stambolic *et al.*, 2001).

p53 activity is mostly regulated at expression levels. There are examples where the transcriptional activity of p53 is uncoupled from its expression levels (Lu *et al.*, 1996). Mdmx protein can bind to the N-terminal transactivation domain of the p53 and conceal its transcriptional activity without targeting it for degradation. Nucleoplasmin (NPM) also binds to the N-terminus of the p53 and suppresses its transcriptional activity (Maiguel *et al.*, 2004). Many activators of p53 like p300 and CBP (CREB binding protein) also bind to the N-terminus (An *et al.*, 2004). The C-terminus of p53 is another region to which many proteins bind. One of the important of them is the parkin-like ubiquitin ligase Parc. This protein interacts with the p53 C-terminus and inhibits its activity by retaining it in the cytoplasm without targeting it for degradation (Nikolaev *et al.*, 2003 a and b).

It is well established fact almost all inactivating mutations in p53 are found in central DNA binding domain. This gives an idea that this region is biologically most

important region in controlling tumor suppression function of the p53. A new class of proteins has been found to interact with the p53 central domain. Large T antigen of the SV40 virus was the first member to be identified to bind to this region (Lane *et al.*, 1979) but the best known cellular proteins of this group are the ASPP (apoptosis-stimulating protein of p53) family consisting ASPP1, ASPP2 and iASPP. ASPP1 and ASPP2 enhance, whereas iASPP inhibits, the apoptotic function of p53 (Samuels *et al.*, 2001; Bergamaschi *et al.*, 2003). So it is clear that ASPP –p53 interaction regulates an important function of the p53 i.e., apoptosis. In conclusion, p53 activity and its expression levels in response to stress signals can be regulated either at mRNA or protein level or through the post-translational modifications by various regulators that are mostly p53-regulated.

2.7 p53 isoforms

p53 transcription factor has been joined by the other two members that together form a family of transcription factors involved in growth, development and tumor suppression. These are the recently discovered p63 and p73 genes (Kaghad *et al.*, 1997 and Yang *et al.*, 1998). All the three transcription factors are sequence specific DNA-binding proteins and share the extensive sequence homology with each other. The functional domains of the three proteins are similar: a) the amino-terminal domain regulates transcriptional activation, b) the central DNA-binding domain regulates the DNA-binding activity and c) the C-terminal domain regulates oligomerization activity. The amino-terminal transactivation domain shares 29% identity between p53 and p73 while in p63 and p53, it shares 22%. The DNA-binding domains share significant homology with 63% identity between p53 and p73, and 60% between p63 and p53. There is approximately 37% homology in the carboxy-terminal domains of the p53 and p63 proteins, while between p53 and p73, it is 38% (**Figure 2**). All the p53 family members function similarly in activating transcription in vitro (Mondal *et al.*, 2005).

All family members of the p53 family have been shown to code for multiple isoforms due to the usage of alternative internal promoter and alternative splicing (Kaghad *et al.*, 1997; Yang *et al.*, 1998). Earlier it was shown that the human p53 gene contains only one promoter, and transcribes only three mRNA splice variants encoding,

respectively, full length p53, p53i9 and delta40 p53. p53i9 is encoded by the alternative splicing at the intron 9 and is truncated of the last 60 amino acids of the full-length p53. This isoform is defective in transcriptional activity and devoid of DNA binding activity. P53i9 isoform has never been reported to be expressed at the protein level. Del40 p53 isoform can be generated either by the alternative splicing of the intron 2 or by the alternative initiation of the translation. It is N-terminally truncated isoform devoid of first 40 amino acids of the full p53 but it still contains second transactivation domain and has been shown to activate p53 responsive genes (Xhu *et al.*, 1998). Del40 p53 can also act in a dominant negative manner after transfection towards wild type p53 and it can also modify p53 cell localization and inhibits p53 degradation by p53 (Ghosh *et al.*, 2004). Recently through an elegant set of experiments it has been shown that p53 gene encodes six isoforms (Bourdon *et al.* 2005) due to the presence of an alternative promoter in the intron 4 and due to the alternative splicing in the intron 9 (**Figure 8**). These p53 isoforms are expressed in normal tissue in a tissue dependent manner. The C-terminus of the full-length p53 can be alternatively spliced in the intron 9 to produce three isoforms: p53 (called as p53 alpha), p53 beta and p53 gamma. The p53 alpha and p53 gamma isoforms lack the oligomerization domains. The usage of the alternative promoter in the intron 4 leads to the expression of an N-terminally truncated isoform called delta133 p53, which is initiated at the codon 133 of the full-length p53. Altogether human p53 gene can encode theoretically nine isoforms: p53 full length, p53i9, del40 p53, p53 beta, p53 gamma, del133 p53, del133 beta, del133 gamma, del40 beta and del40 gamma. All these isoforms are expressed tissue-specifically at mRNA levels (Bourdon *et al.*, 2005). Also very recently it has been shown that two internal ribosome entry sites (IRES) mediate the translation of both full-length and DeltaNp53 isoforms (Ray *et al.*, 2006). IRES leading to the translation of full-length p53 is in 5' untranslated region of the mRNA while as IRES that gives DeltaNp53 extends into the protein-encoding region.

For normal functioning of the p53, it has to bind first to p53-responsive element sequence followed by tetramerization and the pre-initiation complex is formed to initiate transcription at p53-dependent genes. Homotetramerization is one of the very important steps in the regulation of genes by p53. As observed above that in normal cells different p53 isoforms are present, the transcription regulation may be affected. This is because

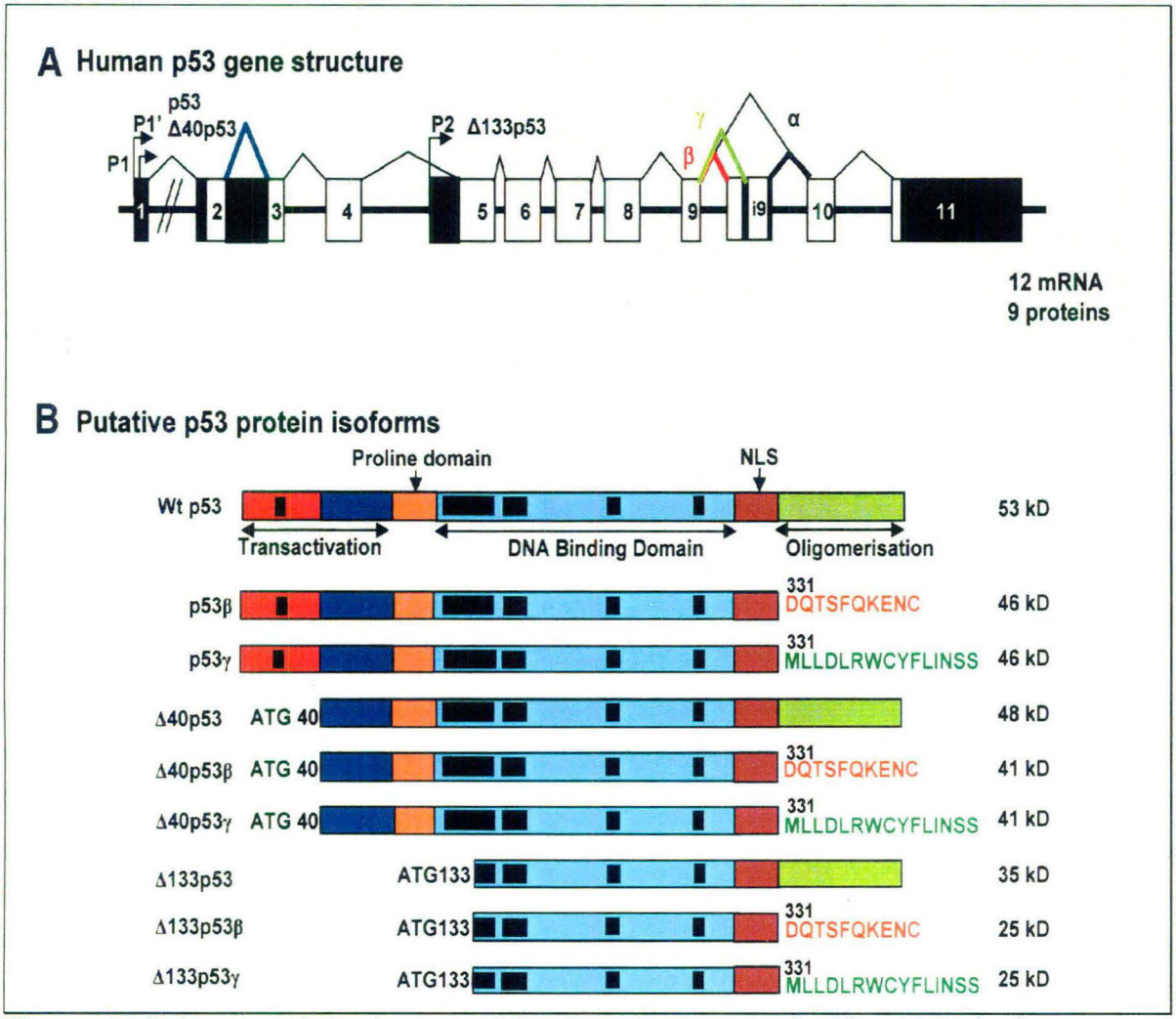

Figure 8. Schemas of the human p53 gene structure and p53 protein isoforms. (A) Schema of the human p53 gene structure. (B) Schema of the p53 protein isoforms theoretically encoded by the human p53 gene. As can be seen, due to the usage of alternative internal promoter, alternative initiation of translation and alternative splicing, nine p53 isoforms are formed. This makes the p53-transcription regulation very complex and interesting. Full-length and deleted versions of isoforms are formed, with additional C-terminal amino acid chains in β and γ isoforms which makes them peculiar in for example binding to specific antibodies. Intron 9 is alternatively spliced while intron 4 contains alternative promoter in human p53 gene which together lead to generation of these isoforms of p53.

many p53 isoforms like del133 p53, lack transactivation domain but have intact DNA-binding and oligomerization domains. So, if p53 homo-tetramerization is affected by these truncated isoforms, p53 response to various stress input signals and the p53 pathways can be affected, and hence prevention of damage to the cell is not proper. This hypothesis can be backed by the observation that only more than 50% of human cancers have p53 mutations and rest have normal p53 gene and its expression scenario. Also these p53 isoforms are differentially expressed in tissues and also under stress conditions like actinomycin D (Bourdon *et al.*, 2005).

In lung cancer, it has been shown that deltaNp63 and deltaNp73 isoforms are expressed together with wild-type p53 and inactivate WTp53 activity in a dominant negative mode (Uramoto *et al.*, 2006). DNp73 is highly expressed in proliferating C2C12 myoblasts, rapidly accumulates in differentiating myocytes and remains elevated in C2C12 myotubes. Specific SiRNA towards DNp73 lead to apoptosis of C2C12 myoblasts. This shows dominant negative effect of DNp73 towards p53 activity (Belloni *et al.*, 2006).

CHAPTER 3

Aims & Objectives

3. Aims and Objectives

It is known that more than 50% of human cancers have a mutation in the p53 gene and rest have a functional p53 gene. For normal functioning of the p53, it has to bind to the p53-response elements in p53-regulated genes. In a normal cell, first a p53 dimmer is formed on the p53-response element which is then co-operatively joined by the other dimmer to produce a homotetramer, and a functional pre-initiation complex is formed which is a critical factor in activating transcription of p53-target genes in response to stress signal. We know that due to the mechanisms of alternative promoter utilization and alternative splicing, in a normal cell different p53 isoforms are generated. N-terminally deleted p53 isoforms lack trans-activation domain but have intact DNA-binding and oligomerization domains. Compared to the normal cell, in tumor cells it has been found that the ratio of some of the p53 isoforms is abnormally high. It is possible that during stress, dis-balance in different isoforms of p53 may lead to the hetero-tetramerization (tetramerization between different isoforms) and dis-regulation.

We hypothesize that if transactivation domain-deleted p53 isoforms replace a wild type p53 subunit in homotetramer, it may lead to repression of p53-regulated gene expression in response to a stress signal. This will therefore, lead to the tumor formation although wild type p53 is present.

Based on the above hypothesis, we would like to access the effect of transactivation-deleted p53 isoforms on the wild type p53 using in vitro system. The objectives of the present study are as under:

1. Cloning of p53 isoforms; del40-p53 and del133-p53 as flag-tagged genes in baculovirus shuttle plasmid pFastBac1.

2. Transposing the cloned p53 isoform genes; full-length p53, del40-p53 and del133-p53, from baculovirus shuttle plasmid to the baculovirus genome in DH10Bac strain through transposition.

3. Transfecting the insect cell line Sf21 with the recombinant bacmids containing flag tagged p53 isoform genes for recombinant virus amplification and protein expression.

4. Purification of recombinant proteins using flag affinity columns.

CHAPTER 4

Materials & Methods

4. Materials And Methods

4.1 Materials

4.1.1 Chemicals and Biochemicals:

Acetic acid, Agaragar (Qualigenes), Agarose, Ampicilline (Sigma), Bromophenol blue, Calcium chloride hexa-hydrate (Sigma), Chloroform (Qualigens), Dimethyl sulfoxide (sigma) DNA molecular weight markers (1 kb ladder) (MBI Fermentas), Ethylene diamine tetra acetic acid (EDTA) (Qualigens), Ethanol (Merk and Bengal Chemicals), Ethedium bromide, Gentamicin, Glycerol (sigma), Hydrochloric acid (SD Fine), Isoamyl alcohol, Isopropanol, IPTG, Kanamycin (Sigma) Manganese chloride, Methanol, Magnesium chloride, Phenol, Potassium acetate, Potassium chloride (Qualigens), RNase A (Sigma), Sodium acetate, Sodium chloride, Sodium dodecyl sulfate, Sodium hydroxide, Glucose (Qualigens), Tetracycline, Trizma base (Sigma), Tryptone, Yeast extract, LB Broth (Qualigens), X-gal (Sigma).

4.1.2 Hotstart DNA polymerase and dNTPs:

The TripleMaster DNA polymerase was supplied by Eppendorff and dNTPs were procured from Sigma Aldrich Co. Taq polymerase was a kind gift from Dr. S.K. Dhar's laboratory, SCMM, JNU, New Delhi India.

4.1.3 Enzymes supplied with buffers:

DNA ligase. BamH1, Xho1, Xba1, HindIII were procured from MBI Fermentas.

4.1.4 Organisms:

DH5α, DH10 β, DH10Bac (Dr. S.K. Dhar,s Lab. SCMM, JNU, New Delhi, India).

DH5α and DH10β were used for cloning of recombinant pFastBac1-flag tagged del40-p53 and pFastBac1-flag tagged del133-p53 isoforms. While as DH10Bac strain was used to generate recombinant bacmids of flag tagged- del40-p53 and –del133-p53 isoforms.

4.2 Methods

4.2.1 Competent cell preparation:

DH5α, DH10β and DH10 Bac strains of *E. coli* were streaked onto LB agar plates and incubated overnight at 37^0C. Next day a single isolated colony of each strain was inoculated into each 10 ml 2XL medium contained in 50-100 ml conical flasks. Then the culture was grown for 16 hrs at 30^0C temperature and 200 rpm. 1% of the above

overgrown culture was inoculated into 50 ml 2XL medium and incubated at 30^0C at 200 rpm till O.D. come to 0.4-0.45. After this culture was kept at ice for 2 hrs and cells were harvested at 5500 rpm for 5 min at 4^0C. The pelletted bacterial cells were resuspended in 1/2 culture volume of fresh acid salt buffer and kept on ice for 45 min. Then resuspension was pelletted at 5500 rpm for 10 min at 4^0C. Pellet was resuspended in $1/20^{th}$ culture volume of acid salt buffer. Glycerol was added at the final concentration of 15%. Then 200 ul aliquots of bacterial suspension were made in eppendorffs and stored at -70^0C for storage and future use.

Composition of 2XL medium:

Yeast Extract	1%
Tryptone	2%
NaCl	0.1%

Autoclaved and added filter sterilized glucose at a final conc. of 0.1%

Composition of Acid Salt Buffer:

CaCl2	100Mm.
MnCl2	70Mm.
CH3COONa	40Mm.

Adjust pH of the buffer by glacial acetic acid exactly at 5.4.

4.2.2 Transformation of competent cells:

An aliquot (50-100 ul) of the competent cells was taken out from -70^0C and thawed on ice for 5 min. Foreign DNA (50-100ng) was added to these cells and mixed very gently by slightly tapping the bottom of the eppendorff tube. Then tube was kept at ice for 30

min and gently tapped every 10 min. Heat shock was given at 42^0C for 90 seconds. Cells were immediately transferred to ice and kept as such for 5 min so that they come to RT. To this, 800 µl of LB medium at RT was added and incubated at 37^0C for half an hour at 200 rpm. Cells were pelletted down at 6000 rpm at 4^0C for 5 min. Bacterial pellet was resuspended in 200 µl of LB medium, and then 80 µl and 20 µl of this suspension was plated onto antibiotic selection LB agar plate. The plate was then incubated in inverted position at 37^0C for 12 hrs. Proper controls were used in order to avoid contamination problems. No DNA was added in the negative control and was plated onto the same plate.

4.2.3 Transposition of recombinant pFastBac1 clone into bacmid of DH10Bac strain:

Since we wanted to generate recombinant viruses of flag tagged- del40-p53 and -del133-p53 clones for transfection and expression of recombinant protein in insect cell line Sf21, it was required to transfer our gene of interest from recombinant transfer vector pFastBac1 to bacmid in DH10Bac cells. For this, LB plates were prepared containing: 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µ g/ml tetracycline, 100 µg/ml X-gal and 40 µg/ml IPTG. DH10Bac competent cells were thawed on ice and 100 µl of it was dispensed into 1.5 ml microcentrifuge tube. Approximately 1ng recombinant donor plasmid (in 5 µl) (i.e. 5ul of 1:100 dilution of minipreparation DNA) was added to the cells and gently mixed by slight tapping of sides of the tube. The mixture was incubated on ice for 30 min. and heat shock was given to the mixture at 42^0C for 45 seconds. The tube was immediately chilled on the ice for 2 min. 900 ul of S.O.C. medium (or LB+ 10mM MgCl2 also worked) was added to the mixture and tube was placed in a shaking incubator at 37^0C with medium agitation for 4 hrs. 10% and 90% of each transformation mix was plated on the above plates. Plates were incubated in inverted position for 24 hrs to 36 hrs at 37^0C. Two types of colonies appeared, blue and white. White colonies contain the recombinant bacmid DNA. Colonies were small and white colonies were visible clearly after 24 hrs of incubation. White colonies were picked (usually large and isolated ones) and streaked on the same plates as above to verify the phenotype, and incubated overnight at 37^0C.

4.2.4 PCR Setup:

PCR amplification was done to amplify the flag tagged del40-p53 and flag tagged del133-p53 genes using the respective flag tagged forward primer and reverse primer. BamHI enzyme site was introduced into the forward primer and XhoI enzyme site in the reverse primer for directional cloning of flag tagged del40-p53 and flag tagged del133-p53 genes into the baculovirus shuttle plasmid pFastBac1. Flag tag was introduced prior to the gene sequence but after the BamHI enzyme site in the forward primer, so that protein will be N-terminally flag tagged and will ease in recombinant protein purification. Sequence of the primers used was as follows:-

1. *Primers for del40-p53*:

   Forward primer: 5' CGGGGATCCATAATGGACTACAAGGACGACGAT GACAAGGGAGATGATTTGATGCTG 3'.

   Reverse Primer: 5' CGGGGCTCGAGTCAGTCTGAGTCAGGCCC 3'.

2. *Primers for del133-p53*:

   Forward primer: 5' CGGGGATCCATAATGGACTACAAGGACGACGAT GACAAGGGAATGTTTTGCCAACTG 3'.

   Reverse primer: 5' CGGGGCTCGAGTCAGTCTGAGTCAGGCCC 3'.

PCR conditions optimized for the amplification of the flag tagged del40p53 and del133-p53 genes: Tm of above primers used for PCR amplification was calculated as below using the formula $4(G+C) + 2(A+T)\ ^0C$.

Tm for flag tagged del40-p53 primers:

Forward primer: $Tm = [4\{(G+C) = 6\}] + [2\{(A+T) = 9\}]\ ^0C =$ **42^0C.**

Reverse primer: $Tm = [4\{(G+C) = 11\}] + [2\{(A+T) = 7\}]\ ^0C =$ **58^0C.**

Tm for flag tagged del133-p53 primers:

Forward primer: $Tm = [4\{(G+C) = 6\}] + [2\{(A+T) = 9\}]\ ^0C =$ **42^0C.**

Reverse primer: $Tm = [4\{(G+C) = 11\}] + [2\{(A+T) = 7\}]\ ^0C =$ **58^0C.**

As we had designed flag tag in our forward primer, there was problem in choosing proper annealing temperature. Initial Tm was 42^0C and as PCR continued, Tm steadily increased. So, when we started with PCR annealing temperatures of 40^0C, 42^0C and 45^0C, we got non-specific bands. To avoid non-specific bands, we optimized our

annealing temperature to 55^{0}C. At this annealing temperature, there were no non-specific bands created during PCR.

So, the PCR conditions optimal for PCR amplification of the above genes were as under:

Step 1. *Initial denaturation.*

	95^{0}C	3 min.

Step 2. (35 cycles).

Denaturation.	94^{0}C	30 sec.
Annealing	55^{0}C	30 sec.
Elongation	72^{0}C	1.30 min.

Step 3. *Final elongation*	72^{0}C		3 min.
	Store	@	4^{0}C.

Preparation of primers: Primers were provided as 40 nmol of lyophilized powder. So this was dissolved in 40 ul of $T_{10}E_1$ so that final concentration of master stock becomes 1nmol/µl. Working stock of the primers was made by diluting 100 times the master stock to get the working stock concentration as 10 pmol/µl.

Reagents for PCR reaction with appropriate concentrations required:

1. Template DNA (pcDNA3 –p53)	1 µl (100ng).
2. Del40 forward primer (10 pmol/ul)	1 µl.
3. Del40 reverse primer (10 pmol/ul)	1 µl.
4 dNTP mix (dATP, dGTP, dCTP, dTTP) (2. 5 mM)	4 µl.
5. 10X PCR buffer containing Mg++	5 µl.
6. TripleMaster DNA polymerase	0.5 µl
7. Autoclaved double distilled water	37.5 µl
Total reaction volume	50.0 µl.

4.2.5 Gel elution of DNA fragments:

Freeze thaw method: DNA was run on 1% agarose gel with marker and agarose block containing the DNA band to be eluted was cut with fresh blade under low intensity UV light. This cut gel slice was chopped and taken in a fresh autoclaved microcentrifuge tube. 10mM Tris (pH 8.0) was added till gel was submerged and the tube was kept in -80^{0}C for half an hour. Tube was removed and thawed at RT for 5-10 min. This freeze-thaw procedure was repeated 4-5 times. Then it was centrifuged at 10000 rpm for 10 min at 4^{0}C. Aqueous phase (upper layer) was taken carefully and put into a fresh microcentrifuge tube. Equal volume of isopropanol and 1/10 volume of sodium acetate (5M, pH 5.5) was added to it and mixed thoroughly by up and down movements. Tube was kept at -80^{0}C for 20-30 min and centrifuged at 13000 rpm for 15-20 min at 4^{0}C. Supernatant was carefully discarded and pellet was washed with 500ul of 70% ethanol, and centrifuged at 13000 rpm for 5-10 min at 4^{0}C. Supernatant was carefully discarded and DNA pellet was air dried for 5-10 min so that last traces of ethanol are evaporated. The DNA pellet was dissolved in 10 µl of double distilled water. An aliquot (0.5 or 1.0 µl) was checked on 0.8% agarose gel with marker to check the purity and concentration.

4.2.6 Restriction digestion:

The gel eluted PCR product and vector DNA were double digested with BamHI and XhoI for ligation of flag tagged- del40-p53 and -del133-p53 into transfer vector pFastBac1. The double digestion was carried out in 2X digestion buffer using restriction enzymes @ of I unit/□g DNA for 5 hrs at 37^{0}C. The restrict digested products were analyzed on 0.8% agarose gel to check the conc., purity and quality.

4.2.7 Ligation reaction set up:

The double digested and agarose gel eluted PCR products were ligated into the double digested and gel eluted vector for directional cloning of flag tagged- del40-p53 and –del133-p53 isoforms into baculovirus shuttle plasmid, pFastBac1. The reaction was carried out in a 10 µl reaction volume with insert: vector ratio of 3:1.The reaction set up as follows:

Insert	300ng.
Vector	100ng.

10x ligation buffer	1 µl.
T4 DNA ligase	1 unit.

Added sterile double distilled water to make reaction volume to 10 µl.

The ligation reaction was carried out at 16^0C for 16 hrs and then whole ligation mix was added to competent DH5α cells in transformation reaction.

4.2.8 Plasmid isolation:

4.2.8.1 Minipreparation:

2.5 ml overnight culture of DH5α strain of *E. coli* harboring the appropriate plasmid was set in 15 ml falcon tubes containing the appropriate antibiotic (for DH5α it is ampicilline (100 mg/ml stock)), to amplify the plasmid. Single isolated colony was inoculated into each falcon tube and grown overnight at 37^0C in incubator shaker at 220 rpm. Cells were harvested by centrifugation at 6000 rpm for 5 min at 4^0C. Bacterial cell pellet was resuspended by vortexing in 200 µl solution I (see recipe below) containing 4 µg/ml lysozyme. 400 µl of freshly prepared solution II was added and mixed well by inverting the tube gently 4-6 times to avoid breaking the plasmid. Chilled 200 µl solution III was immediately added and mixed very gently by pipetting up and down, and the mixture was incubated at 4^0C or in ice for 5 min without shaking. White precipitate was formed. It was centrifuged at 13000 rpm for 15 min at room temperature. Supernatant was carefully transferred to a fresh micro-centrifuge tube avoiding any white material to come out with it. Equal volume of isopropanol and 0.1 volume of 5M (pH 5.5) sodium acetate were added to the supernatant and mixed by inverting the tube up and down several times. The tube was kept on ice for 10 min and centrifuged at 13000 rpm for 15 min at 4^0C. Supernatant was discarded and the pellet was washed with 500 ul 70% ethanol, and centrifuged again for 5 min at 13000 rpm at 4^0C. Pellet was air dried for 5 min and dissolved in 30- 50 µl of double distilled water. 1µl of RNase A was added and incubated at 37^0C for 20 min to digest away all contaminating RNA. An aliquot of 2 µl was checked on 0.8% agarose gel to check the purity and quality of the plasmid preparation. Double distilled water was added to the DNA solution to increase the volume to 200 µl. Equal volume (200 µl) of phenol chloroform isoamyl alcohol (25:24:1) was added to it

and mixed by vigorous vortexing for 12 seconds. It was centrifuged at 12000 rpm at RT for 10 min. Upper aqueous phase was taken into a fresh micro- centrifuge tube and equal volume of isopropanol and 0.1 volume of 5M sodium acetate (pH 5.5) were added to it. Tube was kept at -80^{0}C for 20 min and centrifuged at 13000 rpm for 15 min at 4^{0}C. Supernatant was discarded and pellet washed with 500 µl of 70% ethanol, and then centrifuged at 13000 rpm for 5 min at 4^{0}C. The DNA pellet was air dried for 10 min and dissolved in 30 µl of double distilled water. Tube was labeled properly and kept in -20^{0}C. Again an aliquot of 2 µl was checked on 0.8% agarose gel for purity, quality and concentration of the plasmid preparation.

Recipe of the solutions:

Solution I: 50mM glucose, 25mM Tris-HCl (pH 8.0), 10mM EDTA (pH 8.0). This solution can be autoclaved or filter sterilized and stored at 4^{0}C.

Lysozyme solution: stock of 20 mg/ml in 10mM Tris-HCl (pH 8.0).

Solution II: 0.2M NaOH, 1% (w/v) SDS. Solution II should be freshly prepared from the stocks of 2M NaOH and 10% (w/v) SDS.

Solution III: 5M sodium acetate, pH 5.5. OR

8M ammonium acetate. 100 ml batches can be made and autoclaved and stored at room temperature.

4.2.8.2 Midipreperation:

Single colony of DH5α strain of *E. coli* harboring a desired plasmid was inoculated in 50 ml of LB broth medium containing an appropriate antibiotic concentration (e.g., 100ug/ml final conc. of ampicilline) in a 250 ml flask and incubated at 37^{0}C overnight in an incubator shaker shaking @ 220 rpm. Cells were harvested at 6000 rpm for 7 min at 4^{0}C. All the supernatant was discarded and the bacterial pellet was resuspended in 1.5 ml of solution I (composition same as above for minipreparation protocol) containing 2mg/ml lysozyme, by vigorously vortexing and the tube was kept on ice for 10 min. 3ml of solution II (same as above for minipreparation) was added and mixed gently by slightly rotating the tube 50 times. Tube was kept on ice for exactly 10 min. Chilled 3M sodium acetate (pH 4.6) (solution III) was added to the tube, mixed gently and kept on ice for further 20 min. Cell lysate was pelletted down at 12000 rpm for 15 min at 4^{0}C and supernatant was taken carefully to a falcon tube. 10 µl of 10 µg/ml RNase A was added

to it, mixed well and incubated at 37^{0}C for 1 hr. Tube was taken out and equal volume of phenol chloroform isoamyl alcohol (PCI) (25:24:1) was added and mixed by vigorous vortexing. Centrigugation was done at 12000 rpm at RT for 10 min. Supernatant was transferred to a new falcon and extraction was repeated again with PCI and next time with chloroform: isoamyl alcohol (24:1). To the final aqueous phase 2.5 volumes of ice-cold absolute ethanol was added, mixed thoroughly and incubated in -80^{0}C for half or in -20^{0}C overnight. It was spun at 13000 rpm for 15 min at RT. Supernatant was carefully discarded; pellet was washed with 1 ml 70% ethanol and spun again for 5 min at 13000 rpm at RT. Supernatant was discarded and pellet was marked and air dried for 10 min. Pellet was dissolved in 400 µl of double distilled water. Suspension was taken in 1.5 ml microfuge tube, and 120 µl 4M NaCl and 0.5 ml of PEG (13%) were added to it and mixed well and incubated on ice for 1 hr. It was spun at 13000 rpm for 15 min at 4^{0}C. Supernatant was drained away carefully and pellet washed with 500 µl 70% ethanol. It was spun at 13000 rpm for 5 min at 4^{0}C. Aspirated all the ethanol and air died the pellet for 10 min at RT. Finally pellet was dissolved in 40-50 µl TE or double distilled water. An aliquot of 2 µl was loaded on 0.8% agarose gel to check the purity, quality and quantity of plasmid preparation. Tube was labeled properly and stored at -20^{0}C.

4.2.9 Isolation of recombinant bacmid DNA from DH10Bac cells:

A single, large and isolated white colony of DH10BAC strain harboring the recombinant bacmid was inoculated into 2 ml LB medium supplemented with 50 µg/ml kanamycin, 7 µg/ml gentamicin and 10 µg/ml tetracycline. Cells were grown at 37^{0}C/ 200 rpm in incubator shaker to stationary phase. 1.5 ml culture was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 5000 rpm for 1 min. Supernatant was completely removed by vacuum aspiration and pellet was resuspended (by gently vortexing) in 300 µl of solution I (15mM Tris.HCl (pH 8.0), 10mM EDTA, 100ug/ml RNase A). 300 µl of freshly prepared solution II (0.2N NaOH, 1% SDS) was added to it and incubated at RT for 5 minutes. Slowly 300 µl of 3M potassium acetate (pH 5.5) was added and gently mixed during addition. A thick white precipitate was formed and tube was kept on ice for 5-10 min. After incubation, it was centrifuged at 11000 rpm at 4^{0}C. Supernatant was carefully transferred to a fresh microfuge tube, already containing 800 µl absolute isopropanol, without taking any white material. Sample was gently mixed by inverting

tube 3-4 times and placed on ice for 5-10 min. Bacmid DNA was pelleted down at 12000 rpm at RT for 15 min. Supernatant was removed and pellet washed with 400 µl 70% ethanol. It was again centrifuged at 11000 rpm for 10 min at RT. Supernatant was removed as much as possible very carefully not to disturb the pellet and the pellet was air dried for 5-10 min at RT. DNA was dissolved in 40 µl TE by gently tapping the sides of the tube. Labelled tube was stored at -20^{0}C, however repeated freeze-thaw was avoided as it affects transfection efficiency. An aliquot of 5 µl of DNA solution was run on the 0.5% agarose gel overnight at 24 volts to check the quality and quantity of bacmid DNA. 0.5% agarose gel and low voltage was used because Bacmid DNA is a high molecular weight DNA and will not be resolved clearly on agarose gels greater that 0.5% strength and if voltage is greater than 24 volts, 0.5% gel starts melting.

4.2.10 Colony PCR Procedure.

Colony PCR is the PCR amplification of specific DNA fragment using colony as the source of template. It was done to look for the colonies that could be screened for the isolation of recombinant plasmids to confirm the right clones. The conditions for the colony PCR were same as those for the amplification of genes discussed above except the source of template that in this case was a single isolated colony on antibiotic agar plate. The master mix of dNTPs, forward primer, reverse primer and 10X PCR buffer was made. Reaction volume was limited to 24.5 µl by double distilled water. Single colony was touched gently with autoclaved tip, and it was touched first to fresh antibiotic LB agar plate to keep the replica record and then to the master mix in PCR tube. 0.5 µl of Taq polymerase was added in last and PCR machine was started immediately. After PCR was over, PCR product was run on 0.8% agarose gel to check its location and size. The reagents for the colony PCR were as follows:

1. Template DNA (recombinant colony)	Colony touch.
2. Del40 forward primer (10 pmol/ul)	0.5ul.
3. Del40 reverse primer (10 pmol/ul)	0.5ul.
4 dNTP mix (dATP, dGTP, dCTP, dTTP) (2. 5 mM)	2.0ul.
5. 10X PCR buffer containing Mg++	2.5ul.
6. Taq polymerase	0.5ul

7. Autoclaved double distilled water 19.0ul

Total reaction volume *25.0ul.*

The programme for colony PCR was similar to the initial amplification of the p53 isoforms but it was limited only to 25 cycles of amplification as we only needed to check whether our clone is right or not. The programme was as follows:

Step 1. *Initial denaturation.*

95^0C 3 min.

Step 2. (25 cycles).

Denaturation.	94^0C	30 sec.
Annealing	55^0C	30 sec.
Elongation	72^0C	1.30 min.

Step 3. *Final elongation* 72^0C 3 min.

Store @ *4^0C.*

CHAPTER 5

Results & Discussion

5. Results and Discussion

The gene encoding the tumor suppressor protein, p53, is one of the most commonly mutated genes in human cancers (Hofseth *et al.,* 2004). In normal cells, p53 protein is produced at very low levels and is short lived. But in stress conditions, it is stabilized and activated through post-translational modifications like phosphorylation, methylation, summolation and acetylation, and at the same time other post-translational modifications lead to its degradation like neddylation and ubiquitination (Appela *et al.,* 2001; Xirodimas *et al.,* 2004; Brooks *et al.,* 2003 and Xu *et al.,* 2003). p53 gene is a single copy gene located on the short arm of the chromosome 17 and produces a phosphoprotein which is a sequence specific DNA binding transcription factor. It is well established fact that the tumor suppressor function of the p53 protein occurs via its function as a transcriptional factor that regulates gene expression. As a transcriptional regulator, it must work with other protein factors, known as coactivators/reppressors, in activating/suppressing genes responsible for controlling the growth of the cells. Genetic studies of p53 reveal its importance in genome integrity. Loss of p53 function results in centrosome amplification and karyotype abnormalities (Wang *et al.,* 1998; Bischoff *et al.,* 1990) and also results in defects in DNA repair following UV damage or other genotoxic stresses (Smith *et al.* 1995). In 1997, two p53 homologues, p63 and p73 have been identified which share extensive sequence homology with p53 and act similarly in activating gene expression (Kaghad *et al.,* 1997; Yang *et al.,* 1998; Mondal *et al.,* 2005). p63 and p73 have been shown to produce multiple isoforms. Earlier p53 was thought to produce only full-length protein. But recently in October 2005, Bourdon *et al.* have shown that p53 gene produces altogether nine different isoforms in normal cells due to alternative promoter utilization, alternative initiation of translation and alternative splicing. But only the homotetramer of full-length p53 is functional. Among the isofoms are del40-p53 and del133-p53. In del40-p53 isoform TA1 transactivation sub-domain is deleted whereas in del133-p53 isoform both TA1 and TA2 sub-domains are deleted. Therefore we hypothesize that if these isoforms replace one of sub-units of the full-length p53 homotetramer, repression of transcription will occur at p53 target genes. So to assess the effect of del40-p53 and del133-p53 isoforms, we wanted to clone these two isoforms

initially in baculovirus shuttle plasmid, pFastBac1 and then transferring the genes to the baculovial genome so as to express them in baculovirus expression system.

5.1 Results

5.1.1 Sequence alignment of p53 family members, p53, p63 and p73.

p53 tumor suppressor protein was earlier thought as the unique transcription factor without any homologue. But later it was found that two newly discovered proteins, p63 and p73 share a very good homology with p53 and function similarly in activating transcription. So they were put together in the same family of transcription factors. Here we wanted to see the differences among the three members in humans at the level of amino-acid sequences. Amino acid sequences of the full-length p53, p63 and p73 proteins were aligned using clustal W and we found that three isoforms share a great sequence homology as shown in figures **9a,b, c** and **d**. Sequence homology was more in DNA binding domain followed by oligomerization domain and transactivation domain. The amino-terminal transactivation domain has 29% identity between p53 and p73, and 22% identity between p53 and p63. The central DNA binding domain has greater homology with 63% identity between p53 and p73, and 60% identity between p53 and p63. The C-terminal oligomerization domains share 38% homology between p53 and p73 and 37% between p53 and p63.

5.1.2 Sequence alignment of full-length, delta-40 and delta133-p53 isoforms.

Amino acid sequences of the p53 isoforms; full-length p53, delta40-p53 and delta133-p53; were aligned using clustal W as shown in **figure 10**. We found that three isoforms of p53 differed only in the N-terminal transactivation domain. Full-length TP53 protein isoform has both the sub-domains, TA1 and TA2, present in the transactivation domain. Del40-p53 isoform is deleted of the first TA1 transactivation sub-domain. In del133-p53 isoform, both TA1 and TA2 subdomains are deleted.

5.1.3 Cloning of Flag-tagged del40-p53 isoform

Delta40-p53 isoform was PCR amplified as flag tagged product from the pcDNA3-p53 full-length clone (kind gift from Dr. Gupta SS, NII New Delhi India). The PCR conditions were optimized with respect to the annealing temperature, cycle numbers and the length of the respective phases of the cycle. PCR annealing temperature was taken as

40^0C for PCR amplification. At this temperature lot of non-specific bands were observed (**Figure11**, lane 3). To overcome this problem, PCR amplification was done at three different temperatures 40^0C, 45^0C and 55^0C. At 55^0C non-specific bands were not observed. So for further work, we took 55^0C as the annealing temperature for the PCR amplification (**Figure 12**, lane #3). For negative control we did PCR without p53 template and no bands were observed as shown in **Figure 12**, lane #2. A single band at 1.095 Kb, slightly above 1 Kb was observed, corresponding to flag-tagged del40-p53 isoform.

The PCR product was phenol chloroform isoamyl alcohol (25:24:1) extracted to remove the protein and other impurities. The PCR amplified product was double digested with BamHI and XhoI enzymes. Gel elution of the sample was done by freeze-thaw method to get the double digested flag tagged-del40-p53 isoform insert. The initial cloning vector used was pFastBac1. Because we will ultimately express our clone in baculovirus expression system, it was necessary to use a shuttle vector in which initial cloning can be done and then cloned gene can be transferred to the baculoviral genome with ease. Shutttle plasmid pFastBac1 has baculovirus homologous sites (Tn7R and Tn7L) inserted in it through which homologous recombination between it and baculoviral genome can occur and gene of interest is transferred to bacmid. The cloning vector pFastBac1 was transformed into DH5α strain and the transformation mixture after revival for 30 minutes with added LB medium at 37^0C temperature and 220 rpm speed was plated onto LB-ampicilline-agar plates, and incubated overnight at 37^0C. Colonies were inoculated into 2 ml LB –ampicilline medium and incubated in incubator shaker overnight at 37^0C temperature and 220 rpm speed. Plasmid DNA isolated by standard minipreparation protocol, was double digested with BamHI and XhoI restriction enzymes and gel eluted. The double digested and gel eluted flag tagged-del40p53 isoform was ligated into the double digested and gel eluted pFastBac1 vector in the ratio of 3:1 (insert: vector ratio). Ligation was done at 16^0C temperature for 16 hrs in 10 µl reaction volume. The ligation mixture was transformed into the DH5α competent cells and the whole mixture was plated onto ampicilline-LB agar plate. Plate was incubated overnight at 37^0C temperature in inverted position. 11 colonies were screened for possible recombinant clones. The recombinant clones were first screened by colony PCR. Colony PCR was

done by first preparing the master mixture of primers, buffer, dNTPs and MgCl2. Volume of the reaction mixture was limited to 24.5 µl by adding double distilled water. In each PCR tube a single colony, as source of template, was just touched by autoclaved tip (also replica plate was made on fresh ampicilline-LB agar plates), and finally 0.5 µl taq polymerase enzyme (kind gift from Dr. S.K. Dhar,s laboratory, SCMM, JNU, India) was added to the reaction mixture. PCR machine was set and run immediately and cycle numbers were limited to 25 cycles only as we had to check only size of the amplified product. All the colony PCR products were run on 0.8% agarose gel. In some colonies the PCR amplified product was found to be on the right position of 1.095 Kb corresponding to the flag tagged del40-p53 isoform. Colony number 1, which gave the intense band at the right position of 1.095 Kb on lane number 3 (**Figure 13**), was inoculated into 2 ml of ampicilline-LB medium and grown overnight at 37^0C temperature and 220 rpm speed. Plasmid DNA minipreparation was done by standard protocol. The plasmid DNA preparation was checked on 0.8% agarose gel for quantification. The plasmid DNA was subjected to BamHI and XhoI enzyme digestion to check the presence of the insert. The sample was loaded on to 0.8% agarose gel and flag tagged-del40-p53 isoform fall-out was found to be at right position of 1.095 Kb (**Figure 14**). The flag-tagged del40-p53 isoform clone was also confirmed by the PCR ampilification by using its isolated plasmid DNA as template in the PCR reaction. 25 µl PCR reaction was set same way as for the colony PCR above but in this case flag-tagged del40-p53 isoform plasmid DNA was used as template for the amplification which gave the amplified product at the right position, 1.095 Kb corresponding to the flag-tagged del40-p53 isoform (**Figure 15**). The del40-p53 clone was also confirmed by sequencing data.

5.1.4 Transposing Flag-tagged del40-p53 isoform from pFastBac1 to bacmid to get recombinant Flag-tagged-del40-p53 isoform-baculovirus.

As we will use baculovirus expression system for protein expression, the initially cloned gene was transferred into baculoviral genome. This was done by transforming competent baculoviral genome-containing DH10Bac strain by recombinant baculovirus shuttle plasmid (in our case pFastBac1-flag-tagged del40-p53 isoform). The transformation mixture after revival was plated onto Gentamicin (µg/ml), Kanamycin (50µg/ml), Tetracycline (10µg/ml), X-gal (100µg/ml), IPTG (µg/ml) and LB agar plates because

kanamycin, tetracycline resistance and galactosidase activity is conferred onto the DH10Bac cell by baculoviral genome, and after homologous recombination between pFastBac1 and baculoviral genome through Tn7R and Tn7L sites, gentamicin resistance is conferred to it. IPTG was used as inducer of beta galactosidase enzyme. The plates were incubated for 24 hours at 37^{0}C temperature and 220 rpm speed. Two types of colonies were visible, blue and white. Two large and isolated white colonies were marked and used as template for colony PCR to look for the correct insert. The colony PCR products were run on 0.8% agarose gel. The PCR amplified products were observed at 1.095 Kb, corresponding to the position of the flag-tagged del40-p53 isoform (**Figure 16**). The two colony PCR confirmed colonies were selected and inoculated into 2 ml Gentamicin (7µg/ml), Kanamycin (50µg/ml), Tetracycline (10µg/ml)-LB broth medium to isolate the recombinant bacmid DNA by standard minipreparation protocol. The concentration of recombinant bacmid DNA was checked on 0.5% agarose gel overnight at 24 volts because it is high molecular weight and will not migrate through high strength gels used for smaller plasmids. The Bacmid minipreparation DNA was digested with BamHI and XhoI restriction enzymes and run on 0.8% agarose gel. The flag tagged del40-p53 isoform fall-out was found to be at right position on the gel (**Figure 17**) at 1.095 kb.

5.1.5 Cloning of Flag-tagged del133-p53 isoform:

Del133- p53 isoform was PCR amplified as flag tagged product from the pcDNA3-p53 full-length clone. The PCR conditions were optimized with respect to the annealing temperature, cycle numbers and the length of the respective phases of the cycle. PCR annealing temperature was calculated and taken as 40^{0}C. At this temperature lot of non-specific bands were observed (**Figure 11**, Lane 4). To overcome this problem, PCR amplification was done at three different temperatures 40^{0}C, 45^{0}C and 55^{0}C. At 55^{0}C non-specific bands were not observed. For PCR amplification, 55^{0}C was taken as the annealing temperature. The PCR product was run on the 0.8% agarose gel and the PCR amplified product was running slightly above the 0.75 Kb band in the marker, corresponding to the 0.816 kb size of the flag-tagged del133-p53 isoform. While in negative control (without template) no band was found (**Figure 18**).

The PCR product was phenol chloroform isoamyl alcohol (25:24:1) extracted to remove the polymerase enzyme used in PCR reaction and also the other impurities. The PCR product was subjected to double digestion with BamHI and XhoI restriction enzymes. Gel elution of the sample was done by freeze-thaw method to get the double digested flag-tagged del133-p53 isoform insert. The insert was ligated into BamHI and XhoI enzyme digested, gel eluted pFastBac1 vector. Ligation was done at 16^0C temperature for 16 hrs in 10 µl reaction volume and the ligation mixture was transformed into DH5α competent cells. Whole transformation mixture was plated onto LB-Ampicilline agar plate. Plate was incubated overnight at 37^0C temperature. Next day colonies appeared and colony PCR was done to look for the recombinant colonies. Colony PCR products were run on 0.8% agarose gel (**Figure 19**). Colony PCR was positive for only two colonies and along with the PCR confirmed recombinant colonies, one negative colony was grown overnight in 2 ml LB-Ampicilline medium at 37^0C temperature and 220 rpm speed. Plasmid DNA was prepared by standard minipreparation protocol. The plasmid DNA was used as template for the PCR amplification of the flag-tagged del133-p53 isoform. The PCR products were run on 0.8% agarose gel and flag-tagged del133-p53 isoform amplified product was at the correct position of 0.816 Kb (**Figure 20**). The plasmid DNA was subjected to BamHI and XhoI enzyme digestion. The pFastBac1 backbone and the flag-tagged del133-p53 isoform insert were at right position but double enzyme digestion was incomplete as indicated by the arrows (**Figure 21**, lane 4). The results were cross-checked by increasing the concentration of both the enzymes in the reaction mixture. Increase in the enzyme concentration has no effect as shown in **figure 22**.Then we thought that there may be problem in XhoI enzyme digestion and we looked for another enzyme site in the vector downstream to the XhoI site. We selected HindIII site and after digestion with HindIII and BamHI, fallout came which was more than 1 Kb (our insert was 0.816 Kb). After so many trials we failed to overcome this problem. We also changed the host cloning strain from DH5α to DH10β but could not alleviate the problem of incomplete digestion. After repeated failure, we changed the primers and did the cloning as stated above. After few trials, finally we succeed in getting the flag-tagged del133-p53 isoform-pFastBac1 clone. Two samples

yielded correct and intense fallout at 0.816 Kb which corresponded to flag-tagged del133-p53 isoform insert (**Figure 23**). One of them was taken for further experiments.

5.1.6 Transposing Flag tagged –p53 (full length) from pFastBac1 to bacmid to get recombinant flag-tagged- p53.

The pFastBac1-p53 full-length clone was BamHI and XhoI enzyme digested and the sample was run on 0.8% agarose gel to check the position of the p53 full-length isoform fallout. The fallout came at the correct position at 1.215 Kb. The pFastBac1-flag-tagged full-length p53 recombinant transfer vector was transformed into DH10Bac competent cells. The transformation mixture was plated onto Gentamicin (7µg/ml)+ Kanamycin (50µg/ml)+Tetracycline (10µg/ml)+ X-gal (100µg/ml)+ IPTG (40µg/ml)+LB agar plates. Two types of colonies appeared, blue and white. Two large isolated white colonies were selected, picked and inoculated into 2 ml Gentamicin (7µg/ml), Kanamycin (50µg/ml), Tetracycline (10µg/ml)-LB broth medium and minipreparation of bacmid DNA was done using standard protocol. The Bacmid minipreparation DNA was run at 24 volts overnight to check the concentration. It was digested with BamHI and XhoI restriction enzymes and run on 0.8% agarose gel. The flag-tagged p53-full length isoform fallout was found to be at right position on the gel at 1.215 Kb (**Figure 24**).

5.2 Discussion

TP53 protein has a modular organization comprising N-terminal transactivation, central DNA-binding and C-terminal oligomerization domains (**Figure 4**). Proper functioning of TP53 protein requires that all the domains be intact. Also the N-terminal domain of the p53 is consisted of two sub-domains, TA1 and TA2. The p53 isoforms may contain either both of them, only TA2 or none of them depending upon the mechanisms of alternative translation and alternative promoter utilization. So it seems that transactivation domain deleted isoforms cannot activate transcription of target genes although they are capable of DNA binding and oligomerization. We hypothesize that if these transactivation domain-deleted isoforms (whether fully or partially deleted of TA1 and TA2) replace any of the sub-units of the functional homotetramer, transcription repression may occur.

The aim of this study was to clone the p53 isoforms, del40- and del133-p53 into the initial cloning vector pFastBac1. This initial cloning vector was a baculovirus

shuttle plasmid whose feautures were exploited to introduce our gene of interests into the baculoviral genome so that baculovirus system can be used for protein expression.

Del40-53 isoform was cloned as the flag-tagged gene into the baculovirus shuttle plasmid, pFastBac1 vector. This isoform was PCR amplified with specific primers form the pcDNA3-p53 template containing the full-length p53 gene. The clone was confirmed by colony PCR, BamHI and XhoI enzyme digestion and PCR of recombinant plasmid. Final confirmation of the clone was done with sequencing (by Lab India) After cloning into the shuttle vector, flag-tagged del40-p53 isoform gene was transferred from cloning vector to the baculoviral genome using standard transposition protocol to get the recombinant bacmid DNA for insect cell transfection. The recombinant bacmid DNA was confirmed by colony PCR and BamHI and XhoI enzyme digestion.

Del133-53 isoform was also cloned as the flag-tagged gene into the baculovirus shuttle plasmid, pFastBac1 vector. This isoform was PCR amplified with specific primers form the pcDNA3-p53 template. Initially we encountered huge problems in cloning this isoform. Colony PCR and PCR of plasmid preparations were confirming the recombinant clones, as does the shift of plasmid DNA compared to that of the vector alone. But BamHI and XhoI enzyme digestion was not giving any fall-out. Then we thought that may be there is some problem in XhoI digestion and we took HindIII restriction site downstream to the XhoI in the vector for digestion. On BamHI and HindIII enzyme digestion we got strange result as fallout came at more than 1 Kb position (our insert was 0.816 Kb). We tried hard for long time again and again and got some positive hope. We could clone this isoform but there was a big problem of partial double digestion. When subjected to BamHI and XhoI enzyme digestion, the fallout and the backbone were at correct position on the agarose gel but digestion was incomplete. We changed strains from DH5α to DH10βfor plasmid isolation but could not alleviate partial digestion problem. Also we did phenol chloroform isoamyl alcohol (25:24:1) twice to remove all the proteins as we thought that may be some proteins bind to the restriction sites, but failed to correct this problem. Finally we changed primers and did few cloning trials, and we got the flag-tagged del133-p53 isoform-pFastBac1 clone. We confirmed this clone with colony PCR, double digestion and sequencing.

The full-length p53 isoform in pFastBac1-p53 clone was transferred to the baculoviral genome using standard transposition protocol to get the recombinant p53-bacmid DNA. Recombinant p53-bacmid DNA was confirmed by BamHI and XhoI enzyme digestion.

CLUSTAL W (1.83) multiple sequence alignment

```
p53<        -----MEEPQSDPSVEPPLS----QETFSDLWKLLPENNVLSPLPSQAMDDLMLS----- 46
p73<        MAQSTATSPDGGTTFEHLWSSLEPDSTYFDLPQSSRGNNEVVGGTDSSMDVFHLEGM--T 58
p63<        -MEVPQPEPAPGSALSPAGVCGG-AQRPGHLPGLLLGSHGLLGSPVRAAASSPVTTLTQT 58
                  .*  ..:..          .   .*       .: :   .  :     :

p53<        PDDIEQWFTEDPGPDEAPRMPEAAPRVAPAPAAPTP-AAPAPAPS---WPLSSS-----V 97
p73<        TSVMAQFNLLSSTMDQMSSRAASASPYTPEHAASVPTHSPYAQPS---STFDTMSPAPVI 115
p63<        MHDLAGLGSRSRLTHLSLSRRASESSLSSESSESSDAGLCMDSPSPMDPHMAEQTFEQAI 118
               :      .   .       : .  :.  : .          **    :       :

p53<        PSQKTYQGSYGFRLGFLHSGTAKSVTCTYSPALNKMFCQLAKTCPVQLWVDSTPPPGTR- 156
p73<        PSNTDYPGPHHFEVTFQQSSTAKSATWTYSPLLKKLYCQIAKTCPIQIKVSTPPPPGTA- 174
p63<        QAASRIIRNEQFAIRRFQSMPVR--LLGHSPVLRNITNSQAPDGRRKSEAGSGAASSSGE 176
             : .        * :   :* ..:      :** *.::  . *      :  ..: ....:

p53<        -------VRAMAIYKQSQHMTEVVRRCPHHERCSDSD--GLAPPQHLIRVEGNLRVEYLD 207
p73<        -------IRAMPVYKKAEHVTDVVKRCPNHELGRDFNEGQSAPASHLIRVEGNNLSQYVD 227
p63<        DKENDGFVFKMPWKPTHPSSTHALAEWASRREAFAQRP-SSAPDLMCLSPDRKMEVEELS 235
                   :  *.         *..: . . :.           **    :  : :   : :.

p53<        DRNTFRHSVVVPYEPPEVGSDCTTIHYNYMCNSSCMG-GMN-RRPILTIITLEDSSGNLL 265
p73<        DPVTGRQSVVVPYEPPQVGTEFTTILYNFMCNSSCVG-GMN-RRPILIIITLEMRDGQVL 285
p63<        PLALGRFSLTPAEGDTEEDDGFVDILESDLKDDDAVPPGMESLISAPLVKTLEKEEEKDL 295
                 * *:. .   .: .   . *  . : :...:  **:   .   : ***  . : *

p53<        GRNSFEVRVCACPGRDRRTEEENLRKKGEPHHELPP--GSTKRALP--NNTSSSPQP--- 318
p73<        GRRSFEGRICACPGRDRKADEDHYREQQALNESSAKNGAASKRAFK--QSPPAVPALGAG 343
p63<        VMYSKCQRLFRSPSMPCSVIRPILKRLERPQDRDTPVQNKRRRSVTPPEEQQEAEEPKAR 355
               *   *:  .*.    . .   :.    :.  .      :*:.   :.

p53<        --KKKPLDGEYFTLQIRGRERFEMFRELNEALELKDAQ---------------------- 354
p73<        VKKRRHGDEDTYYLQVRGRENFEILMKLKESLELMELVPQPLVDSYRQQQQLLQRPSHLQ 403
p63<        VLRSKSLCHDEIENLLDS-DHRELIGDYSKAFLLQTVD---------------------- 392
              : :    :     : . :. *:: . .::: *

p53<        ----------------------------AGKEPGGSRAHSS-------------HLKSKK 373
p73<        PPSYGPVLSPMNKVHGGMNKLPSVNQLVGQPPPHSSAATPNLGPVGPGMLNNHGHAVPAN 463
p63<        ----------------------------GKHQDLKYISPET------------MVALLT 411
                                        *:    .   .                    .

p53<        GQSTSRHKKL-----------------------------------MFKTEGPDSD------ 393
p73<        GEMSSSHSAQSMVSGSHCTPPPPYHADPSLVSFLTGLGCPNCIEYFTSQGLQSIYHLQNL 523
p63<        GKFSNIVDKFVIVDCRYP-------------------------YEYEGGHIKTAVNLPLE 446
            *: :.  .                                      :     .:

p53<        ------------------------------------------------------------
p73<        TIEDLGALKIPEQYRMTIWRGLQDLKQGHDYSTAQQLLRSSNAATISIGGSGELQRQRVM 583
p63<        RDAESFLLKSPIAPCSLDKRVILIFHCEFSSERGPRMCRFIRERDRAVNDYPSLYYPEMY 506


p53<        ------------------------------------------------------------
p73<        EAVHFRVRHTITIPNRGGP-GGGPDEWADFGFDLPDCKARKQPIKEEFTEAEIH------ 636
p63<        ILKGGYKEFFPQHPNFCEPQDYRPMNHEAFKDELKTFRLKTRSWAGERSRRELCDRLQDQ 566
```

Figure 9a . Comparison of the amino acids sequences of the p53, p63 and p73 proteins. Amino acid sequences were aligned using clustalW. * indicates identical amino acids in all three isoforms; : indicates strongly conserved amino acids and . indicates weakly conserved amino acid.

CLUSTAL W (1.83) multiple sequence alignment

```
p53<        MEEPQSDPSVEPPLSQETFS-------DLWKLLPENNVLSPLPSQAMDDLMLSPDDIEQW 53
p63<        MEVPQPEPAPGSALSPAGVCGGAQRPGHLPGLLLGSHGLLGSPVRAAASSPVTT--LTQT 58
            ** **.:*:  ..**   ..       .*  **  .: *   * :*  .  ::.  : *

p53<        FTEDPGPDEAPRMPEAAPRVAPAPAAPTPAAPAPAPSWPLSSSVPSQKTYQGSYGFRLGF 113
p63<        MHDLAGLGSRSRLTHLSLSRRASESSLSSESSESSDAGLCMDSPSPMDPHMAEQTFEQAI 118
            : : .* .. .*:.. :    .: :: :. :. .: :    .* .. ..: ..  *. .:

p53<        LHSGTAKSVTCTYSPALNKMFCQLAKTCPVQLWVDSTPPPGTRVRAMA------------ 161
p63<        QAASRIIRNEQFAIRRFQSMPVRLLGHSPVLRNITNSQAPDGRRKSEAGSGAASSSGEDK 178
              :.            ::.*  :*   .**   : .: .*. * :: *

p53<        -----IYKQ------SQHMTEVVRRCPHHERCSDSDGLAPPQHLIRVEGNLRVEYLDDRN 210
p63<        ENDGFVFKMPWKPTHPSSTHALAEWASRREAFAQRPSSAPDLMCLSPDRKMEVEELSPLA 238
                 ::*       ..    :.. ..::*  ::  . **    :  : ::.** *.

p53<        TFRHSVVVPYEPPEVGSDCTTIHYNYMCNSSCMG-GMN-RRPILTIITLEDSSGNLLGRN 268
p63<        LGRFSLTPAEGDTEEDDGFVDILESDLKDDDAVPPGMESLISAPLVKTLEKEEEKDLVMY 298
              *.*:. .   .* ... . *  . : :...:  **:   .   : ***... : *

p53<        SFEVRVCACPGRDRRTEEENLRKKGEPHHELPPGSTKR----ALPNNTSSSPQPKKKPLD 324
p63<        SKCQRLFRSPSMPCSVIRPILKRLERPQDRDTPVQNKRRRSVTPPEEQQEAEEPKARVLR 358
            *   *:  .*.    . .  *::  .*:.. .* ..**    : *:: ..: :** : *

p53<        G-------------------------EYFTLQIRGRER-------------FEMFRELN 345
p63<        SKSLCHDEIENLLDSDHRELIGDYSKAFLLQTVDGKHQDLKYISPETMVALLTGKFSNIV 418
            .                         ::   : *:.:                 * ::

p53<        EALELKDAQAGKEPGG-----------SRAHSSHLKSKKGQSTSRHKKLMFKTEGPDSD 393
p63<        DKFVIVDCRYPYEYEGGHIKTAVNLPLERDAESFLLKSPIAPCSLDKRVILIFHCEFSSE 478
            : : : *.:   *  *              *.*  ***  . .:  :: :::     .*:

p53<        ------------------------------------------------------------
p63<        RGPRMCRFIRERDRAVNDYPSLYYPEMYILKGGYKEFFPQHPNFCEPQDYRPMNHEAFKD 538

p53<        ----------------------------
p63<        ELKTFRLKTRSWAGERSRRELCDRLQDQ 566
```

Figure 9b. Comparison of the amino acids sequences of the p53 and p63 proteins. Amino acid sequences were aligned using clustalW. * indicates identical amino acids in all three isoforms; : indicates strongly conserved amino acids and . indicates weakly conserved amino acid.

CLUSTAL W (1.83) multiple sequence alignment

```
p53<            -----MEEPQSDPSVEPPLS----QETFSDLWKLLPENNVLSPLPSQAMDDLMLS---PD 48
p73<            MAQSTATSPDGGTTFEHLWSSLEPDSTYFDLPQSSRGNNEVVGGTDSSMDVFHLEGMTTS 60
                       .*:...:.*   *    :.*: ** :    ** :   ...:** : *.   ..

p53<            DIEQWFTEDPGPDEAPRMPEAAPRVAPAPAAPTP-AAPAPAPSWPLSSS-----VPSQKT 102
p73<            VMAQFNLLSSTMDQMSSRAASASPYTPEHAASVPTHSPYAQPSSTFDTMSPAPVIPSNTD 120
                 : *:   ..  *: .  . :*.  :*  **..*  :* . ** .:.:      :**:.

p53<            YQGSYGFRLGFLHSGTAKSVTCTYSPALNKMFCQLAKTCPVQLWVDSTPPPGTRVRAMAI 162
p73<            YPGPHHFEVTFQQSSTAKSATWTYSPLLKKLYCQIAKTCPIQIKVSTPPPPGTAIRAMPV 180
                * *.: *.: * :*.****.* **** *:*::**:*****:*: *.:.***** :***.:

p53<            YKQSQHMTEVVRRCPHHERCSDSD--GLAPPQHLIRVEGNLRVEYLDDRNTFRHSVVVPY 220
p73<            YKKAEHVTDVVKRCPNHELGRDFNEGQSAPASHLIRVEGNNLSQYVDDPVTGRQSVVVPY 240
                **:::*:*:**:***:**   * :    **..********   :*:**  * *:******

p53<            EPPEVGSDCTTIHYNYMCNSSCMGGMNRRPILTIITLEDSSGNLLGRNSFEVRVCACPGR 280
p73<            EPPQVGTEFTTILYNFMCNSSCVGGMNRRPILIIITLEMRDGQVLGRRSFEGRICACPGR 300
                ***:**:: *** **:******:********* *****  .*::***.*** *:******

p53<            DRRTEEENLRKKGEPHHELPP--GSTKRALPNNTSSSPQP-----KKKPLDGEYFTLQIR 333
p73<            DRKADEDHYREQQALNESSAKNGAASKRAFKQSPPAVPALGAGVKKRRHGDEDTYYLQVR 360
                **:::*:: *::   :.. .   .::***: :...: *       *::  * : : **:*

p53<            GRERFEMFRELNEALELKDAQ------------------------------------- 354
p73<            GRENFEILMKLKESLELMELVPQPLVDSYRQQQQLLQRPSHLQPPSYGPVLSPMNKVHGG 420
                ***.**:: :*:*:*** :

p53<            ----------AGKEPGGSRAHSS--------------HLKSKKGQSTSRHKKL------- 383
p73<            MNKLPSVNQLVGQPPPHSSAATPNLGPVGPGMLNNHGHAVPANGEMSSSHSAQSMVSGSH 480
                          .*: *  * * :.             *  . :*: :* *.

p53<            ---------------------------MFKTEGPDSD----------------------- 393
p73<            CTPPPPYHADPSLVSFLTGLGCPNCIEYFTSQGLQSIYHLQNLTIEDLGALKIPEQYRMT 540
                                          *.::* :*

p53<            ------------------------------------------------------------
p73<            IWRGLQDLKQGHDYSTAQQLLRSSNAATISIGGSGELQRQRVMEAVHFRVRHTITIPNRG 600


p53<            ------------------------------------
p73<            GPGGGPDEWADFGFDLPDCKARKQPIKEEFTEAEIH 636
```

Figure 9c. Comparison of the amino acids sequences of the p53 and p73 proteins. Amino acid sequences were aligned using clustalW. * indicates identical amino acids in all three isoforms; : indicates strongly conserved amino acids and . indicates weakly conserved amino acid.

CLUSTAL W (1.83) multiple sequence alignment

```
p63<            MEVPQPEPAPG--------SALSPAGVCGGAQRPGHLPGLLLGSHG-----LLGSPVRAA 47
p73<            MAQSTATSPDGGTTFEHLWSSLEPDSTYFDLPQSSRGNNEVVGGTDSSMDVFHLEGMTTS 60
                *  . . .. *        *:*.* ..  .  :..:  . ::*. .     :  . : ::

p63<            ASSPVTTLTQTMHDLAGLGSRSRLTHLSLSRRASESSLSSESSESSDAGLCMDSPSPMDP 107
p73<            VMAQFNLLSSTMDQMSSRAASASPYTPEHAASVPTHSPYAQPSSTFDTMSPAPVIPSNTD 120
                . : .. *:.**.:::. .: :     . :  ..  *  ::.*.: *:       ..

p63<            HMAEQTFEQAIQAASRIIRNEQFAIRRFQSMPVRLLGHSPVLRNITNSQAP-DGRRKSEA 166
p73<            YPGPHHFEVTFQQSSTAKSATWTYSPLLKKLYCQIAKTCPIQIKVSTPPPPGTAIRAMPV 180
                : . : ** ::* :*             ::.:  ::   .*:  :::.. .*  . *   .

p63<            GSGAASSSGEDKENDGFVFKMPWKPTHPS-STHALAEWASRREAFAQRPSSAPDLMCLSP 225
p73<            YKKAEHVTDVVKRCPNHELGRDFNEGQSAPASHLIRVEGNNLSQYVDDPVTGRQSVVVPY 240
                 . *   :.  *.  .. :   ::  :.: ::* :   ... . :.: * :. : : :.

p63<            DRKMEVEELSPLALGRFSLTPAEGDTEEDDGFVDILESDLKDDDAVP----------PG 274
p73<            EPPQVGTEFTTILYNFMCNSSCVGGMNRRP-ILIIITLEMRDGQVLGRRSFEGRICACPG 299
                :      *::.:  . :. :.. *. :.   :: *:  :::*.:.:            **

p63<            MESLISAPLVKTLEKEEEKDLVMYSKCQRLFRSPSMPCSVIRPILKRLERPQDRDTPVQN 334
p73<            RDRKADEDHYREQQALNESSAKNGAASKRAFKQSPPAVPALGAGVKKRRHGDEDTYYLQV 359
                 :   .    :  :  :*..    : .:* *:... . ..: . :*: .: ::    :*

p63<            KRRRSVTPPEEQQEAEEPKARVLRSKSLCHDEIENLLDSDHRELIGDYSKAFLLQTVDGK 394
p73<            RGRENFEILMKLKESLELMELVPQPLVDSYRQQQQLLQRPSHLQPPSYG--PVLSPMNKV 417
                : *...    : :*: *    * :.   .: : ::**:   :    .*.   :*..::

p63<            HQDLKYISPETMVALLTGKFSNIVDKFVIVDCRYPYEYEGGHIKTAVNLPLERDAESFLL 454
p73<            HGGMNKLPSVNQLVGQPPPHSSAATPNLGPVGPGMLNNHGHAVPANGEMSSSHSAQSMVS 477
                * .:: :.. . :.  .  .*. .   :         : .*  : :  ::. .:.*:*::

p63<            KS---PIAPCSLDKRVILIFH-------CEFSSERGPRMCRFIRERDRAVNDYPSLYYPE 504
p73<            GSHCTPPPPYHADPSLVSFLTGLGCPNCIEYFTSQG--LQSIYHLQNLTIEDLGALKIPE 535
                 *   * .*   *  :: ::          *: :.:*  :  : : :: :::*  :*  **

p63<            MY--ILKGGYKEFFPQHPNFCEPQDYRPMNHEAFKD-----------ELKTFRLKTRSW 550
p73<            QYRMTIWRGLQDLKQGHDYSTAQQLLRSSNAATISIGGSGELQRQRVMEAVHFRVRHTIT 595
                 *   :  * :::   *      *  *. *  ::.             *   **::

p63<            AGERS----------------------RRELCDRLQDQ--- 566
p73<            IPNRGGPGGGPDEWADFGFDLPDCKARKQPIKEEFTEAEIH 636
                  :*.                      :: : :.: :
```

Figure 9d. Comparison of the amino acids sequences of the p63 and p73 proteins. Amino acid sequences were aligned using clustalW. * indicates identical amino acids in all three isoforms; : indicates strongly conserved amino acids and . indicates weakly conserved amino acid.

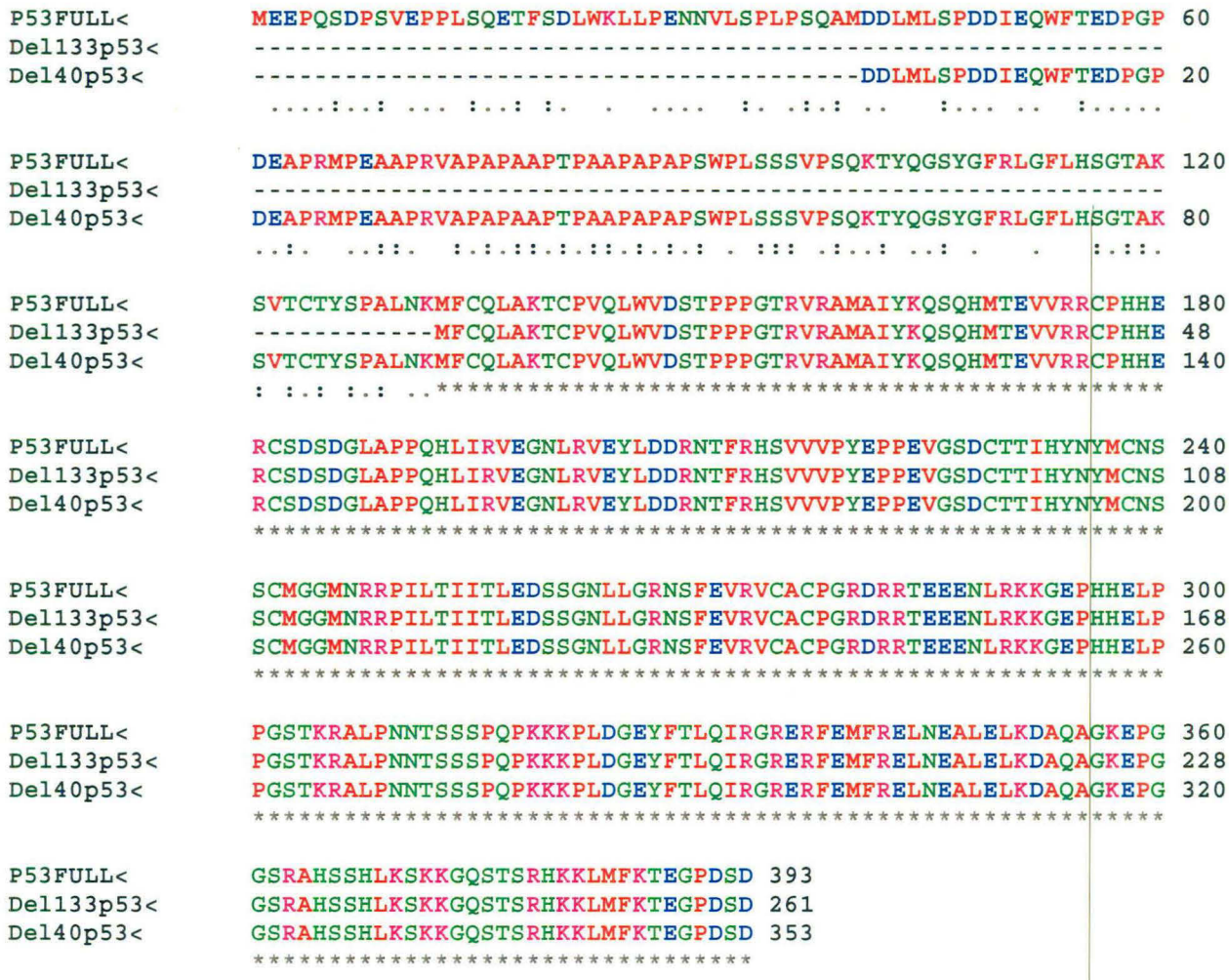

Figure 10. Comparison of the amino acids sequences of the full-length, delta133 and delta40-p53 isofomrs. Amino acid sequences were aligned using clustalW. *indicates identical amino acids in all three isoforms; indicates strongly conserved amino acids and .indicates weakly conserved amino acid.

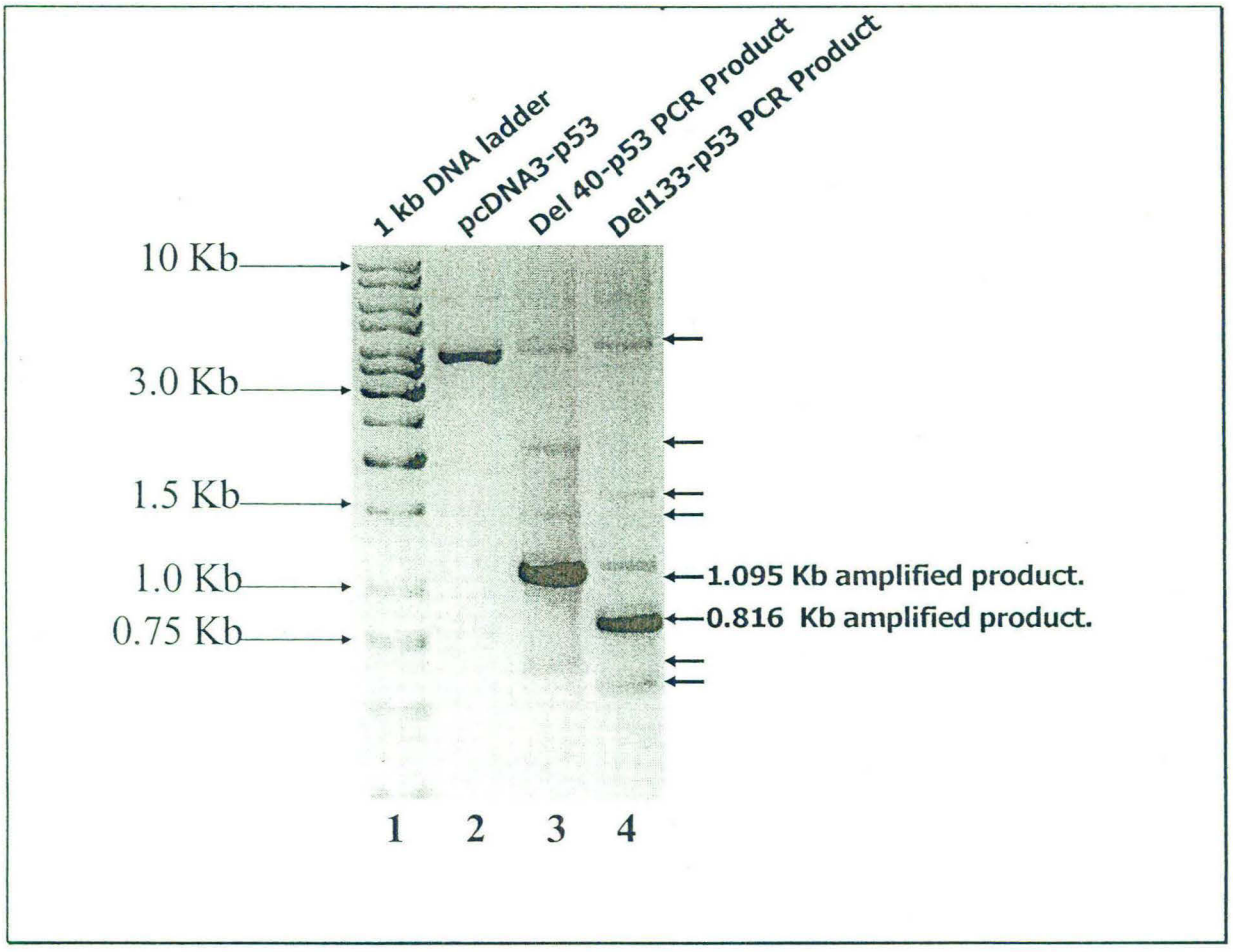

Figure 11. PCR amplification of flag-tagged del40-p53 and del133-p53 isoforms. Using pcDNA3-p53, as the source of template, the PCR amplification of flag-tagged del40-p53 and del133-p53 isoforms was done at 40^0C annealing temparature. Lot of non-specific bands were observed as shown by arrows. Lane 1: 1 Kb DNA marker. Lane 2: pcDNA3-p53 full-length minipreparation DNA (used as template in PCR amplifications for p53 isoform cloning). Lane3: PCR amplified Flag-tagged del40-p53 isoform. Lane 4: PCR amplified Flag-tagged del133-p53 isoform.

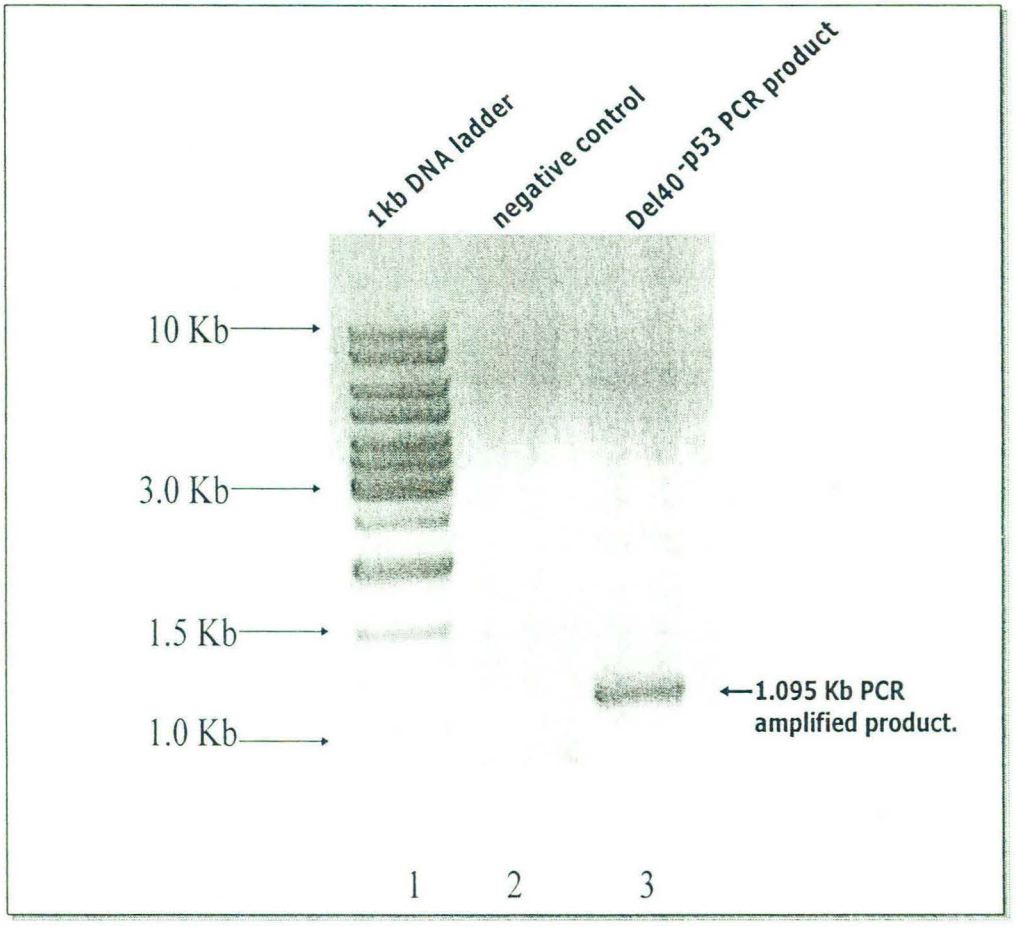

Figure 12. PCR amplification of Flag-tagged del40-p53 isoform.

PCR amplification was done at 55^0C annealing temperature with specific primers to amplify Flag-tagged del40-p53 isoform from pcDNA3-p53 and PCR product was run on 0.8% agarose gel. Lane 1: 1Kb marker; Lane 2: negative control (without template). Lane 3: Flag tagged-del40-p53 PCR product.

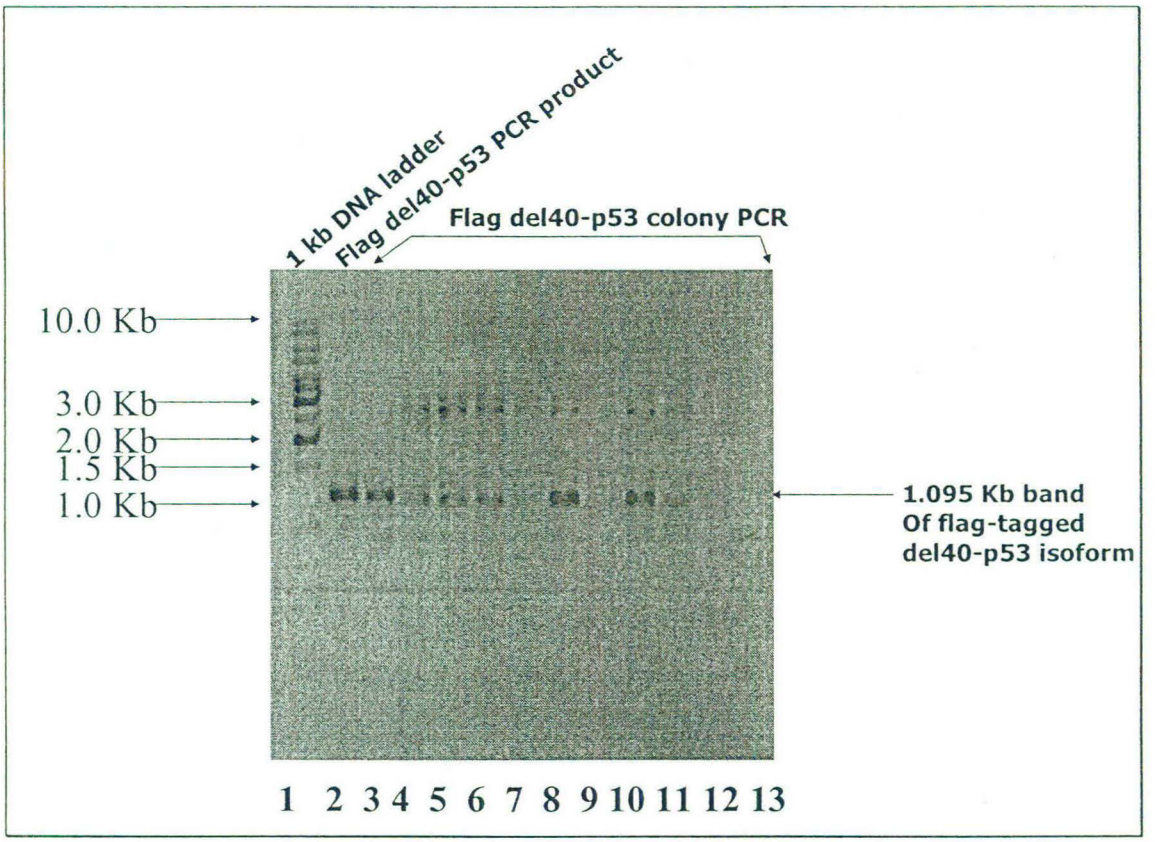

Figure 13. Colony PCR of flag-tagged del40-p53 isoform recombinant colonies. Colony PCR was performed to look for the recombinant clones after ligation mix was transformed in DH5α strains. 11 colonies were picked and used as template source with one colony per PCR reaction by gently touching with autoclaved tip, and PCR reactions were set. In some colony PCR amplified samples, a sharp band was found at 1.095 Kb position on 0.8% agarose gel corresponding to the flag-tagged del40-p53 isoform. Lane 1: 1Kb marker; Lane 2: Flag-tagged del40-p53 PCR product as positive control; Lanes 3-13: Amplified colony PCR products from 11 colonies used as template.

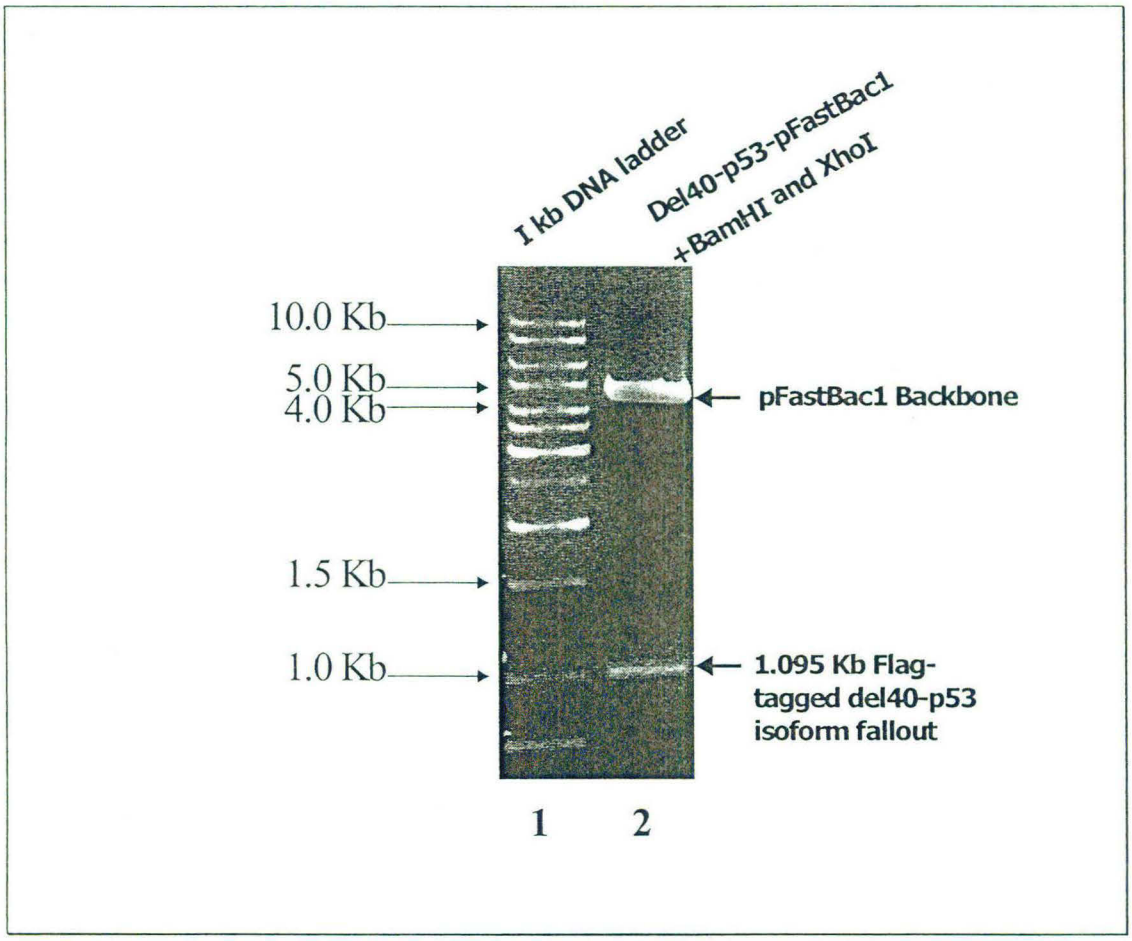

Figure 14. Double digested Flag-tagged-del40-p53 isoform-pFastBac1 clone. Overnight culture of PCR confirmed recombinant colony corresponding to lane #8 of fig. 13 was set in 2ml of ampicilline-LB medium and minipreparation of plasmid DNA was done. Plasmid DNA was subjected to BamHI and XhoI enzyme digestion and the samples were run on 0.8% agarose gel. A sharp band at 1.095 Kb was found corresponding to the flag-tagged del40-p53 isoform. Lane 1: 1Kb marker. Lane 2: Double digested Flag-tagged del40-p53-pFastBac1 clone.

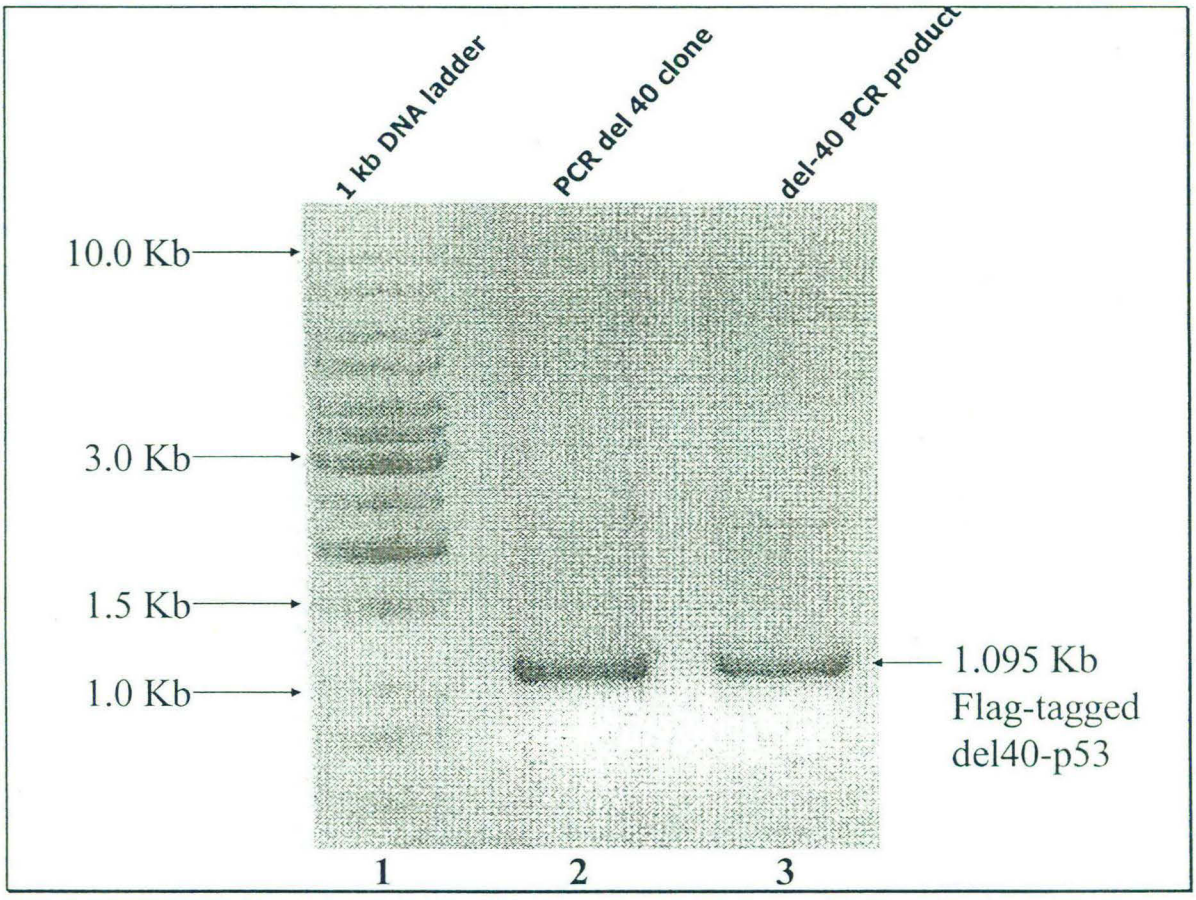

Figure 15. PCR of flag-tagged del40-p53 isoform–pFastBac1 plasmid DNA. Plasmid DNA of Flag-tagged del40-p53 isofrom-pFastBac1 clone was used as template for PCR amplification. Sample was run on 0.8% agarose gel and a sharp band was found to be at 1.095 Kb position corresponding to the flag-tagged del40-p53 isoform amplified product. Lane 1: 1Kb marker. Lane 2: PCR product of Flag tagged-del40-p53 clone. Lane 3: positive control, pcDNA3-p53 was used as the template DNA.

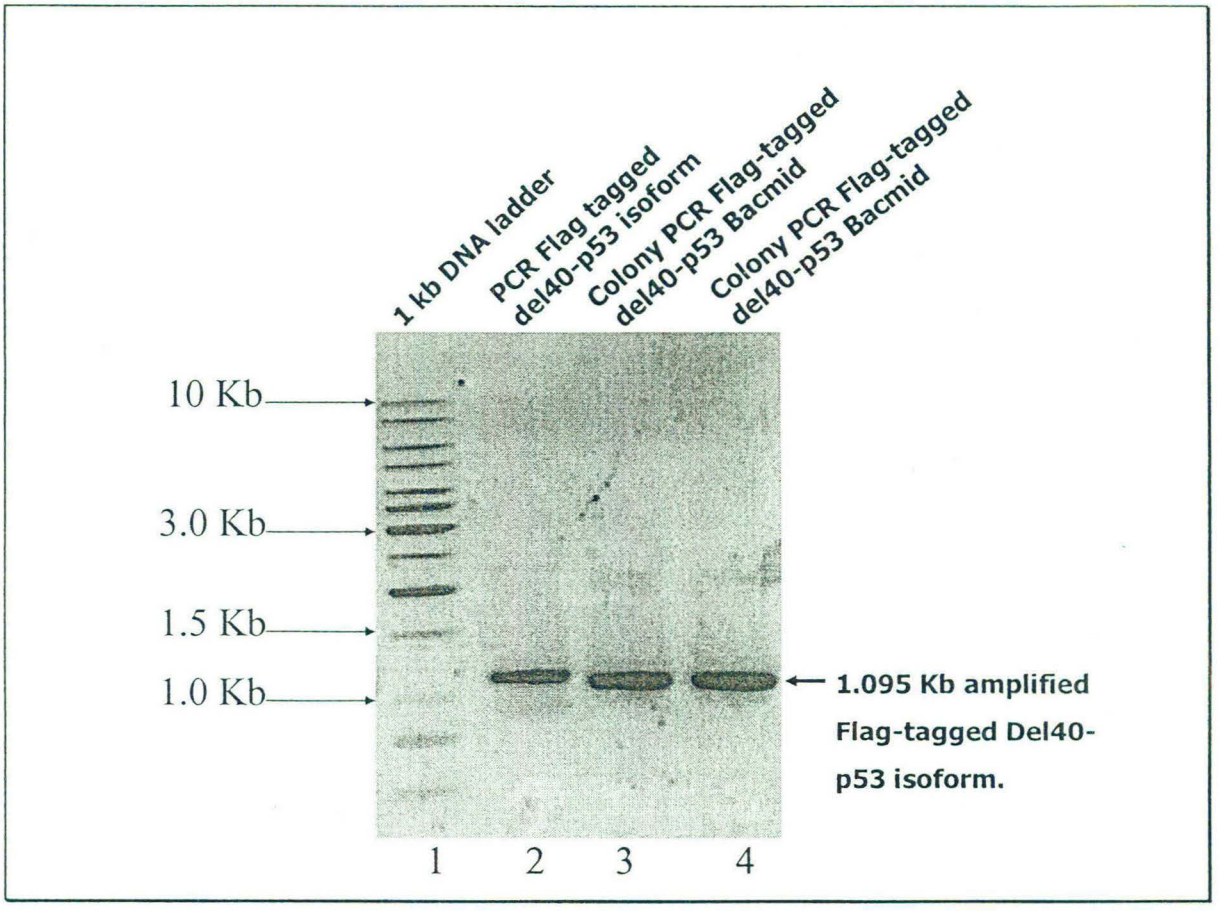

Figure 16. Colony PCR of flag-tagged Del40-p53 isoform recombinant bacmid colonies. DH10Bac strain was transformed with pFastBac1- Flag tagged del40p53 isoform clone. Transformation mixture was plated onto Gentamicin (7µg/ml)+ kanamycin (50µg/ml)+ tetracycline (10µg/ml)+ X-gal (100µg/ml)+ IPTG (40µg/ml) plates and incubated overnight at 37^0C. Two types of colonies appeared, white and blue. Two white colonies were selected as templates for PCR. The PCR amplified product was run on 0.8% agarose gel and a band at 1.095 Kb was found which corresponded to Flag-tagged del40-p53 isoform insert as shown in lanes 3 and 4. Lane 1: 1Kb marker. Lane 2: PCR amplified Flag-tagged del40-p53 from the full-length p53 as positive control.

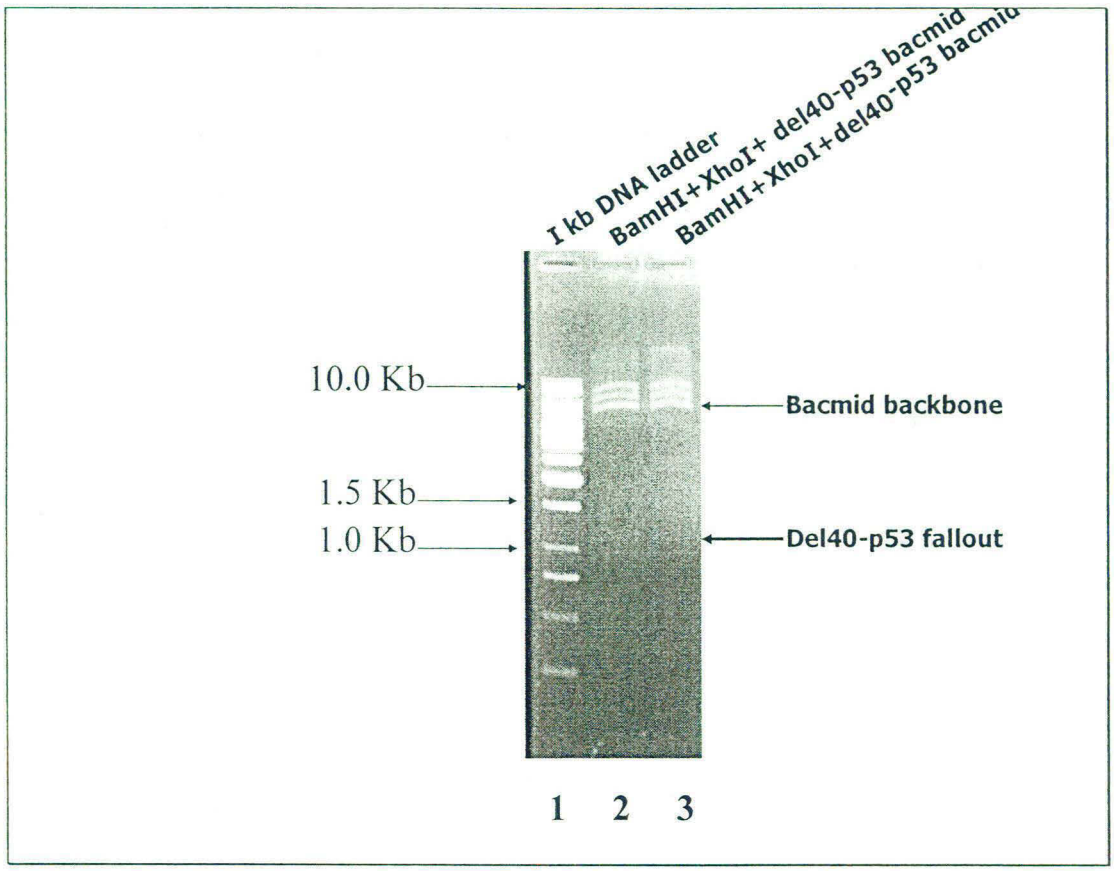

Figure 17. Double digestion of flag tagged del40-p53 isoform bacmid.

PCR confirmed recombinant colonies were grown overnight in LB containing 50 μg/ml kanamycin, 7 μg/ml gentamicin and 10 μg/ml tetracycline. Bacmid were isolated by following the standard minipreparation protocol and double digestion was done with BamHI and XhoI restriction enzymes. Digested DNA was run on 0.8% agarose gel. A band at 1.095 Kb was found which corresponded to Flag-tagged del40-p53 isoform insert. Lane 1: 1Kb marker. Lane 2 and 3: double digested flag del40-p53-bacmid.

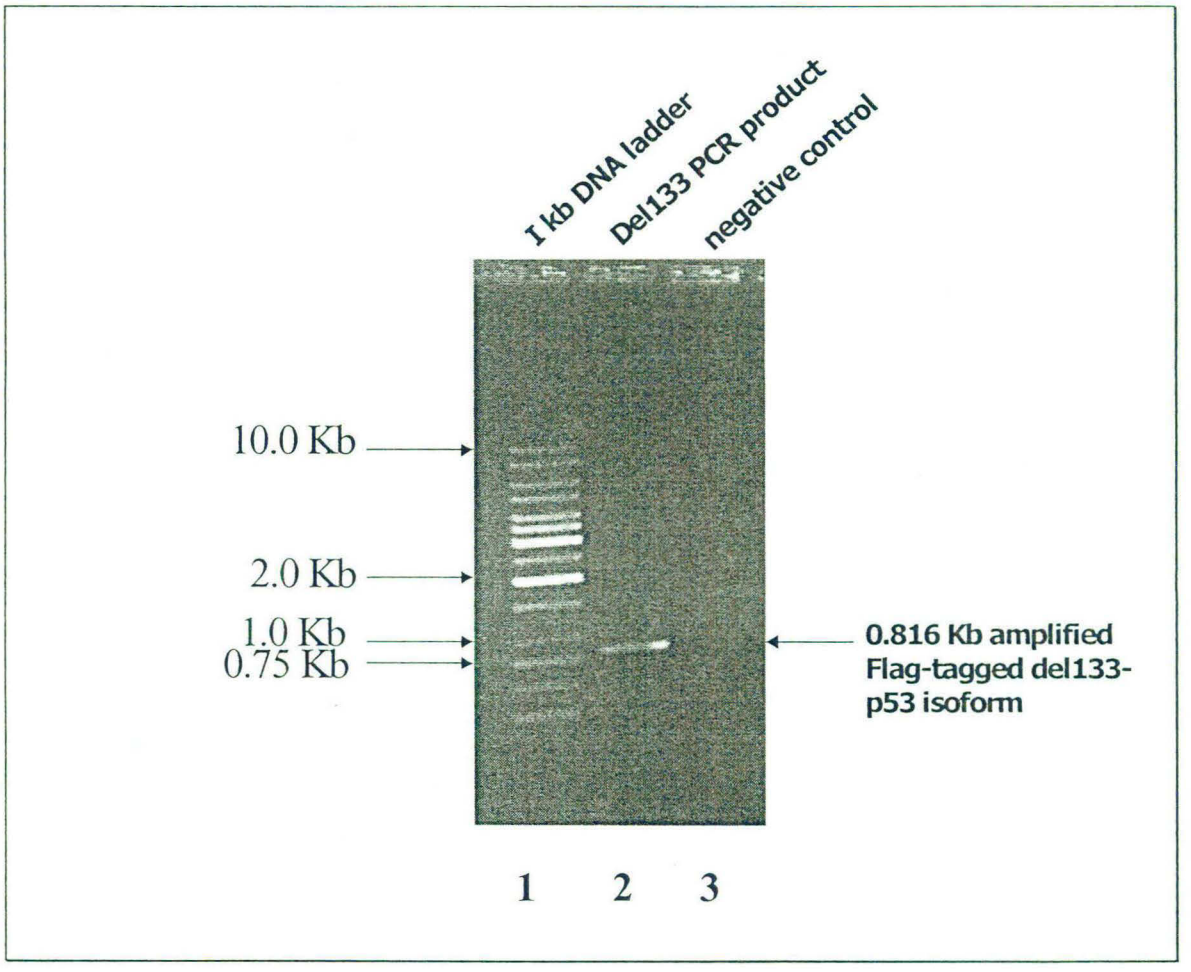

Figure 18. PCR of flag-tagged del133-p53 isoform. PCR was done with specific primers to amplify del133-p53 isoform as flag-tagged gene. 0.8% agarose gel was run and a sharp band was found at 0.816 Kb which corresponded to the flag-tagged del133-p53 isoform. Lane 1: 1 Kb Marker. Lane 2: PCR amplified Flag-tagged del133-p53 isoform. Lane 3: negative control, PCR without template DNA.

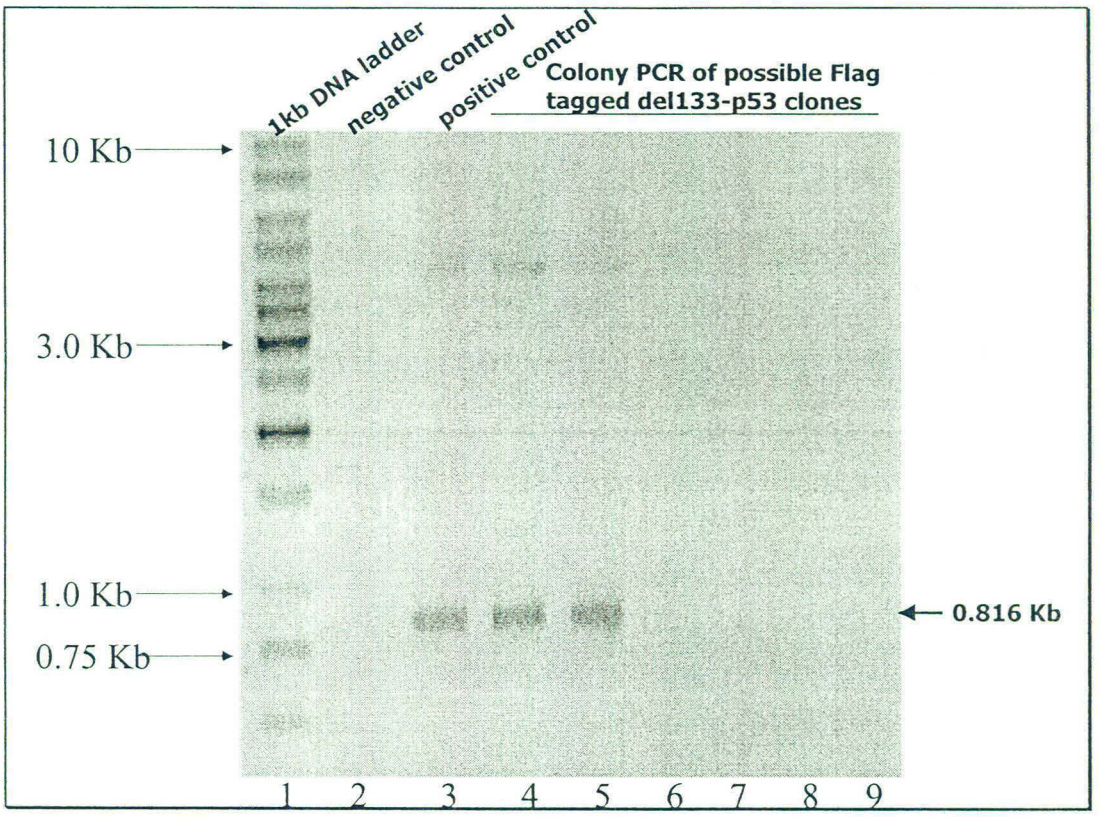

Figure 19. Colony PCR of Flag-tagged del133-p53 isoform-pFastBac1 clones. Colony PCR was performed to look for the recombinant clones after ligation mixture was transformed in DH5alpha strains of *E. coli*. Reactions were set and colonies were used as template source by gently touching with autoclaved tips. Six colonies were picked for recombinant clone screening. Samples were loaded on 0.8% agarose gel. PCR amplified product of 0.816 Kb, which is the size of the flag-tagged del133-p53 isoform, was observed in lane 4 and 5. Lane 1: 1Kb marker. Lane 2: negative control (PCR without template). Lane 3: Flag-tagged del133-p53 isoform PCR product as positive control. Lanes 4-9: Amplified colony PCR products of six colonies used as template in colony PCR reactions.

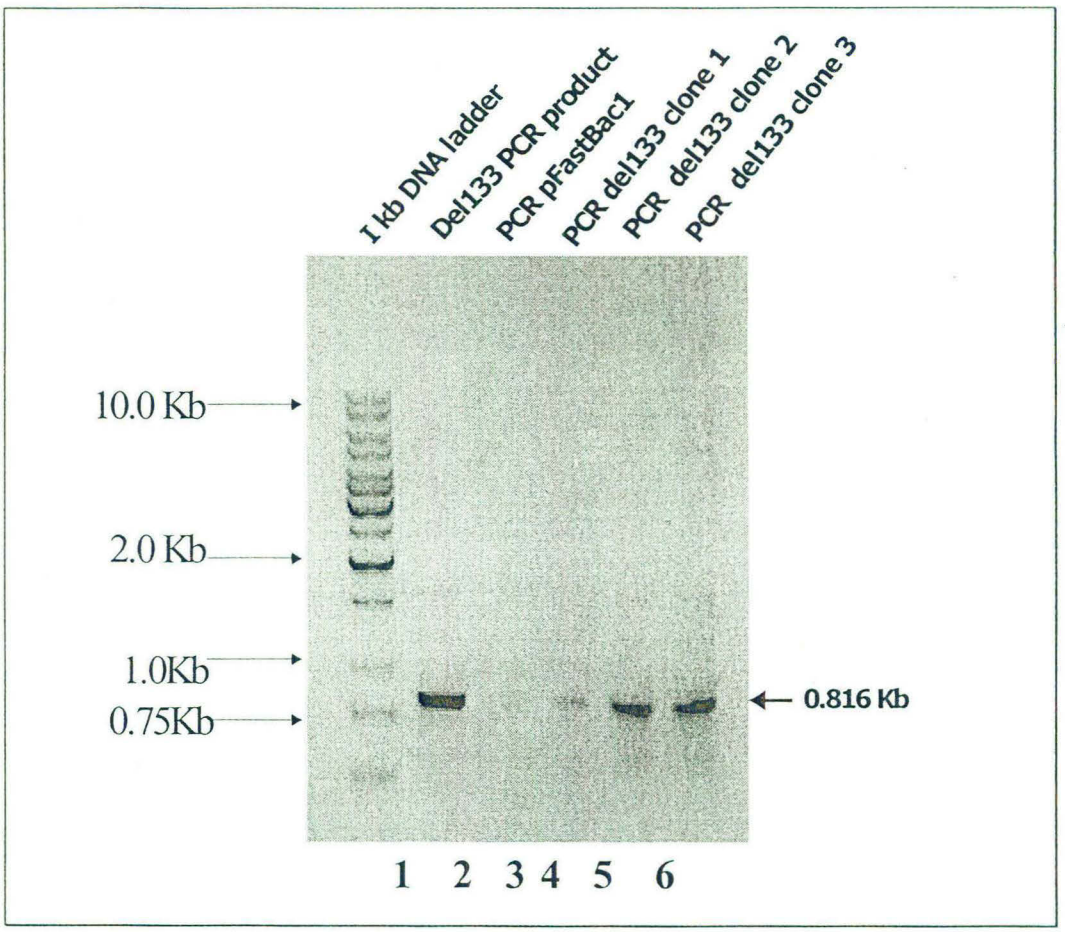

Figure 20. PCR flag-tagged del133-p53 isoform plasmid DNA. Recombinant colonies were inoculated in 2 ml ampicilline LB medium and incubated overnight at 37^0C. Plasmid isolation was done using standard minipreparation protocol and PCR was set with specific primers used for del133-p53 cloning. Plasmid DNA isolated was used as template and Flag tagged del133-p53 was amplified specifically. PCR products were run on 0.8% agarose gel to confirm the size of the amplified PCR product (0.816 Kb). Lane 1: 1 Kb Marker. Lane 2: flag del133-p53 PCR product. Lane 3: negative control 1 (PCR of cloning vector). Lane 4-6: PCR of clones. Only clones that yielded intense PCR products, lane 5 and 6, were taken for further experiments.

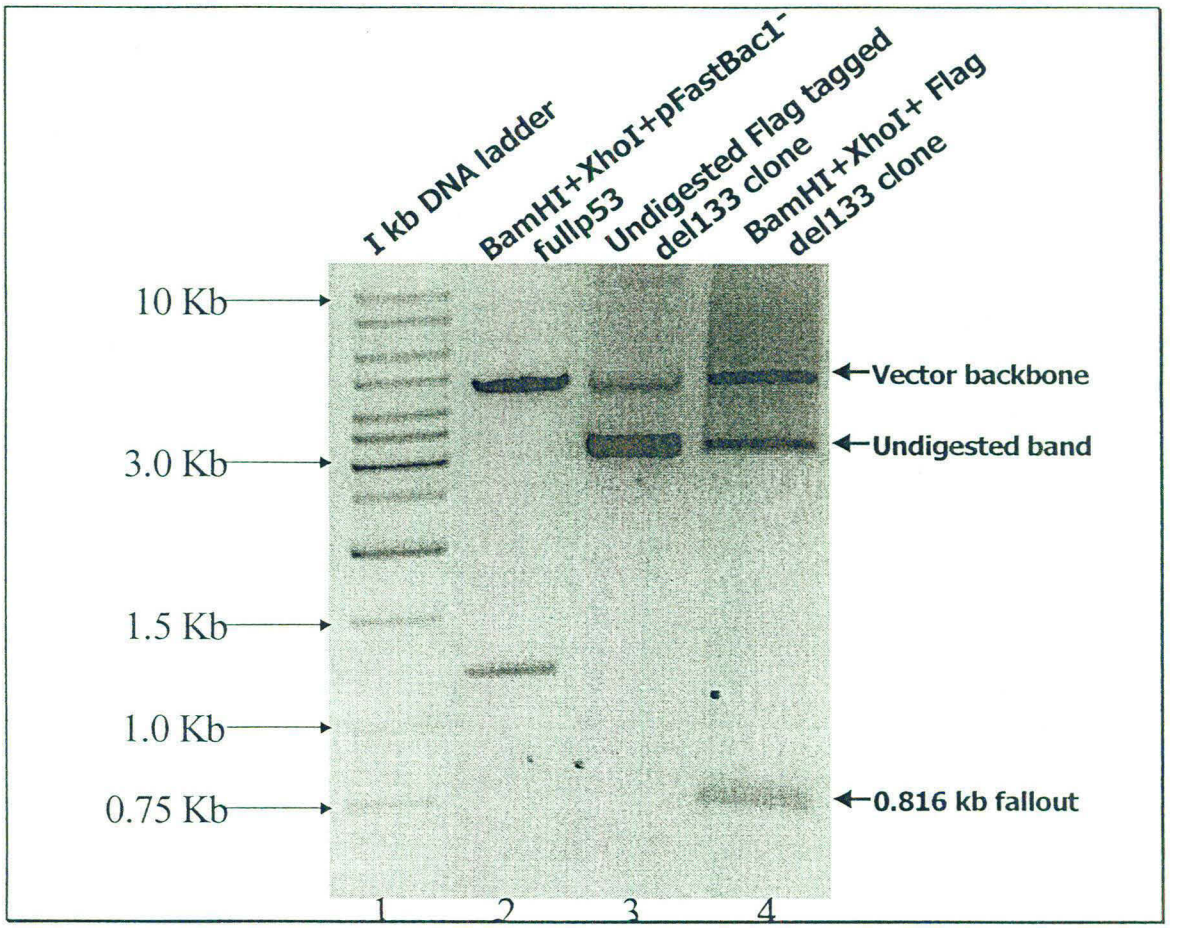

Figure 21. Double digestion of flag-tagged del133-p53 isoform-pFastBac1 clone. Plasmid isolation was done from overnight cultures grown in 2 ml LB-ampicilline medium at 37^0C and 220 rpm speed. Double digestion with BamHI and XhoI enzymes of flag-tagged del133-p53 isoform-pFastBac1 clone yielded a band at 0.816 Kb, corresponding to Flag-tagged del133-p53 isoform. Lane 1: 1 Kb marker. Lane 2: bamHI and XhoI enzyme digested pFastBac1-fullp53. Lane 3: Plasmid DNA of Flag-tagged del133-p53 isoform DNA. Lane 4: BamHI and XhoI enzyme digested flag-tagged del133-p53 clone.

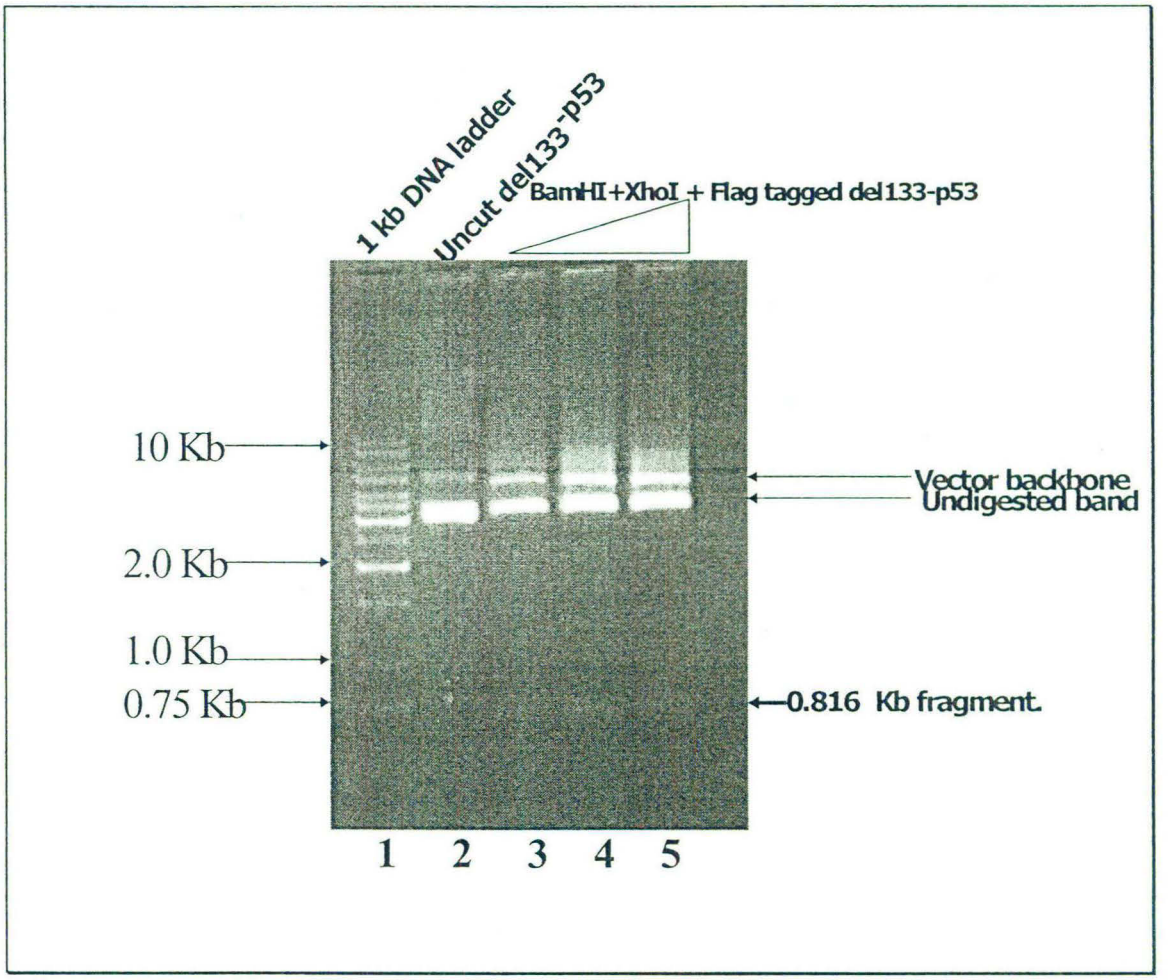

Figure 22. Double digestion of Flag-tagged del133-p53 isoform-pFastBac1 clone with increasing concentrations of restriction enzymes. Double digestion with BamHI and XhoI enzymes of flag-tagged del133-p53-pFastBac1 plasmid DNA at different concentrations of restriction enzymes yielded a band at 0.816 Kb corresponding to flag-tagged del133-p53 isoform. But the digestion by the restriction enzymes was incomplete, even after increasing the concentration of restriction enzymes as shown in lane 3-5. Lane 1: 1 Kb marker.

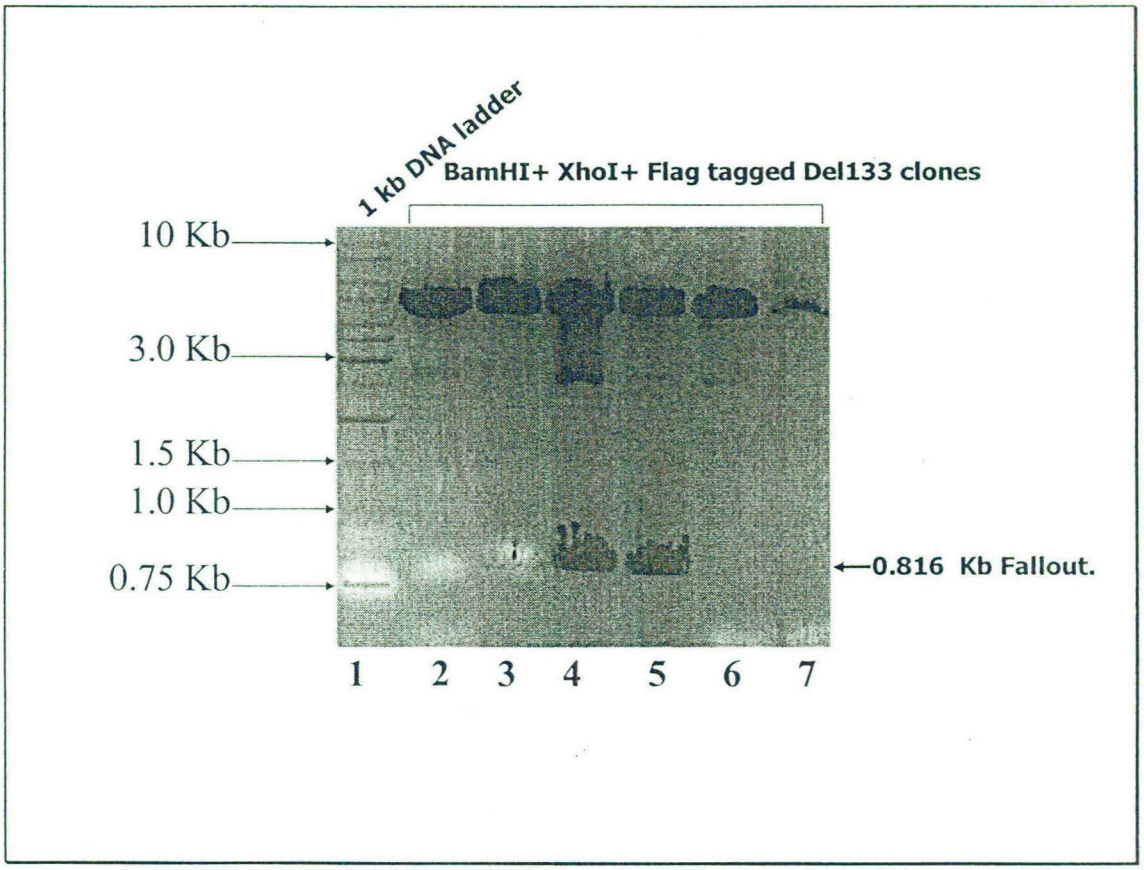

Figure 23. Double digestion of flag-tagged del133-p53 isoform-pFastBac1 clones. Overnight cultures of PCR confirmed recombinant colonies were set in 2ml of ampicilline-LB medium and the plasmid DNA was isolated following the standard minipreparation protocol. Plasmid DNA was subjected to BamHI and XhoI enzyme digestion and the samples were run on 0.8% agarose gel. Lane 1: 1 Kb marker. Lane 2-7: BamHI and XhoI enzyme digested possible flag-tagged del133-p53 isoform clones. Lanes 4 and 5 yielded intense bands at 0.816 Kb, corresponding to flag-tagged del133-p53 isoform insert of flag tagged del133-p53 isoform.

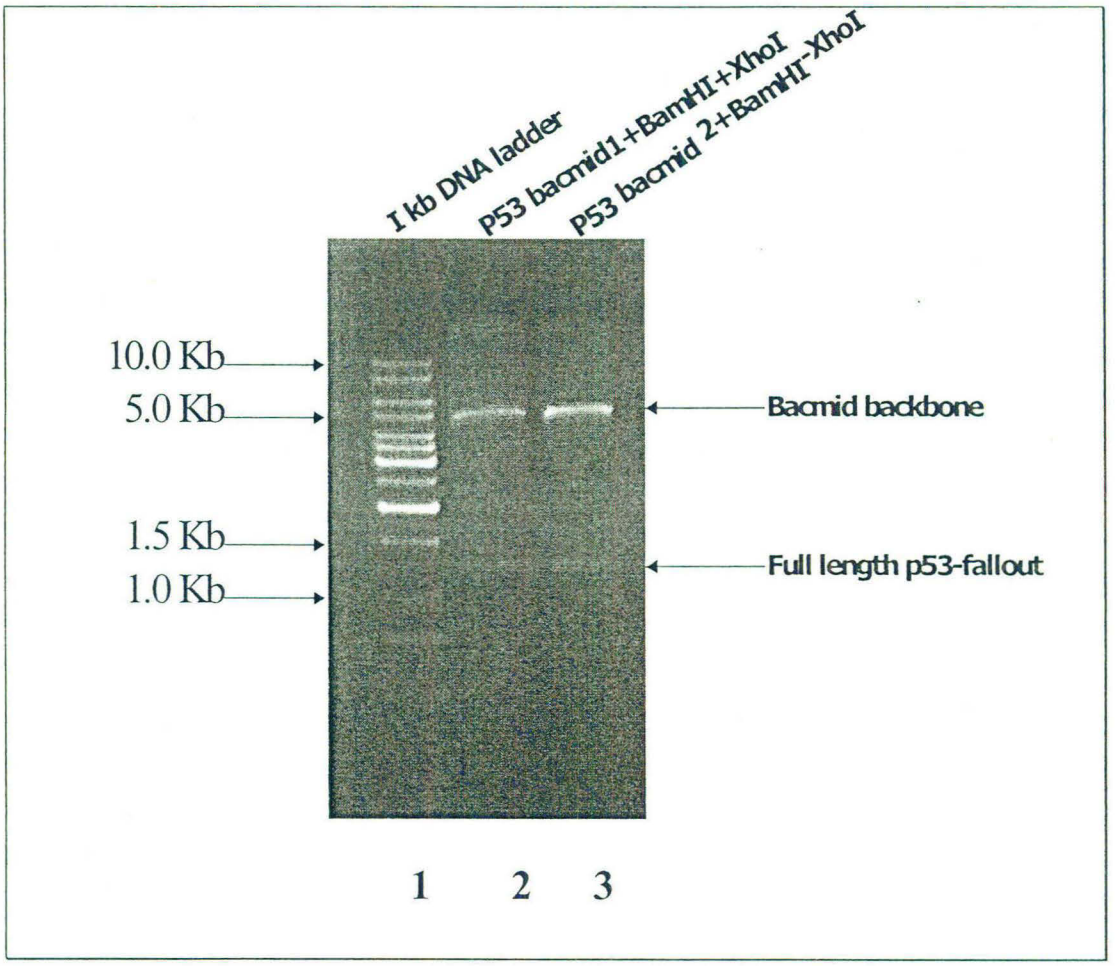

Figure 24. Double digestion full-length flag-tagged p53- bacmid DNA.

The white colonies were grown overnight at 37^0C in LB medium containing 50μg/ml kanamycin, 7μg/ml gentamicin and 10μg/ml tetracycline. Bacmid DNA were prepared by standard minipreparation protocol and subjected to BamHI and XhoI digestion. The Flag tagged-p53 fall-out came out at right position at 1.215 Kb on 0.8% agarose gel. Lane 1: 1Kb marker. Lane 2 and 3: double digested full-length flag-tagged p53-Bacmid DNA.

CHAPTER 6

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CHAPTER 7

Appendix

Yeast Extract	1% (w/v)
Tryptone	2% (w/v)
NaCl	0.1% (w/v)
Glucose	0.1% (w/v)
Water	Add to make 100 ml

Table 4. Composition of 2XL medium for the preparation of competent cells.

Luria Broth	2% (w/v)
Agar	1.5% (w/v)
Water	Add to make 100 ml

Table 5. Composition of Luria Broth agar medium.

CaCl2	100 mM
MnCl2	70 Mm
CH3COONa	40 Mm

Table 6. Composition of Acid Salt Buffer (pH 5.5 glacial with acetic acid) for competent cell preparation.

Glycerol	15 ml
Water	35 ml

Table 7. Preparation of 30% glycerol.

Glucose	50 mM
Tris.Cl pH 8.0	25 mM
EDTA pH 8.0	10 mM
Lysozyme	2 mg/ml (w/v)

Table 8. Composition of solution I for plasmid DNA Isolation (Miniatis *et al.*, 1984).

Sodium Hydroxide	O.2 N
SDS	1%

Table 9. Composition of solution II for plasmid DNA Isolation (Miniatis *et al.*, 1984).

Potassium Acetate pH 4.8 with glacial Acetic Acid.	5 M

Table 10. Composition of solution III for plasmid DNA Isolation (Miniatis *et al.*, 1984).

RNase A	10 mg/ml
Tris.Cl pH 7.5	10 mM
NaCl	15 mM

Table 11. Preparation of RNase A solution.

Tris.Cl	10 mM
EDTA pH 8.0	1mM

Table 12. Preparation of Tris-EDTA buffer (pH 8.0).

Tris base	242 g
Glacial Acetic Acid	57.1 ml
0.5M EDTA (pH 8.0)	100 ml
Water	Add to make 1000 ml volume.

Table 13. 50X TAE for agarose gel electrophoresis.

Bromophenol blue	0.25% (w/v)
Xylene cyanol FF	0.25% (w/v)
Glycerol	30% (w/v)

Table 14. Composition of DNA loading dye.

Tris.Cl pH 8.0	10 mM
Isopropanol	Absolute
Potassium Acetate	5 M pH 4.6
Ethanol	70% (w/v)

Table 15. Composition of reagents for DNA elution from agarose gels.

Tris.Cl pH 8.0	15 mM
EDTA pH 8.0	10 mM
RNase A	100 µg/ml

Table 16. Composition of solution I for bacmid DNA Isolation.

Sodium Hydroxide	O.2 N
SDS	1%

Table 17. Composition of solution II for bacmid DNA Isolation.

Potassium Acetate pH 5.5 with glacial Acetic Acid.	3 M

Table 18. Composition of solution III for bacmid DNA isolation.

Antibiotic	Concentration in stock.	Working concentration.
Ampicilline	100 mg/ml in water.	100 μg/ml.
Kanamycin	50 mg/ml in water.	50 μg/ml.
Gentamicin	14 mg/ml in water.	14 μg/ml.
Tetracycline	10 mg/ml in ethanol.	10 μg/ml.

Table 19. Antibiotic concentrations.

Chemical	Concentration in stock	Working concentration
X-gal	100 mg/ml in DMSO	100 μg/ml
IPTG	40 mg/ml in water.	40 μg/ml

Table 20. Concentrations of reagents for blue-white selection.