IDENTIFICATION OF THE INTERACTING DOMAIN OF p53 FAMILY MEMBERS WITH $p33^{ING1}$ PROTEIN

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By

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January, 2002

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To My Family…

For letting me free to go after my dreams

Whatever they might be…

ABSTRACT

IDENTIFICATION OF THE INTERACTING DOMAIN OF p53 FAMILY PROTEINS WITH $p33^{ING1}$

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p53 is a tumor suppressor gene, which is mutated, in about 50% of human cancers. The product of p53 gene encodes a sequence specific transcription factor. The genes transactivated by p53 code for proteins that are implicated in the negative regulation of cell proliferation and DNA damage repair. Two proteins, p63 and p73 members of p53 family, show striking homology to p53. p53 protein interacts with several viral and cellular proteins and these interactions are important in the regulation and dysregulation of the functions of p53. Another gene named, *ING1* was identified as a candidate tumor suppressor gene due to its functions in apoptosis and cell cycle arrest. $p24^{ING1}$, one of the protein product of ING1, was shown to enhance the growth suppressor functions of p53. Furthermore a physical association between p53 and $p33^{ING1}$, another ING1 transcript, proteins has been detected by immunoprecipitation. In this study, we investigated the physical interaction between p53 family proteins and $p33^{ING1}$ using *in vitro* techniques in order to determine the region(s) of p53 family proteins and $p33^{ING1}$ that enabled this interaction. As a preliminary step for the study, the wild-type p53 cDNA and its several deletion mutant constructs were used in GST pulldown assays and Far Western assays with purified GST-p33 protein to map the interacting region on p53 protein. New deletion mutant constructs of p53 protein were created and cloned into expression vectors for the detailed analysis of the interacting domain of p53 protein. Also the other members of p53 protein family, p63 and p73 were examined *in vitro* for interaction with $p33^{ING1}$. Deletion mutants of these proteins were created and cloned into expression vectors for protein-protein interaction assays. The results of this study shows that p53 protein interacts with $p33^{ING1}$ and suggests that oligomerization domain of p53 protein is needed for this interaction. In addition, for the first time, it was showed that p63 and p73 proteins interact with $p33^{ING1}$ and in p63 the C-terminus region is the primary determinant region, involved in these interactions with $p33^{ING1}$.

Keywords: $p33^{ING1}$, p53, p63, p73, tumor suppressor protein, protein-protein interaction, GST pulldown, Far Western Blotting

ÖZET

p53 PROTEİN AİLESİNİN $p33^{ING1}$ PROTEİN'İ İLE İLİŞKİYE GİREN BÖLGESİNİN BELİRLENMESİ

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p53, insan kanserlerinin yaklaşık %50'sinde mutasyona uğrayan bir tümör baskılayıcı gendir. p53 gen ürünü, DNA dizisi özgünlüğü olan bir transkripsiyon faktörüdür. p53 tarafından transkripsiyonu aktive edilen genlerin protein ürünleri, hücre bölünmesini önleyici yönde görev yapan kontrol elemanları ya da DNA tamir mekanizması elemanlarıdır. İki yeni protein, p63 ve p73 p53 protein ailesinin üyeleri, p53 proteinine hem gen seviyesinde hem de protein seviyesinde büyük benzerlik göstermektedir.p53 proteini bir çok hücresel ve viral protein ile etkileşime girer ve bu etkileşimler p53'ün işlevlerinin düzenlenmesinde ya da düzensizliğinde önemli yer tutar. *ING1* ("Inhibitor of Growth 1":Büyüme İnhibitörü 1) adlı yeni bir gen, apoptoz ve hücre döngüsün durdurulmasında ki rolleri dolayısıyla tümör baskılayıcı gen adayı olarak tanımlanmıştır. *ING1*'ın protein ürünlerinden biri olan $p24^{ING1'}$ ın p53'ün büyüme baskılayıcı işlevlerini arttırdığı gözlemlenmiştir. Dahası, p53 ile $p33^{ING1}$, diğer bir *ING1* proteın ürünü, arasında fiziksel bir etkileşim, immunopresipitasyon yöntemiyle belirlenmiştir. Bu çalışmada, p53 protein ailesinin üyelerinin, $p33^{ING1}$ ile etkileşen bölgelerini belirlemek amacıyla bu proteinler arasındaki fiziksel etkileşimi *in vitro* yöntemlerle inceledik. İlk adım olarak, normal p53 cDNA'sı ve birçok değişik p53 delesyon mutantı cDNA'ları, p53'ün $p33^{ING1}$ ile etkileşen bölgesini belirlemek amacıyla 'GST pulldown' sisteminde ve 'Far Western Blotting' sisteminde kullanılmıştır. Bu çalışmalardan elde edilen sonuçlara bağlı olarak iki yeni p53 delesyon mutantı yaratılmış ve protein-protein etkileşiminin belirlenmesinde kullanılmıştır. p53 protein ailesinin diğer iki üyesi olan p63 ve p73 proteinlerinin $p33^{ING1}$ proteini ile etkileşimi ve etkileşim bölgeleri C-terminali delesyon mutantları yaratılarak incelenmiştir. Bu çalışmanın sonuçları p53 proteinin, $p33^{ING1}$ ile oligomerizasyon bölgesi aracılığı ile etkileşime girdiğine işaret etmektedir. Buna ek olarak, p63 ve p73 proteinlerinin, $p33^{ING1}$ proteini ile etkileşime girdiği ve bu etkileşimin p63 proteininin C-terminalinden olduğu gösterilmiştir.

Anahtar Kelimeler: $p33^{ING1}$, p53, p63, p73, tümör baskılayıcı, protein-protein etkileşimi, GST-pulldown, Far Western Blotting

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ABBREVIATIONS

A_{600}	absorbance at 600 nanometer wavelength
aa	amino acid
AGE	agarose gel electrophoresis
APS	ammonium persulfate
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
cm	centimeter
ddH_2O	deionized, distilled water
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxid
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	Dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
FCS	fetal calf serum
g	gram
Gn	Guanidium
GST	Glutathione S-Transferase
hr	hour
IPTG	isopropylthio-beta-D-galactoside
IVTT	*in vitro* transcription and translation
kb	kilobase
kD	kilodalton
LB	Luria-Bertani medium
M	molar
mA	miliamper
ME	mercaptoethanol
min	minute
MidiPrep	medium-scale isolation of plasmid DNA by alkaline lysis method
MiniPrep	small-scale isolation of plasmid DNA by alkaline lysis method
ml	milliliter
mRNA	messenger RNA

nm	nanometer
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pBsk	pBluescript II KS^- phagemid vector
PCR	polymerase chain reaction
rpm	revolution per minute
RNA	ribonucleic acid
RNase	ribonuclease
RT	room-temparature
SDS	sodium dodecyl sulphate
sec	second
TAE	tris/acetic acid/EDTA buffer
TEMED	N,N,N,N-tetramethyl-1,2 diaminoethane
Tris	2-amino-2-[hydroxymethyl]- 1,3 propandiol
U	unit
V	volt
v/v	volume for volume
wt	wild type
w/v	weight for volume
w/w	weight for weight

CHAPTER 1. INTRODUCTION

1.1 General Introduction

In an adult, the rates at which new cells in a tissue are formed and old cells die are in balance, producing a steady state in which the tissue does not increase in size. If the mechanisms regulating cell division are not functioning properly, the affected cells may produce new cells faster than old cells are removed, forming a growing mass of tissue known as a tumor. If the tumor cells grow into the surrounding tissues, disrupting their functions, and spread to other regions of the body, the tumor is said to be malignant tumor. Malignant tumors are composed of cancer cells, that have lost the ability to respond the normal mechanisms that regulate cell growth (Hanahan & Weinberg 2000).

Two classes of genes are mutated in cancer formation. The first class of genes (gatekeepers) comprising tumor suppressor genes, directly controls cellular proliferation. They can do this either by controlling the rate of cell birth or the rate of cell death. The second class of genes (caretaker genes) does not directly control cell growth, but instead controls the rate of mutation. Cells with defective mutator genes acquire mutations in all genes including oncogenes and tumor suppressor genes at an elevated rate. This higher rate leads to accelerated tumorigenesis (for review see Vogelstein and Kinzler, 1998).

Tumor suppressor gene is a genetic element whose loss or inactivation allows a cell to display one or another phenotype of neoplastic growth deregulation. The

products of the tumor suppressor genes are required for normal cell function, and they antagonize the proliferative actions of oncogenes to prevent tumorigenesis. Tumor suppressor mutations act as a recessive trait in the formation of tumors. Tumor suppressor genes constitute a very heterologous class of genes in terms of functions of their protein products. They can be transcription factors (e.g. p53, WT-1), cyclin dependent kinase inhibitors (e.g. p16) or receptor tyrosine kinases (e.g. RET) (for review see Haber, 1998).

1.2 p53 Tumor Suppressor Gene and Protein Product

1.2.1 Background

p53 protein is known as guardian of the genome, functional inactivation of p53 by gene mutation and deletion, protein degradation or viral oncogene binding renders a mammalian cell susceptible to oncogenic stimuli and environmental factors that promote growth deregulation and malignant progression. More than half of all human cancers shows either absence of p53 protein or mutations in this gene (for reviews, see Wang & Harris, 1997; Adams & Kaelin, 1998; Ko & Prives, 1996) . p53 is a nuclear phosphoprotein. It was originally discovered in SV40-transformed cells, where it is associated with T antigen (Lane *et al*., 1979; Linzer *et al*., 1979). A large increase in the amount of p53 protein is found in many transformed cells or lines derived from tumors. In early experiments, the introduction of cloned p53 was found to immortalize cells. These experiments caused p53 to be classified as an oncogene, However subsequent studies revealed that p53 cDNAs used in these studies were mutant and wild type counterparts were shown to suppress the growth of transformed cells *in vitro* and tumorigenic potential of these cells in animals (Finlay *et al*., 1989). Mutations of p53

alleles were implicated in many human and animal tumors. Indeed, members of Li-Fraumeni cancer-prone families were shown to carry germ-line p53 mutations (Malkin *et al*., 1990). The role of p53 in cancer was more directly accessed by the observation that p53 knock out mice developed tumors at high frequencies (Donehower *et al*., 1992). Therefore, p53 was re-classified as a tumor suppressor gene.

1.2.2 Cellular Functions of p53 protein

The human p53 is located in the short arm of chromosome 17, 17p13.1. The gene has 11 exons and spans 20kb of genomic DNA. Many organisms have p53 homologues, such as monkey, chicken, rat, frog, fruit fly and worm (Derry B. W., *et al*., 2001; Jin S., *et al*., 2000; Soussi T., *et al*.,1988; Rigaudy P., *et al*.,1989). The general organization of the p53 protein is well conserved during evolution.

p53 integrates signals from the cell's internal and external environment to respond to inappropriate growth promoting or growth inhibiting conditions. The tumor suppressor activity of p53 stems from its ability to both inhibit the mitotic cell cycle and promote apoptosis. The functional character of the p53 protein was determined by experiments showing that p53 contains a strong transcriptional activation domain within its amino terminus and that it is a sequence specific DNA-binding protein (El-Deiry *et al*., 1992). Although the p53 protein acts as a transcriptional activator of genes containing p53 binding sites, it is also capable of inhibiting transcription from many genes lacking p53 binding sites. Several oncogenic DNA viruses express viral gene products that associate with and inhibit the *trans*-activation function of p53, notably SV40 large antigen, the adenovirus E1B 55-kD protein, and E6 protein of oncogenic forms of human papillomavirus (HPV E6) (for review, see Prives & Ko, 1996).

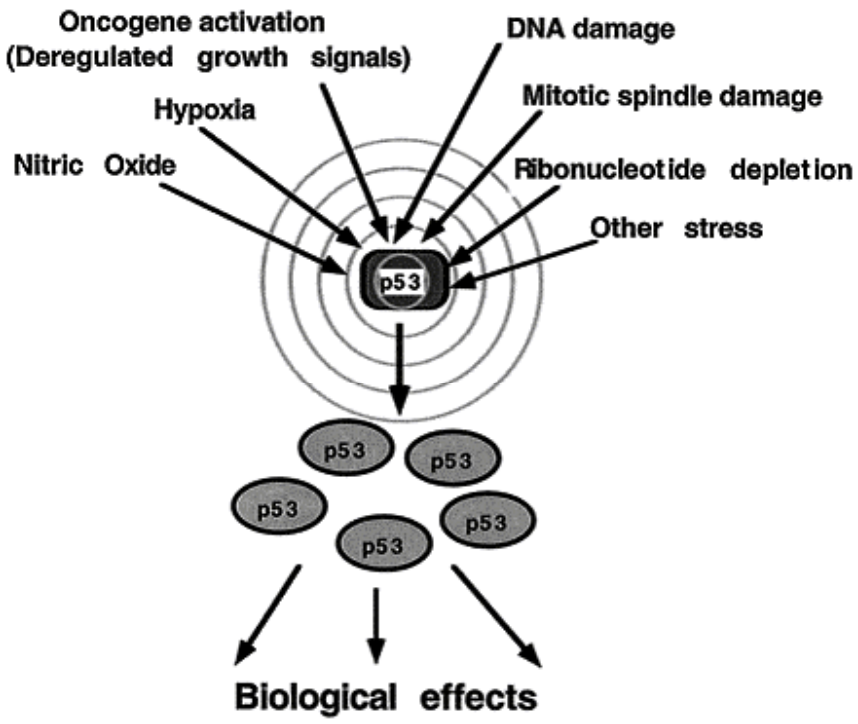


Figure 1.1: Upstream elements activating the p53 (From Oren 1999).

p53 plays multiple roles in cells. Expression of high levels of wild-type p53 has two outcomes: cell cycle arrest, or apoptosis. In unstressed cells, p53 appears to be present at low levels and exist in a latent inactive form that requires modification to become active. The types of modification that p53 is subject to seem to be stress-, species- and cell-type-specific. Levels and/or activity of p53 increase in response to DNA damaging agents (Maltzman and Czyzyk, 1984; Kastan *et al*., 1991), decreased oxygen (Graeber *et al*., 1994), oncogenic stimuli (Debbas and White, 1993), cell adhesion (Nigro *et al*., 1997), altered ribonucleotide pools (Linke *et al*., 1996), and redox stress (Hainaut *et al*., 1993) (**Figure 1.1**). The importance of p53 and modifications that affect its functions are not limited to malignant disease. The activity of p53 can increase in normal tissues when undergoing pathophysiological changes that

result in oxidative or redox stress, such as ischemia, reperfusion injury of the brain, heart, and other tissues.

1.2.3 Structure of p53 protein

The human p53 protein is a single polypeptide of 393 amino acids. It can be divided into different domains (for review, see Ko & Prives, 1996): at the N-terminus, a transactivation domain (residues 1 to 43) and proline-rich domain (residues 62 to 91); in the middle core, a DNA-binding domain (residues 100 to 300); and at the C-terminus, a tetramerization domain (residues 326 to 354) and a regulatory region (363 to 393) (**Figure1.2**).

p53 binds *in vitro* to several proteins through its activation domain. The amino terminus of p53 interacts with many general transcription factors such as the TATA box-binding protein (TBP) component of general transcription factor TFIID, several TBP-associated factors (TAFs), including *Drosophila melanogaster* TAF-40 and TAF-60 (Thut *et al*., 1995), the human TAF-31 (Lu and Levine, 1995) and TFIIH (Xiao *et al*., 1994). p53 transcriptional activation is negatively regulated by the adenovirus E1B-55Kd protein (Kao *et al*., 1990) and human Mdm2 protein (Haupt *et al*., 1997). In both cases, p53 amino acid residues 22 and 23 play a key role in the binding of p53 to E1B-55Kd or Mdm2 protein. Thus the negative regulators of p53-mediated transcription target some of the same p53 amino acids critical to positive regulation of transcriptional activation. This evidence showed that p53 uses a hydrophobic interface in its N terminal domain to interact with the transcriptional machinery of the cell and its negative regulators. Also this domain is the target of phosphorylation by various kinases (for review, see Levine, 1997).

Proline rich region spans the amino acids 62-91. There are five tandemly repeated proline rich motifs in the form of PXXP (where P is proline and X is any amino acid) and these motifs were shown to bind SH3-containing proteins. This region is implicated in both growth arrest and apoptosis (Venot *et al*., 1998).

The sequence-specific DNA-binding domain of p53 is localized between amino acid residues 100 and 300. It is a protease resistant and independently folded domain containing a Zinc ion that is required for its sequence-specific DNA-binding activity.

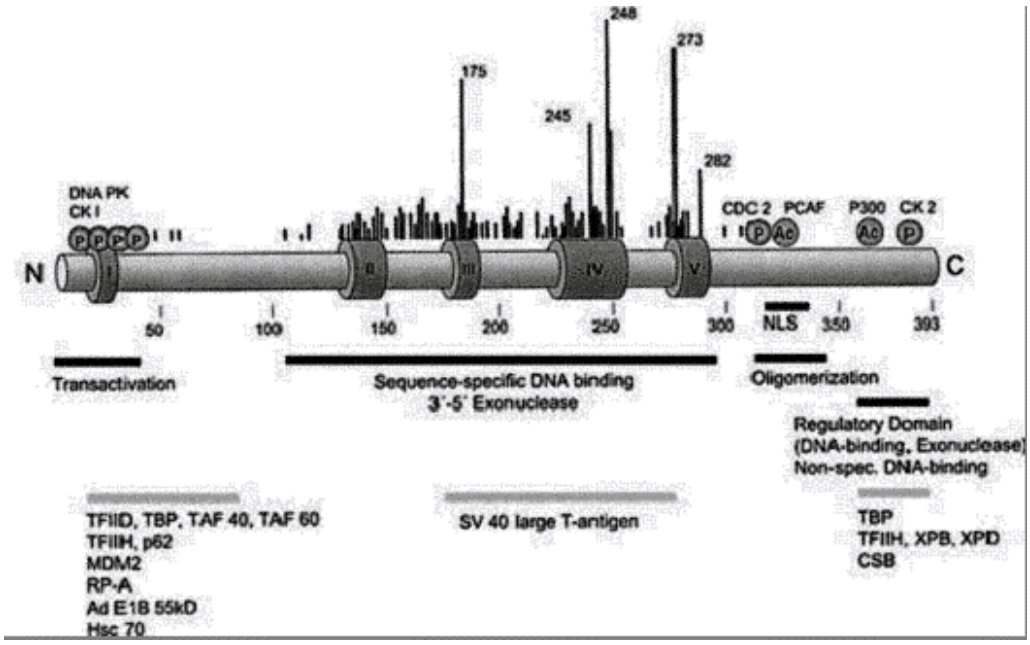


Figure 1.2: Domain structure of p53 , together with mutational hotspots and interacting proteins. *Dark Grey Boxes* I-V: regions conserved among species; at the N-terminus: Transactivation domain; Next to it proline rich domain; In the middle of the protein: DNA binding domain ; At the C-terminus: there is both oligomrization and regulatory domain (Adapted from Ko & Prives 1996).

The p53 protein binds to four repeats of a consensus DNA sequence 5'-PuPuPuC(A/T)-3', and this sequence is repeated in two pairs each arranged as inverted repeats (El-Deiry *et al*., 1992). More than 90% of the missene mutations in the p53 reside in this sequence-specific DNA-binding domain, and these mutations fall into two

classes: mutations which result in defective contacts with the DNA and loss of the ability of p53 to act as a transcription factor, or mutations which disrupt the three dimensional (3D) conformation of the protein (for review, see Levine, 1997).

The native p53 protein functions as a tetramer, and amino acid residues 326-354 are required for this oligomerization of the protein. The structure of this domain contains a dimer of a dimer with two β sheets and two α helices. This tetramerization domain is linked to the sequence specific DNA-binding domain by a flexible linker of 37 residues (Lee *et al*., 1994; Clore *et al*.,1995; Jeffery *et al*.,1995).

The C-terminus 26 amino acids form an open, protease sensitive, domain composed of nine basic amino acid residues that bind to DNA and RNA (Lee *et al*., 1995). There is a considerable evidence showing that the p53 protein requires a structural change to activate it for sequence-specific binding to DNA. This non-DNA binding form can be regulated by basic C-terminus domain. Deletion of this domain, phosphorylation of certain residues by kinases, or by the binding of certain specific antibodies all activate site-specific DNA binding by the central domain (Hupp and Lane, 1994). Short (20-39 nucleotide) single strands of DNA interacting with this C-terminus domain can also activate specific p53 DNA binding. The C-terminus domain helps to catalyze the reassociation of single-stranded DNA or RNA to double strands (Lee *et al*.,1995). It also binds preferentially to DNA ends and to internal deletion loops in DNA as generated by replication errors.

1.2.4 Regulation of p53 Activity

Because p53 is such a critical cellular protein, multiple mechanisms have evolved to regulate its activity. These regulatory mechanisms exist both tightly and

rapidly control the activities of p53 and to provide alternative regulatory mechanisms for different cell types and different physiological stimuli. Normally, in a cell, the p53 protein is kept at a low concentration by its relatively short half-life. In addition to its low protein concentration, in some cells p53 probably also exists in a latent form (Kastan *et al*.,1991). To activate the protein, p53 must receive a signal or alternation. Several stressful situations, such as DNA damage, γ irradiation, presence of DNA repair intermediates after ultraviolet irradiation or chemical damage to DNA, can activate p53. This results in a rapid increase in the level of p53 in the cell and activation of a p53 as a transcription factor. Although levels of p53 mRNA does not change in response to stimuli, the levels of p53 protein increase rapidly (Kastan *et al*., 1991). The half-life of p53 increases several folds after DNA damage. Enhanced translation of p53 mRNA also contributes to this induction. Therefore the regulation is mainly posttranslational. This type of regulation is necessary for very rapid and efficient control of the p53 levels in a cell. In addition to changes in the level of p53, several different modifications are implicated in the activation of p53 protein, such as phosphorylations, dephosphorylations, acetylations, and binding to certain proteins (for review see Meek 1994; Chernov *et al.,* 1998). Both posttranslational modifications and alternations in p53 binding proteins appear to be major contributors to the modulation of p53 activity. Carboxy terminal phosphorylation and incubation with a Carboxy terminal binding anti-p53 antibody markedly enhanced sequence-specific DNA binding of p53 (Hupp *et al*., 1993). This evidence showed that Carboxy terminus domain of p53 is the regulatory region of the protein. Mdm2 protein as a major intracellular regulator of ubiquitin-mediated degradation of p53 protein (Haupt *et al*., 1997; Kubbutat *et al*., 1997). Mdm2 protein can inhibit p53 function by binding to and inhibiting transactivation by p53.

The existence of these various regions of p53 provides a way by which a primary p53 regulatory mechanism could be by specific post-translational modifications or one or more of these domains, or modulation of proteins that bind to these domains. The cell uses different functions and proteins to recognize different classes of DNA damage and different systems of enzymes to repair them. For example, ATM kinase activity is enhanced following ionizing radiation (IR) and this protein is shown to phosphorylate Serine-15 of p53. ATM was also implicated in a radiation-induced dephosphorylation event. IR causes a loss of a phosphate group from Serine 376 in the carboxy terminus of p53 in an ATM dependent manner and this dephosphorylation event results in a creation of a binding for 14-3-3 proteins and enhancement of sequence-specific DNA binding (Waterman *et al*., 1998) (**Table 1.1**).

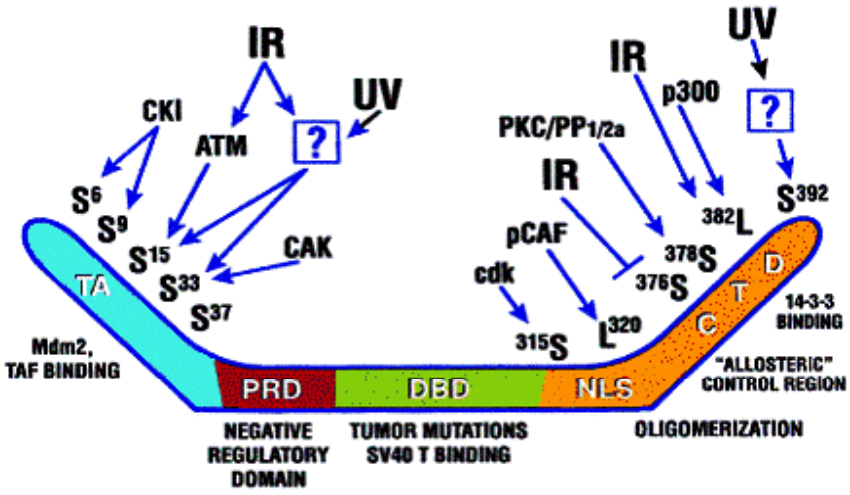


Figure 1.3: Summary of posttranscriptional modifications and domains of p53. (From Giaccia &Kastan, 1998).

Ultra violet (UV) irradiation is also a potent inducer of p53 protein; however, clear differences exist in p53 induction following UV radiation versus IR. UV signaling to p53 does not depend on ATM kinase but lysine residues in the N terminal domain of p53 are acetylated by PCAF and p300/CBP (Lill *et al*., 1997; for review see Giaccia &

Kastan 1998) (**Figure 1.3**) . In addition to DNA damage, hypoxia is able to stimulate p53 levels and activate p53 protein in a different way from UV radiation and IR. It was suggested that hypoxia increases p53 protein levels via induction of another protein, HIF-1β (An *et al*., 1998).

Yet a third signal to activate p53 is sent when ribonucleoside triphosphate pools fall below a critical threshold. Normal nucleoside triphosphate pools are necessary to support DNA replication and progression through the cell cycle (Linke *et al*., 1996). As seen in the examples, activation of p53 is complex and tightly regulated process.

Table 1.1 Representative examples of proteins interacting with p53.

PROTEIN	*CATEGORY / FUNCTION*	*REFERENCE*
SV40Tag	viral	(Lane et al., 1979)
HPV E6	viral	(Scheffner et al., 1992)
Ad E1b-55	viral	(Kao et al., 1990)
EBV EBNA-5	viral	(Szekely et al., 1993)
TBP	Transcription Factor/ Part of TFIID	(Seto et al., 1992)
TAFII60,TAFII 40	Transcription Factor/ Part of TFIID	(Thut et al., 1995)
RPA	Replication	(Dutta et al., 1993)
Mdm2	oncoprotein / inhibitor of p53	(Momand et al., 1992)
WT1	transcriptional activator / tumor suppressor	(Maheswaran et al., 1993)
Sp1	transcriptional activator	(Gualberto et al., 1995)
CK2	protein kinase	(Meek et al., 1990)
DNA-PK	protein kinase	(Lees-Miller et al., 1990)
ATM	protein kinase	(Banin et al., 1998)
p300/CBP	histone acetylase	(Lill et al., 1997)
E6-AP	ubiquitin system protein	(Huibregtse et al., 1993)
HMG1	DNA bending	(Jayaraman et al., 1998)
$p19^{ARF}$	tumor suppressor	(Kamijo et al., 1998)
$p33^{ING1}$	tumor suppressor? modulator of p53	(Garkavtsev et al., 1998)
RAD51	replication/repair	(Buchhop et al., 1997)
REF1	redox/repair	(Jayaraman et al., 1997)
BRCA1	tumor suppressor/repair	(Zhang et al., 1998)
ERCC2	helicase / Part of TFIIH	(Xiao et al., 1994)
XPD	helicase/ Part of TFIIH	(Xiao et al., 1994)

1.2.5 Downstream Events

The transcriptional activating function of p53 is a major component of its biological effects. By binding to specific sites within their promoters, p53 activates the transcription of various genes including, $p21^{WAF1}$, GADD45, mdm2, bax, thrombospondin 1, cyclin G, and insulin-like growth factor binding protein 3 (IGF-BP3) (**Figure1.4**) (for review see Somasundaram & El-Deiry 2000).

The p53-dependent G1 arrest occurs largely through the transactivation of $p21^{WAF11}$ (el-Deiry *et al*., 1993). $p21^{WAF1}$ is a member of small cyclin-dependent kinase inhibitor (CKI) family. It binds to a number of cyclin and cyclin-dependent kinase (cdk) complexes, by this way inhibits the transition to S-phase. $p21^{WAF1}$ also binds to proliferating cell nuclear antigen (PCNA), and inhibits the role of PCNA as a DNA polymerase processivity factor in DNA replication (Waga *et al*., 1994).

More recently, p53 has been implicated in a G2/M phase checkpoint. When mitotic spindle inhibitors are added to cells with wild-type p53, the cells are blocked in G2. In the absence of wild-type p53, these cells reinitiate DNA synthesis. These data suggest that p53 may be part of a G2/M checkpoint, preventing premature entry into another S phase. In addition p53 appears to be an integral part of the process that regulates the number of centrosomes in a cell (Cross *et al*.,1995; Fukasawa *et al*., 1996).

Another way for eliminating abnormal cells is apoptosis. p53 mediates apoptosis in several cell types in response to several stimuli, including DNA damage, adenovirus E1A expression, *myc* expression or withdrawal of growth factors. p53 may use

transcriptional activation or direct protein signaling (protein-protein interactions) (Hupp *et al*., 1995) or both to initiate apoptosis.

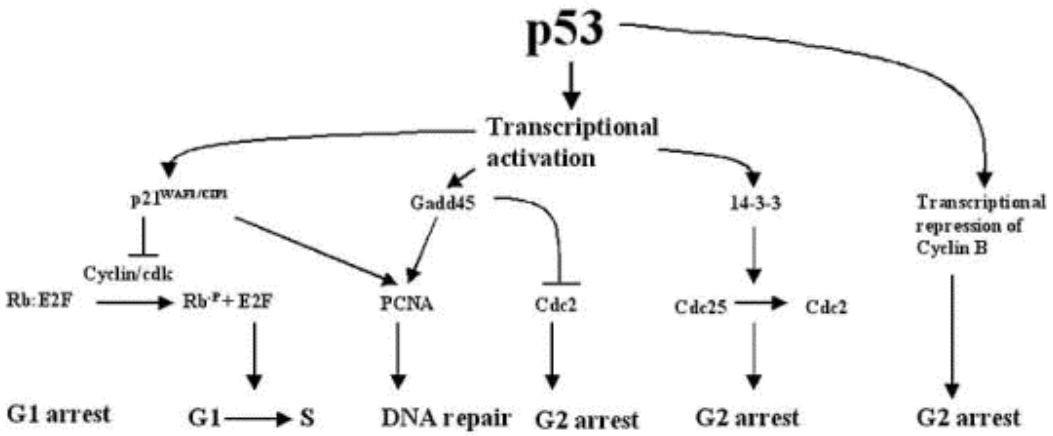


Figure 1.4 Major downstream targets of p53 (From Somasundaram & El-Deiry 2000).

Two of the genes that are regulated by p53 could influence the decision to commit to an apoptotic pathway: *bax* and *IGF-BP3* (Miyashita and Reed, 1995; Buckbinder *et al.,* 1995). *Bax* is a tumor suppressor gene whose protein product interacts with bcl-2 protein. Bcl-2 is an anti-apoptotic factor and increased bax expression block the functioning of this protein. A second p53 regulated gene product that could affect growth regulation is the *IGF-BP3*. IGF-BP3 protein blocks the IGF mitotic signaling pathway by binding to IGF and preventing its interaction with its receptor. Thus, the blocking of IGF activity could enhance apoptosis or lower the mitogenic response of cells.

Number of factors, like extend of DNA damage, certain oncogenic disruptions, availability of survival or growth factors, and cell type, affect the decision of a cell to enter a p53-mediated cell cycle arrest or apoptotic pathway.

1.2.6 The p53 Family

For a long time, p53 was thought to be alone, but two mammalian proteins, p73 and p63, with significant sequence homology to p53 have recently been identified. Its first relative, TP73, which encodes p73, was identified by chance in 1997 (Kaghad *et al*., 1997), and its second, TP63 (which encodes p63 and is also known as KET, P51, P40, chronic ulcerative stomatitis protein (CUSP) and P73L), was identified independently by several groups (Yang *et al*., 1998; Trink *et al*., 1998; Osada *et al*., 1998; Augustin *et al*., 1998; Senoo *et al*., 1998; Lee *et al*., 1999). There are several common features among the p53 gene family members. (1) They all contain very large introns, (2) exon1 is always non-coding, (3) the exon/intron organization of all the family members is highly similar (**Figure 1.5**).

Both p63 and p73 give rise to many differently spliced mRNAs, which are translated into several different proteins. Most of the splicing occurs at the 3' end and creates proteins that have different C-termini. There are at least six alternatively spliced forms of human p73: p73α, p73β, p73γ, p73δ, p73ξ, p73ε (Kaghad *et al*., 1997; De Laurenzi *et al*., 1998) in addition to these COOH-termini splice forms, two additional forms, ΔNp73α, and ΔNp73β, result form the use of an alternative promoter located in intron 3 (Yang *et al*., 1998). The TP73 gene contains 14 exons and is located at chromosome 1p36.33. The TP63 gene is located at chromosome 3q27-29 and contains 15 exons (Yang *et al*., 1998; Osada *et al*., 1998) and can be transcribed from two different promoters, which are located upstream of exon1 and within intron 3 (Yang *et al*., 1998). Recognized variants of TP63 include the α, β, and γ forms of p63. Furthermore p63 utilizes a cryptic promoter located in intron 3 to generate additional

transcripts called ΔNp63α, ΔNp63β, ΔNp63γ (**Figure 1.5**). The three members of the p53 family share very significant homology both at the genomic and at the protein level. Each contains a transcriptional activation domain (TAD), a DNA-binding domain (DBD) and an oligomerization domain (OD). In addition p63 and p73, but not p53, contain long C-termini (for review see Levrero *et al*., 2000).

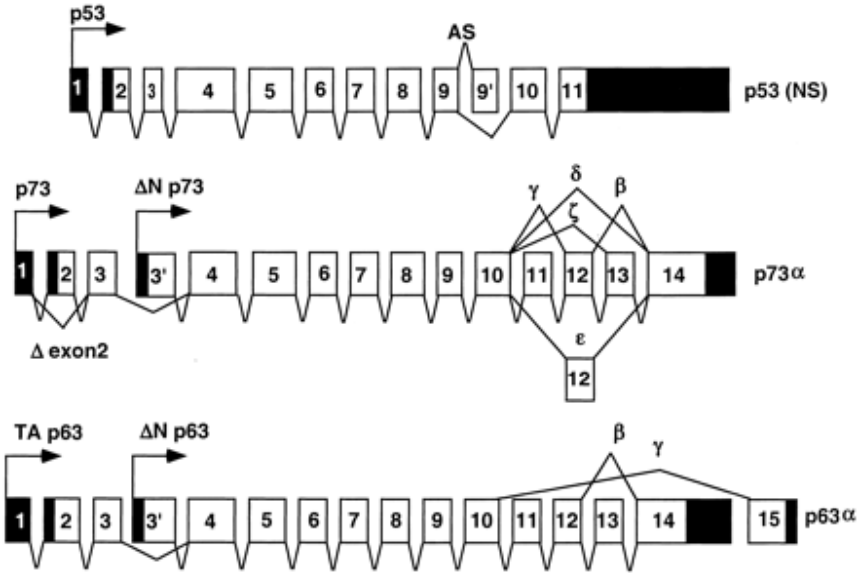


Figure 1.5: Exon-intron organization of p53 family members, showing different transcripts.

The highest level of homology is reached in the DBD (63% identity between p53 and p73, and 60% identity between p53 and p63), which suggests that the three proteins can bind to the same DNA sequences and transactivate the same promoters. The vast majority of missense mutations of p53 found in human tumors are clustered in the sequence specific DNA-binding domain. In addition, p73 and p63 are 29% and 22% identical to the region corresponding to the N-terminus transactivation domain of p53 respectively. Residues in the C-terminus region of p73 and p63 are 38% and 37% identical to the corresponding C-terminus oligomerization domain of p53 (for review see Chen 1999). The nuclear import and nuclear export signals lie within the

oligomerization domain and are conserved among p53 from different species as well as in p63 and p73. The sequences of p63 and p73 diverge from p53 mostly in their C-termini. Furthermore different p63 and p73 C-termini are generated as a result of alternative mRNA splicing. The determination of the three dimensional solution structure of the p73 C-terminus has shown that this region contains a sterile alpha motif (SAM), which is a protein-protein interaction domain (Chi *et al*., 1999). The SAM domain is a globular domain composed of four α-helices and a small 3_{10}-helix. SAM domains are protein-protein interaction modules found in proteins involved in developmental regulation. This domain is also present in p63 C terminus. Although SAM domains are known to participate in homotypic or heterotypic interactions, the p73 and p63 domains bind neither to themselves nor to each other. Nevertheless, it is possible that these domains interact either with a distantly related SAM domain protein or perhaps with a non-SAM containing protein (**Figure 1.6**) (for review see Irvin & Kaelin 2001; Kaelin 1999).

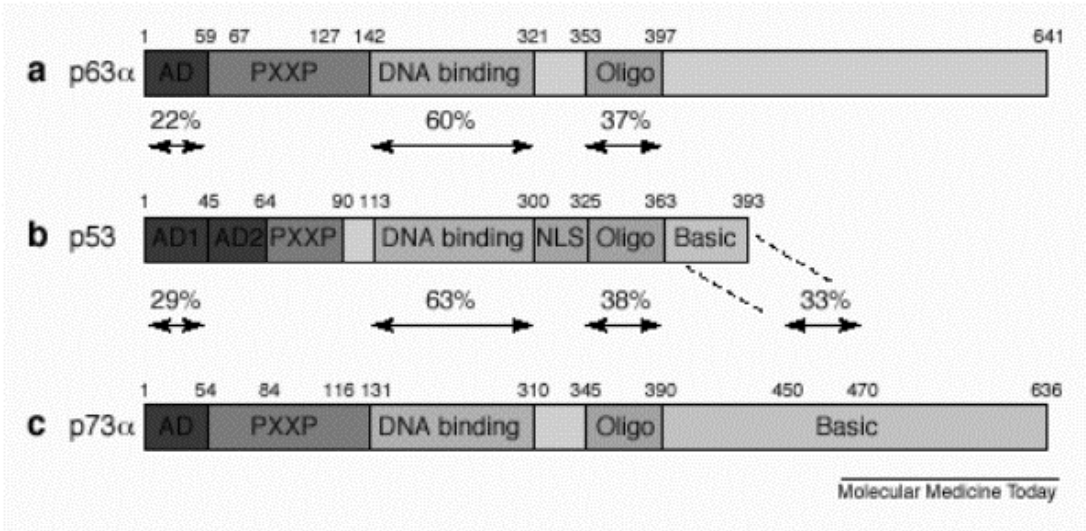


Figure 1.6: Homology between the p53 family members. AD, activation domain, PXXP proline rich repression domain, Oligo, oligomerization domain, Basic, C-terminal basic domain (from Chen, 1999).

p73 was initially proposed as a possible tumor suppressor gene because it is related to p53, it maps to chromosome 1p36.33, a region frequently deleted in neuroblastoma and other human cancers and it has been found to be monoallelically expressed owing to genomic imprinting (Kaghad *et al*., 1997). However the status of p73 as a tumor suppressor gene has been challenged by recent observations. It was found that p73 can be biallelically expressed in both normal or tumor tissues and cell lines, including neuroblastoma and mutation of the p73 gene occurs infrequently in human cancers (Ichimiya *et al*., 1999; Han *et al*., 1999).

It is not also certain whether p63 is a tumor suppressor gene. p63 is located at chromosome 3q27-29, a region that is not a common site of loss of heterozygosity in human cancers. p63 gene was found to be mutated infrequently in both human tumor tissues and cancer cell lines (Osada *et al*., 1998). Although p63 might have functions similar to those of p53 in cell-cycle arrest and apoptosis, ΔNp63, which lacks an activation domain, inhibits the activity of both p53 and p63, thereby exhibiting oncogenic functions (Yang *et al*., 1998).

While p53 is ubiquitously expressed, p73 and p63 are restricted to certain tissues. In the developing mice, p73 is detected in the epidermis, sinuses, inner ear, and brain while p63 is expressed in proliferating basal cells of the epidermis, cervix and prostate. In the $p63^{-}$ deficient mice, the apical ectodermal ridge essential to limb development is defective, and the mice have truncated limbs. In addition, p63 knockout mice have no hair follicles, no teeth, no mammary, lachrymal or salivary glands (Mills *et al*., 1999; Yang *et al*., 1999). Thus the phenotype of p63-deficient mice suggests that the primary biological function of p63 proteins is to regulate development. Recently

mutations of p63 have been detected in children affected by ectrodactyly ectodermal dysplasia and facial clefts (Celli *et al*., 1999). Ectodermal dysplasia is manifested by changes in skin, hair, nails, teethlacrimal duct, and urogenital tract. Similar to p63-deficient mice, p73-deficient mice exhibit severe defects, including hydrocephalus, hippocampal dysgenesis, chronic infections and inflammation, and abnormalities in the pheromone sensory pathway. However they do not develop any spontaneous tumors (Yang *et al*., 2000). According to these findings, both p63 and p73 play very important roles in ectodermal differentiation and neurogenesis respectively. However these findings do not exclude the roles of p63 and p73 as tumor suppressors. Therefore the functions of p53 family may overlap in some tissues because of the requirement for simultaneous activity of p53, p63 and p73 at specific stages of development.

Although there is a high degree of sequence similarity between p53 and p73, neither SV40 TAg, E1B55k, nor E6 oncoproteins bind to p73 (Marin *et al*., 1998; Higashino *et al*., 1998; Roth *et al*., 1998). However the possibility exists that they can be bound and inactivated by other viral proteins. Only one viral oncoprotein, adenovirus E4orf6, was capable of associating with p73 and inhibiting the activity of p73 in one experimental protocol (Steegenga *et al*., 1999), but not in another (Roth *et al*., 1998). Thus further studies are required to address this issue. Whether these viral oncoproteins can regulate p63 remains to be determined.

Like p53, p73 and p63 can induce cell-cycle arrest and apoptosis (Kaghad *et al*., 1997; Yang *et al*., 1998; Osada *et al*., 1998; Jost *et al*., 1997; Zhu *et al*., 1998). Loss of p73 transcriptional activity abrogates its activity in cell-cycle arrest and apoptosis (Kaghad *et al*., 1997; Jost *et al*., 1997; Zhu *et al*., 1998). It is known that p53 upregulates $p21^{WAF1}$, an inhibitor of cyclin-dependent kinases. Both p63 and p73 also

can induce $p21^{WAF1}$ transcription. Although p73 can induce $p21^{WAF1}$, the level of cellular $p21^{WAF1}$ induced by p73 is several times lower than that induced by p53. Two other p53 targets GADD45 and B-cell translocation gene 2 antiproliferative are only weakly activated by p73.

Like p53, p73 can induce G2/M arrest, p73 is capable of inducing 14-3-3σ (Zhu *et al*., 1998). However, p73 induces several fold higher levels of 14-3-3σ gene product than does p53. It remains to be found whether p63 can induce 14-3-3σ and cell-cycle arrest at G2/M.

Although both p53 and p73 can induce apoptosis (Jost *et al*., 1997; Zhu *et al*., 1998), the signaling pathways may be different because of the different abilities of p73 to activate some p53 target genes. While bax and several redox-related genes (PIG2, PIG3, PIG6 and PIG11) are possibly involved in mediating p53-dependent apoptosis (Miyashita *et al*., 1994; Polyak *et al*., 1997), they were not significantly induced by p73 (Zhu *et al*., 1998). Because p73 transcriptional activity is required for inducing apoptosis (Jost *et al*., 1997; Zhu *et al*., 1998), it is possible that p73 can activate a different subset of cellular genes for mediating apoptosis.

Mdm2, an oncogene that negatively regulates p53, and is also induced by p53 (Wu *et al*., 1993), is weakly induced by p73β and p73α. The interaction with Mdm2 leads to the inactivation of transcription and apoptosis functions of p73-β and p73-α (Zeng *et al*., 1999) but does not result in the rapid degradation of p73 protein (Zeng *et al*., 1999; Dobbelstein *et al*., 1999). Disruption of Mdm2-p73 interaction is needed for stimulation of transcription and apoptosis by p73 but does not effect the stability of p73.

Accumulation of p73 in response to certain DNA-damaging stimuli relies on the activity of the c-Abl tyrosine kinase, which prolongs the half-life of p73 (Gong *et al*., 1999). The accumulation of p53 and p73 in response to certain stimuli occurs through independent pathways. The accumulation of p53 is regulated by the inhibition of its degradation, but the accumulation of p73 requires the activity of the stabilizing protein (c-Abl) (Yuan *et al*., 1999; for review see Levrero *et al*.,2000).

Although ultraviolet light, actinomycin D, and methylmethane sulfonate (MMS) do not induce the accumulation of p73, cisplatin, which crosslinks DNA (Gong *et al*., 1999), and taxol, an agent that stabilizes microtubules and prevents completion of mitosis, induce p73 accumulation and increase its half-life. Later it was shown that cisplatin and ionizing irradiation could regulate p73 through protein accumulation or tyrosine phosphorylation, respectively (Gong *et al.,* 1999; Agami *et al.,* 1999; Yuan *et al.,* 1999). These post-translational modifications of p73 occur through its physical interaction with c-Abl kinase. According to these findings, regulation of p73 in response to several types of DNA damage is a complex phenomenon that may be mediated by the recruitment of the different upstream proteins.

1.3 ING1 Gene and its Protein product p33

A novel gene called "Inhibitor of Growth 1, ING1" encoding a 33 kDa protein was identified by subtractive hybridization (Garkavstev *et al*., 1996) (see Appendix A for more info.). By using indirect immunofluorescence, it is shown that the $p33^{ING1}$ protein is located in the nucleus, with its proposed role as a growth regulator. Also a genomic probe to human ING1 localized to chromosome 13 at q33 → q34 by fluorescence *in situ* hybridization (ISH). This area is close to known sites of genomic

alteration in several human cancers: primary gastric cancer, haematologic neoplasms, and head and neck squamous cell carcinomas (Zeremski *et al*., 1997).

All the initial studies of ING1 protein was done by using $p33^{ING1}$, but later the authors reported that the constructs used in these studies were actually the $p24^{ING1}$, one of the three products of human *ING1* gene, due to a cloning defect (Garkavstev *et al*., 1999) (see detailes later).

First studies showed that, $p33^{ING1}$ ($p24^{ING1}$), the protein product of ING1 gene, regulate the growth negatively. Overexpression of transfected ING1 constructs efficiently decreased S-phase fraction by blocking the entry of cells into S-phase. Chronic expression of antisense constructs resulted in tumor induction *in vivo* and in foci formation and ability to grow in soft agar *in vitro*. Further studies suggested that $p33^{ING1}$ may be involved in cell cycle control like many other tumor suppressor genes. Consistent with it playing a growth inhibitory role, the RNA and protein levels of ING1($p24^{ING1}$) increased eight- to ten-fold in senescent cells compared to young proliferation-competent human diploid fibroblasts. Additionally, chronic expression of antisense ING1 ($p24^{ING1}$) RNA resulted in extension of the proliferative life span of normal human diploid fibroblasts (Garkavstev *et al*., 1997a).

The *ING1* gene also appears to be involved in the modulation of some cellular forms of apoptosis including the developmentally programmed certain multipotent embryonic cells. Overexpression of the $p33^{ING1}$ ($p24^{ING1}$) protein conferred sensitivity to apoptosis in different cell models, whereas decreasing ING1 ($p24^{ING1}$) expression using an antisense construct protected cells from apoptosis. These properties of $p33^{ING1}$ ($p24^{ING1}$) are very similar to some tumor suppressor genes, such as p53. Also it was shown that, in response to UV exposure that causes DNA damage, $p33^{ING1b}$ translocates

to the nucleolus. Two stretches of four amino acids act as a nucleolar targeting sequence and mutants of the nucleolar targeting sequence are less effective in inducing apoptosis in primary human diploid fibroblasts (Scott *et al*., 2001). Another study reported that $p33^{ING1}$, is induced at both mRNA and protein levels in melonama cell line after UV irradiation and $p33^{ING1}$ protein enhances nucleotide excision repair of both UV-damaged genomic DNA and exogenous plasmid DNA supporting that $p33^{ING1}$ is a tumor suppressor (Cheung *et al*., 2001).

ING1 gene products contain a zinc finger motif. Zinc finger motifs comprise several structural subfamilies and most of them are thought to participate in recognition of macromolecules, such as DNA, RNA and protein. The carboxyl-terminal 70 amino acid residues of ING1 contain the Cys4-His-Cys3 sequence of a Plant Homeo Domain (PHD) finger domain. PHD finger domains have been found in many different proteins, including transcription factors and other proteins implicated in chromatin mediated transcriptional regulation (Aasland *et al*., 1995).

A $p33^{ING1}$ homolog was identified by a search of a database of randomly sequenced human cDNAs (see appendix B for details). The new gene was called as ING1L (for ING1-like protein). The full-length ING1L cDNA contained an open reading frame of 840bp, encoding a deduced protein of 280 amino acids with a predicted molecular weight of 32.8kDa. ING1L gene is mapped to 4q35.1 by ISH and radiation hybrid mapping techniques. Comparison of the deduced amino acid sequence of ING1L with $p33^{ING1}$, the gene products showed 58.9% identity. A PHD-type zinc finger motif was present in the carboxyl terminal halves of both ING1L and $p33^{ING1}$. Northern blot analyses revealed that ING1L is ubiquitously expressed in various human

tissues and there is a relative increase in the mRNA levels of ING1L in colon cancer tissues compared to normal counterparts (Shimada *et al*., 1998).

Association of *ING1* gene products with growth suppression, replicative senescence, anchorage dependence, and apoptosis raised the question of its relationship with p53. In 1998, Garkavtsev and his collogues reported that $p33^{ING1}$ ($p24^{ING1}$) directly cooperates with p53 in growth regulation by modulating the ability of p53 to act as a transcriptional activator. Reduction of ING1 ($p24^{ING1}$) expression inhibits the growth suppressive activity of p53, suggesting that $p33^{ING1}$ ($p24^{ING1}$) is essential for p53 function. The mechanism of the cooperation between $p33^{ING1}$ ($p24^{ING1}$) and p53 involves physical interaction between these two proteins, which form a complex detectable by immunoprecipitation. Furthermore, it was shown that activation of transcription from the $p21^{WAF1}$ promoter, a key mechanism of p53 mediated control, depends on the expression of $p33^{ING1}$ ($p24^{ING1}$) (Garkavstev *et al*., 1998). These data place $p33^{ING1}$ into a family of p53-interacting proteins, including Mdm2, Ref-1 and p300 which modulate p53 activity through physical interaction. The involvement of $p33^{ING1}$ ($p24^{ing1}$) in this signaling pathway indicates that ING1 may be a potential tumor suppressor gene, the loss or inactivation of which may contribute to alter cell growth control, resistance to apoptosis or establishment of the immortal phenotype in tumors retaining wild type p53.

1.3.1 Structure of *ING1* gene

In 1999, Zeremski and his collaborators reported the detailed structural and expression studies of mouse ortholog of the human *ING1*, *ing1*, gene. Mouse homolog of ING1, ing1, is transcribed from at least three differently regulated promoters, and

resulting transcripts encode at least two different proteins. All of the transcripts share a common region encoded by a common exon but differ in their 5'-exons. Two of the three alternative exons do not contain protein-coding sequences, while the third one does. Therefore one of the *ing1* transcripts encodes a 37-kDa protein ($p37^{ING1}$), while two others are translated into a shorter protein 24 kDa that surprisingly runs as if it was 31 kDa ($p31^{ING1}$). $p37^{ING1}$ is ubiquitously expressed in thymus, spleen, liver, lung, brain, heart, and testis in mouse but $p31^{ING1}$ levels vary among organs and cells. There is a link between the proliferation rate of the cell and *ing1* expression both at the protein and mRNA levels. The most important and interesting finding of this work is the two products of ing1 have opposite effects on p53-dependent transcription regulation; one, $p31^{ING1}$, acts as a p53 cooperator, while the other, $p37^{ING1}$, acts as a p53 suppressor. p53 is found in a complex with the long but not with the short product of ing1 and overexpression of the longer product inhibits accumulation of p53 protein after DNA damage. This may show a new genetic mechanism of promoting cancer that involves an imbalance between the two products of one gene. Overexpression of the longer product of ING1 could be the mechanism of attenuation of p53 activity in tumors that do not require mutations in p53 itself (Zeremski *et al*., 1999).

Alignment of the predicted amino acid sequences with that of human $p33^{ING1}$ revealed 89% similarity between the mouse and human proteins (Zeremski *et al*., 1999).

Human ING1 gene has three exons, from which four mRNA variants are transcribed from three different promoter regions (**Figure 1.7**) (Gunduz *et al*., 2000; Cheung & Li 2001).

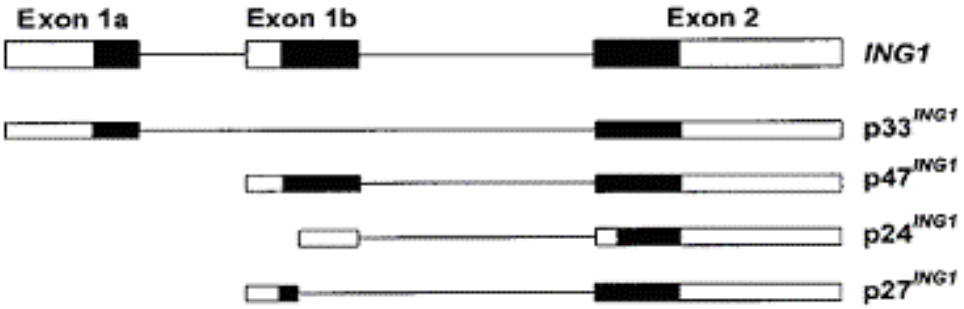


Figure 1.7: Genomic structure of human *ING1* and its alternatively spliced mRNA variants. p47*ING1*, p32*ING1*, p27*ING1*, and p24*ING1* .(■) Coding sequence in exon. (□) Noncoding sequence in exon.

1.3.2 *ING1* Gene products in chromatin remodeling complexes

Recently, the functions of three yeast proteins Yng1, Yng2, and Pho23, which have strong sequence identity in their C-termini PHD finger domains with the human ING1 gene, have been investigated. These three yeast proteins have a role in chromatin remodeling and transcriptional regulation. Yng2 associates with Tra1, a yeast homolog of TRRAP, which associates with PCAF histone acetyl transferase (HAT) complex. TRRAP was identified as a cofactor that interacts with c-Myc and E2F-1 and is required for transformation by c-Myc and E1A (McMahon *et al*., 1998). It has also been shown that Yng1, Yng2 and Pho23 are complexed with HAT activities. HAT activities are often found in large, modular, multiprotein complexes containing known transcriptional regulators. Yng1, Yng2, and Pho23 associate with HAT activities with preferences for different histones, suggesting that they associated with different HAT complexes. The homology between these yeast proteins and $p33^{ING1}$ ($p24^{ING1}$) suggests that these proteins are functionally related. ING1 also associates with HAT activity when it is expressed in yeast. The structure and function of ING1 and Yng2 particularly well

conserved suggesting that human ING1 may be associated with HAT activity and TRRAP, the mammalian homolog of Tra1, in mammals (Loewith *et al*., 2000). A model in which ING1 regulates HAT activity would be consistent with its putative tumor-suppressor role.

Another study showed that alternative transcripts of human $p33^{ING1}$ differentially associate with mSin3 transcriptional corepressor complex and have HDAC1-dependent transcriptional corepressor activity in reporter gene assays. While $p33^{ING1b}$ is associated with the Sin3/HDAC1 mediated transcriptional repression, $p24^{ING1c}$ does not seem to interact with the Sin3/HDAC1 complexes (Skowyra *et al*.,2000). These findings suggest that this interaction is through N-terminal region of $p33^{ING1}$ protein because the three alternative transcripts vary in their N-terminal region.

1.3.3 Cancer Development and p33

ING1 gene emerged as a candidate tumor suppressor gene because: 1) it is located on 13q, a frequent deletion target in many tumor types, including head and neck cancer; 2) it was found to be genetically altered in one neuroblastoma cell line, and decreased rates of protein expression have been detected in several breast cancer cell lines; 3) it interacts with the p53 protein in vitro; and 4) the biological effects of the $p33^{ING1}$ and p53 proteins may be interrelated, since the ING1 gene is implicated in p53 mediated cell growth inhibition. For these reasons scientists started to investigate $p33^{ING1}$ mutations, deletions and rearrangements in different cancer type (for review see; Cheung & Li 2001).

Recently, it has been reported that the ING1 gene is mutated very rarely in primary breast and ovarian cancers and breast cancer cell lines. Also expression of

ING1 gene is reduced in high proportion of breast tumors (44%) and in all breast cancer cell lines. Furthermore it has been suggested that ING1 expression levels correlate strongly with metastasis, with tumors showing reduced levels of ING1 being metastatic at a frequency at least six fold greater than these showing increased levels of ING1 (Garkavstev *et al*., 1999).

However molecular analysis of the ING1 in human head and neck tumors with 13q deletions, showed no somatic mutation in any of the tumors or cell lines (Sanchez-Cespedes *et al*., 1999). Recently another study suggested that of 34 informative cases of head and neck squamous cell carcinoma, 68% of tumors showed loss of heterozygosity at chromosome 13q33-34, where the ING1 gene is located. Three missense mutations and three silent changes were detected in the ING1 gene in some tumors with allelic loss at the 13q33-34 region. These missense mutations were found within the PHD finger domain and nuclear localization motif in ING1 protein. These changes may effect the PHD finger and break the three-dimensional structure of ING1 protein leading, the loss of the function (Gunduz *et al*., 2000). In 31 informative esophageal squamous cell cancer cases, nearly 60% of tumors showed allelic loss at chromosome 13q33-34 and four tumor specific missense mutations detected within the PHD-finger domain and nucleolar localization motif of the ING1 (Chen *et al*., 2001).

The $p33^{ING1}$ status in colorectal carcinomas showed that allelic deletion of, or mutations within, the ING1 gene do not appear to occur during colorectal carcinogenesis (Sarela *et al*., 1999).

1.4 Aim and Scope of the Project

From the evidences presented above, it is obvious that understanding the signaling pathways of p53 family proteins and the proteins involved in these pathways is crucial to solve the cancer puzzle.

This study aims at the identification of the interacting domain of p53 family proteins with $p33^{ING1}$ protein. For this purpose, mapping of the interacting regions of p53 family proteins with $p33^{ING1}$ protein has been investigated, by using *in vitro* methods namely GST pulldown and Far Western Blotting. For this purpose different deletion mutants of p53 family proteins were created and cloned into expression vectors and used in vitro protein-protein interaction assays.

Such a study is thought to highlight the significant points in the interactions among mentioned proteins.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

The laboratory chemicals were analytical grade and supplied from Sigma Biosciences Chemical Company Ltd. (St. Louis, MO, U.S.A) or from Carlo-Erba (Milano, Italy).

2.1.2 Bacterial Strains

The bacterial strain used in this work had the following genotypes:

***E. coli,* DH5α:** F^-, (f80dÊ(*lac*Z)M15), *recA1*, *end*A1, *gyr*A96, *thi*1,*hsd*R17,(r-km-k), *sup*E44,*rel*A1, *deo*R, Ê(*lac*ZYA-ar gF)U169

2.1.3 Enzymes

Restriction endonucleases were purchased either from MBI FERMENTAS Inc. (NY, U.S.A) (*Sal*I, *Xba*I, *Xho*I , *BamH*I, *Not*I, *Apa*I, *EcoR*I, *Nhe*I, *Kpn*I) or from Appligene-Oncor (Illkirch, France) (*Hind*III, *Sac*I). Cloned *Pfu* Polymesase was purchased from Stratagene (CA, U.S.A). T4 Ligase was purchased from MBI FERMENTAS Inc. (NY, U.S.A).

2.1.4 Oligonucleotides

The oligonucleotide primers used for the PCR amplification of $p33^{ING1}$,p63, p73 and for C-terminal deleted forms of these genes were synthesized in the Beckman Oligo 1000M DNA synthesizer (Beckman Instruments Inc. CA. U.S.A) at the Bilkent

University, Faculty of Science, Department of Molecular Biology and Genetics, (Ankara, Turkey).

The primers for p33 are:

Forward: 5'- AGACGTCGACAAATGTTGAGTCCTGCCAACG -3'
*Sal*I

Reverse: 5'- AGACAAGCTTCTACCTGTTGTAAGCCTCTC -3'
*Hind*III

Reverse: 5'-ATATAAGCTTATTAGGGGACGAATCTCGCTC-3'
*Hind*III

The primers for p63 are:

Forward: 5'-GATAGGATCCATGTCCCAGAGCACACAG-3'
*BamH*I

Reverse: 5'-CGCGCTCGAGTCACTTTGTACTGTCCGAAAC-3'
*Xho*I

The primers for p73 are:

Forward: 5'-TATAGGATCCATGGCCCAGTCCACCGCC-3'
*BamH*I

Reverse: 5'-CGCGCTCGAGTCACTTGGCGGAGCTCTCGTT-3'
*Xho*I

Prior to synthesis, the primers were optimized for their annealing temperature, %GC content, hairpin and dimer formation potential with the help of Primer Designer Version 2.0 computer program (Scientific & Educational Software,1990,1991). It was confirmed by the Clone Manager Version 4.0 computer program (Scientific & Educational Software) that $p33^{ING1}$ cDNA contained no apparent *Sal*I, *Hind*III, *Kpn*I

and *EcoR*V restriction enzyme recognition sequences, p63 cDNA and p73 cDNA contained no apparent *BamH*I and *Xho*I restriction enzyme recognition sequences .

2.1.5 Cloning Vectors

PGEM-T Easy is a convenient vector for the cloning of PCR products. This type of vectors have 3'-terminal thymidine at both ends. These single 3' overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into a plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases, like *Taq* DNA Polymerase (**Figure 2.1**).

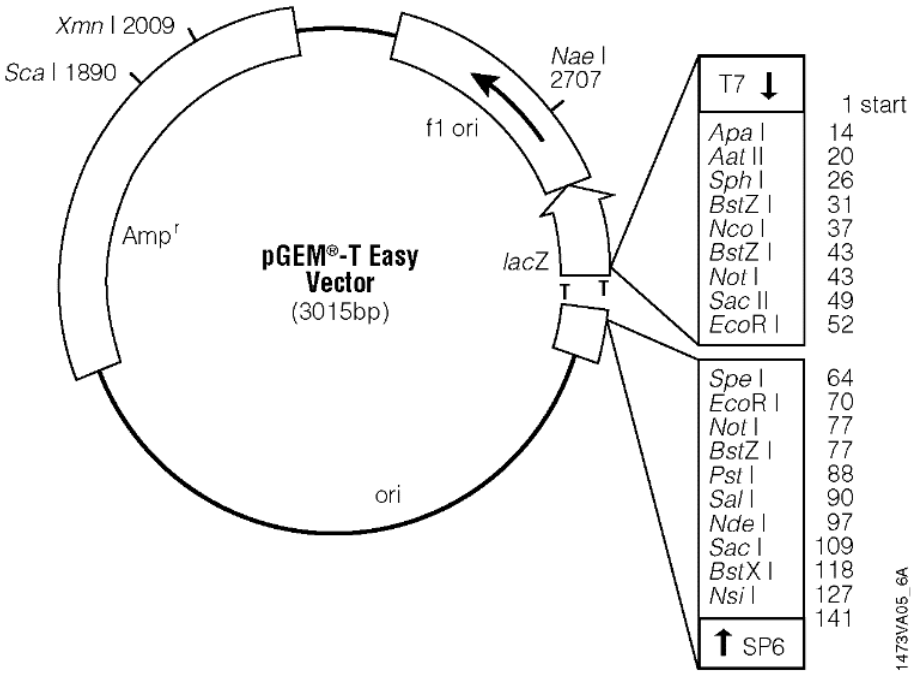


Figure 2.1 Vector map of pGEMT-easy

pBluescript II KS^{-} (GenBank #X52326) is a phagemid that permits expression of cloned genes in bacterial systems or *in vitro* transcription-translation (IVTT) reactions in eukaryotic systems. The vector contains T3 and T7 viral promoters that

span the multiple cloning site and are oppositely oriented. These promoters enable eukaroytic expression of the cloned gene in the presence of respective viral RNA poymerases (**Figure 2.2**).

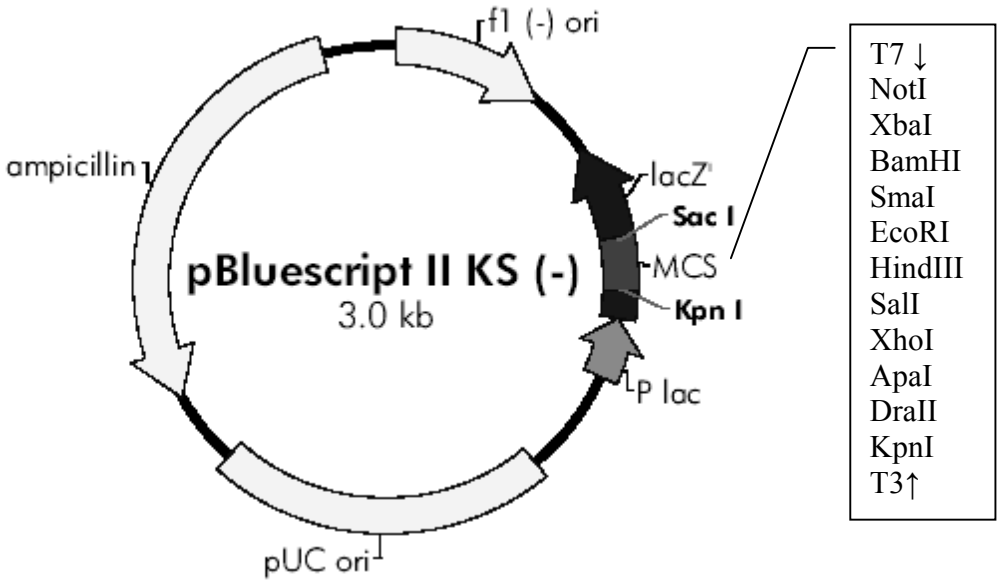


Figure 2.2: Vector Map of pBlueScript II KS-

pcDNA 3 is a mammalian expression vector. The vector contains both T7 and Sp6 viral promoters, spanning the multiple cloning site. These promoters and CMV promoter permits the expression of this plasmid both IVTT reactions and mammalian systems respectively (**Figure 2.3**).

2.1.6 Antibodies

The primary antibodies used in GST pulldown assays are as follows: monoclonal anti p33 antibodies (3G6, 20H6, 15B9, 9H9), produced by Rengül Çetin-Atalay, monoclonal mouse-anti-p53 antibodies (7D3: specific to amino acids 211-220 ;

HR221: specific to amino acids 371-380; 9E4 specific to amino acids 281-290) kindly provided by Dr. Esma Yolcu (Yolcu *et al.,*2001).

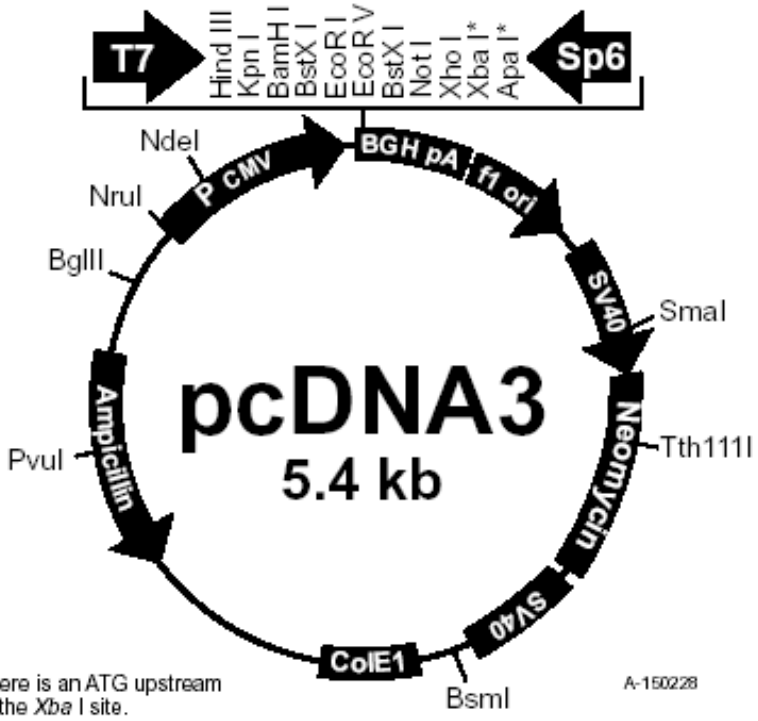


Figure 2.3: Vector Map of pcDNA3

2.1.7 Commercially Available Kits

QIAquick PCR Purification Kit (by Qiagen (Chatsworth, CA, U.S.A)) is used for cleaning nucleic acids from contaminants.

QIAEXII (by Qiagen (Chatsworth, CA, U.S.A)) is for extraction of DNA bands from agarose gels.

QIAGEN Plasmid Purification Kit (by Qiagen (Chatsworth, CA, U.S.A)) is utilized to obtain plasmid DNA at higher amounts and concentrations with higher purity than those achieved by MiniPrep.

pGEM-T and pGEM-T Easy Vector Systems is purchased from Promega (Madison, WI, USA) for the easy cloning of the genes in vectors.

TNT Coupled Reticulocyte Lysate System is purchased from Promega (Madison, WI, USA) for the single step *in vitro* transcription-translation (IVTT) of the cloned genes in an eukaryotic system.

Amplify Solution from Amersham (Uppsala, Sweden) is a flourimetric method to enhance the visualization of the signals generated by radioisotopes on polyacrylamide gels.

2.1.8 Apparatus

Vertical mini gel apparatus for polyacrylamide gel electrophoresis and the power supply are products of E-C Apparatus Corp. (Florida, U.S.A). Horizontal midi gel apparatus used for agarose gel electrophoresis is purchased from Stratagene. Thermal cycler for PCR is a product of Perkin Elmer (CA, USA). Slab Gel Dryer is from Savant Instruments Inc.(N.Y.,USA). Semi-dry transfer unit for Far Western Blotting and GelDoc2000 Image analyzer for agarose gels are purchased from Bio Rad Laboratories (CA, U.S.A).

2.1.9 Materials for Autoradiograghy

Radiolabelled methionine (^{35}S-Methionine) and light-proof film cassette (Hypercassette) and film developing unit are products of Amersham (Uppsala, Sweden). Medical X-ray films are purchased from Fuji (Tokyo, Japan).

2.1.10 DNA and Protein Size Markers

As a DNA size marker, 1 kb DNA Ladder from MBI FERMENTAS Inc. (NY, U.S.A) is used.SDS-PAGE protein size markers are either from Pharmacia (Uppsala, Sweden) or Bio Rad Laboratories (CA, U.S.A) (prestained markers).

2.2 Solutions and Media

2.2.1 Agarose Gel Electrophoresis Solutions

Tris-acetic acid-EDTA (TAE)	40mM Tris-acetate 1mM EDTA
Ethidium bromide:	10 mg/ml in water (stock solution), 30 ng/ml (working solution)
1x Gel loading buffer:	0.25% bromophenol blue, 0.25% xylene cyanol**,** 50% glycerol, 1mM EDTA

2.2.2 Solutions for Plasmid DNA Isolation (MiniPrep)

Solution I	50 mM Glucose, 25 mM, Tris.Cl, pH 8.0, 10M EDTA. Sterilize in autoclave.
Solution II	0.2 N NaOH, 1% (wt/vol) SDS
Solution III	3 M Potassium acetate, pH 4.8 11.5% (v/v) glacial acetic acid
Phenol/ Chloroform	100% equilibrated phenol / 100% chloroform

2.2.3 Solutions for Bacterial Transformation

$CaCl_2$	50 mM in double distilled water, filter sterilized.

Transformation Buffer	10 mM PIPES, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, Filter Sterilized and stored and 4˚C.

2.2.4 Microbiological media and antibiotics

Luria-Bertani medium (LB)	*Per liter:* 10 g nutrient broth, 5 g bacto-yeast extract, 8 g NaCl, 0.52g Tris Base *For LB-agar plates, add 15 g/L bacto agar.* Sterilized by autoclave.
SOB	*Per liter*: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, Autoclaved to sterilize. Then 20 mM $MgSO_4$ and 10 mM $MgCl_2$.
SOC	Per liter: SOB + 20 mM Glucose from filter sterilized 1 M Glucose stock solution in ddH_2O.
DMSO	Commercially available, 100%
Ampicillin	working solution: 100 µg/ml or 50 µg/ml stock solution :100 mg/ml in ddH_2O (stored at -20°C)

2.2.5 Polyacrylamide Gel Electrophoresis Solutions

Resolving (Lower) Buffer (2X)	375mM TrisHCl, 0.2% SDS, pH 8.9
Stacking (Upper) Buffer (2X):	250mM TrisHCl, 0.2% SDS, pH6.8
Running Buffer (1X)	25mM Tris base, 192 mM glycine, 1% SDS

Sample Dye (2X)	50mM TrisHCl pH6.8, 1% SDS, 2mM EDTA, 1% 2-ME, 0.02% Bromophenol Blue,10% glycerol
Acrylamide-Bisacrylamide mix	24% acrylamide, 0.64% bisacrylamide (stock)
Amonium persulphate (APS)	10% (w/v) ammonium persulphate in ddH_2O
Coomassie Staining Solution	Solution1: 10% Acetic Acid Solution2: 0.04% Coomassie G Blue, 0.5% $CuSO_4$ in 27% ethanol *Mix Solution1 and 2 at a ratio of 1:1*
Destain Solution	14% ethanol, 7% acetic acid in ddH_2O

2.2.6 Far Western Buffers

Denaturation Buffer:	Hepes (pH:7.8)	25mM
	NaCl	25mM
	$MgCl_2$	5mM
	GnHCl	6M
	DTT	1mM
Renaturation Buffer:	Hepes (pH:7.8)	25mM
	NaCl	25mM
	$MgCl_2$	5mM
	GnHCl	0.187M
	DTT	1mM
Saturation Buffer:	Hepes (pH:7.8)	25mM
	NaCl	25mM

	$MgCl_2$	5mM
	DTT	1mM
	NP-40	0.05%
	Milk Powder	1%
IVTT Buffer:	NaCl	0.1M
	Tris HCl (pH: 7.6)	20mM
	DTT	1mM
	EDTA	1mM
	Glycerol	10%
	Milk Powder	1%

2.2.7 Solutions for Trichloroacetic acid Precipitation

TCA Solution:	Trichloroacetic acid	5% (w/v)

2.3 Methods

2.3.1 PCR amplification of cDNA

PCRs were performed using Cloned *Pfu* DNA Polymerase from Stratagene (La Jolla, CA, U.S.A) as described in the manual provided by the supplier with some modifications. Ingredients of each 50 µl reaction is as follows: Cloned *Pfu* DNA Polymerase (2.5 units), dNTP mix from MBI FERMENTAS Inc. (NY, U.S.A) (10 nmoles), buffer of Cloned *Pfu* DNA Polymerase (1x), specific forward and reverse primers (250 ng or 125 ng each), DNA template (6µl from MCF7 total cDNA, or 1 µl from pBks plasmid containing the genes of interest), ddH_20 (making the total volume 50µl). Care was taken not to contaminate the reaction tubes with foreign DNA. Every time, a negative control reaction that contains no DNA template was set up. High extension times (2.5 min/cycle) were applied to compensate the low nucleotide incorporation rate of the enzyme. Polymerase was added into the reaction tubes after the initial denaturing step to disallow premature extention ("Hot Start PCR"). PCR products are checked for length and purity by AGE.

2.3.2 Agarose gel electrophoresis of DNA

DNA fragments were fractionated by horizontal electrophoresis by using standard buffers and solutions. DNA fragments less than 1 kb were generally separated on 1.0 % agarose gel, those greater than 1 kb were separated on 0.8 % agarose gels.

Agarose gels were completely dissolved in 1x TAE electrophoresis buffer to required percentage in microwave and ethidium bromide was added to final concentration of 30 ng/ml. The DNA samples were mixed with one volume-loading

buffer and loaded onto gels. The gel was run at room temperature in 1x TAE at different voltage and time depending on the size of the fragments.

Nucleic acids were visualized under ultraviolet light (long wave, 340 nm) and standard DNA size marker, 1 kb DNA ladder, was used to estimate the fragment sizes.

2.3.3 Recovery of DNA Fragments from Agarose Gels

DNA fragments were extracted from agarose gels by using the QIAEX II gel extraction kit according to the manufacturer's instructions.

Gel purification with the QIAEX II kit yields 60-70 % recovery of DNA fragments between 1.0 kb to 6.0 kb in 10-20 µl volume.

2.3.4 Methods for Cloning and Subcloning of Genes

2.3.4.1 PolyA Extension Reaction

Gel-extracted PCR products were polyA extended using *Taq* DNA Polymerase enzyme. Ingredients of polyA extension reaction are as follows: *Taq* DNA Polymerase (1µl), dATP (2mM dATP/µl), *Taq* DNA Polymerase Buffer (1x), DNA extracted from gel (7 µl). Reaction tubes were incubated at 70^{o}C during 30 minutes.

2.3.4.2 Insertion of Products of PolyA Extention Reaction into pGEM-T easy vector

Insertion of products of PolyA extention reaction were performed in 15 µl insertion reaction volume for overnight incubation at 16°C. Reactions were carried out with the appropriate reaction buffer and according to the manufacture's recommendations.

2.3.4.3 Restriction Enzyme Digestion of DNA

Restriction enzyme digestions were routinely performed in 20 μl reaction volume for 1.5 to 4 hours and typically 1-10 μg DNA and 5-15 units of restriction enzyme was used. Reactions were carried out with the appropriate reaction buffer and conditions according to manufacturer's recommendations.

Digestion of DNA with two different restriction enzymes simultaneously was generally possible by using a universal buffer (potassium acetate buffer) that fulfills the activity requirements of both enzymes. In this regard, Y^+/Tango Buffer from MBI Fermestas was usually suitable. The working concentration of this buffer was modified for each enzyme (either 1x or 2x), and this way, it was possible to match the buffer requirements of various enzymes simultaneously.

2.3.4.4 DNA Ligation Reactions

DNA fragments were ligated into plasmid vectors in 15 μl reaction volumes containing 0.3-1.0 μg of linearized plasmid vector and 7-10 times molar excess of insert DNA in the presence of 1-4 Weiss units of T4 ligase and 1x concentration of the standard ligation buffer supplied by with the enzyme. The reaction mix was incubated at either room temperature for 2.5-4 hours or at 16^{o}C overnight.

2.3.4.5 Transformation of *E.coli*

Transformation of plasmid DNA into *E.coli* was achieved by using calcium chloride method. The following procedure is based on Ausubel *et al.* (1991).

Preparation of competent cells

This method is based on a report by Inoue *et al*. 1990. DH5α cells were grown in SOB medium at 30°C to an A_{600} of 0.5-0.6 with vigorous shaking/stirring at 200 rpm and cooled down on ice for 10 minutes. Cells were transferred to 500 ml centrifuge bottles and centrifuged at 300 rpm (Beckman JA10 rotor, pre-cooled at 4°C). The pellet was resuspended in 1/3 of the initial volume of ice-cold transformation buffer by gently swirling and kept on ice for 10 minutes. The suspension was then centrifuged at 3,000 rpm for 10 minutes. The pellet was gently resuspended in 1/12.5 initial volume of ice-cold transformation buffer and DMSO was added while gently swirling to a final concentration of 7 %. Before distribution into tubes, incubation was done on ice for 10 minutes. Tubes were immersed in liquid nitrogen to freeze rapidly and stored at –80°C.

Transformation

Competent cells from –80°C stock were thawed at room temperature and placed on ice as soon as completely thawed. 200 µl of cells were dispensed into plastic tubes (15 ml tubes). DNA to be transformed up to 20 µl was added and incubated on ice for 30 minutes. After heat-shock at 42°C for 30 seconds, tubes were put on ice and 800 µl SOC was added. Following incubation at 37°C with vigorous shaking for 1 hour, cells were plated out on LB+ selective agent(s).

2.3.4.6 Plasmid DNA Preparation

Small scale isolation of plasmid DNA (MiniPrep)

The transformant bacteria strain containing the plasmid of interest was grown in 5 ml LB medium containing 100 µg/ml ampicillin at 37°C, while shaking at 200 rpm overnight. 1.5 ml culture was pelleted in 1.5 ml microfuge at 13,000 rpm for 2 min.

After removal of supernatant, the cells were resuspended in 100 µl ice-cold Solution I. Freshly prepared 200 µl of Solution II was added and mixed in by inverting the tube very gently. Bacterial chromosomal DNA and cell debris were precipitated by the addition of 150 µl of Solution III. Then, 500µl of Phenol/Chloroform was added. The tubes were vortexed vigorously for 2 min and centrifuged for 5 min at 13,000 rpm, 4°C to pellet the host DNA and proteins. Supernatant was transferred into a new eppendorf tube, mixed with 1 ml ice-cold absolute ethanol and the mixture was incubated at -80°C for 20 min. The plasmid was recovered by centrifugation at 13,000 rpm for 25 min at 4°C. The pellet was washed with 300 µl 70% ethanol. Ethanol was aspirated and the pellet was air-dried. The pellet was resuspended in 20-30 µl sterile distilled H_2O containing 10 µg/ml RNase A and incubated at 37°C for 10-20 minutes and resuspended. The sample was kept at 4°C for short-term or at -20°C for long-term storage. This procedure yields approximately 1-1.5 µg of DNA per reaction.

Purification of plasmid DNA using the Qiagen Kit (MidiPrep)

The Qiagen 100 kit was used for large scale isolation of pure plasmid DNA. The method is based on the "midi-prep" instructions supplied with the QIAGEN Plasmid Midi Kit (Cat. No. 12145) by Qiagen (Germany).

This procedure yields approximately 60-150 µg of plasmid DNA from 100 ml of initial bacterial culture.

2.3.5 Quantification of Nucleic Acids

Concentrations and purity of the double stranded nucleic acids (plasmid DNAs and constructs) and oligonucleotides were determined by using the Beckman

Instruments Du Series 600 Spectrophotometer software programs (ds DNA and Oligo DNA Short methods) on the Beckman Spectrophotometer Du 640 (Beckman Instruments Inc. CA. U.S.A) at A_{260} and A_{280}.

2.3.6 Growth and Storage of Bacterial Strains

A single bacterial colony picked from either an agar plate or a loopfull of bacterial glycerol stock was inoculated into 5 ml LB (containing the appropiate antibiotics, if necessary) in 15 ml screw capped tubes. The tubes were incubated at 200 rpm at 37°C overnight in a rotator-incubator.

Bacterial strains were stored in 2 ml cryotubes at -80°C in LB medium containing 40% glycerol for long term storage. Recombinant clones were stored under the same condition in media containing the appropriate antibiotic. Strains were maintained as isolated colonies on LB agar plates at 4°C for short term storage.

2.3.7 SDS Polyacrylamide Gel Electrophoresis

Electrophoretic separation of proteins under denaturing conditions was performed by following the methods described in Sambrook et. al. (1989) with slight modifications. 8%, 10%, 12% gels were made according to the specific purpose. The mini gel glass plates were assembled according to the manufacturer's instructions (E-C Apparatus Corp). In order to prepare a 12% gel, 5 ml of 2x resolving gel, 5 ml of acrylamide-bisacrylamide mix, 140 µl of 10% APS, 8 µl of TEMED were mixed and poured into the gap between the glass plates. Sufficient space (the length of the comb teeth plus 1 cm) was left for the stacking gel. Water saturated 2-propanol was layered onto the top of the gel. After the gel was settled and polymerized at room temperature,

the 2-isopropanol layer was removed and 3% stacking gel (3ml 2x stacking buffer, 0.75 ml acrylamide-bisacrylamide mix, 2.25 ml double distilled water, 100 µl 10% APS, 6 µl TEMED) was poured on top of the resolving gel. The comb was immediately inserted into the stacking gel, being careful to avoid air bubbles. After the gel was polymerized, the comb was removed and the wells were cleaned from the remaining unpolymerized gel by a syringe. 2x sample buffer was added to the samples and the samples were heated for 5 min at 95^{o}C, when necessary prior to loading. After loading the samples to the wells, the gel was mounted in the electrophoresis tank and 1x SDS-running buffer was added to the top and bottom reservoirs. The gel was run such that each gel received around 25 mA of current. The gel was then either fixed and amplified or used in semi-dry tranfer. The gels were dried in slab gel dryer for long term storage

2.3.8 *In vitro* Transcription-Translation (IVTT) Reactions

TNT Coupled Reticulocyte Lysate System from Promega Corp. (WI,USA) provides a conventional system for protein synthesis. Transcription and translation from an open reading frame cloned in a vector having either T7 or T3 viral promoters are facilitated as a single, coupled reaction. All the transcription and translation machinery is supplied by the reticulocyte lysate of rabbit. In addition to its ease and rapidness, this system provides a means to label the protein of interest. Labeling takes the form of radio-labeling, such that exogenously supplied methionine which contains a radioactive sulfur (^{35}S-Methionine) is incorporated into the polypeptide of interest during translation. Radio-labeled proteins can be further used in various experiments, such as GST Pulldown assays.

IVTT was performed as described in the manual supplied by the manufacturer. Care was taken to prevent RNase contamination by using filtered micropippette tips, DEPC ddH_2O, and RNasin (RNase inhibitor from Promega Corp.) when available. Always a negative control lacking the DNA template was used to check foreign DNA contamination. At the end of the reaction, protease inhibitor cocktail was added into each reaction mix to a final concentration of 1X and kept a 4^oC for short term (1-2 days) storage. pBluescript II KS^- and pcDNA3 were used as the cloning vectors from which IVTT reactions took place.

Measuring of Incorporated S-Methionine

The incorporated ^{35}S-Methionine was measured after IVTT reaction before using in Far Western and GST pulldown assays. 2µl from each IVTT reaction product was put on two sets of the glass fiber filters and dried. The first set of these filters were air dried only. The second set of these filters were incubated in 5% TCA solution during 5 minutes, and boiled in TCA solution during 5 minutes. After boiling, the filters were air-dried and both sets were measured using Liquid Scintillation Counter.

2.3.9 GST Pulldown Assays

GST Pulldown Assays were employed to detect the *in vitro* interaction between $p33^{ING1}$ and p53 family proteins and to map the interacting region on p53 family members with $p33^{ING1}$. In this system, a GST-fusion protein is bound to gluthatione-sepharose beads. The next step is to incubate this complex with the labeled polypeptide whose interaction with the GST-fusion protein is investigated. Following extensive washes; the reaction mixture is subjected to SDS-PAGE. If an interaction between the polypeptides is present, the labeled protein persists in the reaction mixture despite

washes, and it can be detected because of its label. Label of the second protein is in the form of radioactivity due to ^{35}S-labeled methionine that is incorporated into the protein during IVTT reaction. In this study, different radiolabeled deletion mutants of p53 protein family and p33^{ING1} were synthesized *in* vitro in order to detect the regions of p53 family proteins required to interact with p33^{ING1}.

The assay was performed as follows: Gluthatione-Sepharose beads (25 μl per reaction) were equilibrated by washing with binding buffer (25 mM HEPES pH 7.5, 10% glycerol,150 mM KCl, 0.1% NP40) twice. Centrifugation of beads between washes and all through the process was for 4 min at 2000 rpm. Beads were resuspended in 25 μl of binding buffer per reaction and GST-fusion proteins were added. For each GST pulldown assay, 3 reaction were set: one with purified GST-p33 (4 μg) which was the test sample; one with purified GST (2 μg) which was a negative control; one with no protein at all which was another negative control. These samples were incubated with the beads overnight at 4^{o}C, on a shaker. Then, reaction mixes were saturated with BSA (final concentration: 3%) for 2 hrs at 4^{o}C, on a shaker, and washed with 1 ml of binding buffer for 3 times. 25 μl of buffer per reaction was left in the tube at the last wash. Following saturation, 10 μl of the second protein (radiolabeled IVTT reaction product) plus DTT (final concentration: 1mM) were added to the reaction mix and incubated for 2 hrs at 4^{o}C, on a shaker. Washes were performed at 4^{o}C with wash buffer (20 mM TrisHCl pH7.5, 200 mM KCl, 1% NP40). Each sample was washed with 1 ml of buffer for 4 times. Microcentrifuge tubes of the reaction mixes were changed after first wash in order to prevent false positives in case radioactivity adsorbs to the tube. After the washes, bead pellets were resuspended in SDS-loading dye (25 μl) and

incubated in boiling water for 5 min to disrupt all protein-protein interactions, followed by brief centrifugation to pellet the beads. The supernatants were subjected to SDS-PAGE, and then the polyacrylamide gels were incubated in fixing solution (25% 2-propanol, 10% acetic acid) for 30 min at RT and incubated in *Amplify* solution to enhance radioactivity intensity for 20-30 min at RT. Gels were then dried in vacuum dryer and exposed to X-ray films overnight to 48 hrs.

2.3.10 Far Western Blotting

Far Western Blotting is a novel technique to show protein-protein interaction *in vitro* (Guichet *et al*., 1997). By the help of this technique, interacting regions of the proteins can also be detected. In this system, protein of interest is first run on SDS-PAGE and then transferred to the nitrocellulose membrane like usual Western Blotting. Then this membrane is first denaturated, then renaturated and saturated with different buffers and finally incubated with a radioactively-labeled IVTT product. Following several times washing, membrane is exposed to X-ray films. If there is an interaction, interacting protein is held on the membrane and can be detected by autoradiography.

The assay was performed as follows: 10μg from GST-p33, GST and purified 6Histidine-PTEN protein run on 10% SDS-PAGE and then transferred to the nitrocellulose membrane by applying 12V, during 1 hour. After transfer, proteins on the membrane is first denaturated at 4^{o}C, 30 minutes in denaturation buffer and then renaturated at 4^{o}C, during 1 hour, in renaturation buffer and then membrane was saturated in saturation buffer 2 hours at 4^{o}C. During these processes, wild type p53, p63, p73 proteins and their C-terminally deleted forms were *in vitro* transcribed-translated and radioactively labeled, each in 25μl reaction volume, by IVTT reaction.

The incorporated radioactivity were measured using liquid scintillation counter for each sample, and equal amounts of the IVTT products were incubated with nitrocellulose filters in IVTT saturation buffer. Following overnight incubation at 4^{o}C, the membrane was washed twice with IVTT saturation buffer and was exposed to X-ray films for 3-7 days.

CHAPTER 3: RESULTS

Former studies carried out in our laboratory, revealed that the possible interacting region of p53 with $p33^{ING1}$ is at the C-terminus of the protein by GST pulldown assays (Emre N. C. T., 1999). Therefore in the first part of our studies, the interacting region of p53 and $p33^{ING1}$ was confirmed by *in vitro* protein-protein interaction assays. For the ultimate mapping of the region of p53 interacting with $p33^{ING1}$; new deletion mutants of p53 were cloned and used in GST pulldown and Far Western assays. The second task of our study was to look for whether the members of the same family also interact with $p33^{ING1}$ gene product, or not. Full-length p63 and p73 proteins were shown to interact with $p33^{ING1}$ by Far Western Blotting. Then C-terminally deleted forms p63 and p73 were amplified and cloned into expression vectors and checked for the interaction with $p33^{ING1}$ by *in vitro* protein-protein interaction assays.

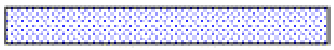
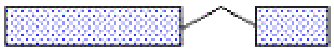
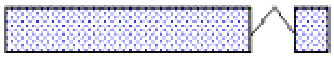
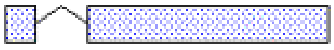
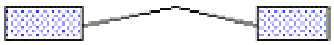
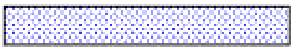
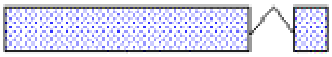
3.1 Examination of Deletion Mutant Constructs of p53

Previous studies in our laboratory have examined the interacting region of p53 protein with $p33^{ING1}$ by using different deletion mutants of p53 *in vitro* analysis. These studies showed that the interacting region of p53 is at the C-terminus of the protein. The results of these studies are summarized in Table 1(Emre N. C. T., 1999).

The first part of our studies concentrated on the confirmation of the former results by GST pulldown assays. Before producing the wt p53 protein and its deletion mutants by the coupled in vitro transcription-translation reaction, all old p53 deletion

mutant constructs were checked for the presence of the desired inserts by single digesting with *Hin*dIII and double digesting with *Hin*dIII and *Sac*I. Agarose gel electrophoresis of the digestion reaction products was shown in **Figure 3.1**, all with the expected inserts.

Table 3.1: Different deletion products of p53 and their interaction status with p33^{ING1}
Transcritional activation domain locates between aa residues 1-43, proline rich domain locates between aa residues 62-91, DNA binding domain locates between aa residues 102-292, oligomerization domain locates between aa residues 320-360, C-terminus domain locates between aa residues 330-393.

P53 constru	Deleted regions (a.a.)	Diagrams	Interaction with p33^{ING1}
WT	-		+
Fu-Rz	361-393		-
F4-R4	207-293		-
F6-R5	300-357		-
F2-R2	44-99		+
F4-R3	98-293		-
Fa-Ru	1-39		+
F5-R5	300-325		+

The full-length human p53 protein and its deletion mutants, as presented in **Table 1**, were produced by the coupled in vitro transcription-translation kit of Promega (see Section 2.3.8)

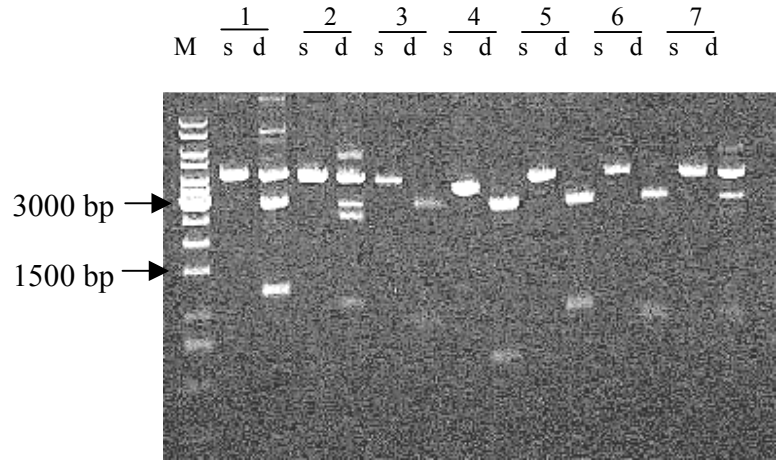


Figure 3.1: Restriction enzyme digestion analysis of MiniPreps of deletion mutant constructs: : *s*: single digestion with *Hind*III; *d*: double digestion with *Hind*III/*Sac*I *1*:WT (1179 bp); *2*:FaRu (1062 bp); *3*: F5R5 (1104 bp); *4*; F4R3 (585 bp); *5*:F2R2 (1014 bp); 6:F6R5 (1008 bp); *7*: F4R4 (921 bp). 1% agarose gel.

Figure 3.2 reveals that there has been protein synthesis from all of the constructs prepared. Some of the lanes contain 2 or more bands, which may be degradation products or premature translation-stop products.

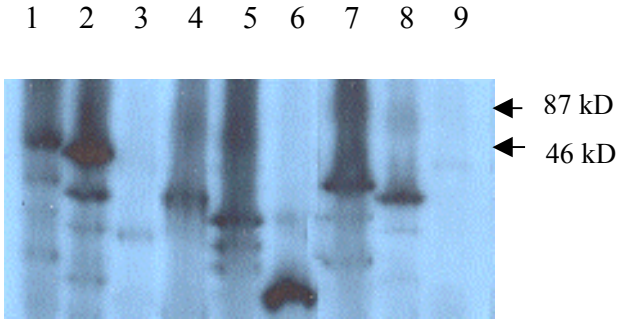


Figure 3.2: SDS-PAGE analysis of IVTT reactions of deletion mutant constructs of p53:
1: wtp53 (393 aa); 2:FuRz (361 aa); 3:F4R4 (307 aa); 4F6R5 (336 aa); 5:F2R2 (338 aa); 6:F4R3 (195 aa); 7:FaRu (354 aa); 8:F5R5 (368 aa); 9:NC. IVTT reaction was performed in 25µl reaction volume for each sample. 12% SDS-PAGE.

3.2 Confirmation of Previously Obtained Results

GST pulldown assays were used in order to map the regions of p53 that interact with $p33^{ING1}$. For each GST pulldown assay, 3 reactions were set: one with purified

GST-p33^{ING1} which was the test sample; one with purified GST, which was a negative control; one with no protein at all which was the other negative control.

Initially, before testing the p53 deletion mutants for mapping purposes, the full-length p53 was shown to bind GST- p33^{ING1}, but not either to GST or the gluthatione beads, *in vitro* by GST pulldown assays.

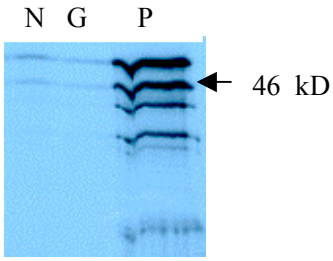


Figure 3.3: GST pulldown assay of wt p53 with GSTp33.
N: no protein (-) control; G: with GST protein (-) control; P: with GST-p33 protein. 12% SDS-PAGE.

In **Figure 3.3**, there are several bands in the last lane, which may be degradation products or premature translation-stop products. After verifying the *in vitro* interaction of the full-length proteins (see **Figure 3.3**), p53 deletion mutant constructs were tested for the confirmation and initial mapping of the p53's interacting region. Eight different constructs (including the wt p53), together with their negative controls, were first *in vitro* transcribed and translated and then used in GST pulldown assays.

The results of this experiment confirmed the previous findings. **Figure 3.4** shows that the interacting region(s) is at the C-terminus region of the p53 protein. The interaction in tubes containing N-terminus mutant forms of p53, F2R2 and FaRu, was not inhibited. However when the C-terminus of the p53 was deleted the interaction was inhibited.

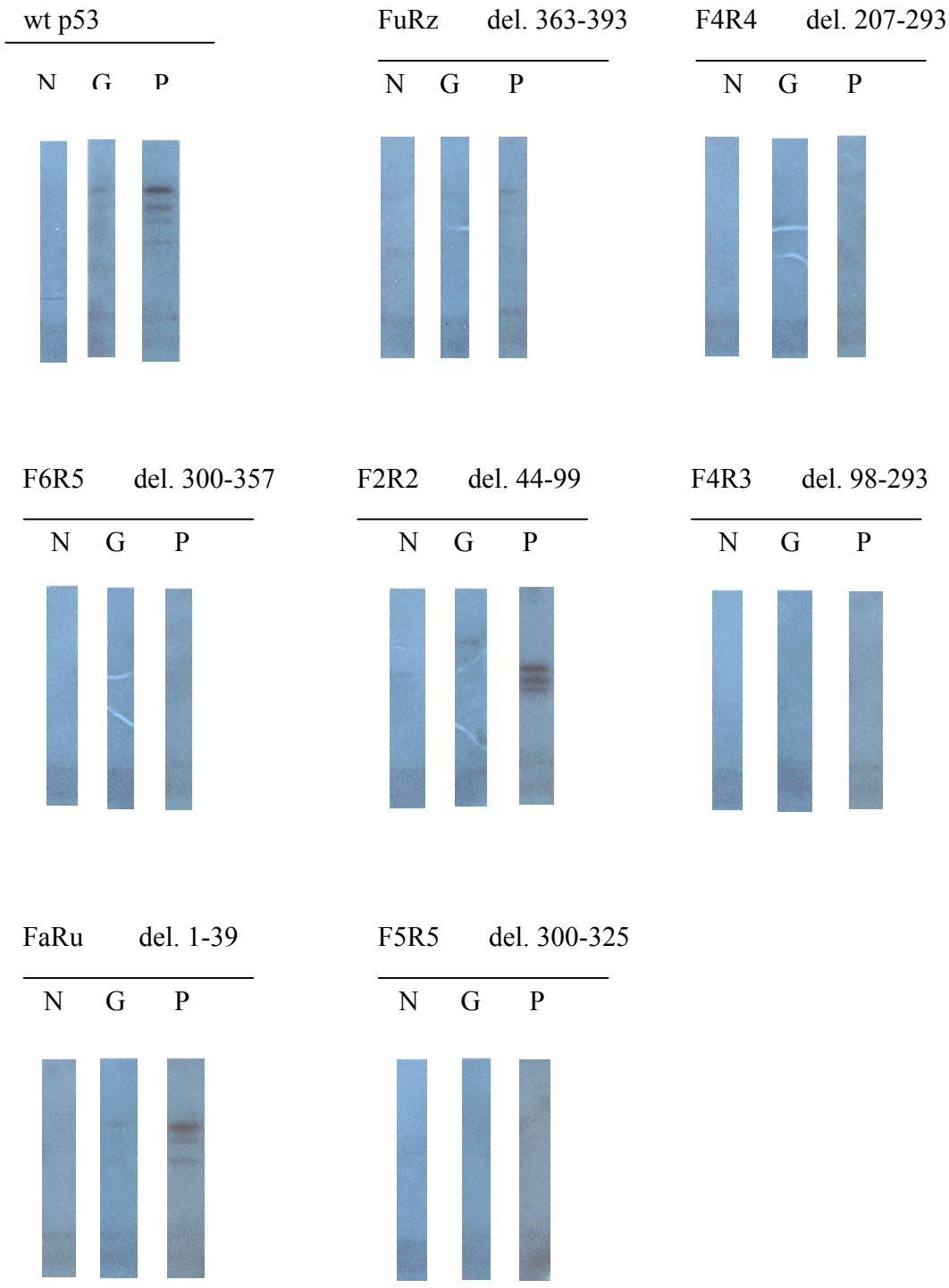


Figure 3.4: GST pulldown with p53 deletion constructs.
N: no protein; *G*: with GST protein; *P*: with GST-p33 protein. 12% SDS-PAGE.

To examine this interaction more precisely, GST pulldown assays were repeated by using different p53 and $p33^{ING1}$ antibodies.

3.3 Specific Monoclonal Antibodies to p53 and $p33^{ING1}$ can not Inhibit the Interaction

Another way to find out the interacting region of p53 with $p33^{ING1}$ was to use the monoclonal antibodies against p53 and $p33^{ING1}$ in conjunction with GST pulldown assays. If the IVTT-p53 (wt) and GST-$p33^{ING1}$ incubated with a monoclonal antibody, reacted with a site that was required for the interaction of these proteins, we would expect a decrease or loss of GST-$p33^{ING1}$-p53 binding compared to control reaction without the antibody.

The GST pulldown assays with antibodies specific to p53, including the C-terminus specific p53 antibody, HR231, and specific to $p33^{ING1}$ were performed as described with the following modifications: just before incubating the IVTT reaction mixture with the bead-bound proteins, the IVTT of wt p53 was incubated with the one of the anti-p53 antibodies or one of the anti- $p33^{ING1}$ antibodies were incubated with bead bound GST-$p33^{ING1}$ protein. p53 and $p33^{ING1}$ antibodies were available as hybridoma supernatants, 8µl from each hybridoma supernatant was added to each reaction mixture and incubated for 30 minutes at 30°C. As a control, one reaction with no antibody was also performed in parallel (supplemented with regular DMEM instead of hybridoma supernatant). **Figure 3.5** shows that the intensity of the signal with the antibodies does not change significantly when compared to control (no antibody) reaction. The C-terminus specific antibody HR231 also could not inhibit the interaction between these two proteins.The interaction between these two proteins is either too

strong to be inhibited by antibodies or the antibodies in hand are not specific to the region which is directly involved in interaction.

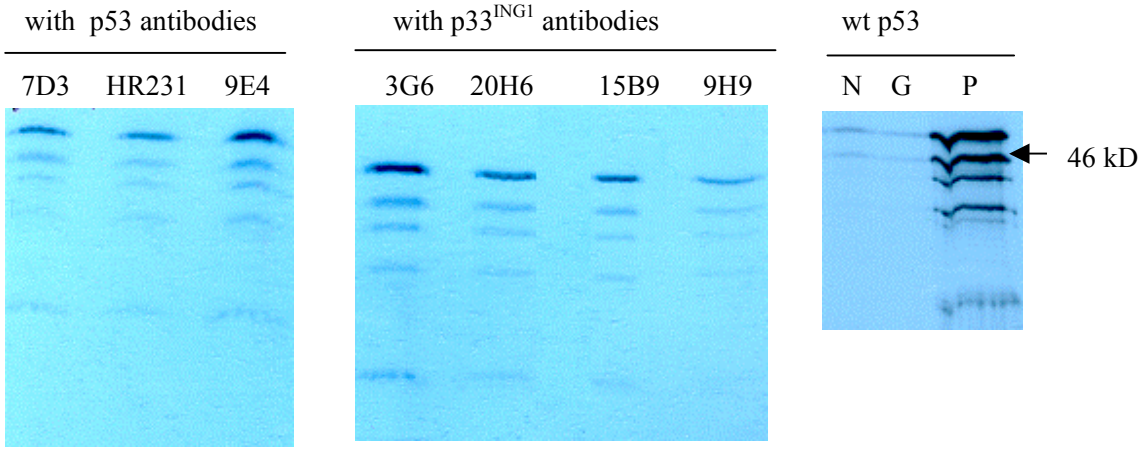


Figure 3.5: GST pulldown assay with p53 and p33^{ING1} antibodies 7D3: specific to aa 211-220; HR231: specific to aa 371-380; 9E4: specific to aa 281-290; specificities of p33^{ING1} antibodies does not known yet; ***N***: no protein**;** ***G*:** with GST protein**;** ***P*:** with GST-p33^{ING1} protein. 12% SDS-PAGE.

3.4 Demonstration of p53 and p33^{ING1} Interaction by Far Western Analysis

As previously mentioned, Far Western assay was used to find out and confirm the interacting regions between p53 and p33^{ING1} Far Western blotting was a method of choice because of its specificity.

Prior to testing the interaction of the proteins, the Far Western assay was optimized using full-length p53. This assay was tried in two different ways: First without the inclusion of the denaturation and renaturation steps and secondly with the inclusion of the buffers by applying the radioactively labeled *in vitro* synthesized wt p53 directly onto the saturated membrane. The results, **Figure 3.6**, showed that denaturation and renaturation steps are vital for this assay, since the interaction between labeled p53 and GST-p33^{ING1} could only be seen with treated membrane.

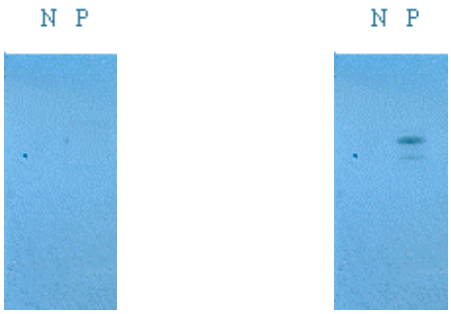


Figure 3.6: Optimization of Far Western assay. *N*: GST protein only (-) control; *P*: GST p33^{ING1} protein. 10µg protein was loaded in each lane. First gel was not treated with denaturation/renaturation buffers, second gel was treated with denaturation/renaturation buffers.

After optimization of the conditions and showing that wt p53 interacts with GSTp33^{ING1}, this experiment was repeated by using the C-terminus deleted mutant of p53, FuRz, which does not have the last 30 amino acids of the wild type p53. For this reason, wt p53 and FuRz were IVTT-produced and incubated with a nitrocellulose membrane containing denaturated/renaturated GST, GST- p33^{ING1} proteins.

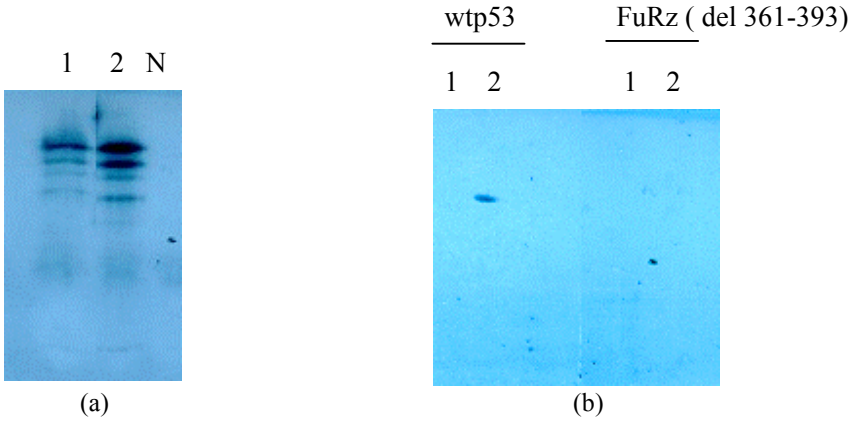


Figure 3.7: Far Western analysis of wt p53 and C-terminally deleted mutant of p53.
(a): 3µl from each IVTT reaction product were loaded on 12% SDS-Polyacrlyamide gel as a positive control. 1:wtp53; 2: FuRz (missing last 30 amino acids from wt p53); N: IVTT reaction without template DNA (negative control). (b) 1: GST protein only (-) control; 2: GSTp33^{ING1} protein.

To ensure that both proteins were produced in IVTT reaction, 3µl from each reaction mixture were loaded an SDS-PAGE gel, fixed, amplified, dried and exposed.

Results of these experiments are showed in Figure 3.7. Although there is no interaction between p53 and GST protein (negative control), there is an interaction between GST-$p33^{ING1}$-p53 and this interaction cannot be seen between C-terminally deleted form of p53 and $p33^{ING1}$.

3.5 Subcloning of p53 cDNA and p53 Deletion Mutant Constructs

In order to determine exactly which regions of p53 protein interact with $p33^{ING1}$, new C-terminally deleted constructs of p53 protein were used and cloned into a vector, such as pBks, which permits IVTT reaction. In one of these constructs, F6R6, only the oligomerization domain was deleted, and in the other construct, FuR6, both oligomerization domain and rest of the C-terminus region of the p53 protein were deleted.

The subcloning experiments were performed as follows. As a starting material, *E. coli* glycerol stocks of the constructs were available. One of these constructs, F6R6, was chosen for the construction of the desired mutant p53 proteins. In this construct, the oligomerization domain of p53 was deleted, starting from 331aa to 357aa. All constructs had been ligated into the pAUCT/CCW vector directionally through *Nhe*I (5' of the construct) and *Hind*III (3' of the construct) restriction enzyme sites by Erden Gökay. Although pBks had a *Hind*III recognition site at its multiple cloning site it did not have an *Nhe*I site. Another restriction enzyme, *Xba*I, creates a compatible DNA overhang with *Nhe*I at its target sequence. *Xba*I recognition site was present in the multiple cloning sites of pBks and this enzyme was used to digest pBks along with *Hind*III. The orientation of the DNA insert in pBks, when ligated at the mentioned restriction enzyme sites, enabled IVTT with the help of T7 viral RNA polymerase.

A different deletion-mutant of p53 was also prepared to better analyze the interacting regions of p53. In this new construct, named FuR6, a bigger deletion was done including the oligomerization domain and rest of the C-terminal region of the p53 gene, starting from aa 331 to the end of the gene. To be able to prepare this mutant construct, F6R6 found in pAUCT/CCW was digested with *Nhe*I and *Xho*I, which make deletion at the desired parts and pBks vector was digested with *Xba*I and *Xho*I.

Following the AGE of the digestion products, desired DNA bands were extracted from the agarose gel. Ligation reactions were set up between purified vector and insert sequences as already mentioned. After transformation, positive colonies were chosen from the plates. Overnight-grown colonies were used for MiniPrep plasmid isolation. Those MiniPreps were subjected to restriction enzyme analysis. In order to perform double digest of the constructs, *Not*I and *Apa*I for F6R6-pBks construct and *Not*I and *Xho*I for FuR6 construct restriction enzymes were used. *Nhe*I-*Xba*I hybrid site

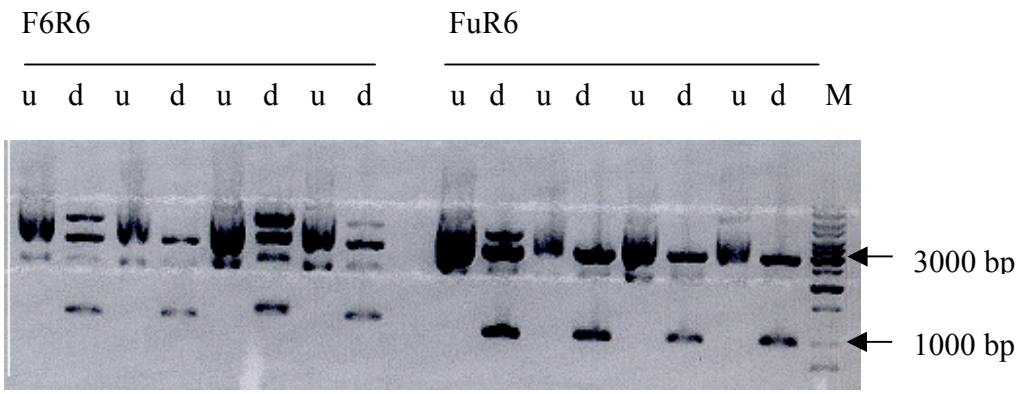


Figure 3.8: AGE analysis of MiniPrep of pBksF6R6 and pBksFuR6 constructs.
2µl of construct MiniPrep from chosen colonies was digested simultaneously with *Not*I and *Xba*I or *Not*I and *Xho*I (10 U each) in the presence of 2X of Y+/Tango Buffer for 4 hours at 35.5 C. 10µl of the digestion mixtures were loaded on the gel. *M:* marker; *u*: undigested; *d*: double digested. 1% AGE.

produces a recognition site for *Not*I restriction endonuclease. Positive colonies were chosen and MidiPreps were prepared. Restriction double digest of MiniPreps of the constructs, presented in **Figure 3.8**, show the presence of the desired cloned inserts.

3.6 Further Detailed Mapping of the Interacting domain of p53 with p33^{ING1} by *In Vitro* Techniques

Newly subcloned C-terminus deletion constructs of p53 protein were used in GST-pulldown assays with their negative controls for the ultimate mapping of the p53's interacting region. Before using in GST pulldown assay, the constructs were checked in IVTT reaction for the presence of the desired proteins. Two different p53 C-terminally deleted mutant constructs, F6R6 and FuR6, were used for further GST-pulldown analysis. In both of these constructs the oligomerization domain of the p53 was deleted but in FuR6 in addition to oligomerization domain the remaining C-terminus region of the protein was deleted.

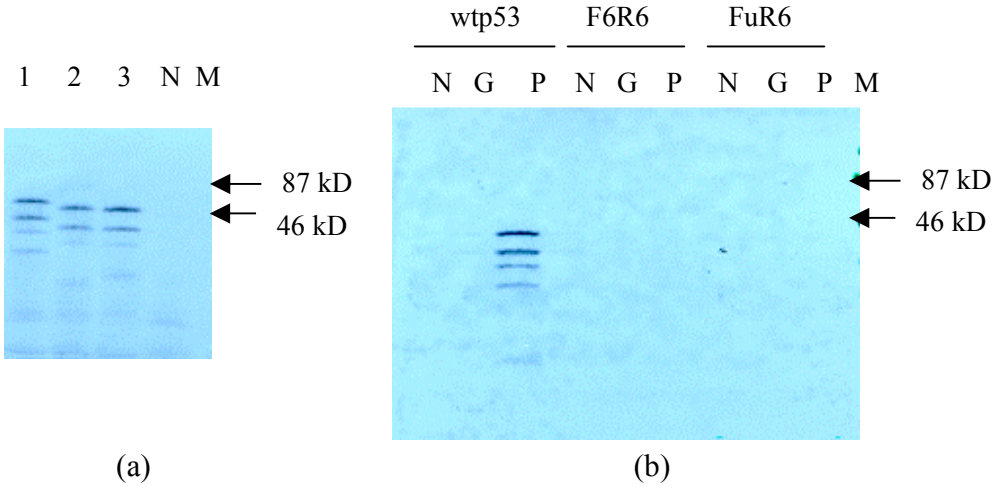


Figure 3.9: GST pulldown with p53 deletion constructs. (a): 3µl from each IVTT reaction product were loaded on 12% SDS-Polyacrlyamide gel as a positive control. *1*:wt p53; *2*: F6R6 (del; 331-357); *3*: FuR6 (del; 331-393); N: IVTT reaction without template DNA (negative control). (b) GST-pulldown assay of different p53 C-terminally deleted mutants; N: no protein (-) control; G: with GST protein (-) control; P: with GST- p33^{ING1} protein.

The results, presented in **Figure 3.9**, showed that there is no nonspecific interaction either between beads and p53 or GST protein and p53. Radioactively labeled p53 protein specifically interacts with $p33^{ING1}$ protein and this interaction is only seen when the oligomerization domain of the protein is intact. Both of the deletion mutant constructs of p53 failed to interact with $p33^{ING1}$. Although FuR6 does not have the last 62 amino acids of p53 protein, in F6R6 the only missing region is the oligomerization domain of the p53. This result suggests that the oligomerization domain is required in p53- $p33^{ING1}$ interaction.

This result was confirmed by Far Western analysis of the same constructs. All the conditions were kept the same with the previous assays with the addition of another negative control. An unrelated protein, 6Histidine-PTEN, was loaded onto SDS-Polyacrlyamide gel along with GST and GST-$p33^{ING1}$. As a positive control 3µl from each IVTT reaction product was run on SDS-Polyacrylamide gel.

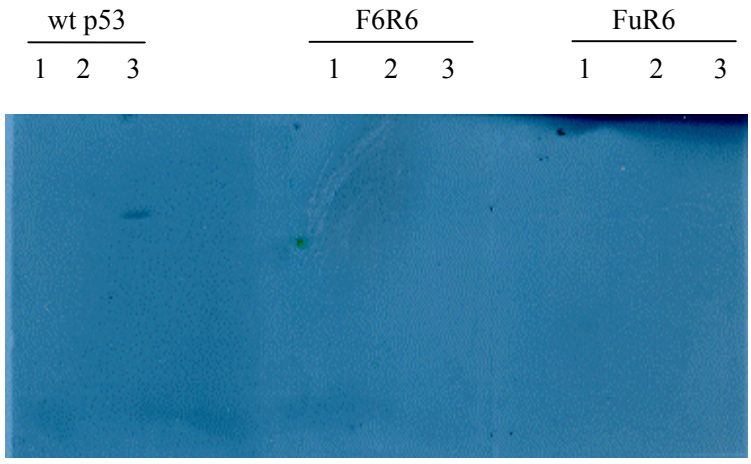


Figure 3.10: Far Western with p53 deletion constructs. Nitrocellulose membranes containing 6Histidine-PTEN , GST and GST-p33 proteins were incubated with indicated in vitro transcribed-translated p53 deletion mutant constructs.*1*: with 6Histidine-PTEN protein (as a negative control); *2*: with GST protein (-) control; *3*: with GST-p33 protein (test sample)

Result of Far Western analysis was confirmed the results of GST-pulldown assay. The interaction was possible only when full-length p53 and $p33^{ING1}$ were present.

3.7 Demonstration of Interaction between $p33^{ING1}$ and other p53 Family Members

Among p53 family members, there are highly conserved functional domains. Oligomerization domain is one of these domains, which is at the C-terminus region. In addition p63 and p73, proteins have SAM domains at their C-termini, which may be involved in protein-protein interactions. In the light of these observations, we decided to make sequence analysis of the C-terminus region of the p53 family members. Based on the high homology between p63 and p73 to p53 sequences having a role in the interaction with $p33^{ING1}$, the alignments were done with the deleted portions of p53 to see whether the deleted parts interfering with the interaction with $p33^{ING1}$ are conserved in p63 and p73. Depending on this conservation, we tried to find any possible interaction between p63 and p73 with $p33^{ING1}$ *in vitro*. Therefore, Far Western assay was repeated by using radioactively labeled IVTT products of wild type p63 and p73 proteins.

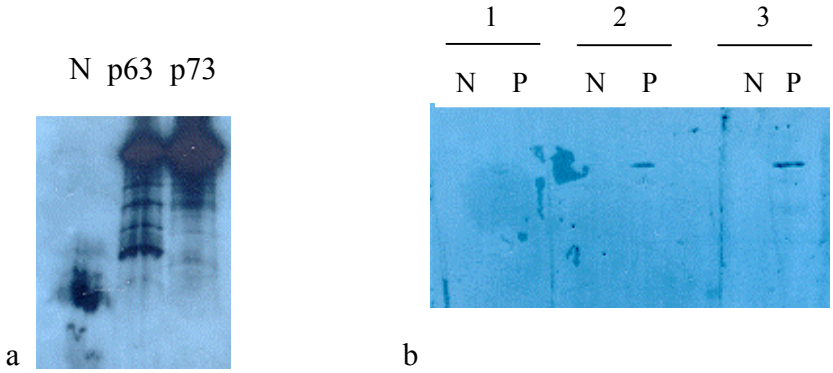


Figure 3.11: Far Western analysis of wt p63 and wt p73. (a): 3µl from each IVTT reaction product were loaded on 12% SDS-Polyacrlyamide gel as a positive control. *N*: IVTT reaction without template DNA (b) Far western analysis of wt p63 and wt p73 with GSTp33. (1): incubated with IVTT reaction product containing no DNA template; (2): incubated with IVTT reaction product containing wt p63 as a template; (3): incubated with IVTT reaction product containing wtp73 as a template. *N*: GST protein only as a negative control; *P*: GST $p33^{ING1}$ protein (10µg from each protein was loaded on the SDS-Polyacrlyamide).

After each IVTT reaction 3µl from each reaction product was loaded onto SDS-PAGE gels as a positive control then used in the Far Western assays. **Figure 3.11 (a)**, shows the IVTT reaction products of the full-length p63 and p73 proteins before adding to the membrane containing GST alone and GST- $p33^{ING1}$ proteins.

The result of this experiment was not surprising. As the **Figure 3.11** shows, there is an interaction between $p33^{ING1}$ and the other members of the p53 family. Since the C-terminus portion of p53 is required for this interaction, and oligomerization domain is the only conserved region at this part of the family members, we hypothesized that oligomerization domain may directly be involved in these interactions. To test this hypothesis, we prepared deletion mutant constructs of p63 and p73 and test these mutant proteins in protein-protein interaction assays *in vitro*.

3.8 Cloning of C-terminally deleted forms of p63, and p73

The oligomerization domain of p63 resides residues between 355-404 and of p73 resides residues between 351-383. Therefore, specific primers, having *BamH*I and *Xho*I restriction enzyme sites, excluding the last 311 aa of p63 and last 316 aa of p73 were designed.

As a preliminary step to the cloning, p63 and p73 C-terminally deleted cDNAs were PCR-amplified from the plasmids containing full-length p63 and p73 genes (p63 in PRc/CMV, p73 in pcDNA3). Specific forward and reverse primers that contained *BamH*I and, *Xho*I for restriction enzyme sites were used in the PCR reaction. PCR reaction mix was prepared as described in Section 2.3.1. Cloned *Pfu* polymerase was preferred over *Taq* polymerase because of its high fidelity due to 3'-5' proofreading mechanism.

PCR products, showed in **Figure 3.12**, were extracted and purified from gel. Extracted DNAs were subjected to the Poly A extension reaction to add adenine residues and ligated into the pGEMT-easy vector. The re-ligation control reaction was set up to see whether a self-ligation of the vector without insert has taken place.

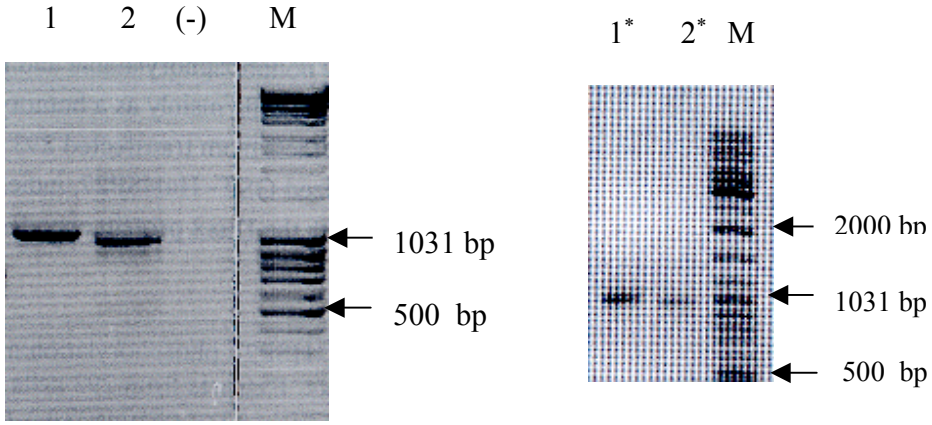


Figure 3.12. AGE of p63ΔC, p73ΔC PCR-products. *M*: DNA size marker; *1*: p63ΔC ; *2*: p73ΔC; (all same conditions, 5µl/lane); *1**: p63ΔC; *2**: p73ΔC; pcr products after purification (3µl/lane); *(-)*: negative control that contains no DNA. 1% agarose gel.

Super-competent E.coli cells were transformed by the ligation product. Along with the ligation product, control transformations were performed: transformation by the religation control (negative control); and by no DNA at all (mock transformation, negative control). Positive colonies were chosen and small-scale plasmid isolation was performed. Chosen colonies were examined by restriction enzyme digestion analysis. Medium-scale plasmid isolation was done from the colonies containing the desired genes. As seen in **Figures 3.13-3.14**, cloned insert is present at the expected size, around 1000bp, after restriction enzyme double digest, implying that the cloning processes was successful.

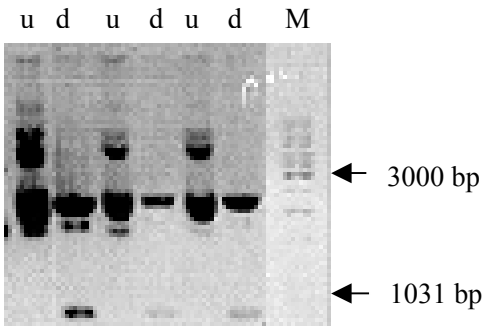


Figure 3.13: AGE analysis of MiniPrep of pGEMTp63ΔC . 2µl of construct MiniPrep from chosen colonies were digested simultaneously with *BamH*I and *Xho*I (10 U each) in the presence of 2X of Y+/Tango Buffer for 4 hours at 35.5 C. 10µl of the digestion mixtures were loaded on the gel. *M:* marker; *u*: undigested; *d*: double digested. 1% agarose gel.

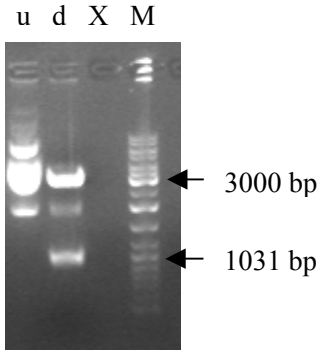


Figure 3.14: AGE analysis of MiniPrep of pGEMTp73ΔC constructs. 2µl of construct MiniPrep from chosen colonies were digested simultaneously with *BamH*I and *Xho*I (10 U each) in the presence of 2X of Y+/Tango Buffer for 4 hours at 35.5 C. 10µl of the digestion mixtures were loaded on the gel. *M:* marker; *u*: undigested; *d*: double digested. 1% agarose gel.

3.9 Subcloning of C-terminally deleted forms of p63 and p73

In order to express mutant and wild-type p63 and p73 in mammalian cells, p63ΔC and p73ΔC were subcloned from pGEM-Teasy to pcDNA3 directly. MidiPreps of p63ΔC, p73ΔC and pcDNA3 were double-digested with *BamH*I and *Xho*I restriction enzymes. The DNA fragments were purified by gel-extraction and AGE analysis performed. Ligation reactions were set up using the indicated vectors and then ligation

reaction mixture along with the indicated controls were transformed into super-competent *E. coli* cells. Positive colonies on the ligation plates were chosen and MiniPreps were prepared and analyzed by restriction enzyme digestion. MidiPreps of the positive colonies were prepared.

As the **Figure 3.15** showed both of the mutant proteins was subcloned successfully from pGEMT-easy to pcDNA3. Restriction enzyme double digest showed that both of the deletion mutant constructs are at the expected size, around 1000bp.

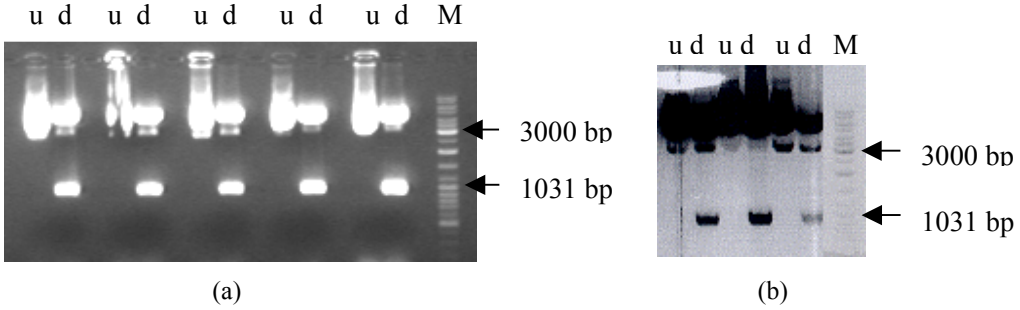


Figure 3.15: AGE analysis of MiniPrep of pcDNA3p63ΔC (a) and pcDNA3p73ΔC (b) constructs. 2µl of construct MiniPrep from chosen colonies were digested simultaneously with *BamH*I and *Xho*I (10 U each) in the presence of 2X of Y+/Tango Buffer for 4 hours at 35.5 C. 10µl of the digestion mixtures were loaded on the gel. *M:* marker; *u*: undigested; *d:*: double digested. 1% agarose gel.

3.10 Production of Radioactively Labeled Deletion Mutant p63ΔC

After cloned and subcloned, the expression of the newly cloned deletion mutant p63ΔC was checked by the coupled in vitro transcription-translation kit of Promega (see Section 2.3.10)

As the **Figure 3.16** shows, p63 deletion mutant, p63ΔC, was produced, *in vitro* by IVTT reaction. The size of the protein is nearly half of the wild type p63 protein, as we expected.

Because our p63ΔC could be expressed the next step is to examine the interaction between p63ΔC and $p33^{ING1}$ *in vitro.*

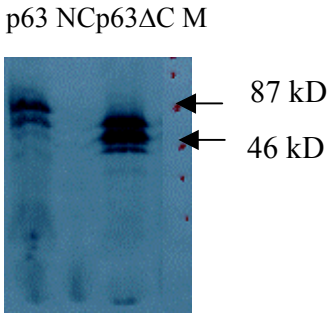


Figure 3.16: SDS-PAGE analysis of IVTT reactions of deletion mutant construct of p63 . 12% SDS-PAGE.

3.11 Examination of Interaction between p63ΔC and p33^{ING1} *in vitro*

This interaction was first analyzed by Far Western assay. Far Western analysis was repeated for wild-type p63 and p63ΔC. All the conditions were kept the same with the previous assays with the addition of another negative control. An unrelated protein, purified recombinant protein purified 6Histidine-PTEN, was loaded onto SDS-Polyacrlyamide gel along with GST and GST-p33^{ING1}. As a positive control 3µl from each IVTT reaction product was run on SDS-Polyacrylamide gel.

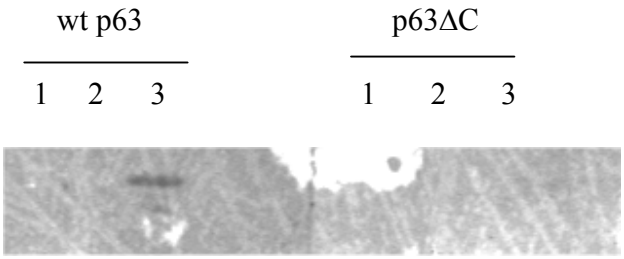


Figure 3.17: Far Western Blotting with p63 deletion constructs. The nitrocellulose membranes containing indicated proteins were incubated with IVTT products of either p63 or p63ΔC.*1*: with 6Histidine-PTEN protein (as a negative control); *2*: with GST protein (-) control; *3*: with GST-p33 protein (test sample).

As the figure shows, wtp63 does not interact neither with GST alone or purified 6Histidine-PTEN protein but the interaction between p63 and GST p33^{ING1} is obvious

and this interaction was disrupted when the C-terminal portion of the protein containing the oligomerization domain was deleted.

Both wt p63 and p63ΔC were used in GST pulldown assay and result of this experiment confirmed what was found in Far Western assay.

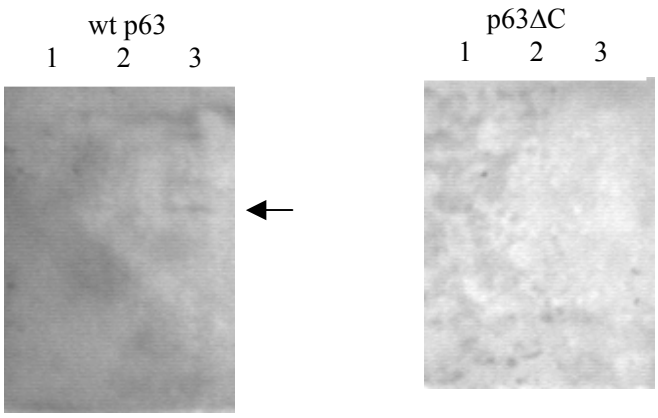


Figure 3.18: GST pulldown with p63 deletion constructs. GST-pulldown assay of either wt p63 or p63ΔC; *1*: no protein (-) control; *2*: with GST protein (-) control; *3*: with GST- $p33^{ING1}$ protein. 12% SDS-PAGE.

3.12 Cloning and the Subcloning of the deletion mutant of $p33^{ING1}$

After mapping the interacting regions of the p53 family proteins, we decided to examine which region of $p33^{ING1}$ is actually involved in interaction with p53 family members. To answer this question, we cloned the C-terminus deletion construct of $p33^{ING1}$. We deleted the region containing the PHD finger domain, which is important for chromatin-mediating interactions and transcriptional regulation. Also PHD finger motifs are thought to participate in the recognition of the macromolecules, such as DNA, RNA and proteins (Aasland 1995). Based on these findings, we examined the C-terminus region of $p33^{ING1}$ in interactions with p53 family.

As a preliminary step to the cloning, $p33^{ING1}$ cDNA was PCR-amplified from the plasmid containing full-length $p33^{ING1}$ gene (p33 in pBks). Specific forward and reverse primers that contained *Sal*I and *Hind*III restriction enzyme sites were designed and used in PCR reaction. PCR reaction mix was prepared as described in Section 2.3.1. PCR conditions were kept the same with the previous cloning experiments.

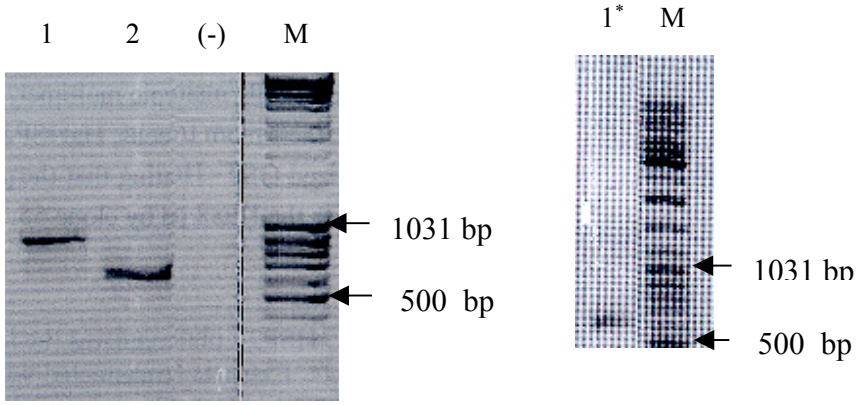


Figure 3.19: AGE of $p33^{ING1}\Delta C$ PCR-product. *M*: DNA size marker; *1*: wt p33(positive control); *2*: $p33^{ING1}\Delta C$; (all same conditions, 5µl/lane); *1**: p33ΔC; pcr product after purification (3µl/lane); *(-)*: negative control that contains no DNA. 1% agarose gels.

PCR product, shown in **Figure 3.19**, was purified by gel and extracted from gel. Extracted DNA was subjected to the Poly A extension reaction to add adenine residues and ligated into the pGEMT-easy vector. The re-ligation control reaction was set up to see if a self-ligation of the vector without insert has taken place.

Super-competent E.coli cells were transformed by the ligation product. Along with the ligation product, control transformations were performed: transformation by the religation control (negative control); by no DNA at all (mock transformation, negative control). Positive colonies were chosen and small-scale plasmid isolation was performed. Chosen colonies were examined by restriction enzyme digestion analysis.

Medium-scale plasmid isolation was done from the colonies containing the desired genes.

As **Figure 3.20** shows, the restriction enzyme double digest of the MiniPreps resulted in the cloned insert, which is at the expected size between 700-800 bp.

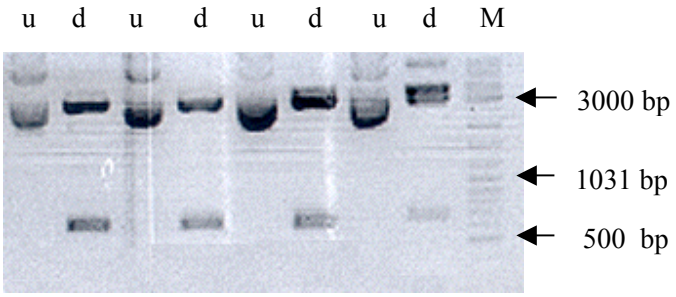


Figure 3.20: AGE analysis of MiniPrep of pGEMT-p33^{ING1}ΔC constructs. 2µl of construct MiniPrep from chosen colonies were digested simultaneously with *Hind*III and *Sal*I (10 U each) in the presence of 2X of Y+/Tango Buffer for 4 hours at 35.5 C. 10µl of the digestion mixtures were loaded on the gel. *M:* marker; *u*: undigested; *d*: double digested. 1% agarose gels.

In order to express mutant and wild-type p33^{ING1} in mammalian cells, p33^{ING1}ΔC was first subcloned from pGEM-Teasy vector to pBks expression vector and then pcDNA3 mammalian expression plasmid. MidiPrep of p33^{ING1}ΔC and pBks vector were double-digested with *Sal*I and *Hind*III and the DNA fragments were purified by gel-extraction and AGE analysis performed. Ligation reactions were set up using the indicated vectors and then ligation reaction mixture along with the indicated controls were transformed into super-competent *E. coli* cells. Positive colonies on the ligation plates were chosen and MiniPreps were prepared and analyzed by restriction enzyme digestion. The results of these analyses are presented in **Figure 3.21**. MidiPreps of the positive colonies were prepared.

At the end of this step, p33^{ING1}ΔC was in the pBks vector, therefore it is subcloned once more from pBks to pcDNA 3. Also full-length p33^{ING1} was in the pBks

vector too. Both full-length $p33^{ING1}$ and $p33^{ING1}\Delta C$ were subcloned from pBks to pcDNA3. $p33^{ING1}$ and also pcDNA 3 were digested with *Kpn*I and *EcoR*V and gel-extracted. Ligation reaction were set up with the purified fragment and vector DNA along with the controls and incubated 16 hours at 16°C.

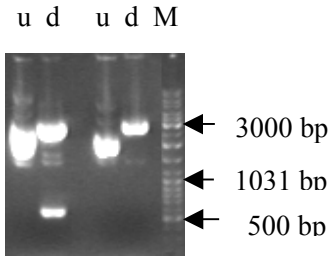


Figure 3.21: AGE analysis of MiniPrep of pBks $p33^{ING1}\Delta C$ constructs. 2µl of construct MiniPrep from chosen colonies were digested simultaneously with *Sal* I and *Hind*III (10 U each) in the presence of 2X of Y+/Tango Buffer for 4 hours at 35.5 C. 10µl of the digestion mixtures were loaded on the gel. *M:* marker; *u*: undigested; *d*: double digested. 1% agarose gel.

Ligation products were transformed into super-competent *E. coli* cells and incubated overnight at 37°C. Positive colonies were selected and analyzed by restriction enzyme digestion. MidiPreps of the positive colonies were prepared.

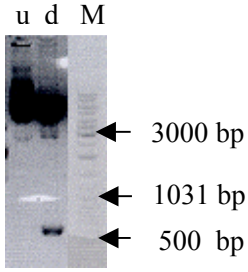


Figure 3.22: AGE analysis of MiniPrep of pcDNA3 $p33^{ING1}\Delta C$ constructs. 2µl of construct MiniPrep from chosen colonies were digested simultaneously with *Kpn*I and *EcoR*V (10 U each) in the presence of 2X of Y+/Tango Buffer for 4 hours at 35.5 C. 10µl of the digestion mixtures were loaded on the gel. *M:* marker; *u*: undigested; *d*: double digested. 1% agarose gel

Restriction digestion analysis showed that our cloning and subcloning was successful. At the end of this step both full-length and C-terminally deleted forms of $p33^{ING1}$ are in the pcDNA3 expression vector, presented in **Figure 3.22**.

DISCUSSION

The result of our study suggests that C-terminus region, particularly oligomerization domain of p53 is involved in interaction with $p33^{ING1}$ *in vitro*. This conclusion can be drawn from results of our *in vitro* protein-protein binding assays assays with different deletion mutants of p53. Oligomerization domain of p53 regulates the formation of p53 tetramer, form of active p53. This domain also contains a nuclear exclusion signal, which is hidden when the protein form tetramer. The results of GST pulldown assays and Far Western Blotting assays with p53 C-terminus deletion mutants, FuR6 and F6R6, suggest that, oligomerization domain of p53 protein is involved in interaction with $p33^{ING1}$. The interaction between p53 and $p33^{ING1}$ was inhibited when FuR6, lacking the C-terminus region of p53 (lacking the last 62 aa of p53), was used *in vitro* protein-protein interaction assays. When F6R6, which lacks only the oligomerization domain of p53 (lacking the aa between 331-357), was used in GST pulldown assay and Far Western blotting, we obtained the same results. Therefore, according to our data, oligomerization domain may be necessary for the interaction between p53 and $p33^{ING1}$. In one of our GST pulldown assays, FuRz, a deletion mutant of p53 which has an intact oligomerization domain, does not interact with GST-p33. This deletion mutant construct of p53 lacks the 30 amino acids, which may hinder the formation of native 3D-structure of C-terminus region of the p53 required for the specific interaction with $p33^{ING1}$. Also we showed that other members of the p53 family, p63 and p73, interacts with $p33^{ING1}$ and this interaction is primarily determined by C-terminus region of these proteins. This is the first time showing that p53 family

proteins are interacting with $p33^{ING1}$. This result is not surprising since there is a 37% homology at oligomerization domain among p53 family members.

p53 activity may be controlled by $p33^{ING1}$. $p33^{ING1}$ may behave as a Mdm2-like protein in the cell. It has been shown that different transcripts of ING1 gene regulates p53 activity differently; while $p33^{ING1}$suppresses p53 activity by physically interacting, $p24^{ING1}$ cooperates with p53 in the cell (Zeremski *et al*., 1999). Therefore by interacting with a oligomerization domain, $p33^{ING1}$ may inhibit the tetramer formation of p53 and its transcriptional activity. Upon a stimulus, this may be a DNA damaging agent, the complex of p53-$p33^{ING1}$ may dissociate and p53 can activate its target genes. It is very early to hypothesize about the place of $p24^{ING1}$ in this scheme but it may help p53 when it is activated.

There are limited information about the regulation of the activities of p63 and p73 proteins and their involvement in growth arrest. Various numbers of proteins interact with p53 but only few proteins found to interact with the other members of the family (see for review Levrero, 2000). We have shown, as a first time, that one of the p53 interacting protein, $p33^{ING1}$, interacts with both p63 and p73. As $p33^{ING1}$ gene products are cell cycle regulated and involved in cell growth regulation, this data support the notion that p63 and p73 have roles in cell cycle regulation.

$p33^{ING1}$ may interact with p63 and p73 through their oligomerization domain by this way it may inhibit the interaction between p53 and ΔNp63 and ΔNp73. Recent data suggests that p53 can be inactivated by ΔNp63 and ΔNp73 by physically interacting (Sayan *et al.,* 2001; Irwin & Kaelin 2001). $p33^{ING1}$ may behave as a “tuner protein” to regulate the activities of p53 family proteins.

Some of the p53 responsive promoters can be activated by p63 and p73 in different degrees (Kaghad *et al.,* 1997, Yang *et al.,* 1998, Jost *et al.,* 1997). $p24^{ING1}$ has been shown to involve in the activation of $p21^{WAF1}$ promoter together with p53 (Garkavtsev *et al*., 1998). Therefore $p24^{ING1}$ may be involved in the regulation of the activation of p53 responsive promoters together with the family members. Also the strength of this activation may depend on these interactions.

Different forms of ING1 gene product have been shown to have chromatin modifying activities or associate with proteins involved in chromatin modification. Therefore $p33^{ING1}$ may be the way to link p53 family proteins to multiprotein complexes functioning in the regulation of chromatin modulation and gene activation. By its C-terminus domain, $p33^{ING1}$ may recruit the multiprotein complexes to chromatin structure, while it's N-terminus domain may interact with p53 family members. By this way, $p33^{ING1}$ may make chromatin structure accessible for p53 family proteins. Mutations in p63 and p73 genes are rarely found in human cancers. Functional inactivation of p63 and/or p73 may occur as a result of decreased expression of $p33^{ING1}$ or loss of function of this gene product rather than a mutation in these genes.

Perspectives

The next step to this study is to use the C-terminus deletion mutant of p73 and test in *in vitro* protein-protein interaction assays. Also finer mapping of the interacting region of p63 is required. In our study we deleted both oligomerization domain and SAM domain of the p63 in the same construct, different deletion mutants of p63 should be prepared to examine the effects of these domains separately. To see whether oligomerization domain of p53 family proteins is the place where they interact with $p33^{ING1}$, only the oligomerization domain of the family members could be cloned into

expression vectors and could be used in protein-protein interaction assays. Our findings are *in vitro* results, these results should be repeated *in vivo* studies using different cell lines.

One further step is to do the whole study the other way around to find out the molecular determinants on the $p33^{ING1}$ protein that enable the interaction. As a first step, we cloned the C-terminus deletion mutant of $p33^{ING1}$ protein. This construct may be used in protein-protein interaction assays together with p53 family proteins. The next region that may have role in these interactions may be the N-terminus of the protein. Therefore cloning the N-terminus deleted mutants of $p33^{ING1}$ may answer this question.

In our studies, we used alpha isoform of both p63 and p73, but there are at least six different isoforms of p63 and p73. Although the oligomerization domain is found in all of the isoforms, the SAM domain differs from one isoform to the other. Therefore examination of interaction of $p33^{ING1}$ with different isoforms of p53 family may be another step to understand the consequences of this interaction.

One further step is to do same studies by using other ING1 gene products, $p47^{ING1}$, $p27^{ING1}$, and $p24^{ING1}$. Since different ING1 isoforms have different effects on p53 regulated gene expression, the interaction between p53 family members and ING1 isoforms may show various patterns.

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APPENDICES

Appendix A:

```
LOCUS       AF044076                911 bp    mRNA    linear   PRI 04-FEB-1998
DEFINITION  Homo sapiens candidate tumor suppressor p33ING1 (ING1) mRNA,
            complete cds.
ACCESSION   AF044076
VERSION     AF044076.1  GI:2829207
KEYWORDS    .
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 911)
  AUTHORS   Garkavtsev,I.A., Kazarov,A.R., Gudkov,A.V. and Riabowol,K.
  TITLE     Suppression of the novel growth inhibitor p33ING1 promotes
            neoplastic transformation
  JOURNAL   Nature Genet. 14 (4), 415-420 (1996)
  MEDLINE   97099452
REFERENCE   2  (bases 1 to 911)
  AUTHORS   Garkavtsev,I.A., Grigorian,I.A., Ossovskaya,V.S., Chernov,M.V.,
            Chumakov,P.M. and Gudkov,A.V.
  TITLE    The candidate tumour suppressor p33ING1 cooperates with p53 in cell
            growth control
  JOURNAL   Nature 391 (6664), 295-298 (1998)
  MEDLINE   98101645
REFERENCE   3  (bases 1 to 911)
  AUTHORS   Grigorian,I.A. and Gudkov,A.V.
  TITLE     Direct Submission
  JOURNAL   Submitted (21-JAN-1998) Molecular Genetics, University of Illinois
            at Chicago, 900 South Ashland Avenue, Chicago, IL 60607, USA
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YNR"
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Some Biophysical Data of p33ING1 calculated from sequence data:

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hydrophobicity: 0.5710
insolubility factor: 1.4121
isoelectric point: 7.13

Appendix B:

```
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VERSION     NM_001564.1  GI:4504694
KEYWORDS    .
SOURCE      human.
  ORGANISM  Homo sapiens
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            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (sites)
  AUTHORS   Shimada,Y., Saito,A., Suzuki,M., Takahashi,E. and Horie,M.
  TITLE     Cloning of a novel gene (ING1L) homologous to ING1, a candidate
            tumor suppressor
  JOURNAL   Cytogenet. Cell Genet. 83 (3-4), 232-235 (1998)
  MEDLINE   99172097
COMMENT     REVIEWED REFSEQ: This record has been curated by NCBI staff. The
            reference sequence was derived from AB012853.1.
            Summary: ING1L encodes inhibitor of growth 1-like, so named
            because of its considerable similarity to the putative tumor-
            suppressor gene ING1. ING1 is thought to exert its effects through
            interaction with p53, while the function of ING1L remains unknown.
            COMPLETENESS: incomplete on the 3' end.
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