ROLE OF c-JUN IN THE REGULATION OF TUMOR SUPPRESSOR p53 HOMOLOGUE, p73

L Boominathan

M. Sc (Medical Biochemistry**),**
Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER),
Pondicherry;
M. Sc (Life Sciences)**,**
The Feinberg Graduate School of the Weizmann Institute of Science (WIS), Rehovot.

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DEDICATION

THIS THESIS IS DEDICATED TO

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LIST OF PUBLICATIONS

INTERNATIONAL CONFERENCE PUBLICATIONS

1. Boominathan L and Sabapathy K, c-Jun is required for stabilization and activation of the p53-homologue, p73***, 5th Beatson International Cancer Conference (sponsored by AARC), Glasgow, UK*****, from July 15 to18th, p36, (2001).**
2. Sabapathy K and Boominathan L c-Jun is required for stabilization and activation of the p53-homologue, p73, ***5th Beatson International Cancer Conference (sponsored by AARC), Glasgow, UK*****, from July 15 to18th, p78, (2001).**
3. Boominathan L and Sabapathy K UV and Cisplatin-mediated p73 stabilization and p53-independent apoptosis requires c-Jun. ***11th International p53 Workshop, Barcelona, Spain*****, from May 15 to 18th , (2002).**
4. Sabapathy K, Boominathan L, Vasantha MN, Lin K.W UV-mediated p73 stabilization, resulting in p53-independent apoptosis**. *11th International p53 Workshop, Barcelona, Spain*****, from May 15 to 18th, (2002).**
5. Sabapathy K and Boominathan L c-Jun is required for stabilization and activation of the p53-homologue, p73**. *p73/p63 workshop 2002, Rome, Italy*****, from June 10 to11th, (2002).**
6. Sabapathy K and Boominathan L **c**-Jun is required for stabilization and activation of the p53-homologue, p73***, Cancer Genetics & Tumor Suppressor Genes, Cold spring harbour laborotary, NY, USA*****, from August 14 –18th, (2002).**

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Boominathan L and Kanaga Sabapathy. The tumor suppressor p53 homologue, p73 increases AP-1 activity in cooperation with c-Jun (will be sent upon request)

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Boominathan L and Kanaga Sabapathy Wild-type p73 can transform immortalized fibroblasts in co-operation with c-Jun (will be sent upon request)

Abbreviations

ALLN	Calpain pathway inhibitor
AP-1	Activator protein
ATM	Ataxia telengectasia.
c-ABL	Abelson leukaemia
COS7	Monkey kidney derived cell line
CRE	Cyclin AMP responsive element
DBD	DNA binding domain
DEPC	Diethyl pyrocarobonate

EDTA	Ethylene diaminetetraacetic acid
EtBR	Ethidium bromide
FCS	Fetal calf serum
H1299	Small cell lung carcinoma
IR	Ionizing radiation
JNK	Jun N-terminal kinase
LOH	Loss of hetrozygosity
LOI	Loss of imprinting
LZ	Leuzine zipper domain
MAPK	Mitogen activated protein kinase
MCF-7	Breast carcinoma cell line
MEFs	Mouse embryonic fibroblasts
MG132	Proteasome inhibitor
MMS	Methyl methane sulphate
MOPS	3-(N-morpholino) propanesulfonic acid
NIH-3T3	Immortalized 3T3 cell line
OD	Oligomerization domain
p53RE	p53 responsive element carrying promoters
PAGE	Polyacrylamide gel electrophoresis.
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Rpm	Revolution per minute
RT-PCR	Reverse transcription and PCR amplification

SAM	Sterile alpha domain
SAOS2	Osteosarcoma cell line
SDS	Sodium dodecyl sulphate
SUMO-1	small ubiquitin-like modifier 1
TA	Transactivation domain
TAD	Tetramerizaiton domain
TEMED	N, N, N', N'- tetramethylethylene-diamine
TRE	TPA responsive element
UV	Ultraviolet radiation

Summary

The study shows for the first time that c-Jun stabilizes and activates p73, in turn, p73 influences c-Jun's function in cell fate decisions. Genetic evidences consolidated the findings, as *c-jun*$^{-/-}$ cells are defective in p73 stabilization, transcriptional activation, and are resistant to cisplatin-induced apoptosis. Introducing c-Jun in *c-jun*$^{-/-}$ cells restores p73 stabilization/levels, activation and sensitivity to cisplatin. The half-life of p73 is extended by c-Jun resulting in enhanced p73 mediated-transactivation. The ability of p73 to transactivate its down stream genes is reduced in *p53*$^{-/-}$*c-jun*$^{-/-}$ cells compared to *p53*$^{-/-}$ cells. Both the amino and carboxy-termini of c-Jun independently are required for increased p73 levels and transcriptional activity. The PY motif is conserved in both p73 and c-Jun, indicating that they have shared functions in regulating various biological processes in the cells. Furthermore, the apoptosis inducing function of p73 is potentiated by c-Jun.

Exposure to UV radiation is shown to induce p73 levels in a variety of cell lines. The UV-mediated p73 stabilization occurs at the post-transcriptional level and is not compromised in cells lacking c-Abl or c-Jun-amino-terminal kinases, Jnks 1, and 2. It was also shown that when p73 is transiently over expressed, UV stabilizes the transfected p73. However, the consecutive exposure of cells to the γ-irradiation and the UV-irradiation enhanced the stabilization of p73 and increased the cell death when compared to cells treated with either or UV irradiation alone. This is exemplified by the absence of colony formation in *p53*$^{-/-}$ cells, indicating that combined signals can induce apoptosis by stabilizing p73.

The ability of TA-p73 to influence c-Jun function was also studied, as both appeared

to be over expressed and co-exist in tumors. This study shows for the first time that p73 increases AP-1 (5XTRE) activity and it synergies with c-Jun to potentiate AP-1 activity. The transactivation domain (TAD1) near the NH2-terminus of p73 is necessary for its ability to synergize with c-Jun. Furthermore, it appears that p73 potentiated AP-1 activity, predominantly dependent on the endogenous c-Jun expression. JNK-mediated c-Jun phosphorylation is required, but not essential for its ability to co-operate with p73. Further, it can increase the expression of AP-1 target genes such as collagenase-1 and MSH-2. P73β shows the best synergistic effects with c-Jun as compared to the other p73 family members. In addition, the basal level of AP-1 activity was lowered by the dominant negative p73 (DD), indicating that p73 is essential for the basal AP-1 activity. Both TA and ΔNp73 promoter encodes AP-1 like responsive elements and it indicates the possible existence of a regulatory loop between ΔN-p73 and TA-p73/c-Jun.

This study also show that p73 could transform immortalized fibroblast cell lines such as NIH 3T3 in co-operation with c-Jun. This indicates that p73 could support transformation in the presence of excessive oncogenic signals, but not in its absence. In addition, p73-induced MDM2 promoter activity observed in $p53^{-/-}$ fibroblasts is reduced in $p53^{-/-}c\text{-}jun^{-/-}$ and $p53^{-/-}Mdm2^{-/-}$ fibroblasts. Correspondingly, p73-β, c-Jun, and MDM2 synergistically increase MDM2 promoter activity. Taken together, these observations suggest that p73 function is modulated in cancer cells. In aggregate, this study has identified for the first time a critical role of c-Jun in the regulation of p73.

CHAPTER I

"The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them" Sir William Bragg (1862 - 1942).

INTRODUCTION

1.1 p53, the tumor suppressor

The p53 protein is a transcription factor that induces both cell cycle arrest and apoptosis, in response to diverse genotoxic and cellular stresses. The p53 gene is frequently mutated in human cancer, being mutated or lost in 55% of all tumors (Oren, 1999; Hollstein et al., 1991; Sengupta, 2005). Hence, p53 is thought to play an important role in maintaining--commonly referred to as guardian of genome--the integrity of the genome (Lane et al., 1992).

1.1.1 p53 structure and targets

The p53 protein transactivates several sets of genes to execute DNA repair, growth arrest and apoptosis (Figure 1.1). It contains an NH2-terminal transactivation domain, a central DNA-binding domain (DBD) and a carboxyl-terminal oligomerization domain (OD). The DBD-- a mutational hot spot that commonly occurs in various human cancers-- facilitates sequence-specific DNA binding to p53 response elements (p53RE) present within the regulatory regions of a number of p53-regulated genes (Ko et al 1996; El-Diery, 1998). The OD facilitates tetramer formation. Post-translational modifications including phosphorylation, acetylation, sumoylation, glycosylation are critical in modulating the binding activity of p53 to its responsive elements (Meek et al., 1999; Wahl et al., 2001; Brooks et al., 2003).

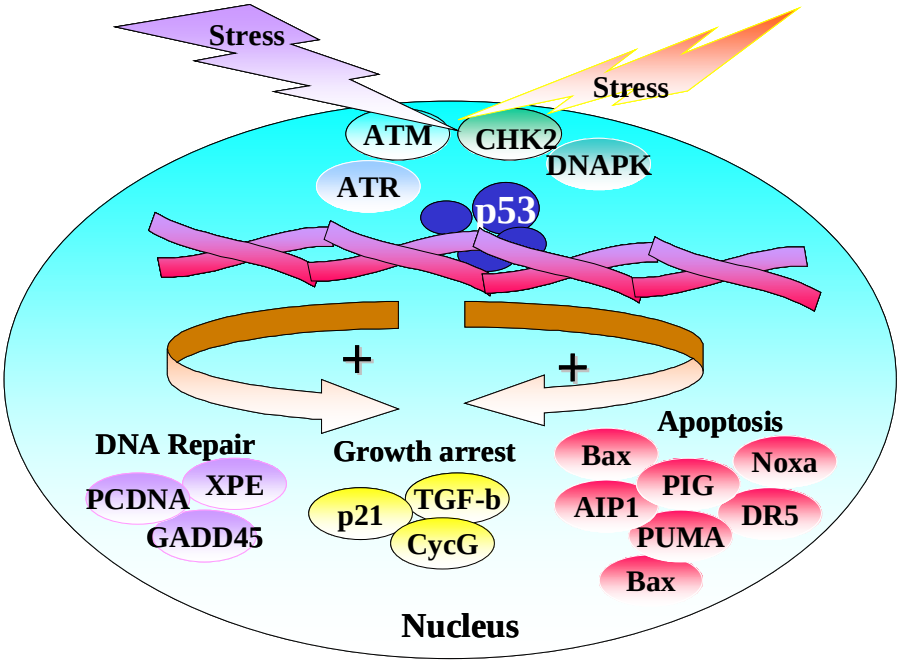


Figure 1.1 Activation of p53 can lead to the expression of DNA repair, Cell cycle arrest and Apoptotic target genes (Ryan et al., 2001; Chen et al., 1999; Lohrum et al., 2000; Ko et al., 1996; Vousden K 2000; El-Diery WS 1998; Sengupta, 2005.)

1.1.2 Regulation of p53 function

Activation of p53 can occur in response to UV, IR, MMS, NO, anti-cancer drugs, hypoxia, nucleotide deprivation etc. (Lakin et al., 1999; El-Diery et al., 1998). The signals and mechanisms that regulate p53 activity are described in several recent reviews (Oren, 1999; 2003 Ko et al., 1996; El-Diery, 1998; Gottlieb et al., 1998; Ryan et al., 2001; Wahl et al., 2001; Haupt et al., 2004; 2003a; 2003b; 2002; Ashcroft et al., 1999a; 1999b; May et al., 1999; Sionov et al., 1999). The p53-MDM2 auto regulatory loop is a well-established regulatory network: MDM2 is a target gene of p53 and is a negative regulator of p53 protein levels. In an undamaged cell, p53 is complexed (through the N-terminus) with Mdm2 and targeted for ubiquitin-dependent proteolysis (Haupt et al.,

1997). In response to DNA damage, p53 is phosphorylated at serine-15 and serine-20, displacing Mdm2 from the N-terminus, leading to an increase in the protein levels of p53 (Figure 1.2). Activated p53 is then capable of inducing the transcription of genes that lead to cell cycle arrest (p21, TGF-b and Cyclin G), apoptosis (Bax, AIP1, PUMA, Noxa, PIG, DR5 etc.) or enhanced DNA repair (PCDNA, XPE and GADD45) (Oren, 1999; Michael et al., 2002; Ryan et al., 2001; Sengupta, 2005).

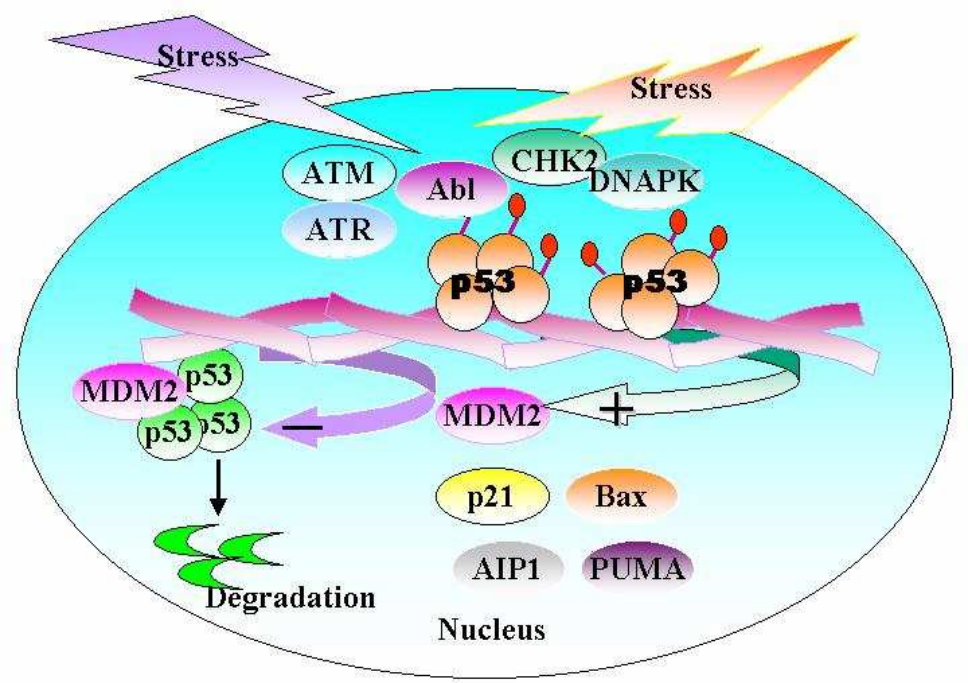


Figure 1.2 The p53-MDM2 auto regulatory loop As with other p53 target genes, transcription of MDM2 is increased when p53 is activated and stabilized (Ashcroft, 1999). In turn, the MDM2 interacts with p53 and target it for ubiquitin-dependent degradation. In response to stress signals, p53 undergoes ser/thr phosphorylation near the MDM2 binding site (N-terminus of p53), which blocks MDM2's ability to target p53 (red) for degradation (Michael et al., 2002; Ryan et al., 2001)

1.2 p53 family members

Given the importance of the p53 gene in human cancers, it is not surprising that a considerable effort has been put forth to identify p53 homologues. Only in late 1990s, two novel family members were identified and termed p73 and p63 (Kaghad et al., 1997; Caput, 1997; Yang et al., 1998). Though they were structurally similar, research in the last 7 years showed surprising diversities. That is, p73 appear to carry out both p53 related functions and completely novel functions (Irwin et al., 2001).

1.3 Introduction to p73

In a search for new interleukins, a cDNA that was predicted to encode p53 like protein, Daniel Caput and co-workers identified p73 in 1997.

1.3.1 Chromosomal localization of p73

. It is located to chromosome 1p36.3, a region that is frequently deleted in variety of human cancers or exhibits loss of hetrozygocity (LOCH) in neuroblastoma, lung cancer, gastric cancer, HCC, breast cancer, ovarian cancer (Khagad et al., 1997; Barrois et al., 2001; Blatt et al., 2001; Casciano et al., 2002; Melino et al., 2002; Yang et al., 2001; Araki et al., 2002; Nakagawara, 2001; Imyanitov et al., 1999; Ichimiya et al., 1999; 2000; 2001). The fact that p73 was located in a long suspected tumor locus, was met with a great expectation, as this gene could potentially function as a tumor suppressor. However, subsequent searches in many types of cancer have found neither mutations nor consistent pattern of loss of hetrozygocity in the remaining allele of p73 gene (Melino et al., 2002).

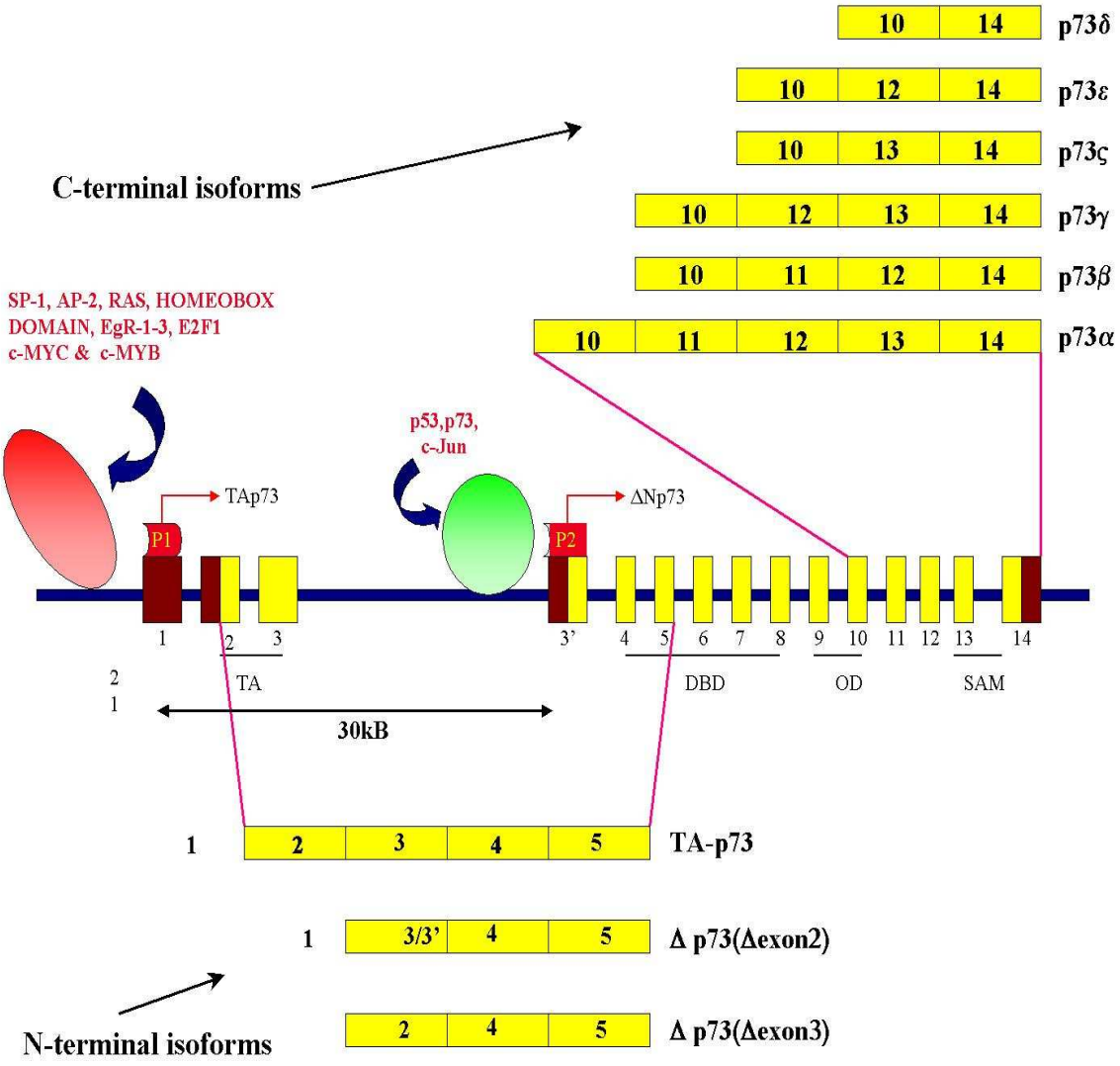
1.3.2 Gene architecture of p73

The structure of the p73 gene is highly complex when compared to that of p53. The gene encoding p73 is approximately 65 KB in size. Human p53 gene has a single promoter, which directs the production of a single mRNA (Strano et al., 2001; Yang et al., 2000). On the contrary, the TP73 gene, contains two independent promoters, P1 and P2, which make use of alternative splicing to generate various isoforms (Yang et al., 2000). The promoter P1 is in the 5'P-UTR, upstream of a non-coding exon 1 and produces full-length proteins containing the TA domain (TAp73). The promoter P2 is located within the 30 KB spanning, Intron 3. It gives rise to TA-deficient-ΔNp73 proteins (Melino et al., 2002).

1.3.3 Structural organization of the p73 promoter

The upstream promoter region of the human p73 gene has been partially characterized (Ding et al., 1999). Unlike the p53 promoter, the p73 promoter contains a TATA-like box (Strano et al., 2001). Initial studies show that the region between nucleotides –119 to +119 relative to the start of exon 1, contains the region required for the basal transcription of p73 (Ding et al., 1999; Seelan et al., 2002). This region contains putative SP1, AP-2, and Egr-1, 2,3 sites and several stretches of CpG di-nucleotides (Ding et al., 1999; Strano et al., 2001; Davis et al., 2001). The region located between position –119 and –2714 contain additional regulatory sites for: E2F and c-Myb (Levrero et al., 1999; Melino et al., 2002; Seelan et al., 2002). Further, a potential p53-binding site was identified in the p73 promoter that is responsive to both p53 and p73 and is auto regulated (Chen et al., 2001). Interestingly, ΔN-p73 promoter does not share any sort

similarity with its counter part TA-p73 promoter and it appears to be regulated by different transcription elements (Melino et al., 2002).



(Adapted and modified from Melino et al., 2002)

Figure 1.3 Splice variants of p73, and transcriptional factors regulate p73 The p73 gene has two promoters, which are divided into two groups. The two groups include those containing the TA domain (containing first three exons), directed by the P1 promoter and the ΔN domain (containing 4-14 exons), directed by the P2 promoter. The use of either alternative splicing or alternative promoters can generate NH2- (due to exons 2, 3 and 3') and COOH-termini isoforms (due to exons 11, 12 and 13). Potential

transcription factors/proteins that are active on both TA (red colour) and ΔN-p73 (yellow colour) promoters are indicated (Melino et al., 2002).

1.3.4 Structure organization of p73

The gene encoding p73 contains 14 exons and two alternative promoters. It is subjected to alternative splicing as well. As a result of the use of alternative promoters and splicing, at least five NH2-terminal and six C-terminal isoforms (Melino et al., 2002) can be produced. (Figure1.3) (Melino et al., 2003; 2002; Lohrum et al., 2000)

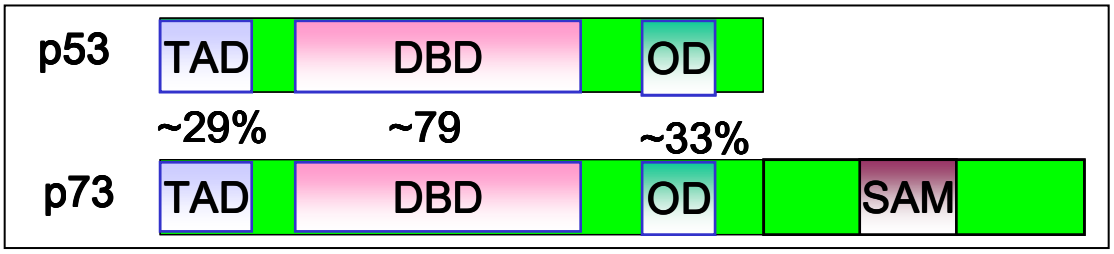


Figure 1.4 Comparison of the protein structures of p53 and p73. Percent homology to the p53 sequence is indicated above. Potential protein-protein interaction domains, include a SAM-like domains found only in p73 (Melino et al., 2003; 2002; Lohrum et al., 2000).

Transactivation domain (TAD)

TAD is subjected to post translational modifications in response to DNA damage. MDM2 binds to TAD and modulates protien functions (Michael et al., 2002; Gu et al., 2000; 2001). MDM2 can bind to TAD and suppress its transcriptional activity under *in vitro* conditions (Chen et al., 1999; Balint, et al., 1999). However, MDM2 failed to promote p73 degradation as observed for p53 (Cox et al., 1999).

DNA binding domain (DBD)

All the 'hot spot residues"(R175, G245, R249, R273 and R282) in the DBD are conserved (Ichimiya et al., 2000; Yang et al., 2000). The DBD binds to promoter DNA for the transactivation of genes, such as p21, MDM2, GADD45, and Bax.

Oligomerization domain (OD)

OD mediates homotetramer formation (Ko et al., 1996). Furthermore, a weak heterotypic interaction between p63 and p73 proteins was suggested (Kojima et al., 2001).

SAM domain and C-terminus (CTD)

The CTD diverges among the isoforms. Structural analysis recently elucidated that p73α has a molecular feature at the C-terminus representing a sterile alpha motif (SAM)-like domain that is not found in p53 (Khagad et al., 1997). The SAM domain is conserved only between p73-α and p63-α and the percentage of homology is 51% (Melino et al., 2003). The SAM domain is hypothesized to play a role in protein-protein interaction (Davison et al., 1999). Furthermore, this region could add specificity to the function of p73.

1.3.5 Expression of p73

1.3.5.1 p73 expression in normal tissues

Most cells express very low levels of p73. In human fetal and adult tissues, TA (transactivation)-p73 isoforms are most abundant (Ishimoto et al. 2002; Grob et al, 2001), while in the mouse neonatal brain and sympathetic ganglia (Pozniak et al., 2000), ΔN-p73 seems to be the most highly expressed isoform.

1.3.5.2 p73 expression in cancerous cells

In normal cells p73 is present at the low levels, while in tumor cell lines (cancers of the breast, lung, esophagus, stomach, colon bladder, ovary, liver, bile ducts, ependymal lining, myeloid and neurons) p73 is over expressed (Moll et al., 2001; Chen et al., 2000; Codegoni et al., 1999; Dominguez et al., 2001; Zaika et al., 1999; 2002).

1.3.6 Regulation of p73

p73 activity is regulated by several of the same molecules as p53, which supports the idea that p73 participates in maintaining genome stability. Recently, it has been shown that p73 induces apoptosis by potentiating the expression of scotin, PUMA and Bax (Rossi et al, 2005)

1.3.6.1 Regulation in response to DNA damage signals

Like p53, p73 is induced in response to various DNA damaging agents (Yang et al., 2002; Melino et al., 2003; Irwin et al., 2003).

1.3.6.2 Role of Post-translational modifications: Regulation of p73 by c-Abl, ATM, and MLH-1 network.

In response to DNA damaging agents such as cisplatin and ionizing radiation (IR) p73 is up regulated (Gong et al., 1999; Agami et al., 1999; Yuan et al., 1999). Although the molecular mechanisms by which p73 is activated in response to DNA damage signals is not clear yet, the presence of the mismatch repair gene (MLH1) and a functional and physical interaction between c-Abl and p73 are important for efficient induction of p73 (Gong et al., 1999; Agami et al., 1999; Yuan et al., 1999). As c-ABL (Abelson leukaemia) is, itself, phosphorylated and activated by ATM (ataxia telangiectasia

mutated), ATM might also be included in this pathway (Yuan et al., 1999). The activation of p73 in response to DNA damage is mainly regulated at the post-translational level. However, recent results suggest that p73 is also activated at the transcriptional level in response to a DNA damaging drug, Campothesin (Chen et al., 2001). In addition, information is lacking about the interactions of the different splicing isoforms with these kinases and acetylases. It seems likely that the response to DNA damage is highly dependent on the cellular context, relative abundance, and modification of each of the p73 isoforms.

1.3.6.3 p38 kinase

It has been shown by Sanchez-Prieto et al., (2002), that the p38 MAP kinase phosphorylates p73 on threonine residues adjacent to prolines. Furthermore, it was shown that p38 mediated p73 stability and transcriptional activation is dependent c-Abl.

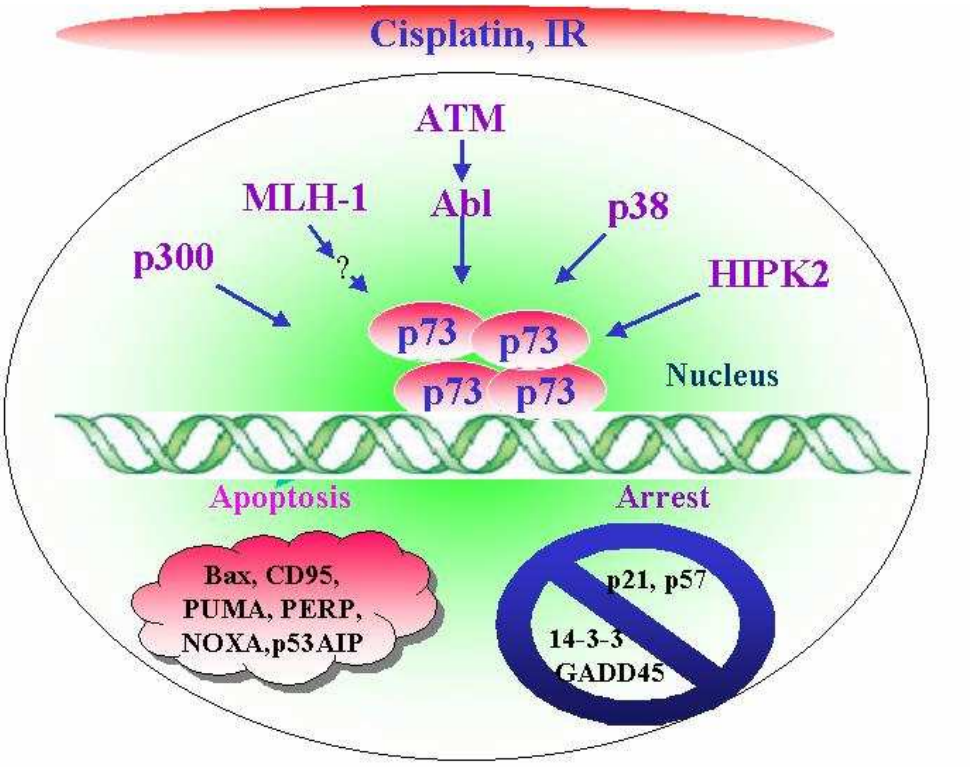


Figure 1.5 Pathway involving p73 in DNA damage DNA damage that is elicited either by cisplatin, or IR-irradiation triggers a p73 pathway-- independent of the p53 status and activation-- that is mediated mainly by MLH1, ATM and c-ABL (Gong et al., 1999; Yuan et al., 1999; Agami et al., 1999). This p53 pathway requires several complex post-translational modifications during its activation. Similarly, there is evidence that p73 is phosphorylated by c-Abl, p38, HIPK2 and acetylated by p300 (Zeng et al., 2000; Kim et al., 2002; Sanchez-Prieto 2002). As with p53, several mechanisms allow p73 to differentially regulate distinct classes of promoters such as cell cycle arrest and apoptosis (Chen et al., 2000; Stiewe et al., 2001).

1.3.6.4 HIPK2 kinase

Another kinase, home domain interacting protein kinase 2 (HIPK2), has been found to bind to p73 and enhance its function (Kim et al., 2002).

1.3.6.5 Acetylases

The ability of p53 to bind to its cognate DNA sequence and activate p53-dependent transcriptional activity is regulated by interaction of p53 with the transcriptional co-activator p300/CREB binding protein (Lill et al., 1997). Similarly, recent studies have demonstrated that the N-terminus (1-156) of p73 directly interacts with N-terminal CH1 domain of p300 to activate transcription and to induce p73-mediated apoptosis (Zeng et al., 2000). More recently, Levrero (2002) and his colleagues reported that p73 was acetylated by p300 on carboxyl-terminal lysine residues and this specifically potentiates the apoptotic function of p73.

1.3.6.6 Sumoylation

p73α, but not p73β has been shown to be covalently modified by the SUMO-1 (small ubiquitin-like modifier 1)(Minty, 2000). The major SUMO-1-modified residue in

p73α is the C-terminal lysine (Lys627). The SUMO-1 modifed p73 is more rapidly degraded by proteasomes than unmodified p73. In addition, it has recently been shown that PIAS-1 binds to p73α and sumoylates it. The PIAS1 mediated sumoylation decreases p73 transcriptional activity on several target promoters (Munarriz et al., 2004).

1.3.6.7 Regulation of p73 by MDM2

It has been shown that several stress signals activate p53. Haut et al., (1997) and Kubbutat et al (1997; 1999) have reported that MDM2—a target gene of p53—is a key player in the regulation of p53 stability (Haut et al., 1997; Kubbutat et al., 1997; 1999). Recent studies have suggested that MDM2 itself shows a specific E3 ubiquitin ligase activity and it covalently attaches ubiquitin groups to p53 as well as to itself (Linares et al., 2003).

p73 was also shown to induce MDM2 at the transcriptional level. Although MDM2 protein binds to N-terminal regions of p73 proteins α and β, it does not degrade p73, but neutralizes the ability of p73 to transactivate (Michael et al., 2002). The p73–MDM2 interaction also affects the sub cellular localization of p73 (Gu et al, 2001), potentially contributing to p73 stability. In fact, MDM2 has been shown to increase the stability of the p73 protein (Ongekoko et al, 1999).

1.3.7 Δ N-p73

ΔN-p73 lacks the transactivation domain and it can be derived either from an alternative promoter in intron 3 or an alternative splicing that originates from the first few exons namely Δ2Np73 and Δ3Np73. As shown in the figure (1.4), both Δ2Np73 and Δ3Np73 are generated from the same promoter as TA-p73 by splicing out exon 2 and

exon 3. ΔN-p73 (P2) promoter has been shown to be transactivated by both TA-p73 and p53 (Ishimoto et al., 2002; Fillippovich, et al. 2001; Melino et al., 2002), indicating an autoregulatory feed back loop (Grob et al., 2001; Kartasheva et al., 2002). Of note, both MDM2 and ΔN-p73 are transcriptional targets of p53. Deregulation of these regulatory loops in cancer cells, resulting in upregulation of either MDM2 or ΔNp73 or both, would effectively inhibit the function of p73 (Melino et al., 2002). In developing brain, ΔN-p73 is highly expressed and appears to play an anti-apoptotic role *in-vivo* (Yang et al., 2002). In human cancers, ΔN-p73 is specifically upregulated (Ishimoto, et al., 2002; Douc-Rasy et al., 2002). This study included 35 cancers (cancers of the ovary, endometrium, cervix, vulval, vagina, breast, kidney, and colon) (Zaika, et al, 2002). Recently, Casciano et al.,(2002) reported that in neuroblastoma patients, expression of the anti-apoptotic ΔN-variant of p73 is strongly associated with reduced survival and predicts a poor outcome.

1.3.8 Regulation by oncogenes

It has been shown recently that various oncogenes such as c-Myc and E1A upregulate the levels of p73 (Irwin et al., 2000; Zaika et al., 2000).

1.3.8.1 c-Myc and E1A

Zaika et al., (2000) have shown that p73 expression is increased by overexpression of c-Myc. In addition, Watanabe *et al.* (2002) showed that the interaction between c-MYC and p73 results in inhibition of p73's transcriptional activity. Flinterman et al., (2004) showed that E1A also increases the expression of endogenous TAp73 mRNA and protein. Both E1A and c-Myc appear to increase p73 levels through E2F1.

1.3.9 The role of p73 in cancer

The fact that the regulatory molecules—ATM, ATR, Abl, p38 etc. —that activate p73 are similar to those known for p53, suggests a comparable function of tumor suppressor genes in human cancers (Stiewe et al., 2002). However, data obtained from knockout mice failed to support its role as a tumor suppressor (Yang et al., 1999). Further, several groups reported increased expression levels of total p73 in tumor tissues compared to the surrounding normal tissue (Zakia et al., 2002). However, the role of increased expression of p73 in tumors is not clear yet. Before making a firm conclusion, one would need to consider the complexity, different transactivation potential and apoptotic activity of p73 isoforms and their ability to interact with each other (Levrero et al., 2000). In the case of hepatocellular carcinomas, overexpression of p73 could be correlated with a poor patient survival prognosis (Qin et al., 2000; Herath, et al., 2000). Another study determined that ΔN-p73 is a strong adverse prognostic marker in neuroblastomas (Casciano et al., 2002).

1.3.10 p73 mutations, Loss of heterozygosity, Imprinting, and promoter silencing

1.3.10.1 p73 mutations and loss of heterozygosity

The human p73 maps to chromosome 1p36.33, which frequently undergoes loss of heterozygosity in breast cancer, neuroblastoma and several other human cancers (Kaghad et al, 1997). The mouse p73 maps to the distal part of chromosome 4, which undergoes frequent loss of hetrozygosity (LOH) in radiation induced T-cell lymphomas (Herranz et al, 1999; Stiewe et al., 2002). The fact that p73 maps to chromosome

1p36.33, which frequently undergoes loss of hetrozygosity, may suggest that p73 could be a tumor suppressor gene. This notion initiated an extensive analysis of the p73 status (Zaika et al., 2002; Stiewe et al., 2002). Unfortunately, loss of function mutations in the p73 ORF is quite uncommon (Melino et al., 2002).

1.3.10.2 Imprinting

Initial studies indicated that p73 is an imprinted gene (Khagad et al. 1997). That is, only one allele is active and other one is silenced by epigenetic mechanisms. However, this appears to be rather infrequent and varies from tissue to tissue (Moll et al., 2001; Zaika et al, 1999; Kovalev et al, 1998). A number of studies have demonstrated loss of imprinting (LOI), biallelic expression of p73 or allele switching (Stiewe et al., 2002). In fact, LOI is exemplified in lung, esophageal and renal carcinoma (Mai et al, 1998 a & b; Cai et al, 2000; Moll et al., 2001).

1.3.10.3 Promoter silencing

Loss of p73 expression due to hypermethylation of promoter appears to be infrequent in general. It is reported only in certain hematological malignancies such as primary acute lymphoblastic leukemia (ALLs) and Burkitt lymphomas (Corn et al, 1999; Kawano et al, 1999; Banelli et al., 2000; Stiewe et al., 2002; Puig et al., 2003). On the contrary, increased expression of p73 was reported in chronic myeloid leukemia, acute myelogenous leukemia, and B-cell chronic lymphocytic leukemia (B-CLL) (Peters et al, 1999; Novak et al, 2001; Stiewe et al., 2002).

1.3.11 p73 alterations in human cancer

Tumor	Mutations	Loss of heterozygosity
Neuroblastoma	2/317(P450R, P425L)	66/307
Central nervous system	2/142 (N204S, E291K)	-
Melanoma	0/68	3/27

Parathyrodi adenoma	0/16	4/16
Lung cancer	1/114(P450K)	32/107
Hypopharyngealcarcinomas	0/17	-
Oesophageal cancer	0/48	25/76
Gastric cancer	0/82	12/32
Colorectal cancer	0/125	8/46
Bladder cancer	0/23	-
Prostate cancer	0/133	2/38
Renal cancer	0/27	-
Cholanglocarcinoma		6/11
Hepatocellular carcinma	0/48	21/71
Leukemia &lymphoma	0/91	-
Breast cancer	1/145	20/194
Ovarian cancer	0/63	33/141
Total	6/1459 (0.46%)	232/1066 (21.76%)

(Adapted from Melino et al., 2002)

Figure 1.6 p73 alterations in cancer.
p73 mutational analysis presented by Melino et al., (2002) suggests that p73 mutations are rare in a variety of tumors. However, there is a significant incidence (40-33%) of Loss of heterozygocity especially in gastric, lung, oesophageal and neuroblastoma cancer types (Melino et al.,2002). (Neuroblastoma (Douc-Rasy et al., 2002; Kovalev et al., 1998; Ichimiya et al., 1999; Ejeskar et al., 1999; Han et al., 1999; Liu et al., 2000; Yang et al. 2000; Kong et al., 1999); Central nervous system (Chi et al., 1999; Lomas et al., 2001; Nozaki et al., 2001 Alonso et al., 2001); Melonoma (Kroiss, et al., 1998; Herbst et al., 1999; Schittek et al., 1999); Parathyroid adenoma (Shan et al., 2001; Lung cancer (Ikeas et al., 1999; Nomoto et al., 1998; Nicholson et al., 2001; Mai et al., 1998; Tokuchi et al., 1999); Hypopharengeal carcinoma (Faridoni-Laurens et al., 2001); Oesophageal cacner (Ryan et al., 2001; Nimura et al., 1998; Cai et al., 2000) ; Gastric cancer (Han et al., 1999 ; Kang et al., 2000; Yokozaki et al., 1999); Colorectal cancer (Han et al., 1999; Sunahara et al., 1998) Bladder cancer (Yokomizo et al., 1999); Prostate cancer (Yokomizo et al., 1999; Takahashi et al., 1998); Renal cancer(Mai et al., 1998); Cholangiocarcinoma (Momoi, et al., 2001); Hepatocellular cancer (Mihara et al., 1999; Peng et al., 2000; Herath et al., 2000); Leukemia and Lymphoma (Corn, et al., 1999; Stirewalt et al, 1999); Breast cancer(Han et al., 1999; Zaika et al., 1999; Shishikura et al., 1999; Schwartz et al; Dominguez, et al., 2000 ; Ahomadegbe, et al., 2000) ; Ovarian cancer (Chen et al., 2000 ; Codegoni, et al., 1999 ; Imyanitov, et al., 1999).

1.3.12 Tumor derived mutants inactivate p73

A moderate degree of interaction between wild-type p53 and p73 has been shown (Kaghad, et al, 1997; De Laurenzi et al, 2000). More than 50% of cancer cells have high

levels of mutant p53 and some tumor derived p53 mutants have a significant ability to bind and interact with TAp73-α. In co-transfection assays, it has been shown that the 175, 248 and 281 mutants of p53 interact with p73-α and inhibits its ability to transactivate reporter genes and apoptosis (DiComo et al, 1999; Gaiddon, et al, 2001; Strano et al, 2001; Melino et al., 2002). In addition, the association between p53 mutants and p73 is regulated by a common polymorphism at codon 72 of p53 that encodes Arg or Pro, with Arg leading to a stronger interaction with p73 (Marin et al, 2000; Melino et al., 2002). Together, inactivation of p73 by mutant p53 seems to provide a selective advantage in promoting tumorigenesis.

1.3.13 Interaction between p73 and viral proteins

Several groups showed that DNA tumor viruses (DTV) interact with p53 (Levrero et al., 2000; Ko, 1996; Levine 1997; Oren 1999). The interaction between DTV and p73 results in inability of p73 to transactivate reporter genes and apoptosis. For example, SV40 T antigen, E1B, HPV proteins bind to p53 and sequester it into an inactive complex. None of these viral proteins interact with p73 (Kaelin, 1999b; Melino et al., 2002). However, the Ad E4 and the HTLV1 tax proteins bind to and inactivate p53 and p73 (Das et al., 2003; Lemasson et al., 2001; Moll et al., 2001; Melino et al., 2002).

1.3.14 Phenotypes of *p73*$^{-/-}$ mice

Unlike p63$^{-/-}$ mice, *TP73*$^{-/-}$ mice survive postnatally, despite having multiple defects (Yang et al., 2001). Given the similarity of the genes, the *TP73* knockout phenotype shows no obvious overlap with that of *TP53*-deficient mice. p53 deficient mice develop thymic lymphoma, fibrosarcoma, other tumors and excencephaly

(Donehowver, 1996). In contrast, p73 null mice show no spontaneous tumors (Yang et al., 2002).

TP73$^{-/-}$ mice appear to suffer from the following:

1. Somatic growth retardation.

2. Malfunctions in fluid control in the central nervous system and respiratory airways.

3. Middle ear inflammation/infections.

4. Defective neurogenesis.

5. Abnormal reproductive and social behavior (Yang et al., 2000).

1.3.15 p73 participates in DNA repair pathways

The following facts may suggest that p73 participates in DNA repair pathways:

1. The ability of p73 to respond to DNA damage signals, just like its counterpart-p53.

2. *MLH-1*$^{-/-}$ cells failed to induce p73 in response to cisplatin treatment (Gong et al, 1999).

3. p73 overexpressing clones have increased levels of DNA repair proteins (Vikhanskaya et al., 2001).

1.3.16 p73 participates in differentiation

The following facts may suggest that p73 participates in differentiation:

1. The overexpresion of p73β induces morphological and biochemical markers of neuroblastoma differentiation (Laurenzi et al., (2000).

2. Laurenzi et al., (2000) have shown that TA-p73 expression is increased during retionic acid-induced and spontaneous differentiation of neurblastoma cells.

3. Human skin keratinocytes have been shown to undergo terminal differentiation when TAp73-γ and ΔNp73 are overexpressed (Laurenzi et al 2000; Kovalev et al 1998).

4. Li (2005) showed that p73α suppresses myogenic differentiation on one hand. On the other hand, p73β fails to have any effect on differentiation.

2.1 c-Jun

The proto-oncogene Jun, represents one of the important components of the AP-1 family of transcription factors (Angel et al., 1991).

2.2 Introduction to c-Jun

The transcription factor, c-jun, is the cellular homologue of v-jun, the transforming oncogene of the avian sarcoma virus 17 (Nishimura et al., 1988). The c-Jun appears to regulate both cellular proliferation and apoptosis.

2.3 Gene structure of the *c-jun*

Hattori et al (1988) and Nishimura (1988) cloned the c-jun gene nearly fifteen years ago. Cloning of the c-jun gene revealed that it is a gene without introns (Nishimura et al., 1988). The human jun gene is located on chromosome 1 at region p31-32 (Haluska et al, 1988). Murine jun is located on chromosome 4 sub-region C5-C7 (Mattei et al, 1990; Vogt, 2001).

2.4 c-jun promoter

The c-jun promoter region is highly conserved between mouse, rat and human (Mechta-Grigoriou et al., 2001). The c-jun promoter contains potential binding sites for several transcription factors, including SP1, Jun, CTF (CCAAT Transcription Factor) and AP1 (Brach et al., 1992; Angel et al., 1988; Mechta-Grigoriou et al., 2001).

2.5 Expression pattern of c-Jun

c-Jun has been shown to be mainly expressed in developing cartilage, gut and the central nervous system (Mechta-Grigoriou et al., 2001). However, c-Jun is over

expressed in several human tumors or transformed cell lines of different cell origins (Zoumpourlis et al., 2000). c-Jun's expression level is elevated both in response to growth and stress stimuli. Its expression level is regulated both at the transcriptional and post-translational level.

2.6 The definition of AP-1

AP-1 is a group of dimeric basic region-leucine zipper (bZIP) proteins that belong to the c-Jun, c-fos family and others, which recognize 12-0-tetradecanoylphobol-13-acetate (TPA) response elements (5'-TGAG/CTCA-3') (Shualin et al., 2002; Angel et al., 1988; O'Shea et al., 1992; Chinenov et al., 2001).

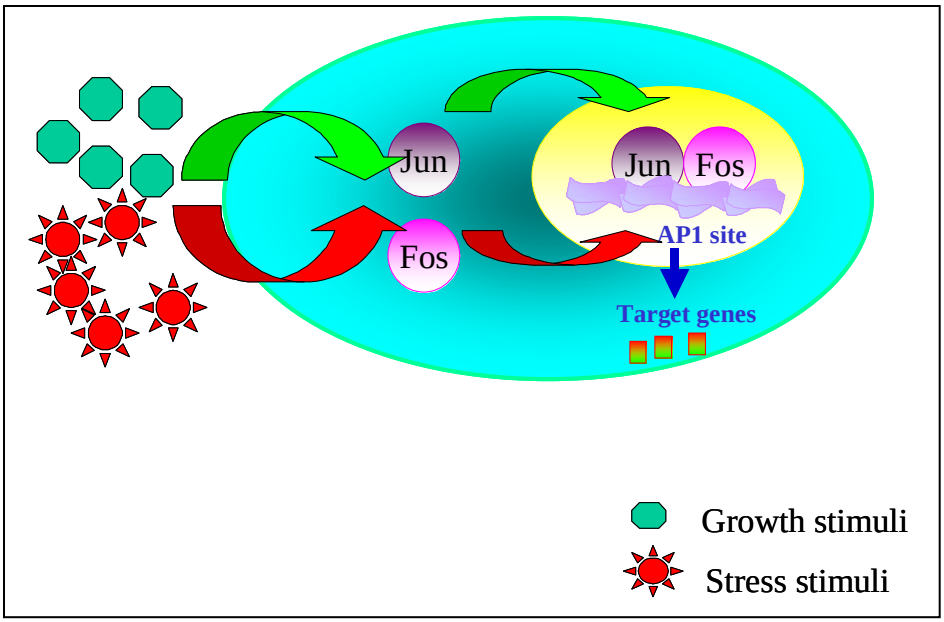


Figure 1.7 AP-1 regulation.

2.7 The structural organization of the c-Jun protein

c-Jun posses a dimerization (280-300), DNA-binding (257-276) and transactivation domains (1-100) and these domains can be exchanged with functionally equivalent domains within the bZIP family (Vogt, 2001).

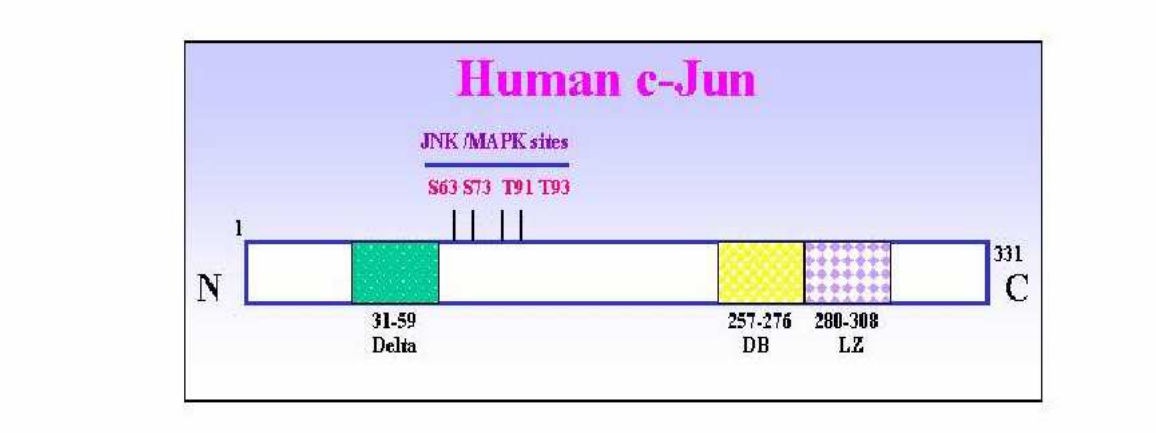


Figure 1.8 Structural domains of c-Jun protein (Morton et al., 2003).

Transactivation (TA) domain

The N-terminal half of Jun contains the transactivation domain.

DNA binding domain (DBD)

The DBD is located immediately N-terminal to the leucine zipper sequence. Residues 252-281 in the c-Jun protein consititues the basic/DNA binding region, which is responsible for the sequence specific DNA recognition site or DNA contact surface (Krebs 1995).

Dimerization domain

This dimerization domain contains five heptad repeats of leucines (Landschulz et al., 1988; Alber et al., 1992). Hence, it is also referred as the leucine zipper (bZIP)

domain. Dimerization is a prerequisite for DNA binding (Halazonetis et al., 1988; Smeal et al., 1989) and the dimerization of Jun and Fos enhances their nuclear translocation (Chida et al., 1999).

The c-Jun is known to homodimerize or heterodimerize with the c-fos protein. However, c-Jun homodimers are less stable than c-Jun/c-fos heterodimers and they have a higher affinity for the DNA target sequence (Allegretto et al., 1990; Halazonetis et al., 1988; Nakabeppu et al., 1988; Smeal et al., 1989). Cellular context and extracellular signalling molecules decide the composition of the AP-1 family of proteins.

2.8 Post-translational modifications of c-Jun

c-Jun mediates transcriptional regulation in response to a variety of stimulants and it is tightly regulated posttranslationally.

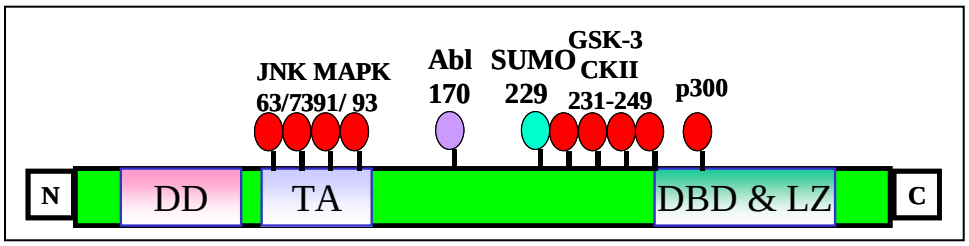


Figure 1.9 Post-translational modifications of c-Jun (Morton et al., 2003; Barilla et al., 2000; Muller et al., 2000).

2.8.1 Phosphorylation

Early studies show that c-Jun needs to be phosphorylated at serine 63 and 73 to become an efficient transcriptional regulator (Adler et al., 1992a; Mechta et al., 1997;

Smeal et al., 1991, 1992; Davis et al., 2000; Bohmann et al., 1989). c-Jun is phosphorylated at two residues proximal to the major transactivation domain. The trasactivation domain is phosphorylated by a family of stress-activated protein kinases (SAPKs) (Adler et al., 1992c; Dai et al., 1995; Hibi et al., 1993; Pulverer et al., 1993; Davis et al., 2000). The corresponding kinase gene, JNK1(Jun N-terminal kinase), was cloned (Derijard et al., 1994). Cloning JNK2 of a second Jun kinase, followed soon after (Kallunki et al., 1994).

Further, c-Jun was shown to be phosphorylated at sites Thr231, Thr239, Ser 243 and Ser249, which are located near the DNA binding domain and known to be phossphorylated by GSK-3 and Casein Kinase-II (Boyle et al., 1991; Lin et al., 1992). Dephosphorylation of these sites in response to growth stimulation augments the binding of c-Jun to DNA (Morton et al., 2003).

2.8.2 Acetylation

It has been shown recently that c-Jun is acetylated *in vivo* and the specific acetylation of c-Jun enhances its ability to trasactivate downstream genes. In addition, mutational analysis identified that Lys 271 in the c-Jun basic region, is acetylated by p300 (Vries et al., 2001).

2.8.3 Sumoylation

It has been shown by Muller et al, (2000) that c-Jun is a new substrate for SUMO-1 both in vitro and in vivo. SUMO-1 targets a single lysine residue in c-Jun (Lys-229), and the SUMO-1 modification decreases transactivation potential on an AP-1-containing promoter compared with wild-type c-Jun, suggesting that SUMO-1 negatively regulates c-Jun activity.

2.9 Biological functions of c-Jun

c-Jun appears to play a role in proliferation, transformation and apoptosis.

2.9.1 c-Jun and cell Proliferation

The following facts support the idea that c-Jun plays a major role in cell proliferation:

1. Fibroblasts/hepatoblasts lacking c-Jun exhibit a severe proliferation defect. This inhibition of cellular proliferation is associated with reduced expression of cyclin D1 and D3. Importantly, c-Jun regulates cyclin D1 promoter activity directly (Eferl et al., 1999; Hilberg et al., 1993; Johnson et al., 1993; Bakiri et al., 2000; Shualin et al., 2000).
2. Its ability to transform cells either alone or in the presence of a cooperating oncogene (Bos et al 1990; 1999; Johnson et al., 1996; Schutte et al., 1989; Leppa et al., 1999; Vogt et al., 2001).
3. Both neutralizing antibodies and anti-sense RNA inhibit the cell's entry into S phase (Kovary and Bravo, 1991; Riabowol et al., 1992; Smith and Prochownik, 1992; Leppa et al., 1999).
4. Over expression of c-Jun alters the proportion of cells in S, G2 and M (Pfarr et al., 1994).

2.9.2 Transformation

2.9.3 Constitutive expression of c-Jun alone causes transformation

It has been shown that both c-Jun and v-Jun could transform primary chicken embryo fibroblasts (Cavalieri et al., 1985; Hartl et al., 1995; 1992; Castellazzi et al., 1990; Wong et al., 1992). The transformed cells are highly tumorigenic and are capable of anchorage-independent growth (Vogt, 2001). Similarly, in mammalian host systems, c- Jun, is able to transform the continuous line of rat fibroblasts known as Rat1a (Schutte et al., 1989; Vogt, 2001). Together, these results support a view that c-Jun plays a significant role in tumor pathogenesis.

2.9.4 c-Jun cooperates with other oncogenes to transform cells

NIH3T3 cells can be transformed by c-Jun in conjunction with Fra-1 (Mechta et al., 1997). In addition, c-Jun can co-operate with mutated ras to transform embryo fibroblasts (Schutte et al., 1987). In the co-transformed cells, ras was shown to act upstream of c-Jun, inducing JNK and constitutively phosphorlylating c-Jun (Vogt et al., 2001; Behrens et al., 1999, 2000).

2.9.5 c-Jun as a mediator of Apoptosis

Although c-Jun is known to induce proliferation, it has the capacity to induce apoptosis, a property that Jun shares with other oncoproteins like Myc, E1A, or E2F. In NIH3T3 cells, over expression of Jun triggers programmed cell death (Bossy-Wetzel et al., 1997). c-Jun can trigger either proliferative or anti-proliferative/apoptotic signals depending on the dimer composition, context, the cell types or concentration of the DNA damaging agents (Leppa et al., 1999).

2.9.6 c-Jun and apoptosis

The following facts support a role for c-Jun in apoptosis:

1. DNA damaging agents such as UV, H_2O_2, MMS, TNF-alpha etc. induce c-jun expression (Mechta-Grigoriou et al., 2001).
2. IL-6 depletion causes apoptosis in lymphocytes. During the execution phase of apoptosis, both c-jun and c-fos expression is increased. Furthermore, addition of c-jun/c-fos antisense oligonucleotides protects these cells from undergoing apoptosis (Mechta-Grigoriou et al., 2001; Colotta et al., 1992).
3. Bossy-Wetzel et al., (1997) showed that increased expression of c-Jun causes apoptosis in immortalized NIH3T3 fibroblasts that can be prevented by Bcl-2 or inhibitors of ICE/CED-3-type cysteine proteinases.
4. Over-expression of c-Jun also induces apoptosis in endothelial cells. The process is preventable by a dominant negative Jun mutant (Wang et al., 1999).

All these results provide evidence that c-Jun is a potent inducer of programmed cell death in various cell types.

2.10 Knockout studies on c-Jun: c-Jun *invivo* functions

c-jun heterozygous mutant mice appear to be normal, but embryos lacking c-Jun exhibit impaired hepatogenesis, altered fetal liver erythropoiesis and generalized edema. These embryos undergo apoptosis in hepatoblasts and erythroblasts lineage (Hilberg et al, 1993; Johnson et al, 1993; Eferl et al, 1999; Mechta-Grigoriou et al., 2001). In contrast, over expression of c-Jun in transgenic mice does not result in any obvious phenotype

(Grigoriadis et al., 1993). Further, Jun phosphorylation mutant mice [c-Jun knock-in mice carrying c-Jun alleles with JNK phosphorylation site mutations (Jun S63A and S73A)] are viable and develop normally, indicating that N-terminal phosphorlylation of c-Jun is not requireed for embryonic development and organogenesis (Behrens et al., 2001).

2.11 The Regulation of c-Jun

2.11.1 Transcriptional mechanisms

Growth factors (EGF, FGF) and tumor promoting agents (TPA/PMA) induce the expression of c-jun at the transcriptional level and they induce it through a TRE- like site present in the murine c-jun promoter regulatory sequences (Mechta-Grigoriou et al., 2001).

2.11.2 *Post-translational mechanisms*

The stimulation of c-Jun expression and post-translation modification seem to be regulated sequentially: endogenous basal c-Jun protein is first activated by phosphorlyation. Next, phosphorylated c-Jun augments its own expression, which results in a positive feed back loop (Angel et al., 1988). The ability of c-Jun to function as a transcription factor is enhanced by JNK dependent phosphorylation mechanism. At least three classes of JNK kinases (JNK1, JNK2 & JNK3) have been identified (Davis R et al., 2000). JNK phosphorylates c-Jun and thereby connects Jun to various signals, generated by mitogens, stress signals, and genotoxic substances (Adler et al., 1995a,b; Derijard et al., 1994; Franklin et al., 1993; Smeal et al., 1991, 1992). It was suggested that phosporylated c-Jun could recruit CBP or histone deacetalase to augment the c-Jun dependent transcription (Arias et al, 1994; Mechta-Grigoriou et al., 2001). The signaling

molecules that originates upstream of JNK and their sequential interactions have not been fully worked out. However, it appears that JNK is itself activated by a signal that originates in Ras (Adler et al., 1992; Minden et al., 1994; Westwick et al., 1994).

2.12 The stability of c-Jun protein

Several studies suggest that multiple proteolytic machineries, including the proteasomes, lysosomes, and ubiquitous calpains, may participate in the destruction of c-Jun. The relative input of each pathway is far from being known. It has been demonstrated that, in certain occurrences, the degradation of c-Jun by the proteasome *in vivo* involves the ubiquitin pathway. Treier et al (1994) showed that c-Jun, but not v-Jun, can be efficiently multiubiquitinated. Consistently, v-Jun has a longer half-life than c-Jun.

2.13 The relationship between p53 and c-Jun

c-Jun null fibroblasts have a proliferation defect. This proliferation defect found in c-Jun null cells was attributed to the accumulation of p53. This data suggests that c-Jun negatively regulates p53 in fibroblasts (Shualin et al., 2001) and it was shown to suppress the expression of p53 at the promoter level (Schreiber et al., 2000). In addition, fibroblasts lacking c-Jun express very low levels of cyclin D1 leading to slow progression from G1 to S phase (Wisdom et al, 1999; Wagner E, 2003). These observations indicate the ability of c-Jun to directly regulate cell cycle and apoptotic machinery. On the one hand, it activates cyclin D1 transcription and on the other hand, it inhibits p21 accumulation through repression of p53 expression (Shualin et al., 2001).

3.1 Hypothesis: c-Jun plays a role in the regulation of p73

3.1.1 c-Abl regulates p73 in response to cisplatin and IR induced DNA damage.

c-Abl is activated by certain DNA-damaging agents such as cisplatin and ionizing radiation. It contributes to the induction of programmed cell death (apoptosis) by p53-dependent and p53-independent mechanisms (Shaul et al., 2000). Cancer cells with mutation in p53 gene are resistant to anti-cancer drugs, however, their resistance is partial, which indicates that alternative apoptotic pathways exist (Gong et al., 1999). The protein p73 is a structural and functional homologue of the p53 tumor-suppressor protein. It can induce apoptosis in the absence of p53 (Jost et al., 1997). The p73 protein level is increased in wild-type fibroblasts but not in $MLH^{-/-}$ and $Abl^{-/-}$ fibroblasts. Also, $MLH^{-/-}$ and $Abl^{-/-}$ fibroblasts were more resistant to cisplatin and the half-life of p73 was extended by cisplatin (Gong et al., 1999). Further, c-Abl has been shown to bind to p73 through its SH3 domain with the carboxyl-terminal homo-oligomerization/PxxP domain of p73. c-Abl phosphorylates p73 on a tyrosine residue at position 99 in cells that have been exposed to IR (Yuan et al., 1999; Agami et al., 1999).

3.1.2 Cisplatin but not IR stabilizes p73

Both ionizing radiation and cisplatin induction have been shown to result in enhanced c-Abl kinase activity. However, only ionizing radiation induces tyrosine phosphorylation of p73. Further, only cisplatin treatment results in the stabilization of p73, but not ionizing radiation (White and Prives 1999). These intriguing observations

raise the possibility that there could be other molecules involved in cisplatin mediated p73 stability. Ionizing radiation induces double stranded DNA breaks. This kind of DNA damage is more amenable to repair; hence, it induces only cell cycle arrest. In contrast, cisplatin interacts with DNA to form intra- and interstand crosslink adducts (Siddik et al., 2003). The cisplatin-modified DNA is not amenable to DNA repair (Jordan et al., 2000). Thus, it is cytotoxic to cells. The fact that p73 is not being activated in *MLH*$^{-/-}$ (Mismatch repair deficient) MEFs in response to cisplatin indicates that MLH is upstream of p73 and it participates in the DNA repair process. Transient activation of p73 in response to ionizing radiation would result in the activation of repair pathways. This clarifies why p73 is only transiently induced in response to ionizing radiation. While in response to cisplatin, the sustained p73 induction, results in stabilization and activation of apoptosis. Hence, it seems clear as to why cisplatin stabilizes p73 and not IR. However, it is essential to identify the molecules that are involved in cisplatin mediated p73 stability.

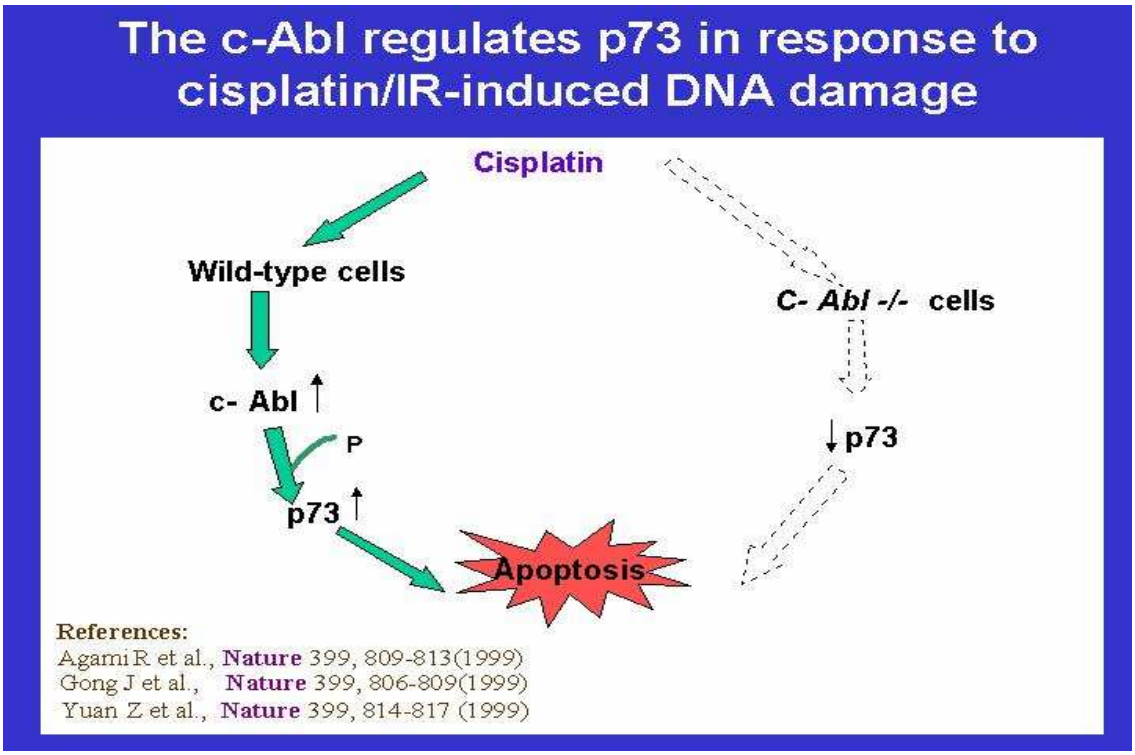


Figure 1.10 c-Abl regulates p73 in response to cisplatin and IR induced DNA damage
The resistance of *c-abl*$^{-/-}$ MEFs to cisplatin treatment was attributed to lack of induction of p73 in these cells. c-Abl has also been shown to interact and phosphorylate p73 in response to IR. Both c-Abl and p73 seem to cooperate with each other in inducing apoptosis in response to cisplatin treatment.

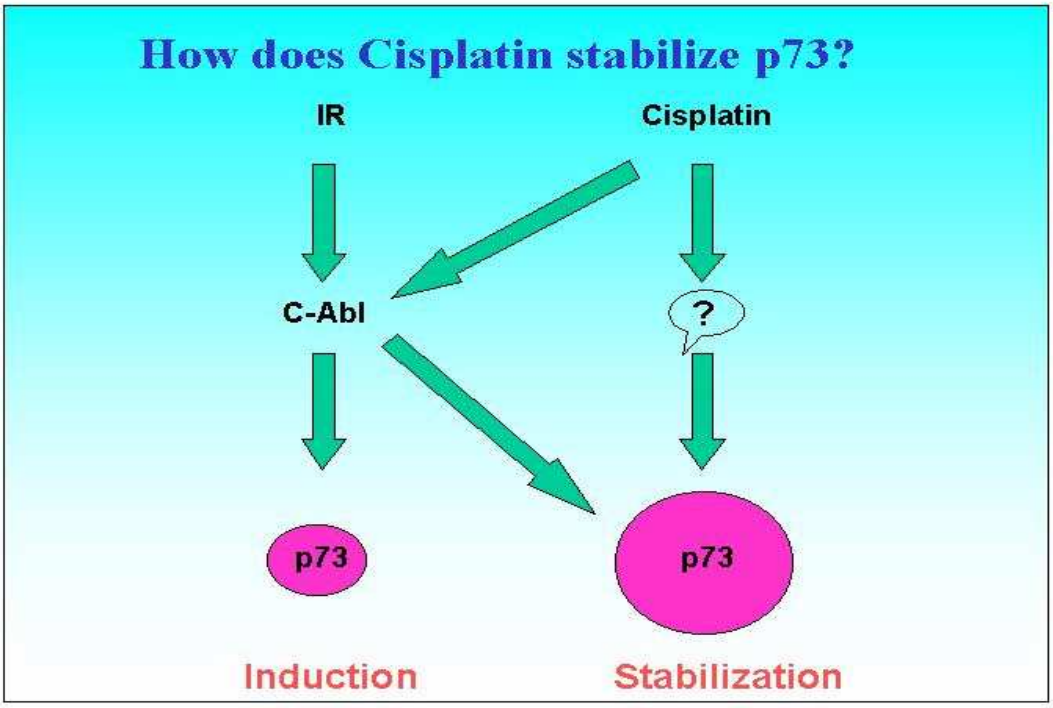


Figure 1.11 cisplatin stabilizes p73 but not IR Both IR and cisplatin induce c-Abl activity, however, only cisplatin stabilizes p73 but not IR. This fact raises the question of how cisplatin stabilizes p73.

3.1.3 Does c-Jun play a role in cisplatin mediated p73 stability?

It has been shown earlier that c-Jun act as a convergence point of many signaling pathways (Karin *et al*., 1997). Its activity is regulated at the transcriptional and post-transcriptional levels. Serine/threonine phosphorylation at Ser63/73 (Phosphorylated by JNKs) and Thr91/93 (Phosphorylated by MAP kinases) sites induces an increase in the DNA-binding and transactivation potential of the protein, as well as an increase in the stability of c-Jun (Treisman, 1996; Karin *et al*., 1997). It has been shown that increased

c-Jun levels increase the activity of c-abl, in turn, c-abl kinase phosphorylates Jun on tyrosine 170 (Barila et al., 2000; Raitano et al., 1995; Renshaw et al., 1996). This positive feedback loop enhances the ability of Jun to activate Abl further. However, whether c-Abl enhances JNK activity or c-Jun enhances c-Abl activation in response to stress signals is not clear. For example, UV and IR do not induce c-Abl activity and c-Jun expression respectively (Liu et al., 1996; Shualin et al., 2000)

Cisplatin induces the expression and activity of c-Abl, JNK and c-Jun, while IR is known to induce only c-Abl, raising a possibility that c-Jun could play a role in cisplatin mediated p73 stability. Furthermore, c-Jun might influence the ability of c-Abl to activate p73. However, the study led by Superti-Furga (2000) failed to indicate the significance of tyrosine phosphorylation of c-Jun by c-Abl and its importance in genotoxic signaling.

3.1.4 The Role of c-Jun in cisplatin resistance and p73 activation.

Most of the anti-cancer drugs exert their action by inducing apoptosis. Cisplatin, a well-known anti-cancer drug, is used to treat a wide variety of cancers, although its efficacy is often limited by its inherently poor activity against many tumor types and by the development of resistance (Young, et al., 1989; Siddik et al., 2003). The mechanism through which cisplatin exerts its toxicity in cancer cells is not clear yet, but it is generally accepted that it acts through the formation of DNA adducts (Zamble, et al., 1995; Jordan et al., 2000). Thus, it is important to understand the mechanism of action of cisplatin and molecules involved in the signaling pathway in order to increase the efficacy of the anti-cancer treatment.

Cisplatin has been shown to activate both p53-dependent apoptosis and p53-

independent apoptosis in cancer cells (Hawkins, 1996. The p53 is not a determinant of cisplatin cytototoxity in ovarian cancer cells (De Feudis, et al., 1997), testicular tumor cell lines (Burger, et al., 1997), breast cancer, and human foreskin fibroblast cell lines (Fan, et al., 1994; Hawkins, 1996).

Furthermore, cisplatin has been shown to activate c-Abl, p38 MAP kinase and JNK. However, the components involved in the cisplatin-mediated p53-independent apoptotic program are not clear yet.

Together, the facts described so far, support the hypothesis that if cisplatin mediated p73 stability/activation requires c-Jun then *c-jun-/-* cells will be resistant to cisplatin mediated apoptosis and this can be attributed to the lack of stabilization of p73 in these cells.

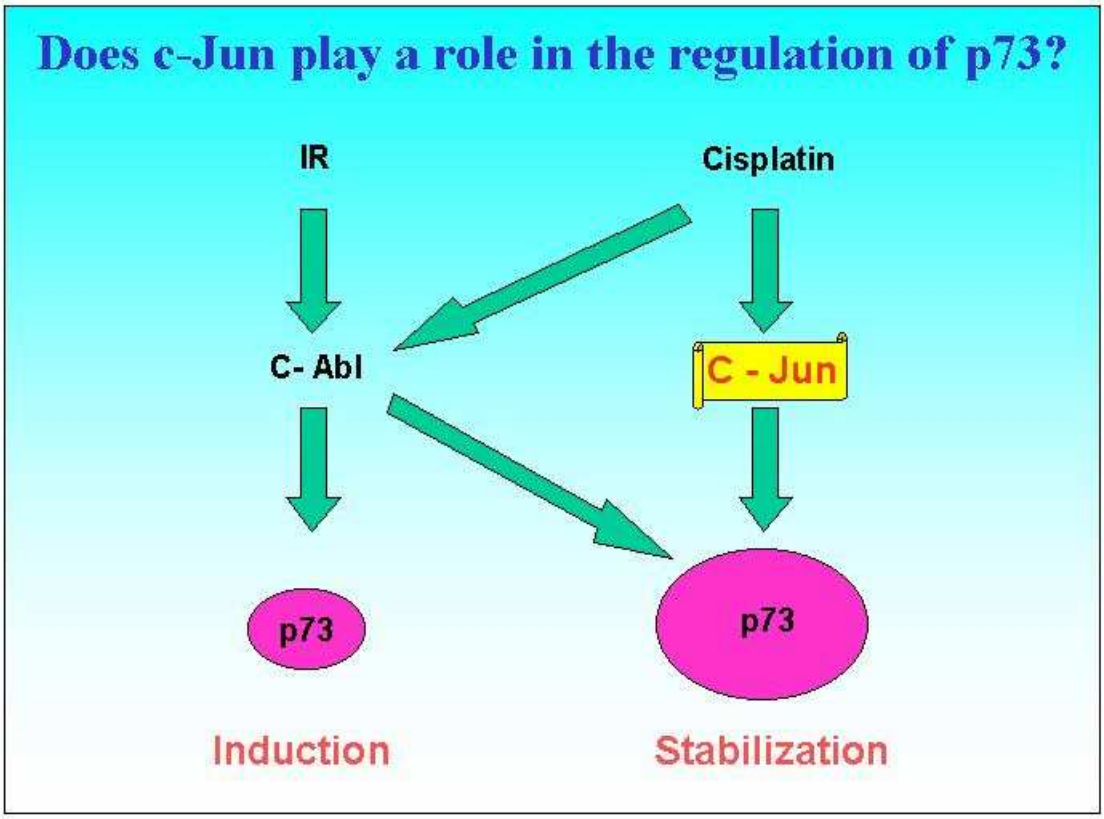


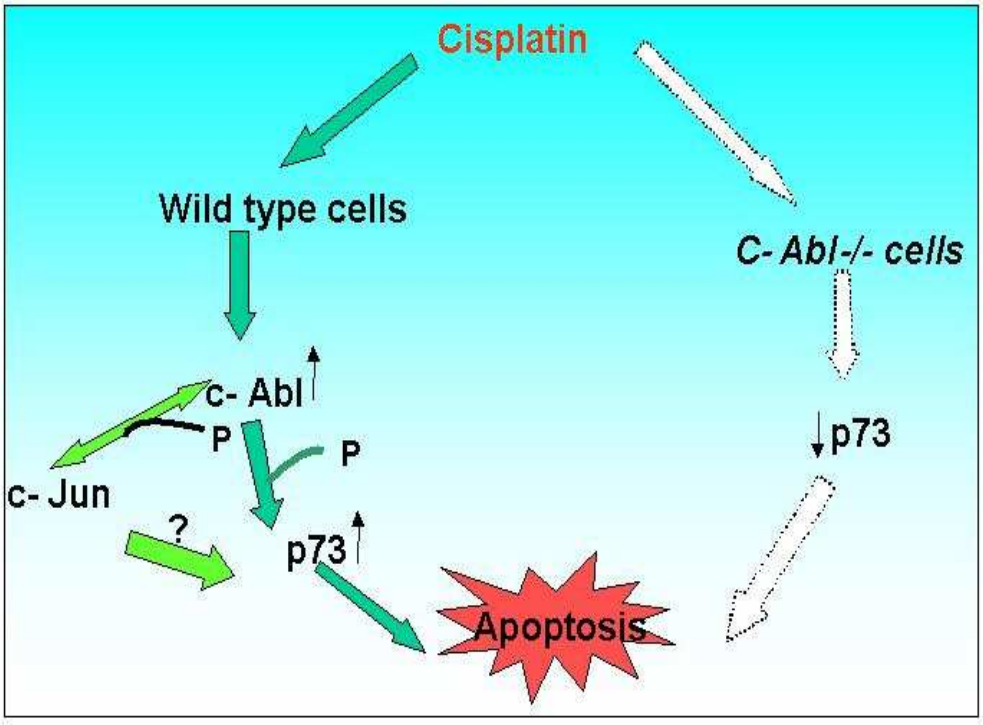
Figure 1.12 C-Jun could play a role in cisplatin mediated p73 stability. A schematic diagram illustrating the difference in p73 stability in response to cisplatin and IR mediated signaling. Although both ionizing radiation and cisplatin induction results in enhanced c-Abl kinase activity, only cisplatin treatment, but not ionizing radiation results in stabilization of p73. This indicates that other molecules play a role in p73 stability. cisplatin has been shown to induce both c-Abl and c-Jun, while IR induces only c-Abl activity, indicating that c-Jun could contribute to cisplatin mediated p73 stability.

Questions:

* Are c-Jun null cells resistant to Cisplatin- mediated apoptosis?

* Is c-Jun required for cisplatin-mediated activation/apoptosis?

A



B.

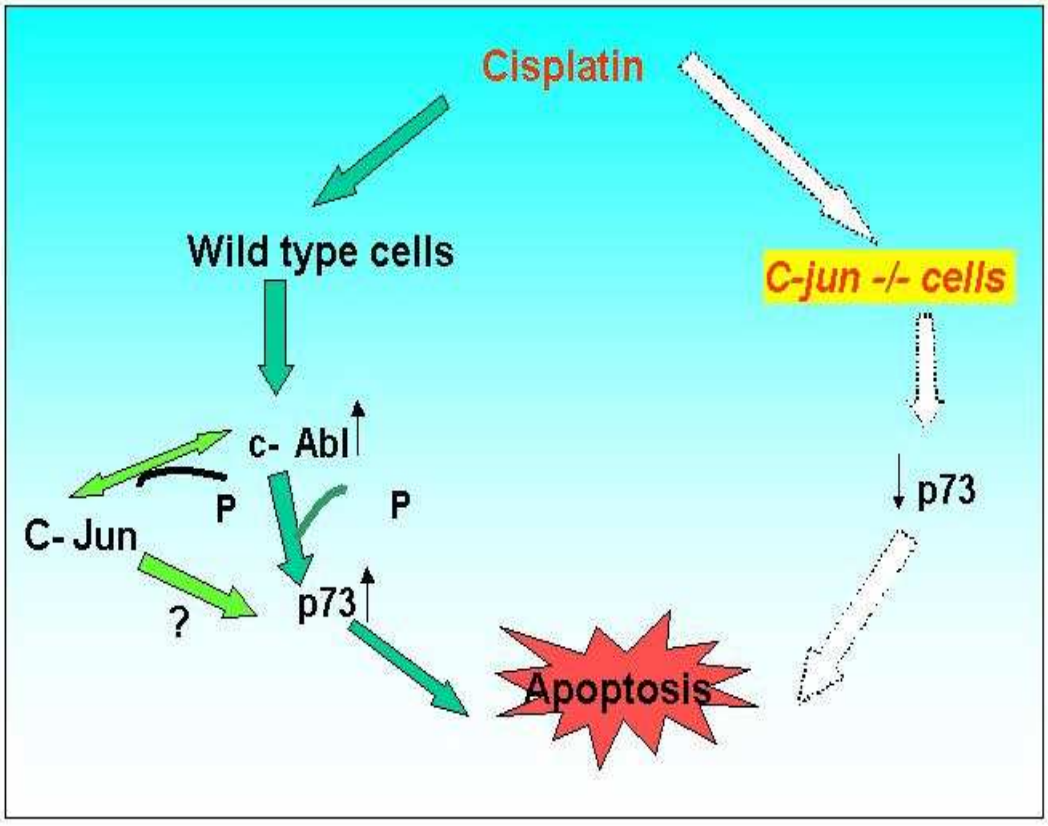


Figure 1.13 A & B, A role for c-Jun in regulating cisplatin mediated p73 stability and apoptosis. Integration of facts derived from various cellular network indicates a possibility that c-Jun could play a role in cisplatin resistance and stabilization of p73. The facts presented so far are illustrated in this circuitry diagram.

4.1 Aims and scope of the thesis

The tumor suppressor, p53 is mutated in at least 55% of cancers, suggesting its pivotal role in the prevention of tumor development. It can promote growth arrest and apoptosis in response to various anti-cancer drugs. The p53-related gene, p73 also induces growth arrest and apoptosis when overexpressed or induced by certain DNA damaging agents like -irradiation and cisplatin treatment. Unlike p53, p73 is not susceptible to mutations. The p73-mediated apoptosis can occur in a p53-independent manner, suggesting that p73 activation can be exploited for the elimination of various cancers with p53 mutations. Hence, identifying molecules that activate or potentiate p73 in tumor cells would have therapeutic benefits. In addition, it would pave the way for better treatment of cancers, specifically those harboring p53 mutations, as these tumors are often resistant to p53-mediated drug induced apoptosis.

The mechanism of p73 stabilization is not clear yet. Besides c-Abl, a non-receptor tyrosine kinase, no other physiological molecular determinant of p73 activation has been identified. Earlier, it was shown that irradiation and cisplatin, a DNA damaging agent and anti-cancer drug could activate p73 in c-Abl dependent manner. c-Abl has been shown to be induced by both cisplatin and irradiation. However, only cisplatin treatment results in p73 stability. This suggests that other molecules are involved in cisplatin mediated p73 stability.

Understanding of the MLH1–ATM-c-Abl–p73 pathway is potentially of great clinical relevance, as it might explain the response to chemotherapy in the majority of cancers that have mutated or functionally inactivated p53. Interestingly, it has been shown that c-Abl phosphorylates c-Jun, which in turn enhances the ability of c-Abl to

enter into the nucleus (Barila et al., 2000). In addition, only cisplatin has been shown to induce c-Jun, but not IR, which raises the possiblity that c-Jun could play a role in cisplatin mediated p73 stability. c-Jun, a well-known player in cell proliferation, can induce apoptosis in certain cellular settings. However, targets for c-Jun mediated apoptosis have not been described so far.

The following goals were set as objectives for my Ph.D thesis:

1. Identifying the molecular component(s) responsible for cisplatin mediated p73 stability.
2. Clarifying whether c-Jun null cells are resistant to cisplatin-mediated apoptosis. Then, identifying molecular component(s) responsible for it.
3. To find out whether c-Jun stabilizes p73.
4. To find out whether c-Jun interacts with p73. If so, identifying domains responsible for the interaction.
5. To find out the physiological significance of c-Jun mediated p73 stabilization.
6. Clarifying how c-Jun influences p73's ability to transactivate its downstream genes (p53 RE promoters).
7. To test whether p73 is stabilized in response to UV irradiation.
8. To test how p73 influences c-Jun's function (on AP-1RE promoters).
9. To test whether phosphorylated c-Jun is required for its ability to cooperate with p73 (on AP-1RE promoters).
10. To test how p73 and c-Jun influence each other's function in transformed cells.

CHAPTER 2

"To engage in experiments on heat was always one of my most agreeable employments". Benjamin Thompson (Count Rumford) (1753-1814) Physicist and diplomat, born in U. S.

MATERIALS AND METHODS

2.1 Cell lines used in this study

NIH-3T3, COS7, HI299, SAOS2, 293 HK, *Jun*$^{-/-}$, *abl*$^{-/-}$, *p53*$^{-/-}$, *p53*$^{-/-}$*MDM2*$^{-/-}$, *p53*$^{-/-}$*Jun*$^{-/-}$ *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$, *p53*$^{-/-}$ *cells and* a number of 3T3 immortalized fibroblasts. Schreiber et al., (1999) generated p53$^{-/-}$*jun*$^{-/-}$ cells by crossing *p53*$^{+/+}$*jun*$^{+/-}$ and *p53*$^{-/-}$ *jun*$^{-/-}$ mice.

2.2 Cell maintenance

Indicated cell lines were cultured in DMEM supplemented with 10% (v/v) fetal calf serum (FCS), antibiotics, pyruvate and glutamate.

2.3 Transient transfection of Cos 7 cells (Calcium phosphate method)

Cells were transfected by a calcium phosphate co precipitation method according to standard Manniatis (book) protocol.

2.4 Transient transfection using Lipofectamine plus reagent (COS7, NIH-3T3, HI299, SAOS2, 293 HK, jun$^{-/-}$, *abl*$^{-/-}$, *p53*$^{-/-}$, *p53*$^{-/-}$*MDM2*$^{-/-}$, *p53*$^{-/-}$*Jun*$^{-/-}$ *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$, *p53*$^{-/-}$)

In this study, Lipofectamine reagent was mainly used for most of the transient and stable transfections (according to standard LifeTechnologies prescribed protocol).

2.5 Establishment of stable cell lines

NIH3T3/MCF7 cells were transfected in a 10cm tissue culture plate with 2 mg of purified DNA following the lipofectamine protocol. 24 hours after transfection cells were reefed with 10ml of fresh medium for overnight. The following day transfected cells were trypsinized and seeded at 1:5 ratio. Once cells adhere to the plates, 1 mg of G418/ml was added. Every 72 hours later, the G418 was resupplied with 10 ml fresh cell

culture medium. Drug resistant clones carrying the expression vector was pooled after 3 weeks and grown as cell lines.

2.6 Cell survival assays

For the analysis of cell survival $0.5x10^6$ cells in a total volume of 1 ml were dispensed into a 6/10 cm tissue culture plate. After 48 hours, the live cell numbers were determined by trypan blue exclusion method.

2.7 Colony formation assays

NIH3T3/MCF7/Cos7 cells were transfected in 10cm tissue culture plate with 2µg of purified DNA (2µg each-PCDNA-p73α/β or PCDNA-p73α/β with PCDNA-c-Jun/c-Jun mutants) following the lipofectamine protocol. 6 hours after transfection, 5ml of complete medium was added. 24 hours after transfection, cells were washed with PBS and reefed with 10ml of fresh medium for overnight. The following day transfected cells were trypsinized and seeded at 1:5 ratio. 1µg of G418/ml was added. Every 72 hours later, the G418 was resupplied with 10 ml fresh cell culture medium. After 2 weeks, colonies were fixed and visualized by staining with crystal violet. Colony numbers were counted in the respective plates.

2.8 Retroviral infection

High titer retroviral stocks were produced by transfecting retroviral construct pBabe-c-Jun (2µg) into a packaging cell line by the lipofectamine method (Schreiber et al., 1999). Virus containing culture supernatants were collected 72 hours post-transfection, at 24 hours intervals, and pooled together. Frozen low passage *Jun*$^{-/-}$ MEF's were thawed and plated at a density of $2x10^5$/10cm dish. After fourteen-hours, cells were infected with

filtered supernatants in the presence of polybrene (5μg/ml; sigma). Fresh supernatants were added three times, at 4h intervals. Forty-eight hours post-infection, cells were trypsinized and replated; twenty-four hours later, fresh medium containing 10% FCS was added to the cells. Cells were selected in puromycin 1.5 μg/ml of the medium for about 3 weeks.

2.9 TRYPAN blue dye exclusion assay

This assay is used to measure the cell viability. Trypan blue dye is impermeable to viable (white) cells, but it is permeable to dead (blue) cells. A cell suspension was mixed with 0.4% trypan blue in PBS to assess the cell viability in haemocytometer. Placed the haemocytometer on microscope and counted the number of blue and white cells.

2.10 Annexin-V binding assay

During the early stages of apoptosis, Phosphatidylserine is translocated from the inner to the outer surface of the plasma membrane (Emoto et al., 1997). AnnexinV protein binds to Phosphatidylserine with high affinity. Therefore, increased AnnexinV staining is used to detect cells in early stages of apoptosis (Martin *et al.,* 1995). The cells were pelleted and washed with PBS, and re-suspendend in 200μL of 1X annexiin binding buffer. To the cell suspension, 5μL of annexin V-FITC (final concentration was 0.5 μg/ml) was added and incubated at room temperature for 15 min in the dark before subjecting it to flow cytometric analysis.

2.11 Preparation of single cell suspension for cell cycle

The cells were washed twice with sample buffer and resuspended in sample buffer. The cells were centrifuged, and the supernatant was removed. For fixing, 1ml ice-

cold 70% ethanol was added to the cell pellet drop by drop while vortexing the cells. The cells were fixed in the ethanol overnight at 4C.

2.12 Propidium iodide (PI) staining and flow cytometry

The fixed cell sample was vortexed and centrifuged to remove the 70% ethanol. Cells were gently resuspended in residual ethanol and 0.5ml PI staining solution added (25μL PI stock (1mg/ml). 50 Kunitz units RNAase A in 0.5 ml sample buffer was added to each sample. Samples were incubated in the dark at room temperature for at least 30 minutes before flow cytometric analysis (Bossy-wetzel et al., 1997).

2.13 Sub-cloning: Restriction enzyme digestion

All the restriction enzymes were purchased from New England Biolabs or Promega. All digestions were carried out at 37^{o}C or 30^{o}C overnight, with proper restriction buffer in a total volume of 20 to 100μL.

2.14 Ligation of DNA fragments

DNA fragments from digestion were ligated to linearized vectors. pBabe-c-Jun and pcDNA plasmids were restriction digested with EcoRI and BamH1 enzymes. Restriction digested plasmids were run on a gel and purified the c-JuncDNA fragment (insert) and linearized pcDNA vector (vector). DNA ligation reaction (1:10 molar ratio of (pcDNA) vector: insert (c-JuncDNA) was carried out typically in 20μL of volume at 4^{o}C for 16 hours.

2.15 Isolation of RNA from cells

Trizol RNA isolation procedure was used to isolate RNA.

2.16 Running RNA samples on denaturing gels

The integrity of RNA was checked in a denaturing formaldehyde-agarose RNA gel before subjecting it for RT-PCR analysis.

2.17 Reverese transcriptase (RT) reaction- PCR

The QIAGEN OneStep RT-PCR Kit (210210) was used to check the expression of p73 and its downstream targets. After checking the integrity of the RNA isolated, cDNAs [e.g. with reverse transcriptase from Moloney murine leukemia virus (M-MuLV) or avian myeloblastosis virus (AMV)] were amplified and quantified carefully with housekeeping primers such as β-tubulin and GAPDH as controls. The following p73 primers were used for RT reaction and PCR analysis (Laurenzi et al., 1998).

5'-TTCTGCAGGTGACTCAGGCTG-3' for RT p73

5'-ACTTTGAGATCCTGATGAAG-3'(sense primer) and

5'-CAGATGGTCATGCGGTACTG-3' (anti sense primer)

2.18 Gene Sequencing

c-Jun, N-c-Jun, C-c-Jun, p73α and β etc. were sequenced and verified using specific primers. The purified DNA was sequenced using a Taq Dye Deoxy TM Terminator cycle sequencing kit (Perkin Elmer, CA) based on he chemical dideoxy-termination method. Sequencing results were analyzed using Biosystems.

2.19 Bio-Rad protein assay

Coomassie Brilliant Blue G-250 dye (Promega) was used to measure the protein concentration. Equal volumes of cell lysate was added to the dye and mixed well.

Known quantities of standard albumin (Sigma) were used as a standard. The absorption at wavelength 595 was measured versus dye.

2.20 SDS PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) and Transfer/ Immunobotting/Western blotting.

Whole cell extracts were prepared in lysis buffer (Sabapathy et al., 1999), and protein content was measured. 30-300μg of protein was run on 10-12% SDS-polyacrylamide gels and transferred the proteins by electroblotting to nitrocellulose membrane for 2 hours (for large gels). Ponceau S fixative dye solution was used to check if transfer has taken place, Transferred filters were incubated with blocking buffer (5% non-fat dry milk/5% BSA in TBST) for 1 hr at room temperature or overnight at 4^0C on a shaker. Antibodies were diluted in washing buffer containing 3% non-specific milk and 0.05% tween-20. After 2-3 hours incubation at room temperature or overnight incubation at 4^0C, membranes were washed three times with TBST, each time for 10-15 min. Diluted (1:1000) the appropriate peroxidase-conjugated secondary antibody in TBST, added to the membrane, incubated at room temperature for 60 min and washed three times in TBST (Bossy-wetzel et al., 1997). Finally, the specific protein of interest was identified using chemiluminescence reaction (ECL-system): incubated the membrane in a 1:1 mix of ECL solutions 1 and 2 or super signal for 1-5 minutes (Pierce).

2.21 Antibodies used in the immunoblot analysis

p73 antibodies

1.mAb ER-15(Santa Crutz).

Immunogen: N-terminus of p73

Specificity: Recognizes p73 α and β.

2. mAbGC-15 (Oncogene science):

Monoclonal GC15 (AB-3 from Oncogene Science; recognizes amino acids 380–499 of human p73 b); WB: 2 micrograms/ml; IP: 5-6 micrograms/tube.

Immunogen: GST fusion-protein corresponding to residues 380-499 of p73β. Clone GC15.

Specificity: Recognizes p73β (MW~70kDa). This antibody does not effectively blot p73β, but will immunoprecipitate p73-β under non-denaturing conditions.

Imgenex antibodies:

3. IMG-246 - recognizes N-terminal epitope; human and mouse.

4. IMG-260 - recognizes C-terminal epitope; human only.

5. IMG-259 - recognizes all protein isoforms; human and mouse

c-Jun antibodies: c-jun (H79) rabbit polyclonal antibody was from Santa Cruz

Biotechnologie.

p53 antibodies: CM5 (Abcam, ab 2433) Rabbit polyclonal to human p53.

GFP: Rabbit polyclonal (clone tech)

Actin: Rabbit polychlorure(Santa crutz)

2.22 Immunoblot stripping

Primary and secondary antibodies were removed from the membrane using stripping buffer. The membrane was incubated in stripping buffer for 30 min at 50^0C. The membrane was washed at least 5 times with TBST and then it was reprobed with new antibody.

2.23 Immunoprecipitation

For immunoprecipitation of cellular proteins, protein G/A sepharose was used. Cell Lysates were first precleared with the proteinG/A sepharose. Then 20 to 50µg Sepharose was incubated with 3-5µg of antibody, 300-500µg precleared lysate and the volume made up to 1 ml in an Eppendorf tube using lysis buffer. Samples were incubated on an incubator for at least 4 to 12 hours at 4^{o}C and then washed with appropriate wash buffers, depending on the stringency required. The immunoprecipitated proteins were boiled in Laemmli buffer and subjected to SDS PAGE and Western blotting.

2.24 Metabolic labeling & Immunoprecipitation

Radiolabelling consists of two steps:

1. Pulse - Short period of incubation with ^{35}S-methionine or cysteine.
2. Chase- Incubation with excess concentration of unlabeled Met+Cys.

Metabolic labeling

Cos7 or *jun*$^{-/-}$ and *jun*$^{-/-}$ +Jun cells were transfected with 2µg of p73/c-Jun pcDNA/ plate. After 7-9h incubation with DNA: lipofectamine mix rinsed once and replaced with fresh medium (10ml/plate). 24h after changed medium, rinsed cells with Labeling Medium (5ml/plate). Added 250µCi of [^{35}S] methionine/cysteine/10cm plate and cultured for another 2 hours at 37°C (pulse). Then added 5ml/well chase medium and cultured for another 4 hours (chase). Harvested metabolically labeled cells at the regular intervals. IP was carried out as described previously. The immunoprecipitated proteins were boiled in Laemmli buffer and subjected to SDS PAGE. Soaked gels into enhancer solution for one hour and then dried completely. p73 expression was detected by autoradiography using intensifying screen.

2.25 Luciferase reporter gene assay

p73, c-Jun, c-Jun mutants expression vectors and 0.5µg of plasmid containing PG13 /MDM2/GADD45/p21/Bax/p53AIP1/5XTRE/Collagenase-I promoters hooked to luciferase reporter gene were transfected either alone or in combination into H1299/ *SaOs2; p53$^{-/-}$; p53$^{-/-}$jun$^{-/-}$; Jnk1$^{-/-}$2$^{-/-}$* cell lines at the indicated ratios in the respective experiments using Lipofectamine transfection method. The PG13 luciferase reporter gene contains 13 tandem repeats of a p53 binding sequence, Bax, MDM2, GADD45, p21 promoters encode p53 binding sequences and 5XTRE contains 5 tandem repeats of TPA responsive elements upstream of the luciferase reporter gene. The transfected cells were harvested and lysed in 100µl of lysis buffer and incubated on ice for 30 min. The crude cell lysates were precleared by centrifugation at maximum speed for 30 min. 50 µl of precleared cell extract was added to Luciferase Assay Buffer. The activity was measured after injection of 50µl of D-luciferin solution on a luminometer. The luciferase activities were normalized against the ß-galactosidase activity of co-transfected 0.5µg gal vector in the ß - galactosidase assay. Results are presented as luciferase units normalized to beta-gal units. Each experiment is performed in duplicates or triplicates. The mean and standard deviations of two independent experiments are shown in the figures.

2.26 β-galactosidase assay

The ß-galactosidase assay was performed according to a standard protocol (Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989: Molecular cloning: A laboratory Manual 2nd Ed., Cold Spring Harbor Laboratory Press, New York). Precleared cell lysate was added to ß-gal Assay Buffer and ONPG-solution and incubated for 1 hour. The ß-gal activity was measured on a luminometer.

CHAPTER III

"I do not know what I may appear to the world; but to myself I seem to have been only like a boy playing on the seashore, and diverting myself in now and then finding of a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me"

Sir Isaac Newton (1642-1727) Physicist, mathematician.

RESULTS

SECTION I: C-JUN IS REQUIRED FOR STABILIZATION AND ACTIVATION OF $_{P}$73

"Theory guides. Experiment decides."
"An old saying in science, seen attributed to many different persons."

3.1.1 Role of c Jun in cisplatin resistance and p73 activation

3.1.1.1 c-Jun null 3T3 fibroblsts are resistant to cisplatin-mediated apoptosis

The feasibility of the hypothesis proposed in Chapter I was examined by treating wild type and *jun* null mouse fibroblasts with cisplatin. In agreement with the hypothesis proposed, 3T3 cells lacking c-*jun* were more resistant (37%) to cisplatin than wild type fibroblasts. This data suggests that c-Jun is required for cisplatin-mediated apoptosis. The c-Jun expression is increased in response to cisplatin (Sanchez-Periz et al., 1998) and its activity is sufficient to trigger apoptotic cell death in NIH 3T3 fibroblasts (Bossy-Wetzel E, 1997), in human vascular endothelial cells (Wang et al., 1999), and in neuronal cells (Ham et al., 2000). However, how c-Jun regulates apoptosis is far from clear.

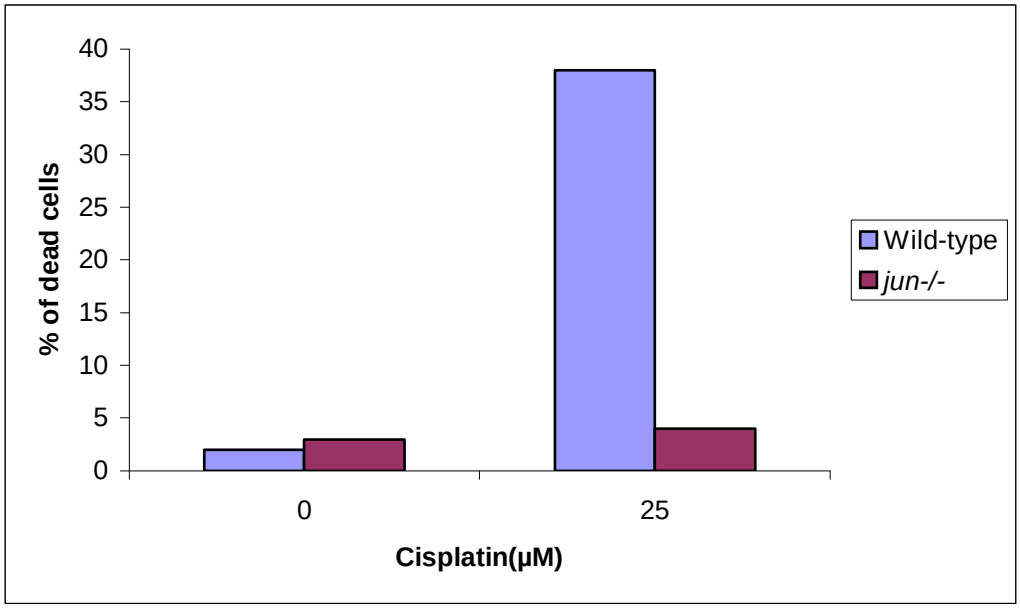


Figure 3.1.1.1 The absence of c-Jun confers resistance to apoptosis after treatment with cisplatin. (TRYPAN blue dye exclusion assay)

Wild type and *jun*$^{-/-}$ fibroblasts were treated with 25µM of cisplatin for 24 hours. Cell viability was estimated as previously described (Chapter 2, 2.9). Each time point is the average of three independent experiments for which the deviation was less than 5%.

3.1.1.2 *p53*$^{-/-}$ cells are more sensitive to cisplatin-mediated apoptosis than *p53*$^{-/-}$*Jun*$^{-/-}$ fibroblasts

Several studies suggest that treatment of cells with cisplatin results in increased levels of p53 in the nucleus and concomitantly increased apoptosis. However, further studies suggest that cisplatin induces both p53-dependent and p53-independent apoptosis. To eliminate the contribution of p53 in cisplatin mediated cell death and evaluate the cisplatin-mediated p53-independent cell death, *p53*$^{-/-}$ and *p53*$^{-/-}$*jun*$^{-/-}$ cells were checked for cisplatin resistance. Furthermore, to rule out the possibility that the reduced rate of proliferation of *jun*$^{-/-}$ cells--rescued by deletion of p53 gene--is responsible for the reduced rate of apoptosis (Shaulian, 2002). As shown in figure 3.1.1.2, *p53*$^{-/-}$ cells were found to be more sensitive to cisplatin compared to the *p53*$^{-/-}$ *jun*$^{-/-}$ cells, indicating that c-Jun is required for cisplatin-mediated-p53-independent apoptosis.

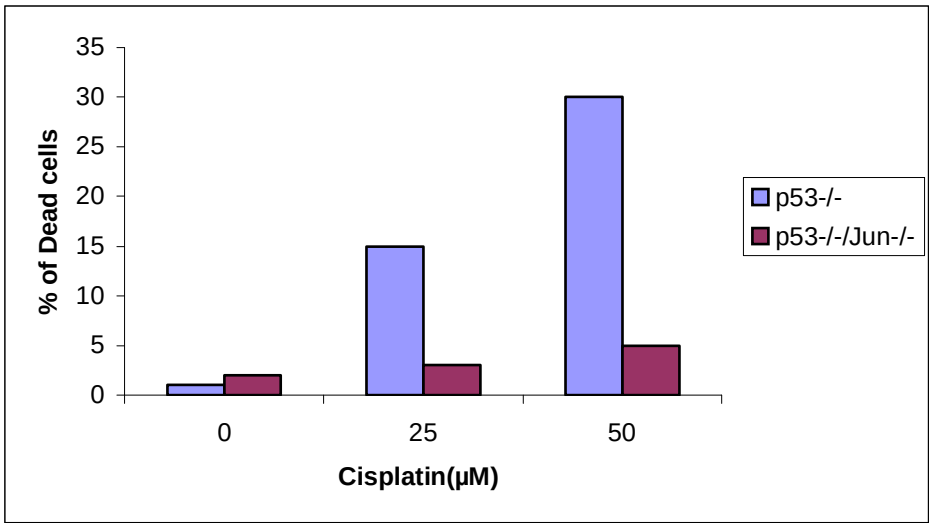


Figure 3.1.1.2 *p53*$^{-/-}$ cells are more sensitive to cisplatin than *p53*$^{-/-}$*jun*$^{-/-}$ cells (TRYPAN blue dye exclusion assay)

Wild type or *jun*$^{-/-}$ fibroblasts were treated with indicated concentrations of cisplatin for 24 hours, and the fraction of dead cells was estimated as previously described (Chapter 2, figure 2.9)

3.1.1.3 *jun*$^{-/-}$ cells are resistant to cisplatin mediated apoptosis

In a detailed analysis, to confirm the earlier results obtained by an independent assay, we measured staining of phosphatidylserine exposed on the outer cell membrane by flow cytometry. In agreement with the data obtained by Tryphan blue dye exclusion assay, annexin V staining showed that only wild type and not mutant cells exhibited a higher percentage of apoptosis. *c-jun*$^{-/-}$, *c-jun*$^{-/-}$+ Jun, and *abl*$^{-/-}$ cells were treated with 4µM and 20µM of cisplatin and collected after 24 hours treatment. As shown previously in figure 3.1.1.3 and Table 2, *jun*$^{-/-}$ mouse 3T3 fibroblasts were found to be more resistant to cisplatin compared to *c-jun*$^{-/-}$ + c-Jun cells (figure 3.1.3) . *c-abl*$^{-/-}$ fibroblasts were used as a negative control (Agami et al., 1999).

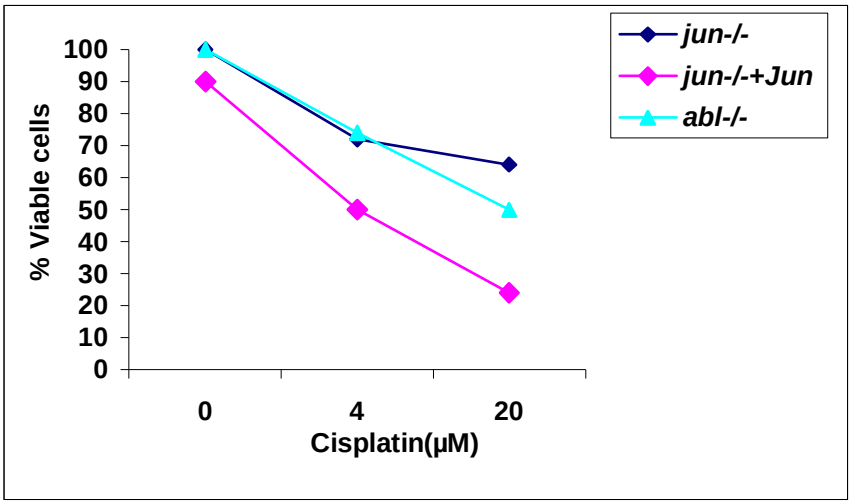


Figure 3.1.1.3 c-Jun is required for cisplatin-induced p73-mediated apoptosis (flow cytometry)

Wild type and *c-jun*$^{-/-}$ 3T3 fibroblasts transduced with the retroviruses directing the expression of c-Jun or the puromycin resistance gene only. These cells were selected with puromycin for two weeks and treated with the indicated concentrations of cisplatin for 24 hours. The extent of apoptosis was determined by staining cells with annexin-V FITC and subsequent flow cytometric analysis. Each time point is the average of few experiments for which the deviation was less than 5%.

3.1.1.4 *p53*$^{-/-}$ cells are more sensitive to cisplatin-mediated apoptosis than *p53*$^{-/-}$*Jun*$^{-/-}$ cells (Flow cytometry)

In a similar annexin-V FITC staining and subsequent FACS analysis, we determined the sensitivity of *p53*$^{+/-}$*c-jun*$^{+/-}$, *p53*$^{-/-}$*c-jun*$^{-/-}$ and *p53*$^{-/-}$ cells to cisplatin. The difference in the number of dead cells between *p53*$^{-/-}$ and *p53*$^{-/-}$ *jun*$^{-/-}$ is ~17% (figure 3.1.1.4). On the other hand, the difference in the number of dead cells between *jun*$^{-/-}$ and *jun*$^{-/-}$ *with* c-Jun or wild type cells is ~35%(figure 3.1.1.1), which means, the contribution of p53 in cisplatin-mediated apoptosis is 18% (35%-17%). Therefore, it appears that the contribution of cisplatin-mediated-p53–independent and c-Jun dependent apoptosis is 17%. Together, the data presented here confirm that c-Jun is required for cisplatin-mediated p53-independent apoptosis.

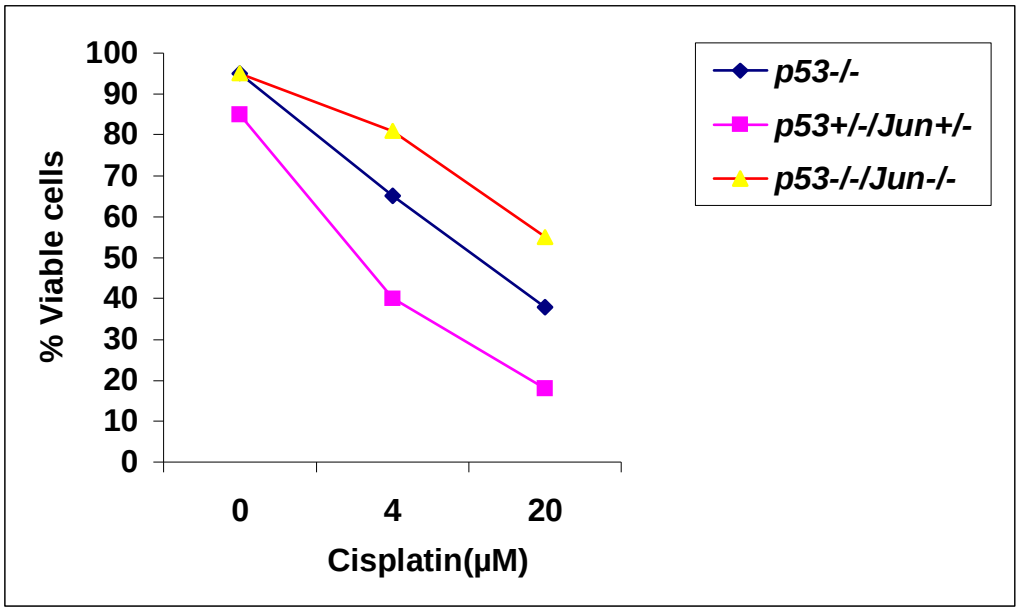


Figure 3.1.1.4 ***$p53^{-/-}$ cells are more sensitive to cisplatin-mediated apoptosis than $p53^{-/-}$ $Jun^{-/-}$ cells***

$p53^{+/-}cjun^{+/-}$, *$p53^{-/-}$ and $p53^{-/-}c\text{-}jun^{-/-}$* fibroblasts were treated with indicated concentrations of cisplatin for 24 hours, and the fraction of viable cells was estimated by flow cytometic analysis as previously described (Chapter 2).

3.1.1.5 c-Jun is required for increased p73 levels in response to cisplatin

In the earlier experiment c-*jun* null cells were compared against wild type fibroblasts to see whether p73 is induced in response to cisplatin treatment. In order to avoid any clonal specific effect in the c-*jun* null background, *jun*$^{-/-}$ cells were transduced with recombinant retroviruses directing the expression of wild-type c-Jun or puromycin resistance gene only (as described in chapter 2). As shown in figure (3.1.1.5), cisplatin induced expression of p73 in *c-jun*$^{-/-}$ + c-Jun cells but not in cells lacking c-Jun. This data suggests that c-Jun augments the expression of p73 in response to cisplatin treatment.

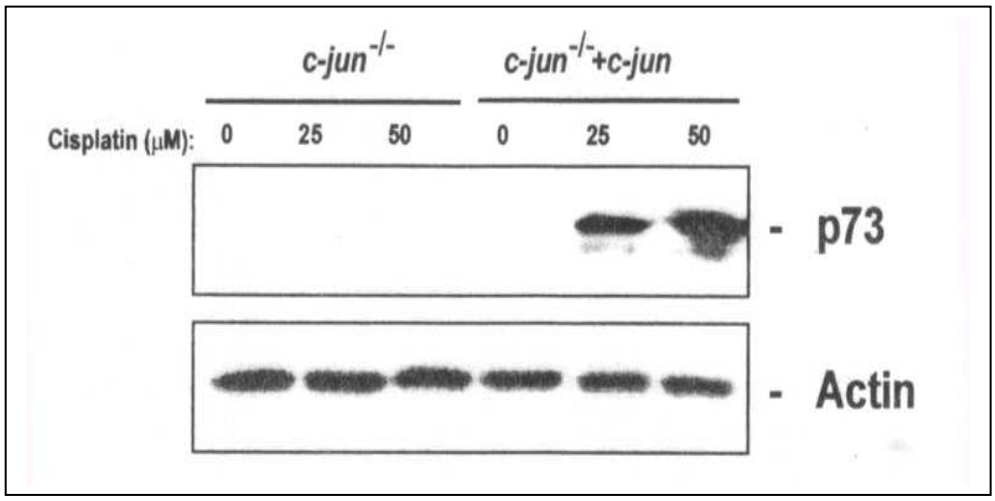


Figure 3.1.1.5 c-Jun is required for p73 induction (GC15)

jun$^{-/-}$ and *jun*$^{-/-}$ + c-Jun cells were treated with cisplatin at different concentrations as indicated above. At 24 hours after cisplatin treatment, cell extracts were prepared and 400μg of proteins per sample was loaded and subjected to Western analysis. The blot was probed with the following: 1. anti-p73-β specific (GC15) mAb (1:1000) 2. anti-actin (1:400) specific mAb.

3.1.2 c-Jun stabilizes p73

Our preliminary analysis suggests that c-Jun is required for increased p73 levels in response to cisplatin treatment. However, it is not clear whether c-Jun is required for p73 induction or stabilization. Thus, experiments were designed to address the question of whether c-Jun stabilizes or induces p73 in detail.

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3.1.2.1 c-Jun is required for increased p73 levels in 3T3 mouse fibroblasts

To check whether transiently transfected c-Jun increases p73 levels, p73 was either transfected alone or in the presence of c-Jun in $jun^{-/-}$ and $jun^{-/-}$ + Jun fibroblasts. Cell extracts were collected 48 hours post-transfection and subjected to Western analysis. As shown in figure 3.1.2.1, p73 protein expression is weak in $jun^{-/-}$ fibroblasts (panel 2, lane 3). While in $jun^{-/-}$ + Jun fibroblasts, p73 expression is seen, indicating that c-Jun augments p73 levels (panel 2, lane 5). On the other hand, p73 expression is seen in $jun^{-/-}$ +Jun cells (panel 2, lane 3), indicating that c-Jun contributes to the increased levels of p73 (panel 2, lane 5). It is also important to note that $jun^{-/-}$+Jun cells express c-Jun at the physiological level (figure 3.1.2.1, panel 2, lanes 4 - 6).

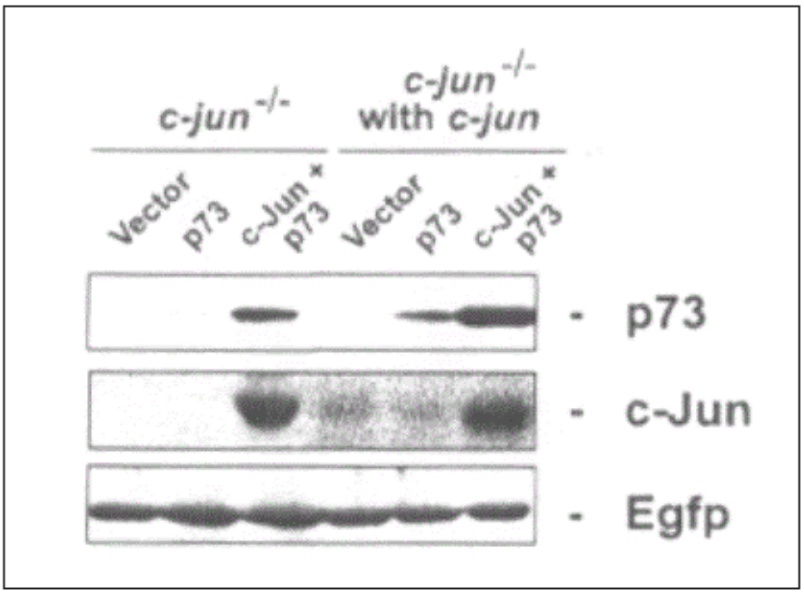


Figure 3.1.2.1 c-Jun is required for increased p73 levels in 3T3 mouse fibroblasts

jun$^{-/-}$ and *jun*$^{-/-}$+*Jun* cells were transfected with the indicated expression vectors. EGFP expression vector was cotransfected to assess the transfection efficiency. The levels of p73β, c-Jun and EGFP proteins were determined by immunoblotting.

3.1.2.2 Expression of c-Jun results in increased p73 levels in COS7 (Monkey kidney (epithelial) cells)

To test whether co-transfection of p73 and c-Jun in an epithelial cell line, increases p73 levels, COS7 cells were transfected with p73, c-Jun, or in combination.. EGFP expression vector was also cotransfected to normalize the transfection efficiency. As shown in figure 3.1.2.2, co-expression of c-Jun and p73β in H1299 cells increases p73 levels compared to expression of p73 alone (compare lanes 2 to 4). In contrast, expression of c-Jun did not affect the levels of green fluorescent protein (Egfp).

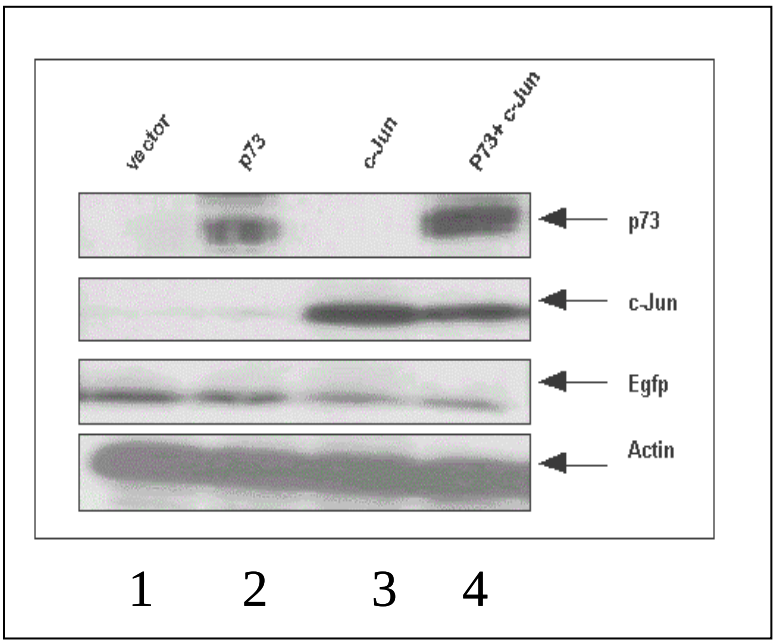


Figure 3.1.2.2 c-Jun increases p73 protein levels in Cos 7 (Monkey kidney cell line) cells

This blot is a representative of three experiments carried out. Cos7 cells were transfected with vectors encoding empty vector (pcDNA), pcDNA-c-Jun, pcDNA-p73β, or in combination as indicated above. Transfected cells were collected 48 hours post-transfection. Antibodies specific for p73-β ((GC15) mAb (1:1000)), c-Jun (H-79) (1:1000), EGFP (1:1000) and actin (1:400) were used for western analysis.

3.1.2.3 Expression of c-Jun results in increased p73 levels in Human non-small cells lung carcinoma cell line (H1299)

To check the universal nature of the observation and to analyze if the earlier observation can be reproduced in a human cell line, H1299 [(p53 null) human epithelial cell line] cells were transfected with the indicated expression plasmids (3.1.2.3). As shown in figure 3.1.2.3, co-expression of c-Jun and p73β in H1299 cells resulted in a dramatic increase in the levels of p73β compared to expression of p73β alone (compare lanes 2 to 4). Thus, this data confirmed our earlier observations in various cell lines of different tissue origin.

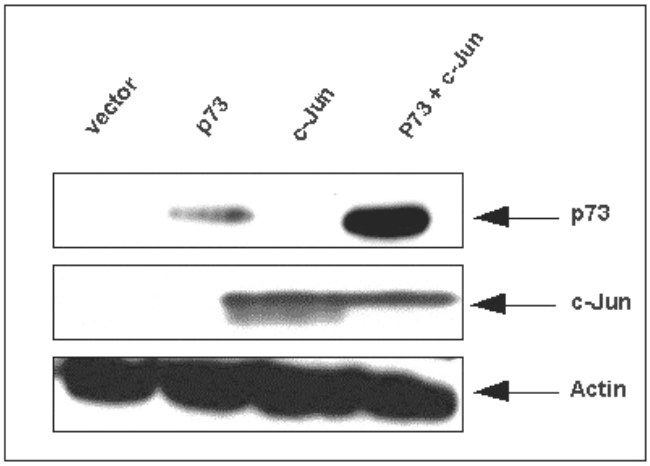


Figure 3.1.2.3 c-Jun increases p73 protein levels in Human non-small cells lung carcinoma (H1299) cells

This blot is a representative of three experiments carried out. H1299 cells were transfected with vectors encoding pcDNA, pcDNA-c-Jun, pcDNA-p73β, or in combination as indicated above. The level of p73, c-Jun and actin proteins were assessed by western analysis using antibodies directed against their protein products.

3.1.2.4 Increasing concentration of c-Jun increases the p73 protein levels

To validate the earlier observations in a more rigorous way, increasing amount of c-Jun (100ng and 300 ng) was co-transfected with constant amount of p73(100ng) in COS7 cell line. As shown in figure 3.1.2.4, transfecting increasing amount of c-Jun increases the levels of p73 compared to the expression of p73 alone (panel 1, lane 2-3). This data clearly confirmed our previous findings that, indeed, c-Jun plays a causative role in the accumulation of p73. EGFP (100ng) was transfected as a measure of transfection efficiency.

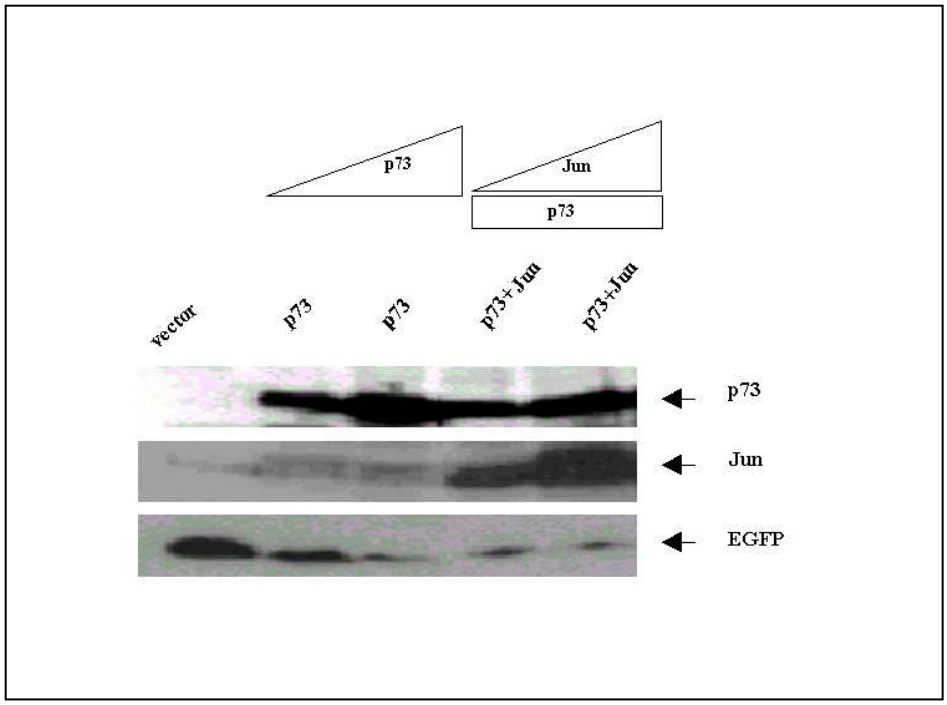


Figure 3.1.2.4 Increasing concentration of c-Jun increases the protein level of p73-β

Cos7 cells were transfected with empty vector pcDNA, increasing concentration of pcDNA-c-Jun and constant amount of p73 as indicated above. Antibodies specific for p73-β ((GC15) mAb (1:1000)), anti-c-Jun (H-79) (1:1000) and anti-EGFP (1:500) were used for Western analysis.

3.1.2.5 c-Jun increases the half-life of p73β

To check whether c-Jun regulates p73β stability, we followed the half-life of over-expressed p73β in COS7 cells. Transfected COS7 cells were pulse labeled with ^{35}S Met/Cys followed by a four-hour chase (Figure 3.1.2.5). Transiently expressed p73β had a half-life of about 1.5 hours (Figure 3.1.2.5: p73). When c-Jun was co-transfected with p73β, its half-life increased to 4 hours (Figure 3.1.2.5: p73 + c-Jun). Furthermore, coexpression of c-Jun and p73 led to the highest levels of p73 expression, thus demonstrating that c-Jun stabilizes p73 protein *in vivo.*

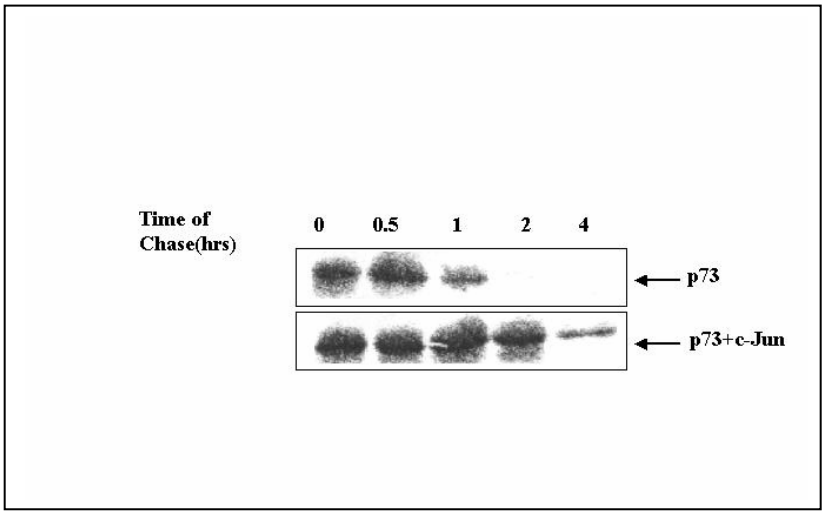


Figure 3.1.2.5 c-Jun enhances the half-life of p73β

Cos 7 cells were transfected with expression vectors as indicated above. Transfected cells were ^{35}S labelled and chased over time as indicated above. 300 μg cell lysate/sample of transfected protein was pre-cleared using mouse Ig-Agarose conjugated. Pre-cleared samples were immunoprecipitated using p73 (5μg/sample) specific mixture of antibodies (p73β specific GC15 and mouse mono clonal antibodies). p73 immunoprecipitates were subjected to Western analysis.

3.1.2.6 c-Jun does not induce p73 mRNA in response to cisplatin treatment

To check whether c-Jun induces or stabilizes p73 mRNA, Cos7 cells were transfected with vector or p73 alone or in the presence of c-Jun. 48 hrs after transfection, RNA was extracted and then reverse transcribed using MMLV Reverse transcriptase. cDNAs were calibrated carefully house keeping primers such as GAPDH and tubulin primers. As shown in figure 3.1.2.6, in response to cisplatin treatment c-Jun does not alter p73 mRNA levels. However, the fact that $jun^{-/-}$ cells have decreased level of p73 protein in response to cisplatin treatment clearly indicates that c-Jun increases p73 protein either at the translational or post-translational level.

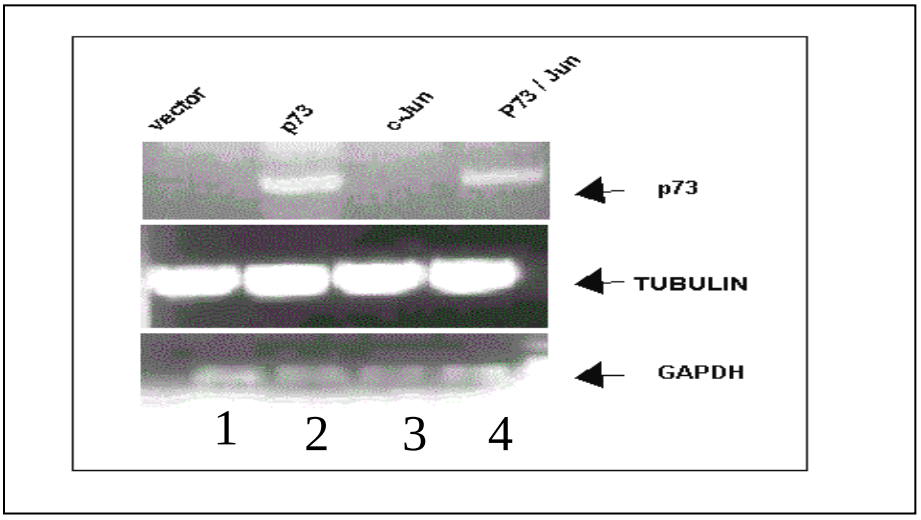


Figure 3.1.2.6 c-Jun does not induce p73 mRNA in response to cisplatin treatment

Cos7 cells were transfected with 2μg of human p73-β expression plasmid, either alone (lane 2) or together with 2μg of c-Jun expression plasmid (lane 3) treated with cisplatin (25 μM). As a negative control parallel cultures were transfected with pCDNA3 empty vector. Total RNA was isolated and from each culture 48 hours after transfection, and 1μg RNA was subjected to RT-PCR analysis with gene specific primers. cDNAs were amplified and quantified carefully with house keeping primers such as β-tubulin and GAPDH.

3.1.2.7 The effect of c-Jun mutants on p73 stability

To determine the domain(s) involved in c-Jun's ability to stabilize p73, Cos 7 cells were transfected with p73, c-Jun and its deletion mutants such as N-c-Jun (Δ194-334) and C-c-Jun (Δ1-194) either alone or together with p73-β. As shown in figure 3.1.2.7, The N-terminus of c-Jun (transactivation and delta domains) is essential for c-Jun's ability to stabilize p73, indicating a possibility that transcription and delta domains could contribute to the increased p73 protein levels. On the other hand, C-terminus of c-Jun (DNA binding and dimerization domains) mutant weakly stabilizes p73 compared to wild type c-Jun (lane 6). Together, these findings suggest that transactivation, DNA binding domains are required for efficient p73 stability.

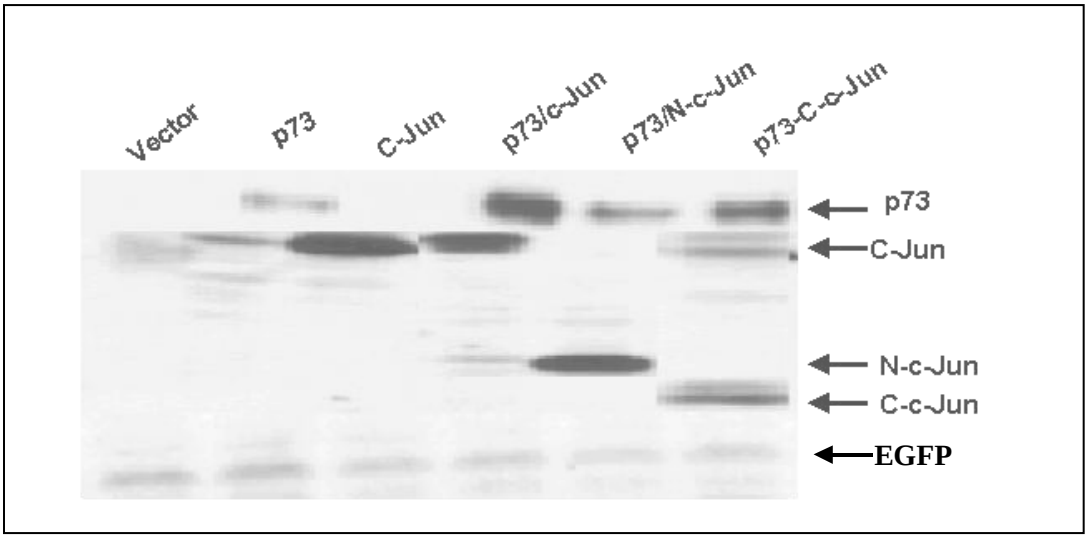


Figure 3.1.2.7 The effect of c-Jun mutants on p73 stability

Cos 7 cells were transfected with indicated combination of vectors encoding empty vector pcDNA, p73β, c-Jun, N-terminus-c-Jun and C-terminus-c-Jun either alone or in combination as indicated above. Transfected cells were collected after 48 hours. Cell lysates were prepared and protein concentration measured. Transferred membranes were sequentially probed with the following antibodies:

1. anti- p73β specific (GC15) mouse monoclonal antibody (1:1000).
2. A mixture of anti-c-Jun antibodies (c-Jun (H-79) mouse polyclonal antibody (1:1000) and c-terminus specific c-Jun antibody)
3. Anti- EGFP specific antibody (1:1000).

3.1.3 Expression of c-Jun modulates transactivation function of p73

It has been shown that the enhanced stability of transcriptional factors can lead to increased transcriptional activity towards its downstream genes. Thus, it was decided to check whether c-Jun mediated p73 stability lead to enhanced functional activation of p73.

3.1.3.1 C-Jun enhances the induction of MDM2 promoter by p73

The effect of c-Jun over expression on the ability of p73 to stimulate transcription from the p53-responsive promoter was tested. H1299 cells were transfected with c-Jun and p73-β or c-Jun alone in addition to a plasmid containing p73-responsive MDM2 promoter, which in turn drives the luciferase reporter gene. As shown in figure 3.1.3.1, while p73 stimulated MDM2 promoter activity (1 fold), over the control vector, the stimulation (about 4 folds) became apparent when c-Jun was co-transfected with p73.

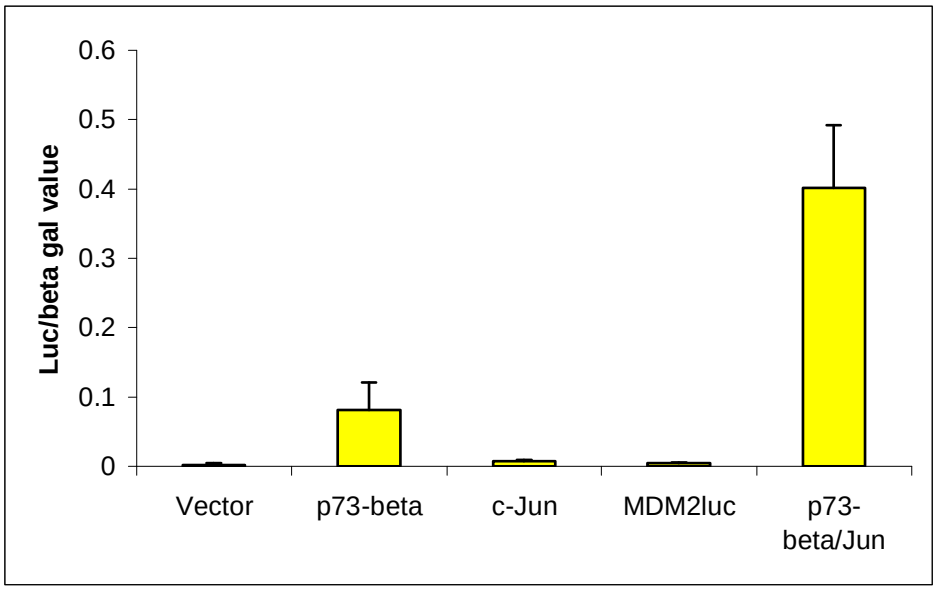


Figure 3.1.3.1 c-Jun enhances the induction of MDM2 promoter by p73

H1299 cells were transiently transfected with the indicated combinations of plasmids (in duplicates) encoding pcDNA (empty vector), p73β, c-Jun, MDM2-luc and beta-gal plasmids. The total amount of plasmid DNA used for transfection kept constant by adding an empty PCDNA3. Luciferase activity measured (in duplicates) 48 hours post-transfection and normalized for transfection efficiency with beta-gal activity. Standard deviation is indicated.

3.1.3.2 c-Jun enhances the induction of GADD45 promoter by p73

To check the consistency of this observation H1299 cells were transiently transfected with a luciferase reporter gene driven by the p53-responsive GADD45 (Growth arrest and DNA damage-inducible gene number 45) promoter. Even though GADD45 has been identified as a downstream target of p53, it is induced in response to several stresses such as MMS, UV and IR, in a p53-independent manner (Takekawa and Saito, 1998). As shown in figure 3.1.3.2, co-transfection of c-Jun and p73 enhance the activity of the GADD45 promoter 5 to 6 folds compared to empty vector (PCDNA3).

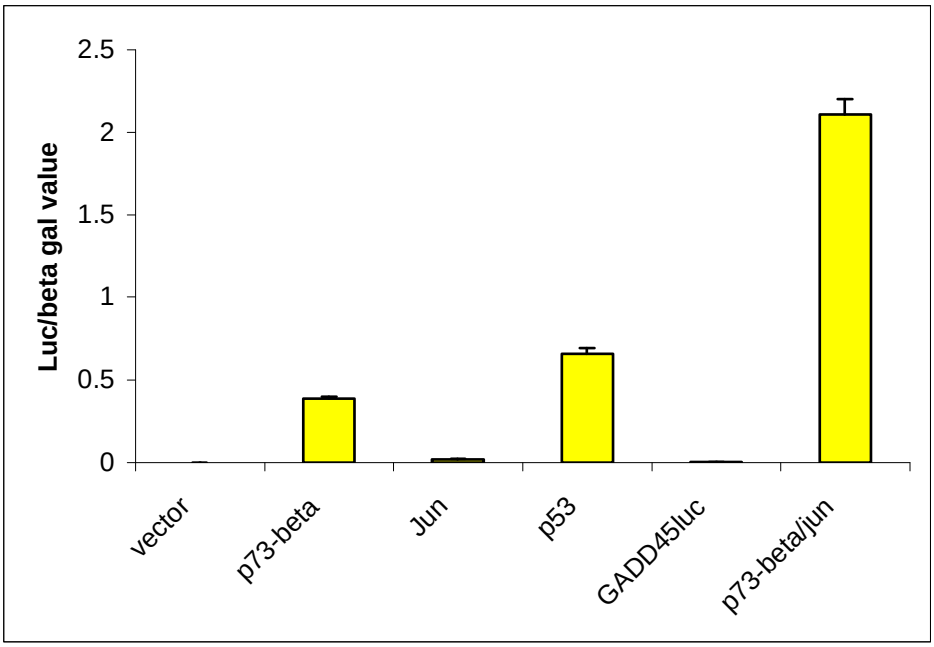


Figure 3.1.3.2 C-Jun enhances the induction of GADD45 promoter by p73

H1299 cells were transiently transfected with the indicated combinations of plasmids (in duplicates) encoding pcDNA, p73β, c-Jun, GADD45-luc and beta-gal plasmids. Cell extracts were prepared 48 hours post-transfection and subjected to the determination of luciferase and beta-gal activity. Results are represented as fold induction of ratio between luciferase and beta-gal activity.

3.1.3.3 *c-Jun has minor effect on the induction of p21 promoter by p73*

The p53 target gene, p21 protein, is a well known inhibitor of cyclin dependent kinases(cdk's) and it is known to be induced in p53-dependent and p53-independent manner. The p53 dependent p21 expression promotes G1 arrest (Dulic et al., 1994). However, cells that lack p53 fail to activate the G1 checkpoint in response to DNA damage (Shaulian, 2002). To check the consistency of this observation H1299 cells were transiently transfected with a luciferase reporter gene driven by the p53-responsive p21 promoter. As shown in figure 3.1.3.3, cotransfection of p73 and c-Jun resulted in less than two fold induction of p21 promoter, relative to the vector (PCDNA) control. However, in comparison to other p53 responsive promoters such as GADD45 and MDM2, c-Jun does not seem to potentiate the function of p73 towards p21 promoter.

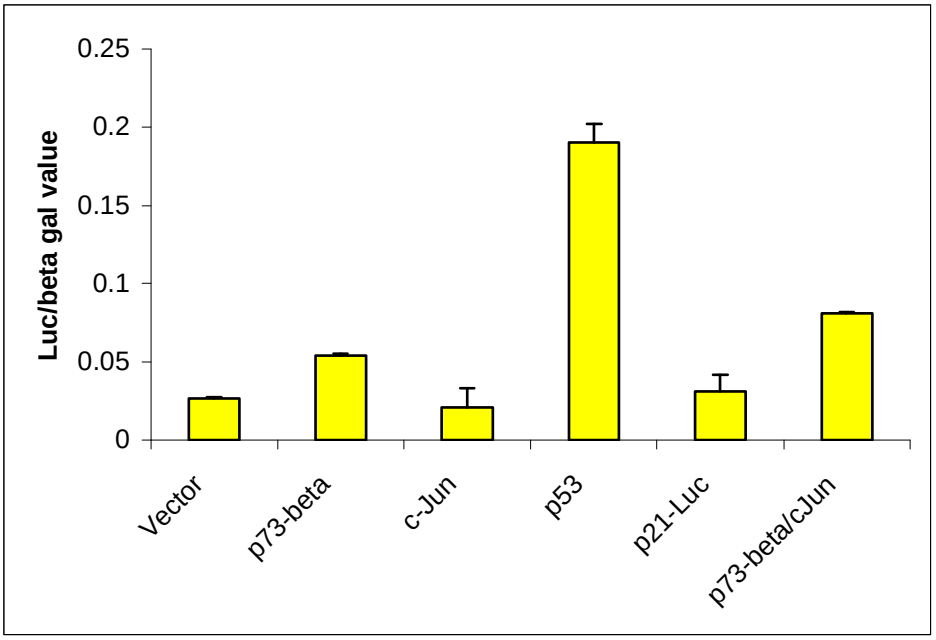


Figure 3.1.3.3 c-Jun has minor effect on the induction of p21 promoter by p73

H1299 cells were transiently transfected with the indicated combinations of plasmids encoding pcDNA, p73β, c-Jun, p21-luc and beta-gal plasmids. The total amount of plasmid DNA used for transfection was kept constant by adding pCDNA3 (3.1λg). Luciferase activity was measured 48 hours after transfection.

3.1.3.4 c-Jun is required for p73 transcriptional activity

To get an insight into how c-Jun enhances p73 transcriptional activity, *p53*$^{-/-}$ *jun*$^{-/-}$ cells were chosen for study, as it is important to confirm the enhanced transcriptional activity of p73, which operates not only on H1299 cell line, but also on an authentic and genetically defined (*p53*$^{-/-}$ *jun*$^{-/-}$ Vs *p53*$^{-/-}$) cell line. In *p53*$^{-/-}$*jun*$^{-/-}$ cells, the ability of p73 to transactivate p53 responsive gene promoter (MDM2-luc, which has an authentic p53 responsive elements) is reduced, while in the *p53*$^{-/-}$ cell line the p73 transcriptional activity enhanced by six folds. This data is consistent with levels of p73 and AP-1 activity seen in these cell lines, as *p53*$^{-/-}$*jun*$^{-/-}$ cells have lower levels of p73 and AP-1 activity compared to *p53*$^{-/-}$ cells (data not shown). Together, the data suggests that an endogenous c-Jun protein is utilized by p73 to execute transcription in cells, and lack of expression of the c-Jun/co-activator impairs p73 transcriptional activity.

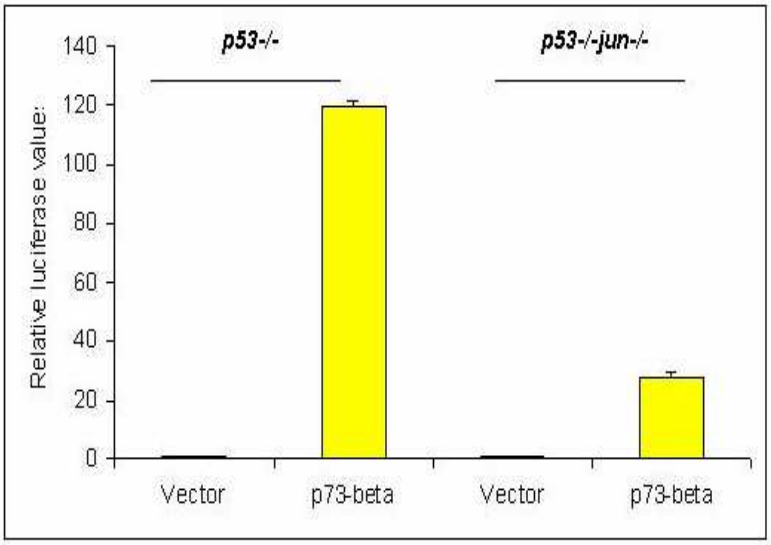


Figure 3.1.3.4 c-Jun is essential for enhanced p73 transcriptional activity

p53$^{-/-}$*jun*$^{-/-}$ and *p53*$^{-/-}$ fibroblasts were transiently transfected with p73-β or pCDNA, p53RE-luc and β-gal plasmids. 100ng of p73 was transfected in *p53*$^{-/-}$*Jun*$^{-/-}$ and in *p53*$^{-/-}$ cell lines (which has an intact c-Jun gene). Total amount of transfected DNA was kept constant by the addition of pCDNA. Cell extracts were prepared 48 hours post-transfection. Results are represented as fold induction of ratio between luciferase and beta-gal activity.

3.1.3.5 c-Jun potentiates p73's ability to induce p53 downstream genes

The various Luc-reporter assays (figure 3.1.3.1 to 3.1.3.4) shown earlier suggest that the enhanced stability of p73 can lead to enhanced transcription of its downstream genes. In order to confirm that whether the c-Jun enhances p73's ability to trasactivate its downstream genes such as GADD45, MDM2, p21 RNAs, p73 was transfected either alone or in the presence of c-Jun into Cos7 cells. Total RNA was collected from each cell pellet and checked for the quality of the RNA before submitting it for reverse transcriptase reaction. As shown in figure 3.1.3.5, co-transfection of c-Jun and p73 lead to enhanced RNA levels of MDM2, GADD45 and p21 in Cos7 cells. This data confirms that c-Jun indeed potentiates p73's ability to transactivate its downstream genes.

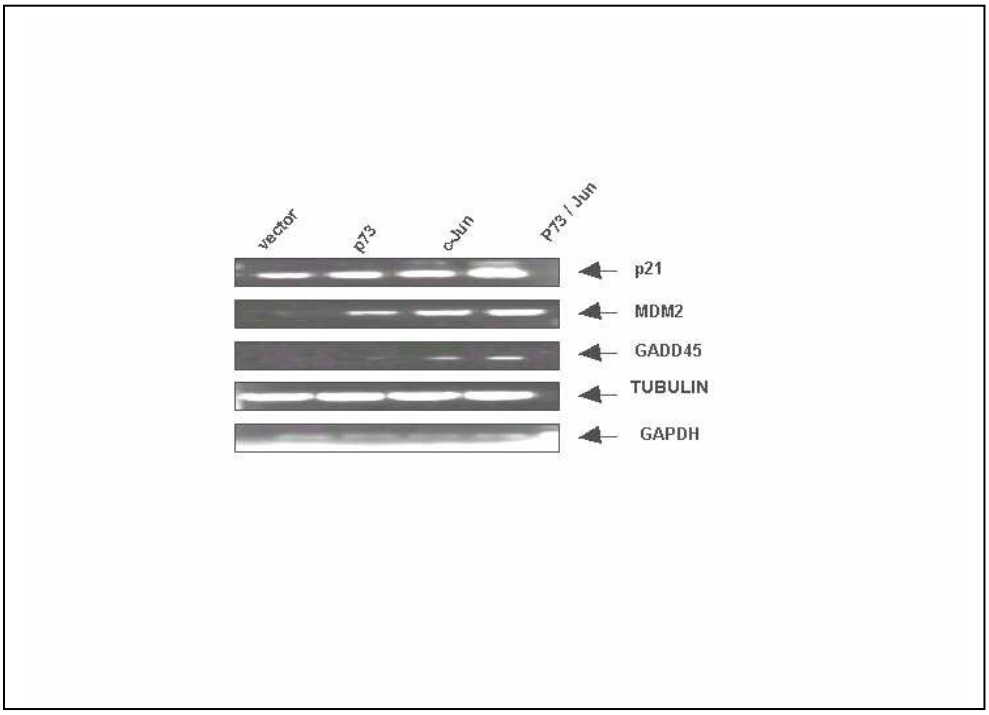


Figure 3.1.3.5 c-Jun potentiates p73's ability to transactivate its downstream genes

Expression of p53/p73 target genes was analysed by RT-PCR analysis. Cos 7 cells were transfected with indicated combinations of expression vectors and then total RNA was isolated. Integrity of the RNA was verified before subjecting it to the reverse transcriptase reaction. cDNAs amplified were quantified carefully with house keeping primers such as β-tubulin and GAPDH before determining p53/p73 target genes expression.

3.1.3.6 c-Jun enhances the induction of MDM2 proteins by p73

To determine whether c-Jun enhances p73 mediated transcriptional activity, not only on artificial promoter constructs but also on endogenous p73-responsive chromosomal genes, MDM2 (a p53/p73 target gene) protein was analysed in Cos7 cells. Cos7 cells were transiently transfected with p73β alone or together with a plasmid encoding c-Jun. As shown in figure 3.1.3.6, in the presence of co-transfected c-Jun steady state levels of MDM2 protein increased compared to p73β alone. Thus, this data strongly suggests that c-Jun potentiates the ability of p73 to transactivate its target genes.

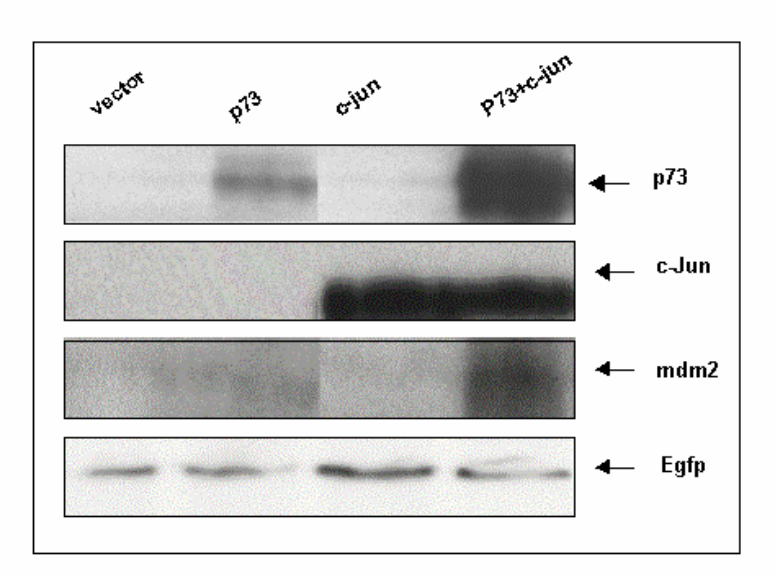


Figure 3.1.3.6 C-Jun enhances the induction of MDM2 protein by p73β

Cos7 cells were transiently transfected with plasmids encoding p73β (2μg/10-cm dish) or c-Jun (2μg/10-cm dish) and EGFP (500ng/10cm)/ dish. Antibodies specific for p73-β ((GC15) mAb (1:1000)), Jun (H-79) (1:1000), MDM2 (1:1000) and EGFP (1:500) were used for Western analysis.

3.1.4 The PY motif conserved in both p73 and c-Jun

PY motif functions as a transcription activation domain in a subset of transcription factors. It utilizes a WW domain— two signature tryptophan residues— containing protein as a co-activator for the efficient stimulation of transcription (Sudol et al., 2001).

The p73α and β contain a PY motif in their C-terminal regions, but not p53. It has been shown that PY motifs present in p73 are required to function as an efficient transcription factor (Strano et al., 2001). The fact that c-Jun stimulates p73 function prompted us to look for conserved sequences in the amino acid sequence of c-Jun and p73. This analysis has identified the PY domain as a conserved motif in both human and mouse c-Jun (figure 3.1.4.1). The conservation of PY motif in both c-Jun and p73 indicate that they have similar/shared functions in regulating various biological processes in cells. In addition, it indicates that common regulators carrying WW domain containing proteins can regulate both c-Jun and p73. For example, any WW domain containing protein, which binds to both p73 and c-Jun through PY domains, could convert a relatively weak transactivator to a strong one (Kristie and Sharp, 1990; Stern and Herr, 1991). Since there are many WW domain-containing proteins with different functions in the cell (Rotin, 1998), it is possible that a WW domain-containing protein(s) associates with the PY motif of transcription factors in certain cellular contexts.

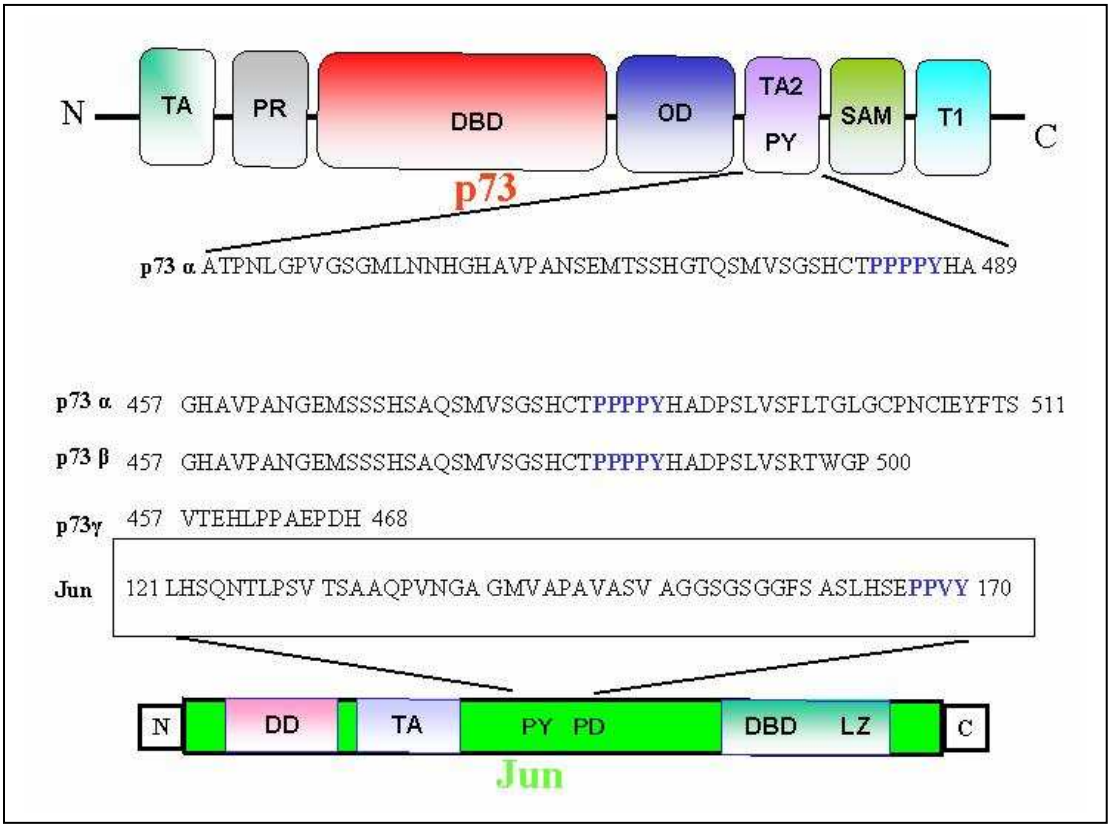


Figure 3.1.4.1 The PY motif conserved in both p73 and c-Jun

A comparison between p73 (α, β and γ) and c-Jun amino acid sequences identified conserved PY domain.

3.1.5 The Role of Phosphorlylation: c-Jun stabilizes and activates p73

3.1.5.1 The effect of c-Jun phosphorlylation mutants on p73 trasncrptional activity

To determine the domain(s) involved in the c-Jun's ability to co-operate with p73β in mediating the transcriptional activation of the p53RE gene promoter, p73, c-Jun and its deletion/point mutants such as N-c-Jun (Δ1-194), C-c-Jun (Δ194-334), Jun S63/73A (JNK phosphorlylation mutants), Jun S91/93A (phosphorylation mutants), 5XASP (five serine residues are replaced with aspartate residues, to mimic the phosphorylation state) and c-Jun delta domain (30-57) and Jun delta NLS (232-334) were transfeted into human lung carcinoma H1299, either alone or together with p73-β. As shown in figure 3.1.5.1, c-Jun cooperates with p73 to transactivate p53RE reporter activity compared to p73β alone. Interestingly, c-Jun point mutants also co-operate with p73, which indicates that the phosphorlyated c-Jun is not an absolute requirement for its ability to co-operate with p73. On the other hand, both N and C-termini of c-Jun mutants included in the assay failed to co-operate with p73. Together, this data indicates that phosphorylation of c-Jun is not essential for its ability to augment the function of p73.

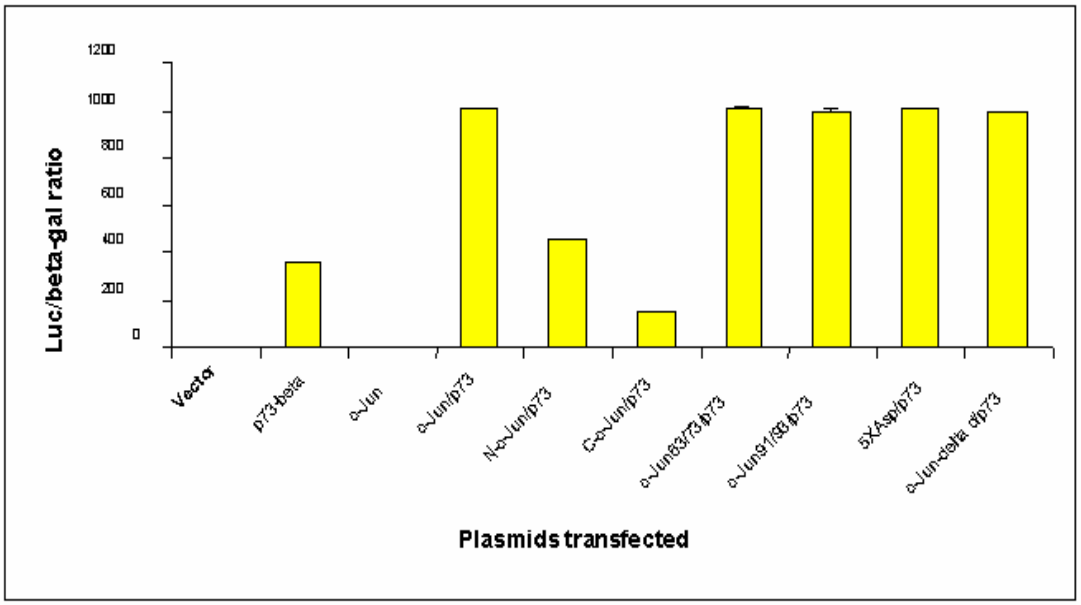


Figure 3.1.5.1 The effect of c-Jun phosphorlylation mutants on p73 transcriptional activity

H1299 cells were transiently transfected with indicated combinations of plasmids encoding pcDNA, p73β, c-Jun, c-Jun phosphorlylation mutants, c-Jun delta domain mutant, 5XASP, MDM2-luc and beta-gal. The total amount of transfected DNA in each dish was kept constant by the addition of empty vector wherever necessary. Cell extracts were prepared 48 hours post-transfection and subjected to the determination of luciferase and beta-gal activity. Results are represented as fold induction of ratio between luciferase and beta-gal activity.

3.1.5.2 p73 is stabilized in *Jnk1*$^{-/-}$*2*$^{-/-}$ fibroblasts

To check whether p73 levels can be increased in the absence of JNK1 and 2, *Jnk1*$^{-/-}$*2*$^{-/-}$ cells were transfected with p73 or c-Jun or in combination. As shown in figure 3.1.5.2, c-Jun increases p73 levels even in the absence of both Jnk1 and Jnk2. This data indicates that JNKs are not essential for c-Jun mediated p73 stability. Of note, c-Jun is normally expressed and phosphorylated in jnk1/2 double knockout embryos, presumably by JNK3 (Hochedlinger et al., 2002). Similarly, in response to UV irradiation or cisplatin treatment, c-Jun is induced in *Jnk1*$^{-/-}$*2*$^{-/-}$ cells, indicating that c-Jun can be activated through alternative pathways (results not shown, Sabapathy K, unpublished).

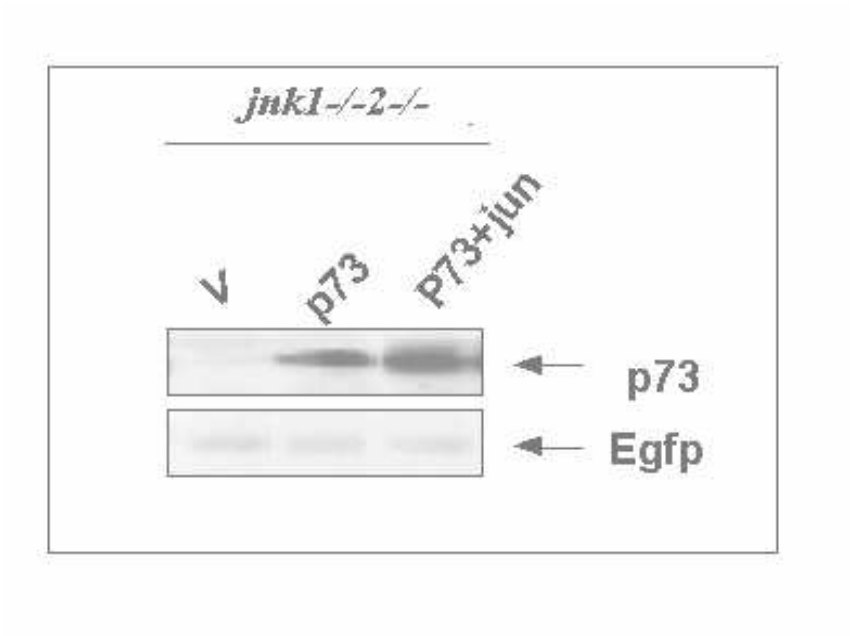


Figure 3.1.5.2 JNK 1 and 2 are not required for c-Jun mediated p73 stability

Jnk1$^{-/-}$*2*$^{-/-}$ cells were transfected with indicated combination of vectors encoding pcDNA, p73β and c-Jun. 48h later, lysates were subjected to SDS-PAGE. Western blots were performed using p73β specific GC15 and EGFP antibodies.

3.1.5.3 p73 transcriptional activity is not compromised in *Jnk1$^{-/-}$2$^{-/-}$* cells.

To determine whether phosporylated c-Jun is essential for its ability to stimulate p73 function, *Jnk1$^{-/-}$2$^{-/-}$* cell lines were chosen for study. p73 was transfected in *Jnk1$^{-/-}$2^{-}* and wild type fibroblast cell lines and its ability to function as a transcription factor was tested in these cell lines. As shown in figure 3.1.5.3, the ability of p73 to transactivate p53 responsive gene promoter MDM2-luc (which has an authentic p53 responsive elements) was not reduced in the absence of JNK1 and 2.

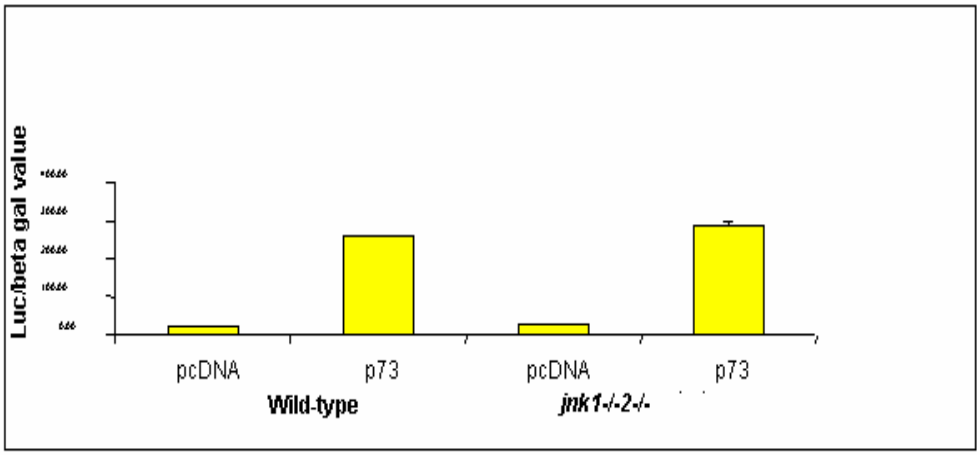


Figure 3.1.5.3 JNKs 1 and 2 are not required for p73 transcriptional activity

Wild type and *JNK1*$^{-/-}$*2*$^{-/-}$ cells were transiently transfected with the indicated combinations of plasmids encoding pcDNA, p73β, MDM2-luc and beta-gal. The total amount of transfected DNA in each dish was kept constant by the addition of pcDNA wherever necessary. Cell extracts were prepared 48 hours post-transfection and subjected to the determination of luciferase and beta-gal activity. Results are represented as fold induction of ratio between luciferase and beta-gal activity.

3.1.6 c-Jun potentiates p73's ability to induce apoptosis in response to Stress

As stated in the introductory sections, there is ample evidence exist that links the c-Jun to the control of cell death. In a few set of experiments, whether p73 and c-Jun co-operate with each other in inducing apoptosis was checked.

3.1.6.1 c-Jun potentiates the ability of p73 to induce apoptosis: AIP1

Our earlier findings presented in this thesis indicate a causal link between c-Jun and p73 in cisplatin-mediated apoptosis. Further, both c-Jun and p73 are induced in response to cisplatin treatment, and p73 is stabilized in c-Jun dependent manner that results in increased activity of p73 towards its downstream genes. Interestingly, it has been shown recently that p300 acetylates p73 in response to DNA damage and thereby potentiates the apoptotic function of p73 by enhancing the ability of p73 to selectively potentiate the transcription of p53AIP in comparison to p21 (Costanzo et al., 2002). These findings indicate that acetylation guides p73 towards apoptotic pathway. Thus, it was decided to determine whether c-Jun mediated p73 stability results in enhanced activity of p73's apoptotic target genes such as p53AIP1. This possibility was checked by co-transfecting p73 and c-Jun in H1299 cells with p53AIP-luc reporter. As shown in figure 3.1.6.1, in the presence of c-Jun, p73 displays enhanced activity towards p53AIP1 promoter, indicating that c-Jun potentiates the ability of p73 to transactivate its apoptotic target genes.

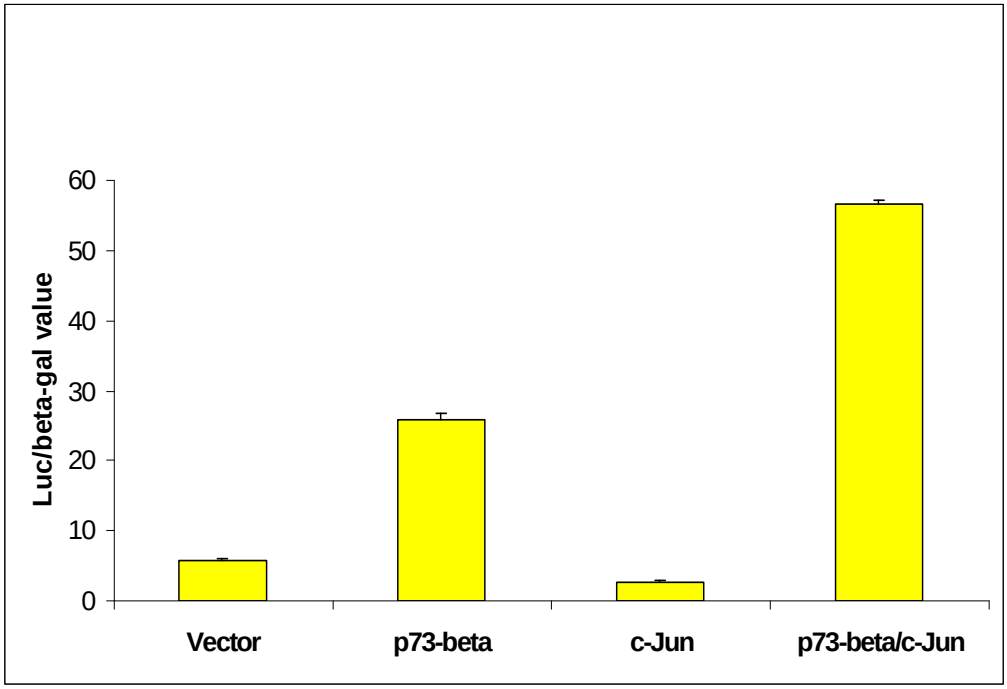


Figure 3.1.6.1 C-Jun enhances the induction of p53AIP promoter by p73

H1299 cells were transiently transfected with the indicated combinations of plasmids encoding pcDNA (empty vector) p73β, c-Jun, AIP-luc and beta-gal. The total amount of transfected DNA was kept constant by the addition of empty vector wherever necessary. Cell extracts were prepared 48 hours post-transfection and subjected to the determination of luciferase and beta-gal activity. Results are represented as fold induction of ratio between luciferase and beta-gal activity.

3.1.6.2 p73 co-operates with c-Jun in transactivating Bax promoter

Recent findings suggest that early induction of apoptosis is associated with mitochondrial changes. Bax, a p53 target gene, has been known to alter mitochondrial events and promote apoptosis by facilitating the release of apoptosis-inducing factor (AIF) and cytochrome C from the mitochondria, which in turn triggers a cascade of caspase activation (Narita et al., 1998; Susin et al., 1999). Bax appears to be essential for p53-mediated cell death in brain tumours (Yin et al., 1997) and fibroblasts (McCurrach et al., 1997) but not thymocytes (Brady et al., 1996). Hence, it was of interest to determine whether c-Jun potentiates the ability of p73 to transactivate, Bax promoter, an apoptotic target. H1299 cells were transfected with p73, c-Jun either alone or in combination. As shown in figure 3.1.6.2, when c-Jun was co-transfected with p73 about 2 fold increase over the p73 transfected alone, indicating that c-Jun potentiates the apoptotic function of p73.

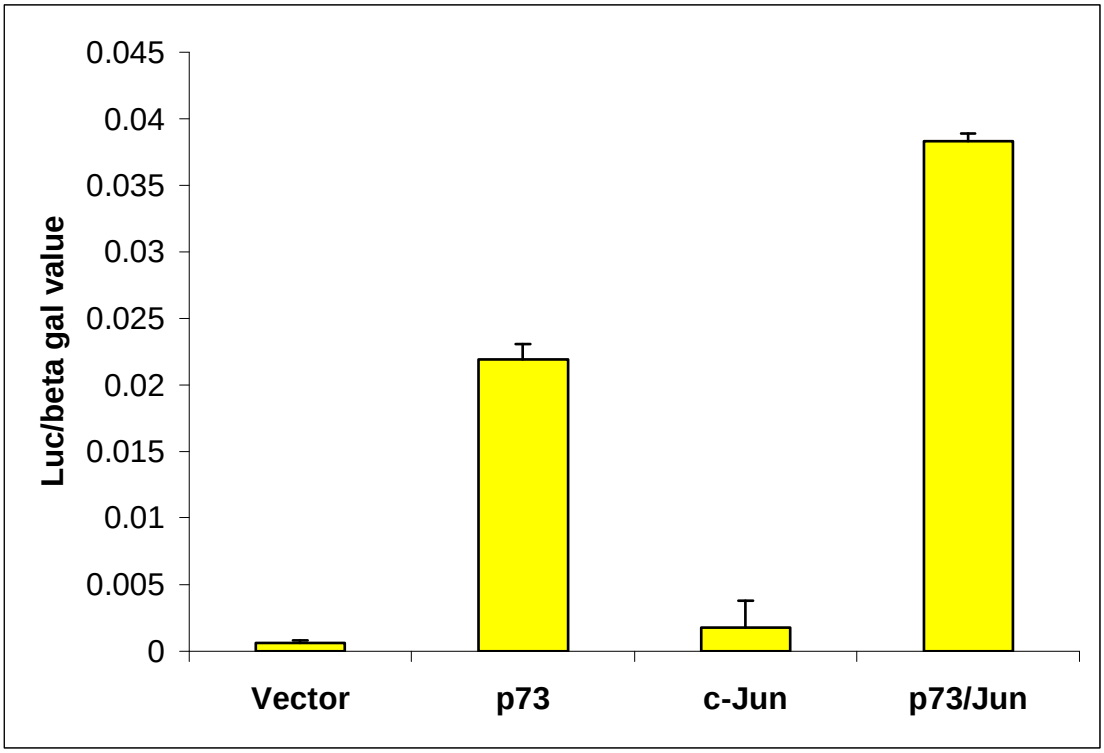


Figure 3.1.6.2 C-Jun enhances the induction of Bax promoter by p73

The indicated plasmids were transfected in H1299 cells with a reporter plasmid containing bax promoter, which drives the luciferase gene, and a plasmid encoding beta-galactosidase gene for evaluating the transfection efficiency. Cell extracts were analyzed 48 hours post transfection for beta-galactosidase and luciferase activity. Results are represented as fold induction of ratio between luciferase and beta-gal activity.

3.1.6.3 c-Jun potentiates the ability of p73 to induce apoptosis

Since c-Jun appears to potentiate the apoptotic function of p73 by enhancing its ability to transactivate its apoptotic target genes such as Bax and p53AIP-1. It was decided to determine whether the co-operation between c-Jun and p73 result in increased apoptosis. Cos7 cells were transfected with p73, c-abl, and c-Jun either alone or in the presence of each other as indicated in figure 3.1.6.3. Transfected cells were analysed by tryphan blue exclusion method and both dead and live cells were counted in duplicates. Number of live cells was plotted against the plasmid combination transfected. As shown in figure 3.1.6.3, co-trasfection of p73 α or β and c-Jun resulted in reduction of number of viable cells by approximately 15%. It has been shown earlier that c-Abl potentiates p73's ability to induce apoptosis (Agami et al., 1999). Thus, c-Abl and p73 were co-transfected to see the reduction of number of viable cells. These results indicate that both c-Jun and p73 collaborate to induce apoptosis.

Further more, p73-β was transfected in *c-jun*$^{-/-}$+ *jun* mouse fibroblasts or those lacking either c-Jun or c-Abl, and followed the fate of transfected cells by including the EGFP expression plasmid in the transfections. Analysis of cells under fluorescence microscopy indicated that unlike *c-jun*$^{-/-}$+ *c-Jun* fibroblasts and similar to c-abl null cells, c-Jun deficient cells were relatively resistant to p73-β-mediated cell death, ascertained by their morphology (data not shown).

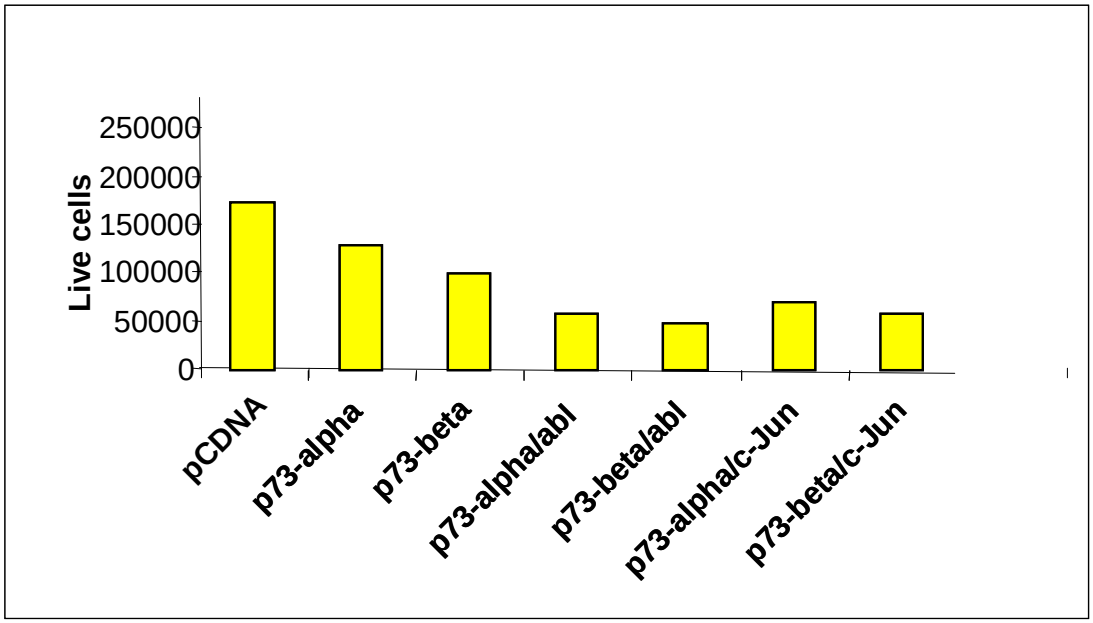


Figure 3.1.6.3 c-Jun potentates the ability of p73 to induce apoptosis

Quantitative representation of c-Jun-induced cell death in Cos7 cells after transient trasfection of indicated expression vectors. 48 hours post transfection, cell viability was analysed by Tryphan blue exclusion method.

CHAPTER 3

RESULTS

Section II: Ultraviolet light (UV) induces p73 levels

3.2.1 Background and Hypothesis

UV can induce p73

Several lines of evidence indicate that UV and Ionizing radiation cause different kinds of DNA lesions and trigger different repair pathways. While UV radiation activates nuclear excision repair, IR radiation results in the activation of the base excision repair machinery. It seems that UV and ionizing irradiation increase p53 levels through different mechanisms (Zeng et al., 2000). Activated p53 can turn on its downstream target genes. It seems that p53 is required for IR, but not UV, mediated G1 arrest. For example, UV, but not ionizing radiation, induced p21, and G1 arrest in p53 deficient fibroblasts from Li-Fraumeni syndrome patients (Loignon et al., 1997) and G1 accumulation was observed in UV-irradiated *p53*$^{-/-}$ T lymphoma cells expressing Bcl-2 (Strasser et al., 1994). Furthermore, several studies suggest that p53 null fibroblasts (both primary and immortalized) are sensitive to UV radiation mediated apoptosis (Al, Mohanna et al., 2001, Lackinger et al., 2001, Lackinger and Kaina, 2000; Mckay and Ljungman, 1999; Wani., 1999), indicating the presence of p53-independent apoptotic program. Thus, the requirement of p53 in UV-dependent G1 arrest and apoptosis is less clear.

UV increases the expression of c-Jun and it is not yet clear whether UV-induced c-Jun participates in DNA repair or apoptosis. Nevertheless, it has been shown that *jun*$^{-/-}$ cells are less responsive to UV-mediated apoptosis. This data suggests that c-Jun could participate in apoptotic response (Shualin et al., 2001). The data presented in chapter I

suggests that c-Jun is required for increased p73 levels. Hence, we propose that UV can stabilize p73.

3.2.2 Role of p73 in UV-induced pG13 luciferase activity in *p53*$^{-/-}$ cell lines

3.2.2.1 p53-independent pG13-luc transcriptional activity

It has been shown that UV-irradiation induces apoptosis in *p53*$^{-/-}$ cell lines (Al, Mohanna et al., 2001, Lackinger et al., 2001, Lackinger and Kaina, 2000; Mckay and Ljungman, 1999; Wani et al., 1999). Recently identified p53 homologues, such as p73 and p63 are shown to be capable of transactivating p53 responsive element containing promoters(Yang et al., 2001). To check whether p53 independent p53RE- activity can be observed in *p53*$^{-/-}$ cells, a panel of *p53*$^{-/-}$ cell lines such as SAOS2 (Osteosarcoma cell line), H1299 (non-small cell lung carcinoma) and *p53*$^{-/-}$*(*fibroblasts) were chosen and transfected with plasmids containing p53 responsive elements, which in turn drive the luciferase gene reporter. As shown in figure 3.2.2.1, p53-independent p53RE-reporter activity was observed in *p53*$^{-/-}$ cell lines. Thus, this result suggests that p53 related proteins exist in *p53*$^{-/-}$ cells that are capable of transactivating p53RE reporter activity.

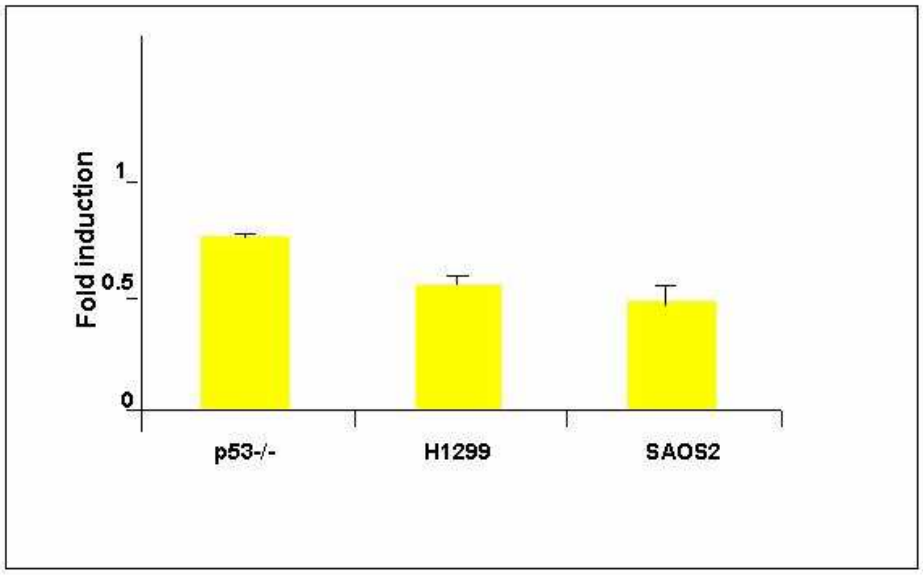


Figure 3.2.2.1 p53-independent pG13-luc transcriptional activity

p53$^{-/-}$, H1299 and SAOS2 cells were transfected with reporter plasmids expressing the luciferase gene under the control of p53RE (pG13) promoter and a plasmid encoding the beta-gal promoter for evaluating the transfection efficiency. Transfected cells were analysed after 48 hours (post-transfection) for both luciferase and beta-gal activity.

3.2.2.2 UV induces p53 reporter activity in *p53*$^{-/-}$ cell lines

Our earlier observation suggests that the p53 reporter activity is present in *p53*$^{-/-}$ cell lines. To check whether UV light can stimulate the p53RE reporter activity further, the same panel of *p53*$^{-/-}$ cell lines used in the earlier study was transfected with pG13-luc reporter. Subsequently, these cells were UV irradiated. As shown in figure 3.2.2.2, in UV-irradiated *p53*$^{-/-}$ cell lines, the p53 reporter activity is augmented. Hence, this observation suggests that the UV can induce p53 reporter activity in *p53*$^{-/-}$ cell lines.

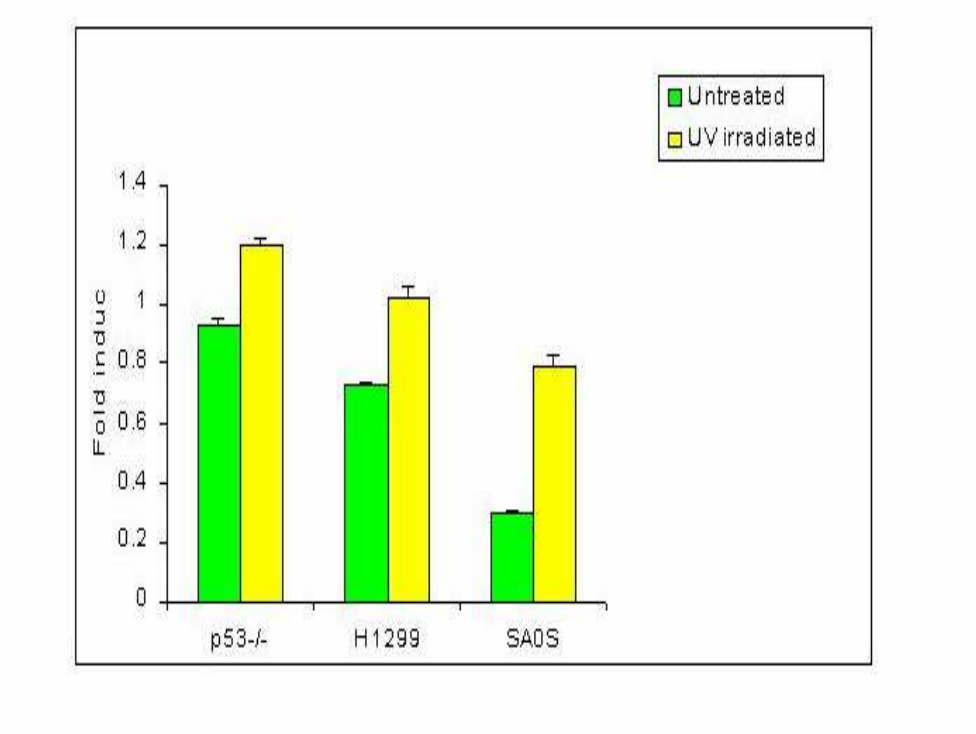


Figure 3.2.2.2 UV induces p53 reporter activity in $p53^{-/-}$ cell lines

$p53^{-/-}$, H1299 and SAOS2 cells were transfected with reporter plasmids expressing the luciferase gene under the control of p53RE (pG13) promoters and a plasmid encoding the beta-gal promoter for evaluating the transfection efficiency. Transfected cells were analyzed 48 hours post-transfection for both luciferase and beta-gal activity.

3.2.2.3 Dominant negative p73 inhibits endogenous pG13 luciferase activity in *p53*$^{-/-}$ cells

In order to clarify whether p53 reporter activity present in *p53*$^{-/-}$ cell line is contributed by p73, p73DD (Δ315-636 a.a), a dominant negative of p73, p53 binding sites driving a luciferase reporter gene, beta-gal plasmids were transfected in H1299 *(p53 null)* cell line. As shown in figure 3.2.2.3, the p53 reporter activity observed in the p53 null background was brought down by the p73 DD (Irwin et al., 2000). Furthermore, p73 transcriptional activity is efficiently brought down by p73 DD. This data suggests that p53RE activity observed in *p53*$^{-/-}$ cells could be attributed to the functional activity of p73.

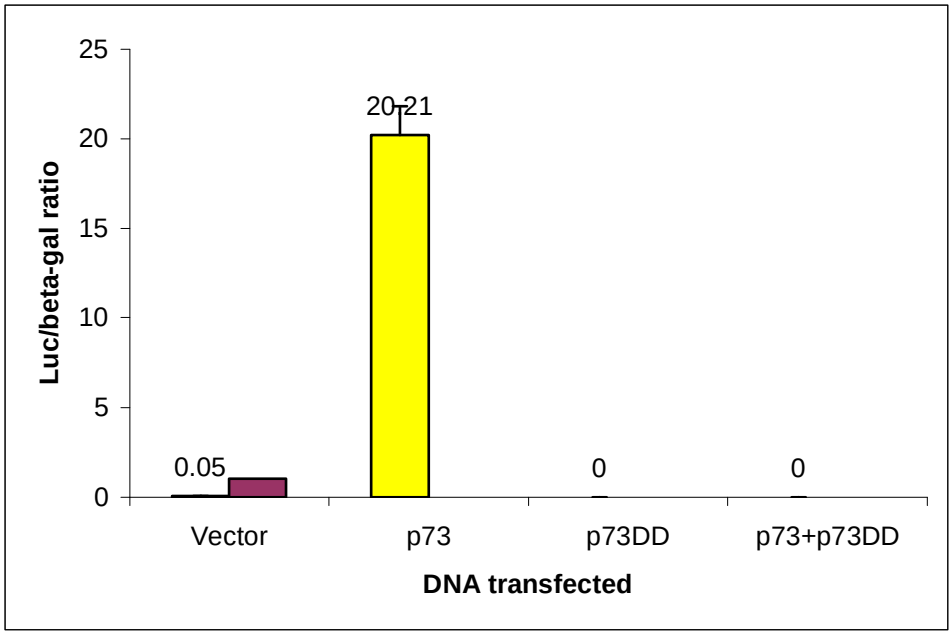


Figure 3.2.2.3 Dominant negative p73 inhibits endogeneous pG13 transcriptional activity in p53$^{-/-}$ cells

H1299 cells were transfected with pcDNA, p73-β, p73DD separately and in combination with p73-β, reporter plasmids expressing the luciferase gene under the control of p53RE (pG13) and the beta-gal promoters for evaluating transfection efficiency. The total amount of DNA was kept constant by using the PCDNA. Transfected cells were collected after 48 hours. Both luciferase and beta-gal activity were measured as described in chapter II.

3.2.3 UV increases p73 levels

3.2.3.1 UV increases transfected p73 levels in cos7 cells

The following facts may suggest that UV-light can stabilize p73 levels:

1. UV is a potent inducer of c-Jun.
2. c-Jun stabilizes p73.
3. UV- can induce p53 reporter activity in *p53*$^{-/-}$ cell lines.

Cos7 cells were transiently transfected with p73β. After 24 hours, cells were irradiated with UV light (50J/m^2). As shown in figure 3.2.3.1, in contrast to the earlier suggestion that UV-light may not induce p73, UV radiation could stabilize p73 further when it was transfected. The blot shown here is a representative of three experiments carried out.

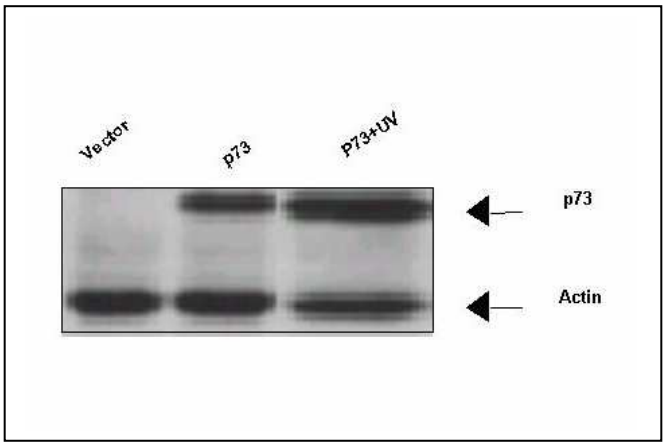


Figure 3.2.3.1 UV increases p73 levels in Cos 7 cells

Western blot analysis of p73 and actin protein levels after transfection into Cos 7 cells. Transfected cells were UV irradiated (50J/m2) for about 12 hours and collected 48 hours post-transfection.

3.2.3.2 UV-induced endogenous p73 is p53 independent

To check whether UV induces endogenous p73 irrespective of the p53 status, *p53*$^{+/+}$ and *p53*$^{-/-}$ 3T3 fibroblasts were UV irradiated (50J/m^2) for two hours. As shown in figure 3.2.3.2, p53 null cells expressed detectable levels of basal p73, which was further induced by UV-irradiation (Figure 3.2.3.2), indicating that UV-mediated p73 induction occurs independent of p53. This data suggests that p53-independent UV-mediated cell death observed in *p53*$^{-/-}$ cell lines could be attributed to the increased p73 levels. In support of this notion, it has been shown that p73 is capable of inducing apoptosis when it is over expressed (Marin et al., 1998).

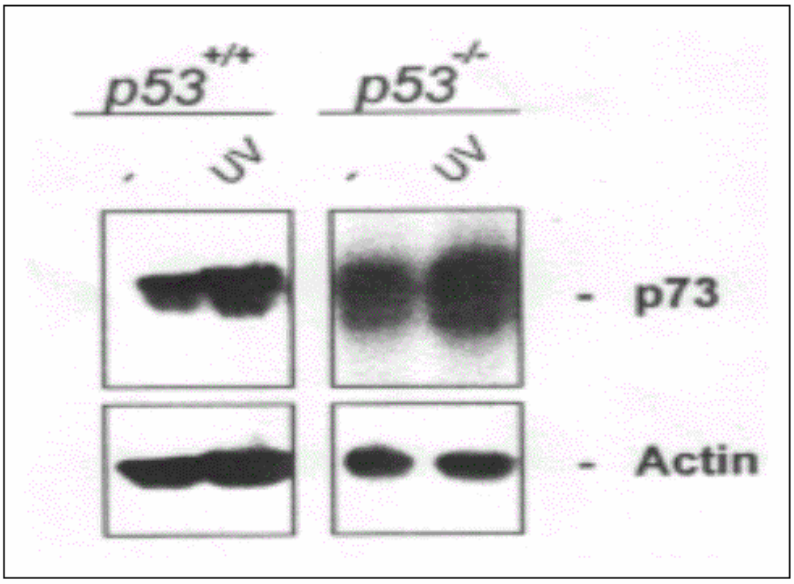


Figure 3.2.3.2 UV-induces endogenous p73 levels independent of p53

p53$^{-/-}$ cells and 3T3 wild-type fibroblasts were UV irradiated (50J/m2) for two hours. After 48 hours, cell lysates were prepared and subjected to Western analysis. Transferred membranes were sequentially probed with antibodies raised against p73-β ((GC15) (1:1000)) and actin (1:400).

3.2.3.3 UV but not IR increases p73 levels

It has been shown that UV can induce c-Jun expression but not IR (Shualin and Karin, 2001). The data presented in this thesis suggests that c-Jun stabilizes p73. Keeping these facts in mind, we proposed that UV irradiation should augment p73 levels but not IR. To test this, H1299 (null for p53 expression) and COS7 (p53 is inactivated by large T antigen) cells were transfected with pCDNA or p73β. These cells were irradiated with UV ($50J/m^2$) or IR (20γ). As shown in figure (3.2.3.3), only UV irradiation stabilizes p73 but not IR.

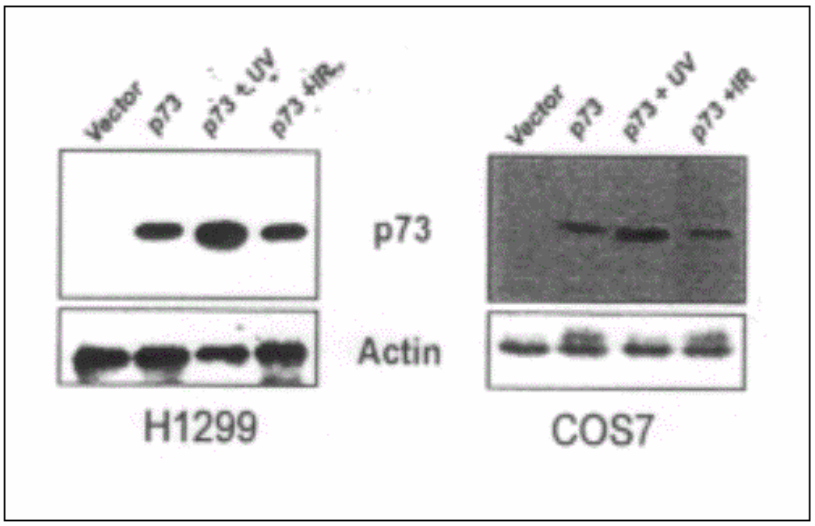


Figure 3.2.3.3 UV increases p73 levels but not IR

Cos7 cells were transfected with pCDNA-3 or p73-β (2λg). 24 hours after transfection, trasfected cells were either UV (50J/m^2) or IR (20λ) irradiated for about 12 hours. The level of p73 and actin proteins were assessed by Western analysis using antibodies directed against their protein products.

3.2.3.4 Cisplatin but not IR induces c-Jun

It has been shown that only cisplatin stabilizes p73 but not ionising radiation (IR) (White and Prives, 1999). Further, it has been shown that cisplatin treatment alone induces c-Jun but not IR irradiation (Shualin et al., 2001). Keeping these facts in mind, 3T3 cells were either irradiated (20γ) or cisplatin (25λM) treated as indicated in figure 3.2.3.4. γ-irradiation did not induce c-Jun, whereas cisplatin treatment resulted in c-Jun induction. The same blot was probed with actin antibody to check the equal loading of proteins in each lane. In line with the earlier observations, this data confirms that c-Jun is induced in response to cisplatin treatment.

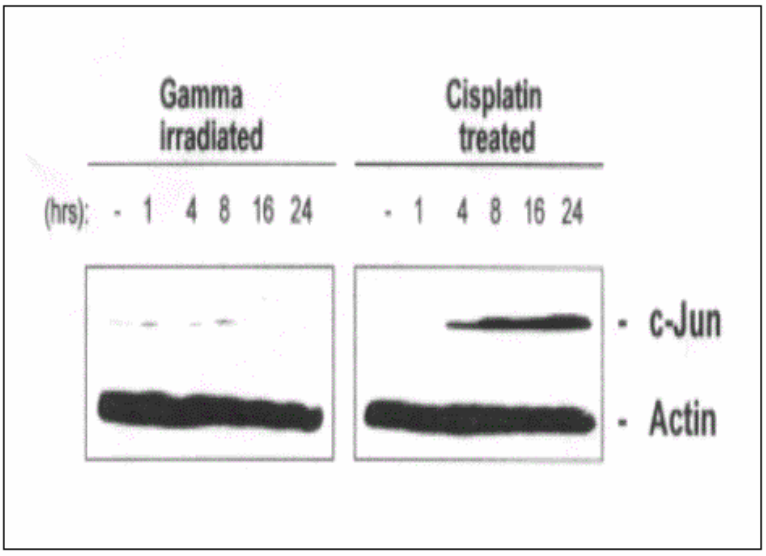


Figure 3.2.3.4 cisplatin but not IR induces c-Jun

3T3 fibroblasts were exposed to cisplatin or IR irradiated (20 γ) as indicated above. Cell lysates were prepared and protein concentration measured. The level of p73-beta and actin proteins were assessed by western analysis using antibodies directed against their protein products.

3.2.3.5 c-Jun over expressing cells increase p73 levels in response to IR

The lack of c-Jun induction in response to IR irradiation could be the cause for lack of p73 stability in response to IR. In order to probe into this issue, human colon carcinoma cells (H1299) stably over expressing c-Jun or the vector were generated and subjected to -irradiations (20 γ). As shown in figure 3.2.3.5, p73 basal level is increased in c-Jun over expressing cell line, which indicates that c-Jun contributes to the increased levels of p73. Further, p73 was induced after 1 hour as expected but the levels had declined by 24 hours post-irradiation in vector expressing H1299 cells. On the other hand, p73 was detected at similar levels at both 1 and 24 hours post-irradiation in c-Jun over expressing cells. This result confirmed that the presence of c-Jun is sufficient to stabilize p73 in response to IR.

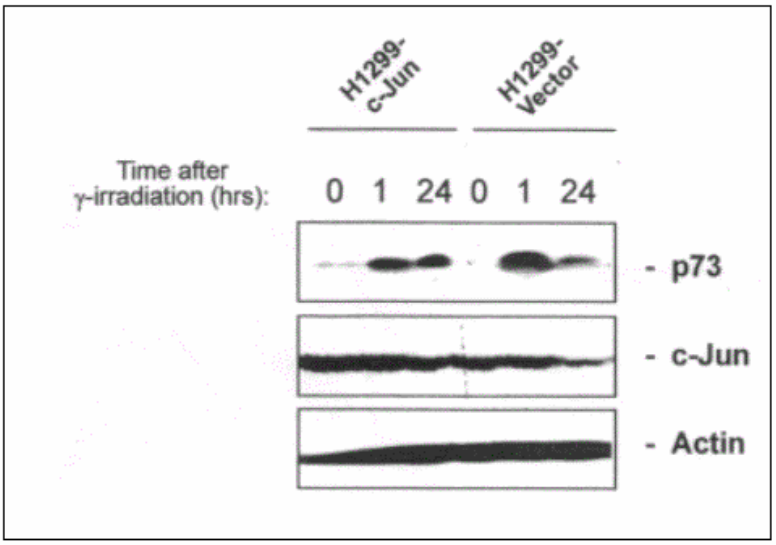


Figure 3.2.3.5 IR stabilizes p73 in c-Jun overexpressing cells

HI1299 cells were exposed to ionising radiation. Cells were harvested at 1 hour and 24 hours after irradiation for western analysis. 300µg of total lysates were used for WB analysis. Transferred membranes were sequentially probed with anti-p73-beta specific (GC15) mouse monoclonal antibody (1:1000), anti-c-Jun (H-79) specific (1: 1000) antibody and an actin (1:400) mouse monoclonal antibody.

3.2.3.6 UV mediated p73 stability is increased in the presence of c-Jun

To determine further whether c-Jun is required for UV mediated p73 induction, *jun*$^{-/-}$ and *jun*$^{-/-}$ + Jun cells were transfected with PcDNA3 and p73. Transfected cells were subjected to UV-irradiation or γ-irradiation. UV, but not γ-irradiation, enhanced both p73 and c-Jun levels in *c-jun*$^{-/-}$+ c-Jun cells (compare lanes 7 with 9 and 12 with respect to actin in figure 3.2.3.6). On the other hand, UV did not effectively increase p73 in *jun*$^{-/-}$ cells (compare lanes 1, 3 and 5), indicating that UV-mediated p73 stability is increased in the presence of c-Jun. Together this data suggests that c-Jun is required, but not essential for UV mediated p73 induction.

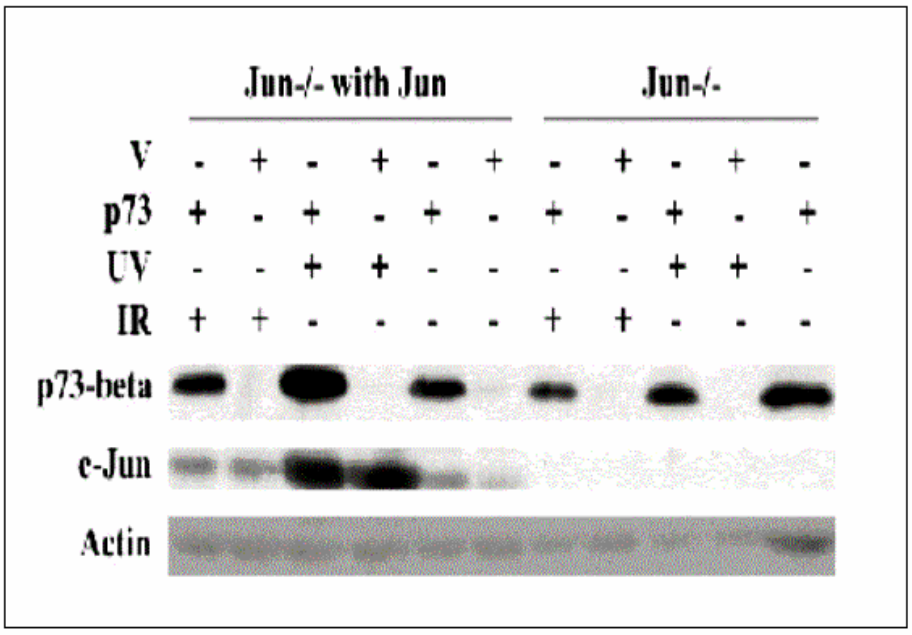


Figure 3.2.3.6 UV enhanced p73 levels is further increased in the presence of c-Jun

The c-Jun null cells and Jun null cells reconstituted with c-Jun were transfected with pCDNA-3(V) or p73-β. 24 hours after transfection, cells were irradiated with either UV (50J/m2) or IR (20λ) for 12 hours. Antibodies specific for p73-β ((GC15) (1:1000)), c-Jun (H-79) (1:1000), and actin (1:400) were used for Western analysis.

3.2.4 Factors influencing UV-induced p73 levels

3.2.4.1 UV increases p73 levels mainly at the post-transcriptional level in MCF7-p73-β clones

To test whether it is due to the increased transcription, MCF-7-p73β cells were treated with Actinomycin D, a transcription inhibitor. As shown in figure 3.2.4.1, UV mediated p73 increase did not decrease in the presence of transcription inhibitor. This data suggests that UV-mediated p73 increase could take place at the post-transcriptional level.

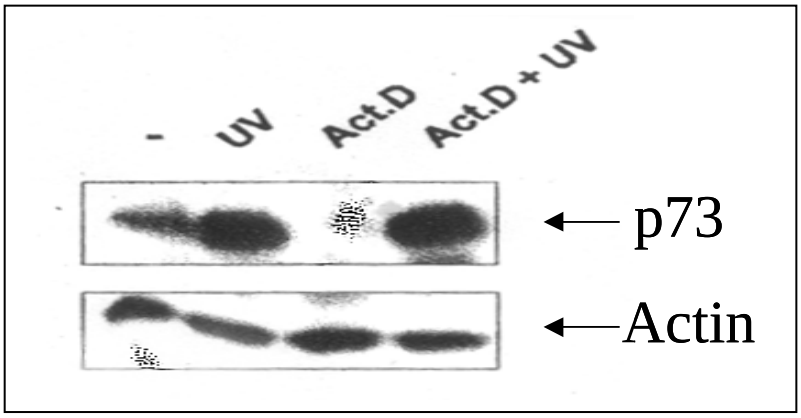


Figure 3.2.4.1 UV increases p73 levels at the post-transcriptional level in stable clones

The MCF7-p73-β stable clones were either untreated, treated with actinomycin D (25ng/ml) for about 15 minutes. Drug treated and untreated plates were UV ($50J/m^2$) irradiated for about 12 hour. Cell lysates prepared and protein concentration measured using Bradford method. Antibodies specific for p73-beta (GC15 (1:1000) and actin (1:400) were used for western analysis.

3.2.4.2 UV increased p73 levels do not depend on JNK 1 and 2 and Abl

It was shown earlier that Jnks are not required for c-Jun mediated p73 stability. To test whether UV mediated p73 induction and stability require JNK 1 and 2 and Abl, *jnk1*$^{-/-}$*2*$^{-/-}$ fibroblasts and *abl*$^{-/-}$ cells were transfected with pCDNA or p73. 48 hours later, cells were UV (50J/m2) irradiated for about 16 hours and subjected to Western analysis. As shown in figure 3.2.4.2, p73 is stabilized by UV irradiation in both *jnk1*$^{-/-}$*2*$^{-/-}$ and *abl*$^{-/-}$ cells This data suggests that both Jnks and abl are not required for UV induced p73 stability. It is not surprising to find p73 stability in the absence of c-Abl, as only ionizing radiation has been shown to activate c-Abl, but not UV irradiation.

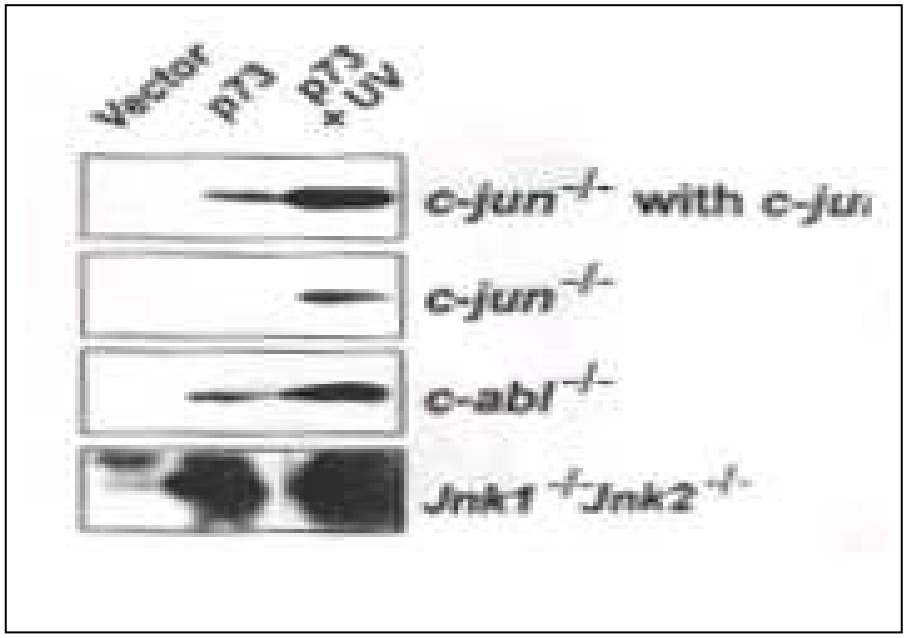


Figure 3.2.4.2 Jnks and abl are not essential for UV increased p73 levels

Jnk1$^{-/-}$*2*$^{-/-}$*; abl*$^{-/-}$*; jun*$^{-/-}$ *and jun*$^{-/-}$+Jun cells were transfected with pCDNA-3 or p73-β. These cells were either untreated or UV irradiated (50J/m2) for 6 hours and subjected to Western analysis using antibodies specific for the p73-β. (GC15) (1:1000). Transferred membrane was stained with ponsue (data not shown).

3.2.4.3 Generation of p73-β expressing MCF-7 stable clones: UV increases p73-β levels in MCF-7-p73 stable clones

To test the ability of UV to induce p73, p73-β expressing stable cell line was generated. For this purpose, MCF7 (human breast carcinoma cell lines) cells were transfected with pcDNA and pcDNA-p73-β. 48 hours after transfection, 250μg G418/ml was added to the transfected cells. After two weeks in drug selection medium, very few surviving clones were selected and developed as cell lines. This is the first demonstration that p73-β expressing stable cell line can be developed. MCF-7-p73-β stable clones were either untreated or UV irradiated (50J/m2). As shown in figure 3.2.4.3, UV increased the p73 levels.

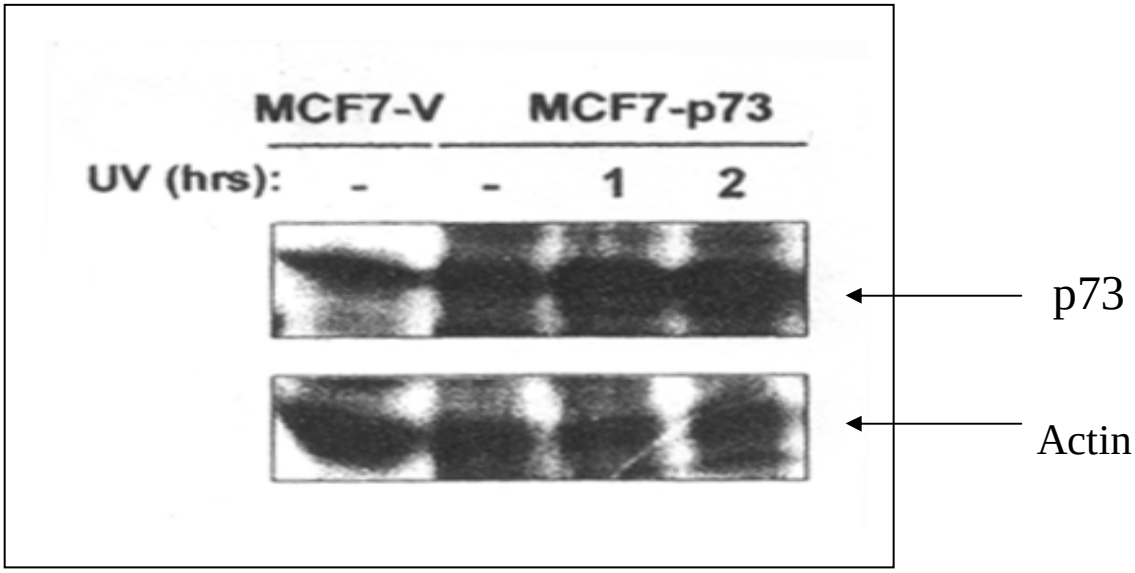


Figure 3.2.4.3 UV increases p73-β levels in MCF-7-p73-β stable clones

The MCF-7-p73-β/vector (V) cells were UV (50J/m2) irradiated as indicated at the top of each lane. 48 hours later, cell lysates were prepared and subjected to SDS-PAGE. Western blot analysis was performed using anti- p73-β specific (GC15) (1:1000), anti-p53 (1:500) and anti- actin (1:400) antibodies.

3.2.4.4 UV induces both p73 and p53 levels in MCF-7-p73-β expressing stable clones

Earlier we have shown that MCF-7 cells stably expressing p73β was further stabilized after UV irradiation. In this experiment, we have decided to compare p73β expression with its counter-part, endogenous p53 expression. MCF-7 cells were UV irradiated for the indicated periods and cell lysates were subjected to western analysis. As shown in figure 3.2.4.4, UV irradiation was able to stabilize both p73 and p53 levels.

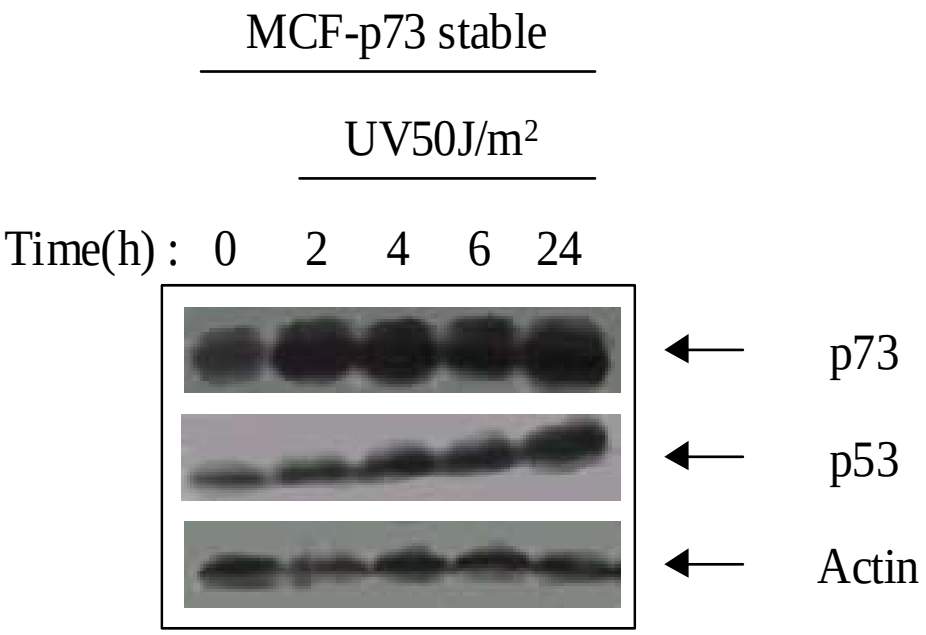


Figure 3.2.4.4 UV increases both p73 and p53 levels in MCF-7-p73 stable clones

The MCF-7 cells stably expressing p73-β were UV (50J/m2) irradiated for the indicated periods. Transferred membranes were sequentially probed with the following:

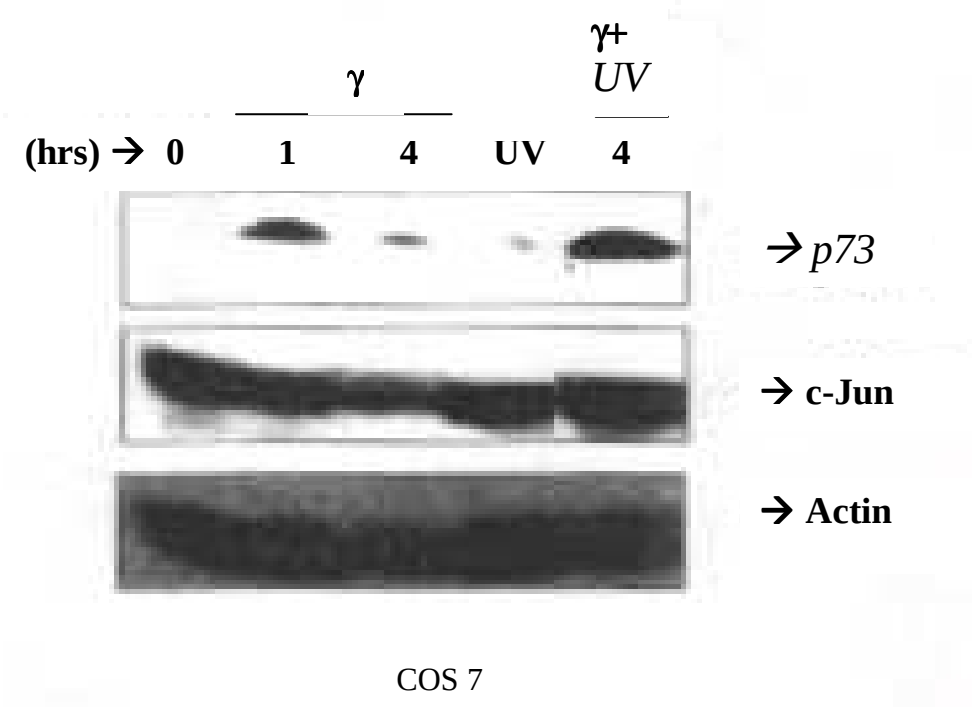
1. anti-p73-β specific (GC15) mouse monoclonal antibody (1:1000)

2. anti-p53 specific polyclonal antibody (rabbit) (1:500)

3. anti-actin (1:400) mouse monoclonal antibody.

3.2.5.1 Synergistic signals (UV+IR) stabilize p73

We proposed that exposure of the cells to inductive/activation signal (Ionizing Radiation-IR) followed by stabilization signal (Ultraviolet radiation-UV) would further enhance the stabilization of p73. To verify this possibility, COS7 cells were either or UV irradiated alone or in combination. γ-irradiation resulted in induction of p73 levels after one hour, but reduced by 4 hours (Figure 3.2.5.1). However, treatment of -irradiated cells one hour later with UV-irradiation rescued the decrease in p73 levels and p73 was detected at 4 hours post -irradiation. In contrast, UV irradiation (l0J/m2) alone did not result in the elevation of p73 levels.



COS 7

Figure 3.2.5.1 Synergistic signals (UV+IR) stabilize p73

Cos 7 cells were either UV (10J/m2) or IR (10γ) irradiated or both as above. Western blot analysis was performed with anti-p73-beta, anti-c-Jun (H-79) and anti-actin antibodies.

3.2.5.2 Synergistic signals (UV+IR) induce apoptosis

IR promotes transient cell-cycle arrest in most of the cell types except thymocytes. It has been shown that IR induces apoptosis in only thymosites but not in fibroblasts (Levrero et al., 2001). To check the possibility of long-term effect of and UV treatment on replicative cell death in cells lacking p53, colony-forming assay was performed. As shown in figure 3.2.5.2, colony number was reduced under either UV or irradiation treatments in comparison to untreated cells; Cells treated with both and UV irradiation did not form any colonies (see figure, untreated: >100; 34; UV: 25; + UV: 0). It is possible that UV irradiation could have further stabilized p73 that is induced by irradiation, which led to p53-independent cell death.

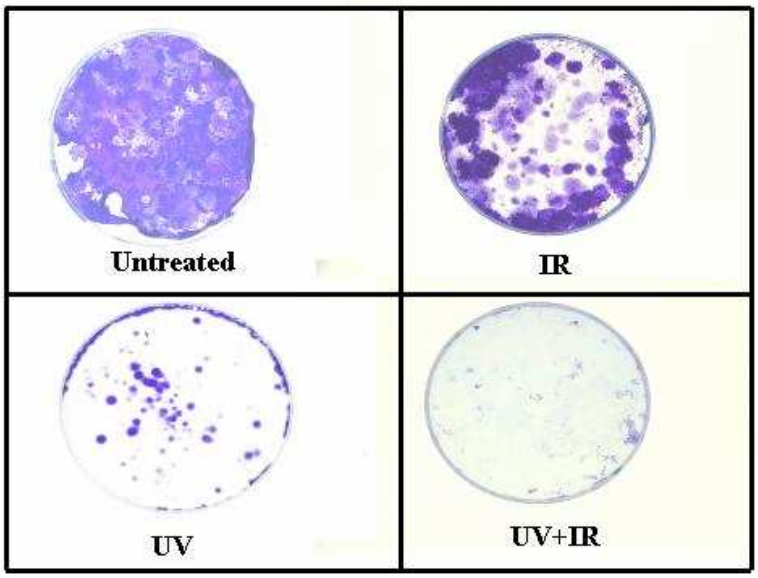


Figure 3.2.5.2 Synergistic signals (UV+IR) led to reduced colony numbers in p53$^{-/-}$ fibroblasts

p53$^{-/-}$ (5X10^4 cells per 10 cm) cells were washed, fixed as described in Materials and methods. Colony formation assay was carried out after UV (10J/m2) or IR (20γ) irradiation or both. After 2 weeks, colonies were visualized by staining with crystal violet.

CHAPTER 3

RESULTS

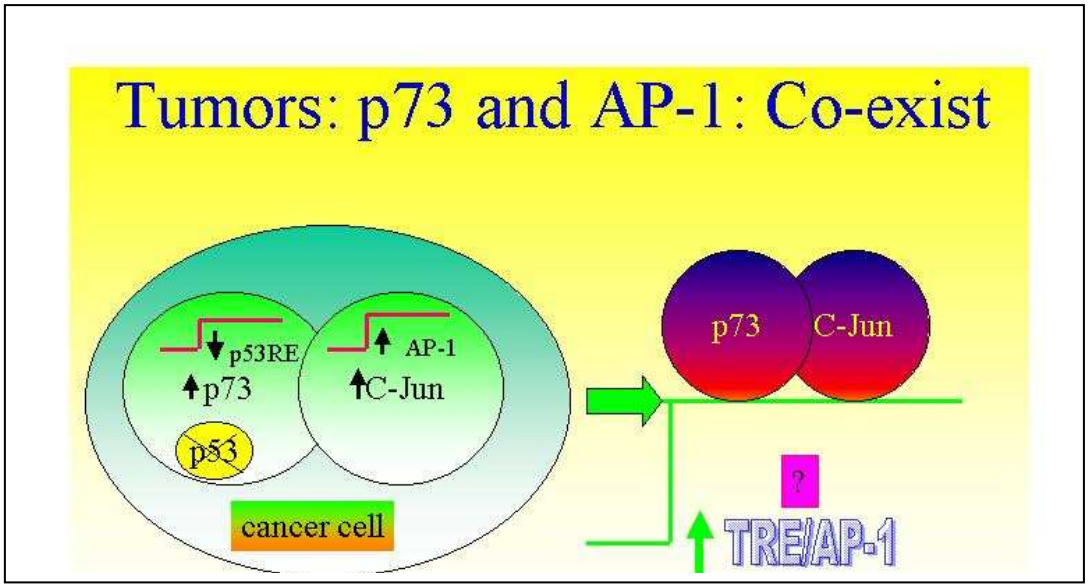
SECTION III: p73 INCREASES AP-1 ACTIVITY

3.3.1 Background and hypothesis: p73 could positively influence AP-1 transactivation.

A structural comparison between p53 and its homologue p73 suggests that DNA binding regions (DBD) of p73 possess a high level of conservation with DBD of p53 (63%) (Levrero et al., 2000). p73 regulates the genes that are regulated by p53 (Yang et al., 2002). However, the striking differences between p53 and p73 knockout mice suggest that *invivo* p73 may regulate different genes. While p53 null mice develop spontaneous tumors, p73-deficient mice have neurological, pheromonal and inflammatory defects and lack spontaneous tumors (Yang et al., 2000), suggesting that *invivo* p73 may regulate different genes. In aggrement with this notion, microarray data from several laboratories suggest that the genes regulated by p53 and p73 are considerably different (Fontemaggi et al., 2002). Further, it seems that p73 may regulate both p53-responsive elements containing genes and other responsive elements containing genes either directly or indirectly. It has been shown that AP-1 activity is highly pronounced in several tumor cell lines. Similarly, it has been recently established that several primary tumor cells and tumor cell lines overexpress wild-type p73 including cancers of the breast, lung, esophagus, stomach, colon, bladder, ovary, liver, bile ducts, ependymal lining, myelogenous leukemia and neuroblastoma (Moll et al., 2001).

The fact that both p73 expression and AP-1 activity are highly pronounced in most of the human tumors indicates a possibility that both p73 and c-Jun could co-exist in human tumors. It appears that most of the tumors have lower levels of p53RE-promoter activity (at least 50% tumors have mutation in p53 gene) but higher levels AP1 activity. This data prompted us to propose that p73 could positively influence the function of c-Jun/AP-1 transcriptional activity.

A.



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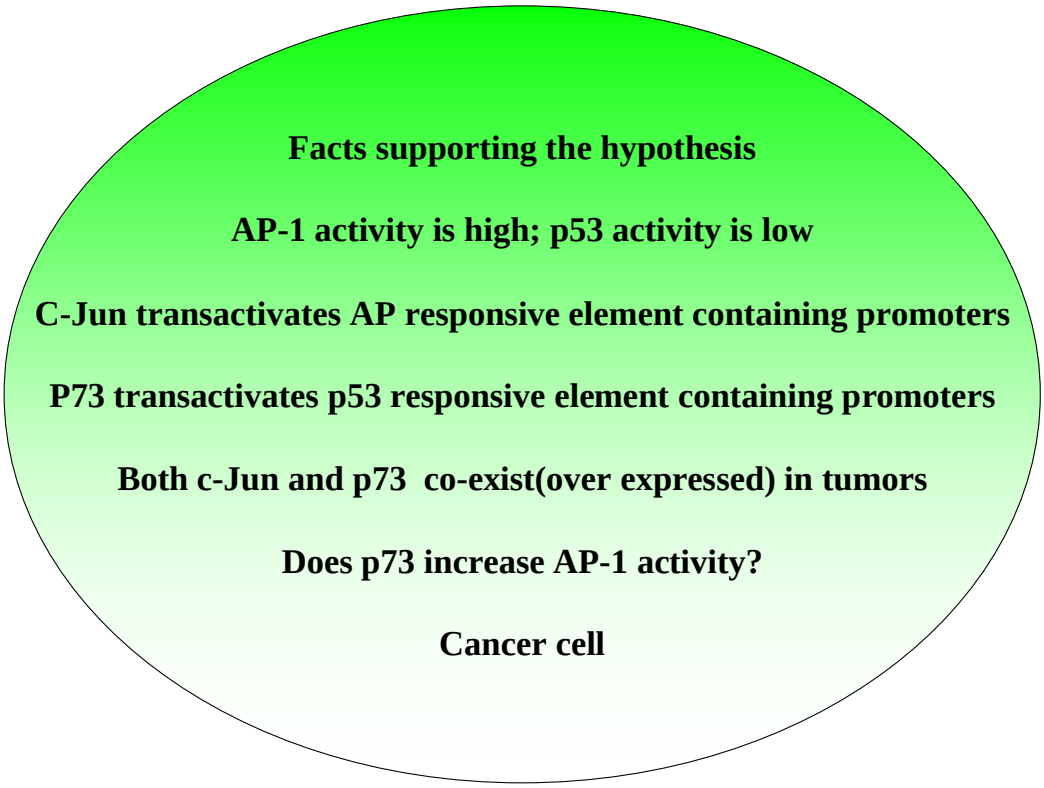

Figure 3.3.1.1 a & b Hypothesis: p73 could augment AP-1 activity

The diagram depicted above indicates the expression status of p73 and c-Jun transcription factors in cancer cells. Although both p73 and c-Jun are upregulated and co-exit in many tumors, only AP-1 transcriptional activity is high, but not p53RE activity. Hence, it was proposed that p73 can increase AP-1 activity.

3.3.2 p73 potentiates AP-1 activity.

3.3.2.1 p73-β increases AP-1 activity.

To check whether p73 influences AP-1 activity, p73 and c-Jun were transfected separately in HI299 cells (a human osteosarcoma cell line, p53 null producer), along with 5XTRE-luc [5X 12-0-tetradecanoyl-13-acetate(TPA)-responsive elements-TRE (5XTRE, five tandem AP-1-binding sites)] and ß-gal promoters. As shown in figure 3.3.2.1, p73 potentiated 5XTRE luciferase activity. c-Jun was used as a positive control for its ability to transactivate AP-1 responsive elements. It was observed that p73 activity is as good as c-Jun mediated AP-1 transcriptional activity. On the other hand, the expression of p53 did not have any effect on 5XTRE elements. Hence, the ability of p73 to stimulate AP-1 elements throws light on its ability to regulate an entirely new set of genes.

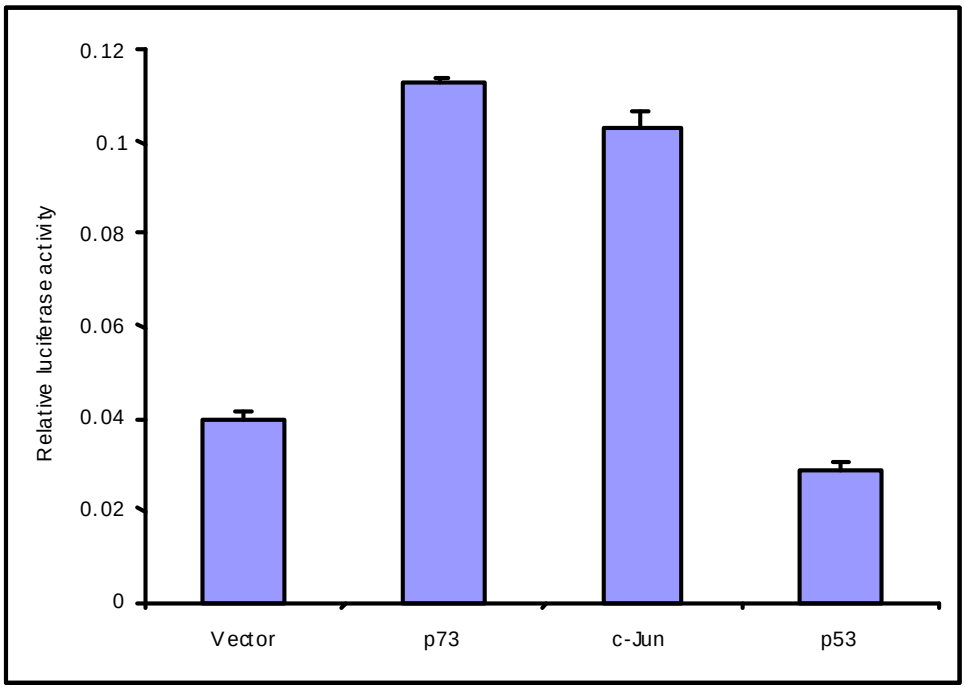

Figure 3.3.2.1 p73β potentiates AP-1 responsive element (5XTRE) promoter activity

H1299 cells were transfected with plasmids encoding 5XTRE-luc (0.5µg), ß-gal (0.5µg), PCDNA3, p73(100ng), p53(100ng) and c-Jun(2µg). The total amount of plasmid DNA used for transfecteion was adjusted to 3.1µg by adding PCDNA3. Luciferase activity was measured 48h after trasfection and normalized for transfection efficiency with ß-gal activity. Results of at least three independent experiments performed in duplicate are shown here.

3.3.2.2 p73β potentiates Collagenase-1 promoter activity.

To generalize the fact that p73 can increase the activity of AP-1 responsive elements containing promoters activity, collagenase-1 promoter was considered as it contains 1X AP-1 responsive elements. p73β was in the presence of collagenase-1 promoter along with ß-gal plasmid in H1299 cells. As shown in figure 3.3.2.2, p73β potentiated collagenase-I promoter in comparison to vector control. This finding strongly suggests that p73β can increase AP-1 responsive elements containing promoter's activity.

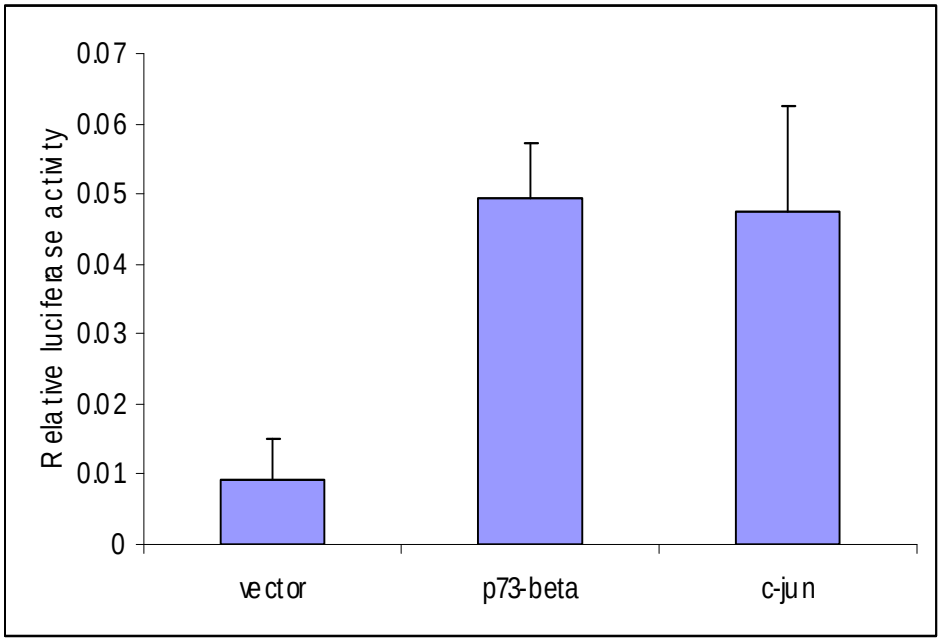

Figure 3.3.2.2 p73β potentiates collagenase-1 promoter activity

H1299 cells were transfected with plasmids encoading collagenase I-pro-luc (0.5µg), ß-gal plasmid (0.5µg), PCDNA3, p73β (100ng), p53 (100ng), and c-Jun (2µg). The total amount of plasmid DNA used for transfection was adjusted to 3.1µg by adding PCDNA3. Luciferase activity was measured 48h after trasfection and normalized for transfection efficiency with ß-gal activity.

3.3.3 The Dose effects of p73β on the reporter expression from 5XTRE promoter: p73β synergizes with c-Jun in potentiating AP-1 activity

To check whether increasing amount of c-Jun increases the ability of p73 to increase AP1 activity, HI299 cells were transfected with an AP-1-driven luciferase reporter gene in the presence of increasing amount of c-Jun (100ng; 2μg) and constant amount of p73β (100 ng). All luciferase activity was normalized using a co-transfected ß-gal expression vector. Over expression of c-Jun caused an increase in the relative activation levels of the AP-1 reporter and the expression of p73 protein further enhanced the AP-1 activity. .

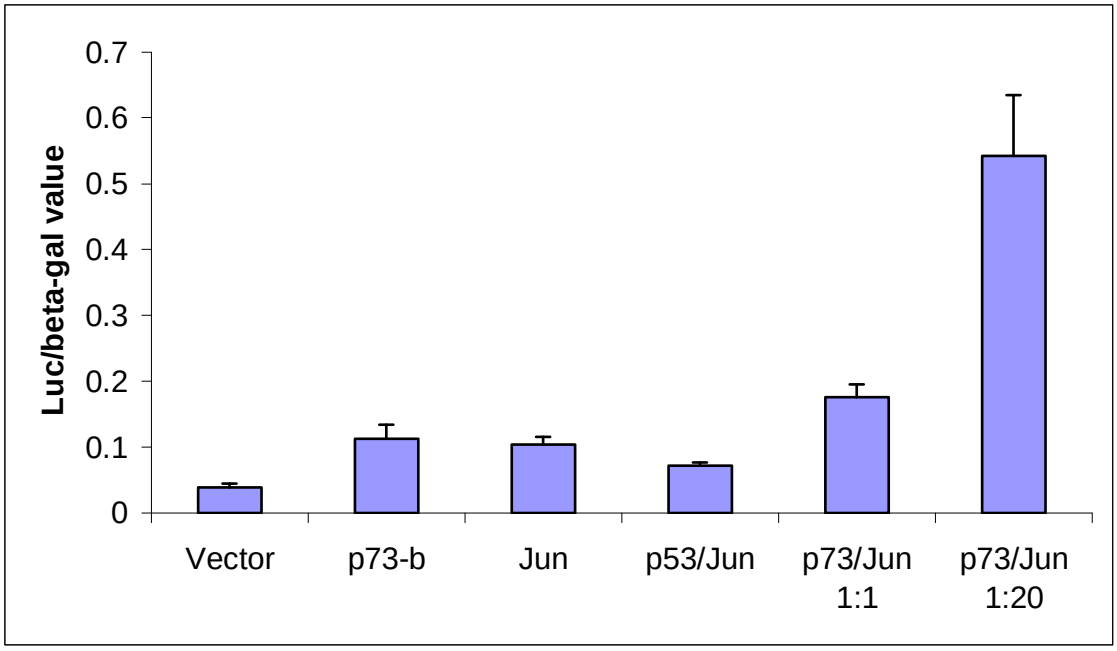

***Figure 3.3.3.1 Synegistic effect of p73*β and Jun on AP-1 promoter activity**

H1299 cells were transfected with 0.5µg of 5XTRE--luc, 0.5µg ß-gal plasmid and 100ng of p73β, 100ng and 2µg of c-Jun. The total amount of plasmid DNA used for transfecteion was adjusted to 3.1µg by adding an empty PCDNA3. Luciferase activity was measured 48h after trasfection and normalized for transfection efficiency with ß-gal activity.

3.3.3.2 p73β synergizes with c-Jun in potentiating the AP-1 activity

Previously, we showed that c-Jun stabilizes p73. The fact that c-Jun homodimers and c-Jun-c-fos heterodimers increases AP1 activity, through their ability to interact with each other, prompted us to ask whether p73 synergies with c-Jun in increasing 5xTRE-luc activity. Both p73 and c-Jun were transfected in the presence of the reporter 5XTRE-luc in H1299 cell line. Remarkably, luciferase activity was much enhanced (5 to 6 folds) than expected in their combination (2 folds), indicating that p73 and c-Jun synergistically increase AP-1 activity and not p53/Jun. These results imply that p73 could be a partner in AP-1 dimers. The importance of AP-1 composition in transcriptional activation is beginning to emerge. c-Jun containing dimers showed distinct promoter specificity in transient-transfection experiments, depending on the partner. It is possible that choice of dimerization partner defines the role of c-Jun in gene activation and cell cycle regulation (Kaminska et al., 2000). It would be interesting to delineate the functions of p73 and c-Jun dimers within a cell. Together, this data suggests that p73 not only synergizes with c-Jun, but also contributes to enhanced AP-1 activity.

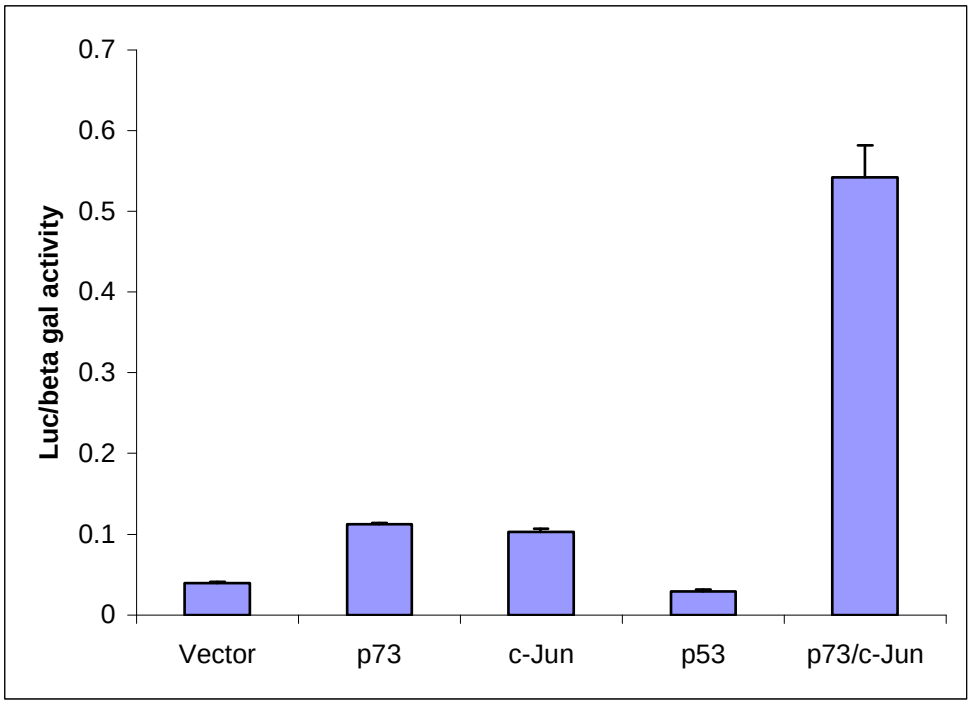

Figure 3.3.3.2 p73 co-operates with c-Jun in potentiating AP1 activity

H1299 (75% confluence in 35mm plates) cells were transfected with 100ng p73β, alone or with 2μg c-Jun, 500ng of the luciferase reporter plasmid driven by the 5XTRE motif and 500ng ß-gal plasmid (as an internal control). The total amount of plasmid DNA used for transfecteion was adjusted to 3.1μg by adding an empty pCDNA3. Transfected cells were collected 48 hours after transfection. Luciferse activity was measured and normalized with β-galactocidase activity.

3.3.3.3 p73-β efficiently synergies with c-Jun but not with other family members

The structure of p73 gene is highly complex when compared to that of p53. The p73-α contains the SAM domain near the C-terminus but not p73-β. To find out whether there is any difference in the ability of p73 isoforms to increase AP1 activity, H1299 cells were transfected with indicated combinations of plasmids in addition to 5XTRE-luc and ß-gal plasmids. As shown in figure 3.3.3.3**,** the co-transfection of both p73-β and c-Jun reproducibly resulted in a moderate increase of the AP1 activity, as measured by luciferase and ß-gal assays. In contrast, none of the other p73 family members increase AP1 activity as much as p73-ß does. Furthermore, p73-α did not co-operate efficiently with c-Jun to increase AP1 activity. Of note, SAM domain may not be essential for p73's ability to potentiate AP-1 activity, as p73-β is much more efficient than p73-α.

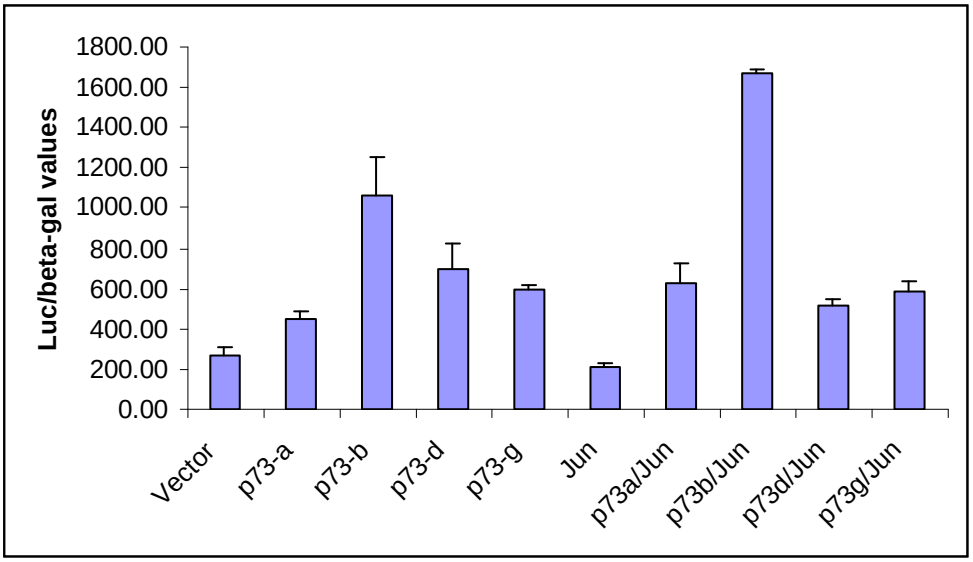

Figure 3.3.3.3 p73-ß synergies with c-Jun better than p73-α and other p73 family members

H1299 (75% confluence in 35mm plates) cells were transfected with p73α (100ng), p73β (100ng) alone or with c-Jun (100ng) and the luciferase reporter plasmid (100ng) driven by the 5XTRE motif and 500ng ß-gal plasmid (as an internal control). The total amount of plasmid DNA used for transfection was adjusted to 3.1µg by adding an empty pCDNA3. Transfected cells were collected after 48 hours and luciferse activity was measured. Luciferase activity was normalized with β-galactocidase activity.

3.3.4 Structural requirements of c-Jun and p73 in potentiating AP-1 activity

3.3.4.1 The effect of c-Jun mutants on the abilty of p73-β to stimulate AP1 activity

To identify which domain of c-Jun is important for its ability to cooperate with p73-β, p73 and a series of c-Jun mutants (Jun N (Δ 1-194); JunC (Δ 194-334); TAM (Δ 142-334); c-Jun-d-domain (Δ30-57); c-Jund-NLS (Δ1-232)) were co-transfected into H1299 cells. As shown in figure 3.3.4.1, although c-Jun mutants synergized with p73-β to potentiate 5XTRE-luc activity, they were not as good as wild-type c-Jun. Interestingly, c-Jun mutant lacking δ domain, which is similar to v-Jun, synergized with p73-β much better than wild-type c-Jun. This could be because c-Jun-δ-domain mutant is constitututively active and more stable than wild-type c-Jun.

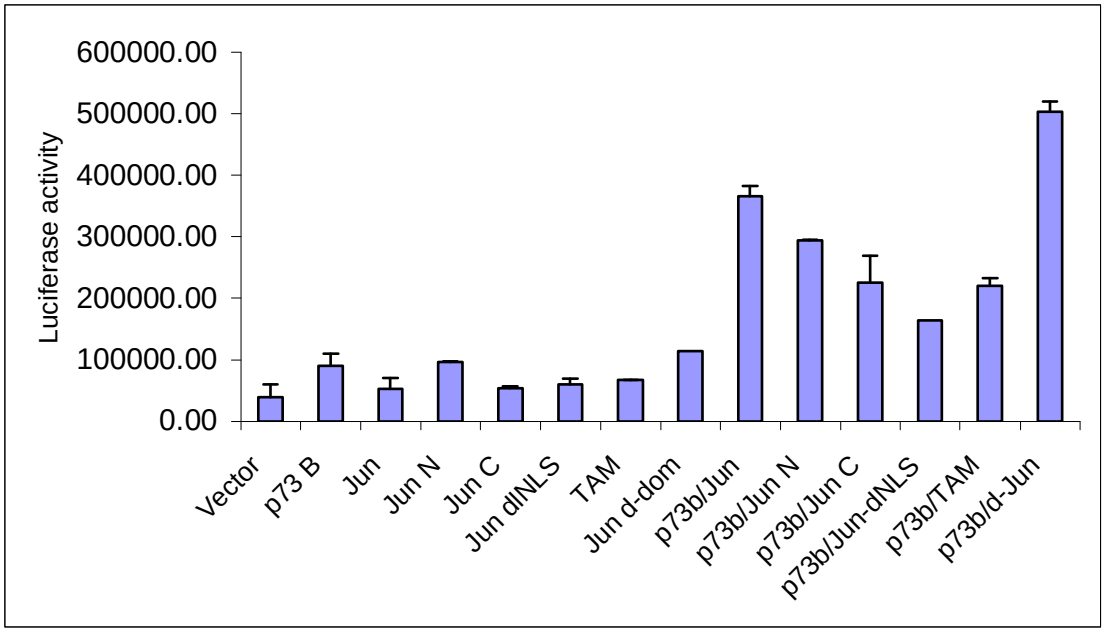

Figure 3.3.4.1 The effect of c-Jun mutants on the abilty of p73-β to stimulate AP1 activity.

H1299 cells were transfected with PCDNA, vector control, p73-β, and c-Jun, N-c-Jun (1-194), C-c-Jun (194-334), TAM (142-334); c-Jun-d-domain (30-57); c-Jun-NLS (1-232) separately, or in combination. In addition, reporter plasmids expressing the luciferase under the control of 5XTRE promoters and a plasmid encoding the ß-gal promoter were co-transfected. The total amount of plasmid DNA used for transfection was adjusted to 3.2µg by adding PCDNA3. Transfected cells were collected 48h after transfection and both luciferase activity and ß-gal activity in cell extracts were measured.

3.3.4.2 TAD1 domain of p73 is essential to cooperate with c-Jun

N-terminus of p73 contains transactivation domain, which shares 34% sequence identity with p53 though it is not the only transactiavtion domain proposed. It seems that p73 has an additional glutamine rich transactivation domain near the c-terminus. In an effort to understand whether N-terminus of p73 is important for its ability to transactivate AP-1 responsive element containing promoters, indicated vectors (figure 3.3.4.2) were transfected either alone or in combination. Although DeltaN-p73 increases 5XTRE activity, it was not as efficient as p73-ß, indicating that N-terminus of p73 is not essential for its ability to transactivate 5XTRE elements. However, delta N-p73 failed to co-operate with c-jun in transactivating 5XTRE elements, indicating a possibility that c-Jun binds to the N-terminus of p73 and thereby augments its ability to stimulate AP-1 elements.

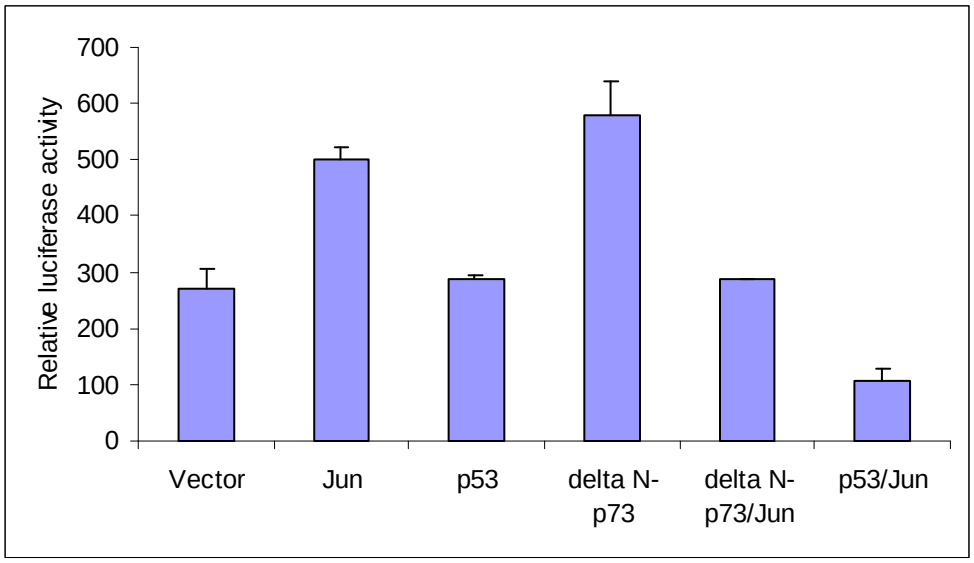

Figure 3.3.4.2 N-terminus of p73 is not essential for its ability to transactivate AP-1 responsive element but for its ability to co-operate with c-Jun

H1299 cells were transfected with vectors encoading PCDNA3, p73-ß, c-Jun, delta N-p73, p53 separately, or in combination with either delta N-p73 or p53 and a plasmid expressing the luciferase gene under the control of 5XTRE promoters and a plasmid encoding the ß-gal promoter for evaluating the transfection efficiency. The total amount of plasmid DNA used for transfection was adjusted to 3.2µg by adding PCDNA3. Transfected cells were collected 48h after transfection and both luciferase activity and ß-gal activity in cell extracts were measured. This reporter assay was part of the earlier experiment, just for the clarity sake it is presented here separately.

3.3.5 The role of Post translational modifications

3.3.5.1 JNK phosporylation is (63/73a.a) required, but dispensable for c-Jun's ability to co-operate with p73

To check whether JNK-mediated c-Jun phosphorylation is required for its ability to co-operate with p73 to transactivate AP-1 (5X TRE) response elements, p73, c-Jun and its phosporylation mutants (Jun63A/73A and 5XASP, serine residues are replaced with aspartate residues) were transfected separately or in combination in HI299 cells, which do not produce p53 protein. As shown in figure 3.3.5.1**,** though c-Jun 63/73 co-operated with p73 in transactivating 5XTRE elements, it was not as much as wild-type c-Jun. On the other hand, phosporylation-mimicking (5XASP) c-Jun co-operated with p73 in comparison to wild-type c-Jun, indicating that phosporylation is important for its ability to transactivate 5XTRE elements.

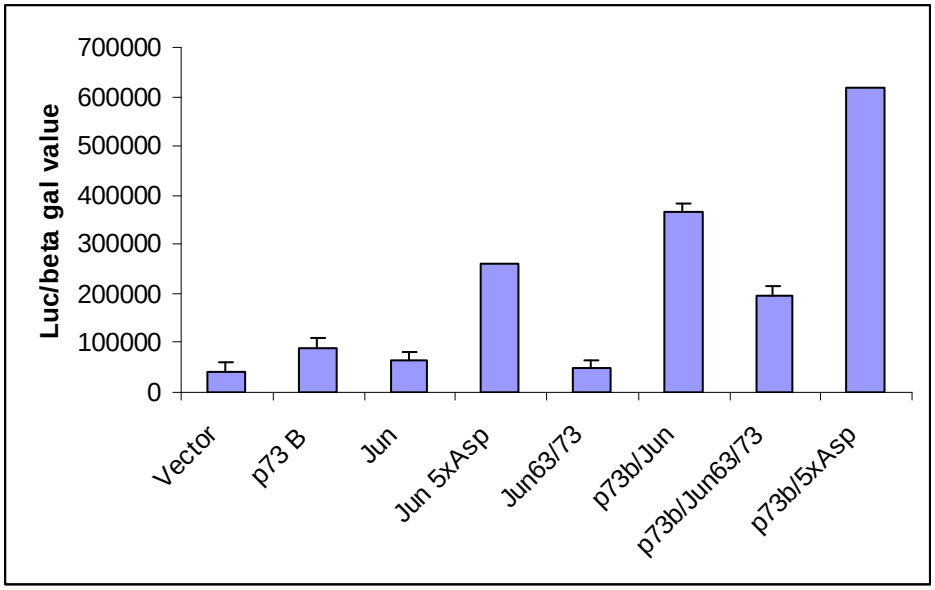

Figure 3.3.5.1 Phosphorlylation of c-Jun is required for its ability to co-operate with p73

H1299 cells were transfected with reporter plasmids 0.5µg 5XTE-luc, 0.5µg ß-gal (for evaluating the transfection efficiency) and expression vectors encoding p73β (100ng), c-Jun (2µg), Jun 5XASP (2µg), Jun S63/73A (2µg), and PCDNA3. The total amount of plasmid DNA used for transfection was adjusted to 3.1µgm by adding PCDNA3. Luciferase activity was measured 48h after transfection and normalized for transfection efficiency with ß-gal activity.

3.3.5.2 c-Abl negatively regulates c-Jun's ability to co-operate with p73 in increasing AP-1 activity

c-Abl has been shown to increase the ability of p73 to induce apoptosis by directly phosphorylating it (Yuan et al., 1999; Agami et al., 1999; Gong et al., 1999). c-Abl is also known to phosporlylate c-Jun at tyrosine 170. The tyrosine phosporlyated c-Jun, promotes a positive feedback loop by enabling c-Abl to enter into the nucleus (Barila et al., 2000). In order to find out whether c-Abl influences c-Jun to co-operate with p73 to increase AP1 activity, H1299 cells were transfected with the indicated vectors (figure 3.3.5.2). c-Jun co-operated with p73 better than tyrosine phosphorlyation mutant of c-Jun (Y170→F170). This data suggests that c-Abl down regulates the ability of p73-β to stimulate AP-1 activity.

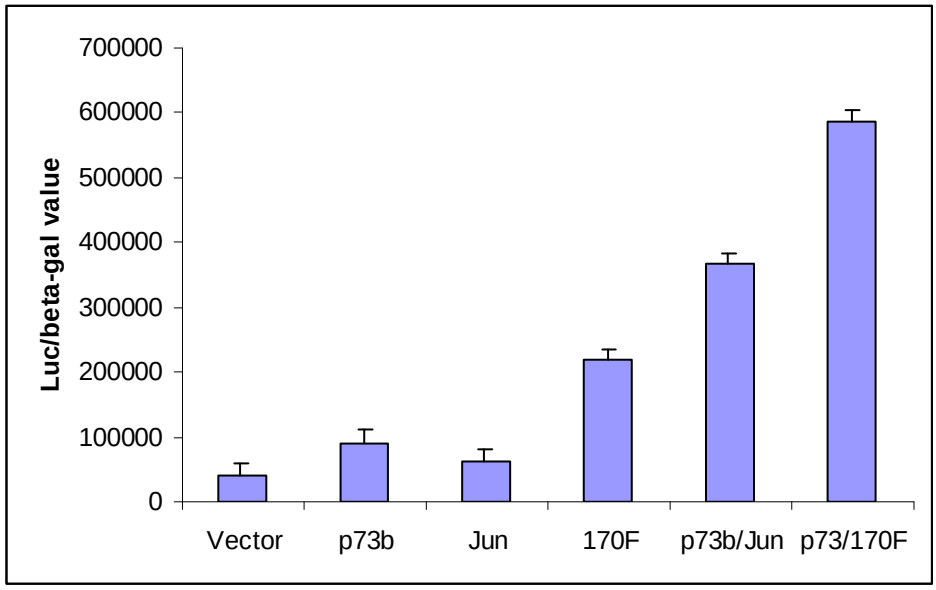

Figure 3.3.5.2 C-Abl negatively regulates the ability of p73 to increase AP1 activity

H1299 cells were transfected with reporter plasmids 5XTRE-luc, beta-gal, expression vectors PcDNA3, PcDNA3-p73-ß, PcDNA3-c-Jun, PcDNA3-Jun170F separately and in combination with either c-Jun or c-Jun 170F. Transfection efficiency was controlled by cotransfection of a ß-gal construct. The total amount of plasmid DNA used for transfection was adjusted to 3.2µg by adding PCDNA3. Transfected cells were collected 48 hours after transfection for luciferase and ß-gal assays. Luciferase activity was normalized by the internal ß-gal activity.

3.3.6 Dominant negative p73 (p73 DD) inhibits AP-1 transcriptional activity.

3.3.6.1 Dominant negative p73 (p73 DD) inhibits AP-1 transcriptional activity.

The p73-DD encodes (315a.a-636 a.a) carboxyl terminus of p73, which retains the oligomerization domain. It has the capacity to oligomerize with wild-type p73 to inactivate its transcriptional activity towards p53 responsive elements (Jost et al., 1997). To clarify whether p73 activity is essential for the AP-1 activity, we checked if the endogenous AP-1 activity was brought down by the p73 DD in H1299 (*p53*$^{-/-}$) cell line. Remarkably, 5X-TRE-luc activity was lowered by the dominant negative p73, indicating that p73 contributes to the AP-1 transcriptional activity (Figure. 3.3.6.1). Intriguingly, the basal AP-1(5XTRE) activity seen with PCDNA3 is diminished in the presence of p73DD. Thus, this data suggests that p73 contributes to the basal AP-1 activity.

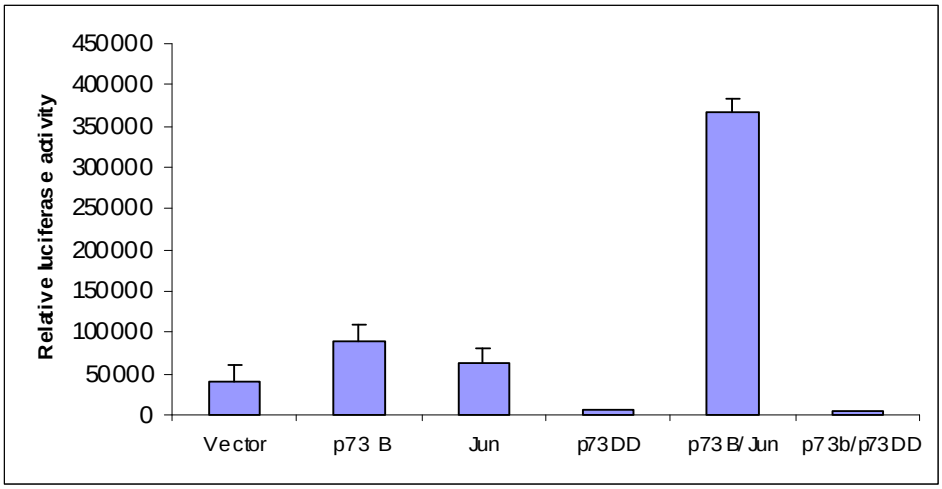

Figure 3.3.6.1 Dominant negative p73 inhibits AP-1 transcrptional activity

H1299 cells were transfected with PcDNA3, p73-ß, c-Jun, p73 DD separately and in combination with either c-Jun or p73DD and reporter plasmids expressing the luciferase gene under the control of 5XTRE promoters and a plasmid encoding the ß-gal promoter for evaluating the transfection efficiency. The total amount of plasmid DNA used for transfection was adjusted to 3.2µgm by adding an empty PCDNA3. Transfected cells were collected 48 h after transfection and both luciferase activity and ß-gal activity in cell extracts were measured.

3.3.6.2 p73's ability to stimulate AP-1 transcription in $p53^{-/-}$ $jun^{-/-}$ null cells

To check whether p73 induces the AP-1 transcriptional activity through c-Jun, increasing concentration of p73 was transfected in *$p53^{-/-}$c-$Jun^{-/-}$* null fibroblasts. As shown in figure 3.3.6.2, increasing concentration of p73β mildly increases luciferase/ß-gal ratio. This data may suggest that the ability of p73 to stimulate AP-1 activity is not completely compromised in the absence of c-Jun. However, it is important to note only mild increase is noted.

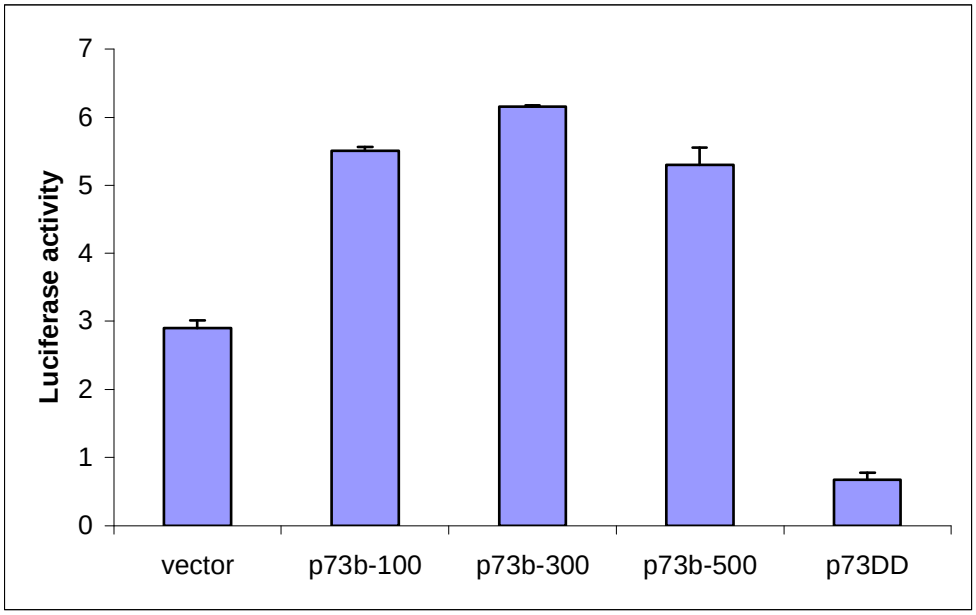

Figure 3.3.6.2 Dose effect of p73β on the reporter expression from 5XTRE in $p53^{-/-}$ $Jun^{-/-}$ fibroblasts

$p53^{-/-}Jun^{-/-}$ cells were transfected with reporter plasmid 0.5µg 5XTE-luc, 0.5µg ß-gal plasmids and increasing amount of p73 and p73DD. The total amount of plasmid DNA used for transfecteion was adjusted to 3.1µg by adding PCDNA3. Luciferase activity was measured 48h after trasfection and normalized for transfection efficiency with ß-gal activity.

3.3.6.3 p73 synergizes with c-Jun in potentiating hMSH2 promoter (p53RE and AP-1) activity.

The following facts support the idea that p73/c-Jun could regulate the expression of hMSH2 promoter:

1. Treatment of cells with tetradecanoylphorbol- 13-acetate (TPA) specifically increases the amount of p73 mRNA (Fontemaggi et al., 2001).

2. p73 increases the AP1 activity.

3. TPA is known to induce AP-1/c-Jun activity.

4. hMSH2 contains AP-1 responsive elements.

As shown in figure 3.3.6.3, the synergism between p73 and c-Jun when they were co-transfected can be explained on the basis of the presence of binding sites for both p53 and AP-1 in the *hMSH2* promoter sequence. However, the *hMSH2* transcription is clearly independent of p53, since the SAOS2 cell line used in this is a p53 null producer.

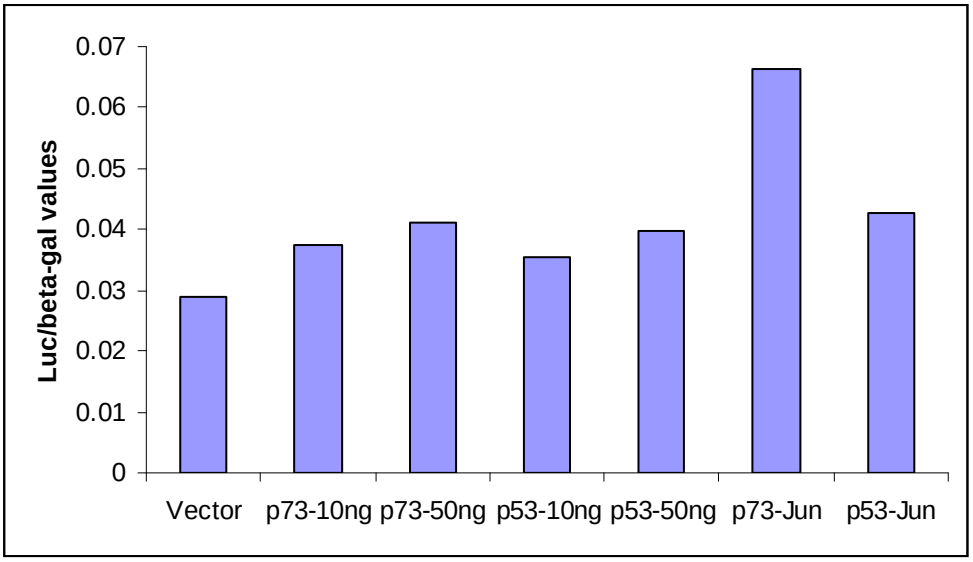

Figure 3.3.6.3 p73 cooperates with c-Jun in potentiating MSH2 promoter (p53RE and AP-1) activity

Saos2 cells were transfected with reporter plasmid hMSH2 promoter-luc(5µg), ß-gal plasmids(0.5µg) and increasing amount of p73(10ng to 100ng), and p53. The total amount of plasmid DNA used for transfection was kept constant by adding PCDNA3. Luciferase activity was measured 48h after trasfection and normalized for transfection efficiency with ß-gal activity. Transfected cells were collected 48 hours after transfection and both luciferase activity and ß-gal activity in cell extracts was measured.

3.3.6.4 p73 promoter contains AP-1 like responsive elements

Fontemaggi et al., (2001) have shown that TPA, a potent inducer of c-Jun/AP-1 activity (Lamph, 1988), increases TAp73 mRNA levels, which indicates a possibility that AP-1/c-Jun could also regulate TAp73 at the transcriptional level. These observations prompted us to analyze TAp73 promoter for any AP-1 like responsive elements. As shown below, promoter analysis ranging from −2713/+1 revealed the presence of a consensus binding site for the transcription factor AP-1. The exact localization of this sequence (5'-TGA C/G TCA-3') is circled in figure 3.3.6.4. Promoter analysis of the human TA-p73 promoter revealed seven putative AP-1-like binding sites. The identification of AP-1 like sequences in the p73 promoter indicates a possibility that c-Jun/AP-1 family of proteins can regulate p73 levels—in a context dependent manner— at the transcriptional level.

p73 promoter sequence accession number:. E51345.

AP1 sites: (5'-TGA G/C TCA-3').

No.	AP-1 like sites	Positions
1	tga a tac	127-134
2	tga g cac	414-420
3*	tga ga tca	830-838
4	tgg a tcg	890-897
6	tga g cca	1411-1417
7	cga g tca	1428-1434

1 gcggccgccc ccggccctgc ccgccgggga cgctggcacc gaggatgtcc tgcccgtggc
61 ccaggtcccc gccgctcacc aggtacttgc cgtccgggga gaacttgcag agtaagctgg
121 agagct tga a tac ctcggag aagttcatgg ccgccgcctg ccgcgggcgc caccctgcgc
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421 ctccagctag ggctgctgat aaaaatgtag aaagcacagt aaaatt tgaatttca gattc
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601 acacatgcat a tata tata t ttatatttata tgtaaatgt atgtttacat gtaaatatat
661 gtttacctac aaatatatct ttaataagta atacggtgtc tgtcgcacat atattatatc
721 gtgtatgtaa tgtataagta tttatttcgt ttgcttgggg ttttgtttgc ttttgctgag
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1381 ccccaccccg tgcgcccagc aaacagtggg tgagcca tga agatg tg cgagtca gccgga
1441 ccctccccgt caggcgcgga cccgctgcgg ccagagaacc cagtctgcgc cagcccggct
1501 cgctcgcgaa gccacgggct tcactgacgc gactttccaa gacgtggggg tcaccatggg
1561 cagaggacat cggttcggag ccagatcacg ggccccataa gcatcagacc ataagcagcg
1621 ccgccactga gagccgctcg gaactcgccc agcatgtcgg gtcccctagc cagggcctgg
1681 tgtacgtggt cgagggccct ggaagccccg atggcctagg aggagcaggc gggcggggcg
1741 gcgggtgtcg ctggccggta gagagcttcg gcctgaccta gcgcaggtct ggtgcgcgca
1801 gagaacaact ccaagcgcac cgacgcccgc gagctccttc caaacaccga acgggatcca
1861 gagcccgagc ccacaggcgg cggccggggg agggagcagg gtgctggccg ccgcccggga
1921 gtgttcgcgt cctgggtgac ccctggaagg acgtggggcc caaactccgg ctggggttgg
1981 gagagcagcc cccagaggct ctccgcggga tcctctgccg ggcgggaccg tggctccaca
2041 ggagaagtgg gtggcaagcc ctgcttggcg gaaagcagcc gttcccctcc tcctgggcct
2101 ggggcggcgc ccctcacccc tgttccccgc ccctcacccc tgttccccgc cggccacatc
2161 ccctgcccct tggattccaa gcgccccgcg cgccgaggag cccagcgcta gtggcggcgg
2221 ccaggagaga cccgggtgtc aggaaagatg ggccgtctgg gggacagcag ggagtccggg
2281 ggaaacgcag gcgtcgggca cagagtcggc accggcgtcc ccagctctgc cgaagatcgc
2341 ggtcgggtct ggcccgcggg aggggccctg gcgccggacc tgcttcggcc ctgcgtgggc
2401 ggcctcgccg ggctctgcag gagcgacgcg cgccaaaagg cggcgggaag gaggcggggc
2461 agagcgcgcc cgggaccccg acttggacgc ggccagctgg agaggcggag cgccgggagg
2521 agaccttggc cccgccgcga ctcggtggcc cgcgctgcct tcccgcgcgc cgggctaaaa
2581 aggcgctaac gcccgcggcc gcctactccc cgcggcgcct cccctccccg cgcccatata
2641 acccgcctag gggccgggca gcccgccctg cctccccgcc cgcgcacccg cccggaggct
2701 cgcgcgcccg cga

Figure 3.3.6.4 p73 promoter possesses AP-1 like responsive elements

The promoter analysis of TA p73 promoter (TAp73 promoter sequence accession number-E51345) has identified at least seven AP-1 sequences (highlighted in blue).

3.3.6.5 ΔNp73 promoter contains AP-1 like responsive elements

Costanzo et al have suggested in abstract presented in the ESDR meeting (2002) that deltaNp73 promoter can be regulated by both c-Jun and p53. However, whether deltaN p73 promoter has AP-1 elements is less clear from this study. Interestingly, deltaN p73 promoter could be analogous to MSH-1 promoter, as it has been shown to encode both p53 and AP-1 RE at the proximal sites and regulated by both c-Jun and p53. Similarly, MDM2 promoter contains both AP1 and p53RE in its promoter and is subjected to the regulation both by AP-1/c-Jun and p53 (Ries et al., 2000). Therefore, deltaNp73 promoter was analyzed carefully for any AP-1 like responsive elements. Interestingly, as shown below, deltaNp73 promoter analysis ranging from - 2940/+1 revealed the presence of a consensus binding site for the transcription factor AP-1. The exact localization of this sequence (5'-TGA C/G TCA-3') is circled in figure 3.3.6.5. This promoter analysis revealed seven putative AP-1-like binding sites. The identification of AP-1 like elements in deltaNp73 promoter analysis suggests that human deltaNp73 promoter can be synergistically potentiated by p73 and c-Jun/AP-1 transcription factors. Further, it indicates the possible existence of regulatory loop between deltaNp73 and p73/c-Jun.

DeltaNp73 promoter sequence accession number: intron 3, which includes the DeltaNp73 promoter.

No.	AP-1 like sites	Positions
1	tca gc tca	60-67
2	tga c aca	235-242
3*	tga g tca	454-460
4	tag c tca	662-668
5	tgc a tca	883-889
6	tga aa tac	1841-1847
7	tga a tac	2547-2553

* AP-1 site ; The highlighted sequence in blue represents AP-1 like site

1 ccagggagga tctgtagctg gaggaagggt ggggtcatgc gtgggagcag ggaggggct
61 cagctcacca cggtcagctc tgagactcca gcccacccgt taccccctcc cagagagccc
121 ccactcagcc tttcctttgg tgggctttcg tgacaaagca ctttggggct gcacagaagt
181 gaaccccacc cagcacccag gtctcagagc cttgcagctt ctgcggcctc ttccatgcgg
241 tgggatgaag ccagctgccc agcagggacc ctgtgccatg agtttggcct tgaactgaca
301 ca tcactggc accaggaaac gaagtccccc tgtctgttct ggcacataac ccctcccact
361 aactggttcc tgaagagtgc cgtggcctgc ggcagcgtcg ttcccccctg tcctgcggcc
421 cagggtcctgcggaaagtca ggcggaatcc ccgg tgag tca gaagcagaa tgaaagcaga
481atggaggacccagcagggag ggaacctgga ggaggcgcta agggccacgc caaggggtg
541 tggccccaga tcccctgtcc ctgtcctctg caaggctggg ccttgggaac gtttgcagaa
601 agctgggtgc cgctctgggg cagaggccag tggttttggg tgcttttgag ttggaaacgt
661gtagctcagc cgcactggga tccccgcagc ctggcccaga tgctaagggt ggagagatgc
721 ggggtctcag gcacggtgcc ctgggcatgg gtggggctcg tgctgaaggc agcctggctg
781 tcttccttcc tcacgtcctt ccacttggcg ctctcctttt ggctatttat aaaaccatca
841 ggccggccctgtgcatggga ctcgcctgagtctccttttc aa tgc a tca t tccctttggc
901 aggagaggac accgcctaca gaggctgagg atgtgccctg tgggggtcgg gagcggaacc
961 caggccccgc ctcggccctg ctctgagggt ctgtccatcc ctggggagcc cgcccccaac

1021 ccaagagggg tcccaggctc agaagcagaa ggcaccctca tccccagggc atccccgatc
1081 ccagcaggag tctcctagtg ctcgccctgg gctctcctgc aaggaggctg ctgctttccc
1141 cagaacatcc agtctgggcc ccagccgacc ccctgcaggg ggcttcccag agacgccctt
1201 cctgaacctg atctaccaga caaaactgtc tttttctcag tcgtctcctc ctgagtgctg
1261 ctgcccttcc tgttgggggc tgagatcctc tgccacagga agagacgggc gtccaggact
1321 cacctgctgc ctcccggccc tagggccctg agctgggctc tccaggcccc agccccttgg
1381 ggcacaacac ctggaatcgt cctttcgtcc tcagcccggc ctgctggtgg ggcagggcgg
1441 gtccccaggg ctcctcaggc agctgcagtc caaacctccc ctgccctcac ccagctctgc
1501 ccgctctccc gggggtgggg gtggggagcg atgaggcccc tgccggctct cggtggggac
1561 gacagggagg aaggaagctg gggagatgga gacaagagaa agcaggcagg tggtttggga
1621 tttggcagga aaaggttgga aggaaagggg aaagggtctc cgcatggatt tctca gctcc
1681 ccatggattt ctca gccctc gtgagagcca cggcgccctg gggactggaa gtgtgggtcc
1741 gcaggcccca gtccccaggt ttgtctgagc atagatgccc tgcctgcttc caggggact
1801 cgggcccctc tgccagggtc aactttgtac ccaagacggc tga aa tacaa tggaaattca
1861 gacggcccaa cagggag tgg cag tca cctc aaaggcccca ctagacgggt gcggggcacc
1921 actgcagagc ccctccctgg ctgtgccaag gccgtccacg cctgcagggg gccccactgc
1981 cgggctgttc tttggcaaca gtggcttgtc cctgtttcct gggggcttgg ccagtgccag
2041 ggtgggctcc aaacgcacgg ctctgggctc ttggactcac ccctgctttg ggcaggcagt
2101 ggaaggcagg ccccacaaga gctgctcact cccgtcacct gtctccctcg ggggtctagg
2161 gtcgaacctc ctgtgagccc ctcctctcca tgcagccctt ggactagtcc tggcggacca
2221 ccgagttccc cgcgcagggg gcaggtgcgc cccacctggg tgccaaggga ggcgacacca
2281 tctctccccc ttggggtggc ccagccttgc ctaccatgat ctccagggcc ggggctcagc
2341 cctcatgcct gggaacagag gctgctttac ggggtgaggg cctggggccc cccgagcctt
2401 ccccaggcag gcagcatctc ggaaggagcc ctggtgggtt taattatgga gccggcgctg
2461 accggcgtcc ccgccctccc cacgcagcct ccttggtgcg gtccaacaca tcaccgggca
2521 agctgaggcctgccccggac ttgga tga a tac tca tgagg aataaagggg tgggccgcgg
2581 gttttgttgt tggattcagc cagttgacag aactaaggga gatgggaaaa gcgaaaatgc
2641 caacaaacgg cccgcatgtt ccccagcatc ctcggctcct gcctcactag ctgcggagcc
2701 tctcccgctc ggtccacgct gccgggcggc cacgaccgtg acccttcccc tcgggccgcc
2761 cagatccatg cctcgtccca cgggacacca gttccctggc gtgtgcagac cccccggcgc
2821 ctaccatgct gtacgtcggt gaccccgcac ggcacctcgc cacggtaggt gtgacgcgcc

Figure *3.3.6.5* DN p73 promoter encoades AP-1 like responsive elements

The promoter analysis of DN p73 promoter (DeltaNp73 promoter sequence accession number ACCESSION AB055067) has identified at least seven AP-1 sequences (highlighted in blue).

CHAPTER III

RESULTS

SECTION IV: P73 AND C-JUN COOPERATE IN TRANSFORMATION

3.4.1 Background and Hypothesis: p73 and c-Jun can cooperate in transformation

3.4.1.1 p73 is over expressed in various human cancers

Whether p73 is a tumor suppressor or a proliferation associated gene is still under immense scrutiny. However, it is certain that p73 is not a classical tumor suppressor gene that would follow Knudson's two hit hypothesis (Melino et al., 2002). Despite the search in 900 human tumors for mutation in p73 gene, the results were unfruitful for cancer biologists (Zaika et al., 2002; Stiewe et al., 2002).

Current studies strongly suggest that p73 appears to be over expressed in a variety of tumors including neuroblastomas, ependymomas, hepatocellular, gastric, breast, bladder, ovarian, and esophageal cancers compared with their normal tissue counterparts (Moll et al., 2001). Importantly, p73 over expression appears to be positively correlated with prognostically relevant parameters (Petrenko et al., 2003; Sun, 2002). The correlations between high-level expression of p73 and various prognostic factors indicate that p73 could function like an oncogene to enhance tumor progression.

3.4.1.2 Role of c-Jun/JNK in supporting transformation events in tumor cells

It has been shown earlier that both c-Jun and JNK play an important role in tumor cells. The following evidence suggests a role for both c-Jun and JNK in transformation and tumor cell survival:

1. The AP-1 activity is required for tumor promotion (Young, et al., 2002).
2. Ras induced transformation requires c-Jun (Johnson et al., 1996)
3. Ras induces c-Jun phosphorlyation on sites that are phosphorlyated by JNK (Pulvrer et al., 1991; Smeal et al., 1991).
4. Knock-in studies demonstrated that mutation of the JNK phosphorlyation sites on c-Jun prevented Ras-induced tumorigeneicity (Behrens et al., 2000)
5. In addition, it has been shown that JNK is constitutively activated in several tumor cell lines and that the transforming actions of several oncogenes (for eg., BCR ABL) have been reported to be JNK dependent (Ip and Davis, 1998).

These accumulated data support the hypothesis that both c-Jun and JNK are relevant to cancer.

3.4.1.3 Hypothesis: p73 and c-Jun can cooperate in transformation

The p73 (wild-type) is over expressed in variety of tumor cells. However, it is unclear why TA-p73 did not prevent tumorigenesis, especially when it is expressed in the wild-type form. Why would tumors need to retain factors or express factors that would make them more sensitive to apoptotic stimuli? Further, it is intriguing to note that *p73*$^{-/-}$ cells slowly exit from S-phase in response to UV irradiation, indicating that it could play a role for p73 in G1-S phase transition/proliferation (Costanzo et al., 2002). Alternatively, it may suggest that p73 does not act as a UV checkpoint but rather counteracting checkpoint function. Indeed, p73 expression is itself modulated during the cell cycle and TAp73 proteins accumulate in S phase. In addition, the function of p73 proteins is also regulated by post-translational modifications and protein-protein interactions in different cellular and pathophysiological contexts (Fulco et al., 2003). Furthermore, it has been shown that cyclin-dependent kinases phosphorylate p73 at threonine 86 in a cell cycle-dependent manner and negatively regulate p73 function. All p73 proteins have a Cyclin Recognition Motif located within the N-terminal portion of the DNA binding domain (KKL; 149) (Gaiddon et al., 2003). Alternatively, over expressed dominant negative p73 or interacting oncogenes could modulate TA-p73 function in tumor cells. Intriguingly, p73 is regulated by oncoproteins such as c-Myc, MMI, c-Jun, MDM2, and mutant p53 etc. All these proteins could effectively bind to p73 in tumors and suppress its p53 related functions. Similarly, during the tumor development Jun/JNK-dependent stress induced apoptosis must be suppressed or adopt mechanisms to suppress Jun/JNK-dependent apoptosis. It is also possible that tumor cells may activate survival pathways

that function dominantly with respect to the JNK/Jun pro-apoptotic pathway. JNK/Jun could even function differently in tumors and normal cells. Evidently, it has been demonstrated that JNK is required for stress-induced apoptosis of primary cells (Garay et al, 2000; Ho et al., 2002). In contrast, induction of apoptosis and inhibition of growth of tumor cells were noted when tumor cells were transfected with anti-sense JNK nucleotides (Bost et al., 1999; Patopova et al., 2000). These results imply that JNK supports transformation in tumor cells.

Together, all these critical facts and questions raise the possibility that the function of p73 is modulated in tumor microenvironment and p73's "p53 related functions" are compromised in cancer cells. Primarily, p73 and other oncogenes such as c-myc and c-Jun have the capacity to induce apoptosis when they are over expressed. However, it does not happen efficiently in tumors, otherwise tumorigenesis would be prevented. Alternatively, the majority of tumor cells would have undergone apoptosis and a few surviving cells could have learnt to modulate the functions of these proteins through protein-protein interactions and developed as clones. This onco-suppressor protein function is tuned or modulated according to the needs of the cancer cells to sustain proliferation and this could one of the reasons why p73 is over expressed in tumors.

Keeping all these facts in mind, it was hypothesized that both p73 and c-Jun can cooperate with each other in transformation.

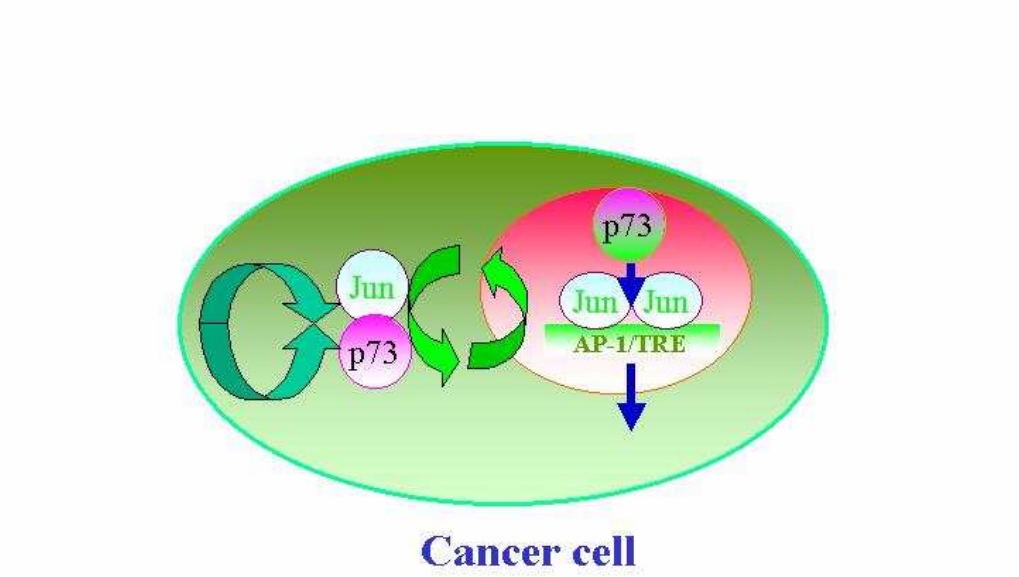


Figure 3.4.1.1 Hypothesis: p73 and c-Jun can cooperate in transformation

It appears that AP-1 activity is required for tumor promotion (Young, et al., 2002) in the mouse model. c-Jun is specifically activated in several tumor cell lines and AP-1 activity is highly pronounced in tumors (Shualin et al., 2002). Furthermore, both p73 and c-Jun synergistically increase AP1 activity. These findings may support the hypothesis that p73 and c-Jun can cooperate with each other in transformation.

3.4.2 p73 transforms fibroblasts in conjuction with c-Jun.

3.4.2.1 p73 α/β inhibits colony formation.

NIH 3T3 cells were transfected with pCDNA, p73 alpha and p73-beta and selected in G418 for about two weeks. As shown in figure (3.4.2.1), transfection of either p73α or p73β did not give rise to any macroscopic colonies. The suppression of colony formation could be due to apoptosis. In contrast, many drug resistant colonies were formed following transfection with the empty vector (pCDNA3). This confirms the earlier reports that p73 induces apoptosis when it is overproduced.

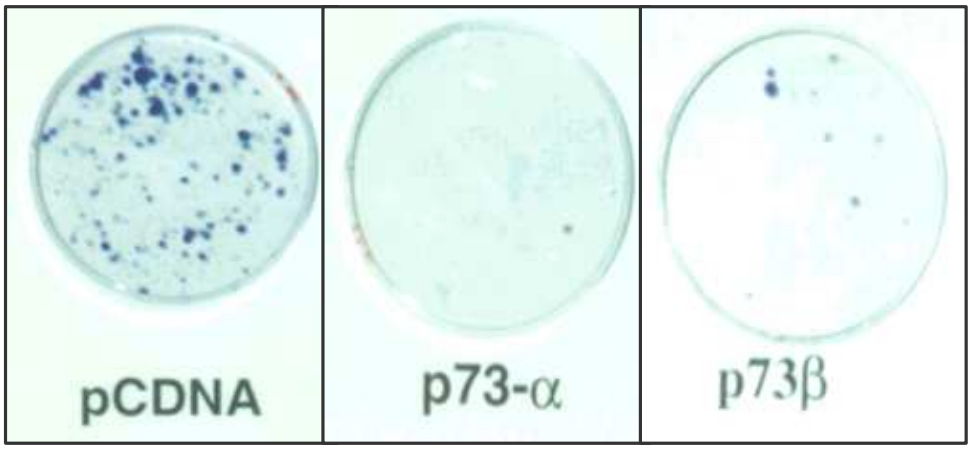


Figure 3.4.2.1 p73α/β inhibit colony formation

NIH 3T3 cells were transfected with a pcDNA that conferred G418 /neomycin resistance and pcDNA plasmids expressing p73α and -β (2 micrograms each). For colony formation assay $5X10^4$ cells per 10 cm plate were transfected with a neomycin plasmid and were grown for 48 h before 750 μgm/ml of neomycin was added. After 2 weeks, colonies were fixed and visualized by staining with crystal violet. Note that the inhibition of colony formation by p73α is much better than p73β.

3.4.2.2 p73 and c-Jun co-operate in transformation

TA-p73 (wild-type) is over expressed in many human tumors (Moll et al., 2002). However, it fails to induce apoptosis to prevent tumor formation. In addition, its putative target genes (p53 responsive element encoding genes such p21, Bax, p53AIP1) seem to be not upregulated in tumors. One could argue that delta N-p73, which has the ability to inactivate both p73 and p53 are also over expressed in tumors. Thus, it was hypothesized that wild-type transcriptionally active p73 (TA-p73) alpha and beta cannot prevent proliferation in the presence of oncogenic signals provided by c-Jun. This hypothesis was tested by transfecting wild-type pCDNA-p73 alpha/beta together with pCDNA-c-Jun in immortalized fibroblast cell lines such as NIH 3T3 cells and selected in G418/Neomycin 750-1000(microgram/ml). As shown in the figure 3.4.2.2, p73 increased the number of colony formation in the presence of c-Jun, in comparison to vector (pcDNA) and c-Jun. This result indicates that c-Jun modulates the function of p73 in such a way that it supports the transformation process, as Jun has been shown to support transformation in conjuction with other established oncogenes.

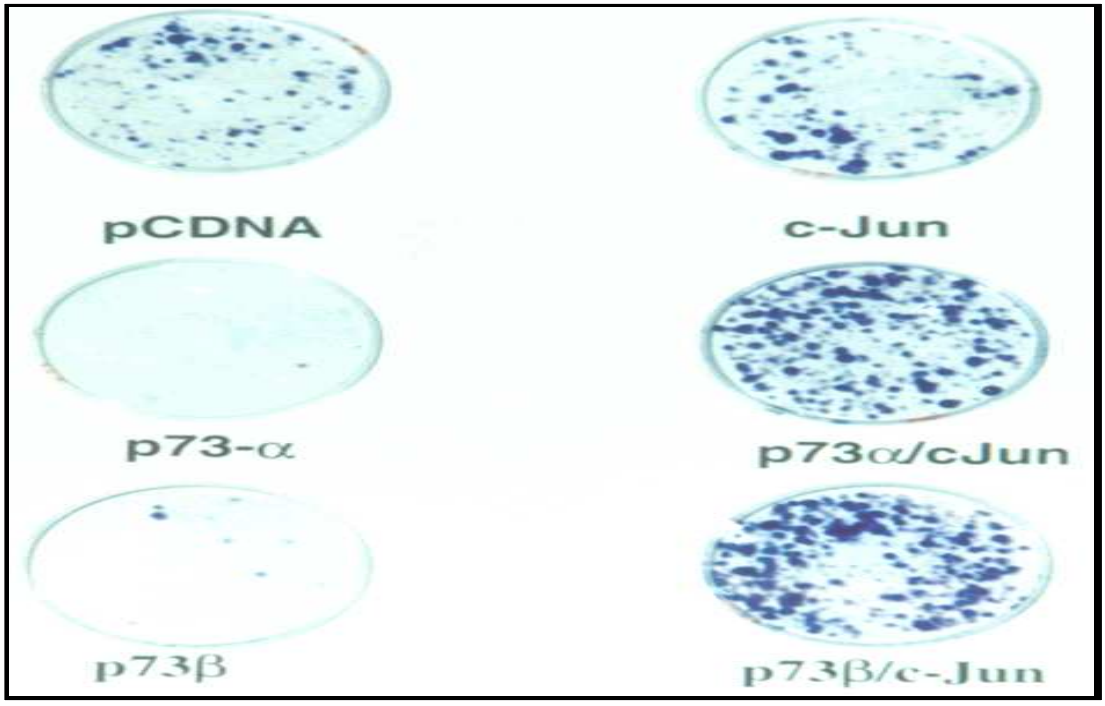


Figure 3.4.2.2 p73α/β transform fibroblasts in co-operation with c-Jun

NIH 3T3 cells were transfected with a pcDNA that conferred G418 /Neomycin resistance and pcDNA plasmids expressing p73α and p73-β (2μg each). For colony formation assay $5X10^4$ cells per 10 cm plate were transfected with a Neomycin plasmid and allowed to grow for 48 h before 750 μg/ml of Neomycin was added. After another 2 weeks, colonies were fixed and visualized by staining with crystal violet. Note that inhibition of colony formation by p73α is much better than p73β.

3.4.3 c-Jun mutants ability to modulate p73 function

3.4.3.1 c-Jun mutants cooperate with p73 in transformation: Either N or C-termini of c-Jun co-operates with p73 in transformation

In the earlier chapter (chapter 2) it was shown that both N and C-termini of c-Jun (1-194 and 194-334, respectively) cooperate with p73 in inducing AP1 activity. To check how the binding of N and C-termini of c-Jun influences p73 in promoting transformation, NIH 3T3 cells were transfected with indicated vectors. As shown in figure, p73 increased the number of colony formation even in the presence of either N or C- termini of c-Jun independently (Figure 3.4.3.1). Interestingly, both N and C-termini of c-Jun by itself reduces the colony number compared to full-length c-Jun, but in the presence of p73 it increased the colony number.

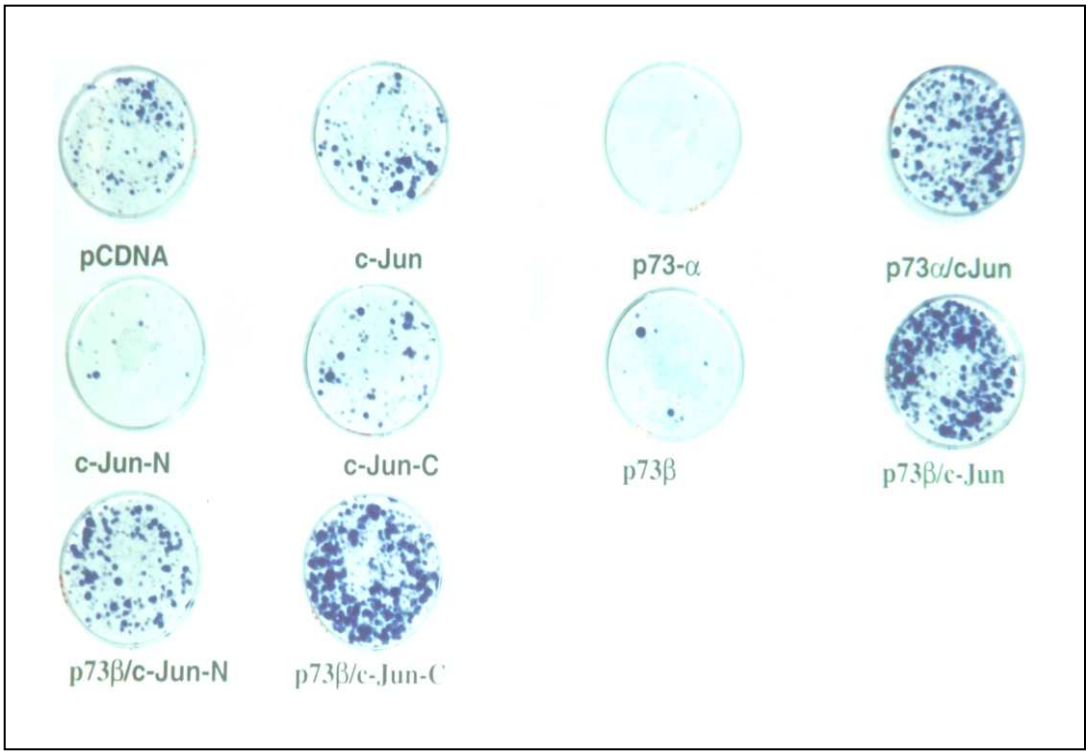


Figure 3.4.3.1 p73 transform fibroblasts in co-operation with both N and C-termini of c-Jun

NIH 3T3 cells were transfected with pcDNA that conferred G418 /Neomycin resistance and pcDNA plasmids expressing p73α and -β, c-Jun, N-c-Jun(1-194), C-c-Jun(194-334) (2μg each) separately and together as shown in the figure. For colony formation assay, transfected NIH3T3 cells were allowed to grow for 48h before 750 μg/ml of Neomycin was added. After 2 weeks, colonies were fixed and visualized by staining with crystal violet. Note that C-terminus of c-Jun is much better than N-terminus of c-Jun to co-operate with p73-β in transformation. In the presence c-Jun, both p73 α and β increased the colony number.

3.4.4 Factors contributing to c-Jun- p73 mediated transformation

3.4.4.1 Both c-Jun and MDM2 are required for enhanced MDM2 promoter activity

In an effort to look at other cellular changes that would have favored the ability of p73 and c-Jun to co-operate in transformation, it was decided to look at changes in the p53 level, as the presence of p53 has already been shown to hinder transformation process. p73 has also been shown to transactivate MDM2 promoter. Unlike p53, MDM2 does not promote degradation of p73, but it has been shown to inhibit transactivation at the highest concentration. Interestingly, MDM2 has been shown to stabilize p73 (Ongkeko et al., 1999) at the protein level. However, the mechanism of action is not yet clear. To clarify this point, the ability of p73 to augment MDM2 promoter activity was checked in genetically defined *$p53^{-/-}$; $p53^{-/-}jun^{-/-}$; $p53^{-/-}Mdm2^{-/-}$* cell lines. Equal amount of p73 and MDM2-luc plasmid and beta-gal plasmids were tranfected and luciferase assays were performed to find out the MDM2 promoter activity in the knockout cell lines chosen. Interestingly, MDM2 activity was high in *$p53^{-/-}$* cell line, in comparison to both *$p53^{-/-}jun^{-/-}$ and $p53^{-/-}Mdm2^{-/-}$* cell lines, indicating that both c-Jun and MDM2 independently are required for maximal MDM2 induction (Figure 3.4.4.1).

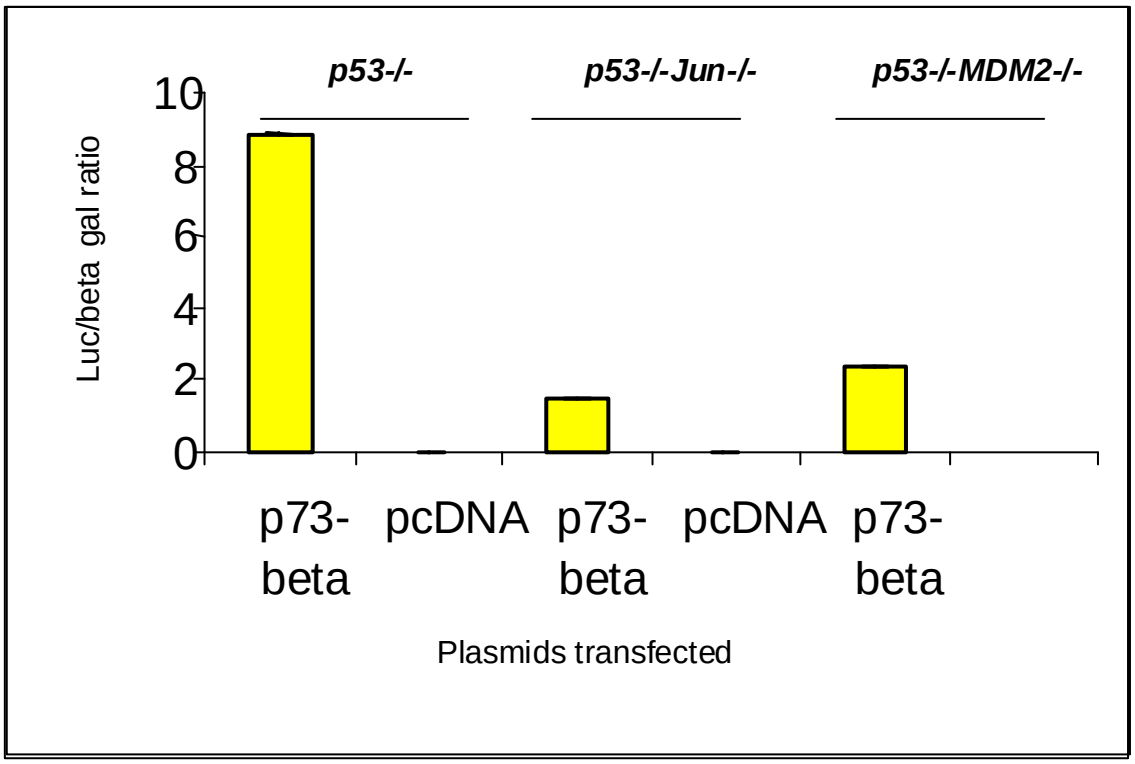


Figure 3.4.4.1 Both c-Jun and MDM2 are required for enhanced MDM2 promoter activity

$p53^{-/-}$; $p53^{-/-}Jun^{-/-}$; $p53^{-/-}MDM2^{-/-}$ cells were transfected with pcDNA,Vector control, p73-beta and reporter plasmids expressing the luciferase gene under the control of MDM2 promoter and a plasmid encoding the beta-gal promoter for evaluating the transfection efficiency. The total amount of plasmid DNA used for transfecteion was adjusted to 1.2µg by adding an empty PCDNA3. Transfected cells were collected 48 hours after transfection and both luciferase activity and beta-gal activity in cell extracts were measured.

3.4.4.2 Immunoprecipitation analysis to find out the interaction between c-Jun, p73 and MDM2: Immunoprecipitation(IP) with c-Jun Ab

Our earlier observations suggest that c-Jun and p73 interact with each other through both N and C-termini of c-Jun. It has already been shown that p73 interacts with MDM2. Unlike p53, MDM2 does not promote the degradation of p73. Further more, when a large amount of MDM2 was over expressed p73 transcriptional activity towards p53 responsive elements was lost. Considering these observations, it was decided to check whether a complex containing these proteins exist in *invivo.* To determine whether c-Jun, p73 and MDM2 interact with each other, cos7 cells were transfected with vector carrying no insert (pCDNA), p73-ß, c-Jun, MDM2 and in combination as shown in figure 3.4.4.2. 48h after transfection, cell extracts were prepared and immunoprecipitated with agarose conjugated c-Jun Ab and c-Jun immunoprecipitates were analyzed by western blotting. The membrane was sequentially probed with, p73, c-Jun and MDM2, indicating a possibility that in *invivo* such a complex could exist. C-Jun antibody efficiently immunoprecipitated p73 when either p73 alone or both c-Jun and p73 were transfected together, indicating that endogenous c-Jun interacts with over expressed p73. When p73.MDM2 or p73.c-Jun.MDM2 was transfected together, again c-Jun antibody efficiently immunoprecipitated p73. Next, the membrane was stripped and re-probed with a mixture of MDM2 antibodies (SAM10 and a mouse monoclonal antibody). When p73/ MDM2 or p73/c-Jun/MDM2 were transfected together c-Jun efficiently pulled down MDM2. Interestingly, when p73 was transfected alone, more MDM2 was brought down by c-Jun, indicating that p73 induces MDM2 expression and is being brought down by

the endogenous c-Jun. In addition, when MDM2/c-Jun was tranfected it did not bring down either MDM2 or c-Jun indicating that this plate was not transfected efficiently. Finally, the membrane was stripped again and re-probed with c-Jun antibody to check whether the agarose conjugated c-Jun antibody is efficiently pulling down overexpressed/endogeneous c-Jun. As can be seen from the figure, c-Jun antibody pulls down c-Jun. Together, this experiment failed to imply c-Jun physically bind MDM2, but that both are in same complex.

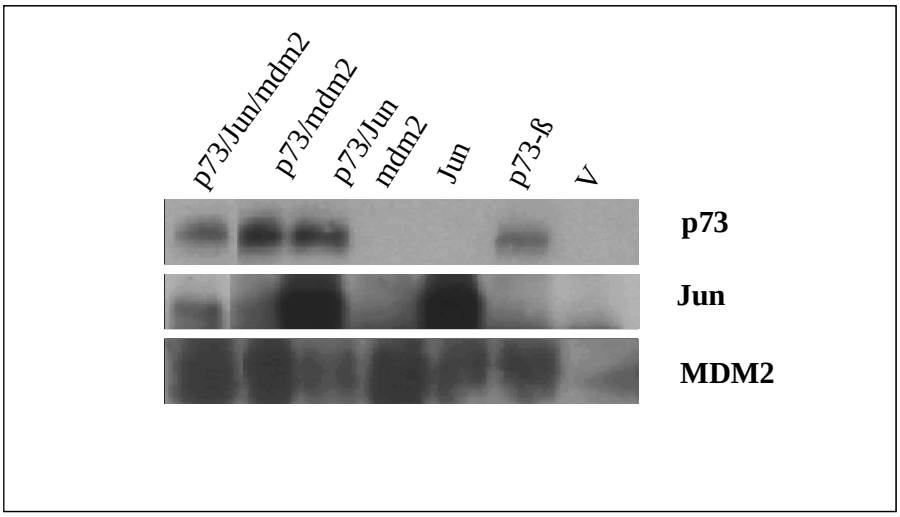


Figure 3.4.4.2 A c-Jun, MDM2 and p73 interact with each other: IP using c-Jun antibody

COS7 cells were transfected with expression vectors as indicated above. Transfected cells were collected 48 h after transfection and cell extracts were prepared and protein concentration measured. Equal amounts of protein lysates (300µg/sample) were first pre-cleared with glutathione agarose beads and then immunoprecipatated with Agarose conjugated polyclonal-c-Jun (H-79) antibody. Immunoprecipitates were analyzed by immunoblotting with p73 (mouse monoclonal antibody), c-Jun (poly clonal antibody-H79) and a mixture of MDM2 (SAM90 plus 12A), the data presented as shown above.

3.4.5 The co-operative effect of c-Jun, MDM2 and p73: a potential mechanism to degrade p53

3.4.5.1 The co-operative effect of c-Jun, MDM2 and p73 on MDM2 promoter

Since these proteins interact with each other and there is a possibility that they may coexist in human tumors, we checked their ability to transactivate MDM2 promoter in human lung carcinoma cell line (H1299). H1299 cells were transfected with the indicated vectors and cells were collected 48h post-transfection. Extracts were analyzed for luciferase and beta-galactisidase activity. As shown in figure (3.4.5.1), when MDM2 was co-transfected with p53, it brings down the activity, while it did not inhibit the transcriptional activity of p73. MDM2 promoter activity was maximal and induced by six folds when p73 and c-Jun were co-transfected together. In addition, when p73, c-Jun and MDM2 expression vectors were transfected together, it augmented the MDM2 promoter activity by fourteen folds, indicating a possibility that coexistence of these factors can effectively inactivate wild-type p53 in the tumors as well as during transformation process.

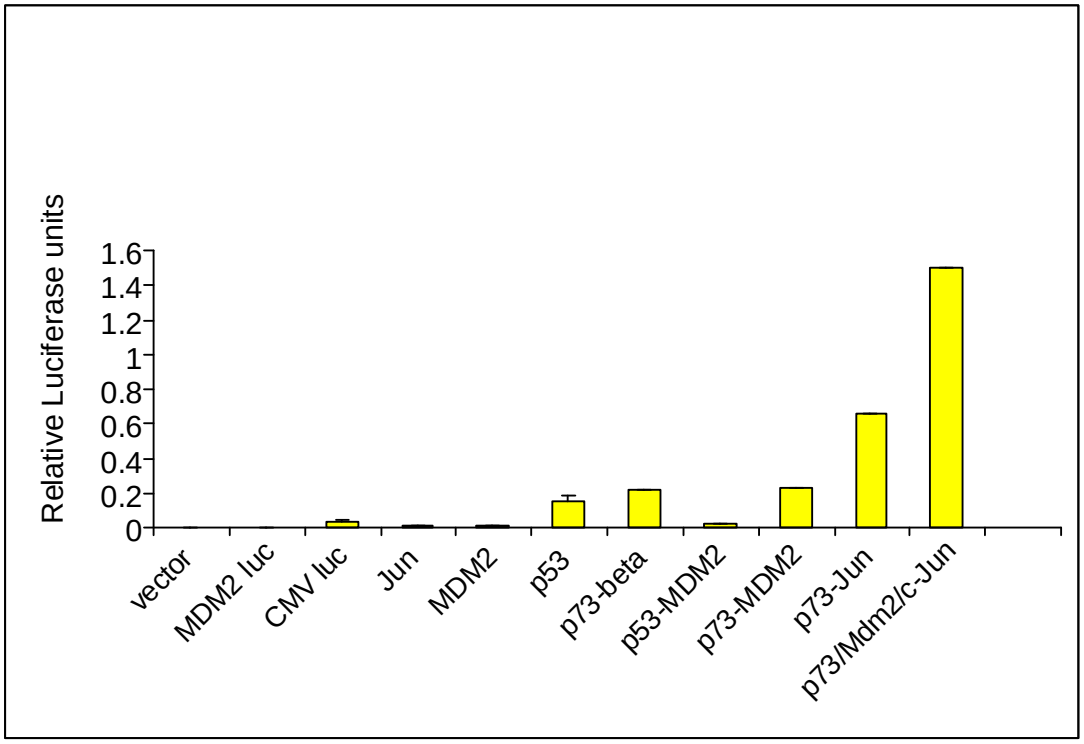


Figure 3.4.5.1 The co-operative effect of c-Jun, p73 and MDM2 on MDM2 promoter

H1299 cells were transfected with the indicated expression vectors. In addition reporter plasmids expressing the luciferase gene under the control of MDM2 (full length) promoter and a plasmid encoding the beta-gal promoter were transfected. The total amount of plasmid DNA used for transfecteion was adjusted to 6μg by adding an empty PCDNA3. Transfected cells were collected 48h after transfection, both luciferase activity and beta-gal activity in cell extracts were measured, and the data presented as shown above.

CHAPTER IV

"Happy is he who gets to know the reasons for things". Virgil (70-19 BCE)

DISCUSSION

"Science does not know its debt to imagination" Ralph Waldo Emerson.

Section I: c-Jun is required for p73 activation and stabilization

1. *jun*$^{-/-}$ and *p53*$^{-/-}$*jun*$^{-/-}$ cells are resistant to cisplatin

The resistance of *jun*$^{-/-}$ cells to cisplatin suggests that c-Jun is required for cisplatin-mediated apoptosis. Further, the resistance of *p53*$^{-/-}$*Jun*$^{-/-}$ cells to cisplatin indicates the importance of c-Jun in cisplatin-mediated p53-independent apoptosis. A number of studies suggest that c-Jun participates in stress responses. It is induced by various stress stimuli such as UV radiation, MMS and anti-cancer drugs etc. Furthermore, it has been shown that over-expression of c-Jun induces apoptosis in 3T3 fibroblasts (Bossy-Wetzel et al., 1997), in human vascular endothelial cells (Wang et al., 1999), and in neuronal cells (Behrens et al., 2001). Studies using sympathetic neurons and PC12 cells have shown that inhibition of c-Jun activity either by microinjection or expression of dominant negative mutant forms of c-Jun protects the cells from Nerve Growth Factor (NGF) and withdrawal-induced apoptosis (Estas et al., 1994; Ham et al., 1995; Xia et al., 1995). c-Jun has also been shown to inhibit oncogene-mediated transformation in primary rat cells (Ginsberg et al., 1991). However, neither the mechanism of c-Jun-mediated apoptosis nor its targets are known at present.

2. c-jun null cells have low levels of p73, and re-expression of c-Jun restores p73 levels and sensitivity to cisplatin

If cisplatin mediated p73 stability occurs through c-Jun then *c-jun*$^{-/-}$ cells would be resistant to cisplatin-mediated apoptosis and p73 stabilization and function will be altered in these cells. Further, a number of studies suggest that ras can augment c-Jun expression. Fokstuen et al.(1997) showed that ras farnesylation inhibitor BZA-5B increases resistance to cisplatin in a human melanoma cell line. Of note, ras also regulates p73 levels (Melino, 2001). Moreover, transfection of a dominant negative c-Jun mutant Δ169 resulted in cisplatin resistance (Sanchez-Perz and Perona, 1999). Thus, these studies indicate a logical relationship between loss of c-Jun and p73 expressions and cisplatin resistance. In line with these studies, low expression of p73 was found in *c-jun*$^{-/-}$ cells in response to cisplatin treatment. In addition, re-expression of c-Jun in c-jun null cells restored p73 levels in response to cisplatin treatment, indicating that c-Jun is required for increased p73 levels and cisplatin-mediated apoptosis. It has been shown that p73 can activate p53-responsive genes and it can induce apoptosis in several cell lines (Khagad et al., 1997; Jost, 1997). Thus, it is possible that c-Jun may stabilize/increase p73 levels and thereby controls cellular fate. Further, Flores et al., (2002) suggested that p53 is upstream of p73 and p63. In the absence of p73, mouse embryonic fibroblasts failed to undergo apoptosis in response to various chemotherapeutic drugs such as cisplatin in comparison to wild-type cells (Flores et al., 2002). Interestingly, c-jun null cells have higher levels of wild-type p53 but are still resistant to cisplatin-mediated apoptosis. This can be explained by considering the fact that in the absence of c-Jun, p73 levels are low and p73 expression may be required for effective induction of cisplatin-mediated

apoptosis (Flores et al., 2002). Thus, these data suggest that the presence of c-Jun is necessary for induction of p73. Further, these studies indicate a possibility that in the absence of p53 protein chemotherapeutic drugs can induce p73 protein to execute apoptosis. The following facts highlight the role of p73 in chemotherapeutic drugs induced apoptosis in various cancers:

1. The ability of p73 to induce p53-independent apoptosis explains the sensitivity of human cancers (in spite of p53 mutations) to chemotherapeutic drugs (Kaelin et al., 2000; 2003).

2. Das et al., (2005) have shown that Ad-p73 is more potent than Ad-p53 in enhancing the chemosensitivity of mutant p53 expressing cancer cells.

2. p73, but not p53, expression differs between the cisplatin-resistant cell line and its isogonics cell line, emphasizing the importance of p73 in cisplatin mediated apoptosis (Ono et al., 2001).

3. p73, but not p53, expression differs in cells defective in mismatch repair, such as those derived from the hereditary cancer syndrome human non-polyposis colon carcinoma (Zheng et al., 1999).

4. SK-N-AS (a human neuroblastoma cell line-p73 negative) cells are more resistant to cisplatin than SK-N-SH (p73-positive) cells (Zheng et al., 1999).

Thus, understanding p73 mediated p53-independent apoptotic pathway is of paramount importance in understanding cisplatin resistance found in various human cancers.

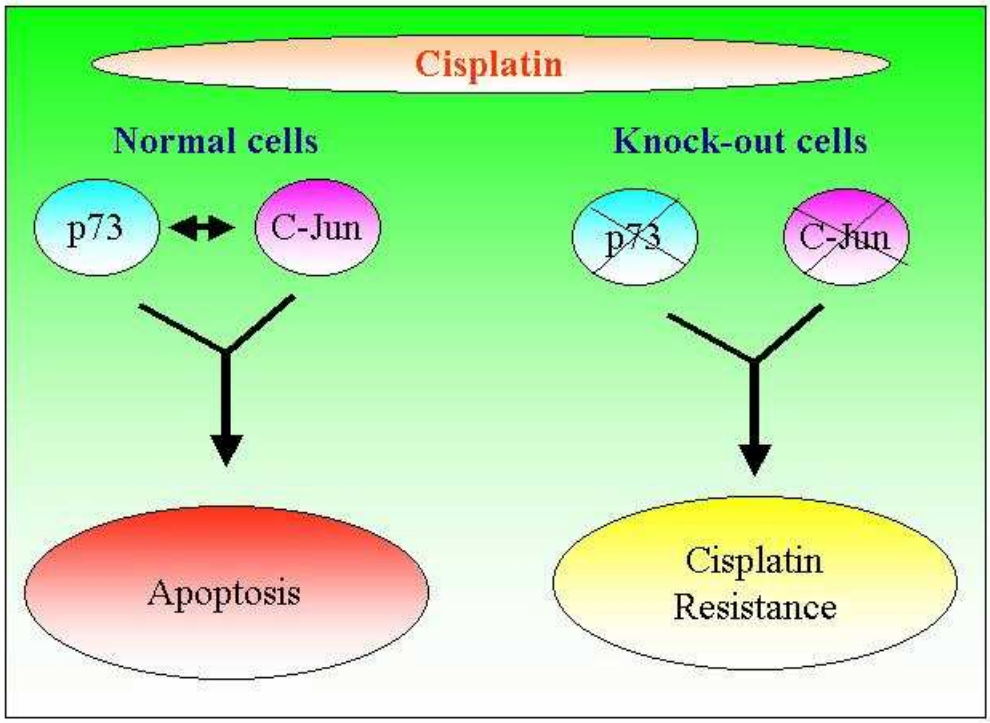


Figure 4.1 Schematic diagram depicted here exemplifies the role of c-Jun and p73 in cisplatin resistance

3. c-Jun stabilizes p73 in response to cisplatin treatment: Possible and potential mechanisms

Cisplatin has been shown to stabilize both p53 and p73 at the post-transcriptional levels (Gong et al., 1999). The data presented here suggest that both exogenously transfected c-Jun and endogenous c-Jun increase p73 levels. c-Jun does not alter p73 RNA in response to cisplatin treatment, but it stabilizes p73 at the protein level. This conclusion is drawn from various experiments (Co-transfection, Western, Pulse chase, RT-PCR etc.) presented in Chapter III. In line with this, Gong et al., (1999) showed that cisplatin does not increase p73 at the RNA level.

The stability of key proteins involved in cell fate decisions is regulated by several degradation mechanisms. Regulatory proteins are often ubiquitinated, depending on their association with auxiliary proteins that serve as adapters of the ubiquitination machinery. Both c-Jun and p73 are degraded by proteasome dependent and independent mechanisms (Muller et al., 2000; Lee et al., 1999; Bernassola et al., 2004). c-Jun is targeted for ubiquitination by its association with inactive JNK (at the basal level). On the other hand, c-Jun is protected from ubiquitination by its association with active JNK (in response to various external and internal stimuli) (Fuchs, et al., 1997). It has recently been shown that Itch, a Hect ubiquitin-protein ligase, binds to p73 and target it to proteasome dependent degradation (Rossi et al., 2005). Furthermore, RanBPM has been shown to bind to p73alpha and stabilzes p73 by inhibiting proteasome mediated degradation (Kramer et al., 2005). Asher et al (2005) have shown that p73 stabilization is regulated by NQO1 as well. It appears that NQO1 interacts with p73 in an NADH-dependent manner and guards them from proteasomal degradation (Asher et al., 2005). However, p73

degradation and/or stabilization mechanisms are not very clear yet. c-Jun has been shown to interact with Ubc9 (E2 ubiquitin-conjugating enzyme) (Gottlicher et al., 1996). Likewise, p73 has been shown to interact with several ubiquitin related proteins such as Ubc-9, SUMO1 (Minty et al., 1999). This data suggests that c-Jun by competing with p73 for binding Ubc9, it could prevent the interaction between p73 and Ubc9. By preventing the interaction between p73 and Ubc9, c-Jun could protect p73 from being degraded. It has been shown that serum is a potent inducer of c-Jun expression (Lamp et al., 1988). Serum also stabilizes p73 with no significant change in p73 mRNA levels (Weiss et al., 2001). These data suggest that serum could stabilize p73 by activating the expression of c-Jun. Alternatively, c-Jun can increase p73 levels by negatively regulating repressors of p73.

Recently, Zaika et al., (2002) and Slade et al., (2004) showed that DN-p73 increases p73 protein levels, but it decreases its functional activity. Intriguingly, deltaN-p73 promoter encoades AP1 responsive elements. In agreement with this data, c-Jun has been shown to increase the deltaNp73 at the promoter level (Costanzo, et al., 2002). Thus, it is also possible that in response to growth stimuli c-Jun can stimulate deltaN-p73 expression, which in turn enhances p73 protein levels.

Further, N-terminus of c-Jun ((Δ194-334) mutant does not stabilize p73. This data suggests that transactivation and delta domains are essential for c-Jun mediated p73 stability. On the other hand, C-terminus ((Δ1-194)) of c-Jun mutant weakly stabilizes p73 compared to wild type c-Jun, indicating that DNA binding and dimerization (leucine zipper) domains are important as well. Thus, both N- and C-termini of c-Jun (containing transactivation, dimerization and DNA binding domains) are required for its ability to

stabilize p73. In support of this finding, it has been shown that c-Jun mediated apoptosis require both bZIP and transactivation domains, suggesting that apoptosis depends on transcriptional regulation (Bossy-Wetzel et al., 1997).

Next, the likelihood of interaction of p73 with c-Jun was revealed in the immunoprecipitation experiments carried out in cells over expressing c-Jun or p73 (data not shown). However, immunopreciptation of endogenous c-Jun with p73 antibody was unsuccessful, indicating that higher levels of p73/c-Jun expression is required for interaction invivo.

The central part of c-Jun protein contains PPXY(Tyr170) domain. This PPXY domain is also conserved in p73 (near the carboxy termini). The domain could bring about PPXY motif-WW domain interaction, which constitutes a part of a complex network of multiple signal transduction pathways. If this indeed the case, then c-Jun may be part of complex in which p73 is constituent or c-Jun and p73 can interact indirectly through WW domain containing proteins. This explains the difficulties in coimmunoprecipitating the endogenous p73/c-Jun protein. Further efforts to identify the binding partners of the PY motif of p73 and c-Jun should contribute to our understanding of the overall network of cellular signaling involved. Evidently, it has been shown that c-abl interacts with the PPXY domain of c-Jun (Barila et al., 2000).

Further, it appears that both the N (Δ1-194) and C-termini of c-Jun (Δ 194-334) bind to p73 independently under over expressed conditions (data not shown). This data implies a possibility that c-Jun interacts with p73 through multiple regions, one near the N-terminus of c-Jun and the other near the C-terminus (possibly through the DNA

binding and leucine zipper domains), but both may be required for efficient interaction and stabilization.

c-Abl has been shown to stabilize p53 by direct protein-protein interaction. . Though c-Abl has been shown to interact with p53, it does not phosphorlyate p53. Therefore, it appears that c-Abl through direct interaction with p53 masks the export and ubiquitination signals, which results in retention and stabilization of p53 in the nucleus (Shaul, 2000; Sionov et al., 2001; 1999). Both c-Jun and Abl haave been shown to interact with each other. The intramolecular interactions between c-Abl, c-Jun and p73 will certainly affect their stability, though the extent may depend on the cellular context. Our data suggests that p73 and MDM2 interact with each other. Also, both Abl and c-Jun have been shown to interact with each other (Agami et al., 1999; Barla et al., 2000) Thus, the intermolecular interactions between c-Abl, MDM2, c-Jun and p73 will have an effect on their stability and function, though the extent may depend on the cellular context.

4. Expression of c-Jun modulates p73-mediated transactivation

The data presented here suggest that c-Jun, by increasing p73 protein levels, augments its ability to transactivate p53 responsive genes such as p21, GADD45 and MDM2 in the reporter and RT-PCR analysis. Further, it also augments p73's ability to increase MDM2 at the protein level. Correspondingly, the ability of p73 to transactivate its downstream genes is markedly reduced in $p53^{-/-}$ cells compared to $p53^{-/-}Jun^{-/-}$ cells, indicating that c-Jun is required for increased p73-dependent transcriptional activity. c-Jun could protect against proteasome dependent degradation by binding to the shared component of proteasome machinery and thereby increase stability and transcriptional activity of p73. It has been shown that Abl by directly binding to p53, potentiates p53's

ability to transactivate its downstream genes. It has been established that CBP/p300, which binds both p73 and c-Jun independently, functions as a co-activator for both. Hence, it is possible that p300 could bring p73 and c-Jun closer to the proximity of the p53 responsive element containing promoters and thereby it could augment the capacity of p73 to transcribe its target genes. On the other hand, c-Jun neither binds to p53 nor stimulates its function (our observation; Shualin et al., 2001). Infact, it has been shown that c-Jun suppresses the expression of p53 and its target gene, p21 at the promoter level (Shualin et al., 2000; Schreiber et al., 1999).

A recent study by Faniello et al., proposes an alternative model of H-ferritin promoter transactivation by p300. p300 can recruit c-Jun to promoters that do not contain AP-1 binding sequences. c-Jun, when over expressed, is able to potentiate the transcription of promoters without binding directly to the DNA. All these observations are consistent with the hypothesis that accessory factors can up- or down-regulate transcriptional activity by influencing the protein-protein interaction, which directly controls the transcriptional properties of a transcription factor (Chan et al., 2001). With similar mechanism, one can predict c-Jun's ability to synergize with p73 to transactivate p53 responsive element containing promoters.

5. The c-Jun potentiates p73 function to induce apoptosis in response to cisplatin treatment

The resistance of *p53*$^{-/-}$*jun*$^{-/-}$ and *jun*$^{-/-}$ fibroblasts to undergo apoptosis in the presence of anti-cancer drugs such as cisplatin highlights the importance of c-Jun and p53 related proteins such as p73 in inducing apoptosis. c-Jun potentiates p73 transcriptional and proapoptotic activities that are markedly impaired in *jun*$^{-/-}$ and *p53*$^{-/-}$

jun$^{-/-}$ cells. These data are consistent with the ability of dominant negative c-Jun to prevent cisplatin mediated cell death (Sanchez-Perez et al., 1999). It has been shown previously that mere transfection can "activate" p53 and p53-responsive downstream genes (Renzing et al., 1995). c-Jun's ability to potentiate p73 proapoptotic activites could be part of the stress response, as transfection itself activates a stress-signalling pathway. Further, it has been shown that *p63*$^{-/-}$*p73*$^{-/-}$ fibroblasts are resistant to undergo apoptosis in the presence of anti-cancer drugs such as doxorubicin and cisplatin. This highlights the importance of p73 and p63 in inducing apoptosis (Flores et al., 2002). Intriguingly, in response to doxorubicin treatment, p53 is induced in *p73*$^{-/-}$*p63*$^{-/-}$ MEFs and it binds to p21 and MDM2 promoters but not to apoptotic promoters such as Bax, PERP, and NOXA. This data suggests that p63 and p73 are important elements in recruiting p53 to promoters at apoptosis-related genes. This proposal is supported by at least two facts. First, p63, possibly p73, has shown to be present at apoptotic-associated target gene promoters, even in the absence of p53. Second, p53 is not present at these promoters in the absence of p73 and p63(Flores et al., 2002). Similarly, in c-Jun null cells, though basal p53 and p21 levels are high and the levels of p53 protein is further induced in response to cisplatin treatment, these cells are relatively resistant to cisplatin mediated apoptosis. The reason for this could be that p73 is neither induced nor stabilized in c-jun null cells in response to cisplatin treatment. As described earlier, p73 is required for p53's ability to transactivate apoptotic target genes such as Bax, PERP etc. Intrestingly, the ability of c-Jun to synergize with p73 on p21 promoter is minimal. On the other hand, c-Jun synergized with p73 very effectively on Bax and p53AIP

promoters. Collectively, c-Jun, a known mediator of apoptosis(Bossy-Wetzel et al., 1999) collaborates with p73 to induce apoptosis in response to cisplatin treatment.

Finally, the ability of c-Jun to potentiate p73's proapoptotic function can be used as a mode of treatment for cancer. Hence, identifying the modulators of pathways involving p73 and c-Jun is of great importance for the following reasons: (1) At least 50% of tumor cells harbor mutations in p53 gene, but its homologue p73 is infrequently mutated in human cancers. (2) p73 is an important player in chemotherapy-induced apoptosis. (3) p73 can induce apoptosis in the absence of p53.

If p73's proapoptotic activity is specifically activated using c-Jun derived peptides (derived from the common interacting regions of p73 and c-Jun) in cancer cells, then this can be used to increase the response of a variety of p53-defective cancer cells to therapeutic agents.

6. Role of JNK in c-Jun-mediated p73 stability and transactivation

The JNK kinases phosphorylate Jun and stimulate its transcriptional activity. JNKs are activated by stress inducing agents and their ability to induce apoptosis or proliferation will vary depending upon the cell type, cellular context and stimuli (Leppa et al., 1999). The data presented in this thesis suggests that c-Jun could promote p73 function and stability independent of its phosphorylation (63/73; 91/93 a.a) status, as p73 can be stabilized further by transfected c-Jun and p73's function is not compromised in *Jnk1*$^{-/-}$*Jnk2*$^{-/-}$ fibroblasts. In addition, we have found that *Jnk1*$^{-/-}$*Jnk2*$^{-/-}$ cells were more sensitive to cisplatin and induced c-Jun protein (data not shown). This data suggests that JNK1 and JNK2 are not essential for cisplatin mediated cell death. In support of this finding, it has been shown that cisplatin treatment did not potentiate JNK activity

(Fokstuen et al., 1997; Liu et al., 1996), but clearly induced c-jun mRNA expression (Liu et al., 1996). Thus, it appears that genotoxic stress-induced transcriptional induction of c-Jun expression (nascent c-Jun) could co-operate with p73 effectively in transactivating apoptotic promoters carrying p53 responsive elements; and, JNK mediated increase in c-Jun(Phosphorylated) protein levels may not co-operate with p73 effectively in transactivating p53-dependent apoptotic promoters. Accordingly, it has been shown that JNK-mediated c-Jun phosphorylation is not important for TNF-α or sorbitol mediated cell death, as *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ fibroblasts were more sensitive to TNF-α and sorbitol-induced apoptosis in fibroblasts (Hochedlinger et al., 2002). Further, this data suggests the following: 1. JNK1 and JNK2 negatively regulate apoptosis in response to certain stress stimuli. 2. The existence of JNK-independent apoptotic pathways in fibroblasts. Further, JNK1 has been shown to degrade p53 by binding to the region near N-terminus (92-112) of p53 (Fuchs et al., 1988), however, this region is not conserved in p73. This may possibly eliminate a direct role for Jnks in p73 regulation. JNK-p53 complexes were found specifically in G0/G1 phases of the cell cycle (Ronai et al., 1999), while MDM2-p53 complexes were preferentially found in S/G2-M phases of the cell cycle. It appears that JNK is an Mdm2-independent regulator of p53 stability in nonstressed cells. On the other hand, the same group has showed earlier that expression of a constitutively active form of JNKK upstream kinase, mitogen-activated protein kinase kinase kinase (DeltaMEKK1), increased the level of the exogenously transfected p53. Increased p53 level by forced expression of DeltaMEKK1 coincided with a decrease in p53 ubiquitination *in vivo* with prolonged p53 half-life. Correspondingly, JNK has been shown to phosphorylate p53 at T81 in response to DNA damage and stress-inducing

agents (Buschmann et al., 2001). Unlike wild-type p53, in response to JNK stimuli p53 mutated on T81 (T81A) did not exhibit increased expression or concomitant activation of transcriptional activity, growth inhibition, and apoptosis. Similarly, transfection of antisense JNK 1- and -2 decreased T81 phosphorylation in response to UV irradiation. Together, the data accumulated from various laboratories in the recent past suggest that JNK augments p53 stability under stressfull conditions, while in non-stressful conditions, JNK appears to degrade p53. How the same protein differentially regulates p53— whether p73 is prone to similar regulation— under different conditions requires detailed investigation.

Taken together, the results presented in this thesis suggest that phosphorylated c-Jun (63/73) is neither essential for p73 stability nor for its transcriptional activity.

7. The role of c-abl in c-Jun enhanced p73 stability and function

The data presented in this thesis suggests that c-Jun can stabilize p73 in the absence of c-Abl at higher concentrations of cisplatin (Chapter 3.1 figure). Thus, c-Jun mediated p73 stability is independent of c-Abl activity. In response to IR, c-Abl phosphorylates p73 and thereby stimulates its transcriptional function (Gong et al., 2000). On the other hand, co-transfection of c-Abl, c-Jun and p73 did not result in enhanced p73 stability compared to the co-transfection of p73 and c-Jun (data not shown). This can be explained if one considers that transfecting eqimolar of c-Jun, c-Abl and p73 plasmids may not demonstrate their in vivo effect, as c-Abl protein level remains unchanged in response to IR, but its kinase activity is increased. Although cisplatin induces c-Abl kinase activity, it does not phosphorylate p73 (White, 1999). Moreover, it has been

shown that cisplatin does not activate JNK at concentrations that produce an increase in c-Abl activity in the HEC59 cell system suggesting that these kinases are not part of the same signaling pathway, and that JNK1 activation is not a universal feature of the cisplatin-induced cellular injury response (Nehme et al., 1999). However, cisplatin can induce c-Jun expression at the transcriptional level and does not depend on JNK to increase the levels of c-Jun (Rabo, et al., 1996; Wang et al., 1996). In fact, c-Jun is increased in $Jnk1^{-/-}2^{-/-}$ cells in response to cisplatin and the $Jnk1^{-/-}2^{-/-}$ cells are more sensitive to cisplatin-mediated apoptosis, indicating that Jnks could function as a negative regulator of apoptosis (data not shown). Evidently, both $Jnk1^{-/-}$ and $Jnk2^{-/-}$ cells have also been shown to be more sensitive to TNF-α and sorbitol induced cell death (Hochedlinger et al., 2002). These results support the idea that c-Jun expression is more important for cisplatin sensitivity but not JNKs. Consistently, it has been shown that activation of c-Abl and JNK represent distinct signaling responses to DNA damage (Liu et al., 1996). For example, UV irradiation induces JNK, but not Abl activity, while IR induces Abl but not JNK activity at lower doses. It is possible that c-Jun activation of Abl and JNK may form part of a phosphorylation circuit that integrates growth and stress-related stimuli for cellular fate decisions. Thus, these observations suggest that the fate of cells exposed to various stress signals does not always correlate with and is not always determined by JNK activity.

Further, it has been shown that activation of c-Abl can contribute to apoptosis by phosphorylating, stabilizing and activating the p73 transcription factor. However, it has been shown recently that c-Abl mediated p73 stability could be pro-apoptotic or anti-apoptotic, as p73 gene can generate a number of splicing variants with pro-apoptotic and

anti-apoptotic functions(Wang., 2003). c-Abl appears to retain the ability to interact and phosphorylate all the isoforms of p73. If c-Abl could stabilize both the pro-apoptotic full-length p73 and anti-apoptotic deltaN-p73, the effect of c-Abl on apoptosis would then be determined by the expression pattern of the p73 gene (Wang, 2003).

Section II: UV light mediated p73 induction/stability

UV-radiation is one of the most biologically relevant inducers of DNA damage. UV-B radiation from the sunlight is the cause for most human skin cancers. Exposure to UV-light causes DNA damage through formation of pyrimidine dimmers and 6-4 photoproducts which effectively blocks RNA polymerase II from transcription (Cullinane et al., 1999; Mello et al., 1995; Selby et al., 1997). UV-induced DNA damage results in either transient cell cycle arrest or apoptosis (Lane, 1992; Lu and Lane, 1993). Transient cell cycle arrest provides cells, an ample amount of time for the DNA repair before replicating damaged DNA (Elledge, 1996). UV-induced DNA lesions are removed by nucleotide excision repair pathway (NER) (Friedberg et al., 1995).

Although exposure to UV-radiation results in rapid p53 stabilization and its role in UV-radiation induced apoptosis is still questionable. Studies in the recent past suggest that primary and immortalized fibroblasts lacking functional p53 can be hypersensitive to apoptosis induced by moderate doses of UV-radiation (Lackinger et al., 2001; Sheikh et al., 1997; Bissonnette and Hunting, 1998). In addition, UV light exposure rapidly stimulates c-Jun and in fact, c-Jun is one of the most UV-responsive genes identified so far (Devary et al., 1991). c-Jun appears to play a role in UV-radiation induced apoptosis,

as c-Jun null cells are resistant to UV-induced cell death and over-expression of c-Jun promotes the death of UV-irradiated cells (Shualin et al., 2000).

1. UV increases p73 levels

Our data suggests for the first time that UV-irradiation can induce/stabilize p73 levels. Endogenous p73 is induced in response to UV irradiation in many cell lines (NIH3T3, Cos7, MCF7, H1299) analyzed. After presenting this data in the 11th-p53 international conference, Rome (2002), Melino et al., (2002) suggested that UV can stabilize p73. In support of these data, Zhang, et al., (2003) showed that UV-radiation can stabilize p73 in human keratinocytes.

Further, it appears that UV-induced p73 expression biphasic in nature and it could play a role both in DNA repair and apoptosis (data not shown). The induction of p73 in the earlier phase could lead the p73 to participate in DNA repair. Correspondingly, induction of p73 at the later phase could potentially involve p73 in p53-independent apoptosis. Further, transiently transfected p73 is stabilized much better in *c-jun*$^{-/-}$+c-Jun cells, compared to *jun*$^{-/-}$ cells. However, endogenous p73 expression is induced to a comparable level in *jun*$^{-/-}$ and *jun*$^{-/-}$ + c-Jun cells. These differences noted highlight the fact that UV-mediated p73 induction could occur in the absence of c-Jun through alternative pathway mechanisms, possibly through p38 kinase pathway. Together, it appears that UV-mediated p73 expression is enhanced in the presence of c-Jun expression, but may not be entirely dependent on c-Jun, as UV-light is known to activate several stress related signaling pathways.

p53 is stabilized at the post-translational level in response to DNA damaging agents. Similarly, our data suggests that UV-light mediated p73 induction and

stabilization can occur independent of transcription. However, considering the fact that p73 promoter encoades AP1 elements, one cannot exclude the transcriptional mechanisms contributing towards its upregulation in response to UV-radiation.

The enhanced p53RE-carrying promoter activity observed in a panel of p53-negative cell lines suggest that p53 family members could act as a compensatory mechanism to activate p53RE promoter activity in the absence of p53. However, it is important to note that UV-light induced p53-RE reporter and p73 transcriptional activity were less than 2 folds in the panel of p53 negative cell lines tested (data not shown). and. DeltaNp73, which does not contain the N-terminal activation domain in p73, has been thought to be transcriptionally inactive. It has been shown to form inactive complexes with TA-p73 and thereby hinder its function. However, it has been found recently that DeltaNp73beta is indeed active in inducing cell cycle arrest and apoptosis (Liu et al., 2004). In addition, p53 has been shown to induce apoptosis by transcription dependent and independent mechanisms (Haput et al., 1995; 1997). The authors further suggested that the existence of two p53-dependent apoptotic pathways--one requiring activation of specific target genes, and the other independent of sequence-specific trans-activation. The latter pathway may actually be totally uncoupled from the binding of p53 to its consensus DNA sites. However, the relative contribution of trans-activation-independent apoptosis to tumor suppression by p53 is less clear. Similarly, p73 can contribute to UV-mediated apoptosis in transcription independent manner.

2. JNK 1, 2 and Abl do not influence UV-radiation induced p73 levels

JNKs are stimulated upon UV irradiation of cells. Thus, the role of JNK1 and 2 in p73 induction in response to UV-radiation was checked. Our data suggests that UV-mediated p73 induction does not depend on JNK1 and JNK2, as the *Jnk1*$^{-/-}$*2*$^{-/-}$ fibroblasts exposed to UV irradiation still induce p73 expression. Further, it has been shown that IR-induced activation of JNK is dependent on the c-Abl function in cell-type specific manner (Kharbanda et al., 1995). So, the role of c-Abl in UV-radiation mediated p73 induction was analysed. Intrestingly, p73 level was not significantly altered in response to UV irradiation of *c-abl*$^{-/-}$ fibroblasts, indicating that c-Abl is unlikely to participate directly in UV mediated p73 induction. In support of this finding, it has been shown earlier that only IR is known to activate c-Abl tyrosine kinase (Liu et al., 1996), but not UV-radiation. Hence, the role of c-Abl in JNK-dependent apoptosis is less apparent in our studies.

3. Consecutive exposure of UV and IR induce apoptosis in *p53*$^{-/-}$ cells

UV-radiation is a potent inducer of c-Jun but not IR. Increased levels of transfected p73 seen in response to UV-radiation, but not IR, indicate that UV-radiation can stabilize p73, but not IR. However, exposing cells to combination of IR and UV-radiation at lower doses increased the stabilization of p73. Furthermore, exposing cells to the combined signals led to the absence of colony formation in *p53*$^{-/-}$ cells. Duration of p73 induction is the determining factor for cell cycle arrest versus apoptosis. Transient p73 induction could lead to cell cycle arrest, whereas sustained p73 activation (leading to stabilization) causes apoptosis.

Ionising radiation induces cell cycle arrest but not apoptosis in most of the cell types except in thymosites (Nagasawa, 1993; Di Leonardo et al., 1994). Zeng et al, (2000) have showed that UV-radiation induces apoptosis of teratocarcinoma cells, but IR induces growth arrest. Further, UV-radiation (10 or 20 J/m2), but not irradiation (7 or 10 Gy), caused a massive apoptosis of human teratoma Tera-2 or murine testicular carcinoma F9 cells. This differential response (UV-radiation versus IR) can be explained by considering the fact that IR induced DNA damage results in double stranded breaks, which can be considered as part of the physiological mechanism in certain cells, as double stranded breaks often occurs during homologous recombination and meiosis. UV radiation mediated DNA damage produces pyrimidine dimers(PDs). The PDs are difficult to repair compared to double stranded breaks (DSB) and therefore induces apoptosis irrespective of the p53 status of the cell. Thus, the observation that only UV radiation provides stabilization signal but not ionising radiation prompted us to propose that combined signals, that is, ionising radiation (induction signal provided by c-Abl) followed by UV irradiation (stabilization signal provided by c-Jun) could result in further stabilization of p73, which in turn, causes p53-independent apoptosis. IR mediated signalling results only in growth arrest in most of the cells. Therefore, it may not be necessary to stabilize p73, which would lead to apoptosis. Transiently induced p73 in response to IR may play a role in DNA repair pathway, as MLH1 appears to be upstream of p73 (Gong et al., 1999).

Section III: The role of p73 in the regulation of AP-1 activity

1. p73 augments AP-1 activity

p53 binds in a squence specific manner to the p53RE containing genes and transactivates its target genes such as p21, MDM2, Bax, GADD45 etc. (Osada et al., 1998; Yang et al., 1998). Similarly, TAp73 isoforms transactivate most of the p53 responsive genes. However, further studies suggest that p73 plays a physiologically distinct role. Knocking out the p73 gene in mice led to inflammatory, neurological, secretory and phremonal defects (Yang et al., 2000), while p53 knockout mice developed normally with an early onset of tumorigenesis in different tissues (Donehowever et al., 1992). These differences between p73 and p53 suggest that they must be regulated through different mechanisms. Thus, the mechanism of regulating p73-dependent transcriptional activation is crucial for a better understanding of the biological role of p73.

p73 shows a degree of specificity for the promoters of target genes that are quantitatively distinct from the response mediated by p53 (Chen et al., 2001). For example, p73 activates the GADD45 gene more efficiently than p53, while the reverse situation was apparent for the p21 gene. These effects are, in part, due to the influence of a regulatory domain present in the extended C-termini of the p73 isoforms (Lee et al., 1999). However, it is important to bear in mind that these differences are noted within the p53 responsive element containing promoters, for eg., p21, MDM2, Bax etc. and not other regulatory elements.

The results presented in this section demonstrate that p73 increases AP-1 transcriptional activity. When p73 was co-transfected with c-Jun, a significant increase in AP1-RE-luciferase activity, compared with construct alone, was noted. Moreover, when p73 was co-transfected with c-Jun at different ratios 1(p73): 1(c-Jun) and 1(p73): 20(c-Jun), it stimulated AP-1 transcriptional activity. Further, the study presented here shows that p73, but not p53 stimulates AP-1 activity. Intrestingly, there seems to be a strong symbiotic relationship between c-Jun (Leuzine zipper transcription factor, transactivates AP1 responsive elements) and p73 (transcription factor, transactivates p53 responsive elements), considering the fact that c-Jun increases p73 levels and in turn, p73 increases AP-1 activity in c-Jun dependent manner. Therefore, it is plausible that depending upon the cellular context and other accessory cellular factors, these transcription factors decide whether to potentiate AP-1 or p53 responsive elements containing promoters or both. However, the mechanism for this exciting observation is not obivious at present, hence several mechanistic possibilities are presented here:

2. Mechanism of action:

Mechanism 1: p73 enhances the affinity of Jun-Jun homodimers to enhance AP-1 activity.

Both fos and jun associate with binding site of activator protein-1 (AP-1) and increase its activity. However, fos did not bind to the AP-1 site on its own, but it acted synergistically with Jun to give enhanced DNA-binding activity. The increased affinity of the Fos-Jun complex for DNA is due to the stabilization of the protein-DNA complex (Rauscher et al., 1988). c-Jun can form homodimers and bind to the AP-1 site but not c-fos. However, it has been shown that c-Jun homodimers are less stable and exhibit low

AP-1 activity (that is evident only at high concentrations of DNA) than Jun:Fos hetrodimers (Smeal et al., 1989). The fact that p73 does not have LZ (leucine zipper) domain may suggest that it may not bind to AP-1 elements directly. Bearing these facts in mind, one could envisage a mechanism in which p73 could serve as a bridging factor to change the conformations of c-Jun homodimers (less stable/active). This conformational change could increase the stability and activity of c-Jun-c-Jun homodimers (Figure 4.2). In support of this mechanism, it has been shown that several cellular and viral modulators of transcription such as transcription factors and coactivators do not bind to DNA by themselves but operate through association with the DBDs of transcription factors (Wagner and Green 1994).

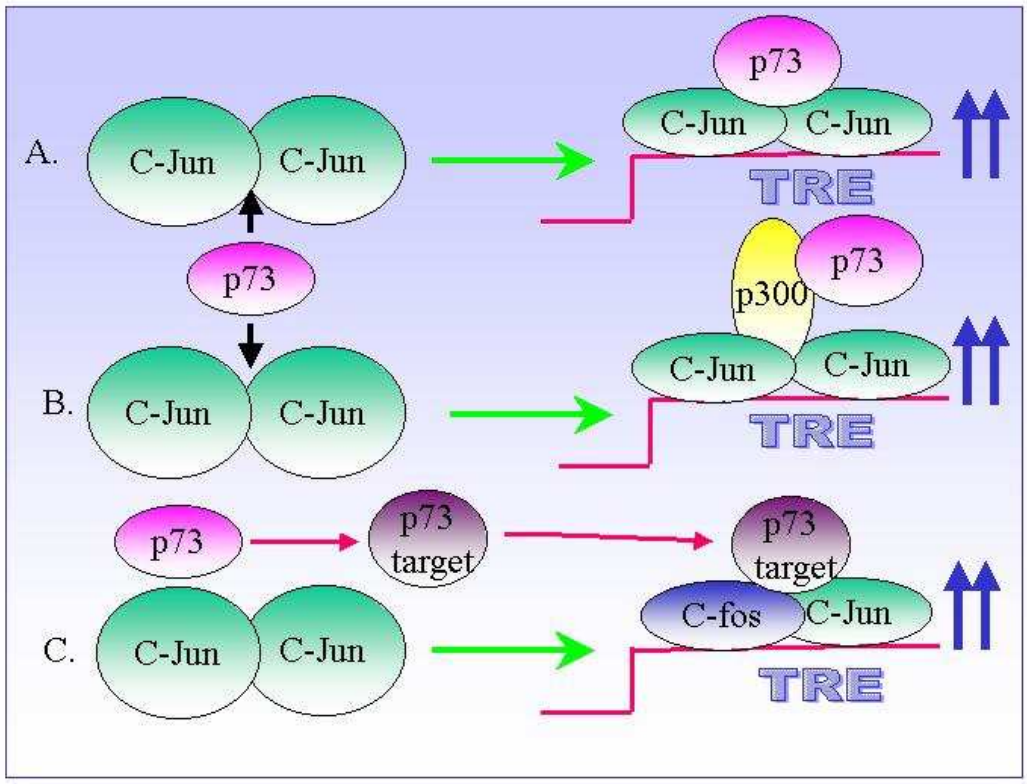


Figure 4.2 p73 stimulated AP-1 transcriptional activity: possible mechanisms

The hypothetical model depicted above presents different scenarios by which p73 could stimulate AP-1 responsive elements. A. p73 does not interact with AP-1 responsive elements directly but it just enhances the stability and DNA binding activity of AP-1 complex. B. The interaction of c-Jun and p73 proteins could bring p300, a co-activator, to the closer proximity of AP-1 promoters, resulting in enhanced AP-1 transcriptional activity. C. p73 transactivates a coactivator of the AP-1 that potentiates the c-Jun homodimers abilty to increase transactivation of an AP-1-dependent promoter.

Mechanism 2: Both c-Jun and p73 proteins could bring p300, a co-activator, closer to the proximity of AP-1 promoters, thereby enhancing AP-1 transcriptional

activity. In support of this view, it has been shown that p300 interacts with p73 when this transcriptional activator binds to its responsive DNA element sequence (Zeng et al., 2001). Furthermore, p300 proteins might be the centre point for the assembly of diverse co-factor proteins into multicomponent co-activator complex by interacting with other HAT's, including pCAF, SRC1, and P/CIP (Chan and Thangue, 2001). By interacting with several transcription related proteins, p300, serves as a scaffold for the assembly of transcription co-factors and increases the relative concentration of these factors in the local transcription environment and thereby facilitate protein-protein and protein-DNA interactions (Chan and Thangue, 2001).

Mechanism 3: p73 induces a target gene, which potentiates the ability of c-Jun to transactivate AP-1 responsive elements

p73 has been shown to transactivate several p53RE containing gene promoters. However, accumulating evidence suggests that p73 will have its own set of target genes, other than transactivating p53RE containing gene promoters (Fummegallie et al., 2001). Thus, it is possible that specific expression profiles of p73-responsive genes could result from combinatorial interactions with other proximal transcription factors. p73 could transactivate a coactivator of the c-Jun transcription factor that potentiates the ability of c-Jun homodimers to increase transactivation of an AP-1-dependent promoters. All of these mechanistic possibilities may not exclude one another, although more studies are necessary for a better understanding of the regulation of the detailed mechanism for p73-mediated regulation of AP-1 activity.

3. Domains required for p73-Jun stimulated AP-1 activity

In an effort to identify the interacting domains required for p73.c-Jun enhanced AP-1 transcriptional activity, transactivation domain (TA) of p73 was found to be not essential for its ability to stimulate AP1 activity. However, it is required for the maximal activation, as ΔN-p73β was not as efficient as TA-p73β in stimulating AP-1 transcriptional activity. Then, how does delta-N-p73 stimulate AP-1 activity? p73 has been proposed to have two transactivation domains, one near the N-terminus and other near the C-terminus(Takada et al., 1999). It is possible that second transactivation domain near the C-terminus of TAp73 could have interacted through c-Jun for its ability to stimulate AP-1 activity. Thus, in the absence of transactivation domain, p73 can stimulate AP-1 activity either through the second transactivation domain or the DNA binding domain (DBD) or both. However, ΔN-p73 failed to co-operate with c-Jun to increase AP-1 activity. This data suggests that transactivation (TA) domain of p73 is required to synergize with c-Jun. This observation strongly suggests that transactivation property of p73 is required to synergize with c-Jun. Thus, p73 could transactivate a coactivator of c-Jun that potentiates the ability of c-Jun to increase transactivation of an AP-1-dependent promoter.

To co-operate with p73, c-Jun might require two regions of p73: One, N-terminus-transactivation domain and the other being the second transactivation domain near the C-terminus. Among the p73 family members (α,β,γ and δ) tested, only p73-β appeared to transactivate p73 efficiently, though, all of them stimulated AP-1 activity more than basal level. On the c-Jun side, the reporter assays pointed that both DNA binding (DBD) and leucine zipper (LZ) domains are important for its ability to synergize with p73.

4. Phosphorylated c-Jun is required to synergize with p73 for maximal AP-1 activation.

The data presented in this thesis suggests that phosphorylation of c-Jun is required for its ability to co-operate with p73 to increase AP-1 activity. As wild-type c-Jun co-operated with p73-β much better than c-Jun63/73a/a (JNK phosphorylation site mutants) and Jun5XASP (serine is replaced with aspartate to mimic the phosphorlyation status) co-operated much better than wild-type c-Jun (figure 4.3). In support of these results, Sanchez Prieto et al., (2002) have suggested a possibility that JNK pathway can also promote p73 phosphorylation and stabilization. Further, *jnk1*$^{-/-}$*2*$^{-/-}$ cells seem to be more sensitive to cisplatin mediated apoptosis (data not shown), indicating that JNKs are not required for cisplatin mediated apoptosis. Therefore, it appears that JNKs might as well decide between apoptosis versus survival, under a given cellular context.

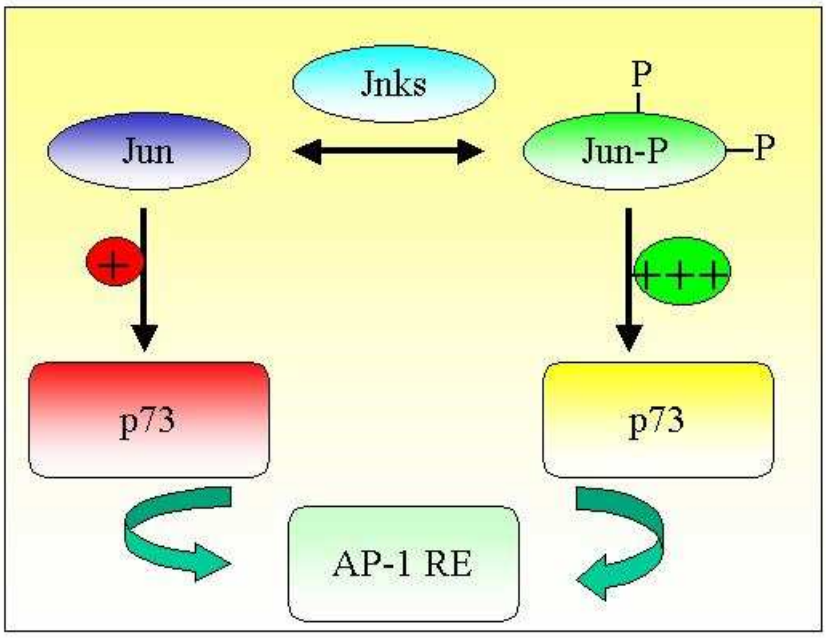


Figure 4.3 Phosphorylated c-Jun is required to synergize with p73 for maximal AP-1 activation

5. Abl negatively regulates p73's ability to potentiate AP-1 activity

As described in chapter I, c-Abl has been shown to phosphorylate both p73 and c-Jun (Agami et al., 1999; Barila et al., 2000) and it potentiates the ability of p73 to induce apoptosis by phosphorylating it (Yuan et al., 1999; Agami et al., 1999). On the other hand, the reporter assays presented in this thesis suggests that c-JunY170F (c-Abl phosphorylation site mutant) co-operated with p73 on AP-1 RE a few folds higher than wild-type c-Jun. This indicates that c-abl by phosphorylating c-Jun negatively regulates c-Jun's ability to transactivate AP-1 responsive element containing promoter activity. Thus, c-abl mediated c-Jun phosphorylation may not be essential for its ability to co-

operate with p73 in potentiating AP-1 activity.

6. Dominant negative p73 (p73DD) inhibits AP-1 transcriptional activity

p73 is essential for increased AP-1 activity is revealed from the fact that dominant negative p73 inhibits both basal AP-1 and c-Jun mediated AP-1 transcriptional activity. This exciting data could mean the following: 1.p73 is required for increased AP-1 activity. 2. p73 contributes to the basal AP-1 transcriptional activity either directly or indirectly by turning on AP-1 coactivator. 3. p73 dominant negative proteins could sequester AP-1 family members and prevent them from binding to AP-1 family members.

7. Identification of AP-1 like responsive elements in both TA and DN-p73 promoters

The ability of c-Jun to regulate p73 levels led us to make an in silico search for promoter regions of the TP73 gene. This analysis suggests that several AP-1 like elements are present in both TA-and DN-p73 promoters. In agreement with this data, it has been shown that promoter region present in the intron 1 of TA-p73 gene and DNp73-promoter encodes AP-1 like responsive elements (Sayan, et al., 2004; Costanzo et al., 2002). Although it is not clear whether AP-1 responsive elements present in the TA-p73 gene and DN-promoters will be used in tissues, it is possible that AP-1 like elements will be actively used in tissues, where AP-1 transcription factors are highly expressed. The ability of c-Jun to stabilize TA-p73 at the protein level in response to cisplatin treatment, and transactivate TA/DN-p73 at the promoter level under basal conditions might add to a

complex regulatory network that would activate/inactivate the function of TA-p73 under different cellular contexts. Further, c-Jun, TA-p73 and deltaNp73 promoters encode AP-1-like responsive elements, indicating a possibility that these proteins can share a positive feed back loop to regulate proliferation and death depending upon the cellular context. This may be an essential mechanism adapted by the cells to exit from the stress response when damaged DNA is repaired. However, how these complex regulatory networks are controlled in response to stress and growth will require a further investigation. In addition, the differential ability of c-Jun to regulate seemingly opposing function could decide the cellular outcome and function of p73 protein.

8. Identification of p73-c-Jun target genes

1. *Collagenase-1:* p73 increases the Collagenase-1 (AP-1 responsive elements) promoter activity

To check the effect of p73 on other AP-1 responsive element containing promoters, collagenase-1 promoter was used. Indeed, p73-β is able to increase collagenase promoter activity as well. This finding opens up new vistas. Firstly, those promoters, which encode AP-1-like responsive elements, will be stimulated by p73. Secondly, collagenase-1, Matrix metalloproteinase's (MMPs) are proteolytic enzymes capable of degrading extra cellular matrix. The MMPs have been shown to play a significant role in tumor invasion, metastasis and tumor-induced angiogenesis. Intriguingly, p73 is over expressed in tumors, however its role in tumorigenisis is not clear yet. The ability of TA-p73-β to increase collagenase-1 (MMP-1) gene promoter activity indicates that it can support metastasis and thereby it could behave like an oncogene in human tumors. In support of p73's role in metastasis, it has been shown

recently that p73 augments the expression of VEGF (Vikanskya et al., 2001), which is known to play a major role in tumor angiogesis and metastasis. Hence, it is plausible that p73 might play an important role in controlling the proteolytic phenotype of fibroblasts, e.g. in tumor invasion by augmenting the expression of collagenease-1.

2. *hMSH1:* p73 increases the hMSH1 (p53 and AP-1 responsive elements) promoter activity

The data presented in this thesis further suggests that MSH-1 promoter, which encodes classic AP-1 and p53 elements adjacent to each other, is stimulated by p73 and c-Jun. In support of this finding, it has been shown recently that treatment of the cells with tetradecanoylphorbol- 13-acetate (TPA), an activator of p73, c-Jun expressions and AP-1 activity, specifically increases the amount of hMSH2 mRNA (Humbert et al., 2003; Fontemagi et al., 2001) in p53 negative cell lines. Further, it was shown that AP-1 binding sites present in the hMSH2 promoter were potentially functional, since these sites were involved in the p53 regulation of hMSH2 associated with UV exposure (Scherer, et al., 2000). The synergism between p53 and c-Jun in response to UV irradiation was explained based on the presence of binding sites for both p53 and AP-1 in the hMSH2 promoter sequence. However, the type of positive up regulation that we describe here for the hMSH2 transcription is clearly independent of p53, since the H1299 cell line used in the study is p53 negative. This opened the possibility that targets for both c-Jun and p73 could exist in cells. For example, when DNA damage is not lethal to the cells then DNA repair mechanism is activated and that could lead to the activation of both p73 and c-Jun to effectively transactivate MSH-1 in repairing the damaged DNA. Thus, it is possible that increased p73 and c-Jun expression could augment Mismatch repair (MMR)

function. Interestingly, AP-1 has been suggested to play a critical role in the cellular response to genotoxic agents. Indeed AP-1 target genes exist among known repair genes, ERCC1 and MGMT (alkyl guanine DNA methyl-transferase) (Humbert et al., 2003). For both the ERCC1 and MGMT genes, AP-1 induced transcription arises in response to genotoxic stress mediated by cisplatin (Li et al., 1998). Therefore given the ability of p73 to stimulate AP-1 target genes, one can undertake studies aimed at examining whether other MMR genes are p73/AP-1 responsive genes.

Section IV: p73 and c-Jun co-operate in transformation

The results presented in section IV suggests that wild type TA-p73 and c-Jun co-operate in transformation. Both p73α and β transform immortalized NIH 3T3 mouse fibroblasts in conjunction with c-Jun. On the other hand, c-Jun rescues the p73-mediated inhibition of colony formation in MCF-7 cells (data not shown), indicating more oncogenes are required to transform human cells. The ability of c-Jun to function as a transcriptional activator varies in different cell types and this partly explains the differences in the ability of c-Jun to transform cells (Imler et al., 1988; Vogt 2001). In addition, mouse primary cells are more easily transformed than primary human cells (in some cases, at least four oncogenes are needed). These results open up many avenues relating to the ability of p73 to transform immortalized fibroblasts in conjunction with established oncogenes.

Both full length TA-p73 and delta-N-p73 have independently over expessed in a variety of tumours. The overexpression of p73 can be co-related with the over expression

of other established oncogenes such as c-Myc, Ras etc. in human cancer (Zaika et al., 2000; Sun et al., 2002). The inability of overexpresed TA-wild-type p73 to prevent neoplastic process can be explained by considering the ability of deltaNp73 to inhibit TA-p73 function.

At least, 30% of human tumors over express c-Myc and in turn induce tumorigenesis, however, conditions favorable for it to induce tumorigenesis or apoptosis is not yet clear. Interestingly, c-Myc over expressing clones seems to over express p73-α/β constitutively. Similarly, ras over expression has been co-related with increased p73 expression in colorectal tumors and there seems to be a positive correlation with increased p73 expression and poor survival of the patients (Sun et al., 2002). The correlations between over expression of p73 and established oncogenes in tumours indicate that p73 function could be modified and tuned in tumours to augment/favour proliferation. Evidently, as stated earlier, c-Myc over expressing clones do over express p73, but its ability to transactivate p53RE containing promoters is reduced (Zakia et al., 2000). The activation of p53RE promoters in cells could result in apoptosis. Further, it has been shown that p73 activity is highly cell-context and promoter-specific. TA--Full-length--p73 expressed in the transformed leukemia cell line Jurkat behaves as a specific dominant negative transcriptional repressor of the cell cycle inhibitor gene p21 and blocks p53-mediated apoptosis (Freebern et al., 2003). These findings provide evidence for a new mechanism in oncogenesis through which the functional properties of p73 can be altered in cell-specific fashion. The p73's p53-related function—ability to transactivate p53RE promoters—needs to be compromised in order to transform fibroblasts. In cell lines or tissues (keratinocytes) that overexpress c-Jun there is an

increase in DN-p73 levels (Costanzo et al., 2002). Similarly, immortalized cell lines that constitutvely express both p73 and c-Jun, TA-p73 would loose its ability to transactivate p53 responsive elements containing promoters. It seems that if a cell line manages to compromise p73's, p53 related function then it can express TA-p73 constitutively. This is evident from the fact that we managed to generate TA-p73β overexpressing NIH 3T3 and MCF cell lines. Similarly, Vikhanskaya (2001) has generated stable p73alpha overexpressing clones from the human ovarian cancer cell line A2780. Further, Vikhanskaya et al., (2001) had shown that the clones overexpressing p73 increases VEGF expression and reduces thrombospondin-1 production. Production of the other angiogenic factors FGF-2, PIGF and PDGF-B was also increased in p73 overexpressing clones. Furthermore, the p73 overexpressing clones were more angiogenic than parental cells, as shown in vitro by their increased chemotactic activity for endothelial cells, and *invivo* by the generation of more vascularized tumors (Vikhanskaya et al., 2001). In support of these findings, our data suggests that p73 augments collagenase-1 or MMP-1 expression in conjuction with c-Jun. Among the multiple MMPs expressed in a wide range of tumors, MMP-1, which is expressed especially in tumor cells with significant invasive properties, is thought to be particularly important for proteolysis (Seiki, 2003). Thus, p73 by increasing the expression of collagenase-1, it could support tumorigenesis.

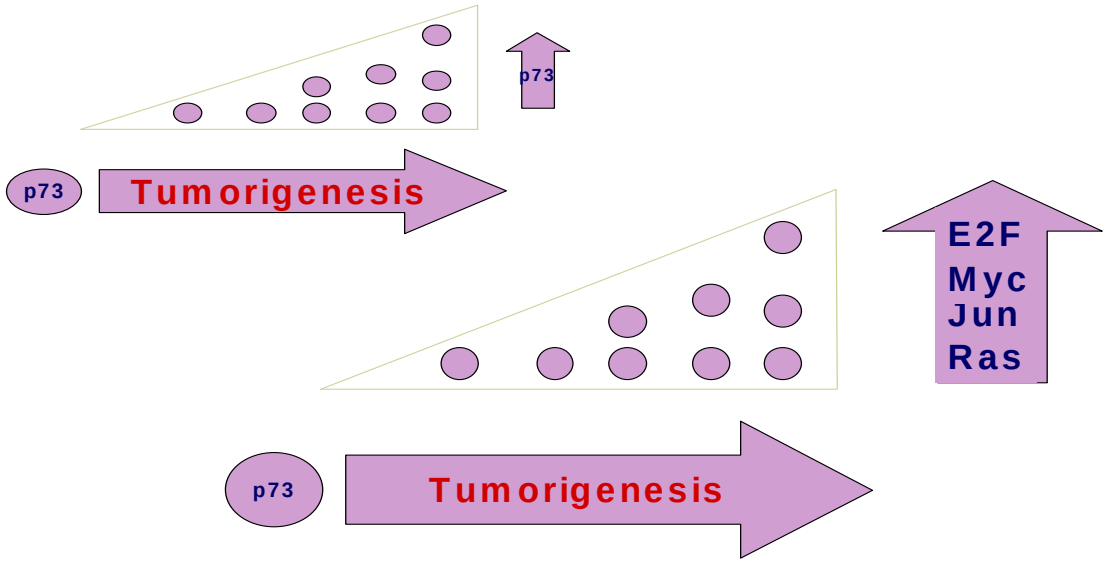


Figure 4.4 Co-existence of p73 and the established oncogenes in cancers

A schematic diagram depicted indicates a seemingly positive co-relation between the over expression of p73 and established oncogenes.

The data presented in section IV is in conjunction with results obtained from various laboratories support the notion that p73 function is modulated in cancer cell microenvironment to support tumorigenesis. Furthermore, the ability of p73 to co-operate and co-exist with established oncogenes explains how TA-p73α/ β failed to prevent cancer cells from aberrant proliferation. Together, these findings indicate a possibility that p73 in conjuction with established oncogenes can promote transformation and tumor angiogenesis.

Further, our data suggests that c-Jun mutants can cooperate with p73 in transformation. Although the discussion regarding how N- and C-terminus of c-Jun co-operate with p73 to transform fibroblasts may not be physiologically relevant, it would indicate the domains (of c-Jun) required for p73 to transform fibroblasts. Both N and C-

termini of c-Jun did not increase the colony number on its own compared to vector control. However, in the presence of p73, both N and C-termini of c-Jun increased the colony numbers. Comparison between the ability of N- and C-termini of c-Jun to co-operate with p73 revealed that C-terminus (194-331) of c-Jun seems to produce more colonies than N-terminus of c-Jun. This difference could be attributed to the fact that C-terminus of c-Jun contains both DNA binding and leucine zipper domain, which is vital for intermolecular interactions.

Although both p73 and c-Jun appear to co-operate with each other in transformation, it is not clear how they bring about transformation. In order to explain this, several mechanistic possibilities are proposed here.

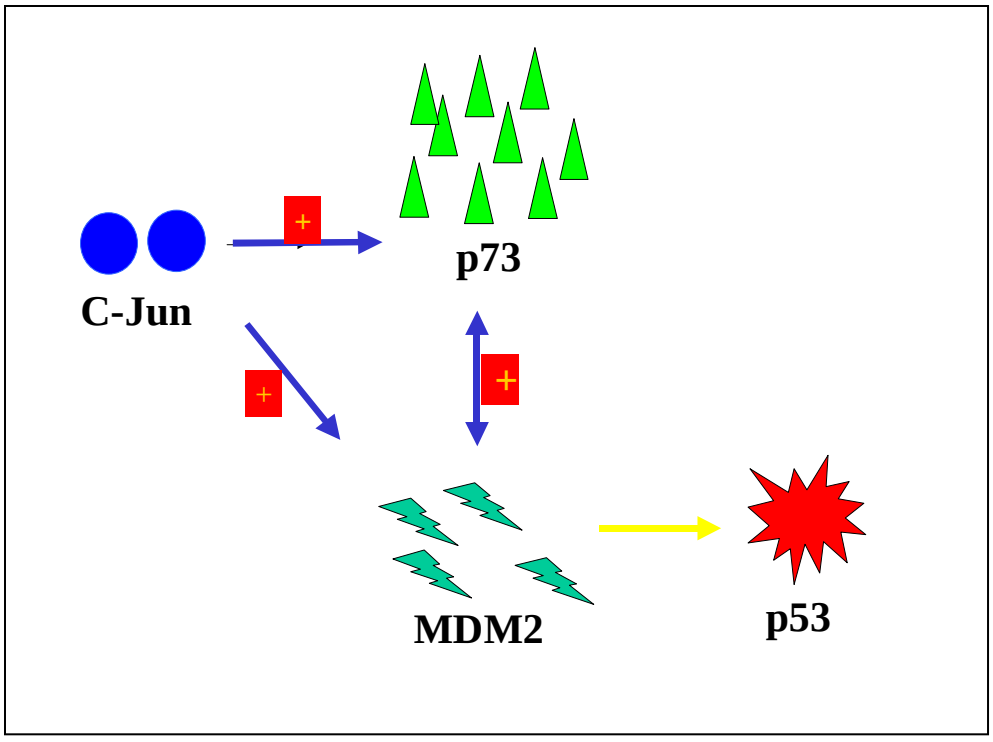


Figure 4.5 A potential mechanism: how the co-operative effect of p73, c-Jun and MDM2 can facilitate transformation

A model proposed here (figure 4.5) explains how p73, c-Jun and MDM2 co-operate with each other in the degradation of p53. c-Jun increases p73 levels, which in turn augment the expression of MDM2. Increased expression of MDM2 degrades p53 on one hand and stabilizes p73 on the other. The degradation of p53 and increased expressions of p73 and c-Jun, would favour transformation.

Mechanism 1: The co-operative effect of p73, c-Jun and MDM2 could promote transformation

Our data suggests that p73, Jun and MDM2 synergistically increase the MDM2 promoter activity. A model depicted above explains how p73, c-Jun and MDM2 co-operate with each other to promote transformation. c-Jun could increase p73 stability. In turn, p73.c-Jun could co-operatively augment the expression of MDM2. Increased MDM2 expression would augment p73 stability, as MDM2 has been shown to stabilize p73 protein (Ongkeko et al., 2000). Sequentially, increased p73 stability can further augment the expression of MDM2, which in turn degrades p53, an important step that promotes transformation.

Mechanism 2: how p73 increases colony number in the presence of c-Jun

The data presented in section III suggests that p73 enhances AP-1 activity. Furthermore, p73 has been shown to increase the transcriptional activity of AP-1 target genes such as MMP-1 and Msh-1 in conjuction with c-Jun. c-Jun has been shown to transactivate Cyclin D1 promoter (Shualin et al., 2001), which encoades AP1 responsive elements in it. Thus, in principle, p73, in concert with c-Jun, can enhance the expression of Cyclin D1 —an AP-1 target gene. The following molecular reaction could occur in cells

sequentially: First, increased cyclin D1 levels would increase Cyclin dependent kinase (CDK4/CDK6) activity. Second, increased CDK4/CDK6 activity would phosphorylate Rb heavily, which results in the release of E2F1. Third, increased E2F1 activity would increase p73 levels, a transcriptional target of E2F1 (Irwin et al., 2001). In turn, p73 would augment the expression of cyclin D1. Thus, increased expression of cell cycle regulatory proteins such as E2F1, p73 and cyclin D1 could decrease the cell cycle time of immortalized cells and increase the colony number. In support of this proposed mechanism, it has been shown recently that various Cyclins (A,B,D and E) and cyclin dependent kinases such as CyclinA-CDK1/2, CyclinB-CDK1/2 and Cyclin E/CDK2 complexes interact with p73 and phosphorylate it at T-79 and thereby inactivate its ability to transactivate p53 responsive element containing promoters such as p21 (Gaiddon et al., 2003). These concurrent events would favor the increased colony numbers seen in the presence of c-Jun and p73. Together, these mechanisms proposed support the idea that p73 and c-Jun indeed co-operate with each other in transformation.

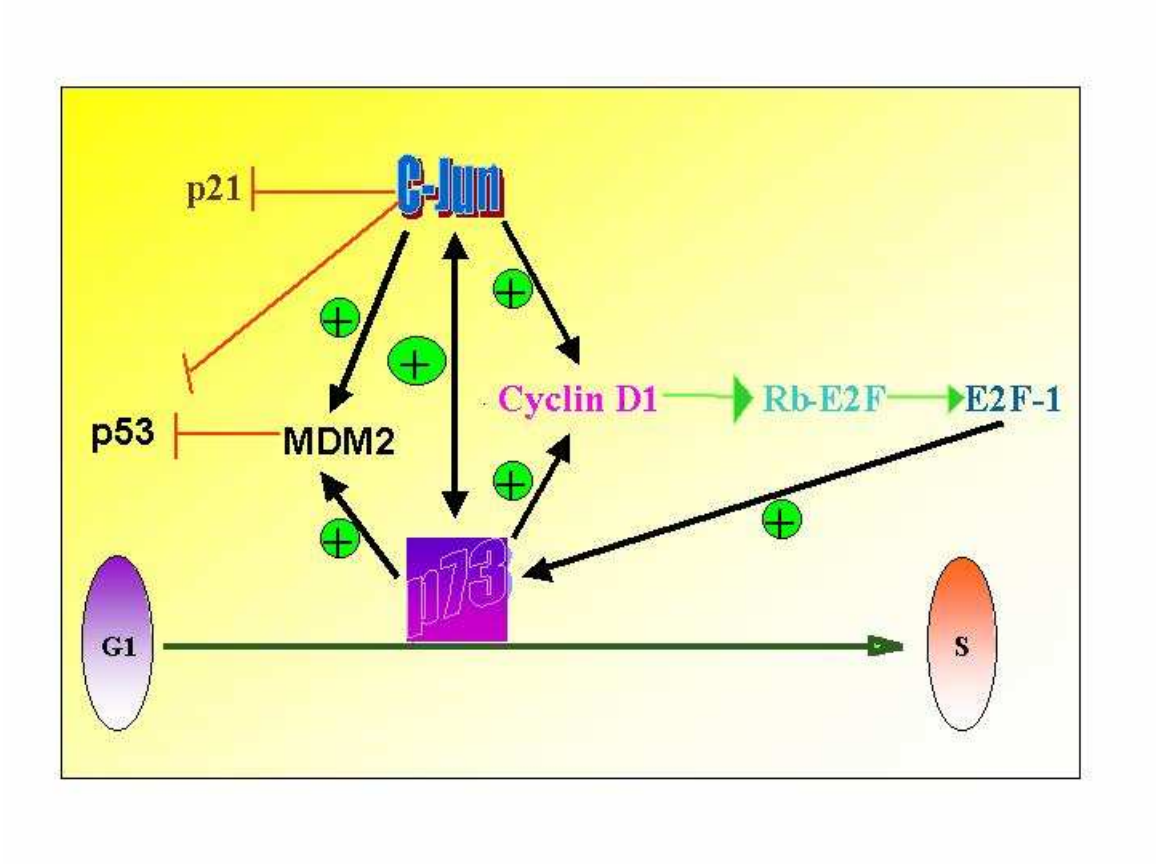


Figure 4.6 How p73 increase the colony number in the presence of c-Jun

c-Jun enhances p73 level. Both p73 and c-Jun synergistically increase cdk4/cyclin D activity. In turn, Rb is heavily phosphorylated, which results in the release of E2F-1. Increased E2F1 activity could agument the transcription of p73. Also, both c-Jun and p73 cooperate with each other to augment the expression of MDM2. In turn, MDM2 enhances p73 level. Hence, co-existence of p73 and c-Jun can increase cyclin D1 levels and suppress p53 function in tumors, thereby increasing colony numbers.

Further, c-Jun has been shown to be over expressed in some human cancers (Mathas et al., 2002). And, the full oncogenic properties of some cancer cells might require elevated c-Jun function (Vogt et al., 2001). Moreover, it is important to realize

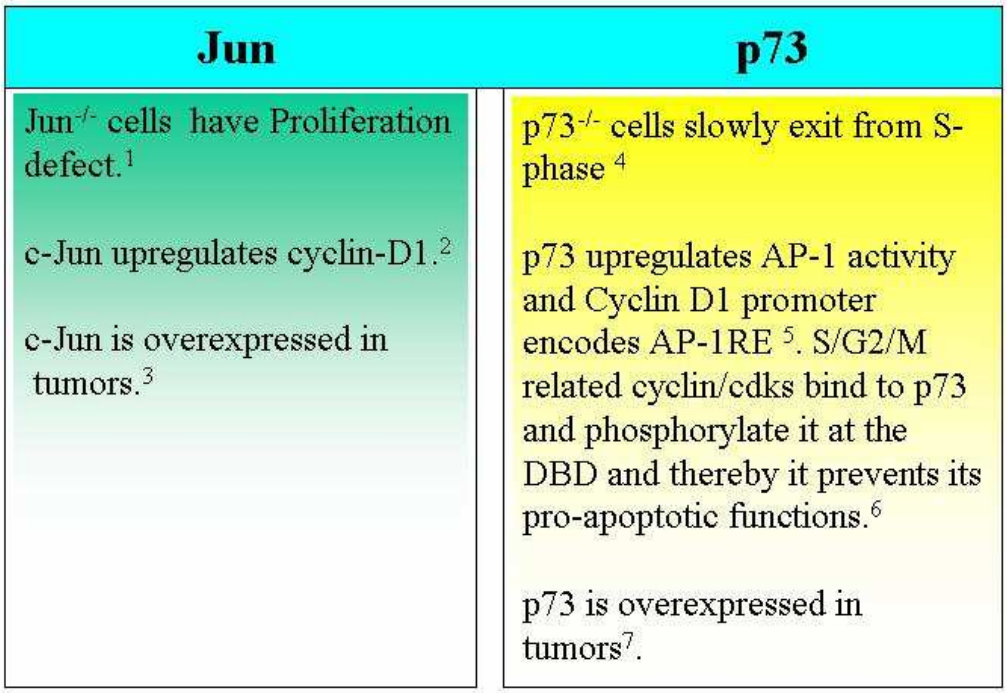
that c-Jun does not have the capacity to increase the colony numbers on its own. This notion is also supported by the fact that transgenic over expression of c-Jun in the absence of additional oncogenic stimulation does not increase tumor incidence. However, c-Jun supports the proliferation in conjunction with other oncogenes such as ras. Intrestingly, Ras causes senescence in primary mouse fibroblasts, but supports tumorigenisis and even serves as a causative factor in colorectal tumors. Similar to oncogenic Ras and c-Myc, p73 induces apoptosis or transformation depending on the absence or presence of survival signals respectively. Intrestingly, it has been shown recently that ras-induced immortalized fibroblasts focus formation without cell cycle regulation (Jacobsen et al., 2002). That is, the growth rate of numerous independent Ras-transformed NIH3T3 cell clones in several NIH3T3 sublines indicated no alteration in doubling times at low cell densities of the transformed cells. It appears that Ras-induced changes do not result in growth advantage at low cell densities. Also, the transforming activity of oncogenic ras is sufficient to relieve cells from contact inhibition, but does not confer a proliferative advantage to cells in low serum (Jacobsen et al., 2002). Moreover, ras appears to regulate p73 levels (Melino, 2003; Sun, 2002). c-Jun has been shown to be downstream of ras. Therefore, p73 mediated c-Jun dependent transformation could result in focus formation, but may not result in increased proliferation rates at low densities.

Furthermore, Pelengaris et al., (2001) showed that switchable c-Myc protein can induce apoptosis and proliferation-- in specific tissues, pancreatic islets cells and keratinocytes-- depending upon the cellular context. Activation of c-Myc in pancreatic beta cells, promotes entry of cells into the cell cycle. However, this is followed by

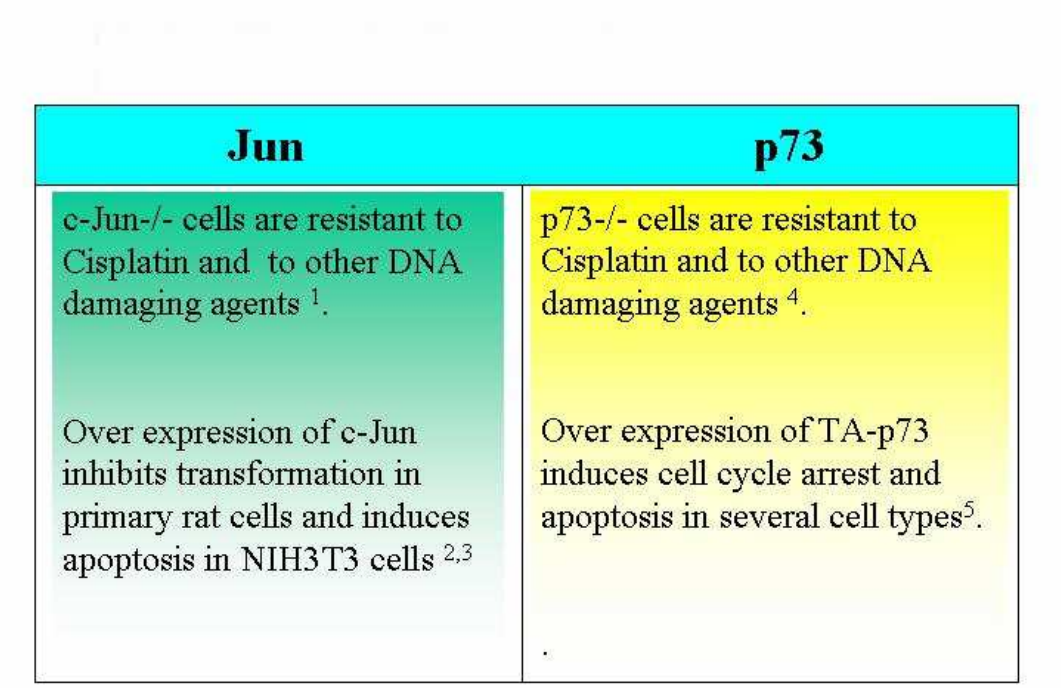
apoptosis that overcomes proliferation. By contrast, activation of c-Myc in skin cells, triggers proliferation, but no apoptosis is evident. This is attributed to the excess of anti-apoptotic survival factors present in skin that acts to suppress the otherwise apoptotic action of c-Myc. Similarly, in tissue culture models, p73 induces apoptosis when it is over expressed in the absence of anti-apoptotic survival factors, while in the presence of excessive oncogenic signals it increases the colony numbers. Hence, these studies suggest that the co-operativity of p73 and c-Jun can promote transformation in continuously growing cell lines.

p73 could be a growth regulatory protein, endowed with the ability to induce apoptosis. The ability of p73 to induce apoptosis and transformation should not be surprising considering the fact that a number of proteins perform two opposing functions: First, TNF-alpha, which elicits two opposing effects, the induction of apoptosis and the transcription of antiapoptotic genes. Second, TGF-beta can switch from a tumor suppressor in the premalignant stages to an ongogenic agent at later stages to result in metastasis (Wakefield et al.,.2003). Third, E2F1, a potent transcriptional activator of p73: mouse model suggests that it is a tumor suppressor, however, in tissue culture it promotes proliferation, Fourth, c-fos has been shown to induce both proliferation and apoptosis in several cell lines etc. Regulatory systems that control proliferation and apoptosis appear to overlap extensively, and indeed, a number of genes identified as oncogene products have been found to mediate apoptosis under certain circumstances. Thus, proteins that regulate both cell cycle and apoptotic machinery are closely connected. This strategy may have been adapted as a ‘fail-safe mechanism’ to prevent aberrant cellular proliferation which could lead to cancer. Finally, it is worth investigating the mechanisms underlying

this phenomenon in order to establish optimum molecular targets whose modulation might trigger analogous regression in human cancers.



Figure/Table 4.7 **Both c-Jun and p73 regulate cellular proliferation.** *(*Ref. 1a. Schreiber et al, 1999; 1b. Wisdom et al., ; 2. Shualin , 2002; 3. Maeda , 2003 ;4. Costanzo , 2002; 5. Bakiri , 2000 6. Gaiddon , 2003 7. Melino , 2003)



Figure/Table 4.8 Both c-Jun and p73 regulate apoptosis (Ref. 1a.Sanchez-Perez , 1999; 1b. Kolbus , 2000 ; 2. Ginsberg , 1991; 3. Bossy-Wetzel , 1997; 4. Flores , 2003; 5. Jost, 1997)

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- E Shaulian, M Karin.**AP-1 as a regulator of cell life and death. Nat Cell Biol. 2002 May; 4(5): E131-6. Review**.

Chapter V

"Science may set limits to knowledge, but should not set limits to imagination". Bertrand Russell (1872 - 1970)"

CONCLUSIONS AND FUTURE DIRECTIONS

This study suggests that both c-Jun and p73 reciprocally regulate each other's stability and function in response to stress and growth stimuli. This fact is strengthened by the following observations:

1. *jun*$^{-/-}$ cells are resistant to cisplatin mediated apoptosis.

2. Transduction of c-Jun in *jun*$^{-/-}$ cells restores sensitivity to cisplatin mediated apoptosis and augments p73 expression. This suggests that p73 and c-Jun may collaborate with each other to induce apoptosis in response to cisplatin mediated apoptosis.

3. UV increases both endogenous and exogenous p73 levels.

4. p73 acts as a positive regulator of AP-1 activity.

5.p73 synergizes with c-Jun to potentiate AP1 target genes such as collagenase-1 and MSH-1. Transactivation domain(TA) of p73 is essential for its ability to synergize with c-Jun.

6. Identification of AP-1 like elements in both TA-and ΔNp73 promoters suggests that both p73 and c-Jun regulate each other in the cell.

7. The PPxY domain is conserved in both p73 and c-Jun.

8. c-Jun synergies with p73 to potentiate p53RE activity. Consecutively, p73 synergizes with c-Jun to increase AP1 activity.

9. The inter-dependence of p73 and c-Jun is evident from the observations that jun null cells exhibit weak p73 transcriptional activity (on p53RE promoters) on one hand, and on the other hand, dominant negative p73 inhibits c-Jun's ability to transactivate AP-1 RE promoters.

10. In the colony formation (long term) assay, wild-type p73α/β and c-Jun co-operate with each other in transformation, indicating that p73 could behave like an oncogene in the presence of excessive oncogenic signals.

1. c-Jun mediated p73 stabilization and activation

This study has identified for the first time, p73 and c-Jun, two key players in the p53 independent apoptotic pathway that collaborate with each other in causing cisplatin sensitivity. p73 has been shown to be induced in response to DNA damage (Agami, et al., 1999; Yuan et al.,1999 and Gong et al., 1999). However, only cisplatin stabilizes p73 but not IR (White et al., 1999). This study explains why cisplatin stabilizes p73 and identifies c-Jun as a component that is responsible for this differential response. Cisplatin is most effective in the treatment of metastatic testicular tumors and is indicated in various combinations of chemotherapeutical regimes for ovarian, head and neck, bladder, cervical and other neoplasms. In addition to its toxic side effects, a major drawback of cisplatin chemotherapy is drug resistance. Hence, understanding the molecular basis of cisplatin mediated apoptosis and drug resistance could significantly improve clinical protocols. Toward this goal, the present study suggests that the absence of c-Jun, could lead to the decreased stability of p73, resulting in cisplaitn resistance in tumor cell lines. Moreover, p73 appears to augment AP1 activity. Several enzymes known to be involved in repair of DNA-cisplatin adducts and implicated in cisplaitn resistance contain AP-1 related sites in their promoters including DNA polymerase β and proliferating cell nuclear antigen (PCNA) (Patopova et al., 1997). It is likely that transcription of these genes can be activated by p73-c-Jun upon stimulation by cisplatin treatment. This

finding opens up new vistas to look for c-Jun and p73 expression in cisplatin resistant human tumours. The following cell lines have been identified to lack p73 expression and are known to be resistant to cisplatin treatment:

1. p53 expression is induced to the similar level in cisplatin resistant human bladder carcinoma cell line such as TCC (transitional carcinoma) cell line and its isogenic cell line. On the other hand, p73 expression is not induced in cisplatin resistant TCC, emphasizing the importance of p73 in cisplatin mediated apoptosis. In addition, these results suggest that loss of p73 induction may lead to CDDP resistance of TCC carcinoma (Ono et al., 2001).
2. p73 expression is not induced in Hereditary cancer syndrome human non-polyposis colon carcinoma (defective in mismatch repair) cells that are resistant to cisplatin (Gong et al., 1999).
3. p73-negative SK-N-AS, a human neuroblastoma cell line, is more resistant to cisplatin than p73 positive-SK-N-SH cells (Gong et al., 1999).
4. *p73*$^{-/-}$ cell lines are more resistant to cisplatin compared to wild type (Flores et al., 2002).
5. The study presented here shows that *jun*$^{-/-}$ and *p53*$^{-/-}$*jun*$^{-/-}$ cell lines are more resistant to cisplatin compared to wild type; and p73 transcriptional activity (p53 RE promoters) is reduced in the absence of c-Jun (*p53*$^{-/-}$ Vs *p53*$^{-/-}$*Jun*$^{-/-}$).

These data generated from various laboratories support the idea that p73 expression plays a major role in cisplatin resistance, which is commonly found in various human cancers. p73 could be an attractive therapeutic target in oncology because its tumor-suppressor mimicking (apoptotic inducing) activity can be activated to eradicate tumor cells.

Promoting the p73–c-Jun interaction could be a promising approach for activating p73, because this association could lead to further stabilization and increased transcriptional activity of p73. It is important to note that a similar strategy has been used to activate p53 by peptides that inhibit the p53–MDM2 interaction (Chene et al., 2001). These peptide inhibitors exhibit an antiproliferative effect in tumor cells over expressing MDM2.

2. UV radiation enhances p73 levels

The results in this study show for the first time that UV radiation enhances p73 levels. Next, it would be worthwhile to find out the kinases responsible for the phosphorylation of p73 in response to UV irradiation. p38, a serine-threonine kinase, could be one of those candidates that need to be verified in detail. p38 kinase has been shown to play a critical role in the initiation of a G2 delay after ultraviolet radiation and inhibition of p38 blocks the rapid initiation of this checkpoint in both human and murine cells after ultraviolet radiation (Boulavin et al., 2001). Although our results suggest that UV radiation increased p73 levels could lead to p53-independent apoptosis, it is worthwhile to understand the molecular mechanisms involved. Given the ability of p73 to respond to UV irradiation, it is possible that p53-independent UV-mediated cell cycle arrest and apoptosis can occur through p73. Studies need to be carried out further to address these issues in detail.

3. p73 synergies with c-Jun to induce AP-1 activity

The results in this study show for the first time that p73 functions as a positive regulator of AP-1 activity. The AP-1 activity is stimulated in response to an incredible array of stimuli, including mitogenic growth factors, growth factors of the TGF-beta

family, UV, cellular stress, antigen binding, and neoplastic transformation, which indicates that p73 could, in principle, participate in regulatory network of all these processes. For example, many of the growth and stress regulatory genes encode AP-1 elements in their promoter, so in principle p73 can play a part in the growth, DNA repair and stress response. The ability of p73 to regulate AP-1 encoding genes can be exploited for pharmacological benefits. Hence, our findings open up new vistas in p73 mediated gene regulatory pathways that differ from its counter part, p53. This study has identified MSH-1, a mis-match repair enzyme and Collagenase-1, Matrix metalloprotease enzyme as targets of the c-Jun-p73. Also, it is possible that p73 could potentiate c-Jun dependent expression of other AP1 target genes. However, it is not clear how exactly p73 stimulates AP-1 activity and how does p73 co-operate with c-Jun to stimulate its activity. Moreover, p73-c-Jun appears to regulate both p53REs and AP-1REs, possibly in a context dependent manner. Thus, studies need to be carried out further to address these questions in detail.

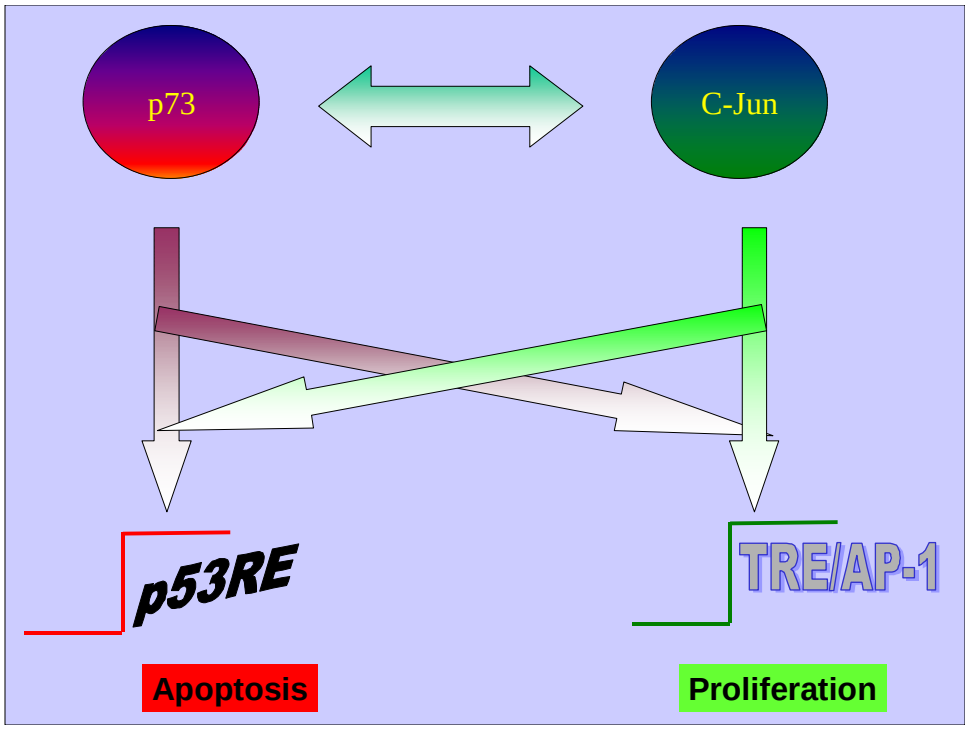

Figure 5.1 Schematic representation of the ability of p73 and c-Jun to choose promoters containing different response elements in a context dependent manner

In a context dependent manner, both p73 and c-Jun synergistically enhance the transcription of either p53 RE (stress) or AP1 RE (growth) containing promoters.

The schematic diagram depicted here (5.1) exemplifies preferential selectivity of transcription factors to particular DNA responsive elements based on the imposed conditions i.e. under growth promoting conditions AP-1 promoter activity is predominantly chosen and under stressful conditions p53RE activity is chosen. In addition, promoter selectivity could rely on the composition of partner proteins responding to a given stimuli. This would play a major role in determining whether to potentiate either p53RE or AP-1 activity, consequently, to favor apoptosis or

proliferation. Furthermore, this partner dependent promoter regulation is applicable to both AP-1 responsive promoters and p53RE containing promoters. The dynamic balance between p73 and c-Jun may play a decisive role in whether the cell survives or undergoes apoptosis. However, it is important to note that AP-1 activity is also induced by various chemotherapeutic agents and stress stimuli and seems to play a role in the induction of apoptosis. Evidently, it has been shown that AP-1 binds directly to the promoter region of the p21WAF1 gene in a p53-deficient human lung carcinoma cell line, H1299 and regulates its expression in response to H_2O_2 (Chung, 2002). This data suggests that p73 could regulate p21 expression in p53 deficient cells in cooperation with c-Jun/AP1. Similarly, the p73-c-Jun complexes could effectively transactivate genes that encode AP-1 responsive elements in their promoters and thereby participate in cellular proliferation, DNA repair and apoptosis, depending upon the cellular context. Therefore, p73, possibly assisted by c-Jun, could control the expression of an even broader set of growth regulatory genes such as cyclin D1, apoptotic and DNA repair genes. In the future, it will be interesting to analyse other candidate genes for p73-c-Jun dependence. Dominant negative p73 (DD) does not only lower the ability of c-Jun or p73 to increase AP-1 activity but it lowers the basal AP-1 activity in cells.

The ability of dominant negative p73 to inhibit AP-1 activity certainly highlights p73's importance in influencing AP-1 activity other than through c-Jun in cells. Moreover, one would also be interested to find out how different JNK members influence the p73 function, as phosphorylated c-Jun is more efficiently synergized with p73 in increasing AP-1 activity.

4. p73 and c-Jun co-operate with each other in Transformation

The results presented here suggest that p73 could support transformation in the presence of excessive oncogenic signals provided by c-Jun. The data from various laboratories indicate that p73 co-operates with several established oncogenes (Zakia et al., 2001; Melino, 2002). These observations support the view that p73 could be a proliferative response gene, which has the capacity to induce proliferation or apoptosis in a context dependent manner.

The p73 (full-length) is overexpressed in a variety of tumour cells (Zakia et al., 2002; Melino, 2002). The results in this study shed new insight on p73 and provide a likely explanation as to why p73 failed to prevent tumorigenesis and in turn how p73 can support transformation in the presence of excessive oncogenic signals provided by c-Jun. Evidently, p73 co-operates with oncogenes such as E2F-1, E1A, c-Jun, c-Myc etc. The ability of p73 to co-operate with several oncogenes would certainly make it a less susceptible candidate for acquired loss of functional mutations during oncogenesis, as there is no selection pressure to mutate p73, unlike tumour suppressor p53. This prediction is strongly supported by the following facts: First, hepatocytes express only delta N-p73, where as the activation of TA-p73 expression in hepatocellular carcinoma (HCC). Second, monoallelic expression of p73 has been noted in normal lung, while the activation of 'p73 silent allele' in lung cancer. Third, monoallelic expression of p73 has been found in gastric mucosa, while the activation of 'p73 silent allele' in gastric cancer. Thus, the acquired expression of TA- p73 in HCC, lung cancer and gastric cancer looks like transformation related rather than a tumour suppressor related function. Together, our results provide a framework for understanding why wild-type TA-p73 (full length)

failed to prevent tumorigenesis in spite of it's over expression seen in several tumors.

Cell proliferation and apoptosis might appear like opposing functions, but proto-oncogene c-Jun and putative tumor suppressor homologue, p73 might be able to initiate or regulate both. The mechanism of oncogenes initiated cell death and its targets are just beginning to emerge. Furthermore, how oncogenes regulate both proliferation and cell death machinery is far from being understood. However, it makes lots of sense now as to why these processes need to be linked. Apoptosis is not only induced by stress stimuli, but also induced by excessive oncogenic signals, and therefore to prevent cancer. In addition, when oncogenes like c-Jun, c-Myc, E1A, E2F, c-fos etc. are ectopically over expressed under conditions not appropriate for cell proliferation, for example, under serum starved conditions (Colotta et al., 1992; Clark et al., 1997) or DNA damaged cells, then they have been shown to be capable of initiating apoptotic cascades. Evidently, it has been shown recently that apoptotic target genes such as Fas L (Kolbus et al., 2000) and caspases (Nahle et al., 2002) are direct targets of proliferation associated genes such as c-Jun and E2F1 respectively.

This study shows for the first time that p73 functions as a co-factor for c-Jun mediated biological responses, in turn, it appears that c-Jun influences p73's stress related activity. In line with our results, Massimo's group showed that deltaN-p73 promoter, which encodes AP-1 responsive elements, is regulated by c-Jun (Costanzo et al, ESDR meeting, 2002). Furthermore, the TA/DN p73 promoter analysis presented in this thesis and data presented by Costanzo et al., suggested that deltaN p73 promoter, which encodes both AP-1 and p53 responsive elements, can be regulated both by c-Jun and p53/p73. It is possible that p73 and c-Jun regulate the deltaN-p73 promoter and inactivate p53/p73 by

complexing with it directly, which would be analogous to p53 mediated MDM2 regulation. It is also important to emphasize here that MDM2 promoter contains both AP1 and p53RE in its promoter and it is subjected to the regulation of both by c-Jun and p73 (Ries et al., 2000). These interesting findings could well suggest that the co-operation between p73 and c-Jun on AP-1 promoters will be very effective in switching off stress related activities especially in tumor cells. While in normal cells the relative regulatory networks could be stringent and it could effectively induce apoptosis in response to stress. Collectively, it appears that both c-Jun/AP-1 and TA/DN-p73 can regulate each other in cells. However, further studies should be extended to understand how c-Jun regulates p73 stability and activation, in turn, how p73 regulates AP1 activity.

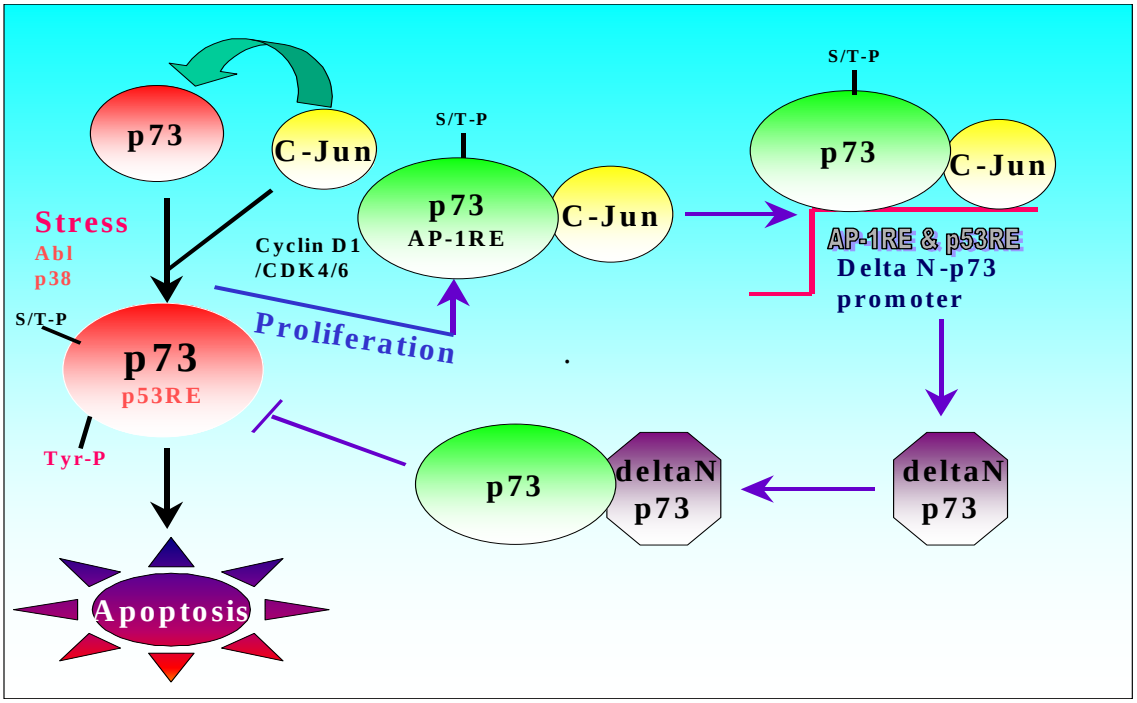

Figure 5.2 Schematic representation of the existence of a regulatory loop between the TA-p73/ΔN-p73 and c-Jun.

In response to cisplatin treatment, both c-Jun and p73 are activated, and p73 is stabilized by c-Jun dependent mechanism and they collaborate with each other to induce apoptosis. The ability of c-Jun-p73 to transactivate deltaN-p73 could be switched off transiently by post-translational modifications in response to stress. In response to growth stimuli or in tumor cells, c-Jun-p73 could increase deltaN-p73 transcripts level and the increased deltaN p73 could compete with p73/p53 protein to form inactive complexes. Increasing the deltaNp73/TA-p73 ratio will favour growth, transformation and tumorigenesis. In addition, deltaN-p73 is proposed to function as growth promoting gene, independent of its ability to inactivate p73/p53.

Together, this study shows for the first time that both p73 and c-Jun regulate each other in regulating life and death of a cell. In addition, this study points out a future direction in which how other AP-1 family members regulate p73 in response to various extra and intra cellular stimuli to regulate cell fate decisions.

“Every science begins as philosophy and ends as art” Will Durant

CHAPTER 6

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