Introduction
p53: Structure and Function

The p53 gene, first described in 1979, was the second tumor-suppressor gene to be identified. It was originally believed to be an oncogene, a cell-cycle accelerator but genetic and functional data obtained ten years after its discovery showed it to be a tumor suppressor (Vogelstein, 2000). The importance of p53 protein lies in the fact that the p53 protein does not function correctly in most human cancers. In about half of these tumors, p53 is inactivated directly as a result of mutations in the p53 gene. While in many others, it is inactivated indirectly through binding to viral proteins, or as a result of alterations in genes whose products interact with p53 or transmit information to or from p53 (Vogelstein, 2000).

In laboratory animals, the absence of p53 permits the occurrence of numerous genetic alterations, including gene amplification and abnormal chromosome numbers, and leads to the development of a variety of tumors in young adults (Donehower, 1992; Fukasawa, 1997; Yin, 1992). These findings underscore the pivotal nature of p53 in preventing tumour development and are consistent with the well established occurrence of loss of p53 function in the development of a wide range of human tumours (Hollstein, 1996). The importance of p53 in the cancer lies in the fact that p53 was the first commercially available gene therapy treatment to be approved (Pearson, 2004).

The human p53 gene is localized to the short arm of chromosome 17 (17p13) and spans about 20 kb of DNA. It is composed of 11 exons, the first of which is non coding and is localized 8-10 kb away from coding exons, 2 to 11 (Benchimol, 1985; Isobe, 1986; Miller, 1986; Oren, 1985). The human p53 gene encodes a 393 amino acid protein that can be divided into four major functional domains (Fig. A). The amino terminal domain of p53 consists of an intrinsically disordered transactivation domain (TAD) and a proline-rich region. It is followed by the central, folded DNA-binding core domain that is responsible for sequence specific DNA binding. The core domain is connected to the tetramerization that regulates the oligomerization state of p53 domain via a flexible linker. At its carboxy terminus p53 contains the negative regulatory
2domain. This domain is natively unfolded and is rich in basic amino acids (mainly lysines) and binds DNA nonspecifically (Joerger, 2008). Cross species comparison of amino acid sequences of the p53 protein showed the existence of five highly conserved regions within the amino acid residues 13-23, 117-142, 171-181, 234-250 and 270-286 (Soussi, 1990; Soussi, 1996). These regions, termed domains I-V, were expected to be crucial for the p53 functions.

Although, p53 is a nuclear protein, but it shuttles between the cytoplasmic and nuclear compartments in a cell cycle dependent fashion (Liang, 2001). For the nucleo-cytoplasmic shuttling of p53 in the cell, it contains nuclear localization signal (NLS) and nuclear export signal (NES). Three NLS are reported in the p53 protein, out of which one is strong and spans between residues 316 -322. A highly conserved leucine-rich NES has been identified in the tetramerization domain of p53 (326-354) (Liang, 2001), a NES is also identified in the transactivation domain (amino acid residues 11-27) (Zhang, 2001). The p53 protein is a transcription factor that enhances the rate of transcription of genes that contain p53 binding site (sites). As transcription factor, p53 regulates the expression of genes involved in a variety of cellular functions, including cell-cycle arrest, DNA repair, and apoptosis (Vogelstein, 2000). The p53 protein was also shown to repress the transcription of a variety of genes including c-MYC, Cyclin B, VEGF, RAD51, and hTERT (Laptenko, 2006). In addition p53 also works in the transcription independent manner in the apoptotic pathway (Chipuk, 2004; Moll, 2005; Leu, 2004).

In normally growing cells, p53 protein levels are very low. In response to different cellular stresses, such as DNA damage, oxidative stress, inappropriate cell proliferation driven by oncogene activation, telomere erosion, nutrient deprivation, radiations and hypoxia, and so on, p53 is stabilized by covalent and noncovalent modifications, succeeding specific protein–protein interactions that in turn, rapidly increase p53 protein levels and induce the expression of genes involved in cell cycle arrest to allow repair processes or failing that, induce the expression of genes promoting apoptosis (Laptenko, 2006). Genotoxic damage caused by chemotherapy or radiotherapy induces p53 overexpression to control the rate of proliferating damaged cells, thus triggering the mismatch repair or apoptotic pathway. The p53 protein also has its darker side, as slight constitutive hyperactivation of p53 results in an alarming premature-ageing phenotype in
mice (Tyner, 2002; Maier, 2004). In humans, a polymorphism in p53 that results in a slight reduction in its activity is associated with an enhanced cancer risk, but also with increased longevity (van Heemst, 2005). How p53 might promote ageing is not yet clear, although a contribution of p53 to cellular senescence and the limitation of the proliferative capacity of stem cells has been proposed (Sharpless, 2004).

In a nutshell, p53 is primarily a sequence-specific transcriptional activator. It binds to cognate p53 responsive elements within the genome and activates the transcription of genes residing in the vicinity of these binding sites. Although p53 can also function in a transcription-independent manner (Fuster, 2007), the best understood functions of p53 have been attributed to its DNA binding and transcription activity.

**Functional regions**

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<tr>
<th>Transactivation SH3</th>
<th>DNA binding domain</th>
<th>Tetramerization domain</th>
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**Conserved region**

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Fig. A. Structural and functional regions of the p53 protein. Functional regions and corresponding amino acid residues are shown on top. The bottom represents the evolutionary highly conserved domains I-V. (May, 1999).

**Amino terminal domain**

The amino-terminal domain of p53 encompassing residues 1-93 (Wells, 2008) of the protein is remarkably acidic and its primary structure is similar to other activation domains. It consists of two ill-defined and contiguous transcriptional activation subdomains (amino acids 1-42 and 43-63) (Candau, 1997; Zhu, 1999) and an adjacent proline-rich domain (amino acids 62-91) containing five copies of the sequence “PXXP” (Cain, 2000) as well as a repressor domain...
between the proline rich domain and the DNA binding domain (residues 100-116) (Curtin, 2005).

Structure of the amino terminal domain

Unlike the DNA binding and tetramerization domain which are well structured the amino terminal domain of p53 is natively unfolded (Bell, 2002; Dawson, 2003), although a residual secondary structure is observed in regions of functionally important hydrophobic residues (Lee, 2000). However recently using paramagnetic relaxation enhancement (PRE) it has been shown that the p53TAD contains some global organization to the transient secondary structures and this global organization is important for the function of p53TAD (Vise, 2007). The unbound full-length p53 transactivation domain is populated by an amphipathic helix and two nascent turns. The helix is formed by residues Thr18-Leu26 (Thr-Phe-Ser-Leu-Trp-Lys-Leu-Leu), whereas the two turns are formed by residues Met40-Met44 and Asp48-Trp53, respectively (Lee, 2000). Amino acid residues 15-29 adopt \( \alpha \)-helical conformation upon binding to a hydrophobic cleft in the N-terminal domain of MDM2 and MDM4 (Kussie, 1996; Popowicz, 2007). In addition Chi et al (2005) reported two mini-motifs (turn I, residues 40-45 and turn II, 49-54) which were shown to bind MDM2.

The regions of the nascent helical structure that are present in the native state of p53 become rigid and fully folded when the transactivation domain binds with the partner proteins. A characteristic is the presence of molecular recognition features, short segments of about 20 residues that undergo disorder-to-order transition upon binding to their protein or nucleic acid binding partners (Mohan, 2006; Vacic, 2007). Regions within TAD2 fold into amphipathic \( \alpha \)-helices upon binding to replication protein A (Bochkareva, 2005) and to the Tfb1 subunit of yeast TFIH (Di Lello, 2006). The amino terminal domain is also reported to take part in the formation of p53 tetramer. The N-terminus (residues 1-63) of one p53 monomer binds with the C-terminus (residues 323-393) of another p53 molecule to form a dimer (Okorokov, 2006).

The proline-rich region that links the TAD to the DNA-binding domain in human p53 contains five PXXP motifs (Walker, 1996) that mediate numerous protein-protein interactions in
signal transduction through binding to Src homology 3 domains (Kay, 2000). The PXXP motifs are not conserved among mammalian p53s, but the prevalence of prolines in this region is maintained (Toledo, 2007), indicating a functional or structural requirement for a certain degree of rigidity. Small angle X ray (SAXS) have shown that the proline-rich region (residues 64–92) was stiffer and has a tendency to adopt a polyproline II (PPII) structure that projects the TAD away from the core domain (Wells, 2008, Fig. B). The mutational studies on mouse models have shown that the putative protein docking sites in this region are dispensable for tumor suppression, whereas the length of the region is crucial (Toledo, 2007). These findings support a modular role of this domain, potentially as a spacer between the different functional domains.

**Fig. B.** Modeling the N-terminal domain in the p53–DNA complex. p53CTetD (gray) and DNA (magenta) are shown in space fill mode. The flexible C-terminal domain is not shown. N-terminal domains forming the four difference monomers are shown in different colors for clarity. Twenty copies are shown for each monomer. (Wells, 2008).

**Functions of the Transactivation Domain**

The transactivation potential of transactivation domain mainly lies in the first transactivation domain, since the deletion of amino acid residues 20 to 42 completely abolishes the transactivation activity, which can be restored by substituting the transactivation domain of the VP16 protein at the same location (Pietenpol, 1994). Functional analysis of this region using
a library of point mutations at various residues revealed that a single point mutation was generally not able to completely abolish transactivational activity (Lin, 1994). However, simultaneous mutation of two hydrophobic residues Leu22 and Trp23 markedly impairs transactivation by p53 ((Lin, 1994). Mutation of residues Trp53 and Phe54 affect specifically the ability of p53 to regulate some of its pro-apoptotic target genes in some settings (Candau, 1997). These findings suggested that the overall structure of the transactivation domain rather than its sequence is important for the transactivational activity. These results also explained why mutations in the transactivation domain cannot be found in human cancers. Also unlike the DNA-binding region, which is highly sensitive to virtually any point mutation, the transactivation domain is sufficiently stable to be tolerant of a single mutational event (Soussi, 1996).

Further the amino terminus (residues Glu17 to Asn29) is the region to which its negative regulator Mdm2 binds and targets p53 for both repression of its transcriptional regulation functions and also for proteasome-mediated degradation (Bond, 2005). CHIP that is responsible for the ubiquitination of wild type as well as mutant p53 and the chaperoning of p53 also binds with p53 amino terminus (Esser, 2005; Tripathi, 2007). The MDM2-binding region of the p53 TAD overlaps with parts of the binding site for the transcriptional coactivator p300, which is essential for the transcriptional function of p53. The p300 acetylates lysine residues in the C-terminal region of p53 (Goodman, 2000).

The p53 amino terminus is a site of post-translational modification of which phosphorylation is major change with 10 being observed within 100 amino acids of the N-terminus (Lavin, 2006) (Fig. C). These phosphorylations are brought about by a number of protein kinases that respond to different stress stimuli including ATM (mutated in ataxia-telangiectasia); ATR (A-T and Rad3-related), the checkpoint kinases, Chk1 and Chk2; Jun NH2-terminal kinase (JNK), p38 and others. The most frequently described phosphorylation is on Ser15 and occurs in response to different stress signals. This phosphorylation occurs rapidly in response to DNA double-strand breaks and is carried out by ATM (Lavin, 2006). ATM also mediates phosphorylation at Ser 6, 9, 15, 20, 46 and Thr 18 on p53 in response to ionizing radiation (Saito, 2002). Ser15 phosphorylation of p53 acts as a precursor for the subsequent
phosphorylation of Thr18 by another kinase such as Chk2 and may also have a bearing on Ser9 and Ser20 phosphorylations (Saito, 2003). Phosphorylations at Ser15, Thr18 and Ser20 stimulate the recruitment of other factors including p300, CBP and P/CAF that promote C-terminal acetylation that may prevent p53 from ubiquitination and subsequent degradation (Ito, 2001).

Fig. C. Post-translational modification of p53. (Lavin, 2006).

Phosphorylation of p53 at amino acid residues Ser15, Thr18 and Ser20 also switches the folded transactivation domain to more open conformations that interact with transcription factors such as p300/cAMP-responsive element-binding protein-binding protein, leading to enhancement of gene expression (Kar, 2002). Furthermore the homedomain-interacting protein kinase 2 (HIPK2) phosphorylates p53 on ser46 and may assist in dissociating p53 from MDM2 and also causes the reduced shuttling of p53 from the nucleus (Di Stefano, 2004). JNK is responsible for phosphorylation of p53 at Thr81, which stabilizes and transcriptionally activates the molecule (Buschmann, 2001).

The phosphorylation of p53 is also responsible for promoter recognition by the molecule. In mice, phosphorylation of serine residues 18 and 23 is necessary for apoptosis and tumour suppression, but not cell cycle arrest and senescence (Chao, 2006). Phosphorylation of human p53 on serine 46 has been shown to contribute specifically to the activation of some apoptotic target genes, and mutation of this phosphorylation site reduces the ability of p53 to induce cell
death but not proliferative arrest (Mayo, 2005; Oda, 2000). Phosphorylation of serine 46 can contribute to the interaction of the second transactivation domain of p53 with the p62/Tfb1 subunit of the general transcription factor TFIIH (Mayo, 2005) resulting in the induction of a specific subset of p53-responsive genes. Phosphorylation at serine 46 has also been linked to the ability of p53 to repress expression of galectin-3, an anti-apoptotic protein (Cecchinelli, 2006).

Functions of the Proline Rich Domain

The p53 proline-rich domain (PRD) contributes to the regulation of p53 stability, transactivation ability, and induction of transcription-independent apoptosis (Toledo, 2007). This region was identified as a suppressor of growth by inducing the apoptosis, it is dispensable for transcriptional activation however its deletion results in the impairment of p53’s ability to suppress tumor cell growth in culture (Walker, 1996). Further experiments indicated that the proline rich domain is necessary for apoptosis (Baptiste, 2002; Chipuk, 2004; Sakamuro, 1997; Roth, 2000) but not cell cycle arrest (Sakamuro, 1997; Zhu, 1999; Venot, 1998). The p53 protein lacking the proline rich region has altered specificity for endogenous target promoters, there is a decrease in the induction of several apoptotic genes: PIG3, PIG6, PIG11, p85, and BTG2 (Zhu, 1999), most of which were implicated in the cellular apoptotic response to oxidative stress (Polyak, 1997; Yin, 1998). However, the induction of other apoptotic genes, such as bax and KILLER/DR5, is unaffected (Zhu, 1999).

The p53 can function as a pro-apoptotic BH3-domain protein that leads to the release of cytochrome c from the mitochondria and induction of caspases and cell death (Vousden, 2007). Although this function of p53 is independent of transcription, an elegant model has been proposed in which activation of expression of the BH3-only protein PUMA by p53 is necessary to dislodge cytoplasmic p53 from an inactivating complex it forms with the anti-apoptotic BH3 domain proteins such as BclxL (Chipuk, 2004). This domain might also ensure optimal p53-p300/CBP interactions through PXXP motifs, and the deletion of the proline repeat motif of p53 prevents DNA-dependent acetylation of p53 by occluding p300 from the p53-DNA complex (Dornan, 2003).
The proline-rich region of p53 also plays a role in the regulation of p53 by Mdm2. The prolyl isomerase Pin1 was proposed to affect proline conformational changes that may reduce Mdm2 binding and enable p53 accumulation (Wulf, 2002; Zacchi, 2002; Zheng, 2002). The Pin1 causes the isomerization of proline 82, enabling the recruitment of CHK2 to phosphorylate serine 20 and consequently reduce MDM2 binding (Berger, 2005). The proline rich region (amino acids 65-71) is the binding site of focal adhesion kinase (FAK) and the mutation in the binding site in p53 reversed the suppressive effect of FAK on p53-mediated transactivation of p21, BAX (Bcl-2-associated X protein) and Mdm2 (murine double minute 2) promoters (Goluboyaskaya, 2008). This region was also shown to be required for p53-dependent cell growth arrest through Gas-1, a plasma membrane protein highly expressed during G0 phase (Ruaro, 1997).

Function of the Repressor Domain

One more functionally divergent region within the N-terminal part of p53 is a repression domain, localized between proline-rich domain and DNA-core domain (residues 100-116) (Curtin, 2005). This domain can function as an independent heterologous repressor, and has been shown to decrease VP16-driven activation up to 20 times in human embryonal carcinoma cells, when being fused to VP16 transactivator (Curtin, 2005). As wild-type p53 is found to be over-expressed in some type of tumors, it has been proposed that the newly identified domain may play an important role in repression of the basal activity of p53 (Laptenco, 2006). Factors that are or can be specifically recruited by this domain, and that might be essential for the repression phenomenon found, are not known at present. Thus, these and other experiments further highlight the likelihood that transcriptional regulation by p53 involves several different regions within its N-terminus.

DNA binding domain

The DNA binding (core) domain of p53 is the largest domain of p53 protein (residues 94-312) and is the location of nearly all of the mutations that inactivate p53 in some 50% of human cancers (Olivier, 2002). The core domain is naturally unstable, with a melting
temperature of 42-44°C (Bullock, 1997), and many oncogenic mutations inactivate the core by either simply destabilizing or distorting it (Bullock, 2001). The structural studies have shown that the p53 core domain consists of an immunoglobulin-like β-sandwich that provides the basic scaffold for the DNA-binding surface (Fig. D). This surface is subdivided into two structural motifs that bind to the minor groove and major groove of target DNA, respectively.

![Fig. D. Structure of the p53 DNA-binding domain. (a) Sequence and secondary structure of the p53 core domain (p53C). DNA-contact residues are marked with asterisks. (b) Structure of DNA-free p53C shown with a rainbow color gradient, from blue at the N terminus to red at the C terminus, Protein Data Bank (PDB) entry 2OCl (c) p53C bound to DNA (PDB entry 2AIH). (d) p53 core domains bound to two consecutive DNA half-site motifs (PDB entry 2AIH). (Joerger, 2008).](image)

The loop-sheet-helix motif, which docks to the DNA major groove, includes loop L1, β-strands S2 and S2, parts of the extended a β strand S10, and the C-terminal helix. The other half of the DNA-binding surface is formed by two large loops (L2 and L3), which are stabilized by a zinc ion (Joerger, 2008). The zinc ion is tetrahedrally coordinated by a histidine and three cysteine side chains (Cys-176, His-179, Cys-238, and Cys-242). Zinc loss results in a significant
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decrease in thermodynamic stability (Bullock, 2000), increases aggregation tendencies, and is accompanied by structural fluctuations in neighboring loops that cause loss of DNA-binding specificity (Butler, 2006; Duan, 2006). The solution structure of the p53 core domain reveals a number of buried polar groups that are not satisfied by hydrogen bonds, which may be one of the key factors contributing to p53’s inherent instability (Canadillas, 2006). Furthermore, less rigid core of the β-sandwich may provide the structural plasticity required for optimal function.

Oligomerization domain

The full-length p53 protein forms tetramers via a tetramerization domain in the C-terminal region of the protein (residues 325–356) (Veprintsev, 2006). The tetrameric structure of the domain is a dimer of dimers as revealed by X-ray crystallography (Jeffrey, 1995; Mittl, 1998) and in solution by NMR (Clore, 1995). The topology of the tetramerization domain is reflected by its folding pathway, which proceeds via a dimeric intermediate (Mateu, 1999a). A study of p53 biogenesis in vitro found that dimer formation occurs cotranslationally on the polysome, whereas tetramers are formed posttranslationally by dimerization of dimers in solution (Nicholls, 2002).

Negative regulatory domain

The negative regulatory domain (amino acid residues 360–393) of p53 protein is intrinsically disordered but may undergo local disorder to order transitions upon binding to other proteins or nonspecific DNA (Bell, 2002, Weinberg, 2004 and Friedler, 2005). This region of the p53 protein is subject to extensive posttranslational modifications, including acetylation, ubiquitination, phosphorylation, Sumoylation, methylation, and neddylation, that regulate p53 function and cellular protein levels (Toledo, 2006; Bode, 2004 and Lavin, 2006). The C terminus of p53 binds DNA nonspecifically in vitro via the electrostatic interactions of several lysine residues, albeit with a comparatively low affinity, which inhibits binding of a specific DNA by the core domain in a competitive manner (Weinberg, 2004). Studies with a C-terminally truncated p53 variant lacking the last 30 amino acid residues show that the C terminus is required for efficient binding and transactivation of target genes in the context of large molecules of DNA or chromatin (Espinosa, 2000; McKinney, 2004). Consistent with its capability to bind DNA
nonspecifically, it was suggested that the p53 C terminus promotes linear diffusion on DNA (McKinney, 2004). The sliding of p53 along DNA may facilitate the search for target sequences.

**DNA binding activity of p53 protein**

Wild type p53 binds to a DNA consensus site containing two or more copies (consecutive or separated by one or two helical turns) of the 10 bp half-site 5'-PuPuPuPuC(A/T) (T/A)PyPyPyPy-3', where Pu and Py represent purines and pyrimidines, respectively (Kern, 1991; El-Deiry, 1992; Funk, 1992). Thus, the consensus site comprises four inverted 5 bp quarter-sites. By mapping p53 target sites in the human genome it was found that more than half of the high-probability binding loci contain consecutive half-sites without insertion (Wei, 2006). X-ray crystallography has revealed that four core domains of the p53 tetramer occupy the four quarter-sites in a full consensus sequence without steric clashes (Cho, 1994). This model is also consistent with solution studies, which shows four p53 core domains can bind cooperatively to a consensus DNA sequence (Balagurumoorthy, 1995; Wang, 1995a). The p53 tetramer is a dimer of dimers and the two monomers within a dimer bind to a half-site (two consecutive quarter-sites, but not to the alternating quarter-sites) (McLure, 1998). Tetramers bind similarly, with the two dimers within each tetramer binding to pairs of half-sites. Thus the four p53 core domains bind to these response elements in a highly cooperative manner to give a complex with 4:1 stoichiometry (Balagurumoorthy, 1995; Wienberg, 2004).

The architecture of the p53 core tetramers bound to DNA has been revealed by crystal structures of p53 core in complex with 20mer double stranded DNAs containing different half-site motifs (Kitayner, 2006). The core domain dimer interface is formed by residues from the L3 loop and the short helical segment within L2 (177,178,181,243, and 244), and is stabilized by both hydrophobic and water-mediated polar contacts (Rippin, 2002; Klein, 2001). In the crystal structures of all the dodecamer DNA complexes studied, the half-site dimers assemble to form a tetramer through protein-protein and base-pair stacking interactions, mimicking the binding mode of four p53 core domains to a full DNA response element with a two base-pair spacer between the half-sites (Kitayner, 2006). The amino acids Lys-120, Ser-241, Arg-248, Arg-273,
Ala-276, Cys-277, and Arg-280 are the key residues in the p53 core-DNA interface that make direct contact with a DNA half-site (Cho, 1994; Kitayner, 2006).

**Loss of p53 protein function**

Since the level of p53 is tightly regulated in the cells, the regulation of its stabilization and degradation are the key steps that make the organism healthy. The loss of p53 function is the greatest cause of the human cancer. The p53 function loss is due to the absence of the protein or the presence of inactive mutant protein. The mutation in the p53 gene is very common, even the p53 first discovered was mutant protein. The p53 gene has the features of a recessive tumor suppressor in its wild-type form, and it can be a dominant oncogene in its mutated form. Over 60% of cancer cases have p53 gene mutations and the core domain contains 95% of p53 mutations and 75% of this occur as single missense mutations rather than deletions, insertions or frameshifts (Soussi, 2005; Beroud, 1998).

So, the oncogenic form of p53 is predominantly a full-length protein with a single amino-acid substitution in its core domain (Bullock, 2001). Most of the remaining tumors although containing wild type p53, are defective in the pathway of p53 induced cell cycle arrest or apoptosis due to virus infection, MDM2 overexpression, ARF or ATM deficiency (Wang, 2003). Deficiency of p53 function also confers a growth advantage under hypoxic conditions, which contribute to tumor progression (Pluquet, 2001). The functional status of p53 has been related to prognosis, progression and therapeutic response of tumors. Tumor cells containing wild-type p53 are usually more sensitive than those bearing mutant p53 (Wang, 2003).

Six mutation hot spots cluster to the DNA-binding surface: two contact DNA directly R248 (L3) and R273 (S10 in LSH) and four stabilize the surrounding structure R175 (L2), G245 (L3), R249 (L3) and R282 (H2 in LSH). These give rise to two classes of mutants ‘DNA contact’ and ‘structural’ (Bullock, 2001). These mutants can also be distinguished by two polyclonal antibodies, PAB1620 (Milner, 1987) and PAb240 (Gannon, 1990), raised against native and denatured p53, respectively. Contact mutants tend preferentially to bind PAB1620 because they have an intact native fold, whereas structural mutants specifically bind to PAb240, which
recognizes a continuous epitope partially buried and inaccessible in the native state (Milner, 1995). Many of the point mutations in the core domain of p53 produce a change in the global conformation of the protein different from the wild type p53 conformation, which can be recognized by PAb240 antibody (Xirodimas, 1999). Further it was shown that a variety of structural consequences result upon mutation: (i) the removal of an essential contact with DNA, (ii) creation of large, water accessible crevices or hydrophobic internal cavities with no other structural changes but with a large loss of thermodynamic stability, (iii) distortion of the DNA binding surface, and (iv) alterations to surfaces not directly involved in DNA binding but involved in domain-domain interactions on binding as a tetramer (Friedler, 2003).

Many of the core domain mutants are thermodynamically unstable at body temperature, these mutants denature within minutes at 37°C, whereas the half-life ($t^{1/2}$) of the unfolding of wild-type p53 core domain was found to be 9 min. Hot spot mutants denatured more rapidly with increasing thermodynamic instability. The highly destabilized mutant I195T had a $t^{1/2}$ of less than 1 min. (Friedler, 2003). These mutations result in the loss of sequence specific DNA binding, as a result mutant p53 proteins fail to activate transcription of p53 target genes and hence, do not trigger a p53-dependent biological response. The mutant p53 protein also works in a dominant negative manner, the mutant p53 when oligomerizes with wild type p53, drives the conformation of the wild-type protein into a mutant state (Milner, 1991).

**Restoration of p53 function**

Several studies have demonstrated the ways to restore the normal function to mutant p53 protein in tumors (Bykov, 2003) and these can be divided into three groups: (a) design of antagonists for negative regulators of p53 in tumors carrying wild-type p53 (Vassilev, 2007), (b) reactivation of mutant p53 (Joerger, 2007; Bullock, 2001), and (c) exogenous p53 expression, e.g., via adenovirus-mediated gene transfer (Roth, 2006). Recently, two elegant studies on transgenic mice, in which p53 expression was reversibly switched on and off, have independently shown that restoration of p53 function can lead to tumor regression in vivo, indicating that reactivating p53 is a promising therapeutic strategy (Martins, 2006; Ventura, 2007).
The introductions of second site suppressor mutations in p53 results in the stabilized folding of the mutant protein and also restored sequence specific DNA binding (Nikolova, 2000). Analyses of the effects of second-site mutations that rescue hot spot mutations of p53 imply that it is possible to stabilize many destabilized mutants of p53 in a generic way. Furthermore, specific mutations that distort the native structure might also be reversed (Nikolova, 2000).

A peptide, CBD3 is reported to stabilize mutant p53 core domain and rescue its sequence-specific DNA binding activity (Friedler, 2002). CBD3 binds the native but not the denatured state of p53 core domain increases the half-life of the p53 core domain and thus stabilizes it (Bullock, 2001; Friedler, 2002). It binds to a surface region of p53 that partly overlaps with the DNA-binding surface. The fluorescein-labeled form FL-CDB3 raises the melting temperature of wild-type p53 and the destabilized R249S mutant in vitro (Friedler, 2002). It induces upregulation of wild type p53 and representative mutants in human cell lines (Issaeva, 2003).

Further by screening a chemical library of 100,000 synthetic compounds, using a protein assay two molecules (CP31398 and CP257042) were identified that can preserve wild-type conformation of p53, rescue its transactivation capacity and restore its antitumor activity in vivo (Foster, 1999). Wild-type p53 core domain loses the PAb1620-specific epitope within several minutes at 45°C. However the compounds that bind specifically to the native state increase the thermostability and protect the core domain of p53 from denaturation and maintain the PAb1620-reactive conformation for 30 minutes at 45°C (Foster, 1999). In the presence of two compounds CP-257042 and CP-31398 four core domain mutants, V173A, R175S, R249S and R273H, also maintain PAb1620 binding. Furthermore, CP-31398 activates p21 transcription in V173A or R249S transfected Saos-2 osteosarcoma cells that are null for p53 (Foster, 1999).

Another compound PRIMA-1 was identified by screening a chemical library, that induces mutant p53-dependent apoptosis and restores native conformation, DNA binding, and transcriptional transactivation to mutant p53, and inhibits tumor growth in vivo (Bykov, 2002). Further a maleimide-derived molecule, MIRA-1 was identified that can reactivate DNA binding and preserve the active conformation of mutant p53 protein in vitro and restore transcriptional transactivation to mutant p53 in living cells. MIRA-1 induced mutant p53-dependent cell death
in different human tumor cells carrying tetracycline-regulated mutant p53. The structural analog MIRA-3 showed antitumor activity in vivo against human mutant p53-carrying tumor xenografts in SCID mice (Bykov, 2005).

**Temperature dependent loss of p53 function**

The conformation of wild-type p53 is very temperature sensitive. It undergoes irreversible conformational changes after short incubations (10 min) at temperatures in a range from 37°C to 45°C by losing its wild-type (PAb1620-positive) conformation. The loss of the PAb1620-reactive epitope correlates with a loss of the tetrameric nature of p53 and with a loss of the p53 DNA binding activity (Hansen, 1996). The thermal instability of p53 protein is due to its core DNA binding domain, which unfolds with a half-life of 9 min. at 37°C and 10 hrs at 30°C (Friedler, 2003). This unfolding was found to be concentration-dependent and results in the rapid aggregation (Friedler, 2003). The inactivation of p53 DBD at higher temperature is due to the rapid accumulation of misfolded species. Elevated temperatures increase the frequency of cycling between folded and unfolded states and the function of p53 DBD is lost because a fraction of molecules become trapped in misfolded conformations with each folding-unfolding cycle (Butler, 2006). However the folding kinetics do not change appreciably from 5°C to 35°C (Butler, 2006).

**Stabilization of p53 functions by antibodies and chaperones**

The thermal stability of p53 protein can be increased by several ways. The deletion of C-terminal 30 amino acid negative regulatory region from the p53 showed a dramatically enhanced intrinsic stability to temperature (Hansen, 1996). The *E. coli* heat shock protein DnaK that binds to the C terminus of p53 (Fourie, 1994) protects PAb1620-positive p53 from a temperature dependent loss of DNA binding activity but cannot renature and activate denatured p53 (Hansen, 1996). The DnaK protein also activates the p53 protein for sequence specific DNA binding (Hupp, 1992). Two monoclonal antibodies to the N terminus of p53, PAb1801 and DO-1 also protect the temperature dependent loss of DNA binding at 37°C (Hansen, 1996). The DNA binding activity of mutant p53 is also protected by PAb 1801 and its Fab fragment at 37°C. The
mutant p53 protein binds DNA at 25°C whereas heating at 37°C irreversibly destroyed its ability to bind DNA. Whereas, the addition of amino terminal antibody or the Fab fragments protected the DNA binding ability at 37°C (Friedlander, 1996).

In the last few years several molecular chaperones are reported to protect the sequence specific DNA binding ability of p53 protein at 37°C. The Hsp90 which is abundant in cells, is important for protecting the cells from stress such as high temperature. Hsp90 was found in a complex with several oncoproteins, including v-Src, c-Erb2, Raf-1, Akt, Bcr-Abl, and tumor suppressor protein p53 (Young, 2001; Zylicz, 2001; Picard, 2002; Prodromou, 2003; Wegele, 2004). It has been known from years that genotypically mutant p53 co-immunoprecipitates with members of the Hsp70 and Hsp90 families. However recent studies have shown that Hsp90 associates with the wild type p53 protein. Using purified proteins and applying several independent methods of interaction such as surface plasmon resonance, immunoprecipitation, ELISA, and cross-linking it was demonstrated that Hsp90 directly, in the absence of any other co-chaperones, associates with genotypically wt-p53 but not with mutant p53 protein (King, 2001). The binding of Hsp90 with p53 provides the stability to the p53 protein, and protects the DNA binding ability at 37°C. It also prevents the denaturation and subsequent aggregation of the p53 protein at 45°C (Muller, 2004). Further studies have also shown that the chaperoning activity of Hsp90 towards p53 for DNA binding is ATP dependent and the Hsp90 inhibitors geldanamycin and radicicol, inhibit the activity of p53 as a transcription factor by dissociating it from the target DNA promoter sequence sites (Walerych, 2004).

Recently our group has reported that CHIP (carboxyl terminus of Hsp70-interacting protein) independently binds with the wild type p53 protein and prevents PAb1620-positive conformation as well as DNA binding activity from temperature dependent loss. CHIP also protects p53 from irreversible thermal denaturation and subsequently aggregation at physiological and elevated temperatures. CHIP binds preferentially to the denatured conformation of wild type p53 protein and restores its DNA binding activity in an ATP independent manner (Tripathi, 2007). The MDM2 protein that is mainly responsible for the ubiquitination and degradation of p53 is also reported to work as a chaperone towards p53.
MDM2 protects the DNA binding ability of p53 protein at 37°C in an ATP dependent manner (Wawrzynow, 2007).

**Role of the amino terminal domain in p53 DNA interaction**

In addition to the transactivation function of the amino terminal domain, it also plays a role in the regulation of p53 binding to its consensus site. Studies have shown that the amino terminus of p53 serves as a self inhibitory region for the DNA binding, it causes the increased dissociation of p53 from the DNA (Cain, 2000). The antibodies that bind in a region from 10 to 55 amino acid residues decrease the dissociation of p53 from DNA and stabilize the interaction. The deletion of 96 amino acid residues further stabilizes the binding (Cain, 2000).

The proline rich region of p53 also plays a role in the regulation of DNA binding by p53, although its role is controversial as different authors have reported contrasting results. The part of proline rich region (amino acids 80-93) of the human p53 is reported to serves as a negative regulator for DNA binding (Muller-Tiemann, 1998). The deletion of this region resulted in the activation of DNA binding by p53 protein, synthetic peptides from this region also activate p53 for DNA binding (Muller-Tiemann, 1998). However it was also reported that, the murine p53 having two deleted proline rich repeats (77-89) binds DNA as efficiently as the wild type p53 protein (Sakamuro, 1997). Interestingly there is another report that shows proline rich domain as a positive regulator for p53 DNA interaction (Roth, 2000). They have studied the binding of p53 protein with three different p53 DBS, the p21, MDM-2, and of PIG3 promoters and found that the deletion of proline rich domain causes substantial decrease in the DNA binding by p53 protein to all three promoters (Roth, 2000). Using gel retardation assay it was revealed that this domain did not alter *in vitro* the specific binding to the p53-responsive element of PIG3, but plays a critical role in transactivation from a synthetic promoter containing this element (Venot, 1998).

A peptide derived from the amino terminus of the core domain of p53 protein (residues 105-126) is reported to inhibit the DNA binding by the p53 protein (Protopopova, 2003). The peptide interfered the p53 DNA binding activated by PAb.421 in a dose dependent manner and
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blocked DNA binding of the constitutively active c-terminal deleted p53 protein (p53Δ30) and it binds with full length p53(1-393), the core domain (100-300) as well as with the C-terminus (320-393) of p53 protein (Protopopova, 2003). Studies have shown that the transactivation domain is responsible for the thermosensitive nature of p53 protein. The binding of N-terminal specific antibodies like DO-1 or PAb 1801 protects the DNA binding ability of wild type and mutant p53 protein at 37°C (Hansen, 1996; Hansen, 1998). Whereas the deletion of 37 amino acid residues from the N-terminus of p53 makes it to be stabilized by every antibody it binds (Hansen, 1998). However further deletion, up to 96 amino acids from amino terminal domain of p53 loses the protection by binding with antibodies, although PAb.1620 protects by 50% (Hansen, 1996).

The amino terminal domain of p53 protein is natively unfolded, the natively unfolded regions of the proteins can be divided into two groups, those with no ordered (secondary) structure and those with some secondary structure; the latter resemble molten globules and lack tertiary structure (Uversky, 2002). The amino terminus of p53 belongs to the second class, which possesses some residual secondary structures but lack a tertiary structure (Bell, 2002; Dawson, 2003). The intrinsically disordered proteins perform different functions including the regulation of transcription and translation, cellular signal transduction, protein phosphorylation, the storage of small molecules, and the regulation of the self-assembly of large multiprotein complexes such as the bacterial flagellum and the ribosome (Dyson, 2005).

Unfolded regions are also present in proteins that function as chaperones for other proteins and for RNA molecules. The protein chaperones like α-synuclein and α-casein are disordered along their entire length (Tompa, 2002), whereas other chaperones like Hsp90, Hsp70, Hsp60, p23, Hsp105 and bacterial GroEL have disordered regions (Tompa, 2004). The structural disorder has been noted as a functional feature of protein chaperones. Removal of the disordered segment by limited proteolysis often abolishes or markedly reduces chaperone activity in other instances, such as for p23 co-chaperone (Weikl, 1999), α-crystallin (Andley, 1996), and Hsp25 (Lindner, 2000). The diminution of chaperone activity by decreasing the flexibility of the disordered segment by a point mutation has been demonstrated for α-crystallin (Smulders, 1996). A reduction in flexibility of the disordered segment upon direct contact with the partially folded
or misfolded substrate protein has been shown by time-resolved fluorescence spectroscopy and NMR in GroEL (Gorovits, 1995) and α-crystallin (Lindner, 1998). These studies indicate that a subtle balance of these regions between structural order and disorder are important in the functional cycle of chaperones. The chaperone function is performed by binding the unfolded regions to misfolded proteins and RNA molecules, such that they function as recognition elements and/or help in the loosening and unfolding of kinetically-trapped folding intermediates including molecular recognition via binding to other proteins or to nucleic acids (Tompa, 2004).

Given the structural flexibility of the amino terminal domain of p53 we have investigated its role in the DNA binding activity of p53 protein. The transactivation function of p53 was found to be responsible for the increased dissociation of p53 to its target DNA and the addition of antibodies against this region or the deletion of this region potentiates the p53 DNA interaction (Cain, 2000). Further the transactivation domain is also responsible for the thermosensitive nature of p53 DNA interaction and the addition of antibodies against this region protects the DNA binding activity of p53 protein at physiological temperature (Hansen, 1996; Hansen, 1998). Whereas the role of other sub-domains of amino terminus the proline rich domain and the repressor domain in the DNA binding activity is not known presently. Further the amino terminal domain of p53 protein is intrinsically disordered and as described above the natively unfolded regions are prevalent in protein chaperones and are important in the chaperoning activity. In this study we explored the role of complete amino terminal domain (NTD) on the p53 DNA interaction as well as on the conformation of p53 protein and its DNA binding activity at physiological and elevated temperatures.
AIMS AND OBJECTIVES

1. Cloning of p53 and its amino terminal domain (NTD) in the bacterial expression vector, protein expression and purification.
2. To investigate the effect of NTD on the DNA binding activity of p53 protein.
3. To study the interaction of NTD with p53 under different temperature conditions.
4. To investigate the role of NTD in the conformational modulation of p53 under heat stress conditions.
5. To study the role of NTD in the protection of DNA binding activity of p53 protein from temperature dependent loss and in the recovery of DNA binding activity of heat denatured p53 by NTD.
6. To develop an ELISA based approach to study sequence specific p53 DNA interaction using different p53 DNA binding sites (DBS).
7. To construct the fluorescent tagged p53 and NTD expression plasmids and study their localization.