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REVIEW

The role of tetramerization in p53 function

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The tumour suppressor gene p53 is extensively studied for its importance in cancer. In its active conformation, p53 is tetrameric and one domain – the tetramerization domain – permits the oligomerization of this protein. Until recently, little attention was given to this domain because, in contrast to the DNA-binding domain, it is not often mutated in cancer. However, various experimental studies have shown evidence that the tetramerization domain is essential for DNA binding, protein–protein interactions, post-translational modifications, and p53 degradation. Moreover, single mutations in the tetramerization domain can inactivate the wild-type protein in a manner similar to that seen with mutations in the DNA-binding domain. Interestingly, the phenotype of several tetramerization domain mutants differs from that observed with DNA-binding domain mutants. In this review, current knowledge about the importance of the tetramerization domain to the function of p53 will be summarized. *Oncogene* (2001) **20,** 2611–2617.

Keywords: p53; tetramerization; cancer

Introduction

The p53 tumour suppressor is a transcription factor involved in different cellular functions, such as cell cycle control, apoptosis, and differentiation (for recent reviews on p53 function see (Bates and Vousden, 1999; Levine, 1997; Oren and Rotter, 1999; Steele *et al*., 1998)). It can be divided into different functional domains. A transactivation and a proline-rich domain (residues 1 to 43 and 61 to 94, respectively) are present at the N-terminus. The DNA-binding domain (DBD) is located in the middle of the protein (residues 110 to 286). Finally, a tetramerization domain (TD) and a regulatory region (residues 326 to 355 and 363 to 393, respectively) are located at the C-terminus. A large part of p53 activity comes from its ability to stimulate the transcription of various genes which contain a p53-binding element in their promoter (Selivanova and Wiman, 1995). Therefore mutations in the DBD, which abolish DNA binding, inactivate its tumour suppressor activity. Such mutations are very frequent in cancer (Hollstein *et al*., 1999; Soussi *et al*., 2000). The function of p53 is also mediated by protein–protein interactions, and various p53-binding proteins have been identified (Hainaut and Hollstein, 2000; Ko and Prives, 1996). Several studies show evidence to suggest that p53 activity depends on its conformation. The protein exists in a latent conformation (inactive for DNA binding) and in an active conformation, where it binds to DNA (Hupp *et al*., 1992). Its conformation can be modulated by redox changes (Hainaut and Milner, 1993), and it can be modified by mutations in the DBD that prevent DNA binding (Gannon *et al*., 1990; Michalovitz *et al*., 1990; Milner and Medcalf, 1990). The p53 protein is active when it is tetrameric, and in this conformation it binds with high affinity to DNA or interacts more efficiently with various other proteins. Moreover, shifts between the active and the latent DNA-binding conformation only occur within p53 tetramers and are mediated by the TD (Halazonetis and Kandil, 1993). This domain is therefore important for p53 function because it ensures that the protein is endowed with its correct conformation.

Identification of the p53 tetramerization domain

Early findings revealed that p53 forms high molecular weight oligomers (Kraiss *et al*., 1988; McCormick *et al*., 1981; Schmieg and Simmons, 1988). The ability of p53 to oligomerise was further supported by the finding that proteins mutated at their DBD are dominant negative over wild-type protein, inactivating it upon hetero-oligomerization (Eliyahu *et al*., 1988; Halevy *et al*., 1989; Milner and Medcalf, 1991). Experiments using gel electrophoresis, chemical cross-linking and zonal velocity gradient centrifugation demonstrated that p53 forms tetramers and complex oligomers in the absence or presence of DNA (Friedman *et al*., 1993; Stenger *et al*., 1992). In contrast to other transcription factors which oligomerize upon interaction with DNA, p53 exists as tetramers in the absence of DNA (Friedman *et al*., 1993). The region of the protein responsible for tetramerization was identified at its C-terminus (Iwabuchi *et al*., 1993; Pavletich *et al*., 1993; Sturzbecher *et al*., 1992; Wang *et al*., 1993), and limited proteolysis experiments located the TD between residues 311 and 363 (Pavletich *et al*., 1993). The free energy (−23.4 kcal/mol) and dissociation constant (2.3 10^{-6} M) for tetramer formation have been determined for the protein fragment 303–393 (Sakamoto *et al*., 1994). These data led Sakaguchi *et al*. (1997) to suggest that because the cellular concentration of p53 is very low in human cells with undamaged DNA (1 to 10 nM), p53 may exist in these cells

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predominantly as monomers. Tetramerization of p53 could be stimulated by post-translational modifications, such as the phosphorylation of serine-392 (Sakaguchi *et al*., 1997). However, these K_D values have been obtained with fragments of the p53 protein, and other regions of the protein may help in stabilizing the tetramers. It has been shown, for example, that several interactions at the interface with the DBD may stabilize the tetramers (Cho *et al*., 1994).

Structure of the tetramerization domain

The structure of the TD has been determined both by X-ray crystallography and by NMR (Chène *et al*., 1997; Clore *et al*., 1994, 1995a; Jeffrey *et al*., 1995; Lee *et al*., 1994; Miller *et al*., 1996; Mittl *et al*., 1998). A monomer contains a β-strand (residues 326 to 333) linked to an α-helix (residues 335 to 355) by a single residue (glycine-334) (Figure 1a). A monomer has a V-shape, and both elements of the secondary structure are a branch of the V. Three amino acids – Ile332, Phe338 and Phe341 – form a small hydrophobic cluster at the hinge region. Estimations with regard to the surface of interaction between monomers and experimental evidence indicate that the TD is a dimer of dimers (Lee *et al*., 1994; Mateu *et al*., 1999). Two monomers associate via their β-strands to form an antiparallel double-stranded sheet and via the antiparallel association of their helices to create a double-helical bundle (Figure 1b). The formation of the antiparallel β-sheet leads to the creation of eight backbone hydrogen bonds. A hydrophobic core, made up of Phe328, Leu330, Ile332 from the β-strands and Phe338, Phe341 and Asn345 from the α-helices, is also created at the interface between the monomers. A salt bridge possibly exists between Arg337 from one monomer and Asp352 from the other monomer. Two dimers interact via their α-helices, but different angles between the two pairs of helices have been reported (Clore *et al*., 1995b; Jeffrey *et al*., 1995; Lee *et al*., 1994; Surridge, 1994). The β-strands are on the outside of the tetramer, and their residues are therefore not directly involved in the association between the two dimers (Figure 1c). The interface between the helices is mainly hydrophobic and involves Met340, Leu344, Ala347, Leu348 and Leu350.

This structural information was completed by the analysis of different alanine mutants of the TD. The most inactivating alanine mutations are: Phe328Ala, Leu330Ala, Ile332Ala, Arg337Ala, Phe338Ala, Met340Ala, Phe341Ala, Leu344Ala and Leu348Ala (Chène *et al*., 1997; Mateu and Fersht, 1998; Waterman *et al*., 1995). This shows that the residues located within the hydrophobic core are crucial for the formation of the tetramers and that the hydrophobic effect is therefore the major force responsible for stabilizing the TD (Johnson *et al*., 1995; Mateu and Fersht, 1998; McCoy *et al*., 1997). The alanine mutations which prevent tetramerization also inactivate p53 *in vitro* and in cellular assays (Chène and Bechter, 1999a; Chène *et al*., 1997; Waterman *et al*., 1995). The TD is unusually sensitive to mutation possibly because for each mutation in a monomer, four amino acids are changed in the tetramer therefore increasing the overall destabilization (Mateu and Fersht, 1998).

Tetramerization domain and evolution

The TD is found in all the p53 proteins cloned so far and in the two other p53 family members p73 and p63. However, its primary sequence is less well conserved than other regions of the protein located either within the DNA-binding domain or at the N-terminus (Soussi *et al*., 1990). A comparison of the primary sequence of p53 proteins from different species (Figure 2) reveals that the residues constituting the hydrophobic core are well conserved, even if some changes occurred during evolution (see below). This is to be expected, since they are essential for tetramerization. Similarly, Gly334 – the only amino acid that can be located for conformational reasons at the hinge between the α-helix and the β-strand – is present in all p53. A more surprising observation is that several residues located at the surface of the TD (Glu326, Arg333, Arg335, Glu336,

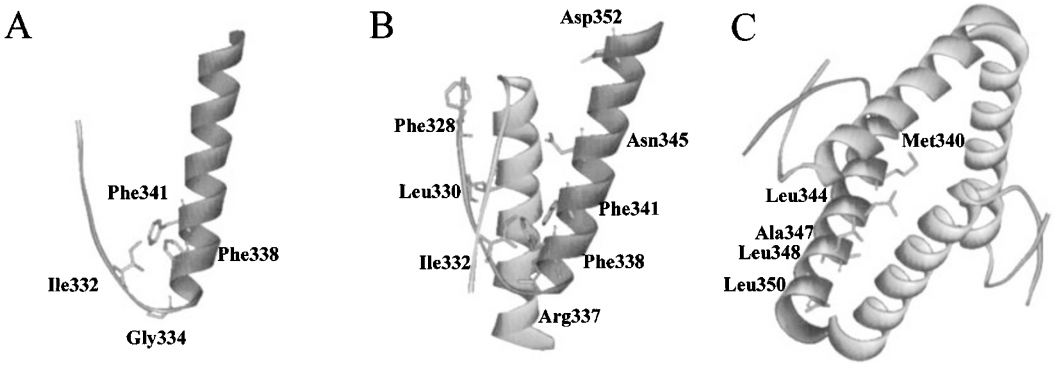

Figure 1 Structure of p53 tetramerization domain. (**a**) Ribbon representation of a monomer (residues 326–356). The residues involved in the stabilization of the monomer are indicated. (**b**) Both chains of a dimer are represented and the residues involved in the formation of the dimer are indicated. (**c**) The four chains of a tetramer are represented. For simplicity, the residues involved in the interaction between the different helices are only indicated for one chain

	332	334	336	340	342	346	352
	326	333	335	340	344	350	355
	6	0	5	0	5	0	5
Homo sapiens	EYFTLQIRGRERFEMFRELNEALELKDAQAG						
Cercopithecus aethiops	EYFTLQIRGRERFEMFRELNEALELKDAQAG						
Macaca muletta	EYFTLQIRGRERFEMFRELNEALELKDAQAG						
Felix catus	EYFTLQIRGRERFEMFRELNEALELKDAQSG						
Canis familiaris	EYFTLQIRGRERYEMFRNLNEALELKDAQSG						
Mesocritus auratus	EYFTLKIRGQERFKMFQELNEALELKDAQAL						
Rattus norvegicus	EYFTLKIRGRERFEMFRELNEALELKDARAA						
Mus musculus	EYFTLKIRGKERFEMFRELNEALELKDAHAT						
Gallus domesticus	EIFYLQVRGRKRYEMLKEINEALQLAEGGSA						
Xenopus laevis	EIFTLRIKGRSRYEMIKKLNDALELQESLDQ						
Salmo irideus	EIYTLQIRGKEKYEMLKKFNDSLELSELVPV						
p73	DTYYLQVRGRENFEILMKLKESLELMELVPQ						
p63	ELLYLPVRGRETYEMLLKIKESLELMQYLPQ						

Figure 2 Comparison of the primary sequence of the tetramerization domain of p53 proteins from different species. The different primary sequences have been aligned using the Clustal W program. The conserved charged and the hydrophobic amino acids are underlined and in bold, respectively. The numbers correspond to the human sequence

Arg337, Glu339, Arg342, Glu346, Glu349 and Asp352) are also well conserved. Arg337, Asp352 and Glu349 are involved in the stabilization of the tetramers, which explains their conservation. In contrast, the other residues do not seem to be involved in tetramer formation (Chène and Bechter, 1999a; Chène *et al*., 1997; Mateu and Fersht, 1998). One possibility is that they may act in a cooperative fashion in the formation of the tetramers, so that single mutations would not prevent tetramerization whereas multiple mutations would. Alternatively, these exposed residues could interact with another region of the p53 protein or with other proteins. Since this interaction may be important for p53 function, these amino acids would be conserved during evolution. The analysis of the primary sequence of the TD also shows that a phosphorylable residue (threonine or tyrosine) at position-329 is well conserved during evolution. This residue is located at the surface of the protein and is not important for tetramer formation (Chène and Bechter, 1999a; Chène *et al*., 1997; Mateu and Fersht, 1998). So far, no phosphorylation of this residue has been reported (Appella and Anderson, 2000; Meek, 1999).

Thermodynamic analysis of the stability of the TD in different species shows that it has been the subject of mutually compensatory mutations in the course of evolution (Mateu and Fersht, 1999). The key residues of the hydrophobic core have been substantially mutated during evolution, and when introduced as single mutations in the human TD most of these changes destabilize the domain. However, if all the relevant changes from a different species are introduced at once, the destabilization observed in most of the cases is lower than the sum of the individual destabilizations. While the core itself has therefore evolved, its overall stability has not been substantially changed over the course of evolution.

The presence of different amino acids within the hydrophobic core suggests that some of the p53 family members may not hetero-oligomerize. This has been observed between the human and the *X. laevis* p53 (Wang *et al*., 1995a) and between p53 and p73 (Davison *et al*., 1999). It has been reported, however, that some p53 mutants associate with p73 (Di Como *et al*., 1999; Marin *et al*., 2000; Strano *et al*., 2000). Nevertheless, various observations suggest that the association between p53 and p73 is not dependent on the TD. A polymorphism at position-72 in p53 affects the interaction between some p53 mutants and p73 (Marin *et al*., 2000). Their interaction is dependent on the presence of an arginine at position-72. Another observation is that the DBD mutant Arg175His, deleted at its N- and C-terminus (Arg175His(74-298)), binds to p73α, which indicates that p53 DBD is sufficient for the association with p73 (Strano *et al*., 2000). This suggests that regions outside the TD influence the association between these proteins. Isolated TD from p53 and p73 may thus not interact with each other (Davison *et al*., 1999) while in some cases the full-length proteins could do so (Di Como *et al*., 1999; Marin *et al*., 2000; Strano *et al*., 2000). Similarly, it has been shown that while human and *X. laevis* p53 do not hetero-oligomerize, a hybrid human p53 protein whose TD has been replaced by the TD from *X. laevis* does hetero-oligomerize with the human p53 protein (Chène, 1999).

Function of the tetramerization domain

Many reports have shown that p53 variants in which the TD has been deleted are able to bind to DNA and stimulate transcription (Balagurumoorthy *et al*., 1995; Bargonetti *et al*., 1993; McLure and Lee, 1998; Nagaich *et al*., 1999; Pavletich *et al*., 1993; Sang *et al*., 1994; Shaulian *et al*., 1993; Stenger *et al*., 1994; Subler *et al*., 1994; Tarunina and Jenkins, 1993; Wang *et al*., 1993, 1995b; Zhang *et al*., 1994). This can be explained by the fact that p53 monomers bind to DNA in a cooperative manner. However, their affinity for DNA is 10 to 100 times lower than that of the full-length protein (Balagurumoorthy *et al*., 1995). Moreover, the isolated DBD and the full-length protein bend and twist DNA with significant differences (Nagaich *et al*., 1999). The TD thus influences both the strength of the interaction and the conformation of the p53-DNA complexes, and it may also help in the pre-organization of the four DBDs. A recent model suggests that one DBD dimer binds first to one half of the consensus DNA-binding site, increasing the probability for the binding of the second dimer to the adjacent half of the site (McLure and Lee, 1998, 1999). The binding of the DBDs is accompanied by conformational changes (Halazonetis *et al*., 1993; Halazonetis and Kandil, 1993; McLure and Lee, 1996, 1999; Waterman *et al*., 1995) which enhance the stability of the overall protein-DNA complex. In addition, the TD ensures that the C-terminus of the protein is in the correct orientation for binding to DNA in a non-specific manner and increasing DNA bending (Nagaich *et al*., 1999).

The TD is also important for protein–protein interactions. Various proteins bind directly to the TD

or have their interaction with p53 regulated by its oligomeric status. The binding site of the casein kinase 2 (residues 325 to 344) (Gotz *et al*., 1999), the Ca^{2+}-dependent protein kinase C (residues 320 to 346) (Delphin *et al*., 1997) and the adenovirus E4orf6 protein (residues 318 to 360) (Dobner *et al*., 1996) have been directly mapped within the TD. For other proteins, such as RelA (binding site between residues 300 and 393) (Ikeda *et al*., 2000) and hepatitis B virus HBx (binding site between residues 300 and 393) (Lin *et al*., 1997), the binding site was located within the p53 C-terminus. However, a direct interaction between the TD and these proteins has not yet been demonstrated. It has also been observed that free and poly(ADP-ribose) polymerase-bound ADP-ribose polymers bind *in vitro* to p53 TD (Malanga *et al*., 1998). Other proteins do not interact directly with the TD, but only bind to p53 tetramers. The TD thus plays an indirect role in the association between these proteins and p53. This is the case for mdm2 (Lomax *et al*., 1998; Marston *et al*., 1995) (binding site at the p53 N-terminus (Chen *et al*., 1993)), HPV-16 E2 (Massimi *et al*., 1999), c-abl (binding site at the C-terminus (Nie *et al*., 2000)) and TBP (binding site at the N-terminus (Liu *et al*., 1993)). The direct (or indirect) involvement of the TD in several protein–protein interactions shows that this domain functions not only to facilitate p53 DNA-binding but also to favour interactions with other proteins. Its presence is important for p53 regulation by other proteins (e.g., phosphorylation of p53 by the casein kinase 2 or the Ca^{2+}-dependent protein kinase C) but also a tetrameric structure may permit the simultaneous interaction of the tetramers with different proteins allowing the integration of the various signals that converge to the p53 protein.

Several post-translational modifications are dependent on the quaternary structure of p53. The phosphorylation of p53 by CHK1 is abolished when residues 334 to 354 (TD deletion) of p53 are deleted (Shieh *et al*., 2000), and the ability of casein kinase 2 to phosphorylate p53 on serine-392 is reduced when the leucine-330 is mutated to histidine, thus rendering p53 monomeric (Chène, 2000). The ubiquitination of p53 requires it to be oligomerized (Maki, 1999), and its degradation is abolished when tetramerization is inhibited (Kubbutat *et al*., 1998).

The TD has also been reported to contain a nuclear export signal located between residues 340 and 351 (Stommel *et al*., 1999). This export signal would be at the surface of the protein when it is monomeric (at low cellular concentration) and buried beneath the surface when it is tetrameric (after accumulation). According to this finding, the cellular localization of the p53 protein could be regulated by changes in its quaternary structure.

Tetramerization and cancer

The distribution of mutations identified in human cancer on the p53 gene shows that few of them occur in the TD (Hainaut and Hollstein, 2000; Hollstein *et al*., 1991, 1999; Soussi *et al*., 2000). Various hypotheses have been postulated to explain this observation. One is that the real number of TD mutations may be artificially minimized, because only the region coding for the DBD has been sequenced in most investigations. However, if the same analysis is carried out with samples where the entire p53 gene was sequenced, the TD still shows very few (4%) mutations (Levine *et al*., 1995). A second hypothesis suggests that the TD is particularly resistant to mutations and that more than one mutation is required to destabilize it. However, functional and thermodynamic analysis of alanine mutants of the TD show that this hypothesis is not tenable (Chène and Bechter, 1999a; Chène *et al*., 1997; Mateu and Fersht, 1998; Waterman *et al*., 1995). This is confirmed by the study of proteins identified in cancer and mutated at their TD. The analysis of p53 mutation databases (Hainaut and Hollstein, 2000) shows that most of the amino acids (20 of the 31 residues) of the TD are mutated in human cancer (Figure 3). These mutations lead to changes in amino acids and chain termination. The residue Arg342 is a particular target for chain termination. The introduction of a stop codon at position-342 corresponds to 22% of all the alterations found in the TD. Two missense mutations – Arg337Cys and Arg337Pro – have been found in Li-Fraumeni patients (Lomax *et al*., 1997; Varley *et al*., 1996), indicating that mutations in the TD can also be of germline origin. Very little work has been done to characterize these mutants (Atz *et al*., 2000; Davison *et al*., 1998; Ishioka *et al*., 1997; Lomax *et al*., 1997, 1998; Rollenhagen and Chène, 1998). The experimental data, however, show that most of these mutants do not oligomerize, do not bind to DNA, do not stimulate transcription of reporter genes under the control of a p53-binding sequence, and do not inhibit growth of tumour cells in colony formation assays. The activity of some of these proteins may be dependent on their level of expression (Atz *et al*., 2000; Rollenhagen and Chène, 1998). At low levels they are inactive, whereas they become active at higher levels of expression. One explanation is that the K_d value for

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                **    *M    *  *    * K*   * *E *
                DCC RHSP PDPCDIC D SDG*D*NGGHG
                GSSIPRTLALGLSGRS*GRTGVFGFRAVRVA
                AFYNHPNHVHAHYATYPAPIADSASTVDPDV
                VDVAVLVGEGVGVVKVLVQDVPWVWMHPLPE
                KNLSFELCWCQCLQVLQQVHQSVQVEYSESW
                QHIPIKFSRSKSIKLIGKMYKTMKMQNTKTR
Secondary Struct bbbbbbbbtaaaaaaaaaaaaaaaaaaaaa
Primary sequence EYFTLQIRGRERFEMFRELNEALELKDAQAG
                3  3  3  3  3  3  3
                2  3  3  4  4  5  5
                6  0  5  0  5  0  5
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Figure 3 Mutations of p53 tetramerization domain. The primary sequence of the TD of the p53 protein is indicated in bold. The secondary structure is also indicated. a, b and t correspond to α-helix, β-strand and turn, respectively. The various possible amino acids that can be obtained by the mutation of one single base per codon for each amino acid are represented. Stop codon are represented by *. The mutations identified in human cancer are underlined

tetramer formation of these mutants is higher than for wild-type and therefore higher amounts of mutant proteins are required for efficient tetramerization.

Altogether, this demonstrates that single mutations in the TD can lead to mutants which have lost the wild-type function, as do mutations in the DBD. The question then is: why are such mutations not identified in cancer more often? One possibility is that only a limited number of amino acids within the TD may be mutated in such a way that p53 becomes inactivated, so that the likelihood of abolishing p53 activity through mutations in its TD would be low. This can be challenged with our knowledge of the p53 TD. Since a good correlation exists between the structural analysis and properties of proteins mutated at their TD (Chène and Bechter, 1999a), the behaviour of TD mutants can be predicted with a reasonable level of confidence. The analysis of all possible mutations (changing one base per codon – Figure 3) for the TD residues reveals that most of them can be mutated in such way that tetramerization will be inhibited. For example, seven of the 20 residues of the α-helix 335–354 can be mutated into proline, a well-known helix breaker (Chou and Fasman, 1974). Similarly, all of the eight hydrophobic core residues can be mutated either to a charged residue (Arg, Lys) or to a small hydrophilic residue (Ser), mutations that would destabilize the tetramers. Another possibility to explain why TD mutations are less selected in cancers than DBD mutations is that they may not give the same phenotype. It has been shown that DBD mutants can inactivate wild-type p53 upon hetero-oligomerization (dominant negative effect) (Brachmann *et al*., 1996; Chène, 1998; Milner and Medcalf, 1991). However, dominant negative DBD mutants singly mutated at their TD so that they become monomeric lose their dominant negative effect (Chène and Bechter, 1999b; Chène *et al*., 1997). The TD is thus essential for the p53 mutant dominant negative effect. Another property of the DBD mutants is their gain of function. Several DBD mutants have some properties (disruption of checkpoint controls and induction of genomic instability, enhancing of tumorigenicity in nude mice, etc.) that enhance neoplasmic transformation (reviewed in (Blagosklonny, 2000; Hixon *et al*., 2000; Roemer, 1999; van Oijen and Slootweg, 2000)). Different groups have shown that the TD and/or the C-terminus regulatory region are necessary for the gain of function by p53 mutants (Chène and Bechter, 1999b; Frazier *et al*., 1998; Lanyi *et al*., 1998). Moreover, it has been reported that when a DBD mutant which shows a gain of function is singly mutated at its TD so that it becomes monomeric, it loses its gain of function (Chène and Bechter, 1999b). Altogether, these data suggest that tumours would preferentially select for DBD mutants because these offer an additional growth advantage over TD mutants which have only lost the wild-type tumour suppressor activity.

Discussion

The TD of p53 has long been ignored because it is not often mutated in cancer. During the last few years, however, a number of structural and functional analyses have revealed its importance for p53 function. On the practical side, detailed knowledge of its structure and of the thermodynamic stability of p53 tetramers has enabled p53 molecules to be designed with modified oligomerization properties that could be used in gene therapy to deliver p53 in tumours (Stavridi *et al*., 1999). Finally, recent findings which show that the TD is required for the gain of function by p53 mutants hold out the prospect that anticancer drugs may be developed which are able to suppress this phenotype in tumours.

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