

REVIEW

Structure–function–rescue: the diverse nature of common p53 cancer mutants

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The tumor suppressor protein p53 is inactivated by mutation in about half of all human cancers. Most mutations are located in the DNA-binding domain of the protein. It is, therefore, important to understand the structure of p53 and how it responds to mutation, so as to predict the phenotypic response and cancer prognosis. In this review, we present recent structural and systematic functional data that elucidate the molecular basis of how p53 is inactivated by different types of cancer mutation. Intriguingly, common cancer mutants exhibit a variety of distinct local structural changes, while the overall structural scaffold is largely preserved. The diverse structural and energetic response to mutation determines: (i) the folding state of a particular mutant under physiological conditions; (ii) its affinity for the various p53 target DNA sequences; and (iii) its protein–protein interactions both within the p53 tetramer and with a multitude of regulatory proteins. Further, the structural details of individual mutants provide the basis for the design of specific and generic drugs for cancer therapy purposes. In combination with studies on second-site suppressor mutations, it appears that some mutants are ideal rescue candidates, whereas for others simple pharmacological rescue by small molecule drugs may not be successful.

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Introduction

Mutation of the p53 gene is the most common genetic alteration in human cancer, affecting more than 50% of all tumors. In most cases, the mutation is located in the DNA-binding core domain of the protein (Olivier *et al.*, 2002; Hamroun *et al.*, 2006) (Figure 1a). According to the latest version of the TP53 mutation database of the International Agency for Research on Cancer (version R11 at www-p53.iarc.fr), about 1300 different cancer-related single amino-acid changes in the core domain

of the protein have been reported. The functional consequences of p53 cancer mutations are complex (Blagosklonny, 2000; Sigal and Rotter, 2000), and several systematic studies have shown that while some mutants result in complete loss of p53 function others exhibit altered transactivation spectra or retain function at lower temperatures (Kato *et al.*, 2003; Resnick and Inga, 2003; Dearth *et al.*, 2007; Menendez *et al.*, 2006). Clinical studies of breast cancer indicate that there is a link between the type of mutation, cancer prognosis and response to drug treatment (Olivier *et al.*, 2006). It is, therefore, crucial to understand the molecular basis of p53 inactivation in cancer by different types of mutation, so as to predict the biological outcome or the response to drug treatment. In this review, we provide insights into the structure of the wild-type protein, in particular the DNA-binding domain, and the diverse structural and functional consequences of mutation. In the light of these data, we evaluate therapeutic concepts that directly target the core domain of common cancer mutants.

The domain organization of full-length p53

The 393-aa p53 protein has a complex domain structure (Figure 1a), which comprises well-defined domains and natively unfolded regions, and undergoes a reversible equilibrium to form tetramers (Sakaguchi *et al.*, 1997; Bell *et al.*, 2002; Veprintsev *et al.*, 2006). Its cellular protein levels are tightly regulated, and the activity of p53 is further modulated by post-translational modifications (Bode and Dong, 2004; Lavin and Gueven, 2006) and through interactions with a multitude of signaling proteins (Braithwaite *et al.*, 2006). The N-terminal region contains the transactivation domain (residues 1–62), which can be further divided into two sub-domains, followed by a proline-rich region (residues 63–94). The transactivation domain interacts with a number of regulatory proteins, such as the negative regulator MDM2, which regulates cellular levels of p53 (Momand *et al.*, 2000; Marine *et al.*, 2006), components of the transcription initiation complex (Lu and Levine, 1995; Thut *et al.*, 1995) and the acetyltransferases p300 and CBP, which act as co-activators and regulate p53 function via acetylation of its C-terminus (Gu *et al.*, 1997; Grossman, 2001). The proline-rich region contains

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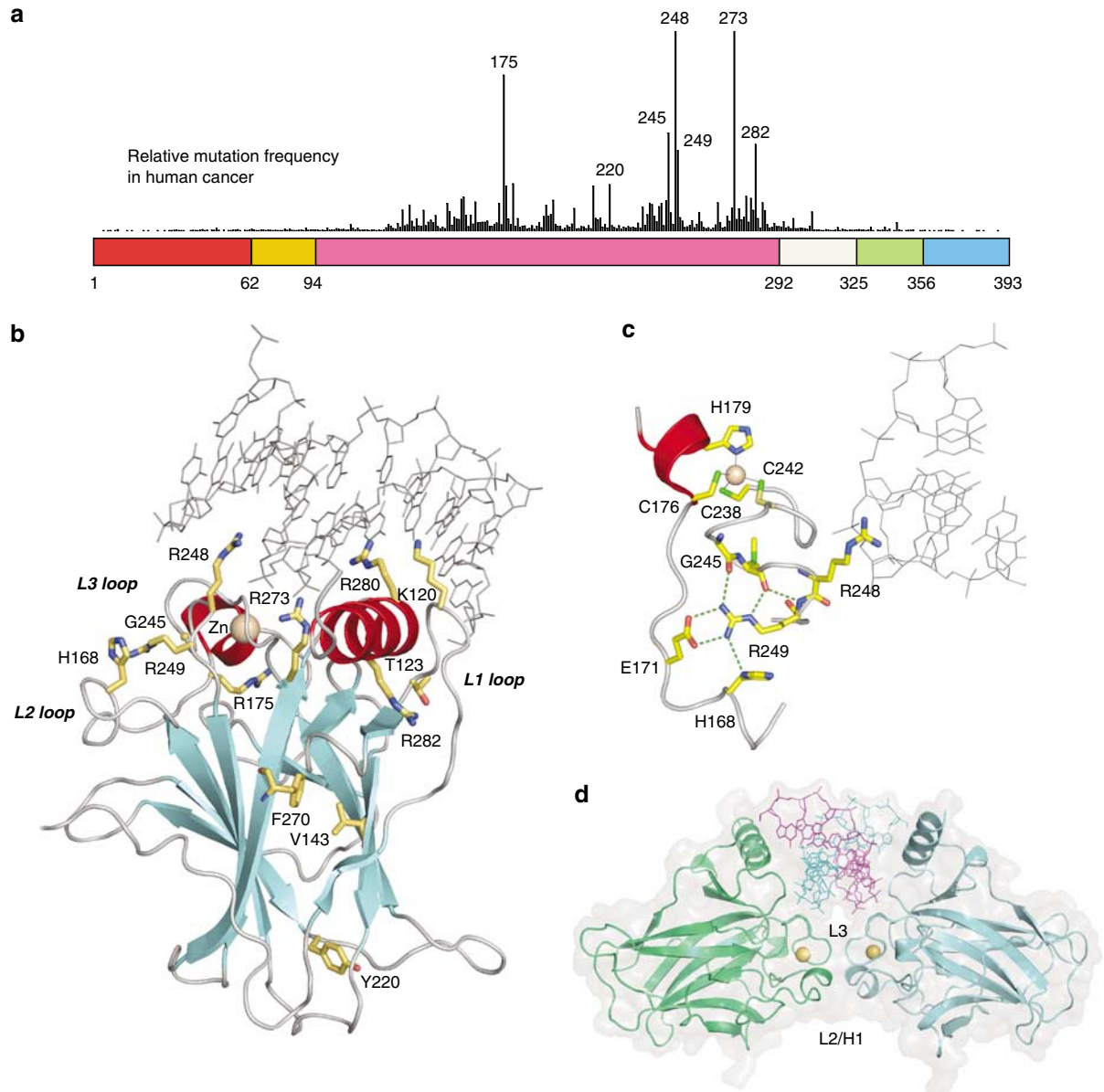


Figure 1 Structure of p53. **(a)** Schematic view of the domain structure of p53. The 393-residue p53 protein comprises an N-terminal transactivation domain (red), followed by a proline-rich region (orange), a central DNA-binding core domain (magenta), a tetramerization domain (green) and a negative regulatory domain (blue) at the extreme C-terminus (see text for further details). The columns indicate the relative frequency of cancer-associated mutations for each residue according to the TP53 mutation database of the International Agency for Research on Cancer (version R10, www-p53.iarc.fr) (Olivier *et al.*, 2002). **(b)** Ribbon diagram of the structure of DNA-bound p53 core domain (PDB ID code 2AHI) (Kitayner *et al.*, 2006). Residues of importance are highlighted in orange (see text). **(c)** Close-up view of the L2/L3 region, including the zinc-binding site, in the structure of DNA-bound wild-type (PDB ID code 2AHI, molecule A). The L3 loop is anchored to the minor groove of the bound consensus DNA via Arg-248, and its conformation is stabilized via the Arg-249 side chain. Arg-249 mediated interactions are depicted as green dashed lines. **(d)** Core-domain dimer bound to a DNA half-site (PDB ID code 2AC0) (Kitayner *et al.*, 2006). The protein–protein interface of the symmetrical dimer is formed by residues from the L3 loop and the H1 helix within the L2 loop. The figure was generated using PyMOL (DeLano, 2002).

SH3-domain binding motifs (PXXP) and is thought to have a regulatory role (Walker and Levine, 1996; Müller-Tiemann *et al.*, 1998). The central (core) domain (residues 94–292) binds specifically to double-stranded target DNA that contains two decameric ‘half-site’ motifs 5′-Pu·Pu·Pu·C·(A/T)·(T/A)·G·Py·Py·Py-3′

(Pu = A/G, Py = T/C) separated by up to 13 base pairs (el-Deiry *et al.*, 1992). Four core domains bind these DNA response elements in a cooperative manner to give a 4:1 complex (Balagurumoorthy *et al.*, 1995; Weinberg *et al.*, 2004b). The affinity with which p53 binds its response elements varies depending on the sequence.

Generally, p53 binds with high affinity to the recognition elements of genes involved in cell cycle arrest, whereas all the lower affinity-binding sites are found in genes involved in apoptosis (Qian *et al.*, 2002; Weinberg *et al.*, 2005). The C-terminal region includes the tetramerization domain (residues 325–356), which regulates the oligomerization state of p53, and the negative auto-regulatory domain at the extreme C-terminus, which contains acetylation sites and binds DNA non-specifically (Prives and Manley, 2001; Weinberg *et al.*, 2004a; Friedler *et al.*, 2005a).

The N-terminal domain is natively unfolded, apart from small regions that exhibit nascent turn or helix formation (Lee *et al.*, 2000; Bell *et al.*, 2002; Dawson *et al.*, 2003). The region with nascent helix formation extends into a full amphipathic α -helix (residues 15–29) upon binding to a hydrophobic cleft on the surface of MDM2 (Kussie *et al.*, 1996) or in a membrane-like environment (Rosal *et al.*, 2004). Similarly, the C-terminal domain is also largely unstructured in its native state (Bell *et al.*, 2002; Weinberg *et al.*, 2004a), although studies on isolated peptides show that parts of it adopt a helical conformation upon binding to the S100B($\beta\beta$) protein (Rustandi *et al.*, 2000). Intrinsic disorder is common in proteins at the center of highly connected protein–protein interaction networks (Dunker *et al.*, 2005). It facilitates binding promiscuity and reflects the ability to interact with a large number of diverse target proteins.

By contrast, the DNA-binding domain and the tetramerization domain each adopt a well-defined conformation, and a wealth of structural information is available for these domains. Crystal structures have been reported for human p53 core domain in complex with cognate DNA (Cho *et al.*, 1994; Kitayner *et al.*, 2006), bound to domains of regulatory proteins (Gorina and Pavletich, 1996; Derbyshire *et al.*, 2002; Joo *et al.*, 2002), bound to a viral oncoprotein (Lavin and Gueven, 2006), and for a multitude of core domain mutants in their DNA-free form (Joerger *et al.*, 2004, 2005, 2006). These structures have recently been complemented by the solution structure of DNA-free wild-type (Canadillas *et al.*, 2006). In addition, the core domain of mouse p53 in its DNA-free and DNA-bound state (Zhao *et al.*, 2001; Ho *et al.*, 2006) and the *Caenorhabditis elegans* ortholog Cep-1 in its DNA-free form (Huyen *et al.*, 2004) have been structurally characterized. The structure of the tetramerization domain has also been solved by X-ray crystallography (Jeffrey *et al.*, 1995; Mittl *et al.*, 1998) and in solution by nuclear magnetic resonance (NMR) (Clore *et al.*, 1995).

The greater picture of how the individual domains are assembled in the full-length tetramer and what dynamic processes occur upon DNA binding or binding of regulatory proteins, however, has still to be revealed. So far, the full-length protein has defied crystallization, which does not come as a surprise considering its tendency to aggregate and its high content of disordered regions. Because of its size (170 kDa), the tetrameric full-length protein is also at the limits of conventional NMR spectroscopy (Veprintsev *et al.*, 2006).

The DNA-binding domain of p53

Structure and DNA-binding mode

In 1994, the crystal structure of the p53 core domain in complex with consensus DNA was solved at 2.2 Å resolution, which provided a framework for understanding the deleterious effects of common cancer mutations (Cho *et al.*, 1994). The crystal form in which the structure was solved contains three molecules in the asymmetric unit with specific packing interactions. One of the three core domains is bound to a DNA consensus site, the second to a non-consensus region of DNA, and the third molecule makes no significant contact with DNA, thus representing a DNA-free state of the core domain. The main structural feature of the core domain is an immunoglobulin-like central β -sandwich of two antiparallel β -sheets, which provides the basic scaffold for the DNA-binding surface (cf. Figure 1b). This surface is formed by two large loops (L2 comprising residues 164–194, which is interrupted by a short helix, and L3 comprising residues 237–250) that are stabilized by a zinc ion, and a loop–sheet–helix motif (loop L1, β -strands S2 and S2', the end of the extended β -strand S10, and the C-terminal helix H2). The zinc ion is coordinated by a histidine and three cysteine side chains (Cys-176, His-179, Cys-238 and Cys-242). Removal of the zinc ion substantially destabilizes the protein, resulting in local structural perturbation and loss of sequence-specific DNA binding (Bullock *et al.*, 2000; Butler and Loh, 2003). Similarly, molecular dynamic simulations predict a high degree of structural fluctuation in the adjacent loops in the absence of zinc (Duan and Nilsson, 2006). Conserved residues from the loop–sheet–helix motif make specific contacts with the major groove of bound target DNA, whereas the L3 loop is anchored to the minor groove via Arg-248. The six residues that are most frequently mutated in human cancer have been classified as 'contact' (Arg-248 and Arg-273) or 'structural' residues (Arg-175, Gly-245, Arg-249 and Arg-282), depending on whether mutation results in the loss of an essential DNA contact or is likely to perturb the structure of the DNA-binding surface (Cho *et al.*, 1994).

Recently, several crystal structures of the core domain bound to different double-stranded DNA dodecamers revealed core domain–core domain interactions upon binding as a tetramer and details of how p53 recognizes different target sequences (Kitayner *et al.*, 2006). In these complexes, two core domain monomers bind to a DNA half-site to form a symmetrical dimer (Figure 1d). Two such dimers are assembled to form a tetramer via protein interactions and base-pair stacking interactions. As such, the assembly in these core domain complexes mimics the scenario of four core domains bound to a continuous DNA double helix containing two half-sites that are separated by a two base-pair spacer. The symmetrical core-domain dimer involves residues from the L3 loop and helix H1 within the L2 loop, which is consistent with earlier studies in solution by NMR (Klein *et al.*, 2001; Rippin *et al.*, 2002). The protein–protein interface is stabilized by hydrophobic interactions as well as polar, often water-mediated contacts.

The surface area that is buried in this interface is relatively small, and a substantial contribution to the stabilization of the dimer is made by the contacts with DNA.

The finer details of the DNA-contact geometry show an interesting variation that depends on the sequence of the bound DNA. The L3 loop is anchored to the minor groove of target DNA via Arg-248 (Figure 1b and c). The conformation of the arginine side chain varies in the different DNA-complex structures from fully extended to folded, and the side chain contacts the DNA backbone either directly or via water-mediated contacts. Arg-273 contacts the DNA backbone at the center of the half-site (Figure 1b). In the major groove, Arg-280 makes invariant contacts with the conserved guanine base, whereas the other major groove contacts via Lys-120, Ala-276 and Cys-277 vary depending on the sequence of the half-site. These findings provide the structural framework for understanding the differences in binding affinities for the various p53 response elements and levels of target gene transactivation (Kitayner *et al.*, 2006).

Structural basis for the low stability of p53 core domain

The stability of full-length p53 is dictated by its core domain, which is only marginally stable and melts at only slightly above body temperature (Bullock *et al.*, 2000; Ang *et al.*, 2006). In addition, the core domain is also kinetically unstable. At 37°C, the core domain is rapidly cycled between the folded and unfolded states, followed by aggregation, even though the protein is thermodynamically stable at this temperature (Friedler *et al.*, 2003; Butler and Loh, 2005). A comparative analysis of void volumes in wild-type p53 and other proteins has excluded poor packing density or unusually large void volumes as the main cause of this low stability (Cuff and Martin, 2004). Novel insights into the structural basis for the relative instability of p53, however, have recently emerged from the analysis of the solution structure of p53 core domain by NMR (Canadillas *et al.*, 2006). This structure revealed a high degree of structural flexibility in various loops regions, such as L1 or the S7–S8 loop, which is also evident from a comparison of the various crystal structures. In addition, dynamic processes that involve buried aromatic residues were discovered. More importantly, an analysis of the location of the hydrogen atoms in the structure revealed several buried hydroxyl and sulfhydryl groups that form suboptimal hydrogen-bond patterns. The buried Tyr-236/Thr-253 pair is such an example, where the hydroxyl groups are not completely saturated with hydrogen bonds. Interestingly, in the thermodynamically more stable paralogs p63 and p73, these two residues are replaced by phenylalanine and isoleucine, respectively. When these residues are introduced into p53, the corresponding double mutant Y236F/T253I is indeed stabilized by 1.6 kcal/mol, as shown by urea denaturation (Canadillas *et al.*, 2006). These data strongly suggest that p53 has evolved to be only marginally stable at body temperature. Further

support for this hypothesis comes from a comparison of the structure of the human p53 core domain with the structure of the *C. elegans* ortholog Cep-1. The latter structure shows the same overall topology as the human variant but a higher proportion of secondary structure, and hence reduced flexibility, in the functionally important L1 and L3 loops (Huyen *et al.*, 2004). An analysis of the structural fluctuations in molecular dynamics simulations suggests that the stability difference between these two variants is caused by the structural and dynamic properties of the peripheral structural motifs, such as the S7–S8 loop, which is much shorter in Cep-1 (Pan *et al.*, 2006). Possibly, having an intrinsically unstable p53 protein has provided an evolutionary advantage. It may facilitate the tight regulation of the cellular protein levels and allow for enough structural plasticity of p53 to fulfil its various and complex functions at the center of a multitude of cellular networks. As a result of being only marginally stable, the p53 protein is very susceptible to inactivation by destabilizing mutations. Many mutations that inactivate the protein by reducing its thermodynamic stability might have only a mild effect in a more stable protein and would result in neutral, wild-type-like phenotypes under physiological conditions.

The quest for stable p53

Quite often accurate biophysical studies of p53 are substantially compromised because of its low intrinsic thermodynamic stability and associated aggregation tendencies, especially when destabilized mutants are studied. These experimental problems, in combination with the desire for a more stable and hence more effective p53 variant for gene therapy purposes, have driven the quest for stable p53. By adopting a semirational design strategy, a superstable quadruple mutant of p53 core domain was developed (*T*-p53C), containing the point mutations M133L, V203A, N239Y and N268D, which is stabilized by 2.6 kcal/mol (Nikolova *et al.*, 1998). These stabilizing mutations are either naturally occurring in p53 from different species or known second-site suppressor mutations. Interestingly, the two mutations that contribute most to the stability increase in *T*-p53C (N239Y and N268D) were also identified by *in vitro* evolution of thermostable full-length p53 variants (Matsumura and Ellington, 1999). *T*-p53C has wild-type-like properties, and the structure is virtually identical to the wild-type, apart from the mutated side chains (Joerger *et al.*, 2004). Overall, the mutations reduce the structural plasticity of the protein by creating a more rigid structural framework. The N268D mutation, for instance, results in an altered hydrogen-bond pattern. Thereby, Asp-268 connects the two sheets of the β -sandwich in an energetically more favorable way than the asparagine in the wild-type (Figure 2). Other approaches for the design of stable p53 mutants on the basis of molecular dynamics simulations include targeting flexible loops, such as L1 or the S7–S8 loop, but have yet to be experimentally verified (Pan *et al.*, 2005, 2006).

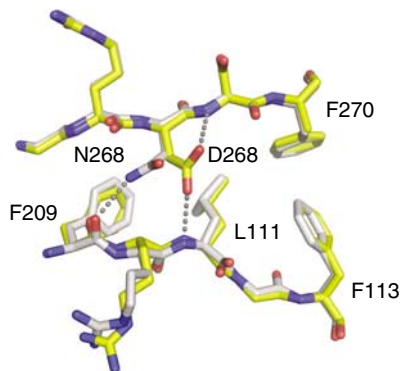


Figure 2 Crystal structure of the stabilized p53 core domain variant *T*-p53C. Close-up view of the N268D mutation site in the structure of *T*-p53C (PDB ID code 1UOL, molecule A; yellow) (Joerger *et al.*, 2004) superimposed on the structure of the wild-type core domain (PDB ID code 1TSR, molecule A; gray) (Cho *et al.*, 1994). Hydrogen bonds formed by the Asn-268 and Asp-268 side chains are highlighted with dotted lines. The figure was generated using PyMOL (DeLano, 2002).

Effects of common p53 cancer mutants

Thermodynamic stability and DNA-binding properties

The core domain of p53 is only marginally stable at body temperature. Hence, any mutation that reduces the thermodynamic stability of the protein will have a profound effect on the amount of folded protein in the cell. The thermodynamic stability determines the folding-unfolding equilibrium of a particular mutant and is not to be confused with the term stability that is often used in the p53-related literature, referring to the cellular levels of p53, regardless of the folding state. p53 mutants often accumulate in tumor cells, in part because of a disrupted feedback regulation of p53 protein levels by Hdm2/Mdm2, which normally sequesters p53 for degradation by the proteasome (Midgley and Lane, 1997; Buschmann *et al.*, 2000).

Equilibrium unfolding studies by urea denaturation combined with DNA-binding studies at low temperatures, at which the mutants are largely folded, show that common p53 cancer mutants can be grouped in several distinct mutant classes, which often coincide with the location of the mutations in the structure (Bullock *et al.*, 2000) (Figure 3). DNA-contact mutants, such as R273H, have only a small effect on the thermodynamic stability of the protein. In contrast, structural hotspot mutations in the DNA-binding surface are destabilized to varying degrees, ranging from 1 kcal/mol for G245S to 2 kcal/mol for R249S to up to more than 3 kcal/mol for R282W, and show different patterns of DNA-binding activity in their folded state. One class of structural mutations that either directly replace a zinc ligand (C242S) or are thought to induce structural perturbation at the zinc-binding site (R175H) destabilize the core domain by about 3 kcal/mol and abolish DNA binding, even at low temperatures. The same effect is observed for the wild-type core domain upon removal of the zinc ion by a chelating agent. All tested β -sandwich mutants (e.g. V143A in β -strand S3, F270C in β -strand

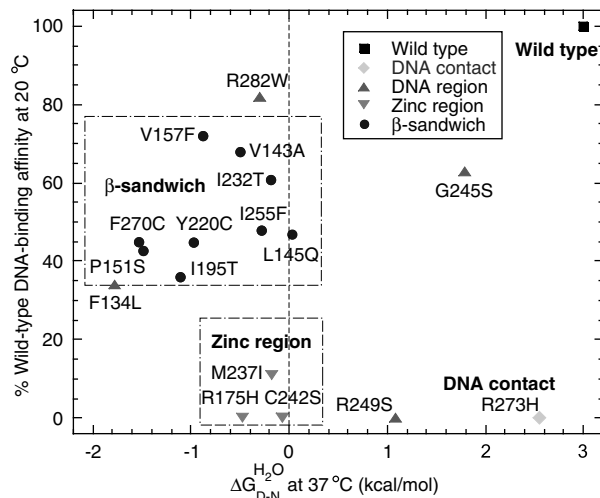


Figure 3 Thermodynamic stability and DNA-binding properties of p53 cancer mutants. The thermodynamic stability of p53 core domain mutants at 37°C is plotted against the binding affinity for *gadd45* DNA at 20°C, a temperature at which all mutants are folded. A free energy of unfolding in water of 0 kcal/mol (shown by a dashed line) corresponds to 50% denatured protein. Data taken from Bullock *et al.* (2000).

S10, or Y220C at the beginning of the loop that connects β -strands S7 and S8) are also highly destabilized by 3.0–4.5 kcal/mol, but unlike the destabilizing mutations at the zinc-binding site, they all show binding of *gadd45* DNA at a level close to the wild-type. Similar effects on stability and DNA binding are observed for the structural mutations in the loop-sheet-helix motif (F134L and R282W). In addition to their thermodynamic instability, many core domain mutants are also kinetically unstable (Friedler *et al.*, 2003; Butler and Loh, 2005). Highly destabilized mutants, such as V143A and C242S, unfold more than 10 times faster than the wild-type core domain, whereas the contact mutant R273H has roughly the same half-life as the wild-type (Friedler *et al.*, 2003).

Implications for the analysis of antibody-binding studies

The folding state of p53 mutants has historically been assessed with antibodies that specifically recognize either the folded or the denatured state of p53. The PAb1620 antibody is specific for the 'wild-type' conformation of human and mouse p53 but does not bind to denatured p53 (Cook and Milner, 1990). It recognizes a specific structural motif on the surface of the core domain that is distant from the DNA-binding site and involves residues Arg-156, Leu-206, Arg-209 and Asn-210 (Wang *et al.*, 2001). Conversely, PAb240 and several other antibodies bind to denatured wild-type and highly destabilized mutants (Gannon *et al.*, 1990; Legros *et al.*, 1994). The antibody PAb240, for example, is directed against an epitope on β -strand S7 (residues 212–217), which is buried within the core domain and becomes accessible only upon unfolding of this region (Stephan and Lane, 1992). Hence, the lower the thermodynamic stability of a mutant, the higher is its reactivity with PAb240 at

physiological temperatures. Reactivity with PAb240, however, has no predictive value for the actual conformation of a mutant in the folded state. While the use of such antibodies is a very powerful tool for assessing the folding state of p53, it has nourished the relatively common misconception that p53 mutants adopt a generic 'mutant conformation', when, in fact, they are simply unfolded.

Structural classes of p53 core domain cancer mutants

A first indication that common p53 cancer mutants exhibit characteristic local structural changes came from heteronuclear single quantum correlation NMR spectroscopy studies (Wong *et al.*, 1999; Friedler *et al.*, 2004). Using the stabilized *T*-p53C variant, the finer details of the structural effects of numerous oncogenic mutations have been elucidated recently by X-ray crystallography (Joerger *et al.*, 2005, 2006). These high-resolution crystal structures paint an intriguingly diverse picture of the effects of common cancer mutations. In all cases, the basic structural scaffold of the wild-type is conserved, while there are mutant-specific local structural changes. These changes include the mere removal of a DNA-contact residue, distortions or conformational changes in various parts of the DNA-binding surface, creation of internal cavities and the formation of surface crevices in regions remote from the DNA-binding site.

DNA-contact mutations

DNA-contact mutations inactivate p53 by replacing residues that form essential contacts with its DNA response elements, such as Arg-248, Arg-273 and Arg-280. Crystallographic studies have shown that the R273H and R273C contact mutations merely remove the DNA-contact residue Arg-273 without inducing structural perturbations in neighboring residues, and that the overall architecture of the DNA-binding surface is preserved in these mutants (Joerger *et al.*, 2005, 2006; Figure 4a). These findings are consistent with (i) the observation that the R273H mutant shows about the same kinetic and thermodynamic stability as the wild-type and (ii) that full-length R273H exhibits residual DNA-binding activity *in vitro*, although binding selectivity is largely lost (Ang *et al.*, 2006). The residual binding affinity, however, is too weak for normal transactivation of p53 response elements (Kato *et al.*, 2003; Dearth *et al.*, 2007). A similar scenario with rather small local changes can be expected for cancer-associated mutations of the DNA-contact residues Arg-248 and Arg-280. Several frequent contact mutations do not only result in a loss of a DNA contact, but may actively prevent sequence-specific DNA binding because a large hydrophobic side chain is introduced (e.g. S241F, R248W and C277F).

Structural mutations in the L3 loop

The L3 loop plays a dual role in DNA binding. It provides a DNA-contact residue in Arg-248, which anchors the core domain to the minor groove of target

DNA, but also forms an integral part of the core domain-core domain dimerization interface upon binding to a DNA half-site (Kitayner *et al.*, 2006) (Figure 1c and d). Loop L3 is the location of the two structural cancer hotspot mutations G245S and R249S. These two mutations perfectly illustrate how the extent of mutation-induced local structural changes determines the energetic and functional response to mutation. The R249S mutation is predominantly found in hepatocellular tumors in eastern Asia and sub-Saharan Africa, and tumorigenesis has been linked to the exposure to aflatoxin-B1, which is a common food contaminant in these regions (Aguilar *et al.*, 1993; Laurent-Puig and Zucman-Rossi, 2006). Structurally, Arg-249 is essential for stabilizing the hairpin conformation of the L3 loop. Via its guanidinium group, it forms a salt bridge with Glu-171 and, more importantly, hydrogen bonds with the main-chain oxygens of Gly-245 and Met-246 (Figure 1c). The crystal structure of *T*-p53C-R249S shows that the loss of these interactions induces a high degree of flexibility and favors a non-native conformation of this loop (Joerger *et al.*, 2005). Most notably, there is a peculiar methionine switch, involving Met-243 and Met-246. Met-243, which is solvent-exposed in DNA-free wild-type and buried in the core domain-core domain interface when bound to target DNA, displaces Met-246 from its buried location within a hydrophobic pocket of the zinc-binding region (Figure 4b). This methionine switch in *T*-p53C-R249S results in formation of a short α -helix and is accompanied by a large conformational change that also substantially displaces the DNA-contact residue Arg248. It is interesting to note that a very similar switch of Met-243 and Met-246 has recently been observed in the crystal structure of the p53 core domain bound to the simian virus 40 large T-antigen (Lilyestrom *et al.*, 2006), which is consistent with the observation that the R246S mutant of mouse p53, the equivalent to R249S in human p53, retains its ability to bind the simian virus 40 large T antigen (Ghebranious *et al.*, 1995).

The structural changes induced by the G245S mutation are much smaller, and the overall conformation of the L3 loop is conserved. In *T*-p53C-G245S, the Ser-245 side chain is accommodated by displacing a structural water molecule and by small structural shifts in the proximity of the mutation site (Joerger *et al.*, 2006). These changes mainly affect residues that are involved in protein-protein interactions upon DNA binding. As a result, the affinity of full-length *T*-p53-G245S for *gadd45* DNA is reduced by about a factor of 15. In accordance with the structural observations, the affinity loss for full-length *T*-p53-R249S is much more dramatic and is in the range of that found for the contact mutant R273H (Ang *et al.*, 2006).

Disruptions of the zinc-binding region

Arg-175 is located in the L2 loop and is the site of the most common cancer mutation, R175H. This mutation results in activity loss at both physiological and subphysiological temperatures *in vitro* and *in vivo*

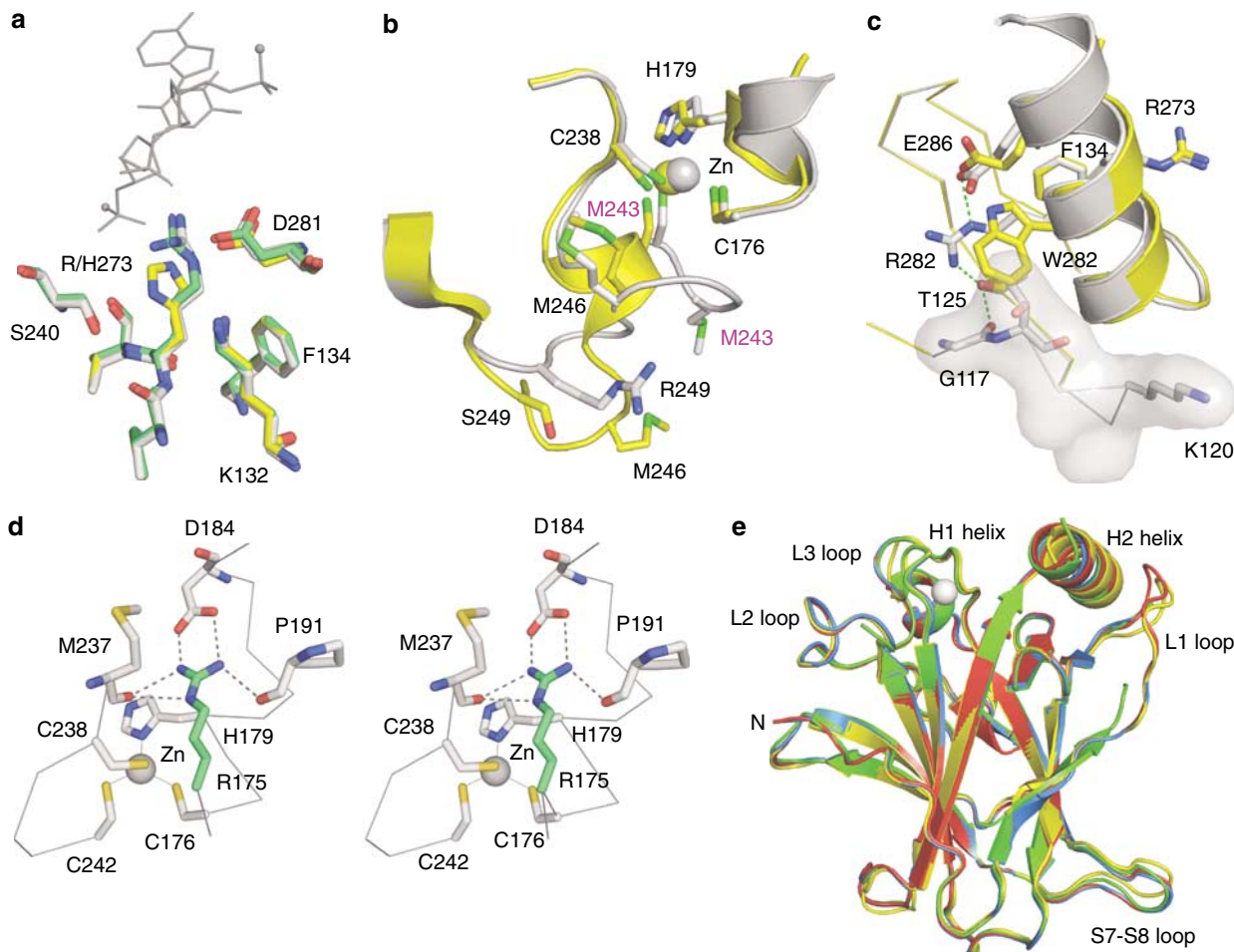


Figure 4 Structural effects of cancer mutations in the DNA-binding surface. (a) Structure of the contact mutant *T*-p53C-R273H (PDB ID code 2BIM, molecule A; yellow) (Joerger *et al.*, 2005) superimposed on the structure of *T*-p53C (PDB ID code 1UOL; green) (Joerger *et al.*, 2004) and DNA-bound wild-type (PDB ID code 2AC0, molecule C; gray) (Kitayner *et al.*, 2006). (b) L3 loop and zinc-binding region in the structure of *T*-p53C-R249S (PDB ID code 2BIO; yellow) (Joerger *et al.*, 2005) superimposed on the structure of DNA-free wild-type (PDB ID code 1TSR, chain A; gray) (Cho *et al.*, 1994), showing the Met-243/Met-246 methionine switch (see text for details). (c) Loop-sheet-helix motif in the structure of *T*-p53C-R282W (PDB ID code 2J21, molecule A; yellow) (Joerger *et al.*, 2006) superimposed on the structure of *T*-p53C (PDB ID code 1UOL, molecule A; gray) (Joerger *et al.*, 2004). The hydrogen-bond network mediated via Arg-282 in *T*-p53C is highlighted in green. Residues 117–121 in *T*-p53C-R282W are disordered and were not detected in the crystal structure. For the corresponding five residues in *T*-p53C, the molecular surface is shown in transparent gray. (d) Stereo view of the Arg-175 hotspot site in the structure of DNA-bound wild-type (PDB ID code 2AHI, molecule A) (Kitayner *et al.*, 2006). (e) Superposition of the structures of *T*-p53C (PDB ID code 1UOL, molecule A; red) (Joerger *et al.*, 2004), *T*-p53C-H168R (PDB ID code 2BIN; green) (Joerger *et al.*, 2005), *T*-p53C-G245S (PDB ID code 2J1Y, molecule A; yellow) (Joerger *et al.*, 2006) and *T*-p53C-R282W (PDB ID code 2J21, molecule A; blue) (Joerger *et al.*, 2006), showing conservation of the overall structural scaffold. The zinc ion in *T*-p53C is depicted as a gray sphere. The orientation of the molecules is similar to that of the DNA-bound wild-type in Figure 1b. The figure was generated using PyMOL (DeLano, 2002).

(Bullock *et al.*, 2000; Kato *et al.*, 2003; Dearth *et al.*, 2007). In the wild-type protein, the Arg-175 side chain is embedded between the L2 and L3 loops, and the guanidinium group forms hydrogen bonds with the carbonyl oxygens of Pro-191 and Met-237, and a salt bridge with Asp-184 (Figure 4d). Most importantly, it is directly adjacent to three of the zinc ligands (Cys-176, His-179 and Cys-238). Even though there are no NMR or crystallographic data available for this mutant yet, the nature of the structural effects can be assessed from analyzing the wild-type structure and functional data on the effects of various mutations at codon 175. The

deleterious effect of the R175H mutation seems to reside primarily in the fact that a bulky histidine residue is introduced that causes structural distortions and may directly interfere with zinc binding rather than in the mere loss of wild-type interactions. The corresponding alanine mutant of the core domain (R175A), for instance, is only moderately destabilized (by 0.7 kcal/mol as compared to 3.5 kcal/mol for R175H) and shows 67% wild-type activity with *gadd45* DNA at 20°C *in vitro* (Bullock *et al.*, 2000). Similarly, it was found that the R175C mutant essentially retains wild-type cell cycle arrest and proapoptotic function when transfected into

p53-null human tumor Saos-2 cells, whereas these functions are lost upon introduction of large bulky side chains (e.g. for mutants R175W and R175Y) (Ryan and Vousden, 1998). A more recent *in vivo* study found that the R175L mutant has a phenotype similar to that of the wild-type, which is in stark contrast to the complete abrogation of DNA-binding activity for R175H in these assays (Dearth *et al.*, 2007). Accordingly, R175H can be grouped with cancer mutants that directly affect the zinc coordination sphere by substituting one of the zinc ligands (e.g. C176F, H179R and C242F).

Structural mutations at the periphery of the DNA-binding surface

The R282W hotspot mutation is located in the C-terminal helix (H2), which is part of the loop-sheet-helix motif that binds to the major groove of DNA response elements. Arg-282 plays an important role in maintaining the structural integrity of the loop-sheet-helix motif. It packs the C-terminal helix against the S2-S2' β -hairpin and thus anchors it to the core of the protein (Figure 4c). The aliphatic part of the Arg-282 side chain makes van-der-Waals interactions with Phe-134, and the guanidinium group is at the center of a network of hydrogen bonds connecting the structural elements of the loop-sheet-helix motif (e.g. it interacts with the hydroxyl group of Thr-125, which in turn forms a hydrogen-bond with Gly-117 from the L1 loop, and the carboxylate group of Glu-286 in the C-terminal helix H2). The crystal structure of *T*-p53C-R282W shows that the mutation induces distinct structural perturbations in the loop-sheet-helix motif but leaves the overall structure of the core domain intact (Joerger *et al.*, 2006). The Arg-282 mediated stabilizing interactions are lost and only in part compensated for by the tryptophan, which explains the observed stability loss. Moreover, the adjacent L1 loop is displaced by Trp-282 because of steric hindrance and lacks a defined conformation in the region containing the DNA-contact residue Lys-120, which leaves the Trp-282 side-chain largely solvent exposed (Figure 4c). Importantly, the overall structure of the DNA-binding surface, including helix H2, is largely preserved, which explains why the mutant retains DNA-binding activity at subphysiological temperatures both *in vitro* and *in vivo*, at least for high-affinity response elements (Bullock *et al.*, 2000; Dearth *et al.*, 2007).

The H168R cancer mutation has a similar effect on protein stability and DNA binding as R282W (Bullock *et al.*, 2000; Nikolova *et al.*, 2000). It is located in the β -turn region at the beginning of the L2 loop on the opposite edge of the DNA-binding surface (cf. Figure 1b). Similarly to *T*-p53C-R282W, the structure of *T*-p53C-H168R shows that the overall fold is largely preserved but exhibits distinct local structural distortions (Figure 4e). In this particular case, introduction of Arg-168 causes substantial disorder at the mutation site, resulting in a lack of a defined conformation for residues 166 to 170 in the L2 loop (Joerger *et al.*, 2005).

Mutations in the L1 loop

The L1 loop is a mutational cold spot in which only a few oncogenic mutations have been identified, even though it contains the conserved DNA-contact residue Lys-120. Several mutations in the L1 loop have been reported that enhance the affinity for specific DNA sequences (Saller *et al.*, 1999; Inga *et al.*, 2001). It is one of the most mobile structural elements of the core domain, which undergoes a conformational change upon DNA binding that mainly affects residues Lys-120 and Ser-121 (Zhao *et al.*, 2001; Joerger *et al.*, 2004). In several crystal structures of core domain mutants (Joerger *et al.*, 2005), and surprisingly also in some wild-type DNA complexes (Ho *et al.*, 2006; Kitayner *et al.*, 2006), parts of this loop are disordered, reflecting its intrinsic flexibility. In all DNA complexes where it adopts a defined conformation, Lys-120 makes specific contacts with bases of the target DNA half-site, with the exact nature of the interactions depending on the actual DNA sequence (Kitayner *et al.*, 2006). As such, the L1 loop may play a role in modulating the affinity of p53 towards different response elements, which may explain the need for high intrinsic flexibility. It is also interesting to note that the corresponding loop in the *C. elegans* ortholog Cep-1 adopts a significantly different conformation, induced by a short helical turn at the beginning of the loop, which makes it unlikely that Lys-237, the equivalent to Lys-120 in human p53, can make the major groove contacts that are observed in human p53 (Huyen *et al.*, 2004). It has, however, an important role in DNA binding as shown by mutagenesis studies. Together with the structural changes in the L3 loop, these observations suggest that the DNA-binding mode of human p53 and the *C. elegans* ortholog show significant differences, despite their conserved binding specificity (Huyen *et al.*, 2004).

A recent systematic mutational analysis of the L1 loop by alanine and arginine scanning confirms that it plays an important, yet highly modular role in DNA binding and that it tolerates structural perturbations to a certain extent without completely abrogating function (Zupnick and Prives, 2006). This study also highlights the importance of regulation of the cellular p53 levels in cell-based activity assays. For only weakly binding mutants, overexpression of the protein may offset the weaker binding and mask activity differences between wild-type and mutants that are detected when expressed at physiological levels, as observed for the K120A mutant.

The T123A mutation was found to increase transactivation both *in vitro* and *in vivo* (Inga *et al.*, 2001; Resnick and Inga, 2003; Zupnick and Prives, 2006). Interestingly, super-transactivation is found mainly with low-affinity proapoptotic response elements, such as AIP-1, Bax1 or PUMA, but not with high-affinity binding sites. On the basis of the available structural data, there is no obvious explanation for this behavior. A core domain mutant containing the T123A mutation shows no structural change at the mutation site in its DNA-free form compared with the corresponding variant lacking this mutation (Joerger *et al.*, 2005).

A possible explanation is that the T123A mutation changes the conformational flexibility of the L1 loop in a way that facilitates binding to proapoptotic response elements (cf. Figure 1b). Clearly, more biophysical data and structural information on the DNA-binding mode are needed to satisfactorily explain why certain L1 mutants enhance the affinity for proapoptotic response elements.

β -Sandwich mutants

Y220C is the most common cancer mutation outside the DNA-binding surface and accounts for 1.4% of somatic p53 missense mutations, which is only slightly less frequent than the R249S and G245S hotspot mutations in the DNA-binding surface (frequency of 1.8 and 1.9%; cf. version R11 of the TP53 mutation database at www.p53.iarc.fr). This number is particularly high, considering that the likelihood of this mutation occurring is much smaller than for most of the other hotspot mutations, as calculated from sequence-dependent substitution models (Lunter and Hein, 2004). The only other hotspot mutation with a low intrinsic mutation rate is the R249S mutation, which has been linked to aflatoxin-B exposure (see above). Tyr-220 is located at the periphery of the β -sandwich at the beginning of the loop that connects β -strands S7 and S8 (Figure 1b). Its aromatic ring is an integral part of the hydrophobic core of the β -sandwich, whereas the hydroxyl group interacts with the solvent. The mutation is highly destabilizing but leaves the overall structure of the core domain intact, as revealed by the crystal structure of *T*-p53C-Y220C (Joerger *et al.*, 2006). There are, however, interesting alterations to the molecular surface of this mutant, which exhibits a large mutation-induced crevice, linking two smaller crevices that preexist in the wild-type and *T*-p53C (Figure 5a and b). Importantly, this surface cleft is distant from the known functional interfaces of the core domain.

Other β -sandwich mutants are less frequent, but taken together, they account for a substantial portion of the cancer-associated mutations in the core domain. In about one-third of reported cancer cases with mutations in the core domain of p53, the mutation is located outside the wider DNA-binding surface, as defined by loops L2, L3 and the loop-sheet-helix motif. Many of the β -sandwich mutations are large-to-small substitutions of buried residues and, as such, they are potentially cavity creating. Systematic mutagenesis studies on the protein barnase have shown that truncation of buried hydrophobic side chains results in an average stability loss of 1.5 kcal/mol per deleted methyl(ene) group. For partly exposed hydrophobic residues, the stability loss is only 0.8 kcal/mol per deleted methyl(ene) group (Serrano *et al.*, 1992). Structural studies on barnase (Buckle *et al.*, 1993, 1996) and T4 lysozyme (Eriksson *et al.*, 1992; Xu *et al.*, 1998) have linked the structural and energetic response to cavity-creating mutations in the hydrophobic core of the protein. On the basis of these studies, the stability loss upon mutation can be approximated to be the additive contribution of two terms. The first term is

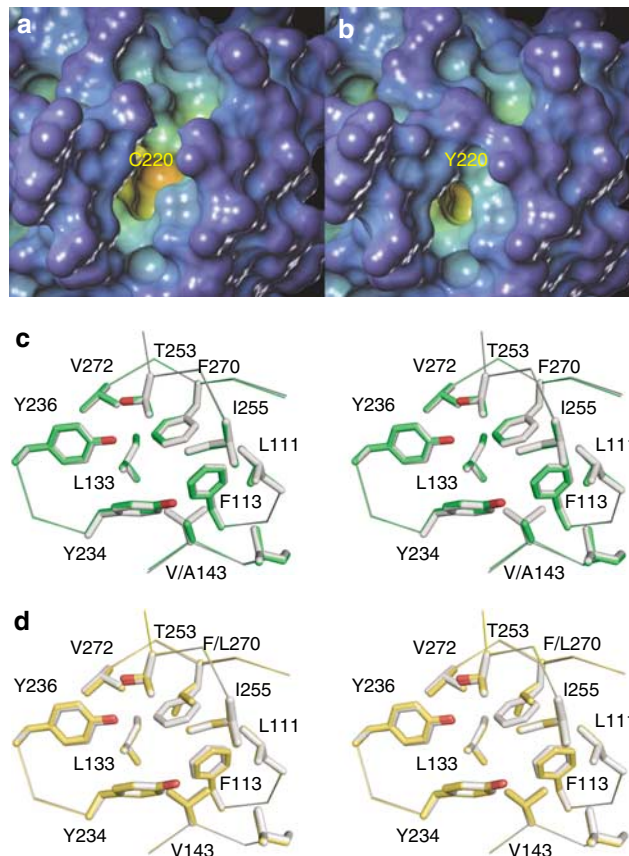


Figure 5 Structural effects of mutations in the β -sandwich region. (a) Molecular surface of *T*-p53C-Y220C around Cys-220, showing the mutation-induced crevice (PDB ID code 2J1X, molecule A) (Joerger *et al.*, 2006). (b) Molecular surface of *T*-p53C around Tyr-220 (PDB ID code 1UOL, molecule A) (Joerger *et al.*, 2004). (c) Structure of *T*-p53C-V143A (PDB ID code 2J1W, molecule A; green) superimposed on *T*-p53C (gray), showing the region of the mutation-induced internal cavity (Joerger *et al.*, 2006). (d) Structure of *T*-p53C-F270L (PDB ID code 2J1Z, molecule A; orange) superimposed on *T*-p53C (gray), showing the region of the mutation-induced internal cavity (Joerger *et al.*, 2006). The figure was generated using SYBYL 6.9 (Tripos Inc., St Louis, MO, USA) and PyMOL (DeLano, 2002).

a constant for a particular amino-acid substitution and corresponds to the difference in the free energy of transferring the two amino acids from the interior of the protein to the solvent, which is commonly described as the ‘hydrophobic effect’. The second term is a function of the loss of favorable van-der-Waals interactions upon mutation (as compared to the wild-type) and depends on the structural response to mutation. For a given mutation, this term is maximal if there is no collapse of the structure upon creation of the cavity. It is substantially reduced, however, if repacking of the hydrophobic core, resulting in partial or total collapse of the cavity, compensates for the loss of wild-type interactions.

In the p53 core domain, two of the cancer-associated large-to-small substitutions, V143A and F270L, have been structurally studied. Val-143 and Phe-270 are located on opposite strands of the β -sandwich

(Figure 1b). Their side chains are within van-der-Waals distance and form an integral part of the hydrophobic core. The high-resolution crystal structures of *T*-p53C-V143A and *T*-p53C-F270L reveal that the mutations create internal cavities in the hydrophobic core of the protein without collapse of the surrounding structure (Figure 5c and d). In both cases, the wall of the mutation-induced cavity is formed mainly by hydrophobic atoms, which explains why no ordered water molecules are detected in these cavities (Joerger *et al.*, 2006). Hence, as a result of the hydrophobic effect and loss of van-der-Waals interactions, the core domain is destabilized by 3.7 and 4.1 kcal/mol, respectively. Most importantly, there are virtually no other structural changes, apart from the formation of the cavity.

Mutations in the tetramerization domain

Mutations outside the core domain of p53 occur only rarely in human cancer. A different scenario is found when germline mutations in the p53 gene are analyzed, as found in patients with Li-Fraumeni (or related syndromes), a rare autosomal hereditary disorder characterized by cancer onset at a very early age (usually diagnosed before the age of 45 years) (Olivier *et al.*, 2003). The R337H mutation in the tetramerization domain accounts for about 20% of the reported cases of germline p53 mutations. As such, Arg-337 is much more frequently mutated than the cancer hotspot sites in the core domain. The profile of the codon distribution is otherwise similar to that found for the somatic mutations. It has to be noted, however, that the total number of reported cases/families with a germline missense mutation is still relatively small (295 cases/families according to version R11 of the TP53 mutation database; www-p53.iarc.fr) and that the cases are regionally clustered (Southern Brazil), suggesting that they might be genealogically linked (see review by Petitjean *et al.*, 2007). By contrast, mutations at codon 337 account for only about 0.1% of somatic p53 mutations.

The tetramerization domain consists of a short β -strand and an α -helix that are connected via a sharp hairpin (Clore *et al.*, 1995; Jeffrey *et al.*, 1995). Through intermolecular β -sheet formation and helix packing, four tetramerization domain chains assemble to form a tightly packed tetramer, which can be best described as a dimer of primary dimers (Figure 6a). Arg-337 forms an intermolecular salt-bridge and is also involved in hydrophobic packing interactions within the primary dimer of the tetramerization domain (Figure 6b). It has been shown that truncation of Arg-337 substantially destabilizes the tetramerization domain (Mateu and Fersht, 1998). In the case of the R337H mutation, the stability of the domain shows a strong pH-dependence in the physiologically relevant pH range, which correlates with the protonation state of the histidine side chain (DiGiammarino *et al.*, 2002). Similarly, other cancer-associated mutations also destabilize the tetramerization domain, for example by introducing a helix breaking proline (L344P) or weakening the hydrophobic

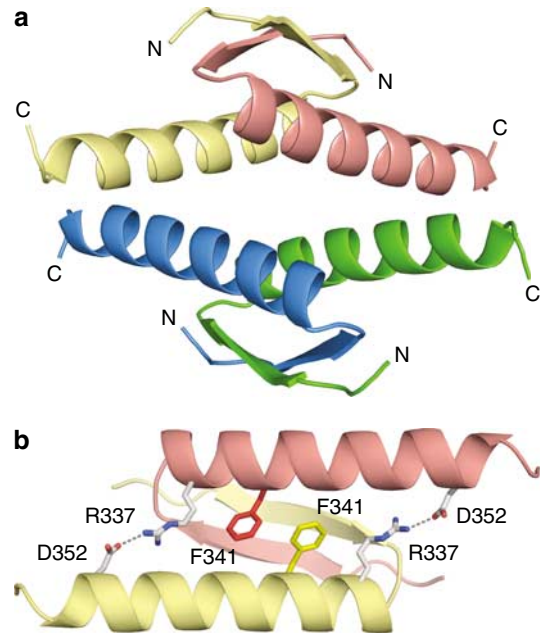


Figure 6 Crystal structure of the p53 tetramerization domain. (a) Structural organization of the tetramer as a dimer of primary dimers with D₂ symmetry (PDB ID code 1C26) (Jeffrey *et al.*, 1995). (b) Inter-subunit contacts within a primary dimer. The figure was generated using PyMOL (DeLano, 2002).

core of the primary dimers (F341L), which either completely abrogates oligomerization or shifts the tetramerization equilibrium, thus preventing tetramer formation at normal cellular levels (Ishioka *et al.*, 1997; Mateu and Fersht, 1999; Chène, 2001; Kawaguchi *et al.*, 2005). The G334V mutant of the tetramerization domain, which is associated with lung cancer, was found to form amyloid fibrils under physiological conditions, although it was still able to form a tetrameric complex at high concentrations at lower temperatures (Higashimoto *et al.*, 2006). In the wild-type tetramerization domain, Gly-334 facilitates the formation of a sharp turn connecting the β -strand with the α -helix and adopts a backbone conformation that would be energetically unfavorable for a non-glycine residue, indicating that structural distortions are to be expected upon mutation. The low frequency of these mutations in cancer has been in part attributed to the fact that mutants with an impaired tetramerization domain do not interfere with the function of wild-type p53 (Chène, 2001).

Towards the understanding of mutation-associated phenotypes

Stability and structure are the key determinants for the phenotypic response to mutation. The stability of a mutant determines the relative amount of folded and potentially functional protein under physiological conditions. Whether a particular mutant is functional in the

folded state – and if so, to what extent – depends on the exact details of the nature of the mutation-induced structural changes and on the dynamic properties in the folded state. The local structure not only determines the intrinsic ability of the mutant to bind to target DNA but also its activity in the wider context of the complex p53 network, involving a multitude of protein–protein interactions. While we are still far away from fully understanding the functional consequences at the cellular level, certain aspects of the phenotypic response to mutation can be rationalized.

Temperature-sensitive phenotypes

Temperature-sensitive p53 mutants have attracted much interest, because they are potential targets for the pharmacological rescue of p53 cancer mutants. The classic example is the V143A mutant, which is inactive at body temperature but shows transactivation activity at subphysiological temperatures in both yeast and mammalian cell-based assays (Zhang *et al.*, 1994; Di Como and Prives, 1998). Similar temperature-sensitive behavior has been reported for the Y220C mutant (Di Como and Prives, 1998). Systematic functional characterization in yeast and mammalian assays has led to the identification of a large number of temperature-sensitive mutants. Shiraishi *et al.* (2004) have isolated 142 temperature-sensitive p53 mutants from a comprehensive missense-mutation library that show transactivation activity at 30°C. This set of temperature-sensitive mutants comprises about 10% of all cancer-related single amino-acid changes in the core domain. The majority of the mutations are clustered in the β -sandwich region of the protein and are predominantly hydrophobic large-to-small substitutions. A more recent systematic study shows that the population of mutants that exhibit temperature-sensitive behavior depends on the actual assay temperature at which the selection for such a phenotype is performed (Dearth *et al.*, 2007). From 76 mutants studied, representing more than 50% of the reported cases of missense mutations in the core domain, five mutants were found to be active at 30°C. When the same assays were performed at 25°C, an additional set of five mutants was found to be active. These observations are consistent with the *in vitro* stability data, showing that different mutants are destabilized to a different extent (Bullock *et al.*, 2000) and hence are expected to have a different threshold temperature (permissive temperature) for the detection of a potentially temperature-sensitive phenotype. The higher the loss of thermodynamic stability, the lower the permissive temperature is expected to be. For example, the temperature-sensitive phenotype of the H168R and R282W mutants, which are destabilized by 3 kcal/mol, was detected at 30°C. By contrast, the temperature-sensitive phenotype of the mutants Y220C and F270C, which are destabilized by more than 4 kcal/mol, was only detected when the DNA-binding assays were performed at 25°C. It is not surprising that mutations that are associated with temperature-sensitive transactivation activity are predominantly located in the

β -sandwich. Generally, internal residues are more important for folding and stability but not necessarily for function. Mutations that perturb the packing of the core of the β -sandwich are more likely to have a highly destabilizing effect than mutations in surface regions. On the other hand, the compact β -sandwich is more capable of quenching the structural effects of mutation, resulting in preservation of a wild-type-like architecture in the DNA-binding surface and restoration of function at lower temperatures. This is exactly what is observed for the structures of T-p53C-V143A, T-p53C-Y220C and T-p53C-F270L, which show mutation-induced formation of an internal cavity or a surface crevice but otherwise perfect conservation of the wild-type conformation (Joerger *et al.*, 2006). Structural mutations in the DNA-binding surface result in temperature-sensitive phenotypes only if the mutation-induced distortion affects just the peripheral region of the interface with DNA, as is the case for H168R and R282W (Joerger *et al.*, 2005, 2006).

The dominant-negative effect

The observation that the transactivation activity of wild-type p53 can be lost in the presence of an inactive mutant with an intact tetramerization domain has been described as the ‘dominant-negative effect’, which often requires overexpression of the p53 mutant (reviewed by Blagosklonny, 2000). Over the years, a number of theories have been put forward to explain this phenomenon, including a conformational, prion-like model, whereby the mutant induces a conformational change in the wild-type. In the light of recent structural and functional data, such a mechanism is not plausible and can be excluded for mutants such as R273H, where the mutation neither affects the folding state nor the structure of the protein, apart from the missing DNA contact. The molecular basis for this effect may be rather simple. Formation of mixed tetramers will effectively reduce the cellular level of fully active wild-type homotetramers, potentially below the levels required for normal transactivation of p53 response elements. Assuming equal expression of both wild-type and mutant alleles, and similar stabilities of the corresponding proteins, the formation of heterotetramers reduces the probability of forming fully active wild-type homotetramers by a factor of 16 if a random assembly of individual chains is assumed (Chan *et al.*, 2004) and by a factor of 2 if stable co-translationally formed homodimers form the basis for heterooligomerization, as suggested by Nicholls *et al.* (2002). This factor increases with increased concentration of the mutant. In a mixed tetramer of wild-type and an inactive mutant, wild-type core domains would still be able to bind to target DNA, but the overall binding energy of the protein–DNA complex would be significantly reduced, because the presence of a non-binding or only weakly binding mutant would result in a loss of binding cooperativity (Nicholls *et al.*, 2002; Weinberg *et al.*, 2004b). So far, there are no quantitative data available on the binding affinity of mixed tetramers. Studies on

R273H and R249S report that at least three mutant molecules are required per tetramer to inactivate the transactivation function (Chan *et al.*, 2004). For individual mutants, this may vary depending on the extent of the structural perturbation. In a recent systematic study, Dearth *et al.* (2007) tested whether cancer mutants interfere with nuclear wild-type function and found a diverse spectrum. About one-third of the tested mutants showed lack of wild-type inhibition at 37°C, despite overexpression and lack of wild-type function of the tested mutants. Interestingly, none of the mutants that exhibit temperature-sensitive DNA binding significantly interferes with the function of wild-type p53. The reported absence of a dominant-negative effect for many cancer mutants highlights the importance of determining whether there is a loss of heterozygosity when assessing the p53 status of human cancer.

Interactions with signaling proteins

The functional consequences of p53 cancer mutations at the cellular level depend on a multitude of factors, because p53 is embedded in a complex regulatory network. Mutations in the core domain not only affect p53's ability to bind DNA but also its interaction with protein binding partners. There is growing evidence that the DNA-binding surface is a rather promiscuous binding site that overlaps with the docking site of a number of regulatory proteins (Friedler *et al.*, 2005b). Since the binding interfaces do overlap in many cases but not match, a particular cancer mutation may have a different effect on the binding to target DNA and the binding to one of these regulatory proteins. The interaction of p53 with the ASPP2 protein is a good example to illustrate the diverse functional consequences of mutation. ASPP2 specifically stimulates the apoptotic function of p53 but not cell cycle arrest (reviewed by Trigianta and Lu (2006). The C-terminal domain of ASPP2, 53BP2 (p53-binding protein 2), binds to the p53 core domain, and the structure of the corresponding complex has been solved by X-ray crystallography (Gorina and Pavletich, 1996). 53BP2 contains an SH3 domain and four ankyrin repeats. The SH3 domain binds the L3 loop of p53, and one of the ankyrin repeats binds the L2 loop. In the p53 core domain–DNA complex, these structural elements of p53 are either anchored to the minor groove of DNA or form the core domain–core domain dimerization interface (Kitayner *et al.*, 2006). The region of the core domain that makes major groove contacts (loop–sheet–helix motif) does not contribute to the interface with 53BP2. These structural observations are consistent with a recent biophysical study showing (i) that binding of the p53 core domain to DNA response elements and 53BP2 is mutually exclusive and (ii) that there are mutations that abrogate only one of the two functions (Tidow *et al.*, 2006). The DNA contact mutation R273H, for instance, which lies just outside the p53–53BP2 interface and does not perturb the surrounding structure, has no effect on the binding of 53BP2. In contrast, no binding of 53BP2 was

detected for the R181E and G245S mutations. Compared with R249S and R273H, these mutations have only a moderate effect on DNA binding, presumably by affecting the core domain–core domain dimerization interface (Dehner *et al.*, 2005; Ang *et al.*, 2006). These results suggest that some mutants may cause cancer by specifically disrupting an important proapoptotic pathway. Similarly, other cancer mutations at the surface of the core domain may specifically disrupt a pathway involving one of the other protein-binding partners.

Reversing the effects of deleterious mutations

Structural basis of rescue by second-site suppressor mutations

Adaptation to deleterious mutations by a compensatory mutation at a different site of the genome is a common occurrence in evolution (Poon *et al.*, 2005). Besides the importance for understanding evolutionary processes that minimize loss of fitness, the analysis of second-site suppressor mutations is also a powerful tool for studying functional interactions within and among proteins (Hartman and Roth, 1973; Poteete *et al.*, 1991; Wray *et al.*, 1999; Sujatha *et al.*, 2001). In the case of p53, studies on second-site suppressor mutations are particularly interesting, because they provide important clues as to whether activity can be restored to common cancer mutants, which has far-reaching consequences for the development of therapeutic anticancer strategies. By using a genetic approach in yeast and mammalian cells, second-site suppressor mutations that restore activity to many oncogenic mutants have been identified (Brachmann *et al.*, 1998; Baroni *et al.*, 2004). Some of these mutations are specific, whereas others rescue a whole subset of cancer mutants. The global suppressor mutations N239Y and N268D increase the thermodynamic stability of the core domain by about 1.5 kcal/mol each and thus directly compensate for the stability loss induced by the oncogenic mutations (Nikolova *et al.*, 2000; Joerger *et al.*, 2004). Accordingly, such mutations can rescue a whole subset of different cancer mutants. Mutants with distinct structural changes in functionally important regions require specific suppressor mutations, which may be deleterious when found in isolation. The R249S hotspot mutant, for instance, is only rescued in combinations that include the H168R mutation. Individually, both mutations are cancerous, because they substantially destabilize the protein and induce specific structural changes in or close to the DNA-binding surface (Nikolova *et al.*, 2000; Joerger *et al.*, 2005). Yet in combination, Arg-168 mimics the structural role of Arg-249 in the wild-type (Figure 7). Its guanidinium group stabilizes the hairpin conformation of the L3 loop that is essential for positioning the DNA-contact Arg-248 and forms an integral part of the core domain–core domain interface upon DNA-binding, concomitant with restoration of DNA-binding to wild-type levels (Joerger *et al.*, 2005). For the DNA-contact mutants R273H and R273C, the

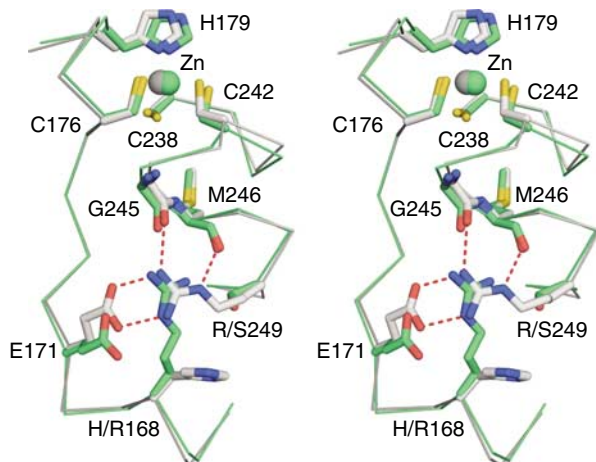


Figure 7 Rescue of mutant R249S by a second-site suppressor mutation. Structure of *T*-p53C-T123A/H168R/R249S (PDB ID code 2BIQ; green) (Joerger *et al.*, 2005) superimposed on the wild-type (PDB ID code 1TSR, molecule A; gray) (Cho *et al.*, 1994). Arg-168 acts as a specific second-site suppressor mutation by mimicking the structural role of Arg-249 in the wild-type. The figure was generated using PyMOL (DeLano, 2002).

second-site suppressor mutations S240R (Baroni *et al.*, 2004) and T284R (Wieczorek *et al.*, 1996) have been reported. Both mutations introduce an arginine close to residue 273 that potentially creates a novel DNA contact. Interestingly, no intragenic suppressors for oncogenic mutations that perturb the zinc-binding site (e.g. R175H, C176F, H179R) have been identified so far.

p53 mutants as drug targets

Many novel therapeutic strategies aim at rescuing the function of p53 by small molecule compounds. In recent years, several p53-activating compounds have been identified in protein- and cell-based screening assays (reviewed by Bykov *et al.*, 2003 and Wiman, 2006). Most of these compounds, however, do not directly interact with p53 but function via different mechanisms, some of which are still a matter of debate. Rational structure-based drug design that directly targets p53 is still in its infancy. The underlying principle for a direct pharmacological rescue of p53 cancer mutants follows simple thermodynamic considerations. Any compound that binds to the folded state but not the denatured state will stabilize the protein by shifting the folding-unfolding equilibrium to the folded state and may hence rescue the function of destabilized mutants. The low kinetic stability of p53, and in particular of destabilized mutants, further requires that such a compound acts immediately upon biosynthesis of the protein and acts as a chemical chaperone.

Studies on the CDB3 peptide provided a proof of principle that pharmacological rescue of p53 mutants by small molecule drugs is a feasible strategy. CDB3, a 9-residue peptide derived from one of the p53-binding loops of 53BP2, and its fluorescein-labelled form FL-CDB3 bind to the p53 core domain, albeit with a strong

electrostatic component, and raise the melting temperature of the wild-type and conformationally destabilized mutants (Friedler *et al.*, 2002). The CDB3-binding site on the surface of the p53 core domain has been mapped using NMR techniques. It partly overlaps with the DNA-binding region but, interestingly, differs from that of the corresponding loop in the p53–53BP2 complex. CDB3 not only raises the melting temperature of p53 and its mutants but also increases the half-life of kinetically unstable mutants *in vitro* (Friedler *et al.*, 2003) and was found to induce upregulation of wild-type p53 and oncogenic mutants in human cell lines (Issaeva *et al.*, 2003; Friedler *et al.*, 2002). An optimized drug for therapeutic purposes would have to be on a non-peptide basis with improved physicochemical and pharmacokinetic properties to allow for sufficient bioavailability.

The availability of high-resolution structural data on p53 mutants, combined with data on the energetic and functional consequences of mutation, has opened novel avenues for the design of small molecule drugs to reactivate p53 mutants. This knowledge is fundamental for assessing the most suitable rescue strategy for each individual mutant. Since p53 mutants have distinct structural and functional characteristics, different rescue strategies can be applied, which can also include drugs that specifically target the folding state of a particular mutant. The ideal candidates for generic small molecule drugs are mutants, such as the β -sandwich mutants V143A and F270L, that are destabilized while retaining the structural features of the wild-type in important functional regions, be it the DNA-binding surface, surfaces involved in domain-domain interactions or surfaces that form the docking sites for signaling proteins. For many β -sandwich mutants and other mutants that show a temperature-sensitive phenotype, restoration of wild-type-like activity could be achieved by a generic stabilizing drug. For the G245S and R249S hotspot mutations in the L3 loop, simple stabilization by a small molecule compound may only result in partial rescue, if any, because of the distinct structural changes in the L3 loop, even in the stabilized *T*-p53C variant. It is difficult to see how DNA-contact mutants, such as R273H and R248Q, can be rescued by generic small molecule drugs. An effective drug would have to create a novel DNA contact that directly compensates for the lost DNA-contact residue, which is without doubt a much greater design challenge. Similarly, rescue of structural mutations that directly perturb the zinc region appears to be equally challenging, as indicated by the lack of intragenic suppressors for these mutations (Baroni *et al.*, 2004). The hotspot mutant Y220C falls into a category of mutants that is a particularly attractive target for structure-based drug design. Its temperature-sensitive behavior makes it a good candidate for a generic small molecule drug, and the mutation-induced crevice could also be specifically targeted. This crevice has its deepest point at the mutation site, Cys-220 (Figure 5a), thus providing a binding pocket for a small molecule drug with a moiety that selectively targets mutant Y220C (Joerger *et al.*, 2006). Such a drug would allow specific targeting of the

mutant without binding to the wild-type protein in a heterozygous scenario. The frequency of this mutation in human cancer is high enough to make the development of a mutant-specific drug an attractive proposition. Currently, the Y220C mutation accounts for about 75 000 new cancer cases worldwide per year (www-p53.iarc.fr). On the basis of the latest predictions on the increase of world cancer rates by the World Health Organization, it can be estimated that this number will increase to more than 100 000 new cases per year by 2020.

Future prospects

The emerging structural and systematic functional information on p53 mutants has in a way dispelled the common misconception that a uniform population of mutant p53 exists. In fact, quite the opposite is true. Common p53 cancer mutations induce distinct energetic and structural responses that directly translate into a whole spectrum of distinct phenotypes. The structural data may be exploited for cancer therapy purposes to rationally design both generic and mutant-specific drugs that rescue the function of p53 mutants. Such development will have to go hand in hand with the development and implementation of standard diagnostic tests that accurately determine the p53 status of cancer patients. Several computational approaches have been reported

to predict the structural and functional impact of oncogenic p53 mutations (Cuff *et al.*, 2006; Yip *et al.*, 2006; Mathe *et al.*, 2006a,b), and in some of these studies the predictions have been validated by experimentally determined transactivation activities. In the future, improved algorithms using both experimental functional data and structural data on p53 mutants may result in even more accurate predictions at the level of individual mutations. A thorough understanding of the effects of mutation is inextricably linked to the understanding of the structural and dynamic features of the wild-type protein. One of the most challenging tasks is undoubtedly to elucidate the structure of the full-length protein, the domain motions involved upon DNA-binding or binding of regulatory proteins and the precise role of the many post-translational modifications. System genomics projects may eventually lead to a more quantitative description of the p53 pathway and help to assemble the individual pieces of the jigsaw. The scope of the structure–function relationships of p53 and its mutants extends beyond the p53 field and may serve as a paradigm for other disease-related proteins and proteins at the center of highly connected networks.

Abbreviations

T-p53C, p53 core domain containing the four point mutations M133L, V203A, N239Y and N268D.

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