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REVIEW ARTICLE

Strategies for manipulating the p53 pathway in the treatment of human cancer

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Human cancer progression is driven in part by the mutation of oncogenes and tumour-suppressor genes which, under selective environmental pressures, give rise to evolving populations of biochemically altered cells with enhanced tumorigenic and metastatic potential. Given that human cancers are biologically and pathologically quite distinct, it has been quite surprising that a common event, perturbation of the p53 pathway, occurs in most if not all types of human cancers. The central role of p53 as a tumour-suppressor protein has fuelled interest in defining its mechanism of function and regulation, determining how its inactivation facilitates cancer progression, and exploring the possibility of restoring p53 function for therapeutic benefit. This review will highlight the key biochemical properties of p53 protein that affect its tumour-suppressor function and the experimental strategies that have been developed for the reactivation of the p53 pathway in cancers.

Key words: cell-cycle checkpoint pathway, degradation, DNA damage, transcription.

INTRODUCTION

Mutational inactivation of the p53 gene product is one of the most common genetic events that occur in human cancers, highlighting the central role of p53 as a tumour suppressor. p53's function as a tumour suppressor is linked to its role as a co-ordinator of a damage-induced cell-cycle checkpoint pathway whose action prevents the propagation of permanently damaged clones by the induction of apoptosis or growth arrest in the perturbed cell [1]. The biochemical activity of p53 most closely associated with tumour suppression is its function as a sequence-specific DNA-binding protein and transcription factor that controls the expression of a large panel of gene products implicated in normal growth control, DNA repair, cell-cycle arrest, engagement of apoptosis, angiogenesis, redox regulation, metastasis, nitric oxide production, and protein degradation [2,3]. These general biological and cellular properties of p53 have been discussed extensively, and we would suggest the reader refers to a collection of reviews published recently on this subject [4,5]. The present review will centre on biochemical research in the p53 field, summarizing some key concepts that have emerged regarding the post-translational regulation of the p53 protein-degradation pathway and the p53-dependent transactivation pathway, with a focus on the experimental strategies that have been developed to successfully manipulate the biochemical function of the p53 pathway in cells. These recent successes have justified the hope of basic scientific research to enhance our understanding of the molecular basis of human cancer and to raise the realistic possibility of targeting the p53 pathway in cancers for therapeutic benefit.

THE TRANSACTIVATION FUNCTION OF p53 PROTEIN

The functional and regulatory domains of p53

Following the discovery of p53 as a transformation-associated protein in 1979 [6,7], approximately 10 years elapsed before it was defined unambiguously as a tumour suppressor [8,9]. These conclusions were based on studies demonstrating that: (a) inactivating mutation and/or deletion of the p53 gene is a common event in distinct types of human cancers [10,11]; (b) wild-type p53 protein is a transcription factor, and mutant forms of p53 protein are defective in this biochemical function [12–14]; and (c) the many gene products induced by wild-type p53 mediate its tumour-suppressor function by actually playing a direct role in modulating growth arrest, apoptosis, membrane signalling, protein degradation or oxidative stress (Figure 1) [2,3,15].

The transcriptional activity of p53 is induced by stresses thought to be important as tumour-suppressing signals in developing cancers, including chemical oxidants, nucleotide-pool perturbation, low extracellular pH, hypoxia and thermal stress. Additionally, common anticancer agents that similarly injure cells, including adriamycin, 5-fluorouracil, ionizing radiation and etoposide, can induce p53 function, indicating that this tumour-suppressor pathway can respond to physiological and exogenous tumour-suppressing agents. Despite the fact that many distinct damaging agents and cell types share similar features in regulating p53 function, no single cell type or damaging agent can be used to generalize all known components of the p53 pathway. Comprehensive gene profiling using the oligonucleotide microarrays clearly demonstrated the global

Abbreviations used: CBP, CREB (cAMP-response-element-binding protein)-binding protein; ATM, ataxia telangiectasia mutated protein; p300, the transcription adaptor protein required to drive p53-dependent gene expression; JMY, p300 junction-mediating regulatory factor; HPV, human papillomavirus; MDM2, murine double minute clone 2 oncoprotein; ATR, ataxia telangiectasia-related; DNA-PK, DNA-dependent protein kinase; CHK2, checkpoint kinase 2; BRCA1, breast cancer susceptibility gene-1; PKR, double-stranded-RNA-dependent protein kinase; JNK, c-Jun N-terminal kinase; HSP, heat-shock protein; CDK, cyclin-dependent kinase; PCNA, proliferating-cell nuclear antigen; BH, bcl-2 homology domain; SUMO, is small ubiquitin-related modifier-1; pRb, retinoblastoma protein; ARF, alternative reading frame protein.

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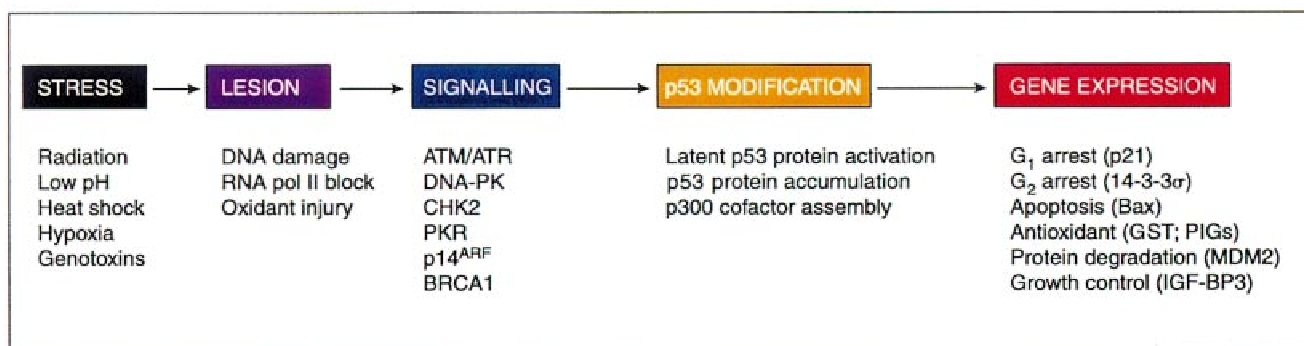

Figure 1 Stages in the activation of p53-dependent transcription

p53-dependent transcription from its target promoters can be stimulated by cellular exposure to a variety of stimuli (black), including radiation and genotoxins [116–118], low extracellular pH [231], thermal stress [232,233] nucleotide-pool perturbation [234] and hypoxia [235]. The 'lesions' (purple) induced by exposure to these agents include DNA damage such as strand breaks thymine dimers and excision-repair products [117,119–121], inhibition of RNA polymerase (pol) II-dependent transcription [49,50,139,141] and chemical-oxidant stress or redox imbalance [127–133]. Epigenetic studies have defined upstream signal-transduction pathways (blue) that mediate by direct or indirect means, p53-dependent transcription and include ATM/ATR [41,42,236], DNA-PK [44], CHK2 [110,111,238,239], PKR [240,241], p14^{ARF} [95–102] and BRCA1 [242–244]. Regulation (orange) of p53 protein's transactivation function and degradation pathways by these upstream lesions and signalling events are the focus of the present review and are summarized in the text. Prototypical gene products induced by p53 protein [15] can affect G_1 growth arrest [190], G_2 growth arrest [245,246], apoptosis [220,221], the antioxidant response [2,247], protein degradation [79–81,167] and growth-factor signalling [249,250] (red). Further abbreviations used: GST, glutathione S-transferase; PIGs, p53-inducible genes; IGF-BP3, insulin-like growth factor binding protein 3.

differences and similarities in the gene products induced or repressed by p53 protein in different cell types under different conditions [15]. Independent studies using a panel of isogenic colorectal-cancer cell lines with and without the p53 gene or the p21^{WAF1} gene demonstrated that there is a pronounced heterogeneity in the magnitude and kinetics in the expression of p53-dependent gene products which depend upon the damaging agent and magnitude of p53 protein induction [2,3]. Most striking was the observation using the same colorectal-cancer cell type that 5-fluorouracil-induced apoptosis requires wild-type p53 but not its effector protein p21^{WAF1}, whereas adriamycin-dependent apoptosis could be inhibited by the p53–p21^{WAF1} axis [17]. Such observations highlight the utility for p53-gene profiling as a form of cancer diagnosis and genotyping to define the best therapeutic treatment for each patient. The cell specificity of p53 protein induction and apoptosis has similarly been highlighted by examining the response of normal murine gut cells *in vivo* to ionizing radiation and the antimetabolite 5-fluorouracil [16,19]. In the small intestine, p53-dependent apoptosis induced by ionizing radiation occurred in the crypt at the position containing stem cells, whereas 5-fluorouracil-induced apoptosis occurred in the transit cells. In the colon, 5-fluorouracil-dependent apoptosis occurred at the base of the crypt, while these same cells resisted ionizing-radiation-induced death. Although such studies do not necessarily assist in defining strategies for treating colorectal cancer cells with either 5-fluorouracil or ionizing radiation, they do provide clues into the common problem of normal gut cell toxicity when administrating these anti-cancer agents.

This cell specificity in the regulation of p53 protein activity as a transcription factor and tumour suppressor will depend upon numerous damage-responsive factors that post-translationally modify p53 in a cell-specific manner (reviewed below). For example, the most comprehensive study to date which addresses the post-translational modification of p53 protein at five distinct phosphorylation sites in response to various stimuli has demonstrated that no condition affects all five protein kinase signalling pathways identically in normal human fibroblasts [18]. This latter work has shown that quiescence, senescence and chemically damaged cells induce strikingly different changes (increases and decreases) in steady-state phosphorylation within the N-terminal and C-terminal regulatory domains of p53, although one common phosphorylation event at the AT-superfamily kinase site was stimulated by all treatments. Together, these studies highlight the cell specificity and damage specificity in p53 regulation and the need in future to develop physiological models to define p53 function accurately as a tumour suppressor in each type of cancer [20,21].

The stress-regulated transactivation function of p53 is driven by its sequence-specific DNA-binding domain and is co-ordinated by specific protein–protein interactions that can in turn be modulated by covalent and non-covalent modifications [22]. p53 protein is composed of at least four functional and regulatory domains that modulate its activity as a stress-induced sequence-specific DNA-binding protein and transcription factor: (I) the central sequence-specific DNA-binding domain drives specific promoter recognition and is the domain containing most of the p53 mutations found in human cancers; (II) the oligomerization domain which ensures assembly of p53 into conformationally active tetramers; and (III and IV) the N-terminal and C-terminal regulatory domains containing heterologous protein docking sites and phosphorylation, SUMOlation or acetylation sites implicated in the modulation of p53 protein–protein interactions (Figure 2) (SUMO is small ubiquitin-related modifier-1).

The central core domain of p53 from amino acids 90 to 295 contains the sequence-specific DNA-binding domain which is highly conserved amongst vertebrates and in two recently identified human homologues: p73 and p63^{KET} [23,24]. The consensus DNA-binding site contains two copies of the 10 bp motif 5′-PuPuPuCWWGPyPyPy-3′, which can be separated from 0 to 13 bp [14] (Pu is purine base, Py is pyrimidine base and W means A or T). Although the p53 homologues p73 and p63 can drive gene expression from promoters similar to that bound by p53, neither of these two homologues have been found to be highly mutated in human cancers, nor is p73 bound to viral oncoproteins that neutralize p53 protein activity, so their function in regulating p53-dependent cancer progression is unclear. The DNA-binding domain of p53 is organized into a β-sandwich that forms a scaffold for a loop–sheet–helix motif and two loops that co-

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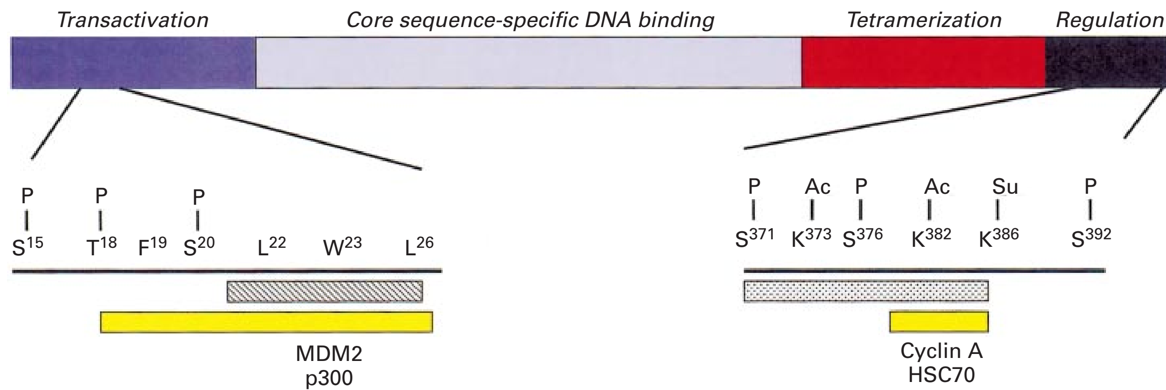

Figure 2 Functional and regulatory domains of p53: key concepts derived from studies on p53-dependent transactivation

Although a more comprehensive review of the p53-binding proteins can be found in other reviews [4,5] listed in this Figure are some key interaction sites defined by structure–function analyses that have affects on the biochemical activity and conformation of p53. p53 protein can be divided into at least four distinct domains that modulate its sequence-specific DNA-binding and transcriptional activation function: (1) the N-terminal transactivation domain (purple box) containing the binding sites (yellow box) for the modulators including p300 and CBP [33–35], TAFs (transactivating factors) [61–63] and MDM2 [83–86], as well as regulatory phosphorylation sites within this protein–protein interaction domain at Ser^{15}, Thr^{18} and Ser^{20} [18,41,42,108–111,113,238]; (2) the core sequence-specific DNA-binding domain (lilac box) containing most of the p53 mutations found in human cancers [251,252]; (3) the tetramerization domain (red box) that assembles the core domain into a fully active DNA-binding protein [32,253]; and (4) a negative regulatory domain (black box) containing an HSC70 (heat-shock cognate 70 stress protein)-binding site [148,254] and a cyclin A docking site (yellow bar) [57], as well as multiple sites of covalent modifications, including phosphorylation (P–) at Ser^{371}, Ser^{376} and Ser^{392} [54,124,255], acetylation (Ac–) at Lys^{373} and Lys^{382} [58,59] and 'SUMOlation' at Lys^{386} [48,60]. The common monoclonal antibodies towards p53 map to the indicated positions and include (i) DO-1 (hatched bar; DO-7), which overlaps the MDM2/p300-binding site [256] and (ii) PAb421 (stippled bar) which overlaps the binding site for cyclin A, HSC70 and protein kinases that phosphorylate at the indicated serine residues [256]. Phospho-specific monoclonal antibodies directed towards the Ser^{15}, Thr^{18}, Ser^{20}, Ser^{315} and Ser^{392} phospho-epitopes have been reviewed previously [257]. Phospho-specific and acetyl-specific polyclonal sera towards epitopes at Lys^{320}, Lys^{373}, Lys^{382}, Ser^{378} and Ser^{376} have been reviewed previously [258]. Amino acids are shown using the one-letter notation.

ordinate a zinc ion [25]. Inactivating mutations in p53 at over 200 different amino acid positions within this core DNA-binding domain have been detected in human cancers, and the codon changes in the p53 gene often provide the fingerprint expected from the environmental factor often associated with cancer development [26]. Biochemical and biophysical characterization of these mutant forms of p53 protein have suggested the existence of at least three distinct classes of mutants with unique biochemical defects in tetramerization, conformational regulation and intrinsic folding and stability (Figure 3).

An oligomerization domain from amino acids 320–356 flanks the conserved core sequence-specific DNA-binding domain and consists of a monomeric core which is organized into a dimer via an antiparallel β-sheet and an anti-parallel helix/helix interface. The dimers associate across a hydrophobic interfaces to form a four-helix bundle arranged orthogonally into tetramers. The need for a tetramerization domain in modulating p53 activity is at first glance unclear, since replacement of this domain with a coiled-coil domain of GCN4 (positive general control of transcription-4) is sufficient to confer transcriptional activity [27]. However, mis-sense mutation of p53 within the tetramerization domain can be detected in human cancers, and these mutations promote a conversion from a tetrameric into a dimeric or monomeric form with reduced specific activity as a DNA-binding protein [28–32]. These data suggest that tetramerization can be important in modulating p53's tumour-suppressor function *in vivo*, at least in certain genetic backgrounds or under select environmental pressures.

The N-terminal regulatory domain of p53 contains the transactivation domain through which p53 interacts with components of the transcriptional machinery, and a smaller, highly conserved, BOX-I domain which directs the binding of p53 to proteins, including p300 (the transcription adaptor protein required to drive p53-dependent gene expression) and $TAF_{II}31$ or $TAF_{II}70$ (Figure 2). p53 protein interaction with the transcriptional co-activator p300 or CBP (CREB-binding protein) can drive both p53-dependent gene expression and apoptosis [33–35] and can promote p53 protein accumulation [36]. The regulation of this protein–protein interaction may in turn be modulated by either phosphorylation within the BOX-I domain or protein–protein interactions that can compete with p300 docking [37]. In addition, p300 or CBP binding to p53 in the N-terminus and subsequent acetylation of the C-terminal domain of p53 can be stimulated by Ser^{15} phosphorylation within the p300 docking site [38]. Consistent with these data, mutation of full-length p53 protein at multiple sites including Ser^{15} can reduce its specific activity as a transcription factor *in vivo* [39,40]. Enhanced phosphorylation of endogenous p53 protein at Ser^{15} following DNA damage, quiescence or senescence [18] can occur through the action of an ATM/ATR/DNA-PK kinase-dependent pathway [41–44] (where ATM is ataxia telangiectasia mutated protein, ATR is ataxia telangiectasia-related protein kinase and DNA-PK is DNA-dependent protein kinase). These studies identified a key signal-transduction cascade that could stimulate p53-dependent transcription via modification of the N-terminal domain of p53.

Flanking the tetramerization domain of p53 in the extreme C-terminus is a negative regulatory domain whose post-translational modification (Figure 2) may play an important role in modulating the specific activity of p53 *in vivo*. One function for this regulatory C-terminal domain is to maintain p53 protein in a latent state for specific DNA binding. Deletion of this domain or stoichiometric phosphorylation at Ser^{392} [by casein kinase 2 and double-stranded-RNA-dependent protein kinase (PKR)] activates the latent specific DNA-binding function of p53 *in vitro* by an allosteric mechanism [45]. Although initial mutagenesis-coupled transient-transfection assays have failed to show that phosphorylation at Ser^{392} can modulate p53 function as a transcription factor *in vivo*, more sophisticated studies have shown that Ser^{392} phosphorylation may facilitate the stabilization of the tetramerization domain [46] and that phosphorylation at the equivalent site on murine p53 may regulate the specific activity of p53 as a transcription factor *in vivo* [47]. Similarly,

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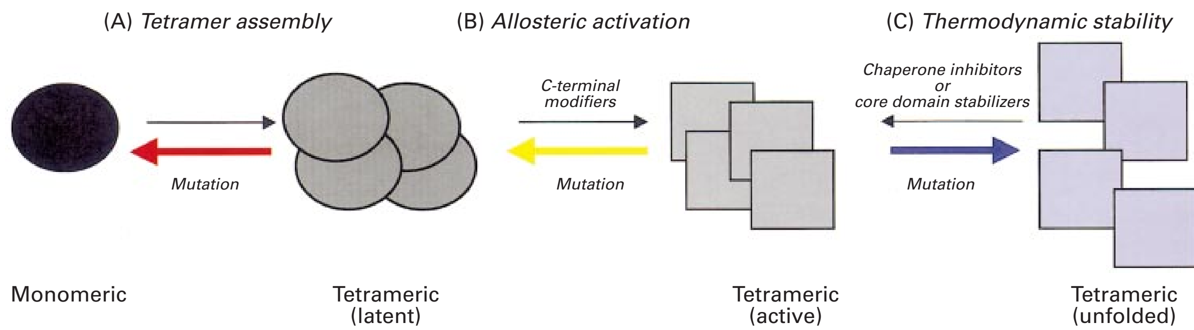

Figure 3 Biochemical defects in mutant p53 protein folding pathways

Naturally occurring tumour-derived p53 mutants can be divided into at least three distinct classes that affect tetramer assembly, allosteric activation and intrinsic thermodynamic stability. (**A**) Tetramer assembly. The tetramerization class of mutant p53 protein contains point mutations within the oligomerization domain (amino acids 320–356) that stabilize the dimeric or monomeric conformers of p53 [30,31]. The red arrow represents the shift in equilibrium promoted by mutation from the latent folded tetramer (dark-grey ellipses) to the unfolded monomeric protein (black ellipse). (**B**) Allosteric activation. The allosteric class of mutant protein with a thermodynamic stability similar to wild-type p53 cannot be activated from the latent state after phosphorylation at Ser^{392} or Ser^{315}, but maintains the capability to be activated for DNA-binding by C-terminal modifiers [146]. The yellow arrow represents the shift in equilibrium promoted by mutation from the folded active tetramer (light-grey squares) to the folded latent tetramer (dark-grey ellipses). The allosteric mutant class of p53 can be activated *in vivo* by agents that target the C-terminal negative regulatory domain [52,53,261,262]. (**C**) Thermodynamic stability. A thermodynamically unstable class of mutant protein has a relatively high degree of intrinsic instability with respect to *cis*-acting protein-folding pathways [143] and can be further subdivided into distinct thermodynamically defined groups [144]. The purple arrow represents the shift in equilibrium promoted by mutation from the folded active tetramer (light-grey squares) to the unfolded inactive tetramer (lilac squares). The thermodynamically unstable class of mutant p53 can be stabilized *in vivo* by compounds that prevent unfolding of the native tetramer, including conformation stabilizers [174] and molecular-chaperone inhibitors [184,185].

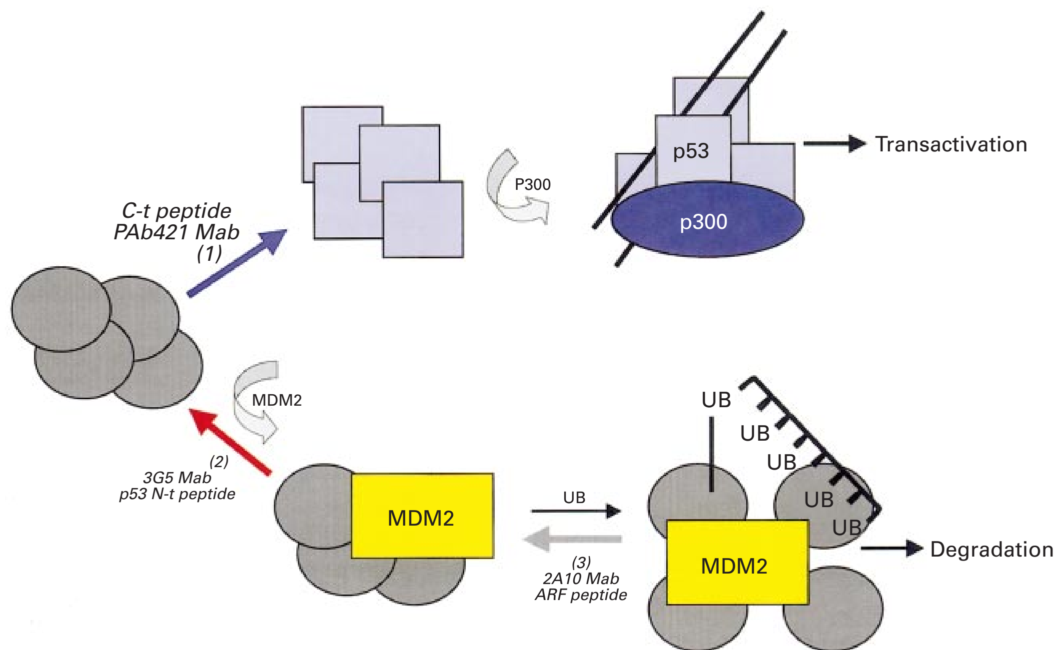

Figure 4 Three rate-limiting steps in the regulation of p53 protein function in normal cells

p53 protein assembles into native tetramers (dark-grey ellipses) [54,259,260] whose function can be stimulated in normal cells by agents that stimulate DNA-binding by p53 or that block degradation by MDM2. (i) Stimulating p53 DNA binding. One class of activating agents (*1*) including a C-terminal peptide (*C-t peptide*) or a monoclonal antibody (*PAb421 MAb*), promotes the conversion of p53 tetramers from a latent (dark-grey ellipses) into an activated (lilac squares) state by neutralizing its C-terminal negative regulatory domain. This increases the specific activity of p53 as a sequence-specific DNA-binding protein and stimulates p53-dependent transcription and apoptosis [45,51,53,150,261,262]. (ii) Inhibiting MDM2 binding to p53. The native tetrameric conformation of p53 (dark-grey ellipses) is important for high-affinity binding by MDM2 protein (yellow) [78,87]. The effects of MDM2 on p53 function can be dissected first into an MDM2-binding event in the N-terminal BOX-I domain of p53 (see Figure 2), which is dependent upon the N-terminal domain of MDM2. This initial docking event can reduce p53 activity by blocking directly p53 protein interactions with transcriptional components. A second class of activating agents (*2*), including a p53-derived BOX-I peptide or a monoclonal antibody that binds to MDM2 protein at the MDM2/p53 interface (*3G5 MAb*) prevents MDM2 binding to p53 and liberates p53 to function as a transcription factor [89,90,92]. (iii) Blocking MDM2 ubiquitination of p53. Following the initial docking of MDM2 in the N-terminus (*N-t*) of p53, a second reaction can occur in which MDM2 can catalyse the polyubiquitination of the C-terminal domain of p53, which is in turn dependent upon the C-terminal domain of MDM2. A third class of activating agent (*3*), including a $p14^{ARF}$-derived peptide or a monoclonal antibody that binds to MDM2 protein at the MDM2/$p14^{ARF}$ interface (*2A10 MAb*) prevents MDM2-dependent polyubiquitination of p53 and liberates p53 from the negative regulation imposed by MDM2 [94]. These models have provided a rationale for identifying the signalling events and enzymes in normal cells that stimulate C-terminal modification of p53, that regulate MDM2–p53 protein interactions and that regulate MDM2–$p14^{ARF}$ interactions. Additionally, these activating agents serve as prototypes for drug-discovery programmes aimed at activating p53 in cancers with a wild-type p53 pathway or with activatable mutant forms of p53. Abbreviations: *MAb*, monoclonal antibody; UB, ubiquitin.

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directed mutation at a single amino acid near the Ser^{392} site (at Lys^{386}) can increase the specific activity of p53 *in vivo*, presumably by neutralization of its negative-regulatory motif [48]. Increased phosphorylation at Ser^{392} of endogenous human p53 protein in cells occurs following UV-C and X-irradiation damage, with enhanced steady-state phosphorylation correlating with enhanced p53-dependent transcription [49,50]. In addition, microinjection or intracellular synthesis of an antibody (PAb421) that binds to the C-terminal negative regulatory domain near the phosphorylation sites (Figures 2 and 4) can activate p53-dependent gene expression *in vivo* [45,51–53], suggesting that C-terminal modification can be a rate-limiting step in stimulating p53 function *in vivo* (Figure 4).

Together these data provide the basis for the paradigm that two distinct signalling pathways modulate the specific activity of p53 by a concerted mechanism: (i) the N-terminal ATM kinase-dependent pathway targeting Ser^{15} (and now other BOX-I phosphorylation sites; see Figure 2) stimulates p53 protein–protein interaction with components of the transcriptional machinery such as p300 and (ii) the C-terminal Ser^{392}-kinase pathway (and now other C-terminal modifications; see Figure 2) stimulates the latent specific DNA-binding function of the p53 by conformational alterations in the tetramer [45,54] and/or by increasing the K_d for non-specific DNA binding [55,56]. Future research using more sophisticated mutagenesis-coupled gene-expression assays *in vivo* and assays for determining the stoichiometry of these multiple post-translational modification events may identify promoters whose ctivation by certain damaging agents can be regulated by specific p53 isoforms, thereby defining how this cluster of covalent adducts modulates p53 function.

Transcriptional partner proteins of p53

The first transcription-dependent protein–protein interaction defined for p53 involved its ability to interact and function with $hTAF_{II}31$ and $hTAF_{II}70$ [61,62]. p53 protein has a series of acidic residues in its transactivation domain, suggesting that it may be a member of the acidic class of activators, including the prototypical VP16. The binding of $hTAF_{II}31$ to the transactivation domain containing the core sequence Phe^{19}-Ser^{20}-Asp^{21}-Leu^{22}-Trp^{23} induces a conformational change within p53 into that of a small α-helix [63]. As transcriptional activation domains do not necessarily share significant homology at the primary amino acid level, these data support the notion, in combination with the structure of other transcription factors, that protein–protein interactions at a eukaryotic promoter are mediated through small amphipathic helical motifs with short regions of structural homology. The most well characterized protein–protein interaction driving p53-dependent gene expression is the p300/CBP–p53 interaction [33–35]. The interaction with the transactivating cofactor p300 may be particularly important, as mutant forms of p53 are also defective in binding to p300, and therefore protein–protein interactions may also be compromised by naturally occurring point mutations in the p53 protein. It seems evident with hindsight that reduced interaction of mutant p53 with transcriptional proteins, including p300 and another p53-binding protein, BP2 [64,65], as well as consensus-site DNA, will be selected for in cancers and play a role in compromising the tumour-suppressor function of p53.

The p300/CBP family of transcriptional co-activators comprises a group of proteins that facilitate gene expression by a wide range of sequence-specific DNA-binding proteins. The function of p300 as an adapter protein is regulated by its interaction with other regulatory proteins, including P/CAF p300/CBP-associated factor), SRC1 (steroid receptor co-activator) and ACTR (activator of thyroid and retinoic acid receptor), which facilitate relatively unique protein–protein interactions that define promoter-specific and cell-specific gene expression [66–68]. The intrinsic acetyltransferase activity associated with the p300/CBP family of proteins [69,70], which is implicated in acetylation of transcriptional components and regulation of transactivation, is also thought to play a role in stimulating the *in vitro* sequence-specific DNA-binding function of p53 [58]. Phosphorylation of p53 at Ser^{15} in the N-terminal domain can further stimulate *in vitro* acetylation in the C-terminus by the p300 or CBP family of proteins, suggesting a link between an N-terminal kinase signalling network and a mechanism for regulating both transactivation and sequence-specific DNA binding of p53 protein. Elevated steady-state acetylation of p53 protein can be detected following DNA damage [59,71], consistent with the concept that covalent modification of the C-terminal domain will play a role in stimulating p53 activity *in vivo*. The sites of acetylation can be mapped to two distinct motifs within the C-terminal regulatory domain: (I) at Lys^{373} and Lys^{382} [59,72], near the cyclin A box from amino acids 381–385 [57] and near the SUMO site at Lys^{386} [48,60]; and (II) at Lys^{320} [59,72] near the cyclin-dependent kinase (CDK) 2/cell-division cycle 2 kinase (CDC2) phosphorylation site at Ser^{315}.

Obviously, the architecture at a promoter will build upon this core p53–p300 protein interaction, and an intriguing cofactor of p300 recently identified and named JMY (for p300 junction-mediating regulatory factor) was cloned by virtue of its interaction with a truncated p300 protein using yeast two-hybrid system [73]. JMY shows little homology with known proteins, with features including two distinct p300-binding domains, a motif resembling the adenovirus E1A protein (conserved region 2), a proline-rich domain and a cluster of potential CDK phosphorylation sites. Reconstitution using purified proteins established that p300 and JMY complexes are due to direct interactions, validating the interaction defined by the two-hybrid system and by co-immunoprecipitation from cell lysates. Attempts to link JMY function to the p53 response revealed that JMY can stimulate p53-dependent gene expression from a *bax* promoter without changes in p53 protein levels, suggesting that JMY stimulation of p53 is due to transcriptional co-activation with p300 and not p53 protein accumulation. An interesting feature of the function of JMY is its ability to induce Bax protein, but not $p21^{WAF1}$, in a p53-dependent manner. In addition, full-length JMY promotes significant increases in p53-dependent *bax* gene expression and apoptosis, while the alternatively spliced form of JMY does not affect *bax* expression and induces a p53-dependent growth arrest. These data identify transcriptional cofactors as a class of proteins that can play a role in regulating promoter occupation by p53, thereby controlling its tumour-suppressor function.

THE DEGRADATION OF p53 PROTEIN

Murine double minute clone 2 oncoprotein (MDM2)-dependent ubiquitination is a key negative regulatory stage in controlling p53 function

One of the first molecular studies on p53 had shown that the protein has a very short-half life in cultured cells and that it accumulates after cellular exposure to DNA-damaging agents [74]. The 'stabilized' or accumulated form of p53 is now known to be active as a transcription factor, and much effort this past decade has since been centred on understanding how p53 protein is normally degraded or accumulated. Independent studies demonstrating the ability of the high-risk human papillomavirus

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(HPV) E6 protein and cellular E3-ubiquitin adapter protein to promote the degradation of p53 had underscored the role that protein-degradation pathways can play in controlling p53 protein function [75,76].

Since the discovery that other viral oncogenes, like the simian-virus-40 (SV40) T-antigen and adenovirus E1B protein can interact with and inactivate p53, identification of the cellular protein(s) whose function these oncogenes presumably mimicked had been an elusive goal in the attempt to understand the function of p53. MDM2 protein was discovered by virtue of its ability to bind to, and co-purify with, p53, and was the first cellular protein identified that could directly inactive p53 function as a transcription factor [77]. Although MDM2 protein does not share structural features with the SV40 T-antigen or HPV E6 protein, nor are the interaction sites identical, MDM2 protein appears to be a functional composite of the two viral oncogenes in that it can either inactive the transcription function of p53 by binding preferentially to the native, tetrameric form of p53 protein [78,87] and it can catalyse the ubiquitination and degradation of p53 [79–82].

p53 contains a primary docking-site for MDM2 in the N-terminal domain overlapping the p300- and TAF-binding sites [83–86], while a secondary interaction site for MDM2 in the central domain of p53 can be stabilized by the binding of MDM2 to polyRNA [87]. Localization of the primary MDM2 docking site on p53 protein in the N-terminal domain was defined using small peptide mimetics, crystallographic analysis, and mutational studies (Figure 2). These studies revealed that a highly conserved hydrophobic series of amino acids within the N-terminal region of p53 interact with a hydrophobic binding pocket in the N-terminal domain of MDM2 protein [83,84,88]. The micro-injection of monoclonal antibodies to the p53-binding interface of MDM2 protein can activate p53-dependent expression (Figure 4), providing direct evidence that MDM2 is normally a negative regulator of p53 function *in vivo* [89,90]. Independent studies showing that a double knockout of p53 in MDM2$^{-/-}$ animals can rescue the embryonic lethality of the animal, further suggested that MDM2 is a negative regulator of p53 in normal cells [91]. The development of a small peptide derived from human p53 into a highly bioactive peptide mimetic (Figure 4), which could activate p53-dependent transcription in cells via the inhibition of the MDM2 binding to p53, not only indicated that MDM2 protein was a key negative regulator of p53 *in vivo*, but provided a novel target for re-activation of the p53 response in cancers where wild-type p53 protein was inactivated by hyperdegradation [92]. A similar accumulation of the transcription factor E2F via inhibition of MDM2 protein by the bioactive peptide mimetic also indicates that the MDM2 degradation pathway may play a role in modulating gene expression by a variety of stress-regulated transcription factors [93] and opened the possibility of both re-activating p53 function and/or driving E2F-dependent apoptosis for therapeutic benefit.

Regulation of MDM2-dependent inactivation of p53 protein

The most well studied regulatory factor that can affect the ability of MDM2 to inactivate p53 is the p14^{ARF} tumour-suppressor gene product that can bind with a high affinity to a distinct site on MDM2 protein compared with p53 protein [94] (ARF is alternative reading frame protein). The p14^{ARF} protein is a component of a signalling pathway whose up-regulation in response to proliferative signals induced by oncogene activation can sequester MDM2 protein, thereby activating the p53-dependent cell-cycle checkpoint pathway [95–102]. Although amplification of this oncogene signalling pathway via loss of p14^{ARF} may be a physiologically relevant driving force for inactivation of the p53 pathway in cancers, stresses that induce DNA damage do not apparently use the p14^{ARF} signalling pathway to stabilize and activate p53. A detailed review on the p14^{ARF} pathway has been described elsewhere [103].

Biochemical studies have further driven the development of additional mechanisms for regulating MDM2–p53 protein complex formation. A secondary docking site on p53 within a conformationally sensitive core domain can be stabilized by the complexing of MDM2 protein to RNA [87] through the action of the RING-finger domain of MDM2 [104]. This conformational regulation of MDM2 protein provides the only known molecular mechanism for stabilizing the MDM2–p53 protein complex *in vitro*, although whether this functions in cells is only suggested by the co-localization of p53 and MDM2 with ribosomal L5 protein and RNA [105]. Phosphorylation of p53 protein itself in response to DNA damage within the BOX-I domain has been proposed to be one mechanism whereby MDM2 binding to p53 protein is disengaged, permitting p53 protein accumulation [106]. Alternatively, p300 protein binding and/or hTAF$_{II}$31 binding to p53 protein has been reported to be required for p53 protein accumulation, presumably because these factors compete with MDM2 binding to the same region within the transactivation domain of p53 [36]. As such, competition between MDM2 and p300 binding to the N-terminus of p53 may be the general protein interaction driving p53 from the degradation pathway to the transactivation pathway. This MDM2/p300–p53 protein interaction can be regulated, in part, by Ser^{15} phosphorylation of p53, which may in itself facilitate such a competitive switch by both reducing MDM2 protein binding and stimulating p300 binding to p53 [38,107]. Mutation of the Ser^{15} residue to alanine can enhance p53 degradation and reduce p53 apoptotic function [40], providing evidence that phosphorylation at Ser^{15} may regulate MDM2-dependent degradation of p53 *in vivo*.

Two other sites of post-translational modification are now known to be clustered within this BOX-I regulatory domains (Figure 2), with a similar paradigm being supported: phosphorylation of p53 within the N-terminal BOXI domain at Thr^{18} or Ser^{20} can affect heterologous protein–protein interactions [108–111]. The phosphorylation sites within the N-terminal regulatory domain of p53 exhibit distinct types of regulation, depending upon the context or damaging agent. Normal human fibroblasts constitutively modify p53 at the Ser^{20} site and oxidant stresses can result in hypophosphorylation at this site [109,112], while the ionizing-radiation-induced form of p53 protein is phosphorylated at Ser^{20} by a checkpoint kinase 2 (CHK2)-dependent pathway [110,111]. The Thr^{18} site is modified in human breast cancers [108], induced during senescence [18] or transiently following ionizing radiation [113]. More strikingly, Ser^{15} phosphorylation increases during quiescence, senescence, UV irradiation or ionizing irradiation, thus identifying signal-recognition pathways that respond to distinct signals in normal human cells [18]. Presumably this cluster of phosphorylation sites that differentially affects MDM2-dependent degradation of p53, p300 binding to p53, or other heterologous protein–protein interactions [114,115] will modulate the specific activity of p53 as a transcription factor and will be important in regulating the rates of p53-dependent tumour suppression in a cell-specific manner.

MODELLING THE SIGNAL-TRANSDUCTION PATHWAYS THAT TARGET p53: THE p53 ACTIVATING LESIONS

Although many distinct stresses can activate p53 function, it appears that at least two distinct signal-transduction pathways

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play a role in p53 activation. The more recently identified is a growth factor/oncogene-mediated signalling pathway that targets and modulates MDM2-dependent degradation by alterations in the tumour-suppressor protein p14^{ARF} [103]. A second, more widely studied, pathway is the 'DNA damage'-mediated activation of p53 [116–118], and one problem that has yet to be satisfactorily addressed in the control of p53 function is the definition of the damaged lesion(s) that leads to p53 activation and/or accumulation. Such a question is more complicated given that agents as diverse as antimetabolites or hypoxic conditions can activate p53 function in the absence of detectable DNA damage. Nevertheless, epigenetic studies have shown that mutating or inactivating putative or established 'tumour-suppressor' proteins that play a role in the DNA damage response, including ATM, ATR, DNA-PK, CHK2, breast-cancer-susceptibility gene-1 (BRCA1) or PKR, can prevent or attenuate the activation of p53 by DNA damage and have formed the basis for an epigenetic map to describe 'upstream' regulators of p53 (Figure 1). However, these studies do not address whether these individual enzymes can 'sense' the damaged lesion and initiate the cascade that can activate p53. Key perturbations that are thought to lead to p53 activation include the production of oxidant radicals (i.e. oxidative stress), damaged DNA structures or repair intermediates, and the inhibition of enzymes that affect RNA polymerase II-dependent transcription (Figure 1). Most of the research in this part of the p53 field has utilized UV-C or ionizing radiation as damaging agents, and the dissection of the upstream pathways controlling p53 have been defined by examining the similarities and differences between these damaging agents in different genetic backgrounds.

Thymidine dinucleotides can activate the p53 response, suggesting that this primary lesion plays a crucial role in mediating the cellular response to UV radiation, but the enzymes that sense the accumulation of this intermediate and transmit this damage to p53 have not yet been mapped out [119,120]. The microinjection of DNA templates representing a DNA lesion [121] and the direct DNA cleavage by the introduction of restriction enzymes to a cell [117] can lead to p53 accumulation and/or activation, suggesting that a DNA break induced by ionizing radiation can be a signalling lesion. Consistent with these data, cells which are sensitive to ionizing radiation owing to defects in enzymes, including the ATM kinase, have a defective p53 response to such type of DNA damage [122]. In addition, as ATM mutant cells are also defective in both N-terminal and C-terminal p53 phosphorylation after ionizing radiation, is it evident that the upstream ATM defect may perturb multiple p53-modifying pathways [123,124]. However, ionizing radiation can also induce oxygen radicals, leading to protein and lipid damage due to oxidative stress, and further evidence for oxidative damage playing a role in p53 protein accumulation and/or activation stem from work aimed at examining the role of antioxidants in the control of the p53 response [125,126]. The accumulation of p53 protein induced by high levels of UV-C radiation can be attenuated by pretreating cells with *N*-acetylcysteine. Under these same conditions, the antioxidants did not quench DNA damage induced by the radiation exposure, suggesting that oxidant radicals and not gross DNA damage are a trigger of p53 protein accumulation after damage [127]. These data are consistent with the concept that antioxidant pathways such as REF-1 [128–130], thioredoxin reductase [131,132] and thioredoxin [133] play important roles in modulating p53 activity by counteracting oxidant stress. The central role for antioxidants in modulating the rate of cancer progression [134,135] and the possibility that chronic exposure to chemical oxidants may place a significant burden on the antioxidant regulators of p53 protein makes an understanding of these factors important for defining modulators of cancer progression.

In addition to the role of oxidant injury as a key lesion in the activation of p53, the use of cell lines with patients from skin diseases such as xeroderma pigmentosa defective in specific stages of DNA repair after UV irradiation have given an interesting insight into the physiology of the p53 response [136–138]. One of the major differences between the damage induced by ionizing radiation or by UV-C irradiation is that ionizing radiation fails to inhibit RNA polymerase II-dependent transcription [139] and, as such, this latter biosynthetic pathway will presumably play a more significant role in the UV response to damage. UV radiation induces long-lived DNA adducts, an inhibition of RNA polymerase II-dependent general transcription and a sustained induction of p53-dependent stress-activated transcription. Furthermore, the UV-C induced p53-response is enhanced in XP-A cells (but not XP-C) in which transcription-coupled repair is specifically defective and the recovery of RNA synthesis following UV is delayed [136,140]. Using selective kinase inhibitors that block the enzymes required for RNA polymerase II-dependent transcription, the accumulation and post-translational activation of p53 can be promoted [49,50,139–142,237]. Future research into the type of lesions that induce the accumulation and/or activation of p53 function will necessitate developing a comprehensive understanding of the types of perturbations initiated by the specific type of damage and how regulatory gene products integrate with such signalling lesions.

THERAPEUTIC STRATEGIES FOR ACTIVATING THE p53 PATHWAY

Exploiting p53 regulatory pathways operating in normal cells (Figure 4)

Activation of allosteric mutant forms of p53 by agents that target the C-terminal negative regulatory domain

Although naturally occurring mutations in p53 protein are generally known to have reduced sequence-specific DNA-binding activity, more refined molecular studies have shown that p53 mutants can be divided into at least three distinct classes with respect to tetramerization, conformational modulation and intrinsic-core-domain folding (Figure 3). One class of p53 mutant containing a point mutation within the core domain (prototype His^{175}) cannot be activated for DNA binding by the allosteric C-terminal modifications and have a relatively high degree of intrinsic thermodynamic instability [143,144]. A second class of p53 mutant protein with a thermodynamic stability similar to wild-type p53 has the capacity to bind to DNA sequence-specifically, but cannot be activated from the latent state by phosphorylation ([146]; prototypes His^{273} and Lys^{285}). A third class contains point mutations within the tetramerization domain, alters assembly of this motif and can be defined as an oligomerization domain mutant [30,31]. Of these three mutant forms of p53, the allosteric class (His^{273} and Lys^{285}) is the type that can be re-activated *in vitro* and *in vivo* by agents that modify the C-terminus, whereas the structural mutants (His^{175}) can be re-activated by agents that promote the stabilization of its intrinsic folding pathway. As such, understanding more about the properties of the allosteric class and structural class of mutant p53 protein may permit more rational drug design programmes being formed to re-activate its function.

The allosteric class of mutant p53, embodied by the His^{273} allele, exhibited structural changes in the core domain at the loop–sheet–helix motif and the L3 loop, where the aromatic mutant residue perturbs the local environment, resulting in disruption of a salt bridge affecting structural changes in helix-2.

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This allosteric class of p53 mutant was first predicted from studies on regulatory properties of the wild-type p53 protein. The regulation of DNA binding of wild-type p53 by phosphorylation in its extreme C-terminus (and now other types of covalent modification; see Figure 2) and has been proposed to result from allosteric means, on the basis of kinetic data showing: (i) an uncoupling of Ser^{392} phosphorylation from latent p53 activation [45] in which the effects of phosphorylation at Ser^{392} on p53 activity may be related to ability of this modification to induce conformational changes in or to stabilize the tetramerization domain [145], which presumably will be directed to changes in the conformation and activity of the core DNA-binding domain; (ii) the inability of some mutants, with the machinery to engage in specific DNA binding and which can be activated by antibodies and peptides *in vitro* and *in vivo* (His^{273} and Lys^{285}), to be activated by phosphorylation [146]; and (iii) on the basis of the stoichiometry of an antibody-activated p53–DNA complex ($[PAb421]_2[p53]_4$) [54], which infers the need to overcome the inherent negative co-operativity built into tetramer regulation via stoichiometric modification. This conformational model for p53 activation is supported by independent approaches that include changes in the proteolytic cleavage products generated in an ATP-dependent manner by some CDKs [147] and the ability of CDC2-site mutation to compromise activation of p53 protein specific DNA-binding function by agents that modify the C-terminal negative regulatory domain [148,149].

These biochemical studies predict that manipulation of p53 function can occur through modification of the C-terminal negative regulatory domain, and a set of cellular criteria has supported this hypothesis. First, the purified latent fractions of p53 protein are inactive using *in vitro* transcription assays with hepatocyte nuclei as a source of transcription factors, and transcription by this latent p53 protein can be activated by the addition of the activating monoclonal antibody PAb421 [150], which itself targets the C-terminal regulatory domain near the phosphorylation sites (Figure 1). In addition, highly purified and activated fractions of p53 protein which are phosphorylated *in vivo* within the PAb421 epitope are intrinsically active using *in vitro* transcription systems and can be inactivated by prior treatment with phosphatases [150]. These studies provided direct evidence that C-terminal phosphorylation or antibody binding to the C-terminal domain can stimulate p53-dependent transcription. Further, the microinjection of the activating monoclonal antibody PAb421 can stimulate p53-dependent gene expression *in vivo* [45,51], presumably through a similar mechanism. Similarly, small peptides that can activate the latent DNA-binding function of p53 *in vitro* can also activate p53 function *in vivo* when coupled to carrier protein or ligand [52,261,262]. The intracellular synthesis of a single-chain PAb421 antibody expression cassette can also activate allosteric p53 mutants, but not structural p53 mutants *in vivo* [53], highlighting the biochemical distinctions that exist between p53 mutant proteins and the need to define the class of mutant p53 existing within cancers if activating drugs are in fact developed and used. These studies have predicted the possibility that cellular enzymes which modify the C-terminal domain may exist, and now a variety of enzymes that target the C-terminal domain are thought to play a stimulatory role in the p53 pathway after cell injury (Figure 2).

Activation of wild-type p53 by inhibiting MDM2-dependent degradation

Many stress-regulated transcription factors, including NF-κB (nuclear factor κB), HIF-1 (hypoxia-inducible factor-1) and HSF-1 (heat-shock factor 1), can be produced as inactive or latent isoforms in unstressed cells via negative regulation by trans-acting factors that ensure their latent function remains under strict control [151–153]. The p53 protein is no exception, being negatively regulated in part by an MDM2-dependent pathway that results in nuclear–cytoplasmic shuttling [154–157] and subsequent ubiquitin-dependent degradation of the protein [48,81]. p53 protein can also be targeted and degraded by a c-Jun N-terminal kinase (JNK)-dependent pathway, although it has been reported that the JNK-degradation pathway functions in G_0/G_1 phase, whereas the MDM2-degradation pathway functions in S-G_2/M phases, of the cell cycle [158]. The negative regulation of wild-type p53 protein by MDM2 identifies a mechanism whereby the specific activity of p53 could be reduced in cancers overamplifying MDM2 [90,159] and provides a potential drug target for re-activating the p53 pathway. The development of small-peptide effectors that can inhibit MDM2 binding to p53 protein in cell lines [92] provides such a precedent for blocking the MDM2/p53 interface (Figure 4). The use of antisense technologies to reduce the levels of active MDM2 protein in cells similarly leads to an activation of p53 function [160,161]. Similar inhibition of JNK binding to p53, either by removing the JNK docking site or by utilizing small peptides derived from the JNK/p53 interface, can reduce the extent of JNK-dependent ubiquitination and degradation [158].

A second molecular mechanism for inhibiting MDM2-dependent degradation of p53 protein involves the interaction between the tumour suppressor protein $p14^{ARF}$ and MDM2, whose binding interface is distinct from that of the MDM2–p53 interaction site [94]. $p14^{ARF}$ is frequently deleted in cancers with a wild-type p53 gene, providing strong evidence for a role for this protein as an upstream regulator of p53 function in some cancer types. $p14^{ARF}$ blocks the degradation of p53 by MDM2 through the inhibition of the ubiquitin ligase-associated function of MDM2 and by preventing nuclear export of p53 protein [95–102]. The use of small peptides derived from $p14^{ARF}$, which map at the $p14^{ARF}$/MDM2 interface, can activate p53 in cell lines, providing an additional target assay for modulating MDM2-degradation pathways [94]. Presumably MDM2 protein will bind to, and degrade, other polypeptides in cells, and the possibility remains that other factors that bind to MDM2 protein at the p53 or the $p14^{ARF}$ interfaces could modulate p53 activity as a tumour suppressor.

Exploiting regulatory pathways dysregulated in cancers (Figure 5)

Activation of structural mutant forms of p53 by agents that reduce p53 protein unfolding

The first molecular defect described for p53 protein in cancers was the frequent 'unfolding' or 'denaturation' of the mutant polypeptide, which was defined by immunohistochemical cell staining and immunoprecipitation of p53 protein using antibodies specific for denatured p53 [162,163]. Although this unfolding of mutant p53 depends upon the 'environment' of the tumour cell [164], possibly involving alterations in the expression of factors such as molecular chaperones and MDM2 [165–167], it is becoming more evident that one class of mutant p53 protein is intrinsically destabilized thermodynamically and that normal conformation in cells can be restored by reducing the temperature [168–170]. Immunochemically, this refolding of mutant p53 involves reduced expression of an antibody specific for unfolded p53 (PAb240) and increased expression of an antibody specific for the native tetramer (PAb1620) [171]. These data have recently encouraged mutagenesis approaches set out to produce a thermostable p53 protein in the hope of defining the amino acid side chains involved in stabilizing the native tetramer [172,173]. Such

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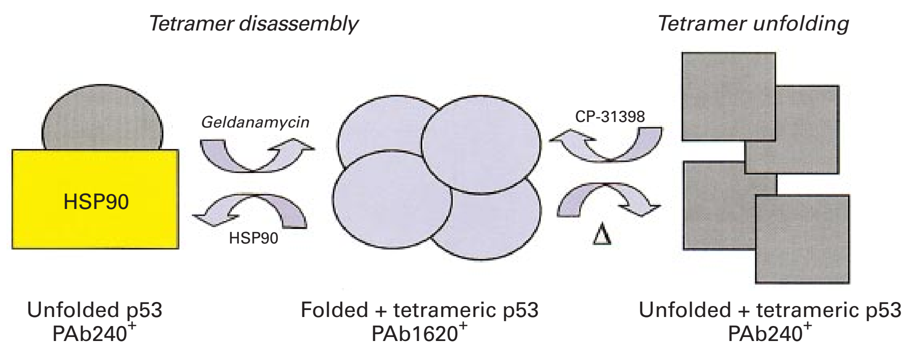

Figure 5 Pharmacological manipulation of p53 protein conformation in cancer cells

P53 protein can be assembled into native active tetramers (lilac), which bind to the conformationally sensitive monoclonal antibody PAb1620^{+} [263], whereas unfolding and inactivation of the p53 tetramer can be quantified using the monoclonal antibody specific for unfolded p53 protein [monomer (grey ellipse) or tetramer (grey squares)], PAb240^{+} [162,264]. Two distinct classes of organic compounds have been shown to preserve the native PAb1620^{+} conformation of p53 *in vitro* and *in vivo*. One class of compounds, comprising the benzoquinone ansamycin class of antitumour fungal antibiotics (geldanamycin), inhibits the HSP90 (yellow)-dependent chaperone holoenzyme complex unfolding of p53 protein [165,184,185] and forms a precedent for developing therapeutically relevant agents that modulate chaperone-dependent anti-apoptotic pathways [182,183]. A second class of compounds (prototype being CP-31,398) presumably binds directly to native p53 protein and prevents its unfolding and inactivation by stabilizing the intrinsic thermoinstability (Δ) of the tetramer [143,168,169,171,174,263].

an immunochemical folding assay provides an ideal platform for a high-throughput screening assay set up to stabilize the native (PAb1620-reactive) conformation of p53 protein. In fact, a recent report has identified classes of low-molecular-mass agents by screening a library of synthetic compounds which could not only maintain structural mutant p53 conformation *in vitro* (elevated PAb1620 reactivity at elevated temperatures), but can stimulate mutant p53 protein activity in cultured cells and *in vivo* [174]. The active compounds identified (prototype CP-31,398) contain a core moiety with a linker tethering a bivalent pairing containing a hydrophobic group and a cation. Such studies demonstrate the striking success that can be achieved from random screening of chemical libraries based on a well-characterized biochemical activity of a target protein and will hopefully drive the discovery of drugs capable of manipulating p53 protein activity for therapeutic effect (Figure 5).

Manipulation of p53 function by targeting the molecular chaperone HSP90

The first cellular protein (i.e. non-viral protein) shown to bind to p53 included a member of the heat-shock protein 70 (HSP70) family of proteins [175], whose associations with p53 have since been extended to include the molecular chaperone holoenzyme complex including HSP40, HSP90, immunophilins and p23 [166]. HSPs or molecular chaperones are implicated in the folding of nascent polypeptide chains, in partial unfolding of polypeptide during intracellular transport and in the repair or degradation of damaged polypeptides [176]. The chaperones function as a co-ordinated holoenzyme or protein-folding machine, with discrete steps in the protein folding and unfolding process. The core polypeptides of the holoenzyme can include HSP90, HSP70, HSP40, $p60^{HOP}$, $p48^{HIP}$, various immunophilins and p23, with each protein having distinct functions, including substrate recognition and release, nucleotide-dependent binding and turnover, and conformational regulation of the holoenzyme complex organization [177]. Chaperones can function as anti-apoptotic effectors in cells exposed to otherwise toxic levels of damaging agent and the ability of antitumour antibiotics of the ansamycin class to bind to chaperones, which are overexpressed in tumour cell lines, to induce cell death further highlights their role in cell survival and the attraction for targeting chaperones for therapeutic effect [178].

The significance of the interaction of p53 with HSPs in tumour cells have been unclear, but recent evidence described below suggests that one component of the anti-apoptotic function of HSPs may be related to the control of the conformation and inactivation of p53. For example, the recently identified HSP70 homologue Mortalin (Mot-2), originally implicated in promoting the senescent phenotype, can inactivate p53 function and its activity is associated with the transformed phenotype [179–181]. The altered folding of mutant p53 protein in tumour cells, presumably catalysed by HSP90-dependent pathways [165], was first detected with the PAb240 monoclonal antibody, which is specific for denatured or unfolded forms of p53 [162]. Although this was not clear at the time, this unfolding of p53 identified the first molecular pathway whose dysregulation in tumours contributed to the inactivation of p53 and now links chaperone activity to p53 unfolding. The elevated stability of the HSP–mutant p53 protein complex in some tumours also provided a compelling link between dysregulation of the protein-folding machinery and the inactivation of p53 protein function. Thus studies aimed at dissecting the nature of the HSP–p53 interaction should shed light on molecular processes that contribute to p53 inactivation and assist in designing strategies to re-activate the normal p53 protein-folding and assembly pathways in cells. A breakthrough in dissecting molecular pathways that regulate mutant p53 protein conformation and stability in tumour cells came from independent studies examining the mechanism of function of the benzoquinone ansamycin class of antitumour compounds, which include geldanamycin and the analogue 17AAG [182,183]. The benzoquinone ansamycins are proving to be useful in identifying novel signalling proteins that interact with HSP90, as a common feature of inhibition of HSP90 function with benzoquinone ansamycins is target protein degradation by the proteosome pathway. Given that molecular chaperones, including HSP90, bound to mutant p53 in tumours, studies were developed to determine whether mutant p53 folding and stability are regulated by chaperones. A reduction in mutant p53 protein levels after treatment of tumours with geldanamycin mediated by ubiquitin-dependent means and the refolding of the mutant p53 into the native, tetrameric conformation [165,184,

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185] demonstrates that HSP90 may play an important role in modulating mutant p53 conformation and steady-state protein levels (Figure 5).

Targeting mutant p53 protein in tumour-specific killing assays: exploiting stabilized p53 protein

Almost half of all cancers have the p53 gene mutated, resulting in high-level expression or accumulation of mutant p53 protein [186]. This nuclear accumulation appears to occur via inhibition of the MDM2-degradation pathway and may involve binding by the anti-apoptotic survival factor HSP90, as the inhibition of HSP90 by the anti-cancer drug geldanamycin can destabilize p53 protein and promote its degradation by the ubiquitin-dependent pathway [165]. Regardless of the molecular mechanism of this accumulation of mutant p53, the elevated level of mutant p53 in cancer cells compared with normal cell types provides an intriguing target in neoplastic cells for the design of anti-proliferative agents.

One such approach of targeting cancer cells containing high levels of mutant p53 protein or without p53 protein using an adenovirus hybrid (called ONYX-015) engineered to kill cells with mutant p53, but not wild-type p53, has been reviewed previously and provides a precedent for the engineering of smart therapies [187,188]. Briefly, the adenovirus E1B gene product that normally binds to wild-type p53 protein has been inactivated by mutation such that the virus cannot replicate in cells with a functional p53 protein. The virus can replicate in cancer cells containing mutant p53, and preliminary success at tumour regression using virus injected into solid tumours has been reported. Although subsequent work has questioned whether in fact ONYX-015 replication is p53-dependent or -independent, possibly due to the types of cell lines used, there can be some selectivity for ONYX-015 replicating in and killing cells with mutant p53 *in vitro* and *in vivo* [265–267]. Additionally, the ability of ONYX-015 to kill synergistic cancer cells in combination with common anticancer treatments provides a hopeful precedent for manipulating the p53 pathway successfully in the treatment of specific cancer types.

Further experimental strategies have demonstrated an intriguing angle on the selective targeting of cancer cells by recruiting the transactivation domain of the stabilized mutant p53 protein to activate the expression of a target 'killer' gene. The factor exploited to recruit the mutant p53 protein which displays both high specificity and affinity is a single-chain antibody chimaera specific for p53 protein fused to the DNA-binding domain of the tetracycline repressor. In cell lines, this concept has been very successful and certainly provides hope for exploiting mutant p53 protein overproduction for therapeutic effect [189]. The development of single-chain antibodies as a new type of therapeutic agent for gene therapy has been reviewed elsewhere. However, this type of transcriptional transactivator, termed a trabody (transcription-activating antibody), provides a novel technology for targeting a protein whose expression plays a role in human disease.

Peptide mimetics in experimental therapeutics: replacing the effectors of p53

The $p21^{WAF1}$ tumour modifier gene product

The first physiologically relevant gene product shown to be induced by p53 was the $p21^{WAF1/CIP1}$ CDK regulator that appears to mediate in part p53's tumour-suppressor function [190]. It has since become clear that $p21^{WAF1/CIP1}$ is capable of contributing to the regulation of cell division on several different levels. These include mediation of negative growth signals, functions in differentiation and senescence, and the more recently defined roles for $p21^{WAF1/CIP1}$ as a modulator of the of apoptotic response [191] and an activator of certain CDKs in response to mitogenic signals [192,193]. However, the biochemical function of p21 was first identified on the basis of independent research in the cell-cycle field. Cell growth and divisions are driven by the CDK family, which is tightly regulated by post-translational mechanisms, including phosphorylation, degradation and the action of low-molecular-mass kinase inhibitors. One of these kinase inhibitors, namely $p21^{WAF1/CIP1}$, was originally identified as a CDK and proliferating-cell-nuclear-antigen (PCNA)-binding protein that was able to inhibit CDK catalytic activity [194,195].

The mechanism by which $p21^{WAF1/CIP1}$ inhibits cyclin–CDK activity has been relatively controversial. It has been reported that $p21^{WAF1/CIP1}$ could be associated with both active and inactive G_1 cyclin–CDK complexes [196,197] and that changes in the stoichiometry of $p21^{WAF1/CIP1}$ protein regulate this transition, recent studies have established that a single molecule of $p21^{WAF1/CIP1}$ is sufficient to completely inhibit the catalytic activity of cyclin A–CDK2 [198]. This correlates with studies showing that all the $p21^{WAF1/CIP1}$-containing CDK2 complexes in a cell are catalytically inactive [199]. In contrast, all the cyclin D–CDK4 pRb (retinoblastoma protein) kinase activity in proliferating cells is in complex with either $p21^{WAF1/CIP1}$ or its close relative p27 [192,193] and, at a molar ratio of 1:1, $p21^{WAF1/CIP1}$ does not efficiently inhibit cyclin D–CDK4 activity [192,200]. In fact it appears from *in vitro* studies that $p21^{WAF1/CIP1}$ can stimulate the assembly of catalytically active cyclin D–CDK4 by stabilizing the preformed complex [192] and in cells $p21^{WAF1/CIP1}$ appears to assemble and target the nuclear localization of cyclin D1–CDK4 [193]. Thus although p21 is a potent inhibitor of CDK2-containing complexes, it is a positive modulator of CDK4 activity.

Structure–function analysis of $p21^{WAF1/CIP1}$ has shown that it contains at least three distinct regulatory or interaction sites that can mediate its biochemical function in cells. The N-terminal half of $p21^{WAF1/CIP1}$ has been shown to be sufficient for both cyclin–CDK binding and inhibition, since it contains a cyclin-binding motif and a CDK interaction site [201–205]. A second cyclin-binding motif lies at the extreme C-terminus of $p21^{WAF1/CIP1}$, and although this domain is sufficient to bind and inhibit some cyclin–CDKs, its function within the full-length protein remains unclear [205,206]. In addition to a second cyclin-binding site, the C-terminus of $p21^{WAF1/CIP1}$ also contains a region which interacts with the replication and repair protein PCNA [207], a phosphorylation site whose modification *in vivo* can inhibit PCNA binding [208], and two cleavage sites for the apoptosis-associated protease caspase 3 [209]. The interaction of $p21^{WAF1/CIP1}$ with PCNA blocks the ability of PCNA to act as a processivity factor for DNA polymerases, modulating the primer-template recognition complex and inhibiting DNA replication *in vitro*. Although there is clear evidence that $p21^{WAF1/CIP1}$–PCNA complexes form in response to DNA damage, it has proved difficult to show a significant affect of $p21^{WAF1/CIP1}$ on DNA replication in cells. However, recent data suggest that the interaction with PCNA is important in preventing endoreduplication [210], inhibiting S-phase progression [211] and promoting DNA repair [212,213].

Although it is not necessarily evident that any one p53-dependent gene product can alone replace the tumour suppressor function of p53, it is notable that the $p21^{WAF1/CIP1}$ gene is as effective as the p53 gene in some experimental models of cancer treatment [214]. Intriguingly, $p21^{WAF1/CIP1}$ gene dosage plays a role in modulating the rate of tumorigenesis in breast cancers

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with an $ATM^{-/-}$ background, suggesting that the specific activity of $p21^{WAF1/CIP1}$ can determine whether developing cancer cells engage proliferative or antiproliferative pathways and, in accordance with this, $p21^{WAF1/CIP1}$ has been proposed to be a tumour-modifying gene [215–217]. Further, the recently identified okadaic acid-sensitive kinase cascade targeting the PCNA-binding site of $p21^{WAF1/CIP1}$ protein provided the first evidence that $p21^{WAF1/CIP1}$ protein specific activity can be regulated by covalent modification [208] and highlights the possibility that post-translational signal-transduction mechanisms can affect its tumour-modifier activity.

Thus it remains possible that experimental strategies developed that by-pass the need for p53 protein by developing low-molecular-mass mimetics of $p21^{WAF1/CIP1}$ or enzyme inhibitors that inhibit the kinases that phosphorylate its C-terminal domain would be exciting leads for drug-discovery programmes aimed at regulating $p21^{WAF1/CIP1}$ tumour-modifier function. In fact, the CDK-inhibitory function of $p21^{WAF1/CIP1}$ has been reconstituted with low-molecular-mass peptides derived from the N- and C-terminal domains, and the introduction of these low-molecular-mass effectors into cells can induce cell-cycle arrest via inhibition of pRb phosphorylation [205]. Later studies have demonstrated the power of the peptide-mimetic approach in replacing $p21^{WAF1/CIP1}$ function and identifying tumour cell lines where cell death can be induced via the introduction of $p21^{WAF1/CIP1}$ peptide linked to carrier polypeptides [218,219].

The *bax* gene product as an apoptotic mediator of p53

The *bax* gene product has been defined as a mediator of p53-dependent apoptosis by antagonizing the Bcl-2-dependent survival pathway [220,221], and intriguing links have been made between alternatively spliced forms of JMY and p53-dependent induction of *bax* gene expression and apoptosis [73]. The Bcl-2 homologues comprise a family of proteins that play an important function in the regulation of apoptosis, with some members promoting apoptosis, and others promoting cell survival, following cell injury [222]. The Bcl-2 family of proteins function in a signal-transduction pathway where competitive dimerization between bcl-2 homology (BH) domains among family members regulates cytochrome *c* dissociation from mitochondria [223,224] and the activation of caspase function leading to nuclear disintegration and cell death. Although a set of other regulatory factors also transduce signals within this apoptotic pathway, including HSP70 [225], links have been made between the tumour-suppressor protein p53 and apoptotic signalling promoted by the Bcl-2 homologue BAX. Binding sites for p53 protein exist in the *bax* gene, and induction of BAX can occur in a p53-dependent manner [221]. Deletion studies have shown that the BH domains are required for the survival of damaged cells via the anti-apoptotic Bcl-2 homologues, while the BH domains are essential for the induction of cell death by the apoptotic-promoting Bcl-2 homologues BAX and BAK [226,227]. The ability of BH3 domains derived from BAK to induce apoptosis by competitive disruption of the anti-apoptotic homodimers or heterodimers provides a foundation for the development of therapeutic strategies to manipulate the cell-death pathway [228]. Using small peptides derived from the pro-apoptotic or anti-apoptotic Bcl-2 homologues, cell-death signalling pathways can be manipulated in extracts from *Xenopus* eggs [229]. The changes induced by the apoptotic BH3-domain peptides mimic that induced by full-length proteins in that they require membrane fractions from the cytoplasm and mitochondria, promote the release of cytochrome *c*, activate caspases and antagonize Bcl-2 function. The introduction of the BH3 domains of BAK into human tumour cells via the *Antennapedia* carrier peptide can promote apoptosis through a cytochrome *c*-independent activation of caspases [230]. The data support a model whereby the apoptotic role of the BAX and BAK proteins may be promoted by their ability to disrupt the anti-apoptotic Bcl-2 homologues and provide the scaffold for the design of peptide mimetics for manipulating the p53-dependent or independent cell-death switch.

PERSPECTIVES

This review has aimed to first highlight some of the fundamental biochemical properties of p53 protein and the key factors implicated in regulating its activity as a tumour-suppressor protein. The 'guardian of the genome' paradigm was an important milestone to highlight the inducible nature of the p53 pathway. The clear definition of p53 as a transcription factor that functions at a nodal point in the cellular stress response helped to explain why p53, and not its numerous gene products, undergoes a high rate of mutation in distinct types of human cancers. Additional concepts that have emerged include the realization that: (i) p53 protein is negatively regulated by degradation pathways; and (ii) cell-specific and damage-specific factors drive alterations in the specific activity of p53 as a transcription factor via covalent and non-covalent modifications. Thus, underlying the fundamental protein–protein interactions that drive p53 function, are regulatory processes such as cycles of phosphorylation and dephosphorylation, protein folding and unfolding, and protein synthesis and degradation, whose components are now being identified in detail. Equally important developments have included the ability to exploit these biochemical properties of p53 protein by manipulating its activity through the development of activating agents or drugs that can replace or modify mutant p53 structure and function *in vivo*. However, this basic research needs to be translated to more clinically relevant models to understand and to treat cancer as a biological disease. Rational exploitation of these biochemical pathways will require the development of quantitative physiological models that allow a greater understanding of the role of p53 as a tumour suppressor *in vivo*.

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