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The role of p53 in chemosensitivity and radiosensitivity

Wafik S El-Deiry*,[1]

[1]*Departments of Medicine (Hematology/Oncology), Genetics, Pharmacology, Abramson Cancer Center, University of Pennsylvania School of Medicine, Philadelphia, PA, USA*

The role of p53 as a central mediator of the DNA damage and other cellular stress responses is well established. The ultimate growth-suppressive function of p53 in part explains its ability to confer chemosensitivity and radiosensitivity upon tumor cells. Recent work in the field has added complexity to our understanding, in terms of identifying novel regulators of p53 stability and function, elucidation of the importance of the p53 family towards p53 function, a growing list of transcriptional targets as well as transcription-independent apoptotic effects and mechanisms, tissue specificity of the p53 response, a molecular understanding of p53-dependent therapeutic sensitization, and efforts towards molecular targeting of the p53 pathway. p53 remains an attractive target for drug development in cancer because its alteration provides a fundamental difference between normal and cancer cells. Strategies are emerging for the identification of mutant p53-specific therapies, therapies targeted at mutant p53-expressing tumors, as well as therapies that target various aspects of the p53 life cycle to enhance chemosensitization. The tools of molecular imaging are beginning to accelerate the pace of discovery and preclinical testing of p53 in animal models. The future holds promise for specific, individualized targeting of mutant or wild-type p53, or its transcriptional targets, in combination therapies with other cancer-specific drugs, to maximize tumor cell killing while protecting normal cells from toxic side effects.
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Introduction

The involvement of the p53 tumor-suppressive protein in response to a variety of cellular stresses has become more clear in the last decade (Vousden and Lu, 2002; Lowe and Sherr, 2003). A number of stresses, including damage to chromosomal DNA incurred by ionizing irradiation and exposure to ultraviolet light, activation of oncogenic signaling, hypoxia or nucleotide depletion, can all signal stabilization of the cellular p53 protein, which in turn mediates a growth-suppressive response (Figure 1). Loss of p53 pathway function commonly occurs in human tumors, and can contribute not only to aggressive tumor behavior but also to therapeutic resistance (Vogelstein and Kinzler, 1992; Velculescu and El-Deiry, 1996; Vogelstein *et al*., 2000). The p53 protein is a major target for mutational inactivation in human cancer, and represents a major difference between normal cells and cancer cells. Understanding of the regulation and downstream effects of p53 as well as efforts to target the pathway for therapeutic gain represent high priorities at present in the fields of Molecular Oncology and Cancer Biology.

In recent years, much has been learned about the details of the normal cellular response to stress (Figure 2). These include the unraveling of the early steps of kinase activation to post-translational modifications of key sensors and transducers such as p53 to the activation of downstream effector responses that control cellular proliferation in order to avoid propagation of damaged cells or the progression of initiated tumors (El-Deiry, 1998; Vousden and Lu, 2002; Lowe and Sherr, 2003; Sax and El-Deiry, 2003). Insights have been gained from the identification of p53 family members whose mechanism of involvement in human cancer has begun to emerge (Melino *et al*., 2002; Soussi, 2003). Discrete phosphorylations of p53 in response to various stressful signals, and a host of other specific modifications such as ubiquitination, deubiquitination, sumoylation, acetylation, and deacetylation, have been observed, and a number of enzymes have been discovered to mediate these activities, that is, MDM2, Pirh2, p300, HAUSP, and Sir2 (Ashcroft *et al*., 2000; Vousden and Lu, 2002; Brooks and Gu, 2003; Leng *et al*., 2003; Grossman *et al*., 2003). Modifications in p53 that direct specific responses, for example, serine 46 phosphorylation, and protein–protein interactions, for example, ASPP1, BRCA1 or PTEN, or the coordinated activity of both p63 and p73, have been found to serve as determinants of whether or not apoptosis occurs (Flores *et al*., 2002; MacLachlan *et al*., 2002; Bergamaschi *et al*., 2003a, b; Freeman *et al*., 2003; Slee and Lu, 2003). Indeed, recent studies suggest a possible role for the p53 family in influencing chemosensitivity responses even in the absence of functional p53 (Irwin *et al*., 2003), as well

*Correspondence: WS El-Deiry, Department of Medicine (Hematology/Oncology), Genetics, Pharmacology, Abramson Comprehensive Cancer Center, University of Pennsylvania School of Medicine, 415 Curie Blvd., CRB 437A, Philadelphia, PA 19104, USA;
E-mail: wafik@mail.med.upenn.edu

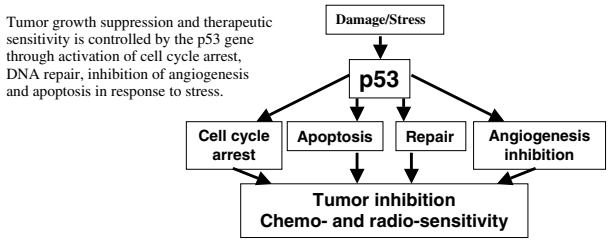

Figure 1 Tumor growth suppression and therapeutic sensitivity is controlled by the p53 gene through activation of cell cycle arrest, DNA repair, inhibition of angiogenesis, and apoptosis in response to stress

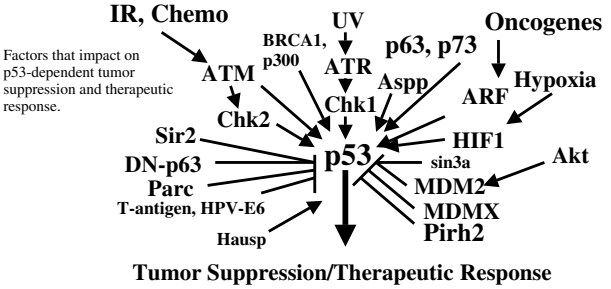

Figure 2 Factors that impact on p53-dependent tumor suppression and therapeutic response

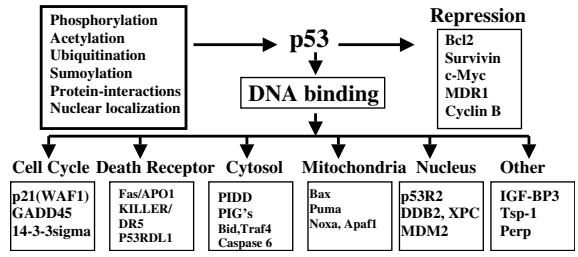

Figure 3 Immediate upstream regulation of p53, and downstream activated (after DNA binding) and repressed effector genes

as differential p73 modulation by polymorphic alleles of p53 (Bergamaschi *et al*., 2003a).

The role of p53 in chemosensitivity is well established, but how p53 mediates its effects has remained less well understood (Sax and El-Deiry, 2003). A number of novel effectors of p53 have been discovered (Figure 3), and some have been suggested to play a role in sensitizing cells to drugs and radiation damage, that is, chemosensitivity genes such as APAF1, caspase 6, and Bid (Sax and El-Deiry, 2003). A more complete understanding of how p53 causes cell death has begun to emerge from an appreciation of the various apoptotic pathways directly activated, including death receptors such as Fas, KILLER/DR5, or p53RDL1 (Burns and El-Deiry, 2003; Tanikawa *et al*., 2003), cytoplasmic proteins such as PIDD, PIGs or Bid (Sax and El-Deiry, 2003), and proteins that act on the mitochondria such as Bax, Bak, Noxa, and Puma (Oda *et al*., 2000; Wei *et al*., 2001; Yu *et al*., 2003). Additional possible effects of transcriptional repression of Bcl2 and survivin (Hoffman *et al*., 2002) or mitochondrial localization of the p53 protein (Dumont *et al*., 2003; Manfredi, 2003; Mihara *et al*., 2003) have been described. A remarkable tissue specificity in the p53 target gene activation response has been uncovered *in vivo* (Burns *et al*., 2001; Fei *et al*., 2002). Further understanding of the control of subcellular localization of p53 has emerged from a number of regulators such as MDM2, ARF, and Parc (Kastan and Zambetti, 2003; Lowe and Sherr, 2003; Michael and Oren, 2003; Nikolaev *et al*., 2003). A greater appreciation of the role of p53 in DNA repair has been realized with the identification of direct targets for transcriptional activation including p53R2, the XPE gene product DDB2 or p48, and XPC, genes whose proteins are directly involved in DNA repair processes (Tanaka *et al*., 2000; Amundson *et al*., 2002; Takimoto *et al*., 2002a; Tan and Chu, 2002).

The wealth of details surrounding the p53 life cycle from its short half-life under normal physiological conditions to its numerous interactions with other proteins, post-translational modifications, subcellular localization, and divergent effector responses of the stabilized p53 protein under various circumstances have led to a number of proposed strategies for therapeutic modulation. Some strategies have focused on the blockade of p53 to reduce toxicity of drugs or radiation (Komarov *et al*., 1999), whereas others have focused on blocking interactions between p53 and negative regulators such as MDM2 (Bottger *et al*., 1996, 1997; Midgley *et al*., 2000). Yet other strategies have been targeted at mutated p53 to restore some wild-type p53-related transcriptional responses, for example, CP-31398 (Foster *et al*., 1999; Takimoto *et al*., 2002b; Wang *et al*., 2003) or Prima1 (Bykov *et al*., 2002), or therapies aimed at preferential killing of p53-deficient cells (Hamid *et al*., 2003; Hecht *et al*., 2003). The identification of novel regulators such as HAUSP, Pirh2, Parc, Akt, or Sir2 has suggested other points of intervention to modulate p53 activity (Zhou *et al*., 2001; Vousden and Lu, 2002; Brooks and Gu, 2003; Grossman *et al*., 2003; Kastan and Zambetti, 2003; Leng *et al*., 2003; Nikolaev *et al*., 2003). With the recent identification of tissue-specific p53 gene activation responses *in vivo* come a number of important insights into drug and radiation toxicity, as well as possible future efforts to further refine p53 blockade or altered specificity (Burns *et al*., 2001; El-Deiry, 2001; Fei *et al*., 2002). The emergence of imaging technologies for noninvasive detection of tumors, as well as the monitoring of molecular changes such as alterations in gene expression or protein–protein interaction, have already begun to impact on the role and activities of p53 in tumor therapy (Doubrovin *et al*., 2001; Massoud and Gambhir, 2003; Wang and El-Deiry, 2003). A number of open questions remain in the field, as does the promise of effective use of p53 in directing patient care either in terms of diagnosis or therapy. While the pathway and its details continue to become increasingly complex, the attractiveness of p53 as a target continues to rest on its extremely frequent involvement in human cancer and its

normal involvement as a potent tumor-suppressor gene and protein.

Stabilization and 'activation' of p53

Very recent insights into the earliest events in p53 activation have come from the laboratory of Michael Kastan (Bakkenist and Kastan, 2003). Using biochemical strategies, it was possible to determine that sites on ATM were early substrates for autophosphorylation and dimer disruption, as the ATM kinase becomes competent for acting on its other substrates including p53. These events occur almost instantaneously, following exposure of mammalian cells to very low doses of ionizing radiation (Bakkenist and Kastan, 2003). ATM-dependent events leading to p53 stabilization represent the initial major mechanism by which cells utilize the p53 pathway for the cellular stress response, leading to cellular growth inhibition and tumor suppression.

It appears that ATM and Chk2 are sequentially activated in the radiation response and phosphorylate serines 15 and 20, respectively, on p53 (Dumaz *et al*., 1999; Hirao *et al*., 2002). MDM2 also becomes phosphorylated in an ATM-dependent manner following ionizing irradiation, and this leads to the reduced binding between p53 and MDM2 and subsequent p53 protein stabilization (Maya *et al*., 2001). MDM2 overexpression either through gene amplification or enhanced translation can lead to defective stabilization of wild-type p53 in tumors without p53 mutation (Deb, 2002). MDM2 possesses E3 ubiquitin ligase activity towards p53, leading to its proteasomal degradation. MDM2 also directly binds to p53, and can inhibit its transcriptional activity. The MDM2-related protein MDMX (Figure 2) also binds to p53 and can inhibit its transcription, but does not ubiquitinate or target p53 for degradation (Badciong and Haas, 2002; Finch *et al*., 2002). Recent results suggest that Akt, which is often constitutively activated in human cancer, can directly phosphorylate MDM2 (Figure 2), leading to more efficient p53 degradation (Zhou and Hung, 2002). Akt has also been shown to influence p73-dependent transcription and apoptosis through a novel target YAP (Basu *et al*., 2003). Recent studies also suggest that PTEN, which has been described as a weak target for p53-dependent transcriptional activation, can also stabilize p53 (Freeman *et al*., 2003). In cells with loss of PTEN, p53 levels are reduced (Freeman *et al*., 2003). In response to ultraviolet light exposure (Figure 2), p53 is stabilized in an ATR- and Chk1-dependent manner (Chehab *et al*., 1999). In response to hypoxia (Figure 2), p53 has been reported to be stabilized in a HIF1-dependent manner (An *et al*., 1998; Carmeliet *et al*., 1998). In response to oncogenic signals (Figure 2), ARF protein is involved in p53 stabilization through interactions with MDM2 that diminish association between p53 and MDM2. In terms of stabilization, a recent report has described a novel protein called Pirh2, which appears to possess ubiquitin ligase activity towards p53 (Leng *et al*., 2003). Like MDM2, Pirh2 (Figure 2) is also a direct transcriptional target of the p53 protein (Leng *et al*., 2003). While MDM2 has been reported to monoubiquitinate p53, the p300 protein (Figure 2) which can serve as a coactivator of p53 has been found to mediate polyubiquitination of p53 (Grossman *et al*., 2003). It is not yet clear if the polyubiquitination activity towards p53 requires MDM2 only for monoubiquitination or whether p300 might also affect the ubiquitin ligase activity of MDM2 (Grossman *et al*., 2003). Clearly, p300 has ubiquitin ligase activity of its own *in vitro*, and its effect on p53 can be blocked by E1A but not an E1A mutant that fails to bind p300 (Grossman *et al*., 2003). The HAUSP protein (Figure 2) was discovered as a deubiquitinating enzyme for p53 (Li *et al*., 2002).

p53 is a nuclear phosphoprotein that can bind to DNA in a sequence-specific manner to activate gene expression. Thus, stabilization, post-translational modification, and nuclear localization of p53 represent necessary steps for its 'activation.' There may be other activities of p53 in gene repression or mitochondrial localization whose pathways are not well understood, but which may ultimately play an important role in p53 'activation' and function. In terms of nuclear localization, the Parc protein has been recently found to be overexpressed in neuroblastoma, and this overexpression correlated with cytoplasmic localization and retention of p53 protein (Kastan and Zambetti, 2003; Nikolaev *et al*., 2003). Nuclear p53 which is stabilized can be 'activated' as a transcription factor by additional events including phosphorylation and acetylation. p53 can be acetylated by p300 and PCAF, and deacetylated by Sir2.

Cellular and tissue-specific effects of p53

The stabilization and activation of p53 in response to cellular stresses leads to a number of cellular effects that collectively contribute to tumor growth inhibition and chemo- and radiosensitivity (Figure 1). These cellular effects include cell cycle arrest, apoptosis, DNA repair, and inhibition of angiogenesis. How p53 can mediate such diverse responses has been the subject of intense research for the past 12 years, because understanding these mechanisms was felt to be essential for understanding tumor suppression and essential as a prelude to therapeutic intervention. The greatest insights into p53 function have come from understanding how it functions as a transcription factor and which genes it regulates (Figure 3).

It is well known, however, that p53 can act to suppress growth or to induce cell death even in the absence of target gene activation. For example, a classical experiment in the laboratory of Michael Karin revealed that a temperature-sensitive p53 can induce apoptosis even if transcription is blocked with actinomycin D (Caelles *et al*., 1994). Later studies showed that transcription-deficient mutants of p53 can induce apoptosis in part through its proline-rich domain located between its N-terminal transactivation domain

and its large central DNA-binding domain (Walker and Levine, 1996). More recent studies have reported that a polymorphism at codon 72 in p53 can, without an apparently markedly altered gene activation profile, promote cell death through apparently direct effects of p53 localized at the mitochondria (Dumont *et al*., 2003). Yet other studies have revealed that p53 can repress gene expression in part through recruitment of histone deacetylases such as sin3a (Murphy *et al*., 1999), and that repression of targets such as Bcl2 or survivin (Figure 3) may be important for the phenotypic outcome in the p53 response (Hoffman *et al*., 2002). It is very interesting that during the cellular response to hypoxia, Amato Giaccia and colleagues have found that while p53 is stabilized it is not competent in transcriptional activation, but retains the ability to repress gene expression (Koumenis *et al*., 2001). These studies also revealed that under the duress of hypoxia, p53 fails to recruit its coactivator p300 but continues to recruit sin3a, and thus can repress gene expression (Koumenis *et al*., 2001).

The evidence for the importance of transcription in p53 function has come from several sources. Studies in the early 1990s demonstrated p53 is a sequence-specific DNA-binding protein and that tumor-derived p53 mutants failed to bind to DNA (Vogelstein and Kinzler, 1992). The crystal structure of p53 with its DNA-binding site revealed that hotspots for mutational inactivation in cancer represent contact points between p53 and DNA (Cho *et al*., 1994). p53 is conserved in Drosophila and binds to DNA, and activates genes involved in programmed cell death (Ollmann *et al*., 2000). A point mutant in the transactivation domain of p53 has been introduced in the germ line to generate a knock-in mouse by Geoff Wahl and colleagues (Jimenez *et al*., 2000). This mouse has a phenotype very similar to the p53 knockout mouse, and cells from this mouse are defective in cell cycle checkpoints and apoptotic responses. Cells derived from the p21 knockout mouse targeting the major cell cycle inhibitory transcriptional target of p53 were also found to be defective in G1 checkpoint arrest in response to cellular stress (Deng *et al*., 1995).

With evidence that transcriptional targets of p53 are indeed relevant to its function, efforts have been directed at the identification and characterization of these effector target genes (El-Deiry, 1998). A number of targets were identified and linked to cell cycle arrest, including p21, GADD45, and 14-3-3sigma (Figure 3). In addition to regulation of cell cycle checkpoints that arrest cells, giving them time to repair damage, p53 also activates genes that are in some way more directly involved in DNA repair responses (Figure 3). These include p53R2, a ribonucleotide reductase involved in replenishing nucleotide pools, and the xeroderma pigmentosum genes XPC and XPE (Tanaka *et al*., 2000; Amundson *et al*., 2002; Takimoto *et al*., 2002a; Tan and Chu, 2002). XPE encodes a DNA damage-binding protein DDB2, also known as p48, which is part of a herterodimer with p125 and involved in DNA damage recognition and binding following ultraviolet light damage. p53 regulates a number of secreted proteins that can influence growth or angiogenesis. These include IGF-BP3, the Insulin-like Growth Factor-Binding Protein #3 which binds to IGF1 and can inhibit growth factor signaling and may have other more direct proapoptotic effects, as well as a number of secreted inhibitors of angiogenesis (El-Deiry, 1998). The activated inhibitors of angiogenesis include Thrombospondin 1 (Tsp1) as well as a glioblastoma-derived angiogenesis-inhibitory factor (GD-AIF) and BAI, the brain-associated angiogenesis inhibitor. p53 appears to directly regulate a number of phosphatases such as Wip1 and Pac1, which may ultimately negatively regulate its activity (Choi *et al*., 2002; Yin *et al*., 2003). p53 also directly regulates proteins that negatively regulate its stability, including MDM2 and Pirh2 (Leng *et al*., 2003).

With respect to apoptosis, the picture that has been emerging in the last several years involves p53-dependent transcriptional activation of a number of genes that have the potential to induce apoptosis (Figure 3). While it has become clear that following anticancer drug exposure, cell death signaling proceeds through the mitochondria and a number of mitochondrial defects can confer therapeutic resistance, it has also become clear that p53 can activate cell death through multiple pathways (Sax and El-Deiry, 2003). For example, p53 appears to directly upregulate the expression of a number of cell surface death receptor proteins including Fas/APO1, KILLER/DR5 (Takimoto and El-Deiry, 2000), and the recently described p53RDL1 (Tanikawa *et al*., 2003). p53 also transcriptionally upregulates a number of cytoplasmic proteins including the death domain containing protein PIDD, the PIG genes (Polyak *et al*., 1997) involved in the generation of reactive oxygen species, as well as Bid which serves as a link between death receptor signals and mitochondrial cytochrome *c* release (Sax *et al*., 2002). Caspase 8-mediated Bid cleavage is followed by Bid myristoylation and insertion into the mitochondrial membrane and subsequent apoptotic events. p53 also directly regulates expression of a number of proteins that act on mitochondria, including Bax, Bak, Puma, and Noxa (Oda *et al*., 2000; Wei *et al*., 2001; Yu *et al*., 2003). Downstream of mitochondria, p53 directly controls expression of APAF1, which is involved in apoptosome activation (Moroni *et al*., 2001), and executioner caspase 6, which specifically cleaves nuclear lamins during nuclear envelope breakdown (MacLachlan and El-Deiry, 2002).

There are several notable developments with respect to our understanding of the p53-dependent apoptotic response, and how the presence of wild-type p53 might sensitize cells to chemotherapeutic drugs or radiation. One of the obvious questions has been why there are so many proapoptotic p53 targets (Figure 3). In the case of G1 cell cycle checkpoint regulation, one target, $p21^{WAF1/CIP1}$, appears sufficient to maintain an arrest and to allow time for execution of the DNA damage checkpoint. However, in the case of apoptosis, the target gene activation profile appears complex with numerous

direct p53 transcriptional targets (El-Deiry, 2001). It is possible that p53-dependent apoptosis is so important that the multiple activated targets represent necessary redundancy to succeed in tumor suppression. It is also possible that all proapoptotic targets are not always induced by p53 all the time, under all circumstances, leading to cell death. Insights into this question began to emerge from studies investigating the induction of various p53 targets in different tissues during p53-dependent apoptosis *in vivo* in irradiated wild-type and p53-null mice (Burns *et al*., 2001; Fei *et al*., 2002). These studies revealed that in response to exposure to ionizing radiation, for example in two different tissues such as the spleen and thymus that undergo similar amounts of cell death, the nature and magnitude of upregulated p53 targets can be quite variable (Burns *et al*., 2001). Recent studies using *in situ* hybridization have revealed remarkable patterns of p53 target gene activation *in vivo* (Fei *et al*., 2002). For example, in the spleen the p53-upregulated mediator of apoptosis Puma is strongly induced in the splenic white pulp, whereas Bid and Noxa appear to be induced in the red pulp. It could be predicted that genes induced in a given compartment are likely to contribute to cell death in that compartment, even though direct evidence for this is still lacking. Other insights emerged from analysis of p53 target induction *in vivo*. For example, the liver appeared resistant to ionizing irradiation-induced apoptosis, whereas the small bowel, thymus, and spleen were highly sensitive (Fei *et al*., 2002). Interestingly, it was observed that there is a strong p53-dependent induction of $p21^{WAF1/CIP1}$ in the liver, but no apparent induction of p53-activated proapoptotic targets examined (Fei *et al*., 2002). In contrast, in tissues with high levels of apoptosis, there was induction of proapoptotic targets.

Many questions remain, including a basic understanding of global gene expression patterns in various tissues treated with chemotherapy or ionizing radiation, whether gene expression patterns truly account for tissue sensitivity as appears to be the case from analysis of a small number of target genes, and whether the induced genes that mediate cell death are the same in a tumor that arises from a given tissue as the parent tissue of origin. Yet other questions remain, including whether a strong p53-dependent apoptotic response translates into a strong tumor-suppressive phenotype in a given tissue. Clearly, this is a complex issue because p53-dependent cell cycle arrest and senescence programs can perform a potent tumor-suppressive function as well. Nonetheless, the three tissues with the highest apparent p53-dependent apoptotic response may depend on p53 for tumor suppression as well. For example, it is well known that p53 knockout mice develop lymphomas of the thymus and spleen, consistent with a potent role of p53 in suppressing such tumors. On the other hand, small intestinal tumors are quite rare in humans, possibly due to the potent tumor-suppressive effect of p53. In the mouse studies, there was significantly less apoptosis in the colon following irradiation, as compared to the small bowel (Fei *et al*., 2002).

The identification of a subgroup of proapoptotic p53 targets has also recently shed some light on the long-observed ability of wild-type p53 to sensitize cells to killing by anticancer drugs or radiation (MacLachlan and El-Deiry, 2002; Sax *et al*., 2002). This group of genes has been referred to as 'chemosensitivity genes' (Sax *et al*., 2002, Sax and El-Deiry, 2003). The idea is that induction of these genes and increased expression of the encoded proteins is not sufficient to induce an efficient cell death. However, if a cell has elevated levels of the protein products encoded by these chemosensitivity genes, then cells undergo cell death very efficiently. The three known chemosensitivity genes directly regulated by p53 are APAF1, Bid, and caspase 6. The first of these genes to be identified as a p53 target was APAF1 (Moroni *et al*., 2001), which was found to be silenced in melanoma by hypermethylation (Soengas *et al*., 2001). Restoration of APAF1 expression led to efficient caspase 9 activation and anti-cancer drug-induced cell death (Soengas *et al*., 2001). The second example to be identified was caspase 6, and the key observation was that the combination of p53 overexpression and adriamycin exposure led to very efficient lamin A cleavage due to caspase 6 activation, and not higher caspase 6 expression levels due to the combination of p53 and drug exposure (MacLachlan and El-Deiry, 2002). The third example Bid was found to be important for sensitizing cells to killing, because Bid-null MEFs were more resistant to adriamycin and 5-FU exposure (Sax *et al*., 2002). Bid was also observed to be highly induced in irradiated mouse colonic epithelia in a p53-dependent manner (Sax *et al*., 2002). The model for the action of chemosensitivity genes is that the increased levels of the chemosensitivity proteins in cells can lower the threshold for death in response to chemotherapy or radiation (Sax *et al*., 2002; Sax and El-Deiry, 2003). Processing and activation of the chemosensitivity proteins, following exposure of a cell to death stimuli, greatly facilitates apoptosis in a manner due to amplification of the death signal. This is in effect a molecular mechanism for chemo- and radiosensitization that appears to depend on p53 (Sax *et al*., 2002; Sax and El-Deiry, 2003).

Directing selectivity of p53 targets and responses

One of the major questions and areas of intense investigation in the p53 field which remains is to unravel the mystery of why p53 activation causes cell cycle arrest in some situations and cell death in other situations (Vousden, 2000; Vousden and Lu, 2002). This is a very important question not only from a cell and molecular biology perspective but also from an anticancer drug development perspective. One would like to protect normal cells while killing cancer cells (El-Deiry, 2001), and it would be much better to kill a cancer than simply to arrest it in a dormant state where it might re-emerge and metastasize at a future date.

Historically, some insight into this arrest-apoptosis decision came from observations that hematopoietic

cells tended to undergo p53-dependent apoptosis more easily and efficiently than fibroblasts (Lowe *et al*., 1993; Schmitt *et al*., 2002). Thus, there has been long-standing evidence that cell type is likely to influence whether a cell is more likely to arrest or die. However, early studies to understand control of this phenotype by analysing what happened to p53 target gene activation did not reveal the answer; for example, $p21^{WAF1/CIP1}$ was induced regardless of whether cells underwent cell cycle arrest or cell death (El-Deiry *et al*., 1994). However, further insights into the arrest-apoptosis decision began to emerge as the upstream regulation of p53 began to be unraveled, along with various post-translational modifications of p53 and as additional transcriptional targets of p53 were discovered and analysed.

While there has been evidence that cells of different origins can undergo different outcomes in terms of cell cycle arrest versus apoptosis in response to cellular stresses, a more complete understanding would have to await the ability to convert a cell which normally undergoes arrest into one that undergoes apoptosis or *vice versa*. In the case of cells of different origins such as hematopoietic versus epithelial cells, one could invoke tissue-specific factors or modifications in p53 that might affect its ability to cause cell cycle arrest or apoptosis. No such tissue-specific factor has been identified yet, although several candidates that can influence the decision are worth investigating. The recent studies of Fei *et al*. (2002) looking by *in situ* hybridization at p53 target gene responses have provided some hint that p53-dependent gene activation profiles may be involved in whether there is apoptosis or arrest. The question remains in this case as to why do cells of different origins that stabilize p53 following irradiation undergo a different cell fate. One possibility is that the level of p53 in different cells may be different; however, that was not apparently the case *in vivo* (Fei *et al*., 2002).

There is now little doubt that the level of p53 achieved in a given cell can directly influence the outcome of arrest versus death (Blagosklonny and El-Deiry, 1996; Wu and El-Deiry, 1996; Blagosklonny and El-Deiry, 1998). A sustained high level of p53 is more likely to lead to cell death, although there are certainly known examples where prolonged high levels of p53 lead to prolonged arrest and senescence. However, in various experimental systems, expression of p53 at higher levels is more likely to lead to death (Blagosklonny and El-Deiry, 1996; Wu and El-Deiry, 1996; Blagosklonny and El-Deiry, 1998). Under conditions where cells are exposed to cytotoxic DNA-damaging agents, this has been rationalized as follows. Cells with irreparable damage are more likely to sustain p53 stabilization and activation, and this is more likely to lead to cell death. There has also been the idea that higher levels of p53 are more likely to lead to activation of proapoptotic targets, some of which contain lower affinity DNA-binding sites for p53. However, compelling evidence to support this notion in a given cell, based only on p53 levels and without other changes such as altered phosphorylation of p53, is still lacking. Interestingly, a recent study using a transgenic mouse model has suggested that a higher dose of p53 may more effectively suppress tumor development without a 'cost' in terms of premature senescence (Garcia-Cao *et al*., 2002).

The pattern of p53 target gene expression could be a determinant of whether cells or tissues undergo cell cycle arrest or cell death. As discussed above, there are certainly some *in vivo* data that can support such a scenario, although the mechanism remains unknown (Fei *et al*., 2002). There are also data to support the idea that p21 which causes cell cycle arrest can also protect cells from cell death. Thus, while p21 is induced by p53 in dying cells, its deletion has been found to result in greater sensitivity to killing by anticancer drugs or radiation (Waldman *et al*., 1997). A number of other experiments support the idea that target gene activation can influence the outcome of cell cycle arrest or apoptosis, and that p53 interaction with other proteins and/or post-translational modifications may influence this decision (Oda *et al*., 2000; Samuels-Lev *et al*., 2001; Flores *et al*., 2002). For example, it has become clear that the presence of p63 and/or p73 is necessary for the localization of p53 to proapoptotic target gene promoters (Flores *et al*., 2002). It is also clear that the presence of adenovirus E1A or association of proteins such as ASPP1 can direct cells to an apoptotic fate (Lowe *et al*., 1993; Samuels-Lev *et al*., 2001). There is some evidence that phosphorylation of human p53 on serine 46 can direct p53 towards activation of proapoptotic genes including p53Aip1 (Oda *et al*., 2000), although it remains unclear whether this pathway is conserved in mouse. Recent studies have also unraveled a connection between the tumor suppressor BRCA1 and the outcome by p53 in terms of arrest and repair or death in a given cell (MacLachlan *et al*., 2002). It appears that in the same cell, for example, human lung cancer or ovarian cancer cells containing wild-type p53, exposure to a cytotoxic anticancer drug such as adriamycin leads to p53 stabilization and cell death, whereas overexpression of BRCA1 leads to similar p53 stabilization and cell cycle arrest. Analysis of p53 target gene activation responses revealed that p53 stabilized by BRCA1 upregulates expression of cell cycle arrest targets and DNA repair targets, but not proapoptotic targets examined (MacLachlan *et al*., 2002). In contrast, p53 stabilized by adriamycin in the same cells leads to transactivation of proapoptotic genes. BRCA1 appears to protect cells from apoptosis induced by drugs or radiation, and this is consistent with its function in repair as a tumor-suppressor gene (El-Deiry, 2002; MacLachlan *et al*., 2002). Thus, there are now a number of experimental systems where one could attempt to determine the molecular changes that influence p53 protein in selecting target genes for activation, or indeed other effects of p53 that could lead to cell cycle arrest versus apoptosis.

Modulating p53 for therapeutic gain

There is evidence that the status of p53 in tumor cells is an important determinant not only of tumor development

and the clinical behavior of the tumor, but also of the therapeutic response (Velculescu and El-Deiry, 1996). Additional evidence linking p53 status and response to therapy has come from studies performed at NCI using a large panel of human cancer and leukemia cell lines (O'Connor *et al*., 1997; Weinstein *et al*., 1997). These studies which examined a large number of potential parameters that could determine response, as well as gene expression profiles of treated and untreated cells, concluded that the vast majority of clinically used chemotherapeutic agents are more effective in killing human tumors with wild-type as compared to mutant p53 (O'Connor *et al*., 1997; Weinstein *et al*., 1997). However, there are some clinically useful agents such as taxol, which were found to be more effective in tumor cells with mutant p53 (Weinstein *et al*., 1997). These types of screens identified chemical compounds that appeared to be more effective in killing mutant p53, but unfortunately many of these agents were also toxic to normal cells. However, it remains a possibility that further work refining structures of potentially active drugs that may be more specific for mutated p53 could be fruitful. A recent study has implicated loss of p53-dependent regulation of polo-like kinase 2 in greater sensitivity of p53-deficient cells to microtubule poisons (Burns *et al*., 2003).

A body of evidence supporting the importance of p53 in therapeutic response has come from studies of p53 wild-type versus p53-null normal cells and lymphomas (Lowe *et al*., 1993; Schmitt *et al*., 2002). The work of Lowe *et al*. (1993) using these systems showed that wild-type p53-expressing mouse thymocytes or mouse embryonic fibroblasts expressing ras and E1A were much more likely to undergo apoptosis following exposure to cytotoxic chemotherapeutic agents or ionizing radiation. More recent studies by Lowe and colleagues have elegantly demonstrated the role of p53 using green fluorescent protein-expressing murine lymphoma cells using the Eμ-myc lymphoma model (Schmitt *et al*., 2002). These studies revealed that p53-deficient lymphoma cells are slow to respond to cytotoxic therapy and invariably relapse and confer a poor survival to the mice. On the other hand, lymphomas that carry wild-type p53 but also express the antiapoptotic protein Bcl2 are also less responsive to chemotherapy, but ultimately the mice have a better survival due to the cell cycle arrest and senescent programs activated by p53 when their lymphoma cells are exposed to cytotoxic therapy (Schmitt *et al*., 2002).

Table 1 Therapeutic strategies targeting p53/p53 family

Strategy	*Reference*
Classical chemotherapy	Lowe *et al*. (1993), Schmitt *et al*. (2002)
Gene replacement	Nishizaki *et al*. (2001)
ONYX-015	Hamid *et al*. (2003), Hecht *et al*. (2003)
CP-31398	Foster *et al*. (1999), Wang *et al*. (2003)
Prima1	Bykov *et al*. (2002)
Ellipticine	Peng *et al*. (2003)
Targeting MDM2	Bottger *et al*. (1997), Midgley *et al*. (2000)
Targeting MDM2	Bottger *et al*. (1997), Midgley *et al*. (2000)
Pifithrin	Komarov *et al*. (1999)

In addition to the use of drugs such as taxol to preferentially kill mutant p53-expressing cells, there are other strategies that have been used to target such cells (Table 1). One strategy that is well into clinical trials involves the use of ONYX-015, a replication-deficient E1B-deleted adenovirus that selectively replicates in p53-deficient cells (Hamid *et al*., 2003; Hecht *et al*., 2003). Normal cells inhibit viral replication by stabilizing their endogenous p53, which is not effectively counteracted by the virus because it lacks E1B proteins. On the other hand, p53-deficient cells support viral replication and expansion, because the tumor cells lack the cellular response which prevents viral replication through p53. There is hope that this strategy may be useful for some tumor therapy, perhaps in combination with other agents. Another gene therapeutic approach involves direct replacement of p53 using a number of different vector systems including Ad-p53. This is also in clinical trials, and may be of use in combination therapy with other agents (Nishizaki *et al*., 2001).

Various strategies for targeting p53 are in preclinical development. One strategy involves the identification of agents that can block p53 : MDM2 interaction (Bottger *et al*., 1996, 1997; Midgley *et al*., 2000). Such agents may be of use in the therapy of tumors where MDM2 is overexpressed, or those in which ARF is deleted or otherwise mutated. Blockade of p53 : MDM2 interaction is expected to raise the cellular levels of wild-type p53, thereby restoring its tumor-suppressive activity in such wild-type p53-expressing tumor cells.

Another strategy that has been of interest for several years involves the identification of small molecules with potential to restore wild-type p53 activity to cells carrying mutated p53. Two such compounds have been found: CP-31398 (Foster *et al*., 1999; Takimoto *et al*., 2002b; Wang *et al*., 2003) and Prima1 (Bykov *et al*., 2002). Both of these compounds have been reported to cause cell death of tumors that carry mutated p53, and to be able to restore a wild-type epitope in mutated p53. There is more information presently about CP-31398, because it was identified earlier and has been under investigation for a longer period of time (Foster *et al*., 1999; Rippin *et al*., 2002; Takimoto *et al*., 2002b; Wang *et al*., 2003). Previous studies have revealed that CP-31398 restores a wild-type epitope to mutated p53 *in vitro*, and can cause regression of tumor xenografts without apparent toxicity to mice (Foster *et al*., 1999). Later studies showed that CP-31398 can cause either cell cycle arrest or cell death in tumor cell lines carrying mutant p53, but can also stabilize wild-type p53 and arrest or kill such wild-type p53-expressing tumor cells (Takimoto *et al*., 2002b). It is clear that higher doses of CP-31398 are required to stabilize wild-type p53, than to restore a low level of p21 expression in mutant p53-expressing cells (Takimoto *et al*., 2002b). CP-31398 appeared to not have significant effects in terms of cell death of p53-null cells, except at high doses (Takimoto

et al., 2002b). However, it has not been possible to document physical binding between p53 and CP-31398 (Rippin *et al*., 2002). Nonetheless, more recent studies demonstrated that the stabilization of wild-type p53 by CP-31398 involves a mechanism whereby ubiquitination of p53 is inhibited and the p53 is stabilized in a manner different from that induced by DNA-damaging agents (Wang *et al*., 2003). Thus, CP-31398 could stabilize p53 in ATM-deficient cells, and without inducing significant phosphorylation changes at a number of N-terminal residues including serine 15, 20, and 46 (Wang *et al*., 2003). Interestingly, CP-31398 appeared to affect the expression levels of p53 family members, further bringing into question the uniqueness of its effect on p53 (Wang *et al*., 2003). It has not been determined whether CP-31398 or any other p53-targeted drug can modulate mutant p53 in a p63-/p73-deficient background, for example, using siRNA. Thus, it is possible that the effects of the available 'mutant p53-targeted' agents may involve effects on the p53 family members p63 and p73. This speculation is of particular interest and relevance, given recent studies suggesting a role for the p53 family in chemosensitivity in the presence of mutant p53 (Bergamaschi *et al*., 2003a; Irwin *et al*., 2003; Soussi, 2003). These published studies, while suggesting that CP-31398 may not be the ideal compound in terms of specificity or efficacy, have suggested a number of avenues to improve p53 targeting. Some of these avenues might involve microarray screens to optimize p53/p53 family specificity, the isolation of agents that might specifically target p53 ubiquitination, and the combination of CP-31398 with other agents (Takimoto *et al*., 2002b; Herbert *et al*., 2003; Wang *et al*., 2003). It is clear that agents that might stimulate the p53 family in mutant p53-containing cells could be very useful. Likewise, agents that can truly restore wild-type p53 function to mutant p53, independent of effects on the p53 family, could also be very useful. With respect to its effect on wild-type p53, CP-31398 appears to alter p53 selectivity towards death receptor induction, and this may be of interest for strategies combining this agent with death ligands such as TRAIL (Takimoto *et al*., 2002b; Wang *et al*., 2003), and also for studies attempting to understand how p53 selectivity in target gene activation responses is controlled. CP-31398 also appears to increase nuclear localization of wild-type p53, and this may be of use as a sensitizing agent, perhaps in combination with p53 gene replacement therapy or in addition to wild-type p53-targeting classical chemotherapy (Wang *et al*., 2003). Much less is known about Prima1 as detailed follow-up studies of its specificity, ability to target mutant p53, and use in combination with other agents are still in progress. Yet another group of compounds including ellipticine and its derivatives may offer mutant p53 selectivity (Peng *et al*., 2003). Despite all of the stated and potentially unknown limitations, mutated p53 remains an important target for therapeutic modulation in cancer therapy.

One recent development worth mentioning here is the possibility of imaging the p53 response and therapeutic outcome *in vivo*. Recent progress in imaging p53 transcriptional activity using PET (Doubrovin *et al*., 2001) or bioluminescence imaging (Wang and El-Deiry, 2003), as well as progress in imaging correlates of therapeutic response such as apoptosis (Laxman *et al*., 2002), should accelerate the pace of drug discovery and pre-clinical testing of novel agents either alone or in combination with available cytotoxic chemotherapeutics.

The flip side of targeting mutated or wild-type p53 for enhancing cell kill are strategies that are aimed at protecting normal cells. Thus, it is well known that therapeutic gain and success results from balancing death of the tumor with death of the normal cells and tissues of the patient. It is desirable to protect normal cells while maximizing tumor cell death. One strategy has attempted to block p53 generically, using a chemical compound known as Pifithrin (Komarov *et al*., 1999). This work performed by Andrei Gudkov and colleagues led to the striking result that a single systemic dose of Pifithrin could protect a mouse from a lethal dose of ionizing radiation. This is truly remarkable and holds promise as a strategy to protect normal tissues (Komarov *et al*., 1999), even if the parent compound is not specific for p53 (Komarova *et al*., 2003). Other compounds may prove to be more p53-specific. Another approach that emerges from understanding the mechanism of action of p53 and the remarkable tissue-specific responses in terms of p53 target gene activation is the possibility of developing cocktails of, for example, siRNA oligonucleotides, for brief suppression of targets involved in tissue toxicity, but not therapeutic response towards tumor cell killing. It is clear that systemic administration of even a single dose of siRNA can alter gene expression and modulate apoptotic responses *in vivo* (Song *et al*., 2003), and that there are good models for stable suppression of gene expression *in vivo* (Hemann *et al*., 2003).

Conclusion

Much has been learned about the regulation and activation of the p53 response. This network is tightly regulated and complex with multiple feedback loops, post-translational changes, downstream targets, and tissue specificity. Both wild-type and mutant p53 remain as important targets for therapeutic modulation in cancer. The p53 family and the downstream targets of p53 also represent targets for modulation. A number of p53-targeted strategies have been under development in recent years, and these continue to hold promise for improving cancer therapeutic outcome. What are also required are greater resources, persistence, and patience to fully explore and develop the therapeutic potential of this major tumor-suppressor gene and pathway. There is still much that is not known about the p53 pathway, but many improved tools for discovery are now available to facilitate the discovery and development of new agents.

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