



Roles for p53 in growth arrest and apoptosis: putting on the brakes after genotoxic stress

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The tumor suppressor gene p53 plays a major role in regulation of the mammalian cellular stress response, in part through the transcriptional activation of genes involved in cell cycle control, DNA repair, and apoptosis. Many factors contribute to control of the activation of p53, and the downstream response to its activation may also vary depending on the cellular environment or other modifying factors in the cell. The complexity of the p53 response makes this an ideal system for application of newly emerging rapid throughput analysis techniques and informatics analysis

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Introduction

Although p53 is dispensable for normal development, it plays a central role in the cellular response to DNA damage from both endogenous and exogenous sources providing a protective effect against tumorigenesis. Indeed, mutations have been found in nearly all tumor types and are estimated to contribute to around 50% of all cancers, making p53 the most commonly mutated gene in human cancer (Hollstein *et al.*, 1991; Levine *et al.*, 1991). Transgenic mice expressing mutant p53 or p53^{-/-} 'knockout' mice with both alleles of p53 disrupted are also very prone to both spontaneous and induced tumors (Donehower *et al.*, 1992, 1995; Lavigne *et al.*, 1989). There are four highly conserved domains in p53, each of which may contribute to the cellular response to DNA damage. These include the N-terminal domain which is required for transcriptional transactivation (Fields and Jang, 1990), a sequence-specific DNA binding domain (Cho *et al.*, 1994; Pietenpol *et al.*, 1994), a tetramerization domain near the C-terminal end (Sturzbecher *et al.*, 1992), and the C-terminal domain which interacts directly with single stranded DNA (Selivanova *et al.*, 1996). Activation of p53 may result in a cell cycle delay, presumably to allow an opportunity for DNA repair to occur before replication or mitosis (Hartwell and Kastan, 1994). In some cell types, however, p53 activation results instead in apoptotic cell death as a means of eliminating irreparably damaged cells. The final outcome of p53 activation depends on many factors, and is mediated largely through the action of downstream effector genes transactivated by p53.

Known targets of p53 include genes associated with growth control and cell cycle checkpoints (e.g. *CIP1/WAF1*, *GADD45*, *WIP1*, *MDM2*, *EGFR*, *PCNA*, *CyclinD1*, *CyclinG*, *TGF α* and *14-3-3 σ*), DNA repair (*GADD45*, *PCNA*, and *CIP1/WAF1*), and apoptosis (*BAX*, *BCL-X_L*, *FAS1*, *FASL*, *IGF-BP3*, *PAG608* and *DR5*). Based on a recent survey of p53 binding sites in the human genome, it has been estimated that there may be a hundred or more p53 regulated genes yet to be discovered (Tokino *et al.*, 1994). As activation of p53 results in a cascade of downstream effects, and it is regulated by the interaction of many factors, p53 is at the nexus of a vast regulatory web. While a great deal of progress has been made in understanding this field in recent years, the complete picture is still emerging.

Modulation of p53 activity in response to DNA damage

DNA damage activates p53 protein

p53 is activated in response to DNA damage, and many factors interact to signal and modulate this response. A single double strand break in DNA is sufficient to activate p53, as has been shown by introduction of damaged DNA substrates (Huang *et al.*, 1996) and restriction enzymes (Wahl *et al.*, 1997) by microinjection. It has been necessary to use microinjection for these studies, as the p53 pathway in most cells is so sensitive it can be activated by the stress of many transfection methods, including calcium phosphate, electroporation and liposome-based reagents. p53 has been shown to bind directly to sites of DNA damage including mismatches (Lee *et al.*, 1995), and single-stranded DNA (Bakalkin *et al.*, 1994; Jayaraman and Prives, 1995), leading to the hypothesis that p53 itself serves directly as the damage detector (Lee *et al.*, 1995; Reed *et al.*, 1995), either alone or as part of the larger TFIIH recognition complex (Wang *et al.*, 1995, 1996b). p53 (Ford and Hanawalt, 1995; Li *et al.*, 1996; Smith *et al.*, 1995) and its downstream effector genes (Ford and Hanawalt, 1997; Smith and Fornace, 1996, 1997) have also been shown to play a direct role in DNA repair. Many signal transduction pathways converge on p53, however, possibly relaying the presence of DNA damage from other molecular sensors.

In contrast to many other cellular responses, induction of transcription is not a major mechanism for the acute up-regulation of p53 following DNA damage. Accumulation of p53 occurs in the presence of inhibitors of transcription and protein synthesis (Caelles *et al.*, 1994; Price and Park, 1994), and has been shown to result from stabilization of the protein

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(Maltzman and Czyzyk, 1984). Activation of increased DNA-binding and increased expression of genes containing p53-binding sites can also occur without increased p53 protein levels (Price and Park, 1994; Selvakumaran *et al.*, 1994). Under normal conditions of cell growth, p53 protein has a relatively short half-life, being rapidly targeted for ubiquitination and degradation. Following cellular stress, p53 is phosphorylated on a number of sites, increasing its half-life and transactivation activity (Meek, 1994). Many different kinase families phosphorylate p53, including DNA-PK, the casein kinase family, MAP kinases, SAP kinases and CDKs (reviewed in Meek, 1998). DNA-dependent protein kinase (DNA-PK), which is activated only in the presence of DNA strand breaks (Nelson and Kastan, 1994), has recently been reported to be required to activate sequence specific DNA binding by p53 following DNA damage (Woo *et al.*, 1998).

Phosphorylation of the sites on the C-terminal end of p53 by CDKs has been shown to activate the sequence specific binding of p53 in a manner specific for the promoters of stress-responsive genes (Hecker *et al.*, 1996; Wang *et al.*, 1995). Recently, serine/threonine protein phosphatase type 5 (PP5) has also been shown to modulate the phosphorylation and DNA binding activity of p53 to alleviate G1 arrest (Zuo *et al.*, 1998). Finally, the Atm kinase phosphorylates p53 on serine-15 and this activity is enhanced in response to ionizing but not ultraviolet radiation (Matsushime *et al.*, 1992). These phosphorylations could relay the signal from

DNA damage, and the multiple sites available for phosphorylation may provide a means for shaping the specificity of p53 activity in response to different types of cellular stress (Figure 1).

ATM, the gene mutated in ataxia-telangiectasia (AT) patients (Savitsky *et al.*, 1995), is one of the major upstream regulators of the p53 response to ionizing radiation-induced damage. AT is an autosomal recessive disease characterized by high cancer predisposition, radiation sensitivity, increased chromosome breakage, and other physiological symptoms. Cells from AT patients show reduced and delayed accumulation of p53 protein in response to ionizing radiation, indicating that *Atm* may play a role in relaying the presence of DNA damage to p53 (Kastan *et al.*, 1992; Khanna and Lavin, 1993). AT cells are also impaired in their ability to induce transcription of p53 downstream genes, including *GADD45*, *CIP1/WAF1*, and *MDM2* (Barak *et al.*, 1993; Canman *et al.*, 1994; Oliner *et al.*, 1993; Papathanasiou *et al.*, 1991). *Atm* also appears to be required for ionizing radiation-induced dephosphorylation of p53 serine 376 which allows specific binding of 14-3-3 proteins to p53 and leads to an increase in the sequence-specific DNA-binding activity of p53 (Waterman *et al.*, 1998). This may represent one of the molecular links in the chain of signal transduction from DNA damage to a p53-directed response (Figure 1). Thymocytes from *atm*^{-/-} mice show increased resistance to γ -ray-induced apoptosis, while fibroblasts from these mice are defective in G1 to S progression following serum

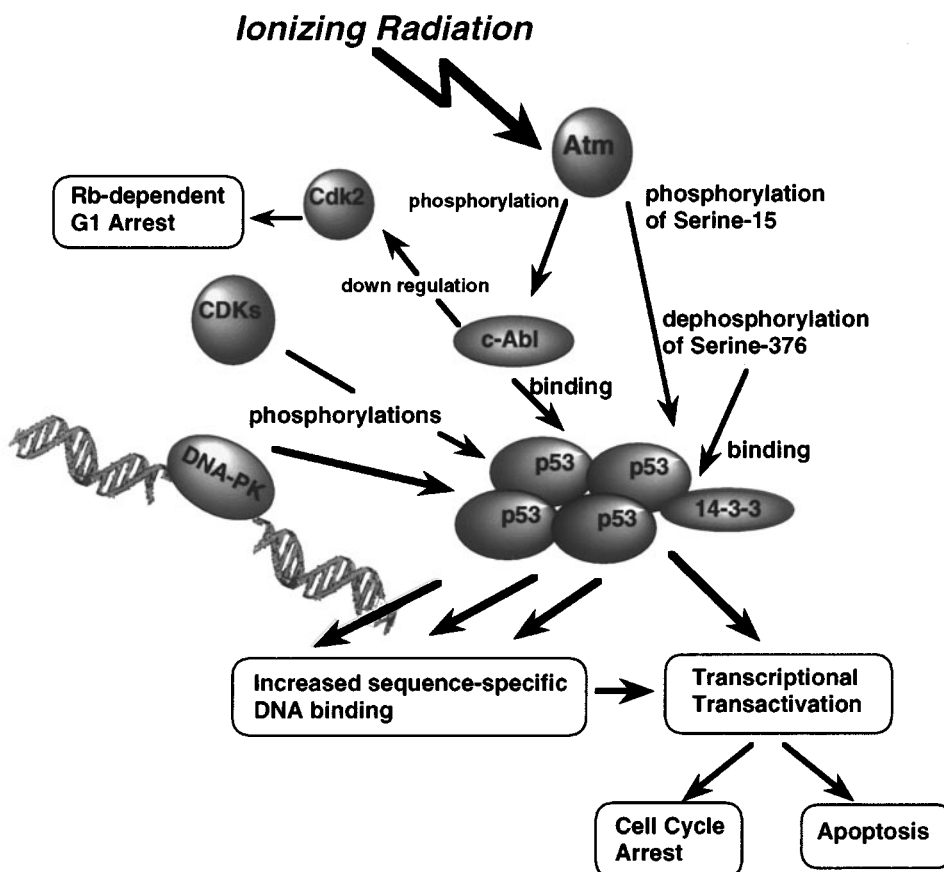


Figure 1 The transcriptional activity of p53 is modulated in response to DNA damage by the activity of a number of kinases, and by protein:protein interactions, resulting in either cell cycle arrest or apoptosis

stimulation, undergo premature senescence, and have elevated basal levels of p21^{Cip1/Waf1} (Xu and Baltimore, 1996). The knockout of *atm* in a different mouse background resulted in no difference in radiation-induced apoptosis or *bax* induction, but did lead to defective cell-cycle arrest and p21^{Cip1/Waf1} induction (Barlow et al., 1997). This suggests that differences in the cellular background may interact with the Atm/p53 pathway to modulate activation of specific downstream p53 functions. Finally, mice doubly null for both *atm* and p53 show complete resistance to radiation-induced apoptosis along with more rapid formation of tumors than seen with the knock-out of either gene alone (Westphal et al., 1997). This observation supports the idea that p53 response is not the only effector of the Atm-regulated radiation response, as the pathways do not completely overlap.

Regulation of p53 activity

p53 levels in the cell must be closely controlled to maintain cell viability. Regulation of cellular localization, active and inactive protein conformations, and protein levels all contribute to this control (reviewed in Kubbutat and Vousden, 1998). One way p53 protein levels are regulated is through an autoregulatory loop with the p53-induced gene product Mdm2. Activated p53 increases transcription from the *MDM2* promoter in a manner recently found to be dependent on interaction between p53 and the p300 transcriptional coactivator (Thomas and White, 1998). Mdm2 protein in turn binds to p53, inhibiting its transcription promoting activity (Chen et al., 1994) and targeting it for nuclear export and degradation (Nevins, 1992), thus damping the induced response, and limiting the duration of cell cycle delay (Figure 2). The importance of this mechanism is highlighted by the initial attempts to knock out *mdm2* in mice, where lack of *mdm2* was found to be a homozygous lethal condition at an early embryonic stage (Jones et al., 1995; Montes de Oca Luna et al., 1995). When *mdm2* knockouts were bred into a p53 deficient background, however, the double knockouts were viable, leading to the hypothesis that dysregulation of p53 in the original *mdm2*^{-/-} mice was the underlying cause of the observed lethality (Jones et al., 1995; Montes de Oca Luna et al., 1995).

Microinjection of anti-Mdm2 antibodies into normal human fibroblasts has recently been shown to induce p53-dependent growth arrest (Blaydes and Wynford-Thomas, 1998), suggesting that Mdm2 control of p53 is also required for normal division of human somatic cells. Recent studies have shown that the human tumor suppressor p14^{ARF} and its murine homolog p19^{ARF} can stabilize p53 by binding and antagonizing Mdm2 (Pomerantz et al., 1998; Stott et al., 1998; Zhang et al., 1998b). Overexpression of p14^{ARF} activates a p53-dependent cell cycle arrest in both G1 and G2/M with elevated levels of Mdm2 and p21^{Cip1/Waf1} (Stott et al., 1998). p19^{ARF} has also been shown to bind p53 directly, and overexpressed p53 was impaired in its ability to induce both p21^{Cip1/Waf1} and G1 arrest in *arf*^{-/-} MEFs, suggesting direct modulation of p53 transactivation activity by p19^{ARF} (Kamijo et al., 1998). Conversely, p19^{ARF} levels are unusually elevated in p53^{-/-} MEFs (Rouault et al., 1998) and can be restored to normal by restoration of functional p53 (Kamijo et al., 1998), suggesting another autoregulatory loop between p53 and p19^{ARF} (Figure 2).

A similar autoregulation operates between p53 and the product of the proto-oncogene c-Myc (Figure 2). c-Myc is a key cellular proliferative signal, and its dysregulation can contribute to cellular transformation. In contrast to Mdm2, c-Myc transcription is down-regulated following p53 activation (Moberg et al., 1992), while activation of c-Myc increases both the transcription of p53 message and the stability of the p53 protein (Hermeking and Eick, 1994), but without promoting the cell cycle arrest usually resulting from p53 accumulation. c-Myc expression also induces p19^{ARF} in MEFs, and *arf*^{-/-} MEFs are resistant to c-Myc mediated apoptosis upon serum withdrawal (Zindy et al., 1998). Furthermore, expression of c-Myc can also modulate the transcription of genes directly involved in cell-cycle arrest, some of which are p53 effector genes. This will be discussed in more detail later.

Another proto-oncogene that interacts directly with p53 to regulate its function is the nuclear tyrosine kinase c-Abl. c-Abl is activated by DNA damage (Kharbanda et al., 1995) in normal cells, but not in cells from AT patients (Shafman et al., 1997). Activation of c-Abl by ionizing radiation has recently been shown to require phosphorylation of c-Abl by the ATM kinase, and cells from AT patients and *atm*^{-/-} mice are defective in this activation (Baskaran et al., 1997). Activated c-Abl binds p53, enhancing its sequence specific transactivation ability (Goga et al., 1995). Overexpression of c-Abl results in G1 arrest, and in mouse embryo fibroblasts (MEFs) this inhibition of entry into S phase was found to require both p53 and Rb wild-type proteins (Wen et al., 1996). This represents a link between the mechanisms of two of the major known tumor suppressors. While c-Abl expression induced expression of *CIP1/WAF1*, overexpression in *waf1*^{-/-} cells still resulted in a G1 arrest, which was associated with down-regulation of Cdk2 (Yuan et al., 1996). Thus, the ATM-dependent interaction of c-Abl with p53 appears to reduce the activity of cyclin dependent kinases specifically required for entry into S phase, in addition to increasing the ability of p53 to transactivate its downstream effector genes. Overexpression of c-Abl

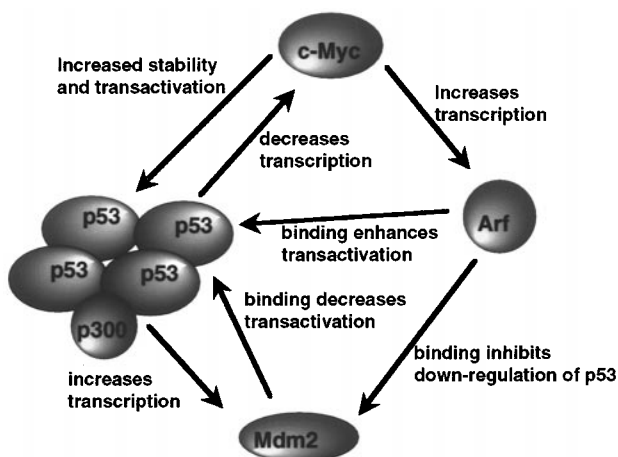


Figure 2 Several autoregulatory loops operate to limit the duration of p53 activation

in certain tumor cell lines has recently been shown to induce apoptosis, even in the absence of p53 or Rb (Theis and Roemer, 1998), perhaps indicating additional downstream targets of c-Abl in this pathway.

p53 and cell cycle checkpoints

G1 arrest

A cell can arrest its progression through the cell cycle at a number of points following the detection of DNA damage. p53 has been associated with delays in transit through both G1 and G2, as well as in a mitotic spindle checkpoint. G1 arrest is a prominent outcome of DNA damage, and is induced in many cell types by expression of exogenous wild-type p53. Activation of the G1 checkpoint in human cells shows a remarkable concordance with functional p53 status (Hartwell and Kastan, 1994). For example, in a panel of established tumor lines strong checkpoint activation was only seen in those with functional p53 (O'Connor *et al.*, 1997). In such tumor lines, checkpoint activation is transient and many of the cells will subsequently progress into S phase. The argument has been made that in normal cells, such as human fibroblasts, p53's role is not to provide a protective delay but rather to permanently remove cells from the cell cycle by a G1 checkout terminal arrest (Linke *et al.*, 1997). However, in the same study normal human bronchial epithelial cells showed a transient G1 checkpoint which was reminiscent of that seen in established lines; this again highlights the importance of cellular context for such studies. In addition, the checkout effect in human fibroblasts appears to be reversible if the cells were trypsinized and replated (Gadbois *et al.*, 1997), suggesting a role for cellular-extracellular matrix interactions in cell cycle control after irradiation.

As cells lacking normal p53 function were found to lack DNA damage-induced G1 but not G2 arrest

(O'Connor *et al.*, 1997), most early studies of p53-mediated cell cycle arrest focused on the G1 checkpoint. Induction of G1 arrest by p53 is mediated largely by the sequence specific transactivation function of p53 (Table 1) (Hartwell and Kastan, 1994). Induction of *CIP1/WAF1* in response to ionizing radiation is dependent on wild-type p53 (El-Deiry *et al.*, 1993), and p21^{Cip1/Waf1} is a G1 cyclin-dependent kinase (*cdk*) inhibitor (Harper *et al.*, 1993), giving it a clear role in cell cycle arrest. Exit from G1 and entry into S phase require the activation of G1-specific cyclin/*cdk* complexes, and p21^{Cip1/Waf1} inhibits phosphorylation and activation of Cdk2 associated with Cyclin D or Cyclin E, preventing the phosphorylation of downstream protein targets required for cell cycle progression (Askew *et al.*, 1991; Radford *et al.*, 1994). One of the critical targets of G1 cyclin-*cdk* phosphorylation is the retinoblastoma protein (pRb), which in its hypophosphorylated state, binds and sequesters E2F, a transcription factor required for entry of the cell into S phase (Chellappan *et al.*, 1991; Johnson *et al.*, 1993). Over-expression of exogenous p21^{Cip1/Waf1} results in growth arrest, presumably through inactivation of cyclin/Cdk2 complexes and the failure of Rb to release sequestered E2F. This model is supported by the fact that overexpression of E2F-1 overrides radiation-induced G1 arrest and relieves p21^{Cip1/Waf1} inhibition of cyclin/*cdk* activity (Johnson *et al.*, 1993). Fibroblasts from p21^{-/-} mice have also been found to be partially defective in radiation-induced G1 arrest (Brugarolas *et al.*, 1995; Deng *et al.*, 1995), suggesting that although p21^{Cip1/Waf1} is a major mediator of G1 arrest, it is not the only one.

Additional modifiers of p53 DNA sequence-specific transcription

Direct binding of p53 to its consensus recognition sequence is not the only mechanism through which p53 can regulate transcription of its effector genes. For

Table 1 p53 effector genes with roles in growth arrest and apoptosis

Gene	Function	Outcome
<i>CIP1/WAF1</i>	Cdk inhibitor	G1 and G2/M Arrest
<i>MDM2</i>	p53 binding	Down-regulation of p53
<i>ClnG</i>	Cyclin	G1 Arrest
<i>ClnD1</i>	Cyclin	G1 Arrest
<i>WIP1</i>	Protein phosphatase	G1 Arrest
<i>EGF-R</i>	Growth factor receptor	Cell cycle arrest
<i>TGF-α</i>	Cytokine	Cell cycle arrest
<i>Rb</i>	Sequesters E2F txn factor	Cell cycle arrest
<i>PCNA</i>	Cell cycle control, DNA replication & repair	Cell cycle arrest
<i>GADD45</i>	Interacts with Cdc2, DNA repair	G2/M arrest
<i>14-3-3σ</i>	Bind and sequester Cdc25C	G2/M arrest
<i>BTG2</i>	Bind mCAF1	G2/M arrest
<i>BAX</i>	Dimerizes with Bcl2	Promotes apoptosis
<i>BCL-X</i>	Competes with Bax for Bcl2 binding	Protects against apoptosis
<i>PAG608</i>	Nuclear zinc-finger protein	Apoptosis
<i>FAS/APO1</i>	Membrane receptor in TNFR family	Apoptosis
<i>KILLER/DR5</i>	Membrane receptor in TNFR family	Apoptosis
<i>TRID</i>	Antagonist decoy receptor	Protects against apoptosis
<i>TRUNDD</i>	Antagonist decoy receptor	Protects against apoptosis
<i>Seven in absentia</i>	Bag-1 binding; zinc-finger protein	Apoptosis or growth arrest
<i>IGF-BP3</i>	Inhibits mitogens & survival factors	Apoptosis or growth arrest
<i>PIG1 to PIG14</i>	Oxidative stress response	Apoptosis or growth arrest

instance, the p53-dependent increase in transcription of *GADD45* in response to ionizing radiation is thought to be mediated through a consensus p53 binding site in the third intron of the gene (Hollander *et al.*, 1993; Kastan *et al.*, 1992). However, p53 has also been shown to play a role in the induction of *GADD45* transcription in response to other DNA-damaging stresses, such as MMS and UV radiation, through a WT1/Egr1 site recently identified in the promoter (Zhan *et al.*, 1998b). Although p53 does not bind to this site directly, activation of transcription through the WT1/Egr1 site does require direct binding by a complex containing both p53 and WT1 (Zhan *et al.*, 1998b), another tumor suppressor. p53 is known to be able to associate *in vivo* with WT1 (Maheswaran *et al.*, 1993, 1995). An important implication for this 'indirect' role for p53 in the increased expression of the *GADD45* promoter is the possibility that p53 contributes to the induction of multiple genes lacking typical p53-binding sites. In this case, the number of genes positively regulated by p53 may be substantially greater than those (Tokino *et al.*, 1994) containing direct p53-binding sites. For instance, the stress genes *GADD153/CHOP* and *GADD34/MyD116*, lack detectable p53-binding sites and are not induced by IR, however, their induction by base-damaging agents, such as UV radiation and alkylating agents, was attenuated after disruption of p53 function (Gujuluva *et al.*, 1994; Zhan *et al.*, 1996b).

In addition to the direct effects on the regulation of p53 already discussed, overexpression of c-Myc can also modulate the basal levels and stress-responsiveness of a number of growth suppression genes, including *gas1* (Lee *et al.*, 1997), *Cyclin D1* (Philipp *et al.*, 1994), *CIP1/WAF1* (Hermeking *et al.*, 1995), *GADD34*, *GADD153*, and *GADD45* (Amundson *et al.*, 1998; Marhin *et al.*, 1997). In the case of *GADD45* we have mapped this effect of Myc to the same Egr1/WT1 binding site that is responsible for the WT1 mediated effect of p53 on *GADD45* transcription (Amundson *et al.*, 1998). The exact mechanism of the interaction of c-Myc with the *GADD45* promoter is not yet known, so it is possible that interaction with additional proteins is required to regulate *GADD45* suppression by c-Myc.

BRCA1 is a tumor suppressor gene, the loss of which is associated with familial predisposition to breast and ovarian cancer. Overexpression of *BRCA1* in cancer cell lines induces G1 arrest through a p53-independent induction of p21^{Cip1/Waf1} (Somasundaram *et al.*, 1997). *Brcal* also binds directly to p53, increasing p53-induced transcription from both the *CIP1/WAF1* and *BAX* promoters (Zhang *et al.*, 1998a). In addition, recent findings from our laboratory indicate that cotransfection with either *BRCA1* or *BRCA2* enhances p53-induced transcription of *GADD45* promoter-reporter constructs (Q Zhan, unpublished results). A recent report (Andres *et al.*, 1998) also indicates that both *BRCA1* and *BRCA2* transcription may be regulated by DNA damage in a p53-dependent manner. A pattern is starting to emerge in which gene products associated with proliferative signals (i.e. c-Myc) dampen the stress-induction of p53-responsive growth arrest genes, while those that slow cell growth (i.e. WT1, *BRCA1*, *BRCA2*), enhance the responsiveness of the same promoters, adding yet another layer of control to the p53 response.

Roles for p53 in G2/M delay

Earlier studies of p53-mediated cell cycle arrest focused on the G1 checkpoint, but it has become increasingly clear that p53 can also contribute to damage-induced checkpoints in other phases of the cell cycle. Although G2 arrest in response to ionizing radiation-induced damage does not require wild-type p53 (Kastan *et al.*, 1991; O'Connor *et al.*, 1993), p53-regulated genes can participate in the regulation of G2 arrest. Inducible systems have demonstrated that p53 overexpression can result in both G1 and G2 arrest, and that prolonged overexpression of p53 could down-regulate CyclinB1 (Agarwal *et al.*, 1995; Stewart *et al.*, 1995). The G2 checkpoint can be suppressed by caffeine and staurosporine related compounds, and this is usually accompanied by increased radiosensitivity (Iliakis, 1997; O'Connor, 1997). Interestingly, p53-deficient cells appear to be more sensitive to these compounds and at some doses both checkpoint activation and radioresistance can be decreased preferentially in the p53-deficient cells (Fan *et al.*, 1995; O'Connor, 1997; Powell *et al.*, 1995; Wang *et al.*, 1996a). This may indicate that while not required, p53 can contribute to the stringency of G2 checkpoint control (O'Connor, 1997). As the CyclinB1/Cdc2 complex is probably the major regulatory factor required for entry into mitosis (Elledge, 1996; O'Connor, 1997), decreased expression of Cyclin B1 (Maity *et al.*, 1995; Muschel *et al.*, 1991) and inhibitory phosphorylations of Cdc2 (Krek and Nigg, 1991) are thought to be the major mediators of G2 arrest. For instance, overexpression of Cyclin B1 in HeLa cells has been shown to over-ride the G2 arrest caused by ionizing radiation (Kao *et al.*, 1997).

The p53-regulated protein Gadd45 has now been shown to participate in G2 arrest. This was first indicated by the observation that microinjection of a *GADD45* expression vector into normal human fibroblasts resulted in a G2/M arrest, which could be attenuated by Cyclin B1 and Cdc25C overexpression (Wang *et al.*, 1997). Gadd45 has more recently been shown to inhibit activity of the CyclinB1/Cdc2 complex *in vitro* through disruption of the complex, probably via a direct interaction with Cdc2 (Zhan *et al.*, 1998a).

Other p53-regulated genes have also been implicated in G2 arrest (Table 1). Over-expression of p21^{Cip1/Waf1}, a major mediator of p53-dependent G1 arrest, causes cells to accumulate in both G1 and G2, and is associated with a reduction of CyclinB-associated kinase activity (Medema *et al.*, 1998; Niculescu *et al.*, 1998). Expression of p21 may also participate in a brief delay late in G2, possibly enabling late cell cycle checkpoints in normally cycling cells (Dulic *et al.*, 1998). Exogenous expression of 14-3-3 σ , another IR-induced p53-dependent gene, results in G2 arrest (Hermeking *et al.*, 1997). 14-3-3 proteins can bind and sequester phosphorylated Cdc25C, thereby preventing its dephosphorylation and activation of Cdc2 (Peng *et al.*, 1997), and providing another link between p53 and the G2 checkpoint. *BTG2* has also recently been shown to be regulated by p53, and its inactivation in ES cells led to the disruption of DNA damage-induced G2/M arrest, perhaps through loss of interaction with mCaf1 and consequent transcriptional regulation of other cell cycle regulatory genes (Rouault *et al.*, 1998).

The mitotic spindle checkpoint and p53

There is also evidence for cell cycle controls in late G2 and early M phase that may involve p53. Cells will usually not progress through mitosis in the presence of spindle inhibitors, but p53^{-/-} mouse embryo fibroblasts will undergo multiple rounds of DNA synthesis without completing chromosome segregation, leading to the formation of tetraploid and octaploid cells (Cross *et al.*, 1995). Centrosome duplication may also be regulated in part by wild-type p53, as MEFs from p53^{-/-} mice frequently produce multiple copies of functionally competent centrosomes during a single cell cycle, thus contributing to genetic instability (Fukasawa *et al.*, 1996). Moreover, expression in normal diploid human fibroblasts of HPV16 E6 (Thompson *et al.*, 1997) or SV40 large T antigen (Chang *et al.*, 1997), both of which disrupt wild-type p53 function, results in a decrease of radiation-induced mitotic delay, increased uncoupling of mitosis from completion of replication, and a reduced ability to arrest in response to mitotic spindle inhibitors, implicating wild-type p53 function in these checkpoints in human cells. Overexpression of the antiapoptotic protein Bcl-X_L has been reported to block this p53-dependent mitotic checkpoint (Minn *et al.*, 1996). The contribution of p53 to a mitotic checkpoint may actually be due to arrest of p53 wild-type cells in the next G1 following release from a transient pause in mitosis. This checkpoint has been shown to require activity of the G1 cyclin cdk inhibitor p21^{Cip1/Waf1} in addition to p53 (Lanni and Jacks, 1998). The differences between normal and p53 deficient cells in their response to mitotic poisons may provide another potential target for experimental therapeutics.

In addition to the effects of loss of p53 function on G2/M control, specific p53 mutations can confer a gain-of-function phenotype that also interferes with regulation of the spindle checkpoint. Dominant gain-of-function mutations in p53 have been shown to increase oncogenic transformation when expressed in p53-null cells (Wolf *et al.*, 1984), and to participate in tumor progression (Dittmer *et al.*, 1993; Iwamoto *et al.*, 1996; Pohl *et al.*, 1988). A specific class of dominant p53 mutations has now been identified in cells from Li-Fraumeni Syndrome patients which specifically interferes with the mitotic spindle checkpoint and promotes genomic instability (Gualberto *et al.*, 1998).

p53-dependent apoptosis

Following exposure to ionizing radiation, lymphoid and myeloid cell lines usually undergo rapid apoptotic cell death, while most non-lymphoid cell lines tend to die by necrosis or by later apoptosis during the first or subsequent mitoses (Radford *et al.*, 1994). Ectopic expression of wild-type p53 in murine myeloid leukemia cells induces rapid apoptosis (Yonish-Rouach, 1991), but there appear to be both p53-dependent and -independent apoptotic pathways. Thymocytes from p53^{-/-} mice are resistant to ionizing radiation-induced apoptosis, but not to apoptosis induced by other stresses, such as

glucocorticoids (Clarke *et al.*, 1993; Lotem and Sachs, 1993; Lowe *et al.*, 1993; Symonds *et al.*, 1994). p53-dependent apoptosis can proceed from signals other than ionizing radiation, and has been shown to be important for the tumor-suppression function of p53 (Gottlieb and Oren, 1996); e.g., p53 is required for the removal of damaged keratinocytes ('sunburn cells') after UV-irradiation of skin (Ziegler *et al.*, 1994).

Several studies indicate that sequence specific transactivation is a required function for p53-mediated apoptosis in some experimental systems (Attardi *et al.*, 1996; Sabbatini *et al.*, 1995), and an increasing number of p53-responsive genes are being associated with apoptotic pathways (Table 1). For instance, a series of 14 new p53-induced genes that are expressed prior to apoptosis was recently identified (Polyak *et al.*, 1997). Another newly isolated p53-induced gene, *PAG608*, can induce apoptosis when transiently overexpressed in human tumor cell lines (Israeli *et al.*, 1997). Further study will be required to determine the mechanism by which these genes contribute to the control of apoptosis, but some p53-regulated genes play known roles in the apoptotic pathway. For instance, Bax is a p53-induced (Miyashita *et al.*, 1994b; Zhan *et al.*, 1994) member of the Bcl-2 family of apoptosis promoting and preventing factors. Bax/Bcl-2 heterodimers suppress apoptosis signaled by a number of stresses, while Bax homodimers promote apoptosis, leading to the idea that the relative level of these two proteins in a stressed cell determines life or death (Oltvai *et al.*, 1993). Although its role in apoptosis is well established, Bax expression is neither required nor sufficient for radiation-induced apoptosis, as this process still occurs in a p53-dependent manner in thymocytes from bax^{-/-} mice, and Bax overexpression did not restore radiation-induced apoptosis to p53^{-/-} cells (Brady *et al.*, 1996).

Another member of the Bcl-2 family, Bcl-X_L, is also induced by ionizing radiation in a p53-dependent manner (Zhan *et al.*, 1996a). Unlike Bax, however, Bcl-X_L protects against apoptosis (Boise *et al.*, 1993; Gottschalk *et al.*, 1994; Schott *et al.*, 1995). Bcl-X_L may exert its anti-apoptotic effect largely through antagonism of Bax (Schott *et al.*, 1995). Thus in response to ionizing radiation, p53 appears to regulate induction of both a promoter and inhibitor of apoptosis which interact directly. Again, the balance of these two members of the Bcl-2 family is likely to play a role in determining the outcome of the apoptotic signal. Bcl-X_L may play an even greater role in protection from apoptosis, as its basal mRNA levels correlate with sensitivity to radiation-induced apoptosis in a panel of lymphoid and myeloid lines (Zhan *et al.*, 1996a) and with sensitivity to 123 standard chemotherapy agents in the cancer cell lines of the NCI anti-neoplastic drug screen panel (Amundson *et al.*, in preparation).

Fas/Apo-1 is another mediator of apoptosis which is upregulated by p53 in several cell types (Owen-Schaub *et al.*, 1995). Fas/Apo-1 is a membrane receptor protein in the tumor necrosis factor receptor (TNFR) family. Binding of the Fas ligand (FasL) to Fas/Apo-1, sets in motion a cascade of signaling events resulting in activation of the ICE-like proteases (caspases) and apoptosis (Enari *et al.*, 1995; Schlegel

et al., 1996; Tewari and Dixit, 1995). Fas-induced apoptosis can be partially abrogated by overexpression of Bcl-2 in some cell types (Itoh *et al.*, 1993), indicating cross-talk between these branches of the apoptotic pathway.

Another member of the TNFR family, *KILLER/DR5*, is also induced by p53 (Wu *et al.*, 1997) and in response to genotoxic stress (Sheikh *et al.*, 1998). Interaction of DR5 with its ligand, TRAIL, activates the cytoplasmic death domain of DR5 which in turn activates the caspase cascade culminating in apoptosis (MacFarlane *et al.*, 1997; Pan *et al.*, 1997; Screaton *et al.*, 1997; Walczak *et al.*, 1997). The antagonist decoy receptors TRID and TRUNDD are highly homologous to DR5, but lack the cytoplasmic death domain of DR5 (Pan *et al.*, 1997; MacFarlane *et al.*, 1997; Screaton *et al.*, 1997). Both TRID (Sheikh *et al.* submitted) and TRUNDD (El-Deiry *et al.* submitted) have also recently been shown to be induced by genotoxic stress and by exogenous expression of wild-type p53. DR5 and its related decoy receptors compete for binding with TRAIL, to enhance or abrogate apoptosis respectively (MacFarlane *et al.*, 1997; Pan *et al.*, 1997). The finding that all three of these receptors are induced by p53 strengthens the link between p53 and the caspase cascades, and also suggests a mechanism for the fine-tuning of apoptosis. Additional factors must influence the relative balance between DR5, TRID and TRUNDD expression, and characterization of these may yield a better understanding of apoptotic regulation.

Non-transcriptional roles for p53 in apoptosis

While transcription of p53-regulated genes can contribute to the regulation of apoptosis, p53 mutants lacking sequence-specific transactivation function have been shown to induce a slower, less efficient apoptotic response (Haupt *et al.*, 1995) in a cell-type specific manner (Haupt and Oren, 1996). p53 can also induce apoptosis without initiating *de novo* protein or RNA synthesis (Caelles *et al.*, 1994; Wagner *et al.*, 1994), and wild-type p53 can promote exit from ionizing-radiation induced G2 arrest resulting in apoptosis (Guillouf *et al.*, 1995). This evidence indicates that other functions of p53 can promote apoptosis, at least in some experimental systems. In addition to its sequence specific transactivation function, wild-type p53 also represses transcription of many genes (Ginsberg *et al.*, 1991; Hall *et al.*, 1996; Mack *et al.*, 1993). Several of these, including Bcl-2 (Miyashita *et al.*, 1994a), IGF-IR (Prisco *et al.*, 1997), and MAP4 (Murphy *et al.*, 1996), can block p53-mediated apoptosis, making their down-regulation by p53 a possible mechanism for the non-transactivational participation of p53 in apoptosis. Direct protein:protein interactions involving p53 could represent another non-transactivational mechanism for p53 regulation of apoptosis. For instance, p53 binds to the transcription/repair complex TFIIH, inhibiting the helicase activity of two of its subunits, XP-B and XP-D (Wang *et al.*, 1995, 1996b). Expression of wild-type p53 in human fibroblasts with either XP-B or XP-D mutations results in an abrogated apoptotic response which can be restored to normal by co-introduction of the appropriate wild-type XP gene (Wang *et al.*, 1996b).

Modulators of outcome – cell cycle arrest or apoptosis?

Wild-type p53 can clearly stimulate both of the major cellular responses to DNA damage, cell cycle arrest and apoptosis. Additional factors must contribute to modulation of the p53 signal to determine the final outcome of p53 activation and accumulation. Factors which influence this decision may be specific to certain cell types or may be triggered by exogenous signals from the cellular environment. The presence of growth factors can be a major determinant of this decision in certain cell types. For instance, IL-6 can protect M1 myeloid leukemia cells from p53-induced apoptosis, while erythropoietin similarly protects DP16 Friend erythroleukemia cells (Gottlieb and Oren, 1996). Following exposure to ionizing radiation, the Ba/F3 murine leukemia cell line undergoes a p53-dependent cell cycle arrest in the presence of IL-3, but rapid p53-induced apoptosis in its absence (Canman *et al.*, 1995; Collins *et al.*, 1992; El-Deiry *et al.*, 1994). Another mouse lymphoma cell line, DA-1, is similarly dependent on IL-3 for its response to radiation. Exposure of DA-1 cells to DNA damage causes p53 accumulation which leads to growth arrest in the presence of IL-3 and apoptosis without IL-3 (Gottlieb *et al.*, 1996). In DA-1 cells, the cooperation of IL-3 and p53 in determining apoptosis appears to be mediated through cleavage of the Rb protein by caspase activation following IL-3 withdrawal (Gottlieb and Oren, 1998).

The cellular context can also influence the outcome of p53 activation and accumulation. Among p53 wild-type cells for instance, thymocytes (Clarke *et al.*, 1993; Lowe *et al.*, 1993) and lymphoid or myeloid cell lines (Radford *et al.*, 1994) tend to undergo apoptosis in response to ionizing radiation, while fibroblasts undergo cell cycle arrest and retain higher viability following similar doses (Di Leonardo *et al.*, 1994). The mechanism for this broad difference between cell types is not yet known, but several genes associated with p53-dependent apoptosis have been shown to be induced by DNA damage in only a subset of cell lines. Lymphoid and myeloid human cancer cell lines, which undergo rapid apoptosis following ionizing radiation, also induce *BAX* (Zhan *et al.*, 1994) and *BCL-X_L* (Zhan *et al.*, 1996a) in a p53-dependent manner, while cells resistant to apoptosis, predominantly fibroblastoid cell lines, do not. *MCL-1* (Zhan *et al.*, 1997), *GADD34* (Hollander *et al.*, 1997), and *c-JUN* (Sherman *et al.*, 1990) are also induced predominantly in cell lines prone to apoptosis, but without the requirement for wild-type p53 function.

Some well-characterized genetic changes can also affect apoptotic propensity. Activation of oncogenes is a common factor changing the regulation of apoptosis. For instance, overexpression of c-Myc induces apoptosis in serum-deprived fibroblasts (Askew *et al.*, 1991; Evan *et al.*, 1992), but only in cells with wild-type p53 (Hermeking and Eick, 1994; Wagner *et al.*, 1994), suggesting cross-talk between p53 and c-Myc in the induction of apoptosis. Conversely, overexpression of activated c-Raf or v-Src in Ba/F3 leukemia cells results in a G1 arrest on irradiation and IL-3 withdrawal, instead of the usual apoptosis (Canman *et al.*, 1995).

The viral oncoprotein HPV-E7 binds to RB and inactivates its growth suppressive function, and human

fibroblasts overexpressing E7 undergo apoptosis in response to ionizing radiation, when without E7, they would normally undergo growth arrest in response to the same treatment (White *et al.*, 1994). This suggests a role for Rb in the diversion of the p53 signal from cell cycle arrest toward apoptosis. The usual role of Rb following stress is the sequestration of E2F, resulting in inability of the cell to enter S phase. Coexpression of E2F-1 and p53 in a fibroblast cell line abolished the p53-induced cell cycle arrest usually seen in these cells, and resulted instead in S phase progression and apoptosis (Wu and Levine, 1994). Coexpression of E2F-1 and mutant p53 or Rb, however, does not result in apoptosis (Qin *et al.*, 1994). In the absence of Rb function, E2F may drive the cell cycle forward into S phase in the presence of additional growth arrest signals from p53. These conflicting signals may result in activation of the apoptotic program. The Rb protein is a member of a family of pocket proteins, including also p107 and p130, which sequester members of the E2F growth promoting transcription factor family (reviewed in Paggi *et al.*, 1996). While pRb is the only member of this family which has frequently been found to be mutated in tumors, it is possible that alterations in the function of other family members may also contribute to the regulation of cell cycle arrest and apoptosis by p53.

It has recently become apparent that p53 is also a member of a family of closely related proteins. p73 was initially described as a candidate tumor suppressor gene in neuroblastomas which could oligomerize with itself or with p53 and which could induce transcription of the p53 target gene *CIP1/WAF1* (Kaghad *et al.*, 1997). Overexpression of p73 in p53-null human cancer cells or in baby hamster kidney cells induced apoptosis, similar to overexpression of p53 (Jost *et al.*, 1997). Unlike p53, however, p73 was found not to be induced by genotoxic damage (Mai *et al.*, 1998). No mutations were found in p73 among a number of lung cancers examined, although overexpression of p73 was noted in 24% of tumors compared to their normal tissue controls (Mai *et al.*, 1998). It is possible that overexpression of wild-type p73 could act as a dominant negative through its binding interaction with p53. Exogenous expression of wild-type but not mutant p73 in cells also expressing the E6 oncoprotein resulted in either cell cycle arrest or apoptosis, and E6 does not target p73 for degradation as it does p53 (Prabhu *et al.*, 1998). A third potential p53 family member, p53CP, binds to consensus p53 binding sites, and this binding is inhibited following genotoxic stress in p53 wild-type cells, but not in p53 mutant cells (Bian and Sun, 1997). Non-p53, p53RE binding protein (NBP) is yet another protein that binds p53 consensus response elements and enhances transcription, but which displays a different sequence preference from p53 (Zeng *et al.*, 1998). Another p53 homolog, p51, was also found to induce apoptosis and suppress colony formation in p53 null human cell lines, and to transactivate the *CIP1/WAF1* promoter, although to a lesser extent than p53 itself (Osada *et al.*, 1998). It appears likely that the status of other p53 family members could contribute to the regulation of p53 cellular responses, in the absence of p53, and that these proteins could modify the activity of p53 through their interactions with p53 or some of its targets. The

interplay between various members of the p53 and Rb families may contribute to the great diversity of stress responses observed in different cell types and tumor cell lines.

Unraveling the complexity: functional genomics approaches?

Given the heterogeneous responses of different cell lines to genotoxic stress, and the cross-talk between the many molecules we currently know to be involved, the classic reductionist approach to molecular biology is becoming inadequate for understanding p53 function. Modern integrative approaches may be necessary for clarifying our understanding of the many factors interacting in the p53 pathway and in cellular stress response in general. Techniques for obtaining expression data simultaneously for thousands of genes, potentially even for the entire genome, are becoming more readily accessible. Sophisticated pattern analysis and database mining methods coupled to rich informatics systems are being developed to digest these database-size experiment result sets. These approaches may allow us to unravel the pathways of stress signal transduction and may help identify the determinants of cell fate following DNA damage in different cell types and in different individuals.

Informatics and database mining

One example of such a database-intensive approach is the National Cancer Institute's antineoplastic drug screen (NCI-ADS) panel (Grever *et al.*, 1992; Monks *et al.*, 1991; O'Connor *et al.*, 1997; Rubinstein *et al.*, 1990; Stinson *et al.*, 1992). This is a collection of 60 human tumor cell lines which have been characterized with respect to over 300 molecular markers measured as individual gene mutations, mRNA expression, protein expression or activity. For instance, there is a striking correlation between p53 status and several markers of integrity of the DNA damage-induced G1 checkpoint among these cell lines (O'Connor *et al.*, 1997), but no correlation with G2 checkpoint. This again emphasizes the central and required role of p53 in G1 checkpoint regulation. In addition, activity 'fingerprint' profiles for over 70 000 compounds have been determined in the NCI-ADS cell lines so far, and more compounds are being tested continually. Selectivity for particular cell lines has been used by the NCI's Developmental Therapeutics Program to prioritize compounds with apparently novel mechanisms of action for further development. Five of the drugs tested in the NCI-ADS have gone on to clinical trials (O'Connor *et al.*, 1997). Characterization of the molecular factors that differentiate the cell lines has been used successfully to decode the selective cytotoxicity observed for some compounds, notably P-glycoprotein substrates (Lee *et al.*, 1994), compounds that appear to exploit differing EGFR expression (Wosikowski *et al.*, 1997), compounds that exploit ras mutation status (Koo *et al.*, 1996), and compounds that exploit the context of nm23 expression (Freije *et al.*, 1997). Continued refinement of this and similar databases may in turn aid in the discovery of

compounds that exploit discernible molecular characteristics in complex model systems allowing specific tailoring of therapy to individual well-characterized tumors (Weinstein *et al.*, 1997).

Several computer algorithms have been developed explicitly for the exploration and interpretation of the NCI-ADS data. One of the earliest was the COMPARE program, which views each chemical compound tested in the screen as a cell line selectivity pattern of 60 IC₅₀ values. Using a Pearson correlation coefficient to measure pattern similarity, COMPARE searches the accumulated database for compounds having similar selectivity patterns to a particular 'seed' compound. Frequently, matching compounds found by COMPARE share mechanism of action characteristics with the seed compound despite a lack of chemical structure similarity (Bai *et al.*, 1991; Paull *et al.*, 1992, 1995). This suggests that encoded within a compound's cell line selectivity pattern are details of its interaction with cellular components. Artificial neural networks, which are 'trained' on a data set from the NCI-ADS database, have proven to have a relatively low error-rate for identification of drugs from specified classifications based on known mechanisms of action (van Osdol *et al.*, 1994; Weinstein *et al.*, 1992). Discriminant analysis has also been successful at using the NCI-ADS patterns to differentiate predetermined mechanism of action classes (Koutsoukos *et al.*, 1994). Finally, the DISCOVERY program package is a set of tools developed for revealing coherent patterns in the data from the NCI-ADS database. For instance, the ClusCor program can cluster drugs or molecular targets by looking for correlations between the most similar patterns, potentially leading to mechanistic insights (Myers *et al.*, 1997; Weinstein *et al.*, 1997). Use of this clustering technique has revealed a strong trend of higher toxicity in cell lines with wild-type p53 for the majority of chemotherapeutic agents in the database (Weinstein *et al.*, 1997). Further characterization of the p53 pathway in the cell lines of the NCI-ADS also showed a correlation between functional measurements of p53 activity (ionizing radiation-induction of *CIP1/WAF1*, *GADD45* and *MDM2* and ionizing radiation-induction of G1 arrest) and sensitivity to 123 standard chemotherapy agents (O'Connor *et al.*, 1997). This set of 123 chemotherapy agents can be divided into about 15 classes based on well characterized mechanisms of action, and only the class of antimetabolic agents did not show a dependence on wild-type p53 for cytotoxicity (O'Connor *et al.*, 1997). This kind of information may aid both in the study of p53-dependent mechanisms of cell killing and in the identification of new chemotherapy agents with selective activity against p53-mutant tumor cells. Analysis using the DISCOVERY package has also revealed an association between topoisomerase inhibitors and a class of p53 wild-type cell lines with aberrant induction of *GADD45* (Bae *et al.*, 1996; Carrier *et al.*, 1998). Further experiments have supported this conclusion, and have led to the investigation of previously unsuspected functions of *Gadd45* in chromatin structure (Carrier *et al.*, 1998) (Smith *et al.* submitted). These examples illustrate how use of sophisticated data analysis techniques can provide new insights for the study of the p53 pathway.

High throughput expression profiling

New techniques are being developed to obtain expression profiling information for thousands of molecular targets in a single experiment. Serial analysis of gene expression (SAGE) allows comparison of relative mRNA abundance in any two samples (Velculescu *et al.*, 1995). Among other applications, SAGE has been used to study p53 as a transcriptional activator, identifying around thirty transcripts apparently induced by p53 expression (Polyak *et al.*, 1997). SAGE analysis of rat embryo fibroblasts expressing temperature sensitive p53 yielded 14 transcripts induced by wild-type p53 and three down-regulated by p53 (Madden *et al.*, 1997). An advantage of the SAGE technique is that it does not require previous identification of genes or sequence representation in any database. This could be crucial if some genes of interest are not expressed under non-stressed conditions, as EST collections are derived primarily from libraries made from untreated cells.

Another functional genomics approach to the study of p53 and stress-response is cDNA microarray hybridization (Schena *et al.*, 1995, 1996). This method requires some pre-existing sequence information, usually in the form of EST clones. It has been successfully applied to characterization of expression patterns in human cancers (DeRisi *et al.*, 1996), and studies are underway to use this technique to characterize expression patterns of the cell lines in the NCI-ADS for up to 10 000 genes (PO Brown, personal communication).

In our laboratory, we are applying cDNA hybridization technology to the study of molecular responses to DNA damage. Our initial studies have demonstrated a good concordance between induced expression measurements obtained from the microarray and by classical single cDNA probe hybridization (Bittner *et al.*, in preparation). In addition, we have identified 48 transcripts in a p53 wild-type cell line that are regulated by ionizing radiation, many not previously known to be radiation-responsive (Amundson *et al.*, submitted). Further analysis of ionizing radiation-induction of these genes in a larger panel of cell lines reveals complex induction patterns, as well as an overall heterogeneity of response in different cell lines. This data led us to explore the possibility that radiation-induction of two of the genes in this set, *FRA-1* and *ATF3*, may be regulated, at least in part, by p53. Our subsequent finding that these transcripts are induced only in the tissues of wild-type but not p53 null mice supports this idea.

The pattern of gene induction by a different stress exposure, the alkylating agent methylmethane sulfonate (MMS) differs from the pattern found with ionizing radiation, implying activation of distinct, if overlapping, response pathways. Even a relatively modest window onto the complexity of cellular stress response makes clear the need for high throughput measurements coupled with sophisticated data management and analysis techniques, such as the recently described ArrayDB (Ermolaeva *et al.*, 1998), for meaningful interpretation of stress-response pathways. It appears that cells have many redundant mechanisms for directing and responding to stress signals, and that

the function of some of these has been lost in different model cell systems. The application of such techniques as SAGE and cDNA microarray hybridization to

systems with defined genetic differences will generate data of challenging complexity, and great potential value.

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