

The p53 Network*

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Loss of control of genomic stability is central in the development of cancer, and p53, by regulating normal responses to DNA damage and other forms of genotoxic stress, is a key element in maintaining genomic stability. Thus, it is no surprise that functional p53 is lost in about half of all human cancers. What about the other half? One possibility is that p53-independent regulatory mechanisms have been lost. Another is that inactivation of p53-dependent pathways can occur at any of several different points and that p53 itself is merely the most common target. For example, the p53 inhibitor Mdm2 is overexpressed in tumors independently of the p53 mutation. Here, we review pathways that signal *in* to p53, in response to different forms of stress, and pathways that signal *out*, triggered by activated p53. It is clear that p53 is the central component of a complex network of signaling pathways and that the other components of these pathways pose alternative targets for inactivation. For additional recent reviews, see Refs. 1 and 2.

Signaling In

The amount of p53 protein increases in response to a variety of signals, such as damaged DNA, arrest of DNA or RNA synthesis, or nucleotide depletion. The same stimuli also activate p53, which is mostly latent in the absence of stress. The increase in the amount of protein is often achieved through an increase in the half-life, from ~30 min in untreated cells to ~150 min in, for example, UV-treated cells (3). However, an increase in the rate of translational initiation of p53 mRNA can also affect the steady-state level of the protein (for example, see Ref. 4). The ubiquitin pathway probably plays an important role in degrading p53 (5), and evidence for a ubiquitin-independent mechanism of degradation has also been presented (6).

Recent evidence has also shown that the Mdm2 protein, which binds to p53, accelerates its degradation, possibly through the ubiquitin pathway (7, 8). The fact that the *mdm2* gene is a transcriptional target of p53 suggests a molecular basis for the commonly observed increased metabolic half-life of mutant p53 proteins defective in transactivation. Thus, the stability of these mutant proteins appears to be due to their inability to up-regulate the expression of Mdm2, a protein involved in their degradation, rather than an intrinsic property conferring resistance to degradation *per se*.

An increase in transactivation due to p53, with no increase in the level of the protein, was found in cells treated with low doses of UV radiation, and microinjection of an antibody to the

C-terminal domain also stimulated p53-dependent transcription, even in the absence of DNA damage (9). Chernov and Stark (10) found that sodium salicylate, which inhibits protein kinases inhibits the activation of p53, with no significant effect on the accumulation of the protein. Several processes might be involved in activating p53 (1), including phosphorylation, glycosylation, binding to regulatory proteins, alternative splicing, and acetylation (11).

How does p53 sense signals? Several known proteins are suspects. The DNA-dependent protein kinase (DNAPK),¹ a plausible candidate, binds to and is activated by broken ends of DNA (12) and can phosphorylate residues 15 and 37 of p53 in a DNA-dependent manner *in vitro* (13). The phosphorylation of serine 15 affects the transactivation and growth arrest functions of p53 in some cells (14). However, cells lacking DNAPK show no defect in the p53-mediated inhibition of the cell cycle, revealing that if DNAPK has any role in regulating p53 at all, other components must be able to compensate for its loss (15).

Many protein kinases have been shown to phosphorylate p53 *in vitro* and are candidates for upstream regulators (1). However, very little *in vivo* evidence exists for the role of phosphorylation in regulating p53. Recent work showing that p53 can be acetylated *in vitro* is intriguing and suggests the possibility of an additional mechanism of regulation (11). However, it is still necessary to show that acetylation occurs in response to stress.

Poly(ADP-ribose) polymerase (PARP) has long been known to have a role in recognizing DNA damage and in DNA repair. PARP-null Chinese hamster cells are defective in activating p53 and resistant to apoptosis induced by DNA damage (16). However, embryo fibroblasts from PARP-null mice have normal DNA repair and DNA damage-induced apoptosis (17), and although there is a significant decrease in the induction of p53 protein after DNA damage or nucleotide depletion, there is no change in p53 activity or in the cellular responses to stress (18). Therefore, although PARP is involved in increasing the amount of p53 protein in mouse fibroblasts, other signaling pathways must be more important in activating p53 in response to DNA damage, consistent with experiments showing at least two levels of p53 regulation (9, 10). Loss of ATM, the product of the ataxia telangiectasia gene, slows the induction of p53 protein in response to the DNA strand breaks caused by γ -radiation but not in response to the pyrimidine dimers caused by UV radiation (19, 20). Similarly, p53 is induced normally in human ATM-null cells after treatment with *N*-(phosphonacetyl)-L-aspartate (PALA), which blocks *de novo* UMP biosynthesis, or adriamycin, which damages DNA.² p53 and ATM may both be components of complexes that function in recombination (21). Similarly, the gene product involved in Nijmegen breakage syndrome (NBS) has also been placed upstream of p53 in the pathway that responds to ionizing radiation but not in the responses to other DNA-damaging agents (22). Because the defects in p53 induction in ATM-null, NBS-null, and PARP-null cells are partial or selective for certain kinds of DNA

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¹ The abbreviations used are: DNAPK, DNA-dependent protein kinase; PARP, poly(ADP-ribose) polymerase; PALA, *N*-(phosphonacetyl)-L-aspartate; NBS, Nijmegen breakage syndrome; MAP, mitogen-activated protein; MAPK, MAP kinase; CAD, carbamyl-P synthetase/aspartate transcarbamylase/dihydro-orotase.

² M. L. Agarwal and G. R. Stark, unpublished results.

damage, these gene products are involved in some but not all of the signals. Double or triple knock-outs should have a more profound (perhaps even a complete) defect in p53 induction in response to DNA damage. Similar partial defects in p53 signaling have been observed in Fanconi anemia syndrome (FAS) and Bloom's syndrome (BLS) fibroblasts, suggesting that many pathways regulate p53 (20, 23).

Recently a role for oncogenic Ras and the mitogen-activated protein (MAP) kinase pathway in p53 modulation and function has been revealed in both human and rodent cells. High expression of Ras or activation of the Mos/MAPK pathway induces wild-type p53 levels and causes a permanent growth arrest, similar to cellular senescence (24, 25). Cells lacking p53 can tolerate high levels of MAPK and display loss of p53-dependent cell cycle arrest and enhanced genomic instability (24). In a cell line defective in the MAP kinase pathway and in p53 expression, increased expression of the MAP kinase ERK2 restores the normal levels of p53, clearly placing ERK2 in a pathway that regulates the steady-state level of p53.³ MAPK has been shown to phosphorylate residue 73 or 83 of murine p53 *in vitro*, and this phosphorylation may be important in stabilizing the protein (26). Other kinases, such as DNAPK II, cyclin A-Cdc2, and cyclin B-Cdc2, are known to phosphorylate the p53 protein *in vitro* and may play a role in stabilizing it (14, 27). The mechanisms of p53 induction in response to different types of stress are still largely unknown.

Signaling Out

p53 is involved in several different aspects of cell cycle arrest, apoptosis, control of genome integrity, and DNA repair (1, 2). How does it regulate so many different processes? p53 is a tetramer that can bind to specific sequences and thus transactivate a group of genes (reviewed in Ref. 1; for example, *p21/waf1*, *gadd45*, *mdm2*, *cyclin G*, *bax*, and *IGF-BP3*). Several groups have found that active p53 is sensed differently at different promoters, resulting in differential DNA binding and transactivation (for example, see Ref. 28). p53 can also inhibit the expression of some genes (for example, see topoisomerase IIa (29)). Furthermore, some p53-dependent phenotypes do not involve transcriptional regulation at all (for example, see Ref. 30).

Cell Cycle Controls

The G₁-S Transition—Antibodies recognizing the C terminus of p53 prevent serum-stimulated fibroblasts from entering S phase (31). This result, originally interpreted as evidence that a positive function of p53 was required, posed a paradox when overexpression of wild-type p53 was found to cause growth arrest (32). The paradox was resolved when it was found that these antibodies activate rather than inhibit p53 (9). It is now understood more clearly that p53 mediates G₁ arrest in response to DNA damage caused by UV or γ -radiation, chemotherapeutic drugs, or nucleotide deprivation (33–35). The cell-type variability in p53-dependent G₁ arrest is illustrated by studies with γ -radiation, which in normal diploid fibroblasts causes long-term, p53-dependent arrest associated with prolonged induction of p21/Waf1 (36). The irreversibility of this arrest depends on the inability of these cells to repair even a small number of double-strand DNA breaks, so that the activating signal persists (37). In contrast, γ -irradiation of HT1080 cells, derived from a fibrosarcoma with wild-type p53, causes a transient G₁ arrest (38), whereas the colorectal tumor line RKO and the breast tumor line MCF7, which also have wild-type p53, fail to arrest in G₁ following irradiation (39).

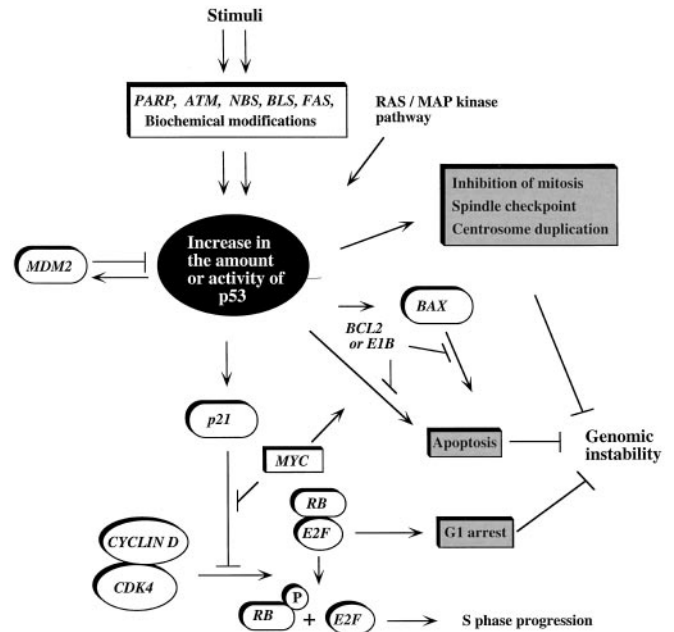


FIG. 1. Components of p53 signaling pathways. p53 accumulates and is modified and activated in response to signals generated by a variety of genotoxic stresses. Several proteins, including ATM, PARP, FAS, BLS, and NBS (see the text for full names), are involved in activation, but the pathways that lead to modification are largely unknown. The RAS-MAP kinase pathway is involved in establishing basal levels of p53 and may also affect function. Some of the cellular functions affected by p53 can be compromised by deregulated expression of Myc, Bcl2, E1B, or E2F. The control of p53 activity includes an autoregulatory loop involving Mdm2. The intact set of p53-dependent pathways helps to maintain genomic integrity by eliminating damaged cells, either by arresting them permanently or through apoptosis. p53 also helps to regulate entry into mitosis, spindle formation, and centrosome integrity, cell cycle checkpoints that are likely to be involved in preventing DNA damage from occurring.

These differences may indicate that tumor formation may involve the inactivation of components upstream or downstream of p53, causing the cellular response to DNA damage to fail. For example, γ -irradiation activates p53 to turn on the transcription of *p21/Waf1*, which binds to and inhibits cyclin-dependent kinases, causing hypophosphorylation of Rb, thus preventing the release of E2F and blocking the G₁-S transition (Fig. 1). Alteration of any of these downstream components may have an effect similar to that of inactivating p53 itself in preventing the pathway from functioning.

The Spindle Checkpoint—p53 is involved in a checkpoint that blocks the re-replication of DNA when the mitotic spindle has been damaged. When the DNA content of embryo fibroblasts was measured after treatment with nocodazole or other inhibitors of microtubule assembly, it was found that normal fibroblasts arrest with a 4N content of DNA, whereas p53-null fibroblasts attain DNA contents of 8 or 16N (40). Spindle destruction might block progression through mitosis, or re-replication might be controlled by blocking entry into S phase. In a murine cell line with wild-type p53, nocodazole causes transient mitotic arrest, followed by entry into G₁ without chromosome segregation (41). p53 is induced after mitosis is complete. The conclusion that the p53 induced in response to spindle damage blocks entry into S phase was also reached by analyzing DNA synthesis in fibroblasts exposed to nocodazole or colcemid (42). Interestingly, fibroblasts from *p21/Waf1*-null mice do not re-replicate their DNA when treated with spindle poisons, consistent with the observation that G₁ arrest in response to a number of agents is only partially abrogated in these cells (43). Therefore, p53 must also utilize p21-independent

³ M. L. Agarwal, R. Chilakamarti, W. R. Taylor, A. Agarwal, and G. R. Stark, manuscript in preparation.

ent mechanisms to arrest cells in G₁ and thus to inhibit replication in response to spindle poisons.

Centrosome Homeostasis—Embryo fibroblasts from p53-null mice acquire more than two centrosomes, leading to mitosis with more than two spindle poles and frequent mitotic failure (44). p53 is associated with centrosomes and thus may affect centrosome duplication directly (45). Alternatively, improper duplication of centrosomes may signal p53 activation, which could in turn cause arrest in G₂ or G₁. It is intriguing that MAP kinase and Cdc2, both capable of phosphorylating p53, are also bound to centrosomes (1, 26, 46, 47) and, like p53, MAP kinase is important for centrosome homeostasis (46).

The G₂-M Transition—In both human and mouse fibroblasts, overexpression of wild-type p53 can inhibit entry into mitosis (48, 49). Recent results show that this property of p53 is important in a novel cell cycle checkpoint that controls entry into mitosis when DNA synthesis is blocked.⁴ In hydroxyurea-treated cells in which synthesis of dNTPs is blocked very rapidly, p53 plays a vital role in inhibiting premature entry into mitosis. Wild-type mouse embryo fibroblasts do not attempt mitosis in hydroxyurea. In contrast, p53-null mouse embryo fibroblasts continue to attempt mitosis, entering metaphase with condensed chromatin and high levels of phosphorylated histone H1. However, cytokinesis cannot occur and mitosis is aborted, presumably because the spindles cannot segregate incompletely replicated DNA. Presumably, these responses are present to prevent the segregation of damaged or incompletely synthesized DNA.

Regulation of Apoptosis

p53 plays a role in triggering apoptosis in certain cell types, *e.g.* cells of hematopoietic origin. Stimuli such as DNA damage, withdrawal of growth factors, and expression of Myc or E1A can also cause p53-dependent apoptosis (50–54). p53 must be able to function as a transcription factor to block the G₁-S transition, but p53-mediated apoptosis does not necessarily require transcriptional activation, because inhibition of transcription by actinomycin D or translation by cycloheximide does not always affect p53-dependent apoptosis (30, 54). Furthermore, inhibitors of protein phosphatases induce p53-dependent apoptosis in the absence of transactivation (55). However, the pro-apoptotic proteins Bax and Igf-Bp3 are transcriptional targets of p53, suggesting that transactivation by p53 is important in inducing apoptosis in some circumstances. In addition, the anti-apoptotic proteins Bcl2 and the adenovirus 19-kDa E1B protein can prevent p53-mediated apoptosis (53, 56).

p53 induces apoptosis in some cell types but cell cycle arrest in others, in response to the same stimulus. Although the mechanisms of such divergent responses are not known, deletion of *p21/Waf1* can cause cells that would otherwise undergo p53-dependent cell cycle arrest to undergo apoptosis instead, underscoring the major role of genetic background in determining these cellular responses (57). Several variables, such as the extent of DNA damage and the levels of p53, also affect the choice between cell cycle arrest and apoptosis (58). Also, cross-talk between the p53 and Rb pathways may be important in determining the biological responses to DNA damage. For example, the inactivation of Rb results in the loss of G₁ arrest and induction of apoptosis after DNA damage (59). This might be explained by the release of E2F (Fig. 1), which when overexpressed on its own can induce apoptosis (60). Furthermore, overexpression of Rb blocks p53-mediated apoptosis (61). Thus,

modulation of Rb and E2F through p53 signaling in response to DNA damage may play a central role in determining the balance between cell cycle arrest and apoptosis.

Genomic Stability

p53-dependent cell cycle control maintains genetic integrity in populations of cells. Gene amplification is a widely used model to study genetic integrity. In most transformed or immortalized cells, drugs such as PALA or methotrexate, which inhibit the synthesis of nucleotide precursors, select for the amplification of target genes whose products overcome the inhibition, carbamyl-P synthetase/aspartate transcarbamylase/dihydro-orotase (CAD) in the case of PALA and dihydrofolate reductase for methotrexate (62). However, normal cells (63) and rare cell lines, such as REF52 (64), do not give rise to resistant colonies in these drugs. The function of p53 is lost frequently during the process of tumorigenesis (65) and in the spontaneous immortalization of primary cells (66), indicating that p53 can be the main factor determining permissivity for gene amplification. Indeed, embryo fibroblasts from p53-null mice are permissive for gene amplification (67), and primary human cells from Li-Fraumeni patients became permissive as soon as they lost their single copy of wild-type p53 (67, 68). Transformation of REF52 cells with either SV40 large T antigen or activated Ras plus E1A abolishes p53-dependent cell cycle control and allows these cells to become permissive for gene amplification (64). What signal is generated as a part of the mechanism of gene amplification that could activate p53-dependent pathways and prevent the propagation of drug-resistant cells? The current model of amplification involves, as an essential early step, multiple bridge-breakage-fusion cycles in which broken DNA is formed throughout an entire lineage of daughter cells (69, 70). The importance of DNA damage in regulating early stages of gene amplification was demonstrated with REF52 cells transfected with a temperature-sensitive mutant of SV40 large T antigen (71). When these cells are selected with PALA at low temperature, active large T antigen inactivates p53, rendering the cells permissive for gene amplification. Restoration of p53 by inactivating large T antigen at a higher temperature very early in the process of forming PALA-resistant colonies stably arrests all cells containing newly amplified DNA.

Human cell lines can achieve resistance to PALA by mechanisms other than gene amplification *in situ*, which is by far the most common mechanism in rodent cells. Most PALA-resistant colonies, from several different human cell lines, either do not contain amplified CAD DNA at all or increase the copy number of CAD as isochromosomes 2p (72). However, in both cases, p53-dependent pathways are still involved. The depletion of pyrimidine nucleotides caused by PALA generates a signal for p53 induction before any DNA damage occurs (35), arresting the cells and preventing PALA-resistant colonies from forming. Recent work has shown that overexpressing endogenous or exogenous N-Myc allows REF52 cells to overcome the p53-dependent cell cycle arrest caused by DNA damage, making these cells permissive for gene amplification (73). This observation emphasizes the fact that p53-dependent pathways can be inhibited at any of several different points (Fig. 1).

Concluding Remarks

p53 signaling pathways connect with tumor suppressors and oncogenes known to influence the cell cycle machinery (Fig. 1). Alterations in components either upstream or downstream of p53 may be analogous to inactivation of p53 itself, preventing all or a part of the entire pathway from functioning and leading to deregulation of cell cycle controls, genomic instability, and the development of cancer. In addition, the recently discovered

⁴ W. R. Taylor, M. L. Agarwal, A. Agarwal, D. W. Stacey, and G. R. Stark, submitted for publication.

protein p73, which has a high degree of structural and functional homology to p53, may be another important target for inactivation during the development of cancer (74). It remains to be determined if p73 affects signals impinging on or emanating from p53 or if it is a central component of its own independent signaling network.

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