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Invited Review

p53 Signaling and Cell Cycle Checkpoints

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1. p53: The Early Years

The p53 protein was first identified more than 20 years ago as a cellular protein that could be immunoprecipitated with the SV40 large T antigen (*1*, *2*), a product of the SV40 viral genome implicated in the oncogenic properties of the SV40 virus (*3*−*5*). On the basis of the association with the oncogenic large T antigen, it was hypothesized that p53 was involved in SV40-mediated transformation and thus would exhibit oncogenic properties. Further studies suggested a role for p53 in tumorigenesis, as p53 was found to co-associate with the adenovirus type 5 ElB-55 kDa viral oncogenic protein (*6*). In addition, p53 protein levels were significantly elevated in several human tumor cell lines when compared with those in primary cells, an observation supporting the proposed oncogenic properties of p53 (*7*). The identification of p53 tumor suppressor function occurred when several groups demonstrated that cells transfected with expression vectors containing wild type (wt)[1] p53 cDNA exhibited growth arrest or apoptosis (*8*−*15*).

Sequence analysis of the *p53* gene isolated from a variety of human tumor types showed that chromosomal deletions and/or point mutations in the *p53* gene are common events during tumorigenesis (*16*−*21*). These studies also showed that the high basal level of p53 expression observed in many tumor cells was indicative of mutant p53. Subsequent protein analyses of nontransformed cells confirmed that p53 is normally present at low steady-state levels in most cell types, and many mutant p53 isotypes have increased half-lives as com-

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[1]Abbreviations: ARF, alternative reading frame; AT, ataxia telangiectasia; ATM, ataxia telangiectasia mutated; ATR, ATM-related kinase; CAK, Cdk-activating kinase; Cdk, cyclin-dependent kinase; CK, casein kinase; DNA-PK, DNA-activated protein kinase; dsDNA, double-stranded DNA; IGF-1R, insulin growth factor-1 receptor; MEFs, mouse embryo fibroblasts; NER, nucleotide excision repair; p21, $p21^{Waf1/Cip1}$; PCNA, proliferating cell nuclear antigen; PI-3K, phosphoinositide-3 kinase; PKC, protein kinase C; pRB, retinoblastoma protein; ssDNA, single-stranded DNA; UV, ultraviolet; wt, wild type; XPB, xeroderma pigmentosum protein B; XPD, xeroderma pigmentosum protein D.

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pared to the wt protein due to the altered structural conformation of the mutant protein (*22*−*28*). The mutant p53 protein can also function in a dominant negative manner by binding and inhibiting the tumor suppressor activity of wt p53 (*9*, *29*−*32*). Overexpression of mutant p53 is sufficient to immortalize rat embryo fibroblasts, and coexpression of mutant p53 with an activated *Ras* or *Myc* gene can transform primary cells (*33*−*36*). Additional insight to the importance of p53 function in vivo was provided by the finding that germ line mutations of the *p53* gene occur in Li-Fraumeni syndrome, a familial disorder in which patients have a significantly elevated incidence of breast cancer, brain tumors, and sarcomas (*37*, *38*). Since these initial findings were published, numerous groups have concentrated on determining the biochemical properties of the p53 protein and the mechanism(s) of p53-mediated tumor suppression.

2. p53 Structure

The human *p53* gene encodes a 393-amino acid protein that can be divided into three distinct functional domains: an acidic amino-terminal transactivation domain, a central DNA-binding domain, and a basic carboxy-terminal oligomerization domain (Figure 1) (*39*−*42*). Each of these domains plays an important role in the regulation of p53 function. The transactivation domain (amino acids 1−73) is bound by proteins that regulate the ability of p53 to function as a transcription factor (*43*). For example, binding of the oncogenic MDM2 protein to the amino terminus of p53 inhibits p53 transactivation by blocking its association with the basal transcription machinery (*44*, *45*). The amino terminus of p53 contains a proline-rich domain (amino acids 64−92) that is necessary for efficient p53-mediated growth suppression (*46*). The proline-rich domain may dictate p53 specificity for transcriptional activation of downstream genes; for example, this domain is unnecessary for transactivation of $p21^{Waf1/Cipl}$ (p21), MDM2, and BAX but is required for PIG3 transactivation (*47*). The amino terminus also contains numerous serine and threonine residues that may be phosphorylated after cellular stress (*48*). These phosphorylations are proposed to play a role in p53 stabilization by inhibiting MDM2 binding and are discussed in more detail in a subsequent section.

The central domain of p53 (amino acids 100−293) is required for sequence-specific DNA binding (*49*, *50*). The importance of DNA binding to p53 tumor suppressor function is underscored by the finding that the majority of tumor-derived p53 mutations occur in the central domain and disrupt the ability of p53 to bind DNA (*21*, *51*). The consensus p53 DNA binding site is composed of two copies of the 10 bp sequence RRRC(A/T)(T/A)GYYY separated by 0−13 bp and is degenerate in 8 of the 10 bases, which may account for the selective activation of downstream targets after various stimuli (*52*). The human genome is estimated to contain 200−300 p53 consensus sites (*53*). Studies of the crystal structure of the central domain of p53 bound to a consensus DNA binding site demonstrate that tumor-derived mutations in the central domain alter either the structural integrity of the central domain or the amino acids that directly contact the DNA (*54*).

The basic carboxy terminus (amino acids 293−393) of human p53 contains three functional motifs: (i) a nuclear

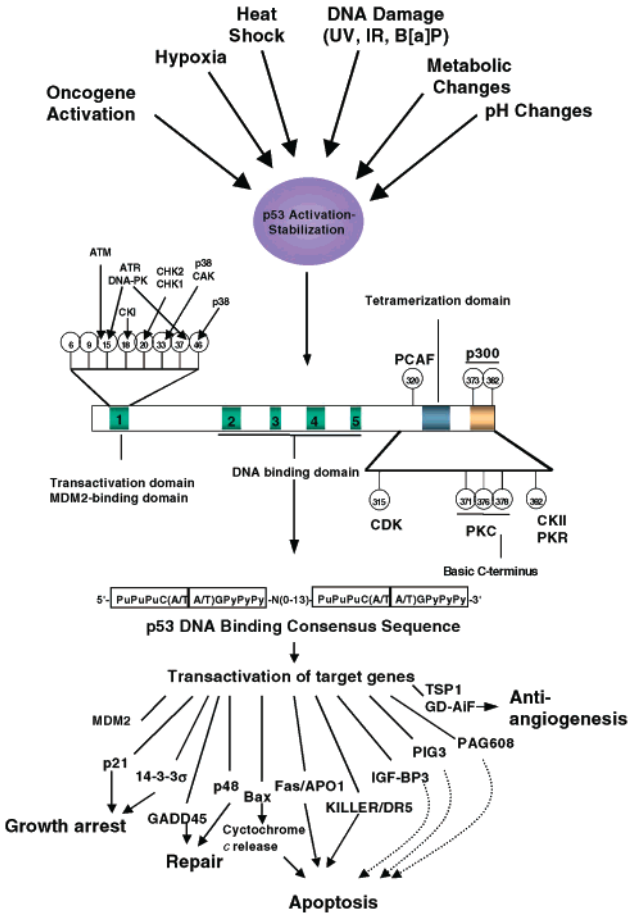

Figure 1. p53 signaling. The p53 protein is stabilized and activated after exposure of mammalian cells to a variety of stresses, including DNA-damaging agents, hypoxia, radiation, nucleotide depletion, or oncogene activation. The schematic diagram shows human p53 domain structure, specific amino acid residues that are subject to phosphorylation or acetylation after DNA damage, as well as downstream targets of p53 that mediate its biological effects. The 393-amino acid human p53 protein is diagrammed from the amino terminus to the carboxyl terminus. The transactivation domain, which includes the MDM2 interacting domain, resides between amino acids 1 and 42. The DNA binding domain resides between amino acids 100 and 300. Specific amino acid residues in human p53 that become phosphorylated and the putative kinases that are responsible are shown. DNA-PK can phosphorylate p53 at both Ser-15 and Ser-37. ATM phosphorylates p53 at Ser-15. When the amino terminus of p53 is phosphorylated after stress, p53 interacts weakly with MDM2 and becomes stabilized. p53 can be acetylated by p300 at Lys-382 and by PCAF at Lys-320. Acetylation of the carboxyl-terminal region activates p53 DNA binding in vitro. The DNA binding consensus sequence for p53 is shown. Although DNA binding occurs with up to a 15 bp space region [N(0−13)], usually for transcriptionally active binding sites, the spacer between the two 10 bp half-sites contains less than three nucleotides. p53 is a sequence-specific DNA binding transcription factor that increases the level of expression of a number of target genes that in part mediate its effects in cell cycle arrest, apoptosis, differentiation, senescence, and antiangiogenesis.

localization signal (amino acids 315−386), (ii) an oligomerization domain (amino acids 337−355), and (iii) a nuclear export signal (amino acids 340−351). The nuclear localization and nuclear export signals regulate the subcellular localization of p53 (*55*). For example, in certain breast cancer cell lines, p53 function is disrupted in the absence of mutation by inappropriate localization of the protein to the cytoplasm instead of the nucleus (*17*, *56*). The oligomerization domain is an amphipathic helix that permits p53 to form transcriptionally active ho-

modimers or homotetramers (*57*, *58*). The oligomerization domain also mediates the dominant negative effect of mutant p53 proteins, as oligomerization of tumor-derived mutant p53 with wt p53 inhibits the ability of DNA to bind and transcriptional activation of the wt protein (*59*). Tetramerization of p53 masks the nuclear export signal, thus retaining active p53 in the nucleus and preventing it from being exported to the cytoplasm where it is subject to degradation (*60*). Finally, the carboxy terminus is the site of phosphorylation and acetylation, and these post-translational modifications may regulate the ability of p53 to function as a transcription factor (*48*).

3. Role of p53 as a Transcription Factor

Since the discovery of p53 as a sequence-specific transcription factor, numerous genes and pathways regulated by p53 have been identified. p53 binds to DNA in a sequence-specific manner (*29*) and regulates the transcription of gene products involved in growth arrest, DNA repair, apoptosis, and the inhibition of angiogenesis (Figure 1) (*58*, *61*, *62*). p53-mediated growth arrest results from the p53-dependent transactivation of p21 (*63*), 14-3-3σ (*64*), and GADD45 (*65*). p53 regulates DNA repair pathways by the transcriptional upregulation of p21 (*66*−*68*), GADD45 (*65*, *69*), and the p48 xeroderma pigmentosum protein (*70*). p53-induced apoptosis is proposed to be mediated by the transactivation of BAX (*71*), Fas/APO1 (*72*), KILLER/DR5 (*73*), IGF-BP3 (*74*), AIP1 (*75*), Pidd (*76*), and the p53-inducible genes (*77*). In addition, the p53-dependent transcriptional upregulation of thrombospondin-1 (*78*, *79*), glioblastoma-derived angiogenesis inhibiting factor (*80*), and hypoxia-inducible factor-1α (*81*) has been implicated in p53-mediated regulation of angiogenesis. Another key target of p53 transcriptional upregulation is MDM2 (*82*, *83*). The MDM2 protein promotes the rapid degradation of the p53 protein, thus forming an autoregulatory feedback loop that tightly regulates p53 protein levels (*44*, *84*, *85*).

p53 also mediates transcriptional repression of proteins implicated in diverse signaling pathways. These proteins include BRCA1 (*86*, *87*), Bcl-2 (*88*), Wee-1 (*89*), c-fos (*90*), interleukin-6 (*91*), topoisomerase IIα (*92*), MAP4 (*93*), presenilin (*94*), hsp 70 (*95*), stathmin (*96*), cyclin B 1 (*97*, *98*), Cdc2 (*97*, *98*), and the insulin-like growth factor-1 receptor (*99*). p53-dependent transcriptional repression may result from p53 binding to the basal transcriptional machinery, thus prohibiting its interaction with other promoters (*100*, *101*). p53 transcriptional repression may also be mediated by its association in vivo with histone deacetylases and the transcriptional corepressor mSin3a (*102*).

4. p53 Stabilization and Activation after Cellular Stress

4.1. Ubiquitin-Mediated Regulation of p53 Protein Levels. In normal, nonstressed cells, the wt p53 protein is maintained at low steady-state levels and has a half-life of ∼20 min (*22*) due to rapid, ubiquitin-dependent degradation of the protein after synthesis (*103*, *104*). Ubiquitin-mediated protein degradation occurs by conjugation of multiple ubiquitin molecules to lysine residues of proteins that target the proteins for degradation by the 26S proteasome (reviewed in ref *105*). p53 is stabilized in cells treated with chemical inhibitors of the 26S proteasome, suggesting ubiquitin-mediated degradation regulates p53 protein levels (*104*, *106*). Further, purified components of the ubiquitin proteolytic system reconstitute the conjugation and subsequent degradation of p53 in vitro (*107*). p53 ubiquitination may also be regulated by the covalent attachment of SUMO-1 to Lys-386 of p53, as this latter modification protects p53 from ubiquitin-mediated degradation (*108*).

The MDM2 protein is a key mediator of p53 protein stability. MDM2 binds to the transactivation domain of human p53 (amino acids 20−40) (*45*) and functions as an E3 ubiquitin ligase, targeting p53 for ubiquitin-mediated proteolysis (*84*, *85*). Mutant MDM2 proteins lacking E3 ligase activity function in a dominant negative fashion, stabilizing p53 by interfering with the degradative function of the endogenous MDM2 (*109*). The in vitro ubiquitin ligase activity of MDM2 toward p53 is enhanced by the covalent attachment of SUMO-1 to Lys-446 of MDM2, although the precise role of this modification in vivo has not been elucidated (*110*). MDM2 may also influence p53 protein stability by regulating the nuclear export of p53, as mutations in the nuclear localization or nuclear export signals of MDM2 eliminate MDM2-mediated p53 degradation (*111*). The importance of MDM2 for maintenance of appropriate p53 levels in vivo is demonstrated by the fact that homologous deletion of MDM2 in mice results in early embryonic lethality that is rescued by a dual knockout of MDM2 and p53 (*112*, *113*). Further in vivo proof of the critical role MDM2 plays in p53 stability is provided by recent studies of mice genetically engineered to express a mutant allele of p53 that does not bind MDM2 (*114*, *115*). Embryonic stem cells and mouse embryo fibroblasts (MEFs) expressing this mutant p53 allele had elevated basal levels of the altered p53 protein and did not have a further increase in p53 protein levels after DNA damage, suggesting that the binding of MDM2 to p53 is a major determinant of both basal and stress-induced p53 protein levels (*114*, *115*).

Another protein that regulates the stability of p53 is JNK. JNK is a stress-activated kinase that participates in a signaling cascade activated by a variety of cellular insults, including DNA damage, osmotic stress, and cytoskeletal disruption (reviewed in ref *116*). Like MDM2, JNK binds to human p53 (amino acids 97−116) (*117*) and targets p53 for ubiquitin-mediated degradation in murine cells (*118*). Overexpression of an amino-terminal JNK fragment in mouse fibroblasts prevented p53 stabilization, reduced the level of p53-dependent transcription, and increased the level of apoptosis after hydrogen peroxide or UV irradiation treatments, providing evidence for the biological relevance of JNK-mediated regulation of p53 protein levels (*119*). Further, p53-deficient rat cells have elevated basal JNK kinase activity and impaired induction of further JNK kinase activity following UV irradiation (*120*). This latter study suggests an autoregulatory feedback loop exists between JNK and p53, which is analogous to that occurring by p53-dependent transcriptional upregulation of MDM2. In this model, JNK activation stimulates its release from p53 and results in p53 stabilization. However, p53-dependent signaling inhibits JNK activity, thus facilitating the binding of JNK to p53 and establishing a regulatory cascade for controling p53 protein levels.

4.2. Phosphorylation of the p53 Protein. Phosphorylation of p53 may be important for p53 stabilization and activation after genotoxic stress or disruption of

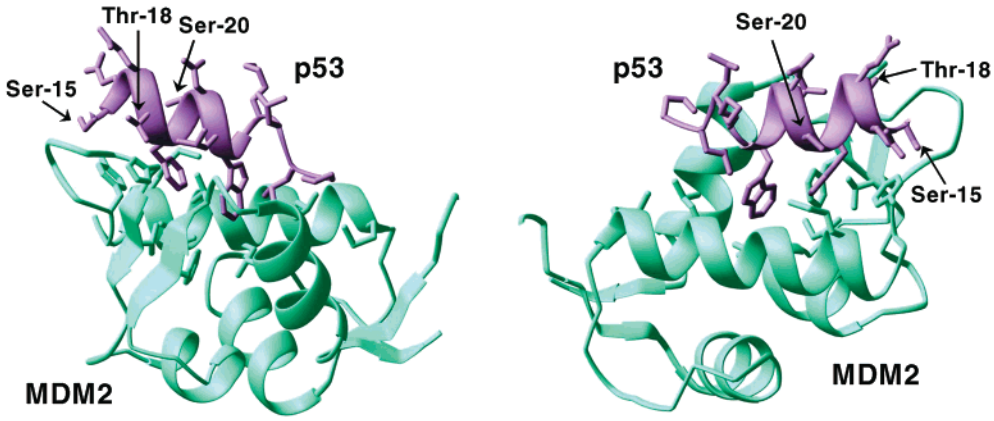

Figure 2. Two views of a human p53 amino-terminal peptide [amino acids 15−29 (purple)] binding to an MDM2 amino-terminal peptide [amino acids 1−109 (green)]. p53 forms an amphipathic helix whose hydrophobic face binds the MDM2 cleft. Only p53 amino acids are labeled. These views were modeled from the structure in the Protein Data Bank (1YCR) (*123*). Amino acids 15 and 16 were modeled as an α-helix.

microtubule dynamics. Studies utilizing phosphopeptide mapping and phospho-specific antibodies have identified eight phosphorylation sites in the amino-terminal transactivation domain (serines 6, 9, 15, 20, 33, 37, and 46 and threonine 18) and five phosphorylation sites in the carboxy-terminal oligomerization domain of human p53 (serines 315, 371, 376, 378, and 392) (Figure 1). DNA damage-induced phosphorylation of the amino terminus of p53 is hypothesized to disrupt the binding of MDM2 to p53, thus allowing stabilization of p53. Phosphorylation of the carboxy terminus of p53 may regulate the nuclear localization of p53 and p53-mediated transactivation (*121*, *122*). Numerous cellular kinases mediate phosphorylation of p53 after ionizing irradiation and UV irradiation. Of note, there are distinct differences between the specific residues that are phosphorylated after ionizing irradiation and UV irradiation, as well as the kinases that mediate these phosphorylations (Figure 1). However, the biological impact of phosphorylation on p53-mediated cell cycle arrest and apoptosis and the interplay among the kinases that phosphorylate p53 in response to different damaging agents remains to be elucidated.

The crystal structure of the amino terminus of MDM2 in complex with the amino terminus of human p53 shows that amino acids 18−26 of p53 form an amphipathic helix that fits into a deep hydrophobic groove in MDM2 (*123*) (Figure 2). Thr-18 is in the region of direct contact with MDM2 in contrast to Ser-15, Ser-20, and Ser-37 which lie outside the region of direct contact (*123*, *124*). Phosphorylation of Thr-18 may introduce a charge−charge repulsion that destabilizes the p53−MDM2 interaction. In contrast, phosphorylation of Ser-15, Ser-20, or Ser-37 is not as likely to have a direct effect on altering the stability of the p53−MDM2 complex. Biochemical evidence in support of these hypotheses is given by the finding that an amino-terminal peptide of p53, phosphorylated at Thr-18, binds weakly to MDM2 as compared to the unphosphorylated peptide or peptides phosphorylated at Ser-15 or Ser-37 (*125*). Furthermore, phosphorylation of Ser-15 stimulates p53 transactivation but does not alter the interaction between p53 and MDM2 (*126*). However, Craig et al. (*127*) concluded that p53 peptides phosphorylated at either Ser-15, Thr-18, or Ser-20 all equivalently disrupted the p53−MDM2 interaction in vitro. Further studies are necessary to determine the in vivo relevance of p53 phosphorylation at specific residues.

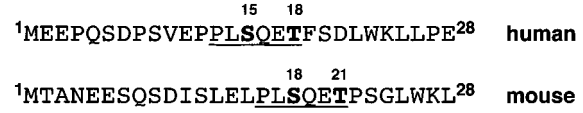

Figure 3. Alignment of the human and mouse p53 protein sequences over the first 28 amino acids. In mouse p53, the homologous residues for human Ser-15 and Thr-18 are Ser-18 and Thr-21, respectively. The underlined residues flanking the phospho residues are 100% conserved across species.

Several members of the phosphoinositide-3 kinase (PI-3K) family directly phosphorylate p53 at amino-terminal residues after both ionizing irradiation and UV treatment, including DNA-activated protein kinase (DNA-PK), ataxia telangiectasia mutated (ATM) kinase, and ATM-related kinase (ATR) (*128*−*132*). DNA-PK is activated in vitro by double-stranded DNA (dsDNA) breaks and phosphorylates human p53 at Ser-15 and Ser-37 in vitro (*128*). However, ionizing radiation induces phosphorylation of murine p53 at Ser-18 (homologous to Ser-15 of human p53, Figure 3) in DNA-PK-deficient MEFs, indicating that either DNA-PK does not mediate Ser-18 phosphorylation in vivo or it can be accomplished independently by another kinase(s) after ionizing radiation (*133*−*136*). Like DNA-PK, ATM is also activated by dsDNA breaks and phosphorylates human p53 at Ser-15 in vitro (*129*, *130*, *137*). ATM function is defective in patients with ataxia telangiectasia (AT), a disorder in which patients have increased sensitivity to radiation and are highly cancer prone (*138*). Cells from AT patients have delayed p53 stabilization and significantly diminished levels of Ser-15 phosphorylation after ionizing irradiation (*129*, *139*, *140*). In contrast, p53 stabilization and Ser-15 phosphorylation are not impaired in AT cells after UV irradiation or treatment with topoisomerase inhibitors (*130*). DNA-PK and ATM also phosphorylate human MDM2, preventing formation of the p53−MDM2 complex in vitro (*141*, *142*); however, the biological significance of MDM2 phosphorylation in p53 regulation is not well defined to date.

ATR also phosphorylates p53 at both Ser-15 and Ser-37 in vitro (*131*, *132*). Overexpression of a catalytically inactive ATR reduces the level of Ser-15 phosphorylation at late times after ionizing radiation, suggesting that ATM mediates early Ser-15 phosphorylation, while both ATM and ATR participate in Ser-15 phosphorylation at later times (*131*). In contrast, ectopic expression of the kinase-defective ATR decreases the level of both early

and late Ser-15 phosphorylation of p53 after UV irradiation, and cells expressing the kinase-defective ATR display increased sensitivity to ionizing and UV irradiation, potentially from defective p53 activation (*131*, *143*). Caffeine, a compound known to induce radiosensitization, inhibits the catalytic activity of both ATM and ATR at drug concentrations similar to those that induce radiosensitization (*132*, *144*). Caffeine also inhibits the ionizing and UV radiation-induced phosphorylation of p53 at Ser-15 (*144*). These data suggest that the radiosensitizing effects of caffeine are related to inhibition of the protein kinase activities of ATM and ATR and the subsequent loss of p53 stabilization mediated by Ser-15 phosphorylation.

After ionizing irradiation, ATM also phosphorylates and activates Chk2 (*145*−*148*), while ATR-dependent signaling mediates activation of Chk1 after UV irradiation (*149*, *150*). Chk1- and Chk2-mediated phosphorylation of human p53 at Ser-20 may be important for p53 activation through stabilization after DNA damage (*151*−*153*). Chk1 and Chk2 phosphorylate p53 at Ser-20 in vitro, and this phosphorylation dissociates p53−MDM2 complexes (*152*, *153*). Ectopic expression of Chk1 or Chk2 increases the level of p53 stabilization in human cells, whereas expression of kinase-defective mutants of either Chk1 or Chk2 abrogates both Ser-20 phosphorylation and stabilization of p53 (*152*, *153*). Chk2−/− embryonic stem cells have defective p53-dependent signaling and cell cycle arrest after ionizing irradiation, a phenotype similar to that of p53−/− cells (*151*). Further, *Chk2* germ line mutations occur in patients with classical Li-Fraumeni syndrome lacking mutations in the *p53* gene, indicating that germ line inactivation of the *Chk2* or *p53* gene results in a similar phenotype (*154*). Since substitution of Ser-20 with alanine abrogates p53 stabilization after exposure to either ionizing or UV irradiation, Chk1 and Chk2 play an important role in p53 activation after DNA damage (*155*).

Since UV radiation induces bulky lesions in the DNA as compared to the dsDNA breaks associated with ionizing irradiation, it is not surprising that distinct kinases phosphorylate p53 after these specific types of DNA damage. One such UV radiation-specific kinase is the p38 stress-activated kinase, which phosphorylates human p53 at Ser-33 and Ser-46 after UV irradiation but not ionizing radiation (*156*). Inhibition of p38 kinase activity or mutation of Ser-33 and Ser-46 to alanines results in a reduced level of p53-mediated apoptosis and decreased sensitivity to UV irradiation, suggesting p38 plays an important role in p53 signaling after UV exposure (*156*). The related JNK kinase mediates phosphorylation of murine p53 at Ser-34, and the corresponding residue in human p53 is Ser-33 (*157*, *158*). However, there is no direct evidence that JNK phosphorylates human p53, providing evidence that there is species-dependent phosphorylation of p53 after various types of DNA damage (*159*).

Members of the casein kinase (CK) family phosphorylate p53. CKI phosphorylates human p53 at Ser-6 and Ser-9 and the corresponding residues of murine p53 in vitro (*160*−*162*). CKI also phosphorylates human p53 at Thr-18 in vitro, a modification that requires prior Ser-15 phosphorylation (*125*, *163*). While the level of phosphorylation at Ser-6, Ser-9, and Thr-18 of human p53 increases after ionizing and UV irradiation, further studies are necessary to determine if CKI mediates DNA damage-induced phosphorylation of human p53 at these residues in vivo (*125*, *160*). CKII phosphorylates human p53 at Ser-392, a modification proposed to control p53-mediated transactivation (*121*, *164*). UV irradiation induces phosphorylation of Ser-392, resulting in an increased level of sequence-specific DNA binding (*165*, *166*). Phosphorylation of the CKII site of p53 does not occur after ionizing radiation treatment, indicating DNA damage-specific post-translational modifications occur (*165*, *166*). Further, mutation of Ser-389 in murine p53 (corresponding to human p53 Ser-392) to alanine inhibits the antiproliferative function of p53, suggesting this residue plays an important role in p53 function in vivo (*167*).

The double-stranded RNA-activated protein kinase PKR also phosphorylates Ser-392 of human p53 (*168*). PKR-dependent activation of p53 may be necessary for TNFα-induced apoptosis, as inhibition of p53 expression in PKR-overexpressing cells abrogated TNFα-mediated apoptosis (*169*). Conversely, overexpression of wt p53 in PKR-deficient cells rendered the cells susceptible to TNFα-induced apoptosis, indicating that p53 induction is a downstream event of the TNFα-induced upregulation of PKR (*169*). Of note, p53-mediated transcription and cell cycle arrest are impaired in PKR−/− MEFs, suggesting PKR plays an important role in p53 activation in vivo (*170*).

Protein kinase C (PKC) mediates phosphorylation of three carboxy-terminal serine residues of human p53: Ser-371, Ser-376, and Ser-378 (*171*). These three serines lie in consensus sites for PKC, and phosphorylation of these residues in vitro by PKC enhances sequence-specific DNA binding by p53 (*172*). However, the in vivo biological relevance of PKC-mediated phosphorylation of these sites has not been fully elucidated. Again, species-dependent phosphorylation of p53 by PKC signaling is observed, as phosphorylation of carboxy-terminal sites of murine p53 is not induced after treatment of cells with 12-*O*-tetradecanoyl phorbol 13-acetate, a known activator of PKC (*173*). In contrast, treatment of both human and murine cells with PKC inhibitors increased the half-life of p53 and led to accumulation of p53 in the nucleus; however, the overall level of p53 phosphorylation decreased, and an increase in the level of p53-specific transcription was not observed (*174*). These results may be partly explained by the finding that Ser-376 and Ser-378 of human p53 are constitutively phosphorylated in unstressed cells (*175*). Further, Ser-376 is dephosphorylated in response to DNA damage in an ATM-dependent manner, resulting in enhanced binding of 14-3-3 proteins to p53 which mediates the increased level of sequence-specific DNA binding by p53 (*175*). Despite these recent findings, the precise role of PKC isoforms in DNA damage-induced dephosphorylation of p53 requires further investigation.

Several kinases that participate directly in cell cycle pathways phosphorylate p53. One such kinase is the Cdk7/cyclin H kinase (CAK), which phosphorylates both Ser-33 and a carboxy-terminal site of human p53 (*176*, *177*). Further, both Cdc2 and Cdk2 phosphorylate human p53 at Ser-315 (*178*, *179*). Phosphorylation of Ser-315 by Cdc2 and Cdk2 may influence the nuclear localization of p53 and regulate p53 binding to a subset of p53 consensus DNA binding sites (*122*, *180*). However, wt p53 and p53 containing Ser-315 mutated to alanine were equally effective at activating transcription when cotrans-

Table 1. Post-Translational Modifications of Human p53

residue	modification	enzyme	stimulus	effect	ref
Ser-6	phosphorylation	CK1	Chemo	ND[a]	*160*
Ser-9	phosphorylation	CK1	Chemo	ND	*160*
Ser-15	phosphorylation	ATM/ATR DNA-PK CHK1	IR UV Taxol	inhibits binding to MDM2	*128−132, 140, 152, 190, 325*
Thr-18	phosphorylation	CK1	IR	follows phosphorylation of Ser-15 inhibits binding to MDM2	*125, 126*
Ser-20	phosphorylation	CHK1 CHK2	IR UV	inhibits binding to MDM2	*151−153, 190*
Ser-33	phosphorylation	p38 CAK	IR UV	may be constitutive	*156, 176*
Ser-37	phosphorylation	DNA-PK CHK1 ATR	IR UV	inhibits p53 transactivation inhibits binding to MDM2	*128, 132, 140, 152, 190*
Ser-46	phosphorylation	p38	UV	may enhance p53-dependent apoptosis	*156*
Ser-315	phosphorylation	CycA/Cdk2 CycB1/Cdc2	ND	may regulate nuclear localization increases level of sequence-specific binding	*178−180*
Lys-320	acetylation	PCAF	IR UV	inhibited by Ser-378 phosphorylation	*186, 190*
Ser-371	phosphorylation	PKC	ND	ND	*174*
Lys-373	acetylation	p300	IR UV	increases level of sequence-specific binding	*186*
Ser-376	phosphorylation	PKC	ND	inhibits 14-3-3 binding; dephosphorylated after IR	*175*
Ser-378	phosphorylation	PKC	IR	enhances 14-3-3 binding; increases level of sequence-specific binding	*172, 175*
Lys-382	acetylation	p300	IR UV	increases level of sequence-specific binding inhibited by Ser-378 phosphorylation	*185*
Ser-392	phosphorylation	CKII p38 PKR	UV	increases level of tetramerization increases level of transactivation increases level of sequence-specific binding	*121, 165, 168, 322*

[a] ND, not determined.

fected into NIH3T3 cells with a reporter construct, suggesting this modification mediates other cellular functions of p53 in vivo (*179*). The Cdc14 phosphatase also interacts with p53 in vivo and specifically dephosphorylates Ser-315 of p53, indicating that the phosphorylation status of Ser-315 may modulate the function of p53 by altering the subcellular localization of p53 (*181*). Taken together, these studies suggest that regulation of p53 phosphorylation by cell cycle kinases and phosphatases plays an important role in mediating the cellular activity of p53 (Figure 1).

Recent studies have suggested that phosphorylation of p53 at the amino terminus is not essential for p53 stabilization and activation after DNA damage (*182*, *183*). In these studies, previously described amino-terminal and carboxy-terminal phosphorylation sites of human p53 were mutated individually and in combination to alanine, and the mutant proteins were examined for their ability to be stabilized and to mediate p53-dependent transactivation after DNA damage. The majority of the mutant proteins were stabilized and retained transactivation function equivalent to that observed for the wt p53 protein after genotoxic stress (*182*, *183*). The only protein with altered function was a mutant protein in which all previously described carboxy-terminal phosphorylation sites were modified to alanine, as this protein exhibited a reduced level of transactivation of endogenous p21 (*182*). These results suggest that phosphorylation of p53 is not essential for DNA damage-induced stabilization of p53; however, p53 function was evaluated under conditions of overexpression in these studies. Phosphorylation of p53 may not be necessary for transcriptional activity of p53 if the level of overexpressed protein is sufficient to overcome MDM2-mediated degradation, which is likely to occur in overexpression systems.

Perhaps the best model systems for evaluating the biological effects of p53 phosphorylation are mice engineered to express specific phospho mutants of p53 in place of wt p53 alleles (*115*, *184*). Chao et al. recently utilized homologous recombination to introduce a Ser-18 to alanine point mutation in both endogenous p53 alleles in murine embryonic stem cells (analogous to mutating human p53 at Ser-15, Figure 3) (*184*). Phosphorylation of murine p53 at Ser-18 was required for complete p53-dependent responses after both ionizing and UV irradiation. The embryonic stem cells carrying phospho mutant p53 alleles had a significantly reduced level of p21 induction and growth arrest after treatment as compared to wt p53-containing cells (*184*). These results suggest that phosphorylation of murine p53 at Ser-18 is required for complete p53-dependent response. Further, the study of Chao et al. exemplifies the need for comprehensive studies of p53 phospho mutant proteins in vivo; however, given the number of residues modified in p53 and in vitro studies that suggest interplay between the various residues, such a study may not be a feasible undertaking without a large collaborative effort.

4.3. Acetylation of the p53 Protein. p53 is also modified by acetylation at the carboxy terminus after DNA damage, a modification proposed to increase the level of sequence-specific DNA binding by p53. Acetylated p53 has enhanced binding to its canonical DNA site in vitro (*185*, *186*). p53 associates with transcriptional coactivators p300/CBP (CBP) and PCAF, both of which possess intrinsic histone acetyltransferase activity (*187−189*) (Table 1). CBP acetylates human p53 at Lys-373 and Lys-382, while PCAF acetylates human p53 at Lys-320 (*185*, *186*, *190*). Studies utilizing antibodies specific to p53 peptides acetylated at Lys-320 or Lys-382 demonstrate that the level of acetylation at both sites increases in vivo after exposure to ionizing or UV radiation (*186*, *190*). Phosphorylation of p53 at Ser-15 also enhanced the ability of p53 to recruit CBP, resulting in an increased level of acetylation of the carboxy terminus of p53 in vitro (*191*). However, mutation of Ser-18 of murine p53 to alanine in embryonic stem cells (corresponding to human

p53 Ser-15, Figure 3) did not prevent acetylation of the carboxy terminus of p53 in these cells after UV irradiation (*184*). Further, human p53 peptides phosphorylated at Ser-33 or Ser-37 differentially inhibited p53 acetylation by CBP and PCAF in vitro (*190*). These results suggest that after DNA damage, a phosphorylation/acetylation cascade occurs, in which phosphorylation of the p53 amino terminus regulates the ability of the carboxy terminus to undergo acetylation by CBP and PCAF, a modification that enhances DNA binding by p53.

4.4. Protein−Protein Interactions. In addition to phosphorylation, protein−protein interactions can disrupt MDM2 binding to p53. These interactions include a protein encoded by the *INK4a/ARF* locus in human cells. This locus encodes the cyclin-dependent kinase inhibitor $p16^{INK4A}$ (p16), as well as an alternative reading frame product, $pl4^{ARF}$ (ARF). While p16 regulation of the retinoblastoma protein (pRB) has been recognized for several years, only recently was the role of ARF in p53 regulation discovered. ARF gene expression is induced by oncogenic stimuli such as viral oncoprotein expression or elevated levels of MYC or RAS (*192−194*). When present in the cell, the ARF protein binds to MDM2 and disrupts MDM2-mediated degradation of p53 (*195−197*). ARF binding to MDM2 also inhibits MDM2 ubiquitin ligase activity (*198*) and results in MDM2 nucleolar localization (*199*). The nucleoloar sequestration of MDM2 blocks MDM2-mediated nuclear export of p53, thus preventing the cytoplasmic degradation of p53. The biological importance of ARF in the activation of p53 signaling pathways is exemplified by the finding that ARF-deficient mice develop spontaneous tumors and have accelerated tumor progression after carcinogen exposure similar to p53-null mice (*200*). These findings provide the molecular basis for the stabilization of p53 observed in cells after oncogenic stimulation and demonstrate that this signaling pathway is distinct from that activated by genotoxic stress.

The pRB protein also forms a tertiary complex with MDM2 and p53, resulting in the inhibition of p53 degradation and enhanced apoptosis (*201*). However, the interaction of pRB with MDM2 does not prevent MDM2 from binding to p53; thus, the pRB−MDM2 complex does not prevent MDM2 from inhibiting p53-dependent transcription (*202*). In summary, regulation of p53 stability in response to cellular stress is mediated by several biochemical mechanisms that ultimately prevent MDM2-mediated p53 degradation.

5. p53 Regulation of Cell Cycle Checkpoints

5.1. Cell Cycle Checkpoints. At key transitions during eukaryotic cell cycle progression, signaling pathways monitor the successful completion of upstream events prior to proceeding to the next phase. These regulatory pathways are commonly termed cell cycle checkpoints (*203*). Cells can arrest at cell cycle checkpoints temporarily to allow (i) cellular damage to be repaired, (ii) the dissipation of an exogenous cellular stress signal, or (iii) availability of essential growth factors, hormones, or nutrients. Checkpoint signaling may also result in activation of pathways leading to programmed cell death if cellular damage cannot be repaired. Defects in cell cycle checkpoints can result in gene mutations, chromosome damage, and aneuploidy, all of which can contribute to tumorigenesis (*204*).

5.2. G1/S Checkpoint. The most extensively studied cell cycle checkpoint occurs at the G1/S transition. Cells will arrest cell cycle progression at the G1/S checkpoint in response to DNA damage to prevent the replication of mutated DNA. Negative regulation of G1 phase cyclin−Cdk complexes plays a key role in the G1/S checkpoint function after DNA damage (*205*, *206*). Cdks are negatively regulated by a group of functionally related proteins called Cdk inhibitors (reviewed in ref *206*). p53 plays a critical role during the DNA damage-induced G1/S cell cycle checkpoint, as p53-deficient cells fail to undergo a G1/S arrest after genotoxic stress (*11*, *12*, *14*). After exposure of cells containing wt p53 to genotoxic agents, p53 is activated and transcriptionally upregulates the Cdk inhibitor p21 (*63*). p21 binds and inactivates cyclin−Cdk complexes that mediate G1 phase progression, resulting in pRB hypophosphorylation, E2F sequestration, and cell cycle arrest at the G1/S transition (Figure 4). p53-dependent induction of p21 also results in an S phase checkpoint response, as p21 binds to the proliferating cell nuclear antigen (PCNA) and prevents PCNA from mediating recognition of the DNA primer−template complex, thus inhibiting the elongation step in DNA replication (*207*, *208*). Mice with homozygous deletion of the *p21* gene develop normally, but p21−/− MEFs are deficient in their ability to undergo a G1/S arrest in response to DNA damage (*209*, *210*). Further, deletion of both p21 alleles from a human colon cancer cell line containing wt p53 abrogated DNA damage-induced G1/S arrest, confirming the requirement for p21 in the p53-dependent G1/S checkpoint (*211*).

5.3. G2 Checkpoint. In addition to activation of the G1/S checkpoint, DNA damage also activates checkpoint pathways that result in G2 arrest. The DNA damage-induced G2 arrest is mediated by signaling cascades that converge to inhibit the activation of Cdc2 (*212*). After DNA damage, members of the PI-3K family, including ATM and ATR, become activated and initiate signal transduction pathways that regulate G2 arrest. ATM phosphorylates and activates Chk2 after ionizing irradiation (*145−148*), while ATR-dependent signaling mediates activation of Chk1 after UV irradiation (*149*, *150*). Activated Chk1 and Chk2 then phosphorylate Cdc25C at Ser-216, generating a consensus binding site for 14-3-3 proteins (*145*, *213−215*). Binding of 14-3-3 proteins to Cdc25C results in the nuclear export and cytoplasmic sequestration of Cdc25C and G2 arrest due to inhibition of Cdc2 activity (*216*) (Figure 5). As previously described, ATM and ATR can directly phosphorylate human p53 (*129−132*, *137*), and recent studies demonstrate that Chk1 and Chk2 mediate phosphorylation and stabilization of p53 after DNA damage (*151−153*).

While the importance of p53-mediated signaling in G1/S checkpoint function is well-documented, the role of p53 signaling at the G2 checkpoint has only recently been defined. Early studies showed that p53-deficient cells maintain the DNA damage-induced G2 arrest, suggesting p53 signaling does not regulate the G2 checkpoint (*217*, *218*). However, expression of p53 in the absence of cellular stress induces cell cycle arrest at both the G1 and G2 checkpoints (*219−221*). Further, p53 and p21 are necessary for maintaining a G2 arrest following DNA damage, as tumor cells lacking these proteins enter into mitosis with accelerated kinetics (*97*, *222*). The mechanism of p53-dependent G2 arrest involves an initial inhibition of cyclin B1−Cdc2 activity by p21 followed by

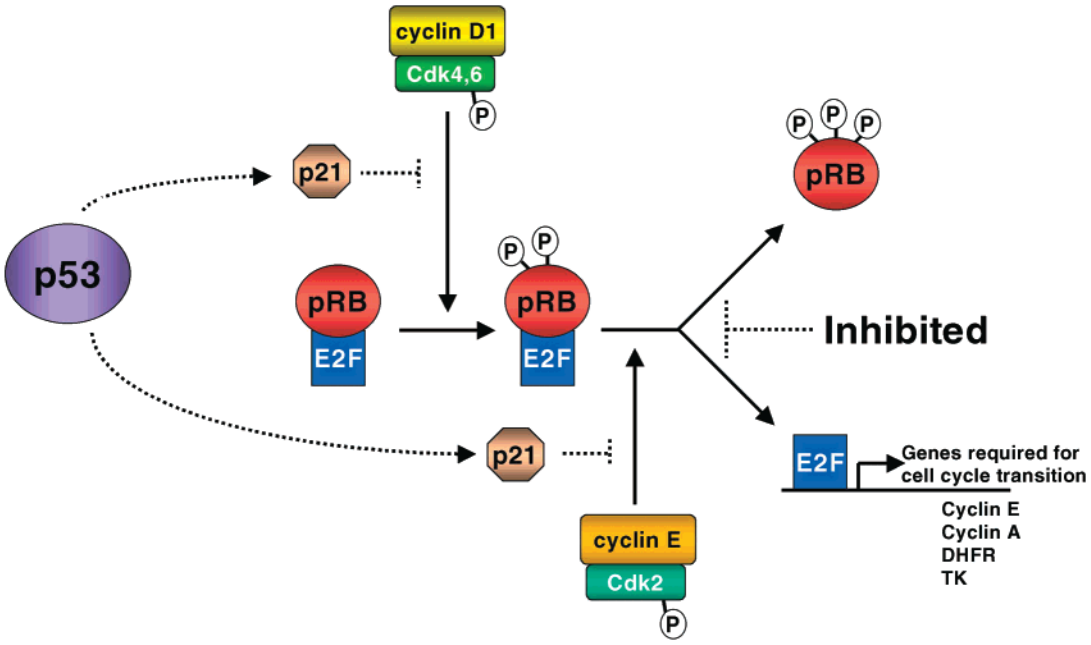

Figure 4. G1/S checkpoint. During G1 progression, there is sequential activation of cyclin D1 and cyclin E complexes. Cyclin D1 binds to Cdk4 and -6, and cyclin E binds to Cdk2. These complexes undergo activating phosphorylation and, once active, phosphorylate pRB. In its hypophosphorylated state, pRB binds to E2F to inhibit S phase entry; however, once hyperphosphorylated, pRB releases E2F. The release of E2F results in activation of genes required for S phase entry. Cdk inhibitors, such as p21 (a p53 downstream target), can inhibit the binding of either cyclin and Cdk subunits or the activated kinase complexes to mediate a G1/S cell cycle arrest. DHFR represents dihydrofolate reductase and TK thymidine kinase.

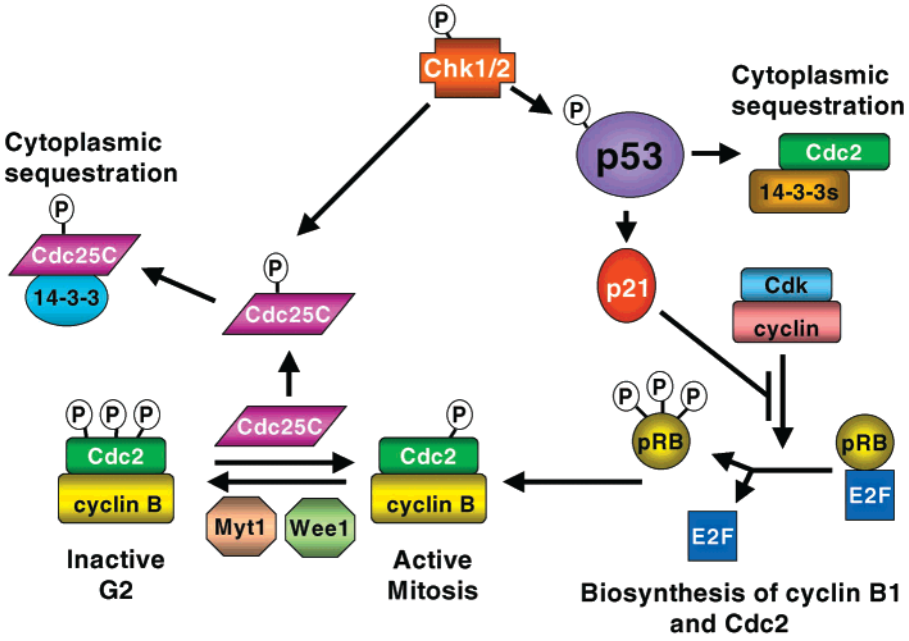

Figure 5. G2 checkpoint response after genotoxic stress. During the G2/M transition, the activity of Cdc2 is tightly regulated by phosphorylation and protein interactions. Cdc2 activation requires association with cyclin partners and phosphorylation by CAK. During S and G2 phases, cells accumulate the cyclin B1−Cdc2 complex in an inactive form due to inhibitory phosphorylations by Weel and Myt1 kinases. The conversion of Cdc2 from an inactive to active form is mediated by the Cdc25C phosphatase. Mitotic events are stimulated by active cyclin B−Cdc2 complexes. In response to genotoxic stress, the Chk1 and Chk2 kinases are activated and phosphorylate Cdc25C. This phosphorylation promotes the interaction of Cdc25C with 14-3-3 adaptor proteins and inhibits the ability of Cdc25C to activate the cyclin B1−Cdc2 complex, resulting in cell cycle arrest at the G2/M transition. After DNA damage, activated Chk1 and Chk2 also phosphorylate p53, resulting in stabilization and activation of the protein. p53-dependent signaling contributes to maintenance of the G2 cell cycle arrest by upregulating the 14-3-3σ protein that binds to Cdc2 and sequesters the kinase in the cytoplasm. p53-dependent transcription also elevates the Cdk inhibitor p21, which binds to cyclin−Cdk complexes to reduce the level of phosphorylation of pRB. Hypophosphorylated pRB remains bound to E2F, preventing E2F from mediating the biosynthesis of cyclin B1 and Cdc2.

a reduction of cyclin B1 and Cdc2 protein levels (*97*, *223*, *224*). The decreased level of expression of the cyclin B1−Cdc2 complex is mediated in part by p53-dependent transcriptional repression of the cyclin B1 and Cdc2 promoters and is pRB-dependent (*97*, *98*) (Figure 5). The importance of p53-dependent regulation of Cdc2 activity

is exemplified by the finding that constitutive activation of the cyclin B1−Cdc2 complex overrides p53-mediated G2 arrest (*225*).

p53 may also exert G2 checkpoint responses through transcriptional upregulation of the downstream target genes, p21, 14-3-3σ, and GADD45. Similar to its regulation of the cyclin−Cdk complexes at the G1/S checkpoint, p21 binds to and inhibits the G2/M regulatory cyclin B1−Cdc2 complex in vitro, although it has significantly lower affinity for this complex than the G1 phase kinase complexes (*223*, *226*). Loss of 14-3-3σ also results in abrogation of the DNA damage G2 checkpoint and premature mitotic entry (*227*). The p53-dependent increase in the level of 14-3-3σ is thought to modulate the subcellular localization of the cyclin B1−Cdc2 complex, as the binding of 14-3-3σ to Cdc2 results in retention of the kinase in the cytoplasm (*227*). The increased level of expression of GADD45 in primary fibroblasts results in a G2 arrest that can be abrogated by the overexpression of cyclin B1 or Cdc25C (*228*). This GADD45-induced G2 arrest is p53-dependent since overexpression of GADD45 in p53-deficient fibroblasts fails to mediate a G2 arrest (*228*). Subsequent studies have shown that GADD45 directly inhibits the cyclin B1−Cdc2 complex after UV irradiation (*229*). Of note, the GADD45-dependent G2 arrest is induced only after specific types of DNA damage, as lymphocytes from GADD45 knockout mice failed to arrest after exposure to UV radiation, but activated the G2 checkpoint after ionizing radiation (*228*).

5.4. Spindle Checkpoint. The proper coordination of mitotic exit and subsequent S phase entry is integral to cell cycle regulation. After DNA synthesis, cells have a tetraploid (4*N*) DNA content that is reduced to a diploid (2*N*) DNA content in each daughter cell after successful completion of mitosis. The mitotic spindle checkpoint monitors spindle microtubule structure, chromosome alignment, and chromosome attachment to kinetochores during mitosis and delays chromosome segregation until defects in the mitotic spindle are corrected (reviewed in ref *230*). Cells with defective spindle checkpoint function can exit from mitosis with a 4*N* DNA content, inappropriately enter the S phase with a 4*N* DNA content, and generate polyploid cells, a process known as endoreduplication (*231*).

p53 was initially hypothesized to function directly in the mitotic spindle checkpoint because p53−/− MEFs endoreduplicate after treatment with microtubule inhibitors, whereas p53+/+ MEFs do not generate polyploid cells under the same conditions (*232*, *233*). p53 also localizes to centrosomes; thus, it was proposed that p53 is necessary for mitotic arrest to occur after disruption of microtubule dynamics (*232*, *234*). However, p53+/+ and p53−/− MEFs exit mitosis with equivalent kinetics after treatment with microtubule inhibitors (*235*). Further, elevation in p53 protein levels in p53-containing cells after microtubule inhibitor treatment occurs only after the cells exit mitosis and proceed to the G1 phase with a 4*N* DNA content (*236*, *237*). Recent experiments demonstrate that p53-dependent inhibition of endoreduplication requires transactivation of p21 which inhibits Cdk2 kinase activity at the G1/S transition to block S phase entry after an aberrant mitotic exit (*237*). Further, ectopic expression of p21 in p53-deficient cells restores Cdk2 regulation and prevents endoreduplication after microtubule inhibitor treatment (*237*). Thus, in addition to regulating checkpoint function after DNA damage, p53 also mediates the G1/S checkpoint to prevent inappropriate S phase entry after an abnormal mitotic exit and is critical for proper coordination of the S phase and mitosis.

6. Role of p53 in DNA Repair

After genotoxic stress, p53 modulates DNA repair through multiple mechanisms, including sequence-specific transactivation and direct interaction with components of the repair machinery (*238*). Initial evidence which shows that p53 could regulate DNA repair pathways came from studies using Li-Fraumeni syndrome-derived cell lines that contain a germ line transmission of a single mutant p53 allele (*239*). Global genomic repair is compromised in Li-Fraumeni fibroblasts and cells in which p53 is disrupted by E6, as repair of UV-induced dimers is slower than in cells with wt p53 (*240*−*243*). A role for p53 in transcription-coupled repair has also been proposed, as Li-Fraumeni fibroblasts and E6-expressing cells exhibit a delay in the recovery of mRNA synthesis after UV irradiation (*244*). However, this delayed mRNA synthesis may represent a defect in a postrepair mRNA recovery process and not transcription-coupled repair.

p53 sequence-specific transactivation-dependent DNA repair occurs in part by transactivation of p21. In addition to mediating a G1/S cell cycle arrest, induction of p21 by p53 results in an S phase checkpoint response, as p21 binds to PCNA and blocks PCNA-mediated recognition of the DNA primer−template complex, thus inhibiting the elongation step in DNA replication (*207*, *208*). While PCNA has a dual role in DNA replication and repair, p21 specifically inhibits PCNA-mediated DNA replication while allowing PCNA-regulated DNA repair (*66*). p53 also induces expression of GADD45 (*65*), another protein that binds to PCNA (*69*). Induction of GADD45 stimulates DNA excision repair in vitro and inhibits the entry of cells into the S phase (*69*). Further, abrogation of GADD45 expression decreases the level of DNA repair and sensitizes human colorectal carcinoma cells to UV radiation, supporting the importance of GADD45-mediated DNA repair in vivo (*245*).

p53 sequence-specific transactivation-dependent DNA repair is also mediated by transactivation of the xeroderma pigmentosum *p48* gene (*70*). The p48 protein functions in global genomic repair of UV-induced cyclobutane pyrimidine dimers, and humans with defective p48 function have increased UV sensitivity and a predisposition to skin cancer (*246*). The efficient repair of UV-generated DNA lesions also requires activation of p53 (*241*, *243*). Further, both p53−/− and p48-deficient cells have impaired global genomic repair, suggesting p48 mediates p53-dependent DNA repair after UV damage (*70*).

p53 also directly interacts with proteins that function in DNA repair pathways. Most of these proteins are members of the TFIIH complex that initiates basal transcription of RNA polymerase II and couples transcription with nucleotide excision repair (NER) (*238*). For example, interaction of wt p53 with xeroderma pigmentosum protein B (XPB) and xeroderma pigmentosum protein D (XPD) inhibits the intrinsic DNA helicase activity of these proteins, while tumor-derived mutant p53 proteins do not inhibit XPB and XPD helicase activity (*240*, *247*). p53 also binds to the Cockayne syndrome B repair helicase (*240*) and replication protein A (RPA), a trimeric protein complex that functions in DNA replica-

tion, homologous recombination, and NER (*238*). After DNA damage, RPA is phosphorylated, causing dissociation of RPA−p53 complexes and allowing RPA to participate in NER (*248*).

p53 may also be involved in DNA repair through direct interaction with DNA. Soon after p53 was discovered, it was shown that p53 binds to short single-stranded DNA (ssDNA) (*249*). Subsequently, Tegtmeyer's laboratory demonstrated that p53 can anneal two ssDNA strands and has strand transfer activity (*250*). These interactions with ssDNA are of interest because such DNA fragments are intermediates of DNA damage and have been shown to activate p53-dependent growth arrest and apoptosis (*251*). Interestingly, Jayaraman and Prives reported that short ssDNA fragments stimulate p53 binding to its consensus site in vitro (*252*). Further proof that p53 interacts with damaged DNA comes from Griffith's group which used electron microscopy and gel shift assays to show that p53 binds insertion/deletion lesions (*253*). These studies suggest that the direct interaction of p53 with damaged DNA is an upstream event in p53 activation. From saturation binding studies, the K_D of p53 binding to insertion/deletion lesions was determined to be 45 pM as compared to a K_D of 31 pM for p53 binding to DNA fragments containing a consensus binding site (*254*). Although p53 has a higher affinity for DNA with a consensus site than for DNA with insertion/deletion lesions, the relative number and availability of each form of DNA in a cell immediately after DNA damage may promote p53 interaction with DNA lesions.

The carboxy terminus of p53 has been implicated in the majority of studies exploring the phenomenon of p53 binding nonspecifically to DNA lesions (*250*, *253*). Initial experiments suggested that the carboxy terminus of p53 binds ssDNA ends, whereas the central DNA binding domain of p53 binds to internal ssDNA segments (*255*). However, Zotchev et al. recently demonstrated that the carboxy terminus of p53 interacts with both ssDNA ends and internal ssDNA gaps (*256*). Association of the carboxy terminus of p53 with ssDNA ends facilitates the binding of the p53 core domain to DNA, whereas interaction with ssDNA internal gaps prevents p53 core domain−DNA complexing, implying that p53 recruits different repair factors depending of the type of DNA lesion (*256*).

p53 also possesses intrinsic 3′→5′ exonuclease activity that is associated with the core domain of the protein (*257*). This exonuclease activity may play an important role in p53-mediated repair, as tumor-derived p53 mutants are exonuclease-deficient and cells expressing mutant p53 are defective in global NER (*238*). p53 may also participate in the repair of endogenous DNA damage through its 3′ → 5′ exonuclease activity, as wt p53 protein, but not mutant p53, enhances the DNA replication fidelity of DNA polymerase α, an enzyme that lacks 3′ → 5′ exonuclease activity (*258*). Clearly, a further understanding how the sequence-specific and nonspecific DNA binding activities of p53 are integrated will contribute to our knowledge of how signaling cascades are initiated after DNA damage.

Numerous studies have utilized p53-deficient mice and cells derived from these animals to examine the role of p53 in DNA repair. Experiments using a transgenic mouse shuttle vector-based mutagenicity assay (Big Blue) have shown similar rates of mutation in p53−/− and p53+/+ mice (*259*). However, only tissues from liver, spleen, and brain were examined in this latter study; thus, a role for p53 in DNA repair in other tissues cannot be excluded (*259*). Similarly, Sands et al. found no difference in the rate of DNA damage-induced point mutations in genomic DNA isolated from fibroblasts and thymocytes derived from p53+/+ or p53−/− mice (*260*). However, examination of basal, undifferentiated keratinocytes derived from p53+/+ and p53−/− mice found a significantly reduced level of NER repair after UV irradiation in p53-deficient keratinocytes (*261*). Since nonmelanoma skin cancers originate from basal keratinocytes, this finding suggests that loss of wt p53 plays an important role in the pathogenesis of such skin cancers (*261*). Assays measuring the rate of DNA repair synthesis after UV irradiation in p53+/+ and p53−/− fibroblasts verified that p53-deficient cells have impaired NER as compared to wt p53-containing cells (*262*). Finally, several independent groups have demonstrated that p53−/− mice have an increased incidence and rate of formation of skin tumors after exposure to UV radiation as compared to p53+/− or p53+/+ mice, providing in vivo evidence for the importance of p53 in DNA repair after UV exposure (*263*−*265*). In summary, p53 plays an integral role in maintaining genomic integrity after DNA damage by its ability to mediate DNA repair and replication processes by multiple mechanisms.

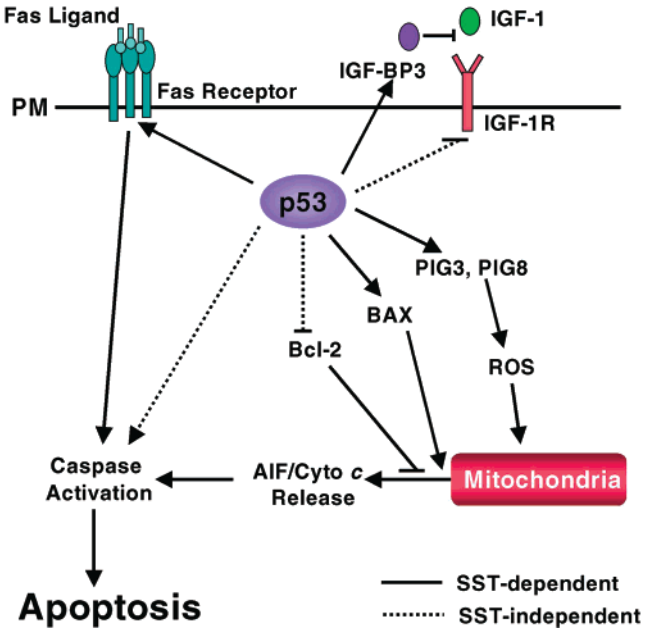

Figure 6. p53-mediated apoptotic signaling. p53-dependent apoptosis is mediated by both sequence-specific transactivation-dependent (—) and sequence-specific transactivation-independent (---) pathways. Several p53 target genes can promote apoptosis, including the pro-apoptotic BAX protein and the Fas death receptor. BAX facilitates the release of AIF and cytochrome *c* from the mitochondria, thus activating the caspase cascade. p53 inhibits expression of the anti-apoptotic Bcl-2 protein, which normally blocks apoptosis by preventing the release of AIF and cytochrome *c* from the mitochondria. In addition, p53-dependent induction of IGF-BP3 induces apoptosis by blocking IGF-1R survival signaling. p53 may mediate mitochondrial signaling by elevating the level of reactive oxygen species through PIG3 and PIG8 induction. SST represents sequence-specific transactivation.

7. Role of p53 in Apoptotic Signaling

Several p53 target genes can promote apoptosis, including the pro-apoptotic BAX protein and the Fas/CD95 ligand (Figure 6). However, significant cellular death is

only observed when several of these genes are expressed in concert, suggesting that p53 apoptotic target genes need to activate parallel apoptotic pathways to induce programmed cell death (*266*). The p53 apoptotic target genes can be divided into two groups; the first group encodes proteins that act through receptor-mediated signaling, and the second group encodes proteins that regulate apoptotic effector proteins. p53-dependent transactivation of IGF-BP3 induces apoptosis by blocking IGF-1 survival signaling to the IGF-1 receptor (IGF-1R). When combined with p53-dependent repression of the IGF-1R, p53 signaling results in a highly efficient block of this survival pathway (Figure 6) (*267*). p53 also mediates apoptosis through activation of the Fas/APO1/CD95 (Fas) and KILLER/DR5 death receptors. Fas is induced by p53 in response to genotoxic stress and is necessary for T-cell-mediated apoptosis in response to anticancer drugs (*267*, *268*). Tumor cells expressing wt p53 are more sensitive to drug-induced apoptosis mediated by Fas-dependent signaling (*269*), and p53-mediated apoptosis is impaired in MEFs derived from Fas-deficient mice and Fas ligand-deficient mice (*270*).

Early cell changes that occur during apoptosis are associated with mitochondrial changes mediated by members of the Bcl-2 family of proteins, including anti-apoptotic Bcl-2 and pro-apoptotic BAX proteins. In addition to the previously described p53 transrepression of Bcl-2 (*88*), p53 may also inhibit Bcl-2 function through transactivation of Cdc42 (*271*). Cdc42 initiates a signaling pathway resulting in Bcl-2 phosphorylation and inactivation, and the expression of a Bcl-2 phosphorylation resistant mutant inhibits both Cdc42- and p53-mediated apoptosis (*271*). In contrast to Bcl-2, BAX expression is upregulated during p53-dependent apoptosis (*71*). BAX facilitates the release of the apoptosis-inducing factor and cytochrome *c* from the mitochondria, thus activating the caspase cascade (*267*). The subcellular localization of the BAX protein may be a critical determinant in p53-dependent apoptosis, as translocation of BAX from the cytosol to the mitochondria is required for p53-dependent apoptosis (*272*). The apoptotic activity of BAX is also directly implicated in tumor suppression, as *BAX* mutations have been observed in human colon tumors (*273*, *274*). Further, BAX expression in breast cancer cell lines expressing wt p53 increased cell death after exposure to genotoxic drugs, demonstrating the importance of BAX activation in p53-dependent apoptosis (*275*).

p53 may also mediate mitochondrial apoptotic signaling through the elevation of the level of reactive oxygen species (Figure 6). A recent study identified a panel of 14 p53-induced genes that are activated after oxidative stress (*77*). One of these genes, PIG3, encodes a protein that is homologous to NADPH-quinone oxidoreductase, an enzyme that generates reactive oxygen species (*77*). However, overexpression of PIG3 in human tumor cells fails to initiate apoptosis, suggesting that other signaling pathways must be activated for programmed cell death (*276*). Another of these p53-inducible genes, PIG8, has been localized to human chromosome 11q23, a region frequently altered in human tumors (*277*, *278*). While the amino acid sequence of PIG8 offers little insight into its function, ectopic expression of PIG8 in cells inhibits cell colony formation and induces apoptotic cell death (*278*). The precise biological functions of the p53-inducible gene products and the roles they play in apoptotic signaling after oxidative stress await further characterization.

p53 may also mediate apoptosis by sequence-specific transactivation-independent mechanisms; however, observations supporting transactivation-independent mechanisms have only been made in cell culture-based, in vitro systems. For example, Caelles et al. demonstrated that cells expressing a temperature-sensitive p53 mutant protein undergo apoptosis in the absence of new RNA or protein synthesis when shifted to the permissive temperature (*279*). While the precise mechanisms by which p53 may mediate sequence-specific transactivation-independent apoptosis have not been elucidated, recent studies suggest that loss of pRB signaling and the subsequent release of E2F family members is required (*280*). E2F-1 and p53 act synergistically to induce apoptosis, and E2F-1 mediated apoptosis is impaired in p53-deficient mice (*281*, *282*). Furthermore, in a transgenic mouse model system in which loss of pRB function results in significantly elevated rates of apoptosis, elimination of either E2F-1 or p53 equivalently reduces the level of apoptosis (*283*).

Although transactivation-independent models of p53-mediated apoptosis are provocative, a recently developed transactivation-deficient p53 mouse model provides insight into p53 regulation and function in vivo as it relates to apoptosis. Using homologous recombination and LoxP/Cre-mediated deletion in murine embryonic stem cells, Jimenez et al. generated mice with a p53 allele encoding changes at Leu-25 and Trp-26, two residues known to be essential for transcriptional transactivation and MDM2 binding in the murine protein (*115*). When the mutant p53 protein was expressed in mice, its level was not affected by DNA damage and it bound DNA; however, it was defective in cell cycle regulation and apoptotic function. In fact, the double-point mutant p53 protein did not induce thymic apoptosis in vivo after exposure of the animals to ionizing radiation, suggesting that transcriptional activation is required for apoptosis in vivo (*115*). Using similar technologies, Chao et al. engineered mouse embryonic stem cells to express p53 with Gln and Ser in place of Leu-25 and Trp-26, respectively, at the native p53 locus (*184*). The derived stem cells did not undergo UV-induced apoptosis, nor did the thymocytes of mice derived from these cells undergo cell death after exposure to ionizing radiation (*184*). These results show that transcriptional activation is required for apoptosis. Future research is necessary to identify the various attributes of the p53 protein required to mediate apoptosis.

8. Role of p53 in Teratogen-Induced Developmental Defects

In addition to its function as a tumor suppressor protein, p53 has also been implicated in regulation of both normal embryonic development and in prevention of developmental defects after teratogen exposure. Despite the viability of p53 knockout mice, p53−/− and p53+/− mice are predisposed to the development of lymphomas and sarcomas (*284*), suggesting that p53 function is critical for response to environmental stresses and regulation of differentiation. The finding that the p53-dependent response to ionizing radiation is intact in early gestation murine embryos provided support for a role of p53 as a teratological suppressor (*285*). In fact, numerous studies have confirmed the ability of p53 to function as an in vivo teratological suppressor. Treatment of pregnant p53+/− and p53+/+ mice with benzo[*a*]-

pyrene at gestation day 10 resulted in twice as many fetal resorptions and a 4-fold increase in embryo lethality in the p53+/− mothers as compared to p53+/+ mothers (*286*). Further, regardless of maternal genotype, in utero death occurred in 26% of p53+/− and 36% of p53−/− embryos in comparison to only 10% of p53+/+ embryos (*286*). In a separate experiment, p53−/− embryos irradiated at day 9.5 had a 70% incidence of developmental abnormalities and an only 7% incidence of deaths, while irradiated p53+/+ embryos had a 20% incidence of developmental abnormalities and a 60% incidence of deaths (*287*). In this latter study, the p53+/+ embryos demonstrated high levels of apoptosis after irradiation not seen in similarly treated p53−/− embryos, suggesting that p53 functions to eliminate embryonic stem cells with teratogen-induced DNA damage through p53-dependent apoptosis (*287*). Further, treatment of p53+/+, p53+/−, and p53−/− embryonic limbs with an activated analogue of cyclophosphamide at day 12 resulted in significantly increased rates of limb malformation in p53−/− embryos as compared to p53+/+ embryos, while heterozygous p53+/− embryos exhibited an intermediate phenotype (*288*). Taken together, these studies suggest that embryos lacking p53 expression continue cell cycle progression despite the presence of teratogen-induced DNA damage, resulting in an increased incidence of developmental anomalies.

9. p53 Signaling and Chemosensitivity

The role of p53 as a determinant of chemosensitivity has been the subject of much controversy. Initial studies in cells derived from p53-deficient mice led to the prediction that tumors that had lost wt p53 function would have reduced responses to radiotherapy and chemotherapy, since p53 may enhance chemosensitivity by promoting apoptosis (*289*−*291*). However, p53 also has the potential to decrease chemosensitivity by promoting cell cycle arrest through induction of p21, a prediction that is substantiated by numerous studies (*292*−*294*). The effect of p53 signaling on chemosensitivity likely depends on the cellular context and other existing gene mutations, an important consideration when comparing and contrasting the results from various studies (*295*, *296*).

9.1. Genotoxic Agents. The effect of p53 status on chemosensitivity has been most extensively studied with genotoxic agents. Transfection of wt and p53-deficient MEFs with adenoviral E1A and E1B proteins and subsequent treatment of the MEFs with various types of genotoxic agents demonstrated that E1A-dependent sensitization to apoptosis is blocked in the absence of p53 (*291*). Thus, loss of p53 may permit the survival of cells harboring an activated oncogene functionally related to the ElA protein, such as *Myc* or *Ras* (*291*). Further, thymocytes from p53-deficient mice have decreased cytotoxicity after ionizing radiation and etoposide treatment as compared to p53-containing thymocytes (*289*, *290*). In addition, a variety of hematopoietic cell lineages derived from transgenic mice expressing mutant variants of the mouse *p53* gene had significantly increased cellular resistance to ionizing radiation, providing in vivo evidence that p53 mutations may confer increased radioresistance (*297*).

Nontransformed human fibroblasts are sensitized to DNA-damaging agents such as cisplatin (*298*) after disruption of p53 function by ectopic expression of the human papillomavirus E6 protein (*299*). E6 binds p53 and targets it for ubiquitin-mediated degradation, thus abrogating p53-dependent signaling (*299*). These results provide evidence supporting the hypothesis that in nontransformed cells lacking other alterations, loss of p53 function may result in enhanced chemosensitivity, presumably because these cells maintain a functional G1/S checkpoint response. In contrast, other groups have reported that E6 disruption of p53 activity in fibroblasts decreases sensitivity to ionizing radiation and fails to alter cellular sensitivity to adriamycin (*300*, *301*). Consistent with these observations, a difference in radiosensitivity was not observed in p53−/−, p53+/−, or p53+/+ fibroblasts (*292*). The apparent discrepancy between the results described in the studies above may be explained by drug type- and cell type-specific effects of p53 status on cellular sensitivity (*302*). For example, ionizing radiation sensitivity may only occur in p53-deficient lymphocytic or hematopoietic cells, while fibroblasts may be specifically affected by p53 status in response to DNA-damaging drugs such as cisplatin.

p53 status as a determinant of chemosensitivity has also been analyzed in numerous cancer cell lines. However, interpretation of these studies is difficult since most tumor cell lines have multiple genetic mutations that are often poorly defined, thus modulation of chemosensitivity may not be solely a consequence of altered p53 function. Examination of a panel of lymphoma tumor cell lines demonstrated that p53 mutation correlates with decreased sensitivity to DNA-damaging agents (*303*). Similarly, p53-deficient testicular cell lines have increased resistance to etoposide-induced apoptosis, while cell lines derived from gliomas with mutant p53 have decreased chemosensitivity to genotoxic drugs (*304*, *305*). Disruption of wt p53 function in MCF-7 breast cancer cells and RKO colorectal cancer cells by expression of either the E6 protein or a dominant negative p53 protein enhanced sensitivity of the cells to cisplatin in clonogenic survival assays, but not to other genotoxic agents (*306*). However, the same disruption of wt p53 function in ovarian carcinoma cell lines resulted in resistance to cisplatin-induced apoptosis, further supporting the hypothesis that there is cell type-specific modulation of chemosensitivity by p53 (*307*, *308*).

Disruption of p53 function in human colon carcinoma cells enhanced sensitivity to DNA-damaging agents but increased resistance to antimetabolic drugs, suggesting there is drug type-specific regulation of chemosensitivity by p53 (*309*). Further, loss of p21 in colon carcinoma cells increased sensitivity to ionizing radiation in vivo (*310*). These latter studies utilized isogenic model systems in which both alleles of either *p53* (*309*) or *p21* (*310*) were disrupted by homologous recombination in a human colon carcinoma cell line, thus generating a matched set of tumor cell lines differing at only the p53 or p21 locus. Since most tumor cells have numerous genetic lesions, these isogenic model systems allow evaluation of how loss of p53 or p21 modulates chemosensitivity in tumor cell lines with the same genetic composition. In conclusion, like nontransformed cell lines, most tumor cell lines show heterogeneous responses to alterations of p53 function, as there are both cell type- and drug type-specific factors mediating cellular sensitivity to genotoxic drugs.

9.2. Microtubule Inhibitors. Microtubule inhibitors such as Taxol are effective in the treatment of many

cancers, including metastatic breast, ovarian, and non-small-cell lung cancer (*311*). Microtubule inhibitor efficacy is correlated with an increased level of tubulin polymerization, tubulin bundling, and G2/M cell cycle arrest; however, the biochemical mechanisms by which these events lead to tumor cell apoptosis are not well understood (*312*−*315*). Several groups have examined if the p53 status of a cell modulates its sensitivity to Taxol and other chemotherapeutic microtubule inhibitors. However, as in studies evaluating genotoxic drugs, contradictory results have been obtained. These discrepancies may be in part explained by cell type specificity, as well as differences in the assays used to assess chemosensitivity. For example, both E6-expressing human fibroblasts and p53−/− MEFs had enhanced sensitivity to Taxol-induced apoptosis (*298*). Further, loss of p53 conferred increased sensitivity to microtubule inhibitor treatment in tumor cell lines (*316*, *317*). In contrast, other studies concluded that p53 or p21 status does not affect the cellular sensitivity to microtubule inhibitors (*318*, *319*). However, in these latter studies, chemosensitivity was determined using a monolayer clonogenic survival assay. Subsequent studies indicate that anchorage-dependent assays may fail to predict chemosensitivity of p21-deficient cell lines in vivo, again demonstrating the caution that must be used when comparing results from various sensitivity studies (*310*, *320*). In fact, recent studies using isogenic model systems in both in vitro and in vivo settings demonstrated that the status of p53-mediated G1/S checkpoint signaling in tumor cells is an important determinant in the efficacy of microtubule inhibitors used clinically (*321*).

10. The Future of p53 Research: Linking Biochemistry to Biology

A current challenge facing the p53 field is linking the biochemistry of p53 to biological outcomes. Numerous signaling pathways converge to regulate p53 activity. The cellular outcome of these signaling pathways is dictated by p53 target gene expression and the contribution of stress type- and cell type-specific factors. One major question that warrants further investigation is the biological role of p53 post-translational modifications. Several studies suggest that phosphorylation of the p53 amino terminus functions to disrupt binding of MDM2 and thus allow increased p53 protein levels (*125*, *127*). Further, two recent reports now indicate that in addition to phosphorylation of p53, MDM2 may also be phosphorylated after genotoxic stress and this phosphorylation of MDM2 contributes to the dissociation of p53−MDM2 complexes (*141*, *142*). Particularly intriguing is the fact that both of the kinases responsible for mediating MDM2 phosphorylation also phosphorylate p53. For example, Mayo et al. found that DNA-PK phosphorylates MDM2, while Shiloh et al. demonstrated ATM phosphorylates MDM2 after treatment with ionizing radiation (*141*, *142*). It will be interesting to re-examine other known p53 kinases to determine if they too have a dual role in phosphorylation of MDM2 and p53 to disrupt the interaction of these proteins.

Adding a further layer of complexity to interpretation of the biological consequences of p53 phosphorylation are recent studies indicating that there is interplay between the phospho sites, such that phosphorylation on one residue may require phosphorylation on a prior site. For example, Bulavin et al. observed that substitution of alanine at Ser-33 completely blocked UV-induced phosphorylation at Ser-37 but did not decrease the level of phosphorylation at Ser-15 (*156*). In contrast, mutation of both Ser-33 and Ser-46 to alanine abrogated UV-induced phosphorylation of Ser-37, and significantly reduced the level of phosphorylation at Ser-15 (*156*). This study suggests that either the presence of Ser-33 and Ser-46 themselves or their ability to be phosphorylated is important for amino-terminal phosphorylation of p53 after cells are exposed to UV light. Similarly, Sakaguchi et al. recently reported that phosphorylation of p53 at Thr-18 required prior phosphorylation at Ser-15 after cells were exposed to ionizing radiation (*125*). This latter study also demonstrated that substitution of alanine at Ser-37 enhanced the level of phosphorylation at Ser-15, Thr-18, and Ser-20 after treatment with ionizing radiation (*125*). Multiple sites may need to be modified to yield a functional p53 molecule. Evidence of this is provided by a recent study reporting that Ser-15 phosphorylation was necessary to disrupt MDM2 binding, but the Ser-15 modification did not induce the DNA-binding activity of p53 (*322*). In contrast, phosphorylation of Ser-392 stimulated the DNA-binding ability of p53 but did not disrupt binding and inhibition by MDM2, suggesting the necessity of modifying multiple residues to modulate p53 activity (*322*). The studies cited above exemplify the need for higher-resolution techniques that will allow analysis of the p53 molecule after cellular stress. Mass spectrometric analysis of the p53 protein was used to identify sites that are covalently modified in vivo, either constitutively or in response to ionizing radiation (*323*). Abraham et al. prepared protein extracts from nonirradiated and irradiated OCI/AML-3 acute myeloid leukemia cells, which express wt p53 (*323*). Following partial purification by immunoaffinity chromatography and enzymatic in-gel digestion, p53 peptides were analyzed by MALDI-TOF and nanoelectrospray mass spectrometry. This analysis identified four amino-terminal sites that are phosphorylated in response to ionizing radiation, as well as a constitutive phosphorylation site at Ser-315 (*323*). By identification specific post-translational modifications after cellular stress, the ability of differentially modified p53 molecules to transactivate specific downstream target genes can begin to be evaluated. Co-analysis of p53 by mass spectroscopy and in vivo promoter trapping (*102*) experiments after a specific treatment will allow important insight to the relationship of post-translational modifications to p53-mediated biological end points.

Another important technology that can be utilized to advance our understanding of p53-mediated signaling is oligonucleotide microarray analyses. Such techniques were employed in a recent study to quantify the mRNA levels of p53-regulated genes (*324*). A cluster analysis of the data demonstrated that genes induced by p53 after ionizing irradiation and UV irradiation form distinct subsets, with a few genes in common, and they were expressed in a cell type- and cell line-specific manner (*324*). Another important finding from the array study was that low levels of p53 in a cell induced or repressed only a subset of genes observed at higher p53 levels; thus, the nature of the p53 response depends on the levels of the p53 protein in a cell, the type of inducing agent or event, and the cell type employed (*324*). In the future, specific p53 phospho mutant proteins could be expressed in cells and downstream target gene expression evaluated

by microarray analysis to determine if the absence of specific residues alters the subset of target genes regulated by p53 in response to various stimuli.

Since disruption of the p53 signaling pathway is one of the most common genetic alterations identified in human tumors and many chemotherapeutic drugs result in either DNA damage or microtubule inhibition, a further understanding of the biochemical pathways mediated by p53 signaling after cellular stress may lead to the identification of novel targets for anticancer therapies. In particular, the finding that microtubule inhibitor treatment of epithelial tumor cells results in distinct patterns of phosphorylation, as compared to genotoxic stress, has important therapeutic implications (*325*). The elucidation of kinases that regulate phosphorylation of p53 after chemotherapeutic treatments may ultimately identify novel targets for therapeutic intervention. For example, loss of p53 function in tumor cells can enhance cellular sensitivity to microtubule inhibitors (*321*). Thus, the development of compounds that inhibit kinases that mediate phosphorylation and activation of p53 after microtubule disruption may lead to enhanced therapeutic efficacy when these compounds are combined with microtubule inhibitors to treat tumors with intact p53 signaling pathways.

During the past 20 years, the role of p53 as a tumor suppressor protein has been elucidated through the discovery and analysis of downstream target genes. The biochemical characterization of p53 has also given us a glimpse of how p53 regulates cell physiology. With the recent completion of the human genome sequence and the rapid advance in biotechnologies for mining this information, several missing links between the biochemistry of p53 and the biology of p53 will be determined. Since the p53 signaling pathway is the most commonly subverted during tumorigenesis, this information will undoubtedly have great benefit in the prevention and treatment of cancer.

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