MINIREVIEW

p21—Negative Regulator of the Cell Cycle

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Abstract. **Progression through the cell cycle is regulated by cyclins and cyclin-dependent kinases (Cdks). The cyclin kinase inhibitor p21 (also known as WAF1, CIP1, SDI1, and MDA-6) can induce G1 arrest and block entry into S phase by inactivating Cdks or by inhibiting activity of proliferating cell nuclear antigen (PCNA). In normal cells, p21 exists in quaternary complexes with cyclin, Cdk, and PCNA. Transcription of the p21 gene is activated by p53-dependent and -independent mechanisms. Mice deficient in p21 exhibit no apparent phenotype, although p21 function has been demonstrated to be necessary for p53-mediated G1 arrest following irradiation of p21-deficient mouse embryonic fibroblasts. Thus, the function of p21 under normal circumstances appears to be redundant. p21 is expressed in terminally differentiating cells of a variety of tissues in a p53-independent manner. Overexpression of p21 results in G1 arrest and has been shown to suppress effectively tumor growth *in vitro* and *in vivo*. We review the recent literature describing the functional characterization of p21. This protein plays a key role in regulating the cell cycle and may have potential gene therapy applications.** [P.S.E.B.M. 1996, Vol 213]

Regulation of cell cycle plays an important role in normal cell growth and differentiation, and in tumor progression. Cell cycle progression is controlled by the cyclin-dependent protein kinases (Cdks), which are the catalytic partners of the cyclins (reviewed in Ref. 1). Each Cdk interacts with a particular subset of cyclins, and each mammalian cyclin can interact with multiple Cdks. Cyclin function is primarily controlled by changes in cyclin levels, while Cdks are activated by phosphorylation of a conserved threonine residue (2). Regulators of G1 progression in mammalian cells include D-type cyclins (D1, D2, D3) and cyclin E (3). D-type cyclins are associated with either Cdk4 or Cdk6, while cyclin E is associated with Cdk2 (4). These cyclins play important, but probably distinct, roles in the regulation of the G1–S phase transition (5).

Substrates of the G1 Cdks include the retinoblastoma protein (pRb) and other pRb-related proteins (6). When it is hypophosphorylated, pRb binds and represses the transcriptional activation by the E2F family of transcription factors that are required for the activation of a variety of genes needed during the G1–S phase transition (7–9). pRb hyperphosphorylation results in the release of E2F, enabling it to activate genes important for S phase entry (Fig. 1; for review see Ref. 10). Cdk-2 also plays a key role in regulating DNA replication during S phase (11–15).

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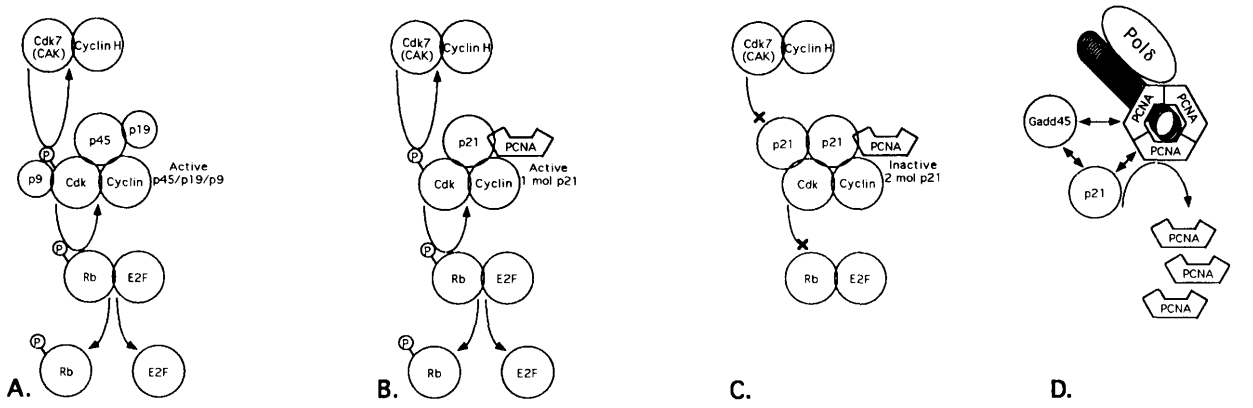

Figure 1. Mechanisms of p21 action. (A and B) When p21 is absent, or present in a stoichiometry of one molecule per complex, Cdk7/cyclin H bind and phosphorylate Cdk2/cyclin E, Cdk4/cyclin D, and Cdk6/cyclin D complexes. These complexes then phosphorylate Rb and release E2F for autoactivation and activation of S phase genes. In Panel A, the p9/Cdk/cyclin/p45/p19 complex characteristic of transformed cells, and essential for entry into S phase in normal cells, is shown. In Panel B, the active unimolar p21 quaternary complex is depicted. (C) When p21 is present in excess, an additional molecule binds the Cdk/cyclin complexes, possibly blocking activation by Cdk7/cyclin H and preventing phosphorylation of Rb, allowing Rb/E2F complexes to inhibit S phase progression. (D) p21 can also inhibit replication by binding to PCNA, dissociating the trimer and causing polymerase δ to lose processivity. Gadd45, another growth-inhibitory protein induced by DNA damage, interacts with p21 and competes with it for PCNA binding.

Cyclin kinase inhibitors are proteins that bind to and inhibit the activity of Cdks. One of the first of these to be identified was p21 which binds and inhibits G_1 cyclin/Cdk complexes (16, 17). The p21 cDNA was cloned independently by several groups and is also known as WAF1 (wild-type p53 activated factor), CIP1 (Cdk interacting protein), SDI1 (senescent cell-derived inhibitor), and MDA-6 (melanoma differentiation associated) (16–20). Thus far, seven cyclin kinase inhibitors have been identified. p27 (21, 22) and p57 (23, 24) are similar to p21 at the amino terminus and have a broad specificity for Cdks (See Fig. 2). p16 (25, 26), p15 (27), p18, and p19 (28, 29) are cyclin kinase inhibitors that form a second family with a more restricted Cdk specificity.

Structure and the Mechanism of Inhibition of Cell Cycle Progression by p21

The human p21 gene consists of three exons of 68, 450, and 1600 bp. The first ATG codon appears at nucleotide 76 in exon 2 and a termination codon appears at nucleotide 570 in exon 3 (16, 18). The protein sequence shares 79% identity with mouse p21, with two regions being highly conserved, human codons 21–60 (95% identity) and 130–164 (89% identity) (30). The first of these regions is cysteine rich and may encode a zinc finger, while the second conserved region contains several putative nuclear localization signals (18, 30). The structure of p21 is depicted in Figure 2.

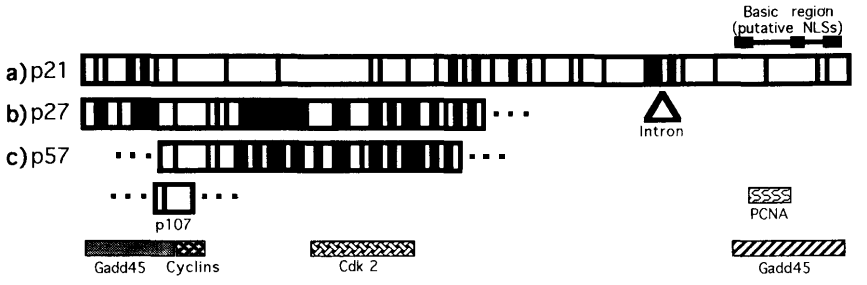

Figure 2. Structural features and homologies of p21. Black bars indicate mismatches and white regions indicate similarity at each amino acid position in each schematic alignment of human p21 to mouse p21 (a), human p27 (b), and human p57 (c). The portions of p27 and p57 depicted are limited to regions with notable homology. Sites of p21 interaction with PCNA, Gadd45, Cdk2, and cyclin are indicated by bars. The boundary between the two coding exons, 2 and 3, is indicated with a triangle. The C-terminal basic region is indicated by a bar and the putative nuclear localization signals (basic amino acids) are indicated by small boxes. Interactions of p21 and the retinoblastoma related protein p107 with cyclin A/Cdk2 or cyclin E/Cdk2 are mutually exclusive, and p107 interaction has been mapped to a short sequence motif with homology to p21 (31).

In normal human diploid fibroblasts, p21 exists in quaternary complexes that contain cyclin, Cdk, and proliferating cell nuclear antigen (PCNA), a processivity factor for DNA polymerase δ (Fig. 1B) (32). In nonvirally transformed cells, the p21 quaternary complexes are replaced by complexes containing Cdk, cyclin, and proteins of 9, 19, and 45 kDa (Fig. 1A) (33, 34). The disappearance of p21 in many tumors may be explained by lack of transcriptional activation of the p21 gene due to mutation of the p53 gene (18).

In the p21-containing complexes, cyclin kinases may exist in both active and inactive forms (35). The transition between active and inactive states occurs through changes in p21 stoichiometry, particularly when multiple p21 molecules as opposed to a single p21 molecule bind to these complexes (35, 36). Inhibitory binding by p21 may prevent phosphorylation of Cdks by Cdk-activating kinase (CAK, also called Cdk7) by making the target inaccessible (37). The affinity of p21 for Cdks is notably increased when the Cdk is associated with a cyclin, probably because of conformational changes that occurs after formation of cyclin/Cdk complexes (36).

p21 effectively inhibits Cdk2, Cdk3, Cdk4, and Cdk6, which have a direct role in the G1–S transition, but it is a poor inhibitor of other known Cdks (36). Early events in DNA replication, including phosphorylation of the single-strand DNA binding protein RP-A, are regulated by Cdk2 and can be inhibited by p21 (11–15). p21 can also bind to PCNA and, when present in excess stoichiometry, can block its ability to activate DNA polymerase δ (Fig. 1D), affecting DNA replication through this interaction (38–40).

PCNA is also important for nucleotide excision-repair (41), but *in vitro* data regarding the role of p21 in regulating DNA repair have been contradictory. In extracts prepared from the RKO human colon carcinoma cell line and healthy human lymphoblasts, addition of recombinant p21 did not influence repair of an ultraviolet (UV)-irradiated plasmid, and in a reconstituted system dependent upon purified PCNA, p21 inhibition appeared to preferentially inhibit long transcripts (39). PCNA-dependent DNA repair also proceeded in Xenopus egg extracts in the presence of excess p21 (42). In contrast, repair of DNA damaged by UV irradiation or alkylating agents was inhibited when p21 was added to Hela cell extracts in a 2- to 4-fold stoichiometry relative to PCNA; PCNA addition reversed this inhibition (43). In all of the *in vitro* studies, p21 was an efficient inhibitor of the synthesis of long DNA templates (39, 40, 43). By using purified proteins, a 10-nucleotide elongation was inhibited with a greater than 40-fold excess of p21 to PCNA (40), leading to the suggestion that PCNA clamp loading is inhibited only by a large excess of p21, a result which is confirmed by *in vitro* cross-linking studies (44). In fact, low levels of p21 appear to stimulate RF-C–mediated PCNA loading by up to 50% (44). In addition, *in vivo* experiments have provided evidence for a repair defect in $p21^{-/-}$ cells; lower levels of reporter gene expression were detected following transfection of damaged constructs into $p21^{-/-}$ cells when compared with $p21^{+/+}$ cells (Dr. Wafik El-Deiry, personal communication).

At physiological levels, p21 is likely to inhibit elongation by decreasing the association of polymerase delta with PCNA or by preventing sliding of the complex. Podust and coauthors find that the polymerase delta core and p21 compete directly for binding to PCNA, while p21 does not prevent the loss by sliding of p21 clamps from linearized DNA (44). These authors suggest that the distinction between excision and replication is simply that long replication requires many cycles of reloading of polymerase delta to the PCNA clamp, and with each loading, polymerase delta must compete with p21. Contradictory results can thus be explained by differences in repair length, pausing sites, and p21 versus PCNA concentration. This is not an "all or none" mechanism for PCNA-dependent replication control but instead allows a graded prolongation of the elongation process.

The Cdk and PCNA inhibitory activities of p21 have been mapped to different domains of the protein (Fig. 2) (45–50). The amino terminus of p21 binds Cdks (17, 46, 47, 51) and cyclins (17, 45, 47, 52). This region plays an important role in suppression of tumor cell growth (45, 46, 50). Amino acids 49–71 of p21 share sequence similarity with residues 60–76 of the cyclin kinase inhibitor p27, and bind Cdk2 (53). Amino acids 21–26 of p21 are also conserved in the related cyclin kinase inhibitors p27 and p57, and were shown to be involved in cyclin D1 and cyclin E binding (45). Mutation of residues 21 and 24 resulted in a lack of binding to cyclin D-Cdk2 and cyclin E-Cdk2 complexes *in vitro* and this mutant p21 protein also failed to suppress growth of tumor cell lines (45).

The carboxy terminus of p21 contains the PCNA binding and inhibitory activity (Fig. 2) (46, 49). Expression of this domain alone in cells has an inhibitory effect on DNA synthesis (47). The binding site for PCNA was localized to eight residues, which when contained within a 20–amino acid peptide retained the ability to bind PCNA and inhibit SV40 DNA replication *in vitro* (48).

Recently, p21 was also shown to interact with Gadd45 (54). Gadd45 (growth arrest and DNA damage) has several features in common with p21. Its expression is induced in response to UV irradiation in a p53-dependent manner (55), and it interacts with PCNA and can compete with p21 for binding to PCNA (56). In an ELISA-based pepscan assay, peptides spanning amino acids 1–20, 141–160, and 144–164 of human p21 interact with Gadd45 (54). The carboxy-

terminal region of p21 that binds Gadd45 overlaps with, but is probably distinct from, the region of p21 that interacts with PCNA (48). In a yeast two-hybrid system, Gadd45 also interacted with the amino-terminus of p21 (54). It is possible that Gadd45 may regulate the availability of p21 and modulate its ability to interact with PCNA.

Expression of p21 during Development and Cell Differentiation *In Vivo*

Examination of the patterns of p21 expression in the developing mouse embryo using *in situ* hybridization provided evidence that induction of p21 expression is correlated with cell cycle arrest that precedes terminal differentiation in a variety of tissues (57, 58). The correlation of p21 induction with terminal differentiation and the cessation of proliferation has also been demonstrated in a variety of *in vitro* systems (57, 59–63). p21 transcripts were first detected in the Day 8.5 post coitum (p.c.) embryo in the midline of the neural tube and hindgut. p21 transcripts were later detected in post-mitotic muscle cells in the myotome by Day 10 p.c., and in a variety of terminally differentiating epithelia between Day 13 and 15 p.c. Although expression of p53 was detected in all cells of the 8.5 to 10.5 p.c. embryo (64), p21 expression was more restricted and detected only in differentiating cells, demonstrating that p53 alone is not sufficient for induction of p21. Expression of p21 appeared normal in embryonic tissues of mice lacking a functional p53 gene ($p53^{-/-}$), indicating that induction of p21 transcription during development of most tissues does not require p53 (58).

Expression of p21 in tissues of the adult mouse was also localized to terminally differentiating cells. In most tissues examined except the spleen, expression of p21 is p53 independent (58, 65). Highest levels of p21 expression were found in tissues with high turnover rates, such as the skin and linings of the gastrointestinal tract (30, 57, 64). The intestinal epithelium continuously regenerates, requiring rapid proliferation of large numbers of cells in the intestinal crypts. Differentiation of the four principal intestinal lineages is accompanied by cell cycle arrest and induction of high levels of p21 transcription. p21 transcripts and protein have been localized to differentiated cells of the small intestinal villus epithelium and upper crypt epithelium in the colon (Fig. 3) (57, 58, 66). This striking compartmentalization of p21 to the nonproliferating cells becomes disorganized early during the development of benign adenomas (66).

The intestinal epithelium was used as a model for examining the role of p21 in regulating differentiation and cell proliferation *in vivo*. Chimaeric mice composed of $p21^{-/-}$ and $p21^{+/+}$ cells were generated and intestinal development and differentiation were studied (67). No changes in morphogenesis, differentiation, or cell proliferation could be detected in $p21^{-/-}$ epithelial cells. Mice deficient in p21 in all tissues were also found to develop normally up to 7 months of age (68). Thus, although p21 appears to play an important role in regulating cell cycle progression, its activity may be functionally redundant in the animal.

The function of p21 during differentiation was also studied in the Drosophila eye (69). Human p21 was expressed posterior to the morphogenetic furrow in the eye imaginal disc, and this inhibited a second wave of mitosis that gives rise to precursors of photoreceptor cells R1, R6, and R7, the cone cells, the pigment cells, and precursors of the sensory bristles. Cell types that were determined early following the second mitosis (R1, R6, R7 cells, cone cells, primary pigment cells) were still present in flies expressing p21, while cell types that were determined later (secondary and tertiary pigment cells, bristle cells) were severely underrepresented (69). Thus, the decrease in the number of precursor cells available as a result of p21 inhibition of

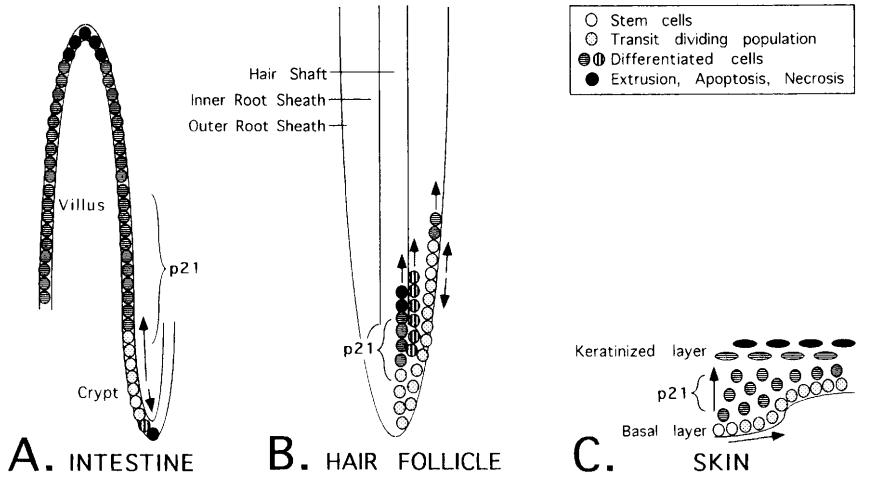

Figure 3. p21 expression patterns in regenerating epithelial tissues. Within epithelia of intestine, hair follicle, and skin, differentiation stages are schematically represented according to the legend at right. In each case, p21 RNA expression (bracketed) corresponds to a population of cells that has begun the process of terminal differentiation and ceased to divide.

mitosis resulted in the absence of some cell types, although ectopic p21 expression did not itself determine cell fate.

p21 Induction following DNA Damage Is Usually p53-Dependent and Responsible for Growth Arrest

The p53 tumor suppressor protein is a transcription factor required for the transactivation of a number of genes involved in growth control (70, 71). Inactivation of wild-type p53 function can lead to a growth advantage and tumor progression. p21 was identified in a screen for genes activated by p53 (18). It has been demonstrated that DNA damage (irradiation) activates p21 transcription in a p53-dependent manner in human diploid fibroblasts (72) and human thyroid epithelial cells (73), leading to cell cycle arrest. Following exposure of mice to ionizing radiation, induction of p21 was also found to be p53 dependent in most tissues, with the exception of the intestine where high levels of p21 are constitutively expressed (65).

Several examples of p53-independent induction of p21 following DNA damage have also been reported. Irradiation of KG-1 human myeloblastic leukemia cells that contain mutant p53 leads to induction of p21, but in this case induction is mediated by tumor necrosis factor–α (TNF-α) (74). It has also been shown that p53 can inhibit cell cycle progression without induction of p21 expression. A temperature-sensitive mutant of human p53 (val 138) is capable of arresting the growth of rat embryo fibroblasts (REF) at the permissive temperature without induction of p21 expression (75). Thus, p21 may arrest cell cycle progression following p53-independent activation, and at the same time p53 may promote cell cycle arrest by activation of genes other than p21.

The redundancy of different pathways that lead to cell cycle arrest following DNA damage was also demonstrated in mice, following targeted deletion of the p21 gene (67, 68). The same number of cells died in intestinal crypts derived from both $p21^{-/-}$ and $p21^{+/+}$ cells, following irradiation of chimaeric mice (67). In addition there was a similar decrease in proliferation in crypts regardless of the p21 status. These data indicated that p21 is not required for DNA damage induced cell death and cell cycle arrest in the mouse intestine, although p53 appears to play a role in normal response to radiation in this tissue (76, 77). p21 was also found not to be required for p53-dependent apoptosis of γ-irradiated thymocytes (68).

In contrast, cultured $p21^{-/-}$ mouse embryonic fibroblasts were significantly deficient in their ability to undergo G1 arrest in response to DNA damage (67, 68). $p21^{-/-}$ cells had a phenotype intermediate between $p53^{-/-}$ and wild-type cells, suggesting the possibility that p53 induces an additional gene that participates in cell growth arrest. Homozygous deletion of p21 in the human colon carcinoma cell line HCT116 completely abrogated the G1 checkpoint following γ-irradiation (66), suggesting that p21 is solely responsible for G1 arrest in these cells. The absolute requirement of p21 for G1 exit as a result of γ-irradiation in human cells, versus the partial requirement for it in murine embryonic fibroblasts, may be explained by differences in species and cell types.

In addition to mediating growth arrest, the p53 protein participates in apoptosis following DNA damage (70, 71) and p21 is also induced in cells undergoing p53-dependent apoptosis (78). Although adenoviral vector directed expression of p53 in tumor cell lines induced apoptosis, expression of p21 did not, indicating that p21 expression alone is not sufficient for inducing apoptosis (79). Several studies suggest that cytokines can block p53-mediated apoptosis and drive cells toward G1 arrest (55, 80). Addition of interleukin-3 (IL-3) to the Baf-3 nonmalignant murine lymphoid cell line while the cells were irradiated resulted in cell cycle arrest (55). When these cells were deprived of IL-3 during irradiation, they underwent apoptotic cell death (55). The p21 gene was induced in both cases, but at higher levels in IL-3–treated cells. p53-dependent apoptosis does not require new RNA or protein synthesis (81), indicating that p21 is not necessary for inducing apoptosis. Still, the levels of p21 that are induced may influence the decision as to whether the cells undergo G1 arrest or apoptotic cell death, and the p21 gene can be induced by a variety of growth factors in a p53-independent manner (62). The *bcl*-2 gene, which usually prevents apoptosis, suppressed p53-dependent activation of p21 in the MCF 10A breast epithelial cell line, but not mitogen-medicated induction (82). Since *bcl*-2 can sequester p53 in the cytoplasm during G1, this is not unexpected (83).

It has also been demonstrated that wild-type p53 is required for Myc mediated apoptosis, but no new synthesis of downstream targets of p53 is needed (84). The Cdk2 inhibition caused by p21 during p53-induced G1 arrest in mouse fibroblasts can be reversed by Myc activity (85). Hermeking and colleagues showed that this is mediated by a heat-labile factor present in cells with Myc activity, which restores Cdk2 activity to the level seen without functional p53 in the cell. On the other hand, rescue by B-myb of GM cells from p21-mediated p53-induced G1 arrest is not associated with a restoration of cyclin E/Cdk2 activity (86).

Levels of p21 were found to increase 10- to 20-fold in senescent human diploid fibroblasts when compared with young cells (19). The increase in p21 levels in senescent cells appears independent of p53 status (87,

88). Nevertheless, p53 seems to play a critical role in growth arrest during cellular senescence (88). Expression of mutant p53 in human diploid fibroblasts grown to near senescence extended the lifespan of the cells over 15 population doublings (88).

Bae and colleagues have suggested that stability of p21 protein is an important factor in determining the duration of G1 arrest in human lymphoma cells (89). Levels of p21 protein were shown to remain elevated after p53 protein and p21 transcripts returned to basal levels in irradiated MCF-7 cells. It was determined that the increased levels of p21 protein were not due to increased p21 stability, but probably to an increase in the rate of p21 translation (90). The correlation between p21 RNA and protein levels and expression of wild-type p53 was examined in breast epithelial cells transfected with a human papilloma virus E6 protein expression construct (90). The E6 protein binds p53 and induces its degradation through an ubiquitin-dependent pathway (91). Decreased levels of both p53 and p21 protein were detected in E6 protein expressing clones, although in some cases p21 RNA levels remained high (90). While p21 RNA levels increased only 2- to 3-fold following differentiation of murine erythroid leukemia cells, protein levels increased approximately 10-fold (65). Thus, expression of p21 appears to be regulated by a variety of posttranscriptional and transcriptional mechanisms (see below).

p53-Independent Induction of p21 during Cell Differentiation

Transcription of the p21 gene can be activated by p53 and by several p53-independent mechanisms (summarized in Fig. 4). Sequence comparison of 4.5 kb of upstream sequence of the rat, mouse, and human genes revealed conservation of two p53 recognition sequences and one region that could possibly bind MyoD (66). At least one of the p53 sites was required for p53 responsiveness (66). A putative ETS-binding site overlaps the proximal p53-binding site in the mouse p21 promoter, and it was speculated may play a role in serum induction of the p21 gene (65).

In differentiating muscle cells, the basic helix-loop-helix transcription factor MyoD activates p21 transcription during the process of muscle cell differentiation. Expression of p21 RNA and protein was induced during differentiation of C2 muscle cells, but not in 10T1/2 fibroblasts (59, 60). Stable introduction of MyoD into 10T1/2 cells converts these cells to myoblasts and leads to p21 induction (58, 59). Although high levels of p21 expression could be detected in wild-type embryonic fibroblasts when they were grown in both high and low serum media, expression of p21 in p53-deficient fibroblasts could be detected only when cells were infected with a retroviral MyoD expression construct and grown in low serum differentiation promoting conditions. In this case, p21 induction appeared MyoD dependent and did not require p53 (59). Interestingly, a role for p21 in promoting differentiation by regulating Cdk activity has also been suggested. Forced expression of cyclin D1 inhibits MyoD mediated transactivation of muscle specific genes, but this can be overcome by overexpression of p21 (90).

Caco-2 cells were derived from a human colon adenocarcinoma and differentiate in the absence of inducers after reaching confluence (93). As these cells are maintained in culture, they polarize, form microvilli, and express increasing levels of brush border enzymes characteristic of differentiated intestinal epithelial cells. During the process of Caco-2 cell differentiation, p21 and p53 mRNA and protein levels also increase (57). In both undifferentiated and differentiated Caco-2 cells, p53 protein was not inducible by DNA-damaging agents. This suggests the absence of functionally wild-type p53 protein and a p53-independent mechanism of p21 induction during differentiation of this intestinal cell line.

In p53-negative hematopoietic and hepatoma cells, p21 was induced during the process of differentiation after these cell lines were exposed to butyrate

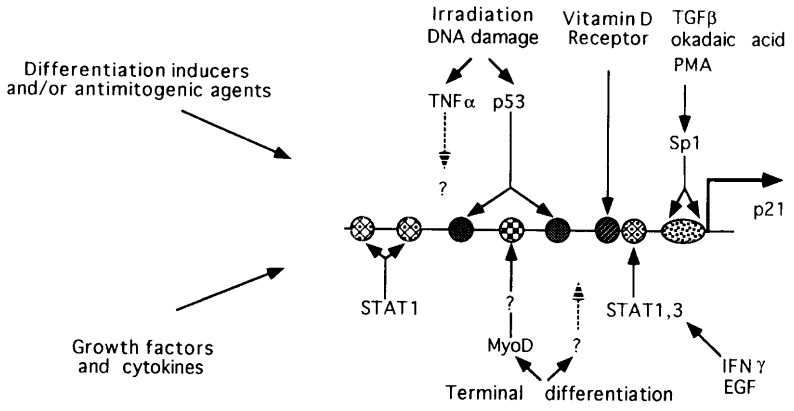

Figure 4. Activation of p21 gene transcription. Activation of the p21 promoter can occur *via* p53, MyoD, and STAT transcription factors, a vitamin D–responsive element, or through Sp1 sites in the proximal promoter that mediate TGF-β, PMA, and okadaic acid induction. A TNF-α–mediated p53-independent pathway for irradiation-induced growth inhibition *via* p21 also exists. See text for references.

or other differentiation agents (61). p53-independent induction of p21 expression at the transcriptional level has been observed as an immediate early response to a variety of physiological and chemical inducers of differentiation. In quiescent embryonic p53-deficient fibroblasts, p21 is induced as an immediate early gene by serum or a variety of purified growth factors including PDGF, FGF, and EGF (62). It has been proposed that p21 acts as an internal timing mechanism in normal cells, controlling the passage of cells past the restriction check point into S phase (16). Interestingly, the growth of A431 cells, which contain high amounts of the EGF receptor, is paradoxically inhibited by EGF, and this growth inhibition is mediated through induction of p21 (94).

Significant insight into the activation of p21 by growth factors and cytokines has been gained through the identification of three STAT transcription factor–binding sites in the p21 promoter (95). Proteins in nuclear extracts from EGF treated A431 cells expressing high levels of STAT1 and STAT3 bandshift and supershift probes for these sites in a manner consistent with STAT1 homodimer binding at the 5′ sites, and binding of STAT1/STAT3 heterodimer or either homodimer at the 3′ site (Fig. 4). Likewise, γ-interferon (IFN-γ) stimulation of three cell types results in STAT1 induction and STAT1 complex formation at all three sites. In each of these cases, p21 is induced and replication is inhibited. Signaling through many cytokine receptors involves the JAK-STAT pathway (reviewed in Ref. 96). The question remains, however, as to whether this induction will normally represent a growth inhibition, since the STAT pathway is normally associated with induction of immediate early genes and stimulation of replication. A possible resolution might be that normal levels of activated EGFR or similar receptors that stimulate growth might lead to lower induction of p21 protein, to the appropriate unimolar level for the normal quaternary complex seen in active replication.

Several additional factors have been demonstrated to induce p21 expression. Nerve growth factor inhibition of growth of NIH 3T3 cells expressing TrkA correlated with induction of p21 protein expression and a 10- to 20-fold increase in levels of p21 complexed with Cdk2 and Cdk4 (97). The treatment of p53-null human leukemia cells by okadaic acid or IFN-γ caused cell growth arrest and monocyte/macrophage differentiation and concomitant induction of p21 (98). The protein kinase inhibitors and antimitogenic agents H7 and staurosporine induced p21 expression in the mammary epithelial MCF-7 cell line, and induction of p21 by staurosporine was p53 independent (99). In the MCF10A breast epithelial cell line, p21 could be activated by p53 or mitogens (82). Treatment of TE85 osteosarcoma and MDAH041 Li-Fraumeni patient derived cell lines, containing no functional p53, with hydroxyurea or hydrogen peroxide leads to transcriptional activation of p21 and growth arrest (100). Administration of a novel synthetic retinoid AHPN to the human breast cell line MDA-MB-231 that contains mutant p53, also induces G0/G1 arrest and simultaneous upregulation of p21 (101). Addition of IFN-β and antileukemic compound mezerin to HO-1 human melanoma cells induces growth arrest and differentiation. This is accompanied by induction of p21 at mRNA and protein level, while a decrease in levels of p53 protein are observed (20).

Diethylmaleate (DEM)-induced oxidative stress diminished the ability of p53 to bind DNA and activate transcription. At the same time, DEM-induced p21 but not Gadd45 in p53-independent way. Addition of DEM to Saos-2 and T98G cells that do not have functional p53 leads to induction of p21 and cell cycle arrest (102).

Addition of calcium to cultured keratinocytes triggers terminal differentiation and p53-independent activation of p21 (60). In these cells, induction of p21 during differentiation was abolished by expression of the adenovirus E1A oncoprotein. Overexpression of a novel nuclear phosphoprotein p300 suppressed the E1A effect, independent of its binding to E1A. In the absence of E1A, exogenous p300 could not significantly activate p21 expression (60).

Transforming growth factor–β (TGF-β) inhibits proliferation of many cell types, including epithelial cells (103). This is in part due to its ability to regulate the cyclin kinase inhibitors p21, p27, and p15 (104). p21 expression is induced by TGF-β by p53-independent pathways in a human keratinocyte cell line (105) and ovarian cancer cell lines (106). The region responsible for the induction in keratinocytes is between −71 and −86 basepairs of the p21 promoter, and is sufficient for TGF-β–induced transcription (107). Gel shift assays demonstrated that several transcription factors, including Sp1 and Sp3 bind this element *in vitro* (107). This element is the third of five Sp1 sites between −119 bp and the TATA box at −48 bp. Deletion of bases −54 to −63, which include an adjacent Sp1 site, reduces TGF-β induction by half.

Sp1 also appears to be necessary for basal, phorbol myristate acetate (PMA)–induced and okadaic acid–induced p21 transcription (108). Basal transcription and induction in response to PMA and okadaic acid are all dramatically decreased by deletion of a promoter construct to −100 bp compared with −117 bp, which eliminates the two upstream Sp1 sites (108). Okadaic acid mediated induction is decreased by a deletion of −62 to −81 bp, which includes the third Sp1

site, and is also decreased 50% by deletion to −117 bp compared with −131 bp.

Induction of CCAAT/enhancer-binding protein–α (C/EBPα) expression in the HT1080 human fibrosarcoma cell line results in inhibition of cell proliferation and a 12- to 20-fold increase in levels of p21 protein (109). This transcription factor appears to regulate p21 levels at the transcriptional and posttranslational levels. The dramatic induction of p21 protein was shown to be due to a transient 3-fold increase in p21 mRNA levels, followed by stabilization of p21 protein.

In the myelomonocytic cell line U937, the hormonal form of vitamin D (1,25-dihydroxyvitamin D_3) activates the p21 promoter in a vitamin D_3 receptor-dependent manner (110). This activation is p53 independent and a functional vitamin D_3–responsive element has been identified, centered at −771 of the p21 promoter (Fig. 4). Addition of 1,25-dihydroxyvitamin D_3 to U937 also leads to the expression of terminal differentiation markers. Transient overexpression of p21 and/or p27 in these cells in absence of 1,25-dihydroxyvitamin D_3 also resulted in induction of terminal differentiation (110). These data suggest that ectopic overexpression of cyclin kinase inhibitors in some cell types is sufficient for induction of terminal differentiation. An important area of research includes the identification of additional *cis*-acting elements and *trans*-acting factors responsible for induction of p21 transcription under diverse conditions.

The Status of p21 in Tumor Cells

Since p21 has properties of a tumor suppressor protein, it was hypothesized that it may be mutated in human cancers. Analysis of the status of p21 in a number of tumor cell lines and tumors of different origin revealed polymorphisms in the p21 gene (111–115). A frequent polymorphism is a single base change of C to A at codon 31 that converts a serine to an arginine. This mutation was not associated with the loss of the tumor suppressor activity of p21 (112) and was not associated with a predisposition to developing colorectal cancer (113). Shiohara *et al.* examined the status of p21 in 351 DNA samples from 14 different kinds of human malignancies and 36 human transformed cell lines using single-strand conformation polymorphism analysis of PCR-amplified regions of these DNAs, and no somatic mutations were found (111).

Recently, a phenylalanine to leucine mutation at codon 63 was identified in the Burkitt's lymphoma cell line DH978. This mutation was shown to affect the growth inhibitory properties of p21 in transfection experiments (89). Gao and co-authors examined the status of p21 in 18 prostate cancer patients with wild-type p53, and they found four mutations in the p21 gene in three patients. Two of these mutations were single base insertions which would produce a frame shift and protein truncation at codon 35 (116). This truncated p21 protein would not be able to inhibit either PCNA or Cdks. The other two mutations were silent mutation in codon 39 and substitution of glutamine for proline in codon 4 (116).

Although the p21 gene is activated by p53, it does not appear that p21 plays a primary role in the tumor suppressor function of p53. The p53 gene is mutated in a wide variety of human cancers (117), and mice lacking a functional p53 gene develop spontaneous tumors at an early age (118–120). In contrast, mutations in the p21 gene are rare and mice lacking a functional p21 gene do not have a propensity for developing tumors (67, 68). The data suggest that other targets of the p53 gene may be responsible for its tumor suppression.

Somewhat paradoxical results were reported by Jung and co-authors (121). They found that p21 expression was low in normal and reactive non-neoplastic brain tissue. In contrast, the majority of gliomas of all malignancy grades with either wild-type or mutant p53 expressed increased levels of p21 (121). It was not determined whether the p21 gene is mutated in gliomas. Although the Cdk inhibitor p16 was found frequently deleted in gliomas, no deletion or rearrangement of p21 was seen in 13 primary brain tumors. The authors proposed that expression of high levels of p21 in gliomas may be the result of a feedback mechanism designed to halt proliferation, and this may account for the slow proliferative rate of gliomas (121). Single-strand polymorphism analysis and direct sequencing of the p21 gene in 158 brain tumors also did not reveal any mutation, although multiple polymorphisms were detected (122).

p21 Suppresses Tumor Cell Growth *in Vitro* and *in Vivo*

p21 acts as a tumor suppressor *in vitro* and *in vivo*. Introduction of p21 expression constructs into normal (16) and tumor cell lines (18) results in cell cycle arrest in G1 (36). Expression of p21 antisense RNA in growth arrested cells resulted in entry into S phase, demonstrating the importance of p21 for maintaining of cells in G0 and G1 (123). Transfection of p21 into the SW480 colon carcinoma cell line in an expression vector conferring hygromycin resistance, resulted in strong growth suppression as demonstrated by a 10- to 20-fold decrease in the number of hygromycin-resistant colonies (18). Overexpression of p21 in a malignant melanoma cell line resulted in accumulation of cells in G0/G1 phase of cell cycle and cell differentiation (124). Infection of lung and breast normal and tumor cell lines with recombinant adenovirus expressing p21 led to a decline in the percentage of cells in S phase, and an accumulation of cells in G1 phase (79). p21 was

overexpressed in colon and prostate carcinoma cell lines using tetracycline-inducible expression system in which transfected gene is expressed in absence of tetracycline, and overexpression of p21 led to suppression of tumor cell proliferation and anchorage-independent cell growth (125). Viral introduction of p21 into a mouse prostate cancer cell line resulted in growth suppression (126).

Several cases illustrate the ability of p21 to inhibit tumor growth *in vivo* (124–126). Following introduction of a viral p21 expression construct into murine renal carcinoma cells, growth of tumors was suppressed when these cells were inoculated into mice (124). Injection of p21 expressing adenoviral vectors into tumor nodules in mice also resulted in striking tumor regression (124). Inducible expression of p21 in colon and prostate carcinoma cell lines resulted in a significant decrease in tumor formation when these cells were injected into nude mice (125).

When p53 or p21 genes were introduced in recombinant adenoviral vector into p53-deficient mouse prostate cancer cells, introduction of p21 was much more effective for growth inhibition *in vivo* and *in vitro* than introduction of wild-type p53 (126). Apparently these two inhibitors do not necessarily behave identically and p21 may be the more powerful proliferation inhibitor in some cell types. Since p21 has recently been demonstrated to be potentially useful in gene therapy strategies directed at cancer treatment, it will be important to understand how this the p21 gene is normally regulated *in vivo*, and what factors regulate its induction.

Conclusion

p21 regulates the cell cycle through a number of independent mechanisms. When multiple p21 molecules bind and inactivate Cdk-cyclin complexes, activation of the E2F family of transcription factors and DNA replication by polymerase α are inhibited. p21 can also compete with the essential replication factor p45 (Skp2) and the target gene p107 for Cdk-cyclin complex binding. p21 can regulate PCNA-related interactions such as elongation by polymerase δ, by binding and dissociating PCNA, which may inhibit replication. It may also bind the G1 growth-inhibitory gene Gadd45 and compete with Gadd45 for PCNA binding.

Thus far, the major nonredundant function identified for p21 *in vivo* appears to be in regulating p53-mediated growth arrest following DNA damage. Nevertheless, p53-independent induction of p21 transcription has been observed in many different types of differentiating cells, both *in vitro* and *in vivo*. Thus, it is possible that a variety of differentiation programs will include activators of p21 or another cyclin kinase inhibitor, because differentiation requires exit from cell cycle. In addition to being regulated at the transcriptional level, p21 is also regulated posttranscriptionally by diverse mechanisms.

Clearly p21 occupies a central role in the control of the cell cycle. While many questions remain to be answered, the practical uses of this protein as a potential tumor suppressor gene and a tool for nontoxic gene therapy to prevent tumors or other tissues from replicating are being investigated.

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1. Morgan DO. Principles of CDK regulation. Nature **374**:131–134, 1995.
2. Kato J, Matsuoka M, Strom D, Sherr C. Regulation of cyclin D-dependent kinase 4 (cdk4) by cdk4-activating kinase. Mol Cell Biol **14**:2713–2721, 1994.
3. Sherr C, Roberts J. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev **9**:1149–1163, 1995.
4. Bates S, Bonetta L, Macallan D, Parry D, Holder A, Dickson C, Peters G. CDK6 and CDK4 are distinct subset of the cyclin-dependent kinases that associate with cyclin D1. Oncogene **9**:71–79, 1994.
5. Dulic V, Lees E, Reed S. Association of human cyclin E with a periodic G1-S phase protein kinase. Science **257**:1958–1961, 1992.
6. Reznitzky D, Reed S. Different roles for cyclins D1 and E in regulation of the G1-to-S transition. Mol Cell Biol **15**:3463–3469, 1995.
7. Hinds PW, Weinberg RA. Tumor suppressor genes. Curr Opin Genet Dev **4**:135–141, 1994.
8. Lam E, Thangue N. DP and E2F proteins: Coordinating transcription with cell cycle progression. Curr Opin Cell Biol **6**:859–866, 1994.
9. Picksley SM, Lane DP. p53 and Rb: Their cellular roles. Curr Opin Cell Biol **6**:853–858, 1994.
10. Muller R. Transcriptional regulation during the mammalian cell cycle. TIG **11**:173–178, 1995.
11. Cardoso MC, Leonhardt H, Nadal-Ginard B. Reversal of terminal differentiation and control of DNA replication: Cyclin A and Cdk2 specifically localize at subnuclear sites of DNA replication. Cell **74**:979–992, 1993.
12. Fang F, Newport JW. Distinct roles of cdk2 and cdc2 in RP-A phosphorylation during the cell cycle. J Cell Sci **106**:983–994, 1993.
13. Adachi Y, Laemmli UK. Study of the cell cycle-dependent assembly of the DNA pre-replication centers in Xenopus egg extracts. EMBO J **13**:4153–4164, 1994.
14. Yan H, Newport J. An analysis of the regulation of DNA synthesis by cdk2, Cip1, and licensing factor. J Cell Biol **129**:1–15, 1995.
15. Jackson PK, Chevalier S, Philippe M, Kirschner MW. Early events in DNA replication require cyclin E and are blocked by $p21^{CIP1}$. J Cell Biol **130**:755–769, 1995.
16. Harper JW, Adami G, Wei N, Keyomarsi K, Elledge S. The p21 cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell **75**:805–816, 1993.
17. Xiong Y, Hannon G, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. Nature **366**:701–704, 1993.
18. El-Deiry W, Tokino T, Velculescu V, Levy D, Parsons R, Trent J, Lin D, Mercer W, Kinzler K, Vogelstein B. WAF1, a potential mediator of p53 tumor suppression. Cell **75**:817–825, 1993.
19. Noda A, Ning Y, Venable SF, Pereira-Smith OM, Smith JR. Cloning of senescent cell derived inhibitors of DNA synthesis using an expression screen. Exp Cell Res **211**:90–98, 1994.
20. Jiang H, Lin J, Su Z, Collart F, Huberman E, Fisher P. Induction of differentiation in human promyelocytic HL-60 leukemia

cells activates p21, WAF1/CIP1, expression in the absence of p53. Oncogene **9**:3397–3406, 1994.
21. Polyak K, Lee M, Erdjument-Bromage H, Koff A, Roberts J, Tempst P, Massague J. Cloning of p27, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell **78**:59–66, 1994.
22. Toyoshima H, Hunter T. p27, a novel inhibitor of G1 cyclin/cdk protein kinase activity, is related to p21. Cell **78**:67–74, 1994.
23. Lee M, Reynisdottir I, Massague J. Cloning of p57, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. Genes Dev **9**:639–649, 1995.
24. Matsuoka S, Edwards M, Bai C, Parker S, Zhang P, Baldini A, Harper J, Elledge S. p57, a structurally distinct member of the p21 cdk inhibitor family, is a candidate tumor suppressor gene. Genes Dev **9**:650–662, 1995.
25. Kamb A, Gruis N, Weaver-Feldhaus J, Liu Q, Harshman K, Tavtigian S, Stockert E, Day R III, Johnson B, Skolnick M. A cell cycle regulator involved in genesis of many tumor types. Science **264**:436–440, 1994.
26. Serrano M, Hannon G, Beach D. A new regulatory motif in cell cycle control causing specific inhibition of cyclin D/cdk4. Nature **366**:704–707, 1993.
27. Hannon GJ, Beach D. $p15^{ink4b}$ is a potential effector of cell cycle arrest mediated by TGF-β. Nature **371**:257–261, 1994.
28. Hirai H, Roussel MF, Kato JY, Ashmun RA, Scherr CJ. Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. Mol Cell Biol **15**:2672–2681, 1995.
29. Chan FK, Zhang J, Cheng L, Shapiro DN, Winoto A. Identification of human and mouse p19, a novel CDK4 and CDK6 inhibitor with homology to p16ink4. Mol Cell Biol **15**:2682–2688, 1995.
30. Huppi K, Siwarski D, Dosik J, Michieli P, Chedid M, Reed S, Mock B, Givol D, Mushinski JF. Molecular cloning, sequencing, chromosomal location and expression of mouse p21 (Waf1). Oncogene **9**:3017–3020, 1994.
31. Zhu L, Harlow E, Dynlacht BD. p107 uses a p21CIP1-related domain to bind cyclin/cdk2 and regulate interactions with E2F. Genes Dev **9**:1740–1752, 1995.
32. Zhang H, Xiong Y, Beach D. Proliferating cell nuclear antigen and p21 are components of multiple cell cycle kinase complexes. Mol Biol Cell **4**:897–906, 1993.
33. Xiong Y, Zhang H, Beach D. Subunit rearrangement of the cyclin dependent kinases is associated with cellular transformation. Genes Dev **7**:1572–1583, 1993.
34. Zhang H, Kobayashi R, Galaktionov K, Beach D. $p19^{Skp1}$ and $p45^{Skp2}$ are essential elements of the Cyclin A-CDK2 S phase kinase. Cell **82**:915–925, 1995.
35. Zhang H, Hannon G, Beach D. p21-containing cyclin kinases exist in both active and inactive states. Genes Dev **8**:1750–1758, 1994.
36. Harper JW, Elledge SJ, Keyomarsi K, Dynlacht B, Tsai L, Zhang P, Dobtowolski S, Bai C, Connell-Crowley L, Swindell E, Fox MP, Wei N. Inhibition of cyclin dependent kinases by p21. Mol Biol Cell **6**:387–400, 1995.
37. Aprelikova O, Xiong Y, Liu ET. Both p16 and p21 families of cyclin dependent kinase inhibitors block the phosphorylation of cyclin dependent kinases by the CDK-activating kinase. J Biol Chem **270**:18195–18197, 1995.
38. Waga S, Hannon G, Beach D, Stillman B. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. Nature **369**:574–578, 1994.
39. Li R, Waga S, Hannon G, Beach D, Stillman B. Differential effects by the p21 CDK inhibitor on PCNA dependent DNA replication and repair. Nature **371**:534–537, 1994.
40. Flores-Rozas H, Kelman Z, Dean FB, Pan ZQ, Harper JW, Elledge SJ, O'Donnell MO, Hurwitz J. Cdk-interacting protein 1 directly binds with proliferating cell nuclear antigen and inhibits DNA replication catalyzed by the DNA polymerase delta holoenzyme. Proc Natl Acad Sci U S A **91**:8655–8659, 1994.
41. Shivji KK, Kenny MK, Wood RD. Proliferating cell nuclear antigen is required for DNA excision repair. Cell **69**:367–374, 1992.
42. Shivji MK, Grey SJ, Strausfeld UP, Wood RD, Blow JJ. Cip1 inhibits DNA replication but not PCNA-dependent nucleotide excision-repair. Curr Biol **4**:1062–1068, 1994.
43. Pan ZQ, Reardon JT, Li L, Flores-Rozas H, Legerski R, Sancar A, Hurwitz J. Inhibition of nucleotide excision repair by the cyclin E-dependent kinase inhibitor p21. J Biol Chem **270**:22008–22016, 1995.
44. Podust VN, Podust LM, Goubin F, Ducommun B, Hubscher U. Mechanism of inhibition of proliferating cell nuclear antigen-dependent DNA synthesis by the cyclin-dependent kinase inhibitor p21. Biochemistry **34**:8869–8875, 1995.
45. Lin J, Reichner C, Wu X, Levine AJ. Analysis of wild-type and mutant $p21^{WAF-1}$ gene activities. Mol Cell Biol **16**:1786–1793, 1996.
46. Chen J, Jackson PK, Kirschner MW, Dutta A. Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA. Nature **374**:386–388, 1995.
47. Luo Y, Hurwitz J, Massague J. Cell-cycle inhibition by independent CDK and PCNA binding domains in p21. Nature **375**:159–161, 1995.
48. Warbrick E, Lane DP, Glover DM, Cox LS. A small peptide inhibitor of DNA replication defines the site of interaction between the cyclic-dependent kinase inhibitor p21WAF1 and proliferating cell nuclear antigen. *Curr Biol* **5**:275–282, 1995.
49. Nakanishi M, Robetorye RS, Pereira-Smith OM, Smith JR. The C-terminal region of p21SDI1/WAF1/CIP1 is involved in proliferating cell nuclear antigen binding but does not appear to be required for growth inhibition. J Biol Chem **270**:17060–17063, 1995.
50. Zakut R, Givol D. The tumor suppression function of p21 Waf is contained in its N-terminal half ('half-WAF'). Oncogene **11**:393–395, 1995.
51. Goubin F, Ducommun B. Identification of binding domains on the p21Cip1 cyclin-dependent kinase inhibitor. Oncogene **10**:2281–2287, 1995.
52. Hall M, Bates S, Peteres G. Evidence for different modes of action of cyclin-dependent kinase inhibitors: p15 and p16 bind to kinases, p21 and p27 bind to cyclins. Oncogene **11**:1581–1588, 1995.
53. Nakanishi M, Robetorye RS, Adami R, Pereira-Smith OM, Smith JR. Identification of the active region of the DNA synthesis inhibitory gene p21Sdi1/CIP1/WAF1. EMBO J **14**:555–563, 1995.
54. Kearsey JM, Coates PJ, Prescott AR, Warbrick E, Hall PA. Gadd45 is a nuclear cell cycle regulated protein which interacts with $p21^{Cip1}$. Oncogene **11**:1675–1681, 1995.
55. Canman CE, Gilmer TM, Coutts SB, Kastan MB. Growth factor modulation of p53-mediated growth arrest versus apoptosis. Genes Dev **9**:600–611, 1995.
56. Chen I-T, Smith ML, O'Connor PM, Fornace AJ. Direct interaction of Gadd45 with PCNA and evidence for competitive interaction of Gadd45 with $p21^{Waf1/Cip1}$ with PCNA. Oncogene **11**:1931–1937, 1995.
57. Gartel AL, Serfas MS, Gartel M, Goufman E, Wu GS, El-Deiry WS, Tyner AL. p21 (WAF1/CIP1) expression is induced in newly nondividing cells in diverse epithelia and during differentiation of the Caco-2 intestinal cell line. Exp Cell Res **227**:171–181, 1996.
58. Parker S, Eichele G, Zhang P, Rawls A, Sands A, Bradley A, Olson E, Harper J, Elledge S. p53-independent expression of p21 in muscle and other terminally differentiating cells. Science **267**:1024–1027, 1995.
59. Halevy O, Novitch B, Spicer D, Skapek S, Rhee J, Hannon G, Beach D, Lassar A. Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. Science **267**:1018–1021, 1995.
60. Missero C, Calautti E, Eckner R, Chin J, Tsai L, Livingston D, Dotto GP. Involvement of the cell-cycle inhibitor Cip1/WAF1 and the E1A-associated p300 protein in terminal differentiation. Proc Natl Acad Sci U S A **92**:5451–5455, 1995.
61. Steinman R, Hoffman B, Iro A, Guillouf C, Liebermann D,

El-Houseini M. Induction of p21 (WAF-1/CIP1) during differentiation. Oncogene **9**:3389–3396, 1994.
62. Michieli P, Chedid D, Lin D, Pierce J, Mercer W, Givol D. Inhibition of WAF1/CIP1 by a p53-independent pathway. Cancer Res **54**:3391–3395, 1994.
63. Jiang H, Lin J, Su Z, Herlyn M, Kerbel R, Weissman B, Welch D, Fisher P. The melanoma differentiation-associated gene mda-6, which encodes the cyclin-dependent kinase inhibitor p21, is differentially expressed during growth, differentiation and progression in human melanoma cells. Oncogene **10**:1855–1864, 1995.
64. Schmid P, Lorenz A, Hameister H, Montenarh M. Expression of p53 during mouse embryogenesis. Development **113**:857–865, 1991.
65. Macleod K, Sherry N, Hannon G, Beach D, Tokino T, Kinzler K, Vogelstein B, Jacks T. p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. Genes Dev **9**:935–944, 1995.
66. El-Deiry WS, Tokino T, Waldman T, Oliner JD, Velculscu VE, Burrel M, Hill DE, Healy E, Rees JL, Hamilton SR, Kinzler KW, Vogelstein B. Topological control of p21 (WAF1/CIP1) expression in normal and neoplastic tissues. Cancer Res **55**:2910–2919, 1995.
67. Brugarolas J, Chadrasekaran C, Gordon J, Beach D, Jacks T, Hannon G. Radiation-induced cell cycle arrest compromised by p21 deficiency. Nature **377**:552–557, 1995.
68. Deng C, Zhang P, Harper J, Elledge S, Leder P. Mice lacking p21 undergo normal development, but defective in G1 checkpoint control. Cell **82**:675–684, 1995.
69. de Nooij JC, Hariharan IK. Uncoupling cell fate determination from patterned cell division in the Drosophila eye. Science **270**:983–985, 1995.
70. Clarke AR, Purdie CA, Harrison DJ, Morris RG, Bird CC, Hooper ML, Wyllie AH. Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature **362**:849–852, 1993.
71. Lowe SW, Schmidt EM, Smith SW, Osborne BA, Jacks T. p53 is required for radiation induced apoptosis in mouse thymocytes. Nature **362**:847–849, 1993.
72. Dulic V, Kaufman WK, Wilson SJ, Tisty TD, Lees E, Harper JW, Elledge SJ, Reed SI. p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. Cell **76**:1013–1023, 1994.
73. Namba H, Hara T, Tukazaki T, Migita K, Ishikawa N, Ito K, Nagataki S, Yamashita S. Radiation-induced G1 arrest is selectively mediated by the p53-WAF1/Cip1 pathway in human thyroid cells. Cancer Res **55**:2075–2080, 1995.
74. Akashi M, Hachiya M, Osawa W, Spirin K, Suzuki G, Koeffler H. Irradiation induces WAF1 expression through a p53-independent pathway in KG-1 cells. J Biol Chem **270**:19181–19187, 1995.
75. Hirano Y, Yamato K, Tsuchida N. A temperature sensitive mutant of the human p53, Val138, arrests rat cell growth without induced expression of cip1/waf1/sdi1 after temperature shift-down. Oncogene **1085**:1879–1885, 1995.
76. Clarke AR, Gledhill S, Hoper ML, Bird CC, Wyllie AH. p53 dependence of early apoptotic and proliferative responses within the mouse intestinal epithelium following gamma-irradiation. Oncogene **9**:1767–1773, 1994.
77. Merritt AJ, Potten CS, Kemp CJ, Hickman JA, Balmain A, Lane DP, Hall PA. The role of p53 in spontaneous and radiation induced apoptosis in the gastrointestinal tract of normal and p53 deficient mice. Cancer Res **5420**:614–617, 1994.
78. El-Deiry WS, Harper JW, O'Connor PM, et al. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res **54**:1169–1174, 1994.
79. Katayose D, Wersto R, Cowan K, Seth P. Consequences of p53 gene expression by adenovirus vector on cell cycle arrest and apoptosis in human aortic vascular smooth muscle cells. Biochem Biophys Res Commun **215**:446–451, 1995.
80. Lin Y, Benchimol S. Cytokines inhibit p53-mediated apoptosis but not p53-mediated G1 arrest. Mol Cell Biol **15**:6045–6054, 1995.
81. Caelles C, Helmberg A, Karin M. p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes. Nature **370**:220–223, 1994.
82. Upadhyay S, Li G, Liu H, Chen YQ, Sarkar FH, Kim H-RC. bcl-2 suppresses expression of $p21^{WAF1/CIP1}$ in breast epithelial cells. Cancer Res **55**:4520–4524, 1995.
83. Ryan JJ, Prochownik E, Gottlieb CA, Apel IJ, Merino R, Nunez G, Clarke MF. c-myc and bcl-2 modulate p53 function by altering p53 subcellular trafficking during the cell cycle. Proc Natl Acad Sci U S A **91**:5878–5882, 1994.
84. Wagner AJ, Kokontis JM, Hay N. Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce p21waf1/cip1. Genes Dev **8**:2817–2830, 1994.
85. Hermeking H, Funk JO, Reichert M, Ellwart JW, Eick D. Abrogation of p53-induced cell cycle arrest by c-Myc: Evidence for an inhibitor of p21WAF1/CIP1/SDI1. Oncogene **11**:1409–1415, 1995.
86. Lin D, Fiscella M, O'Connor PM, Jackman J, Chen M, Luo LL, Sala A, Travali S, Appella E, Mercer WE. Constitutive expression of B-myb can bypass p53-induced Waf1/Cip1-mediated G1 arrest. Proc Natl Acad Sci U S A **91**:10079–10083, 1994.
87. Tahara H, Sato E, Noda A, Ide T. Increase in expression level of p21sdi1/cip1/waf1 with increasing division age in both normal and SV40-transformed human fibroblasts. Oncogene **10**:835–840, 1995.
88. Bond JA, Blaydes JP, Rowson J, Haughton MF, Smith JR, Wynford-Thomas D, Wyllie FS. Mutant p53 reduces human diploid cells from senescence without inhibiting the induction of SDI1/WAF1. Cancer Res **55**:2404–2409, 1995.
89. Bae I, Fan S, Bhatia K, Kohn KW, Fornace AJ, O'Connor PM. Relationships between G1 arrest and stability of the p53 and p21Cip1/Waf1 proteins following gamma-irradiation of human lymphoma cells. Cancer Res **5585**:2387–2393, 1995.
90. Gudas J, Nguyen H, Li T, Hill D, Cowan K. Effects of cell cycle, wild-type p53 and DNA damage on p21 CIP1/WAF1 expression in human breast epithelial cells. Oncogene **11**:253–261, 1995.
91. Scheffner M, Huibregste JM, Vierstra RD, Howley PM. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell **75**:495–505, 1993.
92. Skapek SX, Rhee J, Spicer DB, Lassar AB. Inhibition of myogenic differentiation in proliferating myoblasts by Cyclin D1-dependent kinase. Science **267**:1022–1024, 1995.
93. Pinto M, Robine-Leon S, Appay MD, Kedinger M, Triadou N, Dussaulx E, Lacroix B, Simmon-Assmann P, Haffen K, Fogh J, Zweibaum A. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line CaCo-2 in culture. Biol Cell **47**:323–330, 1983.
94. Fan Z, Lu Y, Wu X, DeBlasio A, Koff A, Mendelsohn J. Prolonged induction of p21Cip1/WAF1/CDK2/PCNA complex by epidermal growth factor receptor activation mediates ligand induced A431 cell growth inhibition. J Cell Biol **131**:235–242, 1995.
95. Chin YE, Kitagawa M, Su W-CS, You Z-H, Iwamoto Y, Fu X-Y. Cell growth arrest and induction of cyclin-dependent kinase inhibitor p21 WAF1/CIP1 mediated by STAT1. Science **272**:719–722, 1996.
96. Ihle JN, Kerr IM. Jaks and STATS in signaling by the cytokine receptor superfamily. Trends Genet **11**:69–74, 1995.
97. Decker SJ. Nerve growth factor induced growth arrest and induction of p21 Cip1/WAF1 in NIH-3T3 Cells expressing TrkA. J Biol Chem **270**:30841–30844, 1995.
98. Zhang W, Grasso L, McClain C, Gambel A, Cha Y, Travali S, Deisseroth A, Mercer W. p53-independent induction of WAF1/CIP1 in human leukemia cells is correlated with growth arrest accompanying monocyte/macrophage differentiation. Cancer Res **55**:668–674, 1995.
99. Jeoung DI, Tang B, Sonenberg M. Induction of tumor suppressor p21 protein by kinase inhibitors in MCF-7 cells. Biochem Biophys Res Commun **214**:361–366, 1995.

100. Johnson M, Dimitrov D, Vojta P, Barrett J, Noda A, Pereira-Smith O, Smith J. Evidence for a p53-independent pathway for upregulation of SDI1/CIP1/WAF1/p21 RNA in human cells. Mol Carcinog **11**:59–64, 1994.
101. Shao ZM, Dawson MI, Li XS, Rishi AK, Sheikh MS, Han QX, Ordonez JV, Shroot B, Fontana JA. p53 independent G0/G1 arrest and apoptosis induced by a novel retinoid in human breast cancer cells. Oncogene **11**:493–504, 1995.
102. Russo T, Zambrano N, Esposito F, Ammendola R, Cimino F, Fiscella M, Jackman J, O'Connor P, Anderson C, Appella E. A p53-independent pathway for activation of WAF1/CIP1 expression following oxidative stress. J Biol Chem **270**:29386–29391, 1995.
103. Derynck R. TGF-β-receptor–mediated signaling. TIBS **19**:548–553, 1994.
104. Reynisdottir I, Polyak K, Iavorne A, Massague J. Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. Genes Dev **9**:1831–1845, 1995.
105. Datto M, Li Y, Panus J, Howe D, Xiong Y, Wang X. Transforming growth factor β induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. Proc Natl Acad Sci U S A **92**:5545–5549, 1995.
106. Elbendary E. Transforming growth factor β can induce CIP1/WAF1 expression independent of the p53 pathway in ovarian cancer cells. Cell Growth Differ **5**:1301–1307, 1994.
107. Datto MB, Yu Y, Wang X-F. Functional analysis of the transforming growth factor β responsive elements in the WAF1/CIP1/p21 promoter. J Biol Chem **270**:28623–28628, 1995.
108. Biggs JR, Kudlow JE, Kraft AS. The role of the transcription factor Sp1 in regulating the expression of the WAF1/CIP1 gene in U937 leukemic cells. J Biol Chem **271**:901–906, 1996.
109. Timchenko NA, Wilde M, Nakanishi M, Smith JR, Darlington GJ. CCAAT/enhancer-binding protein α (C/EBPα) inhibits cell proliferation through the p21 (WAF-1/CIP-1/SDI-1) protein. Genes Dev **10**:804–815, 1996.
110. Liu M, Lee M-H, Cohen M, Bommakanti M, Freedman LP. Transcriptional activation of the Cdk inhibitor p21 by vitamin D_3 leads to the induced differentiation of the myelomonocytic cell line U937. Genes Dev **10**:142–153, 1996.
111. Shiohara M, El-Deiry WS, Wada M, Nakamaki T, Takeuchi S, Yang R, Chen DL, Vogelstein B, Koeffler HP. Absence of WAF1 mutations in a variety of human malignancies. Blood **8484**:3781–3784, 1994.
112. Chedid M, Michieli P, Lengel C, Huppi K, Givol D. A single nucleotide substitution at codon 31 (Ser/Arg) defines a polymorphism in a highly conserved region of the p53-inducible gene WAF1/CIP1. Oncogene **9**:3021–3024, 1994.
113. Li YJ, Laurent-Puig P, Salmon RJ, Thomas G, Hamelin R. Polymorphisms and probable lack of mutation in the WAF1-CIP1 gene in colorectal cancer. Oncogene **10**:599–601, 1995.
114. Mousses S, Ozcelik H, Lee PD, Malkin D, Bull SB, Andrulis IL. Two variants of the CIP1/WAF1 gene occur together and are associated with human cancer. Hum Mol Genet **4**:1089–1092, 1995.
115. Law JC, Deka A. Identification of a PstI polymorphism in the p21Cip1/Waf1 cyclin-dependent kinase inhibitor gene. Hum Genet **95**:459–460, 1995.
116. Gao X, Chen YQ, Wu N, Grignon DJ, Sakr W, Porter AT, Honn KV. Somatic mutations of the WAF1/CIP1 gene in primary prostate cancer. Oncogene **11**:1395–1398, 1995.
117. Hollenstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutation in human cancer. Science **253**:49–53, 1991.
118. Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA, Butel JS, Bradley A. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumors. Nature **356**:215–221, 1992.
119. Harvey M, McArthur MJ, Montgomery CA, Jr., Butel JS, Bradley A, Donehower LA. Spontaneous and carcinogen-induced tumorigenesis in p53-deficient mice. Nat Genet **5**:225–229, 1993.
120. Jacks T, Remington L, Williams BO, Schmitt EM, Halachmi S, Bronson RT, Weinberg RA. Tumor spectrum analysis in p53-mutant mice. Curr Biol **4**:1–7, 1994.
121. Jung J-M, Bruner JM, Ruan S, Langford LA, Kyritis AP, Kobayashi T, Levin VA, Zhang W. Increased levels of $p21^{WAF1/Cip1}$ in human brain tumors. Oncogene **11**:2021–2028, 1995.
122. Koopman J, Maintz D, Schild S, Schramm J, Lousi DN, Wiestler OD, von Deimling A. Multiple polymorphisms, but no mutations, in the WAF1/CIP1 gene in human brain tumors. Br J Cancer **72**:1230–1233, 1995.
123. Nakanishi M, Adami G, Robetorye R, Noda A, Venable S, Dimitrov D, Pereira-Smith O, Smith J. Exit from Go and entry into the cell cycle of cells expressing p21 antisense RNA. Proc Natl Acad Sci U S A **92**:4352–4356, 1995.
124. Yang ZY, Perkins ND, Ohno T, Nabel EG, Nabel GJ. The p21 cyclin-dependent kinase inhibitor suppresses tumorigenicity in vivo. Nature Med **1**:1052–1056, 1995.
125. Chen YQ, Cipriano SC, Arenkiel JM, Miller FR. Tumor suppression by p21 (WAF1). Cancer Res **55**:4536–4539, 1995.
126. Eastham JA, Hall SJ, Sehgal I, Wang J, Timme TL, Yang G, Connell-Crowley L, Elledge SJ, Zhang WW, Harper JW, Thompson TC. In vivo gene therapy with p53 or p21 adenovirus for prostate cancer. Cancer Res **55**:5151–5155, 1995.