

# Lost in Transcription: p21 Repression, Mechanisms, and Consequences

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## Abstract

**The cyclin-dependent kinase inhibitor p21<sup>WAF1/CIP1</sup> is a major player in cell cycle control and it is mainly regulated at the transcriptional level. Whereas induction of p21 predominantly leads to cell cycle arrest, repression of p21 may have a variety of outcomes depending on the context. In this review, we concentrate on transcriptional repression of p21 by cellular and viral factors, and delve in detail into its possible biological implications and its role in cancer. It seems that the major mode of p21 transcriptional repression by negative regulators is the interference with positive transcription factors without direct binding to the p21 promoter. Specifically, the negative factors may either inhibit binding of positive regulators to the promoter or hinder their transcriptional activity. The ability of p21 to inhibit proliferation may contribute to its tumor suppressor function. Because of this, it is not surprising that a number of oncogenes repress p21 to promote cell growth and tumorigenesis. However, p21 is also an inhibitor of apoptosis and p21 repression may also have an anticancer effect. For example, c-Myc and chemical p21 inhibitors, which repress p21, sensitize tumor cells to apoptosis by anticancer drugs. Further identification of factors that repress p21 is likely to contribute to the better understanding of its role in cancer.** (Cancer Res 2005; 65(10): 3980-5)

## Introduction

p21<sup>WAF1/CIP1</sup> is a well-characterized cyclin-dependent kinase (cdk) inhibitor that belongs to the Cip/Kip family of cdk inhibitors (1). It mainly inhibits the activity of cyclin/cdk2 complexes and negatively modulates cell cycle progression (2). In addition, p21 can bind to proliferating cell nuclear antigen thereby blocking DNA synthesis (3). Paradoxically, p21 also stabilizes interactions between cdk4/cdk6 and D-cyclins thereby promoting the formation of active complexes in a concentration-dependent manner (4).

p21 is a transcriptional target of p53 and plays a crucial role in mediating growth arrest when cells are exposed to DNA damaging agents such as doxorubicin and  $\gamma$ -irradiation (5, 6). It has been shown that overexpression of p21 results in G<sub>1</sub>-, G<sub>2</sub>- (7), or S-phase arrest (8, 9). Conversely, p21-deficient cells fail to undergo cell cycle arrest in response to p53 activation after DNA damage (10). Furthermore, p21 and p53 are essential to sustain the G<sub>2</sub> checkpoint after DNA damage in human cells (11). Recently, we discovered a novel alternate p21 transcript, which is highly dependent on p53 for its basal or induced expression (12).

Apart from p53, a variety of other factors including Sp1/Sp3, Smads, Ap2, signal transducers and activators of transcription (STAT), BRCA1, E2F-1/E2F-3, and CAAT/enhancer binding protein  $\alpha$  and  $\beta$  are known to activate p21 transcription (reviewed in ref. 13). In addition to its role in DNA damage response, p21 is also implicated in terminal differentiation, replicative senescence, and protection from p53-dependent and -independent apoptosis (reviewed in refs. 1, 14).

Despite being a central player in cell cycle regulation, unlike the p53 tumor suppressor and the p16 cdk inhibitor, mutations in p21 are extremely rare (15). Although it was suggested earlier that p21 knockout mice undergo normal development (16), a more recent long-term study indicates that these mice develop spontaneous tumors at an average age of 16 months (17), underscoring the importance of this gene in tumor suppression.

Various mechanisms exist to regulate the levels of p21 in a cell including transcriptional regulation, epigenetic silencing, mRNA stability, and ubiquitin-dependent and -independent degradation of the protein. Here, we focus on one mode of regulation—transcriptional repression of p21, discuss its mechanisms and consequences, and highlight its importance in cancer.

## p21 Repression by c-Myc

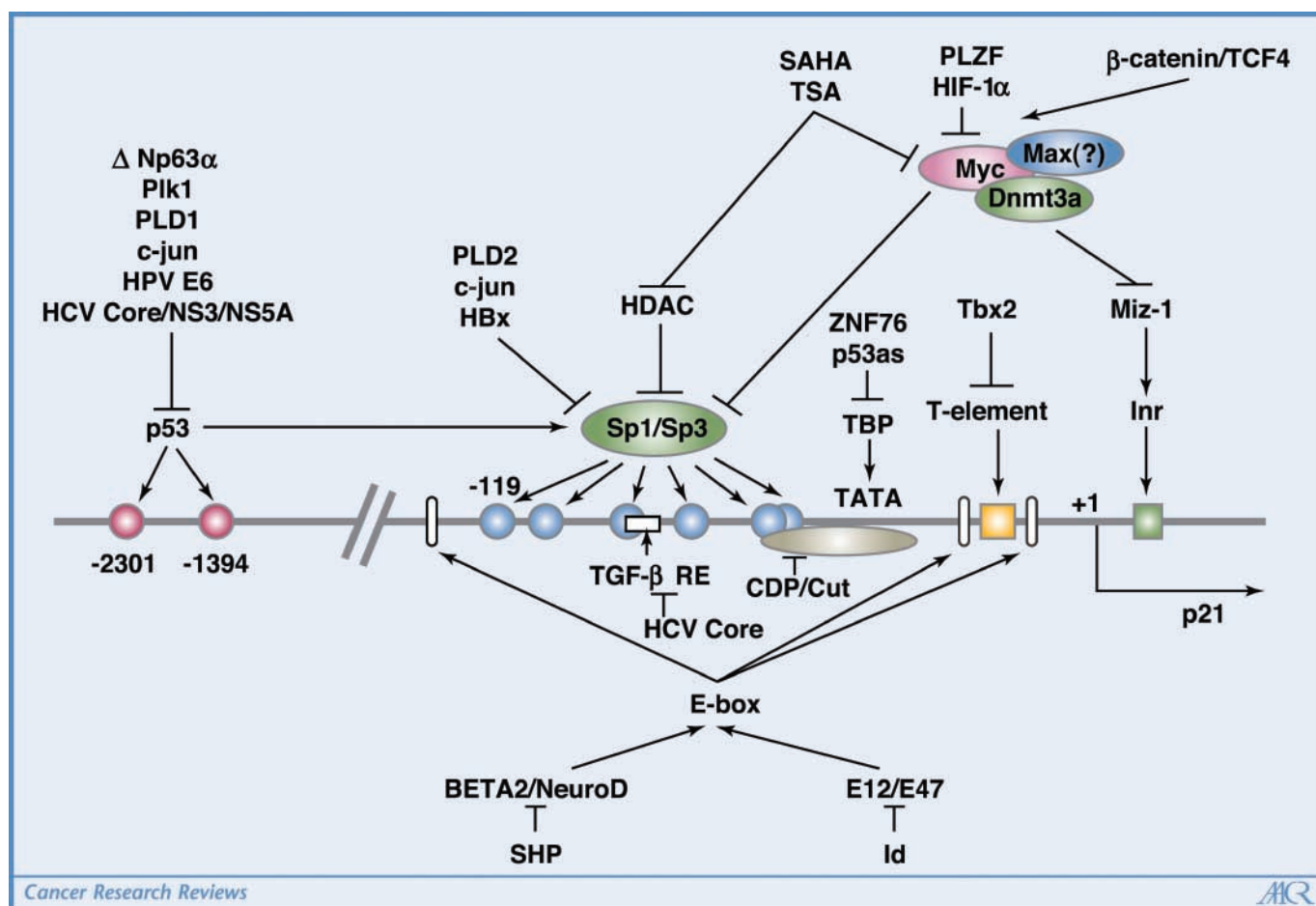
c-Myc is a proto-oncogene which is deregulated in a wide variety of cancers. Apart from activating a number of target genes, Myc also tends to down-regulate several growth arrest genes including gals1, gadds, p15, p27, and p21 (for a review, see ref. 18). A study using microarray analysis has implicated p21 as one of the major targets of c-Myc repression (19).

Down-modulation of p21 levels by c-Myc has been found to be important in a wide variety of circumstances (20–25). For instance, in the case of colorectal cancer, increased  $\beta$ -catenin/TCF4 activity correlated with increased levels of c-Myc, leading to repression of p21 and ultimately to a proliferative phenotype of the cells (25). Understandably, disruption of  $\beta$ -catenin/TCF4 activity led to c-Myc down-regulation, induction of p21, G<sub>1</sub> arrest, and differentiation. These data suggest that constitutive activation of  $\beta$ -catenin/TCF4 pathway in colorectal cells may lead to a cancer phenotype primarily via c-Myc-dependent repression of p21.

Recently, a number of studies have been undertaken to decipher the mechanistic basis of p21 repression by c-Myc. It was found that the proximal promoter region (about 100 bp) of p21 was sufficient for this repression (Fig. 1; refs. 20, 21). Although it is clear that DNA binding by Myc is not essential for this effect, it is still controversial as to what transcription factor(s) Myc binds to on the p21 promoter. We suggested that Myc might bind and inhibit Sp1/Sp3 activity on the basis of interaction studies and further showed that this mechanism may be initiator-independent (18, 21). However, other studies reveal a strong dependence on initiator for p21 repression by c-Myc (23, 24). In this model, the transcription factor Miz-1 binds to the initiator element and

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**Figure 1.** Multiple factors employ distinct mechanisms to repress the p21 promoter. Majority of the factors shown here exert their action through the proximal promoter region. Whereas some of them can bind DNA directly, others tend to interfere with positive regulators of p21 transcription. Also, a few factors can modulate p21 transcription by influencing p53 activity and/or p53 binding to the distal promoter region of p21. Details and references are provided in the text.

up-regulates p21 transcription, whereas c-Myc interferes with Miz-1 activity to repress p21 transcription. As per this model, we would expect p21 levels to go down with decreasing Miz-1. On the contrary, recent microarray experiments showed that after Miz-1 knockdown using small interfering RNAs, p21 levels were up-regulated.<sup>1</sup> Using multiple criteria, Ziegelbauer et al. (26) showed that p21 may not be a bona fide Miz-1 target. Furthermore, in another context, where c-Myc was found to antagonize Ras-mediated induction of p21, it was shown that this effect was Miz-1 independent but relied on the inhibition of Sp1 transcriptional activity (27). However, it is possible that these differences in observation may be due to the difference in cell types or the assay system that was employed. Apart from these passive modes of repression where Myc interferes with Sp1 and/or Miz-1, it was also shown that Myc can actively recruit Dnmt3a DNA methyltransferase corepressor to the p21 promoter (28), thereby demonstrating that Myc may employ a multitude of pathways to repress p21.

Another controversial aspect in the suppression of p21 by Myc is the role of Max protein. Whereas it is known that Myc/Max heterodimerization is required for transactivation of gene targets

by c-Myc, it is still unclear if Max is absolutely essential for its repressive function. In our hands, overexpression of Max seemed to relieve repression of p21 by c-Myc.<sup>2</sup> However, a study by Mao et al. (29), employing genomic-scale chromatin immunoprecipitation techniques, indicates that Max might be required for c-Myc-dependent repression. Interestingly, it was also observed that Max binds to the promoters of the repressed gene targets independently of Myc.

Mechanism aside, repression of p21 by c-Myc has a number of biological implications. For instance, it was shown that Myc is able to switch the cellular response to anticancer drug treatment from p21-dependent cell cycle arrest to apoptosis, which may help eliminate tumor cells. It is known that DNA damage in colon carcinoma cells HCT116 induces stabilization of p53 that leads to cell cycle arrest by transcriptional activation of p21. However, Seoane et al. (23) showed that overexpression of c-Myc in these cells represses p21 and switches the response from cell cycle arrest to apoptosis. Similarly, c-Myc overexpression sensitized LoVo colon cancer cells to camptothecin-induced apoptosis (30). Although ectopic expression of c-Myc in LoVo colon cancer cells increased p53 levels, it potentially suppressed p21 levels, leading to an

<sup>1</sup> J. Ziegelbauer, personal communication.

<sup>2</sup> A.L. Gartel and E. Goufman, unpublished observation.

apoptotic phenotype after camptothecin treatment. All these studies clearly indicate that c-Myc suppression of p21 could be beneficial in cancer therapy in sensitizing tumor cells to anticancer drug-induced apoptosis. Along these lines, identification of compounds such as triptolide (31) and mithramycin A (32), which repress p21, showed that these type of agents may also enhance tumor cell death when used in combination with chemotherapeutic drugs.

As can be expected, down-modulation of c-Myc levels also seems to have a direct effect on p21 mRNA levels. Transcriptional repression of c-Myc by the promyelocytic leukemia zinc finger protein in acute promyelocytic leukemia cell lines leads to a reduction in Myc-induced targets and an increase in Myc-repressed genes including p21 (33). In a similar manner, hypoxia inducible factor  $1\alpha$  displaces c-Myc from the p21 promoter leading to transcriptional activation of p21 and subsequent growth arrest (34). The inverse correlation between c-Myc and p21 expression was apparent again when it was seen that suberoylanilide hydroxamic acid, a histone deacetylase inhibitor, was able to down-regulate c-Myc levels and simultaneously increase p21 levels in multiple myeloma cells (35). Similarly, another histone deacetylase inhibitor, trichostatin A, activates p21 transcription through down-regulation of c-myc expression and release of c-Myc from the p21 promoter in cervical carcinoma cells (36). All of these observations point to a common underlying theme that whereas c-Myc represses basal p21 transcription in different cancer cell lines, agents that can down-regulate c-Myc are able to derepress p21.

### p53-Dependent and -Independent Repression of p21 by Other Cellular and Viral Factors

p53 is a major activator of p21 transcription (5). In theory, anything that inhibits the function of p53 will down-regulate p21. Here, we briefly discuss a few examples of this mode of repression of p21 before we elaborate on the p53-independent pathways.

$\Delta Np63\alpha$ , a splice variant of p63, has been shown to repress p21 in human epidermal keratinocytes by competing with p53 for DNA binding on the p21 promoter (Fig. 1; ref. 37). On the other hand, Polo-like kinase 1, an important regulator of the M phase of the cell cycle, directly binds and inactivates p53, leading to decreased p21 expression (38). Polo-like kinase 1 has been implicated in the genesis and progression of head and neck cell cancer (39), which could in large part be due to its antagonistic effect on p53 and p21. Other factors that down-regulate p21 via p53 include phospholipase D1 by decreasing p53 protein levels (40) and c-jun by repressing p53 promoter (41).

A number of viruses including human papillomavirus and hepatitis C virus also target p53, leading to a decreased p21 expression. Human papillomavirus type E6, a potent oncogene, has long been known to bind and target p53 for degradation (for a recent review, see ref. 42). The hepatitis C virus core protein was seen to exert its effect by decreasing p53 protein levels (43). Whereas it was shown that hepatitis C virus NS5A physically associates with p53 and sequesters it in the perinuclear membrane (44), the manner in which hepatitis C virus NS3 influences p53 was not clear. However, NS3 did not seem to influence mRNA and protein levels of p53, suggesting that protein-protein interaction might be the mechanism behind it (45).

Apart from interfering with p53, repression of p21 may also be mediated by influencing some of its other positive regulators, such

as Sp1/Sp3 and transforming growth factor- $\beta$  (TGF- $\beta$ ), both of which have response elements within the proximal 119 bp promoter region (Fig. 1). For example, phospholipase D2 seems to down-regulate p21 by interfering with Sp1 (40). Phospholipase D2 can also cooperate with phospholipase D1, which operates through the p53-dependent pathway to repress p21 in an additive fashion. Also, c-jun, in addition to its repressive ability on p53 as discussed above, can down-regulate p21 through the Sp1 binding sites (46). However, the effect of c-jun on p21 is not straightforward as it has also been seen that it can interact with Sp1 and induce p21 in some cases (47). It is possible that the differences in cell types used for these studies may explain the seemingly contradictory results. The viral factor, X gene product of HBV (HBx protein), is also able to repress p21 through the Sp1 binding sites (48). Another factor that operates through Sp1/Sp3 is the histone deacetylase 1. Whereas p53 is able to bind and cooperate with Sp1 in p21 induction, histone deacetylase 1 on the contrary was shown to counteract this effect by competing for Sp1 binding (49). Thus, the therapeutic potential of histone deacetylase inhibitors might lie in their ability to relieve this repression coupled with their down-regulation of c-Myc as discussed in the preceding section, both of which contribute to the induction of p21. In addition, HBx is able to cooperate with the hepatitis C virus core protein to cooperatively repress p21 through the overlapping TGF- $\beta$  and Sp1 sites (50).

Another example of p21 repression by interference with activators involves influencing the E-box binding proteins. It was shown that E12/E47 basic helix-loop-helix proteins can induce p21 through the three E-box elements (Fig. 1) close to the transcription start site. However, when the helix-loop-helix motif containing protein Id1 (or Id2) is overexpressed, it heterodimerizes with E12/E47 and precludes its binding to the E-boxes, leading to repression of p21 (51). Similarly, small heterodimer partner protein disrupts the interaction between BETA2/NeuroD basic helix-loop-helix transcription factor and its coactivator p300, which would otherwise activate p21 transcription through the E-boxes (52). Interestingly, small heterodimer partner protein was not able to repress E47-mediated activation of E-box-dependent transcription.

Some factors seem to suppress p21 by interfering with the basal transcriptional machinery. It was shown that ZNF76 displaces the TATA-binding protein from the p21 promoter (Fig. 1) and represses p53-dependent induction of p21 (53). Also, p53as (alternatively spliced p53), a naturally occurring COOH-terminal variant form of p53, which can induce p21 via p53-binding sites, was found to repress p21 by a similar mechanism (54). Here again, the DNA binding ability of TATA-binding protein was diminished in the presence of p53as. Because TATA-binding protein is a basal transcription factor required for transcription of almost all eukaryotic genes, it is hard to imagine as to how selectivity may be achieved by targeting it.

In addition to interfering with its positive regulators, a few other factors repress p21 via direct binding to the proximal promoter (Fig. 1). Tbx2, a T-box transcription factor, which is overexpressed in some breast cancer cell lines, was shown to repress p21 by binding to a T-element (AGGTGTGA) at position -10 (Fig. 1) on the p21 promoter (55). In this study, knockdown of Tbx2 using small interfering RNAs leads to up-regulation of p21, confirming the direct role of Tbx2 in p21 regulation. In a similar manner, transcription factors Runx-1 and Runx-2 repress p21 possibly through the Runx-binding sites on the p21 promoter (56, 57).

Whereas Runx-1 associates with mSin3A, Runx-2 interacts with histone deacetylase 6 to mediate repression of p21.

Another example of a factor that represses p21 by direct DNA-binding is the CCAAT displacement protein/cut (CDP/cut) homeodomain protein, which is involved in development and cell cycle progression. It has been shown that CDP/cut can repress p21 by binding to a region between -60 and -35 bp on the p21 promoter that includes the TATA box (Fig. 1; refs. 58, 59). Recruitment of G9a, a histone lysine methyltransferase (HKMT), to the p21 promoter was found to be essential for this down-modulation of p21 (58). An isoform of CDP/cut, p75, which is generated by alternative transcription, has also been shown to repress p21 promoter (60). Whereas this p75 isoform was detected in primary breast tumors and breast cancer cell lines, it was very weakly expressed in normal breast tissues and mammary epithelial cells. Because aberrant expression of the CDP/Cux p75 isoform in mammary epithelial cells is associated with the process of tumorigenesis in breast cancer, it is tempting to speculate that p75-mediated repression of p21 might contribute to this process.

Epigenetic silencing seems to be another mode of negative regulation of p21 transcription that has been observed in some tumors (see ref. 61 for a review). Whereas in the case of some rhabdomyosarcoma tumors the STAT responsive element on the p21 promoter was found to be methylated (62), in a lung cancer cell line, H719, methylation was found on CpG elements near the Sp1/Sp3 sites (63). Furthermore, in a clinical study involving acute lymphoblastic leukemia patients, 41% of them were found to have hypermethylated p21 promoter in bone marrow cells (64). It is believed that the DNA methyltransferase activity might play a key role in this mode of regulation of p21 (61). In accordance with this notion, it has been observed that treatment of cells containing methylated p21 promoter with a DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (63), or antisense-mediated depletion of DNA methyltransferase (65) led to reexpression of p21. This negative correlation between DNA methyltransferase activity and p21 transcription is in agreement with the involvement of Dnmt3a in Myc-mediated repression of p21 (28) that was discussed in the previous section.

Several other factors exist which have been found to down-regulate p21 but the precise mechanism by which they do so is not completely understood. For example, the hepatitis C virus genomic region, which codes for the core protein, also encodes a small 16 kDa protein called the F protein from an alternate open reading frame (66). Although this F protein does not share major functional properties with the core protein, it still was able to potently repress p21 promoter, although by an unknown mechanism (67).

Similarly, although v-jun was seen to repress through the -60 to +16 bp region of the p21 promoter, no single functional element in this region could be attributed to this repression (68). However, from a functional point of view, this inhibitory effect on p21 by v-jun is not surprising because it was originally identified as an oncogene responsible for the transformation ability of the avian sarcoma virus 17 (69), reinforcing the general notion that oncogenes might down-regulate certain cdk inhibitors to exert their tumorigenic potential. A related scenario involves the case of E5, a weak oncoprotein expressed by human papillomavirus type 11 and 16 (70). This repression of p21 was found to be p53 independent. Also, it was shown that a deletion mutant of E5 that lacks transformation ability was also unable to repress p21,

suggesting that these two biological functions of E5 are intricately connected. However, the exact mechanism by which E5 down-regulates p21 is currently unknown. It could either directly affect p21 promoter or indirectly repress it through c-jun because E5 is known to activate c-jun (71).

Also, p47<sup>ING1a</sup>, a member of the inhibitor-of-growth family of proteins that usually act as negative growth regulators, was able to repress p21 levels in contrast to the other members of the family that can up-regulate p21 (72). However, the mechanism of this repression has not yet been established. Similarly, although it is known that GTPases Rac1 and RhoA can repress p21 transcription (73, 74), the functional elements on the p21 promoter responsible for this repression remain unidentified. Regardless of the mechanism, the result of p21 repression in the majority of the above cases has always been cell cycle progression. However, there are exceptions to this general rule, which will be discussed in the next section.

### Conundrum: The Consequence of p21 Repression is not Always Cell Proliferation

One counterintuitive aspect of p21 is its role as an assembly factor for cyclin D-cdk4/cdk6 (4), which is in complete contrast to its function as a cdk inhibitor. In this case, p21 may act as a positive regulator of cell cycle and, in fact, p21 is induced transiently during G<sub>1</sub>-S progression as a result of mitogenic stimuli. Understandably, when p21 is repressed in this context, it leads to impairment in cell cycle progression due to decreased complex formation of cyclin D-cdk4/cdk6. A few scenarios where repression of p21 results in cell cycle arrest are discussed below.

Although Sp1 is largely known as an activator of p21 transcription (reviewed in ref. 13), it was shown that it also can repress p21 expression in certain circumstances. Kavurma and Khachigian (75) found that in the case of smooth muscle cells, Sp1 can negatively regulate p21 transcription, leading to decrease in cyclin D1-cdk4-p21 complex formation, ultimately resulting in growth inhibition. Further investigation into the mechanism of repression showed that this effect was mediated through the Sp1-binding sites in the proximal p21 promoter and that all of Sp1-binding sites were indispensable for repression (76). Interestingly, overexpression of p21 in smooth muscle cells resulted in cell proliferation and so did the expression of dominant-negative Sp1 that was unable to repress p21. Understanding this unexpected relation between Sp1 and p21 in smooth muscle cells might provide important clues to common vascular disorders like atherosclerosis and restenosis, which involve uncontrolled proliferation of smooth muscle cells in the artery wall.

A similar paradoxical relation was established between the Notch pathway and p21 levels. Whereas Notch signaling has been found to positively regulate p21 levels in mammalian epithelial cells (77), it was also shown that in endothelial cells, Notch activation led to p21 repression and to cell cycle arrest (78). It was found that Notch-mediated p21 repression reduces cyclin D-cdk4 complex formation and nuclear targeting, which inhibits retinoblastoma protein phosphorylation and induces cell cycle arrest. Further, the endothelial cell-cell contact was also seen to activate the Notch pathway leading to p21 repression, suggesting that this could be a primary mechanism by which contact inhibition is implemented in this cell type (78).

These observations clearly show that the consequences of p21 repression are not always a straightforward growth promotion

as one can predict from its function as a cdk inhibitor. The outcome depends on the context and the cell system used. We have only begun to decipher the basics behind the repression of p21 and further studies in this direction will add to our understanding of the role p21 in both normal and cancer cells.

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## Acknowledgments

Received 11/5/2004; revised 1/18/2005; accepted 2/22/2005.

**Grant support:** NIH grant CA91146 (A.L. Gartel).

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We thank Dr. Angela Tyner for comments and helpful discussions.

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*Cancer Res* 2005;65:3980-3985.

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