p53-Mediated Growth Suppression in Response to Nutlin-3 in Cyclin D1–Transformed Cells Occurs Independently of p21

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Abstract

Interaction of cyclin D1 with cyclin-dependent kinases (CDK) results in the hyperphosphorylation of the RB family of proteins, thereby inactivating the tumor-suppressive function of RB. Our previous findings suggest that constitutive cyclin D1/CDK activity inhibits p53-mediated gene repression by preventing the appropriate regulation of CDK activity by the CDK inhibitor p21, a transcriptional target of p53. To study the role of cyclin D1 in driving human mammary cell transformation, we expressed a constitutively active cyclin D1–CDK fusion protein (D1/CDK) in immortalized human mammary epithelial cells. D1/CDK-expressing human mammary epithelial cells grew anchorage-independently in the presence of wild-type p53, consistent with the idea that D1/CDK disrupts downstream p53 signaling. Using this transformation model, we examined the sensitivity of the D1/CDK-expressing cells to Nutlin-3, an HDM2 antagonist that stabilizes wild-type p53. Surprisingly, treatment of D1/CDK-transformed cells with Nutlin-3 prevented their anchorage-independent growth. The Nutlin-3–induced growth arrest was enforced in D1/CDK-expressing cells despite the presence of hyperphosphorylated RB implicating a p53-dependent, RB-independent mechanism for growth suppression. Further analysis identified that CDC2 and cyclin B1, key cell cycle regulators, were stably down-regulated following p53 stabilization by Nutlin-3, consistent with direct interaction between p53 and the CDC2 and cyclin B1 promoters, leading to the repression of transcription by methylation. In contrast to D1/CDK expression, direct inactivation of p53 resulted in no repression of CDC2 and no cell cycle arrest. We conclude that induction of p53 by Nutlin-3 is a viable therapeutic strategy in cancers with constitutive CDK signaling due to the direct repression of specific p53 target genes. [Cancer Res 2007;67(20):9862–8]

Introduction

Cyclin D1 complexes with cyclin-dependent kinase (CDK) to promote the G1-S transition by phosphorylating RB family members, releasing E2F from inhibitory interactions with RB/p130/p107, and de-repressing genes critical for cell cycle progression (1, 2). The p53 tumor suppressor is closely linked with the RB family, playing an essential role in preventing cell cycle progression following oncogenic or genotoxic stress by transcriptionally activating the CDK inhibitor p21, which binds to cyclin-CDK complexes to the inhibit kinase activity that is necessary for cell cycle progression (3). Cyclin D1 is commonly overexpressed in breast cancer. The high levels of cyclin D1–CDK4 and cyclin D1–CDK2 complexes which result drive the constitutive hyperphosphorylation of RB, thereby inactivating its tumor-suppressive functions (4, 5).

To model the effects of hyperactive CDK activity, Chytil et al. (6) created a cyclin D1–CDK2 fusion protein that phosphorylates RB on sites preferred by CDK2 and CDK4, and promotes the anchorage-independent growth of mink lung epithelial cells (6). In addition, we reported previously that the cyclin D1–CDK2 fusion protein could inhibit the repression of a number of genes normally repressed in response to p53 activation. The pathway required for this repression involves the activation of p53 in response to DNA damage or p14ARF induction, transcriptional activation of p21 by p53, CDK inhibition by p21, and finally, RB-E2F–dependent repression of transcription. Inactivation of p53 by overexpression of HDM2 or use of short hairpin RNA (shRNA) to p53, overexpression of cyclin D1 or cyclin E, or inactivation of RB by the adenoviral E7 protein abrogated the repression of genes required for cell cycle progression, including PLK1 (7).

As our knowledge of the roles of specific genes and pathways involved in cancer increases, new strategies for treatment evolve. For instance, rather than developing chemotherapies that kill rapidly dividing cells, including normal cells, efforts are now being directed toward specific mutations. The Nutlin compounds are an excellent example of a directed therapy. They stabilize p53 by competing for binding to the negative regulator HDM2, preventing these two proteins from interacting. The stabilization of wild-type p53 by Nutlin-3 in tumor cell lines and xenographs in nude mice leads to toxicity and prevents tumor growth (8). The Nutlins induce p53-dependent apoptosis in acute myeloid leukemia cells that harbor wild-type p53 and have also been shown to be effective against Kaposi’s sarcoma herpes virus–induced lymphoma (9, 10). Additionally, treatment of neuroblastoma cell lines with Nutlin-3 sensitizes the cells to chemotherapy-induced apoptosis (11). Although this activity of the Nutlins holds great promise for improved therapeutic approaches, we and others have recently shown that normal and transformed cells overexpressing HdmX, a protein with significant homology to HDM2, are resistant to Nutlin-3 (12–14). The failure of Nutlin-3 to affect HdmX-p53 interactions raises important questions regarding the utility of activating wild-type p53 as a therapeutic approach, warranting additional study of the potential use of such therapies.

To construct a physiologic model to examine the effects of cyclin D1 overexpression and to assess the therapeutic value of Nutlin-3 on cyclin D1–expressing cells, we transformed immortalized human mammary epithelial cells (HMEC) using a cyclin D1/CDK2 (D1/CDK) fusion protein. The D1/CDK-expressing cells grew anchorage-independently regardless of whether p53 was present or not, consistent with the inactivation of p53 signaling by...
hyperactive CDK. However, despite the transformed phenotype, the growth of D1/CDK-expressing cells could be prevented by treatment with Nutlin-3, which engaged a p53-dependent repression of important cell cycle regulators, such as CDC2 and cyclin B1, independently of RB. We conclude that compounds which activate wild-type p53, such as the Nutlins, may effectively prevent tumor growth by stabilizing p53, resulting in the direct repression of promoters necessary for cell cycle progression, independently of p53-p21-RB-mediated gene repression.

### Materials and Methods

**Cell lines and culture conditions.** hTERT-HME1 cells were purchased from (Clontech) and grown in a humidified atmosphere containing 5% CO₂ in Medium 171 with mammary epithelial growth supplement (Cascade Biologics) and 50 units/mL of penicillin and 50 µg/mL of streptomycin sulfate (U.S. Biochemical Corp.). HCT116 cells were grown in a humidified atmosphere containing 5% CO₂ in DMEM (with glucose and 1-glutamine; Life Technologies) with 10% fetal bovine serum and 50 units/mL of penicillin and 50 µg/mL of streptomycin sulfate (U.S. Biochemical Corp.). Cells were plated 24 h before Nutlin-3 (Cayman Chemical) or etoposide (Sigma) was added to the fresh medium. For survival and growth assays, medium containing Nutlin-3 or etoposide was removed after 24 or 72 h for HCT116 and hTERT-HME1 cells, respectively, and replaced with fresh medium. The cells were grown to near confluence (~1 week) before staining with methylene blue in 50% methanol. The stain was quantified following extraction with 0.5 mol/L of HCl by reading the absorbance at 595 nm.

**Plasmids and retroviral infection.** WZL-blast-MF-p110a was kindly provided by William Hahn (Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA) and the IRES-blast marker was replaced by an SV40-puro cassette, using the SalI and NheI sites (15). pBlabe-puro-cyclin D1/CDK2 (6) was kindly provided by Brian Law (Department of Pharmacology and Therapeutics, University of Florida, Gainesville, FL). The SV40-puro cassette was replaced with a SV40-bleo cassette from pBabe-Bleo, using the SalI and ClaI sites. The Flag-HdmX and shRNA constructs and protocols for virus production and infection of cells have been described elsewhere (12).

**Soft agar assays.** For hTERT-HME1 cells, 2 × 10⁵ per 60 mm dish, were suspended in 0.6% type VII agarose (Sigma) and plated onto a bottom layer of 1.2% agar. For Nutlin-3 treatment, cells were plated into soft agar and grown for 120 h, after which 1 mL of Medium 171, with 0, 5, or 10 µmol/L of Nutlin-3, was added to the top agar. The medium was changed every 3 days until cells were analyzed (after ~3 weeks). In HCT116 cells, 4 × 10⁵ cells were plated per dish and 1 mL of DMEM with 0, 3, or 9 µmol/L of Nutlin-3 was added 24 h after plating. The medium was changed every 3 days until cells were analyzed (after ~8 days). Plates were analyzed using Metamorph, in which 5 × 5 or 10 × 10 stitched images were counted and multiplied to give colony counts for the whole plate.

**Dose responses.** hTERT-HME1 cells were plated at 1 × 10⁶ per 10 cm dish and treated with 0, 6, 9, or 12 mMol/L of Nutlin-3 for 48 h. Cells were then harvested and analyzed by the Western method.

**Western analysis.** Whole cell extracts were prepared by incubating cell pellets in lysis buffer containing 50 mMol/L of Tris (pH 8.0), 150 mMol/L of NaCl, 1.0% NP40, 10 µg/mL of aprotinin, 100 µg/mL of phenylmethane sulfonyl fluoride, 5 µg/mL of leupeptin, 5 µg/mL of pepstatin, and 1 mMol/L of Na₂VO₄. Cell extracts containing equal quantities of proteins, determined by the Bradford method, were separated by SDS-PAGE (8–12.5% acrylamide) and transferred to polyvinylidene difluoride membranes (Millipore). Antibodies to p53 (DO-1), p21 (C19), CDC2 p34 (C17), cyclin D1 (C-20), cyclin B1 (GNS1), and RB (C-15) were from Santa Cruz Biotechnology; antibodies to HDM2 2A10, IF2, and HBL1 were from Oncogene Research Products; antibodies to β-actin (pan Ab-5) were from Neomarkers; antibodies to phospho-p53 (Ser15) were from Cell Signaling; antibodies to phospho- RB (T249/252) were from Biosource; and antibodies to glyceralddehyde-3-phosphate dehydrogenase were from Calbiochem. Primary antibodies were detected with goat anti-mouse or goat anti-rabbit conjugated to horseradish peroxidase (Hoffman-La Roche), using enhanced chemiluminescence (Perkin-Elmer).

**Results**

**Transformation of immortalized mammary epithelial cells by a constitutively active CDK.** Disruption of the p53 and RB pathways by SV40 large T antigen, together with constitutively activated phosphoinositide-3-kinase (PI3K), transforms immortalized HMECs (15). To avoid the use of viral proteins and to develop a model to study cyclin D1–mediated transformation, we evaluated HME1 cells (nontumorigenic human mammary epithelial cells immortalized with hTERT) expressing a cyclin D1/CDK fusion protein to inhibit RB, a shRNA to inhibit p53 and a constitutively active PI3K. HME1 cells were infected with retroviruses capable of expressing these components, in parallel with control vectors and the expression levels and functionality of the expressed proteins were examined using the Western method (Fig. 1A and B). Cells expressing PI3K had higher levels of phosphorylated AKT, indicating that the PI3K was constitutively active. Inhibition of p53 led to a further increase in phosphorylated AKT, probably due to the inhibition of endogenous PTEN expression (16, 17). Expression of FLAG-labeled D1/CDK was confirmed by Western analysis. The level of p53 was increased modestly in cells expressing D1/CDK, probably due to the replicative stress initiated by inappropriate entry into S phase, as recently described for several different oncopgenes (18, 19). In addition, the level of p21 was significantly higher in cells expressing D1/CDK compared with controls or cells expressing PI3K, probably due to the incorporation of p21 into D1/CDK complexes (6).

Each population of cells was plated into soft agar to test for anchorage-independent growth, a hallmark of transformed cells. Surprisingly, cells expressing only D1/CDK formed colonies in soft agar efficiently, independently of PI3K or p53 status (Fig. 1C). Cells expressing D1/CDK, alone or together with PI3K, with or without shp53, gave rise to 25 times more colonies per field compared with controls or cells expressing PI3K only. We conclude that HME1 cells with constitutive CDK activity have acquired everything needed for transformation in vitro, as judged by the ability to grow anchorage-independently.

**HME1 cells transformed by D1/CDK are susceptible to a p53-dependent, RB-independent growth arrest following treatment with Nutlin-3.** Our previous studies of cancer cell lines...
have delineated a pathway from p53 activation (in response to DNA damage or the expression of p14ARF) to the inhibition of the RB family phosphorylation by the CDK inhibitor p21 (7). Consistent with CDK-mediated RB phosphorylation, the constitutive expression of D1/CDK or cyclin E led to impaired repression of a number of genes normally repressed by the RB family (7). Therefore, we expected that the p53-mediated arrest activated in response to Nutlin-3 would be abrogated in the HME1 cells expressing D1/CDK. To test this, D1/CDK-expressing cells with and without shp53 were plated into soft agar and treated with 5 or 10 μmol/L of Nutlin-3 for 3 weeks (Fig. 2A; Supplemental Fig. S1). There was a 96% decrease in the number of soft agar colonies formed when D1/CDK cells were treated with either dose of Nutlin-3 versus a 50% decrease when D1/CDK-shp53 cells were treated with Nutlin-3. This result is surprising because the p53-p21-RB pathway should be unable to repress genes necessary for cell cycle progression due to the constitutive activity of CDK, which should keep RB in a hyperphosphorylated state, resulting in continued proliferation. Therefore, to confirm that the D1/CDK-expressing cells maintained RB in a hyperphosphorylated state, Western analysis was done using a phosphospecific antibody. As expected, RB remained hyperphosphorylated in D1/CDK-expressing cells following Nutlin-3 treatment (Fig. 2B).

Western analysis revealed an equivalent increase in the levels of p53 and its transcriptional target HDM2 in the control and D1/CDK-expressing cells. This result was also confirmed using a wider range of Nutlin-3 concentrations, indicating that p53 is equivalently activated in control and D1/CDK-expressing cells (Supplemental Fig. S2A). We also confirmed that control and D1/CDK-expressing cells have similar growth rates, as determined in a 6-day growth assay (Supplemental Fig. S2B). Interestingly, although the level of phosphorylated RB did not decrease in D1/CDK-expressing cells, the total amount of RB decreased in both control and D1/CDK cells (Fig. 2B). This effect is probably due to the increased expression of HDM2, which promotes the ubiquitin-mediated degradation of RB (20), and suggests that a higher proportion of the total RB within the cell is phosphorylated. Additional studies will be needed to determine how the limiting levels of RB are phosphorylated more efficiently.

Despite the increased RB phosphorylation, which should provide a greater signal for cell cycle progression, the growth of D1/CDK-expressing cells is still inhibited efficiently. Because HME1 derivatives treated with Nutlin-3 show no signs of apoptosis on tissue culture dishes, it is likely that the mechanism by which Nutlin-3 suppresses anchorage-independent growth also involves the induction of cell cycle arrest. This conclusion is supported by our observation that BCL2 expression does not promote the anchorage-independent growth of HME1 cells (data not shown), indicating that inhibiting apoptosis alone is not sufficient to promote transformation. In contrast to control and D1/CDK-expressing cells, Nutlin-3 treatment of cells expressing shp53 alone, D1/CDK and shp53 together, or HdmX resulted in minimal growth inhibition relative to untreated cells in long-term growth assays (Supplemental Fig. S2C and D; ref. 12). In addition, expression of HdmX or shp53 resulted in no change in the levels of total RB or hyperphosphorylated RB (Fig. 2B and C).

**Figure 1.** hTERT-HME1 cells are transformed by D1/CDK regardless of p53 status. HME1 cells were infected with amphotropic retroviruses encoding D1/CDK, PI3K, or shRNA against p53, or with control vectors. A, cells expressing PI3K with shp53 or shSCR (scrambled control) were examined by the Western method for the levels of FLAG-PI3K and phosphorylated AKT. B, cells expressing PI3K and D1/CDK, separately or together, with the addition of shp53 or shSCR, were analyzed by the Western method for levels of p53, HDM2, FLAG-D1/CDK, and p21. C, cells grown in soft agar were counted.
We next examined the level of CDC2 protein because the CDC2 gene is commonly down-regulated following the induction of p53-p21-RB signaling (21). Surprisingly, CDC2 was repressed similarly in both control and D1/CDK cells, even though differences in the phosphorylation of RB were pronounced. To confirm that cells expressing D1/CDK arrested similarly to control cells, we labeled them with BrdUrd (Fig. 3A). Cells were treated with Nutlin-3 for 48 h and the percentage of cells in S phase was determined after a 4-h pulse with BrdUrd. Again, control cells and cells expressing D1/CDK showed similar decreases in the percentage of cells in S phase, 83% and 77%, respectively. In contrast, the percentage of shp53 cells in S phase did not change and that of cells expressing HdmX decreased by only 25% compared with untreated controls (Fig. 3B), consistent with the inability of Nutlin-3 to alter CDC2 protein expression in these cells (Fig. 2B and C).

Therefore, we conclude that, although constitutively active D1/CDK promoted the constitutive hyperphosphorylation of RB, and was sufficient for anchorage-independent growth, CDC2 was still repressed efficiently following Nutlin-3 treatment, in a p53-independent manner. The 12-fold increase in soft agar growth by the D1/CDK-expressing cells following p53 ablation and Nutlin-3 treatment indicates that the Nutlin-3-mediated effects are at least partially p53-dependent, and although RB is constitutively hyperphosphorylated, p53 can still act efficiently as a tumor suppressor (Fig. 2A). In contrast to the effects of Nutlin-3, whereby the different genetic alterations affected the sensitivity to the treatment, we found that etoposide treatment resulted in similar levels of growth inhibition in all HME1 derivatives tested (Supplemental Fig. S3).

To determine whether the growth inhibition of D1/CDK cells was due to the continued repression of genes even after the removal of Nutlin-3, cells treated with Nutlin-3 for 72 h were examined 24 or 44 h after Nutlin-3 removal, to determine whether CDC2 was de-repressed. Western analysis showed that p53 and p21 levels decreased similarly in control and D1/CDK cells during the period of recovery from Nutlin-3 (Fig. 4), indicating that the induction of p53 in response to Nutlin-3 can be reversed. This conclusion is further substantiated by the increase in hyperphosphorylated RB in control cells following the 24-h recovery period. Based on these observations, we might expect that the cells would re-enter the cell cycle and begin to proliferate. However, the levels of CDC2 did not increase during the recovery period. Failure to up-regulate CDC2, and probably other genes that are repressed in a p53-dependent, RB-independent manner, might explain why control and D1/CDK cells are unable to reverse the growth arrest induced by Nutlin-3. Although there is still a significant growth arrest in D1/CDK-expressing cells following Nutlin-3 treatment, it was noted that there were consistently more D1/CDK-expressing cells at the end of the long-term growth assays relative to the control cells (typically 70–80% growth inhibition for D1/CDK cells relative to 80–95% growth inhibition for controls). Because there were ~2-fold more cells in S phase following Nutlin treatment of the D1/CDK-expressing cells relative to control cells (Fig. 3A), a small subpopulation of cells is probably not arresting completely in
response to Nutlin-3, and therefore, these cells continue to grow during the 7-day assays, resulting in slightly better growth of D1/CDK versus control cells.

Cancer cells are susceptible to a p53-dependent, RB-independent growth arrest following treatment with Nutlin-3. In order to validate the observations made with HME1 cells, we infected HCT116 colorectal cancer cells with retroviruses encoding D1/CDK fusion protein as well as shRNAs to green fluorescent protein (GFP), RB, or p53. These cell lines were treated with Nutlin-3 at 3 or 9 μmol/L for 24 h and then analyzed by the Western method (Fig. 5A). In cells expressing shp53, levels of p53 were significantly decreased whereas other cell lines showed p53 induction upon Nutlin-3 treatment. Levels of HDM2 and p21 also increased as p53 levels were induced. Again, CDC2 levels decreased in the shGFP, shRB, and D1/CDK cells, but there was also a decrease in the level of phosphorylated CDC2 (top band) in all HCT116 cell lines, except the shp53 cells following Nutlin-3 treatment. This result is consistent with studies demonstrating a reduction in phosphorylated CDC2 following p53 activation or overexpression (22). A decrease in cyclin B1 was also observed in Nutlin-3–treated cells following p53 induction. Cyclin B1 influences the phosphorylation status of CDC2 because CDC2/cyclin B1 complexes are a better substrate than CDC2 alone for the kinase Wee1 (23). As seen in the HME1 cells, cyclin D1 expression (both endogenous and exogenous) increased in the control, D1/CDK, shGFP, and shRB cells due to increased p21 protein levels after Nutlin-3 treatment. Next, we examined the growth of HCT116 cells expressing shGFP, shp53, shRB, or D1/CDK on tissue culture plates and in soft agar following treatment with 3 and 9 μmol/L of Nutlin-3 (Fig. 5B). In agreement with the HME1 results, treatment with Nutlin-3 inhibited the growth of all HCT116 derivatives except for those expressing shRNA to p53. Strikingly, <5% of all HCT116 cells formed colonies when p53 was expressed, even in the presence of D1/CDK or the absence of RB (Fig. 5C; Supplemental Fig. S4).

**Discussion**

We describe here the transformation of immortalized HME1 cells by a cyclin D1–CDK fusion protein that models the cyclin D1 overexpression commonly observed in cancer. The transformed phenotype conferred by D1/CDK expression was observed whether or not p53 was inactivated, consistent with the idea that constitutive CDK activity prevents p53-mediated growth suppression by disrupting the p53-p21-RB–mediated repression of genes necessary for cell cycle progression. Although the activity of p53 was insufficient to inhibit anchorage-independent growth, treatment of the D1/CDK-expressing cells with the HDM2 antagonist Nutlin-3 led to a p53-dependent arrest despite the persistent hyperphosphorylation of RB. The growth suppression could be explained by the repression of CDC2 and cyclin B1.

The RB-independent repression of CDC2 and cyclin B1 is consistent with a recent report demonstrating that the p53-dependent decrease in CDC2 levels was due to the direct interaction between p53 and the CDC2 promoter recruitment of DNA methyltransferase 1, which led to promoter methylation and silencing (24). A similar DNA methyltransferase 1–dependent repression was recently reported for the CDC25C and survivin genes (24, 25). The fact that CDC2 expression remained repressed

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**Figure 3.** D1/CDK and control cells arrest in response to Nutlin-3. A, D1/CDK cells were treated with 5 μmol/L of Nutlin-3 for 48 h. BrdUrd was added for 4 h and the cells were harvested and examined for BrdUrd incorporation and propidium iodide staining by using flow cytometry. B, percentages of FITC-positive cells after Nutlin-3 treatment, normalized to untreated samples.

**Figure 4.** Nutlin-3 treatment causes long-term repression of genes needed for cell cycle progression. Cells were treated with 5 μmol/L of Nutlin-3 for 72 h, then given fresh medium and incubated for an additional 24 h. Extracts were analyzed by the Western method.
even after Nutlin-3 was removed and p53 levels decreased, suggests that the repression of CDC2 may be mediated by a stable, epigenetic event consistent with methylation. It would explain why cells with D1/CDK retaining wild-type p53 were unable to resume cell division even after the removal of Nutlin-3, when the level of phosphorylated RB increases. The inability of the cells to demethylate and de-repress specific genes would prevent the expression of specific components that are necessary for cellular division. In contrast to cells having downstream disruptions in the p53 signaling pathway, cells expressing HdmX (which we and others have previously shown to confer resistance to Nutlin-3 or shRNA directed at p53) are unable to induce the p53-mediated repression of this cohort of genes either directly, or by a RB-E2F-dependent mechanism. As a result, these cells show no change in the status of phosphorylated RB or CDC2 expression, and do not arrest appropriately in response to Nutlin-3.

Transformation of normal human cells is far more complicated than that of normal rodent cells (26). Thus, we were surprised to see that D1/CDK expression alone was sufficient to promote anchorage-independent growth in human mammary epithelial cells. The HME1 cells used in our studies are derived from a reduction mammoplasty and are post-selection, meaning that they have spontaneously hypermethylated the p16Ink4a promoter and do not express p16Ink4a protein. In addition, they were immortalized by the expression of exogenous hTERT. Extensive passaging of these cells led to the current HME1 population, which were at passage 140 or greater in the experiments described here. It should be stressed that these cells have probably acquired additional changes during their growth, including increased levels of c-MYC, as described by Zhao et al. (15). Although HME1 cells show no signs of transformation in the absence of additional genetic changes, we now know that the expression of D1/CDK in pre-selection HMECs, with or without shp53, is unable to promote anchorage-independent growth (data not shown). However, these pre-selection cells lack hTERT expression, have normal levels of p16Ink4a, and express low levels of MYC, which may account for at least a part of the difference. The unknown epigenetic changes in the HME1 cells used here probably cooperate with D1/CDK expression to permit transformation, even though the expression of shp53 and PI3K, alone or together, was unable to transform these cells.

The response of cells transformed by specific genetic elements to specific therapies may provide important information regarding the likelihood that a therapy will be successful. p53-dependent signaling typically results in apoptosis or cell cycle arrest (27). In some cases, the growth arrest may be reversible, so that after the stimulus inducing arrest is removed, the cells can reenter the cell cycle and continue to proliferate. Even though the p53-responsive genes that regulate cell cycle arrest, such as p21, 14-3-3z, Reprimo, Gadd45, and B99 have been well studied, how they are repressed and de-repressed after removal of a stress stimulus needs further examination.

In conclusion, we have shown that immortalized mammary epithelial cells are transformed by constitutively activated CDK, where RB activity has been repressed. However, additional p53-dependent mechanisms for growth suppression remain because p53 activated by Nutlin-3 is still functional. Although the levels of hyperphosphorylated RB remained high in D1/CDK cells compared with control cells, the levels of CDC2 were reduced similarly, arguing that a direct mechanism for p53-dependent repression of gene expression exists and can override the effect of deregulated E2F. Other studies indicate that Nutlin-3 can induce growth arrest and apoptosis in some acute myeloid leukemia cell lines and primary multiple myelomas, but is unable to affect cells transformed by HdmX. Continued focus on the sensitivity or resistance of cells transformed by specific alterations, such as the effectiveness of Nutlin-3 in cells transformed by constitutive cyclin D1, will provide important information regarding the practical use of this promising new therapeutic agent (9, 28). Additional studies into the p53-dependent, RB-independent pathway for inducing cellular arrest may also aid us in better understanding how cells are able to undergo growth arrest after a stressful event and whether such events lead to temporary or permanent cellular arrest. Furthering our knowledge of the obstacles that a cell needs to overcome in order to become tumorigenic can suggest new pathways and proteins to target for therapeutic treatment.

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