The human INK4a gene locus encodes two structurally unrelated tumor suppressor proteins, p16INK4a and p14ARF. Although primarily proposed to require a functional p53-Mdm-2 signaling axis, recently p14ARF has been implicated in p53-independent cell cycle regulation. Here we show that p14ARF preferentially induces a G2 arrest in tumor cells lacking functional p53 and/or p21. Expression of p14ARF impaired mitotic entry and enforced a primarily cytoplasmic localization of p34cdc2 that was associated with a decrease in p34cdc2 kinase activity and reduced p34cdc2 protein expression. A direct physical interaction between p14ARF and p34cdc2 was, nevertheless, ruled out by lack of co-immunoprecipitation. The p14ARF-induced depletion of p34cdc2 was associated with impaired cdc25C phosphatase expression and a prominent shift to inhibitory Tyr-15 phosphorylation in G2-arrested cells lacking either p53, p21, or both. Finally, reconstitution of p34cdc2 using a constitutively active, phosphorylation-deficient p34cdc2AF mutant alleviated this p14ARF-induced G2 arrest, thereby allowing cell cycle progression. Taken together, these data indicate that p14ARF arrests cells lacking functional p53/p21 in the G2 phase of the cell cycle by targeting p34cdc2 kinase. This may represent an important fail-safe mechanism by which p14ARF protects p53/p21-deficient cells from uncontrolled proliferation.

In addition to the cyclin-dependent kinase inhibitor p16INK4a, the INK4a locus encodes a second, structurally unrelated protein termed p14ARF (p19ARF in the mouse). p14ARF was identified as a potent tumor suppressor both in vitro and in vivo (1, 2). Although usually expressed at low levels, rapid up-regulation of p14ARF is triggered by oncogenes (3–5) and during replicative senescence (6, 7). In turn, p14ARF mediates the accumulation of p53 via sequestration and subsequent degradation of the p53-antagonist Mdm-2 through the ubiquitin/proteasome pathway (8, 9). Consequently, p53 half-life is prolonged, allowing the consecutive activation of its downstream target genes, such as p21 and Bax.

Most if not all biological activity of p14ARF has been proposed to require a functional p53-signaling axis (10). However, recent data indicate that p14ARF and p53 act in overlapping signaling pathways rather than strictly sequentially (7). The murine homologue p19ARF was shown to exert cell cycle arrest independently from p53, Mdm-2, and Rb (11) as well as p21 (12) and p27KIP1 (13). In the same vein we showed that p14ARF induces p53 and Bax-independent apoptosis, which is preceded by the accumulation of cells in the G2/M phase of the cell cycle (14).

Recently, some of the molecular targets mediating p53-Mdm-2-independent effects of p14ARF were identified (15–17). Those include members of E2F transcription factor family (18, 19), which may account for the induction of a G1 arrest in cells lacking functional p53 and Rb. Furthermore, p14ARF has been shown to delay S phase progression in a p53-independent manner (20). Nevertheless, many aspects of p14ARF signaling remain unclear. In particular, this applies to the mechanisms of p14ARF-induced cell cycle arrest other than G1 and S phase arrest.

We show here that p14ARF induces the accumulation of cells in the G2 but not the M phase of the cell cycle regardless of p53 or p21 deficiency. This G2 arrest was accompanied by inactivation of the p34cdc2 kinase, a key mediator of G2 restriction point control. Furthermore, the concomitant down-regulation of its upstream regulator, the cdc25C phosphatase, increased inhibitory tyrosine phosphorylation of p34cdc2 in G2-arrested cells, and a predominant cytoplasmic localization of p34cdc2 was noted. Nonetheless, an overall decrease in p34cdc2 protein levels in p14ARF-expressing cells through a primarily post-transcriptional mechanism was observed that may further contribute to a reduced p34cdc2 kinase activity. Finally, functional studies reveal that reconstitution of p34cdc2 kinase activity overcomes the p14ARF-induced G2 arrest and triggers progression in the cell cycle. These data establish that p14ARF is capable of inducing a G2 cell cycle arrest by down-regulating p34cdc2 in cells lacking a functional p53/p21-signaling axis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—HEK293 and DU145 were obtained from the ATCC (Manassas, VA) or the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). HCT116 wild type cells and their isogenic knock-out sublines HCT116-p53−/− (21) and HCT116-p21−/− (22) were kindly provided by Dr. Bert Vogelstein, Johns Hopkins Cancer Center, Baltimore, MD. HEK293 and DU145 cells were grown in Dulbecco's modified Eagle's medium, 4.5 gliters glucose, and HCT116 cells were maintained in McCoy's 5A medium; cells were supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 0.1 μg/ml streptomycin (all from Invitrogen) at 37 °C with 5% CO2 in a fully humidified atmosphere. For inhibition of de novo protein synthesis, cycloheximide (Sigma-Aldrich) was added at a final concentration of 10 μg/ml 24 h after adenoviral infection.**

**Adenoviral Gene Expression—Construction of recombinant, replication-deficient Ad5-CMVp14ARF (Ad-p14ARF) was performed as described (14), and high titer adenoviral stocks were generated according to standard procedures as described (23). A binary adenoviral vector system for the conditional expression of a constitutively active p34cdc2 mutant (Ad-cdc2AF/Ad-tTA) was kindly provided by Dr. David Morgan, Johns Hopkins Cancer Center, Baltimore, MD.**
p14ARF Induces G2 Arrest by Targeting p34^{cd2} 

University of California at San Francisco (24). An adenosine expressing β-galactosidase (Ad-lacZ) was used as a control vector.

**Antibodies and Reagents—** Anti-p14ARF (clone 14P02, raised against full-length recombinant human p14^{ARF}) was from NeoMarkers (Fremont, CA). Anti-p21 (clone 6B6, raised against full-length human p21) and anti-p53 (clone DO-1, reactive against amino acids 1–45 of human p53), were from BD Pharmingen, and anti-cyclin B1 (clone D-11, reactive to full-length human cyclin B1) was from Santa Cruz Biotechnology (Santa Cruz, CA). These mouse monoclonal antibodies were used at a dilution of 1:1000. Antibodies against cdc25C (Santa Cruz Biotechnology, reactive against a recombinant 150-amino acid C-terminal fragment of human cdc25C) and β-actin (Sigma-Aldrich; reactive against an 11-amino acid C-terminal fragment of human β-actin) were rabbit polyclonal antibodies used at a dilution of 1:200 and 1:500, respectively. Anti-p34^{cd2}, reactive against amino acids 6–24 of human p34^{cd2}, and anti-phospho-cdc2 (Tyr-15), reactive against a synthetic keyhole limpet hemocyanin conjugated phospho-Tyr-15 peptide corresponding to the residues around Tyr-15 of human p34^{cd2} were both rabbit polyclonal antibodies from Cell Signaling Technologies, Beverly, MA and used a dilution of 1:1000. All antibodies were diluted in PBS supplemented with 0.05% Tween 20 (PBS-T), 3% nonfat dry milk, and 0.1% NaN3. Secondary goat-anti-mouse IgG and goat-anti-rabbit IgG antisera coupled to hors eradish peroxidase from Promega (Madison, WI) were used at a 1:500 dilution in PBS-T. For detection of p34^{cd2}, a polyclonal anti-p34^{cd2} antibody (Santa Cruz) generated by immunizing rabbits with a C-terminal peptide of human p34^{cd2} was used.

**Immunoblotting—** Cells were harvested, washed twice with ice-cold PBS, and lysed in 125 μl of lysis buffer (10 mM Tris/HCl, pH 7.5, 300 mM NaCl, 1% Triton X-100, 2 mM MgCl2, 5 mM EDTA) supplemented with protease inhibitors (1 μM pepstatin, 1 μM leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride) for 30 min on ice. Samples were then centrifuged at 15,000 × g at 4 °C for 15 min, and the concentration of total cellular proteins from the supernatants was determined using the bicinchoninic acid assay (Pierce). Thereafter, samples were mixed with equal amounts of sample buffer (125 mM Tris/ HCl, 288 mM β-mercaptoethanol, 20% glycerol, 2% SDS, 10 μg/ml bromphenol blue), boiled for 5 min, and separated by SDS-PAGE using either 16 or 10% gels. Immunoblotting and visualization of the proteins using enhanced chemiluminescence was performed as described (14).

**Cell Cycle Analysis—** Cells were seeded in 6-well plates at 1 × 10^5 cells per well (Costar; Cambridge, MA), cultured overnight, and infected with adenoviral vectors as indicated. At the indicated time points cells were harvested, and cell cycle analysis was performed as described (25). Cellular DNA contents were measured with a linear amplification in the FL-2 channel of a FACScan flow cytometer (BD Biosciences) equipped with CellQuestPro using the ModFit software.

**5-Bromodeoxyuridine (BrdUrd) Incorporation—** Cells were seeded in 6-well plates at 1 × 10^5 cells per well, cultured overnight, and infected with adenoviral vectors as indicated. After harvest cells were labeled with a mouse monoclonal anti-MMP2 antibody (Upstate Biotechnology; Milton Keynes, UK) at a final concentration of 1 μg/ml as described (27). Thereafter, cells were incubated with a fluorescein isothiocyanate-labeled goat-anti-mouse antibody (Jackson ImmunoResearch; West Grove, PA) at a final concentration of 7.5 μg/ml. Bi-variate analysis of DNA content (propidium iodide) and BrdUrd incorporation (fluorescein isothiocyanate) was performed by flow cytometry, and M phase cells distinct from cells in G2 phase were identified as MMP2-positive events with a 4 N DNA content. Incubation with 0.1 μg/ml nocodazole (Sigma-Aldrich) for 16 h was used as a positive control for cells arrested in mitosis. For chromatin staining, cells were seeded in 6-well plates at 1 × 10^5 cells per well, cultured overnight, and adenovirally infected as indicated. At the end of the culture period cells were washed twice with PBS, fixed with paraformaldehyde and proteinase K for 10 min at room temperature, and stained with Hoechst 33258 (Sigma-Aldrich) at a final concentration of 0.5 μg/ml for 30 min. After three washes with PBS, cells were studied using an Olympus inverted fluorescence microscope.

**p34^{cd2} Kinase Assay—** After adenoviral infection, cells (5 × 10^5 per well) were harvested and lysed in IP-LB (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 1 mM NaF, 0.1 mM Na3VO4, 10 mM β-glycerophosphate, and 0.1% Tween 20) supplemented with 10% complete mini solution (Roche Applied Science). p34^{cd2} was immunoprecipitated from 200 μg (HCT116) or 300 μg (DU145) of cellular extracts by incubation with an anti-p34^{cd2} antibody (Santa Cruz) at 1.4 μg/ml and 20 μl of protein A-Sepharose beads (Sigma-Aldrich) for 2 h at 4 °C in IP-LB buffer without Tween 20. Thereafter, immunoprecipitates were washed twice in IP-LB without Tween 20 and once in kinase buffer (20 mM MgCl2, 10 mM EGTA, and 40 mM Hepes, pH 7.5). The kinase reaction was carried out at 30 °C for 20 min with 0.5 μM [γ-32P]ATP, 25 μM cold ATP, and 5 μg of histone H1 as the substrate in a total volume of 20 μl as described (28). Samples were separated by SDS-PAGE as described above, and gels were subjected to autoradiography.

**Preparation of mRNA and Reverse Transcription-PCR—** A total of 2 × 10^5 cells were plated per 25-cm² flask, cultured overnight, and adenovirally transduced as indicated. RNA preparation and reverse transcription were performed as described (29). Primer sequences were: p14^{ARF} (sense), GCC TCC TGG TCA GAT GG; p34^{cd2} (antisense), TCT GTG GTA CGG ATC ACC. GAT GAC AGC GCC GTC ACC GTG CTC AGG CAC TCT GTG. Amplification consisted of a first denaturation cycle at 94 °C for 4 min followed by 22 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 2 min, and a final extension at 72 °C for 4 min.

**Immunoprecipitation—** A total of 200 μg of cellular proteins were precipitated as described above and precleared with 20 μl of protein A-Sepharose (Sigma-Aldrich) in a total volume of 800 μl of IP-LB at 4 °C for 1 h. After centrifugation at 15,000 × g at 4 °C for 20 s, the supernatants were removed and incubated with 1 μg of antibodies against p14^{ARF}, p34^{cd2}, or cyclin B1 and 20 μl of protein A-Sepharose either in the absence or presence of detergent (1% Triton X-100) in a total volume of 500 μl at 4 °C for 2 h. Removal of the supernatants was accomplished by centrifugation, beads were washed twice in IP-LB, and proteins were eluted by the addition of 20 μl of 2× Western blot sample buffer.

**Immunofluorescence—** Immunofluorescence was performed as described previously (23). Briefly, cells were seeded on sterile coverslips, grown for 24 h, and infected with adenoviral vectors at an m.o.i. of 25 for 48 h. After three washes with PBS, cells were fixed with 3% paraformaldehyde (v/v) for 15 min at room temperature. After permeabilization with 0.5% Triton X-100 in PBS for 2 min, slides were incubated with blocking solution (PBS supplemented with 2% BSA) for 40 min before incubation with anti-p34^{cd2} and anti-histone H3 antibodies in blocking solution at 37 °C for 2 h. After three washes in PBS slides were incubated with Alexa fluor 488-labeled goat-anti-rabbit and Alexa fluor 594-labeled mouse-anti-rabbit antibodies (both from Molecular Probes; 1:1000) in PBS for 1 h. Finally, slides were washed twice in IP-LB, and proteins were mounted with Mowiol solution (Merck) containing Hoechst 33258 (Sigma-Aldrich) at a final concentration of 0.5 μg/ml and subjected to UV microscopy.

**RESULTS**

p14^{ARF} Induces p53-independent Accumulation of Cells in G2/M phase of the Cell Cycle—We previously showed that p14^{ARF}-induced apoptosis is independent of functional p53 (14). Expression of p14^{ARF} also triggered the accumulation of cells in G2/M phase of the cell cycle in p53-deficient cell lines. This was surprising as, so far, the cell cycle regulating capacity of p14^{ARF} had been primarily attributed to the activation of p53 and its downstream effectors, i.e. p21, leading to G1 or combined G1/S/G2/M arrest. To study the mechanism of the p14^{ARF}-induced G2/M arrest by p14^{ARF}, p53-mutated DU145 cells (30) were infected with Ad-p14^{ARF} and analyzed for cell cycle distribution by flow cytometry (Fig. 1A). As compared with mock treatment (Control) or control-vector (Ad-lacZ) infected cells, p14^{ARF} induced a more than 2-fold increase in the number of cells in G2/M phase of the cell cycle. This dose-dependent effect was accompanied by a corresponding decrease of cells in G1 phase (Fig. 1B). Western blot analyses showed a dose-dependent increase in the expression of p14^{ARF}, whereas the level of mutated p53 protein remained stable. Moreover, there was no induction of p21 (Fig. 1).

1 The abbreviations used are: PBS, phosphate-buffered saline; BrdUrd, 5-bromodeoxyuridine; m.o.i., multiplicity of infection; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

---

**References**

1. The abbreviations used are: PBS, phosphate-buffered saline; BrdUrd, 5-bromodeoxyuridine; m.o.i., multiplicity of infection; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
These data indicate that p14ARF induces a p53-independent accumulation of cells in the G2/M phase of the cell cycle. To confirm that the p14ARF-induced accumulation of cells in G2/M is independent of p53, p53-deleted HCT116 cells (HCT116-p53-/-) were subjected to cell cycle analysis in parallel with isogeneic HCT116-p53+/+ cells. As shown in Fig. 2A, p14ARF induced an increase in the percentage of cells in G2/M phase of the cell cycle exclusively in HCT116-p53-/- cells. A time course experiment revealed that p14ARF induces a sustained G2/M arrest in HCT116-p53-/- cells but not in the p53 wild type cells (Fig. 2B). Whereas the relative number of cells in G2/M phase increased in HCT116-p53-/- cells (right panel), no such increase was observed in p53+/+ cells (left panel) at 48 and 72 h. Expression of p14ARF protein increased in a dose-dependent manner in both p53-proficient and p53-deficient HCT116 cells (Fig. 2C). As expected, this was paralleled by the induction of p53 and p21 protein expression in HCT116-p53+/+ cells. No such induction occurred in HCT116-p53-/- cells. These data corroborate that p14ARF induces the accumulation of cells in the G2/M phase of the cell cycle in cells lacking functional p53.

p14ARF-mediated G2/M Arrest Is Independent of p21—The lack of p21 induction upon p14ARF expression in both p53-mutated DU145 and p53-deleted HCT116 cells suggested that the induction of G2/M arrest by p14ARF does not depend on functional p21. Nevertheless, p21 may also be induced by p53-independent mechanisms. To confirm that p21 is dispensable for p14ARF-induced G2/M arrest, p21-deleted HCT116 cells (HCT116-p21-/-) were infected with Ad-p14ARF along with isogeneic HCT116-p21+/+ cells and assayed for cell cycle distribution 48 h after infection in parallel with mock-treated and control vector-infected cells (Fig. 3A). Expression of p14ARF in HCT116-p21-/- induced an almost 2-fold increase in the number of cells in G2/M phase as compared with HCT116-p21+/+ cells. This was accompanied by a corresponding decrease in the percentage of cells in S phase, whereas the number of cells in G1 phase remained stable as opposed to HCT116-p21+/+, where an increase in G1 cells was induced by p14ARF. The p14ARF-induced decrease in S phase cells was, however, also apparent in the p21 wild type cells. Western blot analysis revealed a dose-dependent increase in the expression of p14ARF and p53 in both HCT116-p21+/+ and HCT116-p21-/- cells, whereas an increase in the expression of p21 was detected only in HCT116-p21+/+ (Fig. 3B). These data demonstrate that expression of p14ARF induces the accumulation of cells in G2/M phase of the cell cycle through a p53/p21-independent mechanism, which preferentially occurs in cells with a disabled p53/p21-signaling pathway.
The results described above suggested that the p14ARF-induced G2/M arrest occurs primarily in the absence of either p53 or p21. Yet the p14ARF-induced decrease in S phase cells appears to be independent of the presence or absence of functional p21. To confirm this hypothesis, HCT116-p53+/− and HCT116-p53−/− cells were infected with the indicated adenoviral vectors at 100 m.o.i. or treated mock (Control) for 48 h. Cellular DNA content was determined by flow cytometry, and relative cell cycle distribution is given in %. A, HCT116-p53+/− and HCT116-p53−/− cells were mock-treated or infected with 100 m.o.i. of Ad-p14ARF or Ad-lacZ until the percentage of tetraploid (4 N) cells was determined (open squares, medium control; open circles, Ad-LacZ; filled circles, Ad-p14ARF). B, HCT116-p53+/− and HCT116-p53−/− cells were infected with Ad-p14ARF at increasing m.o.i. for 48 h. Protein expression of p14ARF, p53, and p21 was determined by Western blot analysis.

p14ARF-mediated Inhibition of DNA Synthesis Is Independent of p21—The results described above suggested that the p14ARF-induced G2/M arrest occurs primarily in the absence of either p53 or p21. Yet the p14ARF-induced decrease in S phase cells appears to be independent of the presence or absence of functional p21. To confirm this hypothesis, HCT116-p21+/+ and HCT116-p21−/− cells were infected with Ad-p14ARF for 48 h, pulse-labeled with BrdUrd for 30 min, and subsequently analyzed by flow cytometry in parallel with mock-treated and control vector-infected cells (Fig. 4A). Quantification of BrdUrd-positive cells as percent of cells in S phase (Fig. 4B) confirmed that loss of p21 impairs S phase control as evidenced by the overall higher percentages of BrdUrd-positivity in the p21−/− cells. Nevertheless, the inhibition of DNA synthesis did not differ between p21+/+ and p21−/− cells as indicated by the similar decrease in the number of BrdUrd-positive cells in both cell lines upon p14ARF expression. Thus, p14ARF-mediated G2/M arrest is paralleled by inhibition of DNA synthesis, which is independent of the presence or absence of functional p21, but unlike G2/M arrest is not enhanced upon disruption of p53 (Fig. 2A) or p21 (Figs. 3A and 4) function.

p14ARF Induces G2 But Not M phase Arrest in the Absence of
p14ARF induces G2/M arrest in HCT116-p21−/− cells. A, HCT116-p21−/− and HCT116-p21−/− cells were infected with the indicated adenoviral constructs at 100 m.o.i. or mock-treated (Control). Cell cycle distribution was determined by flow cytometric analysis of cellular DNA contents. Relative numbers of cells in G1, S, and G2/M phase are given in %. B, HCT116-p21−/− and HCT116-p21−/− cells were transduced with Ad-p14ARF or Ad-LacZ or were mock-treated for 48 h. Protein expression of p14ARF, p53, and p21 was determined by Western blot analysis.

Expression of p14ARF Interferes with p34cdc2 Expression and Kinase Activity—Transition of cells from the G2 phase to mitosis after completion of DNA synthesis requires the timely activation of p34cdc2 kinase (cdc2, cdk1). This involves the induction of cyclin B1 expression and its binding to cdc2. Furthermore, the removal of inhibitory phosphorylation at the Tyr-15 residue of the cdc2 molecule is required for its kinase activation. This is carried out by the cdc25C phosphatase before the translocation of cyclin B1-cdc2 complexes from the cytoplasm to the nucleus (31). To clarify whether the G2 arrest upon expression of p14ARF involves the cdc2 kinase-cyclin B1 rheostat, HCT116 wild type, HCT116-p53+/−, and HCT116-p21−/− cells were infected with Ad-p14ARF, and protein expression was studied by Western blot analysis after 48 h. As shown in Fig. 6A, expression of p14ARF induced a decrease in cdc2
protein levels that was even more pronounced in HCT116 cells deleted for either p53 or p21, i.e. the cells that preferentially undergo G2 arrest upon p14ARF expression. This decrease in total cdc2 protein expression in HCT116 wild type cells was paralleled by a corresponding decrease in the expression of inactive Tyr-15-phosphorylated cdc2. In sharp contrast, the levels of inactive Tyr-15-phosphorylated cdc2 remained stable in G2-arrested HCT116 cells void of either p53 or p21, indicating a strong relative increase of the ratio between the inactive Tyr-15-phosphorylated cdc2 and total cdc2. Irrespective of their cell cycle behavior, a substantial decrease in the expression of the cdc25C phosphatase was noted in all HCT116 sublines. This coincided with a shift toward a higher molecular weight indicative of hyperphosphorylated cdc25C species. Both the relative increase in the expression of Tyr-15-phosphorylated cdc2 and the depletion of cdc25C occurred in a dose-dependent fashion (data not shown). Regarding the expression of cyclin B1, p53/p21 wild type cells that preferentially arrest in G2 upon p14ARF expression showed a strong decrease in cyclin B1 protein levels. In contrast, stable or slightly increasing levels of cyclin B1 were detected in both HCT116-p53-/- and HCT116-p21-/- cells. Taken together, these results suggest that the p14ARF-induced arrest in G2 phase of the cell cycle is mediated by the down-regulation of cdc2 protein expression and kinase activity. This coincides with persistence of cyclin B1 expression. In accordance with this, the expression of p14ARF induced a dose-dependent decrease in cdc2 kinase activity in HCT116 cells irrespective of either functional p53 (HCT116-p53-/-) or p21 (HCT116-p21-/-) (Fig. 6B).

In analogy to HCT116 cells, p53/p21-deficient DU145 cells displayed a dose-dependent decrease in total cdc2 protein levels (Fig. 6C) upon expression of p14ARF. Like G2-arrested HCT116-p53-/- and HCT116-p21-/- cells, this was paralleled by a marked increase in the expression of inactive Tyr-15-phosphorylated cdc2 and a decrease in cdc25C protein levels. Noteworthy, an increase in the expression of cyclin B1 upon p14ARF expression was observed in DU145 cells as compared with stable levels of cyclin B1 in both HCT116-p53-/- and HCT116-p21-/- cells. Like in HCT116-p53-/- and HCT116-p21-/- cells, this may be explained by the strong induction of G2 arrest upon expression of p14ARF in DU145 cells void of both functional p53 and p21. In addition, the differential behavior of cyclin B1 expression in p53 wild type and p53-deficient cells precludes that the regulation of cyclin B1 expression is responsible for the G2 arrest induced by p14ARF. In analogy to HCT116-p53-/- and HCT116-p21-/-, DU145 cells showed a decrease in cdc2 kinase activity 48 h after transduction with Ad-p14ARF (Fig. 6D). Taken together with the data obtained in HCT116 cells, these results suggest that, apart from down-regulating cdc2 expression at the protein level, p14ARF modulates cdc2 inhibitory phosphorylation and kinase activity through depletion of the cdc25C phosphatase via a p53/p21-

![Image](image-url)
Expression of p14\textsuperscript{ARF} Precludes Nuclear Localization of the cdc2 Kinase—The transition of active cyclin B1-cdc2 complexes from the cytoplasm into the nucleus during prophase is a prerequisite for the onset of mitosis after completion of G\textsubscript{2} phase (31). Regarding the cellular localization, we observed that cdc2 was almost exclusively localized to the cytoplasm after p14\textsuperscript{ARF} expression (Fig. 7). There was no difference between cells wild-type (upper panel) or homozygously deleted for p21 (lower panel). Cytoplasmic localization of cdc2 upon p14\textsuperscript{ARF} expression was associated with lack of histone H3 staining (middle panel) and the absence of mitotic figures in cells stained with Hoechst 33258 (right panel). Taken together with the data depicted in Fig. 6, these results indicate that p14\textsuperscript{ARF} induces a G\textsubscript{2} arrest in cells lacking either functional p53, p21, or both by inducing a primarily cytoplasmic localization of cdc2 before a p14\textsuperscript{ARF}-triggered decrease in cdc2 expression.

P14\textsuperscript{ARF} Down-regulates cdc2 Protein Expression through a Post-transcriptional Mechanism—To address the mechanism of the p14\textsuperscript{ARF}-induced decrease of cdc2 protein expression, we first investigated whether p14\textsuperscript{ARF} down-regulates cdc2 mRNA levels. Detection of cdc2 expression by semiquantitative reverse transcription-PCR did, however, not reveal differences in cdc2 mRNA levels between mock-treated and p14\textsuperscript{ARF}-expressing cells (Fig. 8A). Therefore, the mechanisms leading to the decrease in cdc2 protein levels observed after expression of p14\textsuperscript{ARF} may reside at the post-transcriptional level, i.e. by interference with protein stability. To further investigate this hypothesis, p14\textsuperscript{ARF} was expressed in DU145 cells followed by the addition of cycloheximide 24 h after infection to inhibit \textit{de novo} cdc2 protein synthesis. As shown in Fig. 8B, a decrease in cdc2 protein levels was observed starting at 8 h after the addition of cycloheximide to the culture medium, further declining over the next 16 h. In contrast, cdc2 levels remained stable in mock-treated cells for up to 24 h. These data indicate that the decrease in cdc2 protein levels (and kinase activity) upon expression of p14\textsuperscript{ARF} may result from interference with cdc2 protein turnover, i.e. through proteasomal degradation. Experiments where the cells were incubated in the presence of proteasome inhibitors, i.e. MG132, were, however, not informative as both DU145 and HCT116 rapidly underwent apoptosis in the presence of this compound.

Lack of a Direct Physical Interaction between p14\textsuperscript{ARF} and cdc2—p14\textsuperscript{ARF} was shown to target a number of cellular proteins, i.e. E2F transcription factors, by direct physical interaction before proteasomal degradation (18, 19). We, therefore, investigated whether the cdc2 protein physically interacts with p14\textsuperscript{ARF}. To this end, DU145 cells were infected with increasing m.o.i. of Ad-p14\textsuperscript{ARF}, and total cellular proteins were subjected to immunoprecipitation with antibodies against either cdc2 or p14\textsuperscript{ARF}. As expected, Western blot analysis (Fig. 8C) revealed that cyclin B1 physically interacts with cdc2 and, therefore, is co-immunoprecipitated with cdc2 under non-denaturing, detergent-free conditions. In contrast, p14\textsuperscript{ARF} did not co-immunoprecipitate with cdc2. Likewise, there was no evidence for co-immunoprecipitation of p14\textsuperscript{ARF} and cdc2 when the immunoprecipitation was performed in the presence of Triton X-100. Under these detergent conditions, co-immunoprecipitation of cdc2 and cyclin B1 was disrupted (Fig. 8D). Similar results, i.e. a lack of co-immunoprecipitation between p14\textsuperscript{ARF} and cdc2, were observed when anti-p14\textsuperscript{ARF} antibodies were employed for the pull-down or when CHAPS was employed as detergent (data not shown). These results indicate that p14\textsuperscript{ARF} and cdc2 do not physically interact, which should, however, be a prerequisite for p14\textsuperscript{ARF}-initiated proteasomal degradation of cdc2.

Fig. 5. \textit{p14\textsuperscript{ARF} induces G\textsubscript{2} and not M phase arrest.} A, HCT116-p21\textsuperscript{−/−} were either mock-treated (Control, upper left), treated with 0.1 \(\mu\)g/ml nocodazole (Nocodazole, upper right), or infected with Ad-LacZ (Ad-lacZ, lower left) or Ad-p14\textsuperscript{ARF} (Ad-p14\textsuperscript{ARF}, lower right) at 25 m.o.i. for 48 h. Cells were stained with a fluorescein isothiocyanate (FITC)-labeled MPM2 antibody. Bi-variate analysis of DNA content (propidium iodide (PI)) from the total number of cells in G\textsubscript{2}/M phase of the cell cycle (\(\square\), control (Co); \(\square\), Ad-lacZ (lacZ); \(\square\), nocodazole (Noc); \(\square\), Ad-p14\textsuperscript{ARF} (p14\textsuperscript{ARF}), C, chromatin staining. Nuclei were stained with Hoechst 33258 dye, and labeled DNA was visualized by the use of fluorescence microscopy (top, medium control (Control); middle, Ad-LacZ; bottom, Ad-p14\textsuperscript{ARF}).
Expression of Constitutively Active p34\textsuperscript{cdc2} Abrogates p14ARF-induced G\textsubscript{2} Arrest—Our results indicate that the expression of p14ARF induces a G\textsubscript{2} arrest in cells lacking either functional p53 or p21 through the down-regulation of cdc2 kinase activity. Thus, functional reconstitution of cdc2 kinase activity should overcome p14ARF-induced G\textsubscript{2} arrest. To test this hypothesis, p53-mutated DU145 cells were infected with either Ad-p14ARF alone or together with an adenoviral expression system, allowing the conditional, tetracycline-dependent expression of the constitutively active cdc2-mutant cdc2AF (Ad-cdc2AF) (24) where the transgene expression is turned on in the absence (-Dox) and shut down in the presence (+Dox) of doxycycline (Tet-off system). In contrast to wild-type cdc2, cdc2AF is refractory to inhibitory phosphorylation at tyrosine residue 15 (Tyr-15) and subsequent sequestration via the proteasome due to targeted mutations at the residues Thr-14 and Tyr-15 to alanine (Ala) or phenylalanine (Phe), respectively (32). As expected, coexpression of p14ARF and cdc2AF in the presence of doxycycline (+Dox) resulted in the accumulation of cells in G\textsubscript{2} phase of the cell cycle (Fig. 9A and B). In contrast, the conditional coexpression of cdc2AF and p14ARF in DU145 cells in the absence of doxycycline (-Dox) abrogated the p14ARF-induced G\textsubscript{2} arrest. This was associated with an increase in the number of cells in G\textsubscript{1} phase of the cell cycle (Fig. 9, A and B) in the absence of doxycycline (-Dox; filled bars).

As shown in Fig. 9C, both the functional activity and expression of cdc2 were increased in DU145 cells transduced with the constitutively active cdc2AF mutant in the absence of doxycycline (-Dox). In contrast, expression of cdc2AF was almost completely suppressed in the presence of doxycycline (+Dox), confirming tight control of the conditional expression system. Expression of p14ARF substantially interfered with both endogenous cdc2 and cdc2AF kinase activities (Fig. 9D). Notably, cdc2AF expression induced a decrease in cyclin B1 expression.

FIG. 6. p14ARF down-regulates p34\textsuperscript{cdc2} kinase activity in p53 and/or p21-deficient cells. A, HCT116-wild type (WT), HCT116-p53\textsuperscript{−/−}, or HCT116-p21\textsuperscript{−/−} were infected with adenoviral vectors at 100 m.o.i. as indicated. Protein expression was determined by Western blot analysis 48 h after infection. p-cdc2, phosphorylated cdc2. B, measurement of p34\textsuperscript{cdc2} kinase activity. HCT116-WT, HCT116-p53\textsuperscript{−/−}, and HCT116-p21\textsuperscript{−/−} cells were infected with Ad-p14ARF or control vector (Ad-lacZ) for 48 h. p34\textsuperscript{cdc2} was immunoprecipitated, and p34\textsuperscript{cdc2} kinase activity was determined in vitro by the use of histone H1 (HH1) as a substrate. C, DU145 prostate cancer cells were infected as described in B for 48 h and subjected to Western blot analysis. D, cells were infected with the indicated adenoviral vectors, and p34\textsuperscript{cdc2} kinase activity was determined using histone H1 as a substrate.
that was even more pronounced in the presence of p14ARF expression (Fig. 9C). This is in accordance with the release from G2 arrest upon restoration of cdc2 kinase activity.

DISCUSSION

Earlier studies indicated that most if not all of the biological effects of p14ARF (and its murine homolog p19ARF) entirely depend on a functional p53/Mdm-2 signaling axis (2, 10, 33). Nevertheless, this paradigm is more and more challenged by recent reports clearly demonstrating that p14ARF also triggers a number of p53-independent effects. These include the induction of p53-independent apoptosis (14, 34) and cell cycle arrest (11, 13, 18–20). In terms of cell cycle regulation, current data are, however, not sufficient to draw a conclusive model of p53-independent cell cycle arrest other than that induced in G1 and S phase.

When investigating the proapoptotic and cell cycle regulatory capacity of p14ARF in a p53-deficient versus wild type background, we and others observed the accumulation of cells in G2/M phase of the cell cycle before apoptotic cell death in p53 mutant cells (14, 18). Here, we show that p14ARF preferentially induces an arrest in the G2 and not the M phase of the cycle in p53-negative cells. Whereas p53 wild type cells arrested primarily in G1, a prominent G2 arrest occurred exclusively in p53-deficient but not in p53 wild type cells. Notably, there was no difference between cells lacking functional p53 due to an inactivating p53 mutation (DU145) or homozygous deletion of the p53 gene (HCT116-p53−/−). This underscores the well established role of p53 in p14ARF-induced G1 arrest programs (10, 35) but nevertheless demonstrates that the G2 arrest programs induced by p14ARF are not only independent of p53 but may require the absence of functional p53 and/or its downstream effector p21 to become apparent. In this regard, our data are neither in contradiction to a number of previous studies demonstrating the requirement of p53 for p14ARF-induced G1 arrest nor do they challenge the observation that p14ARF triggers a G2 arrest in cells with an intact p53/p21 signaling axis (9, 33).

Cell cycle analyses in p53-deficient cells indirectly suggested that p21 is dispensable for p14ARF-induced G2 arrest. Consequently, we observed a prominent G2 arrest in HCT116 cells homozygously deleted for the p21 gene, whereas p53/p21-proficient HCT116 cells preferentially arrested in the G1 phase of the cell cycle. This further underlines the notion that in addition to the p53-dependent mechanisms involving the p53 effector p21 other so far unknown signaling events may mediate the execution of the p14ARF-induced G2 arrest. This is, however, in contrast to the observations of others who recently suggested that p21 is a key effector in p14ARF-induced, cdc2-mediated G2 arrest (36). This latter study showed rather descriptive evidence such as the induction of p21 expression in cells undergoing G2 arrest upon conditional p14ARF expression. This led to the hypothesis that p53-independent induction of p21 is responsible for p14ARF-induced G2 arrest. In contrast, we show here that loss of p21 in a congenic system neither affects targeting of the cdc2 kinase activity nor G2 arrest induced by p14ARF. Moreover, we show that p14ARF induces p21 expression in an entirely p53-independent fashion.

After completion of DNA synthesis, the timely regulation of the p34cdc2 kinase is a prerequisite in G2/M-restriction point control, which licenses entry into mitosis. The activation of cdc2 takes places at several levels including the expression of cyclin B1, its binding to cdc2, and the translocation of cyclin B1-cdc2 complexes from the cytoplasm into the nucleus. Furthermore, the dephosphorylation of inhibitory phosphotyrosine residues, i.e., Tyr-15, within the cdc2 molecule through the cdc25C phosphatase is of central importance for the proper activation of cdc2 (31). Consistent with the dose-dependent reduction of cdc2 kinase activity upon expression of p14ARF, we detected the down-regulation of cdc2 kinase activity in p53-deficient cells arrested in the G2 phase of the cell cycle. On the one hand this down-regulation of cdc2 activity was related to a marked reduction of cdc2 protein levels in cells arrested at the G2/M restriction point. This strongly suggested that the depletion of cdc2 plays a critical role in the induction of cell cycle arrest in G2 phase. On the other hand, a relative p14ARF-induced increase in Tyr-15-phosphorylated cdc2 protein was detected at the same time. This impact of p14ARF on cdc2 Tyr-15 phosphorylation occurred only in p53- or p21-deficient HCT116 cells but not in the congenic wild type controls. The reason for this p53/p21 dependence of cdc2 Tyr-15 phosphorylation remains unclear but appears to be related to the strongly enhanced G2 arrest induced by p14ARF in HCT116-p53−/− and HCT116-p21−/− cells. Tyr-15 phosphorylation was associated with a decrease in cdc25C expression, i.e., might be a consequence of p14ARF-induced depletion of cdc25C phosphatase. Nevertheless, even while p14ARF triggers a decrease in cdc25C phosphatase expression as previously reported by Eymin (36), Tyr-15 phosphorylation of cdc2 does not appear to be regulated by cdc25C alone since no enhanced cdc2 Tyr-15 phosphorylation was observed in HCT116 p53/p21 wild type cells, which show, however, a reduction of cdc25C expression upon p14ARF expression.
The inability of arrested cells to traverse mitosis into G2 was further confirmed by the decrease of MPM2 phosphoepitope exposure upon p14ARF expression. Thus, p14ARF induces a G2 and not M phase arrest. This is supported when the localization of cdc2 is studied. Upon expression of p14ARF, the distribution of cdc2 protein is restricted to the cytoplasmic compartment.
Moreover, cells expressing p14ARF were void of nuclear histone H3 staining and also lacked chromosome condensation and mitotic figures.

The functional relevance of cdc2 inhibition in p14ARF-induced G2 arrest is supported by experiments where cdc2 kinase activity was reconstituted by the use of a constitutively active cdc2 mutant (cdc2AF), which is resistant to inhibitory phosphorylation (24). This alleviated the p14ARF-induced G2 arrest with concomitant accumulation of p14ARF-expressing cells in G1. The kinase activity of cdc2 critically depends on the presence of cyclin B1. In HCT116 cells deficient for either p53 or p21 as well as in p53-mutated DU145 cells lacking p21 induction, cyclin B1 protein levels were stable or even induced upon expression of p14ARF. This clearly supports the hypothesis of a prolonged G2 arrest in these cells as the progression through and completion of mitosis would be paralleled by a decrease of cyclin B1. Furthermore, the fact that cyclin B1 is still abundant at sufficient levels in these cells underlines the notion that the critical G2/M-checkpoint component regulated by p14ARF indeed is cdc2 as shown by the down-regulation of both cdc2 kinase activity and protein expression. In turn, HCT116 cells proficient for p53 exhibited greatly reduced cyclin B1 expression levels. The reason for this might simply reside in the fact that these cells undergo G1 arrest upon p14ARF expression and therefore turn off cyclin B1 expression. Nevertheless, despite the rather moderate decrease of cdc2 protein levels in HCT116-p53 proficient upon expression of p14ARF, this lack of cyclin B1 may well serve to explain the fact that these cells exhibit a reduction in cdc2 kinase activity similar to HCT116 cells deficient for either p53 or p21. Then, however, the question arises of why these cells arrest only to a minor proportion in G2 phase of the cell cycle. In the first place, the presence of functional p53 primarily arrests the cells in the G1 phase. Secondly, because p14ARF interferes with S phase progression, only a minor proportion of cells is enabled to progress toward the G2 restriction point controlled by the cyclin B1/cdc2 rheostat. Taken together, this serves well to explain the fact that p53-proficient HCT116 cells primarily exhibit an arrest in the G1 phase of the cell cycle.
cycle, whereas the G2 arrest is almost entirely absent despite a similar reduction in cdc2 kinase activity as compared with HCT116-p53−/− and HCT116-p21−/− cells, respectively. Finally, the functional data indicate that endogenous cyclin B1 was expressed at sufficient levels in G2-arrested cells to cooperate with the conditionally expressed exogenous cdc2 to overcome G2 arrest. Consequently, re-expression of the constitutively active cdc2AF mutant alleviated p14ARF-induced G2 arrest and reconstituted the propensity of the cells to progress in the cell cycle.

Our data furthermore delineate that neither DU145 nor HCT116-p53−/− cells underwent a clear cut arrest in G2. Thus, proper p53 and p21 function is a prerequisite for executing G2 arrest programs irrespective of the ability of p14ARF to target other cell cycle regulators, such as E2F-1 or c-Myc (18, 19, 34). This also indicates that the signaling requirements for p14ARF-induced cell cycle arrest dissociate upstream of p53-mediated G1 and G2/M checkpoint control. In this vein we favor a model system based on the dual role of p14ARF in the regulation of cell cycle arrest in G1 and G2 phase (Fig. 10). On the one hand, p14ARF targets p53 and its effector p21 to induce an arrest primarily in the G1 phase of the cell cycle. This is the dominant form of arrest triggered in p53/p21-proficient cells. On the other hand, p14ARF targets the cdc2 kinase, thereby arresting cells in G2 phase of the cell cycle, and this arrest is independent of a functional p53/p21-signaling axis. Notably, this model does not challenge the observation that p14ARF may also induce a G2 arrest in cells with an intact p53/p21-signaling axis (9, 33). This may represent a fail-safe mechanism protecting cells from hyperproliferative stimuli or other, yet unknown genetic insults sensed by p14ARF even in the absence of a functional p53 tumor suppressor. However, although we did not further address this question, so far neither we nor others can fully rule out the effects of p14ARF on p53 homologs such as p63 or p73, which might functionally substitute for the loss of p53 function in p53-mutated DU145 cells as well as in p53-negative HCT116-p53−/− cells.

Apart from binding p53-Mdm-2 complexes, p14ARF was demonstrated to physically interact with several other proteins (1). Among those are members of the E2F family of transcription factors (18, 19), which by binding of p14ARF are rapidly targeted for degradation through the ubiquitin/proteasome pathway. However, co-immunoprecipitation studies showed the absence of a direct physical interaction between p14ARF and cdc2 in our setting. Furthermore, data obtained from co-immunoprecipitation under non-denaturing conditions, i.e. in the absence of detergent, strongly argue against the formation of complexes between p14ARF and cdc2 through intermediate proteins. Nevertheless, we cannot formally rule out the induction of proteins by p14ARF that are capable of degrading cdc2, i.e. ubiquitin ligases or F-Box proteins. In the same vein, we did not observe an interference of p14ARF expression with cdc2 mRNA levels, indicating that p14ARF targets cdc2 expression and function at the post-transcriptional level. This is supported by experiments where the de novo protein synthesis was inhibited by cycloheximide. There, expression of p14ARF accelerated the depletion of cdc2 levels. This is well in agreement with the hypothesis that p14ARF interferes with cdc2 protein turnover, thereby tilting the balance toward lower levels. Although the depletion of cdc2 via the proteasome appears likely, the exact mechanism remains so far enigmatic but does not occur through a direct physical interaction between p14ARF and cdc2. However, experiments performed in the presence of proteasome inhibitors were not informative as these compounds rapidly triggered apoptosis. Other possibilities such as the functional inactivation of cdc2 as a consequence of subcellular trapping of the cdc2 molecule (31) might confer to reduced cdc2 protein levels and kinase activity. Because we detected an almost exclusive cytoplasmic localization of cdc2 upon p14ARF expression, further studies are necessary to determine the functional significance of this observation in terms of p14ARF-mediated G2 arrest through inhibition of cdc2 kinase activity and total protein expression. Noteworthy, this cytoplasmic sequestration of cdc2 was observed in both p21 wild type and p21−/− cells. These cells show, however, rather substantial differences in cdc2 inhibitory phosphorylation at Tyr-15, which therefore does not appear to be the sole underlying mechanism for redistribution of cdc2 from the nuclear to the cytoplasmic compartment. Another attractive mechanism how p14ARF could interfere with cdc2 expression might, however, take place at the translational level. Recently, it was reported that p14ARF targets the protein biosynthesis machinery by inhibiting the translation of mRNA through the inhibition of ribosomal RNA processing (17), i.e. by interference with nucleophosmin/B23 (37, 38).

Apart from these mechanistic studies, it should, therefore, be promising to investigate the mechanism of p14ARF-induced cell cycle arrest in G2 for the function of p14ARF as a tumor suppressor, especially in those tumors exhibiting p53 loss of function. Additional prospects of research focused at the elucidation of this cell cycle arrest program might finally provide insight into how p14ARF regulates not only cell cycle arrest but also cellular senescence and induction of apoptosis programs.

Acknowledgments—We thank Antje and Anja Richter and Jana Rossius for expert technical assistance and Dr. Bert Vogelstein, Johns Hopkins Cancer Center, Baltimore, MD for generously providing HCT116 cells and mutants. The conditional adenoviral vector system for cdc2AF expression was kindly provided by Dr. David Morgan, University of California at San Francisco, CA.

REFERENCES

1. Sherr, C. J. (2000) Nat. Rev. Mol. Cell Biol. 2, 731–737
2. Kamijo, T., Zindy, F., Roussel, M. F., Quelle, D. E., Downing, J. R., Ashmun, R. A., Grosfeld, G., and Sherr, C. J. (1997) Cell 91, 649–659
3. Palermo, I., Pantoja, C., and Serrano, M. (1998) Nature 395, 125–126
4. de Stanchina, E., McCurrach, M. E., Zindy, F., Shihe, S. Y., Perbegey, G., Samuelson, A. V., Prives, C., Roussel, M. F., Sherr, C. J., and Lowe, S. W. (1998) Genes Dev. 12, 4239–4242
5. Bates, S., Phillips, A. C., Clark, P. A., Stott, F., Peters, G., Ludwig, R. L., and Vousden, K. H. (1998) Nature 395, 124–125
6. Zindy, F., Eschen, C. M., Randle, D. H., Kamijo, T., Cleveland, J. L., Sherr, C. J., and Roussel, M. F. (1996) Genes Dev. 10, 2444–2453
7. Carnero, A., Henson, J. D., Price, C. M., and Beach, D. H. (2000) Nat. Cell Biol. 2, 148–155
8. Fleissner, J., Schreiber-Agus, N., Liegeois, N. J., Silverman, A., Alland, L., Chin, L., Potes, J., Chen, K., Orlow, I., Lee, H. W., Cordon-Cardo, C., and DePinho, R. A. (1998) Cell 92, 713–723
9. Stott, F. J., Bates, S., James, M. C., McConnell, B. B., Starborg, M., Brooks, S., Palermo, I., Ryan, K., Hera, E., Vousden, K. H., and Peters, G. (1998) EMBO J. 17, 5001–5014
10. Sherr, C. J. (2000) Cancer Res. 60, 3689–3695
11. Weber, J. D., Jeffers, J. E., Behg, J. E., Randle, D. H., Lozano, G., Roussel, M. F., Sherr, C. J., and Zambetti, G. P. (2000) Genes Dev. 14, 2358–2365
12. Modestou, M., Puig-Antich, V., Korgaonkar, C., Eapen, A., and Quelle, D. E. (2001) Cancer Res. 61, 3145–3150
13. Greith, A., Weber, J. D., Willumsen, B. M., Sherr, C. J., and Roussel, M. F. (2000) J. Biol. Chem. 275, 27473–27479
14. Hemmati, P. G., Gillissen, B., van Haefen, C., Wendt, J., Starck, L., Guérin, D., Dirken, B., and Daniel, P. F. (2002) Oncogene 21, 3149–3161
15. Fatoyi, K. and Szalay, A. A. (2001) J. Biol. Chem. 276, 28421–28429
16. Kuo, M. L., Duncavage, E. J., Mathews, R., den Besten, W., Pei, D., Naeve, D., Yamamoto, T., Cheng, C., Sherr, C. J., and Roussel, M. F. (2003) Cancer Res. 63, 1046–1053
17. Sugimoto, M., Kuo, M. L., Roussel, M. F., and Sherr, C. J. (2003) Mol. Cell 11, 415–424
18. Kymion, B., Karayan, L., Seitch, P., Brambilla, C., Brambilla, E., Larsen, J. C., and Gazerri, S. (2001) Oncogene 20, 1033–1041
19. Martelli, F., Hamilton, T., Silver, D. P., Sharpless, N. E., Bardeesy, N., Rokas, M., DePinho, R. A., Livingston, D. M., and Grossman, S. R. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 4445–4449
20. Yarbrough, W. G., Bessho, M., Zanation, A., Bisi, J. E., and Xiong, Y. (2002) Cancer Res. 62, 1171–1177
21. Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., Sedivy, J. M., Kinzler, K. W., and Vogelstein, B. (1998) Science 282, 1497–1501
22. Waldman, T., Kinzler, K. W., and Vogelstein, B. (1995) Cancer Res. 55,
p14ARF Induces G2 Arrest by Targeting p34\textsuperscript{cd2}

23. Gillissen, B., Essmann, F., Graupner, V., Starck, I., Radetzki, S., Dörken, B., Schulze-Osthoff, K., and Daniel, P. T. (2003) *EMBO J.* **22**, 3580–3590
24. Jin, P., Gu, Y., and Morgan, D. O. (1996) *J. Cell Biol.* **134**, 963–970
25. Daniel, P. T., Sturm, I., Ritschel, S., Friedrich, K., Dörken, B., Bendzko, P., and Hillebrand, T. (1999) *Anal. Biochem.* **266**, 110–115
26. Craig, C., Kim, M., Ohri, E., Wersto, R., Katayose, D., Li, Z., Choi, Y. H., Mudahar, B., Srivastava, S., Seth, P., and Cowan, K. (1998) *Oncogene* **16**, 265–272
27. Taagepera, S., Rao, P. N., Drake, F. H., and Gorbsky, G. J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 8407–8411
28. Weber, H. O., Samuel, T., Rauch, P., and Funk, J. O. (2002) *Oncogene* **21**, 3207–3212
29. Radetzki, S., Kohne, C. H., von Haefen, C., Gillissen, B., Sturm, I., Dörken, B., and Daniel, P. T. (2002) *Oncogene* **21**, 227–238
30. Isaacs, W. B., Carter, B. S., and Ewing, C. M. (1991) *Cancer Res.* **51**, 4716–4720
31. Takizawa, C. G., and Morgan, D. O. (2000) *Curr. Opin. Cell Biol.* **12**, 658–665
32. Jin, P., Hardy, S., and Morgan, D. O. (1998) *J. Cell Biol.* **141**, 875–885
33. Quelle, D. E., Zindy, F., Ashman, R. A., and Sherr, C. J. (1995) *Cell* **83**, 993–1000
34. Qi, Y., Gregory, M. A., Li, Z., Brousal, J. P., West, K., and Hann, S. R. (2004) *Nature* **431**, 712–717
35. Sherr, C. J., and Weber, J. D. (2000) *Curr. Opin. Genet. Dev.* **10**, 94–99
36. Eymin, B., Leduc, C., Coll, J. L., Brambilla, E., and Gazzeri, S. (2003) *Oncogene* **22**, 1822–1835
37. Bertwistle, D., Sugimoto, M., and Sherr, C. J. (2004) *Mol. Cell. Biol.* **24**, 985–996
38. Itahana, K., Bhat, K. P., Jin, A., Itahana, Y., Hawke, D., Kobayashi, R., and Zhang, Y. (2003) *Mol. Cell* **12**, 1151–1164