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Harry A. Rogoff  
*University of Massachusetts Medical School*

*Et al.*

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## E2F1 Induces Phosphorylation of p53 That Is Coincident with p53 Accumulation and Apoptosis

Harry A. Rogoff,<sup>1</sup> Mary T. Pickering,<sup>2</sup> Michelle E. Debatis,<sup>2</sup> Stephen Jones,<sup>3</sup> and Timothy F. Kowalik<sup>1,2\*</sup>

*Program in Immunology and Virology<sup>1</sup>, Department of Molecular Genetics and Microbiology,<sup>2</sup> and Department of Cell Biology,<sup>3</sup> University of Massachusetts Medical School, Worcester, Massachusetts 01655*

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**It has been proposed that the E2F1 transcription factor serves as a link between the Rb/E2F proliferation pathway and the p53 apoptosis pathway by inducing the expression of p19ARF, a protein that regulates p53 stability. We find that although p19ARF contributes to p53 accumulation in response to E2F expression, p19ARF is not required for E2F1-mediated apoptosis. E2F1 can signal p53 phosphorylation in the absence of p19ARF, similar to the observed modifications to p53 in response to DNA damage. These modifications are not observed in the absence of p19ARF following expression of E2F2, an E2F family member that does not induce apoptosis in mouse embryo fibroblasts but can induce p19ARF and p53 protein expression. p53 modification is found to be crucial for E2F1-mediated apoptosis, and this apoptosis is compromised when E2F1 is coexpressed with a p53 mutant lacking many N- and C-terminal phosphorylation sites. Additionally, E2F1-mediated apoptosis is abolished in the presence of caffeine, an inhibitor of phosphatidylinositol 3-kinase-related kinases that phosphorylate p53. These findings suggest that p53 phosphorylation is a key step in E2F1-mediated apoptosis and that this modification can occur in the absence of p19ARF.**

The p53 tumor suppressor is an important sensor that responds to various cellular stresses including DNA damage and inappropriate proliferation. DNA damaging agents and gamma-irradiation cause phosphorylation and subsequent accumulation and activation of p53 (23). As a result, a cell will either arrest and allow for DNA repair or commit programmed cell death (73, 78). Additionally, expression of cellular or viral oncoproteins that promote proliferation, such as c-myc or adenovirus E1A, results in p53-dependent apoptosis (13, 27). A role for the E2F family member E2F1 has been well established in activating the p53 apoptotic signaling pathway upon loss of proliferation control, and recent reports suggest that E2F1 may contribute to apoptosis in response to DNA damage (6, 30, 43).

During normal cell proliferation, E2F family members modulate the expression of many genes involved in the transition from G<sub>1</sub> to S phase and DNA replication (53). E2F transcriptional activity is regulated by interaction with members of the retinoblastoma (Rb) family of proteins (24). Inactivation of Rb by cellular or viral oncoproteins or loss of Rb will stimulate cells to bypass G<sub>0</sub>/G<sub>1</sub> and enter S phase (17, 19, 50, 52). S-phase induction also occurs when E2F1, E2F2, or E2F3 is exogenously expressed in quiescent cells (15, 35, 40). There is evidence that ectopic expression of various E2F family members can induce both S-phase progression and apoptosis, depending on the levels of expressed cDNA or cell type context (12, 28, 40, 59, 72, 74, 79). However, in fibroblasts, exogenous

expression of E2F1, but not E2F2 or E2F3, causes apoptosis (15, 39).

The link between E2F1 and p53-dependent apoptosis has been demonstrated by genetic studies in mice. Apoptosis observed in transgenic mouse models expressing a fragment of the simian virus 40 large-T antigen and in the central nervous system of *Rb*<sup>-/-</sup> mouse embryos is p53-dependent and associated with *E2F1* (46, 56, 69). Additionally, expression of an *E2F1* transgene in the skin of K5E2F1 transgenic mice results in increased proliferation and p53-dependent apoptosis (57). Recent reports have also identified a p53-independent mechanism of E2F1-induced apoptosis that is mediated by the p53 homologue p73 (44, 67). E2F1 is able to directly transactivate the *p73* promoter, thereby inducing a cell cycle arrest and apoptosis (33, 67).

E2F1 has been shown to transactivate the expression of the mouse *p19ARF* and human *p14ARF* promoters (4, 32, 60). *p19ARF* encodes a protein that modulates the activity of Mdm2 (58, 77). Mdm2 is an E3-like ubiquitin ligase that regulates the stability of p53 by promoting its degradation by the proteasome (21, 22, 26, 31). By inhibiting Mdm2 activity, p19ARF allows stabilization, activation, and accumulation of p53 protein (58, 77). Thus, it has been hypothesized that p19ARF is a key protein linking the Rb/E2F and p53 pathways (4, 37). One hypothesis for how aberrations in the Rb/E2F pathway are recognized by p53 is that deregulated E2F expression activates p53 by inducing *p19ARF*.

Although the three E2F proteins that induce S phase (E2F1, E2F2, and to a lesser extent, E2F3) also induce *p19ARF* expression (15), only E2F1 induces p53-dependent apoptosis in fibroblasts. These observations suggest that there may be different pathways for E2F1 to signal p53-mediated apoptosis and p53 accumulation. We demonstrate that although p19ARF

\* Corresponding author. Mailing address: University of Massachusetts Medical School, Department of Molecular Genetics and Microbiology, Room S5-710, 55 Lake Ave. N., Worcester, MA 01655. Phone: (508) 856-6035. Fax: (508) 856-5920. E-mail: timothy.kowalik@umassmed.edu.

contributes to p53 accumulation, it is not required for E2F1-mediated apoptosis. We also find that E2F1 expression results in the phosphorylation of N-terminal serine residues on p53, similar to the p53 modifications observed in response to DNA damage. Covalent modification of p53 is required for E2F1-mediated apoptosis and occurs independent of *p19ARF* status. Moreover, E2F2 expression, which does not induce apoptosis in mouse embryo fibroblasts (MEFs), does not lead to the phosphorylation of serine residues on p53 in the absence of *p19ARF*.

## MATERIALS AND METHODS

**Cell culture.** Early passage wild-type and genetically matched *p53*<sup>-/-</sup> and *Mdm2*<sup>-/-</sup>/*p53*<sup>-/-</sup> MEFs were isolated from mouse embryos as described previously (36). MEFs derived from *p19ARF*<sup>-/-</sup> mouse embryos were a generous gift from Charles Sherr (St. Jude Children's Research Hospital, Memphis, Tenn.). MEFs derived from *p19ARF*<sup>-/-</sup>/*p53*<sup>-/-</sup> mouse embryos were a generous gift from Gerard Zambetti (University of Tennessee, Memphis). *ink4a*<sup>-/-</sup> MEFs were kindly provided by Ronald DePinho (Harvard Medical School, Boston, Mass.). MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

**Adenovirus vectors.** Recombinant adenoviruses encoding p53, E2F1, and E2F2 have been described previously (15, 40, 63). An adenovirus encoding an E2F1 DNA binding mutant, E2F1<sub>e132</sub> (35), was created by homologous recombination (40, 54). The p53N/C virus was generated from the pCB6 plus p53N/C construct generously provided by Karen Vousden (National Cancer Institute, Frederick, Md.). Control viruses encode either an empty expression cassette or  $\beta$ -galactosidase. Infection with control virus had no effect on the parameters tested relative to mock infection (data not shown). Viruses were propagated in 293 cells and purified by centrifugation through cesium-chloride gradients. Virus titers were determined by immunohistochemical staining for the adenovirus hexon protein with an anti-adenovirus antibody (Biodesign International) and visualized by using a 3,3'-diaminobenzidine substrate kit from Vector Laboratories (10).

**Virus infection.** Cells were washed once with phosphate-buffered saline (PBS), and serum-free DMEM containing the virus was added to the cells. Infections were carried out at 37°C in 5% carbon dioxide (CO<sub>2</sub>) for 1 h (40). The virus inoculum was then removed and replaced with DMEM containing the appropriate concentration of serum, and the mixture was cultured under the conditions described above.

**Immunoblot analysis.** MEFs were infected with AdCon, AdE2F1, AdE2F1<sub>e132</sub>, or AdE2F2 at a multiplicity of infection (MOI) of 100, except for lanes marked E2F1<sub>low</sub>, which were infected at an MOI of 25. Adp53 was infected at an MOI of 10 unless stated otherwise. Whole-cell extracts from MEFs were harvested at 24 h postinfection (hpi). Cells were washed twice with cold PBS and lysed in whole-cell extract buffer (50 mM HEPES [pH 7.9], 250 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, 1× mammalian protease inhibitor cocktail [Sigma], 1× phosphatase inhibitor cocktails I and II [Sigma]) by incubation for 30 min on ice. Soluble proteins were separated by centrifugation at 13,000 × g in a microcentrifuge, and supernatants were stored at -70°C. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis and transferred to a polyvinylidene difluoride membrane (Millipore) by electroblotting. p19ARF was detected with anti-p19ARF polyclonal antisera (Novus), p53 protein was detected with monoclonal antibody D0-1 or polyclonal antiserum Ab-7 (Oncogene Research Products), phospho-Ser<sup>15</sup> and -Ser<sup>20</sup> forms of p53 were detected with polyclonal antisera specific for each modification (Cell Signaling Technologies). Transduced p53N/C and wild-type p53 were detected with the monoclonal antibody PAb1801 to compare expression levels (Oncogene Research Products) (see Fig. 7A). Actin was detected with polyclonal antisera I-19 (Santa Cruz Biotechnology, Inc.). Immunoreactive proteins were detected by using a chemiluminescence kit (Amersham) according to the manufacturer's recommendations. Relative changes in the levels of p53 were estimated from scanned images of Western blots with Multianalyst software (Bio-Rad).

**Apoptosis analysis by flow cytometry.** At the indicated times postinfection, cells were trypsinized, combined with any floating cells, pelleted, washed with PBS, repelleted, and resuspended in 400  $\mu$ l of PBS. All centrifugations were at 500 × g for 5 min at 4°C. Subsequently, cells were fixed in cold ethanol (final concentration, 70%) and stored at 4°C. Cells were processed for propidium iodide (PI) staining as described previously (40). Flow cytometric analysis was

performed by the University of Massachusetts Medical School Flow Cytometry Core Facility. Cells undergoing apoptosis were defined as the population that contained less than a 2N DNA content.

**Apoptosis analysis by ELISA.** Cells were plated in 24-well plates at a concentration of 1 × 10<sup>4</sup> cells per well. Virus infections were performed, as described above, 24 h after plating, and caffeine was added at the indicated doses following infection. At 72 hpi, cells were centrifuged at 500 × g for 10 min at 4°C, and lysed, and an enzyme-linked immunosorbent assay (ELISA) was performed as described by the manufacturer (Roche).

**Immunohistochemical staining for p53 protein accumulation.** Cells were infected with the appropriate adenovirus constructs and immunohistochemically stained for p53 protein as described previously (39). AdCon, AdE2F1, AdE2F1<sub>e132</sub> were infected at an MOI of 100. At the time of harvest (48 hpi), cells were washed three times with PBS and then fixed for 15 min each in 3.7% formaldehyde followed by methanol. The cells were then washed in PBS-0.5% Tween 20. Cells were then incubated with anti-p53 monoclonal antibody PAb421 (Oncogene Research Products) in the presence of 1% bovine serum albumin in PBS-0.5% Tween 20 for 45 min at room temperature. The cells were washed three times with PBS-0.5% Tween 20, and the bound antibody was detected with a Vectastain 3,3'-diaminobenzidine substrate kit as described by the manufacturer.

**p53 immunofluorescence.** Cells were infected with AdCon, AdE2F1, or AdE2F2 at an MOI of 100, and at 24 hpi, the cells were fixed in 3.7% formaldehyde followed by methanol. Fixed cells were incubated with anti-p53 monoclonal antibody pAb421 (Oncogene Research Products) and polyclonal antisera specific for phospho-Ser<sup>15</sup> p53 (Cell Signaling Technologies). The bound antibodies were detected with fluorescein isothiocyanate (FITC)- or Rhodamine Red-X-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., and Southern Biotechnology Associates, Inc.).

## RESULTS

**p19ARF is not required for E2F1-mediated apoptosis.** Human *p14ARF* and mouse *p19ARF* promoters contain multiple E2F binding sites that are responsive to ectopic expression of E2F1 (4, 32, 60). Because *p19ARF* mRNA levels increase upon E2F1 expression (15), we wanted to determine whether the elevated mRNA levels corresponded to an increase in p19ARF protein levels. Indeed, we found that infecting MEFs with an adenovirus encoding E2F1 (AdE2F1) led to increased levels of p19ARF protein (Fig. 1A). Given that p19ARF expression leads to p53 accumulation via inactivation of Mdm2 functions and that E2F1 expression leads to p53-dependent apoptosis, we determined whether p19ARF was required for E2F1-mediated p53-dependent apoptosis. We find that E2F1 expression induced apoptosis regardless of *p19ARF* status as measured by the percentage of cells with sub-2N DNA content (Fig. 1B). Apoptosis is observed at all MOIs in the wild-type MEFs but only at the highest dose of E2F1 in the *p19ARF*<sup>-/-</sup> MEFs, suggesting that while p19ARF is not required, it may contribute to E2F1-mediated apoptosis. MEFs infected with an adenovirus encoding an E2F1 DNA binding mutant, E2F1<sub>e132</sub>, showed little or no increase in apoptosis compared to the controls. The percentage of apoptotic cells was also measured in *p19ARF*<sup>-/-</sup> and *p19ARF*<sup>-/-</sup>/*p53*<sup>-/-</sup> MEFs to test the requirement for p53 in the apoptosis observed in *p19ARF*<sup>-/-</sup> MEFs. The *p19ARF*<sup>-/-</sup>/*p53*<sup>-/-</sup> MEFs were less susceptible to apoptosis than MEFs lacking only *p19ARF* (Fig. 1C). In addition, expression of E2F1 also induced apoptosis in *ink4a*<sup>-/-</sup> MEFs (unpublished observations). *ink4a*<sup>-/-</sup> mice do not express p19ARF because of the deletion of a shared exon with *p16<sup>INK4A</sup>*, which encodes a cyclin-dependent kinase inhibitor (65). Thus, E2F1 can induce apoptosis in *p19ARF*-deficient MEFs that are generated from independent genetic lesions in mice.

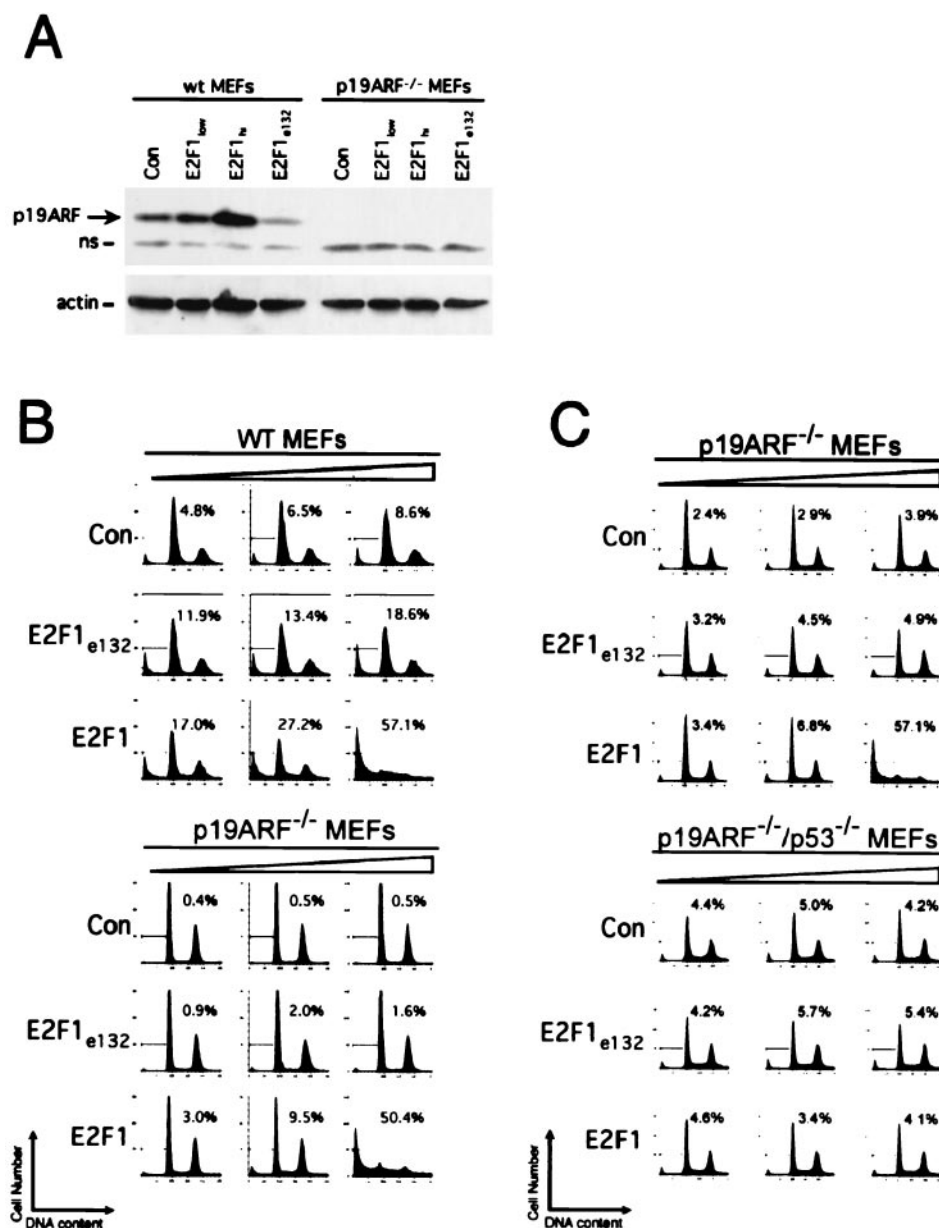


FIG. 1. E2F1 induces apoptosis in the absence of p19ARF. (A) Immunoblot analysis of p19ARF levels in lysates of wild-type (wt) and *p19ARF*<sup>-/-</sup> MEFs. Cells were infected with recombinant adenoviruses encoding E2F1, E2F1<sub>e132</sub>, or a control (Con) virus. Samples were harvested, and lysates were generated at 24 hpi. The actin blot is shown as a protein loading control. ns, nonspecific band. (B) Analysis of apoptosis in MEFs. Wild-type and *p19ARF*<sup>-/-</sup> MEFs were infected at a MOI of 100, 300, or 500 with virus encoding the marked cDNA. Cells were harvested and fixed at 72 hpi, stained with PI, and analyzed by flow cytometry. Apoptotic cells were scored as the percentage of cells with a sub-2N DNA content (marked by the bar in each plot). (C) Analysis of apoptosis in *p19ARF*<sup>-/-</sup> and *p19ARF*<sup>-/-</sup>/*p53*<sup>-/-</sup> MEFs. Infection and harvest conditions were the same as described for panel B. Percentages represent cells with sub-2N DNA content.

**p19ARF and Mdm2 contribute to p53 accumulation.** Given that apoptosis does not require *p19ARF*, we asked whether p19ARF is necessary for E2F1-mediated changes in p53 protein levels. To address this question, we expressed E2F1 in *p19ARF*<sup>-/-</sup> MEFs and assessed p53 protein levels by immunohistochemistry. As shown in Fig. 2A, E2F1 expression resulted in increased levels of endogenous p53 protein in wild-type MEFs. When E2F1 was expressed in *p19ARF*<sup>-/-</sup> MEFs, fewer cells stained positive for p53 protein and more modest changes in p53 protein levels were observed. The changes in

endogenous p53 protein levels observed by immunohistochemical staining following E2F1 expression correlate with the changes observed by immunoblotting for endogenous p53 (see Fig. 5C). Likewise, Nip et al. observed an increase in the half-life of p53 following E2F1 expression in 32D.3 cells, a tumor cell line that lacks p14ARF expression (55). In both MEF genotypes, expressing the E2F1<sub>e132</sub> DNA binding mutant had little or no effect on p53 protein levels. These results are consistent with a model in which p19ARF can contribute to E2F1-mediated changes in p53 protein levels.

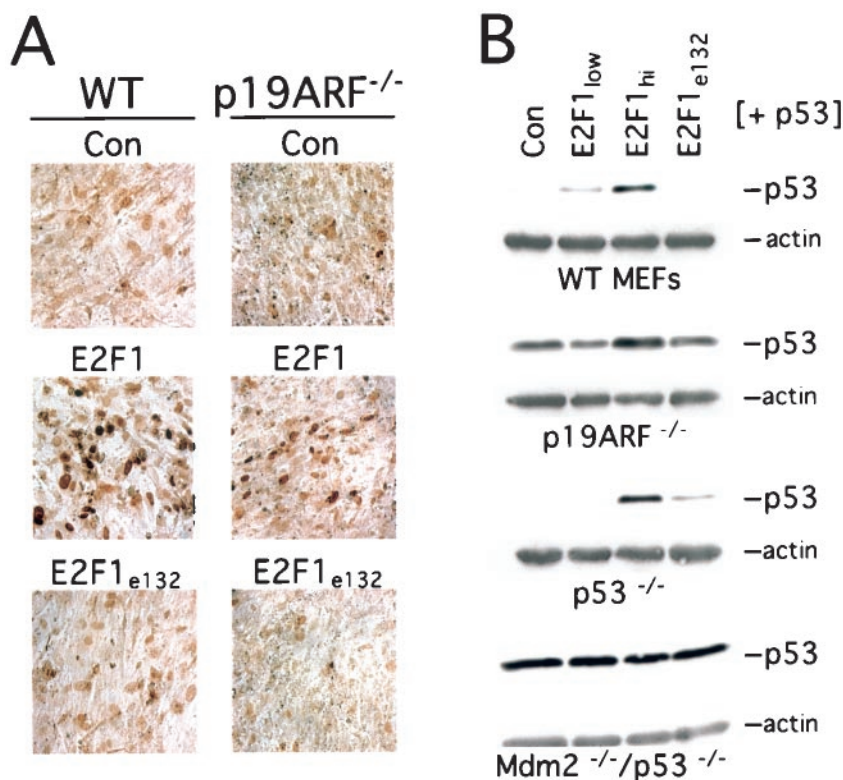


FIG. 2. Contribution of p19ARF and Mdm2 to E2F1-mediated accumulation of p53. (A) Immunohistochemical staining for endogenous p53 protein in wild-type (WT) and *p19ARF*<sup>-/-</sup> MEFs. Cells were fixed for staining at 48 hpi. (B) Immunoblot analysis for p53 in lysates of wild-type, *p19ARF*<sup>-/-</sup>, *p53*<sup>-/-</sup>, and *Mdm2*<sup>-/-</sup>/*p53*<sup>-/-</sup> MEFs. Cells were coinfectd with Adp53 and AdCon, AdE2F1, or AdE2F1<sub>e132</sub>. Samples were harvested at 24 hpi. Actin blots are shown as protein loading controls.

p19ARF affects p53 protein levels by binding to Mdm2 and preventing it from promoting the degradation of p53 (58, 77). Therefore, we determined whether Mdm2 is necessary for E2F1-mediated accumulation of p53. It is not possible to duplicate the experiments described above for Mdm2 because there are no cell lines or MEFs available that lack Mdm2 expression but retain endogenous wild-type p53. However, MEFs can be derived from *Mdm2*<sup>-/-</sup>/*p53*<sup>-/-</sup> embryos (36, 49). To analyze the effects of E2F1 on p53 protein levels in these cells, we introduced p53 in *trans* by infecting cells with an Adp53 recombinant adenovirus, essentially creating a pseudo *Mdm2*<sup>-/-</sup> MEF. To control for the addition of exogenous p53 transcript, wild-type, *p19ARF*<sup>-/-</sup>, and *p53*<sup>-/-</sup> MEFs were also infected with Adp53 at the same low MOI. As observed in the immunohistochemical and immunoblot analysis for endogenous p53, E2F1 expression resulted in a substantial (>6-fold) increase in exogenous p53 levels in wild-type MEFs in comparison to control infected cells when measured by immunoblot analysis (Fig. 2B) (see Fig. 5C). Others have made similar observations for *p19ARF*<sup>-/-</sup> MEFs following retroviral gene transfer of E2F1 (80). p53 also accumulated in response to E2F1 in *p53*<sup>-/-</sup> MEFs that ectopically expressed p53 (Fig. 2B). Therefore, exogenously expressed p53 protein is affected by E2F1 in a manner similar to that of the endogenous protein. Infection with AdE2F1 resulted in a modest (~2.4-fold) increase in total p53 protein levels in Adp53-infected *p19ARF*<sup>-/-</sup> MEFs compared to AdCon infection of the same cell type (Fig.

2B). The basal levels of ectopically expressed p53 were higher in *p19ARF*<sup>-/-</sup> MEFs than in wild-type cells. This observation is consistent with the data obtained by immunostaining and immunoblotting for endogenous p53 in *p19ARF*<sup>-/-</sup> MEFs (Fig. 2A) (see Fig. 5C), although the difference is exaggerated when cells are infected with Adp53 (Fig. 2B). Others have also reported higher basal levels of p53 in *p19ARF*<sup>-/-</sup> MEFs (16). E2F1 expression minimally affected p53 protein levels in Adp53-infected *Mdm2*<sup>-/-</sup>/*p53*<sup>-/-</sup> MEFs. We suspect that the Mdm2 deficiency elevates baseline levels of p53 in these cells due to an absence or greatly reduced level of p53 ubiquitin ligase activity. E2F1<sub>e132</sub> had little or no effect on p53 protein levels in each cell type, consistent with a transcriptional mechanism being at least partly responsible for E2F1-mediated accumulation of p53. The reduced ability of E2F1 to alter p53 levels above those of the controls in *p19ARF*<sup>-/-</sup> MEFs, or in MEFs lacking Mdm2 but expressing p53, is consistent with a model in which E2F1 modulates p53 protein levels, in large part, through the p19ARF/Mdm2/p53 pathway.

**E2F expression leads to the phosphorylation of p53.** In response to DNA damage, p53 is activated by covalent modifications, including the phosphorylation at certain serines and acetylation of lysine residues. Of these modifications, phosphorylation of Ser<sup>15</sup> on human p53, or Ser<sup>18</sup> on mouse p53, is commonly observed in response to DNA damage (3, 7). Given that both DNA damage (37) and E2F1 do not necessarily require p19ARF to activate p53, we determined whether

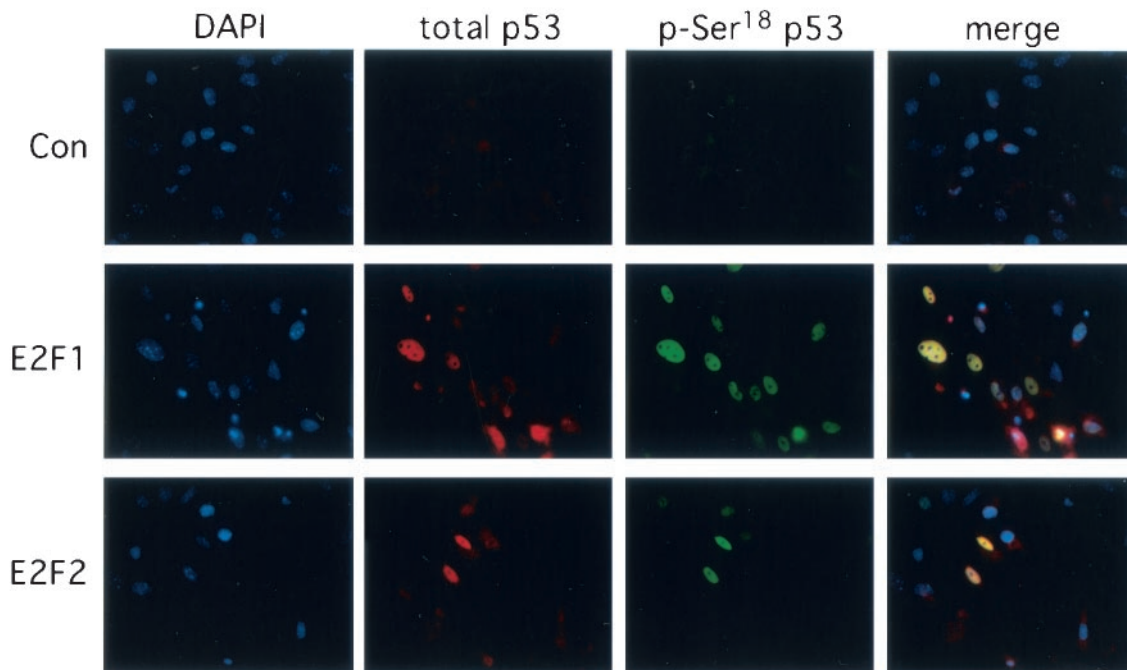


FIG. 3. E2F1 and E2F2 induce endogenous p53 accumulation and phosphorylation of the serine 18 residue of p53 in wild-type MEFs. Immunofluorescent detection of total p53 protein (rhodamine red X) and phospho-Ser<sup>18</sup> p53 (FITC) following E2F1 or E2F2 expression in wild-type MEFs. DAPI (4',6'-diamidino-2-phenylindole) staining is shown in blue. Merged images are also shown. Con, control virus.

E2F1-mediated activation of p53 function is coincident with p53 phosphorylation. To detect phospho-Ser<sup>18</sup>, we used an antibody specific for this modification. We find that E2F1 expression, and to a lesser extent, that of E2F2, in wild-type MEFs results in an increase in endogenous p53 protein levels and the phospho-Ser<sup>18</sup> form of p53 as detected by immunofluorescence (Fig. 3). When merged, it is apparent that only a subset of the cells with an increase in total p53 has increased levels of the phospho-Ser<sup>18</sup> form of p53. Total p53 protein and the phospho-Ser<sup>18</sup> form of p53 were present at low levels in cells infected with the control virus (Fig. 3). These data suggest that E2F1 may signal p53 accumulation and p53 phosphorylation by separate pathways.

**Differential abilities of E2F1 and E2F2 to phosphorylate p53 and induce apoptosis.** To discriminate between E2F1-specific induction of p53 and increased E2F activity in general, we measured the levels of p53 in extracts of cells infected with an adenovirus encoding E2F2 in comparison to E2F1. E2F2 is functionally similar to E2F1. E2F2 is a potent inducer of S phase when expressed in quiescent cells, is normally induced at the G<sub>1</sub> to S transition, specifically interacts with Rb, and induces a similar array of genes when overexpressed (15, 34, 42, 51, 64). However, like other E2F family members, E2F2 is unable to induce apoptosis in REF52 cells (15, 39). We find that E2F2 expression does not lead to apoptosis in MEFs, even though it is capable of inducing the expression of p19ARF protein (see Fig. 5A and B). Ectopic E2F2 expression did result in increased levels of endogenous p53 protein in wild-type MEFs, although not as effectively as E2F1 (Fig. 3) (see Fig. 5C). However, expression of E2F1, but not of E2F2, alters p53 protein levels in *p19ARF*<sup>-/-</sup> MEFs (Fig. 4 and Fig. 5C).

We next compared the ability of E2F1 and E2F2 expression to induce a change in p53 phosphorylation. We find that ectopic expression of either E2F1 or E2F2 leads to an increase in the levels of both endogenous p53 and the phospho-Ser<sup>18</sup> form of p53 in wild-type MEFs, although the levels of phospho-Ser<sup>18</sup> p53 are lower in the E2F2-expressing sample (Fig. 5C). Similar results were observed with immunofluorescence staining for p53 and phospho-Ser<sup>18</sup> p53 in wild-type MEFs (Fig. 3). Expression of E2F1, but not of E2F2, leads to increased levels of the endogenous phospho-Ser<sup>18</sup> form of p53 in *p19ARF*<sup>-/-</sup> MEFs (Fig. 4 and Fig. 5C). In addition to phosphorylation at Ser<sup>15</sup> (the human equivalent to Ser<sup>18</sup> in mouse p53), phosphorylation of Ser<sup>20</sup> (Ser<sup>23</sup> in mouse p53) on human p53 is commonly observed following DNA damage (11, 29, 66). Since we have had difficulties detecting mouse phospho-Ser<sup>23</sup> p53 by immunoblot analysis, cells were coinfecting at a low MOI with an adenovirus encoding human p53 which when phosphorylated can be detected by the anti-phospho-Ser<sup>20</sup> antibody. Using this approach, we find that E2F1 expression results in an increase in the phospho-Ser<sup>20</sup> and phospho-Ser<sup>15</sup> forms of p53 in both wild-type and *p19ARF*<sup>-/-</sup> MEFs (Fig. 6A and B). E2F2 expression results in a slight increase in both the phospho-Ser<sup>15</sup> and phospho-Ser<sup>20</sup> forms of p53 in wild-type MEFs but not in *p19ARF*<sup>-/-</sup> MEFs (Fig. 6A and B). This inability of E2F2 to affect the phosphorylation of p53 in the absence of *p19ARF* was also observed for the endogenous mouse p53 (Fig. 4 and Fig. 5C). The ability of E2F2 to affect p53 protein levels was, unlike that of E2F1, solely dependent on *p19ARF*, since both endogenous and exogenous p53 protein levels were unchanged when E2F2 was expressed in *p19ARF*<sup>-/-</sup> MEFs while E2F1 expression leads to an increase in p53 in both wild-type

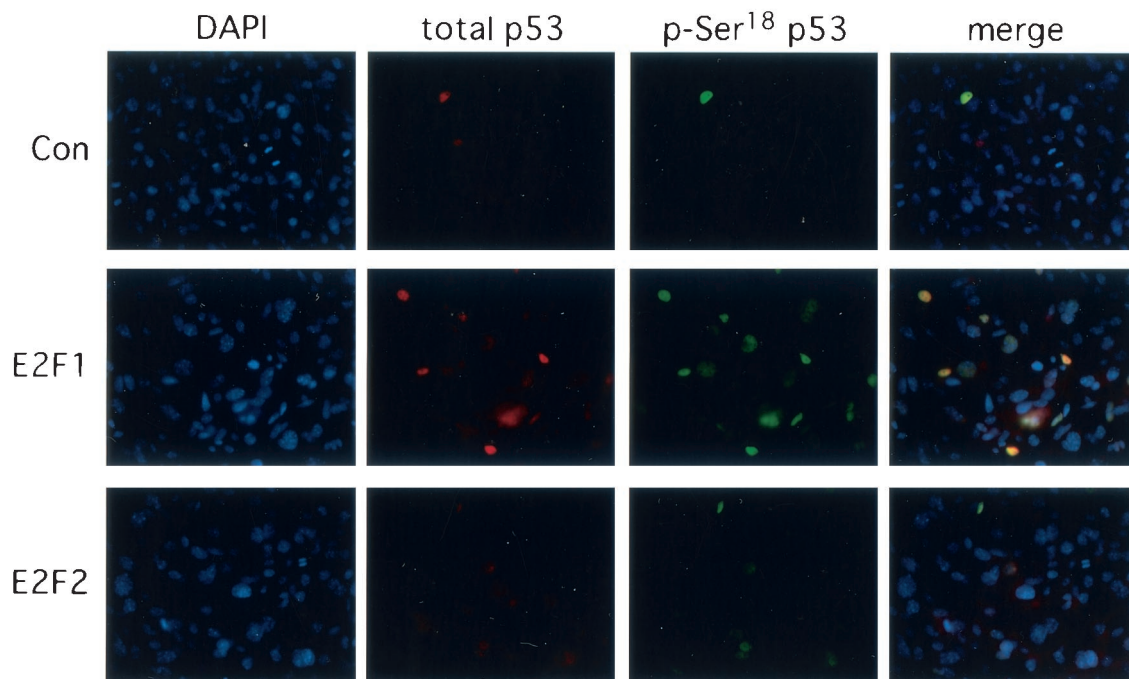


FIG. 4. E2F1 specifically induces endogenous p53 accumulation and phosphorylation of the serine 18 residue of p53 in *p19ARF*<sup>-/-</sup> MEFs. Immunofluorescent detection of total p53 protein (Rhodamine Red-X) and phospho-Ser<sup>18</sup> p53 (FITC) following E2F1 or E2F2 expression in *p19ARF*<sup>-/-</sup> MEFs. DAPI (4',6'-diamidino-2-phenylindole) staining is shown in blue. Merged images are also shown. Con, control virus.

and *p19ARF*<sup>-/-</sup> MEFs (Fig. 6C and 5C). We note that the differential abilities of E2F1 and E2F2 to alter the ectopically expressed p53 in *p19ARF*<sup>-/-</sup> MEFs appears to be exaggerated relative to the endogenous protein in these experiments. These results suggest that E2F2 utilizes the p19ARF/Mdm2 pathway to affect p53 levels.

**p53 phosphorylation contributes to E2F1-mediated apoptosis.** Having found a correlation between E2F1-induced phosphorylation of p53 and apoptosis, we determined if these covalent modifications are required for or contribute to E2F1-mediated apoptosis. Studies of the DNA damage response have suggested that it is unlikely that a single p53 modification is responsible for activating p53-dependent apoptosis (9), so we initially addressed this question by using a p53 mutant in which most of the N- and C-terminal amino acid residues known to be phosphorylated upon DNA damage have been changed to alanines. This mutant, p53N/C, has been shown to activate expression of p21, Mdm2, and Bax reporter constructs as effectively as wild-type p53 and has been reported to induce apoptosis in response to certain stimuli (2). Mdm2 has also been shown to regulate the levels of p53N/C (2). Indeed, we find that the p53N/C protein accumulates at levels similar to those of wild-type p53 when expressed by recombinant adenovirus transduction (Fig. 7A). To measure apoptosis, *p53*<sup>-/-</sup> MEFs were transduced with AdE2F1 or AdE2F2 and either wild-type Adp53 or Adp53N/C (Fig. 7B). Without the addition of p53, levels of apoptosis in E2F1- or E2F2-expressing samples were similar to those of AdCon-infected cells. Cells expressing both E2F1 and wild-type p53 underwent apoptosis. In contrast, apoptosis was greatly reduced in cells transduced with

E2F1- and p53N/C-encoding adenoviruses, suggesting a contribution by these potential phosphorylation sites to apoptosis. As expected, E2F2 did not induce apoptosis when coexpressed with either p53 or p53N/C.

We next examined the role of p53 kinases on apoptosis signaling in response to E2F1 expression by using caffeine, a potent inhibitor of several of these kinases. We find that there is a dose-dependent decrease in apoptosis in E2F1-expressing MEFs following treatment with increasing doses of caffeine (Fig. 8). Treatment of cultured cells with caffeine prior to ionizing radiation results in a delayed and attenuated accumulation of p53 protein (38). This defect in p53 accumulation is due to the inhibition of the ataxia telangiectasia mutated (ATM) and the ATM-Rad3-related (ATR) kinases that phosphorylate p53 on serine 15. Inhibition of ATM and ATR will also block subsequent signals that activate the checkpoint kinases CHK1 and CHK2 and perhaps other kinases that phosphorylate p53 on serine 20 (5, 62). The observations that blocking p53 phosphorylation either by mutating potential phosphorylation sites on p53 or treatment with the p53 kinase inhibitor, caffeine, can significantly compromise apoptosis demonstrates that p53 phosphorylation is a critical step in E2F1-mediated apoptosis.

## DISCUSSION

The signaling mechanism by which the Rb/E2F pathway in general, and E2F1 specifically, communicates with p53 has been thought to involve the p19ARF/Mdm2 pathway. Indeed, we find that p19ARF and Mdm2 are required for the majority

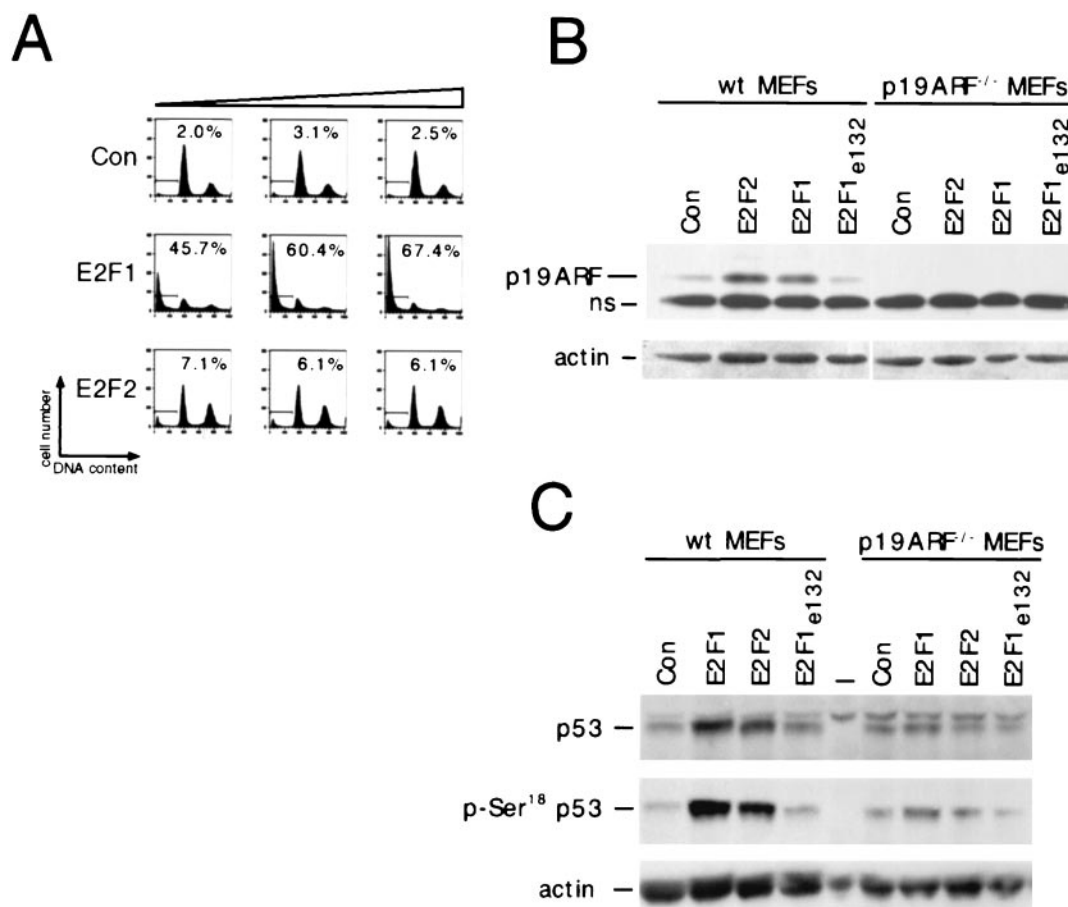


FIG. 5. Induction of apoptosis is specific to E2F1. (A) Apoptosis analysis in wild-type (wt) MEFs infected with AdCon, AdE2F1, or AdE2F2. At 72 hpi, cells were harvested and processed for PI staining and flow cytometry. Percentages represent cells with sub-2N DNA content. (B) Immunoblot analysis for p19ARF in lysates of wild-type and *p19ARF*<sup>-/-</sup> MEFs. Cells were coinfecting with Adp53 and either AdCon, AdE2F1, AdE2F1<sub>e132</sub>, or AdE2F2. Cells were harvested, and lysates were generated at 24 hpi. The actin blot is shown as a protein loading control. ns, nonspecific band. (C) Immunoblot analysis for endogenous p53 and the phospho-Ser<sup>18</sup> form of p53 in lysates of wild-type and *p19ARF*<sup>-/-</sup> MEFs. Cells were infected with AdCon, AdE2F1, AdE2F2, or AdE2F1<sub>e132</sub>. Cells were harvested, and lysates were generated at 24 hpi. The actin blot is shown as a protein loading control.

of p53 protein accumulation observed following E2F1 expression. However, contrary to the role of the p19ARF pathway in p53 accumulation, we find that E2F1 induces apoptosis in MEFs lacking *p19ARF* (Fig. 9). It is unlikely that E2F1 induction of p73 is directly responsible for the apoptosis observed in *p19ARF*<sup>-/-</sup> MEFs since we show that E2F1 failed to induce apoptosis in MEFs lacking both *p19ARF* and *p53*. These results are in contrast to those of earlier studies which concluded that *p19ARF* is important for E2F1-mediated apoptosis (16, 80). However, during revisions to the manuscript, reports published by Tsai et al. (70) and Tolbert et al. (68) showed that *p19ARF* was not required for apoptosis in *Rb*-deficient mouse embryos or following *Rb* inactivation by transgenic expression of a fragment of the simian virus 40 large-T antigen, respectively. Likewise, a report published by Russell et al. showed that crossing an *E2F1* transgenic mouse into a *p19ARF*<sup>-/-</sup> background does not reduce apoptosis caused by expression of the *E2F1* transgene (61). Thus, studies that used several different models arrived at the conclusion that the apoptosis associated with deregulated E2F activity does not require *p19ARF*.

If p19ARF is not required for E2F1 to activate p53, what role does p19ARF have in this context? Expression of p19ARF in *p53*<sup>-/-</sup> MEFs causes growth arrest, and overexpression of E2F1 overcomes this arrest (8), suggesting that p19ARF may act as a negative regulator of the Rb/E2F proliferation pathway. We speculate that induction of the p19ARF/Mdm2/p53 pathway by the E2F family functions primarily to attenuate the proliferation-promoting effects of E2F transcriptional activity (Fig. 9). Indeed, human p14ARF binds E2F1 and inhibits its transcriptional activity (20). Moreover, p19ARF physically interacts with E2F1, E2F2, and E2F3 and promotes their degradation in a proteasome-dependent manner (47). Another possibility is that p19ARF/Mdm2 activates p53 and modulates proliferation by inducing the p21 cyclin-dependent kinase inhibitor, a known transcriptional target of p53 (18, 25, 76). In either case, the p19ARF/Mdm2/p53 pathway would act as a sensor and attenuator of proliferation.

It is conceivable that forcing cells into S phase by ectopic E2F expression can induce the apoptotic response. However, it is not likely to simply be ectopic S-phase induction following



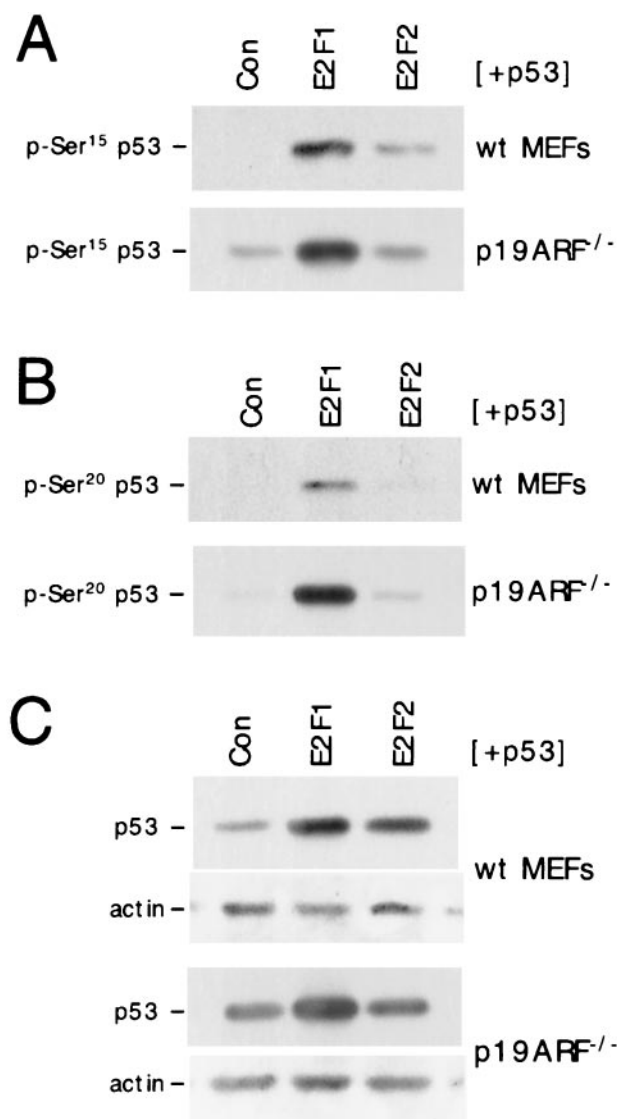


FIG. 6. Induction of p53 phosphorylation by E2F1 and E2F2. Immunoblot analysis for phospho-Ser<sup>15</sup> (A) and phospho-Ser<sup>20</sup> (B) forms of p53 or total p53 in lysates (C) of wild-type (wt) and *p19ARF*<sup>-/-</sup> MEFs coinfecting with Adp53 and either AdCon, AdE2F1, or AdE2F2. Cells were harvested, and lysates were generated at 24 hpi. The blot was sequentially stripped and reprobed. Actin blots are shown as protein loading controls.

E2F expression that triggers apoptosis, since both E2F1 and E2F2 can induce S phase at similar efficiencies (15), and we find that E2F2 does not induce apoptosis in MEFs. Moreover, there does not appear to be a specific phase of the cell cycle in which E2F1 induces apoptosis (14, 40). Our findings that ectopic E2F1, but not E2F2, expression results in increased p53 phosphorylation in the absence of *p19ARF* and that this covalent modification of p53 contributes to E2F1-mediated apoptosis suggest that E2F1 may activate a cellular response similar to DNA damage. Activation of p53 by covalent modification, specifically phosphorylation of serine 15 (serine 18 in the mouse) and serine 20 in response to DNA damage, has been a topic of great interest due to the location of these

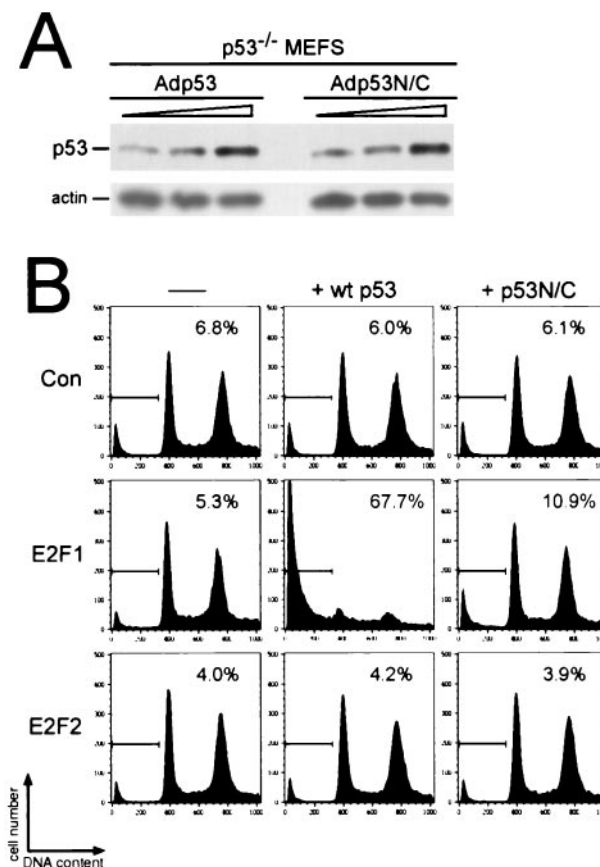


FIG. 7. Covalent modification of p53 contributes to E2F1-mediated apoptosis. (A) Immunoblot analysis of wild-type (wt) p53 and p53N/C in lysates of *p53*<sup>-/-</sup> MEFs infected with Adp53 or Adp53N/C at an MOI of 10, 20, or 50. An actin blot is also shown. (B) Apoptosis analysis in *p53*<sup>-/-</sup> MEFs. Cells were infected with Adp53 or Adp53N/C at an MOI of 20 and AdCon, AdE2F1, or AdE2F2 at an MOI of 500. At 72 hpi, cells were harvested and processed for PI staining and flow cytometry. Percentages represent cells with sub-2N DNA content.

residues within the Mdm2 interaction domain of p53. Mutational analysis of p53 sites known to be covalently modified, including serine 15 and serine 20, have been inconsistent regarding the requirements for phosphorylation at any one of these sites for p53-dependent apoptosis in response to DNA damage (2, 71, 75). While our study examined the phosphorylation status of two N-terminal residues on p53 known to be phosphorylated following DNA damage, we have not established that either of these modifications are causal in p53-mediated apoptosis, nor have we ruled out the involvement or importance of modification(s) to other residues on p53 following E2F1 expression. However, our finding that apoptosis is reduced when E2F1 is coexpressed with a p53 mutant lacking many of these phosphorylation sites demonstrates a contribution by potential p53 phosphorylation targets to E2F1-induced apoptosis. Additionally, the observation that caffeine inhibits E2F1-mediated apoptosis suggests that the action of one or more p53 kinases is likely to be important for E2F1 signaling. Signaling cascades that activate protein kinases responsible for phosphorylating N-terminal residues on p53 upon DNA dam-

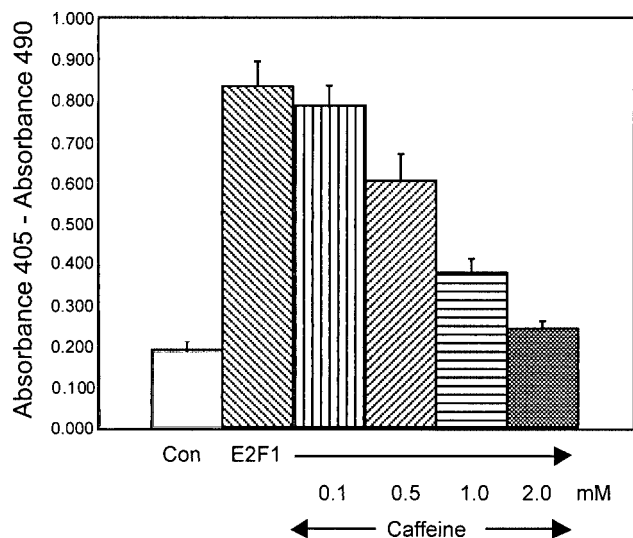


FIG. 8. Caffeine inhibits E2F1-mediated apoptosis. Analysis of apoptosis in wild-type MEFs. MEFs were infected with AdE2F1 at an MOI of 500, and caffeine was added at the indicated doses to the culture medium following infection. Cells were harvested at 72 hpi. Apoptosis was measured by detection of cytoplasmic DNA-histone complexes by ELISA. Error bars represent standard deviations calculated from three separate experiments. Con, control virus.

age are well documented (1). Candidate kinases include ATM, ATR, CHK1, and CHK2. ATM and ATR phosphorylate p53 at Ser<sup>15</sup> and activate the CHK kinases by phosphorylation (3, 7, 41, 45, 48). Active CHK kinases can then phosphorylate p53 at Ser<sup>15</sup> and Ser<sup>20</sup> (66).

Recently, ATM/ATR has been shown to phosphorylate the N terminus of E2F1 but not that of E2F2 or E2F3 (43). This observation, together with the data presented here, leads us to speculate that ectopic E2F1 expression or activation of endogenous E2F1 upon phosphorylation by ATM, leads to increases in the activity and, perhaps, levels of one or more of the p53 kinases, which then phosphorylate p53 and promote apoptosis. Given that the E2F1 DNA binding mutant E2F1<sub>c132</sub> did not induce p53 phosphorylation or apoptosis, transcriptional activation of one or more p53 kinases might provide a mechanism for E2F1 activation of this pathway. However, overexpression

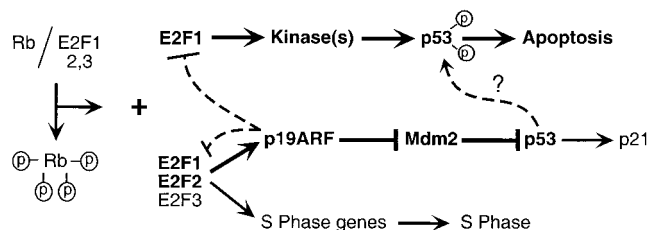


FIG. 9. Model of E2F-mediated p53 accumulation and apoptosis. E2F1, E2F2, and to a lesser extent, E2F3 can signal p53 accumulation through the p19ARF/Mdm2 pathway. p53 phosphorylation contributes to E2F1-mediated apoptosis and can occur in the absence of p19ARF. p19ARF may function to attenuate E2F proliferation-promoting activity as shown by the dashed lines. Activation of the p19ARF/Mdm2 pathway may increase levels of p53, contributing substrate to the p53 kinases induced by E2F1 as shown by the dashed arrow.

of the ATM/ATR, CHK1, or CHK2 kinases has been found to be insufficient for their activation (45, 48). Therefore, an additional signal(s) may be necessary to stimulate their activity.

It is conceivable that E2F1, E2F2, or E2F3 could signal through the p19ARF/Mdm2 pathway to increase p53 protein levels and that the increased pools of p53 would provide more substrate for the p53 kinases activated by E2F1 (Fig. 9). Thus, the p19ARF/Mdm2 pathway may act both as an attenuator of proliferation by targeting E2F family members for degradation and as an amplifier of a DNA damage signal by increasing pools of p53 available for phosphorylation. The decision to undergo growth arrest or apoptosis would then depend on the cellular context or extent of DNA damage.

Our data implicates p53 phosphorylation as a key step in E2F1-mediated p53-dependent apoptosis. These observations raise the possibility that E2F1 signaling and DNA damage response pathways may converge and involve the same or related kinases to activate p53. Alternatively, E2F1 may contribute to or be a component of the DNA damage pathway. Given these possibilities, it is possible that a role of E2F1 may be to amplify DNA damage signals, resulting in p53-mediated apoptosis. Future studies are needed to test this hypothesis and to define the kinase(s) involved in p53 phosphorylation and the mechanisms by which E2F1 activates them.

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