Control of the p53-p21^{CIP1} Axis by E2f1, E2f2, and E2f3 Is Essential for G1/S Progression and Cellular Transformation*

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The E2F family of transcription factors is believed to have an essential role in the control of cellular proliferation by regulating the transcription of genes involved in cell cycle progression. Previous work has demonstrated that the targeted inactivation of E2f1, E2f2, and E2f3 results in elevated p21^{CIP1} protein levels, loss of E2F target gene expression, and cell cycle arrest at G1/S and G2/M, suggesting a strict requirement for these E2Fs in the control of normal cellular proliferation. We now demonstrate that E2f1, E2f2, and E2f3 are also required for oncogene-mediated transformation of mouse embryonic fibroblasts. Analysis of synchronized populations of mouse embryonic fibroblasts revealed that the inactivation of p21^{CIP1} restores the ability of E2f1–3-deficient cells to enter and transit through G1/S (but not G2/M). In contrast, loss of p53 restored the ability of these cells to progress through both G1/S and mitosis, leading to their continued proliferation. The inactivation of p53 (but not p21^{CIP1}) rendered E2f1–3-deficient cells sensitive to transformation and tumorigenesis. These results suggest that the negative regulation of the p53-p21^{CIP1} axis by the E2f1–3 factors is critical for cell cycle progression and cellular transformation.

The timely entry and proper progression through the cell division cycle requires an ordered series of cyclin-dependent phosphorylation events and the execution of a well orchestrated transcriptional program. Growth factor-dependent activation of cyclin-dependent kinases (cdks)3 cdk4/6 and cdk2 results in the sequential phosphorylation of the Retinoblastoma (Rb) family of pocket proteins. This phosphorylation results in the release of E2F transcription factors from Rb-E2F complexes and activation of E2F target gene expression, leading to cell cycle progression (1, 2). In addition to the temporal association with the Rb family of pocket proteins, E2F activity is also regulated through cell cycle-dependent expression, post-translational modifications, interactions with cofactors, and protein degradation (1–3).

Several decades of work support the view that E2F represents an important activity that controls cellular proliferation (4–6). More recent studies suggest a particularly important role for the E2F3 family member in the control of gene expression during the G1/S transition. Inhibitory antibodies against E2F3 (but not E2F1) blocked the entry of cells into S phase (7). Consistent with these results, gene-targeting strategies in mice demonstrated a significant role for E2F3 in the proliferation of MEFs (8, 9). Some functional redundancy among family members is apparent, however, as the combined inactivation of E2f1, E2f2, and E2f3 led to a much more profound proliferation arrest that was manifested at multiple stages of the cell cycle (9). Interestingly, this block was accompanied by the induction of p53 transcriptional activity and the elevation of its target genes including p21^{CIP1} (9, 10). These observations raise the possibility that E2f1–3 activators might regulate cdk activity through a p21^{CIP1}-mediated negative feedback loop, which would then lead to the hypophosphorylation of Rb pocket proteins and the repression of E2F target genes (9, 10, 12). It appears that the activation of p53 in E2f1–3-deficient cells is a relevant event for the control of the cell cycle because its ablation by mutation, viral oncogenes, or gene-targeting approaches mitigated the induction of p53 target genes and the block in cellular proliferation.4 The specific role of p21^{CIP1} in this context, however, has yet to be determined. In the present study, we evaluated the role of E2f1–3 in the control of p21^{CIP1} expression during normal cellular proliferation and oncogene-mediated transformation. Our results demonstrate an important role for the control of the p53-p21^{CIP1} axis by E2f1–3 in cell cycle progression and oncogene-mediated transformation.

**EXPERIMENTAL PROCEDURES**

*Cell Culture—Primary MEFs were isolated from embryonic day 13.5 embryos using standard methods (9). All cells were cultured in DMEM with 15% fetal bovine serum. To bring cells to quiescence, cultures at 40–50% confluency were incubated in DMEM containing 0.2% serum for 60 h. Synchronized cells were then stimulated to proliferate by the addition of DMEM supplemented with 15% fetal bovine serum. Cells were collected at different time points after serum stimulation and were...*
formed in three independent experiments, and representa-

mean plates at the appropriate density were counted, and the crystal violet in 20% methanol. Colonies from three separate were then fixed with 70% ethanol and stained with 5 mg/ml nucleis/35-mm plate were scored for each time point.

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The mice weresacrificed once thetumor masses affected the tumormum length, width, and height using a digital Vernier caliper. was determined every three days by measuring theirmaxi-

four injections were administered per mouse. Tumor volume was isolated using Qiagen RNA miniprep columns as described by the manufacturer, including a DNase treatment before elution from the column. Reverse transcription of 2 g of the total RNA was performed by combining 1 μl of Superscript III reverse transcriptase (Invitrogen), 4 μl of

were harvested at the indicated time points, and total RNA was isolated using Qiagen RNA miniprep columns as described by the manufacturer, including a DNase treatment before elution from the column. Reverse transcription of 2 μg of the total RNA was performed by combining 1 μl of Superscript III reverse transcriptase (Invitrogen), 4 μl of

processed for BrdUrd incorporation and real-time reverse transcription-PCR assays.

Retroviral Infections—The cDNAs for Myc and Ras61L were subcloned into the EcoRI/Sall and BamHI/EcoRI sites respectively of the pBABE-hygro vector. The cre recombinase cDNA was subcloned into the SnaBI site of the pBABE retroviral vector containing either a puromycin (pBABE-puro) or hygromycin (pBABE-hygro) resistance gene. High titer retroviruses were produced by transient transfection of retroviral constructs into the Phoenix-Eco packaging cell line as described previously (13). MEFs were infected three times at 12-h intervals with Phoenix cell supernatants containing 4 μg ml−1 of Sequabrene (Sigma). Infected cells were then selected for a total of 2–5 days in the presence of 2.5 μg ml−1 of puromycin and/or 200 μg ml−1 of hygromycin.

BrdUrd Incorporation Assay—For BrdUrd incorporation assays, serum-stimulated cells were incubated with BrdUrd for 2 h before harvesting and subsequently fixed with methanol and acetic acid in a 1:1 ratio. Cells were then stained in 35-mm dishes with α-BrdUrd antibody (Ab-3, Oncogene) as previously described (14) and counter-stained with 4’,6-diamidino-2-phenylindole. At least 500 4’,6-diamidino-2-phenylindole-positive nuclei/35-mm plate scored for each time point.

Colony Formation Assays—MEFs were infected with supernatants containing various retroviruses as indicated in the leg-

ends to Figs. 1, 4, and 5. Cells were then plated at a density of 1000, 2500, and 5000 cells/100-mm plate. The cells were cultured in DMEM with 15% fetal bovine serum for 2 weeks and were then fixed with 70% ethanol and stained with 5 mg/ml crystal violet in 20% methanol. Colonies from three separate plates at the appropriate density were counted, and the mean±S.D. from one representative experiment is reported unless otherwise stated. Single colonies were isolated from par-

allel plated 96-well culture plates, and genomic DNA was collected and genotyped by PCR.

In Vivo Tumor Model—Animal studies were performed with 8–10-week-old male athymic nude mice. Myc- and Ras61L-transformed primary MEFs of various genotypes, as indicated in the legends to Figs. 2, 4, and 5, were harvested, counted, and resuspended in serum-free DMEM at a concentration of 1 × 107 cells/ml. 100 μl of cells were injected subcutaneously into the right and left shoulders and hips of each mouse; i.e. four injections were administered per mouse. Tumor volume was determined every three days by measuring their maximum length, width, and height using a digital Vernier caliper. The mice were sacrificed once the tumor masses affected the health of the animals. The latency was determined as the period of time required for 50% of the injection sites to form tumors having a volume of 5 mm3. Tumor studies were performed in three independent experiments, and representa-

ive data is shown.

Real-time PCR Analysis—Approximately 1 × 106 cells were harvested at the indicated time points, and total RNA was isolated using Qiagen RNA miniprep columns as described by the manufacturer, including a DNase treatment before elution from the column. Reverse transcription of 2 μg of the total RNA was performed by combining 1 μl of Superscript III reverse transcriptase (Invitrogen), 4 μl of

10× buffer, 0.5 μl of 100 μm oligo(dT) primer, 0.5 μl of 25 μM dNTPs, 1.0 μl of 0.1 m dithiothreitol, 1.0 μl of RNase inhibitor (Roche Applied Science), and water up to a volume of 20 μl. The reactions were incubated at 50 °C for 60 min and then diluted 5-fold with 80 μl of water. Real-time reverse transcription-PCR was performed using the Bio-Rad iCycler

FIGURE 1. E2F1, E2F2, and E2F3 are essential for transformation. Primary 123f/f cells were first infected with retroviruses expressing the Myc and Ras61L oncogenes and then infected with either control (−) or cre (+)-expressing retroviruses as described under “Experimental Procedures.” A, protein was then extracted from these cells, and equal amounts of lysates from Myc- and Ras61L-expressing control (−) or cre-treated (+) 123f/f cells were used for Western blot analysis with antibodies against E2F3 (top panel) and tubulin (bottom panel) as a loading control. B, cells were plated in triplicate on 100-mm plates, and the colonies formed were stained with crystal violet. Representative pictures of crystal violet-stained colonies with the selected genotypes are shown. C, the graph depicts the average number of colonies found in triplicate plates of control- and cre-treated groups. The average number of colonies from the control-treated group was normalized to 100, and the number of colonies found in the cre-treated cell line was adjusted accordingly. D, PCR analysis of genomic DNA isolated from individual colonies. Briefly, genomic DNA was extracted from cre-treated colonies and used for PCR to determine the extent of E2F3 deletion. The floxed allele (E2F3fl) produces a 184-bp fragment, and the knock-out E2F3 allele produces a 416-bp fragment. M, DNA ladder marker.
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RESULTS

E2f1–3 Are Required for Oncogene-mediated Transformation—Previous experiments have shown that E2f1, E2f2, and E2f3 are essential for the proliferation of primary and established MEFs. Our recent observations demonstrating that the inactivation of the p53 tumor suppressor restores the ability of E2f1–3-deficient cells to proliferate raised the possibility that oncogenic insults other than the loss of p53 tumor suppressor function might also relieve the strict requirement for E2f1–3 in cell proliferation.4 We reasoned that the introduction of transforming oncogenes might alter similar or related pathways disrupted by the loss of p53 and help drive the transformation of MEFs. To test this possibility, early passage primary E2f1−/−E2f2−/−E2f3−/− MEFs (123f/f) were first infected with retroviral vectors that contain a hygromycin selection cassette and express Myc and an activated form of Ras (Ras61L). After selection with hygromycin, the cells were further infected with retroviruses that co-express the cre recombinase along with a puromycin selection cassette. 

After two days of selection with puromycin, protein lysates were harvested, and consistent with our previous reports, cre expression in 123f/f MEFs resulted in the loss of both E2f3a and E2f3b proteins (Fig. IA). The ability of Myc-Ras61L-overexpressing E2f1–3-deficient MEFs to be transformed was analyzed by colony formation (15–18). As shown in Fig. 1, B and C, cre treatment of 123f/f MEFs led to a pronounced decrease in the number of transformed foci; the few foci that did form (Fig. 1C, ***), contained at least one non-deleted E2f3′ allele (Fig. 1D). The potential of primary 123f/f MEFs to be transformed was also tested in nude mice assays. To this end, Myc-Ras61L-overexpressing 123f/f MEFs were treated with control- or cre-expressing retroviruses (as described above) and injected subcutaneously into nude mice. Tumor progression was monitored every three days over a period of two months. Myc and Ras61L expression in control-treated cells gave rise to tumors in 75% of the injected sites by three weeks (Fig. 2A). By one month, mice had to be sacrificed, as all of the sites injected contained rapidly growing tumors with a mean tumor volume of 989 mm3 (Fig. 2, B and C). Cre expression in Myc-Ras61L-transformed 123f/f MEFs significantly increased the time of tumor onset and decreased tumor burden relative to control-treated cells. By the
end of two months, only 55% of these injected sites had given rise to tumors. PCR analysis demonstrated that, although >95% of the cre-infected cell population injected into nude mice were deleted for E2F3, 100% of the tumors that eventually developed arose from non-deleted cells (Fig. 2D). Together, these results demonstrate a strict requirement for E2F1–3 in cellular transformation.

Loss of E2F1–3 Leads to Activation of p53 Target Genes and Localization of p53 in the Nucleus—As we have previously shown, real-time PCR analysis of gene expression showed a marked overall reduction of E2F target genes upon ablation of E2F1–3 (Fig. 3A). Because p21<sup>CIP1</sup> is a known transcriptional target of the p53 tumor suppressor, we explored whether other p53-regulated genes were also induced in E2F1–3-deleted cells. Real-time PCR analysis demonstrated that numerous p53 target genes, including killer/dr5, gadd, pidd, and noxa, were significantly elevated in E2F1–3-deficient cells along with p21<sup>CIP1</sup> (Fig. 3B). These results suggest that, in addition to a decrease in E2F transcriptional activity, loss of p53 (but Not p21<sup>CIP1</sup>) Renders E2f1–3-deficient MEFs Competent for Transformation—Considering our recent observations indicating that the inactivation of p53 restores the ability of E2F1–3-efficient cells to proliferate, we sought to assess whether the loss of p53 might also render these cells sensitive to oncogene-mediated transformation. To this end, we intercrossed mice containing a conditional allele of p53 (p53<sup>f/f</sup>) with E2F1<sup>/−</sup>-E2F2<sup>/−</sup>-E2F3<sup>−/−</sup> mice and generated several p53<sup>f/f</sup>/E2F1<sup>/−</sup>-E2F2<sup>/−</sup>-E2F3<sup>−/−</sup> primary MEF lines (p53<sup>f/f</sup>/E2F1–3<sup>−/−</sup>). The capacity of these cells to be transformed by Myc-Ras<sup>G12V</sup> was tested in a similar manner as described above. In contrast to cre-treated 123<sup>f/f</sup> MEFs, both control- and cre-treated p53<sup>f/f</sup>/E2F1–3<sup>−/−</sup> MEFs could be transformed in response to Myc and Ras<sup>G12V</sup> overexpression (Fig. 4, A and B). As shown in Fig. 4B, the capacity of cre-treated Myc-Ras<sup>G12V</sup>-overexpressing p53<sup>f/f</sup>/E2F1–3<sup>−/−</sup> MEFs to form transformed foci was comparable with either control-treated p53<sup>f/f</sup>/E2F1–3<sup>−/−</sup> or cre-treated p53<sup>f/f</sup>-E2F1<sup>/−</sup>-E2F2<sup>/−</sup>-E2F3<sup>−/−</sup> (p53<sup>f/f</sup>-1<sup>/−</sup>-2<sup>/−</sup>-3<sup>/−</sup>) MEFs. PCR analysis of individual colonies confirmed that most colonies that formed were deleted for both E2F3 and p53 (~90%); a few of the colonies were deleted for p53 only (~10%), but no colonies were found to be deleted for E2F3 only (data not shown). Moreover, the injection of cre-treated Myc-Ras<sup>G12V</sup>-transformed p53<sup>f/f</sup>/E2F1–3<sup>−/−</sup> MEFs into nude mice gave rise to tumors in 100% of the injected sites by week 2 (Fig. 4D). PCR genotyping confirmed the ablation of both p53 and E2F3 in the vast majority of these tumors (Fig. 4E). These cells gave rise to aggressive tumors with a similar latency as Myc-Ras<sup>G12V</sup>-transformed cre-treated p53<sup>f/f</sup>-1<sup>/−</sup>-2<sup>/−</sup>-3<sup>/−</sup> MEFs. In either case, transformation of cre-treated p53<sup>f/f</sup>/123<sup>f/f</sup> or p53<sup>f/f</sup>-1<sup>/−</sup>-2<sup>/−</sup>-3<sup>/−</sup> MEFs gave rise to tumors significantly earlier than control-treated 123<sup>f/f</sup> MEFs (compare Figs. 2A and 4C). Hence, both colony and tumor assays in nude mice revealed that the loss of p53 resensitized E2F1–3-deficient cells to oncogene-mediated transformation.

We then assessed whether the specific induction of p21<sup>CIP1</sup> expression in E2F1–3-deficient cells might be responsible for the inability of these cells to be transformed. By similar experimental strategies as described above, we intercrossed mice containing a null allele of p21<sup>CIP1</sup> (p21<sup>−/−</sup>) with E2F1<sup>/−</sup>-E2F2<sup>/−</sup>-E2F3<sup>−/−</sup> mice, generated several p21<sup>−/−</sup>-E2F1<sup>/−</sup>-E2F2<sup>/−</sup>-E2F3<sup>−/−</sup> primary MEFs, and tested them for their capacity to be transformed by Myc-Ras<sup>G12V</sup>. As shown in Fig. 4F, the capacity of p21<sup>−/−</sup>-E2F1–3<sup>−/−</sup> MEFs to form transformed foci was comparable with control-treated 123<sup>f/f</sup> or cre-treated p53<sup>f/f</sup>-E2F1<sup>/−</sup>-E2F2<sup>/−</sup>-E2F3<sup>−/−</sup> (p53<sup>f/f</sup>-1<sup>/−</sup>-2<sup>/−</sup>-3<sup>/−</sup>) MEFs. PCR analysis of individual colonies confirmed that most colonies that formed were deleted for both E2F3 and p53 (~90%); a few of the colonies were deleted for p53 only (~10%), but no colonies were found to be deleted for E2F3 only (data not shown). Moreover, the injection of cre-treated Myc-Ras<sup>G12V</sup>-transformed p53<sup>f/f</sup>-E2F1–3<sup>−/−</sup> MEFs into nude mice gave rise to tumors in 100% of the injected sites by week 2 (Fig. 4G). PCR genotyping confirmed the ablation of both p53 and E2F3 in the vast majority of these tumors (Fig. 4H). These cells gave rise to aggressive tumors with a similar latency as Myc-Ras<sup>G12V</sup>-transformed cre-treated p53<sup>f/f</sup>-1<sup>/−</sup>-2<sup>/−</sup>-3<sup>/−</sup> MEFs. In either case, transformation of cre-treated p53<sup>f/f</sup>/123<sup>f/f</sup> or p53<sup>f/f</sup>-1<sup>/−</sup>-2<sup>/−</sup>-3<sup>/−</sup> MEFs gave rise to tumors significantly earlier than control-treated 123<sup>f/f</sup> MEFs (compare Figs. 2A and 4C). Hence, both colony and tumor assays in nude mice revealed that the loss of p53 rendered E2F1–3-deficient cells resensitized to oncogene-mediated transformation.
lines (p21<sup>−/−</sup> 123<sup>−/−</sup>), and tested the capacity of these cells to be transformed by Myc and Ras<sup>B11</sup>. As for 123<sup>−/−</sup> MEFs, cre treatment of p21<sup>−/−</sup> 123<sup>−/−</sup> MEFs abrogated the capacity of these cells to form transformed foci in vitro and form tumors in vivo (Fig. 5, A–C). Indeed, the expression of cre in Myc-Ras<sup>B11</sup>-transformed p21<sup>−/−</sup> 123<sup>−/−</sup> MEFs resulted in a marked delay in tumor onset relative to control-treated cells (39 versus 27 days, respectively). By the end of two months, only 22% of the sites injected with cre-treated cells gave rise to tumors, and all of the tumors that arose originated from non-deleted cells (Fig. 5, D and E). These results demonstrate that, in contrast to the loss of p53, the inactivation of p21<sup>CIP1</sup> is not sufficient to bypass the requirement for E2f1–3 in transformation. Together, these data suggest that E2f1, E2f2, and E2f3 play an essential role in transformation and tumorigenesis and highlight the dramatic influence that p53 has on whether these E2fs are required for tumor initiation and progression.

**Inactivation of p21<sup>CIP1</sup> Restores the Ability of E2f1–3-deficient MEFs to Enter S Phase**—We then explored why the loss of p53 (but not p21<sup>CIP1</sup>) impacted the transformation of E2f1–3-deficient cells. We initially assessed the ability of control- or cre-treated 123<sup>−/−</sup>, p53<sup>−/−</sup> 123<sup>−/−</sup>, and p21<sup>−/−</sup> 123<sup>−/−</sup> MEFs to respond to mitotic stimuli. To this end, 123<sup>−/−</sup>, p53<sup>−/−</sup> 123<sup>−/−</sup>, and p21<sup>−/−</sup> 123<sup>−/−</sup> MEFs were infected with control- or cre-expressing retroviruses, selected with puromycin, incubated in medium containing 0.2% serum for 60 h, and then restimulated with medium containing 15% serum. As determined by PCR-based genotyping assays, the expression of cre in each case led to the efficient deletion of the conditional E2f3 allele (Fig. 6B). Consistent with previous results (9), the cre-mediated ablation of E2f3 from 123<sup>−/−</sup> MEFs prevented cell cycle entry, as measured by BrdUrd assays (Fig. 6A). Surprisingly, the loss of either p53 or p21<sup>CIP1</sup> restored the ability of quiescent E2f1–3-deficient cells to enter S phase.

The status of E2f target gene expression in these cells was measured by real-time PCR assays. As expected, loss of E2f1–3 prevented the induction of various E2f-responsive genes known to have important functions in the G<sub>1</sub>/S and G<sub>2</sub>/M transitions (cdc6, dhfr, mcm3, pol α, tk, cyclin B1, cyclin A2, and mad2). Importantly, the entry of cre-treated p53<sup>−/−</sup> 123<sup>−/−</sup> and p21<sup>−/−</sup> 123<sup>−/−</sup> MEFs into S phase was accompanied by the induction of most E2f-responsive genes (Fig. 6C and data not shown). These results demonstrate that E2f target gene expression is dependent on the status of the p53 and p21<sup>CIP1</sup> pathway and suggest that the negative regulation of the p53-p21<sup>CIP1</sup> axis by E2f1–3 is critical for the transition of cells through G<sub>1</sub>/S.

**Inactivation of p53 (but Not p21<sup>CIP1</sup>) Restores the Capacity of E2f1–3-deficient MEFs to Proliferate**—Previous work has demonstrated that cells deficient for E2f1–3 arrest at G<sub>1</sub>/S and at
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cellular proliferation (9, 19–21). Each of the E2Fs have been reported to regulate different but overlapping subsets of target genes (22–25). These targets are diverse and include gene products whose activities control DNA replication, such as Cyclin A2, Cdc6, and Mmsn, as well as products involved in DNA repair and G2/M transitions, such as Cdc25a and String. The analysis of MEFs deficient for the entire E2F activator subclass suggests that the control of many of these target genes by E2F1–3 might involve the regulation of a p21CIP1-dependent feedback loop (9).

In this study, gene-targeting approaches were used to evaluate the role of E2f1–3 and p21CIP1 during normal cellular proliferation and oncogene-mediated transformation. Several observations suggest that the induction of p21CIP1 in E2f1–3-deficient cells is likely an important p53-regulated event contributing to the inhibition of E2F target gene expression and the block at G1/S. First, loss of p53 prevents the induction of p21CIP1 expression and restores cdk activity in E2f1–3-deficient cells.4 This leads to Rb phosphorylation, the expression of E2F targets, and continued proliferation. Second, the reactivation of cdk activity by the overexpression of cyclin E/cdk2 can promote the stimulation of quiescent E2f1–3-deficient cells to transiently re-enter the cell cycle and replicate their DNA (9). Finally, the targeted inactivation of p21CIP1 in cells deficient for E2f1–3, as shown by the work presented here, restores the G1/S-specific induction of E2F target genes and the entry of cells into S phase. These data suggest that regulation of the p53-p21CIP1 axis by E2f1–3 is important for the transition of cells through G1/S. Thus, it appears that the function of E2F1–3 in controlling the G1/S transition is mediated, at least in part, by the p53-mediated regulation of p21CIP1. The fact that deletion of p21CIP1 does not fully rescue the growth defect in E2f1–3-deficient cells, however, suggests that other p53 target genes may be involved in controlling the passage of cells through S phase and G2/M. Part of the mechanism by which the induction of p53 may block cells at the G1/S transition could involve the activation of Gadd45 and 14-3-3, two targets with known functions in the regulation of Cdc2 activity (26). The identification of the critical E2F1–3-regulated targets involved in the transition of cells through S and G2/M would be of future interest.

It is commonly believed that E2F is an activity intimately linked to cell cycle progression by virtue of its function in reg-

other stages of the cell cycle, including G2/M (9). To explore the possibility that the negative regulation of the p53-p21CIP1 axis by E2F1–3 also impacts the transition of cells through mitosis, we measured the mitotic index in control- and cre-treated unsynchronized 123f/f, 56) and 123f/f MEFs. The number of mitotic cells was determined by counting at least 2000 4',6-diamidino-2-phenylindole-stained nuclei. The data presented in Fig. 6D demonstrates that cre-treated 123f/f MEFs failed to undergo mitosis. Importantly, the inactivation of p53 (but not p21CIP1) in E2f1–3-deficient cells suppressed the mitotic block in these cells. From these experiments, we conclude that the activation of p21CIP1 in E2f1–3-deficient cells represents only one arm of the p53-dependent program that blocks the proliferation of these cells. Given that 21f–/– and E2f1–3-deficient cells can respond to serum, leading to the induction of a number of E2F target genes, we would suggest that E2F1–3 influence gene expression at G1/S and G2/M by p21CIP1-dependent and -independent mechanisms, respectively.

**DISCUSSION**

A large body of work in plants, insects, and mammals has demonstrated a critical role for E2F activators in the control of

![FIGURE 5. Loss of p21CIP1 in E2f1–3-deficient MEFs does not lead to tumor formation in nude mice. p21f–/–
123f/f primary cells were treated as described in Fig. 1. A, representative pictures of crystal violet-stained colonies with the selected genotypes are shown. B, colony formation assay of p21f–/–
123f/f MEFs that were co-infected with the indicated retroviruses. C, cells were injected into nude mice as described in Fig. 2. The graph depicts tumor volumes in cubic millimeter for each injection site. Shown are p21f–/–
123f/f cells (○) infected with control virus (n = 56) and p21f–/–
123f/f MEFs (▲) infected with cre-expressing retrovirus (n = 56). D, tumor incidence and latency. E, PCR analysis of cre-mediated deletion. To determine the extent of E2f3 deletion in cre-infected cells, PCR genotyping was performed on DNA extracted from the total population of cells prior to injection (left panel) and from each tumor that formed in nude mice (right panel). The appearance of a wild-type E2f3-specific band in samples derived from tumors is due to the presence of contaminating peripheral blood cells.]
FIGURE 6. Genetic deletion of p21\(^{CIP1}\) can restore S phase entry in E2f1–3 deficient MEFs. A, primary 123\(^{-}\), p53\(^{f/f}\)123\(^{-}\), and p21\(^{-/}\)123\(^{-}\) cells were infected with control (−) or cre (+)-expressing retroviruses, selected for puromycin resistance, and then used for BrdUrd incorporation and real-time analysis. Cells were brought to quiescence by serum starvation and stimulated to proliferate by the addition of 15% fetal bovine serum-containing medium. Cells were harvested for BrdUrd incorporation at 0 and 18 h after serum stimulation and processed as described under "Experimental Procedures." A total of at least 500 cells were counted at each time point. B, genomic DNA was extracted from control- and cre-treated cells and used for PCR to determine the extent of E2F and p53 deletion. C, real-time PCR analysis of E2F target genes. Total RNA was extracted from cells treated as in A and subsequently used to produce cDNA. The levels of expression of the indicated E2F target genes were determined by the method described under "Experimental Procedures." The y-axis represents the average fold induction in gene expression, where the level at the 0 h time point is equal to one. D, percentage of mitotic cells in proliferating 123\(^{-}\), p53\(^{f/f}\)123\(^{-}\), and p21\(^{-/}\)123\(^{-}\) MEFs infected with control- or cre-expressing retroviruses. At least 2000 cells for each sample were counted for the presence of mitotic cells.

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In summary, by using a conditional approach to ablate gene function, we have revealed a critical and unexpected role for E2F in the control of a p53-dependent checkpoint, opening the possibility that the requirement for E2F function during development and cancer may not be universal but rather restricted to certain cellular contexts. We propose that the success of strategies designed to block the proliferation of cancer cells by inhibiting E2F1–3 function will therefore be dependent on the status of the p53 pathway.

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This document discusses the role of E2F1–3 in cellular transformation and the regulation of gene expression through both positive (activation) and negative (repression) regulation. E2F activators can thus be viewed to coordinate transcriptional activation in conjunction with transcriptional repression via a p53-dependent pathway. The results presented here argue that E2F1–3 control a p53-p21\(^{CIP1}\)-dependent mechanism that controls E2F-mediated repression and that this feedback mechanism is critical for normal cellular proliferation and transformation.

The data presented in this manuscript have significant implications for future cancer therapies. It has previously been demonstrated that overexpression of E2F1, E2F2, and E2F3a can induce quiescent cells to enter the cell cycle, and each has oncogenic activity in primary murine fibroblasts (22, 27–31). In some human cancers, E2F3 is found to be amplified or overexpressed, suggesting an important role for this particular family member in human malignancies (32, 33). We show here a remarkable difference between the transforming potential of E2f1–3-deficient cells having or lacking p53. Indeed, E2f1–3 function has little effect on the tumorigenic potential of p53-null cells. These results predict that future therapeutic strategies that target E2F1–3 in cancer will only be effective in tumor cells with an intact p53 gene. This also predicts that mutation of p53 may eliminate the need for subsequent genetic or epigenetic alterations that inactivate the Rb pathway and lead to increased E2F1–3 activity. Indeed, our results provide an explanation for previous observations showing that the Rb-inactivating function of the polyomavirus large T oncoprotein is not required for the transformation of primary cells deficient for the p19\(^{ARF}\) tumor suppressor gene (11).

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In summary, by using a conditional approach to ablate gene function, we have revealed a critical and unexpected role for E2F in the control of a p53-dependent checkpoint, opening the possibility that the requirement for E2F function during development and cancer may not be universal but rather restricted to certain cellular contexts. We propose that the success of strategies designed to block the proliferation of cancer cells by inhibiting E2F1–3 function will therefore be dependent on the status of the p53 pathway.
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