Association of p14ARF with the p120E4F Transcriptional Repressor Enhances Cell Cycle Inhibition*

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The p14ARF tumor suppressor is a key regulator of cellular proliferation and is frequently inactivated in human cancer. This tumor suppressor functions in the p53 and pRB cell cycle regulatory pathways and can effectively activate both pathways to induce growth arrest or cell death. We now report that p14ARF forms a complex with the E1A-regulated transcriptional repressor, p120E4F. p120E4F contacts p14ARF and p53 to form a ternary complex in vivo and enhances p14ARF-induced G1 cell cycle arrest in a p53-dependent manner. We suggest that the interaction of p14ARF and p120E4F forms an important link between the p14ARF and p53 tumor suppressor proteins, both of which exhibit enhanced cell cycle inhibitory activity in the presence of this transcriptional repressor.

The INK4a/ARF locus on chromosome band 9p21 is disrupted in a wide variety of human tumors at a frequency comparable with p53 inactivation. The common loss of this locus presumably reflects its dual coding capacity; this single genomic sequence encodes the ARF tumor suppressor and the p16INK4a cyclin-dependent kinase inhibitor (1, 2). Although p16INK4a is frequently and specifically inactivated in tumors and in kindreds with a predisposition to melanoma (3–7), recent evidence confirms that the ARF tumor suppressor is also targeted in human cancers and is implicated in melanoma predisposition. Many mutations inactivating p16INK4a also impair the function of ARF (8, 9) and alterations specifically affecting ARF have been detected in familial melanoma kindreds (10–12).

The ARF tumor suppressor can induce potent growth arrest or cell death in response to hyperproliferative oncogenic stimuli. Mutated Ras, c-Myc, adenovirus E1A, E2F-1 and v-Abl all stimulate ARF expression in normal cells and induce G1 and G2 cell cycle arrest or apoptosis (13–16). p14ARF can activate the p53 tumor surveillance pathway by interacting with and inhibiting the p53-antagonist, Hdm2 (17–22). ARF limits the E3 ubiquitin ligase activity of Hdm2, thus preventing the polyubiquitination, nuclear export, and subsequent cytoplasmic degradation of p53 (22–25). Consequently, the loss of ARF not only diminishes the response of the p53 network to hyperproliferative signals but also reduces the duration of p53 activity in response to DNA damaging stimuli. Animals lacking ARF are highly prone to tumor formation, and their embryonic fibroblasts do not senesce, continuing to cycle after DNA damage (26).

ARF can also inhibit cell growth through p53- and Hdm2-independent mechanisms. Various types of human tumors exhibit simultaneous loss of p53 and ARF (27, 28), and the inactivation of p53 and ARF was not mutually exclusive in immortalized mouse embryonic fibroblasts (MEFs) (29). Mice lacking both ARF and p53, or with or without the mouse Hdm2 homologue, Mdm2, developed a wider range of tumor types than animals lacking either tumor suppressor alone (30). Moreover, the expression of the murine ARF homologue, p19ARF, strongly suppressed colony formation of p53-null MEFs (29) and inhibited DNA synthesis in p53-null/Mdm2-null MEFs (30). Although murine and human ARF display important differences in structure and function, human p14ARF also reduced the rate of DNA synthesis when p53 function was compromised (31).

The p53/Hdm2-independent functions of ARF are poorly understood but presumably involve additional ARF-binding partners. ARF can interact and destabilize the S-phase-inducing transcription factors E2F-1, E2F-2, and E2F-3 (32, 33). Consistent with a role for E2F degradation in the mechanism of ARF growth suppression, p53-null MEFs engineered to over-express E2F-1 were at least partially insensitive to the growth inhibitory action of p19ARF (29, 32). Other proteins that complex with ARF include spinophilin, a regulatory subunit of the protein phosphatase 1 catalytic protein (34), topoisomerase I (35), MdmX (36), HIF-1α (hypoxia-inducible transcription factor 1α) (37), the cytoplasmic peroxisomal protein, pex19p (38), and CARF (novel collaborator of ARF), a novel serine-rich protein (39). Although the extent to which these ARF complexes modulate the cell cycle inhibitory action of ARF is not well established, spinophilin and CARF appear to cooperate with ARF in promoting growth arrest, whereas pex19p retains p19ARF in the cytoplasm and down-regulates the p19ARF-p53 pathway (38, 39).

We now report that p14ARF can associate in vitro and in vivo with the adenovirus E1A-regulated p120E4F transcription factor. In humans and mice, p120E4F is an ubiquitously expressed, low abundance GLI-Kruppel-related zinc finger phosphoprotein that acts as a transcriptional repressor of the adenovirus E4 gene and, presumably, other cellular target genes (40–42). In response to adenovirus E1A or treatment with phorbol ester, p120E4F becomes hyperphosphorylated and undergoes a reduction in both DNA binding and transcriptional repressor activities.
p14ARF Interacts with the p120E4F Transcription Factor

Australia) supplemented with 10% fetal bovine serum and glutamine.

Expression of p120E4F significantly inhibits cellular growth and cell cycle progression, whereas p50E4F has no such effect (42, 43, 45–47). The growth inhibitory activity of p120E4F has been associated with the post-transcriptional elevation of several cell cycle regulatory proteins, including the CDK inhibitors p21Waf1 and p27Kip1, cyclin E, and cyclin B1, with reduced Cdk2, Cdk4/6, and Cdc2 kinase activities and with the down-regulation of cyclin A2 expression (42, 43, 48). Moreover, p120E4F-induced cell cycle arrest is enhanced by its interaction with the p53 transcription factor and hypophosphorylated pRb (46, 47). Although the cyclin A gene is the only cellular target thus far demonstrated to be down-regulated by p120E4F, it is likely that other genes involved in the control of cell cycle progression are regulated by p120E4F, as ectopic expression of p120E4F can induce cell cycle arrest at the G1/S and G2/M transitions even when cyclin A mRNA and protein levels are not reduced (43, 48).

In this study, we demonstrate that p14ARF forms a complex with p120E4F to modulate cell cycle progression. The formation of this complex occurs through residues in p120E4F that are in close proximity to those required for p53 binding and growth suppression activity and through residues in the amino-terminal region of p14ARF, which is also involved in Hdm2, E2F, and topoisomerase binding. Through this interaction, p14ARF sequesters ectopically expressed p120E4F in nucleoli. Importantly, p120E4F contacts both p14ARF and p53 to form a ternary complex in vitro and enhances G1 cell cycle arrest in a p53-dependent manner. We propose that p14ARF co-operates with p53 to induce growth arrest by interacting with and regulating the activity of the E1A-regulated transcription factor, p120E4F.

EXPERIMENTAL PROCEDURES

Mammalian Expression Constructs

The p14ARF FLAG (49), p18NK4ARF, FLAG (8), pCMV-E4F2.5k (encoding full-length p120E4F, amino acids 1–784) (40) and pCMVs-E4Fp501 (encoding p75E4F, amino acids 1–551) (46) constructs have been described elsewhere. The mutant p53 expression plasmid encoding human p53 cDNA with mutations altering amino acids 22, 23 and 281 has also been described previously (50). Truncated p14ARF cDNAs were isolated from the pCMV-FLAG5b clones and fused in-frame with the LexA DNA-binding domain in the displayBAIT vector (Display Systems Biotech) and amplified to characterise the target DNA.

β-Galactosidase Assay—Yeast cells were transformed sequentially with a LexA-BBD-p14ARF construct, a B42-AD-p120E4F construct, and the β-galactosidase reporter plasmid, psH18-34 (Clontech). Transformants were grown on dextrose–ura–his–trp–plates, and five colonies for each transformant type were picked and grown overnight in dextrose–ura–his–trp–liquid medium. β-Galactosidase activity was determined using the Pierce yeast β-galactosidase kit.

Indirect Immunofluorescence—Approximately 40 h after transfection, cells were washed in phosphate-buffered saline and fixed in 3.7% formaldehyde. Cells were immunostained for 50 min with either monoclonal mouse anti-FLAG M2 antibody (Sigma) or rabbit anti-E4F-Nterm (40) followed by a 50-min exposure to either anti-fluorescein isothiocyanate-conjugated anti-mouse secondary IgG (Roche Molecular Biochemicals, Molecular Probes) or a Texas Red-conjugated anti-rabbit secondary IgG (Jackson ImmunoResearch). Subcellular distribution of E4F was determined from a total of at least 250 fluorescent cells from two independent transfection experiments unless otherwise indicated. The standard deviation obtained within transfection experiments was always within ±5%.

Cell Cycle Analysis of Transfected U2OS Cells—Forty hours post-transfection, cells were fixed in 70% ethanol at 4 °C for at least 1 h, washed in phosphate-buffered saline, and stained with propidium iodide (50 ng/μl) containing ribonuclease A (50 ng/μl). EGFP-spectrin was used as a marker for analysis of transfected cells. DNA content from at least 2000 EGFP-spectrin-positive cells was analyzed using ModFIT software. The percentage of S-phase inhibition was calculated as described previously (46).

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Immunoprecipitations and Western Blotting—Expression of endogenous and ectopically expressed proteins was determined 24–40 h after transfecting U2OS cells with the indicated expression plasmids. Proteins were extracted for 20 min at 4 °C using RIPA lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in phosphate-buffered saline) containing protease inhibitors (Roche Molecular Biochemicals, Molecular Probes) or a Texas Red-conjugated anti-rabbit secondary IgG (Jackson ImmunoResearch). Subcellular distribution of E4F was determined from a total of at least 250 fluorescent cells from two independent transfection experiments unless otherwise indicated. The standard deviation obtained within transfection experiments was always within ±5%.

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Immunoprecipitations and Western Blotting—Expression of endogenous and ectopically expressed proteins was determined 24–40 h after transfecting U2OS cells with the indicated expression plasmids. Proteins were extracted for 20 min at 4 °C using RIPA lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in phosphate-buffered saline) containing protease inhibitors (Roche Molecular Biochemicals, Molecular Probes) or a Texas Red-conjugated anti-rabbit secondary IgG (Jackson ImmunoResearch). Subcellular distribution of E4F was determined from a total of at least 250 fluorescent cells from two independent transfection experiments unless otherwise indicated. The standard deviation obtained within transfection experiments was always within ±5%.

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lysed in RIPA lysis buffer containing protease inhibitors (Roche Molecular Biochemicals) for 20 min at 4 °C and precleared using 1 μg of normal IgG for 1 h. Immunoprecipitation was performed overnight at 4 °C with polyclonal E4F antibody and 50 μl protein A-agarose in a total volume of 1.5 ml. Immunoprecipitates were then washed four times with RIPA lysis buffer containing protease inhibitors, resolved using SDS-PAGE, and detected by immunoblot analysis. For immunoprecipitations using M450-tosylactivated DYNALbeads (DYNAL), 2 μg of antibody was adsorbed chemically as described by the manufacturer. Cell lysates were not precleared, and immunoprecipitations were performed at 4 °C for 90 min. Immunoprecipitation washes and protein detection were carried out as described above.

Yeast proteins were extracted as described in the manufacturer's yeast protocols handbook (Clontech). Electrophoresis was performed on SDS polyacrylamide gels, and proteins were transferred to Immobilon-P membranes (Bio-Rad). Western immunoblotting of proteins tagged with the LexA DNA-binding domain or the HA epitope was carried out using the monoclonal mouse anti-LexA antibody (2-12, Santa Cruz) and monoclonal anti-HA antibody (F-7, Santa Cruz), respectively.

**RESULTS**

**Isolation of E4F as a Candidate p14ARF-interacting Protein**—The yeast two-hybrid assay was employed to identify proteins capable of interacting with the p14ARF tumor suppressor. Full-length p14ARF fused to the LexA DNA-binding domain was used as bait to screen a human fetal brain cDNA expression library fused to the B42 activation domain (Display Systems Biotech). Sequence analysis of the 30 positive clones revealed that a single clone corresponded to the carboxyl-terminal half (amino acids 369–784) of the E1A-regulated transcription factor, p120E4F. This region is specific to full-length p120E4F and contains a central zinc finger domain with residues that are involved in the contact of p120E4F with p53 and pRb (46, 47).

**p120E4F Interacts Physically with p14ARF in Vivo**—To verify that E4F interacts with p14ARF in vivo, the full-length p120E4F cDNA cloned in pCMV5 (40) was overexpressed transiently in mammalian cells. Saos-2 cells were transfected with 3 μg of p14ARF-FLAG or p16INK4a-FLAG and 3 μg of pCMV-s-E4F2.5K. Approximately 24 h post-transfection, cell extracts were prepared and immunoprecipitated using protein A-agarose with a polyclonal antibody to E4F. Western analysis of the immunoprecipitates was performed with FLAG-specific M2 antibody (Sigma) to detect FLAG-tagged proteins. IP, immunoprecipitation; WB, Western blot.

**FIG. 1.** p14ARF associates with p120E4F in mammalian cells. Saos-2 cells were transfected with 3 μg of p14ARF-FLAG or p16INK4a-FLAG and 3 μg of pCMV-s-E4F2.5K. Approximately 24 h post-transfection, cell extracts were prepared and immunoprecipitated using protein A-agarose with a polyclonal antibody to E4F. Western analysis of the immunoprecipitates was performed with FLAG-specific M2 antibody (Sigma) to detect FLAG-tagged proteins. IP, immunoprecipitation; WB, Western blot.

**FIG. 2.** p14ARF amino acids 1–64 are sufficient for binding p120E4F. **A**, a yeast two-hybrid binding assay was performed using truncations of p14ARF to identify the region required for binding of p120E4F, and this was compared with p14ARF-Hdm2 binding affinity. Yeast cells were transformed with the indicated truncated p14ARF constructs cloned in-frame with the LexA DNA-binding domain. Nuclear localization sequences (NoLS) and Hdm2-binding domains are highlighted in the p14ARF construct diagrams. Proteins encoded by these expression plasmids were tested for binding to either full-length p120E4F or Hdm2 cloned in-frame with the B42 activation domain. β-Galactosidase activity was determined from five independent yeast colonies, and the binding affinities were expressed relative to full-length p14ARF. **B**, analysis of p14ARF-expression levels in yeast by Western immunostaining. Total yeast proteins were separated using SDS-PAGE and probed with an anti-LexA (2-12, Santa Cruz Biotechnology) monoclonal antibody. The p14ARF-(65–132)-LexA-DBD protein expressed at high levels and was loaded at half the concentration of the other yeast protein extracts.
human Saos-2 cells with either FLAG-tagged p14ARF or FLAG-tagged p16INK4a. Approximately 24 h post-transfection, cell lysates were immunoprecipitated with anti-E4F polyclonal antibody and blotted with anti-FLAG monoclonal antibody. As shown in Fig. 1, p14ARF-FLAG and p120E4F were efficiently co-precipitated, indicating that these two proteins are capable of interacting in vivo. p120E4F was also co-precipitated with p14ARF-FLAG using anti-FLAG or anti-ARF monoclonal antibodies (data not shown). The control immunoprecipitations performed with p16INK4a-FLAG-transfected cells were negative.

Binding to p120E4F Requires the Amino-terminal Half of p14ARF—p14ARF interacts with Hdm2, topoisomerase I, and the E2F transcription factors via its amino-terminal half (amino acids 1–64). This region also contains an arginine-rich nucleolar localization sequence (NoLS), which forms part of the Hdm2-binding domain (Fig. 2) (24, 49, 51). Not surprisingly these amino-terminal 64 amino acids are necessary for the growth inhibitory activity of p14ARF. The carboxyl terminus of p14ARF (amino acids 65–132) is not well conserved but is required for full p14ARF activity; this region also contains an arginine-rich nucleolar localization sequence, a fragment that was shown to interact weakly with Hdm2 using surface plasmon resonance (52).

To define the region of p14ARF that associates with p120E4F, we generated deletion constructs of p14ARF fused to the LexA DNA-binding domain and quantitatively assayed their ability to interact in yeast with full-length p120E4F fused to the B42 activation domain. The amino-terminal half of p14ARF was found to interact with p120E4F, whereas no E4F binding was detected with the carboxyl-terminal p14ARF fragment (Fig. 2). Partial deletion of the amino-terminal Hdm2 binding site (p14ARF-(14–132)) disrupted Hdm2 binding but did not affect its interaction with p120E4F (Fig. 2).

The central p120E4F Zinc Finger Domain Is Required for p14ARF Binding—p120E4F contains six C2H2 zinc finger motifs clustered in two separate regions. Two motifs occur within the amino-terminal domain and are present in the proteolytically derived p50E4F, whereas the remaining four are grouped within a central region (amino acids 493–569) found only in the full-length protein. The interaction of p120E4F with p53 requires residues within the last two of these central zinc finger motifs (amino acids 521–580) and is somewhat impaired by deletion of the last motif (carboxyl-terminal to amino acid 551) (46). The interaction of p120E4F with pRb occurs through two independent regions; one is in the amino-terminal half that is common to both p120E4F and p50E4F, and the other involves residues in the

### Fig. 3. p120E4F central zinc finger motifs are required for binding p14ARF

A, yeast two-hybrid binding assay was performed using truncated mutants of p120E4F to identify the region required for binding p14ARF. Yeast cells were transformed with the indicated truncated p120E4F constructs cloned in-frame with the B42 DNA activation domain. Zinc finger motifs and p53-binding domain are highlighted in the E4F construct diagrams. Proteins encoded by these expression plasmids were tested for binding to full-length p14ARF fused to LexA DNA-binding domain. Binding affinity was determined from five independent yeast colonies. B, analysis of E4F expression levels in yeast by Western immunostaining. Total yeast proteins were separated using SDS-PAGE and probed with an anti-HA (F-7, Santa Cruz) monoclonal antibody. The amounts of yeast protein extracts separated using SDS-PAGE were not equivalent, as the expression of the p120E4F-B42 fusion proteins varied significantly. As a result, the binding properties were not quantitatively expressed.
Co-expression of p120E4F and p14ARF Induces Enhanced Cell Cycle Arrest—The expression of either p120E4F or p14ARF can suppress cell proliferation at the G1-S and G2-M-phase transitions; the growth arrest induced by either protein is enhanced in the presence of functional pRB and p53 pathways (20, 29, 46, 47, 54). To evaluate whether the interaction of p14ARF and p120E4F also influences growth arrest, U2OS cells, which retain wild type p53, Hdm2 and pRB, were transiently transfected with p14ARF and/or p120E4F. At 40 h post-transfection, p120E4F over-expression had a minor effect on the cell cycle distribution of U2OS cells, inhibiting S-phase by ~10% while increasing the percentage of cells in G1 and G2-M each by only 4–5% (Fig. 6). p14ARF expression had a more potent effect, inhibiting S-phase by 40%, with a concomitant increase in the percentage of G2-M cells but no increase in G1 cells (Fig. 6). The simultaneous co-expression of p14ARF and p120E4F caused S-phase inhibition to a consistently greater extent than the expression of p14ARF alone, inhibiting S-phase by more than 65% while increasing the percentage of G2-M cells and to a lesser extent G1 cells. As expected, p14ARF-induced growth arrest was associated with increased p53 protein levels in the U2OS cell line, but the enhanced growth arrest induced when p14ARF was co-expressed with p120E4F was not associated with further increases in p53 or with any detectable changes in cyclin A protein levels (Fig. 7). The ability of p14ARF and p120E4F to induce growth arrest in a cooperative manner was further analyzed by a colony formation assay. U2OS cells were transfected with p14ARF-FLAG or pCMVs-E4F2.5K alone, with the combination of p14ARF-FLAG and pCMVs-E4F2.5K, or with pCMV-FLAG5b vector (as a control). After 2 weeks of selection in hygromycin-containing medium, the number of drug-resistant colonies obtained was determined relative to the vector control. As expected, p14ARF-FLAG (14 ± 3% of the vector control) and p120E4F (32 ± 7% of the vector control) individually inhibited U2OS colony formation (Fig. 6). But again, the co-expression of p14ARF-FLAG and p120E4F in the U2OS cell line produced a stronger inhibitory effect, reducing the number of colonies to 6 ± 3% relative to the vector control (Fig. 6).

p53 Is Required for Cell Cycle Arrest Induced by p14ARF and p120E4F Co-expression—The growth-suppressive function of ARF is enhanced in the presence of p120E4F and p53, and p120E4F can interact with p53 to promote cell cycle inhibition. We therefore investigated the influence of p53 on the cell cycle inhibition induced by the co-expression of p14ARF and p120E4F. In initial experiments, p14ARF-FLAG and pCMVs-E4F2.5K constructs were transiently expressed in the p53-null, pRB+ WMM1175 melanoma cell line. Enforced expression of p14ARF alone in the WMM1175 cell line induced a dose-dependent reduction in S-phase at 40 h post-transfection (data not shown). In contrast, at this time point, overexpression of p120E4F did not alter the cell cycle distribution of WMM1175 cells. Likewise, co-expression of
p120E4F and p14ARF did not significantly enhance the ability of p14ARF to inhibit S-phase entry (data not shown).

To determine the requirement of functional p53 for p14ARF/p120E4F-induced cell cycle arrest, we co-expressed a dominant-negative mutant form of p53 (p53<sup>22,23,281</sup>) in U2OS cells. This p53 mutant lacks a functional transcriptional activation domain and is catalytically inactive but can oligomerize with and inactivate wild type p53 (50). As expected, p14ARF-induced cell cycle arrest in U2OS cells was severely diminished when p14ARF was co-expressed with p53<sup>22,23,281</sup>. The p53 mutant also abolished the cooperative cell cycle inhibitory effect of p14ARF and p120E4F in U2OS cells. When p53<sup>22,23,281</sup> was transiently co-expressed with p14ARF and p120E4F in U2OS cells, there was no detectable S-phase inhibition 40 h post-transfection (Fig. 8).

**DISCUSSION**

The p14<sup>ARF</sup> tumor suppressor is frequently altered in a wide variety of human tumors and is implicated in the genetic predisposition to melanoma. This protein is activated in response to hyperproliferative signals emanating from oncoproteins and inducers of S-phase entry, such as Myc, E1A, Ras, and E2F1. Once activated, p14<sup>ARF</sup> induces G<sub>1</sub>- and G<sub>2</sub>-phase growth arrest or sensitizes cells to apoptosis. The activity of p14<sup>ARF</sup> involves an elaborate series of protein interactions, including binding to the p53-antagonist, Hdm2. In p53-positive cells, it is
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Fig. 7. Effect of p14<sup>ARF</sup> and/or p120<sup>E4F</sup> expression on p53, p21<sup>Waf1</sup>, and cyclin A accumulation. U2OS osteosarcoma cells (2.5 × 10<sup>5</sup>) were transfected with the p14<sup>ARF</sup>-FLAG plasmid (1.5 μg), the indicated E4F plasmid (1.5 μg), and pEGFP-N1 (1 μg). At 40 h post-transfection, ~1 × 10<sup>5</sup> cells expressing GFP were collected using the FACS Vantage cell sorter (BD Biosciences). The sorted populations were tested using flow cytometry and contained at least 95% GFP-positive cells. The sorted cells were lysed, and equivalent amounts of lysates were separated using SDS-PAGE. E4F, cyclin A, p53, CDK4, p21<sup>Waf1</sup>, and p14<sup>ARF</sup>-FLAG levels were determined by Western blotting with polyclonal E4F, C-19, DO-1, DCS-35, C-19, and M2, respectively.

In searching for p14<sup>ARF</sup>-interacting molecules, we isolated a carboxyl-terminal fragment specific to full-length p120<sup>E4F</sup> and localized the p14<sup>ARF</sup>-binding site in p120<sup>E4F</sup> to amino acids 369–566, a region that contains both p53- and pRB-binding domains (46, 47). Although the E4F binding sites for p53 and p14<sup>ARF</sup> are in close proximity, p53 and p14<sup>ARF</sup> can simultaneously bind to p120<sup>E4F</sup> (Fig. 4). Thus p120<sup>E4F</sup>, like Hdm2, can interact with p14<sup>ARF</sup> and p53 in binary (as shown in the p53-null Saos 2 cells; Fig. 1) or ternary complexes (Fig. 4). Not surprisingly, p14<sup>ARF</sup> interacts with p120<sup>E4F</sup> via its amino-terminal domain, a highly basic region that is involved in the p14<sup>ARF</sup>-Hdm2 binding, is less important for E2F and p120<sup>E4F</sup> interactions (Fig. 2) (24, 49, 51, 52, 56–59).

To establish the biological consequence of the p14<sup>ARF</sup>-p120<sup>E4F</sup> interaction, we analyzed the effect of co-expressing these proteins in the U2OS osteosarcoma cell line. The accumulation of exogenous p14<sup>ARF</sup> sequestered exogenous p120<sup>E4F</sup> into the nucleoli (Fig. 5). The significance of nucleolar import remains to be defined; Mdm2 nucleolar targeting is not required for ARF function in response to E2F or Ras (56, 60) but Mdm2 did accumulate with ARF in nucleoli in MEFs undergoing cellular senescence (22). It has been suggested that protein expression levels are the key determinant of nucleolar sequestration by ARF (61), and consistent with this observation, we found that exogenous ARF did not import endogenous Hdm2 or endogenous p120<sup>E4F</sup> into U2OS nucleoli (data not shown). Although the significance of ARF-driven nucleolar sequestration remains unresolved, it is a useful indicator to assess ARF-p120<sup>E4F</sup> binding, which was also confirmed in co-immunoprecipitation assays (Fig. 1) and as such helped to further delineate residues required for p14<sup>ARF</sup> interaction to amino acids 551–566 in p120<sup>E4F</sup>.

At 40 h post-transfection, ectopic expression of p14<sup>ARF</sup> in U2OS cells induced a minor but consistent decrease in S-phase cells, with a concomitant increase in the proportion of cells in the G<sub>2</sub>-phase. Over-expression of p120<sup>E4F</sup> had a lesser effect on the cell cycle distribution of these cells, but because we do not know the nature of the genes regulated by p120<sup>E4F</sup>, a greater effect might have required a longer period of expression. Nevertheless, at this time point, U2OS cells over-expressing both p120<sup>E4F</sup> and p14<sup>ARF</sup> showed a much greater reduction in the percentage of S-phase cells, with a consistent increase in the proportion of cells in G<sub>2</sub>-phase. Increases in G<sub>1</sub>-phase cells were observed occasionally (Fig. 6). S-phase reduction was also noted consistently with either p14<sup>ARF</sup> or p120<sup>E4F</sup> over-expression.
observed by others in Balb/c and NIH3T3 cells over-expressing p120E4F and either p53 or pRb, respectively (46, 47), although the mechanisms underlying this effect, as well as p14ARF-induced G2 arrest, are not yet well defined. With p120E4F, the most relevant changes in cell cycle regulatory proteins would seem to be the post-transcriptional elevation of cyclin B1, p21\textit{Waf1}, and p27\textit{Kip1} protein levels, the reduced levels of cdk2 and cdk2 kinase activities, and the reduced expression of cyclin A2 (42, 43, 48). With p14ARF, both G1 and G2 arrest have been associated with elevated p53 and p21\textit{Waf1} levels (20). Not surprisingly, then, U2OS cells over-expressing p14ARF had increased levels of p53 and p21\textit{Waf1} (Fig. 6). There was, however, no further increase in the amount of p53 or p21\textit{Waf1} in U2OS cells co-expressing p14ARF and p120E4F (Fig. 7), and thus the cooperative effect of p120E4F must be due to the down-regulation of other genes involved in cell cycle progression. To date, only cyclin A has been identified as a target for p120E4F, but we observed no significant change in the levels of cyclin A in U2OS arrested cells (Fig. 7). Therefore, clarification of this mechanism will require identification of other p120E4F cellular target genes.

Defining the role of p53 in the tumor suppressor functions of p120E4F is critical because most human cancers do not accumulate functional p53. As such, we investigated whether p120E4F could enhance the growth-suppressive function of p14ARF in a p53-null background. Although ectopic p14ARF expression induced a dose-dependent S-phase reduction in the p53-null WMM1175 melanoma cell line, this was not enhanced when p120E4F was co-expressed. Further, expression of the dominant-negative mutant p53\textsuperscript{22,23,28} abolished the cooperative growth arrest induced by p14ARF-p120E4F in the U2OS cell line (Fig. 8). This data should be interpreted with caution, however, because the WMM1175 cell line also carries alterations affecting G2 arrest have been associated with elevated p53 and p21\textit{Waf1} (Fig. 9). The formation and transcription of genes involved in G1 and G2 cell cycle arrest or apoptosis. In contrast, we propose that the E4F complex may induce p120E4F, but we observed no significant change in the levels of p120E4F and either p53 or pRb, respectively (46, 47), although the mechanisms underlying this effect, as well as p14ARF-induced G2 arrest, are not yet well defined. With p120E4F, the most relevant changes in cell cycle regulatory proteins would seem to be the post-transcriptional elevation of cyclin B1, p21\textit{Waf1}, and p27\textit{Kip1} protein levels, the reduced levels of cdk2 and cdk2 kinase activities, and the reduced expression of cyclin A2 (42, 43, 48). With p14ARF, both G1 and G2 arrest have been associated with elevated p53 and p21\textit{Waf1} levels (20). Not surprisingly, then, U2OS cells over-expressing p14ARF had increased levels of p53 and p21\textit{Waf1} (Fig. 6). There was, however, no further increase in the amount of p53 or p21\textit{Waf1} in U2OS cells co-expressing p14ARF and p120E4F (Fig. 7), and thus the cooperative effect of p120E4F must be due to the down-regulation of other genes involved in cell cycle progression. To date, only cyclin A has been identified as a target for p120E4F, but we observed no significant change in the levels of cyclin A in U2OS arrested cells (Fig. 7). Therefore, clarification of this mechanism will require identification of other p120E4F cellular target genes.

The activation of p120E4F with p14ARF, p53, and pRb appears to create a complex but effective network for growth control that is disrupted during human tumorigenesis and by viral infection. In vivo, the hypophosphorylated form of pRb promotes viral infection. In vivo, the hypophosphorylated form of pRb, p14ARF, and p53 pathways, and we have shown that the frequent loss of p14ARF would also disrupt the cooperation of p120E4F.
Association of p14ARF with the p120E4F Transcriptional Repressor Enhances Cell Cycle Inhibition
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