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Human Tumor Suppressor ARF Impedes S-Phase Progression Independent of p53[1]

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ABSTRACT

Using alternative reading frames, the human *ARF-INK4a* locus encodes two unrelated proteins that both function in tumor suppression. $p16^{INK4a}$ maintains the retinoblastoma protein in its growth-suppressive state through inhibition of cyclin D-dependent kinase activity, whereas ARF binds with MDM2 and stabilizes p53. The majority of the activity of ARF to date is ascribed to its ability to activate p53, resulting in a G_1 cell cycle arrest or apoptosis. We show here that ARF colocalizes with DNA replication protein A (RPA32) and that overexpression of ARF reduces the rate of DNA synthesis resulting in accumulation of an S-phase cell population. Impediment of DNA synthesis by ARF can occur and becomes more evident in the absence of p53. Hence, the biological consequence of ARF induction varies dependent on cellular p53 status, inducing predominantly a G_1 arrest or apoptosis in p53-positive cells or causing S-phase retardation when p53 function is comprised.

INTRODUCTION

The mammalian *ARF-INK4a* gene locus uniquely encodes two distinct proteins, $p16^{INK4a}$ and ARF, by using separate promoters and alternative reading frames, that both function as cell cycle inhibitors. Compelling evidence from the analyses of a large number of human tumor samples suggests that both ARF and p16 play critical roles in prevention of tumorigenesis. Additionally, mice with disruption of p16 and ARF or ARF alone with retention of p16 expression developed spontaneous tumors, and MEFs[3] derived from the animals had increased rate of spontaneous immortalization and could be transformed by oncogenic *ras*. On the other hand, inactivation of the *p16* gene alone in mice, sparing ARF, resulted in only a subtle to modest increase of spontaneous tumor development and did not allow escape from senescence or ras-induced transformation in MEFs (1, 2). Intriguingly, INK4a-deficient mice with loss of one allele of ARF ($INK4a^{-/-}$-$ARF^{+/-}$) developed tumors at an accelerated rate and with wider spectrum than $INK4^{-/-}$ mice retaining both ARF alleles ($INK4a^{-/-}$-$ARF^{+/+}$) or mice heterozygous for loss of ARF with retention of INK4a ($INK4a^{+/+}$, $ARF^{+/-}$), indicating that INK4a loss can cooperate with ARF heterozygosity in tumorigenesis (2). The molecular mechanism underlying functional INK4a-ARF collaboration is currently under investigation.

ARF binds to and inactivates MDM2, thereby stabilizing p53 (3–5). Transcription of the *ARF* gene can be induced by the ectopic expression of several proliferative oncogenes (6, 7), and loss of ARF abrogates the stabilization and induction of p53 by these oncogenic signals, resulting in greatly diminished apoptosis (6, 7). Taken together, these findings suggest that ARF may mediate a p53-dependent G_1 checkpoint control or apoptosis in response to hyperproliferative oncogenic stimulus.

To date, ARF activity has been primarily ascribed to its ability to stabilize and activate p53, resulting in a G_1 cell cycle arrest. Genetic evidence supporting this notion comes from the observations that tumors arising in ARF-deficient mice lack p53 mutation/deletion, that ectopic expression of ARF inhibits S-phase entry in wild-type MEFs but not in fibroblast lines lacking p53 (8), and that ARF inhibition of cellular transformation requires p53 function (3). *In vivo* genetic analysis, however, suggests that ARF has activity unrelated to p53 function. B cells that overexpress myc and lack both ARF and p53 are more resistant to myc-induced apoptosis and proliferate more rapidly than cells lacking either ARF or p53 singly (9). Furthermore, a p53-independent function of ARF has been recently suggested in promoting cellular senescence and suppressing immortalization (10). The mechanism(s) of these p53-independent ARF activities remains unexplained. We show here that overexpression of ARF results in a growth inhibition that is associated with a delay in S-phase progression and that the S-phase delay can occur in the absence or presence of p53.

MATERIALS AND METHODS

Cell Lines. U2OS and Saos-2 cells were obtained from the American Type Culture Collection. The NHF and its derivative expressing individual viral oncoproteins were described previously and characterized (11). Li-Fraumeni cells (strain LCS087, also known as MDA087) were described previously (12) and were kindly provided by Dr. Michael Tainsky (M. D. Anderson Cancer Center). U2OS and Saos-2 were cultured in McCoy's 5A + 15% FBS, and NHF and Li-Fraumeni cells were cultured in DMEM + 10% FBS.

Cell Cycle and Cell Death Analysis. For cell growth analysis, U2OS and Saos-2 cells were seeded at 2×10^5 cells/well onto six-well dishes. One day after seeding, cells were infected with control, p16, or ARF adenoviruses for 1 h in McCoy's 5A medium containing 2% FBS. Trypsinized cells were stained with 0.4% trypan blue, and viable cells, as determined by trypan blue exclusion, were counted by hemacytometer. For TUNEL, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 (Sigma Chemical Co.). DNA ends were labeled with digoxigenin-conjugated dUTP and TdT, which was then detected by fluorescent microscopy after labeling with anti-digoxigenin fluorescein antibody as described by the manufacturer (Oncor).

BrdUrd Labeling. For BrdUrd indirect immunofluorescence, cells infected with the specified adenovirus 2 days previously were grown in medium containing 20 μM BrdUrd (Calbiochem) for 1 h and then fixed in 70% ethanol. DNA was denatured, and cells were permeabilized in 2N HCl, 0.5% Triton X-100 (Sigma Chemical Co.), neutralized in 0.1 M $Na_2B_4O_7$ (pH 8.5), and then blocked with 0.5% BSA in PBS. Indirect immunofluorescence was performed using anti-BrdUrd (clone 85-2C8; Neomarkers) at a dilution of 1:50 and affinity purified anti-ARF (1 μg/ml) as described (13). Nuclei were stained with DAPI (1 μg/ml) for BrdUrd and/or ARF. For flow analysis, cells were grown in medium containing 10 μM BrdUrd. Trypsinized cells were fixed in 95% ethanol and then incubated in 0.08% pepsin (Sigma Chemical Co.), 0.1 N HCl for 20 min at 37°C to isolate nuclei. DNA was denatured by incubating in 2 N HCl for 20 min at 37°C, followed by neutralization with $Na_2B_4O_7$ (pH 8.5). Incorporated BrdUrd

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[3] The abbreviations used are: MEF, mouse embryo fibroblast; NHF, normal human fibroblast; TUNEL, terminal deoxynucleotide transferase-mediated dUTP nick end labeling; BrdUrd, 5-bromo-2′-deoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; Rb, retinoblastoma.

was stained by indirect immunofluorescence using anti-BrdUrd as above in 10 mM HEPES (pH 7.3), 150 mM NaCl, 4% FBS, and 0.5% Tween 20. DNA was then stained with propidium iodide and analyzed by flow cytometry as described (14).

Antibodies and Indirect Immunofluorescence. Affinity purified rabbit polyclonal antihuman ARF antibody and the procedure for indirect immunofluorescence (13) were described previously. Rabbit polyclonal p16 antibody, mouse monoclonal p21 antibody (clone DCS60.2; Neomarkers), and sheep polyclonal antibody to human p53 (clone Ab-7; Calbiochem) was used for Western blotting. Mouse monoclonal antibodies to human p16 (clone ZJ11; Neomarkers), to human RPA32/SSB (15), to BrdUrd, and rhodamine red-, FITC-conjugated goat secondary antibodies (Jackson ImmunoResearch Laboratories) were used for immunofluorescence.

Adenovirus. The ARF and p16 recombinant adenoviral vectors were generated by Tn7-mediated transposition in *Escherichia coli*. Briefly, the full-length human ARF and p16 coding sequences were subcloned into the pAdCMV transfer vector cDNAs containing a cytomegalovirus-driven mammalian expression cassette flanked by Tn7R and Tn7L (GenVec, Rockville, MD). These plasmids were used as donors to transpose the mini-Tn7 into an adenoviral genome in which lacZattTn7 replaces the E1 region. The recombinant genomes were purified then transfected into HEK293 cells. All recombinant adenoviruses were purified on CsCl gradients and dialyzed. For viral infection experiments, exponentially growing cells were infected with adenovirus in RPMI 1640 (for U2OS and Saos-2) or DMEM (for NHF and Li-Fraumeni cells) media supplemented with 2% FBS and were incubated for 1 h in a 37°C incubator with 5% CO_2. Infected cells were then replaced with fresh medium supplemented with 10% FBS until cell lysis or fixation.

RESULTS

Ectopic Expression of ARF Inhibits the Growth of p53-deficient Tumor Cells. Adenovirus-mediated ectopic expression of human ARF (Ad-ARF) inhibited the growth of p53-positive U2OS cells (Fig. 1*A*). Unexpectedly, Ad-ARF infection of p53-deficient human osteosarcoma Saos-2 cells also inhibited cellular proliferation (Fig. 1*A*). Because growth suppression mediated by the INK4 family of CDK inhibitors requires Rb, Ad-p16 infection inhibited cellular proliferation of U2OS cells but not of Saos-2 cells (Fig. 1*A*). Infection of U2OS or Saos-2 cells with control virus had no significant effect on cell growth as compared with uninfected cells (data not shown). ARF or p16 protein expression in infected cells was confirmed by direct immunoblotting (Fig. 1*B*). Functional activity of ARF was confirmed by an increase in p53 protein levels and the induction of the p53 transcriptional target p21 in U2OS cells.

Although expression of ARF caused growth inhibition in both U2OS and Saos-2 cells, the kinetics of these proliferative arrests in Saos-2 and U2OS cells were distinct. U2OS cells infected with Ad-ARF had a steady decrease in cell numbers, ultimately resulting in a near total loss of viable cells by 4 days (Fig. 1*A*). Trypan blue staining of floating and adherent cells confirmed a significant increase of cell death in Ad-ARF, but not Ad-p16 or control infected U2OS cells, but no increased cell death in Saos-2 cells infected with Ad-ARF (Fig. 1*C*). Cell death in adherent Ad-ARF-infected U2OS cells was a result of apoptosis, as determined by the TUNEL assays (Fig. 1*D*).

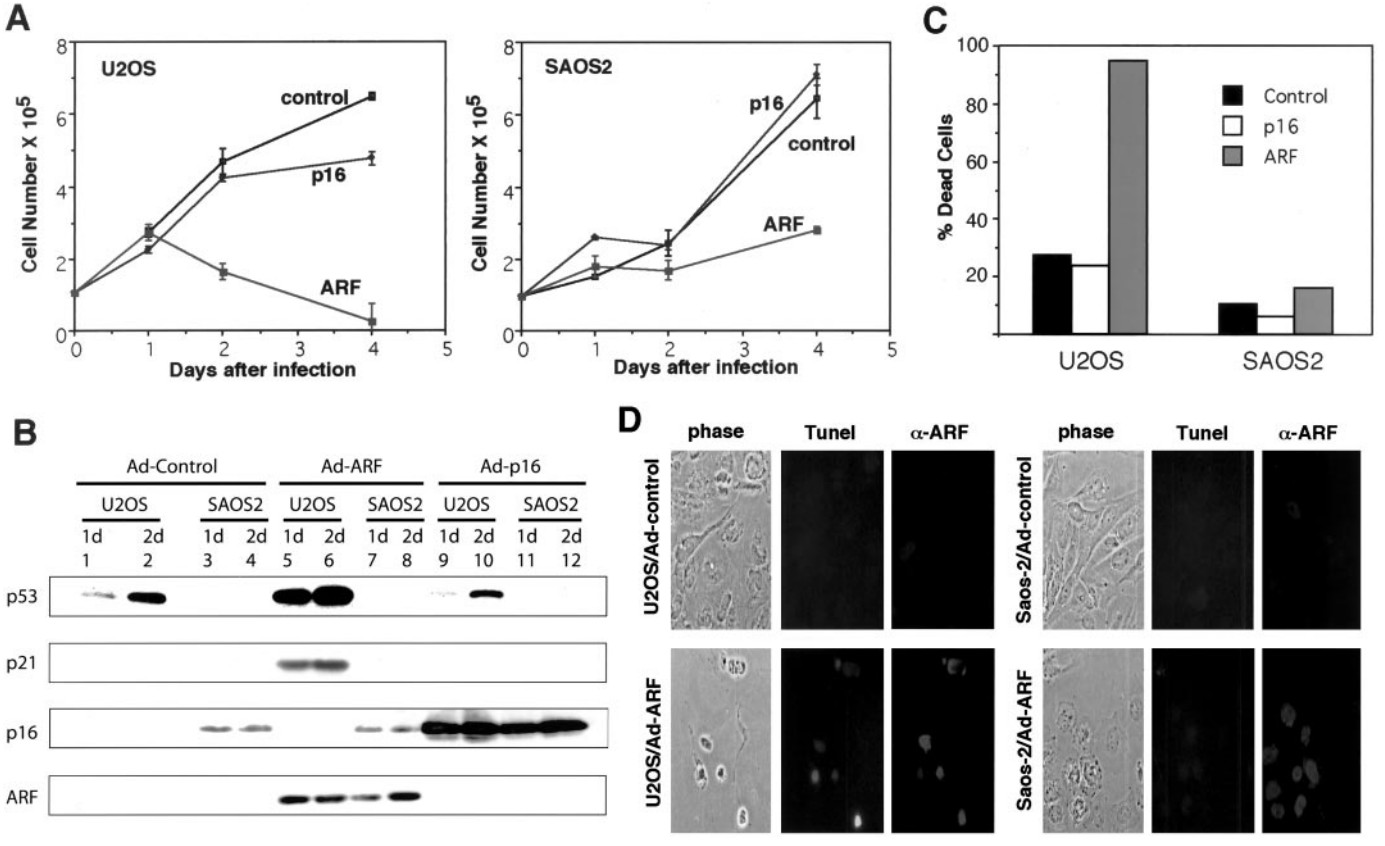
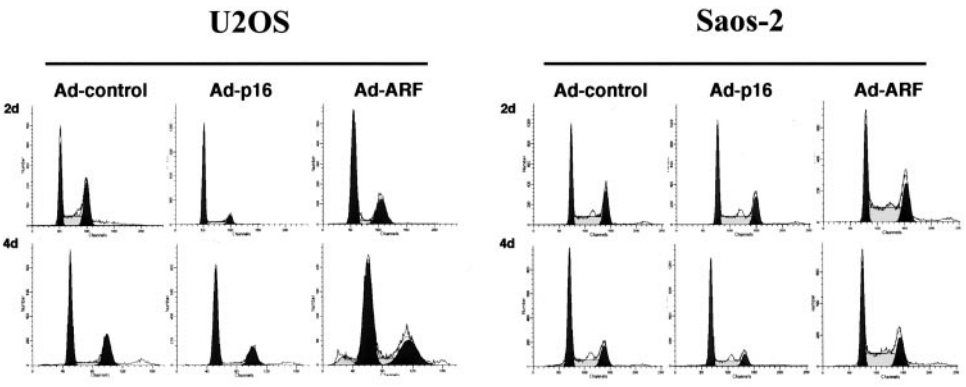


Fig. 1. Cell growth inhibition and death by ARF. *A*, growth curves of cells ectopically expressing p16 or ARF. U2OS and Saos-2 cells were infected with adenovirus directing expression of p16 or ARF or infected with adenovirus without insert (control) at day 0. Adherent, alive cells were counted at days 1, 2, and 4. Cell numbers are the average of two experiments; *bars*, SD. *B*, protein expression after adenoviral infection. Virally infected U2OS and Saos-2 cells were lysed 1 or 2 days after infection, and lysates were electrophoretically separated and immunoblotted with antibodies recognizing p53, p21, p16, or ARF. *C*, death of cells overexpressing p16 or ARF. Cell viability of floating and adherent cells collected 4 days after infection with control, p16, or ARF adenovirus was determined by trypan blue exclusion and direct cell counting. *D*, immunofluorescence and TUNEL assay after control or ARF adenoviral infection. U2OS and Saos-2 cells infected with control or ARF adenovirus were fixed and immunostained for ARF expression and assayed for apoptosis using TUNEL.



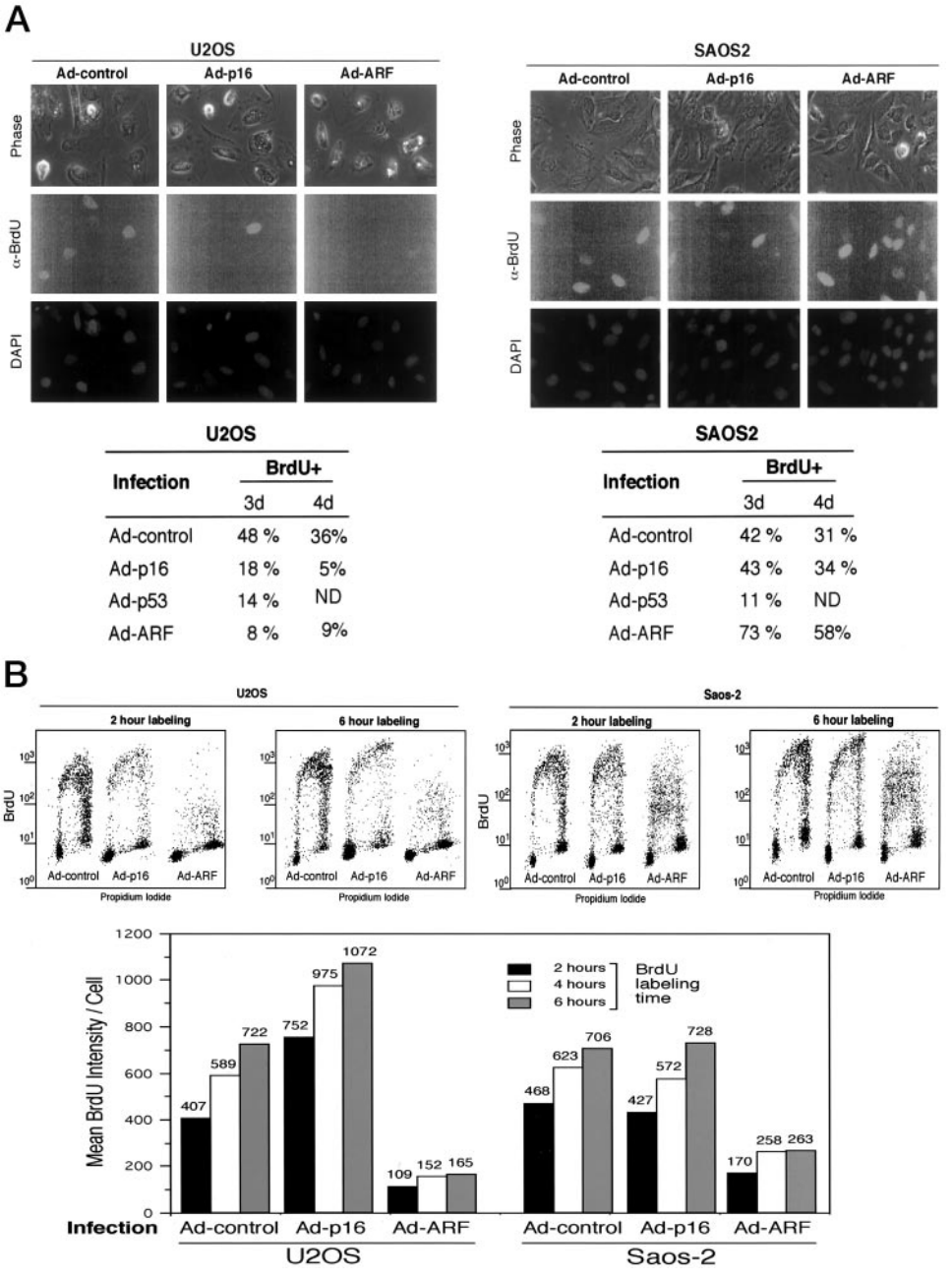
	Infection	2d G1	2d S	2d G2/M	4d G1	4d S	4d G2/M
U2OS	Ad-control	33%	32%	35%	59%	14%	28%
	Ad-p16	72%	17%	11%	72%	10%	18%
	Ad-ARF	65%	11%	25%	60%	14%	26%
Saos-2	Ad-control	38%	36%	26%	52%	29%	19%
	Ad-p16	39%	39%	22%	58%	28%	14%
	Ad-ARF	31%	45%	24%	36%	43%	21%
LCS087	Ad-control	58%	23%	19%	74%	12%	14%
	Ad-p16	82%	08%	11%	84%	06%	10%
	Ad-ARF	43%	48%	09%	36%	53%	11%
NHF/neo	Ad-control	77%	05%	18%	77%	01%	21%
	Ad-p16	85%	02%	13%	88%	01%	11%
	Ad-ARF	58%	29%	13%	59%	25%	16%
NHF/E6	Ad-control	52%	19%	29%	69%	17%	14%
	Ad-p16	78%	07%	15%	85%	04%	11%
	Ad-ARF	42%	45%	13%	55%	32%	13%

Fig. 2. Ectopic expression of ARF causes an S-phase cell cycle arrest in p53-deficient tumor-derived cells. U2OS (human osteosarcoma cells, p53 wild type), Saos-2 (human osteosarcoma cells, p53 deficient), Li-Fraumeni fibroblasts (LCS-087, p53 deficient), NHF cells stably expressing neomycin resistance marker (NHF/neo) and NHF expressing papilloma viral protein E6 (NHF/E6) were infected with control, p16, or ARF adenovirus. At the indicated time points after viral infection, cells were collected and analyzed for cell cycle position by flow cytometry. *2d*, 2 days; *4d*, 4 days.

Expression of ARF in TUNEL-positive cells was confirmed by indirect immunofluorescence staining with an affinity-purified antibody specific to ARF. These results suggest that ARF expression inhibits cellular proliferation in the absence of p53 and that overexpression of ARF in the presence of functional p53 can result in apoptotic cell death.

ARF Expression Results in Accumulation of an S-Phase Cell Population in p53-deficient Cells. To further define the observed cell growth inhibition and cell death induced by ARF, we analyzed the cell cycle distribution of Ad-ARF-infected U2OS and Saos-2, as well as immortalized fibroblasts derived from Li-Fraumeni patients after spontaneous loss of the wild-type *p53* allele during *in vitro* passage (LCS-087) and NHFs selected for expression of a selectable marker (NHF/neo) or the p53-inactivating oncoprotein derived from human papillomavirus type 16 (NHF/E6; Fig. 2; Ref. 11). ARF induced a G_1 accumulation in U2OS cells 2 days after infection, and fluorescence-activated cell sorting analyses indicated an accumulation of a sub-G_1 cell population, a characteristic of apoptotic cell death at 4 days after infection. Remarkably, Ad-ARF-infected Saos-2 cells accumulated an S-phase cell population that was first observed at the 2-day time point, consistent with the first notable growth inhibition by cell counting (Fig. 1). Likewise an S-phase accumulation of cells was observed in LCS-087 and NHF/E6 cells, both of which lack p53 activity (Fig. 2) The S-phase accumulation after Ad-ARF infection of Saos-2 cells persisted at 4 and 6 days (6-day time point not shown), suggesting that growth inhibition of ARF-infected Saos-2 cells may be caused, at least in part, by an S-phase arrest or delay. Consistent with the requirement for functional Rb to exert its growth-inhibitory activity, ectopic expression of p16 induced a G_1 arrest in U2OS, LCS-087, HNF/neo, and NHF/E6 but not in Saos-2 cells. In addition to p53 deficiency, Saos-2 cells also lack functional p73 (16), indicating that the ARF-induced S-phase arrest observed in these cells was independent of p73 as well. There was also an increase of S-phase cells in Ad-ARF-infected NHF/Neo control cells, suggesting that S-phase retarding activity of ARF does not dependent on loss of p53 activity. These data do not exclude the possibility that ARF could have S-phase activity that is p53 dependent in addition to the p53-independent effect observed in Saos-2, Li-Fraumeni, and NHF/E6 cells.

ARF Expression Reduces the Rate of DNA Synthesis. Accumulation of S-phase cells after ectopic gene expression often signifies an S-phase entry, but increased S-phase percentage in ARF-expressing cells was associated with a decrease in cell number, suggesting that ARF expression slowed or halted DNA synthesis. To determine whether cells expressing ARF were capable of synthesizing DNA, Saos-2 and U2OS cells were infected with Ad-ARF, Ad-p16, Ad-p53 or control viruses and 3 or 4 days later pulse-labeled for 1 h with BrdUrd, a thymidine analogue that is incorporated into DNA in place of dTTP. After BrdUrd pulse-labeling, cells were immediately fixed,



U2OS

Infection	BrdU+ 3d	BrdU+ 4d
Ad-control	48 %	36%
Ad-p16	18 %	5%
Ad-p53	14 %	ND
Ad-ARF	8 %	9%

SAOS2

Infection	BrdU+ 3d	BrdU+ 4d
Ad-control	42 %	31 %
Ad-p16	43 %	34 %
Ad-p53	11 %	ND
Ad-ARF	73 %	58%

Fig. 3. Ectopic expression of ARF impedes S-phase progression. *A*, immunofluorescence staining of BrdUrd incorporation. U2OS and Saos-2 cells were infected with the indicated adenoviruses. Forty-eight h after infection, cells were labeled with BrdUrd for 2 h and fixed, and nuclei were stained with DAPI and with monoclonal antibody to BrdUrd. BrdUrd-positive cells were scored microscopically. Infection of 100% of cells was confirmed by indirect immunofluorescence using antibodies specific to ARF, p16, or p53 in an adjacent well (data not shown), and at least 250 infected cells at each time point were scored for BrdUrd staining. Percentages of cells incorporating BrdUrd were calculated from the mean of two independent experiments, except in the case of p53 infection where only one experiment was performed. *B*, quantification of BrdUrd incorporation. Control adenovirus, Ad-p16-, and Ad-ARF-infected U2OS and Saos-2 cells were pulse-labeled with BrdUrd for the indicated times and analyzed by flow cytometry for DNA content and BrdUrd incorporation. Cells incorporating BrdUrd were gated and further analyzed to determine the relative cellular intensity of BrdUrd staining.

and nuclei were stained with the DNA intercalating agent DAPI and BrdUrd. Infection of 100% of cells was confirmed in a separate well by indirect immunofluorescence using antibodies specific to p16, ARF, or p53 (data not shown). Fig. 3*A* shows BrdUrd staining in representative fields 3 days after control, Ad-p16, or Ad-ARF infection, as well as the calculated percentage of cells incorporating BrdUrd 3 and 4 days after adenoviral infection (Fig. 3*A*). At least 250 cells at each time point were scored to determine percentages of cells incorporating BrdUrd. Three or four days after Ad-ARF infection, 73 or 58% of Saos-2 cells incorporated BrdUrd, whereas only 48 or 36% of similarly labeled Ad-control infected cells were BrdUrd positive, indicating that ARF expression did not completely halt DNA synthesis. ARF-infected U2OS cells had a decrease in the percentage of cells incorporating BrdUrd, 8 and 9% at 3 and 4 days after infection relative to control-infected cells and 48 and 36% at 3 and 4 days, consistent with cell death or G_1 arrest as observed by flow, TUNEL, and cell counting (Figs. 1 and 2). As expected, 3 days after Ad-p53 infection, the percentage of BrdUrd-positive cells relative to control-infected cells decreased from 48 to 14% in U2OS and from 42 to 11% in Saos-2 cells. Three days after adenovirus infection, the percentage of BrdUrd-positive U2OS cells was decreased from 48% in control-infected cells to 18% in Ad-p16-infected cells and from 36 to 5% for cells infected 4 days before analyses. As expected, Ad-p16 infection had virtually no effect on BrdUrd incorporation in Rb-deficient Saos-2 cells.

In a separate experiment (data not shown), we infected U2OS and Saos-2 cells with Ad-ARF at a low multiplicity to allow for an examination of both ARF-positive and ARF-negative cells in the same microscopic viewing field 4 days after adenoviral infection pulse-labeled with BrdUrd for 1 h. Cells were indirectly immunostained using anti-ARF and anti-BrdUrd antibodies and secondary antibodies with different fluorescent tags to allow detection of ARF and BrdUrd in the same cell. In agreement with the above results, 60% of ARF-positive *versus* 35% of ARF-negative Saos-2 cells incorporated

BrdUrd. The percentage of ARF-positive U2OS cells incorporating BrdUrd was dramatically decreased (9%) relative to ARF-negative cells (50%).

Given that overexpression of ARF caused an accumulation of cells in S-phase and an increase in the percentage of cells synthesizing DNA but a decrease in total cell number, we wanted to determine whether ARF expression slowed DNA synthesis. Consistent with this notion, we observed that pulse labeling with BrdUrd of cells ectopically expressing ARF stained relatively weaker for BrdUrd than control or p16-expressing cells, as indicated by decreased intensity and longevity of the BrdUrd signal after exposure to the laser causing the excitation (data not shown). To directly test whether ARF impeded DNA synthesis, we compared the relative rate of DNA synthesis in cells infected with Ad-control, Ad-p16, or Ad-ARF viruses by pulse labeling with BrdUrd and then measuring BrdUrd incorporation. Analyses of cells that incorporated BrdUrd revealed that the mean cellular BrdUrd intensity of Ad-ARF-infected Saos-2 cells after BrdUrd pulse labeling for 2, 4, or 6 h was 170, 258, and 263, respectively, significantly lower than similarly labeled control infected (468, 623, and 706) or Ad-p16 infected (427, 572, and 728) Saos-2 cells (Fig. 3*B*). Interestingly, even in U2OS cells where ARF expression resulted primarily in a p53-dependent G_1 cell cycle arrest or apoptosis (Figs. 1*D* and 2), the small fraction of cells that escaped into S-phase had significantly lower mean cellular BrdUrd intensity after 2, 4, or 6 h of BrdUrd labeling (109, 152, and 165, respectively) relative to similarly labeled control-infected (407, 589, and 722) or Ad-p16-infected (752, 975, and 1072) cells. The ability of ARF expression to slow BrdUrd incorporation in p53-positive U2OS cells confirms that functional p53 does not inhibit the S-phase activity of ARF and further suggests that the S-phase activity of ARF may be masked by an overwhelming p53-dependent G_1 cell cycle arrest or apoptosis in p53-positive cells. As an indicator of the magnitude of the decreased DNA synthesis rate caused by ectopic expression of ARF of Saos-2 cells, the mean BrdUrd intensity of ARF-infected cells labeled for 6 h was lower than that of Ad-p16- or Ad-control-infected cells labeled for 2 h, suggesting that the rate of DNA synthesis was decreased $>$3-fold. Similar results were obtained in Li-Fraumeni cells, where ARF infection of LCS087 cells resulted in a decrease of mean cellular BrdUrd incorporation by 31%, after either 4 or 6 h BrdUrd pulse labeling relative to control-infected cells (data not shown). These observations suggest that ARF-expressing cells have a slowed rate of DNA synthesis.

ARF Colocalizes with Replication Protein A (RPA32). To further explore the mechanism by which ARF slowed DNA synthesis, we examined subcellular localization of ARF and other proteins known to be involved in replication of DNA. We and other groups have noted previously that ectopic and endogenous ARF localizes to microscopically visible nuclear components known as nuclear bodies (13, 17). The function of these ARF nuclear bodies remains ill defined, but their relatively large size suggests that additional proteins and possibly chromatin may localize to the bodies. To test whether ARF protein in nuclear bodies was associated with proteins implicated in DNA synthesis, nuclear body formation was induced in U2OS, SJSA, and NHF cells by infection with adenovirus directing expression of p53, mdm2, and ARF. Indirect immunostaining of ARF and the replication protein A (RPA32) revealed that both ARF and RPA32 staining was punctate, and neither protein localized to nucleoli (Fig. 4*B*). The punctate staining of ARF and SSB colocalized, as indicated by yellow color after overlay of ARF and RPA32 staining. In contrast, cells infected with control adenovirus revealed no nuclear bodies, and RPA32 was homogeneously located within the nucleus, whereas endogenous ARF was weakly expressed in nucleoli of SJSA and NHF cells (Fig. 4*A*). U2OS cells do not express detectable ARF, despite retention of the p16-ARF gene attributable to, at least in part, the existence of functional p53. Formation of nuclear bodies altered both ARF and RPA32 localization from nucleoli and homogeneous nuclear, respectively, to nuclear bodies.

To rule out the possibility that ARF and RPA32 localization to nuclear bodies was a result of supraphysiological expression or ARF, we wanted to determine whether endogenous ARF localized with RPA32. Endogenous ARF is expressed at very low levels unless cells are oncogenically stimulated; we infected U2OS, SJSA, and NHF with adenovirus directing expression of E2F1 to induce endogenous ARF. Thirty-six h after infection, cells were fixed and immunostained using antibodies specific for ARF and RPA32. As expected, ARF protein was induced in SJSA and NHF but not in U2OS cells (data not shown). After oncogenic stimulation, endogenous ARF and RPA32 colocalized within nuclear bodies in SJSA and NHF cells (Fig. 4*C*). Slowing of DNA synthesis after ARF expression may be explained by the subnuclear localization of ARF into nuclear bodies, where it may interact directly or indirectly with proteins involved in DNA synthesis.

DISCUSSION

If all tumor suppressor activity of ARF were dependent solely on functional p53, then predictably, the tumor phenotype of $p53^{-/-}$ mice would be more severe and encompass the $ARF^{-/-}$ tumor phenotype. However, comparison of tumor formation in $p53^{-/-}$ and $ARF^{-/-}$ mice reveals carcinomas and neurogenic tumors in $ARF^{-/-}$ mice rarely or never observed in $p53^{-/-}$ mice, suggesting that for tumor formation, loss of p53 and ARF are not equivalent. Likewise, if all ARF activity was dependent on p53, then loss of ARF in addition to p53 should have no effect on tumor progression or cellular growth characteristics. However, myc-induced lymphomas in mice were more aggressive in ARF/p53 double nullizygous mice relative to mice singly nullizygous for either p53 or ARF, and primary lymphoblasts lacking both p53 and ARF grew faster and resisted myc-induced apoptosis better than cells lacking only ARF or p53 (9, 18). Additionally, the induction of growth arrest after restoration of endogenous ARF in immortalized cells is reversed by simultaneous inactivation of both p53 and Rb but not by inactivation of p53 alone (10). The later results suggest that ARF has a p53-independent activity acting through the Rb pathway. These independent systems that do not rely on overexpression of ARF, *i.e.*, restoration of ARF expression by removal of antisense sequences (10) and comparison of ARF null with p53/ARF double null cells (9), strongly suggest p53-independent functions of ARF exist. Although these systems acutely highlight the biological relevance of p53-independent activities of ARF in slowing cellular growth, increasing cellular apoptosis, and inducing cellular senescence, the molecular mechanism(s) underlying these p53-independent ARF activities remains to be elucidated.

To determine the biochemical activity of ARF in the absence of p53 function, we used an adenovirus-mediated ectopic expression system and multiple cell lines lacking p53 activity. Because the cells in our system were immortalized, the possible p53-independent activities of ARF in cellular senescence were not addressed. Our efforts were focused on the effect of overexpression of ARF on cell cycle progression and apoptosis. In three different human cell lines with comprised p53 activity, we uncovered a novel p53-independent function of ARF, *i.e.*, its ability to impede S-phase cell cycle progression that is distinct from its p53-dependent G_1 arrest activity. Parallel infection by control adenovirus or adenovirus expressing p16 did not induce an accumulation of S-phase cell population or reduce the rate of DNA synthesis in Saos-2 (Figs. 1–3), Li-Fraumeni cells, or NHF/E6 cells (Fig. 2). These results argue against the possibility that observed S-phase delay in Ad-ARF-infected cells is caused by the adenovirus infection *per se*

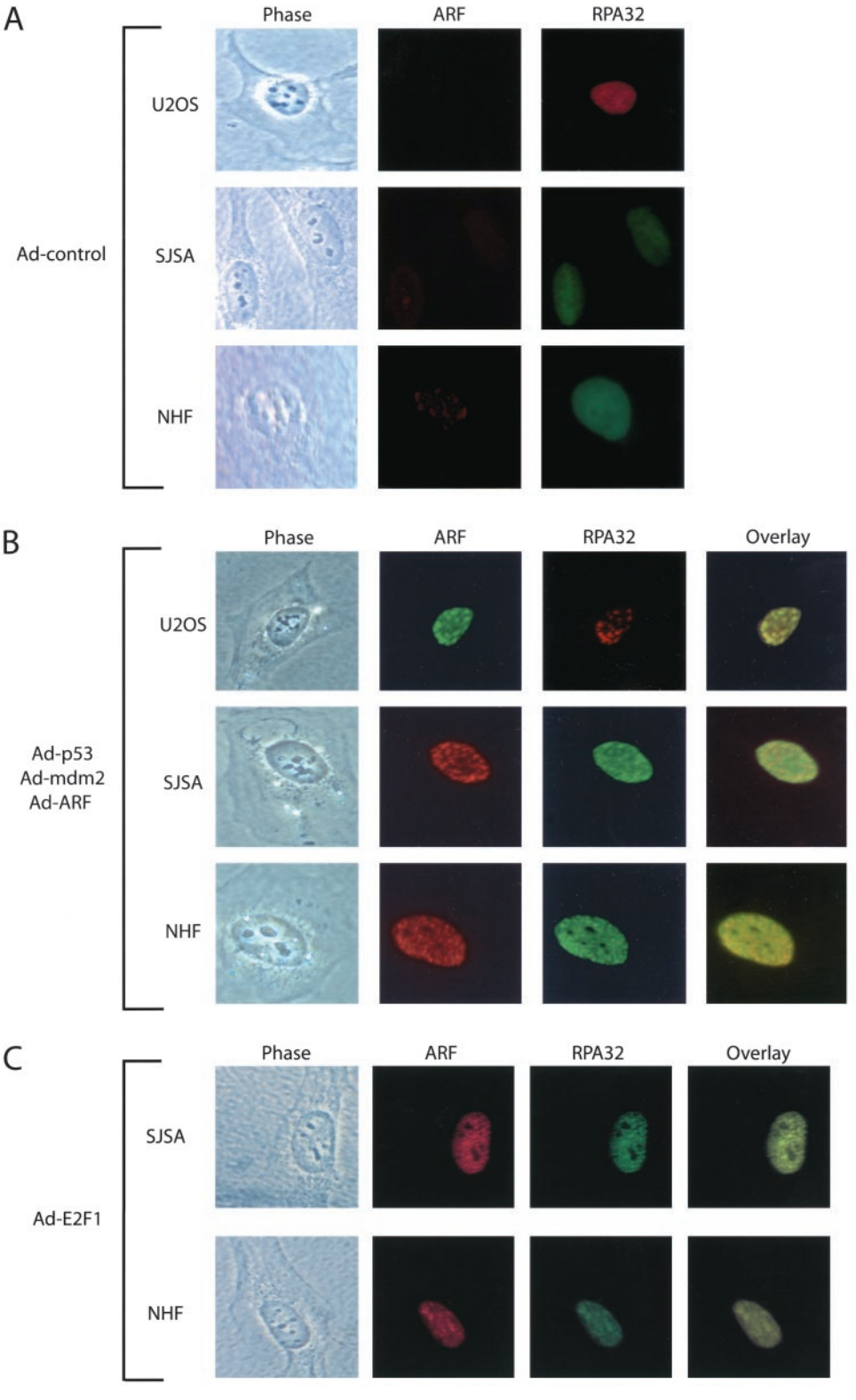


Fig. 4. Colocalization of RPA with ectopically or endogenously expressed ARF. *A*, expression of ARF and RPA32 in cells infected with control adenovirus. U2OS, SJSA, and NHF cells were infected with control adenovirus and 36 h later fixed and indirectly immunostained with affinity purified rabbit polyclonal antibody to ARF or mouse monoclonal antibody to M_r 32,000 subunit of RPA (*RPA32*). *B*, localization of ARF and RPA in cells ectopically expressing p53, mdm2, and ARF. U2OS, SJSA, and NHF cells were infected with adenovirus directing expression of p53, mdm2, and ARF and 36 h later fixed and immunostained for ARF and RPA32. Colocalization indicated by *yellow* in the overlay of ARF and RPA32 staining. *C*, localization of endogenous ARF and RPA. Adenovirus E2F1-infected U2OS, SJSA, and NHF cells were fixed 36 h after infection and immunostained for ARF and RPA32. Colocalization of ARF and RPA32 is indicated by *yellow* in the overlay of ARF and RPA32 staining.

or by the expression of an adenoviral protein(s). Corroborating a direct role in DNA replication, ARF protein was found to colocalize with M_r 32,000 subunit of human replication protein A (RPA32). RPA is a heterotrimeric single-stranded DNA-binding protein that plays essential roles in many aspects of DNA synthesis, including DNA replication, nucleotide excision repair, and homologous recombination. A number of cellular and viral proteins have been found to affect DNA replication through their interaction or colocalization with RPA (reviewed in Ref. 19). One such protein is p53, which has been identified to interact with RPA *in vitro* as well as colocalize with RPA in similar nuclear bodies *in vivo*. The mechanistic details concerning how ARF-RPA colocalization affects DNA synthesis remains to be determined, but the presence of ARF in proximity to RPA suggests a direct role of ARF in DNA synthesis. It should also be pointed out that the experiment system we have used involves ectopic expression of high levels of ARF protein in cultured cells *in vitro*. A p53-independ-

ent, ARF-mediated S-phase delay at physiological levels of ARF expression has yet to be demonstrated and is thus far only inferred from genetic analyses (9, 10, 15).

Hence, the biological consequences of ARF expression vary dependent on cellular p53 status: G_1 cell cycle arrest or apoptosis in the presence of p53 function, and S-phase retardation when p53 function is inactivated. It should be noted that the S-phase delay seen after ARF expression is not inhibited by p53 and was observed in both p53-positive and p53-negative cells. The exact biochemical mechanism and downstream effectors of the p53-independent S-phase delay of ARF is unclear at present but could conceivably involve MDM2 (and its closely related homologue MDMX) and/or E2F1. Evidence that MDM2 expression in both $p53^{+/+}$ and $p53^{-/-}$ backgrounds causes development of multinucleated polyploid cells and an increase of tumor formation suggests that MDM2 can function independently of p53. Furthermore, ectopic expression of MDM2 rescued transforming growth factor β-induced growth arrest in a p53-independent manner. Alternatively, recent evidence that ARF and E2F1 interact suggest that ARF could exert its p53-independent S-phase inhibitory role through E2F1 protein (20). Elucidating the mechanism underlying this p53-independent function ARF should aid our understanding on the functional connection between p16 and ARF.

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