Physical and Functional Interaction of the p14\textsuperscript{ARF} Tumor Suppressor with Ribosomes* 

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Alterations in the p14\textsuperscript{ARF} tumor suppressor are frequent in many human cancers and are associated with susceptibility to melanoma, pancreatic cancer, and nervous system tumors. In addition to its p53-regulatory functions, p14\textsuperscript{ARF} has been shown to influence ribosome biogenesis and to regulate the endoribonuclease B23, but there remains considerable controversy about its nucleolar role. We sought to clarify the activities of p14\textsuperscript{ARF} by studying its interaction with ribosomes. We show that p14\textsuperscript{ARF} and B23 interact within the nucleolar 60 S preribosomal particle and that this interaction does not require rRNA. In contrast to previous reports, we found that expression of p14\textsuperscript{ARF} does not significantly alter ribosome biogenesis but inhibits polysome formation and protein translation \textit{in vivo}. These results suggest a ribosome-dependent p14\textsuperscript{ARF} pathway that regulates cell growth and thus complements p53-dependent p14\textsuperscript{ARF} functions.

The \textit{INK4a/ARF} locus on chromosome 9 is frequently altered in human cancer, and inherited \textit{INK4a/ARF} mutations are associated with melanoma susceptibility in 20 – 40% of multiple case melanoma families (1). This complex sequence encodes the melanoma tumor suppressor proteins, p16\textsuperscript{INK4a} and p14\textsuperscript{ARF} from alternative spliced transcripts in different reading frames (2, 3). Both proteins are centrally involved in the regulation of cell cycle and apoptotic programs in response to oncogenic stimuli and are therefore frequently targeted in tumor development and progression (4). \textit{INK4a/ARF}-deficient mice are tumor-prone (5–7), and those with melanocyte-specific expression of activated H-ras develop cutaneous melanomas with high penetrance (8). High density mapping on chromosome 9p in human melanomas has identified p14\textsuperscript{ARF} as the most commonly deleted \textit{INK4a/ARF} gene (9), and individuals with altered ARF, but apparently wild type p16\textsuperscript{INK4a}, are melanoma-prone (10–13).

p14\textsuperscript{ARF} interacts with the p53 negative regulator hdm2, and inhibits its p53-specific E3 ubiquitin ligase activity (14–17). It has been proposed that ARF physically sequesters hdm2 in nucleoli, thus relieving nucleoplasmic p53 from hdm2-mediated degradation (18). Recent data, however, suggest that nucleolar relocation of hdm2 is not required for p53 activation (19) and that the redistribution of ARF into the nucleoplasm enhances its interaction with hdm2 and its p53-dependent growth-suppressive activity (20, 21). Accordingly, increasing nucleolar localization of ARF reduces ARF p53-dependent functions and diminishes ARF-hdm2 complex formation (21). This current model of ARF function supports the concept that nucleolar disruption contributes to p53 signaling (22) because many stress signals perturb the nucleolus, causing the release of nucleolar proteins (including ARF, L11, L23, L5, and B23) that activate the p53 pathway (23–27).

Nucleolar ARF, rather than residing in inactive “storage” (20, 21, 28), may regulate ribosome biogenesis by retarding the processing of early 47 S/45 S and 32 S rRNA precursors (29). These effects do not depend on hdm2 or p53 but may involve the interaction of ARF with nucleophosmin/B23 in complexes of very high molecular mass (30, 31). B23 is an abundant nucleolar endoribonuclease that is required for the nucleolar targeting of ARF (21) and for the maturation of 28S rRNA (32). Consistent with its role in inhibiting rRNA processing, ARF promotes the ubiquitination and degradation of B23 (31).

Because melanoma-associated ARF mutants have altered nucleolar localization (11) and ARF can mobilize out of the nucleolus in response to stress signals (14, 33), we sought to refine the functional role of p14\textsuperscript{ARF}. We now show that endogenous p14\textsuperscript{ARF} and B23 fractionate with the nucleolar 60 S preribosomal particle. Moreover, p14\textsuperscript{ARF} and B23 interact within the 60 S complex in a rRNA-independent manner. p14\textsuperscript{ARF} does not significantly alter the amount of nuclear preribosomal particles but inhibits polysome formation and retards protein translation. We propose that the p14\textsuperscript{ARF} tumor suppressor functions to integrate cell growth and cell cycle progression with nucleolar ARF regulating ribosome function, whereas nucleoplasmic ARF responds to cellular stress by activating p53-dependent growth arrest.

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3 The abbreviations used are: E3, ubiquitin-protein isopeptide ligase; IPTG, isopropyl \(\beta\)-thiogalactopyranoside; eIF, eukaryotic initiation factor.
**Experimental Procedures**

**Cell Culture**—Human U2OS osteosarcoma cells (ARF-null, p53 wild type), Saos 2 osteosarcoma cells (ARF wild type, p53-null) and WMM1175 melanoma cells (ARF-null, p53-null) were grown in Dulbecco’s modified Eagle’s medium (Trace Scientific, Sydney, Australia) supplemented with 10% fetal bovine serum and glutamine. All of the cells were cultured in a 37 °C incubator with 5% CO₂.

The U2OS_ARF, WMM1175_ARF, or WMM1175_p16<sup>INK4a</sup> cell clones carrying the stably integrated p14<sup>ARF</sup> gene or p16<sup>INK4a</sup> gene under IPTG-inducible expression control have been described previously (35, 36). The NARF2-E6 cells were initially provided by Dr. Gordon Peters (Cancer Research UK London Research) and have been described elsewhere (28).

Briefly, these cells express an IPTG-inducible form of human p14<sup>ARF</sup> and constitutively accumulate human papillomavirus E6 protein, which promotes p53 degradation. Stable cell clones were seeded 24 h prior to induction in the absence of antibiotics and were induced with 1–5 mM IPTG.

**Western Blotting**—Total cellular proteins were extracted for 1 h at 4 °C using radioimmune precipitation assay lysis buffer containing protease inhibitors (Roche Applied Science). Proteins (30–50 μg) were resolved on 12% SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore). Western blots were probed with antibodies against p53 (DO-1; Santa Cruz), p21<sup>Waf1</sup> (C-19; Santa Cruz), p14<sup>ARF</sup> (DCS-240; Sigma), E2F-1 (C-20; Santa Cruz), B23 (C19; Santa Cruz), L28 (A16; Santa Cruz), topoisomerase II (Oncogene Research), tubulin (Molecular Probes), actin (AC-74; Sigma), and eIF2α (Cell Signaling). Antibodies against S6, S7, and L37 were prepared according to Ref. 37.

**Indirect Immunofluorescence**—Cultured cells (1 × 10⁵) seeded on coverslips in six-well plates were washed in phosphate-buffered saline and fixed in 3.7% formaldehyde. The cells were immunostained for 1 h at 4 °C with the primary antibodies followed by a 50-min exposure to Texas Red- or FITC-conjugated secondary IgG (Sigma).

**Cell Cycle Analysis**—The 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 RNase A (50 ng/μl). DNA content from at least 2000 cells was analyzed using ModFIT software.

**Sucrose Density Gradient Fractionation**—Cytosolic ribosomes were isolated as previously described (38), except that the lysis buffer contained only 0.2% Nonidet P-40. The lysates were centrifuged at 10,000 rpm for 10 min, and postmitochondrial supernatant was usually layered on 10–45% or 10–25% (w/w) sucrose density gradients in 10 mM Tris-HCl, pH 7.2, 60 mM KCl, 1 mM MgCl₂, 1 mM dithiothreitol, 0.1 mg of heparin/ml. To disrupt the 80 S cytoplasmic ribosome, MgCl₂ was omitted during lysis, and the lysates were separated in 5–25% (w/w) sucrose gradients in 10 mM Tris-HCl, pH 7.2, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mg of heparin/ml. The gradients were centrifuged at 36,000 rpm for 2 h 40 min at 5 °C in a Beckman SW41Ti rotor and fractionated through a Bio-Rad EM-1 UV monitor for continuous measurement of the absorbance at 254 nm.

**Isolation of nuclear extracts** was performed as described (38). Nuclear proteins (1–1.5 mg) were overlaid on 15–30% (w/w) sucrose gradients in 25 mM Tris, pH 7.5, 100 mM KCl, 1 mM dithiothreitol, 2 mM EDTA and centrifuged at 38,000 rpm for 3 h 20 min at 5 °C in a Beckman SW41Ti rotor. Where indicated, the nuclear extract was treated with RNase A (100 μg/ml) for 10 min at room temperature. The gradients were analyzed as above. The proteins were precipitated from the fractions with trichloroacetic acid prior to Western blotting, and RNA was extracted from fractions using TRI-reagent LS (Sigma).

**Immunoprecipitations**—For immunoprecipitation analysis, U2OS_ARF cells were left untreated or exposed to 1 mM IPTG for up to 96 h. Nuclear protein extracts (1.5 mg) were fractionated on sucrose gradients as detailed previously. Immunoprecipitations were performed overnight using 1 μg of antibody chemically adsorbed to M450-tosylactivated DYNALbeads (DYNAL) as described by the manufacturer. Immunoprecipitates were washed four times with NET2 (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Nonidet P-40 containing protease inhibitors (Roche Applied Science)) lysis buffer, resolved using SDS-PAGE, and detected by immunoblot analysis. Extraction of RNA post-immunoprecipitation was performed using TRI-reagent LS (Sigma).

**Transcription Analysis**—To measure RNA polymerase I transcription after p14<sup>ARF</sup> expression, U2OS_ARF cells were induced with 1 mM IPTG for 72 h. The cells were permeabilized on ice in 20 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 25% glycerol, 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 25 units of RNaseOUT (Invitrogen), and 0.05% Triton X-100. After permeabilization, the cells were resuspended in transcription buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 150 mM NaCl, 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 25 units of RNaseOUT (Invitrogen), 0.5 mM ATP/CTP/GTP (Promega), and 0.5 mM bromouridine (Sigma)), and run-on transcription was performed at room temperature for 30 min. The cells were fixed and immunostained as detailed above.

**Analysis of RNA Processing**—Mammalian cells (2 × 10⁴) were starved for 30 min in methionine-free medium, labeled for 30 min with 50 μCi/ml [methyl-³H]methionine (85 Ci/mmol; Amersham Biosciences), and chased in medium containing excess unlabeled methionine. RNA was extracted with TRI-reagent (Sigma) and analyzed essentially as described (29).

**Biochemical Fractionation**—Cultured cells were permeabilized in buffer A (10 mM HEPES, pH 7.9, 15 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, and protease inhibitors (Roche Applied Science)) for 10 min and then supplemented with 0.2% Nonidet P-40. The cytosol fraction was collected, and the cells were washed in buffer A prior to solubilization using equal volumes of buffer B (50 mM HEPES, pH 7.9, 50 mM KCl, 0.1 mM EDTA, and protease inhibitors) and buffer C (50 mM HEPES, pH 7.9, 0.8 M KCl, 0.1 mM EDTA, and protease inhibitors). The soluble nuclear fractions were collected after centrifugation. Soluble nuclear and cytosolic fractions derived from equal cell numbers were analyzed by immunoblotting.

**Protein Synthesis Measurements**—The cells (1 × 10⁴) were plated in methionine-free, Dulbecco’s modified Eagle’s medium in 96-well FLASH plates (PerkinElmer Life Sciences). Approximately 24 h post-seeding, the medium was replaced...
with methionine-free Dulbecco’s modified Eagle’s medium containing [3H]methionine at 10 μCi/ml, and protein synthesis was measured 72 h later using a TopCount luminometer (Packard).

RESULTS
Impact of Induced p14ARF Expression—To evaluate the influence of p14ARF accumulation on cell proliferation, the U20S osteosarcoma cell line, which is wild type for both p53 and pRb (39), was engineered to express wild type p14ARF. In this U20S_ARF cell line, p14ARF expression was induced with 1 mM IPTG. Three days post-induction these cells behaved as expected, with strong nuclear expression of p14ARF (Fig. 1, A and B), increased p53 levels, increased expression of the p53-transcriptional target p21Waf1, decreased levels of the transcription factor E2F-1 (40) (Fig. 1B), and potent G1 phase cell cycle arrest (Fig. 1C). In addition, although p14ARF has been reported to induce the degradation of B23 (31) and inhibit rRNA processing (29) (which would limit the production of mature rRNAs), we found that the presence of p14ARF had no detectable effect on the accumulation of B23 (Fig. 1B), on the steady-state levels or processing of mature 28S and 18S rRNA species (Fig. 1, D and E), on nuclear transcription activity when RNA polymerase I transcription was visualized by pulse labeling with bromouridine and immunostaining with anti-bromodeoxyuridine antibody (Fig. 1F), or on cell size (Fig. 1G).

p14ARF Associates with the 60 S Preribosomal Particles—The association of tandem affinity purification-tagged murine p19ARF with ribosomal proteins and the fractionation of p19ARF in large nuclear multiprotein complexes (2–5 MDa) (30) suggested that the ARF protein associates with preribosomal particles within the nucleolus. To examine this possibility, nuclear extracts derived from Saos 2 osteosarcoma cells, which express endogenous p14ARF, were subjected to sucrose gradient centrifugation to separate preribosomal particles. Under these conditions three detectable peaks were resolved, and assignment of 40S and 60S preribosomal subunits was confirmed by resolving the extracted RNA species and Western immunoblotting for ribosomal proteins, including antibodies of the small ribosomal subunit, S7 and the large ribosomal subunit, L28, and L37. Western blot detection of endogenous p14ARF in Saos 2 cells confirmed that the majority of p14ARF cosedimented with the 60 S preribosomal particles (Fig. 2A). Likewise, sucrose density gradient ultracentrifugation of IPTG-treated U20S_ARF nuclear cell extracts followed by Western immunodetection confirmed that a large proportion of the induced p14ARF cosedimented with the 60 S preribosomal particles (Fig. 2, B and C).

To verify that p14ARF is a component of preribosomes, nuclear Saos 2 extracts were either left untreated or treated with RNase A before being subjected to fractionation on a sucrose gradient. As expected, treatment with RNase A disrupted ribosomal particles and resulted in the loss of preribosome peaks, and ribosomal protein S7, B23, and p14ARF remained at the top of the sucrose gradient (Fig. 2D).

p14ARF Interacts with B23 in the 60 S Preribosome Particles—To investigate whether p14ARF is bound to known binding partners within preribosomes, we examined the sedimentation behavior of hdm2, p53, and B23. As shown in Fig. 2, a significant pool of B23 cosedimented with p14ARF within the 60 S preribo-
some in both Saos 2 and induced U20S_ARF cells. It is worth noting that B23 also cosedimented with the 60 S preribosomal particles in the absence of p14ARF (Fig. 2B). In contrast, hdm2 and its ubiquitination target, p53 were restricted to smaller molecular weight complexes near the top of the sucrose gradients, and this sedimentation behavior was not altered by p14ARF (Fig. 2, B and C).

Considering that B23 and p14ARF cosediment with the pre-60 S particle, we investigated whether these proteins are complexed within the preribosome. Nuclear extracts derived from IPTG-induced and uninduced U20S_ARF cells were separated using sucrose gradient fractionation. The fractions corresponding to the top of the column (containing small molecular weight complexes), the 40 S preribosome, and the 60 S preribosome were collected. The sucrose fractions (top, 40 and 60 S) were precipitated with antibodies to p14ARF, and recovered immune complexes were denatured, separated on polyacrylamide gels and blotted with antibodies to B23, hdm2, and p53. As expected, the preribosomal fraction of p14ARF was found complexed to B23 (Fig. 3A, lower panel) and not bound to hdm2 or p53 (data not shown).

We next examined whether the ARF-B23 interaction within the preribosome particles occurs independently of rRNA, because both proteins bind the 5.8 S rRNA found in the 60 S ribosome (29, 30). Expression of p14ARF was induced in U20S_ARF cells with 1 mM IPTG for 96 h, and nuclear extracts were prepared and separated using sucrose density centrifugation. The fractions corresponding to the 60 S preribosome were collected and treated with RNase A. Extraction of the 60 S fraction post-immunoprecipitation with the RNA isolation reagent TRI-Reagent LS confirmed that no detectable RNA was present in the RNase A-treated 60 S preribosomal fractions (Fig. 3B, upper panel). In the absence of RNA, endogenous B23 was still found in association with p14ARF in the 60 S preribosomal fraction (Fig. 3B, lower panel).

**FIGURE 2. p14ARF fractionates with 60 S preribosomes.** A, nuclear extracts of Saos 2 cells were fractionated on 10–30% sucrose density gradients with continuous monitoring of absorbance at 254 nm (top panel). RNA extracted from each fraction was resolved by electrophoresis on a formaldehyde-containing agarose gel to demonstrate the sedimentation of the 28 and 18 S rRNA species (middle panel). Individual sucrose fractions were precipitated and analyzed by immunoblotting (bottom panel). B, the inducible U20S_ARF cell line was grown in the absence (+IPTG) of IPTG for 48 h. Preribosomes were fractionated and analyzed as detailed above. C, the inducible U20S_ARF cell line was grown in the presence (+IPTG) of IPTG for 48 h. Preribosomes were fractionated and analyzed as detailed above. **D**, nuclear extracts of Saos 2 were either treated with RNase A or left untreated and analyzed as detailed above.

p14ARF Expression Does Not Influence Preribosome Production—To examine the influence of p14ARF accumulation on nucleolar ribosome assembly, U20S_ARF cells were grown in the absence or presence of 1 mM IPTG for up to 96 h. Equal amounts of nuclear extracts were separated on 15–30% sucrose density gradients. Analysis of 40 and 60 S preribosomal particles revealed no significant changes in the ratio of 60 to 40 S preribosomes subunits at 48, 72, and 96 h post-induction (Fig. 2, A and B). In particular, the peak height ratio of 60 to 40 S subunits in untreated and treated U20S_ARF cells, 96 h post-induction, was estimated to be 1.6 ± 0.1 and 1.6 ± 0.1, respectively (the means of three independent experiments).
p14ARF Expression Inhibits Translation Initiation—The association of p14ARF with preribosomal particles suggested that this tumor suppressor protein might influence cytoplasmic ribosome assembly and/or function. When equal numbers of U205_ARF cells were extracted and the cytoplasmic lysates were separated on 10–30% sucrose density gradients (upper panel, induced U205_ARF fractionation shown), fractions corresponding to small molecular weight complexes (top of gradient), 40 S preribosomes and 60 S preribosomes were immunoprecipitated with a monoclonal ARF or isotype-matched antibody (IgG). The immunoprecipitates were analyzed for the presence of B23 by Western blotting (lower panel). To confirm that rRNA was effectively degraded by RNase treatment, the post-immunoprecipitation lysates were extracted using TRI-reagent LS. Extracted RNA and total cell RNA were resolved in a formaldehyde-containing agarose gel and stained with ethidium bromide (upper panel). Immunoprecipitates (IP) were analyzed for the presence of B23 by Western blotting (lower panel).

p14ARF and Ribosomes

The expression of p14ARF in HeLa cells results in a shift in the distribution of ribosomes to smaller polysomes and larger pools of 80 S monosomes was consistently observed (Fig. 4, A and B). To determine whether the ARF-induced increase in monosome 80S particles was due to reduced polysome levels, we separated all 80 S subunits, including polysomes, into their 40 and 60 S components by preparing...
cytoplasmic lysates in the absence of MgCl2. The disruption of 80 S ribosomes resulted in similar levels of 40 and 60 S subunits in the induced and uninduced U20S_ARF cells (Fig. 4C). Thus, p14ARF did not alter the accumulation of 40 and 60 S ribosome subunits within the cytoplasm but altered the complexes that they produced. In particular, p14ARF expression caused a significant increase in the proportion of monomeric 80 S subunits (Fig. 4A). This was accompanied by a significant reduction in protein translation; U20S_ARF cells induced to express p14ARF for ~72 h incorporated 26% less [3H]methionine than the uninduced control cells (Fig. 5A). Further, these ARF-expressing cells accumulated less of the translationally regulated c-Myc and cyclin D1 proteins (41) (Fig. 5B).

The rate of synthesis of any given protein is determined primarily by the level of translation initiation, and the increase in monosome 80 S subunits in p14ARF-expressing cells was indicative of a block in the initiation phase of translation. The appearance of a large population of nontranslating 80 S couples, consisting of 40 and 60 S subunits joined in the absence of mRNA, is typical of an initiation defect. High levels of salt can readily dissociate these inactive 80 S particles (42), and we examined the effect of including 0.8 M NaCl in the sucrose gradient. As expected, dissociation of inactive couples led to an increase in the levels of cytoplasmic 40 and 60 S ribosome subunits in the p14ARF-expressing U20S_ARF cells. Thus, inactive 80S monosomes are dramatically increased in the presence of p14ARF (Fig. 4A) and constitute a major proportion of the ribosome population in the ARF-expressing cells (Fig. 4D).

To gain some insight into the mechanism of this translation defect, the accumulation of 3-subunit of the eukaryotic initiation factor 2 (eIF2) was examined. eIF2 facilitates binding of the initiator tRNA to the 40 S subunit during translation initiation. Translation can be inhibited by the rapid phosphorylation of the α-subunit (reviewed in Ref. 43), by limiting its expression and accumulation (44), or by specific caspase-mediated eIF2α cleavage during apoptosis (45). Further, phosphorylation of eIF2α has been associated with an increase in the formation of monosome 80 S subunits (46). In this study we found that ARF-expressing U20S cells showed a marked reduction in the accumulation of the eIF2α subunit (Fig. 5B).

The observed changes in cytoplasmic ribosomes, in particular the increase in the monomers, was not an artifact of IPTG exposure, because addition of IPTG to the parental U20S_lac17 cell line, which expresses the lac repressor, did not alter the ribosomal subunit profile (data not shown). To investigate whether p14ARF-induced effects on polysome production were a result of cell cycle arrest and p53-dependent, we utilized the WMM1175_ARF melanoma and NARF2-E6 osteosarcoma cell lines. Both cell lines express an IPTG-inducible form of p14ARF but are functionally null for p53 function (Fig. 6A). The induction of p14ARF in the WMM1175_ARF and NARF2-E6 cells did not induce growth arrest (Fig. 6A) but did promote an increase in 80 S monomeric ribosomes and a reduction in polysomes (Fig. 6, B and C). This ARF-associated decrease in the polysome formation in the WMM1175_ARF cell line is substantial, considering that only 40% of the WMM1175_ARF cells express detectable levels of ARF upon IPTG induction.

To further examine the relationship between cell cycle arrest and inhibition of polysome production, we promoted G1 cell cycle arrest in the WMM1175_p16INK4a cell line by inducing the expression of the cyclin-dependent kinase inhibitor p16INK4a with 5 mM IPTG for 72 h (36). Although, p16INK4a expression induced potent G1 cell cycle arrest (Fig. 7A), it led to an increase in cell size (Fig. 7B) that was accompanied by an increase in the level of cytoplasmic ribosomes and polysome production (Fig. 7C).

Considering that p14ARF reduced cytoplasmic ribosome function, we also examined whether p14ARF cosedimented with ribosomes within the nucleolus and cytoplasm. We found that the majority of p14ARF in Saos 2 cells and induced U20S_ARF cells occurred in the nuclear fraction, with no detectable cytoplasmic p14ARF component (Fig. 8).

**DISCUSSION**

The ARF tumor suppressor plays a central role in limiting cell cycle progression in response to hyperproliferative signals. Cells exposed to oncogenes, such as E2F-1, Ras, or Myc (47–49), accumulate nucleolar ARF, and yet its core function of stabilizing p53 requires its association with hdm2 in the nucleoplasm (21). In this study we sought to explore the interaction of ARF with the nucleolus and have shown that p14ARF is a component of the 60 S preribosome, where it interacts with B23 independently of rRNA. The association of B23 with the 60 S particle is not unexpected, because this protein binds the 28 S rRNA, a major component of the pre-60 S ribosome (50). We also found that ARF did not influence the production of the preribosomes or the steady-state levels of cytoplasmic ribosome subunits but inhibited ribosome function. The impact of p14ARF on ribosome activity may involve the translation initiation factor eIF2α. ARF-expressing U20S cells accumulated low levels of this subunit. Reduced expression of eIF2α in quiescent cells correlates with low levels of protein synthesis (44), whereas induction of eIF2α expression via oncogenes such as c-Myc contributes to the pronounced stimulation of protein production (51). eIF2α is also involved in neoplastic transformation when its function is up-regulated (reviewed in Ref. 52). We are currently investigating whether repression of eIF2α in response to p14ARF expression involves the c-Myc transcrip-
tion factor; p14\textsuperscript{ARF} has been shown to interact with and inhibit the transcriptional activity of the c-Myc oncogene (53, 54).

Our findings extend and help clarify recent reports that describe the ARF-B23 association and thus implicate ARF in the regulation of protein synthesis. Data demonstrating that the murine homologue of p14\textsuperscript{ARF}, p19\textsuperscript{ARF}, promoted the degradation of B23 (31) and inhibited rRNA processing is not supported by our findings with p14\textsuperscript{ARF} or indeed with other studies (21, 55). We found that B23 and ARF associate within the nucleolar pre-60 S ribosome, and it has been reported that nucleolar B23 is resistant to ARF-induced degradation (31). It has also been suggested that the interaction of p19\textsuperscript{ARF} and B23 retains both proteins in the nucleolus, effectively impeding the nucleocytoplasmic shuttling of B23 to induce growth arrest (55) but also blocking p19\textsuperscript{ARF}-mediated p53 activation (21). Importantly, hdm2 and B23 compete for ARF binding, and thus silencing of B23 expression enhanced the ARF-mdm2 association within the nucleoplasm and induced p53-activated growth arrest (19). The implications of these findings are that p19\textsuperscript{ARF} directly accesses ribosome function through its nucleolar association with B23 and that it regulates the p53 cell cycle pathway via its nucleoplasmic interaction with hdm2.

This model is supported by our studies with human p14\textsuperscript{ARF}. We have shown that the predominantly nucleolar p14\textsuperscript{ARF} was associated with B23 within the 60 S preribosome subunit, and this interaction did not involve hdm2 or p53, both of which were excluded from nucleoli and the preribosomes. Further, the ARF-mediated inhibition of protein translation in vivo supports the notion that ARF modulates ribosome function. We propose that ARF is a highly mobile protein, and as it accumulates in response to oncogenic activity, the increased nucleoplasmic fraction complexes with mdm2, in preference to B23 (55), and activates p53-mediated growth arrest. Likewise, a fraction of nucleolar ARF, possibly a fraction tethered to B23, may impact ribosomes within the nucleolus. This work extends the already diverse functional repertoire of ARF to that of effects on protein translation; thus, ARF may serve to coordinate the regulation of cellular growth with that of proliferation. When these programs become uncoupled, as reported in T-ALL cells reconstituted to express p16\textsuperscript{INK4a} (56), cells undergo proliferative arrest but continue to grow and differentiate.

There is strong precedent for the involvement of nucleolar proteins in the regulation of cell proliferation and cell growth. Nucleolar release of the ribosomal proteins L23, L11, and L5 promotes their interaction with hdm2, resulting in hdm2 inactivation and stabilization of p53. Similarly, the nucleolar protein Bop1 inhibits rRNA formation and also activates p53-dependent cell cycle arrest (38, 57). Analogous to ARF, nucleolar B23 contributes to multiple steps in ribosome biogenesis (58), and its nucleoplasmic redistribution activates p53 by inhibiting hdm2 (27). In all cases, these nucleolar regulators can rapidly halt cell cycle progression by leaking out of the nucleolus and signaling through the p53 pathway and also regulate the activity of ribosomes. We found that p14\textsuperscript{ARF} promoted p53 activity and cell cycle arrest within 16 h of induction (the percentage of S phase inhibition in U205_ARF cells induced for 16 h was 47%), whereas the ability of ARF to limit translation was not observed until 3 days after it was induced. This is consistent with the slower growth inhibitory impact of p14\textsuperscript{ARF} expression in p53-null cells (59).

Although p14\textsuperscript{ARF} can alter ribosome function in the absence of p53, it is likely that inhibition of translation is enhanced in the presence of both p14\textsuperscript{ARF} and p53, because both tumor suppressors independently inhibit ribosome function (60). The impact of p53 on translation is minor, however, and requires the inter-
of p14ARF in response to hyperproliferative oncogenic signals may account for its ribosome regulatory function. Expression of p14ARF is induced by the same oncogenes (Myc and Ras) that rapidly increase RNA transcription rates (62, 63). Thus, in cells expressing activated oncogenes, the increased expression of p14ARF would dampen the aberrant up-regulation of ribosome subunits. In contrast, in the absence of p14ARF, oncogenic stimulation would promote increased ribosome biogenesis, enhanced translation rates, and ultimately transformation.

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