Physical and Functional Interactions of the Arf Tumor Suppressor Protein with Nucleophosmin/B23

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The Arf tumor suppressor inhibits cell cycle progression through both p53-dependent and p53-independent mechanisms, including interference with rRNA processing. Using tandem-affinity-tagged p19Arf, we purified Arf-associated proteins from mouse NIH 3T3 fibroblasts undergoing cell cycle arrest. Tagged p19Arf associated with nucleolar and ribosomal proteins, including nucleophosmin/B23 (NPM), a protein thought to foster the maturation of preribosomal particles. NPM is an abundant protein, only a minor fraction of which binds to p19Arf; however, a significant proportion of p19Arf associates with NPM. The interaction between p19Arf and NPM requires amino acid sequences at the Arf amino terminus, which are also required for Mdm2 binding, as well as the central acidic domain of NPM and an adjacent segment that regulates NPM oligomerization. The interaction between p19Arf and NPM occurs in primary mouse embryonic fibroblasts, including those lacking both Mdm2 and p53. In an NIH 3T3 derivative cell line (MT-Arf) engineered to conditionally express an Arf transgene, induced p19Arf associates with NPM and colocalizes with it in high-molecular-weight complexes (2 to 5 MDa). An NPM mutant lacking its carboxyl-terminal nucleic acid-binding domain oligomerizes with endogenous NPM, inhibits p19Arf from entering into 2- to 5-MDa particles, and overrides the ability of p19Arf to retard rRNA processing.

The Arf tumor suppressor protein binds to the p53 negative regulator Mdm2 to induce a p53 transcriptional response that can lead to either cell cycle arrest or apoptosis (37). Arf can also arrest the proliferation of Mdm2- and p53-null fibroblasts, albeit relatively inefficiently, indicating that p19Arf can interact with other targets (48). Arf is an unusually basic protein (pI > 12) that is highly rich in arginine residues. Apart from Mdm2, a series of proteins have been reported to physically interact with Arf, including the E2F-1 and HIF-1α transcription factors (7, 9, 22), the phosphatase-binding protein spinophilin (46), the peroxisomal protein Pex19P (40), topoisomerase I (18), and cyclin G1 (52), although the biological significance of these interactions, if any, remains unclear. Enforced Arf expression antagonizes the transcriptional activity of NF-κB in a p53- and Mdm2-independent manner (35). In the latter case, direct interactions between p19Arf and NF-κB were not documented. Indeed, Arf can induce many antiproliferative genes and repress others, even in cells that lack functional p53 (19).

The vast majority of Arf protein expressed in cells localizes within nucleoli (30, 33, 50), an organelle responsible for ribosome biogenesis. Ribosomal proteins synthesized in the cytoplasm are imported into nucleoli, where they assemble with rRNA precursors to form preribosomal particles that undergo a series of maturation steps before they are exported to the nucleus and then the cytoplasm. Precursor rRNA transcribed by polymerase I within the nucleolus (47S in mammalian cells) assembles into 90S preribosomal particles, which allow several rapid rRNA cleavage steps separate the precursors into large and small ribonucleoprotein subunits, whose maturation continues within the nucleolus (6, 8, 24, 47). rRNA transcription and processing, ribosome assembly, maturation, and transport require hundreds of accessory proteins and small nucleolar RNAs not found within mature ribosomes in the cytoplasm (1, 8, 14, 24, 39, 47), and the functions of most of these regulatory molecules remain obscure.

Expression of p19Arf in nucleoli suggested that it might play a role in ribosome biogenesis, and consistent with this idea, enforced Arf expression was revealed to retard two steps of rRNA processing (41). Specifically, p19Arf slows the processing of early 47S/45S rRNA precursors and also inhibits cleavage of a 32S rRNA intermediate that contains the segmented sequence modules encoding both 28S and 5.8S rRNAs. These effects do not depend on Mdm2 or p53 or on Arf's ability to induce cell cycle arrest per se.

Most of the p19Arf protein expressed in mouse fibroblasts is contained in complexes of very high molecular mass (2 to 5 MDa), consistent with the idea that Arf might enter early preribosomal particles within the nucleolus. We show that p19Arf interacts with several nucleolar proteins implicated in ribosome biogenesis. Among these is an acidic protein, nucleophosmin/B23 (NPM), which oligomerizes with itself and with p19Arf to form complexes that can affect rRNA processing.

MATERIALS AND METHODS

Cells and culture conditions. NIH 3T3 fibroblasts and MT-Arf cells expressing a zinc-inducible Arf gene (19) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 units each of penicillin and streptomycin. Where indicated, MT-Arf cells were treated with 80 μM ZnSO4 for 24 h to induce p19Arf. Mouse embryo fibroblasts (MEFs) from wild-type C57BL/6 mice (17) and from a C57BL/6 × 129 strain lacking the Arf, p53, and Mdm2 genes (48) were cultured in complete medium supplemented

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with 0.1 mM nonessential amino acids, 55 μM 2-mercaptoethanol, and 10 μg of gentamicin per ml instead of penicillin and streptomycin.

**Retrovirus production and infection.** Human kidney 293T cells were transfected as described with helper retrovirus plasmid together with pRS vectors encoding proteins tagged with p19Arf or with murine stem cell virus (MSCV)-internal ribosome entry site (IRES)-green fluorescent protein (GFP) vectors encoding tandem affinity purification (TAP)-tagged Arf or Flag-tagged wild-type or mutant human NPM proteins (53) (see also below). Viruses were harvested 24 to 60 h posttransfection, pooled, and stored on ice. Exponentially growing cells (2 × 10⁶ cells per 10-cm-diameter culture dish) were infected twice at 32°C overnight containing 2 × 10⁶ cells per dish. Following fresh virus-containing supernatant in complete medium containing 8 μg of Polybrene (Sigma, St. Louis, Mo.) per ml. For large-scale purifications with TAP-tagged Arf, 10⁶ cells in 15-cm-diameter culture dishes were infected once with 7 ml of virus-containing culture supernatant. Where applicable, infection efficiencies were confirmed by flow cytometric analysis of GFP-stained cells.

**Tandem affinity purification of tagged Arf protein.** A TAP tag cDNA cassette consisting of two stavblylococcus protein A-immunoglobulin G (IgG) binding domains fused to a tobacco etch virus protease site and a calmodulin-binding domain allows sequential affinity binding of TAP-tagged proteins to an IgG column, their recovery by tobacco etch virus protease digestion, and their further purification via C4–dependent calmodulin binding (34). The TAP tag cassette (a kind gift from Dr. Guido, Vanderbilt University School of Medicine, Nashville, Tenn.) was amplified by PCR and inserted as an EcoRI/Xhol fragment into the cloning sites of the MSCV-IRES-GFP retroviral vector. Full-length, N-terminally hemagglutinin (HA)-tagged Arf cDNA (33) was subcloned into the EcoRI site of the derivative plasmid, fusing the TAP tag sequence in frame with the Arf 3’ end. Deletion mutants lacking sequences encoding N-terminal amino acids 2 to 14 (Δ2-14) or 2 to 62 (Δ2-62) (50), each including the HA tag at the N terminus, were subcloned in place of full-length Arf in the same retrowiral expression vector.

Cells infected with expression vectors encoding TAP-tagged p19Arf proteins were harvested by trypsinization, neutralized with complete medium, and washed twice in phosphate-buffered saline (PBS). Harvested cells were divided into aliquots containing 2 × 10⁶ cells, pelleted at 1,000 × g, snap frozen in liquid nitrogen, and stored at –80°C until used. Cell pellets were resuspended on ice, each in 1 ml of Tween 20 lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.2 U of aprotinin per ml, 10 mM β-glycerophosphate, 1 mM NaF, and 0.1 mM NaVO₄], sonicated in 2-ml batches twice for 7 s each (at 14% power with a Virsonic 475 sonicator) with a 30-s pause between bursts, and then left on ice for 30 min. Lysates were centrifuged at 14,000 × g at 4°C for 15 min, and the resulting supernatants were filtered through 0.45-μm cellulose-aceate filters to remove insoluble material.

In some experiments, lysates were incubated at 30°C for 10 min with or without 100 μg of Rnase A per ml, and insoluble material was again removed. Following protein precipitation with the BCA assay (Pierce, Rockford, Ill.), equal quantities of different protein preparations were diluted to 8 ml in Tween 20 lysis buffer. Tandem affinity purification was performed essentially as described by Puig et al. (31) except that Tween 20 lysis buffer was used to equilibrate and wash the IgG column. Following elution from the second calmodulin column, proteins were concentrated by trichloroacetic acid and deoxycholate precipitation (21) and stored as pellets at –80°C until analyzed.

**Identification of TAP-tagged Arf-containing complexes.** Arf-containing complexes were separated on 4 to 12% gradient Bis-Tris gels (Amersham) separated on 4 to 12% gradient Bis-Tris gels (Amersham) and transferred to Hybond N membranes (Millipore). Proteins were detected with antibodies to p19Arf (33), p53 (Ab-7, Oncogene Research Products, San Diego, Calif.), p21Cip1 (C-19, Santa Cruz Biotechnology, Santa Cruz, Calif.), NPM (C-19 Santa Cruz), Flag tag (M2, Sigma Chemicals), or antibodies to p19Arf (Sigma Chemicals). Immune complexes were recovered with protein G (Zymed) or protein A-Sepharose (Amersham Biosciences) and washed four times with NET2 buffer. Immunoprecipitated complexes and total cell lysates were electrophoretically separated on denaturing polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore). Proteins were detected with antibodies to p19Arf (33), p53 (Ab-7, Oncogene Research Products, San Diego, Calif.), p21Cip1 (C-19, Santa Cruz Biotechnology, Santa Cruz, Calif.), NPM (C-19 Santa Cruz), Flag tag (M2, Sigma Chemicals), or ribosomal protein I7 (Novus Biologicals, NB200-308).

For analysis of corepressing RNAAs, immune complexes were suspended and incubated for 30 min at 37°C in 7 ml of Tn 2HCl, pH 7.5, containing 0.7 mM EDTA, 50 mM NaCl, 0.7% sodium dodecyl sulfate, and 30 μg of proteinase K per ml (Sigma Chemicals). RNAs were recovered by phenol-chloroform extraction followed by ethanol precipitation in 0.3 M sodium acetate containing 20 μg of glycyogen (Roche, Basel, Switzerland), and end-labeled with 40 μCi of [32P]jej-tidine 3' 5'-bisphosphate ([32P]Pcp) (3,000 Ci/mmol; ICN, Costa Mesa, Calif.) with T4 RNA ligase (Amersham Biosciences) as described (45).

**Analysis of newly synthesized RNAAs.** Cells were metabolically labeled with 2.5 μCi of [3H]uridine per ml (30 to 60 Ci/mmol, Amersham Biosciences) in complete medium for 30 min, washed twice with PBS, and incubated in complete medium without the radioactive precursor for 2 h. Total RNAs were isolated with Trizol (Invitrogen, Carlsbad, Calif.), and quantitated by absorbance measurement, and their specific activities were determined by liquid scintillation. RNAs (2,000 cpm per lane) loaded into 15% agarose gels containing 4% formaldehyde were electrophoretically separated and transferred to Hybond N membranes (Amersham). Dried membranes were treated with En3Hance (Perkin Elmer Life Sciences, Boston, Mass.) and subjected to autoradiography at –70°C with intensifying screens.

## RESULTS

Arf associates with proteins involved in ribosome biogenesis. In an effort to identify proteins that associate with p19Arf in growth-arrested cells, we infected NIH 3T3 fibroblasts with a retrovirus expressing p19Arf fused to a tandem affinity purification (TAP) epitope tag (34). Expression of TAP-tagged p19Arf induced p53-responsive genes (Mdm2 and p21Cip1)
of the tandem affinity purification (TAP) tag, transferred to a membrane, and subjected to metabolic labeling. Two days later, cell lysates were immunoblotted with antibodies to Mdm2, Mdm1, and p21Cip1 (A). Like p19Arf, although to a lesser extent, TAP-tagged Arf induced expression of Mdm2 and p21Cip1. Due to the C-terminal tag, the electrophoretic migration of TAP-tagged Arf on the denaturing gel was retarded. (B) Cells were pulse labeled with [3H]uridine for 30 min and then chased for 2.5 h. Total RNA extracted from cells was separated on a gel, transferred to a membrane, and subjected to fluororadiography. RNA in the 5-FU lane was extracted from uninfected NIH 3T3 cells which were exposed to 5-fluorouridine for 20 min prior to metabolic labeling. Expression of Arf and TAP-tagged Arf and inclusion of 5-fluorouridine in the medium all inhibited rRNA processing.

(Fig. 1A), caused cell cycle arrest (reduction of S-phase fraction from 23% to 4%), and inhibited rRNA processing (Fig. 1B), indicating that the fusion protein remained biologically active. Two days after infection, arrested cells were lysed, and TAP-tagged Arf complexes were purified from 33 mg of protein recovered from \( \approx 2 \times 10^6 \) cells by use of a modified version of the tandem affinity purification protocol (31, 34). As a control for nonspecific binding, the same amount of protein recovered from uninfected NIH 3T3 cells was carried through the same purification procedure.

Recovered proteins concentrated by trichloroacetic acid-deoxycholate precipitation were electrophoretically resolved in denaturing 4 to 12% gradient polyacrylamide gels (Fig. 2A). Small portions of purified, gel-separated proteins were stained with silver (sensitivity, \( \approx 1 \) ng of protein per band) to pinpoint bands that were specifically recovered with TAP-tagged p19Arf. The bulk of the protein (80%) was resolved on gels separately and stained with Coomassie blue (sensitivity, \( \approx 100 \) ng of protein per band), and specific bands were excised, digested with trypsin, and sequenced by mass spectrometry. Peptides from TAP-tagged p19Arf (GPHYLLPPAR and KNPFAVSAANR) and 28 other proteins were identified. Of these, 13 were large ribosomal subunit protein, three were nonribosomal proteins involved in ribosome biogenesis, including nucleolin, nucleophosmin (NPM), and polymerase I and transcript release factor (PTRF), and three were importins (Fig. 2A and Table 1). The fact that many of these proteins localize to the nucleolus gave us some confidence that TAP-tagged p19Arf did not bind indiscriminately to proteins normally resident in the nucleoplasm or cytoplasm.

PTRF binds to the 3′ end of pre-rRNA and is required for the termination of transcription by RNA polymerase I, allowing release of both the pre-rRNA transcript and the polymerase I complex from the rDNA template (16). Nucleolin binds to specific RNA stem-loops at several positions in pre-rRNA, and its interaction with one such structure within the 5′ external transcribed spacer sequence is required for the first step of ribosomal processing in vitro (10–12). NPM has been proposed to function in ribosome biogenesis, but its exact role remains unclear (28, 36, 51). Such data suggest that the ability of p19Arf to inhibit rRNA processing (41) may reflect its ability to associate with early preribosomes in the nucleolus.

Mutant forms of Arf lacking residues 2 to 62 (Δ2-62) or 2 to 14 (Δ2-14) are impaired in causing cell cycle arrest (32, 48, 49), and Arf Δ2-14, although localizing to nucleoli, is unable to efficiently inhibit rRNA processing (41). Further purification were therefore conducted with TAP-tagged Arf Δ2-14 and Arf Δ2-62 in tandem with full-length TAP-tagged Arf in order to determine whether the mutant proteins would associate with nucleolin, PTRF, and NPM. A caveat is that Arf Δ2-14 is a hypomorphic mutant not completely devoid of activity (49). Purified proteins were again resolved on denaturing gels, but were blotted onto a polyvinylidene difluoride membrane and stained with Sypro Ruby to visualize proteins (Fig. 2B). Sypro Ruby is more sensitive than Coomassie blue, and unlike silver, it stains different proteins to the same degree. A band in the position of nucleolin was recovered with all three TAP-tagged Arf “baits,” but mass spectroscopy also identified another protein in this region (importin β1) that occluded our ability to detect quantitative differences in nucleolin itself. By contrast, several p19Arf-associate proteins, including NPM, PTRF, and ribosomal proteins, were seemingly absent or were recovered in significantly lower abundance after purification with mutant TAP-tagged Arf proteins.

To determine whether the association of TAP-tagged Arf with these interacting proteins depended upon the presence of RNA, NIH 3T3 cells were infected with the vector encoding TAP-tagged Arf, and detergent lysates were treated with RNase A before we performed tandem affinity purification. Following RNase treatment, some of the protein in the extracts, including about half of the TAP-tagged Arf, became insoluble and precipitated out of solution. Nonetheless, tandem affinity purification of the remaining protein again revealed recovery of the bands containing nucleolin, PTRF, and NPM at similar molar ratios to the TAP-tagged Arf protein (Fig. 2C, upper panel). By contrast, the intensities of bands corresponding to several ribosomal proteins (e.g., L7) were diminished, whereas others (e.g., L5) were not. By immunoblotting these samples with antibodies to ribosomal protein L7, we were able to confirm its markedly reduced recovery after RNase treatment (Fig. 2C, lower panel). Therefore, the interaction of TAP-tagged Arf with nucleolin, PTRF, NPM, and some ribosomal proteins does not appear to depend upon...
RNA. The experiments that followed focused on the NPM-Arf interaction.

**Nucleophosmin associates both with p19Arf and 5.8S rRNA.** NIH 3T3 fibroblasts which have sustained a deletion of the endogenous Ink4a-Arf locus were previously engineered to express an Arf transgene under control of the zinc-inducible mouse metallothionein promoter (19). Antibody precipitates of NPM from lysates of these zinc-induced and growth-arrested MT-Arf cells contained p19Arf (Fig. 3A, top two panels, lane 8), whereas antibodies to p19 Arf reciprocally coprecipitated NPM (lane 12). NPM is a relatively abundant nucleolar protein, so that under the conditions used, several sequential rounds of precipitation are required to recover it all. We estimated that only $\frac{30}{100}$ of NPM was recovered in the initial antibody precipitation shown (compare input lanes 1 to 4 with immune precipitates in lanes 5 to 8). Still, $>10\%$ of the total p19Arf in the cells coprecipitated with NPM (lane 4 versus 8). Note that p19Arf was not efficiently precipitated with the cognate antibody from mammalian cells either. Only $\approx 10\%$ of the total p19Arf pool was recovered by direct antibody precipitation in this experiment (Fig. 3A, lane 12 versus 4), indicating that the amount of NPM in complexes with Arf is also likely to be underestimated by this approach. Nonetheless, we could conclude that only a relatively small proportion of the total NPM pool is associated with p19Arf, whereas a significant proportion of p19Arf is bound to NPM.

Because p19Arf coprecipitates with 5.8S rRNA (41), we next tested whether NPM can also associate with this RNA species. RNAs eluted from immunoprecipitated complexes were end labeled with T4 RNA ligase and separated on denaturing gels. As expected, 5.8S rRNA was recovered from p19Arf immunoprecipitates obtained from zinc-induced cells (Fig. 3A, bottom panel, lane 12), but not from control antibody precipitates (lanes 13 and 14). Under the same conditions, NPM coprecipitated with 5.8S rRNA whether or not Arf was induced (Fig. 3A, bottom panel, lanes 5 to 8), although somewhat more 5.8S rRNA was recovered when p19Arf was present (lane 8). Hence, both proteins can interact with rRNA, potentially within the same complex. Whether p19Arf or NPM can also associate with unprocessed 5.8S sequences found within rRNA precursors remains unclear (41).

To ensure that the Arf-NPM interaction observed in MT-Arf cells did not depend upon p19Arf overexpression, we next determined whether endogenous p19Arf and NPM could be coprecipitated from lysates of primary mouse embryo fibroblasts (MEFs). MEFs established from embryonic day 15 embryos were propagated in culture on a 3T3 protocol, a process during which p19Arf progressively accumulates as the prolifer-
Table 1. Arf-associated proteins

| Type                        | Protein | Accession no. | Peptide(s) | Predicted size (kDa) | pI  |
|-----------------------------|---------|---------------|------------|----------------------|-----|
| Ribosomal subunits          | RP P0   | gi13277927    | GHELNNPALEK | 34                   | 5.9 |
|                             | RP L3   | gi16807136    | NNASTDYDLSDK | 46                  | 10.2|
|                             | RP L4   | gi22001911    | KLDELYEGRW  | 47                   | 11.0|
|                             | RP L5   | gi22002065    | HIMGQNVDYMYR | 34                  | 9.8 |
|                             | RP L7   | gi31981515    | KAGNYVPÆPÆK | 31                   | 10.9|
|                             | RP L7a  | gi30410942    | TNYNDKYREDI | 31                   | 10.6|
|                             | RP L8   | gi6755385     | ASGNYATVISHPETK | 28                  | 11.0|
|                             | RP L9   | gi1414997     | KFLDGIYYSEK | 22                   | 10.0|
|                             | RP L10a | gi6755350     | KYDAAFLASELKL, FSPILTHNENMVAK | 25 | 10.0|
|                             | RP L12  | gi17390751    | HSGNITFDEIVNIAR | 18 | 9.5  |
|                             | RP L18  | gi25050299    | TNSTFNQVLK  | 22                   | 11.8|
|                             | RP L19  | gi6677773     | VNLDPNLTIAANNSR | 24 | 11.5|
|                             | RP L21  | gi31566385    | VYNTOHAVGIVNK  | 19                  | 10.5|
|                             | RP S19  | gi12963511    | RVLOALEGLK  | 16                   | 10.4|
| Ribosome biogenesis associated | Nucleolin  | gi128843      | GLSEDIDTEETLK | 77                  | 4.7 |
| nonribosomal                | Nucleophosmin | gi6679108    | VDNDEQHQLSLR | 33                  | 4.6 |
|                             | PTRF    | gi6679567     | SFTPDHVYVYAR | 44                   | 5.4 |
| Importins                   | Importin 7 | gi31581595    | KOLZCADLDAQ, ENIVAEJISPELIR, SDQNLQTALELTR, DGALHMIGLSAEILK | 119 | 4.7 |
|                             | Importin 4 | gi19745156    | YVRPDDVSLAR, SSSDPSSPSVLOTLSLR | 119 | 4.9 |
|                             | Importin β1 | gi31543051    | GDQENVHPDMVLQPR | 97 | 4.7 |

Arf interacts with nucleophosmin

We next examined if the NPM-Arf interaction occurs independently of Mdm2 and p53. MEFs prepared from Arf/Mdm2/p53 triple knockout mice were infected with retroviral vectors encoding full-length Arf or Arf (Δ2-14) proteins. Four days after infection, cells expressing wild-type p19Arf ceased proliferating, but those infected with an empty control vector or with mutant Arf seemed to associate more avidly than wild-type NPM with the one encoding mutant Arf Δ2-14 continued to divide (48). Arf-NPM complexes were then analyzed by sequential immunoprecipitation and immunoblotting (Fig. 3C). Endogenous NPM coprecipitated with the wild-type p19Arf protein (lane 5) but not with Arf Δ2-14 (lane 6). Since this Arf mutant does not bind to 5.8S rRNA either (41), physical interactions between Arf, NPM, and 5.8S rRNA correlate with p19Arf's ability to inhibit cell proliferation in a p53-independent manner.

NPM acidic and oligomerization domains are required for Arf binding in vivo. Several functional domains have been mapped within NPM (Fig. 4A) (15). The N-terminal portion of NPM includes an oligomerization domain. The central domain, while highly acidic, is punctuated by two nuclear localization signals. A heterodimerization domain that mediates interactions with other nucleolar proteins lies in the C-terminal moiety and is followed by a nucleic acid-binding domain at the extreme C terminus (15).

We generated a series of deletion mutants of Flag-tagged NPM and transiently expressed them in 293T cells. Analysis of the overexpressed proteins by immunofluorescence revealed that wild-type NPM and the C1 mutant lacking the nucleic acid-binding domain localized to nucleoli (Fig. 4B) just like endogenous NPM, which is frequently used as a nucleolar marker. Further C-terminal deletions (C2 and C3) dispersed the protein throughout the nucleus; mutants with N-terminal deletions were also mislocalized to the nucleoplasm (N2) or cytoplasm (N3).

When cotransfected with an Arf expression plasmid into 293T cells, all NPM variants were detected with antibodies to the Flag tag (Fig. 4C, top panel); p19Arf was coexpressed with all forms of NPM (Fig. 4C, middle panel). Antibodies to Flag-tagged NPM coprecipitated p19Arf, as expected (Fig. 4C, bottom panel, lane 2). Arf also bound to the C1 and C2 mutants (Fig. 4C, bottom panel, lanes 3 and 4) but not to the others (Fig. 4C, bottom panel, lanes 5 to 7). Note that the C1 mutant seemed to associate more avidly than wild-type NPM with p19Arf (Fig. 4D, bottom panel, compare lanes 3 and 2). Reciprocally, antibodies to p19Arf selectively coprecipitated wild-type NPM, C1, and C2 but not the other NPM variants (Fig. 4D). More C1 and C2 than wild-type NPM coprecipitated with p19Arf (Fig. 4D, bottom panel, compare lanes 3 and 2), despite the fact that the expressed levels of full-length NPM exceeded those of the C1 mutant (Fig. 4D, top panel, lanes 3 and 4 versus 1 and 2). These results are summarized in Fig. 4A.

The difference between C2 and C3 is the presence of the central acidic domain (Fig. 4A), and because p19Arf is a highly basic protein (pI > 12), it likely requires this region for binding. Although p19Arf did not detectably interact in vivo with the N2 mutant retaining the NPM acidic domain but lacking the oligomerization domain, this protein was excluded from nucleoli (Fig. 4B). Nonetheless, the NPM oligomerization domain may also contribute to p19Arf binding, given evidence that Arf interacts with NPM oligomers (see below).

NPM isoform lacking its nucleic acid-binding domain can override Arf-induced inhibition of rRNA processing. To assess a role for NPM in modulating Arf function, NIH 3T3 and MT-Arf cells were infected with retroviruses encoding wild-type or mutant forms of NPM. Immunoblotting confirmed that...
similar quantities of Flag-tagged NPM proteins were expressed in both cell lines (Fig. 5A). The expression levels of exogenous proteins except N3 were similar to that of endogenous NPM (data not shown, but see Fig. 7 below). Treatment of MT-Arf cells with zinc sulfate induced p19Arf, p53, and the p53-responsive proteins.

FIG. 3. Arf forms complexes with NPM. (A, upper two panels) Lysates prepared from NIH 3T3 and MT-Arf cells treated with zinc sulfate for 24 h (+) or not (−) were precipitated with antibodies to NPM (lanes 5 to 8), p19Arf (lanes 9 to 12), or control IgG (lanes 13 and 14). Denatured immune complexes electrophoretically separated on gels were transferred to a membrane and blotted with the same antibodies. Cell lysates (10%) separated on denaturing gels were directly blotted with antibodies to NPM and p19Arf in order to estimate levels of the expressed proteins (lanes 1 to 4; see text). (A, lower panel) RNAs eluted from immune complexes were labeled with [32P]pCp and T4 RNA ligase, separated on a denaturing gel, and detected by autoradiography. (B) Primary MEFs established from day 15 embryos were propagated on a 3T3 protocol for seven passages. Cell lysates expressing both p19Arf and NPM (lane 1) were precipitated with control antibodies (lane 2) or with antibodies to NPM or Arf (lanes 3 and 4). Proteins separated on denaturing gels were immunoblotted with the same antibodies, indicated at the right of the panel. (C) Lysates prepared from 293T cells transiently transfected with NPM and Arf expression plasmids were separated on denaturing gels and blotted with antibodies to the Flag tag (top panel) or Arf (middle panel) to demonstrate expression of the respective proteins. Immune complexes recovered from lysates precipitated with antibodies to the Flag epitope, and nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI). (C) Transfected cells as in panel C were similarly immunoblotted to demonstrate expression of NPM variants (top panel) and p19Arf (middle panel). Immune complexes recovered from lysates precipitated with antibodies to p19Arf were blotted with antibodies to the Flag tag (bottom).
sive gene p21Cip1 (Fig. 5A, right, bottom panels). Interestingly, the levels of p19Arf were increased in cells expressing wild-type NPM, C1, or C2 (Fig. 5A, compare lanes 18, 20, and 22 to others). These differences appear to reflect NPM-induced retardation of p19Arf turnover (Mei-Ling Kuo and C. J. Sherr, unpublished observations).

Expression of exogenous NPM, C1, or C2 did not affect DNA synthesis in control NIH 3T3 or in uninduced MT-Arf cells (Fig. 6). As expected, Arf induction by zinc led to proliferative arrest of MT-Arf cells within 24 h. The reduction in DNA synthesis in Arf-induced cells was somewhat attenuated in the presence of excess wild-type NPM, whereas the C1

FIG. 5. NPM modulates Arf functions. (A) NIH 3T3 and MT-Arf cells infected with the indicated retroviruses encoding NPM variants were treated (+) with zinc sulfate for 24 h or left untreated (−). Expression of NPM and p19Arf (in induced MT-Arf cells only) was confirmed by immunoblotting. The same lysates were blotted with antibodies to p53 and the p53-inducible gene product p21Cip1. Lower levels of p53 were detected in cells expressing NPM mutants C1 and C2 (lanes 20 and 22 versus 16), and this may explain the observed modest attenuation of Arf-induced proliferative arrest (Fig. 6). (B) NIH 3T3 cells (top panel) and MT-Arf cells (bottom panel) infected with the indicated retroviruses encoding NPM variants as in A were treated with zinc sulfate (+) or not (−). Newly synthesized rRNAs were analyzed by pulse-chase labeling with [3H]uridine. The indicated rRNA species separated on a gel and detected by fluororautoradiography are indicated at the right. Note that enforced expression of C1 (lanes 5 and 6) and C2 (lanes 7 and 8) overrides the effects of Arf expression (lanes 6 and 8), whereas wild-type NPM (lane 4) and the other NPM mutants do not.
FIG. 6. Enforced expression of NPM mutant C1 attenuates Arf-induced cell cycle arrest. NIH 3T3 and MT-Arf cells transfected with NPM and mutants C1 and C2 were pulsed for 1 h with bromodeoxyuridine (BrdU), and incorporation of bromodeoxyuridine into S-phase cells was determined by immunofluorescence. To induce p19\textsuperscript{Arf} in MT-Arf cells, cultures were treated for 24 h with zinc (+) or were left untreated (−) prior to bromodeoxyuridine labeling. Incorporation into control virus-infected cells not treated with zinc was normalized to 100% in each group.

mutants, which bound to p19\textsuperscript{Arf} more efficiently (Fig. 4C and 4D), had a greater effect in overriding p19\textsuperscript{Arf}-induced arrest (Fig. 6). In contrast, C2 was inactive in this respect. Immunofluorescence confirmed that wild-type NPM and C1 colocalized with Arf to the nucleolus, whereas C2 was again delocalized across the nucleus (Fig. 4B and data not shown). In MT-Arf cells, Arf’s ability to rapidly inhibit cell proliferation depends upon p53, and indeed, the p53 level achieved in cells coexpressing C1 was somewhat lower than those seen in cells expressing either a control GFP protein, wild-type NPM (which showed a slight p53 reduction), or the other NPM mutants (Fig. 5A, compare lanes 16, 18, and 20). Since Arf’s ability to interact with NPM does not depend upon Mdm2 or p53 (Fig. 3C), the simplest interpretation is that NPM can sequester p19\textsuperscript{Arf} from Mdm2, thereby accelerating p53 turnover, with the C1 mutant being more efficient than full-length NPM in doing so. Despite the modulating effects of overexpressed NPM on Arf-induced arrest, p19\textsuperscript{Arf} remained able to elicit a p53 response.

We next tested whether the NPM mutants might affect Arf’s ability to inhibit ribosome biogenesis. NIH 3T3 and MT-Arf cells were infected with MSCV-IRES-GFP retroviruses encoding NPM variants, and 3 days later, infected cells were >95% GFP positive. Cells taken postinfection and incubated for 24 h in medium containing or lacking zinc were metabolically labeled with [\textsuperscript{3}H]uridine for 30 min and incubated for another 2 h in the absence of the labeled precursor. Radiolabeled RNA was isolated, separated on a formaldehyde-agarose gel, and analyzed by fluorautoradiography. As reported previously (41), most of the newly synthesized rRNA (47S/45S) was processed into mature 28S and 18S rRNA within 2 h, and expression of exogenous NPM proteins did not affect rRNA synthesis in NIH 3T3 or uninduced MT-Arf cells (Fig. 5B). Treatment with zinc did not significantly affect rRNA production in NIH 3T3 cells (Fig. 5B, upper panel) but strongly inhibited rRNA maturation in MT-Arf cells, with concomitant expression of p19\textsuperscript{Arf} protein (Fig. 5B, lower panel, compare lanes 2 and 1). Wild-type NPM (Fig. 5B, lower panel, lanes 3 and 4) and mutants that do not bind to p19\textsuperscript{Arf} (C3, N2, and N3, Fig. 5B, lower panel, lanes 9 to 14) did not prevent p19\textsuperscript{Arf} from inhibiting RNA maturation. However, expression of the C1 and C2 mutants significantly blocked Arf’s ability to inhibit rRNA processing (Fig. 5B, lower panel, lanes 5 to 8), with C1 (localizing in nucleoli) exhibiting a more profound effect than C2 (pan-nuclear localization).

NPM mutants form oligomers with full-length NPM. As indicated above, the levels of expression of exogenous NPM proteins achieved were marginally greater than that of endogenous NPM in MT-Arf cells (Fig. 7A). Immunoblotting with antibodies to the Flag tag selectively detected the exogenous NPM variants, which exhibited characteristic electrophoretic mobilities on denaturing gels (Fig. 7A, bottom panel). Antibodies to NPM itself recognized both the endogenous and exogenous forms of NPM (Fig. 7A, middle panel), and p19\textsuperscript{Arf} was detected only in induced cells (Fig. 7A, top). Control antibodies precipitated none of these proteins (Fig. 7B), whereas antibodies to p19\textsuperscript{Arf} coprecipitated Flag-tagged (C1 and C2) and untagged forms of NPM (Fig. 7C). In turn, antibodies to NPM coprecipitated p19\textsuperscript{Arf} (Fig. 7D). Moreover, antibodies to the Flag tag also precipitated the endogenously expressed NPM protein whether Arf was induced or not (Fig. 7E), indicating that exogenous NPM as well as the C1 and C2 mutants formed oligomers with endogenous NPM. The p19\textsuperscript{Arf} protein joined these complexes upon induction (Fig. 7E), again with a greater propensity to bind to C1- and C2-containing complexes than those containing only wild-type NPM. Therefore, the NPM C1 and C2 mutants do not sequester p19\textsuperscript{Arf} protein from endogenous NPM but instead exist together in a complex with endogenous NPM and p19\textsuperscript{Arf}.

NPM mutant C1 displaces p19\textsuperscript{Arf} from a high-molecular-weight complex. From the results above, we reasoned that the C1 mutant might interfere with Arf’s inhibition of rRNA processing by causing some qualitative change in the Arf-NPM complex. Cell lysates prepared from induced or uninduced MT-Arf cells expressing either exogenous wild-type NPM or the C1 mutant were subjected to gel filtration through Superose-6. Fractions were analyzed for the presence of the various proteins by immunoblotting. Endogenous NPM was seen to reside in a complex of ~500 kDa in uninduced cells transduced with a control GFP vector (Fig. 8A, top panel). Because the parental NIH 3T3 cells have sustained deletions of the Arf locus, the 500-kDa NPM complex must lack p19\textsuperscript{Arf}. Following Arf induction, however, a small proportion of NPM protein moved into a protein complex of much greater mass that was delivered near the column void volume (~5 MDa). Exogenous wild-type NPM behaved like the endogenous protein (Fig. 8A, middle panel); the C1 mutant exhibited a similar distribution, although the smaller complex was more heterodispersed (Fig. 8A, bottom panel).

Figure 8B shows the distribution of p19\textsuperscript{Arf} in the same column fractions. Most of the induced Arf protein cochromatographed with the minor fraction of NPM in the high-molecular-mass complex (~5 MDa). This is entirely consistent with our previous experiments, which revealed that only a small proportion of total NPM associated with p19\textsuperscript{Arf} (Fig. 3A). However, in the presence of the C1 mutant, more than 60% of the Arf protein was present in the smaller C1-containing complex. These results suggest that an inability of p19\textsuperscript{Arf} to asso-
 ciate with the larger NPM-containing complex prevents its ability to interfere with rRNA processing.

**DISCUSSION**

By counteracting the activity of Mdm2, p19Arf can trigger p53-dependent cell cycle arrest or apoptosis, depending on cell type and collateral signals. Although it was initially thought that Arf’s ability to arrest cell proliferation depended exclusively on p53, subsequent studies showed that p19Arf could also retard the growth of cells lacking p53 (2) or both p53 and Mdm2 (48), albeit much less efficiently than in cells that retain p53 function. The bulk of p19Arf normally resides within the nucleolus (30, 33, 50), Arf being as robust a nucleolar marker as fibrillarin (50) or NPM (20), implying that p19Arf might play a distinct role in this compartment. Indeed, recent experiments indicated that p19Arf could inhibit rRNA processing by retarding the initial cleavages of the 47S/45S rRNA precursors and also by inhibiting a later processing step involving the 32S rRNA precursor that eventually leads to the formation of both 28S and 5.8S rRNAs (41). As we demonstrated previously, Arf can associate with 5.8S rRNA, but we do not know whether it might also interact with larger rRNA precursors. Moreover, the mechanism by which Arf affects rRNA processing remains unclear.

To identify other proteins with which p19Arf may interact, we introduced a TAP-tagged Arf protein into cultured mouse fibroblasts and copurified complexes containing a number of p19Arf-associated proteins. These included NPM, nucleolin, and PTRF (as well as many ribosomal proteins), all of which localize to the nucleolus of mammalian cells. Parallel purifications performed with Arf mutants which are unable to induce either p53-dependent or -independent cell cycle arrest recovered less NPM or PTRF. Since tandem affinity purification was performed with proteins obtained from unfractionated cell lysates, these results provide some assurance that p19Arf physically interacts either directly or indirectly with these nucleolar proteins in situ. Treatment of cell lysates with RNase A prior to tandem affinity purification did not disrupt the interaction of TAP-tagged Arf with NPM, PTRF, or nucleolin, although it did limit the recovery of some ribosomal proteins. Thus, despite the fact that p19Arf, NPM, PTRF, and nucleolin can all interact with RNA, the association of Arf with these other proteins appears not to be RNA dependent. PTRF regulates rRNA transcriptional termination (16), whereas nucleolin controls the earliest processing steps within the 5’ external transcribed spacer sequence of 47S pre-rRNA (10–12). The interaction of p19Arf with complexes containing these proteins therefore implicates Arf as an inhibitor of early preribosome maturation, consistent with its ability to retard 47S/45S rRNA processing.

NPM is also thought to function in preribosome maturation (5, 29, 55). Full-length NPM/B23.1 binds to nucleic acids (4), a function which depends upon the presence of 35 amino acid residues at its C terminus (15). A second NPM isoform, B23.2, formed by alternative splicing of 3’ exons, has a substitution of two amino acids for the C-terminal 37 residues of B23.1; hence, B23.2 does not bind directly to RNA (15). NPM has been reported to act via an associated RNase activity to direct endonucleolytic cleavage of rRNA precursors at a site within the second internal transcribed spacer sequence (ITS-2) located 3’ to the 5.8S rRNA domain in 32S pre-rRNA (36). Like Arf, NPM coprecipitated in immune complexes with 5.8S rRNA from MT-Arf cells whether p19Arf was induced or not.
although more 5.8S rRNA was recovered when Arf was expressed. Since, apart from its effects on 47S/45S rRNA, p19Arf also interferes with processing of the 32S intermediate containing 28S and 5.8S rRNAs (41), the Arf-NPM interaction also interferes with processing of the 32S intermediate complexes. Since, apart from its effects on 47S/45S rRNA, p19Arf becomes insoluble and precipitated out of solution. Thus, although NPM itself can prevent thermally induced aggregation under the same conditions of extraction, induced p19Arf was recovered near the void volume of Superose-6 columns, and this mobilized some NPM into these higher-molecular-weight complexes (2 to 5 MDa). These results are consistent with observations that only a small proportion of NPM associated with p19Arf, although a significant pool of p19Arf coprecipitated with NPM. However, complexes containing C1, which, like C1 itself, should be impaired in RNA binding (27), redistributed the vast majority of p19Arf from the high-molecular-weight (2- to 5-MDa) to lower-molecular-weight (∼500-kDa) complexes. Importantly, enforced expression of C1 at levels that modestly exceeded the level of endogenous NPM also overrode the ability of p19Arf to retard rRNA processing. Clearly, the simplest interpretation is that Arf joins some NPM in preribosomal particles to negatively regulate RNA processing and that C1, like B23.2, generates lower-molecular-weight p19Arf-containing complexes that can no longer interact with RNA.

These results beg the question of whether NPM’s nucleic acid binding domain is required for it to enter the larger particles. We attempted to address this question by treating extracts with RNase prior to molecular sieving. Under these conditions, however, much of the Arf protein and some NPM became insoluble and precipitated out of solution. Thus, although NPM itself can prevent thermally induced aggregation of other proteins in vitro (15, 42), this in itself is insufficient to maintain p19Arf solubility. We also used low doses of actinomycin D to selectively inhibit RNA synthesis, conditions under which nucleolar structures are disrupted and NPM is dispersed (13, 38). Actinomycin D treatment redistributed NPM and p19Arf from 2- to 5-MDa complexes into those of lower molecular mass (∼500 kDa) (data not shown), again consistent with the idea that the larger complex localizes to nucleoli, where its formation is RNA dependent.

Functions other than preribosomal maturation have been attributed to NPM. For example, NPM is phosphorylated by cyclin-dependent kinases (Cdks), specifically during mitosis by cyclin B-Cdk1 (27) and during interphase by cyclin E-Cdk2 (26, 44). Although most NPM localizes to nucleoli, some associates with unduplicated centrosomes during G1 phase, where its cyclin E-Cdk2-mediated phosphorylation takes place. Phosphorylation dissociates NPM from centrosomes and leads to centrosome duplication, a process that can be inhibited by expression of a nonphosphorylatable NPM mutant (26, 44).
Although it is conceivable that the binding of p19\(^{Arf}\) to NPM might somehow affect this process, primary MEFs lacking Arf tend to remain diploid, whereas cells lacking p53, which are defective in expressing the p53-responsive Cd2 inhibitor p21\(^{Cip1}\) in response to DNA damage, rapidly become tetraploid (17, 54). Thus, unlike the case for p53, we have no evidence that p19\(^{Arf}\) plays a role in regulating centrosome duplication.

Within the first 24 h following zinc induction of p19\(^{Arf}\) in MT-Arf cells up to the time that cells underwent proliferative arrest, we observed that a major proportion of induced p19\(^{Arf}\) associated with NPM, whereas a considerably smaller fraction stably bound to Mdm2, a much less abundant protein. In this setting, Arf’s ability to arrest cell proliferation rapidly is p53 dependent. We noted that p53 accumulation in response to Arf induction was modestly attenuated by enforced NPM expression and even more so by the NPM C1 mutant. Hence, by associating with p19\(^{Arf}\), NPM might limit Arf’s interaction with Mdm2 to some degree. In contrast, others reported that NPM could bind directly to p53 to increase its stability and activity following stress (3). The C-terminal heterodimerization and nucleic acid binding domains of NPM were reported to be necessary and sufficient for p53 binding, whereas, as shown here, the nonoverlapping central oligomerization and acidic domains are required for NPM’s interaction with p19\(^{Arf}\). Such data raise the formal possibility that NPM might simultaneously form complexes with p19\(^{Arf}\) and p53 and, in principle, with Mdm2 as well. However, by molecular sieving analyses, we found that Arf-NPM and Arf-Mdm2 complexes chromatographed in different peak fractions; moreover, antibodies to NPM did not precipitate Mdm2. Although induction or introduction of p19\(^{Arf}\) into naive cells can localize Mdm2 to nucleoli, activated p53 remains nucleoplasmic (43, 50). Furthermore, the interaction of NPM and p19\(^{Arf}\) occurs equally well in primary MEFs lacking both Mdm2 and p53.

Whether Arf can form complexes with preribosomal particles in the nucleolus and interfere with certain stages of their maturation warrants further study. The ability of p19\(^{Arf}\) to interact with NPM and other nucleolar proteins and the finding that certain NPM mutants can override the effects of Arf on rRNA processing reinforce the idea that Arf can play a role in regulating ribosome biosynthesis and, by inference, cell growth as well as cell proliferation.

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Addendum in Proof

After acceptance of our paper, Itahanu and coworkers also reported that ARF physically interacts with B23/NPM/nucleophosmin (Mol. Cell 12:1151–1164, 2003).
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