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A Non-Tumor Suppressor Role for Basal p19ARF in Maintaining Nucleolar Structure and Function

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The nucleolus is the center of ribosome synthesis, with the nucleophosmin (NPM) and p19ARF proteins antagonizing one another to either promote or inhibit growth. However, basal NPM and ARF proteins form nucleolar complexes whose functions remain unknown. Nucleoli from Arf−/− cells displayed increased nucleolar area, suggesting that basal ARF might regulate key nucleolar functions. Concordantly, ribosome biogenesis and protein synthesis were dramatically elevated in the absence of Arf, causing these cells to exhibit tremendous gains in protein amounts and increases in cell volume. The transcription of ribosomal DNA (rDNA), the processing of nascent rRNA molecules, and the nuclear export of ribosomes were all increased in the absence of ARF. Similar results were obtained using targeted lentiviral RNA interference of ARF in wild-type MEFs. Postmitotic osteoclasts from Arf-null mice exhibited hyperactivity in vitro and in vivo, demonstrating a physiological function for basal ARF. Moreover, the knockdown of NPM blocked the increases in Arf−/− ribosome output and osteoclast activity, demonstrating that these gains require NPM. Thus, basal ARF proteins act as a monitor of steady-state ribosome biogenesis and growth independent of their ability to prevent unwarranted hyperproliferation.

Cellular growth (i.e., macromolecular synthesis) is an essential function during the early parts of the cell cycle. For cells to transit the G1 restriction point, they must duplicate nearly their entire protein content; failure to do so would result in smaller daughter cells (12). Only recently has an emphasis been placed on the fundamental control of cell growth and its link to the cell cycle. Developments in the understanding of how the cell senses environmental nutritional cues has led to a flurry of research on understanding the mechanisms underlying growth control (40). Not surprisingly, several of these pathways converge on the synthesis of new ribosomes in the cell nucleolus and the regulation of translation.

Approximately half of the cell’s energy expenditure is directed toward ribosome biogenesis (26). The nucleolus, long recognized as a marker for active cellular growth, was first described in the early 1960s as the center of ribosomal DNA (rDNA) transcription and ribosome biogenesis (6, 32). This organelle is composed of three regions, on the basis of morphology at the ultrastructural level: the fibrillar centers, the dense fibrillar compartment, and the granular zone. rDNA transcription occurs in the junction region between the fibrillar centers and the surrounding dense fibrillar component, and the resulting rRNA is further processed in the periphery of the dense fibrillar component. Further posttranscriptional modifications and assembly into subunits occur in the surrounding granular region (18).

While the primary mechanisms regulating these processes have been well studied in Saccharomyces cerevisiae (13), multicellular organisms demand more complex regulatory mechanisms, in that proliferative capacity is determined not only by the relative abundance of nutrients but also by complicated extracellular signals and growth factors. Indeed, previous studies have demonstrated convergence between the growth and proliferation pathways via regulation of the tumor suppressor gene products Rb and p53 (9, 17, 43, 48). Both products are known to negatively regulate the activity of polymerase I in rDNA transcription. Oncogenes such as c-Myc also regulate the transcription of rDNA and the genes that encode ribosomal proteins, implying that an intricate network exists within the nucleolus to ensure the proper synthesis of ribosomes (7, 15, 16).

The tumor suppressor p19ARF represents an attractive candidate for coupling proliferation to growth. Given its nucleolar localization (39, 44, 45) and potent induction by hyperproliferative signals (19, 20, 31, 50), ARF represents a potential nucleolar integrator of growth signals coming into the cell. It has been regarded classically as an activator of p53 through its ability to sequester Mdm2, the E3 ubiquitin ligase for p53 (5, 17, 43). However, recent data have demonstrated a role for ARF in binding to and affecting the function of the ribosomal chaperone nucleophosmin (NPM), independent of its ability to regulate p53 (4, 8, 21). Furthermore, these data are consistent with those from a growing number of studies with mice and humans that describe p53-independent functions for ARF tumor suppression (35).
Given ARF's nucleolar localization, its role in suppressing cellular growth and proliferation, and its ability to bind to a protein involved in ribosome biogenesis, we were inclined to explore ARF's function in this process. To this end, we utilized targeted Arf knockout mice and selective ARF knockdown via lentiviral interference. Cells derived from Arf-null mice displayed significant alterations in gross nucleolar morphology and abundance and had a marked increase in basal protein synthesis levels compared to that in wild-type cells. Furthermore, this increase in protein synthesis was correlated to increased ribosome biogenesis and cytoplasmic ribosome content, implying a regulatory role for ARF in these processes. Importantly, though ARF levels are nearly undetectable in low-passage mouse embryonic fibroblasts (19), the knockdown of endogenous ARF via short hairpin RNA (shRNA) constructs mimicked the Arf-null nucleolar and ribosomal phenotype, implying an important ribosome homeostatic role for basal ARF proteins in wild-type cells. The progrowth phenotype of the Arf loss was not limited to proliferating cells, as fully differentiated osteoclasts from Arf-null mice exhibited tremendous gains in protein synthesis and overall activity in vivo. Mechanistically, all of the ribosome gains exhibited by the loss of Arf were reversed by the removal of the nucleolar NPM proto-oncogene, indicating that NPM, when present, is required for the maintenance of normal nucleolar function. Together, these data strongly argue for a moment-to-moment "thermostat"-like role for basal ARF molecules in controlling NPM-directed ribosome biogenesis and protein synthetic rates.

MATERIALS AND METHODS

Mice. Arf−/− mice were rederived from triple-knockout heterozygous mice (Arf+/−; Mdm2+/−; p53−/−); a generous gift from G. Zambetti, St. Jude, Memphis, TN) to a pure C57BL/6 background by several generations of backcrosses to wild-type C57BL/6 mice, followed by breeding to homogamy. Age-matched wild-type C57BL/6 littermates were used as controls where indicated. Organs were harvested from mice 4 days postnatally.

Cell culture, reagents, and antibodies. Low-passage (2–5) MEFs were isolated and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1× nonessential amino acids, 1 mM sodium pyruvate, and 2 mM glutamine.

Antibodies: Mouse anti-ARF (H-100, sc-144) or a scrambled control were cloned into these sites. The resultant ARF shRNA vector, pLKO-GFP was digested with AgeI/MluI, and annealed oligonucleotides were cloned as described above into pLKO-GFP, the sequences of which were previously reported (27). RNA interference for endogenous c-Myc was performed with siRNAs recognizing the 3' untranslated region (UTR) of c-Myc (5'-AACGTTTATAACAGT1CAA3'-3') (Qiagen). Myc-ER retrovirus was generated and used to infect wild-type and Arf-null MEFs as previously described (30).

AgNOR staining. MEFs were seeded onto glass coverslips overnight and were fixed and stained the following day. A silver nucleolar organizing region (AgNOR) staining method was modified from the protocol presented by Aubele et al. (1). Briefly, cells were fixed in 2% glutaraldehyde, followed by postfixation in a 3:1 ethanol-acetic acid solution. Cells were stained with a 0.33% formic acid-33.3% silver nitrate solution in 0.66% gelatin and mounted on slides with Vectashield (Vector Labs).

Histomorphometry. Histomorphometric analysis was performed with OsteoQuant Nova Prime software (Bioquant Image Analysis Corp.) on images captured at ×200 magnification by an Optitrons Magnifire camera on a Nikon TE300 microscope. Total numbers and total areas (μm²) of AgNOR regions per nucleus from 100 nuclei were assessed, and statistical significance was determined using Student’s t test.

Electron microscopy. Asynchronously growing wild-type and Arf−/− MEFs were trypsinized and fixed with 2% glutaraldehyde in phosphate-buffered saline for 10 min. Samples were further processed by the Washington University Department of Cell Biology’s Electron Microscopy Core. Pictures of nuclei and nucleoli were taken at magnifications of ×3,000 and ×7,000, respectively.

Materials and Methods

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Cell culture, reagents, and antibodies. Low-passage (2–5) MEFs were isolated and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1× nonessential amino acids, 1 mM sodium pyruvate, and 2 mM glutamine. Rabbit anti-p16INK4A (sc-1207), goat anti-rat antibodies (Jackson ImmunoResearch) and ECL technology, rabbit anti-p16INK4A (Santa Cruz Biotechnology), and rabbit anti-L5 antibody (Santa Cruz Biotechnology) were used for Western blotting and serial immunoprecipitation.

Lentiviral production and infection. Viral supernatants were collected and pooled. Wild-type MEFs (8 × 10⁵) were plated and infected with viral supernatant containing 10 μg/ml of the protamine sulfate. Cells were infected again on the following day, checked for green fluorescent protein expression, and allowed to express the shRNA construct for 48 h.

Serum assays. Levels of terminal-resistance acid phosphatase (TRAP) 5b were measured in serum collected from wild-type or Arf−/− mice, using a TRAP 5b enzyme-linked immunosorbent assay (ELISA) system (IDS, Fountain Hills, AZ).

Osteoclast formation assays. Whole bone marrow was extracted from femurs and tibias of wild-type or Arf−/− mice and plated in CMG-14-12 culture supernatant (1/10 vol) in α-minimal essential medium (α-MEM) containing 10% fetal calf serum (FCS) to generate primary bone marrow macrophages (BMM), as described previously (49). Cells were fed every day with α-MEM containing 10% FCS, CMG-14-12 supernatant (1/20 vol), and glutathione S-transferase-RANK ligand (RANKL) (100 ng/ml) and incubated for 8 days to form mature osteoclasts (49). TRAP staining was performed according to the manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO). Five fields at a magnification of ×4 were captured with the Magnafire system, and the TRAP-positive cells with three or more nuclei were counted by one blinded to the genotype. A quantitative TRAP solution assay was performed by adding a colorimetric substrate, 5.5 mM p-nitrophenyl phosphate, in the presence of 10 mM sodium tartrate at pH 4.5.

Macrophage proliferation assays. BMMs (1 × 10⁵) were plated in α-MEM containing 10% FCS and CMG-14-12 supernatant (1/10 vol). Cells were starved in α-MEM containing 0.1% FCS for 12 h. At this time, α-MEM containing 10% FCS and CMG-14-12 supernatant (1/10 vol) was added back to the cells. Cells were labeled with bromodeoxyuridine (BrdU) for 24 h, and proliferation was measured using the chemiluminescent cell proliferation ELISA (Roche Diagnostics, Mannheim, Germany).

Western blotting and serial immunoprecipitation. MEF cell extracts (30 μg) were loaded onto 4 to 20% sodium dodecyl sulfate (SDS)-polyacrylamide gels (ISC Biosciences), transferred to polyvinylidene difluoride membrane (Millipore), and probed with antibody to rat anti-p16INK4A (Novus Biologicals), goat anti-γ-tubulin (Santa Cruz Biotechnology), rabbit anti-Myc (Santa Cruz Biotechnology), rabbit anti-p16INK4A (Santa Cruz Biotechnology), and rabbit anti-L5 (ILAMM). Secondary horseradish peroxidase-conjugated anti-rabbit, anti-goat, or anti-mouse antibodies (Jackson ImmunoResearch) and ECL+ (Amersham) were used to visualize the bands. For serial immunoprecipitation, 200 μg of wild-type MEF lysate was immunoprecipitated with GammaBind (Amersham) by a custom-made rabbit NPM polyclonal antibody (Sigma Genosys) (46). The final supernatant was concentrated with a Vivaspin column (Vivascience), and all

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RESULTS

p19ARF is required for proper nucleolar morphology. A common theme in ARF biology is its intrinsic localization within the nucleolus, under both basal and oncogene-induced settings (31, 39, 44, 45). Many of ARF’s binding partners either reside in the nucleolus or are relocalized there by ARF itself (25, 35). Of the resident nucleolar ARF binding proteins, nearly all are involved in some facet of ribosome biogenesis (e.g., transcription, processing, or export) (34, 35). We hypothesized that basal nucleolar ARF proteins, even at low levels, might exert a subtle activity on these nucleolar proteins to continuously monitor their function. To this end, we adapted an AgNOR staining protocol (1) for use with MEFs derived from wild-type and from Arf−/− mice. Staining methods utilizing the reduction of silver on argyrophilic proteins surrounding the nucleolar organizing region have been used for decades as a prognostic factor with certain carcinomas, wherein increases of the AgNOR index tend to correlate with poor prognoses (28). AgNOR staining of Arf−/− MEFs demonstrated markedly higher numbers of AgNORs per nucleus and a distinctly irregular shape than the numbers and the more rounded, symmetrical shape of the wild-type AgNOR counterparts (Fig. 1A). At the ultrastructural level, we also observed multiple, elongated, irregular nucleoli in Arf−/− cells compared to the round nucleoli of the wild-type cells (Fig. 1B, top panels). These irregularities in Arf-null cells were also associated with larger fibrillar centers, the sites of rDNA transcription (Fig. 1B, bottom panel, arrows). We quantitated the total nucleolar area per nucleus (a common pathological definition of the AgNOR index) (42) and observed a 20% increase in Arf−/− cells (31.6 μm² versus 26.4 μm²; n = 100; P < 0.001) (Fig. 1C). A significant increase in the AgNOR number per nucleus was also observed (5.78 versus 3.49; n = 100; P < 0.001). Additionally, nucleolar morphology changes were observed in vivo. Intestine and liver tissues harvested from newborn wild-type and Arf-null mice and stained for AgNORs recapitulated our earlier in vitro findings in that the loss of Arf resulted in dramatic gains in both AgNOR numbers and overall area (Fig. 2A and B). Moreover, we also observed a moderate increase in the number of larger multinucleolar cells in the livers of Arf-deficient mice (Fig. 2A, right panels). Taken together, these data suggest a role for p19ARF in maintaining a proper nucleolar structure in vitro and in vivo.

Loss of Arf enhances protein synthesis and ribosome biogenesis independent of proliferation. The loss of Arf resulted in dramatic alterations in nucleolar structure (Fig. 1 and 2), suggesting that basal ARF may function in the maintenance of this organelle. To determine whether changes in nucleolar structure result in altered nucleolar function, we assessed ribosome output from the nucleolus. First, we performed [35S]methionine pulse-labeling experiments, measuring the amount of radioactivity incorporated into newly translated proteins over time. As shown, Arf−/− MEFs had an approxi-
ultimately a fourfold increase in incorporated [35S]methionine compared to that of wild-type cells after 24 h (Fig. 3A). Furthermore, this increased protein synthesis was not related to any increase in proliferation rates, as the proliferation of the low-passage wild-type and the Arf–/– MEFs was virtually identical (Fig. 3B). To determine if the protein synthesis differences were due to increased ribosomal output, we performed sucrose density gradient rate-zonal ultracentrifugation of cytoplasmic lysates from wild-type and Arf–/– cells to separate ribosomes. Ribosome subunits and actively translating polysomes were detected by real-time monitoring of absorbance at 254 nm to detect the relative amounts of ribosomal RNAs present in each fraction, indicating a relative abundance of these ribosomal components (Fig. 3C). Consistent with gains in ribosome production and protein synthesis, we observed a significant increase in the overall volume of low-passage Arf-deficient MEFs as well as a robust increase in protein content per cell (Fig. 3D and E). Moreover, gains in ribosome biogenesis were also seen in vivo. Livers were isolated from newborn wild-type and Arf–/– mice, minced, and immediately placed in [35S]methionine-

89 lysates of the Arf–/– cells had significantly more (nearly 40%) cumulative absorbance in the actively translating polysome fraction, indicating a relative abundance of these ribosomal components (Fig. 3C). Consistent with gains in ribosome production and protein synthesis, we observed a significant increase in the overall volume of low-passage Arf-deficient MEFs as well as a robust increase in protein content per cell (Fig. 3D and E). Moreover, gains in ribosome biogenesis were also seen in vivo. Livers were isolated from newborn wild-type and Arf–/– mice, minced, and immediately placed in [35S]methionine-containing medium to measure protein synthesis rates. Cells isolated from Arf–/– livers exhibited a nearly 15-fold increase in incorporated [35S]methionine compared to that of wild-type livers, minced, and immediately placed in [35S]methionine-lysates of the Arf–/– cells, with a significant increase in both the number of AgNORs per nucleus (6.6 versus 3.3; n = 100; P < 0.01) and the total AgNOR-stained area per nucleus (49.8 μm² versus 36.8 μm²; n = 100; P < 0.001) (Fig. 5C). Similarly, the ARF knockdown MEFs displayed tremendous gains in protein synthesis rates as determined by [35S]methionine incorporation, nearly 10-fold higher than that of scrambled control MEFs (Fig. 5D) and almost twice as high as that of Arf-null MEFs (compared to Fig. 3A). The ARF knockdown MEFs also produced significantly more actively translating polysomes (55% more) as determined by UV monitoring of cytosolic rRNAs (Fig. 5E), suggesting that the acute loss of ARF has a greater impact on nucleolar functions.

Genetic disruption of Arf results in increased osteoclast numbers in vitro and elevated levels of TRAP protein in vitro and in vivo. To demonstrate a physiological function for ARF’s baseline regulation of ribosome biogenesis and protein synthesis, we focused on bone-resorbing osteoclasts as a model of a differentiated cell with high protein synthesis demands. Osteoclasts are formed by the fusion of hematopoietically derived macrophages into multinucleated giant cells with a specialized ruffled border containing thousands of vacuolar H⁺-ATPases. The osteoclast forms a sealing zone against the area of bone resorption and, in doing so, allows the specialized ruffled membrane to secrete collagenase and dramatically lower the pH through the activity of the proton pumps. As a result, the osteoclast has a high demand for protein synthesis, since the H⁺-ATPases are specific to the mature osteoclast and are not 60S, and 80S and polysome content (Fig. 4B), demonstrating elevated ribosome output in these tissues. We therefore postulated that basal ARF proteins might act as negative regulators at a certain step(s) in nucleolar ribosome biogenesis.

The acute knockdown of ARF mimics the phenotype of Arf–/– cells. Since ARF’s role in sensing hyperproliferative signals and concomitantly inducing p53-dependent cell cycle arrest have been well established (19, 20, 31, 50), it has been assumed that basal ARF has little, if any, function in the normal day-to-day regulation of cellular homeostasis. However, ARF levels in asynchronously growing wild-type cells are detectable by Western blotting analysis and immunohistochemistry (5). Given our finding that Arf–/– cells exhibit chronic nucleolar morphology changes and increased ribosome output (Fig. 1 to 4), we were poised to reexamine this question in an acute setting by knocking down basal ARF in wild-type cells. This was accomplished by using lentiviral constructs containing a shRNA duplex that recognized bases 157 through 177 in the ARF-specific exon 1β of the Ink4a/Arf locus. To verify the specificity of this construct, we infected wild-type MEFs with lentivirus containing either shRNA specific to ARF or a scrambled control sequence. As shown by Western blotting analysis, infection with viruses containing the ARF shRNA sequence produced a robust knockdown of the level of ARF (96%) without decreasing levels of p16INK4A (Fig. 5A). Moreover, expression of other nucleolar proteins, such as NPM and ribosomal protein L5, also remained unchanged following the ARF knockdown (Fig. 5A). As observed first with the Arf–/– MEFs, the ARF knockdown MEFs also exhibited dramatic nucleolar morphology alterations as depicted by AgNOR staining (Fig. 5B). These acute changes were of greater statistical difference than those originally observed with Arf-null cells, with a significant increase in both the number of AgNORs per nucleus (6.6 versus 3.3; n = 100; P < 0.001) and the total AgNOR-stained area per nucleus (49.8 μm² versus 36.8 μm²; n = 100; P < 0.001) (Fig. 5C). Similarly, the ARF knockdown MEFs displayed tremendous gains in protein synthesis rates as determined by [35S]methionine incorporation, nearly 10-fold higher than that of scrambled control MEFs (Fig. 5D) and almost twice as high as that of Arf-null MEFs (compared to Fig. 3A). The ARF knockdown MEFs also produced significantly more actively translating polysomes (55% more) as determined by UV monitoring of cytosolic rRNAs (Fig. 5E), suggesting that the acute loss of ARF has a greater impact on nucleolar functions.
found in macrophage precursors (41). Furthermore, since the mature osteoclast is a postmitotic cell, it affords an excellent opportunity to examine ARF’s effects on protein and ribosome metabolism independent of proliferation.

We first determined whether the proliferation rates varied between wild-type and \( \text{Arf}^{-/-} \) BMM, osteoclast precursors. BrdU labeling of BMMs demonstrated no significant differences in the proliferation rates between wild-type and \( \text{Arf}^{-/-} \) osteoclast precursors (Fig. 6A), similar to the equal proliferation rates of early passage MEFs (Fig. 3B). Next, BMMs from \( \text{Arf}^{-/-} \) and wild-type mice were induced to produce mature osteoclasts by the addition of macrophage colony-stimulating factor (M-CSF) and RANKL. After 3 days of stimulation with RANKL, cells were fixed and stained with a TRAP substrate, an osteoclast-specific stain that relies on the abundance of TRAP protein produced by the osteoclast. An increased number of mature osteoclasts derived from the \( \text{Arf}^{-/-} \) precursors was observed compared to that of the wild-type controls (Fig. 6B). TRAP-positive cells with greater than five nuclei were counted as a way to differentiate maturing osteoclasts from immature precursors and resulted in a significant increase in the \( \text{Arf}^{-/-} \) genotype (149 versus 91 per well; \( n = 5; P = 0.01 \)) (Fig. 6C).

To determine if the differences seen with osteoclastogenesis were functionally relevant, we compared the TRAP activities (a marker of osteoclast function) of equal numbers of TRAP-positive cells, as determined above. Cell lysates were incubated from day 4 post-RANKL addition (for wild-type cells) and day 3 post-RANKL addition (for \( \text{Arf}^{-/-} \) cells), where approximately equal numbers of multinucleated TRAP-positive cells were observed, with \( p \)-nitrophenyl phosphate, a colorimetric substrate for TRAP. A twofold increase in TRAP activity was seen with \( \text{Arf}^{-/-} \) cells compared to that with wild-type cells (\( P < 0.01 \)) (Fig. 6D), indicating that the \( \text{Arf}^{-/-} \) osteoclasts are far more active than their wild-type counterparts on a per cell basis. In vivo analysis of osteoclast function of \( \text{Arf}^{-/-} \) mice mimicked our in vitro findings of osteoclast hyperactivity, as there was an 18% increase in the level of
were immediately harvested, and cytosolic fractions were
creasing sucrose density.

Graph B shows an

that is effectively associated with NPM (Fig. 7B, lane 1) and a
second pool that is free from NPM (Fig. 7B, lane 6). This
implies that ARF’s effects on ribosome biogenesis may not be
relegated to only NPM-dependent processes and is consistent
with the idea that ARF antagonizes rDNA transcription
through other unique, physically interacting proteins. Accord-
ingly, the loss of Arf resulted in a fourfold increase in 47S
rRNA transcription (Fig. 7C), a process thought to be inde-
pendent of direct NPM regulation (as NPM does not localize
to the fibrillar compartment of the nucleolus).

Newly transcribed 47S rRNAs are further processed in the
nucleolus into their mature 28S, 18S, and 5.8S rRNAs (34).
These processes are known to be readily antagonized by over-
expressed ARF (37). To determine the effect of the Arf loss on
these events, newly synthesized 47S rRNAs were followed
through nucleolar processing. rRNA processing was greatly
accentuated in the Arf+/− MEFs over a 2-h period (Fig. 7D).
While the wild-type and the Arf-null cells clearly start with
different amounts of 47S rRNA (Fig. 7C), the Arf-null cells are
capable of churning out more processed rRNAs (15-fold more
than the wild type), which is nearly a threefold amplification
over the starting amount of 47S transcripts. This suggests that
while levels of 47S rRNA are certainly permissive for greater
processing of rRNAs, they cannot entirely account for the
sheer magnitude of increases in processed rRNAs observed in
the Arf+/− cells.

To determine the precise step at which ARF might influence
rRNA processing, we labeled cells with [methyl-3H]methi-
onine, which labels rRNA, and loaded equal amounts of the
radioactive label to examine processing intermediates after
short time periods of chase with label-free media. We observed
only a modest increase of the 32S rRNA precursors in cells
lacking Arf at early time periods, indicating that ARF may
interfere with the processing steps between the 47S transcript
and the 32S intermediate (Fig. 7E). However, after 2 h of
chase, we saw no differences in the relative amounts of radio-
activity in the final 18S and the 28S products, indicating that
the loss of Arf had no impact on these downstream processing
steps. These results exactly mirror what Sugimoto and col-
leagues observed when they overexpressed ARF, namely, an
accumulation of improperly processed rRNA intermediates
between the 47S and 32S stages (37), albeit to a far lesser
extent in our experiments.

As a final step in ribosome biogenesis, mature ribosome
subunits are exported to the cytosol in a process that we have
previously attributed to NPM-directed nuclear export (27, 46).
The Arf-null MEFs exhibited a more robust (~25-fold) nuclear
export of newly processed rRNAs than the wild-type cells did
(Fig. 8A). Again, this extreme difference between the wild-type
and the Arf-null cells was far greater than any previous step in
ribosome biogenesis (e.g., transcription or processing), imply-
ing that each step represents an amplification of the previous
step. This was most evident when the real-time nuclear export
of rRNAs, as monitored by scintillation counting of newly
exported 3H-labeled rRNA, revealed that the absolute rates of
rRNA export were threefold increased in cells lacking Arf (Fig.
8B). Taking these data together, we believe this reflects the
ability of ARF to regulate moment-to-moment steps in ribo-
some biogenesis, such that alterations in ARF levels may pro-
duce robust and rapid responses that effect cytoplasmic ribo-
somal content.
Myc is not responsible for the rDNA transcription increases in the Arf−/− cells. Previous reports have shown that the c-Myc transcription factor, in part, localizes to the nucleolus to positively regulate the transcription of rDNA (15, 16). Moreover, ARF has been shown to antagonize Myc functions through direct interactions (30). Thus, we sought to determine whether basal ARF proteins might be regulating nucleolar Myc to prevent the aberrant transcription of the rDNA loci. We utilized siRNAs targeting the 3′ UTR of c-Myc to successfully knock down endogenous Myc nearly 20-fold (Fig. 9A). While lower Myc protein levels greatly reduced 47S rRNA transcripts in wild-type MEFs, it had little impact on 47S copies in the Arf−/− MEFs (Fig. 9B). While the former result is consistent with previous studies showing a role for Myc in rDNA transcription (15, 16), the latter result suggests that Myc is not absolutely required for rDNA transcription in cells lacking Arf. However, when Myc levels were restored by using an siRNA-resistant (lacking the targeted 3′ UTR sequence) Myc-ER construct and 4-hydroxytamoxifen treatment (50), 47S transcript levels significantly increased in the Arf-null MEFs, suggesting that Myc proteins can positively direct rDNA transcription in the absence of ARF (Fig. 9A and B).

NPM is required for the growth gains seen in the absence of Arf. Having shown that nearly half of the basal ARF proteins are bound to NPM in wild-type MEFs (Fig. 7B), we hypothesized that NPM is a critical nucleolar target of basal ARF and that the loss of Arf resulted in unregulated NPM activities. To test this hypothesis, we knocked down NPM expression in MEFs lacking Arf to determine the effects on ribosome biogenesis and protein synthesis. Using lentiviruses carrying shRNAs targeting mouse NPM, we were able to achieve greater than 90% NPM knockdown efficiency (Fig. 10A). How-
ever, the reduction in NPM protein expression led to a dramatic increase in 47S rRNA transcripts in cells that also lacked Arf (Fig. 10B), indicating that NPM might actually inhibit rDNA transcription. The increase in 47S rRNA did not result in a similar increase in rRNA processing. In fact, we observed a slight but notable accumulation of 32S rRNA for cells lacking both ARF and NPM (Fig. 10C). We also noticed the appearance of an rRNA species above the 18S rRNA only in the absence of ARF and NPM, which may be the result of an additional processing defect (Fig. 10C). Furthermore, nuclear exportation of processed 18S rRNA was significantly attenuated (55% reduction) in the Arf-null cells lacking NPM (Fig. 10D), demonstrating the requirement for NPM in trafficking mature rRNAs out of the nucleus and into the cytosol. In response to decreased ribosome export to the cytosol, the Arf−/− MEFs with reduced NPM expression exhibited significantly attenuated protein synthesis rates (Fig. 10E). Thus, gains in rDNA transcription are not realized in terms of overall protein synthesis in the absence of NPM. This could be a result of a ribosome biogenesis feedback loop, where reduced ribosome export causes a shift in rDNA transcription to compensate for the lack of cytosolic ribosomes. However, in the absence of NPM, these ribosomes cannot be properly exported.

To determine whether protein synthesis gains observed with the absence of Arf were caused by the deregulation of NPM and were independent of proliferation, we lowered the levels

FIG. 6. Loss of p19ARF has functional consequences on osteoclast biology. (A) BrdU incorporation in the wild-type (WT) and Arf−/− macrophages. (B) Representative TRAP staining of equal numbers of BMMs following 3 days of treatment with M-CSF and RANKL reveals an increase in multinucleated osteoclasts formed from the Arf−/− precursors. (C) The graph shows increases in TRAP-positive osteoclasts with greater than five nuclei derived from the Arf−/− bone marrow. *, P = 0.01. (D) TRAP solution assay of equal numbers of TRAP-positive cells. Cells from the wild-type (day 4 post-RANKL addition) or the Arf−/− (day 3 post-RANKL addition) precursors were lysed and incubated in a colorimetric assay with p-nitrophenyl phosphate, a substrate for TRAP. The graph shows an A405*, P = 0.01. (E) Levels of serum TRAP 5b in Arf−/− compared to that in wild-type mice (P = 0.03; n = 5 mice in each group) as measured by ELISA.
of NPM in maturing osteoclasts. We reasoned that by reducing NPM expression in osteoclasts, we would mimic a restoration of ARF activity without the complicating effects of cell cycle arrest (i.e., osteoclasts are postmitotic) or of ARF binding to Mdm2 (i.e., a p53 response). This provided us with an experimental system with which to test the hypothesis that a balance exists between ARF and NPM in determining the ribosome output from the nucleolus. Lentiviral shRNAs targeting NPM in BMMs significantly reduced NPM protein expression levels (Fig. 11A). Concomitant with decreases in NPM expression, the Arf-/- osteoclasts were dramatically reduced in levels of TRAP staining (Fig. 11B) and activity (Fig. 11C), indicating a sensitivity of osteoclast differentiation to lower NPM levels. However, wild-type osteoclasts were far less sensitive to decreases in NPM expression, showing no statistically significant difference in TRAP activity. These data suggest that, in the absence of Arf, amplified ribosome biogenesis requires a set amount of NPM (for processing or export) and further implicates NPM as a target of basal ARF proteins in the maintenance of proper ribosome output.

DISCUSSION

While ARF has been long appreciated for its abilities to positively regulate p53 levels in the cell (22, 29) and serve as a sensor of hyperproliferative signals (19, 20, 31, 50), the relatively low abundance of ARF in interphase cells implied that ARF functioned only as a cellular checkpoint against aberrant growth and proliferation signals. In this manner, only signals powerful enough to elicit increases in ARF protein expression would trigger an actual ARF response. This implies that basal ARF molecules, even at their low levels, must be antagonized or held in check for the cell to undergo proper cell cycle progression and cell growth regimens. Teleologically, this model seems justified, given the genomic organization of the Ink4a/Arf locus where “leakiness” in p16INK4a or p19ARF...
transcription would have dire effects on the growth and survival of the cell (14). It is widely held that this locus is repressed in mice and that only under conditions of extreme stress or oncogenic signaling is the locus transcribed to elicit a growth and proliferative arrest phenotype (51). Here, we provide evidence that the physiologically low level of ARF has a regulatory role in nucleolar function and ribosome biogenesis. Indeed, as early as 4 days post-ARF knockdown by lentiviral shRNA infection, we observed changes in nucleolar morphology and function that are reminiscent of data from the Arf embryonic cells. This strongly supports the hypothesis that basal ARF consistently monitors and dynamically alters the nucleolar growth/suppression pathway on a day-to-day basis.

We would now argue that basal ARF proteins must be maintained at some steady-state level to provide constant surveillance of nucleolar function. Given the great energy demands of the nucleolus (ribosome biogenesis and protein synthesis account for nearly 50% of the cell’s energy), dysfunctional nucleolar processes may need to be adjusted at a moment’s notice (26). In support of this contention, a recent report (33) demonstrated that selective disruption of the nucleolus by either UV radiation or a number of “stress” responses induced cell cycle arrest and markedly enhanced p53 stability. While we did not observe any gross disruption of nucleoli in cells either lacking or overexpressing ARF, we did observe numerous qualitative changes in the size and number of nucleoli in cells lacking Arf. This would suggest that basal ARF might play a vital role in determining the protein composition of nucleoli, acting to prevent the release of specific ribosomal proteins from the nucleolus or to prohibit the entrance of unwanted (potentially oncogenic) nuclear proteins into the nucleolus.

In the past few years, numerous p53-independent functions have been ascribed to ARF (35). We found that nearly half of the basal ARF in the cell is in a complex with NPM, a protein previously shown to interact with human and mouse ARF proteins (4, 8, 21). While much of the work concerning the ARF-NPM interaction has focused on the ability of each protein to antagonize the function of the other (4, 8, 21, 24, 46), our findings suggest that the baseline interaction functions to maintain a controlled level of ribosome biogenesis. We propose a model where basal ARF antagonizes a small pool of NPM, either directly or enzymatically (8, 38), and thereby constantly limits ribosome output from the nucleolus. Importantly, levels of NPM did not change in the absence of ARF, but rather NPM activity was greatly increased as measured by its ability to promote ribosome nuclear export. Consistent with this model, the knockdown of basal NPM proteins resulted in dramatic reductions in protein production independent of cell proliferation, again underscoring the need for a consistent level of “ARF-free” NPM to promote ribosome synthesis.

While the mechanism and nature of such inhibition are still unclear, our data are consistent with a “thermostat” function for ARF, in that small changes in the abundance of ARF cause

![FIG. 8. Nucleocytoplasmic shuttling of newly synthesized ribosomes is enhanced in the absence of Arf.](image)

![FIG. 9. Myc is not required for the enhanced rDNA transcription of the Arf-null MEFs.](image)
its binding partners to either dampen or enhance ribosome synthesis and export and, ultimately, lead to global changes in protein synthesis. It is apparent from our data that basal ARF can act in three distinct steps: (i) rDNA transcription, (ii) rRNA processing, and (iii) rRNA nuclear export. While NPM has been ascribed roles in both rRNA processing and nuclear export (36, 47), we are uncertain of its ability to regulate rDNA transcription. In fact, NPM and ARF are both found in the granular region of the nucleolus, relatively far removed from the sites of nucleolar rDNA transcription (8). However, we did observe significantly enhanced transcription of 47S rRNA in the absence of Arf, implying that ARF proteins might regulate this process either directly or indirectly. This is not unprecedented, given recent findings that human ARF interacts with topoisomerase I to inhibit rDNA transcription (3, 23). Additionally, nearly half of the basal ARF protein is not bound to NPM, and we therefore cannot rule out the possibility that this pool of ARF is bound to proteins involved in rDNA transcription.

We suggest that ARF is expressed at a low level in interphase cells to ensure that proper growth control is achieved. This would serve to keep the cell in metabolic check, prevent-
FIG. 11. Loss of NPM expression inhibits osteoclastogenesis in the Arf-/- cells. (A) Western blotting of macrophages infected with either control or lentivirus-targeted shRNA specific to NPM to confirm the gene knockdown. (B) TRAP staining of osteoclasts differentiated in vitro with RANKL and M-CSF for 6 days. (C) TRAP activity assay of equal numbers of osteoclasts from the indicated genotypes. *, P < 0.01. WT, wild type.

Our results suggest a novel and important role for basal ARF in maintaining protein synthetic homeostasis in nonmalignant cells. While NPM is certainly required for much of the ribosome biogenesis gains observed for Arf-deficient cells, other interesting nucleolar targets of basal ARF must certainly exist. Precise details of how they may be affected remain elusive. Understanding the nucleolar integration of disparate requirements for proliferation, growth, and ribosome biogenesis will deepen our knowledge of how proteins like ARF adapted from regulators of cellular homeostasis to bona fide tumor suppressors.

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