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The Opposing Roles of Nucleophosmin and the ARF Tumor Suppressor in Breast Cancer

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14. ABSTRACT
The ARF tumor suppressor protein plays an important role in the tumor surveillance of human cancer. In the search for novel ARF binding proteins, we uncovered NPM. Despite the important role ARF plays in the regulation of tumorigenesis, alterations selectively affecting its ability to negate NPM function have not been studied. In our proposed study, we aimed to determine the impact of ARF-NPM interactions in the pathogenesis of breast cancer. To this end, we have found that overexpression of NPM in the absence of ARF is a powerful transforming event. NPM promotes tumorigenesis without affecting genomic stability, implying that the subsequent tumors should remain diploid, a hallmark of ARF-null breast cancers. Indeed, when we analyzed sixty breast carcinomas, NPM was highly overexpressed in 50% of cases. We have begun further analyses of how NPM promotes tumor formation and have discovered that it does so through ribosome dysregulation, opening up the door to new therapeutic targets in breast cancer: protein synthesis.

15. SUBJECT TERMS
Nucleophosmin, ARF, Tumor Suppression, MMTV, Breast Cancer

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INTRODUCTION

As the most prominent of subnuclear structures, the nucleolus has long been recognized as the site for active transcription of ribosomal RNAs (rRNA) and ribosome assembly (6). Various nucleolar proteins, RNAs, and other factors have been suggested to be involved in this complex process of ribosome production and maturation (10). Recently, several groups reported the successful isolation and mapping of the mammalian nucleolar proteome (1, 2, 20). While the nucleolar proteome contains many proteins and ribonucleoproteins proposed to be involved in ribosome biogenesis, a remarkable number of proteins identified (>100) have no known function. The difficulty in assessing nucleolar protein function stems from early assumptions that all nucleolar proteins must be involved, in some way, with static ribosome biogenesis by virtue of their unique subcellular localization. However, a more contemporary view of the nucleolus as a dynamic nuclear organelle capable of regulating numerous cellular processes has led to a re-evaluation of nucleolar protein function(s) (14).

The ARF tumor suppressor is localized to nucleoli in mammalian cells and plays an important role in preventing tumor development. Our initial studies have focused on identifying targets for ARF tumor suppression. One such target, NPM, was recently identified by our lab. Nucleophosmin (NPM/B23) is an abundant phosphoprotein localized in the granular regions of the nucleolus (22). NPM was found to be highly expressed in proliferating cells (7, 8), and has been associated with a variety of cellular phenomena, including ribosomal biogenesis, protein chaperoning and centrosome duplication (8, 13, 18, 19). Structurally, NPM can exist in both a monomeric and multimeric state, although NPM multimers seem to dominate in the nucleolus and may be crucial for the assembly of maturing ribosomes (16, 17, 24). More importantly, NPM, along with other nucleolar proteins, has been suggested to actively mobilize into distinct subcellular pools, supporting the notion that NPM trafficking may contribute to some of its essential functions (4). Indeed, NPM exit from the nucleolus/nucleus is an essential event in S phase progression; inhibition of this trafficking by the nucleolar tumor suppressor ARF results in cell cycle arrest (5). Additionally, NPM is an essential nucleolar protein with loss of its expression resulting in severe attenuation of cellular proliferation and increased apoptosis (3, 5, 9, 11), underscoring NPM’s importance to the cell.

If nuclear exit of NPM plays a positive role in promoting cell growth and proliferation, what necessary function is it performing? While numerous proteins, such as Mdm2, cdc14p and TERT, are topologically restrained in the nucleolus following defined cellular cues, synthesis and export of newly synthesized ribosomal subunits from the nucleolus remains the only known nucleolar-specific event conserved throughout evolution (21). Recent work from Xenopus laevis and Saccharomyces cerevisiae has shown that nuclear export of ribosomes utilizes the CRM1-RanGTP export receptor pathway (12) as well as a nuclear adaptor protein NMD3 that is conserved from yeast to man (23).

Despite the seemingly important role ARF plays in breast tumor prevention, with over half of all breast cancers lacking ARF expression, studying the interplay between ARF and its targets, like NPM, has remained a largely unexplored theme. In my original proposal, I aimed to use a variety of molecular and genetic methods to more accurately address the broad question of how ARF restrains breast cancer progression.
Nucleophosmin (NPM/B23) is a key regulator in the regulation of a number of processes including centrosome duplication, genomic integrity and ribosome biogenesis. While the mechanisms underlying NPM function are largely uncharacterized, NPM loss results in severe dysregulation of developmental and growth-related events. We show that NPM utilizes a conserved CRM1-dependent nuclear export sequence in its amino-terminus to enable its shuttling between the nucleolus/nucleus and cytoplasm. In search of NPM trafficking targets, we biochemically purified NPM-bound protein complexes from HeLa cell lysates. Consistent with NPM’s proposed role in ribosome biogenesis, we isolated ribosomal protein L5 (rpL5), a known chaperone for the 5S ribosomal RNA. Direct interaction of NPM with rpL5 mediated the co-localization of NPM with maturing nuclear 60S ribosomal subunits, as well as newly exported and assembled 80S ribosomes and polysomes. Inhibition of NPM shuttling or loss of NPM blocked the nuclear export of rpL5 and 5S rRNA, resulting in cell cycle arrest and demonstrating that NPM and its nuclear export provide a unique and necessary chaperoning activity to rpL5/5S (See attached paper, Yu et. al. 2006).

The nucleolus, a highly specialized and structured organelle, has been described as the cell’s control center for ribosomal synthesis, maturation and assembly, with a host of proteins, RNAs and other factors being implicated in these processes. Recently, numerous proteins (cdc14, NPM, cyclin E, Mybbp1a, TERT and others) have been shown to continuously shuttle from the nucleolus to various subcellular compartments in a regulated manner, providing evidence that the nucleolus is a dynamic site of multiple cellular events.

One such protein, NPM/B23, has been linked to a variety of important cellular processes, both in and out of the nucleolus, including ribosome processing, molecular chaperoning, genomic integrity, centrosome duplication and transcriptional regulation. Initially, NPM which was imported into the nucleolus from the cytoplasm was presumed to move about the various compartments of the nucleus, a feature shared by many critical cell cycle regulators. This shuttling of proteins between the nucleus and cytoplasm is now recognized as a key mechanism for ensuring proper cell cycle progression. In previous reports, we and others identified NPM as a novel p53-independent target of the ARF tumor suppressor protein. We have since shown that, in response to hyperproliferative signals, nucleolar ARF directly binds NPM, effectively inhibiting NPM’s nucleocytoplasmic shuttling. Here, we have further explored the mechanism and significance of NPM intracellular trafficking. First, we have described the CRM1-dependent nuclear export of NPM, identifying the two leucine residues (42 and 44) that are critical to this process. In addition, we have shown that alteration of the NPM NES resulted in the failure of wild-type NPM to be exported out of the nucleolus, providing evidence that these mutations function in a dominant-negative fashion, through the formation of NPM-NPMdL hetero-multimers. Thus, NPMdL mimics the effects of ARF induction by directly impeding the nucleocytoplasmic shuttling of NPM through direct interaction, further demonstrating that NPM must exit the nucleolus/nucleus to maintain and promote cell growth.

We have previously proposed that targets of nucleolar sequestration might in fact “ride the ribosome” from the nucleolus to the cytoplasm to engage in growth promoting events. In agreement with this hypothesis, our findings reveal a direct interaction between NPM and rpL5, providing the first physical link between NPM and ribosomal subunits. Much of the fields’ focus has been on the putative role of rpL5 in delivering 5S rRNA to the nucleolus, following the initial transcription of 5S rRNA by RNA polymerase III in the nucleoplasm. However, it is also possible that rpL5 is a critical player in the export of the large ribosomal subunit (60S), containing 5S rRNA, from the nucleolus/nucleus to the cytoplasm after its assembly. Clearly, these latter events would render
themselves sensitive to NPM regulation, given that NPM provides the necessary export signals and chaperoning capabilities (via rpL5) required to transport components of the ribosome to the cytosol. Indeed, inhibition of NPM nuclear export via deletion or mutation of its NES prevented the trafficking of rpL5, an integral component of the 60S ribosomal subunit. Moreover, reduction of NPM expression through RNA interference completely abolished the cytosolic stores of rpL5, underscoring the absolute requirement for NPM in rpL5 nuclear export. Thus, our initial hypothesis of “riding the ribosome” should be revised to “taking the ribosome for a ride”.

While many components of the ribosome, including rpL5, encode their own NES, it is clear that a single NES forms a relatively weak interaction with CRM1, suggesting a requirement for additional NESs in the efficient export of complexes. Consequently, proteins like NPM and NMD3 may have evolved to serve this purpose. Additionally, NPM and rpL5 were found, in reduced amounts, in cytosolic 40S and 60S complexes, respectively, after LMB treatment implying that either these particular protein-ribosome complexes are fairly stable or that a minor fraction of NPM and rpL5 utilize CRM1-independent modes of transport from the nucleus. Considering that the predominant function of rpL5 is to bind and mobilize 5S rRNA molecules, it was not surprising that 5S transport was also NPM-sensitive, and thus NPM contributes to the efficient nuclear export of rpL5-5S rRNA complexes. However, NPM was present in 40S, 60S, 80S and polysomes in the cytoplasm, implying that NPM, free (within the 40S subunit) or bound to rpL5, remains associated with the mature ribosome as it assembles and forms actively translating polysomes in the cytosol. Taken together, these findings open up the possibility that NPM might transmit additional cues (beyond nuclear export) to cytosolic ribosomes during translation, consistent with nucleolus’ proposed role in dictating translation rates.

While it has been appreciated for several decades that changes in nucleolar structure are reliable markers of cellular transformation, experiments that provide a direct link between nucleolar dysfunction and tumorigenesis remain to be conducted. In fact, the nucleolus has largely been dismissed as a static organelle, having little-to-no impact on the overall well-being of the cell. However, this “nucleolar stigma” recently has been challenged with the discovery that tumor suppressors, such as p53 and ARF, play a direct role in regulating nucleolar processes. Interestingly, rpL5 is also a binding partner of Mdm2 and p53, suggesting that rpL5 may provide an intriguing mechanistic link between ARF and ARF-binding partners. Clearly, through its interaction with NPM, ARF is capable of inhibiting nuclear export of rpL5-5S rRNA complexes. Inhibition of NPM-directed rpL5-5S nuclear export by ARF or NPM defective shuttling mutants results in cell cycle arrest, demonstrating the importance of rpL5-5S export in maintaining cell proliferation. Moreover, NPM itself is a unique player in both the p53 and ARF responses, providing us with a glimpse of how this network of protein interactions may inevitably lend itself sensitive to oncogenic and tumor suppressive signals in determining tumorigenic cell fates.

Cellular growth (macromolecular synthesis) must be coupled to cell proliferation for proper transit through the cell cycle. The factors underscoring cell cycle control have been well studied. However, our knowledge of mechanisms that control cell growth in response to environmental cues is lacking. The nucleolus is at the center of growth sensing; it is the site of ribosome assembly, with nucleolar nucleophosmin (NPM) and p19ARF proteins antagonizing one another to either promote or inhibit growth, respectively. While ARF vigorously responds to hyperproliferative signals to shunt growth, we first noticed that nucleoli from Arf-/- MEFs displayed increased nucleolar area, suggesting that ARF might regulate key nucleolar functions in a pre-malignant cell. Ultrastructural analysis of Arf-/- nucleoli revealed increased irregularity and larger, more numerous fibrillar centers.

In accord with these dysmorphic nucleoli, ribosomal content and total protein synthetic rates were dramatically elevated in the absence of Arf. Similar results were obtained using targeted lentiviral RNA interference of Arf in wild-type cells, further implicating basal ARF proteins in the regulation of nucleolar structure and function. Finally, Arf-/- osteoclasts, post-mitotic cells whose activities are
intimately tied to their protein synthesis rates, exhibited enhanced differentiation and resorptive functions, demonstrating a physiological function for ARF in maintaining proper basal protein synthesis in vivo. Taken together, these data indicate that disruption of Arf greatly impacts ribosomal biogenesis and translational control, providing a significant teleological role for ARF as a monitor of cellular growth independent of its ability to prevent unwarranted cell cycle progression (see attached article, Apicelli et al., 2007).

I have also completed a novel nucleolar screen to identify novel regulators of cell growth in the absence of ARF. As seen in Figure 1, I identified the p68DDX5 RNA helicase as a protein whose nucleolar expression was increased in cells lacking Arf. Additionally, I have shown that DDX5 is amplified and overexpressed in nearly 50% of ER+ breast cancers (Figure 2). This opens up the possibility that DDX5 may provide a unique breast cancer therapeutic target. It is required for cell growth in Arf-null cells and it is overexpressed or amplified in primary breast cancers.

**KEY RESEARCH ACCOMPLISHMENTS**

- NPM shuttles rpL5 to the cytosol
- NPM actively shuttles rRNA from the nucleolus to the cytoplasm
- ARF inhibits NPM shuttling
- ARF inhibits rRNA nuclear export
- Loss of Arf results in tremendous gains in rRNA synthesis
- Loss of Arf causes severe changes in nucleolar morphology
• Osteoclasts lacking Arf exhibit amplified protein synthesis rates
• Basal ARF proteins have a role in regulating the homeostasis of the nucleolus
• Identification of p68DDX5 as a new potential therapeutic target

REPORTABLE OUTCOMES


• Received Ph.D. May 20, 2007.

CONCLUSIONS

This proposal was designed to investigate the opposing roles of ARF and NPM in the pathogenesis of breast cancer. In the first year of support, we generated a significant amount of data that helped our lab and others understand the intricate mechanism(s) by which ARF targets NPM to suppress tumor formation. In the second year, we discovered that NPM is overexpressed in human breast carcinomas and that in this context, it is a potent oncogene. In the third and final year of this award, I identified a novel role for basal ARF proteins in regulating ribosome biogenesis. I have published two papers on the significance of the ARF-NPM interaction. The final paper in 2007 could be quite important as it opens the door to a whole new array of putative anti-cancer targets that might be involved in protein translation. I have additionally identified a novel oncogenic RNA helicase that is amplified in breast cancers on chromosome 3. This protein is negatively regulated by ARF and forms active complexes with NPM. The lab is now carrying on this work in an attempt to understand the contribution of this novel helicase to breast cancer pathobiology. It is our hope that this helicase will present itself as a novel therapeutic target in breast cancer and current high throughput studies are underway in the lab to identify novel DDX5 inhibitors.
REFERENCES


APPENDICES


Nucleophosmin Is Essential for Ribosomal Protein L5 Nuclear Export

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Nucleophosmin (NPM/B23) is a key regulator in the regulation of a number of processes including centrosome duplication, maintenance of genomic integrity, and ribosome biogenesis. While the mechanisms underlying NPM function are largely uncharacterized, NPM loss results in severe dysregulation of developmental and growth-related events. We show that NPM utilizes a conserved CRM1-dependent nuclear export sequence in its amino terminus to enable its shuttling between the nucleolus/nucleus and cytoplasm. In search of NPM trafficking targets, we biochemically purified NPM-bound protein complexes from HeLa cell lysates. Consistent with NPM’s proposed role in ribosome biogenesis, we isolated ribosomal protein L5 (rpL5), a known chaperone for the 5S rRNA. Direct interaction of NPM with rpL5 mediated the colocalization of NPM with maturing nuclear 60S ribosomal subunits, as well as newly exported and assembled 80S ribosomes and polysomes. Inhibition of NPM shuttling or loss of NPM blocked the nuclear export of rpL5 and 5S rRNA, resulting in cell cycle arrest and demonstrating that NPM and its nuclear export provide a unique and necessary chaperoning activity to rpL5/5S.

As the most prominent of subnuclear structures, the nucleolus has long been recognized as the site of active transcription of rRNA and ribosome assembly (8). Various nucleolar proteins, RNAs, and other factors have been implicated in the complex process of ribosome production and maturation (18). Recently, several groups reported the successful isolation and mapping of the mammalian nucleolar proteome (1, 2, 44). While these studies clearly identified proteins and ribonucleoproteins with purported roles in ribosome biogenesis, a surprising number of proteins within the nucleolar proteome (>100) have no known function. In previous decades, it was assumed that all nucleolar proteins must somehow contribute to static ribosome biogenesis simply by virtue of their localization. However, more-recent findings have demonstrated that the nucleolus is a dynamic subnuclear organelle which regulates numerous cellular processes, prompting a broadened view of the potential functions of nucleolar proteins (28).

Nucleophosmin (NPM/B23) is an abundant phosphoprotein that resides within the granular regions of the nucleolus (46). Proliferating cells express NPM at high levels (9, 13), and NPM has been associated with a variety of cellular events, including ribosomal biogenesis, protein chaperoning, and centrosome duplication (13, 23, 35, 36). Structurally, NPM is present in both monomeric and multimeric states, although NPM multimers appear predominant in the nucleolus and may be crucial for the assembly of maturing ribosomes (33, 34, 53). Furthermore, NPM, along with other nucleolar proteins, is believed (or has been shown) to actively mobilize into distinct subcellular pools, supporting the notion that NPM trafficking may be essential for its (proper) function (6). Indeed, NPM’s transit from the nucleus/nucleus is an essential event in S phase progression; when NPM export was inhibited by the nuclear tumor suppressor ARF, cells arrested in G₁ (7). Moreover, loss of NPM expression results in severe attenuation of cellular proliferation and increased apoptosis (5, 7, 16, 19), underscoring NPM’s indispensable role within the cell.

Given that nuclear export of NPM promotes cell growth, we aimed to further elucidate the crucial roles of NPM’s trafficking. While numerous proteins, such as Mdm2, cdc14p, and telomerase reverse transcriptase, are topologically restrained in the nucleolus following receipt of defined cellular cues, newly synthesized ribosomal subunits must be exported from the nucleolus to promote proper protein translation in the cytosol (45). Recent work with X. laevis and S. cerevisiae has shown that nuclear export of ribosomes utilizes the CRM1-RanGTP export receptor pathway (20) as well as the conserved nuclear adaptor protein NMD3 (51). While investigating the critical nature of NPM trafficking, we noted that NPM’s exit from the nucleus also involved the classical CRM1-dependent nuclear export pathway. In search of proteins that are targeted for NPM-mediated nuclear export, we observed that nuclear and cytosolic NPM proteins directly bound to the ribosomal L5 protein (rpL5), a 60S subunit protein that chaperones the 5S rRNA into the nucleolus and out into the cytosol (31). Here we report that NPM mediates rpL5/5S nuclear export through a CRM1-dependent mechanism, allowing NPM to directly access the maturing ribosome and potentially regulate the protein translational machinery.

MATERIALS AND METHODS

Cell culture. HeLa and NIH 3T3 cells and wild-type (WT) mouse embryonic fibroblasts (MEFs) (ArtisOptimus, Carlsbad, CA) were maintained in Dulbecco’s...
modified Eagle’s medium with 10% fetal bovine serum, 2 mM glutamine, 0.1 mM nonessential amino acids, and 100 U penicillin and streptomycin.

**Plasmid constructs.** Vectors encoding full-length His-tagged murine NPM are described elsewhere (7). The His epitope-tagged NPM coding sequence was subcloned into pcDNA3.1 (Invitrogen) and pEGFP (Clontech) vectors. His-NPMdL, His-NPMdLs, or His-NPMdL mutants were generated using the primers 5′-GAAATTAGCAACGGCAAGCAAGCAATGAC-3′ (sense) and 5′-GTCAATTTATGCTTGGCTGTGTTCTATTGTTTTCT-3′ (antisense); 5′-GTAACACATCTGAGGACACCAAGAAGATGTTCAAGGCGTACTGATGTAACATGACTTCC-3′ (sense) and 5′-GAAATTAGCTTGTTGGCTGCTTAGTGTAACATGACTTCC-3′ (antisense); or 5′-GAAATTAGCTTGTTGGCTGCTTAGTGTAACATGACTTCC-3′ (antisense), respectively, by QuikChange mutagenesis (Stratagene). A myc-tagged NPC-M9 (40) in pcDNA3 and a green fluorescent protein (GFP)-tagged rpl5 plasmid (41) were generous gifts from Alan Dichi (University of Pennsylvania) and Jochem Hauber (Universität Erlangen-Nürnberg).

**Heterokaryon assay.** HeLa cells (2 × 10⁶) were seeded onto glass coverslips and transfected with plasmids. NIH 3T3 cells (6 × 10⁵) were seeded onto the HeLa cells 24 h posttransfection. Co cultured were then incubated for 30 min with cycloheximide (100 μg/ml), followed by incubation with 50% polyethylene glycol in phosphate-buffered saline for 105 s. Cocultures were incubated with Dulbecco’s modified Eagle’s medium containing cycloheximide (100 μg/ml) for an additional 4 h. Heterokaryons were fixed and stained with a rabbit anti-His antibody (Santa Cruz) or mouse anti-myc antibody (Zymed), followed by either fluorescein isothiocyanate-conjugated or rhodamine X-conjugated anti-rabbit or anti-mouse immunoglobulin (Pierce) as described previously (7). Nuclei were stained with Hoechst (Sigma). Fluorescence signals were detected using a Nikon epifluorescence microscope (Ni-2000) fitted with a Nikon FDX-35 camera.

**Immunoprecipitation and Western blot analysis.** Cells were translated as recommended by the manufacturer (Amaxa) with vectors encoding His-NPM, His-NPMdL, and GFP-rpl5 and lysed in binding buffer (25 mM Tris-Cl, pH 8, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40) 48 h after the Nucleofector process. Primary antibody to the NPM N terminus (custom rabbit; Sigma Genosys), GFP (Santa Cruz), His (Santa Cruz), rpl5 (12), or nonimmune rabbit serum (NRS) was added to the binding reaction mixtures. Antibodies were precipitated with protein A-Sepharose (Amersham). The precipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. NPM, His-tagged proteins, and GFP-tagged proteins were visualized by direct immunoblotting with NPM (Zymed), His (Santa Cruz), and rpl5 and GFP (Santa Cruz) antibodies, respectively.

**Fluid phase liquid chromatography.** For affinity chromatography, a rabbit polyclonal antibody recognizing the N terminus of NPM (Sigma) was coupled to N-hydroxysuccinimide-activated Sepharose (Amersham). HeLa cells were lysed in 20 mM Tris, pH 7.4, and 0.1% Tween 20 and sonicated. Lysates (600 μg) were injected onto the NPM N terminus column (custom rabbit; Sigma Genosys), GFP (Santa Cruz), His (Santa Cruz), rpl5 (12), or nonimmune rabbit serum (NRS) was added to the binding reaction mixtures. Immune complexes were precipitated with protein A-Sepharose (Amersham). The precipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. NPM, His-tagged proteins, and GFP-tagged proteins were visualized by direct immunoblotting with antibodies recognizing NPM (Zymed) and rpl5.

**RESULTS**

**NPM nuclear export requires a CRM1-dependent nuclear export signal involving leucines 42 and 44.** NPM is a ubiquitously expressed nuclear phosphoprotein capable of regulated nuclear import (6). When NPM is transiently expressed in mammalian cells, it localizes predominantly to the nucleolus. Moreover, using in vivo heterokaryon shuttling assays (50), we have previously shown that NPM readily shuttles between the nucleolus/nucleus and cytoplasm (7). NPC-M9, a nuclear hnRNP that readily shuttles in a CRM1-dependent manner, was used to investigate the underlying export mechanism of NPM both in the presence and absence of leptomycin B (LMB), a potent inhibitor of CRM1-mediated nuclear export (24). In the absence of LMB, NPM readily migrated from human nucleoli to human cephalic nuclei (speckled pattern, Fig. 1, Hoechst). As shown in Fig. 1A, NPM readily shuttles out of the human nucleolus, into the fused cytoplasm, and back into the mouse acceptor nucleus/nucleolus. Given that a wide range of shuttling proteins utilize the CRM1 transport protein for their nuclear export, we further investigated the underlying export mechanism of NPM both in the presence and absence of leptomycin B (LMB), a potent inhibitor of CRM1-mediated nuclear export (24). In the absence of LMB, NPM readily migrated from human nucleoli to mouse cephalic nuclei (Fig. 1A). In the presence of LMB, NPM failed to shuttle and was restricted to human nucleoli within heterokaryons (92% inhibition; Fig. 1B). The addition of LMB did not hinder the nucleocytoplasmic trafficking of Myc-NPC-M9, an hnRNP that readily shuttles in a CRM1-dependent nuclear export pathway (38).
short leucine-rich hydrophobic stretches of amino acids characteristic of CRM1-dependent nuclear export sequences (NESs) (Fig. 1C) (14, 15). In order to identify which region(s) of NPM contains its NES, we generated deletion mutants of NPM lacking either of the two potential NESs (NPM/H9004 42–61 and NPM/H9004 62–83). Using these NPM constructs, we again conducted interspecies heterokaryon assays. As shown in Fig. 2A, deletion of amino acids 42 to 61 of NPM (His-NPM/H9004 42–61) prevented its shuttling (100% inhibition) to mouse nucleoli. Importantly, a myc-tagged NPC-M9 shuttling control readily shuttled in the same human-mouse heterokaryon, indicating that these heterokaryons formed for each transfection condition in three independent experiments. The percentages of His-NPM shuttling in heterokaryons are given. DIC, differential interference contrast; α, anti. (C) Sequence alignment of putative NPM NESs with known NESs of CRM1-dependent nuclear export proteins (p53, protein kinase inhibitor [PKI], rev, and Mdm2). Critical hydrophobic residues are indicated in yellow.

FIG. 1. Nuclear export of NPM is CRM1 dependent. NIH 3T3 cells were seeded onto HeLa cells that had been transfected with His-NPM in combination with Myc-NPC-M9 (shuttling control) in the (A) absence or (B) presence of LMB. Heterokaryons were incubated in media containing cycloheximide for an additional 4 h before fixation. Heterokaryon formation was verified by phase-contrast microscopy, while His-NPM and Myc-NPC-M9 proteins were visualized with antibodies against His (red) and Myc (green), respectively. DNA was stained with Hoechst. Mouse nuclei are demarcated with dotted circles. Human and mouse nuclei are labeled h and m, respectively. These data are representative of at least five independent heterokaryons formed for each transfection condition in three independent experiments. The percentages of His-NPM shuttling in heterokaryons are given. DIC, differential interference contrast; α, anti. (C) Sequence alignment of putative NPM NESs with known NESs of CRM1-dependent nuclear export proteins (p53, protein kinase inhibitor [PKI], rev, and Mdm2). Critical hydrophobic residues are indicated in yellow.

NPM (His-NPM462–83) did not prevent NPM from shuttling between human and mouse nucleoli (6% inhibition; Fig. 2B), revealing that the putative NES resides within amino acids 42 to 61 of the NPM protein.

Since the type of NES recognized and bound by the CRM1 export receptor is dependent on closely spaced hydrophobic amino acids (particularly leucines) (14, 15), we introduced point mutations into the corresponding leucine residues within the NES of NPM (Leu-42 and Leu-44 to Ala-42 and Ala-44). First, we tested this NPM mutant (designated NPMdL for double-leucine mutant) with Myc-NPC-M9 for double-leucine mutant) with Myc-NPC-M9 as a shuttling control. As expected, NPMdL was unable to transit from a human nucleus to the cytoplasm and into a murine nucleus (100%
FIG. 2. Leucine 42 and leucine 44 are identified as critical nuclear export residues. NIH 3T3 cells were seeded onto HeLa cells that had been transfected with (A) His-NPM\(_{42-41}\), (B) His-NPM\(_{62-83}\), or (C) NPMdL in combination with Myc-NPC-M9. Ectopic NPM proteins and Myc-NPC-M9 proteins were visualized with antibodies against His (red) and Myc (green), respectively. DNA was stained with Hoechst. Mouse nuclei are demarcated with dotted circles. Human and mouse nuclei are labeled h and m, respectively. These data are representative of at least five independent heterokaryons formed for each transfection condition in three independent experiments. The percentages of His-NPM shuttling in heterokaryons are given. DIC, differential interference contrast; α, anti. (D) Sequence alignment of NPM homologues throughout evolution. Identical residues in all species are marked yellow, identical residues in at least seven species are highlighted blue, and conserved residues are marked green. Crystal structure features are identified above the sequences. The consensus NPM sequence for all 11 identified homologues is given, with conserved nuclear export leucines 42 and 44 marked with arrows (NES).
inhibition), indicating that these two leucine residues are critical for nuclear export of the NPM protein (Fig. 2C). Sequence alignment of numerous nucleophosmin homologues underscores the evolutionary importance of this amino-terminal export motif as it is nearly identical from zebrafish to humans (Fig. 2D).

**Heterogeneous complexes containing NPM NES mutants and wild-type NPM fail to shuttle.** Because NPM readily self-oligomerizes (32, 33, 34, 53), we considered the possibility that mutant NPM molecules could form hetero-oligomers with wild-type NPM proteins. To test this hypothesis, HeLa cells were transduced with His-tagged NPMdL expression vectors. Immunoprecipitation of His-NPMdL proteins revealed the coprecipitation of wild-type endogenous NPM proteins, demonstrating the formation of mutant–wild-type hetero-oligomers in cells (Fig. 3A, right). Additionally, cells transduced with His-NPMdL and GFP-NPM displayed formation of hetero-oligomers, as observed by coprecipitation of both proteins using antibodies directed at either epitope tag (His or GFP; Fig. 3A, left). Given our finding that mutant NPM forms oligomers with wild-type NPM, we next examined whether the NPM shuttling mutant NPMdL could also block wild-type NPM from shuttling. In the absence of the shuttling mutant, GFP-tagged NPM readily shuttled from human to mouse nucleoli (Fig. 3C). However, in the presence of His-tagged NPMdL, GFP-NPM was retained in human nuclei (Fig. 3D; 96% inhibition). Although we were unable to determine the exact stoichiometry between mutant proteins and wild-type proteins in the NPM oligomer, it is clear that overexpression of NPMdL severely impaired the shuttling activity of nearly all NPM oligomers.

**NPM associates with cytoplasmic and nuclear rpl5 ribosome complexes.** Previous studies have indicated that NPM might function as an integral component of ribosome maturation through its RNA binding activities (36). However, most hypotheses in this regard are largely based on the fact that NPM is nucleolar and, thus, most likely to be involved in the major process in the nucleolus: ribosome biogenesis. To formally test the nucleolar function of NPM, we examined the composition of in vivo NPM protein complexes in HeLa cell lysates. We generated a custom NPM polyclonal antibody affinity column and used a control nonimmune immunoglobulin column to preclear protein lysates. NPM complexes were eluted with increasing salt concentrations and visualized following SDS-PAGE and SYPRO-Ruby staining. As seen in Fig. 4A, we observed very little protein bound to our non-

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**FIG. 3.** NPM shuttling mutants act as dominant negative inhibitors of NPM nuclear export. (A, left) HeLa cells transduced with His-NPMdL and GFP-NPM were lysed, and the whole-cell lysate was subjected to immunoprecipitation (IP) with NRS or antibodies recognizing His and GFP epitopes. Precipitated protein complexes were separated by SDS-PAGE, and ectopic NPM proteins were visualized with antibodies against GFP and His epitopes. (A, right) HeLa cells transfected with His-NPMdL were lysed, and the whole-cell lysate was subjected to immunoprecipitation with NRS or antibodies recognizing His epitopes. Precipitated protein complexes were separated by SDS-PAGE, and ectopic mutant and endogenous wild-type NPM proteins were visualized with antibodies against NPM. Untransfected HeLa whole-cell lysate was loaded as a marker for endogenous NPM expression (lane 1). IB, immunoblot; α, anti; DIC, differential interference contrast. NIH 3T3 cells were seeded onto HeLa cells that had been transfected with GFP-NPM (B) alone or (C) in combination with His-NPMdL. Heterokaryon assays were performed, and His-NPMdL and GFP-NPM proteins were visualized with antibodies against His (red) or naturally emitting GFP spectra (green). DNA was stained with Hoechst. Mouse nuclei are demarcated with dotted circles. Human and mouse nuclei are labeled h and m, respectively. These data are representative of at least five independent heterokaryons formed for each transfection condition in three independent experiments. The percentages of GFP-NPM shuttling in heterokaryons are given.
immune rabbit serum column (lane 1). However, some proteins (lane 110118) were specifically eluted from the NPM antibody column (lane 3), including NPM and the previously known binding protein nucleolin. To determine whether the eluted proteins were in fact bound to the column through their interaction with NPM, we depleted NPM from HeLa cells using NPM-targeted RNA interference. Knockdown of NPM resulted in a loss of specific proteins bound and eluted from the NPM antibody column (lane 2). Protein bands were excised and identified using MALDI-TOF and tandem mass spectrometry analyses. Among those proteins bound to NPM, a cluster of proteins associated with ribosome biogenesis, including rpL5 and nucleolin, as well as the nuclear pore complex proteins Nup50 and Nup62, were identified (Fig. 4A and B), with nucleolin (C23) being the only known NPM binding protein (26, 27). Western blot analysis of NPM protein complexes verified the presence of these proteins in salt-eluted fractions (data not shown).

Given the novelty and potentially significant ribosome biology of finding rpL5 in the NPM complex, we focused on verifying the NPM-rpL5 interaction. Purified recombinant NPM, NPMdL, and rpL5 proteins (Fig. 5A, left panels) mixed overnight were coprecipitated (Fig. 5A, middle panels), demonstrating that the NPM-rpL5 interaction is direct and independent of the NPM nuclear export signal. To show that the interaction of recombinant proteins was specific, NPM and rpL5 were mixed overnight with recombinant p27kip1 proteins (equally charged proteins not bound to the NPM antibody column). Precipitated proteins exhibited no complex formation between NPM and p27kip1 or rpL5 and p27kip1 (Fig. 5A, right panels). Both NPM and rpL5 readily interact with RNAs through conserved nucleic acid binding domains. To determine whether RNA binding is required for the NPM-rpL5 interaction, HeLa lysates were subjected to RNase A treatment prior to coprecipitation of NPM-rpL5 complexes. Even in the presence of RNase A, NPM and rpL5 visibly formed in vivo protein complexes (Fig. 5B) indistinguishable from those from untreated cells and consistent with our earlier finding that the interaction can be recapitulated with purified recombinant proteins (Fig. 5A). While NPM and rpL5 formed complexes in vivo, serial immunoprecipitation of NPM proteins from HeLa lysates showed that NPM and rpL5 are not exclusive partners. We failed to detect rpL5 in some NPM complexes (Fig. 5C, 3o and 4o), and we also noted that there was a significant amount of rpL5 free from NPM complexes in the remaining supernatant (Fig. 5C, Sup), indicating that both NPM and rpL5 can exist in complexes independent of one another.

Having identified a critical member of the 60S ribosomal subunit, namely, rpL5, in NPM complexes, we wanted to evaluate the colocalization of NPM with ribosomes in vivo. In order to follow the spatial control of NPM-rpL5 complexes in vivo, we utilized the UV absorbance of the ribosome. Ribosomal protein L5 is known to supply the maturing 60S ribosomal subunit with 5S rRNA prior to nucleolar/nuclear export of the 60S subunit (47), providing NPM an ideal time to form nucleolar complexes with rpL5. Cytoplasmic and nuclear extracts of HeLa cells were subjected to sucrose gradient centrifugation, and the gradients were fractionated with continuous UV monitoring. As shown in Fig. 6, NPM associates with the 40S, 60S, 80S, and polysome fractions in the cytoplasm while nuclear pools of NPM associate with the 40S/pre-60S and 60S fractions in the nucleus. Consistent with previous reports (30), we found rpL5 associated with the 60S, 80S, and polysome fractions in the cytoplasm and the 40S/pre-60S and 60S fractions in the nucleus (Fig. 6). These data demonstrate that NPM and rpL5 are localized with the maturing 60S ribosomal subunits in the nucleus and are maintained in the mature ribo-
some once it reaches the cytosol. They also indicate that NPM also associates with the 40S subunit, which is devoid of rpL5 (Fig. 6) (30).

Transduction of HeLa cells with His-NPMdL resulted in a dramatic redistribution of rpL5 in cytosolic ribosomes; rpL5 was maintained in the 60S subunits but severely reduced in 80S ribosomes (Fig. 6, middle left panels). Given these findings, we cannot rule out the possibility that rpL5 proteins are still capable of some NPM-independent shuttling. However, it is more likely that rpL5 association with cytosolic ribosomes in the presence of NPMdL is a result of preexisting, stable cytosolic rpL5 complexes. This notion is further substantiated by the treatment of HeLa cells with LMB. LMB treatment yielded results that were consistent with NPMdL overexpression (Fig. 6, lower panel). Both NPM and rpL5 proteins were found in the cytosol of LMB-treated cells (at reduced levels), even though the nuclear export of both proteins is LMB sensitive. This finding suggests that some preexisting cytosolic NPM and rpL5 ribosome complexes are fairly stable (−24 h) and that, if either protein utilizes CRM1-independent export, it is minimal.

**NPM is required for rpL5 nuclear export.** Having demonstrated a reduction of rpL5 associated with cytosolic ribosome subunits in the absence of NPM nuclear export signals, we next

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**FIG. 5.** NPM interacts directly with rpL5. (A, left) Recombinant NPM, NPMdL, and rpL5 were purified from bacterial lysates using Ni-nitrilotriacetic acid affinity chromatography. Purified proteins were separated by SDS-PAGE and detected with Coomassie blue stain. (A, middle) Purified NPM or NPMdL proteins were incubated overnight with rpL5 and immunoprecipitated (IP) with NRS or antibodies recognizing NPM or rpL5. Precipitated proteins were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with NPM and rpL5 antibodies. (A, left) Purified NPM or rpL5 proteins were incubated overnight with recombinant p27 and immunoprecipitated with NRS or antibodies recognizing NPM, rpL5, or p27. Precipitated proteins were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with NPM, rpL5, and p27 antibodies. (B) Proteins from HeLa cell lysates were immunoprecipitated with NRS, rpL5 antibody, or NPM antibodies. Precipitated proteins were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with NPM and rpL5 antibodies. Alternatively, HeLa lysates were pretreated for 1 h with RNase A prior to immunoprecipitation as described above. (C) HeLa lysates were subjected to serial immunoprecipitation with NPM antibodies (lanes 1 to 4). Precipitated proteins and proteins in the final supernatant (unbound) were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with antibodies recognizing NPM and rpL5.

**FIG. 6.** NPM and rpL5 colocalize with nuclear and cytosolic ribosome subunits. HeLa cells transduced with vector (top panels) or His-NPMdL (middle panels) or treated with LMB (bottom panels) were divided into cytoplasmic and nuclear fractions and subjected to sucrose gradient centrifugation. Absorbance was monitored at 254 nm, and fractions containing 40S, 60S, 80S, and polysome units were collected. Proteins from each fraction were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with antibodies recognizing NPM, the His epitope, and rpL5. α, anti.
FIG. 7. NPM nuclear export signals are required for the efficient export of GFP-rpL5. (A) HeLa cells either untransfected or transfected with GFP-tagged L5 for 48 h were harvested and lysed. Proteins were immunoprecipitated (IP) with NRS or a rabbit GFP antibody. Precipitated proteins were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with GFP and NPM antibodies. α, anti; DIC, differential interference contrast. Loading inputs are indicated. (B to E) NIH 3T3 cells were seeded onto HeLa cells that had been transfected with GFP-rpL5 in combination with (B and C) His-NPM, (D) His-NPMΔ42-61, and (E) His-NPMdl. Additionally, HeLa cells in panel C were treated with LMB for 18 h prior to fusion. Heterokaryon assays were performed with NPM and GFP-rpL5 proteins being visualized with antibodies against His (red) and naturally emitting GFP spectra (green), respectively. DNA was stained with Hoechst. Mouse nuclei are demarcated with dotted circles. Human and mouse nuclei are labeled h and m, respectively. These data are representative of at least five independent heterokaryons formed in three independent experiments. The percentages of heterokaryons exhibiting GFP-rpL5 shuttling are given.
FIG. 8. NPM is essential for rpL5 nuclear export. (A) HeLa cells (−) or cells transduced with siRNAs encoding either scrambled control or NPM-specific sequences were harvested 72 h posttransduction for Western blot analysis. Proteins separated by SDS-PAGE were transferred to PVDF membranes and immunoblotted with antibodies recognizing NPM and γ-tubulin. α, anti. (B) HeLa cells (−) or cells transduced with siRNAs encoding either scrambled control or NPM-specific sequences were harvested 72 h posttransduction for cellular fractionation. Proteins from nuclear (N) and cytosolic (C) fractions were analyzed by SDS-PAGE and immunoblotted with antibodies recognizing rpL5, SOD (cytoplasm control), and lamin A/C (nuclear control). (C) HeLa cells were transfected with His-NPM or His-NPMDL, and 24 h later equal numbers of cells were subjected to fractionation into cytoplasmic (C) and nuclear (N) extracts. L5 protein was detected by Western blot analysis (top panels; WB). Lamin A/C and SOD are shown as nuclear and cytoplasmic fractionation controls, respectively (top panels; WB). 5S rRNA was detected by Northern blot analysis of total RNA extracted from the nuclear and cytosolic fractions (bottom panel; NB). The ratios of nuclear to cytoplasmic
examined the direct influence of NPM shuttling mutants on rpL5 nuclear export using a previously characterized GFP-tagged rpL5 protein (41). To confirm that GFP-rpL5 retained the NPM-binding properties of the endogenous rpL5 protein, we transiently overexpressed GFP-rpL5 in HeLa cells and performed Western blot analysis of GFP-immunoprecipitated complexes. As shown in Fig. 7A, precipitated GFP-rpL5 complexes contained endogenous NPM, confirming that the GFP moiety does not adversely affect the formation of NPM-rpL5 complexes in vivo. GFP-rpL5 and His-NPM readily migrated from human nucleoli to mouse nucleoli, as visualized in interspecies heterokaryons (Fig. 7B). However, in the presence of LMB, both GFP-rpL5 and His-NPM failed to shuttle (95% inhibition; Fig. 7C). Introduction of NPM shuttling mutant NPMΔ42–61 or NPMΔL inhibited GFP-rpL5 shuttling into mouse nucleoli, restricting its expression to human nuclei (Fig. 7D and E; 96% and 100% inhibition, respectively), establishing that NPM nuclear export is required for the export of rpL5. To more definitively show that NPM is required for rpL5 nuclear export, we knocked down NPM expression in HeLa cells (Fig. 8A). Cells lacking NPM protein expression failed to accumulate rpL5 in the cytoplasm, while cells transduced with scrambled small interfering RNA (siRNA) as a control exhibited an equal distribution of rpL5 between the nucleus and cytoplasm (Fig. 8B). These data underscore the necessity of NPM proteins for the efficient transport of rpL5 out of the nucleus and into the cytoplasm.

Ribosomal protein L5 is known to bind specifically to the mature 5S rRNA and aid in its nucleocytoplasmic transport (31, 37, 47). We hypothesized that NPM export, through its influence on rpL5, is the critical determinant for 5S rRNA nuclear export. To test this hypothesis, we performed Northern blot analysis of similar cellular fractions in the presence and absence of NPM shuttling. Indeed, in the presence of dominant negative NPM shuttling mutants, 5S rRNA failed to accumulate in the cytosol and instead was retained in the nucleus in a ratio similar to that for rpL5 (Fig. 8C). In addition to our fractionation studies, we performed RNA FISH to visualize the localization of steady-state levels of 5S rRNA. As shown in Fig. 8D, 5S rRNA was distributed throughout the nucleoli/nuclei and cytoplasm of HeLa cells transduced with empty vector as well as with wild-type NPM. Consistent with our fractionation data, inhibition of NPM and rpL5 nuclear export with LMB or NPMΔL resulted in a severe attenuation of 5S rRNA export to the cytosol (Fig. 8D).

To further expand on this theme, we transduced wild-type MEFs with ARF, a known inhibitor of NPM nuclear export (7). Again, in the presence of the ARF tumor suppressor, 5S rRNA failed to transit to the cytosol and instead was retained in the nucleoplasm (Fig. 8E). These data imply that ARF and NPM mutants defective in shuttling act similarly to prevent rpL5-5S rRNA nuclear export. To determine whether NPM shuttling mutants also prevent cell cycle progression, HeLa cells transduced with NPM expression constructs were labeled with 5-bromo(deoxy)uridine (BrdU) to measure active DNA synthesis. Similar to ARF’s known cell cycle arrest properties (7), cells expressing NPMΔL or NPMΔ42–61 failed to enter S phase (Fig. 8F). Thus, NPM shuttling activity is not only required for the nuclear export of the rpL5-5S rRNA complex but also necessary for continued cell proliferation.

**DISCUSSION**

The nucleolus, a highly specialized and structured organelle, has been described as the cell’s control center for ribosomal synthesis, maturation, and assembly, with a host of proteins, RNAs, and other factors being implicated in these processes (8). Recently, numerous proteins (cdc14, NPM, cyclin E, Mybbpl, telomerase reverse transcriptase, and others) have been shown to continuously shuttle from the nucleolus to various subcellular compartments in a regulated manner, providing evidence that the nucleolus is a dynamic site of multiple cellular events (4, 7, 21, 22, 52).

One such protein, NPM/B23, has been linked to a variety of important cellular processes, both in and out of the nucleolus, including ribosome processing, molecular chaperoning, maintenance of genomic integrity, centrosome duplication, and transcriptional regulation (9, 10, 13, 16, 23, 35). Initially, NPM which was imported into the nucleolus from the cytoplasm was presumed to move about the various compartments of the nucleus (6), a feature shared by many critical cell cycle regulators. This shuttling of proteins between the nucleus and cytoplasm is now recognized as a key mechanism for ensuring proper cell cycle progression (39, 43). In previous reports, we and others identified NPM as a novel p53-independent target of the ARF tumor suppressor protein (5, 7, 19). We have since shown that, in response to hyperproliferative signals, nuclear ARF directly binds NPM, effectively inhibiting NPM’s nucleocytoplasmic shuttling. Here, we have further explored the mechanism and significance of NPM intracellular trafficking. First, we have described the CRM1-dependent nuclear export of NPM, identifying the two leucine residues (42 and 44) that are critical to this process. In addition, we have shown that alteration of the NPM NES resulted in the failure of wild-type NPM to be exported out of the nucleolus, providing evidence that these mutations function in a dominant-negative fashion,
through the formation of NPM-NPMDL heteromultimers. Thus, NPMDL mimics the effects of ARF induction by directly impeding the nucleocytoplasmic shuttling of NPM through direct interaction, further demonstrating that NPM must exit the nucleolus/nucleus to maintain and promote cell growth.

We have previously proposed that targets of nucleolar sequestration might in fact “ride the ribosome” from the nucleolus to the cytoplasm to engage in growth-promoting events (45). In agreement with this hypothesis, our findings reveal a direct interaction between NPM and rpL5, providing the first physical link between NPM and ribosomal subunits. Much of the field’s focus has been on the putative role of rpL5 in delivering 5S rRNA to the nucleolus, following the initial transcription of 5S rRNA by RNA polymerase III in the nucleoplasm (31, 37, 47). However, it is also possible that rpL5 is a critical player in the export of the large ribosomal subunit (60S), containing 5S rRNA, from the nucleolus/nucleus to the cytoplasm after its assembly. Clearly, the latter events would render themselves sensitive to NPM regulation, given that NPM provides the necessary export signals and chaperoning capabilities (via rpL5) required to transport components of the ribosome to the cytosol. Indeed, inhibition of NPM nuclear export via deletion or mutation of its NES prevented the trafficking of rpL5, an integral component of the 60S ribosomal subunit. Moreover, reduction of NPM expression through RNA interference completely abolished the cytosolic stores of rpL5, underscoring the absolute requirement for NPM in rpL5 nuclear export. Thus, our initial hypothesis of “riding the ribosome” should be revised to “taking the ribosome for a ride.”

While many components of the ribosome, including rpL5, encode their own NESs, it is clear that a single NES forms a relatively weak interaction with CRM1 (25), suggesting a requirement for additional NESs in the efficient export of complexes. Consequently, proteins like NPM and NMD3 may have evolved to serve this purpose. Additionally, NPM and rpL5 were found, in reduced amounts, in cytosolic 40S and 60S complexes, respectively, after LMB treatment, implying either that these particular protein-ribosome complexes are fairly stable or that minor fractions of NPM and rpL5 utilize CRM1-independent modes of transport from the nucleus. Considering that the predominant function of rpL5 is to bind and mobilize 5S rRNA molecules, it was not surprising that 5S transport was also NPM sensitive, and thus NPM contributes to the efficient nuclear export of rpL5-5S rRNA complexes. However, NPM was present in 40S, 60S, 80S, and polysomes in the cytoplasm, implying that NPM, free (within the 40S subunit) or bound to rpL5, remains associated with the mature ribosome as it assembles and forms actively translating polysomes in the cytosol. Taken together, these findings open up the possibility that NPM might transmit additional cues (beyond nuclear export) to cytosolic ribosomes during translation, consistent with nucleolus’s proposed role in dictating translation rates (28).

While it has been appreciated for several decades that changes in nucleolar structure are reliable markers of cellular transformation, experiments that provide a direct link between nucleolar dysfunction and tumorigenesis remain to be conducted. In fact, the nucleolus has largely been dismissed as a static organelle, having little to no impact on the overall well-being of the cell. However, this “nucleolar stigma” recently has been challenged with the discovery that tumor suppressors, such as p53 and ARF, play a direct role in regulating nucleolar processes (5, 7, 42, 49). Interestingly, rpL5 is also a binding partner of Mdm2 and p53 (12, 17, 29), suggesting that rpL5 may provide an intriguing mechanistic link between ARF and ARF-binding partners. Clearly, through its interaction with NPM, ARF is capable of inhibiting nuclear export of rpL5-5S rRNA complexes. Inhibition of NPM-directed rpL5-5S nuclear export by ARF or NPM mutants defective in shuttling results in cell cycle arrest, demonstrating the importance of rpL5-5S export in maintaining cell proliferation. Moreover, NPM itself is a unique player in both the p53 and ARF responses (10, 11), providing us with a glimpse of how this network of protein interactions may inevitably become sensitive to oncogenic and tumor-suppressive signals in determining tumorigenic cell fates.

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Therapeutic Targets in the ARF Tumor Suppressor Pathway

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Abstract: One of the outstanding fundamental questions in cancer cell biology concerns how cells coordinate cellular growth (or macromolecular synthesis) with cell cycle progression and mitosis. Intuitively, rapidly dividing cells must have some control over these processes; otherwise cells would continue to shrink in volume with every passing cycle, similar to the cytoreductive divisions seen in the very early stages of embryogenesis. The problem is easily solved in unicellular organisms, such as yeast, as their growth rates are entirely dependent on nutrient availability. Multicellular organisms such as mammals, however, must have acquired additional levels of control, as nutrient availability is seldom an issue and the organism has a prodigious capacity to store necessary metabolites in the form of glycogen, lipids, and protein. Furthermore, the specific needs and specialized architecture of tissues must constrain growth for growth’s sake; if not, the necessary function of the organ could be lost. While certainly a myriad of mechanisms for preventing this exist via initiating cell death (e.g. apoptosis, autophagy, necrosis), these all depend on some external cue, such as death signals, hypoxia, lack of nutrients or survival signals. However there must also be some cell autonomous method for surveying against inappropriate growth signals (such as oncogenic stress) that occur in a stochastic fashion, possibly as a result of random mutations. The ARF tumor suppressor seems to fulfill that role, as its expression is near undetectable in normal tissues, yet is potently induced by oncogenic stress (such as overexpression of oncogenic Ras or myc). As a result of induced expression of ARF, the tumor suppressor protein p53 is stabilized and promotes cell cycle arrest. Mutations or epigenetic alterations of the INK4a/Arf locus are second only to p53 mutations in cancer cells, and in some cancers, alterations in both Arf and p53 observed, suggesting that these two tumor suppressors act coordinately to prevent unwarranted cell growth and proliferation. The aim of this review is to characterize the current knowledge in the field about both p53-dependent and independent functions of ARF as well as to summarize the present models for how ARF might control rates of cell proliferation and/or macromolecular synthesis. We will discuss potential therapeutic targets in the ARF pathway, and some preliminary attempts at enhancing or restoring the activity of this important tumor suppressor.

Keywords: ARF, Mdm2, p53, nucleophosmin, nucleolus, ribosome biogenesis.

INTRODUCTION

Since its discovery as a product of the alternate reading frame of the mouse INK4a/Arf locus [1], the ARF tumor suppressor has been identified as a key sensor of hyperproliferative signals such as those emanating from the Ras and Myc oncoproteins [2-4]. p16INK4a and ARF are transcribed from separate and unique first exons (over 10 kilobases apart) which splice into two shared exons [1] (Fig. (1)). While INK4a and ARF share considerable homology at the DNA level (nearly 70%), the translated proteins are completely distinct from one another. This is due to the unprecedented splicing utilized by ARF which causes a frame shift (alternate reading frame) in the coding region of exon two (and thus providing the ARF moniker). The INK4a/Arf locus is frequently targeted for loss of function in diverse human cancers and both p16INK4a and ARF function as tumor suppressors despite a lack of sequence similarity. ARF is a highly basic (predicted pI=11), insoluble protein which exhibits little structure apart from a pair of alpha helices at its amino terminus [5]. Both mouse and human ARF have been widely studied in the decade since their discovery. Although they differ in size (mouse ARF is 19 kDa and human ARF is 14 kDa) and exhibit only 49% sequence identity, the functions of the ARF proteins appear to be conserved in man and mice. ARF is a bona fide tumor suppressor. Ectopic ARF is capable of arresting immortal rodent cell lines as well as transformed human cells [6, 7], a classic and requisite property of tumor suppressors. The ability of ARF to inhibit cell cycle progression in numerous cell types, suggested that ARF had powerful growth-inhibitory functions in the cell and prompted many researchers to study the in vivo ability of ARF to prevent tumorigenesis.

LOSS OF ARF IN CANCER

Animal studies have been very valuable in elucidating the function of murine p19ARF as a tumor suppressor. Arf-null mice, generated by specifically targeting exon 1β, exhibit spontaneous tumor formation as early as 8 weeks of age [3]. Sarcomas and lymphomas are the most common tumors observed in Arf-deficient mice. Tumor development is also accelerated in newborn Arf-null mice treated with carcinogens when compared to wild-type mice [3, 8], demonstrating that ARF protects cells against aberrant cell growth and proliferation caused by increased mutagenesis. Another interesting facet of ARF biology is the observed immortal phenotype of cultured Arf-null mouse endothelial fibroblasts (MEFs). Unlike its wild-type counterparts which senesce after 10-15 passages in vitro, Arf-deficient MEFs are capable of growing indefinitely in culture [3]. Moreover, immortal Arf-null MEFs are susceptible to transformation by oncogenic Ras alone, indicating that loss of Arf can be substituted for Myc overexpression in classic cooperating transformation assays with Ras [3]. This finding was further refined through experiments that showed the acute loss of Arf as a major event in Myc-induced cellular immortalization in vivo [9].

Consistent with initial findings in mice, frequent mutation or deletion of the INK4a/Arf locus have been found in numerous human cancers was discovered. It is difficult, however, to assess the relative importance of p16INK4a and ARF individually since mutation or deletion at the INK4a/Arf locus frequently affects both proteins. Mutation of exon 1β, which would specifically affect only ARF, is a relatively rare event. However, a germline deletion of a region containing exon 1β of p14ARF but leaving the INK4a gene intact was identified in a family prone to melanoma and neural system tumor development [10]. An exon 1β mutation that altered the growth-inhibitory properties and intracellular localization of human p14ARF was observed and characterized in a melanoma patient [11]. Building on these early reports, ARF haploinsufficiency due to a germline mutation in exon 1β.
Fig. (1). INK4a/Arf locus and effector pathways. A. INK4a inhibits the activity of cyclin D-cdk4 holoenzymes to keep Rb hypo-phosphorylated and active. ARF blocks the activity of Mdm2 thereby activating p53 as well as inhibiting NPM shuttling activity to prevent ribosome biogenesis. In addition, ARF attenuates the activity of several other proteins although the biological outcomes of these interactions are still under intense study. B. The INK4a/Arf locus. Using an uniquely conserved arrangement of exons, INK4a (Exon 1α, light green) and ARF (Exon 1β, dark green) splice into common 2nd and 3rd exons but in alternate reading frames to produce to distinctive amino acid sequences and structurally unrelated proteins.

was observed in a family of three individuals with melanoma or breast cancer. However, somatic changes at the INK4a/Arf locus discovered in one of the melanoma samples resulted in inactivation of both p14<sup>ARF</sup> and p16<sup>INK4a</sup> [12]. Recently, a germline deletion of exon 1β was discovered in two patients from a family predisposed to cutaneous malignant melanoma. A heterozygous germline missense mutation in exon 1β was also found in another individual with melanoma [13]. More commonly, however, exon 2 is the site of mutation, affecting either p16<sup>INK4a</sup>, ARF, or both proteins. Some of these exon 2 mutations alter ARF localization and affect its regulation of downstream target proteins [14-16]. Silencing of the Arf gene promoter through hypermethylation is frequently observed in low-grade diffuse astrocytomas [17], oligodendroglial tumors [18, 19], ependymal tumors [19, 20], kidney cancer [21], hepatocellular carcinoma [22], and oral squamous cell carcinomas [23]. Simultaneous methylation of both Arf and INK4a is also a common occurrence in samples from the accelerated phase of chronic myeloid leukemia (CML) [24]. In one study, loss of p14<sup>ARF</sup> expression was observed in 38/50 glioblastomas, with 29 displaying either homozygous deletion or hypermethylation of Arf. While deletion of both p14<sup>ARF</sup> and p16<sup>INK4a</sup> was common, Arf was specifically deleted in nine of the samples [25], indicating that ARF alone is often a major target in human tumor progression (for a complete list of ARF-specific alterations in human cancers, see Table 1).

NUCLEAR LOCALIZATION

ARF is predominantly localized to the nucleolus [26, 27], a dynamic, membrane-less, subnuclear organelle which controls ribosome biogenesis [28] (Fig. (2A)). Within the nucleolus, ARF resides in the granular region, which contains maturing ribosomes. During mitosis, the nucleolus disintegrates causing nucleolar proteins to disperse throughout the nucleoplasm [29]. Interestingly, nucleolar dissociation is linked with an increase in p53 [30], suggesting that the nucleolus may be an important structure involved in regulating the p53 pathway. Nucleolar breakdown due to mitosis or stress may allow transient ARF activity in the nucleoplasm [31, 32], however non-nucleolar ARF exhibits decreased stability [33]. Importantly, the last two years have been marked with increased understanding of the role of the nucleolus in sensing both environmental and oncogenic stress within the cell [30, 28].
<table>
<thead>
<tr>
<th>Disease</th>
<th>ARF alteration</th>
<th>Occurrence</th>
<th>ARF specificity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>acute lymphoblastic leukemia</td>
<td>deletion</td>
<td>40% ; 45%</td>
<td>No</td>
<td>[97, 98]</td>
</tr>
<tr>
<td>adult acute myelogenous leukemia</td>
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<td>[99]</td>
</tr>
<tr>
<td>adult T-cell leukemia / lymphoma</td>
<td>methylation</td>
<td>6%</td>
<td>ND</td>
<td>[100]</td>
</tr>
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<td>methylation</td>
<td>25%</td>
<td>ND</td>
<td>[101]</td>
</tr>
<tr>
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<td>1. loss of mRNA expression</td>
<td>71%</td>
<td>20%</td>
<td>[102]</td>
</tr>
<tr>
<td></td>
<td>2. mutation OR deletion</td>
<td>67%</td>
<td>No</td>
<td>[103]</td>
</tr>
<tr>
<td></td>
<td>3. methylation</td>
<td>50%</td>
<td>ND</td>
<td>[104]</td>
</tr>
<tr>
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<td>methylation OR deletion</td>
<td>40%</td>
<td>25%</td>
<td>[105]</td>
</tr>
<tr>
<td>atypical meningioma</td>
<td>methylation</td>
<td>26%</td>
<td>40%</td>
<td>[106]</td>
</tr>
<tr>
<td>bladder cancer</td>
<td>1. methylation</td>
<td>56% ; 31%</td>
<td>67% ; N/D</td>
<td>[110, 111]</td>
</tr>
<tr>
<td></td>
<td>2. deletion</td>
<td>43% ; 14%</td>
<td>Yes ; No</td>
<td>[112, 113]</td>
</tr>
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<td>19%</td>
<td>60%</td>
<td>[114]</td>
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<tr>
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<td>33%</td>
<td>ND</td>
<td>[115]</td>
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<tr>
<td>breast carcinoma</td>
<td>1. methylation</td>
<td>24% ; 19%</td>
<td>54% ; 58%</td>
<td>[117, 118]</td>
</tr>
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<td></td>
<td>2. deletion OR methylation</td>
<td>20%</td>
<td>ND</td>
<td>[119]</td>
</tr>
<tr>
<td>cholangiosarcoma</td>
<td>methylation</td>
<td>25% ; 38%</td>
<td>62% ; ND</td>
<td>[120, 121]</td>
</tr>
<tr>
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<td>1. methylation</td>
<td>40%</td>
<td>17%</td>
<td>[24]</td>
</tr>
<tr>
<td></td>
<td>2. mutation</td>
<td>23%</td>
<td>71%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3. methylation AND mutation</td>
<td>17%</td>
<td>60%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4. methylation OR missense mutation</td>
<td>47%</td>
<td>14%</td>
<td></td>
</tr>
<tr>
<td>clear cell sarcoma</td>
<td>deletion or mutation</td>
<td>14%</td>
<td>No</td>
<td>[122]</td>
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<tr>
<td>colon cancer</td>
<td>methylation</td>
<td>33% ; 22% ; 33%</td>
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<td>[118, 123, 124]</td>
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<td>methylation</td>
<td>32% ; 40%</td>
<td>ND ; ND</td>
<td>[125, 126]</td>
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<td>28% ; 38% ; 51%</td>
<td>52% ; 50% ; 70%</td>
<td>[126-128]</td>
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<tr>
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<td>deletion</td>
<td>67% ; 46%</td>
<td>9% ; No</td>
<td>[129, 130]</td>
</tr>
<tr>
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<td>8%</td>
<td>No</td>
<td>[131]</td>
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<td></td>
<td>2. methylation</td>
<td>40%</td>
<td>75%</td>
<td></td>
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<tr>
<td></td>
<td>3. mutation OR methylation</td>
<td>43%</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>EBV-associated gastric carcinoma</td>
<td>methylation</td>
<td>100%</td>
<td>No</td>
<td>[132]</td>
</tr>
<tr>
<td>ependymoma</td>
<td>methylation</td>
<td>21% ; 28%</td>
<td>96% ; Yes</td>
<td>[20, 19]</td>
</tr>
<tr>
<td>epithelial ovarian cancer</td>
<td>mutation, methylation, OR loss of mRNA expression</td>
<td>22%</td>
<td>40%</td>
<td>[133]</td>
</tr>
<tr>
<td>esophageal squamous cell carcinoma</td>
<td>1. deletion</td>
<td>33% ; 14%</td>
<td>Yes ; ND</td>
<td>[134, 135]</td>
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<td>2. methylation</td>
<td>15% ; 52%</td>
<td>No ; 73%</td>
<td></td>
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<td></td>
<td>3. mutation</td>
<td>ND ; 19%</td>
<td>ND ; No</td>
<td></td>
</tr>
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<td>Ewing sarcoma</td>
<td>1. deletion</td>
<td>13%</td>
<td>No</td>
<td>[136, 137]</td>
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<tr>
<td></td>
<td>2. methylation, deletion, OR mutation</td>
<td>13%</td>
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### Table 1. continued

<table>
<thead>
<tr>
<th>Disease</th>
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<th>Occurrence</th>
<th>ARF specificity</th>
<th>References</th>
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<tbody>
<tr>
<td>gall bladder / bile duct carcinomas</td>
<td>methylation</td>
<td>46%</td>
<td>50%</td>
<td>[138]</td>
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<td>methylation</td>
<td>24% ; 10%</td>
<td>Yes ; ND</td>
<td>[139, 140]</td>
</tr>
<tr>
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<td>deletion OR methylation</td>
<td>32%</td>
<td>No</td>
<td>[141]</td>
</tr>
<tr>
<td>glioblastoma</td>
<td>1. deletion</td>
<td>55%</td>
<td>No</td>
<td>[142, 25, 143]</td>
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<td></td>
<td>2. deletion OR methylation</td>
<td>58% ; 67%</td>
<td>45% ; ND</td>
<td></td>
</tr>
<tr>
<td>glioma</td>
<td>deletion</td>
<td>41%</td>
<td>No</td>
<td>[144]</td>
</tr>
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<td>head and neck squamous cell carcinoma</td>
<td>1. methylation</td>
<td>19% ; 16%</td>
<td>85% ; ND</td>
<td>[145, 146]</td>
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<td></td>
<td>2. mutation</td>
<td>35%</td>
<td>6%</td>
<td>[147]</td>
</tr>
<tr>
<td></td>
<td>3. methylation, mutation, OR deletion</td>
<td>43%</td>
<td>16%</td>
<td>[146]</td>
</tr>
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<td>hepatocellular carcinoma</td>
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<td>25%</td>
<td>No</td>
<td>[148]</td>
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<tr>
<td></td>
<td>2. methylation</td>
<td>42%</td>
<td>ND</td>
<td>[22]</td>
</tr>
<tr>
<td></td>
<td>3. deletion OR mutation</td>
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<td>[149]</td>
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<td>4. deletion, methylation, OR mutation</td>
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<td>No</td>
<td>[150]</td>
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<td>methylation</td>
<td>33%</td>
<td>ND</td>
<td>[151]</td>
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<tr>
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<td>methylation</td>
<td>70%</td>
<td>86%</td>
<td>[152]</td>
</tr>
<tr>
<td>intracranial germ cell tumor</td>
<td>deletion OR mutation</td>
<td>71%</td>
<td>No</td>
<td>[153]</td>
</tr>
<tr>
<td>kidney tumors</td>
<td>hypermethylation</td>
<td>17% ; 18%</td>
<td>71% ; ND</td>
<td>[21, 154]</td>
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<td>deletion</td>
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<td>No</td>
<td>[155]</td>
</tr>
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<td>deletion</td>
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<td>[156, 157]</td>
</tr>
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<td>deletion</td>
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<td>No</td>
<td>[158]</td>
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<td>14%</td>
<td>ND</td>
<td>[159, 160]</td>
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<td></td>
<td>2. methylation, OR deltion</td>
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<td>33%</td>
<td></td>
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<td>2. mutation</td>
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<td>[11, 13, 161, 162]</td>
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<td>familial</td>
<td>ND</td>
<td>[12]</td>
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<td>38%</td>
<td>33%</td>
<td>[163]</td>
</tr>
<tr>
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<td>11%</td>
<td>ND</td>
<td>[164]</td>
</tr>
<tr>
<td></td>
<td>2. homozygous deletion</td>
<td>6%</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3. mutation</td>
<td>21%</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>nasal adenocarcinoma</td>
<td>1. deletion</td>
<td>45%</td>
<td>No</td>
<td>[165]</td>
</tr>
<tr>
<td></td>
<td>2. methylation</td>
<td>67%</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>neurofibromas and neurofibrosarcomas</td>
<td>methylation</td>
<td>5%</td>
<td>ND</td>
<td>[166]</td>
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<td>non-Hodgkins lymphoma</td>
<td>deletion OR mutation</td>
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<td>No</td>
<td>[167]</td>
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<tr>
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<td>1. methylation</td>
<td>8% ; 8% ; 30%</td>
<td>ND ; ND ; ND</td>
<td>[168-170]</td>
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<td>2. deletion</td>
<td>18%</td>
<td>ND</td>
<td>[171]</td>
</tr>
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<td>methylation</td>
<td>39%</td>
<td>ND</td>
<td>[107]</td>
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<tr>
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<td>methylation</td>
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<td>78% ; variable</td>
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<tr>
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<td>oral carcinoma</td>
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<td>No</td>
<td>[174]</td>
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<td>1. methylation</td>
<td>20%</td>
<td>30%</td>
<td>[175]</td>
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<tr>
<td></td>
<td>2. deletion</td>
<td>24% ; 30%</td>
<td>Yes ; ND</td>
<td>[176, 177]</td>
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<tr>
<td></td>
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<td>9%</td>
<td>No</td>
<td>[178]</td>
</tr>
<tr>
<td></td>
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<td>12%</td>
<td>[23]</td>
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<td>1. methylation</td>
<td>47%</td>
<td>93%</td>
<td>[179]</td>
</tr>
<tr>
<td></td>
<td>2. methylation, deletion, OR mutation</td>
<td>9%</td>
<td>No</td>
<td>[136]</td>
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</table>
**ACTIVATION OF P53**

ARF is most commonly known for its well-characterized activation of the p53 pathway (Fig. 1A). The p53 gene is the most common target of mutations which inactivate protein function or compromise its expression in human cancers. In fact, p53 is disrupted in greater than 50% of all human cancers. In response to cellular stress, p53 is activated to induce cell cycle arrest or trigger apoptosis depending on the setting. These stress cues include DNA damage, nucleotide depletion, viral infection, heat shock, and oncogenic stimuli. The crucial negative regulator of p53 is the E3 ubiquitin ligase, Mdm2 (Hdm2 in humans). Mdm2 binds to p53 and promotes its nuclear export and degradation through post-translational ubiquitin modification [35]. In the absence of Mdm2, p53 activity is unchecked, resulting in unrestrained apoptosis in cells and mice [36, 37]. Conversely, coinciding loss of p53 and Mdm2 rescues the apoptotic phenotype and mimics the loss of p53 alone [36-38].

In response to oncogenic signals such as those emanating from Ras and Myc, ARF is up-regulated and accumulates in the nucleolus. ARF interacts with Mdm2, preventing its nucleocytoplasmic shuttling and drawing it into the nucleolus. ARF interacts with Mdm2, preventing its nucleocytoplasmic shuttling and drawing it into the nucleolus.

Interestingly, the amino acid residues responsible for the nucleolar localization of mouse p19\(^{ARF}\) and human p14\(^{ARF}\) are somewhat different [34]. While the nucleolus is not partitioned from the nucleoplasm by a membrane, entry into this organelle is not thought to be a passive event. Rather, proteins that reside within the nucleolus often contain arginine and lysine rich domains reminiscent of nuclear localization signals that somehow target them to the nucleolus. However, these positively charged tracts are not obligatory for protein nucleolar localization. In fact, many proteins utilize protein-protein and protein-RNA interactions to “hitch” a ride into the nucleolus. Both mouse and human ARF proteins contain arginine-rich sequences (in fact, both proteins are nearly 25% arginine), albeit in different moieties along ARF’s amino acid sequence. In particular, residues 26-37 are critical for the nucleolar localization murine p19\(^{ARF}\) [27] (Fig. 2B)). In humans, amino acids 2-14 and 82-101 of p14\(^{ARF}\) are important for its nucleolar localization [34, 15, 16] (Fig. 2B)). Note, deletion of the nucleolar localization signal within either mouse or human ARF results in a loss of ARF’s ability to promote cell cycle arrest, revealing that the biological function of ARF might be intimately tied to its ability to properly localize to the nucleolus. However, this simplistic model is complicated by the observation that the regions of ARF that are important for its nucleolar localization also mediate most of the interactions that are critical for its functions. Thus, the critical determinant of functional ARF resides in its ability to interact with numerous oncoproteins.

**Table 1. contd.....**

<table>
<thead>
<tr>
<th>Disease</th>
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<th>Occurrence</th>
<th>ARF specificity</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>primary central nervous system lymphoma</td>
<td>1. deletion OR methylation 2. deletion OR mutation</td>
<td>56% ; 48% 90%</td>
<td>20% ; 13% No</td>
<td>[180, 181] [182]</td>
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<td>deletion OR methylation</td>
<td>13%</td>
<td>No</td>
<td>[183]</td>
</tr>
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<td>methylation</td>
<td>27%</td>
<td>69%</td>
<td>[184]</td>
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<td>deletion or methylation</td>
<td>5%</td>
<td>No</td>
<td>[185]</td>
</tr>
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<td>salivary gland carcinoma</td>
<td>1. deletion 2. methylation</td>
<td>8%</td>
<td>67%</td>
<td>[186]</td>
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<tr>
<td>small bowel adenocarcinoma</td>
<td>hypermethylation</td>
<td>9%</td>
<td>ND</td>
<td>[187]</td>
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<td>sporadic colorectal cancer</td>
<td>methylation</td>
<td>50%</td>
<td>ND</td>
<td>[151]</td>
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<td>mutation</td>
<td>14% ; 55%</td>
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<td>[188, 189]</td>
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<tr>
<td>supratentorial primitive neuroectodermal tumor</td>
<td>methylation</td>
<td>50%</td>
<td>ND</td>
<td>[159]</td>
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<td>T-cell acute lymphoblastic leukemia</td>
<td>mutation OR deletion</td>
<td>100%</td>
<td>3%</td>
<td>[190]</td>
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<td>deletion</td>
<td>25%</td>
<td>No</td>
<td>[191]</td>
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<td>50%</td>
<td>ND</td>
<td>[192]</td>
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<td>Wilms’ tumors</td>
<td>methylation</td>
<td>15%</td>
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<td>xeroderma pigmentosum-associated skin carcinoma</td>
<td>mutation</td>
<td>29%</td>
<td>No</td>
<td>[195]</td>
</tr>
</tbody>
</table>

ARF specificity signifies incidences where INK4a status is unaffected by the ARF alteration. ND=Not determined.
Interestingly, the residues in ARF (both mouse and human) that are critical for binding to Mdm2 also regulate ARF’s nucleolar localization and cell cycle arrest [16, 34]. The amino-terminal 37 residues of p19ARF (contained within exon 1β) are sufficient for all of its known properties including its binding to Mdm2 and localization to the nucleolus [27, 34]. Mdm2 also contributes to its nucleolar co-localization with ARF through a cryptic nucleolar localization signal which is revealed upon binding to ARF [44]. The notion that nucleolar sequestration of Mdm2 by ARF is necessary for its activation of p53 has been challenged by reports showing that ARF-mediated regulation of p53 can occur independent of Mdm2 nucleolar re-localization [45, 46]. Observations of ARF function in the nucleoplasm, particularly in regards to its interaction with Mdm2, opens a new set of possibilities as to how ARF can suppress growth under diverse circumstances. Thus, despite its steady-state localization to the nucleolus, ARF may exhibit growth-inhibitory processes that are independent of its ability to sequester Mdm2 in the nucleolus. However, nucleolar sequestration of Mdm2 by ARF is necessary for its activation of p53 [34, 45, 46].

MDM2 INHIBITORS

Through its inhibition of Mdm2 in response to oncogenic stimulation, ARF plays a key role in p53 pathway activation. In cells where ARF expression or function is lost through mutation or deletion, the aberrant activation of oncoproteins does not induce a typical p53 response, but rather results in cellular transformation [3, 48]. Mdm2 gene amplification, which occurs in tumors expressing wild-type p53 [49], is capable of overriding the suppressive effects of ARF [50]. Thus, Mdm2 represents a promising target for p53-positive tumors. Direct targeting of Mdm2 with pharmacological inhibitors has the potential to increase p53 protein levels and activity. Furthermore, the use of Mdm2 inhibitors would bypass the normal requirement for ARF in p53’s response to oncogenic stimuli, making it an effective therapy in tumors lacking functional ARF.

Several attempts have been made to identify molecules that target the p53-inhibitory activities of Mdm2 with a few promising candidates emerging. The nutlins are a class of Mdm2 inhibitors, identified in a synthetic chemical library screen, which occupy the hydrophobic p53-binding pocket of Mdm2. Nutlins inhibit the interaction between p53 and Mdm2 in a dose-dependent manner in vitro. In cancer cell lines that retain wild-type p53, nutlins inhibit cell cycle progression and induce p53 expression and subsequent apoptosis. Nutlin-3 inhibited growth of tumor xenografts in nude mice without any reported side effects over a three week treatment regimen [51, 52]. Further, in non-transformed fibroblasts and primary human mammary epithelial cells, nutlins produce a growth-inhibitory response without eliciting apoptotic toxicity [52, 53]. The HLI98 class of Hdm2 inhibitors was identified from a screen for

Fig. (2). ARF Nucleolar Localization. A. Wild type mouse embryonic fibroblasts show ARF (green) localized to the subnuclear organelle, the nucleolus. Nuclear DNA (blue) and cytoplasmic actin (red) are also shown. B. Alignment of Exon 1β and Exon 2 is shown for mouse and human ARF with Mdm2 and NPM binding sites (green) and nucleolar localization signals (blue) shown.
small molecules which inhibited auto-ubiquitination of Hdm2. Dose-dependent inhibition of p53 ubiquitination and an increase in p53 protein levels and transcription were observed with HL198. Additionally, HL198 molecules induced apoptosis and inhibited colony formation. Unlike nutlins, HL198 molecules do not inhibit the interaction of Hdm2 with p53 [54], but rather the E3 ligase activity of Mdm2. However, HL198 molecules exhibit limited specificity. Thus, further refinement is needed to improve the feasibility of specifically targeting Hdm2 ubiquitin ligase activity.

Problems surrounding the therapeutic use of Mdm2/Hdm2 inhibitors include potential toxicity in normal tissues due to uncontrollable p53 activity. Moreover, successful inhibition of Mdm2 may well lead to stabilization of p53 but may not elicit a therapeutic response due to other possible mutations in downstream components of the p53 signaling pathway. It seems likely that prolonged treatment with Mdm2 inhibitors may elicit unfavorable responses given a recent report demonstrating severe pathologies in Mdm2-null mice conditionally expressing p53 [53]. Deletion of Mdm2 is embryonic lethal in mice expressing wild-type p53, however p53/Mdm2 double-null mice are viable, indicating that unrestrained p53 activity is fatal during development [36, 37]. To overcome this hurdle, Ringhausen et al. used a previously described p53 knock-in mouse model, in which p53 expression was induced by tamoxifen [55], in the context of an Mdm2-null background [53]. Tamoxifen administration induced apoptosis and atrophy in radiosensitive tissues and tamoxifen-treated mice died within a week [53]. Therefore, despite great interest in the development of Mdm2 inhibitors, unrestrained p53 activity is a potentially dangerous consequence.

P53-INDEPENDENT TARGETS

Mounting evidence suggests that ARF has a second, p53-independent, function [56, 57]. The most convincing data presented to date involved the use of mouse genetics to confirm that p53 and ARF could contribute independently to suppressing tumorigenesis. Mice lacking p53 or Arf are highly tumorous with mean latencies to tumor development of 19 and 32 weeks, respectively [56]. In mice lacking p53, T-cell lymphomas predominate (~70%), with the remainder being sarcomas. In contrast, Arf-null mice develop far fewer cases of lymphoma (~25%) and primarily develop poorly differentiated sarcomas (~50%), with the remainder appearing as rare carcinomas and gliomas [8]. Surprisingly, mice deficient for both p53 and Arf showed a wider range of tumor types than animals lacking either gene alone, and many developed multiple primary tumors without affecting the mean latency of survival (~16 weeks) [56]. To date, more than half of the p53/Arf-null animals have developed wide-ranging multiple-type tumors strongly demonstrating that ARF has additional p53-independent functions. Cells devoid of both p53 and Arf grow at a faster rate and are more resistant to apoptotic signals than cells lacking only p53 or Arf [9], demonstrating a cooperative effect of p53 and Arf loss on cell proliferation. This also implies that ARF may functionally interact with proteins other than p53 and Mdm2 to prevent cell growth (see below). While p53-null mouse embryo fibroblasts are fairly resistant to ARF overexpression, cells deficient for both p53 and Mdm2 are sensitive to ARF-induced growth arrest. This indicates that ARF can act as a bona fide tumor suppressor independent of p53 and that Mdm2 can antagonize this effect.

Additionally, in mouse eye development, proper hyaloid vascular regression is dependent upon ARF, but not p53. Arf-null mice exhibited accumulation of a retrolental mass, lens degeneration, and lens capsule disruption, symptoms characteristic of the human eye disorder persistent hyperplastic primary vitreous [57]. Induction of p53-independent apoptosis by ARF in colon cancer cells occurs via degradation of CtBP [58]. ARF has also been reported to regulate the transcriptional activities of MYC and E2F1 through direct binding to Myc, E2F1 and DP1, respectively. Regulation of these transcription factors by ARF appears to be independent of p53 or Mdm2 [59, 60]. To date, numerous binding partners for ARF have been discovered [61-68]. Many of these have been shown to regulate ARF function in p53-dependent growth inhibition. For others, the potentially diverse functional consequences of their interactions with ARF are still being characterized. One recently identified interactor, ARF-BP1/Mule, is a ubiquitin ligase which is Inhibited by ARF. Inhibition of ARF-BP1 facilitates growth through both p53-dependent and p53-independent mechanisms making ARF-BP1 a promising potential therapeutic target for future investigation [61].

The addition of a small ubiquitin-like SUMO molecule, in a process known as sumoylation, is a post-translational modification that can alter stability and function of the target protein. Recent evidence has shown that ARF promotes the p53-independent sumoylation of numerous proteins, including Mdm2 [69, 70]. Werner’s helicase is sumoylated by ARF, resulting in its redistribution from the nucleus to other sites within the nucleoplasm [71]. Binding of pi4ARF to a SUMO-conjugating enzyme facilitates sumoylation of several proteins including Hdm2, E2F-1, and HIF-1α. Interestingly, point mutations in pi4ARF associated with melanoma altered the ability of ARF to promote sumoylation of Hdm2 or E2F-1 [72], implying that the sumoylation activity of ARF may be a critical component of both its p53-dependent and independent tumor suppressive properties. As such, novel compounds aimed at promoting or mimicking sumoylation of ARF targets may provide a unique mechanism for restoring ARF activity to tumor cells lacking functional ARF.

THE ARF-NPM INTERACTION

Some of the most exciting ARF work in recent years involved the independent discovery of NPM as a nuclear ARF binding partner having functional consequences [73, 74, 50, 75]. Nucleophosmin (NPM) is implicated in cancer biology, with both oncogenic and tumor suppressive functions attributed to this relatively abundant protein [76, 77]. Nucleophosmin undergoes CRM1-dependent nucleocytoplasmic shuttling and regulates the nuclear export of ribosomal protein L5 in order to promote ribosome nuclear export [78, 50, 79]. In fact, one p53-independent function of ARF is to inhibit the transport of ribosomal RNAs to the cytosol by sequestering NPM in the nucleolus [79, 50], reiterating the notion that NPM shuttling is a crucial event in cell cycle progression. Mutations that confer additional nuclear export signals onto NPM, such that NPM rapidly shuttles to the cytoplasm, are associated with acute myeloid leukaemia (AML) [80]. Additionally, chromosomal translocations involving NPM are common in hematological malignancies, while NPM overexpression is observed in diverse tumors [77]. The importance of NPM in maintaining growth and proliferation is underscored by the embryonic lethality observed in Npm1-null mice [76, 81].

NPM interacts with ARF in an association that has apparent functional consequences for both proteins. NPM maintains the stability and nuclear localization of ARF [81, 82, 75] and a cytoplasmic NPM mutant associated with AML redistributes ARF to the cytoplasm and reduces its stability [83, 82], suggesting that while ARF can target the function of NPM, ARF itself can be influenced by NPM oncoproteins [83, 82, 84, 77]. While ARF is stabilized by its interaction with NPM, adenoviral expression of ARF decreased NPM protein levels [73], although other studies have shown that overall levels of NPM remain
largely unchanged in cells despite large differences in ARF expression [74, 50]. The interaction of ARF with NPM is mediated by the amino terminus of p14ARF or p19ARF proteins [74, 50, 73]. Notably, this is the same region required for the formation of ARF-Mdm2 complexes. Indeed, ARF prefers to bind to Mdm2 under conditions of equal molar Mdm2 and NPM, arguing that p53-independent functions of ARF might be sensitive to Mdm2 inhibition [50]. This would provide an additional mechanism by which targeted therapeutics against Mdm2 might also reinstate p53-independent functions of ARF.

ROLE OF ARF IN RIBOSOME BIOGENESIS

NPM presents itself as a more teleological target of ARF tumor suppression, one that allows nucleolar ARF to interfere with proper ribosome assembly and export. Recent hypotheses place the nucleolus as a relaying center for the interpretation of growth and proliferation signals. In this sense, ribosome biogenesis is a critical step in both the regulation of mRNA translation and cell cycle progression with alterations in nucleolar function resulting in huge gains in protein synthesis and eventually, cell growth [28, 30]. How ARF might be involved in these dynamic processes has been debated in recent years. Chromatin immunoprecipitation experiments identified p14ARF at the promoter of rDNA loci and further established a functional interaction between ARF and UBF, a potent inducer of rDNA transcription [85]. Additionally, ARF may act as a checkpoint protein in ribosome biogenesis via inhibition of ribosomal RNA processing [86], resulting in fewer mature cytosolic ribosomes. This potential role seems likely given the

Fig. (3). ARF and Ribosome Biogenesis. The processes of ribosome biogenesis from transcription of rDNA loci to translating polysomes with the known steps sensitive to ARF inhibition are shown.
localization of ARF in the nucleolus and its ability to inactivate NPM, a key player in ribosome biogenesis. This is further supported by the observation that either ARF overexpression or mutation of the NPM nuclear export signal increased nuclear retention of 5S rRNA [79]. One might conclude that ARF could perform all three functions to ensure that ribosome biogenesis was completely inhibited (transcription, processing and export) (Fig. 3) during conditions where ARF is hindering the oncogenic signals presented by Ras and Myc. Loss of Arf or overexpression of NPM could increase ribosome biogenesis and accelerate tumorigenesis through tremendous gains in protein synthesis. Thus, the involvement of ARF in the regulation of translation provides a unique opportunity and potential blueprint as to how small molecule inhibitors against NPM might be used to target the ribosome synthetic machinery to prevent tumorigenesis originating from nucleolar dysfunction.

SYNTHETIC ARF PEPTIDES

Recently, peptide delivery has begun to show promise as a legitimate therapeutic strategy, with several studies showing beneficial anti-cancer activity of peptides in vivo. Injection of a peptide from the von Hippel-Lindau (VHL) tumor suppressor inhibited the growth and invasiveness of renal tumor implants in nude mice [87]. A peptide containing the D-isomer of a p53 C-terminal fragment was able to activate endogenous p53, inhibit tumor growth, and prolong survival of tumor-bearing mice [88]. Shepherdin, a peptide from survivin that inhibits Hsp90, inhibited tumor growth when injected into mice bearing prostate cancer xenografts [89].

Several studies have indicated that all of the known biological functions of ARF are mediated by the N-terminal amino acids 2-14. Deletion of these residues from mouse and human ARF blocks its recruitment of Mdm2 to the nucleolus, impairs its binding to NPM, and prevents its ability inhibit cell growth and proliferation in both p53 wild-type and p53/Mdm2-null cells [56, 34, 16, 74, 50]. ARFA2-14 (lacking residues 2-14) is unable to bind to 5.8S rRNA and subsequently unable to inhibit rRNA processing and proliferation of p53/Mdm2/Arf-null MEFs [86]. Residues 2-14 of ARF are sufficient for binding Mdm2 and NPM [34, 50] and are required for the sumoylation of ARF target proteins [70], suggesting that this short stretch of conserved amino acids (from mice and man) has considerable potential for use in reconstituting ARF function in vivo.

Therapeutic delivery of a small ARF peptide, such as ARF (amino acids 2-14) may mimic the growth-inhibitory effects of full-length ARF expression. In cancers where Mdm2 is overexpressed or where ARF expression is lost through mutation, deletion, or hypermethylation of the Arf locus, introduction of a synthetic ARF peptide might restore its regulatory effects on Mdm2. Inhibition of Mdm2 by synthetic ARF peptides may restore p53 activity in these tumors or, in tumors lacking p53, inhibit ribosome biogenesis (through NPM inactivation) and subsequent cell growth. In fact, expression of the peptide p14ARF (amino acids 1-20) induced p53 expression and prevented its ubiquitination [90], demonstrating the huge potential of this strategy.

It remains to be determined whether intra-tumoral delivery of ARF peptides is feasible. The unusual amino acid sequence and relative lack of structural information about ARF makes it a challenging candidate as a peptide-based therapeutic. Attachment of a Protein Transduction Domain (PTD) may facilitate delivery of an ARF peptide into the cell, but may also alter its localization. A basic PTD, like that of the HIV TAT protein, is less likely to interfere with the nucleolar localization of an ARF peptide. Additionally, isomers of ARF peptides may
enhance its stability and potency without affecting its native nucleolar localization. However, specific targeting of ARF is also a concern, as unregulated p53 activity would be toxic to both tumor and normal cells. Proof-of-principle remains to be established regarding the possible efficacy of ARF peptides as therapeutic anti-cancer agents, but ongoing mutagenesis studies of ARF residues 2-14 could reduce the number of critical amino acids required for ARF function. This would essentially provide chemists with the opportunity to mimic short ARF peptides with the goal of generating chemical compounds that would be capable of inhibiting Mdm2 and NPM function in a manner analogous to ARF.

CONCLUDING THOUGHTS

As a nuclear tumor suppressor, ARF is positioned to sense and regulate growth in the cell (Fig. 4). In response to hyper-growth or hyper-proliferative signals, ARF protein levels increase in the nucleolus leading to cell cycle arrest. Current interest in ARF biology for pharmaceutical companies certainly lies within the selective inhibition of Mdm2 molecules, as numerous compounds are under pre-clinical and clinical investigation for their efficacy in this regards. While these compounds, from a structural standpoint, may not be true ARF mimics, they could be viewed as functional ARF substitutes with their activities serving to potentiate a p53 response in tumor cells. The major drawback to this approach is in its inherent reliance on an intact p53 gene. However, genetic evidence suggests that p53-independent targets of Hdm2 may also contribute to the oncogenic capabilities of Hdm2 [91, 92]. If this holds true, then Hdm2 inhibitors may have profound effects in tumors regardless of their p53 status.

Given its nucleolar localization, it is not surprising that ARF can inhibit numerous steps in ribosome biogenesis. In fact, one could argue that this might be ARF’s teleological role in the cell: maintaining ribosome homeostasis, although definitive experiments in this regard are currently lacking. While it is easy to envisage the effect that inhibiting ribosome production would have on the growth of tumor cells, the side effects of inhibiting these processes in normal cells might be too great to utilize this approach clinically. However, new trials with known inhibitors of ribosome production could reverse this pessimistic view. Rapamycin and its chemical analogues are currently in various phases of clinical trials based largely on their ability to inhibit protein synthesis signaling pathways mediated by mTOR [93]. Inhibition of mTOR selectively inhibits both CAP-dependent and TOP-dependent translation as well as RNA polymerase I and transcription, effectively stopping ribosome production and protein translation [94, 95]. In fact, in some inherited cancer pre-disposition syndromes normal cells are unaffected by rapamycin while tumor cell growth and proliferation is halted [96], suggesting that tumor cells might be far more sensitive to translation inhibition. Viewed another way, tumor cells might simply require greater protein production in order to maintain their proliferative capacity, making them super-sensitive to slight reductions in ribosome output. Under these conditions, ARF mimics (either peptides or small molecules) or ribosome production inhibitors (transcription, processing or export) might be potent inhibitors of tumorigenesis, again regardless of p53 status, without inadvertently affecting normal tissues. Thus, restoring ARF function in tumor cells to activate both p53-dependent and -independent pathways, some of which are only beginning to be elucidated, would provide a formidable block to tumor growth.

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REFERENCES

Therapeutic Targets in the ARF Tumor Suppressor Pathway

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

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Abstract

The nucleolus is the center of ribosome synthesis, with nucleophosmin (NPM) and p19ARF proteins antagonizing one another to either promote or inhibit growth. However, basal NPM and ARF proteins form nucleolar complexes whose function remains 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. Transcription of rDNA, processing of nascent rRNA molecules and 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 mouse embryonic fibroblasts. Post-mitotic osteoclasts from Arf-null mice exhibited hyperactivity in vitro and in vivo, demonstrating a physiological function for basal ARF. Moreover, 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.
**Introduction**

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 nearly duplicate their entire protein content; a 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 as the center of rDNA transcription and ribosome biogenesis in the early 1960s (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. Ribosomal DNA 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 post-transcriptional modifications and assembly into subunits occur in the surrounding granular region (18).

While the primary mechanisms regulating these processes have been well-studied in yeast (13), multicellular organisms demand more complex regulatory mechanisms, in that proliferative capacity is not only determined 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 genes, Rb and p53 (9, 17, 43, 48). Both 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 for 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 classically regarded as an activator of p53 through its ability to sequester Mdm2, the E3 ubiquitin ligase for p53, in the nucleolus (39, 44, 45). 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 the growing number of studies describing p53-independent functions for ARF tumor suppression in mice and humans (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 the functional and physiological consequences of ARF disruption on growth and ribosome biogenesis. Through in vitro and in vivo assays, we utilized targeted Arf knockout mice and selective ARF knockdown via lentiviral RNA 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 when 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), knockdown of endogenous ARF via 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 pro-growth phenotype of 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 loss of Arf were reversed by removal of the nucleolar NPM proto-oncogene, indicating that NPM, when untethered from ARF, promotes unrestrained ribosome biogenesis. Taken 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 re-derived from TKO heterozygous mice (Arf +/-, Mdm2 +/-, p53 +/+, a generous gift from G. Zambetti, St. Jude, Memphis, TN) onto a pure C57/Bl6 background by several generations of backcrosses to wild-type C57/Bl6 mice, followed by breeding to homozygosity. Age-matched wild-type C57/Bl6 littermates were used as controls where indicated. Organs were harvested from mice four days postnatal.

**Cell culture, reagents, and antibodies**
Low passage (2-5) mouse embryonic fibroblasts were isolated and maintained in DMEM supplemented with 10% fetal bovine serum, 10 μg/ml gentimycin, 1X non-essential amino acids, 1 mM sodium pyruvate, and 2 mM glutamine. Rabbit anti-p16INK4A (sc-1207), goat anti-γ-tubulin (sc-7396), and rabbit anti-Myc (sc-764) were purchased from Santa Cruz. Rat anti-p19ARF (NB 200-169A) was purchased from Novus Biologicals. Mouse anti-nucleophosmin (32-5200) was purchased from Zymed.

**Plasmid constructs**

pLKO-GFP, a lentiviral short hairpin RNA (shRNA) expression vector was a generous gift from Dr. Sheila Stewart (Washington University). To construct the ARF shRNA vector, pLKO-GFP was digested with AgeI/MluI and annealed oligonucleotides containing the shRNA target (nucleotides 157-177 of exon 1β of p19ARF) or a scrambled control were cloned into these sites. Resultant clones verified by sequencing. Oligonucleotides are as follows: siARF (sense) 5’-CCGGGCTCTGGCTTTCTGAAACATGCTCGAGCATGTTTACGAAAGCAGGCTTTTTA-3’, siARF (antisense) 5’-CCGGTACGACCTGAACTGCTTAGGAACATGCTCGAGCATGTTTACGAAAGCAGGCTTTTTA-3’, siScrambled (sense) 5’-CGCGTACGACCTGAACTGCTTAGGAACATGCTCGAGCATGTTTACGAAAGCAGGCTTTTTA-3’, siScrambled (antisense) 5’-CGCGTACGACCTGAACTGCTTAGGAACATGCTCGAGCATGTTTACGAAAGCAGGCTTTTTA-3’. The underlined portion represents the 21 nucleotide hairpin sense and antisense strands. For NPM knockdown, annealed oligonucleotides were cloned as above into pLKO-GFP, the sequence of which were previously reported (27). RNA interference for endogenous c-Myc was performed with siRNAs recognizing the 3’-UTR of c-
Myc; 5’-AACGTTTATAACAGTTACAAA-3’ (Qiagen). Myc-ER retrovirus was generated and used to infect wild-type and Arf-null MEFs as previously described (50).

**AgNOR staining**

MEFs were seeded onto glass coverslips overnight and were fixed and stained the following day. The AgNOR staining method is modified from the protocol presented by Aubele et al. (1). Briefly, cells were fixed in 2% glutaraldehyde, followed by a post fixation 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 Corporation) on images captured at 200X by an Optitronics Magnifire camera on a Nikon TE300 microscope. Total number and total area (µm²) of AgNORs 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 PBS for 10 minutes. Samples were further processed by the Washington University Department of Cell Biology’s Electron Microscopy Core. Pictures of nuclei and nucleoli were taken at 3,000X and 7,000X, respectively.
**S-methionine incorporation assay**

Cells (1x10⁵) were seeded in triplicate and then starved of methionine and cysteine. Cells were pulsed with 14.3 μCi of ³⁵S-methionine (Amersham) and then immediately washed twice with cold PBS and lysed with 1% Triton X-100 buffer. Total protein was precipitated from lysates with 10% trichloroacetic acid. Pellets were subjected to liquid scintillation counting to measure incorporated cpm.

**Ribosome fractionation**

Cells (2x10⁶) were treated with 50 μg/ml cycloheximide prior to trypsinization and lysis, and fractionation was carried out over a 10–45% sucrose gradient (46). Gradients were fractionated and RNA absorbance at 254 nm was continuously monitored to detect ribosomal subunits.

**Lentiviral production and infection**

293T cells (5x10⁵) were transfected with one μg of pLKO-GFP containing either scrambled or ARF shRNA cassettes along with the pH8.2ΔR packaging vector and the pCMV-VSV-G envelope vector. Viral supernatants were collected and pooled. Wild-type MEFs (8x10⁵) were plated and infected with viral supernatant containing 10μg/ml protamine sulfate. Cells were infected again the following day, checked for GFP expression, and allowed to express the shRNA construct for 48 hours.

**Serum assays**

Levels of TRAP 5b were measured in serum collected from wild-type or Arf⁻/⁻ mice using a TRAP 5b 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 supernatant (1/10 vol) in α-MEM media containing 10% fetal calf serum (FCS) to generate primary bone marrow macrophages (BMM), as previously described (49). Cells were fed every day with α-MEM containing 10% fetal calf serum (FCS), CMG-14-12 supernatant (1/20 vol) and GST-RANKL (100 ng/ml) and incubated for five days to generate osteoclasts (49). TRAP staining was performed according to the manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO). Five 4X fields were captured with the Magnafire system and the number of TRAP positive cells with three or more nuclei were counted blinded to 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

Bone marrow macrophages (1 x 10⁴) were plated in α-MEM containing 10% fetal calf serum (FCS), CMG-14-12 supernatant (1/10 vol). Cells were starved in α-MEM containing 0.1% fetal calf serum (FCS) for 12 hours. At this time, α-MEM containing 10% fetal calf serum (FCS) and CMG-14-12 supernatant (1/10 vol) was added back to the cells. Cells were labeled with BrdU for 24 hours and proliferation was measured using the chemiluminescent cell proliferation ELISA (Roche Diagnostics, Mannheim, Germany).

Western blot and serial immunoprecipitation
MEF cell extracts (30 μg) were loaded onto 4-20% SDS-PAGE gels (ISC Biosciences), transferred to PVDF membrane (Millipore), and probed with rat anti-p19ARF (Novus Biologicals), goat anti-γ-tubulin (Santa Cruz), rabbit anti-Myc (Santa Cruz), rabbit anti-p16INK4A (Santa Cruz), and rabbit anti-L5 (ILAMM). Secondary HRP-conjugated anti-rabbit, -goat or –rat antibodies (Jackson ImmunoResearch) and ECL+ (Amersham) were used to visualize 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 samples were loaded onto 10% SDS-PAGE gels for immunoblot analysis.

47S rRNA real-time RT-PCR

Levels of 47S rRNA transcripts were performed as previously described by Cui and Tseng (10). Briefly, total RNA was reverse transcribed with a mouse rRNA-specific primer (5’-CGTGGCATGAAACCTTGG-3’). Real time PCR was performed with iQ SYBR Green Supermix (BioRad) according to manufacturer’s protocol with forward primer 5’-CTGACACGCTGTCCTTTCCC-3’ and reverse primer 5’-GTGAGCCGAAATAAGGTGGC-3’ on an iCycler thermal cycler (BioRad). Absolute copy number was obtained by comparison to serial dilutions of a known amount of plasmid containing the mouse rDNA repeat.

rRNA labeling experiments

Equal numbers of wild-type and Arf−/− MEFs were starved in methionine-free media containing 10% dialyzed fetal bovine serum. For uridine labeling, cells were labeled in media containing 2.5 μCi/ml [3H]-uridine (Amersham) and then chased in label-free media. Where noted, cells were
treated with 50 μCi/ml [methyl-³H]-methionine (Amersham) for 30 minutes and chased in unlabeled methionine-containing (10μM) media in the nuclear/cytoplasmic fractionation experiments. Total RNA was isolated using TRIZOL reagent (Invitrogen) and loaded onto 1% agarose-formaldehyde gels for the uridine experiments. Cellular fractionation was carried out using a nuclear extraction kit according to the manufacturer’s protocol (Active Motif). Total RNA was isolated from the nuclear and cytoplasmic fractions using TRIZOL and loaded onto 1% agarose-formaldehyde gels. RNA was transferred to Hybond N+ membranes (Amersham), cross-linked, sprayed with EN³HANCE (Perkin Elmer) and subjected to autoradiography.

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 re-localized 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 (silver nucleolar organizing region) staining protocol (1) for use on mouse embryonic fibroblasts (MEFs) derived from wild-type and Arf⁻/⁻ mice. Staining methods utilizing reduction of silver on argyrophilic proteins surrounding the nucleolar organizing region have been used for decades as a prognostic factor in certain carcinomas, wherein increases of the AgNOR index tend to correlate with poor prognoses (28). AgNOR staining of Arf⁻/⁻ MEFs demonstrated markedly increased numbers of
AgNORs per nucleus and a distinct irregular shape when compared to the fewer numbers and more rounded symmetric shape of AgNORs in wild-type counterparts (Fig. 1A). At the ultrastructural level, we also observed multiple, elongated, irregular nucleoli in Arf<sup>−/−</sup> 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<sup>−/−</sup> cells (31.6 μm<sup>2</sup> vs. 26.4 μm<sup>2</sup>, n=100, P<0.001) (Fig. 1C). A significant increase in AgNOR number per nucleus was also observed (5.78 vs. 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 loss of Arf resulted in dramatic gains in both AgNOR number and overall area (Fig. 2A & B). Moreover, we also observed a moderate increase in the number of larger multi-nucleolar cells in the livers of Arf-deficient mice (Fig. 2A, right panels). Taken together, these data suggest a role for p19ARF in maintaining proper nucleolar structure in vitro and in vivo.

**Loss of Arf enhances protein synthesis and ribosome biogenesis independent of proliferation**

Loss of Arf resulted in dramatic alterations in nucleolar structure (Figs. 1&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 <sup>35</sup>S-methionine pulse labeling experiments, measuring the amount of incorporated radioactivity into newly translated proteins over time. As shown, Arf<sup>−/−</sup>
MEFs had an approximately 4-fold increase in incorporated $^{35}$S-methionine over wild-type cells after 24 hours (Fig. 3A). Furthermore, these increased protein synthesis was not related to any increase in proliferation rates, as the proliferation of low passage wild-type and $Arf^{-/-}$ 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 $Arf$-null cells to separate ribosomes. Ribosome subunits and actively translating polysomes were identified by real-time absorbance monitoring at 254 nm to detect the relative amounts of ribosomal RNAs present in each of the subunit fractions. When compared to wild-type lysates, $Arf^{-/-}$ 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&E). Moreover, gains in ribosome biogenesis were also seen in vivo. Livers were isolated from newborn wild-type and $Arf$-null mice, minced and immediately placed in $^{35}$S-methionine-containing media to measure protein synthesis rates. Cells isolated from $Arf$-null livers exhibited a nearly 15-fold increase in protein synthesis compared to wild-type littermates (Fig. 4A). Additionally, cells freshly isolated from $Arf^{-/-}$ mouse spleens also showed dramatic increases in 40S, 60S, 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 certain step(s) in nucleolar ribosome biogenesis.
**Acute knockdown of ARF mimics the phenotype of Arf<sup>−/−</sup> 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 plays 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 blot analysis and immunohistochemistry (5). Given our finding that Arf<sup>−/−</sup> cells exhibit chronic nucleolar morphology changes and increased ribosome output (Figs. 1-4), we were poised to re-examine this question in an acute setting by knocking down basal ARF in wild-type cells. This was accomplished using lentiviral constructs containing a short hairpin RNA (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 blot analysis, infection with viruses containing the ARF shRNA sequence produced a robust knockdown of the level of ARF (96%) without decreasing p16INK4A levels (Fig. 5A). Moreover, expression of other nucleolar proteins, such as NPM and ribosomal protein L5, also remained unchanged following ARF knockdown (Fig. 5A). As first observed in Arf<sup>−/−</sup> MEFs, 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 in Arf-null cells with a significant increase in both number of AgNORs per nucleus (6.6 vs. 3.3, n=100, \( P<0.001 \)) and total AgNOR area per nucleus (49.8 μm<sup>2</sup> vs. 36.8 μm<sup>2</sup>, n=100, \( P<0.001 \)) (Fig. 5C). Similarly, ARF-knockdown MEFs displayed tremendous gains in protein synthesis rates as determined by <sup>35</sup>S-methionine incorporation, nearly 10-fold over scrambled control MEFs (Fig. 5D) and almost twice as high as Arf-null MEFs (compared to Fig.
ARF-knockdown MEFS also produced significantly more actively translating polysomes (55% more) as determined by UV monitoring of cytosolic rRNAs (Fig. 5E), suggesting that 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 collagenases and dramatically lower the pH through the activity of the proton pumps. As such, the osteoclast has a high demand for protein synthesis, since the H⁺-ATPases are specific to the mature osteoclast and are not found in macrophage precursors (41). Furthermore, since the mature osteoclast is a post-mitotic cell, it affords an excellent opportunity to examine ARF’s effects on protein and ribosome metabolism independent of proliferation.

We first examined whether or not the proliferation rates varied between wild-type and Arf⁻/⁻ bone marrow-derived macrophages (BMM), osteoclast precursors. BrdU labeling of BMMs demonstrated no significant difference in the proliferation rates between wild-type and Arf⁻/⁻ osteoclast precursors (Fig. 6A), similar to the equal proliferation rates of early passage MEFs (Fig. 3B). Next, BMMs from Arf⁻/⁻ and wild-type mice were induced to produce mature osteoclasts by the addition of M-CSF and RANK ligand. After three days of stimulation with
RANK ligand, cells were fixed and stained with tartrate resistant acid phosphatase (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 \textit{Arf}^{-/-} precursors was observed compared to 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 increases in the \textit{Arf}^{-/-} genotype (Fig. 6C, 149 vs. 91 per well, n=5, \(P=0.01\)).

To determine if the differences seen in osteoclastogenesis were functionally relevant, we compared the TRAP activity (a marker of osteoclast function) of equal numbers of TRAP-positive cells as determined above. Cell lysates were incubated from day four post-RANKL addition (for wild-type) and day three post-RANKL addition (for \textit{Arf}^{-/-}), where approximately equal numbers of multi-nucleated TRAP-positive cells were observed, with \(p\)-nitrophenyl phosphate, a colorimetric substrate for TRAP. A two-fold increase in TRAP activity was seen in \textit{Arf}-null cells compared to that in wild-type cells (\(P<0.01\)) (Fig. 6D), indicating that \textit{Arf}^{-/-} osteoclasts are far more active than their wild-type counterparts on a per cell basis. \textit{In vivo} analysis of osteoclast function in \textit{Arf}-null mice mimicked our \textit{in vitro} findings of osteoclast hyperactivity, as there was an 18% increase in the level of serum TRAP activity over wild type controls (Fig. 6E).

**Loss of \textit{Arf} increases rRNA transcription, rRNA processing, and ribosome nuclear export**

Previous reports have demonstrated a role for ARF in rRNA processing (37). Furthermore, our lab has previously demonstrated ARF’s inhibitory activity on the shuttling of NPM (8) and NPM’s nucleolar cargo, rpL5 and 5S rRNA (46). Additional reports have demonstrated a role
for nucleolar ARF in preventing rDNA transcription through both Myc-dependent and -independent mechanisms (2, 3, 30). Taken together, nucleolar ARF could prevent all three steps in ribosome biogenesis: transcription, processing, and export. Loss of Arf had no impact on the levels of either NPM or rpL5, suggesting that ARF’s effect in this pathway was not due to altered synthesis and/or destruction of these proteins (Fig. 7A). Moreover, serial immunodepletion of NPM revealed two distinct pools of ARF: one that is effectively associated with NPM (Fig. 7B, lane 1), and a second pool 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. Accordingly, loss of Arf resulted in a 4-fold increase in 47S rRNA transcription (Fig. 7C), a process thought to be independent 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 overexpressed ARF (37). To determine the effect of Arf loss on these events, newly synthesized 47S rRNAs were followed through nucleolar processing. Ribosomal RNA processing was greatly accentuated in Arf −/− MEFs over a two hour period (Fig. 7D). While wild-type and Arf-null cells clearly start with different amounts of 47S rRNA (Fig. 7C), Arf-null cells are capable of churning out more processed rRNAs (15-fold more than wild-type) which is nearly a 3-fold amplification over the starting amount of 47S transcripts. This suggests that while levels of 47S rRNA are certainly permissive to greater processing of rRNAs, they cannot entirely account for the sheer magnitude of increases in processed rRNAs observed in Arf −/− cells.
To determine the precise step at which ARF might influence rRNA processing, we labeled cells with [methyl-\(^3\)H]-methionine, which labels rRNA, and loaded equal amounts of radioactivity to examine processing intermediates after short time periods of chase with label-free media. We observed only a modest increase of 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 two hours of chase, we saw no difference in the relative amounts of radioactivity in the final 18S and 28S products, indicating that loss of Arf had no impact on these downstream processing steps. These results exactly mirror what Sugimoto and colleagues observed when overexpressing 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). Arf-null MEFs exhibited more robust (~25-fold) nuclear export of newly processed rRNAs than wild-type cells (Fig. 8A). Again, this extreme difference between wild-type and Arf-null cells was far greater than any previous step in ribosome biogenesis (e.g. transcription or processing), implying that each step represents an amplification of the previous step. This was most evident when real-time nuclear export of rRNAs, as monitored by scintillation counting of newly exported \(^3\)H-labeled rRNA, revealed that the absolute rates of rRNA export were increased 3-fold in cells lacking Arf (Fig. 8B). Together, we believe this reflects the ability of ARF to regulate moment-to-moment steps in ribosome biogenesis, such that alterations in ARF levels may produce robust and rapid responses effecting cytoplasmic ribosomal content.
Myc is not responsible for the rDNA transcription increases in 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 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 the lowering of Myc protein levels greatly reduced 47S rRNA transcripts in wild-type MEFs, it had little impact on 47S copies in Arf−/− MEFs (Fig. 9B). While the former 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 using an siRNA-resistant (lacking the targeted 3′-UTR sequence) Myc-ER construct and 4-hydroxytamoxifen treatment (50), 47S transcript levels significantly increased in Arf-null MEFs, suggesting that Myc proteins can positively direct rDNA transcription in the absence of ARF (Fig. 9A&B).

Nucleophosmin is required for the growth gains seen in the absence of Arf

Having shown that nearly half of 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, with loss of Arf resulting 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 encoding shRNAs targeting mouse NPM, we were able to achieve greater than 90% NPM knockdown efficiency (Fig. 10A). However, reduction in NPM protein
expression led to a dramatic increase in 47S rRNA transcripts in cells also lacking 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 in 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 export of processed 18S rRNA was significantly attenuated (55% reduction) in Arf-null cells lacking NPM (Fig. 10D), demonstrating the requirement of NPM in trafficking mature rRNAs out of the nucleus and into the cytosol. In response to decreased ribosome export to the cytosol, 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 proteins 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 in the absence of Arf were caused by de-regulation of NPM and were independent of proliferation, we lowered the levels 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 post-mitotic) or of ARF binding to Mdm2 (i.e. a p53 response). This provided us with an experimental system to test the hypothesis that a balance exists between ARF and NPM in determining ribosome output from the nucleolus. Lentiviral shRNAs targeting NPM in bone marrow-derived macrophages significantly reduced NPM protein expression levels
(Fig. 11A). Concomitant with decreases in NPM expression, Arf<sup>−/−</sup> osteoclasts were dramatically reduced in 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 statistical 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 long appreciated for its ability 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 to 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 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 physiological low level of ARF has a regulatory role in nucleolar function and ribosome biogenesis. Indeed, as early as four days post ARF knockdown by lentiviral shRNA infection, we observed changes in nucleolar morphology and function that is reminiscent of data from Arf<sup>−/−</sup> 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 prohibiting 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 nucleophosmin, 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), thereby constantly limiting 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,
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 is still unclear, our data are consistent with a “thermostat” function for ARF, in that small changes in the abundance of ARF affects 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: 1) rDNA transcription, 2) rRNA processing, and 3) 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, preventing the cell from wasting energy on unnecessary protein synthesis. Disruption of this exquisite basal ARF control would then have a two-fold effect: 1) cells would produce far too many ribosomes resulting in tremendous gains in protein synthesis and 2) the resultant cells would be highly susceptible to oncogenic signals. This setting would seem to provide a selective advantage to
pre-malignant cells by ramping up their growth and, in the presence of appropriate signals, proliferation. In support of this hypothesis, a recent study on the methylation of key loci involved in colorectal carcinogenesis demonstrated that 32% of the adenomas (pre-malignant lesions) isolated from patients with sporadic colorectal cancer demonstrated abnormalities at the \textit{Arf} locus (11). Our findings represent a novel and important role for basal ARF in maintaining protein synthetic homeostasis in non-malignant cells. While NPM is certainly required for much of the ribosome biogenesis gains observed in \textit{Arf}-deficient cells, other interesting nucleolar targets of basal ARF must certainly exist. Precise details of how they may be affected still 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 \textit{bona fide} tumor suppressors.

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References


FIGURE LEGENDS

Figure 1. Loss of ARF results in nucleolar morphological changes. (A) AgNOR staining of representative wild-type and \( \textit{Arf}^{+/−} \) MEFs (40X). Increases in number and irregularity of the AgNORs in the \( \textit{Arf}^{-/-} \) cells. (B) Ultrastructural features of nuclei from wild-type and \( \textit{Arf}^{-/-} \) MEFs. Top row: 3,000X magnification. Bottom row: 7,000X magnification. (C) Quantification of AgNOR indices from (A). Left panel: Number of AgNORs per nucleus (n=100). Right panel: Total nucleolar area (in \( \mu m^2 \)) per nucleus as determined by histomorphometric analysis (n=100). * \( P<0.01 \)

Figure 2. Tissues from newborn \( \textit{Arf}^{-/-} \) mice display altered nucleolar morphology reminiscent of the \textit{in vitro} findings. (A) AgNOR staining of representative sections from the intestine and liver. (B) Quantification of total AgNOR area per nucleus (n=100). * \( P<0.01 \)

Figure 3. Disruption of ARF enhances protein synthesis independent of cellular proliferation. (A) Cells were starved of methionine and cysteine for 30 minutes prior to addition of \( ^{35} \text{S-methionine} \) label for the indicated times, followed by lysis, TCA precipitation of proteins, and liquid scintillation counting. (B) Equal numbers of cells (1x10^5) were plated in triplicate at day 0 and then were trypsinized and counted via a hemocytometer at various time points. (C) Cycloheximide (50 \( \mu g/ml \)) was added for 10 minutes prior to lysis and ultracentrifugation of cleared lysate on 10-40% sucrose gradients. Graph depicts \( A_{254} \) of ribosome subunits over increasing sucrose density. (D) Equal passage MEFs (1 x 10^5) were trypsinized and analyzed by a Coulter Vi-Cell Counter for cell volume. (E) Equal passage MEFs (1 x10^6) were harvested and analyzed for protein content by a standard colorimetric DC-Assay.
Figure 4. ARF regulates protein synthesis and ribosome biogenesis in vivo. (A) Livers were isolated from three wild-type and Arf-null littermates and briefly trypsinized. Cells (5 x 10⁶) were immediately cultured in methionine-free media for 15 minutes and then incubated with ³⁵S-methionine for the indicated times. Proteins were TCA-precipitated and labeled proteins quantified by liquid scintillation counting. (B) Spleens were isolated from three wild-type and Arf-null littermates. Cells (1 x 10⁷) were immediately harvested and cytosolic fractions were loaded onto 7-47% sucrose gradients for ultracentrifugation separation. Graph B depicts A₂₅₄ of ribosome subunits over increasing sucrose density.

Figure 5. Acute depletion of p19ARF results in nucleolar morphological and functional changes reminiscent of Arf⁻/⁻ cells. (A) Western blot confirmation of p19ARF knockdown in wild-type MEFs 96 hours post-infection with lentiviral shRNA constructs using antibodies recognizing γ-tubulin, NPM, rpL5, p19ARF and p16INK4a. Fold expression change is marked under each panel. (B) AgNOR staining of representative MEFs infected with control (scrambled) or p19ARF-specific shRNA virus (40X). (C) Quantification of AgNOR indices. Left panel: Number of AgNORs per nucleus (n=100). Right panel: Total nucleolar area (in μm²) per nucleus as determined by histomorphometric analysis (n=100). * P<0.01 (D) Total radioactivity incorporated after ³⁵S-methionine pulse. Cells were starved of methionine and cysteine for 30 minutes prior to addition of label for the indicated times, followed by lysis, TCA precipitation of proteins, and liquid scintillation counting. (E) Cycloheximide (50 μg/ml) was added for 10 minutes prior to lysis and ultracentrifugation of cleared lysate on 10-40% sucrose gradients. Graph E depicts A₂₅₄ of ribosome subunits over increasing sucrose density.
Figure 6. Loss of p19ARF has functional consequences on osteoclast biology. (A) BrdU incorporation in wild type and \textit{Arf}^{-/-} macrophages. (B) Representative TRAP-staining of equal numbers of bone marrow-derived macrophages following three days of treatment with M-CSF and RANK ligand reveals an increase in multi-nucleated osteoclasts formed from \textit{Arf}^{-/-} precursors. (C) Graph depicting increases in TRAP-positive osteoclasts with greater than five nuclei derived from \textit{Arf}^{-/-} bone marrow. * \( P=0.01 \) (D) TRAP solution assay of equal numbers of TRAP-positive cells. Cells from wild-type (day 4 post-RANKL addition) or \textit{Arf}^{-/-} (day 3 post-RANKL addition) precursors were lysed and incubated in a colorimetric assay with \( p \)-nitrophenyl phosphate, a substrate for TRAP. Graph depicts absorbance at 405 nm. * \( P=0.01 \) (E) Levels of serum TRAP 5b in \textit{Arf}^{-/-} compared to wild-type mice \((P=0.03, n=5 \text{ mice in each group})\) as measured by ELISA.

Figure 7. ARF exerts its effects through control of rRNA synthesis and processing. (A) Western blot demonstrating that \textit{Arf}^{-/-} MEFs do not have alterations in the levels of nucleolar proteins NPM and ribosomal protein L5. (B) Serial NPM immunoprecipitation. Wild-type cells were lysed and serially immunoprecipitated (5X) with mouse NPM antibodies. The final supernatant was concentrated and included as a control for non-NPM binding proteins. (C) Total RNA was collected from equal numbers of asynchronously dividing cells, and quantitative real-time RT-PCR was performed with a primer specific to the mouse 47S transcript. (D) Wild-type and \textit{Arf}^{-/-} cells were pulsed with labeled \(^3\text{H}-\text{uridine}\) for 30 minutes followed by chase in label-free media for the indicated times. Total RNA was isolated from equal cell numbers, loaded onto formaldehyde-containing agarose gels, and transferred to membranes for fluorography. (E)
Cells were labeled with [methyl-$^3$H]-methionine followed by chase in media containing excess unlabeled methionine for the indicated times. Total RNA was isolated, and equal radioactive counts were loaded onto gels and transferred to membranes for fluorography.

**Figure 8. Nucleocytoplasmic shuttling of newly synthesized ribosomes is enhanced in the absence of Arf.** (A) Equal numbers of cells were pulsed with [methyl-$^3$H]-methionine and chased in unlabeled methionine-containing media for the indicated times. Total RNA was isolated from nuclear, ‘N’, and cytoplasmic, ‘C’, fractions and subjected to fluorography. (B) Cytoplasmic fractions from the indicated times were also subjected to liquid scintillation counting to obtain a quantitative estimate of total cytoplasmic rRNA. Inset: Scatter plot of data presented in (B) with best-fit lines to indicate velocity of export. m=slope.

**Figure 9. Myc is not required for the enhanced rDNA transcription of Arf-null MEFs.** Arf$^{-/-}$ MEFs (2 x $10^6$) transduced with siLuc control siRNAs or Myc siRNAs in the absence or presence of MycER expressing retroviruses and 4-hydroxytamoxifen were harvested and (A) immunoblotted with antibodies recognizing c-Myc or γ-tubulin. (B) RNA was isolated from the above cells and real-time PCR using 47S rRNA probes was performed in triplicate.

**Figure 10. NPM is required for ribosome gains in the absence of Arf.** Arf$^{-/-}$ MEFs (2 x $10^6$) infected with lentiviruses encoding scrambled or NPM shRNAs were (A) lysed and immunoblotted with antibodies recognizing NPM and γ-tubulin; (B) lysed and RNA isolated for real-time PCR using 47S rRNA probes; (C) labeled with [methyl-$^3$H]-methionine followed by chase in media containing excess unlabeled methionine for the indicated times, total RNA
isolated, and equal radioactive counts loaded onto gels and transferred to membranes for fluorography; (D) fractionated into nuclear, ‘N’, and cytosolic, ‘C’, lysates, immunoblotted with Lamin A/C and SOD or Northern blotted with probes recognizing the 18S rRNA; (E) starved of methionine and cysteine for 30 minutes prior to addition of label for the indicated times, followed by lysis, TCA precipitation of proteins, and liquid scintillation counting. * $P<0.01$

**Figure 11. Loss of nucleophosmin expression inhibits osteoclastogenesis in Arf-/- cells.** (A) Western blot of macrophages infected with either control or lentiviral-targeted hairpin specific to nucleophosmin to confirm gene knockdown. (B) TRAP stain of osteoclasts differentiated *in vitro* with RANKL and M-CSF for six days. (C) TRAP activity assay of equal numbers of osteoclasts from the indicated genotypes. * $P<0.01$
A

WT       Arf^{−/−}

![Images of cellular structures with magnifications 40X, 3000X, and 7000X]

B

WT       Arf^{−/−}

![Images of cellular structures with magnifications 40X, 3000X, and 7000X]

C

Nucleolar Statistics

<table>
<thead>
<tr>
<th></th>
<th># AgNOR/nucleus</th>
<th>Total area (μm²)</th>
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<tbody>
<tr>
<td>WT</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Arf^{−/−}</td>
<td>8</td>
<td>30</td>
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</table>

* indicates significant differences.
**A**

<table>
<thead>
<tr>
<th>Intestine</th>
<th>Liver</th>
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</thead>
<tbody>
<tr>
<td><strong>WT</strong></td>
<td><strong>WT</strong></td>
</tr>
<tr>
<td><strong>Arf⁻⁻</strong></td>
<td><strong>Arf⁻⁻</strong></td>
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</tbody>
</table>

**B**

![Bar chart showing Total AgNOR Area (μm²) for Liver and Intestine with WT and Arf⁻⁻ conditions.](Apicelli_Fig2)
A

Liver

\[ ^{35} \text{S-Methionine Incorporation (cpm \times 10^4)} \]

\[ \text{Time (Hours)} \]

Arf^{-/-}

WT

B

\text{Absorbance @ 254 nm}

\[ \text{Sucrose} \]

\text{Polysomes}

\text{40S} \quad \text{60S} \quad \text{80S}
A B C D E

**A**

<table>
<thead>
<tr>
<th>RNAi</th>
<th>Scr</th>
<th>Arf</th>
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<tbody>
<tr>
<td>γ-Tubulin</td>
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<tr>
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<td>![Image]</td>
<td>![Image]</td>
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**B**

Scrambled siRNA  
ARF siRNA

40X

**C**

Nucleolar Statistics

<table>
<thead>
<tr>
<th>AgNOR Number/nucleus</th>
<th>Total Area (μm²)</th>
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</thead>
<tbody>
<tr>
<td>Scrambled siRNA</td>
<td>![Image]</td>
</tr>
<tr>
<td>ARF siRNA</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

**D**

³⁵S-Methionine Incorporation cpm (x 10⁶)

Time (Hours)

**E**

Absorbance @ 254 nm

[Sucrose]
**A**

- WT
- Arf^-/

siLuc  siMyc  siMyc +

IB:Myc

Myc-ER

c-Myc

γ-Tubulin

Fold: 1.0 0.05 0.57

**B**

- WT
- Arf^-/

mWTS copy # \( \times 10^6 \) per cell

siLuc  siMyc  siMyc + MycER