RNA Helicase DDX5 Is a p53-Independent Target of ARF That Participates in Ribosome Biogenesis

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Abstract

The p19ARF tumor suppressor limits ribosome biogenesis and responds to hyperproliferative signals to activate the p53 checkpoint response. Although its activation of p53 has been well characterized, the role of ARF in restraining nucleolar ribosome production is poorly understood. Here we report the use of a mass spectroscopic analysis to identify protein changes within the nucleoli of Arf-deficient mouse cells. Through this approach, we discovered that ARF limited the nucleolar localization of the RNA helicase DDX5, which promotes the synthesis and maturation of rRNA, ultimately increasing ribosome output and proliferation. ARF inhibited the interaction between DDX5 and nucleophosmin (NPM), preventing association of DDX5 with the rDNA promoter and nuclear pre-ribosomes. In addition, Arf-deficient cells transformed by oncogenic RasV12 were addicted to DDX5, because reduction of DDX5 was sufficient to impair RasV12-driven colony formation in soft agar and tumor growth in mice. Taken together, our findings indicate that DDX5 is a key p53-independent target of the ARF tumor suppressor and is a novel non-oncogene participant in ribosome biogenesis. Cancer Res; 71(21): 6708–17. ©2011 AACR.

Introduction

The role of ARF in regulating p53 is well established, but the mechanisms by which it exerts its p53-independent tumor suppressor function are yet to be fully characterized. A common theme in p53-independent activity of ARF is its ability to regulate nucleolar ribosome biogenesis (1, 2), but mechanistic details of its involvement have remained elusive. Understanding the p53-independent functions of ARF in the nucleolus is an increasingly important focus in cancer biology.

The nucleolus is a dynamic organelle that assembles around ribosomal DNA (rDNA) repeats and is the cellular center for ribosome biogenesis. Characterization of the nucleolar proteome has revealed the broad spectrum of resident proteins (3). As nucleoli lack membranes, proteins freely diffuse into and out of nucleoli in response to varying conditions (4). Some of the most important residents of nucleoli are proteins that regulate ribosome production, including p19ARF (p14ARF in humans).

The canonical function of ARF is to activate p53 by binding and sequestering the p53 inhibitor Mdm2 (5–8). Arf-null mice develop spontaneous tumors consisting of predominantly fibrosarcomas and lymphomas (9, 10). However, ARF also possesses p53-independent roles that contribute to its growth-inhibitory function and suppression of tumorigenesis (11). For example, basal ARF maintains nucleolar structure and function (12), at least in part, through its ability to interact with nucleophosmin (NPM; refs. 1, 13–16). The ability of ARF to regulate the nucleolar localization of Mdm2 (6) and the nuclear export of NPM (15) suggests that ARF may monitor nucleolar function by regulating the composition of the nucleolar proteome. To determine how the presence or absence of basal ARF affects nucleolar protein composition, we conducted a proteomic screen using isolated nucleoli from wild-type and Arf+/− mouse embryonic fibroblasts (MEF). Among the proteins enriched in nucleoli in the absence of Arf was DDX5, a DEAD-box protein also known as p68 RNA helicase.

The DEAD-box family of RNA helicases is defined by a conserved Asp-Glu-Ala-Asp motif that interacts with Mg2+ and is involved in ATP hydrolysis (17). DEAD-box proteins also contain several conserved motifs that have been shown to function in ATP binding, ATPase activity, and helicase activity (18). Many cellular functions of DEAD-box RNA helicases have been attributed to RNA duplex unwinding and ribonucleoprotein (RNP) complex remodeling (19). In yeast, several RNA helicases have been shown to facilitate ribosome biogenesis (20), which involves both the processing of rRNA as well as its assembly into functional RNP complexes. Given that the cellular center for ribosome synthesis is the nucleolus, it is
not surprising that many RNA helicases have been identified as components of the nucleolar proteome (1, 4). The involvement of several known oncogenes and tumor suppressors in the regulation of protein synthesis underscores the importance of ribosomes and mRNA translational control in cancer (21). Thus, the ability of ARF to direct balanced RNA metabolism in the nucleolus could provide insights into how this major cellular axis might impact tumorigenesis. Apart from its classical function as a sensor of hyperproliferative signals (22–24), we now show that ARF limits non-oncogene-driven ribosome biogenesis to inhibit cellular transformation.

Materials and Methods

Cell culture and reagents
Primary MEFs were isolated and cultured as described (15). Rabbit anti-DDX5 (A300-523A) was purchased from Bethyl Laboratories. Mouse anti-NPM (catalog no. 32-5200) was purchased from Zymed. Rat anti-p19ARF (ab26696) was purchased from Abcam. H-Ras, p21, and γ-tubulin antibodies were purchased from Santa Cruz Biotechnology.

Chromatin immunoprecipitation
Wild-type and Arf−/− MEFs were cross-linked with formaldehyde and cell lysates were immunoprecipitated with the indicated antibodies at 4°C overnight. Samples were then washed with low salt, high salt, LiCl, and TE buffers, prior to elution. Cross-links were reversed by addition of NaCl and samples were subjected to RNase A and proteinase K treatments. DNA was purified from samples using QIAquick PCR purification kits (Qiagen). Quantitative real-time PCR (qRTPCR) was done as detailed above with primer sets specific to rDNA loci. Additional details for the chromatin immunoprecipitation (ChiP) protocol are provided in the Supplementary Material.

rRNA immunoprecipitation
Arf−/− MEFs were starved as described above and labeled with [methyl-3H]-methionine for 4 hours. Cells were harvested, lysed, and subjected to immunoprecipitation and RNA extraction as previously described (26).

Ribosome fractionation
Cells were treated with cycloheximide, collected, and fractionated by sucrose gradient centrifugation as previously described (26). Total protein was precipitated from individual fractions with trichloroacetic acid and analyzed by Western blot.

Foci formation and proliferation assays
MEFs were plated in triplicate at the indicated concentration and foci formation and proliferation assays were conducted as previously described (27).

Soft agar
Arf−/− MEFs were infected with short hairpin RNAs (shRNA) against luciferase or DDX5, prior to infection with either RasV12 or empty vector (pBabe). Cells were seeded onto soft agar at 10^4 cells per 6-cm² dish and grown for 3 weeks. Cells

Quantitative real-time PCR
Total RNA was isolated from wild-type and Arf−/− MEFs using Illustra RNAspin columns (GE Healthcare) according to manufacturer’s protocol. First-strand cDNA synthesis and real-time PCR were done as previously described (26).

[methyl-3H]-methionine labeling of rRNA
Equal numbers of MEFs were subjected to starvation in methionine-free media containing 10% dialyzed FBS for 15 minutes. Cells were treated with 50 μCi/mL [methyl-3H]-methionine and chased in complete media containing an excess of unlabeled methionine (10 μmol/L) for the indicated times. Samples were lysed in RNASolv reagent (Omega Biotek) and extracted RNA was separated on agarose-formaldehyde gels and transferred to a Hybond XL membrane (GE Healthcare). The membrane was cross-linked and sprayed with En3Hance (Perkin-Elmer) prior to autoradiography. Band intensities were quantitated using ImageQuant TL (Amersham Biosciences).

Immunofluorescence
Cells were fixed with 4% paraformaldehyde in PBS for 10 minutes. Cells were permeabilized with 1% NP-40, blocked in 5% FBS, and stained with rabbit anti-DDX5 and mouse anti-NPM, followed by fluorescein isothiocyanate (FITC)-conjugated anti-mouse and Rhodamine-X–conjugated anti-rabbit (both from Jackson Immunoresearch). Samples were counterstained with 4',6-diamidino-2-phenylindole and mounted with Vectashield (Vector Labs). Four independent MEF isolates were used to assay localization of DDX5. Images were acquired using a ×100 oil immersion lens on a Zeiss LSM5 Pascal Vario Two UGB coupled to Axiovert 200 confocal microscope.
were relayered with soft agar on a weekly basis and visible colonies were counted after 3 weeks.

**Tumorigenesis assay**

Arf−/− MEFs were infected with RasV12 and either shDDX5 or shSCR. Fibroblasts were trypsinized and resuspended in PBS at a concentration of 2 × 10^5 cells/mL. Athymic nude mice were injected s.c. with 2 × 10^6 cells along their left flank, with sample sizes of 5 mice per condition. Tumor size was monitored over an 18-day time course using calipers to measure the tumors in 2 dimensions. Tumor volume was calculated using the formula:

\[
\text{Volume} = \frac{[(\text{height})^2 \times \text{length}]}{2}, \text{in which height equals the smallest of the 2 measurements.}
\]

**Results**

**p19ARF interferes with the nucleolar localization of DDX5 RNA helicase**

A proteomic screen was conducted to identify targets that displayed differential nucleolar localization in the presence or absence of basal ARF. Adapting a protocol from Andersen and colleagues (3), we isolated nucleoli from wild-type and Arf−/− MEFs. Isolated nucleoli maintained in vivo morphology (Fig. 1A) and were positive for nucleolar proteins by immunofluorescence (Fig. 1B) and were free of nucleoplasmic contaminants (Fig. 1C). Nucleolar isolated were subjected to comparative 2D differential gel electrophoresis (2D-DIGE) proteomic analysis. Twenty-six spots which showed differences greater than 2.5 SDs from the mean change were excised, and 19 were positively identified by mass spectroscopy (Supplementary Table S1). Among the differences between wild-type and Arf−/− MEFs, enhanced nucleolar expression (10-fold) of DDX5 RNA helicase was revealed (Fig. 1D). The earliest observed effect of DDX5 on ribosome biogenesis was at the level of 47S pre-rRNA concurred with its aforementioned association of DDX5 with the 40S and 60S pre-ribosomal fractions (32). By immunoprecipitation, we observed an interaction between DDX5 and the 40S and 60S pre-ribosomal fractions (Fig. 1E). Biochemical fractionation confirmed the increased presence of DDX5 in Arf−/− nuclei relative to wild-type nucleoli (Fig. 1F).

To investigate whether nucleolar exclusion of DDX5 is mediated by ARF through its activation of p53, we treated Arf−/− MEFs with nutlin-3, a pharmacologic inhibitor of Mdm2. Instead of stimulating DDX5 nucleolar exclusion, nucleolar localization of DDX5 persisted in the presence of nutlin-3 (Supplementary Fig. S1). This shows that p53 activation is not responsible for the ARF-dependent nucleolar exclusion of DDX5 observed in wild-type MEFS, consistent with a p53-independent role for ARF in regulating DDX5 localization.

**ARF regulates the association of DDX5 with rDNA, rRNA, and nuclear preribosomes**

The nucleolar localization of DDX5, along with its function as an RNA helicase, suggested that DDX5 might be involved in the biogenesis of rRNA. The regulation of DDX5 by basal ARF and the role of DDX5 in the biogenesis of rRNA. The regulation of DDX5 by ARF led us to investigate whether ARF could control ribosome biogenesis through regulation of DDX5 function. Both p19ARF (mouse) and p14ARF (human) inhibit rRNA transcription (12, 28, 29), and DDX5 has been ascribed roles as a transcriptional regulator (18). However, it is unknown whether DDX5 regulates transcription at nucleolar rDNA loci.

We conducted ChIP experiments to determine whether DDX5 associated with the rDNA promoter at 2 previously identified binding sites of the RNA polymerase I transcription factor UBF (30). ARF regulated DDX5 association with these sites, such that DDX5 occupancy at the rDNA promoter was over 2-fold greater in Arf−/− MEFs compared with wild-type MEFS (Fig. 2A).

In addition, DDX5 has been shown to be involved in processing of the 5.8S rRNA (31) and the 28S RNA from their respective rRNA precursors (32). By immunoprecipitation, we observed an interaction between DDX5 and the 40S and 60S preribosomal fractions was observed in the Arf−/− nuclear lysates relative to the corresponding wild-type fractions (Fig. 2D). These changes were due to ARF expression because wild-type and Arf−/− MEFS expressed similar levels of DDX5 protein in both whole-cell lysate (Fig. 1F) and nuclear extract (Fig. 2C).

**DDX5 enhances the synthesis and processing of ribosomal RNA**

To determine whether DDX5 could accelerate ribosome biogenesis, wild-type MEFS were transduced with a Flag-epitope-tagged DDX5 or a mutant (K144N) deficient in ATP binding (Fig. 3A). The K144N mutation in the Walker A motif abrogates not only ATP binding but also the ATPase and helicase activities of DDX5 (32). The earliest observed effect of DDX5 on ribosome biogenesis was at the level of 47S pre-rRNA transcription, in which both Flag-DDX5 and Flag-DDX5-K144N increased the amount of 47S transcript per cell (Fig. 3B). The ability of DDX5 to regulate transcription of 47S pre-rRNA concurred with its aforementioned association at the rDNA promoter. Monitoring the processing of the 47S pre-rRNA transcript by pulse-chase analysis, we discovered a more rapid accumulation of mature 28S and 18S rRNAs in cells expressing Flag-DDX5 or Flag-K144N versus vector-transduced cells (Fig. 3C and D). To determine whether the accelerated production of rRNA equated with increased protein synthesis, cytosolic fractions were collected for ribosome profile analysis. Both Flag-DDX5 and Flag-DDX5-K144N enhanced the amplitude of the actively translating polysome fraction (Fig. 3E), indicating that ectopic expression of Flag-DDX5 ultimately increases ribosome availability for translation, and that helicase activity is not required for this induction. These results indicate that DDX5 stimulates the production of functional ribosomes by increasing the total amount of mature rRNA.
DDX5 stimulates proliferation in MEFs

The ability of DDX5 to stimulate rRNA synthesis suggested that it might also be critical for growth and proliferation. The enhanced ribosome biogenesis caused by DDX5 overexpression corresponds to an increased proliferative capacity, as evidenced by the ability of Flag-DDX5 and Flag-DDX5-K144N to stimulate foci formation in wild-type MEFs (Fig. 4A). Furthermore, using 2 different shRNA constructs, we showed that knockdown of DDX5 reduced proliferation of Arf−−−/− MEFs in a dose-dependent manner (Fig. 4B and C). The dependency on DDX5 for unrestricted growth was not exclusive to Arf−−−/− MEFs, as foci formation in p53−−−/− MEFs was impaired by shRNAs targeting DDX5 (Supplementary Fig. S2A and B). DDX5 has been linked to p53 function in several reports, either as a transcriptional coactivator (33) or as a partner of p53 in microRNA processing (34). Whereas these relationships suggest that DDX5 could inhibit growth through its interactions with p53, our data point to the opposite conclusion, specifically that the dominant role of DDX5 is not growth inhibition, as would be inferred from the aforementioned studies, but rather growth stimulation.

 Knockdown of DDX5 phenocopies the p53-independent functions of ARF on ribosome output

DDX5 stimulates ribosome production, whereas ARF inhibits ribosome biogenesis at several stages: 47S transcription, rRNA processing, and rRNA export (12, 29, 35). Ultimately, the effects of Arf loss are exhibited by the enhanced ribosome profiles of Arf−−−/− MEFs relative to wild-type MEFs (12). It was unclear, however, whether these effects of ARF on the cellular ribosome profile were truly p53-independent. To characterize the p53-independent functions of ARF on ribosome biogenesis, we utilized TKO (p53−−−/−, Mdm2−−−/−, and Arf−−−/−) MEFs, in which the entire ARF–Mdm2–p53 axis has been removed (11). By adding ARF back into TKO MEFs, we investigated growth-inhibitory effects of ARF that are completely independent of p53. HA-ARF expression reduced cytosolic ribosomes in the actively translating polyribosome fraction (Fig. 5A), showing a p53-independent role for ARF in the regulation of ribosome output.
ribosome output. Knockdown of DDX5 in TKO MEFs mimicked the effects of ARF overexpression on cytosolic ribosome content (Fig. 5B), causing a decrease in polyribosome peak amplitude. Thus, a DDX5 loss-of-function is equivalent to a p53-independent ARF gain-of-function on ribosome output.

**ARF inhibits the interaction between DDX5 and NPM**

We previously identified an interaction between NPM and DDX5 while probing for NPM binding partners (26). Like DDX5, NPM is a multifunctional protein, with key roles at multiple stages of ribosome biogenesis. NPM associates with the rDNA locus (36), regulating transcription and processing of the rRNA (1). Furthermore, NPM functions as a nuclear export chaperone for ribosomes (26), a function that is antagonized by ARF (15). Interestingly, early embryonic lethality is a phenotype of both Npm1−/− and Ddx5−/− mice (13, 31, 37). We hypothesized that ARF impaired DDX5 function through regulation of its interaction with NPM.

Given the ability of ARF to regulate both proteins individually, we tested whether ARF effected the interaction between DDX5 and NPM. Comparison of wild-type and Arf−/− MEFs revealed that ARF significantly reduced the interaction of DDX5 with NPM (Fig. 6A). We then sought to determine the NPM-binding domain on DDX5 to assess whether this interaction was critical for the growth-stimulatory abilities of DDX5. Little has been reported on the proteins that interact with DDX5 through its C-terminal domain. Given the possibility that core domain mutations might directly impair conserved features that are critical in the DEAD-box helicase family and complicate any interpretations of its overall importance, we instead focused on mutations in the C-terminus. A panel of overlapping C-terminal deletion mutations was introduced to DDX5 in a GST-fusion
protein expression vector. In vitro immunoprecipitation reactions using His-tagged NPM and GST-DDX5 or its mutants mapped an NPM interaction motif to residues 500 to 610 at the C-terminus of DDX5 (Fig. 6B). For further experiments, we chose a smaller mutant within this domain, DDX5D520–550. Whereas ectopically expressed Flag-DDX5 interacted with endogenous NPM in Arf−/− MEFs, the D520–550 mutant displayed no visible interaction (Fig. 6C). Flag-DDX5D520–550 also had reduced occupancy of the rDNA promoter compared with wild-type Flag-DDX5 (Fig. 6D) and did not stimulate 47S pre-rRNA transcription (Fig. 6E). Furthermore, whereas Flag-DDX5 associated with nuclear pre-ribosomal fractions containing the 40S and 60S ribosomal subunits, Flag-DDX5D520–550 was almost completely absent from the 60S fractions containing the large ribosomal protein rpL7a (Fig. 6F). Finally, in transduced Arf−/− MEFs, Flag-DDX5Δ520–550 expression did not affect proliferation compared with the empty vector control, whereas Flag-DDX5 expression enhanced proliferation (Fig. 6G). Thus, it seems that DDX5 cooperates with NPM, through a direct interaction that is antagonized by ARF, to stimulate rRNA synthesis and proliferation.

RasV12-induced transformation of Arf−/− MEFs requires DDX5

Transduction of wild-type MEFs with oncogenic RasV12 results in ARF induction and growth arrest (22). Conversely, transduction of RasV12 transforms Arf−/− MEFs, as determined by colony formation in soft agar. To determine whether DDX5 meets the criteria of a classic oncogene, wild-type MEFs expressing Flag-DDX5, alone or in combination with RasV12,
were plated in soft agar to evaluate anchorage-independent growth. Whereas RasV12-transduced Arf/C0/C0 MEFs plated in parallel formed robust colonies, wild-type MEFs expressing Flag-DDX5 and RasV12 did not form colonies (Supplementary Fig. S3A). Furthermore, unlike RasV12, Flag-DDX5 was unable to stimulate transformation of TKO MEFs (Supplementary Fig. S3B). This suggests that DDX5 is not an oncogene as it cannot, in combination with Arf loss, p53 loss, or RasV12 overexpression, drive transformation.

Despite not being sufficient to transform cells, it remained possible that DDX5 was necessary for transformation. To determine whether DDX5 is required for oncogenic transformation in the absence of Arf, we transduced Arf−/− MEFs with shRNA against DDX5 followed by ectopic expression of RasV12 (Fig. 7A). Knockdown of DDX5 impaired the ability of RasV12 to stimulate colony formation and anchorage-independent growth (Fig. 7B), suggesting that transformation of MEFs by RasV12 requires the cooperation of DDX5.

To determine whether Ras-transformed fibroblasts could form tumors in vivo, Arf−/− MEFs transduced with RasV12 and shDDX5 or a scrambled shRNA were s.c. inoculated into the flanks of nude mice. RasV12-induced tumor growth in nude mice was reduced by knockdown of DDX5 (Fig. 7C and D). The dependence on DDX5 for the growth of these Arf null tumors suggests that DDX5 may function as a non-oncogene by sustaining the levels of ribosome production required by transformed cells to maintain their accelerated proliferation rates.

Discussion

The role of ARF in regulating p53 is well established, but the mechanisms by which it exerts its p53-independent tumor suppressor function are yet to be fully characterized. Our group and others have recently shown the regulation of translation by ARF, but mechanistic details of its involvement are limited. Both mouse and human ARF interact with nucleolar proteins involved in ribosome biogenesis as well as ribosomal components themselves (1, 38). Furthermore, ectopic expression of human p14ARF decreases polyribosomes in a p53-independent manner (38). ARF has recently been linked to ribosome biogenesis through its regulation of TTF-1 (29) and its ability to inhibit ribosome export via its nucleolar interaction with NPM (15, 38). Here we have shown that ARF can control the protein composition of the nucleolus, the central organelle in ribosome biogenesis. Our observation that ARF can regulate DDX5 RNA helicase provides a mechanistic explanation for the inhibitory effects of ARF on 47S rRNA transcription and processing (35).
Our data suggest that most of the endogenous pool of DDX5 may be excluded from nucleoli and inactive in ribosome biogenesis, until a cellular perturbation stimulates this activity. Consistent with this model, upon loss of Arf a substantial increase in nucleolar DDX5 was observed, accompanied by tremendous gains in ribosome production. Surprisingly, both DDX5 and the helicase-dead DDX5 mutant (K144N) were able to stimulate 47S transcription and cellular ribosome output. The ability of DDX5-K144N to increase 47S pre-rRNA transcription is consistent with reports that helicase activity may be dispensable for the activities of DDX5 as a transcriptional coregulator (33, 39). NPM was important for DDX5 to associate with the rDNA promoter and to facilitate 47S pre-rRNA transcription. The DDX5-NPM-binding mutant was also unable to associate with the nuclear 60S pre-ribosomal fraction or enhance proliferation, further underscoring the link between the effects of DDX5 on ribosome biogenesis with those on growth and proliferation. Clearly, the formation of DDX5–NPM complexes, enhanced in the absence of Arf, is necessary for the nucleolar gain-of-function activity reported here for DDX5.

Our results provide a new perspective for understanding the tumor suppressor function of ARF, which has classically been thought of as a checkpoint sensor of hyperproliferative signals. The data presented here suggest that an equally important mechanism by which ARF functions as a tumor suppressor is to limit ribosome output as a defense against oncogene activation and the attendant enhanced cellular protein synthesis requirements. Therefore, in the absence of Arf, DDX5 becomes a...
requisite non-oncogene effector that promotes an increased translational output, in accord with the higher demand for protein production required upon oncogene activation. The ability of ectopic DDX5 expression to stimulate ribosome biogenesis and growth further proves the central role of DDX5 in regulating this translational output.

Our data showing the growth-stimulatory functions of DDX5 in ribosome biogenesis provides a strong rationale to explain the link between DDX5 and cancer. Although this concept is still in its infancy, most non-oncogenes are thought of as critical regulators of cellular stress responses and that their expression provides cancer cells the means to tolerate multiple stresses (40). It is unclear how DDX5 and ribosome biogenesis fit into this stress tolerance model. Rather, DDX5 may represent a class of non-oncogenes whose activities are unleashed in the absence of crucial tumor suppressors. In this setting, the role of the DDX5 non-oncogene is to make a required cellular process, such as ribosome biogenesis, more efficient or prolific in preparation for the tremendous protein synthesis demands following malignant transformation. It remains to be determined whether DDX5 will be an efficacious target in the treatment of cancer; however, our results validate its importance in supplying the sustained ribosome output required for oncogenic transformation. In summary, DDX5 participation in ribosome biogenesis is negatively regulated by ARF, which inhibits the DDX5–NPM interaction, suggesting a dynamic interplay through which ARF and DDX5 duel for nucleolar growth control.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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ARF Opposes DDX5 RNA Helicase

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