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Hypergrowth mTORC1 Signals Translationally Activate the ARF Tumor Suppressor Checkpoint

Alexander P. Miceli, Anthony J. Saporita and Jason D. Weber

The ARF tumor suppressor is a potent sensor of hyperproliferative cues emanating from oncogenic signaling. ARF responds to these cues by eliciting a cell cycle arrest, effectively abating the tumorigenic potential of these stimuli. Prior reports have demonstrated that oncogenic RasV12 signaling induces ARF through a mechanism mediated by the Dmp1 transcription factor. However, we now show that ARF protein is still induced in response to RasV12 in the absence of Dmp1 through the enhanced translation of existing Arf mRNAs. Here, we report that the progrowth Ras/tuberous sclerosis complex (TSC)/mTORC1 signaling pathway regulates ARF protein expression and triggers ARF-mediated tumor suppression through a novel translational mechanism. Hyperactivation of mTORC1 through Tsc1 loss resulted in a significant increase in ARF expression, activation of the p53 pathway, and a dramatic cell cycle arrest, which were completely reversed upon Arf deletion. ARF protein induced from RasV12 in the absence of Dmp1 repressed anchorage-independent colony formation in soft agar and tumor burden in an allograft model. Taken together, our data demonstrate the ability of the ARF tumor suppressor to respond to hypergrowth stimuli to prevent unwarranted tumor formation.

Regulatory checkpoints are key for maintaining homeostasis in the cell. Transit through the mammalian cell cycle is tightly regulated by a series of essential checkpoints that prevent progression in the presence of hyperproliferative signals or genotoxic insults, such as DNA damage, a stalled replication fork, or improper spindle assembly (7, 9, 22). These and several other regulatory checkpoints are so critical for cellular homeostasis that their loss contributes to the deleterious events that are among the hallmarks of cancer (12).

The ARF tumor suppressor functions as an important checkpoint in the cell, acting as a key sensor of hyperproliferative signals. ARF is one of the two tumor suppressors encoded by the CDKN2A (Ink4a/Arf) locus (37). ARF functions in both p53-dependent and p53-independent manners (42). Arf−/− mice are highly tumour prone, predominantly developing spontaneous fibrosarcoma and lymphoma malignancies (20, 21). Deletion or silencing of the Ink4a/Arf locus through hypermethylation of the promoters is extremely common in a multitude of human tumors; among these are numerous examples where ARF function is specifically abrogated independently of p16INK4a (40). These observations underscore the significance of the antitumorigenic functions of ARF and the necessity of cancer cells to evade ARF tumor suppression.

Basal expression of ARF is nearly undetectable. However, ARF protein levels are robustly upregulated in response to excessive proliferative cues, such as those emanating from the RasV12, Myc, E1A, v-Abl, and E2F oncogenes (3, 8, 34, 38, 56). Upon induction, ARF binds MDM2, the E3 ligase responsible for targeting p53 for proteasome-mediated degradation (52). ARF’s sequestration of MDM2 in the nucleolus allows p53 to accumulate in the nucleoplasm and to activate downstream targets that trigger cell cycle arrest (53).

Cell proliferation and cell growth are intimately linked. As such, proliferative and growth stimuli often invoke cross talk at key signaling networks to properly regulate the timing of cell cycle progression and protein synthesis. A key player in this regulation is the mammalian target of rapamycin (mTOR) signal transduction pathway (36). mTOR is a conserved serine/threonine kinase that assembles into two major multiprotein-containing complexes, mTORC1 and mTORC2 (57), each of which is reported to serve a unique function in the cell (29). mTORC1 contains Raptor, LST8, Deptor, PRAS40, and mTOR and is critical for regulating protein synthesis; mTORC2 includes Rictor, LST8, Deptor, Protor, Sin1, and mTOR and plays a role in cytoskeletal organization (57). mTOR responds to several upstream stimuli, including growth factors and nutrients. Upstream signaling is propagated through Ras and phosphatidylinositol 3-kinase (PI3K) (41). In addition, the tuberous sclerosis complex (TSC) gene products are critical upstream negative regulators of mTORC1 signal transduction (15); loss of either Tsc1 or Tsc2 results in constitutive mTORC1 signaling and increased phosphorylation of S6K1 (ribosomal protein S6 kinase 1) and initiation factor 4E binding protein 1 (4E-BP1). This has direct consequences for the protein translation machinery and the downstream gene targets that are regulated by this pathway (14). Mutations among pathway members are common in hamartoma-forming syndromes and a broad spectrum of human cancers (11, 13).

Given ARF’s central role in sensing hyperproliferative signals, we hypothesized that ARF might also be sensitive to hypergrowth cues emanating from mTORC1 signaling. In this report, we investigated ARF gene expression and function in response to hyperactivation of the progrowth mTORC1 signal transduction pathway. Importantly, we also interrogated ARF function in the absence of...
collaborating signals from the Dmp1 transcription factor, the only known regulator of ARF induction from RasV12. RasV12 expression in murine embryonic fibroblasts (MEFs) lacking Dmp1 resulted in increased ARF protein levels, suggesting that (i) Dmp1-mediated transcription of Arf is not obligatory for ARF induction and (ii) another pathway downstream of Ras must modulate ARF expression. Using pharmacological and genetic manipulation, we now show that the Ras/TSC/mTORC1 pathway regulates ARF through a novel translational mechanism. Based on our findings, we propose that ARF can respond to hypergrowth signals emanating from a hyperactivated mTORC1 pathway to prevent tumor formation.

**MATERIALS AND METHODS**

**Mice and cell culture.** Tsc1flox/flox mice were a generous gift from Jeffrey Arbeit (Washington University, St. Louis, MO) (23), with permission from David Kwiatkowski (Harvard University, Cambridge, MA). Tsc1flox/flox and Arfl−/− mice were intercrossed for several generations to generate Tsc1flox/flox, Arfl−/− mice. Inbred homozygous female athymic nude mice (Foxn1tm1/Jax/Foxn1tm1) were purchased from Jackson Laboratory (Bar Harbor, ME). Nude mice were 5 weeks old at the time of purchase and were housed in our facility until they were approximately 7 weeks of age to acclimate to the new facility before injections were performed. Low-passage (passage 3 [P3] to P5) primary murine embryonic fibroblasts for all described genotypes were established as previously described (21) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 0.1 mM nonessential amino acids, 2 μg/ml gentamicin. Etoposide (Sigma, St. Louis, MO) and rapamycin (LC Laboratories, Woburn, MA) were respectively used at final concentrations of 50 μM and 100 nM.

**Virral production and infections.** Pbabe-puro-H–RasV12 was a generous gift from Martine Rousseau (St. Jude Children’s Research Hospital, Memphis, TN). Pbabe-HA-ARF (where HA is hemagglutinin), pWZL-GFP-IRES-blast (where GFP is green fluorescent protein and blast is blasticidin), and pWZL–RasV12–IRES-blast have been previously described (4, 51). Retroviral production was performed as previously described (4, 39). Retroviral helper DNA was kindly provided by Charles Sawyer’s (University of California Los Angeles, Los Angeles, CA). Collected retrovirus was used to infect MEFs in the presence of 10 μg/ml Polybrene. Infected MEFs were selected in 2 μg/ml puromycin and were harvested for analysis at 5 days postinfection. For the production of lentiviruses encoding short hairpin RNAs for Tsc1, 2×105 293T cells were cotransfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with pHCMV.G, CMV/Neo, and pBabe-HA-ARF (where HA is hemagglutinin), pWZL-GFP-IRES-blast (where GFP is green fluorescent protein and blast is blasticidin), and pBabe-HA-ARF (where HA is hemagglutinin), pWZL–RasV12–IRES-blast were, respectively, used to infect MEFs in the presence of 10 μg/ml Polybrene. Infected MEFs were selected in 2 μg/ml puromycin and were harvested for analysis at 5 days postinfection. For the production of lentiviruses encoding short hairpin RNAs, 5×105 293T cells were cotransfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with pHCMV.G, CMV/Neo, and pBabe-HA-ARF. Lentiviruses were packaged, and MEFs were infected as described above.

**For RNAi against Raptor and Rictor,** short hairpin RNA oligonucleotides were purchased from Qiagen (Valencia, CA) and were transduced using the Nucleofector system (Amara, Walkersville, MD) according to the manufacturer’s instructions. Sequences for the short hairpin RNAs recognizing Raptor and Rictor, respectively, are 5’-CCGGTCATGACTTACGGAGA-3’ and 5’-CAGAAGAATGATCTTACTGTA-3’.

**Western blotting.** Harvested cells were resuspended and sonicated in radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid) containing protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride [PMSF], 0.4 U/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM β-glycerophosphate, 0.1 mM NaF, 0.1 mM NaVO4). Proteins (30 to 80 μg) were separated on 12.5% sodium dodecyl sulfate (SDS)-containing polyacrylamide gels. Separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Boston, MA). Membranes were probed with the following antibodies: rabbit anti-Rictor (A300-459), rabbit anti-TSC1 (A300-316), and rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) (A300-641) (all from Bethyl Laboratories; Montgomery, TX); rat anti-Riap (ab26696; Abcam, Cambridge, MA); mouse anti-MDM2 (sp115; Calbi- ochem/EMD Chemicals, Gibbstown, NJ); mouse anti-Akt (sc8342), mouse anti-p21 (sc6246), rabbit antinucleoposin (anti-NPM; sc6013), mouse anti-γ-tubulin (sc17787), and rabbit anti-Ras (sc5290) (all from Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-p53 (2524), rabbit anti-phospho-extracellular signal-regulated kinase 1 and 2 (anti-phospho-ERK1/2), Thr 202/Tyr 204 (4377), rabbit anti-ERK1/2 (9102), rabbit anti-phospho-S6, Ser 244/244 (2115), rabbit anti-S6 (2317), rabbit anti-Raptor (4978), rabbit anti-phospho-4EBP1, Thr37/46 (2855), rabbit anti-YB1 (4952), and rabbit anti-p70 S6K1 (9202) (all from Cell Signaling Technologies, Danvers, MA). Secondary horseradish peroxidase-conjugated anti-rabbit, anti-rat, or anti-mouse antibodies (Jackson ImmunoResearch, West Grove, PA) were added, and Amersham ECL Plus (GE Healthcare, Piscataway, NJ) was used to visualize the bands.

**Quantitative RT-PCR and endpoint PCR.** Total RNA was extracted from cells with a Nucleospin RNAII system (Clontech, Mountain View, CA) according to the manufacturer’s instructions. Reverse transcription (RT) reactions were performed using a SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA) with an oligo(dT) primer. Real-time PCR was performed on an iCycler apparatus (Bio-Rad, Hercules, CA) using iQ Sybr Green Supermix (Bio-Rad, Hercules, CA). Fold change was calculated using the ΔΔCt (where Ct is threshold cycle) method (28). To measure Arf mRNA, the following primers were used: forward, 5’-GA GTACAGAGCGGAGGACAT-3’; reverse, 5’-ATCATCATACCTGGTGC GAGACTCC-3’. To measure Gapdh mRNA, the following primers were used: forward, 5’-GCCTGGGCTCTACCTGAAGGG-3’; reverse, 5’-GGA TGACCTTTGCCACAGCCGC-3’. To assess the presence of Dmp1 mRNA in MEF samples, total RNA was isolated, first-strand synthesis was used to generate cDNA with an oligo(dT) primer, and endpoint PCR analysis was performed. Primers used for detecting Dmp1 were the following: forward, 5’-CTGATACCTGAAAG AATCGCTA-3’; reverse, 5’-TGTATTATCTTCCAAGGGGCG-3’ (19). PCRs were separated on an agarose gel and stained with ethidium bromide.

**RNA and protein stability.** Infected MEFs were treated with either 4 μg/ml actinomycin D (Sigma, St. Louis, MO) to assess mRNA stability or 25 μg/ml cycloheximide (Sigma, St. Louis, MO) to assess protein stability. Cells were harvested over a time course of 0, 2, 4, 6, or 8 h posttreatment and subjected, respectively, to RNA isolation, cDNA synthesis reaction, and qualitative reverse transcription-PCR (qRT-PCR) analysis or to Western blot analysis.

**Immunoprecipitation.** Infected MEFs were freshly harvested, and cells were resuspended and sonicated in EBC lysis buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 0.5% NP-40, 1 mM EDTA). Then, 300 μg of protein lysate was immunoprecipitated overnight with a rabbit anti-ARF polyclonal antibody or normal rabbit IgG (sc2027; Santa Cruz Biotech-
nology, Santa Cruz, CA). Immune complexes recovered by protein A-Sepharose (GE Healthcare, Piscataway, NJ) were washed three times with EBC buffer and were denatured. Proteins were separated on 12.5% sodium dodecyl sulfate (SDS)-containing polyacrylamide gels and were transferred onto PVDF membranes (Millipore, Boston, MA) and subjected to direct immunoblotting as indicated.

**Indirect IF and BrdU incorporation.** Infected MEFs were plated onto coverslips. Cells were washed with PBS and fixed at room temperature using 10% formalin–10% methanol, followed by incubation with 1% NP-40 at room temperature for 5 min. Cells were stained with antibodies recognizing ARF (ab26696; Abcam, Cambridge, MA) or MDM2 (op113; Calbiochem/EMD Chemicals, Gibbstown, NJ), followed by the corresponding secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen, Carlsbad, CA, respectively). Cells were then counterstained for nuclei with SlowFade Gold Antifade mounting reagent with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA). Fluorescence signals were detected using a Nikon epifluorescent compound microscope fitted with a Nikon FDX-35 charge-coupled-device camera. For measurement of DNA replication, 5-bromodeoxyuridine (BrdU) (BrdU) (Sigma, St. Louis, MO) was added to the culturing medium for 2, 18 or 24 h, as indicated in the figure legends, at a final concentration of 10 μM. Cells were then treated for immunofluorescence (IF) analysis as noted above and additionally incubated with 1.5 N HCl at room temperature for 10 min. A mouse monoclonal antibody recognizing BrdU (Amer-sham/GE Healthcare, Piscataway, NJ) was used.

**Cell proliferation assay, focus formation, and soft-agar formation.** For cell proliferation assays, equal numbers of cells (5 × 10^4 Dmp1−/− MEFs; 1 × 10^5 Tsc1ol/fl or Tsc1ol/fl; Arf−/− MEFs) were replated in triplicate. Every 24 h thereafter, cells were harvested and counted using a hemacytometer. For focus formation, 5 × 10^3 infected cells were plated in triplicate onto 10-cm dishes. Cells were grown for 14 days in complete medium and then were fixed with 100% methanol and stained for 30 min with 50% Giemsa. For soft-agar colony formation, 1 × 10^3 infected cells were seeded in triplicate on 60-mm dishes and allowed to grow for 21 days in complete medium supplemented with fetal bovine serum and Noble agar.

**Apoptosis analysis.** Infected MEFs were stained with fluorescein isothiocyanate (FITC)-annexin V and propidium iodide using a Dead Cell Apoptosis Kit (V13242; Molecular Probes/Invitrogen, Carlsbad, CA) according to the manufacturer’s specifications. Cells were analyzed by flow cytometry using a Becton Dickinson FACScalibur cell sorter with CELLQuest Pro (version 5.2) analytical software.

**Ribosome fractionation, RNA isolation, and qRT-PCR.** Cells were treated with cycloheximide (10 μg/ml) for 5 min before being harvested. Equal numbers of cells (3 × 10^6) were lysed, and cytosolic extracts were subjected to ribosome fractionation as previously described (33, 46) using a density gradient system (Teledyne ISCO, Lincoln, NE). RNA was isolated from monosome/disome and monosome/subcloned fractions using RNAseolv (Omega Bio-Tek, Norcross, GA) according to the manufacturer’s specifications. Reverse transcription reactions were performed using a SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA) with an oligo(dt) primer. Real-time PCR was performed on an iCycler apparatus (Bio-Rad, Hercules, CA) using SoFast EvaGreen Supermix (Bio-Rad, Hercules, CA) to amplify Arf or Gapdh from monosome/disome and polysome fractions. Numbers of Arf or Gapdh transcripts per fraction were calculated from a standard curve generated from serial dilutions of a known quantity of subcloned Arf or Gapdh cDNA. *Arf* or *Gapdh* mRNA distribution per fraction was calculated as a percentage of the total number of transcripts in all collected fractions. For the ribosome profiling analysis shown in Fig. 6, cells were treated with puromycin (Sigma, St. Louis, MO) at a final concentration of 1 mM for 3 h.

**Tumorigenic assay.** Infected MEFs were trypsinized and counted. A total of 2 × 10^5 cells were resuspended in PBS and injected subcutaneously into the left flank of athymic nude Foxn1nu/Foxn1nu mice. A sample size of five mice per condition was used. Tumor growth was monitored every day by palpation at the injection site, and the diameter of the tumors was measured in two different planes using a digital caliper. Tumor volume was calculated with the following formula: (height^2 × length)/2, where height represents the smaller of the two measurements.

**Densitometry, image, and statistical analysis.** Autoradiograms and immunoblot films were scanned using an ImageScannerII apparatus (GE Healthcare, Piscataway, NJ), and densities were determined using ImageQuant, version 2005 (GE Healthcare, Piscataway, NJ). Statistical analyses were performed using a Student’s t test.

**RESULTS**

ARF is responsive to RasV12 and is functional in the absence of Dmp1. Previous reports have demonstrated that ARF responds to the RasV12 oncoprotein through a mechanism mediated by the Dmp1 transcription factor (17, 18, 44). However, it was also noted that ARF’s induction from RasV12 is compromised, but not completely lost, in the absence of Dmp1 (16, 18). We sought to further understand the putative regulation and function of ARF in the absence of cooperating transcriptional signals. Dmp1+/− MEFs were infected with a retrovirus encoding RasV12 and harvested at 5 days postinfection for gene expression analysis; confirmation of Dmp1-null status of the MEFs was performed by PCR analysis of reverse transcribed cDNA (Fig. 1A). Consistent with prior find-ings (16, 18), we observed that ARF protein is still increased in response to RasV12 overexpression in the absence of Dmp1 (Fig. 1B). Strikingly, Arf mRNA levels were not significantly altered from RasV12 overexpression in Dmp1-deficient cells (Fig. 1C).

Collectively, these data indicate that transcriptional activation of Arf gene expression is not obligatory for inducing ARF protein levels in response to RasV12. These observations also indicate that the Ras/Dmp1 pathway is not the only mechanism by which ARF can sense the oncogenic cues of RasV12 signaling.

Since ARF is sensitive to the oncogenic stimulus of RasV12 in Dmp1-null cells, we hypothesized that basal ARF could still exert its important antiproliferative functions in these cells. To test this, we infected Dmp1+/− MEFs with a lentivirus encoding a short hairpin targeting a scrambled control or Arf exon 1β (siScramble and siARF, respectively) (1), the ARF-specific exon of the CDKN2A locus. As shown by Western blot analysis, ARF protein levels were dramatically reduced (~90%) compared to those of the scrambled control (Fig. 2A). To determine the effect of acute knockdown of ARF on cellular proliferation, equal numbers of Dmp1+/− MEFS expressing the short hairpin against Arf or scrambled control were seeded in triplicate, and total numbers of cells were counted over 5 days. Acute knockdown of ARF significantly increased the rate of proliferation of Dmp1+/− MEFS (Fig. 2B). Additionally, 5-bromodeoxyuridine (BrdU) incorporation was also monitored to measure the extent of cells entering S phase (Fig. 2C and D). Acute knockdown of ARF caused a significant increase in the percentage of cells undergoing DNA replication; this was observed with both a short (2 h) and a longer (18 h) pulse of BrdU (Fig. 2D and C, respectively). Acute knockdown of ARF did not dramatically alter the amount of cells undergoing apoptosis (4.6% for Dmp1+/− MEFS infected with siScramble-encoding virus and 3% for siARF-encoding virus) (Fig. 2E).

**Ras/TSC/mTORC1 pathway can regulate ARF.** Our data indicate that ARF is induced in response to oncogenic RasV12 independently of Dmp1 transcriptional activity. We hypothesized that the mTORC1 signal transduction pathway could potentially regulate ARF expression. This critical cell growth regulatory pathway coordinates ribosome biogenesis and mRNA translation. Regula-
tion by this pathway is often associated with translational control of target genes whose protein levels, but not mRNA levels, are modulated in particular cellular contexts (10, 24, 43). To begin evaluating this pathway, wild-type and Dmp1−/− MEFs were transduced with a retrovirus encoding RasV12 and subsequently treated with rapamycin, the pharmacological inhibitor of mTORC1 signaling, for 24 h prior to harvesting (Fig. 3A and B). Repressed levels of phospho-S6K1 (Thr 389) and phospho-S6 (Ser 240/244) revealed that mTORC1 signaling was disrupted by rapamycin exposure (Fig. 3A and B). For strains of both genotypes, the induced levels of ARF protein expression were sensitive to rapamycin treatment (Fig. 3A and B), suggesting that mTORC1 signaling is essential for ARF induction from Ras.

We next wanted to interrogate the involvement of the Ras/mTORC1 pathway in regulating ARF protein levels using genetic manipulations. Tuberous sclerosis complex 1 (TSC1) is an upstream member of the mTORC1 pathway. TSC1 forms a complex with TSC2 that negatively regulates mTORC1 signal transduction (48). We hypothesized that activation of the mTORC1 pathway by acute knockdown of TSC1 would induce ARF protein levels. To test this, wild-type MEFs were infected with lentiviruses encoding Raptor or Rictor to ensure that this finding was not a nonspecific effect of Cre recombinase or the adenoviral infection rate of Arf mRNA decay that was nearly identical in Ad-LacZ- and Ad-Cre-infected cells (Fig. 4C and D), suggesting that a higher rate of ARF protein must be synthesized in order to induce ARF protein expression to a greater extent (Fig. 3F).

To investigate whether the ARF induction observed from the loss of Tsc1 is dependent on TSC/mTORC1 signaling, we infected Tsc1flox/flox MEFs with Ad-Cre or the Ad-LacZ control and then treated them with rapamycin for 24 h prior to harvesting. Diminished levels of phospho-S6K1 (Thr 389) and phospho-S6 (Ser 240/244) demonstrated that rapamycin successfully blocked mTORC1 signaling (Fig. 3G). As seen before with infection with a retrovirus encoding RasV12 (Fig. 3A and B), ARF protein levels induced from the loss of Tsc1 were sensitive to rapamycin treatment (Fig. 3G).

To confirm the contributions of mTORC1 signaling following Tsc1 deletion to regulation of ARF, RNA interference was used to acutely knockdown Raptor or Rictor (Fig. 3H and I). Acute knockdown of Raptor, but not Rictor, abrogated the induction of ARF expression from the ablation of Tsc1 (Fig. 3H and I). These data provide further support that mTORC1, but not mTORC2, is necessary for mediating the induction of ARF from the loss of Tsc1. Taken together, these data demonstrate that hyperactivation of Ras/TSC/mTORC1 pathway can regulate ARF protein levels.

**ARF induction from mTORC1 hyperactivation uses a novel translational mechanism.** Given that mTORC1 signal transduction plays a crucial role in the translational regulation of specific mRNA transcripts, we hypothesized that this might be an underlying mechanism responsible for inducing ARF protein levels. To test this, we assessed different aspects of ARF gene expression in the face of mTORC1 hyperactivation. For each of these experiments, Tsc1flox/flox MEFs were infected with Ad-Cre or Ad-LacZ as before. Despite the increases in ARF protein expression, no significant changes were observed in Arf mRNA levels following Tsc1 loss (Fig. 4A). Next, we evaluated Arf mRNA stability and observed a rate of Arf mRNA decay that was nearly identical in Ad-LacZ- and Ad-Cre-infected Tsc1flox/flox cells (Fig. 4B). Moreover, the rate of ARF protein decay was faster in Ad-Cre-infected Tsc1flox/flox MEFs than in Ad-LacZ-infected cells (Fig. 4C and D), suggesting that a higher rate of ARF protein must be synthesized in order to induce ARF protein expression.
crease steady-state levels in the cell. We also assessed the rate of protein decay of ectopic HA-ARF expressed in Ad-Cre-infected Tsc1flox/flox;ArfH11002/H11002/H11002 MEFs (Fig. 4E and F) and noted a similarly accelerated half-life for HA-ARF (4 h). This observation supports the notion that ARF protein is being degraded at a high rate in the absence of Tsc1 compared to ARF’s normally observed half-life of 6 h (25).

To further test the hypothesis that translational regulation could be the molecular mechanism responsible for eliciting ARF’s induction from mTORC1 hyperactivation, we assessed the association of Arf mRNA with actively translating polyribosomes. To accomplish this task, cytosolic ribosomes were isolated by sucrose gradient centrifugation from equal numbers of Dmp1−/− MEFs infected with a retrovirus encoding either RasV12 or an empty vector control (Fig. 5A and B). Ribosomal subunits were detected by measuring RNA absorbance at 254 nm by continuous UV monitoring (Fig. 5B). To assess the distribution of Arf mRNA transcripts in individual fractions comprising isolated monosomes, disomes, or polysomes, total RNA was isolated from each sucrose gradient fraction, and Arf mRNA levels were determined with qRT-PCR. Strikingly, Arf mRNA transcripts associated with different polyribosome fractions in Dmp1-null cells infected with retroviruses encoding RasV12 and empty vector (Fig. 5C). In Dmp1−/− MEFs infected with a RasV12-encoding retrovirus, Arf mRNA was pooled to a heavier polyribosome fraction, indicating that there is a greater extent of Arf mRNAs being actively translated by multiple ribosomes (more ribosomes associated per mRNA) in these cells (Fig. 5C). These data support the hypothesis that ARF is translationally regulated in the presence of oncogenic RasV12 signals.

To address the possibility that general gains in global protein translation could account for the increased translation of Arf mRNA transcripts, we evaluated the distribution of Gapdh mRNA in sucrose gradient fractions in Dmp1−/− MEFs infected with a retrovirus encoding either RasV12 or an empty vector control (Fig. 5D). No dramatic differences in the distribution of Gapdh mRNA transcripts were observed across isolated monosomes or polyribosomes, in contrast to the distribution observed for Arf mRNA (Fig.
This suggests that the gain in \( \text{Arf} \) mRNA association with actively translating polyribosomes is a selective phenotype caused by Ras\(^{V12} \) oncogenic signaling in the absence of \( \text{Dmp1} \).

To confirm that \( \text{Arf} \) mRNA transcripts are actually associating with actively translating polyribosomes, we assessed whether puromycin could release \( \text{Arf} \) mRNA transcripts from the polyribosome fractions. Puromycin treatment causes a block in translation elongation and a premature release of the nascent polypeptide chain from actively translating polyribosomes (2, 45). To accomplish this, \( \text{Dmp1}^{-/-} \) MEFs were infected with blasticidin-resistant retroviral constructs encoding either GFP or Ras\(^{V12} \). Consistent with earlier findings, ARF protein is increased in response to Ras\(^{V12} \) overexpression in the absence of \( \text{Dmp1} \) (Fig. 6A). \( \text{Dmp1}^{-/-} \) MEFs infected with a retrovirus encoding GFP or Ras\(^{V12} \) were treated with 100 nM rapamycin (R) or vehicle (V) control for 24 h prior to harvesting. (H and I) Ad-Cre-infected cells were then transduced with viruses encoding short hairpins recognizing \( \text{Raptor} \) (siRaptor) or \( \text{Rictor} \) (siRictor) or a luciferase control (siLUC) at 5 days postinfection and then harvested at 9 days postinfection for Western blot analysis. P, phosphorylated.
and infected with GFP- and RasV12-encoding retroviruses showed dramatic increases in the amplitude of the 80S peak, along with the complete disappearance of the polysome peaks (Fig. 6B). Arf mRNA distribution in fractions was then determined (Fig. 6C and D). Arf mRNA distribution in puromycin-treated, GFP-expressing cells mimicked the distribution of Arf mRNA in untreated GFP-expressing cells (Fig. 6C). This surprising finding suggests that Arf mRNA found on the polysome peaks in these GFP-expressing cells could in fact be "pseudo-polysomes" as opposed to actual polyribosomes (49). In contrast, puromycin treatment released Arf mRNA from the polysome peaks in Dmp1−/− MEFs infected with RasV12-encoding retrovirus (Fig. 6D), indicating that Arf mRNA transcripts are indeed associating with actively translating polyribosomes in response to oncogenic RasV12 signaling.

To determine whether inhibition of mTORC1 signaling could similarly displace Arf mRNA distribution from polysome peaks, Dmp1−/− MEFs were transduced with a retrovirus encoding
FIG 5 Arf mRNA association with actively translating polyribosomes increases from RasV12 signaling in the absence of Dmp1. Dmp1−/− MEFs were transduced with retroviruses encoding an empty vector control (EV) or RasV12 and were harvested at 5 days postinfection. Cytosolic extracts from equal number of cells (3 × 10⁶) treated for 5 min with cycloheximide (10 μg/ml) were separated on 7 to 47% sucrose gradients with constant UV monitoring (254 nm). (A) Excess cells were lysed, and separated proteins were immunoblotted for the indicated proteins. (B) A representative graph depicts the A₂₅₄ absorbance of ribosome subunits over increasing sucrose density. (C) Total RNA was isolated from each sucrose gradient fraction, and first-strand cDNA was synthesized for each fraction. Monosome-, disome-, and polysome-associated Arf mRNA levels were measured with qRT-PCR and were calculated as a percentage of total Arf mRNA collected in all fractions. Data are the mean ± standard error of the mean of three independent experiments. (D) Monosome-, disome-, and polysome-associated Gapdh mRNA levels were measured as described for panel C.
RasV12 and subsequently treated with rapamycin for 24 h prior to harvesting. Ribosomal subunits were monitored as before (Fig. 7A and B). Although rapamycin did not completely displace Arf mRNA from translating polyribosomes, rapamycin treatment did shift Arf mRNA away from the heavy polyribosome fractions, where it accumulates in response to RasV12 (Fig. 7C). This finding demonstrates the sensitivity of Arf mRNA association with translating polyribosomes to rapamycin exposure. To further interrogate the effects of mTORC1 signaling on the association of Arf mRNA with actively translating polyribosomes, Tsc1flx/flx MEFs

FIG 6 Arf mRNA association with actively translating polyribosomes caused by hypergrowth stimuli can be disrupted with puromycin exposure. Retroviruses were generated with pWZL-GFP-IRES-blast or pWZL-RasV12-IRES-blast. Dmp1flx/flx MEFs were transduced with these retroviruses, and infected cells were analyzed at 5 days postinfection. Cells were treated with 1 μM puromycin for 3 h, and then cytosolic extracts from equal numbers of cells (3 x 10⁶) treated for 5 min with cycloheximide (10 μg/ml) were separated on 7 to 47% sucrose gradients with constant UV monitoring (254 nm). (A) Excess untreated cells were lysed, and separated proteins were immunoblotted for the indicated proteins. (B) A representative graph depicts the A₂₅₄ absorbance of ribosome subunits over increasing sucrose density. (C) Total RNA was isolated from each sucrose gradient fraction, and first-strand cDNA was synthesized for each fraction. Monosome-, disome-, and polysome-associated Arf mRNA levels were measured with qRT-PCR and were calculated as a percentage of total Arf mRNA collected in all fractions. (D) Monosome-, disome-, and polysome-associated Arf mRNA levels were measured as described for panel C.
were infected with Ad-Cre or the Ad-LacZ control and were subjected to ribosome profiling (Fig. 8A and B). We found that more Arf mRNA pooled to heavier polyribosome fractions upon the loss of Tsc1 (Fig. 8C). Taken together, these findings support the hypothesis that ARF is translationally regulated in the presence of hyperactivated Ras/TSC/mTORC1 signaling.

**ARF induction activates a p53 response.** To determine whether the ARF protein translationally induced from Tsc1 loss is functional, we assessed several aspects of ARF biology. ARF binds to and sequesters MDM2 in the nucleolus, allowing p53 protein levels to accumulate and become active in the nucleoplasm (53). 

Dmp1-/- MEFs infected with Ad-Cre or the Ad-LacZ control were analyzed for MDM2 and ARF colocalization (Fig. 9A and B). In both Ad-Cre- and Ad-LacZ-infected cells, ARF exhibited nu-
cleolar subcellular localization (Fig. 9A and B). Furthermore, we found that ARF and MDM2 had increased colocalization in nucleoli in Ad-Cre-infected Tsc1flox/flox MEFs compared to levels in Ad-LacZ-infected cells (Fig. 9A and B). Next, ARF-MDM2 complexes were immunoprecipitated from infected Tsc1flox/flox lysates with a polyclonal antibody recognizing ARF and immunoblotted for MDM2 (Fig. 9C). Induced ARF protein displayed strong binding to MDM2 in Ad-Cre-infected Tsc1flox/flox MEFs (Fig. 9C). Collectively, these data suggest that the loss of Tsc1 increases ARF protein expression and its ability to bind to and relocalize MDM2 into the nucleolus.

To examine whether this increase in ARF-MDM2 binding resulted in p53 activation, infected Tsc1flox/flox lysates were probed for p53 and two of its downstream target genes, p21 and MDM2. p53, MDM2, and p21 displayed 2-fold increases in protein levels following Tsc1 loss (Fig. 9D). Similarly, the induction of p53, p21, and MDM2 was completely abrogated in Ad-Cre-infected Tsc1flox/flox; Arf−/− MEFs (Fig. 9D), implying that ARF is necessary for facilitating the induction of p53 and its target genes in response to Tsc1 loss. Alternatively, infected Tsc1flox/flox MEFs were treated with rapamycin 24 h prior to harvesting. The induction of ARF caused by the loss of Tsc1 was disrupted due to rapamycin expo-
ARF induced from hypergrowth stimuli activates a p53 response. Tsc1^fl/fl^ MEFs were infected with adenoviruses encoding β-galactosidase (LacZ) or Cre recombinase and were harvested at 9 days postinfection for analysis. (A) Infected cells were seeded onto coverslips, fixed, and stained for indirect immunofluorescence analysis with specific primary antibodies for ARF and MDM2 and for Alexa Fluor 594 and Alexa Fluor 488, respectively. Cells were counterstained for nuclei with SlowFade Gold Antifade mounting reagent with DAPI. (B) Quantification of nuclear ARF-MDM2 colocalization is depicted from indirect immunofluorescence analysis as described for panel A. Representative data are expressed as the mean ± standard deviation of 50 nuclei counted in triplicate, and P values were calculated using the Student t test. (C) Lysates from infected cells were immunoprecipitated with a rabbit polyclonal antibody directed against ARF or normal rabbit IgG. Proteins immune complexes were separated, transferred to PVDF membranes, and immunoblotted with the indicated antibodies. (D and E) Infected cells were lysed, and separated proteins were immunoblotted for indicated proteins. Expression fold change over the LacZ control is indicated (D). Ad-Cre-infected cells were treated with 100 nM rapamycin (R) or vehicle (V) control for 24 h prior to harvesting (E).

FIG 9 ARF induced from hypergrowth stimuli activates a p53 response. Tsc1^fl/fl^ MEFs were infected with adenoviruses encoding β-galactosidase (LacZ) or Cre recombinase and were harvested at 9 days postinfection for analysis. (A) Infected cells were seeded onto coverslips, fixed, and stained for indirect immunofluorescence analysis with specific primary antibodies for ARF and MDM2 and for Alexa Fluor 594 and Alexa Fluor 488, respectively. Cells were counterstained for nuclei with SlowFade Gold Antifade mounting reagent with DAPI. (B) Quantification of nuclear ARF-MDM2 colocalization is depicted from indirect immunofluorescence analysis as described for panel A. Representative data are expressed as the mean ± standard deviation of 50 nuclei counted in triplicate, and P values were calculated using the Student t test. (C) Lysates from infected cells were immunoprecipitated with a rabbit polyclonal antibody directed against ARF or normal rabbit IgG. Proteins immune complexes were separated, transferred to PVDF membranes, and immunoblotted with the indicated antibodies. (D and E) Infected cells were lysed, and separated proteins were immunoblotted for indicated proteins. Expression fold change over the LacZ control is indicated (D). Ad-Cre-infected cells were treated with 100 nM rapamycin (R) or vehicle (V) control for 24 h prior to harvesting (E).

sure (Fig. 9E), and, consequently, the induction of p53, p21, and MDM2 was similarly abrogated in the absence of ARF induction (Fig. 9E).

**ARF/p53 response causes cell cycle arrest.** Given that hyperactivation of mTORC1 signaling increases ARF protein expression and that ARF induces p53 and its downstream targets, we hypothesized that ARF was responsible for eliciting a cell cycle arrest in response to mTORC1 hyperactivation. To test this, cell proliferation was monitored each day for 6 days. The rate of proliferation of Ad-Cre-infected Tsc1^fl/fl^ MEFs was markedly reduced compared to that of Ad-LacZ-infected cells (Fig. 10A and B), consistent with the ARF-dependent activation of p53 (Fig. 9). However, this proliferation defect was absent upon Tsc1 loss in cells also lacking Arf (Fig. 10B). Of note, changes in cell death (Fig. 10C) do not account for the decrease in total cell number observed in Ad-Cre-infected Tsc1^fl/fl^ MEFs. Additionally, BrdU incorporation was measured (Fig. 10D). As seen before, Ad-Cre-infected Tsc1^fl/fl^ MEFs exhibited a significant decrease in BrdU incorporation compared to Ad-LacZ-infected cells (Fig. 10D). Notably, this decrease was completely rescued in the absence of Arf (Fig. 10D). Furthermore, acute knockdown of TSC1 reduced BrdU incorporation in wild-type MEFs (Fig. 10E), corresponding with their dose-dependent induction of ARF protein (Fig. 3C).

Since ARF serves to prevent proliferation in response to loss of Tsc1, we hypothesized that removal of ARF would permit cells with hyperactivated mTOR to proliferate long-term without being properly checked. To test this, Tsc1^fl/fl^; Arf^−/−^ MEFs were infected with Ad-Cre or the Ad-LacZ control and subjected to long-term focus formation analysis (Fig. 10F and G). Significantly more foci formed by hyperactivating mTOR signaling in Arf^−/−^ cells, and there was an increase in total focus area (Fig. 10H and I). Taken together, this indicates that ARF keeps cell proliferation in check by responding to heightened levels of mTORC1 signaling to induce cell cycle arrest.

**Translationally regulated ARF represses transformation and tumorigenesis.** The observation that ARF induces a p53-mediated cell cycle arrest in response to hypergrowth cues emanating from hyperactivation of mTORC1 signal transduction led us to test the hypothesis that ARF could inhibit transformation and tumorigenesis in response to these hypergrowth cues. We infected Dmp1^−/−^ MEFs or Arf^−/−^ MEFs with a retrovirus encoding RasV12 or an empty vector control and assessed anchorage-independent growth in soft agar (Fig. 11A). In MEFs infected with RasV12-encoding virus, Dmp1^−/−^ cells formed significantly fewer colonies in soft agar than Arf^−/−^ cells (Fig. 11B). To determine if the induced levels of ARF in Dmp1^−/−^ MEFs infected with RasV12-encoding virus were responsible for the inhibition of colony formation, infected Dmp1^−/−^ MEFs were also transduced with virus encoding an siRNA recognizing ARF or a scrambled
control. Knockdown of ARF restored the ability of Dmp1−/− MEFs infected with RasV12−encoding virus to form colonies in soft agar, thereby phenocopying the colony-forming potential of infected Arf−/− MEFs (Fig. 11B). No dramatic changes in apoptotic cell death were observed, suggesting that changes in cell death do not account for the differences observed in colony formation (Fig. 11C).

To determine whether translationally regulated ARF could repress tumorigenesis in an allograft model, we assessed tumor formation and burden of Dmp1−/− or Arf−/− MEFs infected with RasV12−encoding virus by subcutaneously injecting MEFs into the flanks of nude mice (Fig. 11D and E); as before, Dmp1−/− MEFs were also infected with a virus encoding an siRNA recognizing ARF or a scrambled control (siScramble) in order to determine the specificity of ARF’s involvement in preventing tumorigenesis (Fig. 11E, inset). Strikingly, tumor onset and growth were markedly reduced in mice injected with Dmp1−/− MEFs infected with siScramble-encoding virus compared to Arf−/− MEFs (Fig. 11D and E). Furthermore, acute

FIG 10 ARF/p53 response induces a cell cycle arrest. (A to D) Tsc1flox/flox or Tsc1flox/flox; Arf−/− MEFs were infected with adenoviruses encoding β-galactosidase (LacZ) or Cre recombinase as indicated. Infected cells were lysed, and separated proteins were immunoblotted for indicated proteins (A). A total of 1 × 10⁵ cells were seeded in triplicate for each indicated time point at 5 days postinfection. Cells were then trypsinized and counted with a hemacytometer each day for 6 days thereafter (B). Infected cells were harvested and stained with FITC-annexin V and propidium iodide and subjected to flow cytometry analysis (C). Representative data are depicted as the mean ± standard deviation of 10,000 events performed in triplicate. (D) Infected cells were seeded on coverslips at 9 days postinfection. On day 10 postinfection, cells were pulsed with BrdU for 4 h. Indirect immunofluorescence analysis was used to score BrdU incorporation. Representative data are expressed as the mean ± standard deviation of 50 nuclei counted in triplicate, and P values were calculated using the Student t test. (E) Wild-type MEFs were infected with lentiviruses encoding short hairpins against Tsc1 or the siScramble control and were seeded on coverslips at 7 days postinfection for BrdU incorporation. Cells were pulsed with BrdU for 18 h, and analysis was performed as described for panel D. (F to I) Tsc1flox/flox; Arf−/− MEFs were infected with adenoviruses encoding β-galactosidase (LacZ [L]) or Cre recombinase (C) as indicated. A total of 5 × 10⁴ cells were seeded in triplicate onto 10-cm² dishes for focus formation analysis. Infected cells were lysed, and separated proteins were immunoblotted for the indicated proteins (F). Cells were grown for 14 days in complete medium and were fixed and stained with Giemsa (G). Panels H and I show, respectively, the quantification of the total number of foci and total focus area of representative images from panel G. (P), phosphorylated.
knockdown of ARF in Dmp1−/− MEFs restored the tumorigenic potential of these cells, partially phenocopying the tumor burden observed in Arf−/− MEFs infected with RasV12-encoding virus (Fig. 11D and E). Collectively, these data support the model that ARF acts as a critical checkpoint against hypergrowth stimuli and that in response to these stimuli, ARF can repress cellular transformation (Fig. 12).

**DISCUSSION**

ARF is a key tumor suppressor responsible for safeguarding the cell against oncogenic stimuli. While it has long been appreciated that ARF can inhibit cell cycle progression, both through p53-dependent and p53-independent mechanisms, the context of stimuli to which ARF responds has predominantly been categorized as hyperproliferative cues. Our results now demonstrate that...
ARF has a novel and important role sensing unwarranted hypergrowth stimuli, such as those emanating from robust activation of the mTORC1 signaling pathway. Given that cellular growth and proliferation are in fact two distinct biological processes, albeit highly integrated, we envision a broader range of oncogenic stimuli to which ARF can respond in its antitumorigenic efforts. Since oncogenic stimuli provide the selective pressure for the outgrowth of cancer cells that evade ARF tumor suppression (50), it is important to better understand the array of oncogenic stimuli that are susceptible to ARF tumor surveillance.

In agreement with other groups, we observed that ARF is still capable of responding to RasV12 without transcriptional induction of Arf mRNAs by Dmp1. We found that the mTORC1 pathway regulates ARF protein levels through a novel translational mechanism; Arf mRNA showed enhanced association with actively translating polyribosomes in response to RasV12 and Tsc1 loss. ARF induced from Tsc1 loss facilitated p53 pathway activation and cell cycle arrest. Furthermore, translationally regulated ARF protein repressed anchorage-independent colony formation in soft agar and tumor burden in an allograft model. Therefore, we propose that the cell utilizes this ARF checkpoint as a means to keep excessive progrowth cues under scrutiny.

Of note, Tsc1<sup>−/−</sup> MEFs have been reported to display a lower proliferative rate than Tsc1<sup>+/−</sup> or Tsc1<sup>+/+</sup> MEFs (26). Also, Zhang et al. have shown that primary Tsc2<sup>−/−</sup> MEFs display early senescence in conjunction with a higher expression of p21 (55). Our data suggest that this increase in p21 and the resultant decrease in proliferation could be facilitated in part by the translational ARF induction that ensues from the activation of mTORC1; we observed that p21 induction was abrogated upon the removal of Arf in Tsc1<sup>−/−</sup> cells and that loss of Arf rescued the proliferation defect observed in cells lacking Tsc1.

We envisage collaboration between the Ras/TSC/mTORC1 and the Ras/Dmp1 pathways which together coordinate ARF induction from oncogenic RasV12 overexpression. The involvement of the mTORC1 pathway could explain why RasV12-mediated ARF induction is compromised, but not completely lost, in a Dmp1<sup>−/−</sup> setting. Given the absolute necessity for cancer cells to bypass ARF’s checkpoint against oncogenic stimuli, it is not surprising that multiple regulatory mechanisms would allow ARF to sense as many oncogenic cues as possible.

Deregulation of the members of the mTOR pathway is implicated in the mechanism driving hamartoma-forming diseases. Tuberous sclerosis complex is characterized by the potential for hamartoma formation in a wide spectrum of organs (14). Loss or reduction in function of the TSC1-TSC2 protein complex and the resulting constitutive mTOR signaling are the contributing factors for this disease (6). Our finding that loss of Tsc1 induces an ARF response could give some insight as to why benign hamartomas, as opposed to more aggressive neoplastic tumors, arise in this disease. It is possible that the ARF growth checkpoint could play a putative role in repressing the proliferation of hamartoma-forming cells, thereby inhibiting their progression to a more aggressive neoplastic tumor; these hypotheses would need to be formally tested. It is of note that analysis of pleomorphic xanthoastrocytoma (PXA), a rare astrocytic tumor in the cerebral hemispheres of children and young adults, was reported to have homozygous deletion of the CDKN2A/p14<sup>ARF</sup> and CDKN2B loci as well as reduced Tsc1 mRNA expression as defining molecular alterations (54). This finding suggests that concomitant loss of Tsc1 and Arf can contribute to the mechanisms driving tumorigenesis.

In the current study, we have described the involvement of the mTORC1 pathway in the regulation of the ARF tumor suppressor via a translational mechanism. It has been readily shown that mTORC1 signaling can induce the selective translation of specific mRNA targets. One such example is the stimulation of p53 translation that occurs upon the loss of Tsc1 in response to stress conditions (27). It was shown that mTOR can regulate p53 protein synthesis and that hyperactivation of the mTOR pathway can increase sensitivity to DNA damage and energy starvation. In fact, other reports have further elucidated potential mechanisms by which p53 can be translationally regulated (5, 47). Additionally, mTORC1 signaling has been reported to specifically modulate the translation of myeloid cell leukemia sequence 1 (Mcl-1) (32). Loss of Tsc2 in Eμ-Myc cells increases the translation of Mcl-1, and this modulation of Mcl-1 by mTORC1 is relevant to the chemosensitivity of these tumors. Also, mTORC1 signal transduction modulates the translation of nucleophosmin through a mechanism mediated by FBP1 acting as a regulatory RNA binding protein (33, 35). Here, we show that ARF is another translationally regulated gene product as Arf mRNA has enhanced association with actively translating polyribosomes in response to enhanced mTORC1 signal transduction. Translational control of ARF, as well as these other translationally regulated mRNAs, can serve as a versatile and robust mode of regulation for essential cellular functions.

Further elucidation of the molecular mechanism driving ARF’s responsiveness to mTORC1 signaling is of great significance. The implications include the potential identification of novel downstream players not otherwise thought of in the context of the ARF/p53 regulatory network whose interrogation could potentially open avenues to new cancer therapeutics.

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REFERENCES


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