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TSC1 Sets the Rate of Ribosome Export and Protein Synthesis through Nucleophosmin Translation

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Abstract

Nucleophosmin (B23) is a nucleolar phosphoprotein that has been implicated in numerous cellular processes. In particular, nucleophosmin interacts with nucleolar components of newly synthesized ribosomes to promote ribosome nuclear export. Nucleophosmin is a classic mitogen-induced protein, with changes in its expression correlating with growth factor stimulation. In this study, we examined the underlying mechanism of nucleophosmin induction and showed that hyperproliferative signals emanating from oncogenic H-Ras^{V12} cause tremendous increases in nucleophosmin protein expression. Nucleophosmin protein accumulation was dependent on mammalian target of rapamycin (mTOR) activation, as rapamycin completely prevented nucleophosmin induction. Consistent with this finding, genetic ablation of *Tsc1*, a major upstream inhibitor of mTOR, resulted in nucleophosmin protein induction through increased translation of existing nucleophosmin mRNAs. Increases in nucleophosmin protein accumulation were suppressed by reintroduction of TSC1. Induction of nucleophosmin through *Tsc1* loss resulted in a greater pool of actively translating ribosomes in the cytoplasm, higher overall rates of protein synthesis, and increased cell proliferation, all of which were dependent on efficient nucleophosmin nuclear export. Nucleophosmin protein accumulation in the absence of *Tsc1* promoted the nuclear export of maturing ribosome subunits, providing a mechanistic link between TSC1/mTOR signaling, nucleophosmin-mediated nuclear export of ribosome subunits, protein synthesis levels, and cell growth.

Introduction

Nucleophosmin/B23 is a 38-kDa protein localized to the granular regions of the nucleolus (1). Numerous studies have linked nucleophosmin to cell proliferation (2–6). Activation of murine lymphocytes and immortalized fibroblasts by various growth factors is associated with a rapid and prominent increase in nucleophosmin protein expression (3). In addition, nucleophosmin is present at elevated levels in various lymphoma and malignant cell lines, correlating nucleophosmin protein induction with the transduction of mitogenic signals (2). Recent knockout studies have revealed that nucleophosmin is essential for embryonic

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development, specifically for proper development of the central nervous and hematopoietic systems (7,8). Moreover, loss of nucleophosmin greatly diminishes cell growth and proliferation, underscoring its critical role in these two processes (6,7).

In addition to its role in mitogenesis and embryonic development, nucleophosmin has been recognized as having roles in protein chaperoning (9), regulation of p53 (10), and centrosome duplication (7,11). Additionally, nucleophosmin binds nucleic acids (12–14), cleaves pre-rRNA (15), and associates with maturing pre-ribosomal ribonucleoprotein particles in the nucleolus (16), promoting rRNA processing. Based on these activities and its localization in the nucleolus, nucleophosmin has been implicated as a critical regulator of the general process of ribosome biogenesis. In eukaryotic cells, ribosome biogenesis is a highly coordinated multistep process that begins in the nucleolus (17). During rRNA processing, the rRNA particles associate with numerous ribosomal proteins and the 5S rRNA to assemble the large (L, 60S) and small (S, 40S) ribosomal subunits, which are then transported to the cytoplasm and assembled with nascent mRNAs to direct protein synthesis (18). Recent findings from our laboratory have shown that nucleophosmin interacts directly with maturing ribosomal subunits and aids in their efficient nuclear export (19), placing nucleophosmin in a key position to relay growth cues to protein synthesis.

Appropriate regulation of ribosome biogenesis and translation is necessary to maintain accurate cellular growth and proliferation. A key signaling component known to regulate growth, at least in part, by coordinating protein biosynthesis, is the mammalian target of rapamycin (mTOR). TOR is an evolutionarily conserved protein serine-threonine kinase that controls translation efficiency, cell cycle progression, and ribosomal biogenesis through its ability to integrate nutrient and mitogenic signals (20). When complexed with its cellular receptor FK-506-binding protein 12, the immunosuppressive drug rapamycin directly binds to mTOR (21). This inhibits mTOR-dependent downstream signaling, resulting in delayed cell cycle progression in most cell types (22). Recent insight into upstream regulators of mTOR have revealed a tumor suppressor complex composed of two proteins, TSC1 and TSC2, that function by acting as a GTPase-activating protein for the activator of mTOR, Rheb, a Ras-like protein (23–26). In patients with tuberous sclerosis complex (TSC), loss of either *Tsc1* or *Tsc2* gene expression leads to hyperactivation of the mTOR pathway and results in the widespread formation of benign and malignant tumors.

To understand how growth signals are relayed to the ribosome biogenesis machinery, we hypothesized that nucleophosmin might act as a key nucleolar sensor of growth signals. Indeed, nucleophosmin protein levels increased in response to proliferative signals and this induction was regulated not at the level of transcription but rather solely at the translational level. We also found that induction of nucleophosmin by growth signals was sensitive to rapamycin, clearly placing nucleophosmin translation downstream of mTOR activation. Additionally, we show that the TSC1 protein is a critical regulator of this pathway, with loss of *Tsc1* promoting dramatic increases in nucleophosmin protein, thereby leading to an increased pool of actively translating ribosomes and a higher overall rate of protein synthesis. These processes were dependent on functional nucleophosmin, firmly establishing nucleophosmin at the interface between TSC function and ribosome biogenesis.

Materials and Methods

Cell culture and reagents

Low-passage (3–6) primary mouse embryonic fibroblasts (MEF; ArtisOptimus, Carlsbad, CA) were established and maintained as described (27). *Tsc1*^{-/-}/*p53*^{-/-} MEFs were isolated as described previously (28). Quiescence was achieved by maintaining cells that were ~ 30% confluent in medium containing 0.1% fetal bovine serum (FBS) for 48 h. Cells were

stimulated with either human recombinant platelet-derived growth factor (PDGF)-BB (10 ng/mL; Calbiochem) or 10% FBS. LY294002 and rapamycin (Cell Signaling Technology) were used at final concentrations of 15 μ mol/L and 100 nmol/L, respectively. Total RNA was isolated using Trizol (Invitrogen) following the manufacturer's specifications.

Plasmid constructs

pBabe-H-Ras^{V12} was a gift from Martine Roussel (St. Jude Children's Research Hospital, Memphis, TN). His-NPM and His-NPMdL constructs (19) were subcloned into the retroviral pSR α -MSCV-tkNEO plasmid for production of retrovirus as described previously (6). Full-length human *Tsc1* cDNA cloned into the MSCV retroviral vector was used to express TSC1 in *Tsc1/p53*^{-/-} MEFs. pRK7-HA-S6K1 and pRK7-3HA-eIF4E were provided by John Blenis (Harvard University, Boston, MA).

Viral production and infection

Collected retrovirus (6) was used to infect target cells for 1 to 5 days in the presence of 10 μ g/mL polybrene (Sigma, St. Louis, MO). For pBabe.puro retroviral vector infections, MEFs were selected in 2.0 μ g/mL puromycin. For pSR α retroviral vector infections, MEFs were selected in 0.8 mg/mL G418. The efficiency of infections was generally 100% as assessed by puromycin or G418 selection and β -galactosidase staining. For the production of lentiviruses encoding short hairpin RNAs targeting either luciferase or nucleophosmin, 293Ts were transfected with Fugene 6, using a three-plasmid system: pHCMV.G (envelope), CMV Δ R8.2 (packaging), and either pFLRu-YFP-shLuc or pFLRu-YFP-shNPM. The following oligonucleotides (hairpin loop in italics and antisense in bold) were annealed and cloned into the pFLRu-YFP vector,⁴ to yield short hairpin RNAs targeting the 3'-untranslated region of nucleophosmin: 5'-GCCAAGAATGTGTTGTCAAATTCAGAGATTAGACAAACACATTCTTGCGCTTTT T-3'(forward oligonucleotide) and 3'-CGGTTCTTACACAACAGTTTAAAGTTCCTAATC **TGTTGTGTAAGAACCGAAAAA**-5'(reverse oligonucleotide). MEFs were infected for 4 h with lentiviral-containing culture medium, supplemented with 8 μ g/mL protamine sulfate. MEFs were cultured in the presence of 2 μ g/mL puromycin for 48 h. Following puromycin selection, the cells were trypsinized, counted, and replated for subsequent Western blot, growth curve, foci formation, and soft agar assays.

Western blots

Harvested cells were resuspended and sonicated in lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 120 mmol/L NaCl, 0.5% NP40, 1 mmol/L EDTA (pH 7.4), 10 μ g/mL aprotinin, phosphatase inhibitors, 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF)]. Proteins (100 μ g) were separated on 10% to 12.5% SDS-containing polyacrylamide gels. Separated proteins were transferred to polyvinylidene difluoride membranes (Millipore, Boston, MA). Membranes were probed with the following antibodies: γ -tubulin, cyclin D1, nucleophosmin, Ras, HA, and His (Santa Cruz Biotechnology, Santa Cruz, CA) and AKT, phospho-AKT (Thr³⁰⁸), TSC1, and phospho-S6 (Cell Signaling Technology). Sheep anti-goat (Zymed), goat anti-rabbit, and goat anti-mouse (Bio-Rad, Hercules, CA) IgG (H + L) horseradish peroxidase conjugates were added as the secondary antibodies, and specific protein bands were visualized using enhanced chemiluminescence (Amersham, Piscataway, NJ).

⁴Y. Feng and G. Longmore, unpublished observation.

Northern blot analysis

Total RNA (8–10 µg) was denatured and fractionated by gel electrophoresis using a 1% agarose gel containing 2.2 mol/L formaldehyde. RNA was transferred by capillary action in 10 × saline-sodium phosphate-EDTA [1.5 mol/L NaCl, 100 mmol/L NaH₂PO₄, and 10 mmol/L EDTA (pH 7.4)] to Hybond-NX membrane (Amersham). The membrane was cross-linked with a Hoeffer UV autolinker and stained with methylene blue for visualization of 18S and 28S RNAs. Membranes were blocked with Rapidhyb buffer (Amersham) containing 100 µg/mL salmon sperm DNA (Sigma) for 1 h at 65°C before hybridization to a ³²P-labeled probe specific for nucleophosmin in Rapidhyb buffer with 100 µg/mL salmon sperm DNA for 4 h at 65°C. Membranes were washed once with 2 × SSC [0.3 mol/L NaCl and 30 mmol/L sodium citrate (pH 7.0)]/0.1% SDS at room temperature for 20 min and twice with 1 × SSC/0.1% SDS at 65°C for 15 min. Gels were autoradiographed at –80°C with intensifying screens. Methylene blue–stained 18S and 28S rRNAs were used as internal loading controls.

The complete mouse nucleophosmin cDNA (accession no. M33212) was used as a probe and radiolabeled with [α -³²P]dCTP by random priming using the Rediprime II kit (Amersham) according to the manufacturer's specifications.

Labeling of cellular protein with [³⁵S]methionine and immunoprecipitation of nucleophosmin

Cells (1 × 10⁵) were seeded in six-well plates in triplicate, cultured in DMEM without methionine (Life Technologies) for 30 min, and incubated in the presence of 100 µCi ³⁵S-protein labeling mix (Amersham) for various time points. Cells were washed twice with PBS and lysed with 1% Triton X-100 PBS buffer. For the [³⁵S]methionine incorporation assay, total protein was precipitated from lysates with 10% trichloroacetic acid and pelleted. Pellets were subjected to liquid scintillation counting to measure incorporated cpm. For nucleophosmin pulse label experiments, nucleophosmin proteins were immunoprecipitated from 500 µg total cellular protein lysates with a monoclonal antibody recognizing nucleophosmin (Zymed) and protein A/G-Sepharose. Precipitated nucleophosmin proteins were separated by SDS-PAGE and detected by autoradiography.

Ribosome fractionation

Cells (3 × 10⁶) were treated with 50 µg/mL cyclohexamide for 10 min before trypsinization and lysis, and fractionation was carried out over a 10% to 45% sucrose gradient in a Beckman SW41 rotor at 36,000 rpm (Palo Alto, CA). Gradients were fractionated, and RNA absorbance at 254 nm was continuously monitored to detect ribosomal subunits.

Subcellular fractionation

Equal numbers of wild-type (WT) MEFs were resuspended in HEPES buffer [10 mmol/L HEPES (pH 7.4) with 4 mmol/L MgCl₂, 1 mmol/L PMSF, 10 µg/mL leupeptin, 10 µg/mL aprotinin, 1 µg/mL pepstatin, 1 mmol/L NaF, 10 mmol/L NaVO₄, β-glycerophosphate] followed by passage (15 ×) through a 25-gauge needle. Lysed cells were pelleted and the supernatant was saved as the cytoplasmic fraction. The pellet was resuspended in fractionation buffer [10 mmol/L Tris (pH 7.5), 10 mmol/L NaCl, 1 mmol/L EDTA, 0.5 mmol/L EGTA, 4 mmol/L MgCl₂, 1 mmol/L PMSF, 10 µg/mL leupeptin, 10 µg/mL aprotinin, 1 µg/mL pepstatin], dounced (20 ×) with a B pestle, layered over a 1-mL cushion of sucrose (45% w/v in fractionation buffer), and centrifuged. The pellet was washed thrice by overlaying the pellet with double-distilled water and by centrifuging. The pellet was resuspended in Trizol.

rRNA export

MEFs were infected with retroviruses encoding His-NPM or His-NPMdL and incubated at 48 h at 37°C after infection. Cells were incubated in methionine-free medium for 15 min and labeled with 50 $\mu\text{Ci/mL}$ L-[methyl- ^3H]methionine (Amersham Biosciences) for 30 min. The radiolabel was washed away, and the cells were incubated in a 10-fold excess of cold methionine-containing medium for 2 h. Cells (2×10^6) were harvested and counted, and equal numbers of cells were fractionated into cytoplasm and nuclei as described above. Total RNA was isolated from the cytosolic and nuclear fractions with Trizol and fractionated by gel electrophoresis using a 1% agarose gel containing 2.2 mol/L formaldehyde. RNA was transferred by capillary action in $20 \times \text{SSC}$ to Hybond-NX membrane. The membrane was cross-linked, sprayed with EN 3 HANCE spray (Perkin-Elmer), and subjected to autoradiography.

Foci formation and proliferation assays

Tsc1 $^{-/-}$ /*p53* $^{-/-}$ MEFs were infected with control or His-NPMdL retroviruses, and siLuciferase or siNPM lentiviruses. For foci formation, cells were seeded (1.5×10^3) on 100-mm dishes, grown for 12 days in complete medium, fixed in 100% methanol, and stained for 10 min with Giemsa (Sigma). To assay proliferation, infected cells were seeded (1×10^4) in triplicate wells of a six-well plate and counted once a day over the course of 6 days.

Densitometry and image analysis

Autoradiograms were scanned using a FluoroChem8900 (Alpha Innotech), and densities were determined using NIH ImageJ version 1.33 software.

Results

Nucleophosmin protein is induced by serum, PDGF, and H-Ras $^{\text{V12}}$ in an LY294002-sensitive manner

To investigate whether nucleophosmin protein expression is associated with proliferation of low-passage WT MEFs, cells were synchronized in G_0 by serum deprivation and then induced to enter the cell cycle simultaneously by addition of serum (FBS; ref. 29). Cyclin D1 expression increased on serum stimulation, indicating that the WT MEFs were successfully synchronized in G_0 and simultaneously induced to enter the cell cycle (G_1 -S transition is at 12 h for MEFs; Fig. 1A; ref. 30). A 3-fold increase in nucleophosmin protein was observed by 8 h after serum stimulation, with levels peaking at a 6-fold increase 24 h poststimulation. The heterogeneous nature of serum signaling presents a significant barrier in defining the upstream activator or activators of nucleophosmin induction. Thus, in an effort to more clearly define a growth factor signaling pathway, PDGF was used to stimulate cells into cell cycle entry. Once again, low-passage WT MEFs were starved of mitogen to synchronize them and subsequently driven into cycle by the addition of PDGF. PDGF induced cellular proliferation as well as a significant induction of nucleophosmin protein expression (Fig. 1A). Because H-Ras is a common activator of a multitude of PDGF- and serum-stimulated signaling pathways (31,32), we tested whether expression of a constitutively active H-Ras (Ras $^{\text{V12}}$) would increase expression of nucleophosmin in WT MEFs. On 48 h of Ras $^{\text{V12}}$ expression, nucleophosmin protein was significantly induced (10-fold; Fig. 1B).

In an attempt to define which pathway downstream of growth factor activation was responsible for nucleophosmin induction, pharmacologic inhibition was used to block phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinase signals. WT MEFs were serum starved and then released into cycle by serum stimulation in the presence

and absence of inhibitor. LY294002, an inhibitor of the PI3K pathway (33,34), significantly blocked nucleophosmin expression after serum stimulation (Fig. 1C), whereas addition of UO126 did not affect nucleophosmin protein expression (data not shown). Unphosphorylated AKT was used as a loading control and phospho-AKT (Thr³⁰⁸) showed that PI3K, which phosphorylates AKT at this site (35), was effectively inhibited by LY294002 (Fig. 1C). Given that serum-induced expression of nucleophosmin is sensitive to LY294002, we tested whether Ras^{V12}-induced increases in nucleophosmin protein expression were sensitive as well. LY294002 was added to both empty vector and Ras^{V12}-infected MEFs, and samples were collected 48 h postinfection. As expected, LY294002 caused nucleophosmin levels to decrease in asynchronously growing, vector-infected cells (Fig. 1D). In addition, the presence of LY294002 significantly inhibited the Ras^{V12}-induced increase in nucleophosmin protein expression (Fig. 1D), showing that both mitogenic and oncogenic stimulation of nucleophosmin protein expression are sensitive to PI3K inhibition.

Rapamycin decreases nucleophosmin levels in asynchronously growing and Ras^{V12}-infected cells

To assess the role of downstream PI3K effectors, specifically mTOR, in regulating nucleophosmin protein induction, we used the selective mTOR inhibitor, rapamycin. Addition of rapamycin to WT MEFs grown in serum caused a dramatic decrease in nucleophosmin protein expression (Fig. 2A). Levels of phospho-S6, a downstream target of mTOR activity, also declined in the presence of rapamycin, as expected (Fig. 2A; ref. 36). We next tested whether rapamycin was able to block nucleophosmin induction in response to oncogenic Ras^{V12}. Similar to results observed in asynchronous cells growing in serum, Western blot analysis showed that nucleophosmin protein induction by oncogenic Ras^{V12} was sensitive to rapamycin treatment (Fig. 2B).

Nucleophosmin mRNA is unaffected by serum, Ras^{V12}, or rapamycin

To investigate whether changes in nucleophosmin mRNA induction were responsible for the observed increases in nucleophosmin protein expression, low-passage WT MEFs were synchronized by serum starvation and subsequently induced to proliferate by serum addition. RNA was isolated poststimulation and subjected to Northern blot analysis. Staining of 18S and 28S rRNA was done to ensure equal loading of total RNA (Fig. 3A). Surprisingly, whereas nucleophosmin protein levels increased on serum stimulation (Fig. 1A), nucleophosmin mRNA levels remained constant (Fig. 3A). In addition to the Northern blot, parallel cells at identical time points were harvested for Western blot analysis of cyclin D1 to ensure successful synchronization and release into the cell cycle (Fig. 3A; ref. 29).

To determine whether nucleophosmin mRNA levels responded to oncogenic stimuli, WT MEFs were retrovirally infected with Ras^{V12}. Whereas nucleophosmin protein levels were elevated at this time point (Fig. 1B), mRNA levels again remained unchanged (Fig. 3B). We next tested whether nucleophosmin mRNA levels were sensitive to rapamycin by treating Ras^{V12}-infected MEFs for 48 h. Whereas our earlier results showed that Ras^{V12}-induced nucleophosmin protein expression was sensitive to rapamycin treatment (Fig. 2B), rapamycin had no effect on nucleophosmin mRNA expression in the presence of Ras^{V12} (Fig. 3B). Notably, addition of the proteasome inhibitor MG132 had no appreciable effect on nucleophosmin protein expression levels, indicating that protein stability was not a major cause for the observed increases in nucleophosmin protein levels (data not shown). Taken together, these data indicate that nucleophosmin mRNA levels remain constant regardless of stimulation or treatment, suggesting that overall regulation of nucleophosmin protein expression is regulated posttranscriptionally through a mechanism independent of protein stability.

TSC1 regulates the translation of nucleophosmin mRNAs

Given our finding that nucleophosmin protein is positively regulated by growth factors and that this effect is abrogated on treatment with rapamycin, we hypothesized that nucleophosmin levels would be elevated in cells with a hyperactive mTOR pathway. Recent studies in both *Drosophila* and mammalian systems have shown that the TSC1/TSC2 complex functions upstream of mTOR in the PI3K pathway to suppress cell growth and proliferation (37–40). Primary MEFs lacking either component of the complex initially proliferate faster than their WT counterparts but ultimately undergo premature senescence in a p53-dependent manner (28). Loss of p53 rescues the premature senescence, and MEFs lacking both *Tsc1* and *p53* exhibit greater growth rates than those lacking p53 alone (28). Consistent with these observations, we observed a 20-fold increase in nucleophosmin protein expression in *Tsc1*^{-/-}/*p53*^{-/-} MEFs compared with only modest increases in the absence of *p53* alone (Fig. 4A). The addition of rapamycin inhibited the increase in nucleophosmin protein expression observed in *Tsc1*^{-/-}/*p53*^{-/-} cells (Fig. 4B, left), consistent with current models placing the TSC signaling complex upstream of mTOR. Reexpression of TSC1 in *Tsc1*^{-/-}/*p53*^{-/-} cells reduced nucleophosmin protein expression below basal expression levels (Fig. 4B, right), indicating that TSC1 is a potent suppressor of nucleophosmin protein expression.

Inactivation of the TSC1/TSC2 complex results in mTOR activation and leads to phosphorylation of two main mTOR substrates, the eukaryotic initiation factor 4E binding protein-1 and the ribosomal S6 kinase. These two mTOR-dependent cascades stimulate mRNA translation through distinct mechanisms to coordinate overall protein synthesis in the cell (41,42). To investigate whether these downstream mTOR targets regulate nucleophosmin protein expression, WT MEFs were transduced with constructs encoding WT eIF4E and S6 kinase 1. Western blot analysis showed that nucleophosmin was induced on overexpression of S6 kinase 1 but was unaltered by eIF4E transduction (Fig. 4C), implying that directed translation by S6 kinase could provide a mechanism for nucleophosmin protein regulation. To follow up on this result, we did a pulse-label analysis to assess the rates of nucleophosmin translation in WT cells versus those lacking *p53* or *Tsc1*^{-/-}/*p53*^{-/-}. Our results show that *p53*^{-/-} cells exhibit a similar rate of nucleophosmin translation as WT cells but that cells lacking *Tsc1*^{-/-}/*p53*^{-/-} synthesize more nucleophosmin in the same time period and at an accelerated rate (Fig. 4D). Importantly, the increases in nucleophosmin translation seen in the absence of *Tsc1* were abolished with rapamycin (Fig. 4D). These data show that, in the absence of *Tsc1*, nucleophosmin mRNA translation is significantly increased, resulting in dramatic accumulation of nucleophosmin proteins.

MEFs lacking *Tsc1* have more polysomes and a higher rate of overall protein synthesis than their WT counterparts

Previous findings from our laboratory have shown that NPM is required for the nucleolar/nuclear export of 28S, 18S, 5.8S, and 5S rRNAs in cells and that nucleophosmin is rate limiting in this process, with increases in nucleophosmin protein expression amplifying the amount of cytosolic ribosomes available for mRNA translation (19).⁵ Given that nucleophosmin levels are higher in cells lacking *Tsc1*, we hypothesized that *Tsc1*^{-/-} cells would also exhibit a larger pool of actively translating ribosomes. To test this, ribosomal profiles were generated for WT, *Tsc1*^{-/-}/*p53*^{-/-}, or *p53*^{-/-} MEFs. mRNAs that are being actively translated have variable numbers of ribosomes associated with them, forming large structures called polysomes. These polysomes can be separated from inactive 40S, 60S, and 80S subunits by sucrose gradient densities and detected by RNA absorption at 254 nm. Our

⁵L.B. Maggi, Jr. and J.D. Weber, unpublished observation.

findings show that *Tsc1*^{-/-}/*p53*^{-/-} MEFs have significantly more actively translating ribosomes than WT MEFs and that this difference is ~ 50% greater than *p53* loss alone (Fig. 5A). The larger polysomal fraction in MEFs expressing higher levels of nucleophosmin protein is consistent with our previous finding that overexpression of nucleophosmin causes a similar increase in polysomes.⁵

To determine whether the increased polysomal fraction in MEFs lacking *Tsc1* exhibited increased overall protein synthesis, newly synthesized proteins were labeled over time to measure protein synthesis rates. The results from this experiment are consistent with our polysome profiles in that *Tsc1*^{-/-}/*p53*^{-/-} cells exhibited the greatest amount of overall protein synthesis compared with equal numbers of *p53*^{-/-} and WT MEFs (Fig. 5B).

Expression of a dominant-negative nucleophosmin reverses the effects of *Tsc1* loss on ribosome biogenesis and cell proliferation

To more directly test the hypothesis that higher levels of nucleophosmin protein expression caused the increased polysome fractions and greater rates of protein synthesis in MEFs lacking *Tsc1*, we used a previously described mutant of nucleophosmin (NPMdL) that lacks the ability to shuttle ribosomes between the nucleus and cytoplasm and acts in a dominant-negative fashion by forming hetero-oligomers with WT nucleophosmin molecules (19). As seen previously, the polysomal fraction was significantly larger in *Tsc1*^{-/-}/*p53*^{-/-} MEFs relative to WT cells. However, retroviral introduction of NPMdL into *Tsc1*^{-/-}/*p53*^{-/-} MEFs lowered the polysome peaks below the baseline of WT cells (Fig. 6A), showing that nucleophosmin function is a rate-limiting factor for the larger pool of actively translating ribosomes observed in MEFs lacking *Tsc1*.

To further investigate the mechanism behind the extremely low levels of 40S, 60S, 80S, and polysomes in the cytoplasm of *Tsc1*^{-/-}/*p53*^{-/-} MEFs overexpressing the NPMdL mutant, we decided to examine the ribosomal nuclear export rates in the presence or absence of the dominant-negative nucleophosmin in WT and *Tsc1*^{-/-}/*p53*^{-/-} MEFs. Consistent with our previous findings (19),⁵ expression of the NPMdL mutant prevented a majority of newly synthesized rRNAs from being exported into the cytoplasm in WT and MEFs (Fig. 6B, lanes 1 and 3). In *Tsc1*^{-/-}/*p53*^{-/-} MEFs, a tremendous increase was observed in rRNA export compared with WT cells (Fig. 6B, lanes 1 and 5) and this increase was greatly diminished in the presence of the NPMdL mutant, indicating that increased rRNA nuclear export in the absence of *Tsc1* required functional nucleophosmin proteins.

To determine whether the loss of ribosome export had an effect on the hyperproliferative phenotype of *Tsc1*^{-/-}/*p53*^{-/-} MEFs, cells were infected with retroviruses encoding NPMdL mutants and assayed for cell proliferation. Consistent with the correlation between increased nucleophosmin shuttling activity and loss of *Tsc1*, ectopic expression of NPMdL severely attenuated short-term (Fig. 6C, top) and long-term (Fig. 6C, bottom) proliferation of *Tsc1*^{-/-}/*p53*^{-/-} MEFs. These data indicate a requirement for increased nucleophosmin nuclear export of newly synthesized ribosomes to maintain the proliferative advantage of cells lacking *Tsc1*. Additionally, reduction of nucleophosmin protein expression to nearly WT levels using siRNAs targeting nucleophosmin in *Tsc1*^{-/-}/*p53*^{-/-} MEFs (Fig. 6D, inset) resulted in nearly identical reductions in *Tsc1*^{-/-}/*p53*^{-/-} MEF short-term and long-term proliferation (Fig. 6D). Together, these findings imply a crucial role for both increased nucleophosmin protein expression levels and shuttling activity in setting the proliferative capacity of cells lacking *Tsc1*.

Discussion

Nucleophosmin (B23) is a multifunctional nucleolar protein involved in the processes of ribosome assembly, nucleocytoplasmic shuttling, and cellular proliferation (2,4–6,10,43–45). Consistent with the role of nucleophosmin in promoting proliferation are the findings that reduced nucleophosmin protein expression in primary mouse embryonic fibroblasts or genetic ablation of *Npm1* in the mouse germ-line causes severe defects in cell cycle progression and cellular growth (6,7). These findings point to an active role for nucleophosmin in promoting cell cycle progression and replace the adage that nucleophosmin protein levels are passive indicators of cell proliferation. Nucleophosmin has positioned itself as a critical mediator of cell cycle progression and as an essential integrator of growth-promoting signals. Additionally, its nucleolar localization reinforces the idea that the nucleolus itself is a critical sensor of growth and proliferation (46). Here, we extend these findings and show that nucleophosmin protein expression is induced on stimulation with mitogenic factors to show that nucleophosmin functions as a nucleolar growth factor sensor (47).

Given our observations that nucleophosmin protein expression is induced on stimulation with growth factors, it was not surprising to find that nucleophosmin protein levels increased on over-expression of oncogenic Ras^{V12} or loss of *Tsc1* (hyperactive mTOR signaling). However, increases in nucleophosmin protein in response to hyperactive mTOR signals were not the result of increased mRNA induction but were instead the result of dramatic increases in protein translation. This suggests that nucleophosmin levels are tightly controlled by the cell: a pool of extremely stable mRNA exists whether serum is present or not and a direct, immediate mechanism is used by the cell to induce high levels of nucleophosmin protein expression under either growth or hyper-proliferative signals.

Although much has been elucidated about mitogen-activated signaling pathways and their ability to stimulate cell cycle progression (through induction of cyclins), little is known about how these proliferative signals might also be interpreted by the nucleolus to appropriately couple progrowth signals to the protein synthesis machinery. We found that induction of nucleophosmin, a nucleolar protein important for ribosome export, is sensitive to rapamycin, showing mTOR regulation of nucleophosmin expression. Importantly, this finding now provides a mechanistic link between mTOR signaling and regulation of nucleolar processes capable of modulating protein synthesis. Increasing evidence in mice and humans points to a more direct role of aberrant mTOR signaling in promoting tumorigenesis, specifically, linking the disruption of mTOR-mediated translation to alterations in the cell cycle and cell growth (48). For example, the TSC inherited cancer syndrome is caused by germ-line mutations in either the *Tsc1* or *Tsc2* gene (49). Interestingly, we have showed that genetic ablation of *Tsc1* not only causes nucleophosmin protein to accumulate but also has the biological consequence of promoting nucleolar/nuclear export of maturing ribosomal subunits, increasing the cytoplasmic pool of translating ribosomes and elevating the overall levels of protein synthesis. Our findings imply participation of the mTOR pathway in determining the rate of ribosome export through its effects on nucleophosmin translation rates and place the TSC1/TSC2 complex in an important position to actively suppress ribosome biogenesis (through nucleophosmin down-regulation). Interestingly, much work has shown that mTOR regulates ribosome biogenesis by controlling the transcription of Pol-I-dependent rRNA and Pol-II-dependent ribosomal protein genes (50). Here, we show that mTOR modulates ribosome biogenesis not only by regulating rRNA synthesis but also by controlling nucleophosmin-induced ribosome nuclear export.

As an essential mediator of G₁ progression, nucleophosmin functions as a key nucleolar growth factor sensor, one that readily responds to growth factor or oncogenic signals relayed by mTOR. The responsiveness of nucleophosmin to mTOR activity places it in an ideal position to control mTOR-dependent increases in protein translation. Likewise, given its ability to increase proto-oncogenic nucleophosmin protein levels, mTOR then establishes a direct growth target that might help explain its ability to stimulate tumor formation. With ribosome processing and export originating in the nucleolus and being obvious targets in the transition from normal to tumorigenic cell states, it has been difficult to imagine how mTOR signals might gain access to this isolated subcellular compartment to stimulate increases in this process or how the TSC1/TSC2 complex might do tumor-suppressive functions. The ability of nucleophosmin to sense increased mTOR activity in the absence of TSC1/TSC2 suppression provides a novel link between the growth signals that ultimately increase protein synthesis in proliferating cells and the trafficking protein that increases ribosome output from the nucleolus.

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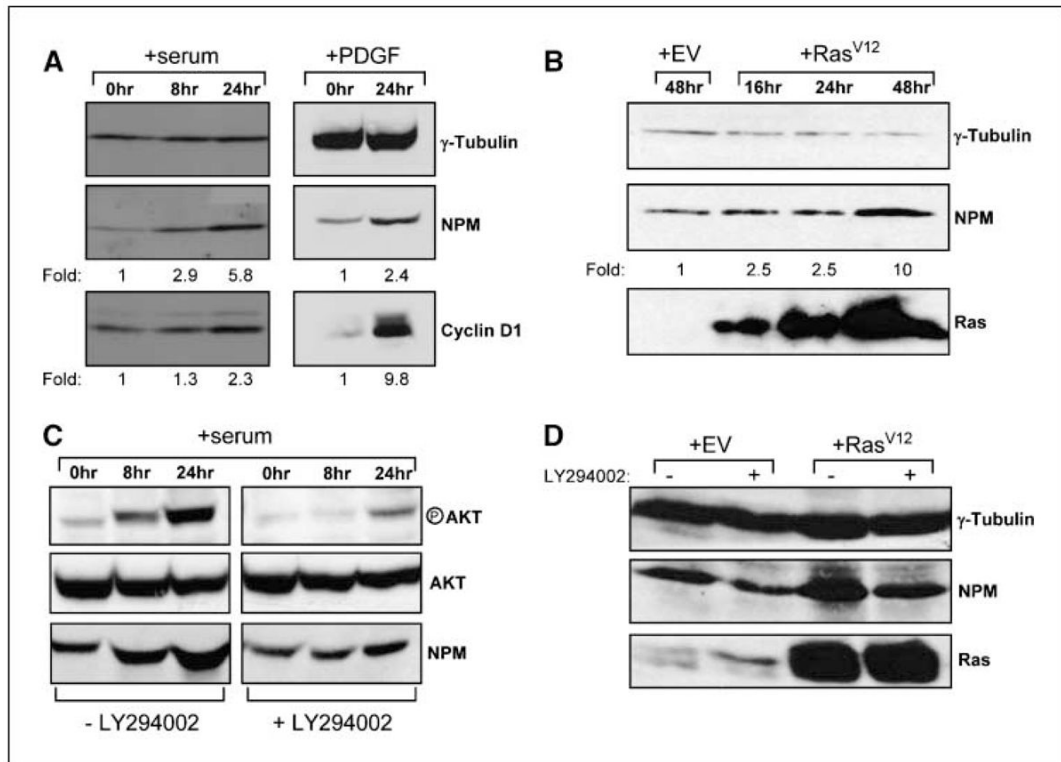
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**Figure 1.**

Nucleophosmin protein expression is induced by progrowth signals and is sensitive to LY294002. **A**, serum-starved low-passage WT MEFs were incubated with 10% serum or 200 ng/mL PDGF for the durations indicated. Proteins were resolved by SDS-PAGE and immunoblotted with antibodies to γ -tubulin, nucleophosmin (*NPM*), and cyclin D1. **B**, MEFs were infected with retroviruses encoding β -galactosidase (*EV*) and Ras^{V12}, collected at the indicated times, and analyzed by Western blot with the indicated antibodies. **C**, MEFs were serum starved and pretreated with 15 μ mol/L LY294002 for 1 h (+) before being stimulated with 10% serum for the indicated times. Proteins were separated by SDS-PAGE and immunoblotted with antibodies specific for nucleophosmin, AKT, and phospho-AKT (\textcircled{P}). **D**, WT MEFs were transduced with the retroviruses encoding β -galactosidase (*EV*) and Ras^{V12}. LY294002 (15 μ mol/L) was added 24 h postinfection. All samples were collected 48 h postinfection and lysates were immunoblotted with antibodies recognizing nucleophosmin, γ -tubulin, and Ras.

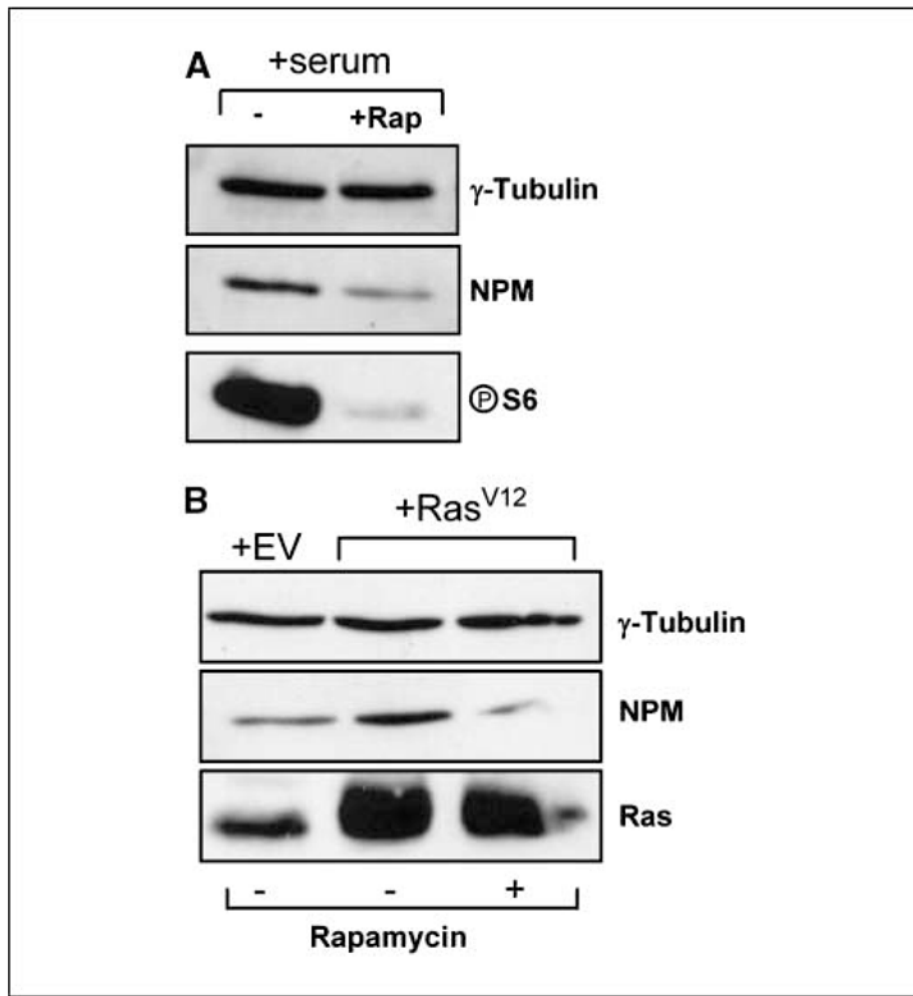


Figure 2.

Effects of rapamycin on nucleophosmin expression. *A*, rapamycin (*Rap*; 100 nmol/L) was added as indicated to asynchronous WT MEFs. Forty-eight hours after rapamycin treatment, cells were harvested and proteins were separated by SDS-PAGE and immunoblotted with antibodies specific for nucleophosmin, γ -tubulin, and phospho-S6 (Ⓟ). *B*, WT MEFs were infected with retroviruses encoding β -galactosidase (*EV*) and Ras^{V12}. Rapamycin (100 nmol/L) was added as indicated 24 h postinfection. All samples were collected 48 h postinfection and proteins were immunoblotted with antibodies against γ -tubulin, Ras, and nucleophosmin.

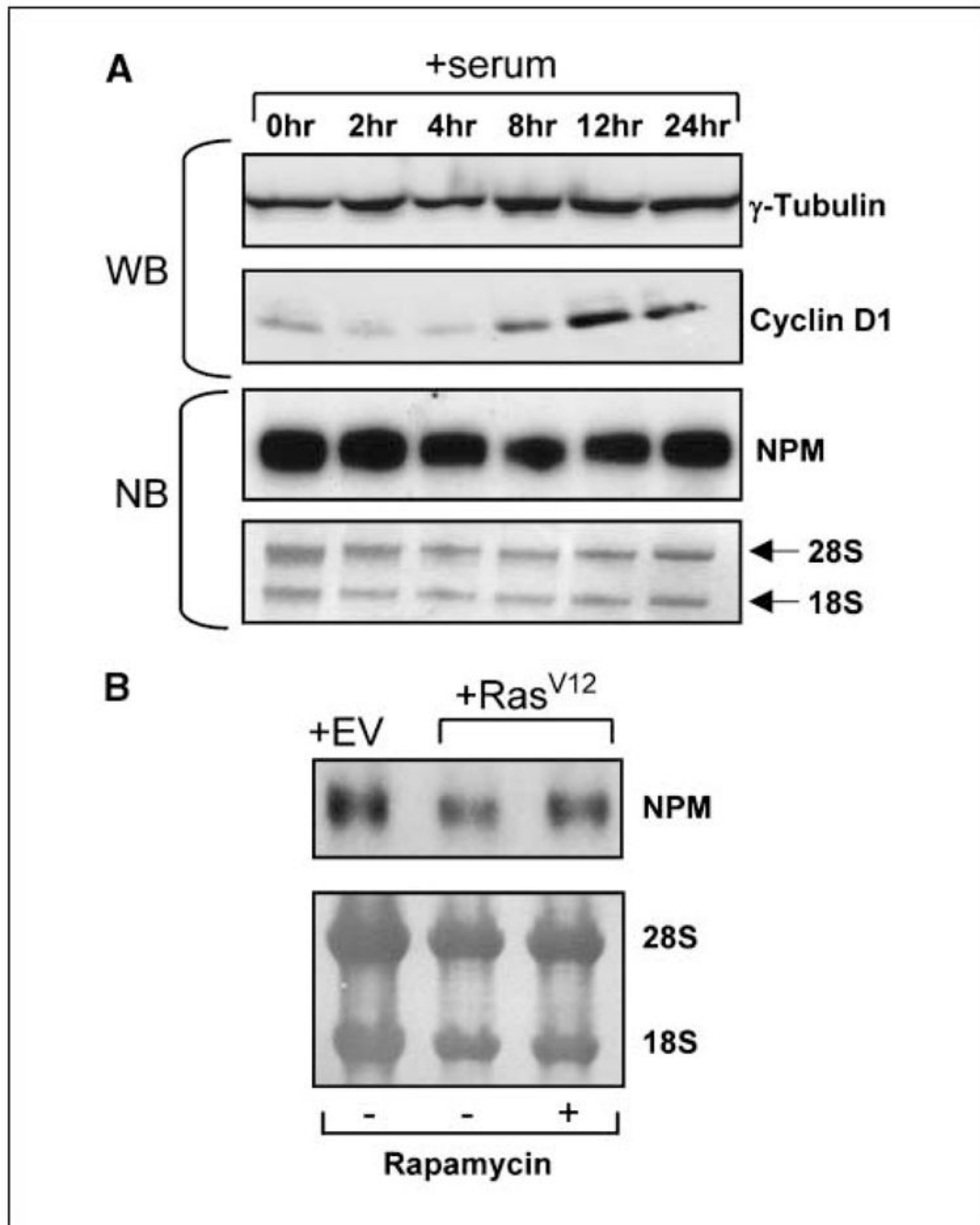
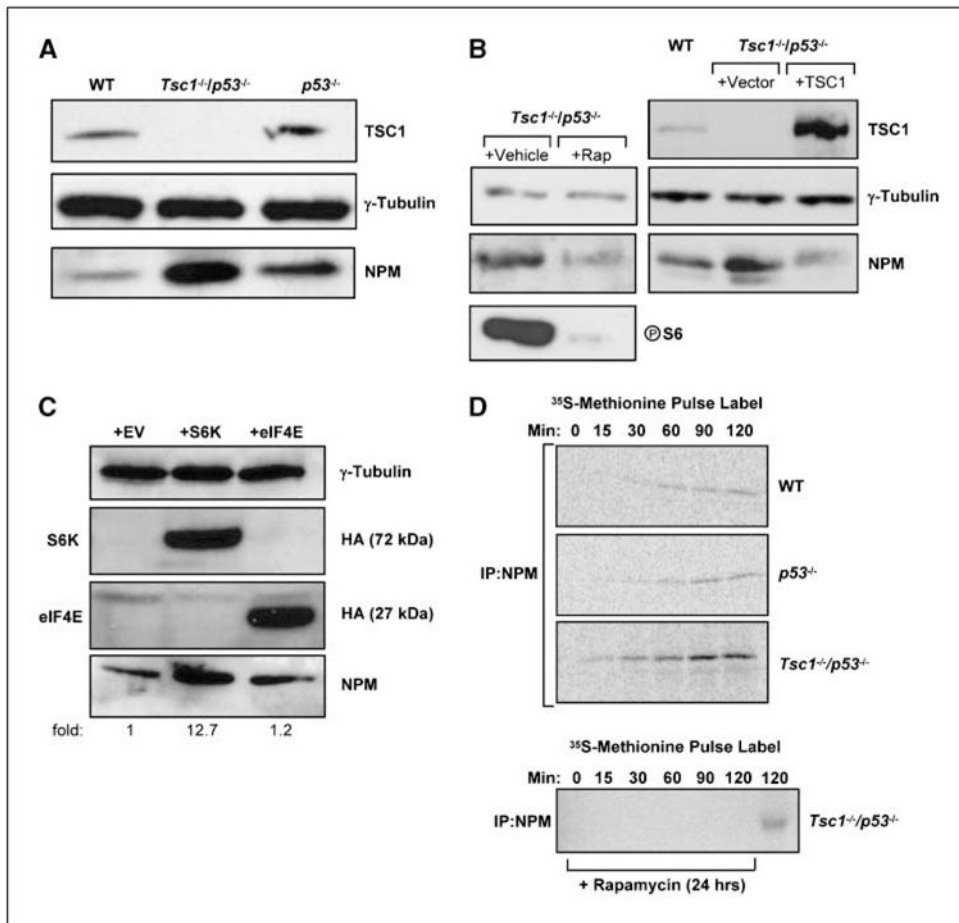


Figure 3.

Effects of progrowth signals and rapamycin on nucleophosmin mRNA levels. *A*, WT MEFs were serum starved and subsequently incubated with 10% serum for the durations indicated. Proteins were resolved by SDS-PAGE and antibodies recognizing γ -tubulin and cyclin D1 were used. Total RNA was isolated at the indicated times and nucleophosmin mRNAs were visualized by Northern blot analysis (NB) with murine nucleophosmin probes. Ribosomal 28S and 18S rRNAs were visualized by methylene blue staining as a loading control. *B*, WT MEFs were infected with retroviruses encoding β -galactosidase (EV) and Ras^{V12}. Rapamycin (100 nmol/L) was added as indicated 24 h postinfection. All samples were

collected 120 h postinfection and analyzed by Northern blot with probes specific for murine nucleophosmin. Ribosomal 28S and 18S rRNAs were detected by methylene blue staining.

**Figure 4.**

Nucleophosmin protein expression and rate of nucleophosmin mRNA translation is higher in MEFs lacking *Tsc1*. *A*, equal amounts of protein lysate from WT, *Tsc1*^{-/-}/*p53*^{-/-}, or *p53*^{-/-} MEFs were resolved by SDS-PAGE and analyzed with antibodies specific for TSC1, γ -tubulin, and nucleophosmin. *B*, *Tsc1*^{-/-}/*p53*^{-/-} MEFs were treated with vehicle or rapamycin (100 nmol/L) and harvested 24 h later for Western blot analysis. Proteins were resolved by SDS-PAGE and antibodies recognizing γ -tubulin, nucleophosmin, and phospho-S6 were used. WT and *Tsc1*^{-/-}/*p53*^{-/-} MEFs were transduced with plasmids encoding β -galactosidase (+ *Vector*) or TSC1 and harvested 24 h later for Western blot analysis. Proteins were resolved by SDS-PAGE and antibodies recognizing γ -tubulin, nucleophosmin, and TSC1 were used. *C*, WT MEFs were transduced with pRK7 plasmids encoding HA-tagged S6 kinase (*S6K*) 1 or eIF4E. Cells were harvested (48 h later) and proteins were separated by SDS-PAGE and immunoblotted with antibodies specific for HA, γ -tubulin, and nucleophosmin. *D*, WT, *Tsc1*^{-/-}/*p53*^{-/-}, or *p53*^{-/-} MEFs were starved of methionine, and [³⁵S]methionine was subsequently added for the indicated times before harvesting to allow for incorporation of label into newly translated proteins in the absence or presence of rapamycin (100 nmol/L). Immunoprecipitated (*IP*) nucleophosmin proteins were separated by SDS-PAGE and detected by autoradiography.

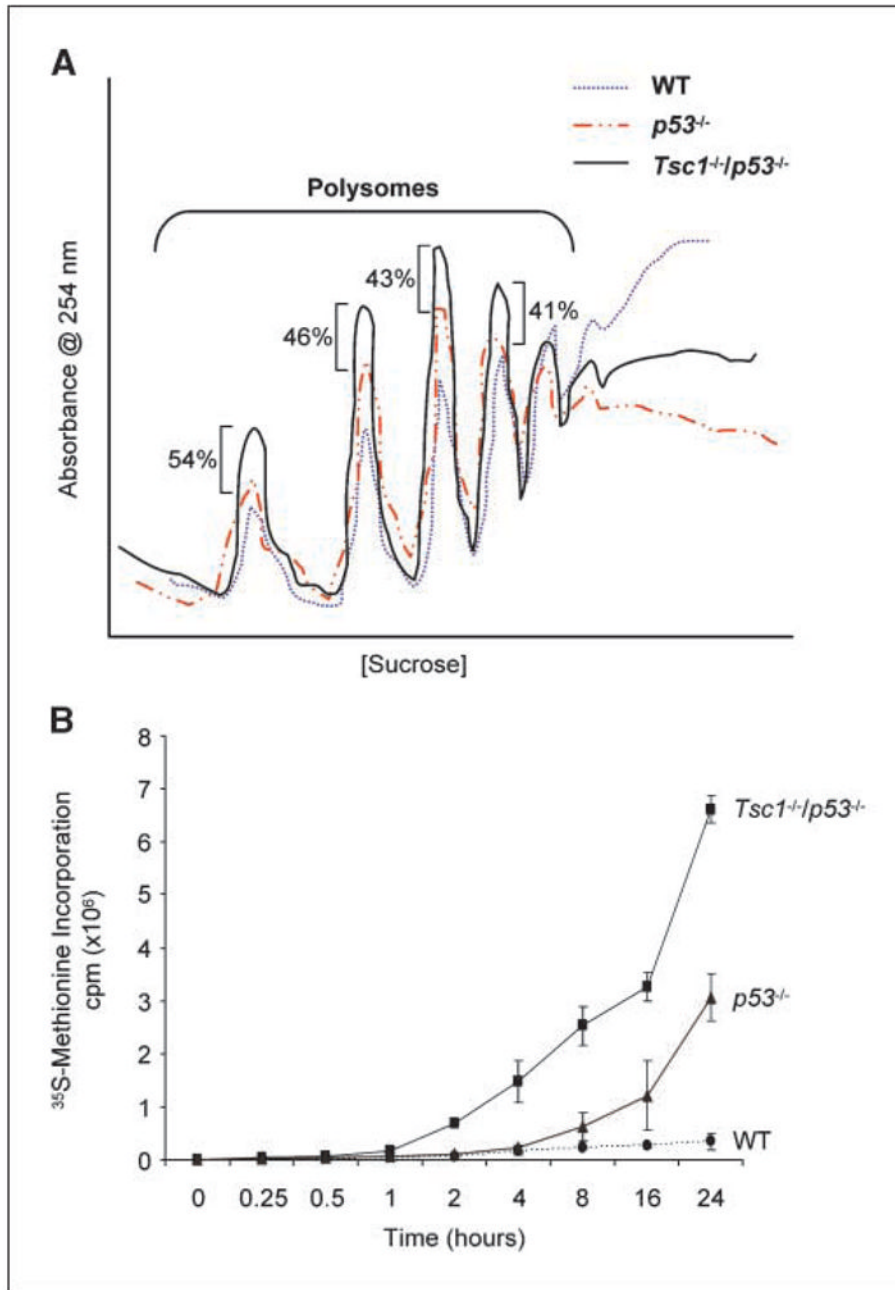


Figure 5.

Loss of *Tsc1* results in greater polysomes and increased rates of protein synthesis. *A*, 3×10^6 cells for each genotype were lysed and fractionated over a 10% to 45% sucrose gradient. Gradients were fractionated and ribosomal subunits were detected by measuring RNA absorbance at 254 nm. Percentages are given for differences in calculated areas under each polysome peak. *B*, [³⁵S]methionine was added to methionine-starved cells of each genotype for the indicated times. Cells were immediately lysed and total protein was precipitated with TCA, pelleted, and subjected to liquid scintillation counting to measure incorporated cpm.

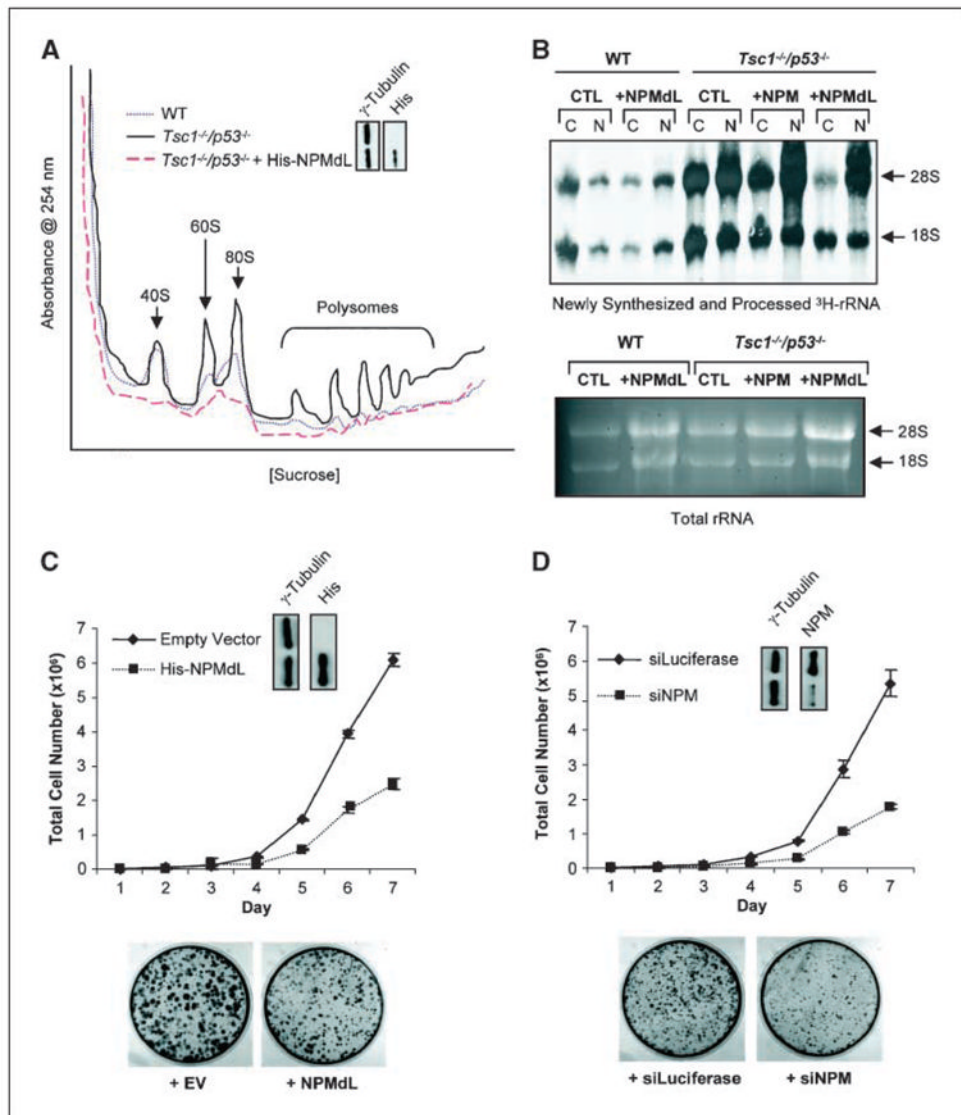


Figure 6. Expression of nucleophosmin export mutant suppresses polysome formation and abrogates enhanced export of newly synthesized RNAs caused by *Tsc1* loss. *A*, WT and *Tsc1*^{-/-}/*p53*^{-/-} MEFs were infected with retroviruses encoding β -galactosidase or His-tagged NPMdL, a nucleophosmin shuttling mutant. Cells (3×10^6) for each condition were lysed and fractionated over a 10% to 45% sucrose gradient. Gradients were fractionated and ribosomal subunits were detected by measuring RNA absorbance at 254 nm. *Inset*, Western blot analysis of proteins harvested from each condition. Separated proteins were immunoblotted with antibodies recognizing γ -tubulin and the His epitope. *B*, WT and *Tsc1*^{-/-}/*p53*^{-/-} MEFs were infected with retroviruses encoding β -galactosidase [control (CTL)] His-nucleophosmin or His-NPMdL. Cells were labeled with [methyl- ^3H]methionine and chased. Equal numbers of cells were subjected to nuclear and cytoplasmic fractionation. Total RNA extracted from each fraction was separated, transferred to membranes, and subjected to autoradiography. Ethidium bromide staining of total rRNA loaded from each sample condition was given to ensure equal rRNA loading from identical sample cell numbers (*bottom*). *C*, *Tsc1*^{-/-}/*p53*^{-/-} MEFs were infected with retroviruses encoding pSR α

(*Empty Vector*) or His-NPMdL and selected in G418. Cells (1.5×10^3) were seeded on 100-mm dishes to assess foci formation (*bottom*). Cells grew for 12 d in complete medium, fixed with methanol, and stained with Giemsa. Proliferation rates were measured by seeding cells (1×10^4) in triplicate and by counting total cell numbers daily over the course of 6 d (*top*). *Inset*, Western blot analysis of proteins harvested from each condition. Separated proteins were immunoblotted with antibodies recognizing γ -tubulin and the His epitope. *D*, *Tsc1*^{-/-}/*p53*^{-/-} MEFs were infected with lentiviruses encoding siRNAs designed to knockdown expression of luciferase (*siLuciferase*) or nucleophosmin (*siNPM*) and selected in puromycin. Ninety-six hours postinfection, the ability to form foci and proliferation rates were assessed as described in (C). Efficient knockdown of nucleophosmin is shown by Western blot analysis.