Supporting Information

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SI Materials and Methods

Cell Culture and Treatments. A549 and U2OS cells were obtained from the American Type Culture Collection. Mouse embryonic fibroblasts (MEFs) were prepared from embryos at embryonic day 13.5 (E13.5) of development. Only early passage (3–6) MEFs were used in our experiments. More information about YFP-L11 (170407pl2D10), YFP-L4 (170407pl2c11), and YFP-S6 (200208pl1c3) H1299 cell lines can be found at www.weizmann.ac.il/mcb/UriAlon/. Each cell-line clone contains an endogenous ribosomal protein (RP) fused to YFP, expressed from its endogenous chromosomal location with its natural regulation. Labeling with YFP was done by exon tagging, where YFP, flanked by splice signals, was delivered into the genome using a retrovirus (1). YFP is integrated into intron 1 of the L4, L11, or S6 gene and then spliced into the protein as a new exon. Protein identity was established by sequencing the cDNA. YFP-L4 and YFP- L11 show strong nucleolar and cytoplasmic staining, which is identical to endogenous RPs. YFP-S6 shows strong nucleolar staining and weaker nuclear and cytoplasmic staining.

All cell types were cultured in Dulbecco modified Eagle medium supplemented with 10% (vol/vol) FCS 2 mM L-glutamine, 5 μM 2-mercaptoethanol, 10^5 U of penicillin/L, 0.1 g of streptomycin/L, and 0.035 g of gentamicin/L. Cells were treated with 5 nM actinomycin D (ActD), 385 μM 5-fluorouracil (5-FU), 25 μM cycloheximide (CHX), 20 μM MG132, or 20 nM rapamycin for the indicated times before harvesting. These chemicals were obtained from Sigma Chemical.

RNAi. The Whitehead siRNA Selection Web Server (http://jura.wi.mit.edu/bioc/siRNA) was used to design short oligonucleotides that specifically down-regulate expression of target genes. HPLC-purified and annealed oligonucleotide targeting the indicated sequences relative to translation initiation codon (Table S1) were used (Ambion). Promyelocytic leukemia (PML) protein siRNA SMARTpool was purchased from Dharmacon. A549 and U2OS cells were transfected with the indicated siRNAs for 48 h using Lipofectamine RNAiMAX transfection reagent (Invitrogen) at a final siRNA concentration of 20 nM according to the manufacturer's instructions. As a nonsilencing control we used scrambled (scr) siRNA.

RNA Isolation and Quantitative RT-PCR. Total RNA was isolated from A549 or U2OS cells using TriReagent (Applied Biosystems). A total of 2 μg of RNA was retrotranscribed by an RT-PCR kit (Applied Biosystems). PCR was performed with Power Sybr Green (Applied Biosystems) according to the manufacturer's instructions on an ABI Prism 7300 sequence detector (Applied Biosystems). The fluorescent signal from SYBR green was detected immediately after the extension step of each cycle, and the cycle at which the product was first detectable was recorded as the cycle threshold. For an internal control, primers specific for GAPDH were used. Primer sequences are detailed in Table S2. The data were imported into an Excel database and analyzed using the comparative cycle threshold method with normalization of the raw data to GAPDH. The results are presented as *n*-fold changes versus the values in the untreated sample. The mean value was calculated from three independent measurements.

Western Blot Analysis. A549 and U2OS cells were lysed in radioimmunoprecipitation assay buffer containing 50 mM Tris·HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% (vol/vol) Nonidet P-40, 0.5% sodium deoxycholate and protease and phosphatase inhibitors (1 μg of leupeptin/mL, 1 μg of aprotinin/mL, 50 μg of PMSF/mL, 1 mM Na_3VO_4, 1 mM NaF) for 30 min at 4 °C and centrifuged at 13,000 × *g* for 30 min. The protein concentration was determined by the Bio-Rad protein assay according to the manufacturer's instructions (Bio-Rad). Total proteins (10–20 μg) were resuspended in Laemmli buffer (63 mM Tris·HCl, 10% (vol/vol) glycerol, 2% (vol/vol) SDS, 0.0025% bromophenol blue, pH 6.8) and electrophoresed on SDS-polyacrylamide gels. The following primary antibodies were used in Western blot analysis: mouse monoclonal β–anti-actin (Chemicon International), mouse monoclonal anti-p53 (DO-1 Santa Cruz Biotechnology), mouse monoclonal anti-p21 (Santa Cruz Biotechnology), mouse monoclonal anti-Mdm2 (SMP-14; Santa Cruz Biotechnology), rabbit polyclonal anti-PML (Abcam), rabbit polyclonal anti–p53-acetyl-Lys382 (Cell Signaling Technology), mouse monoclonal anti-B23 (Sigma), mouse monoclonal anti-UBF (Santa Cruz Biotechnology), mouse monoclonal antitubulin (Santa Cruz Biotechnology), rabbit polyclonal antifibrillarin (Abcam), rabbit polyclonal anti-PARP (Cell Signaling Technology), goat polyclonal anti-lamin B (Santa Cruz Biotechnology), and various mouse monoclonal and rabbit polyclonal anti-RPs developed in our laboratory (*Material and Methods*). Primary antibodies were detected by horseradish peroxidase-conjugated secondary antibodies to rabbit and mouse immunoglobulins (Santa Cruz Biotechnology) and the ECL Prime Western Blotting Detection System (Amersham Biosciences).

Immunoprecipitation. Total cell extracts were prepared in immunoprecipitation (IP) buffer containing 50 mM Tris·HCl (pH 8.0), 150 mM NaCl, 1% (vol/vol) Nonidet P-40, 1 mM DTT, 1 mM EDTA, and protease and phosphatase inhibitors (1 μg of leupeptin/mL, 1 μg of aprotinin/mL, 50 μg of PMSF/mL, 1 mM Na_3VO_4, 1 mM NaF). Total cell extracts were precleared with protein A Sepharose (Sigma Aldrich). For each sample, 1.5 mg of protein extract was incubated with antibodies against Mdm2 (SMP-14 and H221; Santa Cruz Biotechnology), L11 (2), and L5 (rabbit polyclonal) at 4 °C for 3 h before incubation with protein A for 60 min. Immunoprecipitates were washed four times in ice-cold IP buffer, resuspended in Laemmli buffer, and subjected to Western blot analysis.

Isolation of Total Ribosomal and Nonribosomal Fractions. A549 cells (3×10^8) were homogenized in 5 mL of buffer containing 20 mM Tris (pH 7.4), 10 mM MgCl2, 300 mM KCl, 10 mM DTT, 100 U of RNasin/mL, 0.5% Nonidet P-40, and 100 μg of CHX/mL After centrifugation at 10,000 × *g* for 15 min to remove mitochondria and debris, the supernatant was layered over a sucrose [20% (wt/vol)] cushion containing 100 μg of cycloheximide/mL and centrifuged at 149,000 × *g* for 2 h. The ribosome-containing pellet and nonribosomal supernatant were collected. The supernatant was subjected to a second centrifugation at 149,000 × *g* to remove ribosome contaminants. For each sample, 1.5 mg of protein from the nonribosomal fraction was immunoprecipitated with the indicated antibodies.

Isolation of Ribosomal and Nonribosomal Cytoplasmic and Nuclear Fractions. The procedure is a modification of a previously published method (3). A549 cells or MEFs (3×10^7) were suspended in 3 mL low-salt buffer (LSB: 10 mM Hepes-NaOH pH 7.5, 10 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA), incubated for 10 min on ice, pelleted at 1,200 × *g* for 5 min at 4 °C, resuspended in 3 mL LSB containing protease and phosphatase inhibitors (1 μg

of leupeptin/mL, 1 μg of aprotinin/mL, 50 μg of PMSF/mL, 1 mM Na_3VO_4, 1 mM NaF), and lysed by adding Nonidet P-40 to 0.3% and Na deoxycolate to 0.2% followed by vigorous vortexing for 30 s. The samples were centrifuged at 2,800 × *g* for 5 min, and the supernatant was retained as the cytoplasmic fraction. The pellet (nuclei) was lysed with 900 μL high-salt buffer [10 mM Tris·HCl (pH 7.2), 0.5 M NaCl, 50 mM $MgCl_2$, 0.1 mM $CaCl_2$] containing 20 U RNase inhibitor (Ambion) and 150 U DNase I (Invitrogen) for 10 min at room temperature. After centrifugation at 12,000 × *g* for 10 min, the pellet was rinsed with 300 μL of ice-cold nuclear extraction buffer [10 mM Tris·HCl (pH 7.2), 10 mM NaCl, 10 mM EDTA] and centrifuged for 1 min 12,000 × *g*. The two supernatant fractions were pooled (nucleoplasmic fraction). The cytoplasmic and nucleoplasmic fractions were layered over sucrose [20% (wt/vol)] cushions containing 100 μg of CHX/mL and centrifuged at 149,000 × *g* for 2 h. The ribosome-containing pellets and nonribosomal supernatants were collected. To remove any ribosome contaminants, the supernatant was subjected to a second centrifugation at 149,000 × *g*. Ribosome pellets were resuspended in Laemmli buffer, and nonribosomal cytoplasmic and nucleoplasmic fractions were concentrated by precipitation with 10% (vol/vol) trichloroacetic acid and mixed with Laemmli buffer. Fractions were subjected to immunoblotting with the indicated anti-RP antibodies. The purity of the nuclear and cytoplasmic fractions was determined by immunoblotting with antibodies against PARP (nucleoplasmic) and tubulin (cytoplasmic).

Isolation of Highly Purified Nucleoli. Nucleoli were prepared from 5×10^6 A549 cells essentially as previously described (4). Isolated nucleoli were resuspended in Laemmli buffer and analyzed by Western blotting. The purity of isolated nucleoli was confirmed by immunoblotting with antibodies against lamin B (a nuclear protein that does not accumulate in nucleoli), which showed no detectable expression in the purified nucleolar fractions (Fig. S4*C*). For immunoprecipitations, highly purified nucleoli were resuspended in IP buffer.

Immunofluorescence Analysis. Cells were seeded on coverslips and transfected with various siRNAs or treated with the indicated pharmacological inhibitors as described above. Coverslips were then washed in PBS, fixed with 4% (vol/vol) paraformaldehyde for 10 min, permeabilized with 0.5% Triton X-100-PBS for 7 min, and then blocked for 30 min with 3% (vol/vol) BSA-0.2% Triton X-100-PBS. Primary rabbit polyclonal antifibrillarin (Abcam), rabbit polyclonal anti-p53 (FL-393; Santa Cruz Biotechnology), rabbit polyclonal anti-Mdm2 (H221; Santa Cruz Biotechnology), mouse monoclonal anti-PML (PGM3; Santa Cruz Biotechnology), mouse monoclonal anti-Flag (Sigma), mouse monoclonal anti-HA (Cell Signaling Technology), mouse monoclonal anti-UBF (Santa Cruz Biotechnology), mouse monoclonal anti-B23 (Sigma), mouse monoclonal anti-S6, and mouse monoclonal anti-S12 were incubated with cells at appropriate dilution in 3% (vol/vol) BSA-0.2% Triton X-100-PBS for 3 h at room temperature. Secondary antibodies were conjugated with either Alexa 594 or Alexa 488 (both from Molecular Probes) and used in a 1:1,000 dilution for 45 min at 37 °C. 4′,6′-Diamidino-2-phenylindole (DAPI) was added for 5 min in the dark before inclusion in ProLong Gold antifade reagent (Invitrogen). When indicated, H1299 cells expressing YFP-L11 grown on coverslips were permeabilized with 0.1% Triton X-100 in PBS for 5 min, washed with PBS, and treated for 10 min at 37 °C with RNase A (100 μg/mL). After washing with PBS, cells were prepared for immunofluorescence as described above. Images were taken with Zeiss LSM700 confocal laser scanning microscopy (CLSM) (Carl Zeiss) using a 63× Plan Apochromat objective.

Fluorescence Recovery After Photobleaching. H1299 cells (3×10^5) expressing YFP-L11 were cultured on 35-mm glass-bottomed dishes (Ibidi), treated with ActD for 5 h in the presence or absence of CHX. The experiment was done using the LSM 700 (Zeiss) and a 40× Plan Apochromat objective (Carl Zeiss). Briefly, the entire cell area of 15 randomly picked cells was photobleached using a 488-nm laser. Nucleolar recovery of fluorescence was measured at time intervals of 30 min for 3 h. Image acquisition was made under nonconfocal conditions using 0.2% laser power for excitation to avoid bleaching and cytotoxicity. The average intensity of the YFP signal in all bleached nucleoli ($n = 15$) was measured using the Zen imaging software. Values of single nucleoli were averaged and normalized against the average value of unbleached ($n = 15$) nucleoli in the same acquisition frame to compensate for signal loss due to repeated acquisition. Fluorescence recovery after photobleaching (FRAP) values in the nucleolus are shown as average nucleolar FRAP ± SD.

Cell Cycle Analysis. A549 cells were transfected with siRNAs and treated with ActD as described above. Cells were resuspended in PBS and fixed with an equal volume of ice-cold absolute ethanol. After centrifugation cells were incubated with RNase (100 μg/mL) for 15 min at 37 °C, stained with propidium iodide (0.036 mg/mL) for 30 min at room temperature, and analyzed for DNA content using a BD Biosciences FACSalibur flow cytometer. Data were analyzed using the CellQuest and FlowJo software Programs.

1. Cohen AA, et al. (2008) Dynamic proteomics of individual cancer cells in response to a drug. *Science* 322(5907):1511–1516.
2. Sulic S, et al. (2005) Inactivation of S6 ribosomal protein gene in T lymphocytes activates a p53-dependent checkpoint response. *Genes Dev* 19(24):3070–3082.
3. Lapik YR, Fernandes CJ, Lau LF, Pestov DG (2004) Physical and functional interaction between Pes1 and Bop1 in mammalian ribosome biogenesis. *Mol Cell* 15(1):17–29.
4. Andersen JS, et al. (2002) Directed proteomic analysis of the human nucleolus. *Curr Biol* 12(1):1–11.

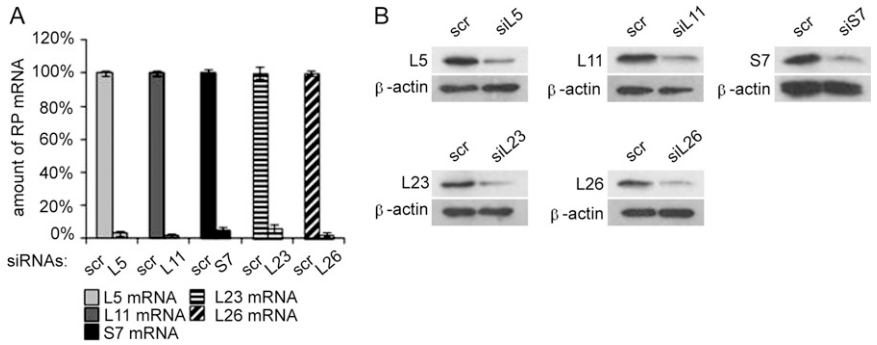

Fig. S1. Efficacy of silencing of RPs. (*A*) A549 cells were transfected with the indicated siRNAs for 48 h and the effective silencing of RPs was confirmed by qRT-PCR (*A*) or Western blot analysis (*B*). Each value in *A* represents the mean ± SD of three independent experiments.

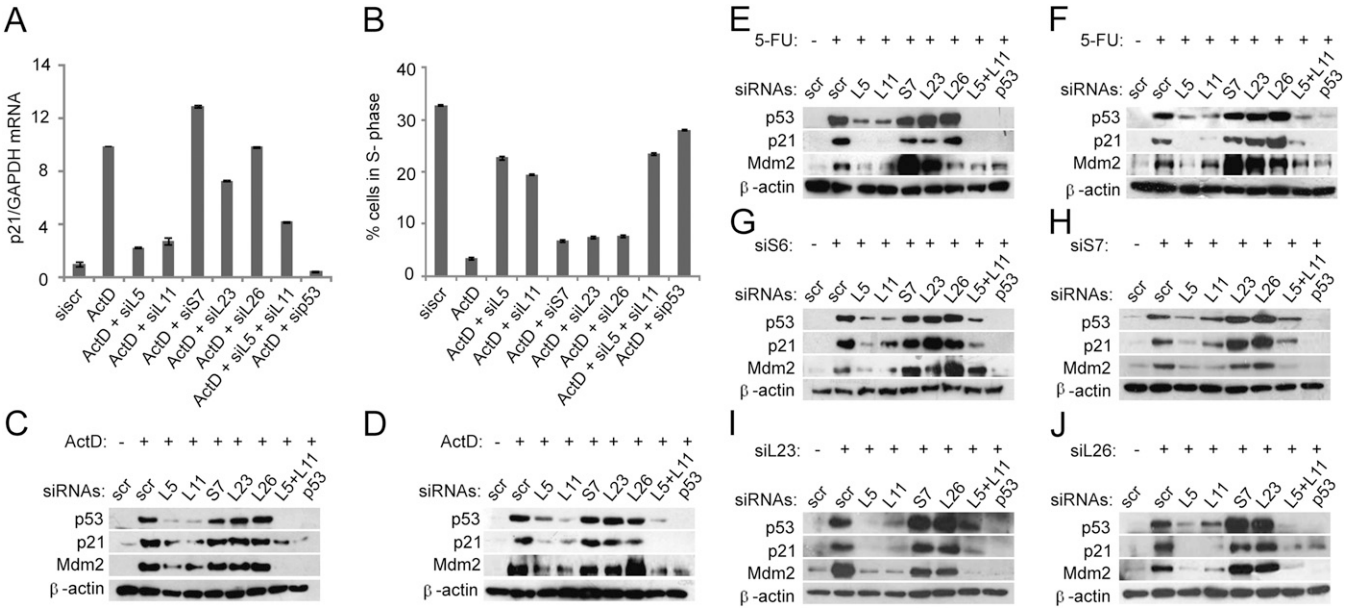

Fig. S2. L5 and L11 are critical for p53 up-regulation in response to various ribosomal stressors. (*A*) A549 cells were transfected with the indicated siRNAs for 48 h, followed by ActD treatment for 16 h. The $p21^{WAF1}$ mRNA expression was determined by qRT-PCR. Each value represents the mean ± SD of three independent experiments. (*B*) A549 cells were transfected and treated with ActD as in *A*, followed by staining with propidium iodide. Cell-cycle-phase distributions were analyzed using flow cytometric analysis. Each value represents the mean ± SD of three independent experiments. (*C*) U2OS cells were transfected with the indicated siRNAs for 48 h and treated with 5 nM ActD for 16 h. Induction of the indicated proteins was verified by Western blot. (*D*) A549 cells were transfected with a second set of siRNAs against the indicated RPs or scr siRNA for 48 h and then treated with 5 nM ActD for 16 h. Expression of the indicated proteins was determined by immunoblotting. A549 (*E*) and U2OS (*F*) cells were transfected with the indicated siRNAs for 48 h and treated with 5-FU for 16 h. Expression of the indicated proteins was determined by Western blot analysis. (*G–J*) A549 cells were transfected with siRNAs against S6, S7, L23, or L26 in combination with the indicated siRNAs for 48 h. Expression levels of p53, $p21^{WAF}$, Mdm2, and β-actin were determined by immunoblotting.

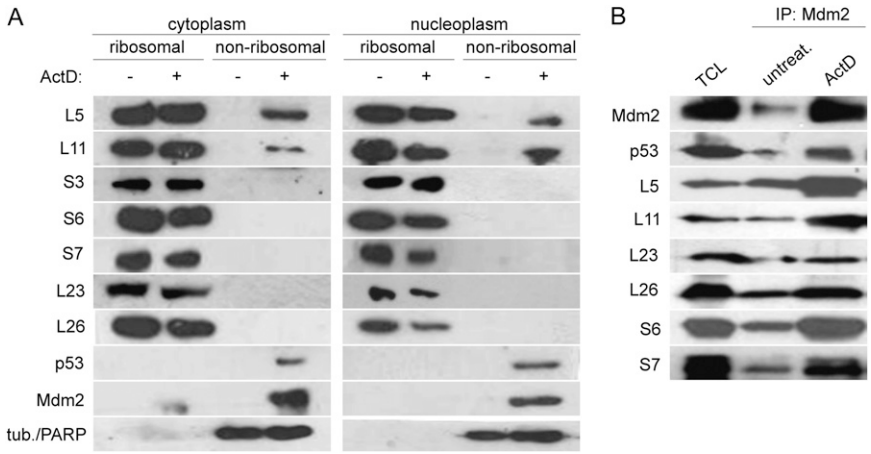

Fig. S3. Specific accumulation of L5 and L11 in the nonribosomal fraction upon ActD treatment. (*A*) E13.5 MEFs were treated with 5 nM ActD for 16 h. Ribosomal and nonribosomal fractions were analyzed by Western blot with antibodies against the indicated proteins. Tubulin (tub.) was used as a cytoplasmic marker and PARP as a nucleoplasmic marker. (*B*) Nonspecific interactions of various RPs with Mdm2 in the total cell lysate (TCL). TCL from untreated and ActD-treated (16 h) A549 cells were immunoprecipitated with anti-Mdm2 antibodies. These immunoprecipitates and TCL from ActD-treated A549 cells were immunoblotted with the indicated antibodies.

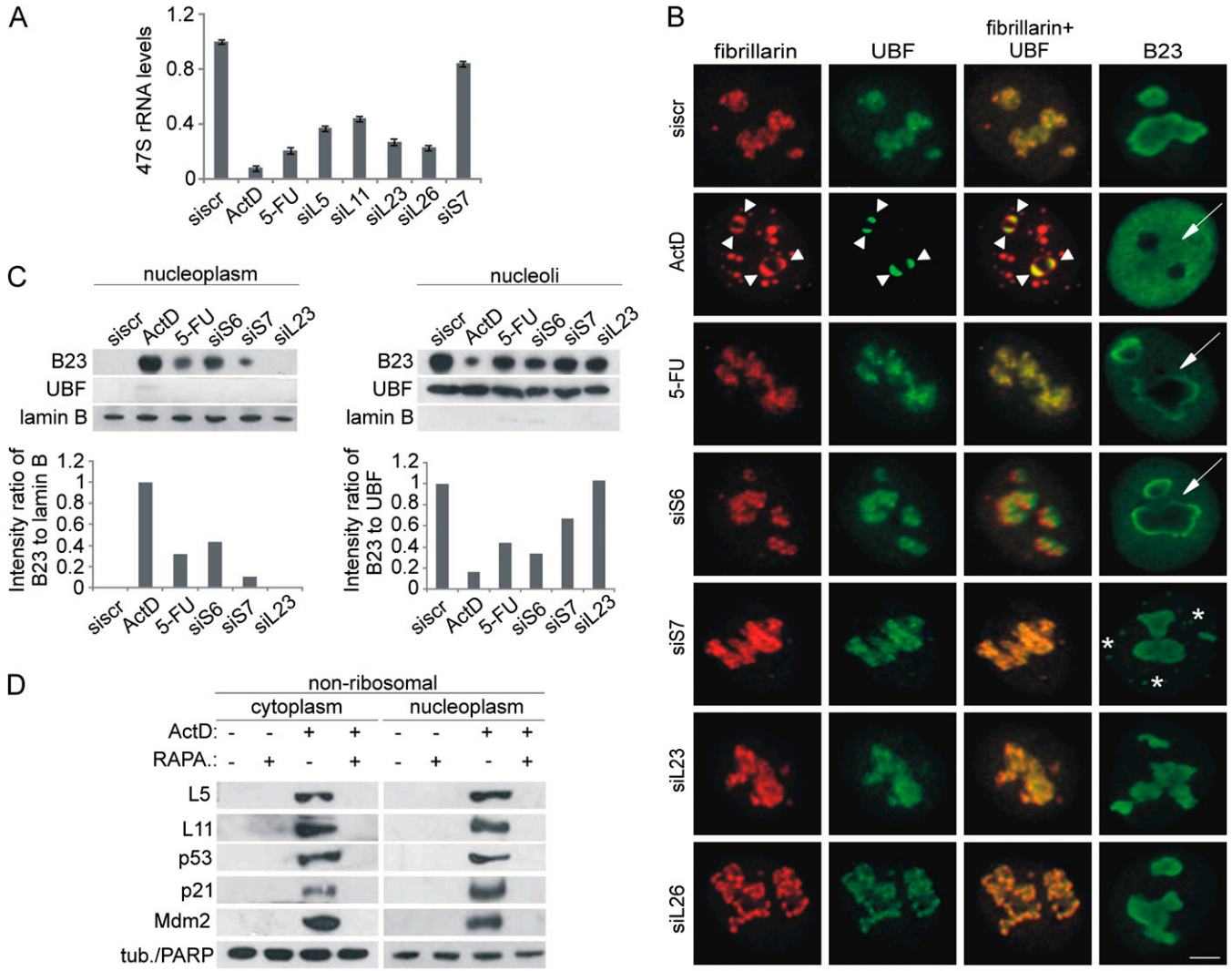

Fig. S4. Nucleolar changes after exposure to different types of ribosomal biogenesis stress. A549 cell were transfected with the indicated siRNAs for 48 h or treated with ActD or 5-FU for 5 h. (*A*) Quantitative RT-PCR analysis of the 47S rRNA expression. The data were normalized to GAPDH. Each value represents the mean ± SD of three independent experiments. (*B*) Untreated and treated cells were fixed and subjected to immunofluorescence staining with antibodies against the indicated nucleolar markers. Diffused nucleoplasmic B23 staining is indicated by an arrow, B23 nuclear spot structures by an asterisk, and fibrillarin and UBF nucleolar cap structures by arrowheads. Yellow represents colocalization of the two stainings. Fluorescence signals were analyzed by CLSM. (Scale bar, 5 μm.) (*C*) Nucleolar and nucleoplasmic extracts from untreated and treated cells were immunoblotted with the indicated antibodies (*Upper* panels). Quantification of ratio of B23 to lamin B or B23 to UBF using ImageJ Software (*Lower* panels). (*D*) Cells were treated with ActD for 5 h in the presence or absence of rapamycin (RAPA), and expression of the indicated proteins was analyzed in cytoplasmic and nucleoplasmic nonribosomal fractions by immunoblotting.

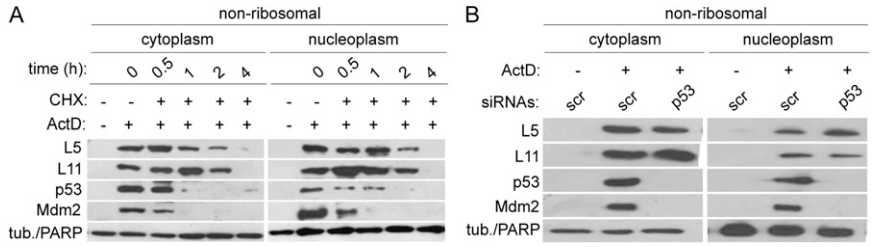

Fig. S5. Ribosome-free L5 and L11 accumulate independently of Mdm2 and p53 upon ActD treatment. (*A*) ActD-triggered accumulation of ribosome-free L5 and L11 through an increase in protein stability. A549 cells were treated with ActD for 5 h, and then CHX was added for the indicated periods of time. Nonribosomal fractions were analyzed for expression of the indicated proteins by immunoblotting. (*B*) A549 cells were transfected with p53 or scr siRNA for 48 h and then treated with ActD for 5 h. Cytoplasmic and nucleoplasmic nonribosomal fractions were subjected to immunoblotting with the indicated antibodies. Tubulin (tub.) was used as a cytoplasmic marker and PARP as a nucleoplasmic marker.

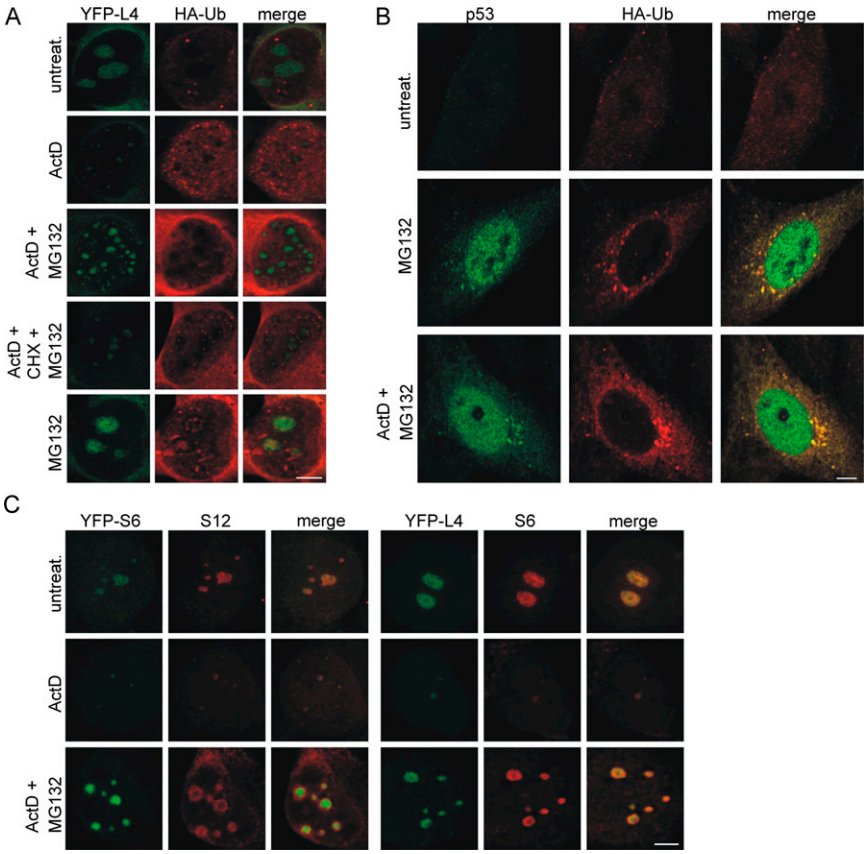

Fig. S6. Similar to YFP-S6 and YFP-L4, endogenous S6 and S12 form nucleoplasmic aggregates in the presence of ActD and MG132. (*A*) H1299 cells containing YFP-L4 (green) were transfected with HA-ubiquitin (HA-Ub) for 24 h and then treated as indicated for 10 h, followed by immunofluorescence staining with anti-HA antibody (red). (*B*) A549 cells were transfected with HA-Ub for 24 h and then treated with MG132 for 6 h, followed by immunofluorescence staining with anti-HA (red) and anti-p53 antibodies (green). (*C*) H1299 cells containing YFP-L4 (green) or YFP-S6 (green) were treated with ActD or ActD in the presence of MG132 for 10 h. Cells were then fixed with methanol and stained with monoclonal antibodies against S12 or S6 (red). Fluorescence signals in *A*–*C* were analyzed by CLSM. Yellow represents colocalization of the two stainings (*B* and *C*). (Scale bar, 5 μm.)

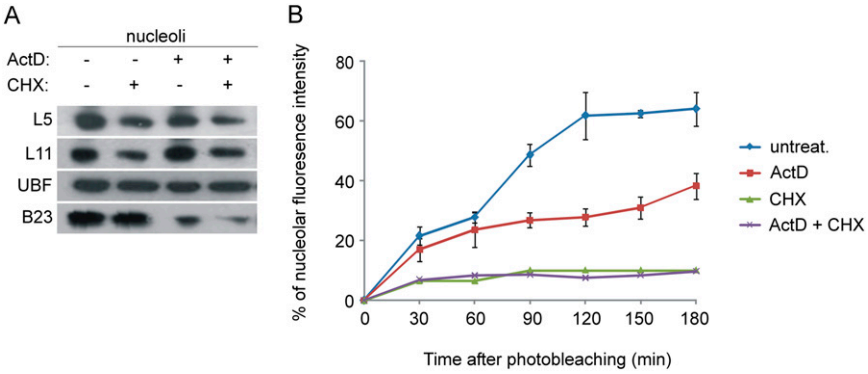

Fig. S7. Newly synthesized L5 and L11 accumulate in nucleoli upon ActD treatment. (*A*) A549 cells were treated with ActD for 5 h in the presence or absence of CHX, and purified nucleoli were isolated and analyzed by Western blot with the indicated antibodies. (*B*) Quantification of the nucleolar fluorescence signal from the experiment shown in Fig. 5*A*. Each time point is expressed as the relative measured intensity divided by the prebleached signal. All values were corrected to the background signal. Error bars indicate SD (n = 15 cells).

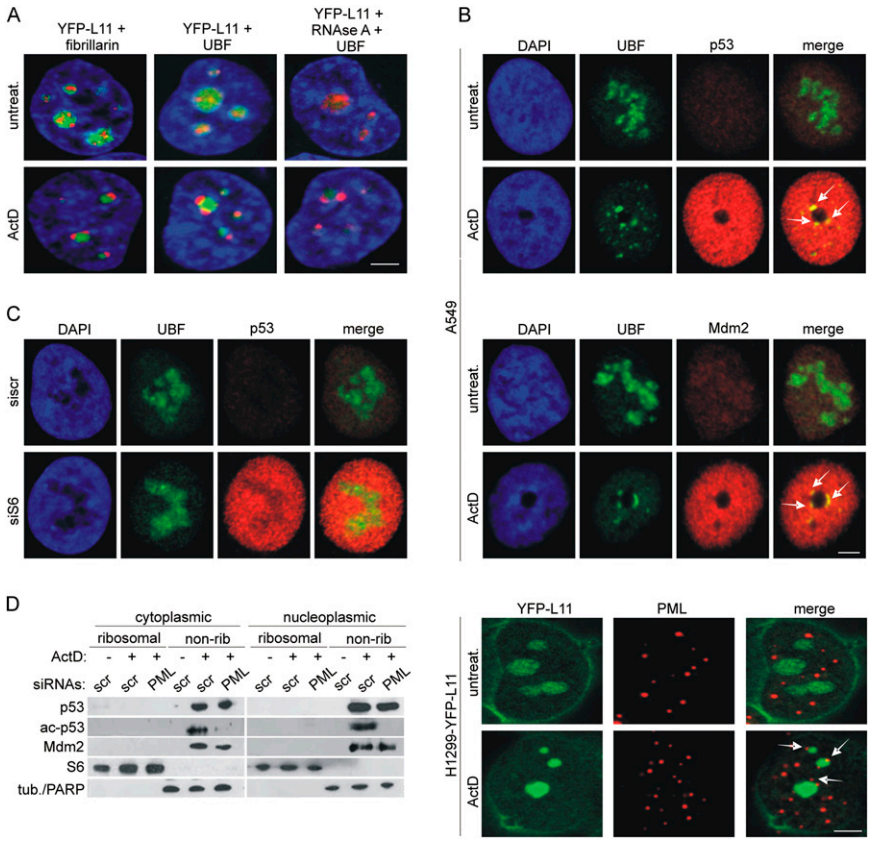

Fig. S8. Ribosome-free L5 and L11 colocalize with Mdm2, p53, and PML in nucleoli upon ribosomal biogenesis stress. (*A*) H1299 cells expressing YFP-L11 were treated as in Fig. 5*A*. At 3 h of the recovery after photobleaching of the nucleolar YFP fluorescence signal (green), cells were stained with antibodies against UBF (red) or fibrillarin (red) in the presence or absence of RNase and analyzed by CLSM. (*B*) A549 or H1299 cells expressing YFP-L11 were treated with ActD for 5 h, fixed, and stained with DAPI (blue) or the indicated antibodies and analyzed by CLSM. Arrows point to nucleolar PML, p53, or Mdm2 nucleolar localization. (*C*) A549 were transfected with siRNA against S6 for 48 h, fixed, and stained with DAPI (blue) or the indicated antibodies and analyzed by CLSM. Yellow represents colocalization of the two stainings (*B* and *C*). (Scale bar, 5 μm in *A*–*C*.) (*D*) A549 cells were transfected scr siRNA or siRNA against PML for 48 h and treated with ActD for 5 h. Ribosomal and nonribosomal (nonrib) cytoplasmic (*Left*) or ribosomal and nonribosomal nucleoplasmic fractions (*Right*) were analyzed by immunoblotting with the indicated antibodies. The per-cell ratio of the amount of protein loaded onto a gel for cytoplasmic ribosomal, nucleoplasmic ribosomal, cytoplasmic nonribosomal, and nucleoplasmic nonribosomal fractions was 1:10:3:10. Tubulin (tub.) was used as a cytoplasmic marker and PARP as a nucleoplasmic marker.

Table S1. Primer sequences for qRT-PCR: Human

Primer name	Sequence
Cdkn1A F	GGACAGCAGAGGAAGACCATGT
Cdkn1A R	TGGAGTGGTAGAAATCTGTCATGC
p53 F	GTTCCGAGAGCTGAATGAGG
p53 R	TCTGAGTCAGGCCCTTCTGT
L5 F	GCACACGAACTGCCAAAATA
L5 R	TTCATCAACCAGTCACCTCCA
L11 F	GAAAAGGAGAACCCCATGC
L11 R	CATTTCTCCGGATGCCAA
L23 F	CTGACAACACAGGAGCCAAA
L23 R	ACACGCCATCTTTTCTACGG
L24 F	CAAATTCCAGAGGGCCATTA
L24 R	TTTGCTTAGGTGCTGCCTTT
L26 F	GGAAAAGGCTAATGGCACAA
L26 R	TCCTTTCCTACTTGGCGAGA
S6 F	TGCTCTGAAGAAGCAGCGTA
S6 R	GGAAAGTCTGCGTCTCTTCG
S7 F	GTGGGAAGCATGTCGTCTTT
S7 R	TGTTCTGCTGTGCTTTGTCC
prerRNA F	CCTGTCTGTTCTCTCGCGCGTCCGAA
prerRNA R	AACGCCTGACACGCACGGCACGGA
GAPDH F	GACAAGCTTCCCGTTCTCAG
GAPDH R	GAGTCAACGGATTTGGTCGT
PML F	CCTCCCCTTCAGCTTCTCTT
PML R	CGCAGAAACTGGAACTCCTC

Table S2. siRNA target sequences: Human

siRNA name	Target sequence
L5 #1	5′-AAGGTTGGCCTGACAAATTAT-3′
L5 #2	5′-AACTACCACTGGCAATAAAGT-3′
L11 #1	5′-AAGGTGCGGGAGTATGAGTTA-3′
L11 #2	5′-TACGGCCTGGACTTCTATGTG-3′
S7 #1	5′-GCAAGGATGTTAATTTTGA-3′
S7 #2	5′-GGAAGCATGTCGTCTTTAT-3′
L23 #1	5′-AAGGCGAGATGAAAGGTTCTG-3′
L23 #2	5′-AAAGATGGCGTGTTTCTTTAT-3′
L26 #1	5′-AAGAGCTGAGACAGAAGTACA-3′
L26 #2	5′-AACCGAAAGGATGATGAAGTT-3′
S6 #1	5′-AAGAAAGCCCTTAAATAAAGA-3′
L24 #1	5′-AAAGGGACAGTCGGAAGAAAT-3′
p53 #1	5′-CAGCATCTTATCCGAGTGGAA-3′