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Phosphorylation of Eukaryotic Translation Initiation Factor 2α Coordinates rRNA Transcription and Translation Inhibition during Endoplasmic Reticulum Stress▽†

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The endoplasmic reticulum (ER) is the major cellular compartment where folding and maturation of secretory and membrane proteins take place. When protein folding needs exceed the capacity of the ER, the unfolded protein response (UPR) pathway modulates gene expression and downregulates protein translation to restore homeostasis. Here, we report that the UPR downregulates the synthesis of rRNA by inactivation of the RNA polymerase I basal transcription factor RRN3/TIF-IA. Inhibition of rRNA synthesis does not appear to involve the well-characterized mTOR (mammalian target of rapamycin) pathway; instead, PERK-dependent phosphorylation of eIF2α plays a critical role in the inactivation of RRN3/TIF-IA. Downregulation of rRNA transcription occurs simultaneously or slightly prior to eIF2α phosphorylation-induced translation repression. Since rRNA is the most abundant RNA species, constituting ~90% of total cellular RNA, its downregulation exerts a significant impact on cell physiology. Our study demonstrates the first link between regulation of translation and rRNA synthesis with phosphorylation of eIF2α, suggesting that this pathway may be broadly utilized by stresses that activate eIF2α kinases in order to coordinately regulate translation and ribosome biogenesis during cellular stress.

All living organisms in their lifetimes are subject to fluctuations in their environments. The evolution of systems to sense and respond to stressful conditions allows the organism to quickly counter the action of the stress, minimizing potential damage or possible death. A common strategy among stress responses is modulation of gene expression programs at all stages, including protein synthesis, to facilitate a return to homeostasis. Currently, two major mechanisms have been described for regulation of protein production rates: one via regulation of the mammalian target of rapamycin (mTOR) pathway and the other by phosphorylation of eukaryotic translation initiation factor 2α (eIF2α).

In response to growth signals and nutrients, mTOR signaling is responsible for coordinately regulating global translation initiation and ribosome biogenesis. mTOR modulates the rate of translation by phosphorylation of the eIF4E binding protein (4E-BP) (3, 8). In addition, mTOR activity modulates ribosome biogenesis by altering both synthesis of ribosomal proteins (7, 12, 47) and rRNA gene transcription by RNA polymerase I (Pol I) (26, 33, 41). Building new ribosomes consumes an enormous amount of energy and represents a significant investment in the protein biosynthetic capacity of the cell. Thus, regulating translation levels is critical to cell physiology. Regulation by mTOR can be thought of as a dial that can tune the efficiency of translation initiation and rRNA transcription up or down in order to balance the demand for resources to sustain cellular functions with the need for cell growth. While the mTOR pathway balances growth signals with nutrient availability, cells must also adjust the rate of protein synthesis in response to other challenges and stressful conditions.

The other major pathway that regulates translation is through phosphorylation of eIF2α and can be thought of as an emergency brake rather than a dial for modulating protein synthesis. The importance of this pathway is demonstrated by the evolution of at least four distinct eIF2α kinases in mammalian cells, each responding to a different set of stress conditions (4, 10, 28, 43, 50, 54). Under normal conditions eIF2α kinases are inactive, and upon stress stimulation they phosphorylate eIF2α, preventing recycling of the eIF2 complex and thus inhibiting the formation of the 43S translation initiation complex (11, 13, 19, 52). This allows cells to rapidly downregulate protein synthesis during stress even under conditions where growth signaling and nutrient availability are not immediately limiting, for example, during the unfolded protein response (UPR).

The UPR pathway monitors protein folding in the endoplasmic reticulum (ER). The synthesis, folding, and modification of proteins targeted to membranes or the secretory pathway takes place within the ER. When the protein-folding capacity of the ER is perturbed by environmental insult or when protein-folding demands are increased during developmental changes, unfolded proteins accumulate within the ER, resulting in activation of the UPR pathway. In mammals, there are three sensor molecules spanning the ER membrane that are

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responsible for initiating the UPR pathway: ATF6, IRE1, and PERK (PKR-like endoplasmic reticulum kinase). Each initiator senses ER stress through its luminal domain and transduces the signal across the ER membrane to the cytosol. All three UPR branches cooperate to increase production of ER chaperones and facilitate clearance of unfolded proteins from the organelle. However, PERK, the UPR-specific eIF2α kinase, has an additional function: to repress global translation initiation, thereby reducing the influx of nascent proteins into the stressed ER (27, 28, 50). In contrast to nutrient deprivation, where mTOR coordinately regulates translation and ribosome biogenesis, translation repression through eIF2α phosphorylation during ER stress has not been reported to be associated with alteration in ribosomal concentration, although a recent study suggests that ribosomal components may alter their association with cellular proteins during ER stress (57a). However, similar to nutrient deprivation, the continued investment in ribosome biogenesis during ER stress may also impose an unnecessary drain on cellular resources while translation is inhibited.

In this report, we have demonstrated that downregulation of rRNA gene transcription takes place immediately after the onset of ER stress. We have shown that rRNA transcription repression occurs simultaneously with (rather than as a consequence of) translation repression. We have found that eIF2α phosphorylation by PERK is necessary for rRNA transcriptional repression and that PERK activation results in dissociation of Pol I and RRN3/TIF-IA, a major Pol I transcription initiation factor, from the rRNA promoter. Our data suggest that in addition to mTOR, there may be a second major pathway induced by eIF2α phosphorylation that coordinately regulates translation and ribosome biogenesis, ensuring proper adjustment of cellular resources during stress.

MATERIALS AND METHODS

Cell culture and treatment. All cells were cultured in Dulbecco's modified Eagle's medium (Cellgro) supplemented with 10% fetal bovine serum (Cellgro) and maintained at 5% CO_2 and 37°C. Cells were incubated for 1 to 2 h in fresh medium before being treated with stress-inducing agents at concentrations indicated in the figure legends. Thapsigargin (Tg) and tunicamycin (Tm) were purchased from Calbiochem, while anisomycin (ANS), H_2O_2, and rapamycin (Rap) were purchased from Sigma.

Northern blotting. Total RNA isolated from whole cells or nuclei was analyzed on a 1% agarose gel containing 1% formaldehyde. Gels were transferred to zeta-probe membranes (Bio-Rad) in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) by capillary action and assayed with radiolabeled DNA probes following UV cross-linking.

RPA. RNase protection assay (RPA) probe templates were PCR amplified from mouse cDNA or genomic DNA with the following primers (where F is forward and R is reverse): for *Xbp1*, TTCCGGATTTACAAACGGAAACTGA AAAACAGAGTAGCAG (F) and TAATACGACTCACTATAGCAGAGGT GCACATAGTCTGAG (R); for pre-rRNA, TTCCGGAAAAACAATCTTCA GTCGCTCGTTGTGTTCTC (F) and TAATACGACTCACTATAGGAGGG CCCGCTGGCAGAACG (R); for *7SL* RNA, TTCCGGATTTTCAAGCGAT CGCTTGAGTCCAGGAG(F) and TAATACGACTCACTATAGAGCACGG GAGTTTTGACCTGC (R). For RPA in HeLa cells, pre-rRNA probe from +309 to +500 was cloned into pCRII (Invitrogen). Probes were transcribed in vitro in the presence of [α-^{32}P]UTP (Perkin Elmer) and gel purified. Probes (1×10^5 to 2×10^5 cpm) were hybridized to total RNA (2 μg) and digested with RNase A/T_1 (Ambion), followed by proteinase K (Invitrogen), phenol-chloroform extraction, and ethanol precipitation. Isolated RNAs were analyzed on 6% acrylamide gels containing 7 M urea.

Nuclear run-on. Nuclear run-on transcription was modified from Banerji et al.(2). Briefly, nuclei were isolated from 6×10^6 NIH 3T3 cells lysed by repeated pipetting in 0.5% NP-40, 10 mM Tris (pH 7.4), 10 mM NaCl, and 3 mM $MgCl_2$, followed by centrifugation. Supernatant was discarded, and nuclei were resuspended in 40% glycerol, 50 mM HEPES (pH 8.5), 5 mM $MgCl_2$, and 0.1 mM EDTA. Nuclei were added to an equal volume of reaction mixture such that the final concentration was 25 mM HEPES (pH 7.5); 5 mM $MgCl_2$; 2 mM dithiothreitol; 75 mM KCl; 25% glycerol; a 2.8 mM concentration of ATP, GTP, and CTP; 3.2 μM UTP; and 50 μCi of [α-^{32}P]UTP (3,000 Ci/mmol). Transcription reactions (20 min at 27°C) were stopped with DNase (Promega) and digested with proteinase K (2 h at 45°C) in 10 mM Tris, 2% sodium dodecyl sulfate (SDS), 7 M urea, 0.35 M NaCl, and 1 mM EDTA. RNA was isolated, denatured, and hybridized to DNA immobilized on zeta-probe membranes (42°C for 36 h) and exposed to phosphor screens.

Labeling nascent proteins. Cells were treated for the time indicated in the figures. At 10 min prior to collection, cells were incubated with ^{35}S-labeled methionine-cysteine mix (50 μCi/ml; Trans Label; MP Biomedicals) to label nascent proteins. Whole-cell extracts were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and exposed to phosphor screens.

Chromatin immunoprecipitation. *Perk*$^{+/+}$ or *Perk*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were treated as indicated and cross-linked in 1% formaldehyde. Isolated nuclei were lysed in 1% SDS, 10 mM EDTA, 50 mM Tris (pH 7.4), and 1 mM phenylmethylsulfonyl fluoride (PMSF) and sonicated to yield DNA fragments averaging 500 bp. Samples were diluted in 1% Triton, 2 mM EDTA, 150 mM NaCl, 20 mM Tris (pH 7.4), and 1 mM PMSF and immunoprecipitated with antibodies against RRN3, Pol I (RPA194), upstream binding factor (UBF) (all from Santa Cruz Biotechnology), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Research Diagnostics). Washed beads were eluted in 1% SDS and 0.1 M $NaHCO_3$ at room temperature and cross-links were reversed by incubation at 65°C followed by ethanol precipitation. DNA was digested with proteinase K, phenol-chloroform extracted, and ethanol precipitated. DNA was PCR amplified with primers against the murine rRNA promoter GTCGACCAGTTGTTCCTT TGAG (F) and CCCGGGAAAGCAGGAAGCGTG (R) or internal transcribed spacer 1 (ITS-1) DNA GGCTCTTCCGTGTCTACGAG (F) and GAGGCCA GAAAAGCGTGGCATC (R). Samples were analyzed on agarose gels stained with ethidium bromide. Relative levels of PCR products from chromatin immunoprecipitation (ChIP) samples were determined using a standard curve generated from PCR-amplified input DNA.

Western blotting. Whole-cell extracts were resolved by SDS-PAGE and transferred to nitrocellulose. Membranes were probed with antibodies against RPA194, RRN3/TIF-IA, UBF, GAPDH, β-actin (Sigma), phospho-S6, and total S6 (both from Cell Signaling). Membranes were developed with ECL Plus Western blotting detection reagent (GE Healthcare).

In vitro transcription. Nuclear extracts were prepared from exponentially growing untreated, Tg-treated (200 nM, 2 h), or ANS-treated (10 μM; 2 h) MEFs as described in Dignam et al. (17). In a standard reaction mixture, 25 μg of nuclear extract, 10 ng of ribosomal DNA (rDNA) template pU5.1E/X (53), and exogenous transcription factors were preincubated on ice. Transcription was initiated by adding 30 mM HEPES (pH 7.9); 0.1 mM EDTA; 10 mM creatine phosphate; 10% glycerol (vol/vol); 5 mM $MgCl_2$; 97 mM KCl; 200 ng/ml α-amanitin; 0.6 mM each of ATP, GTP, and CTP; 0.05 mM UTP; and 10 μCi [α-^{32}P]UTP (3,000 Ci/mM). The reaction was allowed to proceed at 30°C for 30 min. RNA was extracted and analyzed on 4% polyacrylamide gels containing 7 M urea.

Purification of cellular UBF and recombinant RRN3/TIF-IA. HeLa cells were transfected with FLAG-tagged RRN3 plasmid (30) with Effectene (Qiagen), and expressed proteins were purified from cell lysates in 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 1 mM PMSF using anti-FLAG agarose beads (Sigma). Beads were eluted with 0.5 mg/ml FLAG peptide (Sigma) and dialyzed with 20 mM HEPES (pH 8.0), 20% glycerol, 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM PMSF, and 0.5 mM dithiothreitol. UBF protein was isolated from Novicoff hepatoma cells as previously reported (53).

Detection and quantification. Chemifluorescence of Western blots, ethidium staining of agarose gels, and phosphor screens were visualized with a Typhoon 9400 imager (GE Healthcare). Bands were quantified with ImageQuant, version 5.2, software (GE Healthcare).

RESULTS

Activation of UPR inhibits Pol I transcription. To test if changes in rRNA levels accompany UPR-induced translation repression, we measured their levels in RNA isolated from nuclei of NIH 3T3 cells upon UPR induction. We reasoned that this would give us a better estimate of nascent rRNA as it

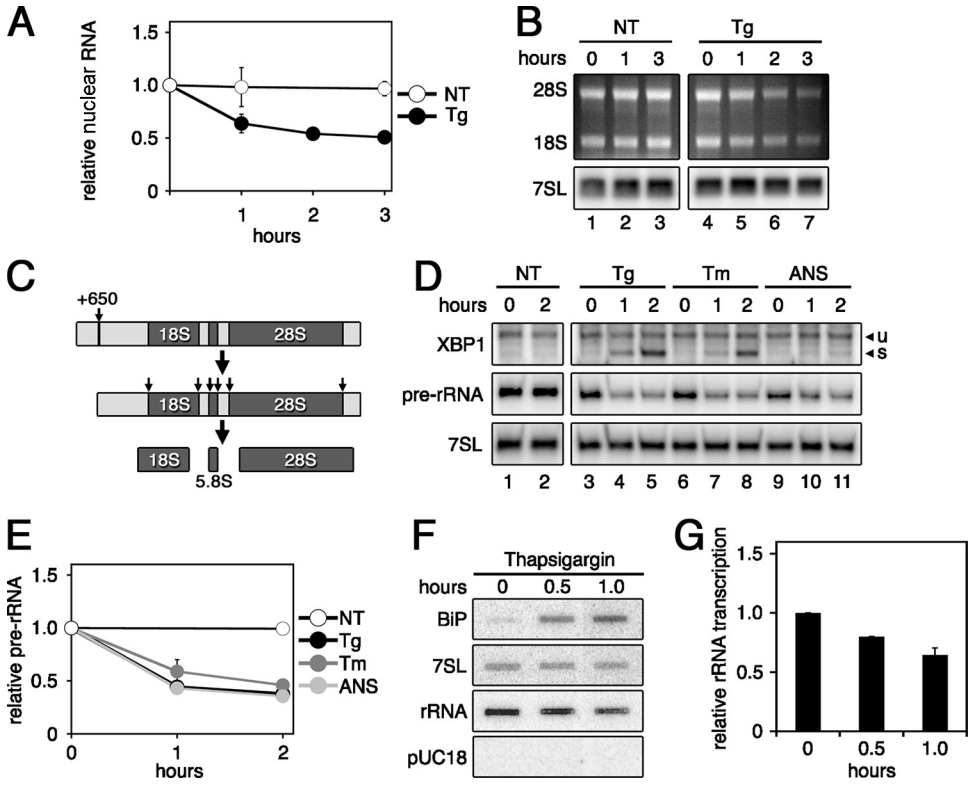

FIG. 1. rRNA transcription is downregulated upon UPR activation. (A) Quantitation of total RNA isolated from an equal number of NIH 3T3 cells during Tg (200 nM) treatment. NT, untreated cells. The amount of RNA at 0 h was given the value of 1, and each point represents the mean ± standard deviation of a minimum of three independent experiments. (B) Northern blotting of nuclear RNA as in panel A. The top panel shows ethidium staining of mature 28S and 18S rRNAs. The bottom panel shows an autoradiograph of the same gel after transfer to zeta-probe membrane and probing for *7SL* RNA. (C) Graphic representation of the 47S primary rRNA transcript showing mature rRNA sequence (dark gray) and transcribed spacers (light gray) (not to scale). Small arrows indicate the majority of endonucleolytic cleavages that occur to produce the mature 18S, 5.8S, and 18S rRNAs. (D) UPR-induced downregulation of pre-rRNA. Cellular RNA was isolated from MEFs were either untreated (NT) or treated with Tg (200 nM), Tm (10 μg/ml), or ANS (10 μM). At the indicated time points, total RNA was analyzed by RNase protection using probes against *Xbp1*, pre-rRNA, and *7SL*. U and S indicate unspliced and spliced Xbp1 protected fragments, respectively. (E) Quantitation of pre-rRNA relative to *7SL* during Tg, Tm, or ANS treatment as shown in panel D. The level of each transcript at 0 h was given the value of 1, and each point represents the mean ± standard deviation of a minimum of three independent experiments. (F) Autoradiograph of labeled transcripts elongated in vitro from nuclei isolated from NIH 3T3 cells treated with Tg (200 nM) for the indicated times. Labeled transcripts were hybridized to DNA fragments corresponding to *BiP* mRNA, *7SL* RNA, rRNA, and pUC18. No hybridization to pUC18 DNA was observed, indicating that there was little to no random interaction of labeled transcript to DNA on the membrane. (G) Measurement of labeled rRNA transcripts relative to *7SL* as shown in panel F. The level of the pre-rRNA at 0 h was given the value of 1, and each bar represents the mean ± standard deviation of two independent experiments.

eliminates the majority of stable steady-state rRNA in the cytoplasm. We isolated nuclear RNA from an equal number of cells upon treatment without or with Tg, an inhibitor of the ER calcium ATPase that perturbs ER protein folding and induces the UPR. We found a striking decrease in the amount of RNA isolated from Tg-treated nuclei compared to untreated nuclei (Fig. 1A). When the nuclear RNA was run on a Northern gel and stained with ethidium, the 18S and 28S rRNA decreased within the first hour of Tg treatment and was barely visible after 3 h (Fig. 1B, lanes 5 to 7). When we probed Northern blots from the same gel for *7SL* RNA, an RNA Pol III transcript that is unchanged during the UPR, we found that its level remained constant, suggesting that not all transcripts are decreased in nuclear RNA extracts. In addition, quantitative PCR analysis of *BiP* mRNA revealed that the level of this UPR target gene was increased, as expected, in nuclear RNA from Tg-treated cells compared to untreated cells while the level of β-*Actin* mRNA did not significantly change during either treatment (unpublished data). Together, these results indicate that activation of the UPR specifically decreased the level of rRNA in the nucleus, which likely occurred by either decreasing its synthesis or promoting its degradation.

To determine whether the UPR pathway regulates rRNA synthesis, we measured the level of newly synthesized rRNA in total RNA isolated from MEFs during a UPR time course. Here, we chose to follow the disappearance of the rRNA primary transcript (pre-rRNA) to further substantiate our findings. Pre-rRNA is synthesized as a long 47S RNA which undergoes a number of cleavages and modifications before assembly into ribosomes (Fig. 1C). The nascent rRNA undergoes processing which begins with an endonucleolytic cleavage in the 5′ external transcribed spacer at the +650-nucleotide (nt) position (44). This processing of the 5′ external transcribed spacer occurs so rapidly that the vast majority of precursor rRNA in the nucleolus has already been cleaved; thus, the level of uncleaved pre-rRNA closely approximates that of

the most newly synthesized rRNA (44). We designed a probe for an RPA that encompasses this initial cleavage site such that the uncleaved pre-rRNA generates a protected fragment that is 232 nt, whereas the possible postcleavage protected fragments are half the size and migrate much faster during gel electrophoresis (see Fig. S1A in the supplemental material). We found that nascent pre-rRNA decreased rapidly within the first hour of Tg treatment and decreased by nearly threefold after a 2-h Tg treatment relative to *7SL* RNA (Fig. 1D, lanes 3 to 5, and E). The result of this RPA was confirmed by Northern blotting for full-length pre-rRNA (see Fig. S2 in the supplemental material) and was linear over a fourfold range of input RNA (see Fig. S1B in the supplemental material). The extent of UPR induction was measured by an RPA probe detecting both the spliced and unspliced forms of *Xbp1* mRNA (see Fig S1A in the supplemental material). The unconventional splicing of the UPR transcription factor *Xbp1* is mediated by the ER-resident transmembrane UPR component IRE1 (14, 45, 51, 56, 57). Splicing of the UPR intron results in a frameshift that is crucial for the formation of a fully active XBP1 transcription factor and upregulation of UPR target genes (57). *Xbp1* splicing is a hallmark of UPR activation and was rapidly induced by Tg treatment (Fig. 1D, lanes 3 to 5). The decrease of pre-rRNA occurred with similar kinetics to the appearance of the spliced form of *Xbp1* mRNA, suggesting that downregulation of rRNA occurs at an early point upon UPR induction (Fig. 1D, lanes 3 to 5).

The observed decrease of pre-rRNA also occurred when MEFs were treated with agents that induce UPR by a different mechanism, such as Tm, which perturbs ER protein folding by inhibiting glycosylation rather than releasing ER calcium (Fig. 1D, lanes 6 to 8, and E). The extent of rRNA downregulation during UPR was significant as it was comparable to treatment of MEFs with ANS, a ribotoxic drug that is a well-characterized inhibitor of rRNA transcription (Fig. 1D, lanes 9 to 11, and E). ANS treatment does not induce UPR, as indicated by the lack of *Xbp1* splicing compared to untreated cells (Fig. 1D, compare lanes 1 and 2 with 9 to 11). These data suggest that loss of 28S and 18S processed forms of rRNA in nuclear RNA pools (Fig. 1B) was likely the result of a decrease in pre-rRNA available for processing in the nucleus. Furthermore, after examining the levels of the most nascent rRNA, we reasoned that the decrease in pre-rRNA observed in Fig. 1D was likely caused by a reduction of transcription.

In order to further investigate the effect of UPR on rRNA gene transcription, we performed a nuclear run-on assay to measure the transcriptional activity of the rRNA genes. In this assay, engaged RNA Pols continue transcription of nascent RNAs in nuclei isolated from UPR-induced cells in the presence of radiolabeled UTP. The level of labeled RNA was measured by hybridization to DNA fragments complementary to rRNA, the UPR target gene *BiP*, and *7SL* RNA (Fig. 1F). Nuclei isolated from untreated cells generated significant levels of rRNA, consistent with robust transcriptional activity of RNA Pol I. In contrast, the level of radiolabeled rRNA generated from nuclei isolated from cells treated with Tg for 1 h was significantly reduced, correlating with the reduction of pre-rRNA detected by RPA (Fig. 1E and G). Production of *7SL* RNA by Pol III in the run-on assay was not reduced during the UPR. In addition, we did not detect a significant decrease in transcription of a number of additional housekeeping genes by Pol II and Pol III during Tg treatment, suggesting that Pol I is specifically regulated during the UPR (see Fig. S3 in the supplemental material).

During the UPR, the decrease in rRNA transcription was accompanied by an increase in *BiP* transcription. BiP is an ER-resident chaperone and is a well-characterized transcriptional target of the UPR. Typically, an increase in BiP mRNA becomes detectable by Northern gels at a minimum of 2 h after Tg treatment (unpublished data). Thus, the ability to detect an increase in *BiP* mRNA and a decrease in pre-rRNA within 30 min of Tg treatment demonstrated the sensitivity of our assay. Taken together, these results suggest that activation of the UPR pathway leads to a decrease in rRNA transcription. Since most transcriptional changes associated with UPR reported to date have been those which increase, our observation that rRNA transcription decreases would be one of the few exceptions. Furthermore, the abundance of rRNA relative to all other transcripts (80 to 90%) suggests that a reduction in rRNA transcription leading to a nearly threefold decrease in pre-rRNA may have a significant impact on the production of ribosomes and, hence, the protein synthesis capacity of the cell.

The PERK signaling branch regulates rRNA synthesis during UPR. To investigate how the ER transduces the signal to downregulate rRNA transcription in the nucleolus, we analyzed the level of pre-rRNA in *Ire1* and *Perk* knockout MEFs upon UPR treatment. First we followed the level of pre-rRNA in wild-type and $Ire1^{-/-}$ MEFs upon Tg or Tm treatment and found that rRNA was downregulated equally well in both wild-type and knockout MEFs (Fig. 2A and B). While $Ire^{-/-}$ MEFs were unable to splice *Xbp1* RNA, activation of UPR was evident from the increase in the level of unspliced *Xbp1* transcript itself (Fig. 2A, lanes 7 to 12), presumably mediated by activation of the ATF6 branch of the UPR (57). These data suggest that the IRE1 signaling pathway is not required for rRNA downregulation during the UPR.

We then tested the involvement of the PERK pathway in regulating rRNA transcription. Upon treatment of wild-type MEFs ($Perk^{+/+}$) with Tg, we found that the level of pre-rRNA decreased nearly threefold within 2 h and remained repressed over the following 12 h (Fig. 2C, lanes 4 to 8, and D). In contrast, the level of pre-rRNA remained unchanged in $Perk^{-/-}$ MEFs treated with Tg for the first 4 h (Fig. 2C, lanes 12 to 14, and E). Ultimately, after 12 h of Tg treatment, the level of pre-rRNA was reduced to the same extent as in wild-type MEFs treated for 1 to 2 h (Fig. 2C, compare lane 16 with lane 5). The decrease in pre-rRNA at later times was not due to a loss in viability, as >95% of all cells excluded the vital dye, trypan blue, over the entire 12-h time course (unpublished data). These results suggest that PERK is the major regulator of rRNA transcription during the first 4 h of UPR activation, resulting in a very rapid decrease in rRNA transcription that is sustained over time. Interestingly, the PERK-independent decrease of pre-rRNA in $Perk^{-/-}$ MEFs correlated with an increase in eIF2α phosphorylation between 8 and 12 h (see Fig. S4A in the supplemental material). The phosphorylation of eIF2α that occurs late in the UPR time course is presumably mediated by another eIF2α kinase, GCN2 (25).

Simultaneous downregulation of translation and pre-rRNA is independent of mTOR pathway. UPR activation resulted in

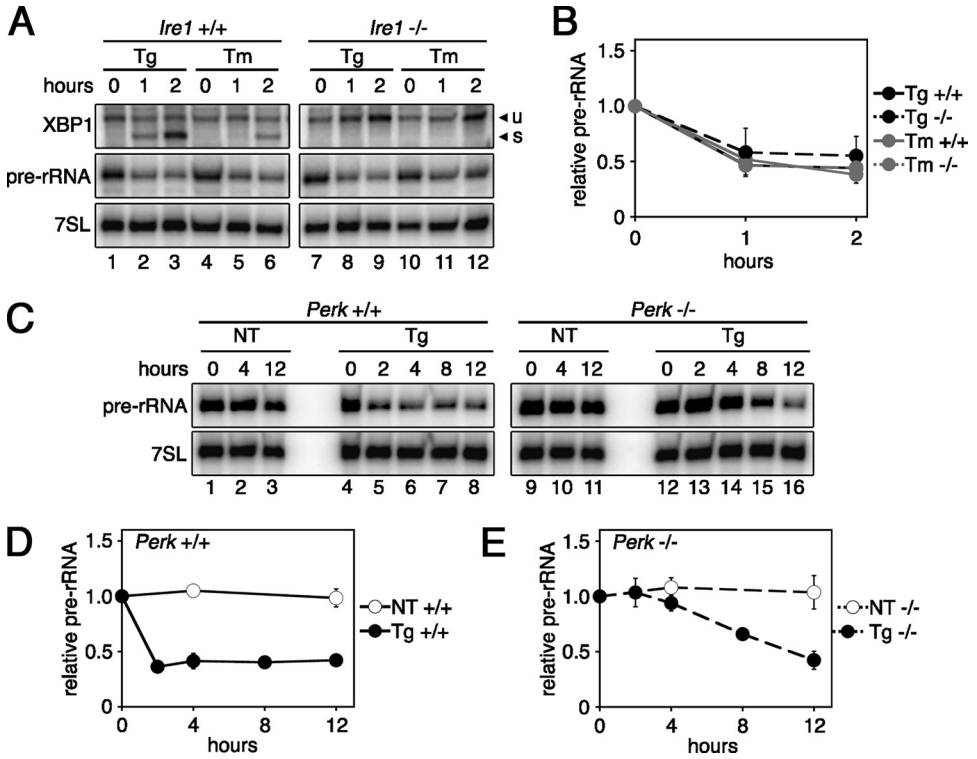

FIG. 2. PERK downregulates rRNA during the UPR. (A) IRE1 is not involved in rRNA regulation. RPA probing for pre-rRNA, *Xbp1* mRNA, and *7SL* in total RNA isolated from *Ire1*$^{+/+}$ and *Ire1*$^{-/-}$ MEFs treated with Tg (200 nM) or Tm (10 μg/ml). U and S indicate unspliced and spliced Xbp1-protected fragments, respectively. (B) Level of pre-rRNA relative to *7SL* RNA in *Ire1*$^{+/+}$ MEFs (solid lines) and *Ire1*$^{-/-}$ MEFs (dashed lines). Cells were treated with Tg (200 nM) and Tm (10 μg/ml) as shown in panel A. The level of each transcript at 0 h was given the value of 1, and each point represents the mean ± standard deviation of a minimum of three independent experiments. (C) RPA probing for pre-rRNA, *Xbp1*, and *7SL* in total RNA isolated from *Perk*$^{+/+}$ and *Perk*$^{-/-}$ MEFs treated with Tg (200 nM) or dimethyl sulfoxide (NT; 0.1%) over a 12-h time course. (D and E) Quantitation of pre-rRNA relative to *7SL* in *Perk*$^{+/+}$ and *Perk*$^{-/-}$ MEFs. Cells were treated with Tg (200 nM) or vehicle (0.1% dimethyl sulfoxide [NT]) as shown in panel C. The level of each transcript at 0 h was given the value of 1, and each point represents the mean ± standard deviation of a minimum of three independent experiments.

a rapid repression of translation through PERK-mediated eIF2α phosphorylation; thus, it is possible that reduced synthesis of components necessary for ribosome biogenesis are feeding back on rRNA transcription. We therefore measured the kinetics of pre-rRNA decrease and translation inhibition upon treatment with UPR inducers Tg and Tm. To measure global translation, we briefly pulsed cells with [^{35}S]methionine-cysteine in order to label nascent proteins at the times indicated in the figures. In this assay, the level of ^{35}S incorporation in whole-cell extracts is used as a measure of global protein synthesis. Based on our previous kinetic studies of UPR activation, we know that PERK is activated within 15 min of Tg treatment and 60 min of Tm treatment (18). We found that the level of cellular translation was decreased rapidly as incorporation of [^{35}S] methionine-cysteine decreased by 20% within 15 min of Tg treatment and by 50% within 60 min of Tm treatment (Fig. 3). For each sample, we also examined pre-rRNA levels by RPA and found that the reduction in pre-rRNA occurred at a similar rate as translation inhibition (Fig. 3A, lanes 2 and 9). In some samples, the decrease in pre-rRNA was detected prior to the decrease in ^{35}S incorporation. Thus, the similarity of the kinetic decrease of both pre-rRNA and translation is consistent with the idea that rRNA transcriptional repression is a primary response to UPR induction rather than the result of reduced translation of necessary components.

Coordinate regulation of rRNA transcription and cellular translation has been reported to take place via the mTOR signaling pathway. Inhibition of mTOR activity by Rap downregulates protein synthesis by inhibiting phosphorylation on ribosomal protein S6 kinase (7, 12, 47), and 4E-BP (3, 8). The mTOR signaling pathway also affects rRNA transcription initiation by a mechanism involving S6K (26, 41). To test for possible involvement of the mTOR pathway in UPR-induced rRNA transcriptional repression, we followed the phosphorylation of ribosomal protein S6 by S6K. Rap treatment resulted in a mild decrease in cellular translation (45%) while the levels of phospho-S6 and total S6 were significantly decreased, as expected (Fig. 3A, Rap). In contrast, we found that phosphorylation of S6 was unchanged upon Tg and Tm treatment. In addition, we found no change in the phosphorylation of 4E-BP upon Tg or Tm treatment (unpublished data), suggesting that activity of mTOR and its downstream substrates 4E-BP and S6K are unchanged during UPR. Together, these results suggest that the canonical substrates of the mTOR pathway are not involved in downregulating rRNA transcription during the

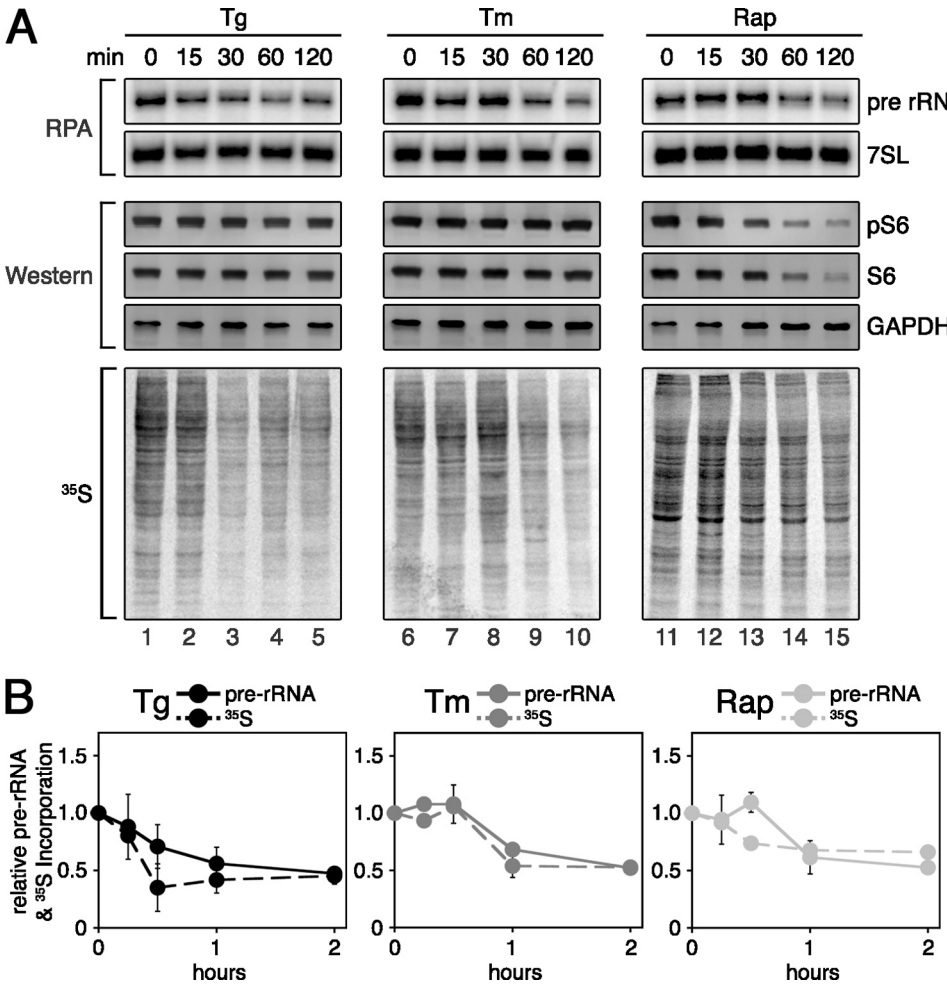

FIG. 3. rRNA downregulation and translation inhibition occurs simultaneously and is independent of mTOR signaling. (A) Wild-type MEFs were treated with Tg (200 nM), Tm (10 μg/ml), or Rap (20 nM). Total RNA was analyzed by RPA against pre-rRNA and *7SL* (top); levels of phospho-S6 (pS6), total S6, and GAPDH were analyzed by Western blotting (middle); and translation inhibition was analyzed by autoradiography of ^{35}S pulse-labeled whole-cell extracts subject to SDS-PAGE (bottom). Ten micrograms of total protein was loaded into each lane. (B) Graphic representation of pre-rRNA relative to *7SL* compared to relative ^{35}S incorporation as shown in panel A. The level at 0 h was given the value of 1, and each point represents the mean ± standard deviation of a minimum of three independent experiments.

UPR and that there may be a novel signaling pathway from the ER to the nucleolus.

Phosphorylation of eIF2α is necessary for rRNA downregulation. Since mTOR is likely not involved, what is the mechanism of rRNA transcription repression during ER stress? The most-well-characterized substrate of the PERK kinase is eIF2α, which functions as a regulatory subunit of the eIF2 complex. In order to address whether eIF2α phosphorylation is involved in rRNA regulation during the UPR, we measured pre-rRNA levels in MEFs carrying a homozygous serine-to-alanine mutation at the conserved eIF2α phosphorylation site, serine 51 (*eIF2*$\alpha^{A/A}$). It has been shown that upon UPR induction, PERK is activated in *eIF2*$\alpha^{A/A}$ cells; however, translation attenuation does not occur (48). We found that both wild-type (*eIF2*$\alpha^{S/S}$) and *eIF2*$\alpha^{A/A}$ MEFs induced a UPR, as indicated by *Xbp1* splicing during Tg or Tm treatment (Fig. 4A). In *eIF2*$\alpha^{S/S}$ MEFs, Tg or Tm treatment resulted in a rapid downregulation of pre-rRNA in coordination with translation inhibition (Fig. 4A, lanes 1 to 3 and 7 to 9, and B). In contrast, we found that *eIF2*$\alpha^{A/A}$ MEFs displayed impaired translation attenuation, as expected, and also failed to downregulate pre-rRNA in response to ER stress (Fig. 4A, lanes 4 to 6 and 10 to 12, and B). Upon treatment with the rRNA transcription inhibitor ANS, the level of pre-rRNA decreased regardless of eIF2α phosphorylation, indicating that the lack of rRNA regulation in *eIF2*$\alpha^{A/A}$ MEFs during Tg and Tm treatment is UPR specific (Fig. 4A, lanes 13 to 18, and B). In contrast to *Perk* knockouts, *eIF2*$\alpha^{A/A}$ MEFs failed to downregulate rRNA over the entire 12-h Tg time course (Fig. 2C, *Perk*$^{-/-}$; see also Fig. S4B, *eIF2*$\alpha^{A/A}$, in the supplemental material). These results indicate that phosphorylation of eIF2α is necessary for both the early and late phases of pre-rRNA downregulation during ER stress. It has been shown that the PERK-independent phosphorylation of eIF2α that occurs late after UPR induction is mediated by GCN2 (25), suggesting that perhaps multiple eIF2α kinases can regulate rRNA transcription. We found that stresses that are known to regulate rRNA synthesis through the mTOR pathway do not require eIF2α phosphorylation, as

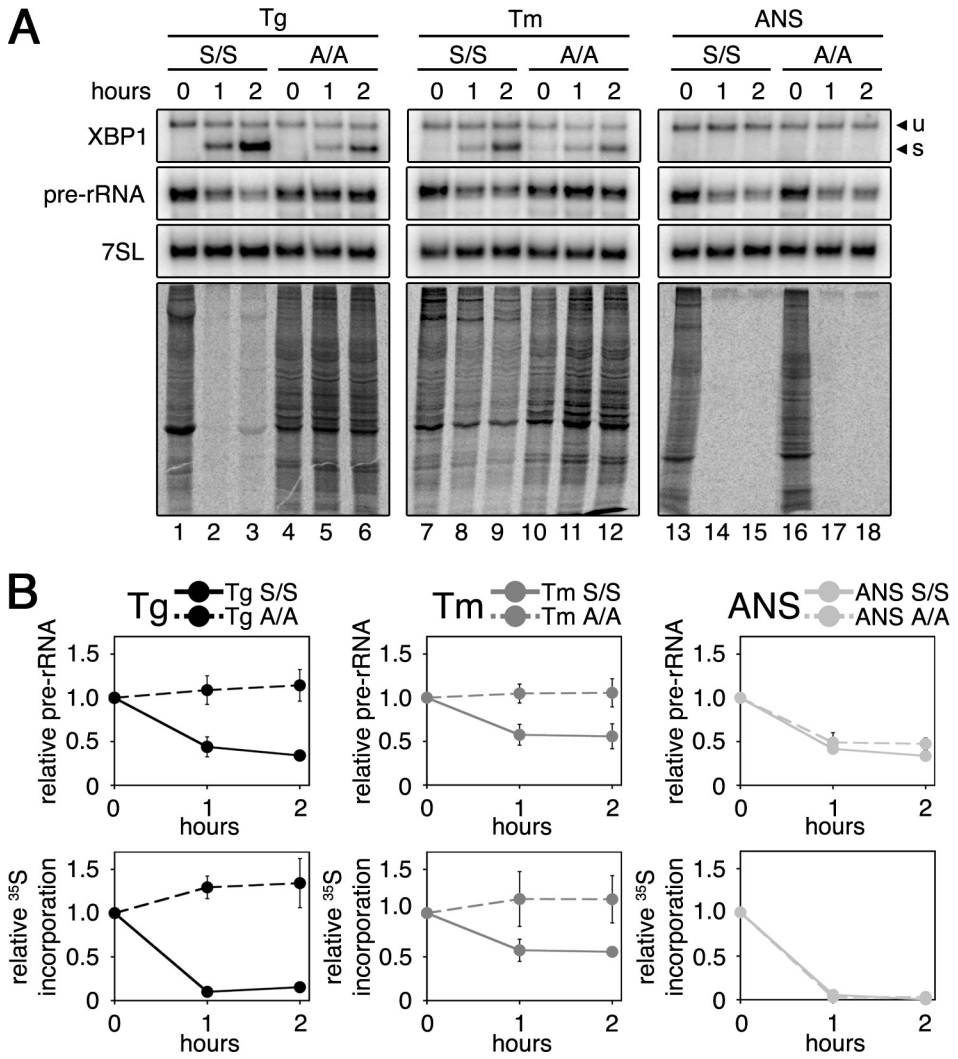

FIG. 4. eIF2α phosphorylation is necessary for rRNA downregulation. (A) Time course of *eIF2*$\alpha^{S/S}$ and *eIF2*$\alpha^{A/A}$ MEFs treated with Tg (200 nM), Tm (10 μg/ml), or ANS (10 μM). Total cellular RNA and protein were isolated at the indicated times. The top three panels are RPAs probing for *Xbp1*, pre-rRNA, and *7SL*. U and S indicate unspliced and spliced Xbp1-protected fragments, respectively. Bottom panels are autoradiographs of ^{35}S-labeled whole-cell extracts analyzed by SDS-PAGE. Ten micrograms of total protein was loaded into each lane. (B) Quantitation of pre-rRNA and ^{35}S incorporation in *eIF2*$\alpha^{S/S}$ (S/S) and *eIF2*$\alpha^{A/A}$ (A/A) MEFs as shown in panel A. Top panels are graphs of pre-rRNA levels relative to *7SL*, and bottom panels are graphs of relative ^{35}S incorporation. Levels at 0 h were given the value of 1, and each point represents the mean ± standard deviation of a minimum of three independent experiments.

Rap treatment was still capable of downregulating pre-rRNA in *eIF2*$\alpha^{A/A}$ MEFs (see Fig. S4C, *eIF2*$\alpha^{A/A}$, in the supplemental material). Together, these results imply that eIF2α phosphorylation-induced translation repression and rRNA transcription inhibition are intimately coregulated during ER stress, suggesting a unique regulatory mechanism that is likely independent of mTOR.

PERK is required for disruption of the rRNA preinitiation complex. As a translation initiation factor, the vast majority of eIF2α is located in the cytoplasm while the rRNA transcriptional machinery is in the nucleolus. So what is the mechanism of eIF2α phosphorylation-dependent rRNA transcription inhibition? To explore how eIF2α phosphorylation downregulates rRNA transcription, we monitored the formation of the preinitiation complex at the rRNA promoter. The major proteins involved in rRNA transcription initiation identified to date are the DNA-binding protein UBF and selectivity factor (SL1) and the non-DNA binding protein RRN3/TIF-IA (Fig. 5A). In order to measure promoter occupancy of preinitiation complex components, we employed a ChIP assay. In this assay *Perk*$^{+/+}$ or *Perk*$^{-/-}$ MEFs were treated with Tg for the times indicated in the figure, and cross-linked chromatin was immunoprecipitated with antibodies against the large subunit of Pol I, RRN3/TIF-IA, and UBF. In addition, we treated cells with ANS as it is known to disrupt the interaction of Pol I and RRN3/TIF-IA with the rRNA promoter, resulting in an inhibition of transcription initiation (40). We found that association of promoter DNA with both Pol I and RRN3/TIF-IA was decreased upon treatment with ANS, indicating that our assay is functioning as expected (Fig. 5B and C). Upon Tg treatment of wild-type MEFs, we found a decrease in the association of promoter DNA with both Pol I and RRN3/TIF-IA, suggesting

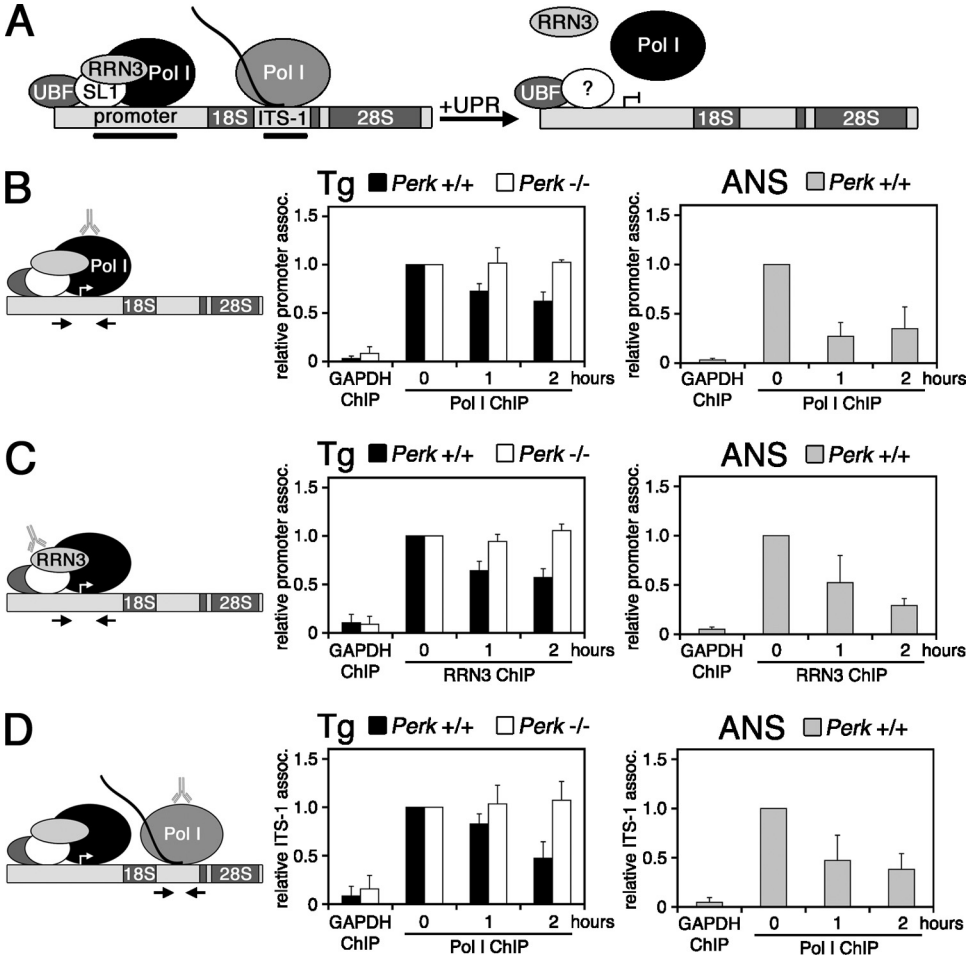

FIG. 5. Disruption of rRNA preinitiation complex is PERK dependent. (A) The right panel depicts a cartoon of the rRNA gene during normal conditions (not to scale). Major rRNA transcription factors UBF, SL1, and RRN3/TIF-IA are shown associated with Pol I in the preinitiation complex on the rRNA promoter, and elongating Pol I is shown associated with ITS-1 DNA. The promoter region and ITS-1 DNA assayed in the following ChIP experiments are indicated by black underlining. The left panel depicts the dissociation of RRN3/TIF-IA and Pol I from the rRNA promoter and ITS-1 after UPR activation. (B) Quantitation of rRNA promoter DNA amplified from ChIP samples using antibody against the large subunit of Pol I (RPA194). *Perk*$^{+/+}$ MEFs were treated with Tg (200 nM) or ANS (10 μM). *Perk*$^{-/-}$ MEFs were treated with Tg (200 nM). Control ChIP was performed with samples from wild-type MEFs using antibodies against GAPDH. Each bar represents the mean and standard deviation of three independent experiments. Promoter DNA amplified from untreated cells was given the value of 1. (C) The same experiment as in panel B except that ChIP was performed with antibodies against RRN3/TIF-IA. (D) The same experiment as in panel B except that PCR was performed against ITS-1 DNA. assoc, association.

that the rRNA preinitiation complex is disrupted during UPR activation (Fig. 5B and C). The decrease in Pol I and RRN3/TIF-IA promoter occupancy was PERK dependent as there was no change in *Perk*$^{-/-}$ MEFs (Fig. 5B and C). We did not detect any change in the promoter occupancy of UBF after Tg treatment in either cell line (see Fig. S5A in the supplemental material). We were unable to determine the promoter occupancy of SL1 because antibodies against subunits of SL1 were not suitable for ChIP assays. Our assay was specific to the targeted proteins, as precipitation with a control antibody against GAPDH did not pull down significant quantities of rRNA promoter DNA (Fig. 5B to D). In addition to the preinitiation complex, we monitored the level of elongating Pol I by its association with the ITS-1 of the rRNA gene and found that there is a PERK-dependent decrease in the association of elongating Pol I with ITS-1 DNA upon Tg treatment (Fig. 5D). The same reaction was performed with antibodies against RRN3/TIF-IA, which did not significantly pull down ITS-1 DNA as expected as RRN3/TIF-IA is not known to travel with the elongating Pol (see Fig. S5B in the supplemental material). The PERK dependence of pre-rRNA downregulation and disruption of the preinitiation complex was specific to the UPR. Treatment of *Perk*$^{-/-}$ MEFs with ANS reduced pre-rRNA to the same extent as wild-type cells (see Fig. S5C and D in the supplemental material). Furthermore, a ChIP assay of *Perk*$^{+/+}$ and *Perk*$^{-/-}$ MEFs treated with hydrogen peroxide (H_2O_2), a known rRNA preinitiation complex disrupter, showed similar levels of Pol I and RRN3/TIF-IA promoter dissociation (see Fig. S5E and F in the supplemental material). Together, these results suggest that PERK activation decreases the promoter occupancy of both RRN3/TIF-IA and Pol I, leading to a decrease in the number of elongating

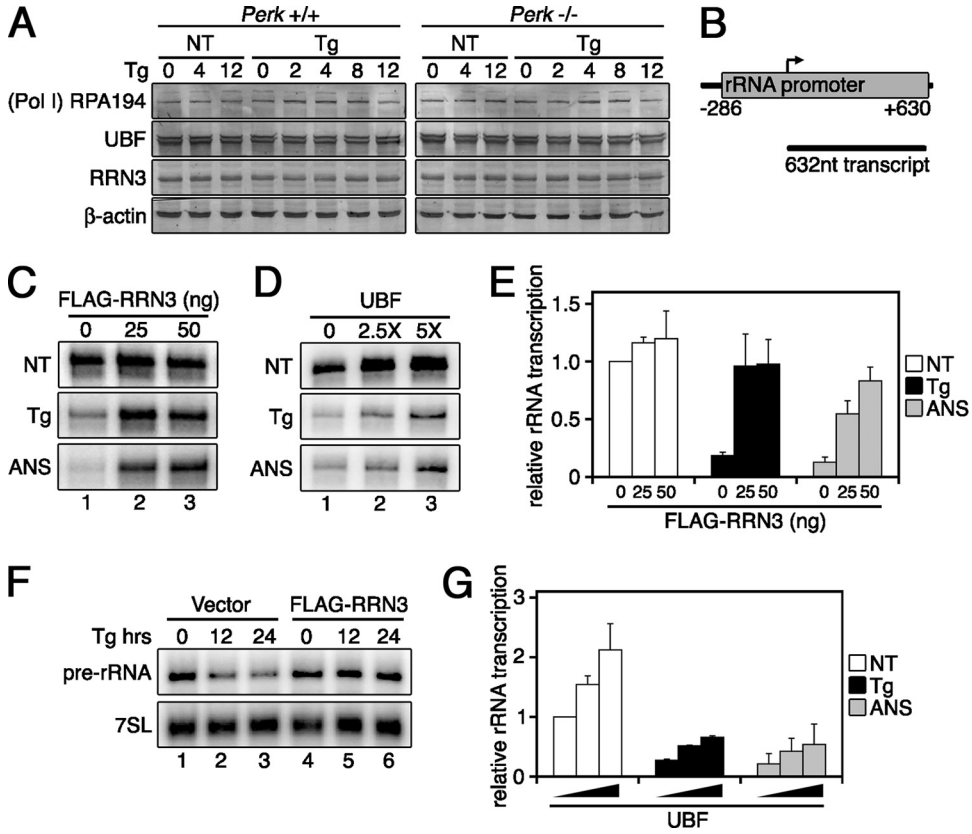

FIG. 6. RRN3/TIF-IA is inactivated during the UPR. (A) Western blots from *Perk*$^{+/+}$ and *Perk*$^{-/-}$ MEFs treated with Tg (200 nM) or dimethyl sulfoxide (NT; 0.1%) over a 12-h time course. Protein samples were run on denaturing polyacrylamide gels and probed with antibodies against RPA194 (Pol I large subunit), both isoforms of UBF, RRN3/TIF-IA, and β-actin. Note that the UBF antibody recognizes both isoforms of UBF protein. (B) Depiction of the rRNA transcription template (gray box) and the 632-nt transcript that results from correct initiation. The correct site of transcription initiation is indicated by an arrow. (C) Autoradiograph of 632-nt in vitro transcribed product resulting from transcription reactions using 25 μg of nuclear extracts and 10 ng of rDNA template in the presence of [α-^{32}P]UTP. Purified RRN3/TIF-IA protein from untreated cells was added to reaction mixtures at the indicated concentrations (lanes 2 and 3). Nuclear extracts were isolated from MEFs subjected to 2 h of Tg (200 nM) or ANS (10 μM) treatment and compared to untreated (NT) cells. (D) The same experiment as in panel C except that partially purified UBF protein was added to the reaction mixture in a concentration of 2.5- and 5-fold excess (2.5× and 5×, respectively) of the endogenous level of UBF (lanes 2 and 3). (E) Quantitation of transcription products as shown in panel C relative to the level of transcript produced by untreated extracts in the absence of exogenous RRN3/TIF-IA. Each bar represents the mean and standard deviation of three independent experiments. (F) HeLa cells were transfected for 24 h with empty vector or FLAG-RRN3 before treatment with Tm (0.5 μg/ml). Total cellular RNA was collected and analyzed by RPA against pre-rRNA and *7SL*. (G) Quantitation of transcription products as shown in panel D relative to the level of transcript produced by untreated extracts in the absence of exogenous UBF. Each bar represents the mean and standard deviation of three independent experiments.

transcription complexes on the rRNA gene. This correlates with results shown in Fig. 1, where the overall level of pre-rRNA and the synthesis of rRNA from isolated nuclei are decreased upon UPR induction.

RRN3/TIF-IA is inactivated during UPR to reduce rRNA transcription. Since PERK is responsible for translation repression, a potential mechanism of the decrease in Pol I and RRN3/TIF-IA association with the rRNA promoter is a reduction in their cellular concentrations. Although antibodies to the TAF_I proteins of the SL1 complex were unsuitable for Western blotting, we found that the levels of the large Pol I subunit (RPA194), both isoforms of UBF, and RRN3/TIF-IA remain constant during UPR in both wild-type and *Perk*$^{-/-}$ MEFs, suggesting that translation inhibition is not leading to depletion of these essential rRNA transcription factors (Fig. 6A). Thus, the decrease in transcription factor association with the rRNA promoter may be a result of a decrease in their activity.

To identify the downstream effectors of PERK-mediated regulation of rRNA synthesis, we assayed Pol I transcription activity in nuclear extracts from untreated and Tg- and ANS-treated MEFs in vitro. In this assay, nuclear extracts were allowed to form preinitiation complexes on an rDNA template, and transcription was allowed to proceed after addition of radiolabeled nucleotides. The rDNA template contains the rat rRNA promoter from −286 to +630 and, when linearized with EcoRI, produces a specific 632-nt transcript (Fig. 6B). The level of transcript produced in vitro correlates with the transcriptional activity of Pol I in vivo. In our assay we found that untreated extracts robustly transcribe from the rDNA template (Fig. 6C and D, lanes 1), correlating with the high level of Pol I transcription from isolated nuclei before UPR induction (Fig.

1F). In contrast, we found that the Pol I transcriptional activity is reduced by 5- to 10-fold in nuclear extracts from Tg- or ANS-treated cells (Fig. 6C and D, lanes 1). The extent of reduction in Pol I transcription in Tg-treated extracts was comparable to extracts isolated from cells treated with ANS. To determine which factors may be inactivated during the UPR, we performed an in vitro transcription complementation assay using purified transcription factors. Similar experiments have been used to identify RRN3/TIF-IA as the target of inactivation during ANS treatment (40). Upon addition of FLAG-RRN3 affinity purified from untreated MEFs to Tg extracts, Pol I transcription was restored, suggesting that RRN3/TIF-IA is inactivated during the UPR (Fig. 6C, lanes 2 and 3). Again, this was comparable to the complementation observed by adding FLAG-RRN3 to ANS-treated extracts. Our affinity-purified FLAG-RRN3 protein was assayed by Coomassie staining and Western blotting, and no visible contamination by other proteins or rRNA transcription factors was detected (see Fig. S6A and B in the supplemental material). We found that addition of 25 to 50 ng of FLAG-RRN3 protein is sufficient to fully complement the level of rRNA transcript produced in Tg extracts compared to untreated extracts (Fig. 6E). Thus, these results pointed to RRN3/TIF-IA as a major target of UPR regulation. If this were the case, we reasoned that ectopic expression of RRN3/TIF-IA might prevent rRNA transcription repression during UPR. In fact, we found that this was the case. Overexpression of FLAG-RRN3 in HeLa cells significantly delayed the decrease in pre-rRNA observed upon Tg treatment compared to mock-transfected cells (Fig. 6F). For the first 24 h of Tg treatment, pre-rRNA levels do not decrease in FLAG-RRN3-transfected cells; however, after 48 h the levels decrease to the same extent as mock-transfected cells (unpublished data). One possible explanation for these results is that the high level of FLAG-RRN3 initially overwhelms the pathways necessary for downregulation of RRN3 during the UPR. Together, these results are consistent with the idea that RRN3/TIF-IA activity is decreased during the UPR, leading to a downregulation of rRNA transcription.

In order to determine if other rRNA transcription factors are involved in the regulation of UPR-induced transcription repression, we performed the same in vitro transcription complementation assay with UBF protein partially purified from Novikoff hepatoma cells (Fig. 6D). The concentration of UBF protein added to the reaction mixture was estimated by Western blotting to be 2.5- and 5-fold over the level of endogenous UBF (see Fig. S6C in the supplemental material). When partially purified UBF was added to untreated extracts, the transcriptional activity was increased by 2.5-fold and did not saturate even after a 10-fold excess of UBF protein was added to the reaction (Fig. 6D and G; also unpublished data). When partially purified UBF protein was added to Tg or ANS extracts, we observed a proportional increase in transcriptional activity with respect to the untreated extracts (Fig. 6G). This is in contrast to the effect of adding FLAG-RRN3 to Tg- and ANS-treated extracts, where activity increased to the same level as in untreated extracts (Fig. 6C and D, compare lanes 3). Together, these results suggest that while other transcription factors exert influence on rRNA transcription, RRN3/TIF-IA is likely the major regulator of rRNA synthesis repression during UPR.

DISCUSSION

All organisms depend on their ability to maintain cellular homeostasis in order to ensure their survival. As such, it is essential that organisms adjust their metabolic states according to external conditions. For example, when growing cells are deprived of nutrients, they downregulate their translational capacity by inhibiting translation initiation and ribosome biogenesis (24, 29, 39). While downregulation of these processes may come at the cost of cellular growth, given the amount of energy and resources they consume, it is likely to be highly beneficial when nutrients are limiting. Conversely, when ample nutrients are available, cells will increase their rates of ribosome biogenesis and protein synthesis, promoting growth. In either case, the mTOR pathway predominantly controls global changes of translational capacity in response to nutrients or growth factor signaling.

In this report we have identified an alternate pathway for coordinately regulating ribosome biogenesis with protein synthesis (Fig. 7). The downregulation of rRNA that we see upon ER stress appears to occur independently of the mTOR pathway. We have demonstrated that during ER stress, PERK signaling is necessary for downregulating rRNA synthesis in addition to its well-documented effects on inhibiting global translation initiation. When PERK is activated by ER stress, it phosphorylates eIF2α, preventing the formation of active ternary complexes and thereby inhibiting initiation of translation (28, 50). Under these conditions, we found that the rRNA preinitiation complex is disrupted by a PERK-dependent dissociation of Pol I and the basal factor RRN3/TIF-IA from the rRNA promoter. In addition, we found that the decrease of pre-rRNA occurs with the same kinetics as translation inhibition and that phosphorylation of eIF2α is required for rRNA downregulation during ER stress. Synthesis of rRNA comprises the majority of transcriptional activity in growing cells, with rRNA constituting 80 to 90% of all RNA within the cell. Thus, changes in rRNA transcription are likely to have significant consequences, particularly during stress. Mutation of the conserved eIF2α phosphorylation site rendered cells incapable of responding to ER stress, suggesting that phosphorylation of eIF2α is a key molecular event in regulating rRNA transcription. This is the first time that phosphorylation of eIF2α has been implicated in the regulation of rRNA synthesis.

To date, four eIF2α kinases have been identified in mammals (PKR, HRI, PERK, and GCN2), and each is activated by a distinct set of stresses. Our finding that rRNA downregulation during ER stress requires eIF2α phosphorylation raises the question of whether all eIF2α kinases utilize this pathway to regulate rRNA transcription. While an eIF2α-dependent rRNA transcription inhibition has never been reported, there is evidence in the literature that stresses leading to eIF2α phosphorylation also downregulate rRNA transcription. We found that for each stress where eIF2α phosphorylation is known to occur, there was at least one independent study that found that rRNA transcription was inhibited under the same conditions (Table 1). This is consistent with the idea that phosphorylation of eIF2α is associated with inhibition of rRNA transcription although none of the studies to date has reported the connection. If other eIF2α kinases signal through eIF2α phosphorylation to regulate rRNA transcription, this pathway

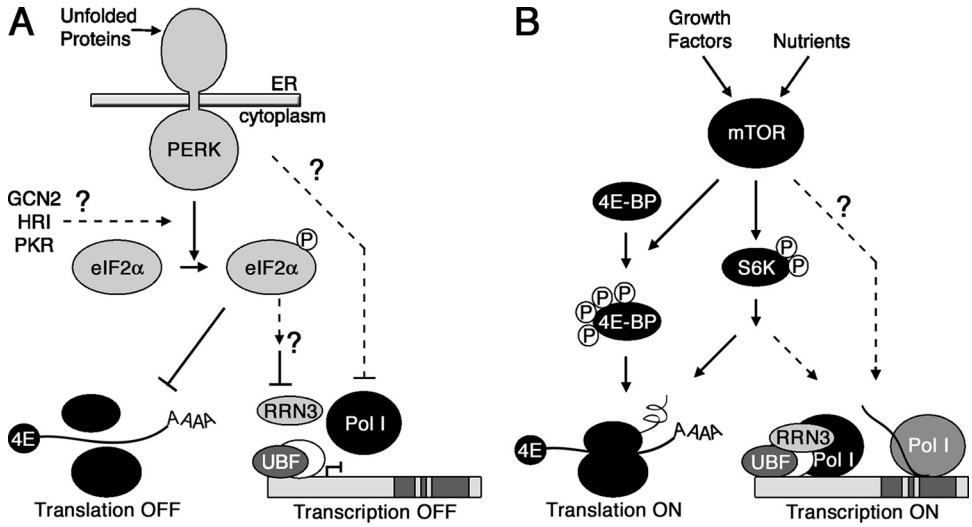

FIG. 7. Model of PERK pathway controlling rRNA transcription and translation in comparison to mTOR. (A) PERK pathway. Unfolded proteins in the ER lumen activate the PERK kinase. PERK phosphorylates eIF2α leading to inhibition of translation initiation and inhibition RRN3/TIF-IA activity, resulting in dissociation of RRN3/TIF-IA and Pol I from the preinitiation complex. Dashed lines indicate that direct interaction of components has not been demonstrated. (B) mTOR pathway. Under favorable conditions, signaling from growth factors and nutrients activates mTOR leading to phosphorylation of 4E-BP and S6K. Inhibition of 4E-BP and activation of S6K by mTOR phosphorylation cooperate to increase the efficiency of translation initiation. While the entire pathway has yet to be elucidated, mTOR and S6K activity lead to phosphorylation of rRNA transcription factors increasing transcription of rRNA.

may actually represent a major mode of regulating rRNA synthesis during stress in general.

Transcription of rDNA in the nucleolus requires at least three basal factors: RRN3/TIF-IA, UBF, and SL1. While all three of these transcription factors are highly regulated, RRN3/TIF-IA is the major target of environmental stresses such as nutrient deprivation, oxidative stress, and treatment with translation inhibitors (9, 30, 40, 41). Here, we found that during ER stress there is a PERK-dependent dissociation of RRN3/TIF-IA and Pol I from the rRNA promoter; however, the concentration of these proteins did not appear to change, suggesting that their activity was altered during the UPR. Additionally, we were able to restore transcriptional activity of UPR-treated nuclear extracts by the addition of active affinity-purified RRN3/TIF-IA but not with UBF, which is consistent with the idea that RRN3/TIF-IA is inactivated during UPR.

The major mechanism by which RRN3/TIF-IA activity is regulated is through phosphorylation. To date, at least eight phosphorylation sites on RRN3/TIF-IA have been identified, and we are now just beginning to understand how the pattern of RRN3/TIF-IA phosphorylation affects its activity (5, 16, 23, 40, 41, 46, 49). For example, inhibition of mTOR by Rap leads to inactivation of RRN3/TIF-IA through hypophosphorylation of serine 44 and hyperphosphorylation of serine 199 (41). Oxidative stress or treatment with the peptidyl transferase inhibitor ANS results in RRN3/TIF-IA inactivation through an inhibitory phosphorylation on threonine 200 catalyzed by JNK2 (40). It is not exactly clear why so many different phosphorylation sites regulate RRN3/TIF-IA activity, but perhaps it allows the rRNA transcription machinery to more tightly integrate signals coming from multiple pathways. Different patterns of phosphorylation may result in different levels of activity or may differentially alter cellular localization. In growing cells RRN3/TIF-IA localization is restricted to nucleoli; however, its localization has been reported to shift to the nucleoplasm during ANS treatment (40) or to the cytoplasm during Rap treatment (41). Perhaps this is an indication that RRN3/TIF-IA has additional functions during stress other than simply regulating Pol I transcription. Future work to determine the UPR-induced phosphorylation sites of RRN3/TIF-IA and identifying potential kinases or phosphatases that regulate RRN3/TIF-IA downstream of phosphorylated eIF2α will be one of the critical steps toward understanding the mechanistic details of how signals are transmitted from the ER to the nucleolus.

One of the major questions remaining to be answered is exactly how eIF2α phosphorylation in the cytoplasm is communicated with the nucleolus to regulate rRNA transcription. One interesting possibility is that the eIF2 complex itself directly communicates ER stress to the nucleolus. It is well established that the vast majority of eIF2 is localized in the

TABLE 1. Comparison of activation of eIF2α kinases and rRNA transcription during stress

Stress source or type	eIF2α kinase		rRNA transcription	
	Name	Reference	Increase or decrease[a]	Reference
Oxidative	HRI	38	↓	40
Amino acid deprivation	GCN2	58	↓	24
Heat shock	HRI	38	↓	21
Hypoxia	PERK	35	↓	42
UV irradiation	GCN2	34	↓	1
Viral infection	PKR	20	↑ or ↓	31
Rap	GCN2	36	↓	41
ANS	None reported		↓	40
UPR	PERK	28	↓	This study

[a] ↑, upregulation; ↓, downregulation.

cytoplasm; however, an increasing amount of evidence suggests that eIF2 is present in the nucleus and may be directed to the nucleolus upon eIF2α phosphorylation. A number of studies have described that a small proportion of the eIF2 complex is localized to the nucleus or nucleolus (6, 15, 22, 32, 37, 55). Specifically, two independent studies have indicated that during postischemic brain reperfusion, phosphorylated eIF2α accumulates in the nucleus and the nucleolus (15, 22). In another study, nearly all cytoplasmic eIF2α relocalized to discrete puncta within the nucleus that resemble nucleoli within 30 min of treatment with the CRM1 nuclear export inhibitor leptomycin B (6). While clear demonstration that these puncta are indeed nucleoli will be required, these results strongly suggest that the eIF2 complex rapidly shuttles between the cytoplasmic and nuclear compartments under normal conditions. In this regard, it would be interesting to examine if the shuttling of the eIF2 complex was altered in response to stresses that induce eIF2α phosphorylation and whether nuclear eIF2 plays a role in downregulating rRNA transcription during stress.

The UPR has provided a unique opportunity to uncover the effects of eIF2α phosphorylation on rRNA transcription as many of the stresses that activate eIF2α kinases also affect mTOR activity. While this may complicate study of the role of other eIF2α kinases in regulating rRNA gene transcription, the UPR has allowed us to begin dissecting the molecular details of how PERK-mediated eIF2α phosphorylation can regulate rRNA transcription in the nucleolus during ER stress. Further understanding of the regulatory mechanisms and ability to uncouple eIF2α phosphorylation and rRNA synthesis will be necessary to provide answers to such questions.

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J.B.D. and M.N. conceived and designed the experiments. J.B.D. performed the experiments. L.I.R. provided recombinant RRN3/TIF1A, UBF, and advice on the in vitro Pol I transcription reaction. D.S. and R.J.K. provided eIF2α$^{A/A}$ mutant MEFs. J.B.D. and M.N. analyzed the data and wrote the paper.

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