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Maintaining ATP levels via the suppression of PERK-mediated rRNA synthesis at ER stress

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ABSTRACT

Currently, [^{3}H]uridine is most often used to monitor rRNA synthesis in cultured cells. We show here that radiolabeled ribonucleoside triphosphates, such as [α-^{33}P]UTP, in culture medium were also incorporated efficiently not only into cells but also into *de novo* RNA, particularly rRNA. Using this method, we first revealed that endoplasmic reticulum (ER) stress inducers such as tunicamycin and thapsigargin suppressed *de novo* rRNA synthesis, and that PERK, but not IRE1α or ATF6, mediated the suppression. PERK is known to mediate the suppression of *de novo* protein synthesis via phosphorylation of eIF2α. Consistently, other translational inhibitors such as PSI, proteasomal inhibitor, and cycloheximide suppressed *de novo* rRNA synthesis. eIF2α knockdown also suppressed both *de novo* protein and rRNA syntheses. Furthermore, ER stress reduced cellular ATP levels, and the suppression of rRNA synthesis apparently mitigated their reduction. These observations provided a close link between ATP levels and suppression of *de novo* rRNA synthesis at ER stress, and we proposed a novel feedback mechanism, in which ATP levels were maintained via suppression of *de novo* rRNA synthesis in ATP-demanding stresses, such as ER stress.

Feedback mechanisms are the core machinery for organisms and cells to remain healthy in the face of changes in the environment. In cellular levels, unfolded protein response (UPR) in endoplasmic reticulum (ER) provides good examples of "feedback mechanisms" [1,2]. ER is an intracellular organelle, in which membrane and secreted proteins are properly folded and modified. When proteins that pass through the ER are not correctly modified or folded, such misfolded proteins are easily accumulated in the ER [3,4]. The accumulation of misfolded proteins triggers several cellular responses, including transcriptional induction of chaperons, stimulation of ER-associated degradation (ERAD) [5], and translational inhibition of mRNAs [6,7]. These responses are collectively called UPR, and all aim to reduce the amounts of accumulated misfolded proteins in the ER [2]. In general, translational inhibition apparently occurs at the earliest time point during UPR, followed by the induction of chaperons and stimulation of ERAD, and thus the translational inhibition, namely suppression of *de novo* protein synthesis, might be a primary demand for cells in handling ER stress.

In UPR, ATF6, IRE1α, and PERK have been shown to function as sensor proteins [6,8–10], which are able to recognize the accumulation of misfolded proteins in the ER, and after the recognition, these proteins trigger signaling cascades for the UPR. Among the three, PERK, a PKR-like kinase, is known to mediate translational inhibition [6]. When PERK is activated at ER stress, activated PERK mediates phosphorylation of Ser51 of the alpha subunit of eukaryotic translation initiation factor (eIF2α), which in turn leads to translational inhibition [6,7]. This provides an elegant feedback system, which prevents further supply of misfolded proteins in the ER. It is noteworthy that the Ser51 phosphorylation of eIF2α has also been observed in several other stress conditions, such as virus infection, oxidative stress, ultraviolet exposure, amino-acid starvation, etc. [11–14]. However, in some of these stress conditions, there seems to be no need of suppression of *de novo* protein synthesis as a feedback mechanism. Thus, yet unknown meaning of the suppression of *de novo* protein synthesis in these stress conditions, including ER stress, might exist.

ATP is necessary in essentially all cellular activities, and its needs increase in stress conditions such as ER stress, in which chaperons utilize ATP in unfolding [15], VCP in ERAD [16,17], proteasome in the degradation of misfolded proteins [18]. Ubiquitination also utilizes ATP for marking unnecessary proteins to be degraded [19]. ATP levels are maintained in the balance between its synthesis and consumption. Although basic mechanism for ATP production by F1F0 ATPase and its consumption by other ATPases are well documented, regulatory mechanisms in keeping ATP levels are only poorly understood [20]. It is noteworthy that large amounts of ATP are also consumed in RNA synthesis, especially in ribosomal RNA (rRNA) synthesis [21]. In this study, we report profound couplings among ATP-demanding conditions, translational

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inhibition, and suppression of *de novo* rRNA synthesis, and propose a novel feedback mechanism.

Materials and methods

Antibodies. Anti-CBP, anti-UBF, anti-TIF-IA, and anti-c-myc antibodies were purchased from Santa Cruz. Anti-eIF2α antibodies were purchased from Cell Signaling. An anti-actin antibody was purchased from Chemicon. An anti-ATF6 antibody was a gift from Dr. Kazutoshi Mori.

Cell Culture. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and penicillin and streptomycin. Indicated amounts of tunicamycin (Nacalai Tesque), Thapsigargin (Nacalai Tesque), PSI (Peptide Institute), Actinomycin D (Nacalai Tesque), and α-amanitin (Invitrogen) were added 2 h prior to the addition of [α-^{33}P]UTP to the medium.

Visualization of rRNA synthesis (VRS). When cells grew at 70–80% confluency, 20 μCi [α-^{33}P]UTP (3000 Ci/mmol) (GE Helthcare) was added to the culture medium. Total RNA was isolated by the acid guanidine phenol chloroform method using Trizol Reagent (Invitrogen). Five micrograms of total RNA was separated in 1% agarose gel, and then transferred onto a nylon membrane (Hybond-N+ membrane) (GE Healthcare). Dried membranes were subjected to autoradiography.

RNAi experiments. Sequences of siRNAs used in this study are listed in Supplementary Table 1. HeLa cells (3×10^5) were transfected with 25 nM siRNAs using Oligofectamine (Invitrogen), and were cultured for 3 days. Cells were then cultured for 2 h in the absence or presence of chemicals, followed by the additional 8 h culture with [α-^{33}P]UTP in the absence or presence of chemicals, respectively, and then total RNA was recovered and analyzed.

Measurement of de novo protein synthesis. HeLa cells (3×10^5) were cultured with methionine- and cysteine-free DMEM containing 10% FBS for 30 min. Then, 25 μCi [^{35}S]methionine/cysteine (37 TBq/mmol) (GE Healthcare) were added to the medium for 30 min. Then cells were washed with ice-cold PBS three times and lysed with RIPA buffer. After centrifugation at 15,000 rpm for 30 min at 4 °C, the supernatants were collected and their protein concentrations were determined. Ten micrograms of lysates was separated by SDS–PAGE, and the gel was fixed with methanol. Then the gel was dried and exposed onto an X-ray film (Fuji film). Alternatively, their radioactivity was determined with a liquid scintillation counter.

Measurement of ATP in cultured cells. ATP was measured by luciferase activities by ARVO multilabel counter (Wallac), using Cellno ATP assay reagent (Toyo B-net Co., Ltd.).

Establishment of HeLa cells expressing eIF2α. A full-length human eIF2α cDNA (No. NM_004094) was obtained from RIKEN BRC DNA BANK [22]. Using a lenti virus system, HeLa cells continuously expressing eIF2α were established. These cells, but not normal HeLa cells, allowed the eIF2α phosphorylation to be detected by Western blot.

Statistical analysis. Each experiment was conducted at least three times with consistent results. The representative gel or blot from each experiment is presented in this study. Mean values and standard deviations were obtained from triplicate experiments.

Results

Visualization of rRNA synthesis

It is generally thought that ribonucleoside triphosphates cannot pass through the plasma membrane due to their hydrophilic or water-soluble nature [23], and thus that it is impossible to radiolabel newly synthesized rRNA in cultured cells by adding radiolabeled ribonucleoside triphosphates, such as [α-^{33}P]UTP, to the culture medium. In contrast, [^{3}H]uridine, a more hydrophobic and smaller molecule than UTP, has been shown to pass through the plasma membrane, and thus it has been used to radiolabel the RNA in many experiments using cultured cells [24]. We cultured a variety of cell lines (HeLa, HEK293A, HEK293T, CV-1, COS1, COS7, PC12, etc.), added 20 μCi [α-^{33}P]UTP (3000 Ci/mmol) to 2 ml of culture medium, incubated for 8 h, harvested the cells, and recovered the RNA. Surprisingly, RNAs from all of the cells exhibited radioactivity. Among the cell lines, RNA from HeLa cells was the most highly radioactive, indicating that UTP was being efficiently incorporated. To the best of our knowledge, this simple method allowed to radiolabel the RNA in cultured cells, for the first time, by using a radiolabeled ribonucleoside triphosphate, namely [α-^{33}P]UTP, although molecular basis as to how UTP passes through the plasma membrane remains to be clarified. [α-^{33}P]UTP contains [^{33}P], a high-energy radioisotope, and thus newly synthesized RNA was expected to be detected much more easily by autoradiography than using [^{3}H]uridine.

We then pulse-labeled HeLa cells for 30 min and analyzed the RNA by agarose gel electrophoresis, followed by membrane transfer and autoradiography. [α-^{33}P]UTP was first incorporated into rRNA precursors and then shifted to mature 28S, 18S, and 5.8S rRNAs in a time-dependent manner (Fig. 1A). Although mRNA could also be visualized as smears in the gel after long periods of labeling, after 30 min of labeling, the several bands predominated at the expected positions for pre-rRNA and mature rRNA (Fig. 1A). These bands were destroyed by RNase A treatment, but not by DNase I treatment (data not shown). Incorporation of [α-^{33}P]UTP into the RNA in these bands was consistently suppressed by addition of actinomycin D (ActD), an inhibitor of RNA polymerase I, but not α-amanitin, an inhibitor of RNA polymerase II to the culture medium (Fig. 1B).

ER stress suppressed rRNA synthesis

We then searched for stressors or compounds that affected rRNA synthesis, and found that tunicamycin (Tm), thapsigargin (Tg), and proteasome inhibitor (PSI) treatments efficiently suppressed *de novo* rRNA labeling (Fig. 1C). Under the same conditions, c-myc levels were slightly decreased by Tm treatment, but were not affected by Tg or PSI treatment (Fig. 1D). c-myc has been shown to increase rRNA synthesis [24]. However, Tm, Tg, and PSI are known to induce endoplasmic reticulum stress (ER stress). Indeed, these three compounds induced CHOP expression, a well-known ER stress marker (Fig. 1D) [25]. We thus hypothesized that ER stress could induce the suppression of *de novo* rRNA synthesis.

We pulse-labeled cells with or without Tm treatment, and examined the profile of rRNA synthesis suppression. Tm treatment did not appear to change the pattern of the bands labeled by [α-^{33}P]UTP, but the strength of each band was greatly reduced (Fig. 1E). Treatment with Tg or PSI produced results that were essentially identical to those observed in Tm treatment (data not shown). We further examined the effects of these compounds after incorporated [α-^{33}P]UTP had shifted completely into mature 28S and 18S rRNA, and found that none of the compounds affected the incorporated [α-^{33}P]UTP patterns in mature 28S or 18S rRNA (Fig. 1F). These results demonstrate that the rRNA transcription was suppressed by Tm treatment, and that maturation or degradation of rRNA did not appear to be affected by Tm treatment.

PERK-mediated rRNA synthesis suppression at ER stress

To obtain more evidence in support of the idea that ER stress induces rRNA synthesis suppression, we examined the effect of blockading the major ER stress signaling pathways, namely, IRE1α,

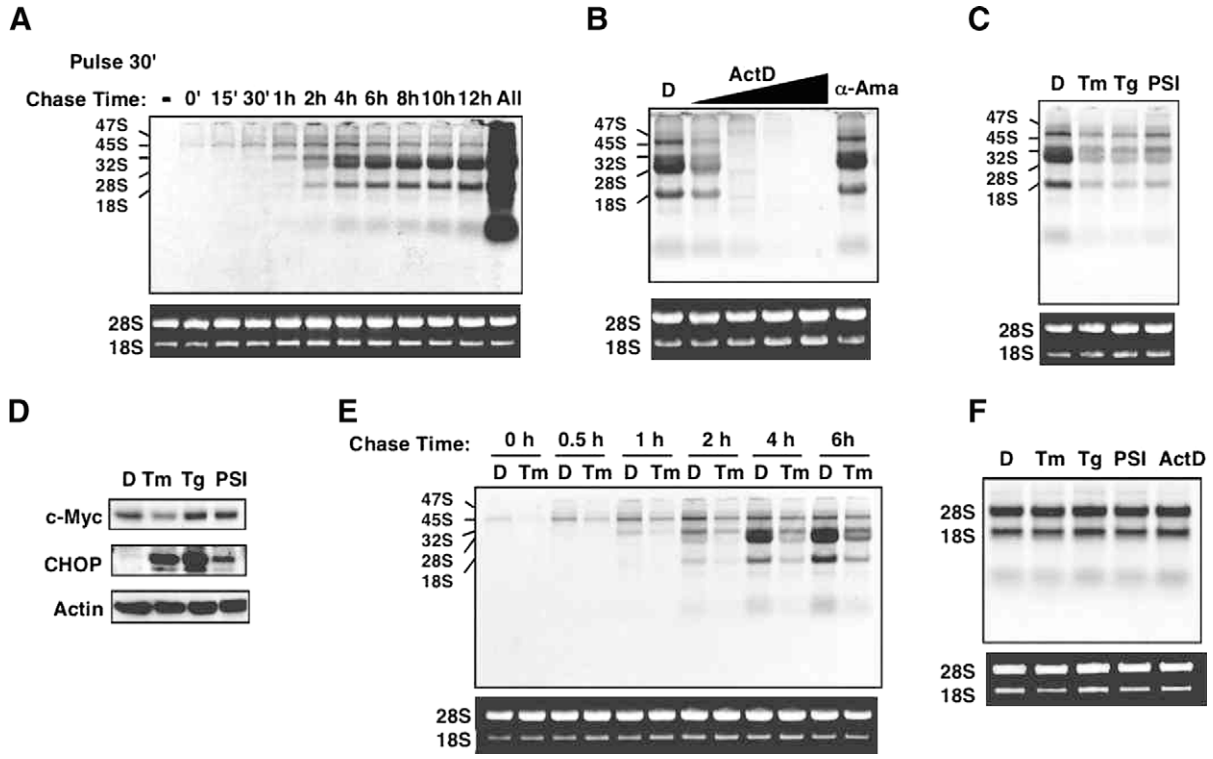

Fig. 1. Visualization of *de novo* rRNA synthesis and its suppression following ER stress. (A) HeLa cells were labeled with [α-^{33}P]UTP for 30 min or 12.5 h (All). Following the 30 min labeling, cells were washed, and cultured for the indicated time periods (from 0 to 12 h). Newly synthesized RNA was detected, as described in "Materials and methods". rRNA patterns are shown as a loading control. (B) HeLa cells were cultured for 8 h with 20 μCi of [α-^{33}P]UTP in the absence (D) or presence of ActD (1, 10, 100, or 1000 μg/ml) or α-amanitin (α-Ama) (50 nM). RNA was then recovered and analyzed as described in (A). (C) HeLa cells were cultured for 2 h in the absence (D) or presence of Tm (2 μg/ml), Tg (500 nM), or PSI (10 μM). RNA was radiolabeled and analyzed as described in (A). (D) HeLa cells were cultured for 8 h in the absence (D) or presence of Tm (2 μg/ml), Tg (500 nM), or PSI (10 μM), and the cell lysates were analyzed by western blot using anti-c-myc, anti-CHOP, and anti-actin antibodies. (E) HeLa cells were cultured for 2 h in the absence (D) or presence of 2 μg/ml Tm, and then 20 μCi of [α-^{33}P]UTP was added to the medium for 30 min. After extensive washing, cells were cultured for the indicated additional time periods (from 0 to 6 h), and RNA was recovered and analyzed as described in (A). (F) HeLa cells were cultured for 3 h with 20 μCi of [α-^{33}P]UTP. Twenty-four hours after the medium change, cells were cultured for an additional 8 h in the absence (D) or presence of Tm (2 μg/ml), Tg (500 nM), or PSI (10 μM). RNA was recovered and analyzed as described in (A).

ATF6, and PERK pathways [6,8,9]. Neither of the two different IRE1α siRNAs inhibited the suppression of rRNA synthesis by Tm (Fig. 2A and Supplementary Fig. S1). Consistent with this, we could not detect inhibition of rRNA synthesis suppression in MEF from IRE1α (−/−) mice (Supplementary Fig. S2). Furthermore, neither of the two different ATF6 siRNAs inhibited the rRNA synthesis suppression (Fig. 2A), although both ATF6 siRNAs efficiently reduced endogenous ATF6 protein levels (Fig. 2B). However, four different PERK siRNAs did inhibit the suppression of rRNA synthesis by Tm (Fig. 2A), although all of the PERK siRNAs effectively destroyed PERK mRNA (Supplementary Fig. S3). Indeed, in the cells treated with PERK siRNAs, induction of CHOP by Tm was inhibited (Fig. 2B). We could rule out the involvement of CHOP in rRNA synthesis suppression, because we observed clear suppression of rRNA synthesis in MEF from CHOP (−/−) mice (Fig. 2C) [26]. PERK siRNAs also induced a similar inhibition of rRNA synthesis suppression in cells treated with Tg, but not in those treated with PSI (Fig. 2D), suggesting that, in addition to PERK, other as-yet unknown molecule(s) may be involved in suppressing *de novo* rRNA synthesis when misfolded proteins or ubiquitinated proteins accumulate in the cytoplasm, as observed after PSI treatment.

Translational inhibition and rRNA synthesis suppression

During UPR, PERK is involved in the translational inhibition of mRNA via eIF2α phosphorylation at Ser51 [7], in addition to the induction of CHOP [25]. Thus, we next examined the possible involvement of translational inhibition in the suppression of rRNA synthesis. Indeed, we observed that 10 μg/ml cycloheximide (CHX) treatment caused the suppression of not only *de novo* protein synthesis but also rRNA synthesis. Both suppressions were not dependent of PERK (Fig. 3A and B). Although CHX treatment slightly induced eIF2α phosphorylation, it was not PERK-dependent (Fig. 3C). Likewise, PSI treatment caused not only suppression of *de novo* protein synthesis but also eIF2α phosphorylation (Fig. 3A and C), which appeared to be partly dependent on PERK (Fig. 3C). Furthermore, two eIF2α siRNAs markedly suppressed *de novo* protein and rRNA syntheses (Fig. 3D and E, and Supplementary Fig. S4). In cells treated with eIF2α siRNAs, Tm was not able to further enhance the suppression of *de novo* protein or rRNA synthesis (Fig. 3D and E). These data, altogether, indicated that suppression of *de novo* protein synthesis profoundly couples with the suppression of *de novo* rRNA synthesis.

Suppression of rRNA synthesis apparently contributed to maintaining ATP levels

We observed that 10 ng/ml ActD showed slightly stronger suppressive effect on *de novo* rRNA synthesis than 2 μg/ml Tm (Fig. 3B), and this concentration of Tm suppressed more than 60% of *de novo* protein synthesis. However, this dose of ActD only marginally suppressed *de novo* protein synthesis for up to 24 h (Fig. 4A). These results indicate that the observed suppression of *de novo* rRNA synthesis affected *de novo* protein synthesis very mildly. How, then, does suppression of *de novo* rRNA synthesis benefit cells with large accumulations of misfolded proteins in

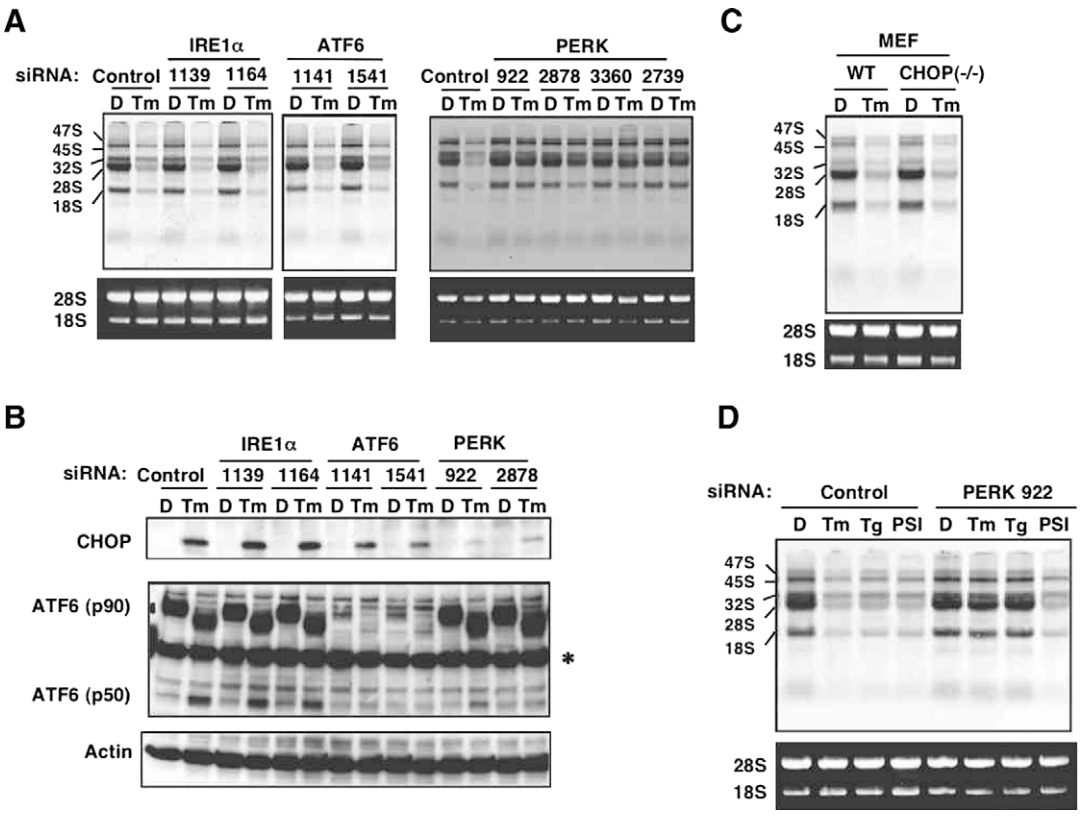

Fig. 2. PERK mediates ER stress-induced suppression of *de novo* rRNA synthesis. (A, C, D) HeLa cells were transfected with indicated siRNAs, and cultured for 3 days. Cells were then cultured for 2 h in the absence (D) or presence of Tm (2 μg/ml), and 20 μCi of [α-^{33}P]UTP was added to the medium. Cells were cultured for an additional 8 h, and RNA was recovered and analyzed as described in Fig. 1A. (B) HeLa cells were transfected with indicated siRNAs and cultured for 3 days. Cells were then cultured for 8 h in the absence (D) or presence of Tm (2 μg/ml), and the cell lysates were analyzed by western blot using anti-CHOP, anti-ATF6, and anti-actin antibodies. * indicates nonspecific bands. (C) MEF cells from wild-type mice (WT) or from CHOP knockout mice (CHOP (−/−)) were cultured for 2 h in the absence (D) or presence of Tm (2 μg/ml). RNA was radiolabeled and analyzed as described in Fig. 1A. (D) HeLa cells were transfected with PERK siRNA (922), and cultured for 3 days. Cells were then cultured for 2 h in the absence (D) or presence of Tm (2 μg/ml), Tg (500 nM), or PSI (10 μM). RNA was radiolabeled and analyzed as described in Fig. 1A.

the ER (at ER stress) or in the cytoplasm (at PSI treatment)? Large amounts of ATP are consumed continuously during rRNA synthesis [21]. In these cells, ATP is required by chaperons and the ubiquitin–proteasome system for unfolding and ubiquitination-degradation, respectively, of the accumulated proteins [5]. Thus, the observed suppression of *de novo* rRNA synthesis likely maintains the supply of ATP in these cells by reducing the consumption of ATP for rRNA synthesis so that it can be used instead to power chaperons and the ubiquitin–proteasome system. We then examined this possibility. Tm treatment indeed reduced ATP levels in the cells, and this reduction was further enhanced with PERK knockdown (Fig. 4B). Furthermore, addition of ActD significantly recovered ATP levels in cells treated with Tm but not in cells cultured in a normal condition (Fig. 4B).

Discussions

In this study, we first showed a novel method to analyze *de novo* RNA synthesis, especially rRNA synthesis, in cultured cells by simply adding radiolabeled ribonucleoside triphosphate, such as [α-^{33}P]UTP in culture medium, followed by RNA extraction, gel electrophoresis, RNA blotting, and autoradiography. This simple method allows to visualize entire steps of rRNA synthesis, e.g. precursor transcription of 47S rRNA, processing, and appearance of mature 18S and 28S rRNA, in a quantitative manner. Thus, we call this method VRS (visualization of RNA synthesis) method.

Using VRS method, we next revealed that ER stress-inducing drugs, such as Tm, Tg, and PSI dramatically suppressed *de novo* rRNA synthesis. It has been reported that ER stress induces various cellular responses via using ER stress sensors, such as ATF6, PERK, IRE1α [6,8,9]. Among the knockdown of these three sensors, only PERK knockdown abolished Tm- and Tg-mediated but not PSI-mediated suppression of rRNA synthesis. These observations evidenced that PERK is involved in the suppression of rRNA synthesis at ER stress. PERK has been known to phosphorylate Ser51 in eIF2α, which in turn causes translational inhibition. Consistently, PSI induced the phosphorylation of Ser51 in eIF2α, even in cells treated with PERK siRNAs. Thus, the suppression of rRNA synthesis observed apparently coupled with the eIF2α phosphorylation.

Recently, DuRose et al. showed very similar observations and suggested that eIF2α phosphorylation couples with RRN3/TIF-IA being released from RNA polymerase I at ER stress, leading to the suppression of rRNA synthesis [27]. They speculated that RRN3/TIF-IA phosphorylation might be responsible for the release [27]. In our experiments, RRN3/TIF-IA knockdown was indeed able to suppress the rRNA synthesis (Supplementary Fig. S5). However, knockdown of eIF2α also induced similar suppression of rRNA synthesis in concomitant with the suppression of *de novo* protein synthesis, indicating that eIF2α phosphorylation is not necessary for the suppression of rRNA synthesis. Furthermore, CHX treatment was able to strongly suppress both protein and rRNA syntheses. These observations could provide an alternative, but not necessarily a mutually exclusive mechanism of the suppression of rRNA synthesis. Namely, translational suppression itself is responsible for the suppression of rRNA synthesis. In this mechanism, it is

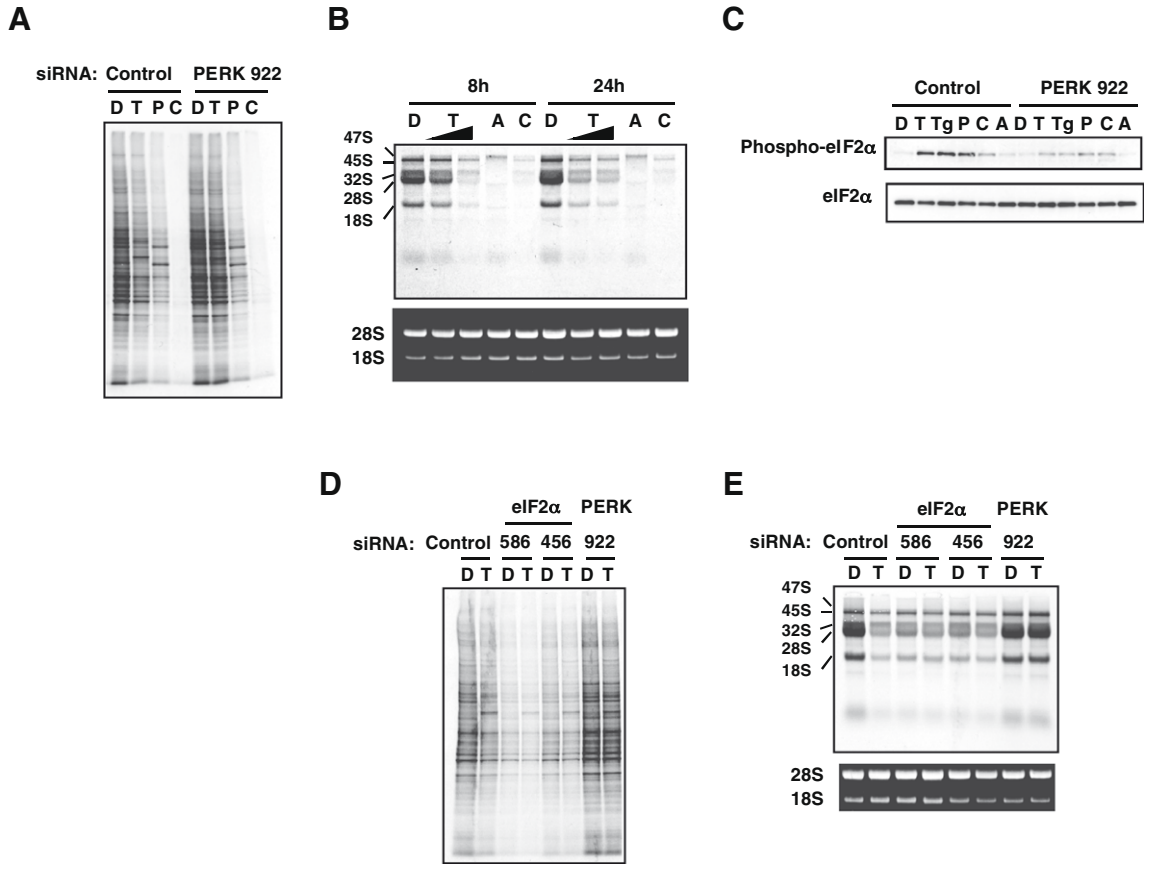

Fig. 3. Translational inhibition and the suppression of rRNA synthesis. (A and D) Measurements of *de novo* protein synthesis (see "Materials and methods") in the absence (D) or presence of Tm (T) (2 μg/ml), PSI (P) (10 μM), CHX (C) (10 μg/ml) for 8 h from cells treated with indicated siRNAs. (B and E) HeLa cells were transfected with indicated siRNAs and cultured for 3 days. Cells were then cultured for 8 h in the absence (D) or presence of Tm (T) (0.2 or 2 μg/ml), PSI (P) (10 μM), ActD (A) (10 ng/ml), and CHX (C) (10 μg/ml). RNA was radiolabeled and analyzed as described in Fig. 1A. (C) Western blot analysis using anti-phospho eIF2α and eIF2α antibodies on cell lysates from HeLa cells overexpressing eIF2α. Cells were transfected with PERK siRNA (922), and cultured for 3 days. Cells were then cultured for 2 h in the absence (D) or presence of Tm (T) (2 μg/ml), Tg (500 nM), PSI (P) (10 μM), CHX (C) (10 μg/ml), and ActD (A) (10 ng/ml).

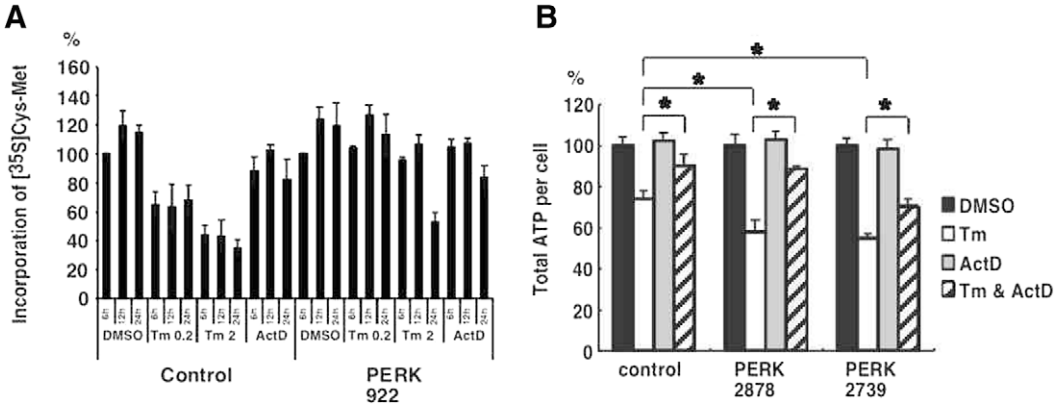

Fig. 4. Suppression of rRNA synthesis and ATP levels at ER stress. (A) Measurements of the relative amounts of *de novo* protein synthesis (see "Materials and methods") in the absence (DMSO) and presence of Tm (0.2, or 2 μg/ml) and ActD (10 ng/ml) for 6, 12 or 24 h. (B) Measurements of the relative amounts of ATP per cell. HeLa cells were treated in the absence (DMSO) and presence of Tm (2 μg/ml), ActD (10 ng/ml), or both for 24 h, and were harvested. Then, ATP amounts from 1.5×10^5 cells were measured (see "Materials and methods"). *, $p < 0.05$.

speculated that protein(s) with very short half-lives or those that are quickly ubiquitinated would play important roles in rRNA synthesis. These possibilities remain to be clarified.

It was evident that simple suppression of rRNA synthesis by ActD did not appear to contribute to the suppression of *de novo* protein synthesis. Instead, the observed suppression of *de novo* rRNA synthesis maintained the ATP levels in cells with ER stress by reducing the consumption of ATP for rRNA synthesis so that it can be used by other systems, such as ER chaperons, ERAD, and the ubiquitin–proteasome system. It is noteworthy that eIF2α phosphorylation occurs not only at ER stress, but also in several other stress conditions, such as amino-acid starvation, oxidative

stress, virus infection, etc. [11–14]. These conditions apparently all require large amounts of ATP, and thus the cells use eIF2α phosphorylation to suppress the supply of newly synthesized proteins, which in turn suppresses the *de novo* rRNA synthesis, leading to the maintenance of ATP levels in the cell. Thus, this system would be a fundamental feedback system for the cells to adapt or survive against several stressful conditions via efficiently utilizing intracellular pools of ATP.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.02.065.

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