Ribosomal Stress Couples the Unfolded Protein Response to p53-dependent Cell Cycle Arrest*

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Protein misfolding in the endoplasmic reticulum (ER) triggers a signaling pathway termed the unfolded protein response pathway (UPR). UPR signaling is transduced through the transmembrane ER effectors PKR-like ER kinase (PERK), inositol requiring kinase-1 (IRE-1), and activating transcription factor 6 (ATF6). PERK activation triggers phosphorylation of eIF2α leading to repression of protein synthesis, thereby relieving ER protein load and directly inhibiting cyclin D1 translation thereby contributing to cell cycle arrest. However, PERK−/− murine embryonic fibroblasts have an attenuated G1/S arrest that is not attributable to cyclin D1 loss, suggesting a cyclin D1-independent mechanism. Here we show that the UPR triggers p53 accumulation and activation. UPR induction promotes enhanced interaction between the ribosome proteins (rPL5, rPL11, and rPL23) and Hdm2 in a PERK-dependent manner. Interaction with ribosomal proteins results in inhibition of Hdm2-mediated ubiquitination and degradation of p53. Our data demonstrate that ribosomal subunit:Hdm2 association couples the unfolded protein response to p53-dependent cell cycle arrest.

Environmental insults that disrupt endoplasmic reticulum (ER) homeostasis, including glucose deprivation and hypoxia, or pharmacological agents that prevent glycosylation (tunicamycin), inhibitors of ER calcium influx (thapsigargin, inhibitor of sarcoplasmic/ER Ca2+-ATPase (SERCA)) or ER-to-Golgi vesicle transport (Brefeldin A), all lead to accumulation of misfolded proteins within the ER triggering a coordinated adaptive program called the unfolded protein response (UPR) (1, 2). The UPR is proximally sensed and transduced by four major ER-transmembrane effector proteins: protein kinases IRE1α and IRE1β, PERK, and a membrane-bound transcription factor ATF6 (activating transcription factor-6) (1, 3, 4).

The UPR coordinates two major functions: 1) transcriptional regulation of specific targets and 2) translational repression. The transcriptional arm of the UPR is mediated largely by IRE1-dependent signaling in concert with ATF6; together these factors induce ER chaperones to remedy protein misfolding (5, 6). Activation of X-box binding protein 1 by IRE1-mediated mRNA splicing induces ER degradation-enhancing α-mannosidase-like protein to degrade misfolded proteins (7), and IRE1 independently mediates the rapid degradation of ER-localized mRNAs (8). Translational regulation is executed by PERK, which phosphorylates eukaryotic translation initiation factor-2α (eIF2α) on serine 51 to block assembly of an active 43 S translation initiation complex thus contributing to translation repression to reduce ER protein load (4, 9). Independent of eIF2α, PERK also phosphorylates NF-E2-related factor 2 to regulate expression of redox enzymes necessary to restore redox homeostasis and thereby contribute to cell adaptation to ER stress (10).

PERK-dependent activities, eIF2α-dependent and -independent, are critical for cell survival following exposure to ER stress (10–15). One critical PERK function is the maintenance of cellular redox homeostasis, which is mediated by transcriptional effectors activating transcription factor-4 and NF-E2-related factor 2 (10, 16). PERK signaling also coordinates cell cycle arrest, which reduces energy consumption and allows cells to divert resources to re-establish homeostasis. The UPR-associated cell cycle arrest correlates with PERK-dependent attenuation of cyclin D1 protein synthesis. Cyclin D1 loss leads to subsequent repression of cyclin D1-dependent CDK4 activity. Loss of D1/CDK4 complex also allows for the redistribution of p21CIP1 to CDK2, thereby triggering the G1/S checkpoint (17, 18).

Because ribosome biogenesis is the major biosynthetic and energy-consuming activity of eukaryotic cells, it is sensitive to the availability of nutrients and growth factors (19). Emerging evidence suggests that ribosome biogenesis is intimately coupled to cell cycle progression through regulation of the Hdm2/p53 signaling (20, 21). Induction of the tumor suppressor Arf (alternative reading frame) contributes to inhibition of rRNA processing, while simultaneously binding to and inhibiting Hdm2, which leads to p53 induction and cell cycle arrest (22). Therefore, Arf coordinates ribosome biogenesis with the cell
cycle in response to oncogenic stress (23). In addition, overexpression of a mutant Bop1, a nucleolar protein critical for rRNA processing and ribosome assembly, triggers p53 activation and induces cell cycle arrest at G1 phase (20, 21). More tellingly, low doses of actinomycin D, which inhibit rRNA synthesis thereby interfering with ribosome assembly, leads to enhanced association between ribosome proteins (rpL5, rpL11, and rpL23) and Hdm2. The increased binding to Hdm2 inhibits p53 ubiquitination thereby triggering cell cycle arrest (24–28).

Because the UPR reduces active polysomes through elf2α phosphorylation, we considered this signal might also lead to increased association between ribosomal proteins and Hdm2 thereby leading to p53 activation. We have evaluated the ability of both physiological (glucose deprivation) and pharmacological (tunicamycin) triggers of ER stress to activate p53. Our data reveal that UPR induction by glucose deprivation or tunicamycin treatment triggers p53 accumulation and activation in a PERK-dependent manner. Activation of p53 is due to reduced Hdm2-mediated p53 ubiquitination and degradation. Inhibition of Hdm2 correlates with increased association of Hdm2 with the ribosomal proteins (rpL5, rpL11, and rpL23). Activation of p53 contributes to cell cycle arrest, because cells lacking a functional p53 allele or its downstream target p21 are refractory to cell cycle arrest in response to ER stress. Therefore, UPR induction activates ribosomal stress, which directly contributes to p53-dependent cell cycle arrest.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HCT116 p53+/+ , HCT116 p53−/− , HCT116 p21cip1+/+ , and p21cip1−/− cells were gifts from Dr. Bert Vogelstein (John Hopkins University, Baltimore, MD) and cultured in McCoy5A media with 10% fetal bovine serum and penicillin/streptomycin. PERK-Flox/Flox mice were a gift from Dr. Douglas Cavener (Dept. of Biology, Pennsylvania State University). U2OS and Saos-2 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and penicillin/streptomycin. Murine embryonic fibroblasts (MEFs) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin/streptomycin, nonessential amino acids, l-glutamine, and β-mercaptoethanol.

**Plasmids**—GFP-rpL5, T7-Hdm2 and FLAG-rpL23 plasmids were previously described (22, 24, 29). FLAG-rpL23 was detected by using anti-FLAG (Sigma); T7-Hdm2 was detected by using anti-Hdm2 (2A10, a gift from Dr. Jason D. Weber, Washington University); Hdm2 (4B11, Calbiochem), heterogeneous nuclear ribonucleoprotein K antibody (a gift from Dr. Gideon Dreyfuss, Dept. of Biochemistry and Biophysics, University of Pennsylvania Medical Center); and L11 rabbit polyclonal antibody (a gift from Karen H. Vousden, Beaton Institute for Cancer Research). FLAG-rpL23 was detected by using anti-FLAG (Sigma); T7-Hdm2 was detected by using T7 tag antibody (Novagen); GFP-rpL5 was detected by using anti-GFP (JL-8, Clontech). For quantification of immunoblot, the secondary antibody used were AlexaFluor 680-conjugated anti-mouse or anti-rabbit antibody ( Molecular Probes, Eugene, OR), and bands were scanned and quantified using the Odyssey version 1.0 software on the LI-COR Odyssey Infrared Imager. Protein levels for p53 or p21 were normalized with loading control and represented % or -fold increase over basal. Data represent at least three independent experiments done in triplicate, with error bars representing standard deviations.

**Quantitative Real-time PCR Analysis**—HCT116 p53+/+ or p53−/− cells were cultured in glucose-free, or low glucose (0.5 mm glucose) media for 16 h. RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer’s protocol. cDNA was made from 2 μg of total RNA using Superscript II First Strand cDNA Synthesis System for reverse transcription-PCR (Invitrogen). These cDNA were then analyzed using quantitative PCR. Gene expression was standardized to 18 S RNA levels using a commercially available primer/probe set (ABI), labeled with a fluorescent dye (6-carboxyfluorescein or JOE) and a quencher (6-carboxytetramethylrhodamine). Expression of p21cip1, p27, and Noxa was then determined using SYBR fluorescence and the following primers: p21cip1 F, AGCAGAGGAACGACATGGGAC; p21cip1 R, TTTGCCAAGCTGaGTCATCCCCAG; p27 F, TTGGAGAGCTGCAGACAGCA; p27 R, TCCACCTTGGCCACTGCTACT; Noxa F, AAAAACTGCAATTCCCCGCCAGAA; and Noxa R, TTTGAGGAGTTCCTCCTCG.

Tagman and SYBR green master mix solutions were obtained from Applied Biosystems (Foster City, CA). SYBR green-labeled
primer/probe pairs for p21<sup>−/−</sup>, p27, and Noxa were custom-designed and obtained from Integrated DNA Technologies (University of Pennsylvania Cell Center). All reactions were performed in triplicate, and all experiments were performed at least three times in a 7900HT sequence detection system (Applied Biosciences). Changes in threshold signal versus 18 S RNA control (ΔC<sub>T</sub>) calculations were used to calculate relative mRNA levels. Error bars represent standard deviation.

Cre-retrovirus Infection and Genotyping of Cre-floxed PERK—The retrovirus construct for pBabe-Cre was a gift from Dr. Kay-Uwe Wagner (University of Nebraska). Retrovirus production and infection were carried out as previously described (18). PERK Flox/Flox MEFs (1 × 10<sup>6</sup>/60-mm dish) were derived as previously described (30). Cells were infected with control pBabe-puro or pBabe-Cre retrovirus 72 h before harvesting for genotyping or replaced with glucose-free media for the indicated intervals followed by harvesting and immunoblotting. For genotyping the WT or Cre-floxed PERK allele, a combination of the primer/probe pairs for p21Cip1, p27, and Noxa was custom-designed and obtained from Integrated DNA Technologies (University of Pennsylvania Cell Center). All reactions were performed in triplicate, and all experiments were performed at least three times in a 7900HT sequence detection system (Applied Biosciences). Changes in threshold signal versus 18 S RNA control (ΔC<sub>T</sub>) calculations were used to calculate relative mRNA levels. Error bars represent standard deviation.

Cell Lysate Fractionation—Nucleolar and nucleoplasmic fractions were isolated from MEFs or U2OS cells as previously described with slight modification (31). Control or glucose-starved MEFs or U2OS cells from two 150-mm dishes were trypsinized, pelleted, washed with PBS, and centrifuged at 2,000 rpm for 5 min. The cell pellet was then resuspended in 1 ml of RSB buffer (0.01 M Tris-HCl, 0.01 M NaCl, 1.5 mM MgCl<sub>2</sub>, pH 7.4) and placed on ice for 10 min. Swollen cells were then collected by centrifugation at 1,500 rpm for 8 min, and resuspended in 1 ml of RSB buffer containing 0.5% Nonident P-40. Cell membranes were then broken using a Dounce homogenizer (tight pestle) by 10 up and down strokes. Nuclei (pellet) were collected by centrifugation at 1,500 rpm for 8 min and resuspended in 0.75 ml of 0.25 M sucrose/10 mM MgCl<sub>2</sub>, overlaid onto 1 ml of 0.88 M sucrose/0.05 mM MgCl<sub>2</sub>, and purified by centrifugation again at 2,500 rpm for 10 min. The pelleted nuclei were resuspended in 0.5 ml of 0.34 M sucrose/0.05 mM MgCl<sub>2</sub> and sonicated for 60 s with 10-s intervals. The sonicated cell lysate was then overlaid onto 1 ml of 0.88 M sucrose/0.05 mM MgCl<sub>2</sub> and centrifuged at 25,000 rpm for 20 min. The pellet contained the purified nucleoli, and the supernatant represented the nucleoplasm. All buffers contained protease inhibitors added freshly before use.

BrdUrd/PI Fluorescence-activated Cell Sorting Analysis—Cells were treated with tunicamycin, or shifted to glucose-free or low glucose media for the indicated time, and incubated with 10 µM BrdUrd for the final hour. After washing with PBS, cells were trypsinized, suspended in 300 µl of PBS and 700 µl of 100% ethanol, and fixed overnight at −20 °C. Cells were incubated with anti-BrdUrd monoclonal antibody and fluorescein isothiocyanate-labeled anti-mouse antibody before being incubated with propidium iodide (PI, 10 µg/ml in PBS) and scanned by flow cytometry using a FACSCalibur (BD Biosciences, San Jose, CA) to determine the proliferating S phase (BrdUrd-positive population) and DNA content (by PI staining).

RESULTS

ER Stress Triggers p53 Activation and p21<sup>−/−</sup> Induction—To evaluate the impact of ER stress on p53 accumulation and induction of downstream targets, p53-deficient HCT116 cells were cultured in glucose-free media or in complete media supplemented with 5 µg/ml tunicamycin and analyzed by immunoblotting. In the absence of glucose, p53 accumulation was apparent by 3 h, with strong induction by 16 h (Fig. 1A). p53 accumulation was accompanied by induction of the p53 target p21<sup>−/−</sup> (Fig. 1A, middle panel). Tunicamycin-dependent p53 induction was also associated with p21<sup>−/−</sup> accumulation (Fig. 1A). Consistent with the published data (32) we noted increased Ser-15 phosphorylation of p53 following glucose deprivation (Fig. 1, B, C, and E). Tunicamycin treatment also promoted p53 accumulation by 16 h, although we noted that at early time points (3 h) p53 levels were slightly decreased, but were clearly induced at later time points (16 h) (Fig. 1A). The p21<sup>−/−</sup> induction was significantly attenuated in p53<sup>−/−/−</sup> HCT116 (Fig. 1B, right panel), suggesting p53-dependence. We also evaluated p53 induction in p53 wild-type U2OS and primary MEFs to
ensure our results did not reflect cell type specificity. Glucose deprivation triggered p53 accumulation and p21Cip1 induction in both U2OS cells and MEFs (Fig. 1, C–E), accompanied by increased phospho-p53 (Ser-15), demonstrating that ER stress by glucose deprivation leads to p53 accumulation and activation in multiple types of cells.
The induction of p21<sub>Cip1</sub> upon p53 accumulation suggests that p53 is transcriptionally active. To determine whether p21<sup>Cip1</sup> induction occurs at the level of gene expression and to evaluate the role of p53 in this induction, quantitative real-time PCR was performed to measure both p21<sub>Cip1</sub> and Noxa, another p53 target gene (33) in either p53<sup>/−/−</sup>/HCT116 cells. A 5-fold induction of p21<sub>Cip1</sub> mRNA was evident by 16 h of glucose deprivation (Fig. 1F). This induction was significantly attenuated in p53<sup>/−/−</sup> cells. Noxa mRNA was also stimulated by glucose-deprivation in a p53-dependent manner (Fig. 1F). As a negative control, we assessed mRNA levels of a non-p53 target, p27<sup>kip1</sup> (Fig. 1F). These data demonstrate that glucose restriction triggers p53 accumulation and p53-dependent activation of downstream targets including p21<sup>Cip1</sup> and Noxa.

### Glucose Deprivation Activates p53 in a PERK-dependent Manner

We have previously demonstrated that the ER-associated kinase, PERK, plays a critical role in the regulation of cell cycle progression following ER stress (17, 18). We used two approaches to assess whether PERK regulates p53 induction. In the first, we utilized a PERK mutant that lacks the C-terminal kinase domain but retains the N-terminal dimerization domain allowing it to inhibit endogenous PERK activity. Dominant negative PERK (DN PERK) was expressed in p53<sup>/−/−</sup>/HCT116 cells, and p53 and p21<sub>Cip1</sub> protein levels were assessed by immunoblot following a time course of tunicamycin treatment (data not shown) or glucose starvation. Induction of eIF2α phosphorylation was blocked by DN PERK expression demonstrating efficacy of this molecule (Fig. 2A, second panel). The increase in basal phospho-eIF2α likely

![FIGURE 2. Glucose deprivation activates p53 in a PERK-dependent manner.](image-url)
reflects an increase in oxidative stress due to inhibition of PERK signaling (10). UPR-triggered accumulation of both p53 and p21Cip1 was attenuated by DN PERK (Fig. 2, A and B), strongly suggesting that PERK is essential for UPR-induced p53 activation.

As a complementary approach, the PERK Flox/Cre system was utilized to delete PERK in primary MEFs (34). PERK Flox/Flox MEFs were infected with retrovirus encoding Cre or empty virus control. PERK was indeed excised as determined by PCR (data not shown). PERK protein levels were reduced by ~80% as determined by Western analysis (Fig. 2C and data not shown). Accumulation of phospho-eIF2α was blocked in pBabe-Cre-infected cells consistent with loss of PERK (Fig. 2C). In the control mock-infected cells, p53 levels were significantly increased by ~4 h of glucose deprivation, and remained elevated at 8 h; however, p53 levels in pBabe-Cre-infected cells did not increase throughout the time course (Fig. 2, C and D). The absence of p53 elevation is not due to increased basal p53 levels as actinomycin D (5 nM) still triggered a robust p53 accumulation in Cre-infected cells as it did in the control cells (data not shown). Taken together, these data strongly support the PERK dependence of p53 accumulation and activation by the UPR.

Glucose Deprivation Inhibits Hdm2-mediated p53 Ubiquitination and Degradation—To determine whether p53 accumulation reflects decreased p53 ubiquitination, we assessed p53 polyubiquitination following induction of ER stress. U2OS cells transfected with a vector encoding His-tagged ubiquitin were mock treated or subjected to ER stress and treated with the proteasome inhibitor, MG132 (20 μM) for 6 h and subjected to glucose deprivation (−G), tunicamycin (Tu), or thapsigargin (TG) for 4 h. The in vivo ubiquitination assay was performed as described under “Experimental Procedures.” p53 levels were assessed using an anti-p53 antibody, and T7-Hdm2 using the anti-T7 antibody. B, HCT116 WT cells treated with cycloheximide (100 μg/ml) for the indicated intervals were harvested and immunoblotted with anti-p53 antibody. Heterogeneous nuclear ribonucleoprotein K (hnRNP) levels were used as the loading control. C, quantification of p53 levels in C. Results represent three independent experiments with similar results. D, HCT116 p53−/− cells were pulse-labeled for the indicated intervals with [35S]methionine after treatment with 2.5 μg/ml tunicamycin for 6 h and subjected to immunoprecipitation with either normal rabbit antisera (NRS) or anti-p53 antibody. Precipitated proteins were resolved by SDS-PAGE and visualized by autoradiography.

FIGURE 3. UPR induction suppresses Hdm2-mediated p53 ubiquitination and degradation. A, following transfection of His-ubiquitin (His-Ub) and T7-Hdm2 in U2OS cells, they were treated with the proteasome inhibitor MG132 (20 μM) for 6 h and subjected to glucose deprivation (−G), tunicamycin (Tu), or thapsigargin (TG) for 4 h. The in vivo ubiquitination assay was performed as described under “Experimental Procedures.” p53 levels were assessed using an anti-p53 antibody, and T7-Hdm2 using the anti-T7 antibody. B, HCT116 WT cells treated with cycloheximide (100 μg/ml) for the indicated intervals were harvested and immunoblotted with anti-p53 antibody. Heterogeneous nuclear ribonucleoprotein K (hnRNP) levels were used as the loading control. C, quantification of p53 levels in C. Results represent three independent experiments with similar results. D, HCT116 p53−/− cells were pulse-labeled for the indicated intervals with [35S]methionine after treatment with 2.5 μg/ml tunicamycin for 6 h and subjected to immunoprecipitation with either normal rabbit antisera (NRS) or anti-p53 antibody. Precipitated proteins were resolved by SDS-PAGE and visualized by autoradiography.
UPR-dependent Activation of p53

**FIGURE 4.** UPR promotes PERK-dependent interaction between ribosomal protein rpL5/rpL11/rpL23 and Hdm2. A, T7-Hdm2- and FLAG-rpL23-expressing U2OS cells were treated with actinomycin D (Act, 5 nM), or cultured in glucose-free media (−G) for 4 h. Complexes were precipitated with an anti-FLAG antibody, and co-precipitating T7-Hdm2 was detected with the T7 antibody. Expression of FLAG-rpL23 and T7-Hdm2 was confirmed by immunoblot with corresponding antibodies. B, time-dependent interaction between rpL23 and T7-Hdm2. Transfected U2OS cells were cultured in glucose-free media as indicated, and complexes were collected with the anti-FLAG M2 antibody and associated T7-Hdm2 was detected with the T7 antibody as in A. C, FLAG-rpL23 and T7-Hdm2 were expressed in the absence or presence of the dominant negative K618PERK-myc, and complex formation was assessed as in A. D, following transfection of 293T cells with GFP-rpL5 and T7-Hdm2, cells were treated with actinomycin D (Act), tunicamycin (Tu), or cultured in glucose-free media (−G) for 4 h, and total cell lysates were precipitated with the anti-T7 antibody, and GFP-rpL5 association was assessed by immunoblot with a GFP-specific antibody. Expression of GFP-rpL5 and T7-Hdm2 was confirmed by anti-GFP or anti-T7 immunoblotting. E, GFP-rpL5 and T7-Hdm2 were expressed in the absence or presence of PERK K618L in U2OS cells. Transfected cells were treated with actinomycin D (Act), tunicamycin (Tu), or replaced with glucose-free media (−G) for 4 h as in D, and total cell lysates were precipitated with anti-T7 antibody, and blotted with a GFP antibody as in D. F, increased interaction between endogenous Hdm2 and rpL11. U2OS cells were treated with actinomycin D (Act), or cultured in glucose-free media (−G) as indicated. Endogenous Hdm2 was precipitated with anti-Hdm2 antibody, and associated endogenous rpL11 was detected with an anti-rpL11 antibody. G, MEFs were switched to glucose-free media for the indicated intervals, followed by cellular fractionation into nucleolar and nucleoplasmic fractions. Fractions were analyzed for the levels of endogenous Mdm2 (the specific Mdm2 band was marked by the arrow), and fibrillarin was used as a nucleolar marker.

Glucose Deprivation Increases Hdm2:Ribosomal Protein Association in a PERK-dependent Manner—Ribosomal stress in response to low dose actinomycin D treatment enhances binding of large ribosomal subunit proteins (rpL5, rpL11, and rpL23) with Hdm2 triggering decreased Hdm2 ubiquitin ligase activity (24, 25, 27). We therefore determined whether the UPR-induced inhibition of Hdm2 ubiquitin ligase activity is associated with increased Hdm2:ribosome protein interaction. We initially focused on the potential interaction of rpL23 and Hdm2. U2OS cells transfected with plasmids encoding FLAG-rpL23 and T7-Hdm2 were treated with actinomycin D (5 nM) as a positive control or subjected to glucose deprivation. Although rpL23-Hdm2 complexes were detectable in the absence of stress, as previously noted (28), actinomycin D treatment increased the rpL23-Hdm2 complex abundance (Fig. 4A, lane 5). Similarly, UPR induction by glucose deprivation also increased rpL23/Hdm2 association (Fig. 4A, lane 6), in a time-dependent manner (Fig. 4B, lanes 7–10). UPR activation also triggered increased binding between rpL5 (and rpL11, data not shown) and Hdm2 (Fig. 4D and E). The increased association was not unique to glucose deprivation, because tunicamycin, a classic UPR inducer, also triggered increased rpL5-Hdm2 association (Fig. 4D, compare lanes 5 and 7, Fig. 4E, compare lanes 7 and 9). Because these interactions were examined using ectopically expressed proteins, we next confirmed that binding of endogenous proteins was also sensitive to ER stress. Due to the availability of suitable antiserum for endogenous ribosome proteins, we focused on rpL11-Hdm2 binding. U2OS cells were treated with either actinomycin D (control) or cultured in glucose-free media for 3 or 5 h. Hdm2 complexes were immunoprecipitated from cell lysates, and co-precipitating rpL11 was detected by immunoblot. As expected, increased rpL11 co-precipitation was noted by 5 h of glucose restriction (Fig. 4F, compare lanes 10 and 7 in the lower panel). These data suggest that UPR promotes the association between ribosomal proteins (rpL5, rpL11, and rpL23) with Hdm2, mimicking the ribosomal biogenesis stress elicited by actinomycin D.

To determine whether the observed increase in Hdm2 association with ribosomal proteins is PERK-dependent, we co-transfected cells with T7-Hdm2, FLAG-rpL23, or GFP-rpL5 together with a dominant negative allele of PERK, PERK K618A (4). Indeed, co-expression of PERK K618A abolished UPR-induced rpL23-Hdm2 association (Fig. 4C, compare lanes 6 and 5) and rpL5-Hdm2 complex formation (Fig. 4E, compare lanes 11 and 12 with lanes 9 and 10). Taken together, these data strongly suggest that UPR-promoted Hdm2:ribosome protein (rpL5, rpL11, and rpL23) association is PERK-dependent.

Nucleolar Relocalization of Hdm2—Inhibition of Hdm2 function is frequently associated with its relocalization to the nucleolus (22, 26, 35). To determine whether the UPR induction is associated with an increase in nucleolar Hdm2 levels, a fractionation assay was carried out to separate cell lysates into the nucleolar and the nucleoplasmic fractions before and after UPR induction by glucose deprivation for 3 or 5 h. In wild-type MEFs glucose deprivation triggered nucleolar accumulation of Hdm2 (Fig. 4G, upper panel, arrow marked). The nucleolar protein fibrillarin remained unchanged (Fig. 4G, lower panel).
panel). The nucleolar localization of Hdm2 was also independently confirmed by confocal microscopy (data not shown). These results suggest that ER stress inhibits Hdm2 activity via ribosomal protein binding and relocation of Hdm2 to the nucleolus.

**Activation of p53 Contributes to G_{1}/S Arrest**—Activation of p53 and concomitant accumulation of p21^{Cip1} results in cell cycle arrest at the G_{1}/S boundary in many cell types (36). We therefore determined whether p53 activation and accumulation of p21^{Cip1} contributes to G_{1} arrest in response to the UPR. We assessed the role of UPR-dependent induction of p53 in asynchronously proliferating p53^{+/+} or p53^{-/-} MEFs. Tunicamycin treatment, glucose deprivation, or culturing in low glucose for 16 h resulted in a significant reduction in S-phase cells in wild-type MEFs (Fig. 5A, left panels). In contrast, G_{1}/S arrest was significantly attenuated in p53^{-/-} MEFs (Fig. 5A, right panels). To determine whether the p53-dependent cell cycle arrest is mediated by the p53 target p21^{Cip1}, we assessed the response of HCT116 p21^{Cip1+/+} or p21^{Cip1/-/-} cells to ER stress. Glucose deprivation-triggered G_{1} arrest was abrogated in p21^{Cip1+/+}/HCT116 cells (Fig. 5B), suggesting p53 induction of p21^{Cip1} is critical for mediating the G_{1}/S checkpoint. We noted cyclin D1 loss upon stress ruling out the possibility that lack of arrest was an indirect result of failure to down-regulate this cyclin (data not shown). We also examined the effect of UPR induction on cell cycle progression in both U2OS (p53^{+/+}) and Saos-2 (p53 and RB^{-/-}) cells. Asynchronously proliferating U2OS and Saos-2 cells were subject to multiple ER stress inducers, including tunicamycin, thapsigargin, 2-deoxy-D-glucose, the non-hydrolyzable glucose analogue and low glucose for 20 h, and G_{1} and S-phase population were analyzed by BrdUrd/PI labeling. UPR induction by all these ER stresses triggers G_{1}/S arrest, as reflected by increased G_{1}/S ratio in p53 wild-type U2OS cells, and this arrest was abrogated in p53-deficient Saos-2 cells (Fig. 5C). Taken together, these data demonstrate that UPR-triggered cell cycle arrest is p53- and p21^{Cip1}-dependent.

**DISCUSSION**

Induction of ER stress is associated with reduced cell proliferation due to G_{1} arrest. Previous work revealed loss of the G_{1} cyclin, cyclin D1, to be critical for this arrest (17). However, the cyclin D1/CDK4 kinase is one of two kinases that promote G_{1} to S-phase transition; the cyclin E/CDK2 kinase also participates in this process. Physiological stresses that induce G_{1} arrest typically target both CDK complexes (37), and indeed the CDK2 kinase is inactive following ER stress (17). Paradoxically, cyclin E levels are maintained following ER stress suggesting that inhibition of CDK2 likely results from increased association with inhibitors such as p21^{Cip1} or p27^{kip1}. Increased p21^{Cip1}, CDK2 association is observed upon ER stress suggesting a role for p21^{Cip1} in the G_{1} arrest checkpoint (17). The present study reveals the p53-dependent induction of p21^{Cip1} and provides additional data demonstrating that this induction is critical for G_{1}-phase arrest. Using a variety of methods, p53 induction is placed downstream of PERK activation consistent with previous work suggesting that the PERK branch of the UPR regulates cell proliferation following exposure of cells to stress (Fig. 6). Our data demonstrate that ER stress-associated cell cycle arrest results from direct inhibition of both G_{1} CDK complexes.

**Induction of p53 Correlates with Increased Binding of Ribosomal Proteins with Hdm2**—Ribosome biogenesis stress triggered by low dose of actinomycin D promotes the association of ribosome proteins rpL5, rpL11, and rpL23 with Hdm2; this
association inhibits the E3 ubiquitin ligase activity of Hdm2 thereby promoting accumulation of transcriptionally active p53 (24, 25). UPR activation is accompanied by decreased polysome formation due to eIF2α phosphorylation leading to increased accumulation of free ribosomes (38). Indeed we now demonstrate that UPR induction also triggers increased Hdm2-ribosomal protein binding. Under conditions of PERK activation, it is likely that the reduction in formation of mature polysomes (12), resulting from PERK-dependent eIF2α phosphorylation, promotes a net increase in the concentration of free ribosomal proteins. The free rpL5/11/23 proteins are then available to associate with Hdm2 and thereby inhibit Hdm2-mediated p53 ubiquitination. Consistent with this hypothesis, p53 accumulation and activation were blocked by overexpression of GADD34, which mediates eIF2α dephosphorylation (data not shown), supporting that p53 activation through the ribosome stress pathway is dependent on eIF2α phosphorylation. We also observed nucleolar sequestration of Hdm2 by UPR (Fig. 4G). Our data cannot establish whether Hdm2 associates with nucleolar ribosomal proteins and remains sequestered, or if ribosomal proteins target nucleoplasmic Hdm2 and together shuttle to the nucleolus. However, in support of the latter notion, ribosome protein rpL11 overexpression can drive Hdm2 into the nucleoli and the nucleolar relocation of Hdm2 by rpL11 depends on both the nucleolus localization signal in Hdm2 and rpL11 binding to Hdm2 (26). We also observed that rpL5 overexpression drove nucleolar sequestration of Hdm2 (data not shown). By analogy with rpL11 recruitment of Hdm2, the rpL5 recruitment of Hdm2 into the nucleoli could be mediated by increased rpL5-Hdm2 interaction. Our data are consistent with a model wherein PERK-dependent eIF2α phosphorylation triggers increased accumulation of free ribosomal proteins that in turn associate with Hdm2 resulting in its inhibition and subsequent p53 accumulation and activation (Fig. 6).

**Contribution of the UPR to Cell Cycle Arrest following Nutrient Limitation**—To assess the physiological significance of p53 activation by the UPR, we examined whether the G1/S cell cycle arrest in response to UPR is p53-dependent (36). Both glucose restriction and tunicamycin promote cell cycle arrest at the G1/S boundary in the p53+/+ MEFs; however, this G1/S arrest was largely attenuated in p53−/− MEFs, suggesting the UPR-triggered G1/S checkpoint is p53-dependent. This is consistent with recent work demonstrating that activation of S'-AMP-activated protein kinase (AMPK) contributes to glucose restriction-mediated p53 phosphorylation on Ser-15 (39). Our data are consistent with a model wherein glucose deprivation activates p53 via two mechanisms: AMPK-mediated p53 phosphorylation and PERK-mediated inhibition of p53 polyubiquitination and degradation. This p53-dependent cell cycle arrest by glucose restriction requires the p53 downstream target p21Cip1, because G1/S arrest is abolished in cells lacking p21Cip1. Taken together, these data strongly suggest that p53 activation plays a critical role in cell cycle arrest in response to glucose restriction-triggered UPR.

Recently published work has suggested that ER stress accelerates p53 degradation within 2–4 h of ER stress treatment (32, 40). We also observed a modest decrease in p53 immediately following tunicamycin treatment of HCT116 cells (see Fig. 1A), but p53 accumulation was clearly evident at later time points. Consistent with our observations of p53 activation, Li et al. (41) found that p53 was elevated by 16 h in multiple lines of MEFs and MCF-7 cells in response to tunicamycin or thapsigargin and suggested that p53 functions to promote apoptosis upon chronic UPR activation. We have also observed p53-dependent apoptosis in primary MEFs during chronic exposure of cells to ER stress (data not shown). Therefore, p53 triggers apoptosis at a later stage after cells fail to remedy homeostasis during cell cycle arrest. Taken together, these data suggest that p53 plays a critical role in the cellular response to ER stress.

In summary, our data strongly suggest that perturbation of the ribosome biogenesis pathway plays an essential role in coupling the UPR to cell cycle regulation in response to glucose deprivation. Hdm2/p53 signaling mediates the cross-talk between ribosome biogenesis and the cell cycle. In future work, it will be important to evaluate how Hdm2/p53 signaling contributes to pathological conditions wherein the UPR is implicated.

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UPR-dependent Activation of p53

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Ribosomal Stress Couples the Unfolded Protein Response to p53-dependent Cell Cycle Arrest

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