



# Induction of REDD1 gene expression in the liver in response to endoplasmic reticulum stress is mediated through a PERK, eIF2 $\alpha$ phosphorylation, ATF4-dependent cascade

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## ABSTRACT

Since the endoplasmic reticulum (ER) plays a vital role in hepatocyte function, it is not surprising that a variety of liver-related diseases are associated with ER stress. As in other tissues, ER stress in the liver leads to generation of the unfolded-protein response resulting in activation of a transcriptional program that promotes restoration of homeostasis within the lumen of the ER. Previous studies using cells in culture demonstrated that ER stress induces expression of REDD1 (regulated in development and DNA damage responses), a potent repressor of signaling through the protein kinase referred to as the mechanistic target of rapamycin in complex 1 (mTORC1). In the present study, the results from the cell culture experiments were extended to show that tunicamycin-mediated ER stress in the liver in vivo also induces REDD1 gene expression. Moreover, the induction of REDD1 gene expression was shown to require the protein kinase PERK and enhanced phosphorylation of its substrate, the  $\alpha$ -subunit of eukaryotic initiation factor 2.

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## 1. Introduction

The protein referred to as regulated in development and DNA damage responses (REDD1; aka DNA-damage-inducible transcript 4, DDIT4) is rapidly induced in response to a wide variety of conditions including hypoxia [1], oxidative stress [2], glucocorticoid treatment [3], food deprivation [4], and conditions that deplete intracellular ATP [5] or cause DNA damage [2]. More recent studies (e.g. [6,7]) have shown that REDD1 expression is also induced under conditions that promote endoplasmic reticulum (ER) stress in cells in culture through increased expression of the transcription factor, ATF4 (activating transcription factor 4). Although its exact mechanism of action is unclear, REDD1 has been shown to repress signaling through a pathway involving the protein kinase known as

the mechanistic target of rapamycin (mTOR) in complex 1 (mTORC1) [1]. Because activation of mTORC1 can promote ER stress [8], induction of REDD1 expression is thought to represent a feed-back mechanism to limit mTORC1 signaling, and thereby constrain the ER stress response.

The ER plays a vital role in liver function not only because of the large number and quantity of proteins and lipoproteins synthesized in and secreted from the organ, but also because lipid metabolism and detoxification of chemicals depend upon it [9]. Thus, it is not surprising that ER stress in the liver has been linked to a variety of diseases including viral hepatitis B and C infection, alcohol-induced injury, and acetaminophen-induced toxicity [10,11]. Animal models of obesity [12] and obese humans [13] also display ER stress and this is attenuated by the selective mTORC1 inhibitor rapamycin [14]. The latter observation suggests a possible role for mTORC1 in the generation of ER stress in the liver. However, whether or not ER stress might modulate mTORC1 signaling in the liver through alterations in REDD1 expression is unknown.

The generation of ER stress leads to enhanced signaling through three independent pathways originating at the ER membrane: activation of the eukaryotic initiation factor 2 $\alpha$  (eIF2  $\alpha$ ) kinase PERK-like ER-localized kinase (PERK, aka eIF2AK3), transport of activating transcription factor 6 (ATF6) to the Golgi where it is cleaved to release the transcriptionally active 50 kDa cytosolic N-terminal domain, and activation of the inositol-requiring enzyme 1 (IRE1, aka ER to nucleus signaling 1) [15]. Activated IRE1 removes a

**Abbreviations:** ASNS, asparagine synthetase; ATF, activating transcription factor; DDIT4, DNA-damage-inducible transcript 4; ER, endoplasmic reticulum; eIF2, eukaryotic initiation factor 2; eIF2  $\alpha$ ,  $\alpha$ -subunit of eIF2; GADD34, growth arrest and DNA damage-inducible protein; IRE1, inositol-requiring enzyme 1; mTOR, mechanistic target of rapamycin; mTORC1, mTOR in complex 1; PERK, PKR-like ER-localized kinase; REDD, regulated in development and DNA damage responses; XBP1, X-box binding protein 1.

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26-base domain from the mRNA encoding X-box binding protein 1 (XBP1), generating the active form of the transcription factor [16]. Phosphorylation of eIF2 $\alpha$  by PERK results in repression of global rates of protein synthesis, but also leads to enhanced translation of a few mRNAs containing upstream open reading frames in their 5'-non-coding regions; one such mRNA encodes the transcription factor ATF4 [17]. Combined, activation of the three transcription factors, ATF4, ATF6, and XBP1, induces the expression of proteins that function to relieve the stress in the ER. One such protein is GADD34 (growth arrest and DNA damage-inducible protein 34), a regulatory subunit of protein phosphatase 1 (PP1) that targets the phosphatase to eIF2 $\alpha$  leading to its dephosphorylation and consequently to restoration of protein synthesis [18,19].

In the present study, we assessed the effect of ER stress on REDD1 expression in the liver by treating mice with a well-characterized initiator of ER stress, tunicamycin [20]. We show that tunicamycin treatment generates ER stress in the liver as assessed by enhanced phosphorylation of the  $\alpha$ -subunit of eIF2 and enhanced splicing of the mRNA encoding XBP1, concomitant with induction of REDD1 mRNA expression. The effects of tunicamycin on eIF2 $\alpha$  phosphorylation and REDD1 expression were attenuated in mice ectopically expressing the active domain of GADD34 in the liver as well as in mice lacking the eIF2 $\alpha$  kinase, PERK, indicating that ER stress acts through a mechanism involving PERK-mediated eIF2 $\alpha$  phosphorylation to induce REDD1 gene expression.

## 2. Materials and methods

### 2.1. Materials

Tunicamycin was purchased from Sigma–Aldrich (St. Louis, MO). Primers for quantitative real-time PCR (qRT-PCR) were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Anti-phosphorylated eIF2 $\alpha$  (Ser51) antibody was obtained from Cell Signaling Technology Inc. (Danvers, MA) and the anti-eIF2 $\alpha$  monoclonal antibody was purified in house from cell culture medium obtained from dishes containing hybridoma cells provided by the late Dr. E.C. Henshaw [21].

### 2.2. Animals

All studies involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University College of Medicine. GADD34 transgenic mice and PERK knockout mice and their corresponding wild-type littermates were a kind gift from Dr. David Ron (University of Cambridge Metabolic Research Laboratories, Cambridge, UK) and Dr. Douglas R. Cavener (Department of Biology, The Pennsylvania State University, PA), respectively. Mice weighing approximately 20–25 g were administered 1  $\mu$ g tunicamycin (50  $\mu$ g/ml in 5% dextrose in sterile water)/g body weight intraperitoneally [22]. Two or four hours later, mice were deeply anesthetized using isoflurane and livers were quickly removed. A portion of the liver was frozen between aluminum blocks pre-cooled to the temperature of liquid nitrogen and the remainder was homogenized as described previously [23].

### 2.3. Western blot analysis

Phosphorylation of eIF2 $\alpha$  on Ser51 was assessed by a slight modification of the method described previously [7]. Briefly, liver supernatants were resolved by SDS–polyacrylamide gel electrophoresis on Bio-Rad Criterion 4–15% gels, and proteins in the gel were subsequently transferred to PVDF membrane. Blots were

incubated with an anti-phospho-eIF2 $\alpha$  (Ser51) antibody, and after development, the blots were stripped and re-probed using an antibody that recognizes the protein regardless of phosphorylation state. Results using the anti-phospho-eIF2 $\alpha$  antibody were normalized for the total expression of the protein.

### 2.4. RNA isolation and quantitative real-time PCR (qRT-PCR) analysis

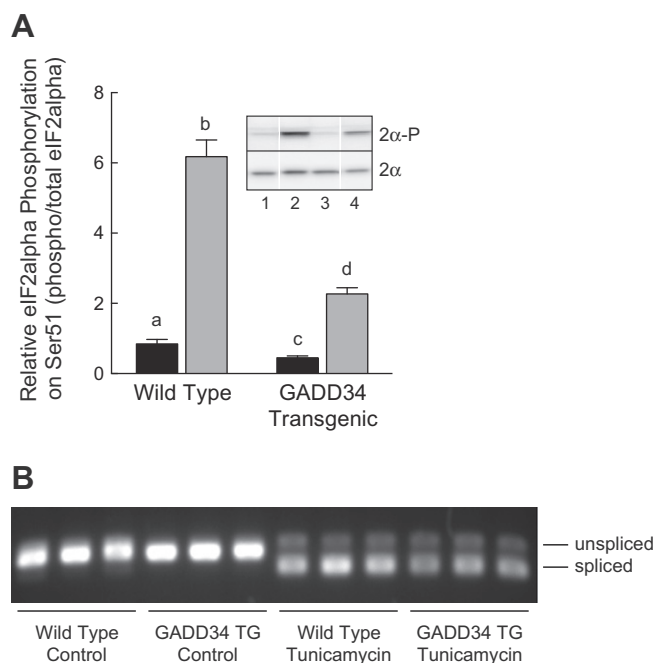
RNA was extracted from liver using TRIzol reagent according to the manufacture's protocol (Invitrogen). qRT-PCR for REDD1 [7], GAPDH [7], and ASNS [24] mRNA quantitation was performed as described previously.

### 2.5. PCR analysis of XBP1 mRNA splicing

Analysis of XBP1 mRNA splicing was performed as described previously [25]. The PCR products were resolved on a 3% agarose gel containing Sybr Safe DNA Gel Stain (Life Technologies, Grand Island, NY) along with a 1 Kb Plus DNA Ladder (Life Technologies).

## 3. Results

In order to reproducibly generate ER stress in the liver, mice were administered tunicamycin, a nucleoside antibiotic that inhibits the first step in glycoprotein synthesis, leading to generation of ER stress [20]. To assess the effectiveness of the antibiotic in generating ER stress in the liver in vivo, eIF2 $\alpha$  phosphorylation on Ser51 was assessed by Western blot analysis. Compared to wild-type mice, basal phosphorylation of eIF2 $\alpha$  was reduced in transgenic mice ectopically expressing the active domain of GADD34 in the liver (Fig. 1A), consistent with its role in targeting PP1 to eIF2 [18,19]. In contrast, the relative phosphorylation of eIF2 $\alpha$  on



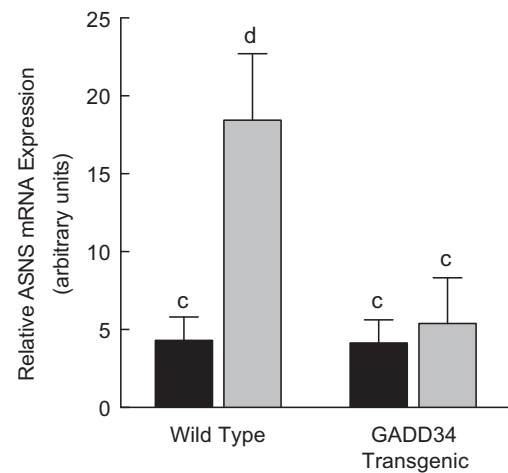
**Fig. 1.** Tunicamycin-generated ER stress is attenuated in GADD34 transgenic compared to wild-type mice. Mice were treated with tunicamycin (grey bars) or vehicle (black bars) for 2 h and the liver was then removed and analyzed for (A) eIF2 $\alpha$  phosphorylation and (B) XBP1 mRNA splicing by Western blot and PCR analysis, respectively, as described under Section 2. A typical Western blot is depicted in an insert in panel A. All samples were analyzed on the same gel, but not in contiguous lanes; white lines separate non-contiguous lanes.  $N=9-10$  per condition. Bars not sharing the same letter are significantly different,  $p < 0.05$ .

Ser51 was more than 3.5-fold greater in the liver of wild-type mice administered tunicamycin compared to mice administered vehicle alone. Moreover, in GADD34 transgenic mice, tunicamycin-mediated eIF2 $\alpha$  phosphorylation was significantly reduced compared to wild-type mice. One possible explanation for the attenuated response of eIF2 $\alpha$  phosphorylation in the transgenic mice is that tunicamycin was less effective in generating ER stress in transgenic compared to wild-type animals. To assess this possibility, a second biomarker of ER stress, splicing of XBP1 mRNA [16], was assessed. As shown in Fig. 1B, in the absence of tunicamycin treatment, the majority of the XBP1 mRNA was in the inactive, unspliced form in the liver of both wild-type and GADD34 transgenic mice. Importantly, tunicamycin treatment was equally effective in promoting a shift from the unspliced to the spliced form in wild-type and transgenic animals, demonstrating that the response to the antibiotic was equivalent in the two genotypes.

In the liver of wild-type mice, tunicamycin treatment produced an 11-fold induction in REDD1 mRNA expression (Fig. 2A). In contrast, in GADD34 transgenic mice the response was greatly attenuated, suggesting that the induction was a consequence of eIF2 $\alpha$  phosphorylation. Qualitatively similar results were observed four hr after tunicamycin administration (Fig. 2B). Thus, the tunicamycin-induced increase in REDD1 mRNA expression was significantly greater in wild-type compared to GADD34 transgenic mice after both two and four hours of treatment.

In cells in culture, ER stress induction of REDD1 mRNA expression has been linked to the transcription factor ATF4 [6,7]. Unfortunately, we have been unable to find an antibody that reliably detects the ATF4 protein in mouse liver supernatants, so possible changes in its expression could not be assessed. Instead, as a surrogate marker for ATF4 upregulation, expression of the well-characterized ATF4 target, asparagine synthetase [26], was assessed. As shown in Fig. 3, tunicamycin treatment led to a 4-fold induction in asparagine synthetase mRNA expression compared to control mice. Moreover, no change in expression was observed in GADD34 transgenic mice.

The results described above are consistent with the idea that the induction of REDD1 expression is a consequence of eIF2 $\alpha$  phosphorylation. However, an alternative explanation is that GADD34 is targeting PP1 to a protein other than eIF2 that is able to modulate REDD1 gene transcription. To confirm that the induction of REDD1 expression was a result of elevated eIF2 $\alpha$  phosphorylation, the effect of tunicamycin was assessed in mice lacking the ER-resident eIF2 $\alpha$  kinase, PERK. In wild-type mice, tunicamycin treatment caused a significant elevation in eIF2 $\alpha$  phosphorylation,

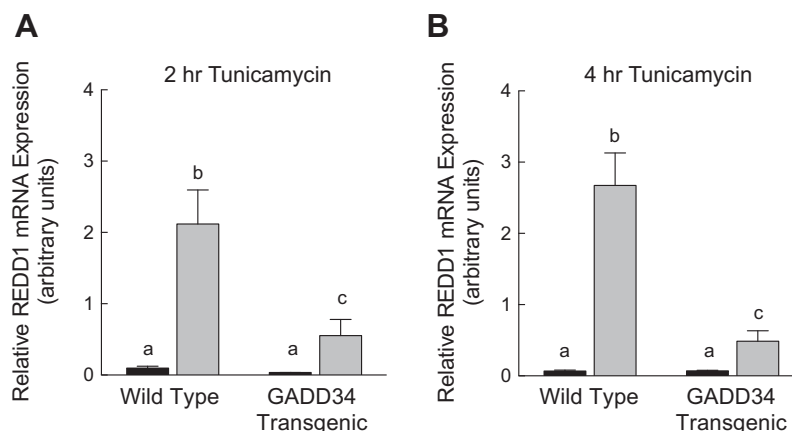


**Fig. 3.** ER stress-mediated induction of asparagine synthetase mRNA expression is attenuated in GADD34 transgenic compared to wild-type mice. Mice were treated with tunicamycin (grey bars) or vehicle (black bars) for 2 h and the liver was then removed and analyzed for asparagine synthetase (ASNS) mRNA expression as described under Section 2.  $N = 4-5$  per condition. Bars not sharing the same letter are significantly different,  $p < 0.005$ .

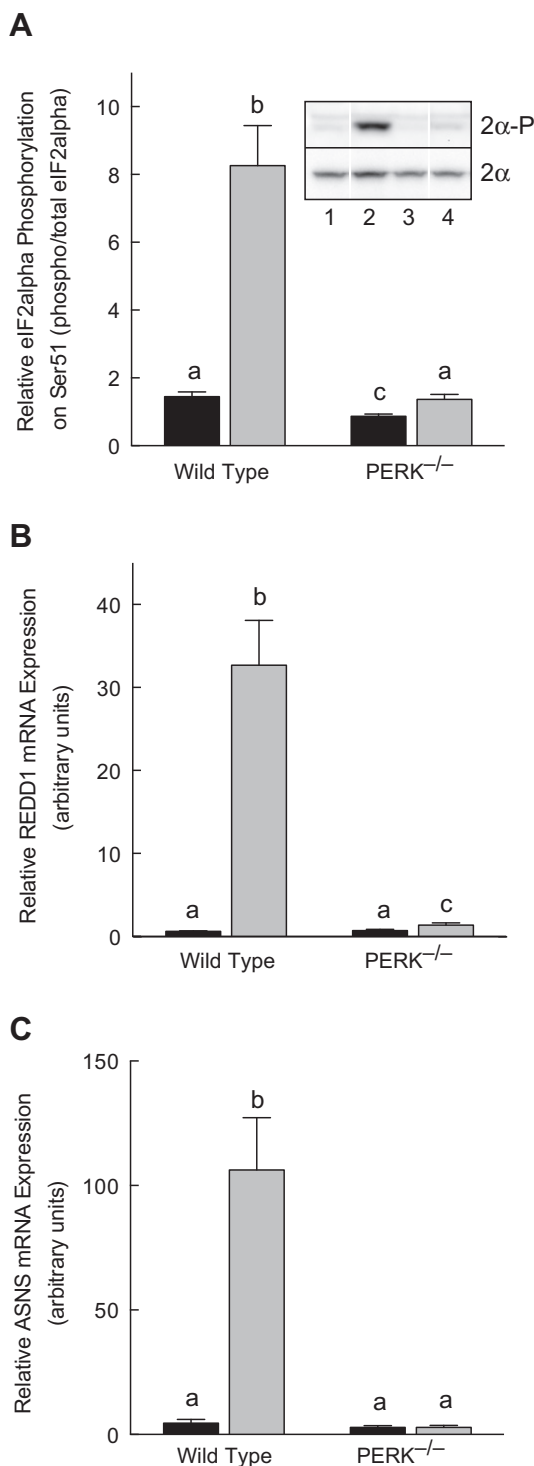
and the effect was almost completely eliminated in PERK $^{-/-}$  mice (Fig. 4A). Similarly, REDD1 mRNA expression was dramatically induced in the liver of wild-type mice in response to tunicamycin treatment, but little change was observed in mice lacking the kinase (Fig. 4B). Moreover, in contrast to wild-type mice, tunicamycin had no effect on asparagine synthetase expression in PERK $^{-/-}$  mice (Fig. 4C). Overall, the results strongly suggest that ER stress mediates upregulation of REDD1 expression in the liver through a mechanism involving activation of PERK and elevated eIF2 $\alpha$  phosphorylation.

#### 4. Discussion

In cells in culture, treatment with tunicamycin or thapsigargin leads to the generation of ER stress, as assessed by elevated eIF2 $\alpha$  phosphorylation and splicing of the XBP1 mRNA [7]. Generation of ER stress also results in induction of REDD1 mRNA expression, an effect that is not observed in mouse embryo fibroblasts lacking ATF4 [7] or in HeLa cells in which ATF4 expression is silenced using siRNA [6]. Moreover, exogenous overexpression of ATF4 in mouse embryo fibroblasts leads to induction of REDD1 mRNA expression



**Fig. 2.** ER stress-mediated induction of REDD1 mRNA expression is attenuated in GADD34 transgenic compared to wild-type mice. Mice were treated with tunicamycin (grey bars) or vehicle (black bars) for (A) 2 h or (B) 4 h and the liver was then removed and analyzed for REDD1 mRNA expression as described under Section 2.  $N = 7-8$  per condition. Bars not sharing the same letter are significantly different,  $p < 0.05$ .



**Fig. 4.** ER stress-mediated induction of eIF2α phosphorylation and induction of REDD1 and asparagine synthetase mRNA expression is attenuated in PERK<sup>-/-</sup> compared to wild-type mice. Mice were treated with tunicamycin (grey bars) or vehicle (black bars) for 2 h and the liver was then removed and analyzed for (A) eIF2α phosphorylation, (B) REDD1 mRNA expression, and (C) asparagine synthetase (ASNS) mRNA expression, as described under Section 2. A typical Western blot is depicted in an insert in panel A. All samples were analyzed on the same gel, but not in contiguous lanes; white lines separate non-contiguous lanes.  $N = 14$ –15 (eIF2α),  $N = 13$ –14 (REDD1), or  $N = 13$  (ASNS) per condition. Bars not sharing the same letter are significantly different,  $p < 0.05$ .

ATF4 expression is rapidly induced following generation of ER stress due to phosphorylation of eIF2α by PERK [17]. Phosphorylation of eIF2α represses the translation of most mRNAs, but paradoxically stimulates the translation of mRNAs, such as the one encoding ATF4, that have multiple upstream open reading frames in the 5'-non-coding region [27]. In the present study, REDD1 mRNA expression was induced in wild-type mice in response to treatment with tunicamycin. However, the effect of the drug on REDD1 expression was greatly attenuated in the liver of mice exogenously expressing GADD34 or in mice lacking PERK, such that the reduction in REDD1 expression was directly proportional to the diminution in eIF2α phosphorylation. The possibility that increased eIF2α phosphorylation resulted in induction of ATF4 expression could not be directly tested in this study because of the lack of an antibody that reliably detects the mouse protein. However, ER stress-mediated induction of asparagine synthetase expression, a well-characterized target of ATF4, was increased in proportion to changes in eIF2α phosphorylation. Thus, the results are consistent with a model in which ER stress induces a cascade of events that includes activation of PERK, elevated phosphorylation of eIF2α, enhanced ATF4 mRNA translation, and ATF4-mediated induction of REDD1 gene transcription.

Overall, the results of the present study extend earlier ones using cells in culture to show that ER stress leads to induction of REDD1 gene expression in the liver in vivo. They also extend the earlier studies to show that the ER stress-mediated induction of REDD1 requires both activation of PERK as well as elevated phosphorylation of eIF2α.

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