TBL2 Is a Novel PERK-Binding Protein that Modulates Stress-Signaling and Cell Survival during Endoplasmic Reticulum Stress

Yoshinori Tsukumo1, Satomi Tsukahara1, Aki Furuno1, Shun-iichiro Iemura2, Toru Natsume2, Akihiro Tomida1*

1 Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Koto-ku, Tokyo, Japan, 2 Biomedicinal Information Research Center, National Institute of Advanced Industrial Science and Technology, Koto-ku, Tokyo, Japan

Abstract

Under ER stress, PKR-like ER-resident kinase (PERK) phosphorylates translation initiation factor eIF2α, resulting in repression of global protein synthesis and concomitant upregulation of the translation of specific mRNAs such as activating transcription factor 4 (ATF4). This PERK function is important for cell survival under ER stress and poor nutrient conditions. However, mechanisms of the PERK signaling pathway are not thoroughly understood. Here we identify transducin (beta)-like 2 (TBL2) as a novel PERK-binding protein. We found that TBL2 is an ER-localized type-I transmembrane protein and preferentially binds to the phosphorylated form of PERK, but not another eIF2α kinase GCN2 or ER-resident kinase IRE1, under ER stress. Immunoprecipitation analysis using various deletion mutants revealed that TBL2 interacts with PERK via the N-terminus proximal region and also associates with eIF2α via the WD40 domain. In addition, TBL2 knockdown can lead to impaired ATF4 induction under ER stress or poor nutrient conditions such as glucose and oxygen deprivation. Consistently, TBL2 knockdown rendered cells vulnerable to stresses similarly to PERK knockdown. Thus, TBL2 serves as a potential regulator of the PERK pathway.

Introduction

The unfolded protein response (UPR) is a survival stress response enabling the cell to cope with the accumulation of unfolded proteins in the endoplasmic reticulum (ER), causing ER stress. Three ER-membrane sensor proteins, PERK, activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1), play important roles in the UPR signaling [1,2]. These sensor proteins are activated in response to ER stress and transmit the signals to activate both transcriptional and translational gene expression programs. The UPR occurs under such pathophysiological conditions as hypoxia, nutrient starvation and low pH. The UPR activation has also been seen in some human diseases, including diabetes, neurodegenerative disease and cancer, and their progressions [1–3].

PERK has been known to induce a response that represses general mRNA translation and promotes translation of a subset of mRNAs as represented by activating transcription factor 4 (ATF4) [4,5]. Under conditions where eIF2α is phosphorylated, most mRNA translation is suppressed while translation of a particular subset of mRNAs is increased by activating transcription factor 4 (ATF4) is elevated [6]. ATF4 expression is increased in response to ER stress and a variety of tumor microenvironmental stresses including low glucose, hypoxia, amino acid depletion [7]. In tumors, ATF4 expression is detected in hypoxic- and nutrient-deprived regions where it plays an important role in maintaining metabolic homeostasis and promoting cancer cell survival by transcriptionally regulating amino acid uptake and biosynthesis, autophagy, redox balance and angiogenesis [7]. Thus, the PERK-eIF2α-ATF4 axis is well characterized. However, the existence of the additional effectors of the PERK pathway has not been fully addressed. Here we show transducin (beta)-like 2 (TBL2) as a novel PERK-binding protein.

TBL2 is a ubiquitously expressed protein with a predicted transmembrane region, WD40 repeats, and a coiled coil domain [8]. TBL2 has been associated with some disorders like Williams-Beuren syndrome (WBS), in which the TBL2 gene is typically deleted. Patients with WBS suffer a developmental disorder caused by deletion of 26–28 genes at chromosome 7q11.23 [8,9]. They
exhibit several common features, including cardiovascular abnormality, hypercalcemia, characteristic facial appearance, mental retardation [8–10]. Although the cardiovascular abnormality in WBS has been explained by the loss of an elastin (ELN) allele, the phenotypic consequences of losing other alleles, including the TBL2 gene, are much less clear. The Tbl2 knockout mouse exhibited increased mean body weight, length, and change in bone metabolism [11]. These observations in knockout mice, however, are not necessarily consistent with phenotype in WBS patients. In addition to genetic loss of TBL2, SNP in the human TBL2 is a new player on the PERK signaling pathway.

Using mass spectrometry, we identified TBL2 as a novel PERK-interacting protein. Our experiments revealed that TBL2 is a type 1 ER transmembrane protein and preferentially associates with phospho-PERK. Importantly, TBL2 was involved in induction of ATF4 expression under stress conditions such as glucose/oxygen deprivation and the cell growth. Thus, our results indicate that TBL2 is a new player on the PERK signaling pathway.

Materials and Methods

Chemicals and antibodies

2-Deoxyglucose (Sigma, St Louis, MO), histidinol (Sigma) and DTT (Nacalai Tesque, Kyoto, Japan) was dissolved in distilled, sterilized water. Tunicamycin (Nacalai Tesque) and thapsigargin (Wako Pure Chemical Industries, Osaka, Japan) were dissolved in dimethyl sulfoxide. Hydrogen peroxide was purchased from WAKO. These compounds were added to culture medium, with the solvent being less than 0.5% of the medium’s volume. The following commercially available antibodies were used: rabbit anti-TBL2 and anti-AF4 (ProteinTech, Chicago, IL), anti-PERK, anti-eIF2 alpha (abcam, Cambridge, MA), anti-phospho-PERK (BioLegend, San Diego, CA), anti-phospho-eIF2 alpha (Ser51), anti-calnexin (Cell Signaling Technology, Danvers, MA), anti-KDEL for GRP78 (StressGen, Victoria, BC, Canada) anti-FLAG M2 (Sigma), and HRP- or FITC-conjugated anti-V5 (Invitrogen), HRP-conjugated anti-rabbit or mouse IgG (GE Healthcare BioSciences Corp, Piscataway, NJ).

Cell lines and treatment

We used following cell lines: Human fibrosarcoma HT1080 cells [14], human renal cell carcinoma 786-O cells [14], human embryonic kidney 293T cells [15] and 293 cells (CRL-1573). HT1080 and 786-O cells were maintained in RPMI 1640 medium and 293 and 293T cells in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 100 μg/mL of kanamycin. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO2. Glucose-free RPMI 1640 medium was obtained from Invitrogen (Carlsbad, CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells were placed in a plastic box with Anaero Pack Kenki for Cell serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use. To create hypoxic conditions, cells CA) and supplemented with 10% heat-inactivated fetal bovine serum for experimental use.
Subcellular fractionation

Subcellular fractionation was modified according to a previously reported procedure [17]. 293T cells were harvested, disrupted in buffer A (50 mM Tris-HCl [pH7.5], 5 mM EDTA, 1 mM DTT) using a Dounce-type homogenizer and then centrifuged at 100,000 xg for 1 h at room temperature. The supernatant was mixed with the same volume of 880 mM sucrose-containing buffer A and then centrifuged at 100,000 xg for an additional 1 h to separate the soluble cytosolic fraction (C) in suspension from mitochondria and microscopic fraction (M) in pellet.

Trypsin digestion assay

Trypsin digestion assay was modified according to a previously reported procedure [17]. 293T cells were transfected with pTBL2 (C-terminal V5-tag) and then the cells were disrupted in a buffer (50 mM Tris-HCl [pH7.5], 5 mM EDTA, 1 mM DTT) using a Dounce-type homogenizer. The nucleus was removed by centrifugation, and then the remaining crude cell extract, which contained microsome [M] and cytosol [C] fractions, was digested with 0.25% trypsin solution for 5 min at room temperature. The reaction was terminated by adding equal volume of 4% SDS buffer, and each sample was subjected to immunoblot analysis.

\[^{35}S\]Methionine incorporation assay

293 cells were transfected transiently with stealth siRNA (4 x 10^5/well in a 6-well plate). After 48 h, cells were incubated for 5 min in Met-Cys-free DMEM supplemented with 2 mM glutamine, 10% dialyzed FBS. After pretreatment with thapsigargin for 20 min, the cells were labeled for 20 min using the 100 µCi/ml easy tag EXPRES \[^{35}S\]S protein labeling mix (PerkinElmer, Waltham, MA) in the presence of thapsigargin. Then, each sample was boiled for 5 min in SDS buffer and subjected to SDS-PAGE. After gel drying, the incorporated \(^{35}S\)Met/Cys was visualized with the Typhoon 9410 (GE Healthcare).

Establishment of shRNA-expressing cells

Lentivirus particles encoding individual shRNA (SHC002 shRNA Control, TRCN0000323118, 0000323032, 0000323108, 0000323235 against TBL2, TRCN0000262374 against PERK) were purchased from Sigma-Aldrich. Stable shRNA-expressing 786-O cells were established according to the manufacturer’s protocol and were selected using puromycin.

Measurement of cell growth and viability

Control-, TBL2- and PERK-shRNA-expressing 786-O cells were incubated for 12 h under glucose- or O$_2$-deprived conditions or under both. Immediately thereafter, cells were resedeed onto 12-well plates and incubated in normal culture medium for 3 days. Relative cell numbers after 3 days were measured using an MTT assay.

Statistical analyses

Statistical analysis was performed using student’s t-test. We considered a P-value of <0.05 statistically significant.

Results

TBL2 is an ER-localized type-I transmembrane protein

To address the molecular mechanisms of the PERK signaling pathway, we screened novel PERK-binding partners by analyzing PERK-coprecipitated proteins in transiently PERK-overexpressed 293T cells using direct nano-flow liquid chromatography/tandem mass spectrometry [16]. As a result, we identified TBL2 (also termed WS-beta-TRP, WBSCR13), the function of which is unknown [8]. The SMART protein domain prediction program (http://smart.embl.de/) suggested that TBL2 contained the N-terminal proximal transmembrane region (TM: 9-31aa), the WD40, and the C-terminal coiled coil domains (Figure 1A). In general, the WD40 and the coiled coil domains are known to engage in protein-protein interaction. In addition, hydropathy analysis of TBL2 with the TMHMM algorithm (http://www.cbs.dtu.dk/services/TMHMM/) predicted one transmembrane domain corresponding to 9-31aa (Figure 1A, bottom). We examined the subcellular localization of TBL2 and found it in the ER, as shown by co-localization with PERK and ER-GFP (GFP with an ER localization signal, see Materials and Methods). In contrast, the del 1-31aa TBL2 mutant that lacked the putative TM region exhibited a broadly diffused staining pattern in the cell, suggesting that the TM region functions as ER-anchor region (Figure 1B). We further analyzed the intracellular distribution pattern of TBL2 in a cell fractionation experiment. Consistent with type 1 ER transmembrane proteins PERK and calnexin, TBL2 was enriched in the M fraction, which contains the ER and mitochondria, under both thapsigargin-treated or non-treated conditions (Figure 1C). The TBL2 protein, as well as PERK and calnexin, was also recovered in the N fraction probably because nuclear outer membrane is contiguous with ER membrane (Figure 1C).

To determine the membrane orientation of TBL2, we examined its sensitivity to trypsin digestion using crude cell extracts that contained microsome and cytoplasm (Figure 1D). TBL2, as well as cytoplasmic protein eIF2α, disappeared completely after trypsin treatment, while the ER lumenal segment of calnexin or the ER luminal protein GRP78 was protected from trypsin digestion (Figure 1D). These results strongly suggest that TBL2 localized in the ER via the TM region and that the C-terminal segment (32-447aa) of TBL2 faces the cytoplasm, that is, TBL2 is a type I ER transmembrane protein.

TBL2 interacts with PERK in response to ER stress

To confirm binding of TBL2 to PERK, we performed immunoprecipitation and immunoblotting after cotransfection of PERK and TBL2 plasmids into 293T cells. As shown in Figure 2A, PERK coprecipitated with TBL2 when cells were
treated with thapsigargin, a representative ER stress-inducing agent. Similarly, immunoprecipitation of PERK protein also showed thapsigargin-stimulated binding of endogenous or exogenous TBL2 protein (Figure 2B). The PERK-TBL2 interaction was also stimulated by treatment with other ER stress inducer DTT, but not amino acid starvation-mimicking agent histidinol [18] (Figure 2C). In contrast to PERK, TBL2 did not interact with GCN2, another eIF2α kinase (Figure 2C).

**Preferential binding of TBL2 to phospho-PERK**

As seen in Figure 2, the electrophoretic mobility of the coprecipitated PERK protein corresponded with its autophosphorylated form. To verify whether TBL2 preferentially interacted with phospho-PERK, we investigated the interaction with the PERK kinase-dead form K621A (PERK-KD) or another type-I ER transmembrane kinase, IRE1, which is another important sensor of the UPR [1,2]. As shown in Figure 3A (right panel), immunoprecipitation showed that PERK was dominantly detected in thapsigargin-dependent manner while PERK-KD or IRE1 was not or only faintly detected. Moreover, the interaction with phospho-PERK was confirmed using phospho-specific antibody (Figure 3B). The interaction was also induced by several kinds of ER stress-inducing agents including thapsigargin, tunicamycin, 2-deoxy-glucose, hydrogen peroxide and observed in several cell
Identification of PERK- and eIF2α-binding region

We constructed a number of TBL2 deletion mutants to determine which regions would be required for interaction with PERK (Figure 4A). In this analysis, we also examined whether TBL2 interacts with eIF2α because it is a well-characterized PERK substrate [4,5]. Interestingly, TBL2 also associated with eIF2α under both normal and thapsigargin-treated conditions (Figure 4B right panel, lanes with “WT”). The mutants that lacked part of the WD40 domain, 131-447aa and 1-350aa, completely lost the ability to associate with eIF2α (Figure 4B), suggesting that a large region of the WD40 domain is required for interaction with eIF2α. Similar requirement of large region of WD40 domain for proper activity has been shown in previous reports on WD40 proteins, UAF1 and COP1 [19,20]. Given that the WD40 domain forms a circularized, propeller structure consisting of each blade of WD40 repeats [21], all of the WD40 repeats may be required for correct folding of TBL2. Next, we found that the 32-447aa TBL2 mutant lacking the N-terminal TM region exhibited impaired interaction with phospho-PERK, probably due to its inability to be retained in the ER membrane (Figure 4B and 1B). The 75-447aa mutant completely lost the PERK interaction ability, suggesting that 32-74aa was crucial for phospho-PERK binding (Figure 4B). The C-terminal deletion of TBL2 (the 1-350aa mutant) also weakened the interaction with PERK but the mutant was still able to bind to PERK (Figure 4B). To determine which region binds to phospho-PERK, we constructed the del32-74aa mutant, which lacked the 32-74aa region only, and compared it with the 1-350aa (Figure 4C). Both mutants localized in the ER (Figure 4D). The del32-74aa mutant kept the association with eIF2α but completely lost the ability to interact with phospho-PERK (Figure 4E). In contrast, while the 1-350aa mutant could not associate with eIF2α (Figure 4E), it still had phospho-PERK binding ability (Figure 4E). Therefore, we concluded that TBL2 interacts with phospho-PERK via the 32-74aa region and also associates with eIF2α via the WD40 domain. Thus, TBL2 forms the complex via its distinct regions. In addition, a PERK mutant lacking its cytoplasmic region (PERK-DN) barely bound to TBL2 despite a greater expression levels than those of PERK-WT (Figure S1), suggesting that TBL2 interacts likely with the cytoplasmic region of PERK.

TBL2 knockdown impairs ATF4 induction under stress conditions

Next, we examined whether TBL2 plays a role in the PERK signaling pathway. For this purpose, we conducted knockdown analysis using siRNA against TBL2. In control siRNA transfected cells, eIF2α phosphorylation and ATF4 protein induction were induced in a thapsigargin-dependent manner while PERK knockdown reduced the eIF2α phosphorylation and the following ATF4 protein induction (Figure 5A). TBL2 knockdown also impaired ATF4 induction at the similar level to PERK knockdown; however, it did not affect the stress-induced eIF2α phosphorylation (Figure 5A). Next, we examined whether TBL2 knockdown had an effect on global protein synthesis since PERK-mediated eIF2α phosphorylation leads to translational repression [4,5]. As assessed by [35S]Met/Cys radiolabeling, thapsigargin treatment clearly reduced protein synthesis in control or TBL2 knockdown cells but not in PERK knockdown cells (Figure 5B top and bottom). Thus, TBL2 was unlikely involved in either eIF2α phosphorylation or general translational repression upon ER stress. We also investigated the effects of TBL2 knockdown on XBP1 splicing and GRP78 induction, which are representative down-stream indicators of activation of IRE1 and ATF6 pathways, respectively (Figure S2A and S2B) [22,23]. Both XBP1 splicing and GRP78 induction occurred in TBL2 knockdown cells at a similar level to control cells. Thus, TBL2 appears to be a selective regulator of the PERK pathway.
TBL2 plays an important role in cell growth after exposure to glucose and oxygen deprivation. We also examined the role of TBL2 under low glucose and hypoxic conditions, which are physiological cell conditions observed in the tumor microenvironment or during ischemia and that cause the UPR [2,3,24,25]. As expected, glucose withdrawal (glc(−)) induced a PERK-TBL2 interaction (Figure 6A). Although hypoxia alone did not trigger the interaction at this time point (4 h), hypoxia combined with glc(−) enhanced PERK-TBL2 interaction compared with glc(−) alone (Figure 6A). We examined the response to glc(−) and hypoxia using stably TBL2-shRNA-expressing cells. In control shRNA-expressing cells, the glc(−)/hypoxia combination stimulated a ATF4 protein expression more strongly than each stressor alone (Figure 6B). By contrast, but similarly to the results of siRNA experiments (Figure 5), ATF4 induction in TBL2-shRNA-expressing cells was impaired and the eIF2α phosphorylation was similar level to that in control cells (Figure 6B). In addition, impaired ATF4 induction in TBL2 knockdown cells was observed even in the presence of a proteasome inhibitor MG132 (Figure 6C), suggesting that TBL2 is unlikely involved in protein degradation of ATF4. In contrast to decrease in the expression at the protein level, ATF4 mRNA expression was largely unchanged in TBL2-shRNA-expressing cells compared to control cells (Figure 6D). Thus, TBL2 appears

Figure 3. Preferential binding of TBL2 to phospho-PERK. (A) 293T cells were transiently co-transfected with pTBL2 (V5-tag) and either pFLAG-PERK, pFLAG-PERK(K621A) or pFLAG-IRE1 and then were treated with 300 nM thapsigargin (Tg) for 2 h. The cell lysates were immunoprecipitated with anti-V5 antibody and immunoblotted with anti-FLAG or anti-V5 antibody. (B) 293T cells were transiently transfected with pFLAG-TBL2 and then were treated with 300 nM thapsigargin (Tg), 4 µg/ml tunicamycin (Tu) or 10 mM 2-deoxyglucose (2DG) for 2 h. Endogenous PERK protein was detected with anti-PERK or anti–phospho-PERK antibody. (C) 293T cells were transiently transfected with pFLAG-TBL2 and then were treated with the indicated doses of hydrogen peroxide (H₂O₂) for 4 h. After immunoprecipitation with anti-FLAG antibody-conjugated beads, each protein was immunoblotted with the indicated antibody. (D) 786-O, 293 and 293T cells were transiently transfected with pFLAG-TBL2 and then were treated with 300 nM thapsigargin (Tg) for 1 h. After immunoprecipitation with anti-FLAG antibody-conjugated beads, each protein was immunoblotted with the indicated antibody.

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to mediate the post-transcriptional process of ATF4 expression under glucos(\textsuperscript{2})/hypoxia. We further investigated growth or viability of TBL2-shRNA-expressing cells exposed to stress using several assays, including MTT assay (Figure 7A), ATP-based cell viability assay (Figure 7B) and counting cell numbers (Figure 7C). Consistent with impairment of ATF4 protein induction, TBL2-shRNA-expressing cells exposed to glucos(\textsuperscript{2})/hypoxia stress for 12 hours exhibited delayed growth compared to control cells (Figures 7A). Likewise, the cell viability assay revealed that TBL2-shRNA-expressing cells become more sensitive to thapsigargin treatment than control cells (Figure 7B). In addition, we investigated cell growth by counting cell numbers of each shRNA-expressing cells after exposure to glucos(\textsuperscript{2})/hypoxia stress for 12 hours. TBL2-shRNA-expressing cells showed a delayed growth compared to control cells while each cells proliferated at similar level in the case of non-exposure to stress (Figure 7C left and right). Thus, we found that TBL2 plays an important role in cell protection, especially under low nutrient conditions such as glucos(\textsuperscript{2})/hypoxia.
Discussion

Upon ER stress, activated PERK inhibits protein synthesis by phosphorylating eIF2α while it activates the transcription factor ATF4. ATF4, in turn, activates transcription of a variety of genes to adapt to stress conditions [26]. In this report, we have identified TBL2 as a protein selectively binding to phosphorylated form of PERK on the ER (Figures 1–3). Moreover, we showed TBL2 interacts with phospho-PERK via the 32-74aa region and also associates with eIF2α via the WD40 domain (Figure 4). Furthermore, we have provided evidence suggesting that TBL2 is involved in ATF4 induction and cell growth under stress conditions (Figures 5–7). These findings indicate that TBL2 is a potential regulator of the PERK pathway.

A limited number of effector or regulator of PERK pathway has been reported until now [27–29]. eIF2α is a well-characterized PERK substrate and a subunit of the heterotrimeric protein eIF2, which mediates the binding of methionyl-tRNA to the ribosome in a GTP-dependent manner [27,28]. Phosphorylation of eIF2α inhibits the guanine nucleotide exchange activity of eIF2B by forming a complex with eIF2B [28], thus impairing the eIF2B-mediated recycling of eIF2 and leading to global inhibition of translation. Besides, an ER luminal molecular chaperone, GRP78/BiP has been reported to prevent PERK from autoactivation through binding to ER luminal region of PERK [29]. During ER stress, GRP78/BiP dissociates from PERK, resulting in allowing PERK to oligomerize and autoactivate [29]. On the other hand, TBL2 does not seem to modulate activation of PERK or PERK-mediated eIF2α phosphorylation. Indeed, TBL2 knockdown or its overexpression had little effects on phosphorylation of PERK and eIF2α and, in consistent, on general translational attenuation (Figures 2B, 5 and 6). Nevertheless, TBL2 knockdown impaired ATF4 induction at similar level to PERK knockdown under stress conditions (Figures 5 and 6). Therefore, TBL2 appears to regulate ATF4 induction in a manner that cannot be explained by the conventional PERK-mediated model.

Figure 5. Effects of TBL2 knockdown on the PERK pathway. (A) 293 cells were transiently transfected with non-silencing siRNA, two TBL2 siRNAs (#1, #2) or PERK siRNAs (#1, #2). After 48 h, the cells were treated with 300 nM thapsigargin for 90 min and analyzed by immunoblot analysis. (B) 293 cells were transiently transfected with non-silencing siRNA, two TBL2 siRNAs (#1, #2) or PERK siRNAs (#1, #2). The protein synthesis rate was measured by incorporating [35S]methionine/cysteine. The pulse labeling was carried out during the last 20 min of the 40-min thapsigargin (Tg) treatment (100 or 300 nM). Upper: autoradiography image of SDS-PAGE. Lower panel: TCA precipitation sample was measured using a scintillation counter.

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A recent study identified a small compound ISRIB that selectively inhibits PERK branch, but not IRE1 and ATF6 branches [30]. ISRIB treatment results in suppressing ATF4 induction under ER stress without affecting eIF2α phosphorylation, which has been considered crucial for general translational attenuation and subsequent ATF4 induction [4,30–32]. These observations may imply the presence of additional factors, like TBL2 shown here, that can be involved in PERK-mediated ATF4 induction. At present, it remains largely unknown about precise molecular mechanisms how TBL2 regulates ATF4 induction through the binding to PERK. Importantly, TBL2 also associated with eIF2α via the WD40 domain under both stress and non-stress conditions (Figure 4). Given that TBL2 is an ER-membrane protein, TBL2 would be able to form a complex with eIF2 on the ER. Conceivably, the interaction of the TBL2-eIF2 complex with PERK during ER stress may have a role in facilitating translation of specific targets by locally and spatially enhancing the availability of eIF2 on the ER. In this regard, it would be noteworthy that ATF4 mRNA can be distributed not only in the cytoplasm but also on the ER [33]. Further study on the TBL2 complex will be helpful to understand the mechanism of PERK-mediated gene expression under ER stress conditions.

We have shown, herein, that under ER stress, TBL2 is a new player that can be involved in ATF4 induction of the PERK pathway and can mediate cell survival. Similarly to TBL2 knockdown, depletion of ATF4 also has been reported to reduce cell survival under stress conditions such as glucose or amino acid deprivation, and hypoxia, which are cell conditions seen in solid tumor [24,25]. These phenotypic similarities may imply that TBL2 is involved in tumor cell adaptation to poor nutrient conditions through induction of ATF4. ATF4 expression is not only induced by PERK activation but also by three other cytosolic
eIF2α-kinases (PKR, HRI and GCN2), which are activated under viral infection or nutrient starvation conditions [34–36]. Given that TBL2 preferentially interacts with PERK, but not GCN2, each eIF2 kinase may have TBL2-like unique binding partner. Therefore, our study could provide important information to help elucidate how, under stress conditions, these eIF2α-kinases achieve the translation of specific mRNAs.

Supporting Information

Figure S1 PERK kinase domain is important to bind to TBL2. 293T cells were transiently transfected with pFLAG-PERK-WT or pFLAG-PERK-DN and then were treated with 300 nM thapsigargin (Tg) for 1 h. The cell lysates were immunoprecipitated with anti-FLAG antibody and immunoblotted with the indicated antibody.

Figure S2 TBL2 knockdown has little effects on XBP1 splicing and GRP78 induction. (A) Analysis of XBP1 transcript in TBL2 knockdown cells. The cells were transiently transfected with non-silencing siRNA, TBL2 siRNA or PERK siRNA. After 48 h, the cells were treated with 300 nM thapsigargin for the indicated times. To detect XBP1 mRNA splicing variant, we amplified each cDNA using a specific primer pair that produces amplicon sizes of 441 bp (unspliced form) and 415 bp (spliced form). (B) The cells were transiently transfected with non-silencing siRNA, TBL2 siRNA or PERK siRNA. After 48 h, the cells were treated with 300 nM thapsigargin for the indicated times. Each lysate sample was subjected to immunoblot with the indicated antibody.

Author Contributions

Conceived and designed the experiments: YT AT. Performed the experiments: YT ST AF. Analyzed the data: YT SI TN AT. Contributed reagents/materials/analysis tools: YT ST AF. Contributed to the writing of the manuscript: YT AT.

References

1. Matsumo KI, Trajkovic J, Gisto P, Datta AK, Bhowmick NA, et al. (2007) Loss of PERK in liver confers resistance to steatosis and apoptosis. Mol Cell 28: 619–633.
2. Haze K, Yoshida H, Yanagi H, Yura T, Mori K (1999) Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. Mol Biol Cell 10: 3767–3779.
3. Cohn MA, Kowal P, Yang K, Haas W, Huang TT, et al. (2007) A UAP1-containing multisubunit protein complex regulates the Fanconi anemia pathway. Mol Cell 28: 786–797.
4. Holm M, Hardtke CS, Gaudet R, Deng XW (2001) Identification of a structural motif that confers specific interaction with the WD40 repeat domain of Arabidopsis COP1. EMBO J 20: 118–127.
5. Smith TF, Gratzek C, Saxena K, Neer EJ (1999) The WD repeat: a common architecture for diverse functions. Trends Biochem Sci. 24: 181–185.
6. Yoshida H, Matsu T, Yamamoto A, Okada T, Mori K (2001) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell. 107: 881–891.
7. Kim YJ, Go MJ, Hu C, Hong CB, Kim YK, et al. (2011) Large-scale genome-wide association studies in east Asians identify new genetic loci influencing metabolic traits. Nat Genet 43: 990–995.
8. Pe´rez Jurado LA, Wang YK, Francke U, Cruces J (1999) TBL2, a novel transducin family member in the WBS deletion: characterization of the complete sequence, genomic structure, transcriptional variants and the mouse ortholog. EMBO J 24: 3470–3481.
9. Ye J, Kumanovka M, Hart LS, Sloane K, Zhang H, et al. (2010) The GCCN2-ATF4 pathway is critical for tumour cell survival and proliferation in response to nutrient deprivation. EMBO J 29: 2082–2096.
10. Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD, et al. (2003) Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. Nat Cell Biol 2: 118–127.
11. Kim YJ, Go MJ, Hu C, Hong CB, Kim YK, et al. (2011) Large-scale genome-wide association studies in east Asians identify new genetic loci influencing metabolic traits. Nat Genet 43: 990–995.
12. Cohn MA, Kowal P, Yang K, Haas W, Huang TT, et al. (2007) A UAP1-containing multisubunit protein complex regulates the Fanconi anemia pathway. Mol Cell 28: 786–797.
13. Holm M, Hardtke CS, Gaudet R, Deng XW (2001) Identification of a structural motif that confers specific interaction with the WD40 repeat domain of Arabidopsis COP1. EMBO J 20: 118–127.
14. Smith TF, Gratzek C, Saxena K, Neer EJ (1999) The WD repeat: a common architecture for diverse functions. Trends Biochem Sci. 24: 181–185.
15. Tang T, Li L, Tang J, Li Y, Lin WY, et al. (2010) A mouse knockout library for the writing of the manuscript: YT AT.
16. Tang T, Li L, Tang J, Li Y, Lin WY, et al. (2010) A mouse knockout library for the writing of the manuscript: YT AT.
17. Huang TT, Li S, Huang H, Xu J, Zhang Y, et al. (2008) The GCN2-ATF4 pathway is critical for tumour cell survival and proliferation in response to nutrient deprivation. EMBO J 29: 2082–2096.
18. Chen JJ (2007) Regulation of protein synthesis by the heme-regulated eIF2α kinase, HRI. Biochemistry and Molecular Biology Education. 35: 403–410.
19. Cohn MA, Kowal P, Yang K, Haas W, Huang TT, et al. (2007) A UAP1-containing multisubunit protein complex regulates the Fanconi anemia pathway. Mol Cell 28: 786–797.
20. Holm M, Hardtke CS, Gaudet R, Deng XW (2001) Identification of a structural motif that confers specific interaction with the WD40 repeat domain of Arabidopsis COP1. EMBO J 20: 118–127.
21. Smith TF, Gratzek C, Saxena K, Neer EJ (1999) The WD repeat: a common architecture for diverse functions. Trends Biochem Sci. 24: 181–185.
22. Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K (2001) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell. 107: 881–891.
23. Kim YJ, Go MJ, Hu C, Hong CB, Kim YK, et al. (2011) Large-scale genome-wide association studies in east Asians identify new genetic loci influencing metabolic traits. Nat Genet 43: 990–995.