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TBL2 Associates With *ATF4* mRNA Via Its WD40 Domain and Regulates Its Translation During ER Stress

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ABSTRACT

PKR-like ER-resident kinase (PERK) phosphorylates eukaryotic translation initiation factor 2 α (eIF2α) under endoplasmic reticulum (ER) stress; this results in repression of general translation and induction of specific gene expression, such as activating transcription factor 4 (ATF4). We previously showed that, upon ER stress, transducin (β)-like 2 (TBL2) was an ER-localized transmembrane protein and interacted with PERK and that TBL2 was involved in ATF4 expression and cell survival. Here, we show that TBL2 is able to associate with *ATF4* mRNA and regulate its translation. The RNA-immunoprecipitation analysis using several TBL2 deletion mutants revealed that the WD40 domain was essential for association with *ATF4* mRNA. Importantly, suppression of TBL2 by knockdown or overexpression of the TBL2 mutant with a defective WD40 domain diminished ATF4 induction at the translational level. Thus, our findings indicate that, under ER stress, TBL2 participates in ATF4 translation through its association with the mRNA. J. Cell. Biochem. 117: 500–509, 2016.

KEY WORDS: ER stress; PERK; ATF4

The unfolded protein response (UPR) is a survival stress response that enables a cell to cope with the accumulation of unfolded proteins in the endoplasmic reticulum (ER) lumen [Kaufman, 2002; Ma and Hendershot, 2004; Hotamisligil, 2010]. PKR-like ER-resident kinase (PERK) is an important transducer of the UPR signaling, and it plays a role in reducing the ER protein load by attenuating general translation under ER stress conditions [Harding et al., 2000a,b]. During the UPR, PERK is activated by oligomerization and autophosphorylation, and the activated PERK subsequently phosphorylates eIF2α at Ser-51 [Harding et al., 2000a,b]. Phosphorylation of the eukaryotic translation initiation factor 2 α (eIF2α) reduces the availability of the eIF2-GTP-Met-tRNA^{Met} ternary initiation complex, thus suppressing global translation initiation [Gebauer and Hentze, 2004]. Under eIF2α phosphorylation, translation of most mRNAs was attenuated while translation of a particular subset of mRNAs was enhanced through the action of upstream, open reading frames (uORFs) in the 5′-untranslated regions (5′UTR) [Harding et al., 2000a,b; Calvo et al., 2009].

In mammalian cells, the best-characterized example of uORF-containing mRNA is the transcription factor ATF4, which plays a significant role in regulating the UPR during ER stress, as well as other types of cellular responses, including viral infection and nutrient starvation [Harding et al., 2000b, 2003]. In *ATF4* mRNA, two uORFs (uORF1 and 2) play essential roles in ATF4 protein expression during the UPR [Vattem and Wek, 2004]. When eIF2-GTP is readily available in unstressed cells, the scanning 40S ribosomal

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Yoshinori Tsukumo and Akihiro Tomida conceived and designed the experiments. Yoshinori Tsukumo, Satomi Tsukahara, Aki Furuno, and Shun-ichiro Iemura performed the experiments. Yoshinori Tsukumo, Shun-ichiro Iemura, Toru Natsume, and Akihiro Tomida analyzed the data. Yoshinori Tsukumo, Satomi Tsukahara, and Aki Furuno contributed reagents/materials/analysis tools. Yoshinori Tsukumo and Akihiro Tomida wrote the manuscript.

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subunit launches reinitiation from the downstream, inhibitory uORF2 immediately after uORF1 translation [Harding et al., 2000a, 2003; Vattem and Wek, 2004]. Whereas, when the available functional eIF2-GTP is decreased by eIF2α phosphorylation, the 40S ribosomal subunit can skip the uORF2 and reach the start codon at the main ATF4 ORF, thus producing the ATF4 protein [Harding et al., 2000a, 2003; Vattem and Wek, 2004].

Transducin (β)-like 2 (TBL2) is a ubiquitously expressed protein with a predicted transmembrane region, WD40 repeats, and a coiled coil domain [Pérez et al., 1999]. Although TBL2 dysregulation could be involved in the phenotypes of several diseases, such as Williams-Beuren syndrome and lipidemia [Meng et al., 1998; Pérez et al., 1999; Pober, 2010; Kathiresan et al., 2008; Tang et al., 2010; Kim et al., 2011; Blattmann et al., 2013], the molecular function has been largely unknown. Of note, our recent study revealed that TBL2 is a type I ER transmembrane protein that preferentially interacts with phospho-PERK upon ER stress [Tsukumo et al., 2014]. We have also shown that TBL2 was involved in ATF4 expression induction and cell survival under stress conditions [Tsukumo et al., 2014]. Here, we show that TBL2 is able to associate with *ATF4* mRNA via the WD40 domain. Moreover, overexpression of the TBL2 mutant with the defective WD40 domain or depletion of TBL2 diminished ATF4 induction at the translational level. Our results indicate that TBL2 may be involved in ATF4 translation via its association with the mRNA.

MATERIALS AND METHODS

CELLS AND TREATMENT

For this study, we used human embryonic kidney 293T [Tsukumo et al., 2007] and 293 (CRL-1573) cell lines. 293 and 293T cells were cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum and 100 μg/ml of kanamycin, at 37°C in a humidified atmosphere containing 5% CO_2.

CHEMICALS AND ANTIBODIES

Thapsigargin (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in dimethyl sulfoxide and added to the 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-ATF4 (ProteinTech, Chicago, IL), anti-PERK, anti-eIF2 α, (abcam, Cambridge, MA), anti-phospho-PERK (BioLegend, San Diego, CA), anti-phospho-eIF2 α (Ser51), anti-FLAG M2 (Sigma–Aldrich, Tokyo) and HRP-conjugated anti-rabbit or anti-mouse IgG (GE Healthcare Bio-Sciences Corp, Piscataway, NJ)

PLASMIDS

pFLAG-TBL2 WT or each of the mutants was constructed by ligating each cDNA amplified by RT-PCR at the HindIII/NotI site into the pFlag-CMV-5c vector (Sigma). An ATF4 5′UTR reporter plasmid was constructed in a pRL-TK–based vector, as follows. Renilla luciferase in the pRL-TK vector was replaced by inserting PCR-amplified firefly luciferase into the HindIII/XbaI site and then the PCR-amplified ATF4 5′UTR, which fully covered the uORF1 and 2 (1-376 of ATF4 mRNA), was inserted upstream of the firefly luciferase. Transient transfections were performed using Lipofectamine 2000 (Invitrogen) or Lipofectamine RNAi MAX (Invitrogen), according to the manufacturer's protocol.

siRNAS

Stealth siRNAs against TBL2 (HSS146816) or PERK (HSS114059) were purchased from Invitrogen. For transient transfection of siRNA, cells were seeded at a density of 4×10^5/well in a type I, collagen-coated, 6-well plate and were cultured overnight. The cells were transfected for 6 h with siRNA (10 nM) using the Lipofectamine RNAi MAX reagent, according to the manufacturer's protocol. Then, the cells were reseeded from 1 well to 3 wells. Two days after transfection, the cells were used for experimentation. Sequences are TBL2 siRNA: 5′- UCA UCU UGA AGA CAC GGA GGG UGU C -3′, 5′- GAC ACC CUC CGU GUC UUC AAG AUG A -3′; PERK siRNA: 5′- UUU ACU GUG AAG AAA CUC CAC UGC C-3′, 5′- GGC AGU GGA GUU UCU UCA CAG UAA A-3′.

IMMUNOBLOT ANALYSIS

Immunoblot analysis was performed as described previously [Tsukumo et al., 2007]. Briefly, cells were lysed in 1× SDS sample buffer, and protein concentrations of the lysates were measured with a BIO-RAD protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of proteins were resolved on a 10% SDS-polyacrylamide gel and transferred by electroblotting to a nitrocellulose membrane. Membranes were probed with antibodies, as indicated, and the specific signals were detected using an enhanced chemiluminescence detection system (GE Healthcare Bio-Sciences Corp., Tokyo, Japan).

IMMUNOPRECIPITATION

Immunoprecipitation was performed as described previously [Tsukumo et al., 2007]. Briefly, cells were washed with ice-cold PBS and lysed in 50 mM Tris-HCl (pH 8.0), 1% Triton X-100, 150 mM NaCl, 1 mM EDTA supplemented with protease inhibitors and a phosphatase inhibitor cocktail (Sigma). The lysates were cleared by centrifugation at 13,000× *g* for 10 min at 4°C and immunoprecipitated by anti-FLAG– or anti-V5–conjugated beads (Sigma) in lysis buffer. Immunoprecipitates were prepared for immunoblot analysis by washing three times with lysis buffer and eluting with 3× FLAG peptide (Sigma) or boiling in SDS sample buffer.

CO-PRECIPITATION OF mRNA:PROTEIN COMPLEXES (RIP ASSAY)

HEK293T cells were transfected with TBL2 or the indicated mutant (FLAG-tag) plasmid for 24 h and then lysed for 10 min on ice in NT2 buffer (50 mM Tris–Cl (pH 7.4), 150 mM NaCl, 1 mM MgCl2, 0.3% NP-40, 200 U/ml RNasin (Promega), phosphatase inhibitor cocktails (Sigma) and protease inhibitors (Sigma)). The lysate was cleared from the nuclei by 10 min centrifugation at 3500× *g*. Using Trizol-LS (Invitrogen), 10% of the lysate was removed to isolate the input RNA. The remaining lysate was incubated with anti-FLAG-M2–conjugated beads for 1 h and washed four times with NT2 buffer. TBL2-bound RNA was extracted with 3 volumes of Trizol-LS and precipitated with isopropanol. After washing with 70% ethanol, samples were dried and dissolved in DNase- and RNase-free water.

For relative comparison, we analyzed the abundance of mRNA in the immunoprecipitates, using RT-PCR or qPCR, and normalized that with the total expression level. For comparison of relative binding affinity of *ATF4* or *TOP1* mRNA to TBL2, the background noise, each mRNA abundance from a mock transfection, was subtracted from each mRNA abundance in the TBL2 immunoprecipitate and then the ratio of *TOP1* to *ATF4* was calculated.

RT-PCR AND qPCR ANALYSIS

Total RNA was isolated from cells using the RNeasy Mini Kit with DNase digestion (Qiagen, Tokyo, Japan) and converted to cDNA using SuperScript III reverse transcriptase (Invitrogen). The cDNAs for *ATF4, b-actin* and *TOP1* were then amplified by PCR with PfuTurbo DNA polymerase (Stratagene, La Jolla, CA). PCR products were separated by electrophoresis on 1.2% agarose gels and visualized by ethidium bromide staining. To quantify *ATF4,* β-*actin* or *TOP1* mRNA level, we used the PCR LightCycler 480 (Roche). Reaction volumes of 20 μL contained cDNA, 0.1 μM Universal Probe Library probe (Roche Applied Science), 0.4 μM forward primer, reverse primer and 10 μl 2 × LightCycler 480 Probes Master (Roche Applied Science). After denaturation for 10 min at 95°C, amplification was performed using 35 cycles of denaturation (95°C for 10 s), followed by annealing (58°C for 15 s) and elongation (72°C for 15 s). Results were analyzed using the LightCycler 480 software. cDNA library synthesized from 293T cells used as reference to calculate expression level (CP value) of each mRNAs.

REPORTER ASSAY

293T cells were transfected with a firefly luciferase–containing reporter plasmid (pATF4-uORF-Luc) together with the renilla luciferase–containing plasmid pRL-TK (Promega) as the internal control. Relative activity of firefly luciferase to renilla luciferase (mean ± SD of triplicate determinations) was determined using the Dual-Glo Luciferase Assay System (Promega).

POLYSOME PROFILING

Polysome profiling was analyzed according to a standard method described by Thomas and Johannes [Thomas and Johannes, 2007]. 293 Cells were prepared at 80% confluence. After a 1 h thapsigargin treatment, cells were incubated for 5 min in 0.1 mg of cycloheximide/ml and collected. Then the cells were lysed on ice in 1 ml of polysome extraction buffer (0.3 M NaCl, 15 mM $MgCl_2$, 15 mM Tris-HCl (pH 7.6), 1% Triton X-100, 1 mg of heparin/ml, 0.1 mg of cycloheximide/ml) for 10 min. Nuclei were pelletized at 10,000× *g* for 10 min, and the resulting supernatant was layered onto a 10–50% linear sucrose gradient. The gradients were centrifuged for 3 h at 150,000× *g* in the SW41 rotor (Beckman Coulter, Inc., Tokyo, Japan). Each fraction was manually collected stepwise. RNA was isolated from gradient fractions using 3 volumes of Trizol LS reagent (Invitrogen) and purified with isopropanol and 70% ethanol precipitation. Purified RNA samples were measured using the NanoDrop (Thermo Fisher Scientific, Waltham, MA) and Bioanalyzer (Agilent, Santa Clara, CA). Equal volumes of RNA from a heavy- or light-fraction pool were used for RT-PCR analysis.

STATISTICAL ANALYSES

Statistical analysis was performed using the Student's *t*-test. We considered a *P* value of <0.05 to be statistically significant.

RESULTS

TBL2 ASSOCIATES WITH *ATF4* MRNA

As shown in Figure 1A and in our previous work [Tsukumo et al., 2014], TBL2 interacts with the phosphorylated form of PERK induced when treated with ER stress-inducing agents, thapsigargin (Tg) or DTT. We also found that TBL2 is involved in ATF4 expression at the post-transcriptional level [Tsukumo et al., 2014]. In addition to these findings, previous reports showed that several proteins with WD40 repeats, including TBL2, can function as a potential RNA-binding protein [Lau et al., 2009; Castello et al., 2012]; therefore, we tested whether TBL2 was able to associate with *ATF4* mRNA. For this purpose, we performed an RNA immunoprecipitation (RIP) assay under transient expression of TBL2. In the immunoprecipitates prepared from the TBL2-transfected cells, but not mock (empty) vector, *ATF4* mRNA was detected to a greater extent than *topoisomerase 1 (TOP1)* mRNA, one of the housekeeping genes (Fig. 1B). *GADD34* mRNA, which is upregulated translationally under ER stress as well as *ATF4* mRNA [Novoa et al., 2001; Kojima et al., 2003; Lee et al., 2009], was also detected abundantly in the TBL2 immunoprecipitates (Fig. 1B). As shown by densitometry analysis, the abundance of TBL2-associated *ATF4* mRNA was hardly changed by thapsigargin treatment (Fig. 1C). Moreover, the association of TBL2 with *ATF4* mRNA was confirmed by qPCR analysis (Fig. 1D). Thus, the RIP assay revealed preferential binding of TBL2 with *ATF4* mRNA.

THE WD40 DOMAIN IS REQUIRED FOR TBL2-A*TF4* mRNA ASSOCIATION

We previously identified the PERK-binding region on TBL2 using a series of TBL2 deletion mutants, as shown in Figure 2A [Tsukumo et al., 2014]. Using the same mutants and the same transfection methods as in that study [Tsukumo et al., 2014], we investigated which regions would be required for interaction with *ATF4* mRNA. The 32-447aa mutant exhibited an increased association with ATF4 mRNA compared to the TBL2-WT (Fig. 2B). Although the underlying mechanisms were not known, this increased association might be attributed to the diffused localization of the 32-447aa mutant [Tsukumo et al., 2014], thereby allowing the mutant to associate not only with *ATF4* mRNAs on the ER but also with the mRNAs in cytoplasm. On the other hand, the 131-447aa and 1-350aa mutants partly lacking the WD40 domain lost the ability to associate with *ATF4 mRNA* (Fig. 2B). Thus, a partial deletion of the WD40 domain completely abrogated the mRNA-binding ability of TBL2. In general, the WD40 domain forms a circularized propeller structure, each blade consisting of a WD40 repeat [Smith et al., 1999]; therefore, all of the WD40 repeats would be required to interact with ATF4 mRNA. A similar requirement for the proper activity of the large region of the WD40 domain has been previously reported on the WD40 protein UAF1 [Cohn et al., 2007].

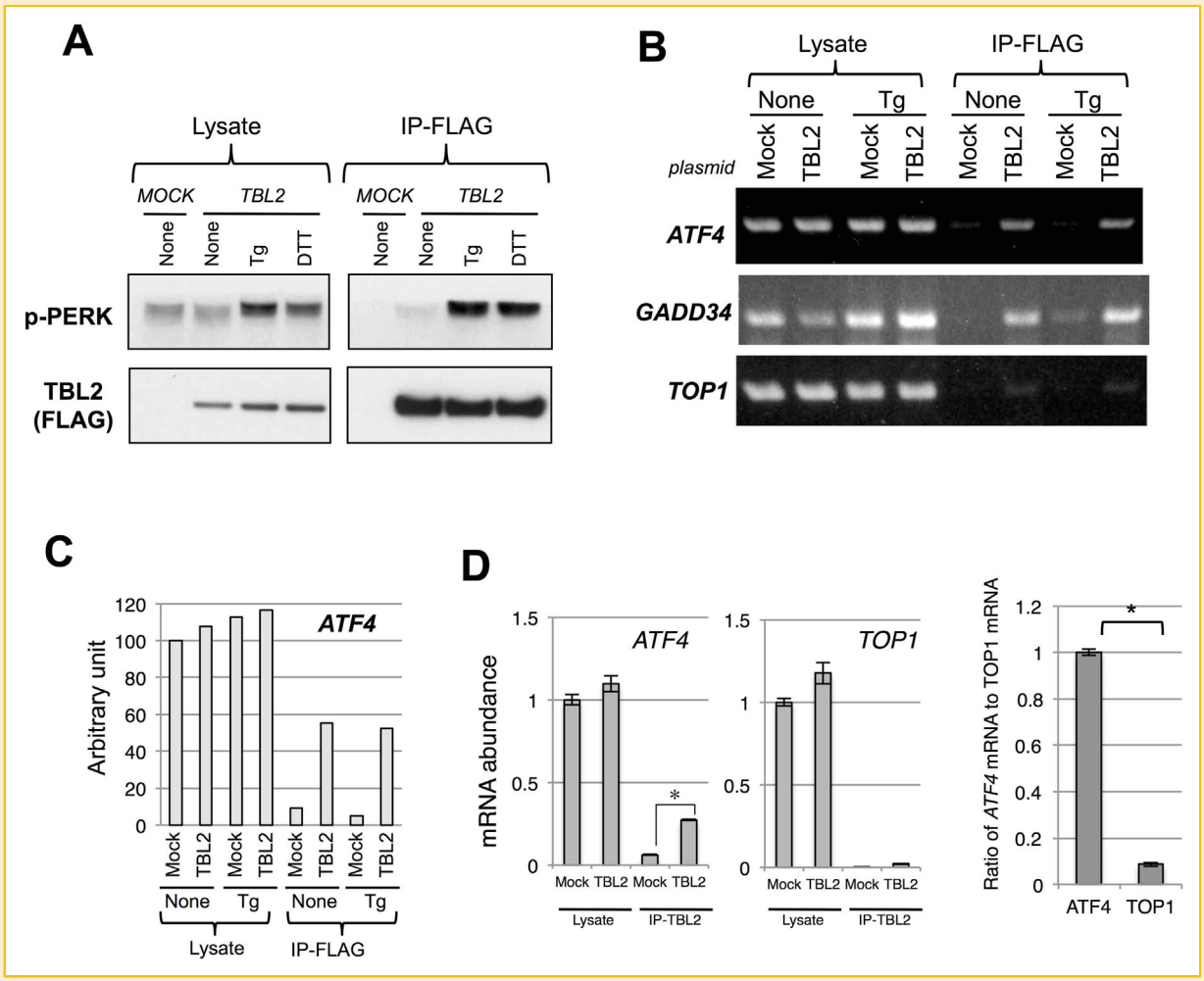

Fig. 1. TBL2 associates with *ATF4* mRNA. (A) 293T cells were transiently transfected with pFLAG-TBL2 and then treated with 300 nM thapsigargin for 1 h, 1 mM DTT for 30 min. After immunoprecipitation with anti-FLAG antibody–conjugated beads, each protein was immunoblotted with the indicated antibody. (B) 293T cells were transiently transfected with pTBL2 (FLAG-tag). After immunoprecipitation of the TBL2 protein, the immunoprecipitates were treated with proteinase K, and the associated RNAs were extracted by Trizol LS. Total or TBL2-associated *ATF4, GADD34* or *TOP1* mRNA was analyzed by RT-PCR (B) or qRT-PCR (C). (C) The *ATF4* and *TOP1* mRNA abundance in the immunoprecipitates was normalized with their abundance in a lysate sample of mock-transfected cells. (C, right panel). To compare TBL2-binding affinity between *ATF4* and *TOP1* mRNA, background noise (mRNA abundance in immunoprecipitates from mock transfection) was subtracted from the related mRNA abundance in each TBL2 immunoprecipitate. The ratio of *TOP1* to *ATF4* mRNA in the immunoprecipitates was shown ($^{*}P < 0.01$, n = 3).

OVEREXPRESSION OF THE TBL2 MUTANT PARTLY LACKING WD40 DOMAIN BUT REMAINING THE PERK-BINDING REGION REDUCES ATF4 PROTEIN INDUCTION

Next, we examined whether overexpression of TBL2 mutants affected expression of ATF4 protein. For this purpose, we used the luciferase reporter gene under control of ATF4-5′UTR because ATF4 expression is regulated by the uORFs in its 5′UTR (Fig. 3A) [Vattem and Wek, 2004]. Similar to previous reports [Harding et al., 2000a, Vattem and Wek, 2004], thapsigargin treatment increased the luciferase activity by more than 4-fold (Fig. 3B). Of the TBL2 mutants we used, only 1-350aa suppressed luciferase activity (Fig. 3B). Moreover, the 1-350aa mutant, as well as a PERK dominant-negative mutant (PERK-DN), suppressed thapsigargin treatment–induced luciferase activity in proportion to its expression level (Fig. 3C). Such suppressive effects of the TBL2 1-350aa mutant on ATF4 expression were also confirmed by immunoblot analysis (Fig. 3D).

The 1-350aa mutant lost its ATF4 mRNA-binding ability but still contained the PERK-binding region (32-74aa), as shown in our previous study [Tsukumo et al., 2014]. Therefore, we hypothesized that overexpression of the mutant might have blocked interaction between the intact TBL2-ATF4 mRNA complex and PERK, thus suppressing ATF4 induction (Fig. S1). To confirm this, we used the TBL2 1-179aa mutant, which has a PERK-binding region but lacks a large part of the WD40 domain (Fig. 4A). This mutant was localized to the ER as well as TBL2 (Fig. 4B). Immunoprecipitation analysis showed that the mutant's overexpression impaired the thapsigargin-stimulated interaction of TBL2 with endogenous PERK (Fig. 4C). Thus, overexpression of the mutant with a defective WD40 domain prevented the interaction of intact TBL2 and PERK during ER stress

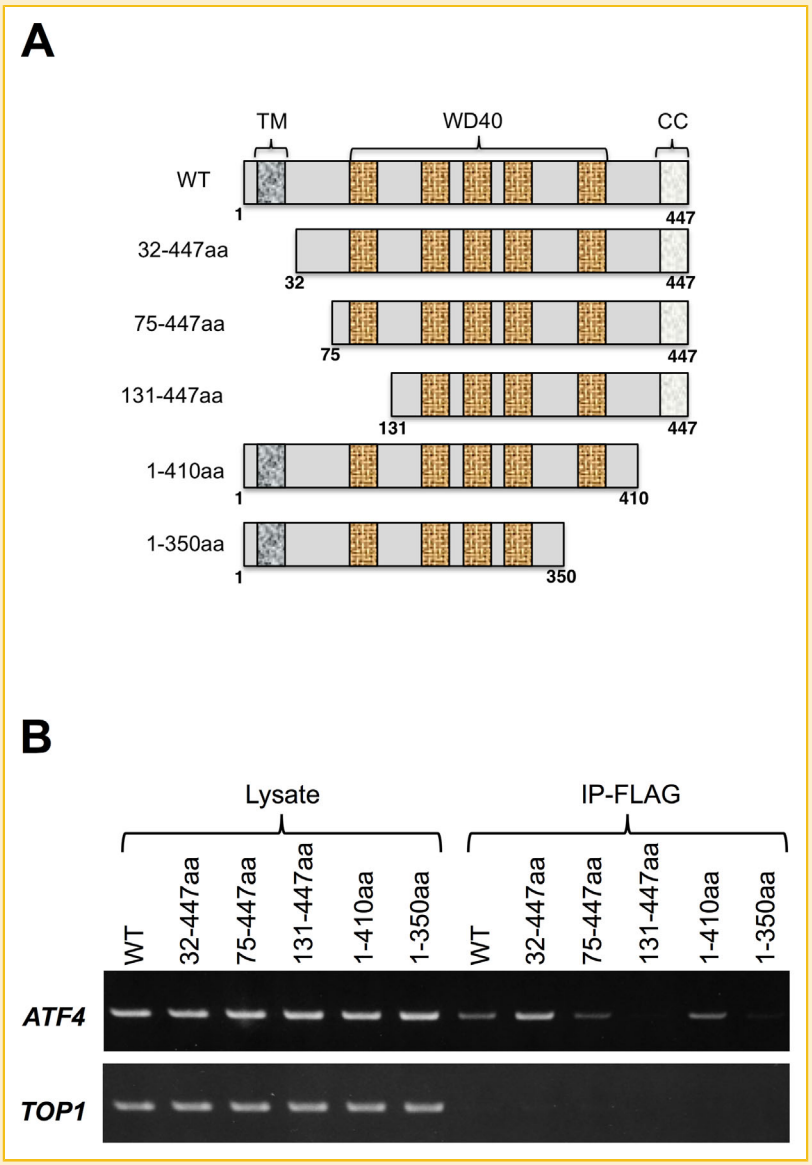

Fig. 2. The WD40 domain is required for TBL2–*ATF4* mRNA association. (A) Schematic representations of each TBL2 mutant. (B) 293T cells were transiently transfected with each TBL2 mutant plasmid. After immunoprecipitation of the TBL2 protein, the immunoprecipitates were treated with proteinase K and the associated RNAs were extracted by Trizol LS. Total or TBL2-associated *ATF4* and *TOP1* mRNA was analyzed by RT-PCR.

corresponding to suppression of ATF4 protein induction, suggesting that the TBL2-PERK interaction may be important for ATF4 protein induction (Fig. S1).

TBL2 KNOCKDOWN IMPAIRS ATF4 INDUCTION AT THE PROTEIN LEVEL

To examine whether TBL2 mediates ATF4 protein induction, we tried a knockdown analysis using siRNA. TBL2 depletion using siRNA suppressed ATF4 protein induction upon thapsigargin treatment at a similar level to PERK depletion, while TBL2 depletion had little effect on eIF2α phosphorylation (Fig. 5A and S2). We also examined whether TBL2 depletion affected *ATF4* mRNA expression. Upon thapsigargin treatment, *ATF4* mRNA expression was increased by approximately 2-fold, but there was no significant difference in *ATF4* mRNA expression between the control and the TBL2-depleted cells (Fig. 5B).

TBL2 KNOCKDOWN DIMINISHES ATF4 TRANSLATION BUT NOT GENERAL TRANSLATION UNDER ER STRESS CONDITIONS

To see the effect of TBL2 depletion on translation, we performed polysome profiling using sucrose density gradient centrifugation. Under normal cell conditions, polysome peaks were observed in heavier fractions (nos. 14–16) in each of the controls, TBL2- and PERK-depleted cells, indicating that these cells are actively translating (Fig. 6A–C, open circles). Upon thapsigargin treatment, the polysome peaks in both the control and TBL2-depleted cells were

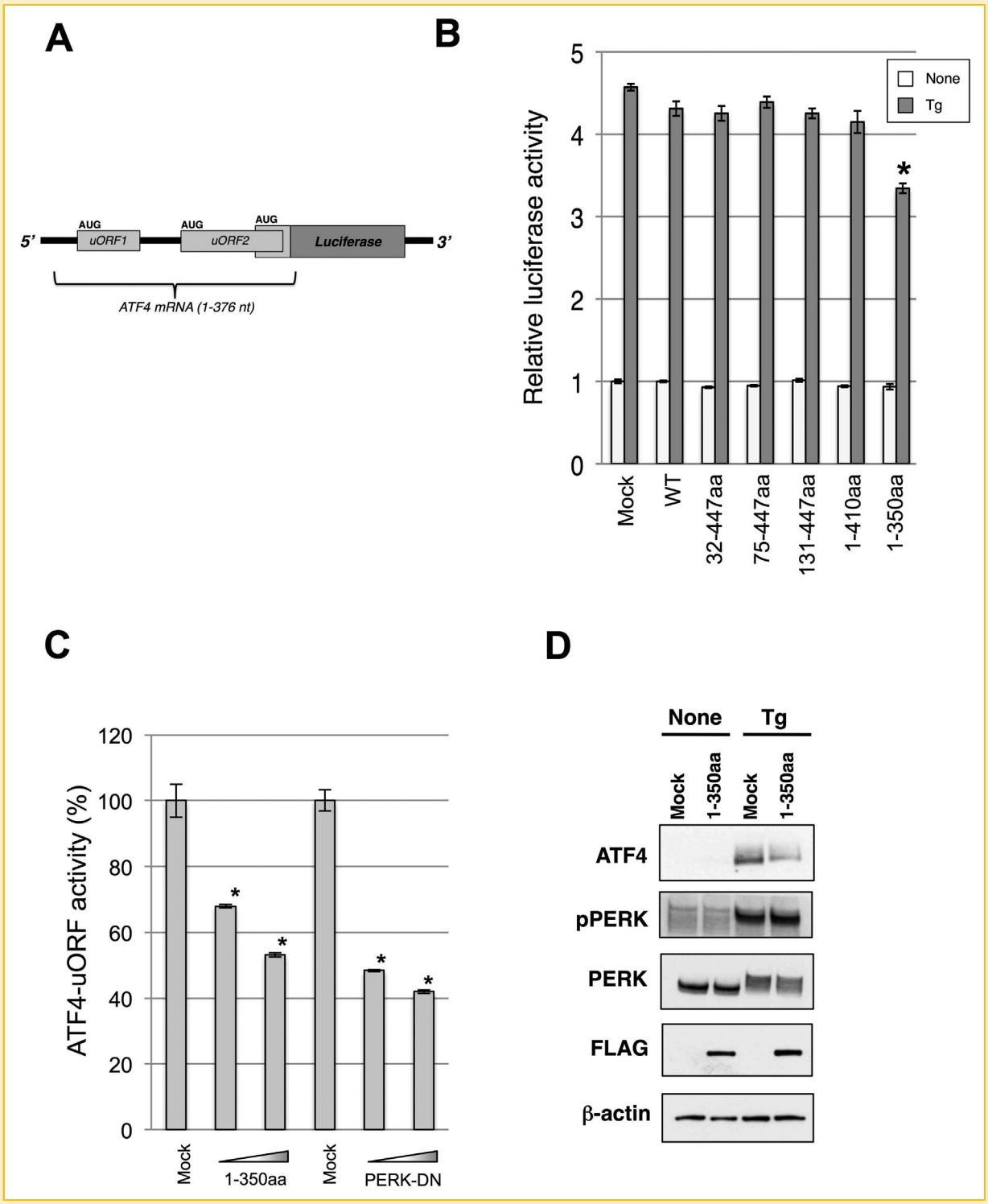

Fig. 3. Overexpression of the TBL2 mutant 1–350aa reduces ATF4 protein induction. (A) Schematic representation of the ATF4–uORF–luciferase reporter gene. (B, C) 293T cells were transiently transfected with the indicated TBL2 mutant plasmid (B) or 0.02, 0.08 μg of pTBL2 (1–350aa) or 0.02, 0.08 μg of pPERK–DN (C). Reporter plasmids pATF4–uORF–Luc and pRL–TK as the internal control were cotransfected. The cells were then treated with thapsigargin (300 nM) for 6 h. Luciferase activity was measured using a dual luciferase assay kit. (D) 293T cells were transiently transfected with a TBL2 1–350aa mutant plasmid and then treated with 30 nM thapsigargin for 1 h.

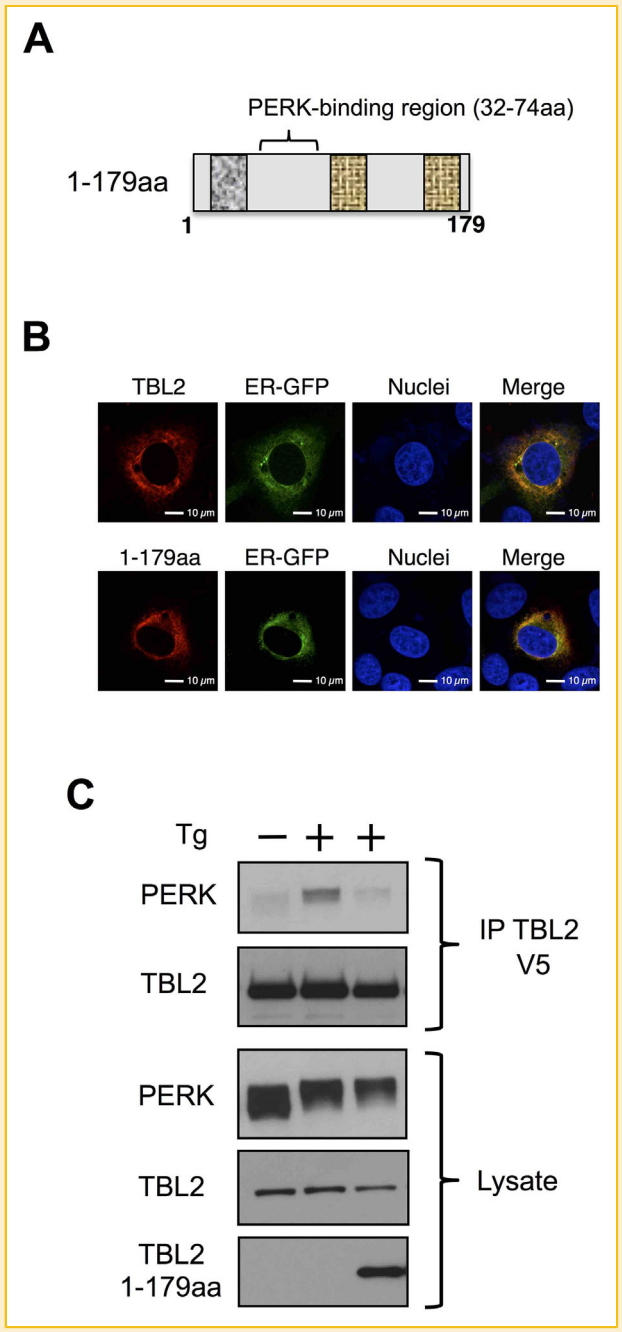

Fig. 4. Overexpression of the TBL2 mutant 1-179aa prevents the interaction between PERK and TBL2. (A) Schematic representation of the TBL2 mutant (1-179aa) (B) HT1080 cells were transiently transfected with pFLAG-TBL2 or pFLAG-1-179aa and pShooter ER-GFP plasmid and then were fixed and analyzed by immunofluorescence using a confocal microscope. (C) 293T cells were transiently transfected with pV5-TBL2-wt (full length) alone or together with the TBL2 1-179aa mutant plasmid (FLAG-tag) for 24 h. Then the cells were treated with 300 nM thapsigargin for 1 h. After immunoprecipitation using anti-V5–conjugated beads, each sample was subjected to immunoblot analysis using the indicated antibodies.

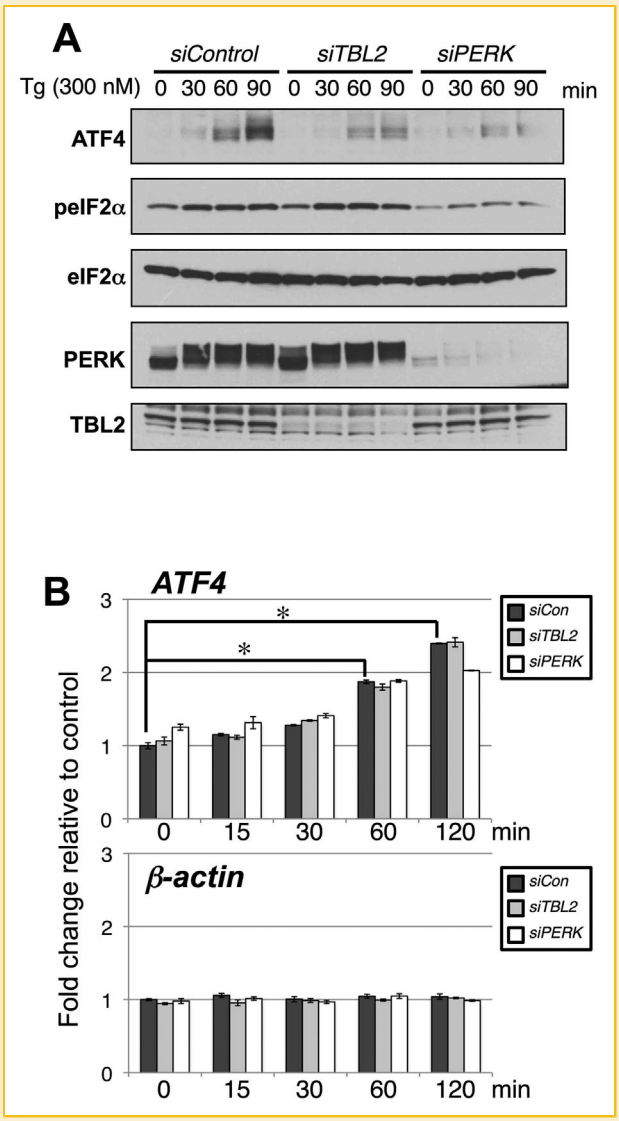

Fig. 5. TBL2 knockdown impairs ATF4 induction at the protein level. (A, B) 293 Cells were transiently transfected with non-silencing siRNA, TBL2 siRNA or PERK siRNA. After 48 h, the cells were treated with 300 nM thapsigargin for the indicated times. Each sample was subjected to immunoblot analysis (A) or each mRNA expression level of ATF4 and β-actin was measured by qRT-PCR ($^{*}P < 0.01$, n = 3) (B). Each expression level was calculated on the basis of the expression in non-treated siCon sample.

reduced, and the subpolysome peaks in the light fractions (nos. 4–6) were increased, suggesting that general translation was attenuated in these cells (Fig. 6A, B, closed circle). On the other hand, in PERK-depleted cells, changes in polysome and subpolysome peaks were mild (Fig. 6C), indicating that the cells continued to translate. Thus, consistent with the little effect on eIF2α phosphorylation, TBL2 depletion hardly affected general translation attenuation under ER stress conditions.

We then examined whether TBL2 depletion affects translation of *ATF4* mRNA. We compared *ATF4* mRNA abundance in polysome fractions (nos. 11-17) to that in subpolysome fractions (nos. 4-10) (Fig. 6D–F). Corresponding to Figure 5B, *ATF4* mRNA expression was increased by thapsigargin treatment, compared with non-treatment (Fig. 6D-F). In thapsigargin-treated control cells, *ATF4*

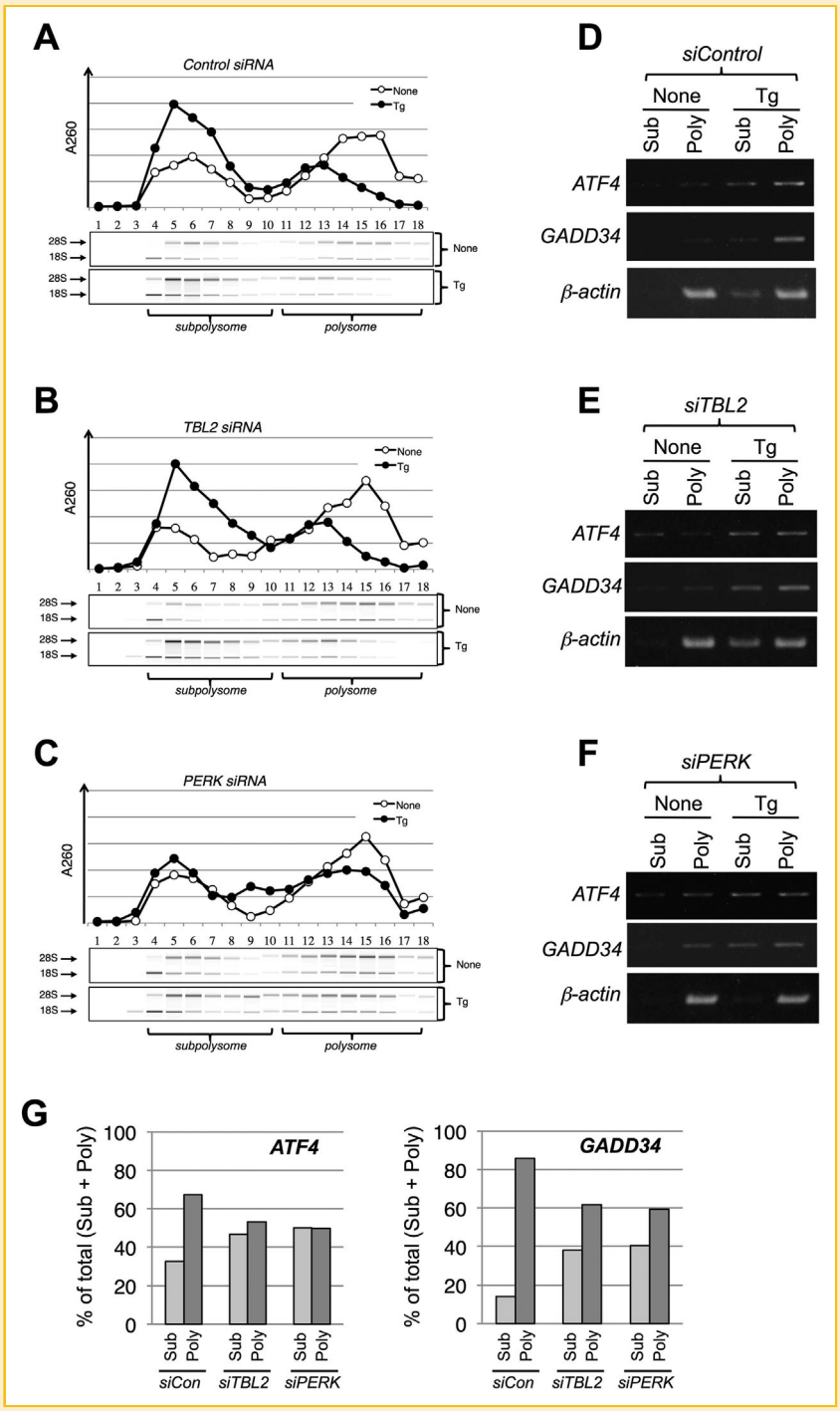

Fig. 6. TBL2 knockdown diminishes ATF4 translation but not general translation under ER stress conditions. (A–C) Polyribosome profiles in cell lysates fractionated by sucrose density gradient centrifugation. 293 Cells were transiently transfected with non-silencing siRNA, TBL2 siRNA or PERK siRNAs. After 48 h, the cells were treated with or without thapsigargin (300 nM) for 1 h and were incubated in the presence of cycloheximide (100 μg/ml) for 5 min prior to lysis in Tris-based buffer (see Materials and Methods). The samples were ultracentrifuged in 10–50% sucrose density gradient, and each fraction was collected stepwise and lysed in Trizol-LS for RNA purification. RNA samples purified from each fraction were measured using the NanoDrop (absorbance at 260 nm) and plotted. 28S and 18S rRNAs were analyzed using the Bioanalyzer. (D–F) The abundance of *ATF4* or *β-actin* mRNA in the subpolysome or polysome pool. The subpolysome or polysome pool was prepared by mixing fraction no. 4–10 or no. 11–17 shown in (A–C), respectively. Each mRNA abundance was analyzed by RT-PCR. A representative data of two independent analyses was shown. (G) Densitometry analysis of PCR products in polysome and subpolysome under thapsigargin-treated conditions. Each percentage of total (Poly + Sub = 100%) was shown here.

mRNA in the polysome was more abundant than that in the subpolysome, suggesting *ATF4* mRNA was translated actively in the cells (Fig. 6D and G). In TBL2- or PERK-depleted cells, ATF4 mRNA in polysome under thapsigargin-treated conditions was distributed at the similar levels to that in subpolysome as shown by the densitometry analysis (Fig. 6E, F and G), suggesting that its translation was diminished in these cells, compared with control cells. Another stress-inducible gene GADD34 mRNA also exhibited similar pattern to ATF4 mRNA (Fig. 6D-G). On the other hand, translation of β-actin mRNA was decreased by thapsigargin treatment in both control and TBL2-depleted cells but not PERK-depleted cells, as shown by detection in the subpolysome (Fig. 6D-F). Thus, under ER stress, TBL2 depletion diminished ATF4 translation without affecting general translation attenuation.

DISCUSSION

In this study, we showed that TBL2 preferentially associated with *ATF4* mRNA, but not *TOP1* mRNA, via the WD40 domain (Figs. 1–2), and regulated ATF4 translation during ER stress (Figs. 3–6). TBL2 also associated with GADD34 mRNA and TBL2 knockdown reduced its translation (Figs. 1B and 6E, G). Similar to ATF4, GADD34 has functional uORFs and its translation is enhanced under eIF2α phosphorylation [Novoa et al., 2001; Kojima et al., 2003; Lee et al., 2009]. Thus, TBL2 may be a regulatory protein for uORF-mediated translation under eIF2α-phosphorylated conditions.

We tried to see whether TBL2 directly activate the uORF-mediated translation using in vitro rabbit reticulocyte lysate system. However, the purified TBL2 protein was barely able to activate the translation in the presence of the purified PERK protein (data not shown), suggesting that TBL2 needs co-factors to activate the translation or it is easily denatured once extracted from the ER-membrane. Therefore, it remains unclear whether TBL2 activates the translation through the direct interaction with the target mRNAs, and it may be possible that TBL2 associates with the mRNAs through the proteins such as eIF3, which has been reported to directly interacts with reinitiation promoting element (RRE) surrounding uORF of some stress-inducible mRNAs GCN4 and YAP1 in yeast and promotes the reinitiaiton by controlling scanning 40S ribosome [Munzarová et al., 2011].

Under not only stress conditions but also normal conditions, TBL2 associated with ATF4 mRNA and TBL2 knockdown reduced the amount of ATF4 mRNA in the polysome (Figs. 1B, 6G and S3), suggesting that TBL2 may be also important ATF4 translation at basal level although the protein expression was very low because of the uORF-mediated regulation (Fig. 5A) [Harding et al., 2000a]. On the other hand, ATF4 protein expression is significantly increased by ER stress (Fig. 1) [Harding et al., 2000a, 2003, Tsukumo et al., 2014]. Therefore, the interaction of TBL2 with PERK, which is triggered by ER stress, may stimulate translation of TBL2-associated *ATF4* mRNA. Indeed, overexpression of the WD40-truncated TBL2 mutant 1-350aa or 1-179aa, which is able to bind to PERK but not to *ATF4* mRNA, diminished ATF4 induction and blocked PERK-TBL2 interaction, suggesting that the mutant was able to act as a dominant-negative form that prevents the TBL2-ATF4 mRNA complex from interacting with endogenous PERK (Figs. 3B–D, 4C and S1). These results indicate that TBL2-mediated assembly of *ATF4* mRNA and PERK on the ER membrane may be important for the preferential translation of ATF4 under the PERK-induced eIF2α phosphorylation conditions.

Previous studies have shown the essential role of PERK-mediated eIF2α phosphorylation in ATF4 mRNA translation [Harding et al., 2000a, Lu et al., 2004]. During ER stress, PERK-induced eIF2α phosphorylation allows the 40S subunit scanning on the ATF4 mRNA to skip the translation initiation at the inhibitory uORF and to reinitiate the translation at the downstream main ORF where non-phosphorylated eIF2α and the 60S subunit are joined [Harding et al., 2000a; Lu et al., 2004; Vattem and Wek, 2004]. Interestingly, we previously found that TBL2 was able to associate with eIF2 and the 60S subunit [Tsukumo et al., 2014, Tsukumo et al., 2015], in addition to ATF4 mRNA as shown in this study. Although the detailed mechanism remains unclear at present, during ER stress, TBL2 may be also involved in the 60S subunit and eIF2 joining for the reinitiation on the ATF4 mRNA. Such a system that efficiently translates target mRNAs would be very important in a situation where the energy source was limited, such as stress conditions.

Although translation of nuclear proteins, including ATF4, is thought to occur generally in the cytoplasm, recent studies showed that various mRNAs encoding cytoplasmic proteins and nuclear proteins including ATF4 have been detected and translated on the ER membrane as well [Stephens et al., 2005; Jagannathan et al., 2014; Reid et al., 2014]. Therefore, it is conceivable that an ER membrane protein TBL2 is able to regulate *ATF4* mRNA translation. In contrast to no alteration in TBL2-ATF4 mRNA association between under non-stressed and stressed conditions, TBL2 interacted with PERK only under ER stress conditions (Figs. 1–3 and [Tsukumo et al., 2014]). Such dynamic change in the TBL2-associated proteins must play an important role to achieve TBL2-mediated specific translation. Therefore, further comprehensive studies to identify additional components of the TBL2 complex could help elucidate how TBL2 contributes to translation of the specific mRNA.

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