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The endoplasmic reticulum-localized protein TBL2 interacts with the 60S ribosomal subunit

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ABSTRACT

Transducin (beta)-like 2 (TBL2) is a poorly characterized protein comprising the N-terminal transmembrane region and the C-terminal WD40 domain. We previously showed that TBL2 is an endoplasmic reticulum (ER)-localized protein that interacts with PKR-like ER-resident kinase (PERK), and under ER stress, it mediates protein expression of activating transcription factor 4 (ATF4). However, further molecular characterization of TBL2 is useful to better understand the function of this molecule. Here, we show that TBL2 associates with the eukaryotic 60S ribosomal subunit but not with the 40S subunit. The association of TBL2 with the 60S subunit was ER stress independent while the TBL2-PERK interaction occurred upon ER stress. Immunoprecipitation analysis using TBL2 deletion mutants revealed that the WD40 domain was essential for the 60S subunit association. These results could provide an important clue to understanding how TBL2 is involved in the expression of specific proteins under ER stress conditions.

1. Introduction

The ribosome is a large molecular complex that catalyzes protein synthesis. The complete eukaryotic ribosome is also known as 80S (Svedberg unit) ribosome, which consists of two subunits, 40S (small) and 60S (large), according to their sedimentation coefficients [1]. Both contain dozens of ribosomal proteins arranged on a scaffold composed of ribosomal RNA (rRNA) [1]. mRNA translation occurs primarily on two populations of ribosomes in the cytosol and those bound to the endoplasmic reticulum (ER) [1]. The ER-bound ribosomes have been known to function in the translation of proteins destined to enter the secretory pathway as well as playing an important role in cytoplasmic protein translation, in particular, under stress conditions [2,3].

Transducin (beta)-like 2 (TBL2) has been originally identified as a ubiquitously expressed protein with a predicted transmembrane region, WD40 repeats, and a coiled coil domain [4]. Although TBL2 dysregulation could be involved in the phenotypes of several diseases, such as Williams–Beuren syndrome and lipidemia [5–10], the molecular function has been largely unknown. We previously showed that TBL2 was localized at the ER and interacted with PKR-like ER-resident kinase (PERK), which, under ER stress, controls translation by phosphorylating eukaryotic initiation factor 2 alpha (eIF2α) [11–14]. We also showed that TBL2 mediated ATF4 induction at the post-transcriptional level [11]. However, it is unknown how TBL2 contributes to specific protein induction. In this study, we show that TBL2 was able to associate with the 60S ribosomal subunit via the WD40 domain. Our results provide an important clue to understanding how the ER-localized protein TBL2 participates in specific protein expression.

2. Materials and methods

2.1. Cells and treatment

We used the human embryonic kidney 293T cell line and human fibrosarcoma HT1080 cells [15]. The 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. All cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Chemicals and antibodies

Thapsigargin (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in dimethyl sulfoxide and added to the culture

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medium; the solvent was less than 0.5% of the medium's volume. The following commercially available antibodies were used: rabbit anti-TBL2 (ProteinTech, Chicago, IL), anti-PERK, anti-RPS16 (abcam, Cambridge, MA), anti-phospho-PERK (BioLegend, San Diego, CA), anti-RPL7, anti-calnexin (Cell Signaling Technology, Danvers, MA), anti-FLAG M2 (Sigma–Aldrich, Tokyo) and HRP-conjugated anti-rabbit or anti-mouse IgG (GE Healthcare Bio-Sciences Corp, Piscataway, NJ).

2.3. Plasmids

pFLAG-TBL2 WT or each of the mutants was constructed by ligating each cDNA amplified by RT-PCR into the pFlag-CMV-5c vector (Sigma) at the HindIII/NotI site. pShooter pCMV/Myc/ER/GFP (Invitrogen), which produces GFP with the N-terminal ER signal peptide and the C-terminal ER retention signal sequence, was used as an ER marker. Transient transfections were performed using the Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

2.4. Immunoblot analysis

Immunoblot analysis was performed as described previously [15]. 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).

2.5. Immunoprecipitation

Immunoprecipitation was performed as described previously [15]. 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 13,000 × *g*

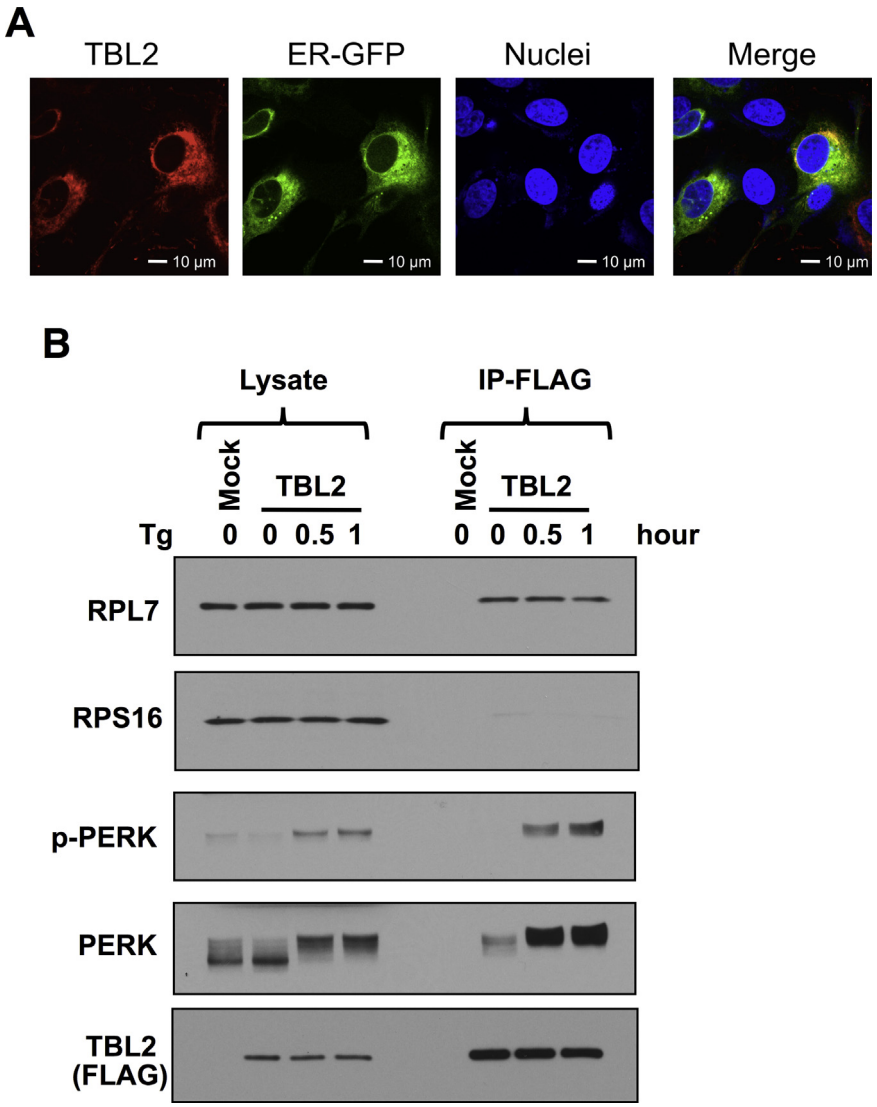

Fig. 1. The protein component of the 60S ribosomal subunit is detected in TBL2 immunoprecipitates. (A) HT1080 cells were transiently transfected with pFLAG-TBL2 and pShooter ER-GFP plasmid and then fixed and analyzed by immunofluorescence using a confocal microscope. (B) 293T cells were transiently transfected with pTBL2 (FLAG-tag) and then treated with 300 nM thapsigargin for 0.5 or 1 h. After immunoprecipitation, each sample was subjected to immunoblot analysis using the indicated antibodies.

centrifugation for 10 min at 4 °C and immunoprecipitated with anti-FLAG-conjugated beads (Sigma) in a lysis buffer. Immunoprecipitates were prepared for immunoblot analysis by washing 3× with lysis buffer and eluting 3× with a FLAG peptide (Sigma) or boiling in SDS sample buffer. For TBL2-ribosome interaction, HEK293T cells were transfected with TBL2 or the mutant (FLAG-tag) plasmids for 24 h and then lysed for 10 min on ice in an NT2 buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM $MgCl_2$, 0.3% NP-40, 200 U/ml RNas in (Promega), phosphatase inhibitor cocktails (Sigma) and protease inhibitors (Sigma)). The lysate was cleared from the nuclei by 3500 × *g* centrifugation for 10 min. To isolate the input RNA, 10% of the lysate was removed. The remaining lysate was incubated with anti-FLAG-M2–conjugated beads for 1 h and washed 4× with NT2 buffer. The immunoprecipitates were treated with proteinase K, and RNAs in the samples were 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. Each 28S and 18S rRNA was visualized using the Bioanalyzer (Agilent Technology, Santa Clara, CA).

3. Results

3.1. The protein component of the 60S subunit is detected in TBL2 immunoprecipitates

As shown in Fig. 1A, and in our previous report [11], TBL2 was localized to the ER via its N-terminal transmembrane region. On the basis of two factors, that 1) TBL2 regulated ATF4 expression at the post-transcriptional level and 2) various WD40-repeat proteins, including TBL2, could function as a potential RNA-binding proteins [11,16,17], we used an RNA immunoprecipitation (RIP) assay to see whether TBL2 had RNA-binding ability. In the process, we found that TBL2 associates with the 60S ribosomal (large) subunit. As shown in Fig. 1B, the 60S ribosomal protein L7 (RPL7) was detected abundantly in TBL2 immunoprecipitates while the 40S ribosomal protein 16 (RPS16) was only marginally detected. The amount of RPL7 in TBL2 immunoprecipitates was unchanged by thapsigargin treatment, unlike PERK (Fig. 1B).

3.2. The rRNA component of the 60S subunit is detected in TBL2 immunoprecipitates

Next, we show the data from the rRNA components detected in the RIP assay. The RNA samples purified from TBL2 immunoprecipitates were analyzed using the Agilent 2100 Bioanalyzer system, which is a nanofluidics device that preforms size fractionation and quantification of small samples of DNA, RNA, or protein. As shown in Fig. 2, the 28S rRNA, an integral RNA component of the 60S ribosomal subunit, was significantly detected in the TBL2-immunoprecipitated sample (Fig. 2A, B (gel image of A)), but a peak of the 18S rRNA, a component of the 40S subunit, was detected to lesser extent (Fig. 2A, B). Thus, both RNA and protein components of the 60S ribosomal subunit were detected in TBL2 immunoprecipitates, indicating that TBL2 preferentially associates with

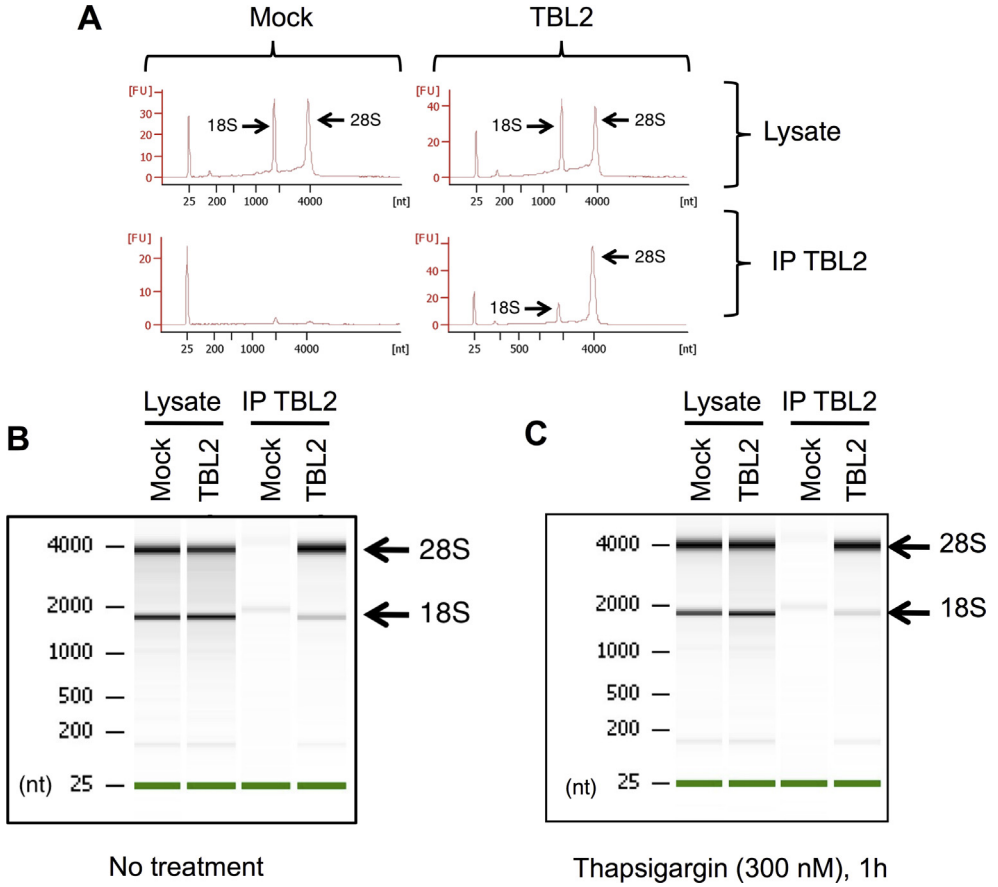

Fig. 2. The RNA component of the 60S subunit is detected in TBL2-immunoprecipitates. (A–C) 293T cells were transiently transfected with pTBL2 (FLAG-tag). After 24 h, the cells were treated with or without thapsigargin (300 nM) for 1 h and then lysed in NT2 buffer (see Materials and methods). After immunoprecipitation of the TBL2 protein, the immunoprecipitate was treated with proteinase K, and RNA was eluted using Trizol LS, then each 28S and 18S rRNA was visualized using the Bioanalyzer. Gel-like images (B, C) were created computationally on the basis of the electrogram. The green line is a loading control RNA, which was added to each sample at equal volumes just before electrophoresis in a Bioanalyzer. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

that subunit. Similar to RPL7, the amount of 28S rRNA in TBL2 immunoprecipitates was unchanged by thapsigargin treatment (Figs. 1B and 2B, C), thereby showing that the TBL2-60S subunit association was independent of ER stress, in contrast to the TBL2-PERK interaction.

3.3. TBL2 preferentially associates with the 60S subunit but not with the 40S subunit

Fig. 2 shows that a small amount of 18S rRNA was also detected in the TBL2 immunoprecipitates, although it was faint compared with 28S rRNA. In order to determine that TBL2 specifically associates with the 60S but not the 40S subunit, we tested the association with each subunit in the absence of magnesium ion (Mg^{2+}) where the 80S ribosome dissociates into the 60S and 40S subunits [18–21]. In the absence of Mg^{2+}, 18S rRNA was not detected in the TBL2 immunoprecipitates (Fig. 3). Thus, TBL2 had the ability to specifically bind to the 60S but not to the 40S subunit.

3.4. The WD40 domain is essential for the TBL2-60S subunit association

Previously, we identified the PERK-interacting region on TBL2 by using several TBL2 deletion mutants [11]. Using the same series of TBL2 deletion mutants and the same transfection methods, we investigated which regions would be required for interaction with the 60S subunit (Fig. 4A). The 32-447aa mutant exhibited an increased association with the 60S subunit compared with the TBL2-WT (Fig. 4B). Although the underlying mechanisms were not known, this increased association might be attributed to the diffused localization of the 32-447aa mutant, thereby allowing the mutant to associate not only with ER-ribosomes but also with cytoplasmic or nuclear ribosomes [11]. On the other hand, two TBL2 mutants that lacked part of the WD40 domain (131-447aa and 1-350aa) lost the ability to associate with the 60S ribosomal subunit, as 28S rRNA was not detected in the immunoprecipitates (Fig. 4B). Partial deletion of the TBL2 WD40 domain profoundly weakened the 60S subunit association. In general, the WD40 domain forms a circularized propeller structure, each blade consisting of a WD40 repeat [22]; therefore, all of the WD40 repeats would be required to maintain the binding ability of TBL2 protein. A similar requirement for the proper activity of the large region of the WD40 domain has been previously reported on the WD40 protein UAF1 [23]. These results indicate that the WD40 domain is essential for the TBL2-60S subunit association.

4. Discussion

In this study, we showed TBL2 specifically associated with the 60S subunit, but not the 40S subunit, via its WD40 domain. Why does TBL2 associate with the 60S subunit? A mammalian eIF6 has been reported to specifically interact with the 60S subunit as well

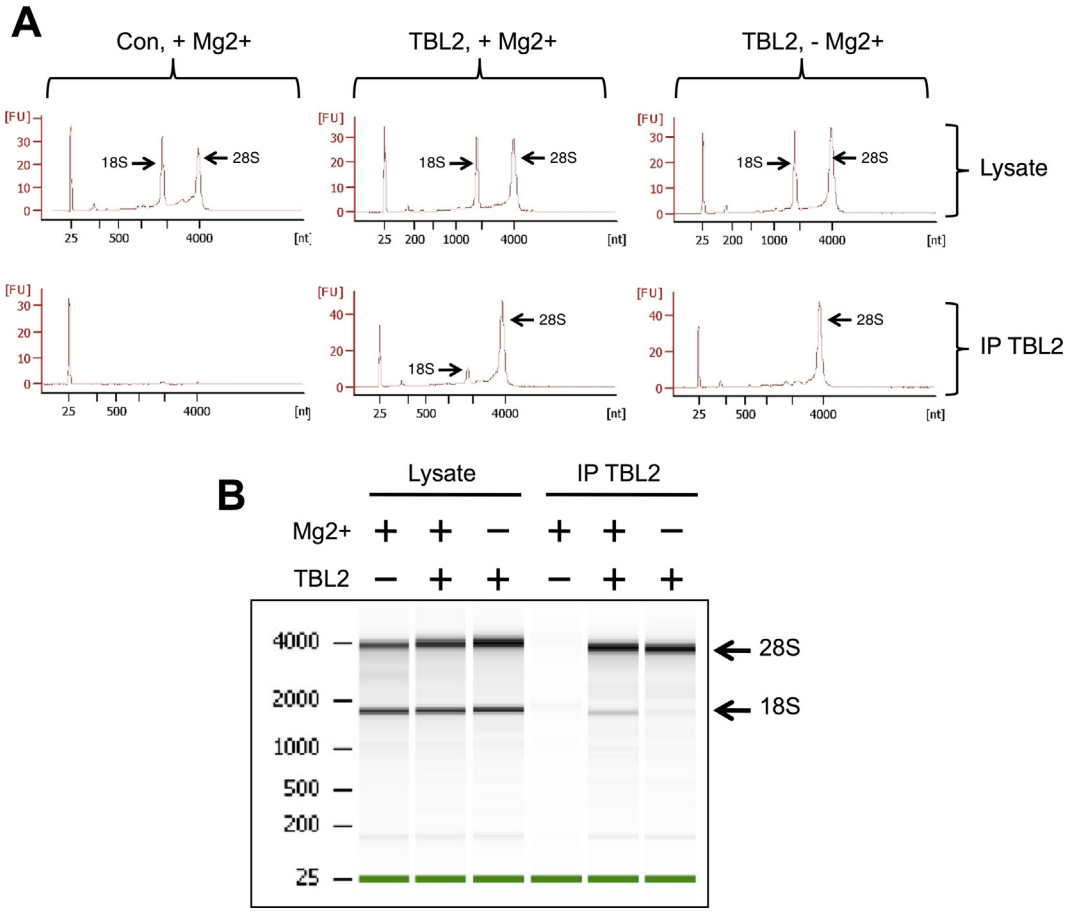

Fig. 3. TBL2 preferentially associates with the 60S but not with the 40S subunit. (A, B) 293T cells were transiently transfected with pTBL2 (FLAG-tag). After 24 h, the cells were lysed in the presence or absence of Mg^{2+} in an NT2 buffer. The TBL2 immunoprecipitate was treated with proteinase K, and RNA in the immunoprecipitate was eluted by Trizol LS. Then, each 28S and 18S rRNA was visualized using the Bioanalyzer. The green line is a loading control RNA, which was added to each sample at equal volumes just before electrophoresis in a Bioanalyzer. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

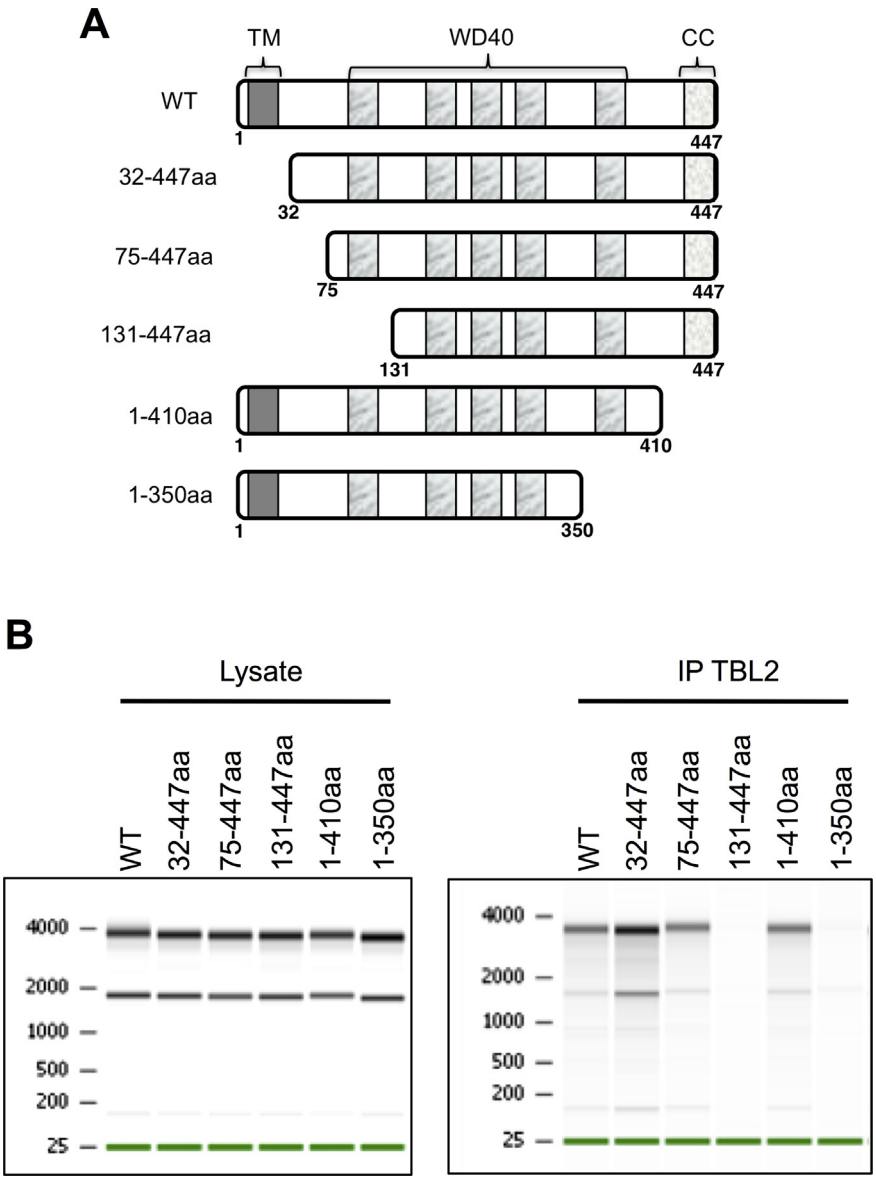

Fig. 4. The WD40 domain is essential for the TBL2-60S subunit association. (A) Schematic representations of each TBL2 mutant. (B) 293T cells were transiently transfected with each TBL2 mutant plasmid. Each sample was analyzed as shown in Fig. 2.

as TBL2 did [21,24,25]. eIF6 is localized in both nucleus and cytoplasm. The nuclear eIF6 is involved in biogenesis of 60S subunits, including their maturation and export from the nucleus to the cytoplasm, while the cytoplasmic eIF6 prevents joining of the 40S and 60S subunits to limit protein synthesis [21,24,25]. On the other hand, TBL2 does not appear to be involved in this fundamental cellular event because neither global protein synthesis nor cell survival was affected by TBL2 depletion, unlike eIF6 [11,21,24,25].

In contrast to normal conditions, under ER stress, depletion of TBL2 reduced cell viability and post-transcriptional expression of a stress protein ATF4 [11]. Therefore, TBL2-associated ribosomes may engage in translation of specific target proteins under stress conditions. While the TBL2-60S subunit association is independent of ER stress, TBL2-PERK interaction occurs under that condition (Figs. 1 and 2) [11]. Therefore, under ER stress, translation of specific proteins mediated by the TBL2-60S complex may be stimulated by interaction with PERK. Given that TBL2 is an ER membrane protein, it may modulate the availability of ribosomes on the ER membrane via its interaction with the 60S subunit and also may contribute to secretory/membrane protein maturation, as well as Sec61, which translocates nascent polypeptides into the ER lumen via association with the 60S subunit [26,27]. Further comprehensive studies to identify additional components of the TBL2 complex would be helpful to elucidate how it participates in gene expression and protein synthesis.

Conflict of interest

The authors declare that they have no conflict of interest.

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