Additional Information for Methods

Cell culture, drug treatment and transfection

BALB/c 3T3 cells, IRE1α$^{+/+}$ and IRE1α$^{-/-}$ MEFs (Lee et al., 2002) were cultured in DMEM (low glucose) supplemented with 10% fetal bovine serum (FBS; Dainippon Sumitomo Pharma Co. Ltd, Osaka, Japan). HEK293 cells were cultured in DMEM (high glucose) supplemented with 10% FBS (JRH Biosciences Inc., Lenexa, KA). ER stress was induced by treatment with 5 μg mL^{-1} tunicamycin (Sigma-Aldrich, St. Louis, MO), 7 μM A23187 (Sigma-Aldrich) and 12.5 mM 2-deoxyglucose, and cytosolic stress with 100 μM arsenite. Cells were heat-shocked for 90 min at 42 °C, and allowed to recover for 2 h at 37 °C. Activity of proteasome was inhibited by treatment with 20 μM lactacystin (Kyowa Medics Co. Ltd, Tokyo, Japan). In order to inhibit anterograde transport from the ER to the Golgi due to osmotic stress, cells were treated with DMEM with 10% FBS and 0.6 M sorbitol. Plasmids and siRNAs were transfected into cells using Lipofectamine 2000 (Invitrogen Life Technologies Inc., Carlsbad, CA) according to the manufacturer's instructions. In the pulse-chase experiments for examining the effects of RNAi, the shRNA vector was transfected three times over the expression vector for the substrates. In siRNA transfection experiments, 0.5 μg of expression vectors for NHK and 200 pmol of siRNA were co-transfected into HEK293 cells on 35 mm cell culture dishes. All RNAi experiments were performed at 72 h after transfection.

Northern blot and hybridization

Total RNA was extracted from cultured cells by the acid-guanidium-phenol-chloroform method using TRIzol reagent (Invitrogen) and 5 or 10 μg RNA was separated by 1% agarose / 2.2 M formaldehyde-denaturing gel electrophoresis, blotted onto nylon membranes (Gene Screen Plus; PerkinElmer Life and Analytical Sciences Inc., Wellesley, MA), and

UV-crosslinked. DNA fragments containing full-length open reading frames (ORF) were labeled with [α-^{32}P]-dCTP (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) by random-priming, according to the manufacturer's instructions (Roche Applied Science, Penzberg, Germany). Free nucleotides were removed using ProbeQuant™ G-50 Micro Columns (GE Healthcare) and the probes hybridized in PerfectHYB™ hybridization solution (TOYOBO Co. Ltd., Osaka, Japan) for 16 h. The membranes were washed and radioactive signals detected by exposure to HR-HA30 X-ray film (Fuji Film, Tokyo, Japan) or the STORM phosphoimaging analyzer (GE Healthcare). Hybridized probes were removed from membranes by boiling for 10 min in stripping buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% SDS) prior to re-hybridization.

Plasmid construction and siRNA

pSUPER (OligoEngine, Seattle, WA) was used to deliver short hairpin RNAs (shRNAs). Three independent 19 bp target sequences were designed against each TRAP subunit and the shRNA sequence for non-specific (NS) RNAi was obtained from Dharmacon (GE Healthcare). To analyze RNAi efficiency using Northern hybridization, HEK293 cells were transfected with pSUPER expressing constructs or non-specific shRNA (pSUPER-NS); two constructs were chosen for each subunit. Target sequences are indicated in supplementary Fig 3A. We constructed vectors expressing human TRAP subunits with C-terminal HA-tags by performing reverse transcription of polyA$^+$ RNA isolated from HEK293 cells, followed by PCR amplification of partial cDNAs encoding human TRAP subunits and PCR subcloning into pcDNA3.1+ (Invitrogen), which introduced the HA tag upstream of the stop codon. The vector expressing C-terminal HA-tagged rat ribophorin I-332 mutant (RI_{332}; a ribophorin I mutant containing only the N-terminal 332 amino acids of the luminal domain) (de Virgilio et al., 1999, 1998) was constructed by PCR amplification of rat ribophorin I cDNA (I.M.A.G.E.

ID 5622466, Invitrogen) using the forward and reverse primers RI_{332}-HA 5'-GGGGTACCACGCGTCCGCGCGGTCATGGAG -3' and 5'- CGGAATTCCTAAGCGT AATCTGGAACATCGTATGGGTATACAAACCGCATCTTCAGTGCATAC-3', respectively, which replaced Asp333 by a stop codon and introduced the HA-tag upstream of the new stop codon. DNA fragments were digested with KpnI and EcoRI, and subcloned into pcDNA3.1+. The DNA sequences of all constructs were verified by PCR sequencing (BigDye Terminator v3.1 Cycle Sequencing Kit and ABI3100 Capillary Sequencer; Applied Biosystems, Foster City, CA). The TCRα expression vector was gifted by Dr. R. R. Kopito (Yu et al., 1997) and wt α1AT, NHK and C-terminal HA-tagged mouse EDEM1 expression vectors were obtained from use in a previous study (Hosokawa et al., 2001). Small interfering RNA (siRNA) specific to human Ribophorin I (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and non-specific siRNA (Dharmacon; GE Healthcare) were used for RNAi experiments of Ribophorin I.

Antibodies

For western blotting, we used rabbit polyclonal antibodies against Sec61β (Upstate Cell Signaling Solutions, Lake Placid, NY) and calnexin (SPA-860; Stressgen Biotechnologies, Victoria, Canada), mouse monoclonal antibodies against TRAPδ (Abnova Corporation, Taipei City, Taiwan), actin (C4; Chemicon International, Temecula, CA), calnexin (PA3-027; Affinity BioReagents, Golden, CO), p97 (MA3-004; Affinity BioReagents) and BiP (BD transduction Laboratories, Franklin Lakes, NJ), sheep polyclonal antibodies against α1-antitrypsin (The Binding Site, Birmingham, UK), as well as goat polyclonal antibodies against HA-probe (Y-11; Santa Cruz Biotechnology Inc.). For immunoprecipitation, rabbit polyclonal antibodies against α1-antitrypsin (DAKO, Denmark), HA-probe (Y-11; Santa Cruz Biotechnology Inc.) and calnexin (Stressgen Biotechnologies). Antibodies against C-terminal

15 amino acids of TRAPα (LPRKRAQKRSVGSDE) (Prehn et al., 1990) were raised by immunizing rabbits (Hokudo Co. Ltd., Hokkaido, Japan) with cysteine was added to the antigenic peptide in order to conjugate keyhole limpet hemocyanin with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester. Rabbit immune antisera were partially purified by precipitation with 33-50% ammonium sulfate. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibodies (Biomedical Technologies Inc., Stoughton, MA), HRP-conjugated goat anti-mouse IgG antibodies (Cappel, ICN Pharmaceutical Inc., Aurora, OH), HRP-conjugated rabbit anti-sheep IgG antibodies (Cappel, ICN Pharmaceutical Inc.) and HRP-conjugated donkey anti-goat IgG antibodies (Santa Cruz Biotechnology Inc.) were used as secondary antibodies.

Western blotting analysis

Proteins were extracted from cultured cells using NP40 lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP40 and protease inhibitors [1 μg mL^{-1} leupeptin, 1 μg mL^{-1} pepstatin A, 2 mM *N*-ethylmaleimide and 0.2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF)]) or digitonin lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 3% digitonin [Calbiochem, EMD Biosciences, Inc., Darmstadt, Germany] and protease inhibitors) and incubated on ice for 20 min. Following centrifugation at 13,000 *g* for 20 min, soluble protein was quantified using the Bradford method and equal amounts of protein were separated by 15% SDS-PAGE (Sec61β and TRAPδ) and 10% SDS-PAGE (other proteins), and blotted onto PVDF membranes (Hybond-P; GE Healthcare). 50 μg of total cell lysate was used for detection of endogenous TRAPδ, and 5 μg for detection of TRAPα, Sec61β, calnexin and β-actin. Immune complexes were detected by ECL analysis (ECL Western Blotting Detection Reagents; GE Healthcare). ECL plus (GE Healthcare) was used for detecting TRAPδ and proteins interacted with endogenous TRAP complex in immunoprecipitation and western

blotting analysis.

Preparation of the microsomal fraction and Blue-Native PAGE (BN-PAGE)

The microsomal fraction was prepared and separated by BN-PAGE as described previously (Schagger et al., 1994; Wang and Dobberstein, 1999). In brief, cells were disrupted at 4 °C in a buffer containing 50 mM Tris-HCl, 5mM EDTA (pH 7.4), 0.25M sucrose and protease inhibitors, using a Dounce homogenizer and centrifuged at 1,000 *g* for 10 min at 4 °C. The supernatants were subjected to centrifugation and ultracentrifugation at 4 °C, using 12,000 and 120,000 *g* for 30 min and 1 h, respectively. Microsome-containing pellets were resuspended in 3% digitonin lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 3% digitonin and protease inhibitors) and incubated on ice for 30 min, followed by centrifugation and ultracentrifugation at 4 °C, using 13,000 and 400,000 *g* for 20 min and 1 h, respectively, in order to remove ribosomes and their tightly-associated proteins. Each protein sample was loaded onto 5-18% Blue Native gel in 0.5 volumes of 3x BN sample buffer (150 mM aminocaproic acid, 30 mM BisTris-HCl pH 7.0, 30% glycerol and 1.5% Coomassie Brilliant Blue [CBB] G-250) (Schagger et al., 1994). A high molecular weight calibration kit for native gel electrophoresis (GE Healthcare) was used. The cathode buffer (containing dye) was replaced by a colorless buffer once the upper half of the gels contained dye. Gels were soaked in BN-PAGE transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.05% SDS) for 30 min prior to blotting onto PVDF membranes (Immobilon^{TM}-P, Millipore Corporation, Bedford, MA). Blots were washed briefly with methanol to remove the CBB dye prior to immunodetection. Quantification analyses were performed using ImageJ 1.37v software. In quantification analysis, signal intensity of native TRAP complex was normalized by that of calnexin complex.

Metabolic labeling and immunoprecipitation

Following preincubation with methionine- and cysteine-free DMEM supplemented with 10 % dialyzed FBS for 30 min at 37 °C, cells were radiolabeled with [^{35}S]-EXPRESS protein labeling mixture (PerkinElmer) and chased in normal DMEM / 10% FBS. Cellular proteins were extracted with 1% NP40 lysis buffer or 3% digitonin lysis buffer. Cell lysates were incubated with antibodies, then immunoprecipitated using protein A-coupled sepharose beads (GE Healthcare). Beads were washed twice in high ionic extraction buffer (concentration of NaCl in lysis buffer was raised to 400 mM), then washed once in 10 mM Tris-HCl (pH 7.4) before elution of the immune complex by boiling in Laemmli's sample buffer. For TRAPγ subunits, immunoprecipitated samples were extracted in Laemmli's sample buffer for 10 min at 37 °C, to avoid aggregation of TRAPγ. Proteins were separated by 10 or 12% SDS-PAGE, and if necessary, gels were overloaded to separate NHK from non-specific bands. Gels were fixed in 50% (w/v) trichloroacetic acid, soaked in 1 M sodium salicylate and dried. Radioactive signals were detected as described for Northern blot analysis. In pulse-chase experiments shown in Fig 3A, the amount of transfected plasmids encoding substrates examined and/or exposure time to X-ray films were controlled for acquiring similar signal intensity at chase 0 h in the different RNAi.

Sequential immunoprecipitation

Metabolically-radiolabeled cells were disrupted in digitonin lysis buffer, and the first immunoprecipitation performed as described above. Immune complexes were released from beads by boiling in elution buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP40, 2% SDS and protease inhibitors) for 10 min. Supernatants were collected and diluted in NP40 lysis buffer until the final concentration of SDS reached 0.1%. The second immunoprecipitation was performed using 1% NP40 as the detergent.

References for supplementary Methods

de Virgilio, M., Kitzmuller, C., Schwaiger, E., Klein, M., Kreibich, G. and Ivessa, N.E. (1999) Degradation of a short-lived glycoprotein from the lumen of the endoplasmic reticulum: the role of N-linked glycans and the unfolded protein response. *Mol Biol Cell*, **10**, 4059-4073.

de Virgilio, M., Weninger, H. and Ivessa, N.E. (1998) Ubiquitination is required for the retro-translocation of a short-lived luminal endoplasmic reticulum glycoprotein to the cytosol for degradation by the proteasome. *J Biol Chem*, **273**, 9734-9743.

Hosokawa, N., Wada, I., Hasegawa, K., Yorihuzi, T., Tremblay, L.O., Herscovics, A. and Nagata, K. (2001) A novel ER alpha-mannosidase-like protein accelerates ER-associated degradation. *EMBO Rep*, **2**, 415-422.

Lee, K., Tirasophon, W., Shen, X., Michalak, M., Prywes, R., Okada, T., Yoshida, H., Mori, K. and Kaufman, R.J. (2002) IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. *Genes Dev*, **16**, 452-466.

Prehn, S., Herz, J., Hartmann, E., Kurzchalia, T.V., Frank, R., Roemisch, K., Dobberstein, B. and Rapoport, T.A. (1990) Structure and biosynthesis of the signal-sequence receptor. *Eur J Biochem*, **188**, 439-445.

Schagger, H., Cramer, W.A. and von Jagow, G. (1994) Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. *Anal Biochem*, **217**, 220-230.

Wang, L. and Dobberstein, B. (1999) Oligomeric complexes involved in translocation of proteins across the membrane of the endoplasmic reticulum. *FEBS Lett*, **457**, 316-322.

Yu, H., Kaung, G., Kobayashi, S. and Kopito, R.R. (1997) Cytosolic degradation of T-cell receptor alpha chains by the proteasome. *J Biol Chem*, **272**, 20800-20804.