TMEM33: a new stress-inducible endoplasmic reticulum transmembrane protein and modulator of the unfolded protein response signaling

Isamu Sakabe1 · Rong Hu1 · Lu Jin1 · Robert Clarke1 · Usha N. Kasid1

Abstract Endoplasmic reticulum (ER) stress leads to activation of the unfolded protein response (UPR) signaling cascade and induction of an apoptotic cell death, autophagy, oncogenesis, metastasis, and/or resistance to cancer therapies. Mechanisms underlying regulation of ER transmembrane proteins PERK, IRE1α, and ATF6α/β, and how the balance of these activities determines outcome of the activated UPR, remain largely unclear. Here, we report a novel molecule transmembrane protein 33 (TMEM33) and its actions in UPR signaling. Immunoblotting and northern blot hybridization assays were used to determine the effects of ER stress on TMEM33 expression levels in various cell lines. Transient transfections, immunofluorescence, subcellular fractionation, immunoprecipitation, and immunoblotting were used to study the subcellular localization of TMEM33, the binding partners of TMEM33, and the expression of downstream effectors of PERK and IRE1α. Our data demonstrate that TMEM33 is a unique ER stress-inducible and ER transmembrane molecule, and a new binding partner of PERK. Exogenous expression of TMEM33 led to increased expression of p-eIF2α and p-IRE1α and their known downstream effectors, ATF4-CHOP and XBP1-S, respectively, in breast cancer cells. TMEM33 overexpression also correlated with increased expression of apoptotic signals including cleaved caspase-7 and cleaved PARP, and an autophagosomes protein LC3II, and reduced expression of the autophagy marker p62. TMEM33 is a novel regulator of the PERK-eIF2α-ATF4 and IRE1-XBP1 axes of the UPR signaling. Therefore, TMEM33 may function as a determinant of the ER stress-responsive events in cancer cells.

Keywords TMEM33 · Endoplasmic reticulum stress and unfolded protein response · PERK · IRE1α · Caspase-7 · Autophagy · Breast cancer

Abbreviations
ATF4 Activating transcription factor 4
ATF6 Activating transcription factor 6
ATF6α/c Cytosolic domain of ATF6
CHOP C/EBP(CCAAT/enhancer-binding protein) homologous protein
eIF2α Eukaryotic translation initiation factor 2α
ER Endoplasmic reticulum
GRP78/ BiP Glucose-regulated protein 78
IRE1α Inositol-requiring enzyme 1α
LC3II Microtubule-associated protein 1 light chain 3
PERK Protein kinase RNA-like ER kinase
TG Thapsigargin
TMEM33 Transmembrane protein 33
TN Tunicamycin
UPR Unfolded protein response
XBP1 X-box binding protein 1
XBP1-S Active (spliced) XBP1

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✉ Usha N. Kasid kasidu@georgetown.edu
1 Georgetown Lombardi Comprehensive Cancer Center and Georgetown University Medical Center, Washington, DC, USA
Introduction

The endoplasmic reticulum (ER) is involved in several fundamental cellular processes including synthesis and sorting of secretory and membrane proteins, detoxification, and intracellular calcium homeostasis [1]. Correct folding of proteins in the ER lumen is regulated by folding and oxidizing enzymes in the presence of chaperones and glycosylating enzymes dependent on ATP and high Ca2+ levels. Misfolded proteins are exported to the cytoplasm for proteosomal degradation by a process known as the ER-associated degradation (ERAD) [2]. ER stress, as defined by the accumulation of misfolded or unfolded proteins above the threshold levels in the ER lumen, leads to activation of an ER-to-nucleus unfolded protein response (UPR) signaling cascade. The ER transmembrane proteins protein kinase RNA-like ER kinase (PERK), inositol-requiring enzyme 1α (IRE1α) and activating transcription factor 6 (ATF6α/β) sense ER stress, each then regulating one of the three distinct axes of the UPR signaling cascade [3]. ER stress-activated PERK phosphorylates serine 51 of eukaryotic translation initiation factor 2α (eIF2α), followed by the suppression of protein synthesis and a selective increase in ATF4 activity. Downstream effectors of PERK signaling include both pro-survival factors such as the transcription factor NRF2 and microRNA miR-211, which are associated with adaptation response, and pro-apoptotic factors including CHOP that can induce cell death [4, 5]. Stress-induced phosphorylation and activation of IRE1α results in activation of the transcription factor X-box binding protein 1 (XBP1) and increased expression of ER chaperones such as GRP78/BiP which, in turn, increase protein folding capacity in the ER lumen. Activated IRE1α also reduces protein load in the ER lumen by cleaving mRNAs encoding secretory and membrane proteins through regulated IRE1α-dependent decay (RIDD). In the ATF6α/β arm of the UPR signaling, ATF6α/β is transported to the golgi where it undergoes cleavage; cleaved ATF6 (ATF6f/ATF6c) functions as a transcription factor for several ER chaperones.

Mechanisms underlying regulation of PERK, IRE1α, and ATF6α/β, and how the balance of these activities determine outcome of the activated UPR, remain largely unclear. Indeed, depending on the acute or chronic ER stress and cellular context, activation of the UPR may lead to apoptotic cell death, senescence, autophagy, oncogenesis, metastasis, and/or resistance to chemotherapeutics and endocrines [6–14]. Identification of new molecules regulating UPR signals may advance understanding of the mechanistic and functional significance of UPR in cancer biology and therapy.

Here, we report characterization of transmembrane protein 33, TMEM33 (also known as SHINC-3) as a novel ER stress-inducible and ER transmembrane molecule and regulator of two main drivers of the UPR: PERK and IRE1α. Our data show that TMEM33 is a new binding partner of PERK. TMEM33 overexpression led to increased expression levels of both p-eIF2α and p-IRE1α and of their respective downstream effectors, ATF4 and XBP1-S in breast cancer cells. TMEM33 overexpression also led to increased expression of CHOP, cleaved caspase-7, and the autophagosome marker LC3II in these cells. Collectively, this work provides new mechanistic insights into the regulation of PERK and IRE1α signaling pathways via TMEM33 in cancer cells.

Materials and methods

Antibodies, reagents, and chemicals

Rabbit polyclonal antibody was custom generated against a TMEM33-specific peptide, KKVLDARGSNSLPLLR (amino acids 127–143; Covance Research Products Inc., Denver, PA). Polyclonal anti-GAPDH antibody (2275-PC-1) was purchased from Trevigen (Gaithersburg, MD, USA). Monoclonal anti-α-tubulin antibody (TU-02), monoclonal anti-Myc antibody (9E10), monoclonal horseradish peroxidase-conjugated anti-cMyc antibody (9E10HRP), polyclonal anti-PERK antibody (H-190), polyclonal anti-GRP78/BiP antibody (C-20), polyclonal anti-IRE1 antibody (H-190), polyclonal anti-Calnexin antibody (C-20), monoclonal anti-PARP antibody (F-2), polyclonal anti-ATF4 antibody (9711), polyclonal anti-ATF-6α antibody (H-280), monoclonal anti-Cyclin D1 antibody (sc-20044); polyclonal anti-β-actin antibody (sc-1616) and Protein A/G PLUS-Agarose immunoprecipitation reagent were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal anti-COX4 antibody (12C4) was obtained from Molecular Probes (Carlsbad, CA, USA). Monoclonal anti-Myc antibody (2276), monoclonal anti-PERK antibody (5683), polyclonal anti-phospho-eIF2α (Ser51) antibody (9721), polyclonal anti-eIF2α antibody (9722), monoclonal anti-phospho-eIF2α antibody (3597), monoclonal anti-ATF4 antibody (11815), monoclonal anti-ATF-6α antibody (12741), monoclonal anti-CHOP antibody (2895), polyclonal anti-cleaved-caspase-7 antibody (9491), polyclonal anti-caspase-7 antibody, and polyclonal anti-cleaved PARP antibody were all purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Additional antibodies and reagents used were as follows: monoclonal anti-ATF6 antibody (IMG-273, Imagex); polyclonal anti-XBP1 antibody (GWB-BACB31, Genway); polyclonal anti-phospho-IRE1α antibody (PA1-16927, Thermoscientific); monoclonal anti-p62 antibody (610832, BD-Bioscience);
FITC-conjugated monoclonal anti-Calnexin antibody (BD Transduction Laboratories); horseradish peroxidase-conjugated mouse and rabbit secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ, USA); Lipofectamine 2000, Lipofectamine LTX, and Lipofectin (Invitrogen Life Technologies, Carlsbad, CA, USA); Fu Gene HD (Roche); proteinase inhibitor cocktail tablets (Roche Diagnostics, Indianapolis, IN, USA); ECL Plus Western blotting detection system (Amersham Biosciences); Coomassie protein assay reagent and Surface-Amps NP-40 (Pierce Biotechnology, Inc., Rockford, IL, USA); Tween 20 (Bio-Rad Laboratories, Inc., Hercules, CA, USA); Re-Blot plus mild antibody stripping solution (Chemicon International, Inc., Temecula, CA, USA); Restore Western blot stripping buffer (21059, ThermoScientific), and thapsigargin, tunicamycin from Streptomyces sp., dimethyl sulphoxide Hybri-Max Sterile filtered (DMSO), and etoposide (Sigma-Aldrich, St. Louis, MO, USA).

**Cell lines and cultures**

MCF-7 human breast cancer cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and Tissue Culture Shared Resource of the Georgetown Lombardi Comprehensive Cancer Center. HEK293T human embryonic kidney cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Human prostate cancer cells (PC-3 and DU-145), breast cancer cells (MDA-MB231), HeLa, and COS-1 cells were obtained from the Tissue Culture Shared Resource of the Georgetown Lombardi Comprehensive Cancer Center. All cell lines were grown as monolayers in Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 5 or 10 % heat-inactivated fetal bovine serum. Endocrine-sensitive (LCC1) and endocrine-resistant breast cancer cells (LCC9), and antiestrogen-resistant MCF-7 cells (MCF7RR) were obtained and established as reported earlier [15–17]. LCC1, LCC9, and MCF7RR cells were maintained in IMEM without phenol red and supplemented with 5 % charcoal-stripped calf serum (CCS). All cell cultures were maintained at 37 °C under 95 % relative humidity and 95 % O2:5 % CO2 atmosphere.

**Construction of Myc-TMEM33 expression vector**

**TMEM33** cDNA (741 bp) was amplified by RT-PCR using total mRNA from human testes (Ambion, Foster City, CA) and cloned into the pCR2.1 vector (Invitrogen). N-terminal Myc-tagged **TMEM33** ORF (771 bp) was amplified by PCR using **TMEM33** in pCR2.1 as template. The forward primer sequence containing the translation initiation codon, the Myc epitope (underlined), and Bgl II primer (bold) was 5'-GAGATCTGCCATGGAGCAGAAACTCATCTCTCTG AAGAGGACCTGTGCGAGATACGCCACGACCGAAC-3', and the reverse primer sequence containing the Mull primer (bold) was 5'-GACGCCTTCTATGGAACTGTG GTGCC-3' as described earlier [18]. The PCR conditions were as follows: 95 °C for 4 min; 40 cycles of denaturation at 94 °C for 30 s; annealing at 65 °C for 1 min; extension at 72 °C for 1 min; and a final extension at 72 °C for 5 min. The amplified product was subjected to electrophoresis in 1 % agarose gels and cloned into the pCR3.1 expression vector. **TMEM33** cDNA sequence was verified by automated DNA sequencing of both strands using vector-based forward and reverse primers as detailed earlier [18, 19].

**Transient cDNA transfections**

COS-1, HEK-293T, and PC-3 prostate cancer cells were transiently transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). HeLa cells were transiently transfected using FuGene HD (Roche), and MCF-7 and MDA-MB231 breast cancer cells were transiently transfected using Lipofectamine LTX (Invitrogen) as described in Supplementary Materials and methods.

**Immunofluorescence and immunostaining**

COS-1 cells were grown overnight on coverslips placed in a six well plate, one coverslip/well. Approximately, 3 x 10⁴ cells were seeded/well. Next day, cells were transfected with 1 μg of **Myc-TMEM33** or empty vector using Lipofectamine 2000. Forty eight hours post-transfection, the medium was removed and cells were immediately fixed in 3.7 % paraformaldehyde, followed by immunofluorescence and immunostaining using various antibodies as described in Supplementary Materials and methods.

**Subcellular fractionation**

Approximately, 5 x 10⁶ MCF-7 cells were seeded per 150 mm tissue culture dish. Next day, the cells were collected by trypsinization and washed once with ice-cold phosphate-buffered saline (PBS). The cytosolic, mitochondrial (heavy membrane), microsomal (light membrane), and nuclear fractions were isolated as described in Supplementary Materials and methods.

**Immunoprecipitation and immunoblotting**

The whole cell lysate (approximately 2 mg protein) was incubated with 25 μL of agarose-conjugated anti-Myc antibody on a rotator at 4 °C overnight. The antibody-conjugated agarose beads were washed 1x in cell lysis
buffer and used for immunoblotting as reported earlier [20] and detailed in Supplementary Materials and methods.

**Thapsigargin and tunicamycin treatments**

Stock solutions of thapsigargin (TG, 2 mM) and tunicamycin (TU, 2 mg/mL) were made in DMSO and stored at −20 °C. Cells from approximately 80 % confluent monolayers were used. The culture medium was removed and fresh DMEM containing 10 % FBS and the desired final concentration of TG or TU was added to the cells and incubation continued for various periods, followed by cell lysis and Western blotting as described in Supplementary Materials and methods.

**Results**

**TMEM33 is a novel endoplasmic reticulum transmembrane protein**

We identified TMEM33 as a novel cDNA fragment (191 bp) in a differentially displayed mRNA screen of cancer cells treated with antisense raf oligonucleotide or control mismatch oligonucleotide [GenBank accession number AF403224]. Subsequent sequential homology search of the human expressed sequence tag (EST) database [21] led to identification of the full-length TMEM33 cDNA sequence (7717 bp) as shown in Fig. 1a. The longest open reading frame of the full-length TMEM33 cDNA encodes a new 247 amino acids (aa) protein (approximately 28 kDa M, Fig. 1b, Supplementary Fig. 1). The TMEM33 amino acid sequence revealed three transmembrane helices (32–52 aa, 101–121 aa, and 156–176 aa), at least one phosphorylation site (T65) and two ubiquitination sites (K148 and K221) [22–24] (Fig. 1b). Using northern hybridization, four major TMEM33 transcripts (7.7, 4.0, 2.5, and 1.5 kb) were detected in most normal human tissues and cancer cell lines tested (Fig. 2).

Based on prediction of its likely subcellular localization, the TMEM33 protein appeared to be localized to ER [25]. Subcellular localization of TMEM33 was demonstrated using a combination of immunofluorescence and biochemical fractionation analyses. COS-1 cells were transiently transfected with either a Myc epitope-tagged TMEM33 expression vector (Myc-TMEM33) or empty vector. Immunofluorescence staining showed co-localization of Myc-TMEM33 with Calnexin and ER-tracker but not mito-tracker or WGA staining (Fig. 3a). Expression of Myc-TMEM33 in COS-1 transfectants was verified by immunoblotting (Fig. 3b).

Subcellular localization of the endogenous TMEM33 protein was examined by cell fractionation and immunoblotting using a custom-generated rabbit polyclonal antibody against a TMEM33-specific epitope (aa 127–143). The anti-TMEM33 antibody recognized an approximately 28 kDa protein in human prostate cancer cells (PC-3 and DU-145); pre-immune serum had no immune reactivity at the corresponding location (Supplementary Fig. 2a). The anti-TMEM33 antibody was further validated by sequential immunoblotting of cell lysates from COS-1 Myc-TMEM33 transfectants with anti-Myc and anti-TMEM33 antibodies. These two antibodies recognized an overlapping band at ~28 kDa in COS-1 transfectants (Supplementary Fig. 2b). Using the custom-generated anti-TMEM33 antibody, human endogenous TMEM33 (~28 kDa) was detected in several human cancer cell lines including A549, Aspc-1, Colo-357, MDA-MB435, MCF-7, and HeLa cells (data not shown). In the combined cell fractionation and immunoblotting assay, Calnexin was detected in both the heavy membrane pellet (HM) and the ER-containing microsomal fractions (MS) of MCF-7 cells; heavy membrane pellets contain mitochondrial, lysosomal, and ER-resident proteins. Similar to Calnexin, endogenous TMEM33 expression was seen in both the HM and MS fractions of MCF-7 cells but not in the nuclear or cytosolic fractions (Fig. 3c). Together with the predicted 2D topology of TMEM33 [26, 27] (Fig. 3d) these data establish TMEM33 as a novel ER transmembrane resident protein.

**TMEM33 is an ER stress-inducible molecule**

The ER localization of TMEM33 prompted us to ask whether TMEM33 expression is stimulated in response to known inducers of ER stress including thapsigargin (TG), an inhibitor of ER Ca2+ ATPase, tunicamycin (TU), an inhibitor of N-linked glycosylation, and low glucose. Expression of TMEM33 was analyzed in HeLa cells exposed to TG, TU or low glucose. TMEM33 protein expression was increased in response to ER stress (Fig. 4a, b). Consistent with these observations, a significant induction of the largest TMEM33 transcript (7.7 kb) was seen in HeLa cells treated with thapsigargin (Fig. 4c). As expected, GRP78/BiP, a well-known ER stress-inducible protein chaperone in the ER lumen, was also induced in HeLa cells under similar conditions (data not shown). In addition, ER stress-inducible expression of TMEM33 and GRP78/BiP was also noted in PC-3 prostate cancer cells (data not shown). Thus, TMEM33 is a new ER stress-inducible protein.

**TMEM33 overexpression is associated with enhanced stimulation of the ER stress-responsive PERK/p-eIF2α/ATF4 signaling pathway**

To determine whether TMEM33 is a component of the ER stress-inducible UPR signaling pathway, we first looked for
interaction(s) between TMEM33 and the ER membrane-resident molecules PERK, IRE1α, and ATF6. HEK293T cells were transiently transfected with the Myc-TMEM33 plasmid and treated with 1 μM TG for various times. Using a reciprocal co-immunoprecipitation assay, our findings show an ER stress-related interaction between TMEM33 and PERK (Fig. 5a). Myc-TMEM33 did not interact with either IRE1α or ATF6 under similar conditions (data not shown). Next, we examined the effects of TMEM33 overexpression on downstream effectors of activated PERK (p-eIF2α; ATF4). As shown in Fig. 5b and c, and Supplementary Fig. 3, overexpression of TMEM33 was associated with enhanced basal and ER stress-induced expression of p-eIF2α and ATF4 in Myc-TMEM33.
transfectants, relative to empty vector control transfected HEK293T cells. These data establish TMEM33 as a novel binding partner of PERK and demonstrate that TMEM33 overexpression correlates with enhanced activation of the ER stress-inducible PERK/p-eIF2α/ATF4 signaling.

**TMEM33 overexpression and increased expression of ER stress-induced cell death signals**

We next determined the effects of TMEM33 overexpression on various cell death signals. HeLa cells were transiently transfected with a Myc-TMEM33 or control vector and exposed to TG (1 μM) for indicated times, followed by immunoblotting of whole cell lysates with various antibodies (Fig. 6). For quantification of relative levels of immunoblotting of whole cell lysates with various antibodies and exposed to TG (1 μM), 48 h, 5.0 fold; Cl. PARP, TG (1 μM, 48 h), 3.0 fold). Increased expression levels of cleaved caspase 7 and cleaved PARP were also observed following TG treatment of COS-1 cells transiently transfected with Myc-TMEM33 versus control vector transfec-
tants (Supplementary Fig. 4a). In addition, Caspase 2 expression was found to be increased in Myc-TMEM33 transfec-
tant COS-1 cells versus control vector transfected cells (data not shown). Furthermore, constitutive expression of cleaved caspase 7 was also seen in TMEM33-transfected

![Fig. 3](image-url) **Fig. 3** TMEM33 is an ER transmembrane resident protein. **a** Immunofluorescence analysis of ER localization of TMEM33 in COS-1 transfectants. After 48 h of transfection with Myc-TMEM33 cDNA expression vector, COS-1 transfectants were fixed and immunostained as described in "Materials and methods" section. For ER-tracker or Mito-tracker staining, transfected cells were first incubated with these dyes. Right subpanels: a DAPI; d anti- Calnexin antibody; g ER-tracker; j Mito-tracker; m, WGA. **b** Expression of Myc-TMEM33 protein in transiently transfected COS-1 cells was confirmed by immunoblotting with anti-Myc antibody. The blot was reprobed with anti-GAPDH antibody. **c** The subcellular localization of endogenous TMEM33. MCF-7 cells were homogenized and fractionated into nuclear, heavy membrane (HM), microsomal (MS), and cytosolic fractions, followed by sequentially immunoblotting of the cell fractions using anti-TMEM33, anti-Calnexin (ER membrane), anti-PARP (nucleus), anti-COX4 (mitochondria), and anti-tubulin (cytosol) antibodies. WCE, whole cell extract. **d** The ER membrane structure of TMEM33. TOPO2, transmembrane protein display software, was used to display 2D topology of TMEM33. ER lumen, N-terminus 1–31 aa; 122–155 aa; ER membrane, 32–52 aa; 101–121 aa; 156–176 aa; Cytoplasm, 53–100 aa; 177–247 aa—C terminus; Potential phosphorylation site (T65) (red) and two ubiquitination sites (K148 and K221) (blue) are shown

![Fig. 2](image-url) **Fig. 2** Expression analyses of TMEM33 transcripts in normal human tissues and human cancer cell lines. The mRNA blots (Clontech) were sequentially probed with a radiolabeled TMEM33 cDNA probe, followed by β-actin or GAPDH cDNA probe. S.M., smooth muscle; PBL, peripheral blood lymphocytes; HL-60, promyelogenous leukemia; K-562, chronic myelogenous leukemia; MOLT-4, lymphoblastic leukemia; BL-Raji, Burkitt’s lymphoma; SW480, colorectal adenocarcinoma; A549, lung carcinoma; G361, melanoma; DU-145, prostate cancer
MCF-7 breast cancer cells but not in control pCR3.1-transfected cells (Fig. 7, Supplementary Fig. 4b).

**TMEM33 overexpression and constitutive activation of the PERK-p-eIF2α-ATF4 and IRE1α-XBP1 axes of the UPR signaling and autophagy in breast cancer cells**

To address the consequences of TMEM33 overexpression in human cancer cells, MCF-7 breast cancer cells were transiently transfected with either the Myc-TMEM33 or pCR3.1 vector, followed by immunoblotting of the cell lysates with antibodies against various components of the UPR signaling pathways. Figure 7 shows that exogenous expression of TMEM33 was sufficient to increase basal levels of p-eIF2α, ATF4, CHOP, cleaved caspase 7, p-IRE1α, and XBP1-S but not cleaved ATF6. In addition, (5.5 mM) DMEM containing 10% FBS for the times indicated, followed by sequential immunoblotting with anti-TMEM33 antibody and anti-GAPDH antibody. Normalized expression levels of TMEM33 were quantified as in panel a. Data shown are representative of two independent experiments. c Northern blot analysis of TMEM33 mRNA in HeLa cells treated with thapsigargin as shown. The blot was reprobed with radiolabeled GAPDH cDNA. The normalized expression levels of 7.7 kb TMEM33 transcript at various time points were quantified using ImageQuant software (Molecular Dynamics).
**Fig. 5** TMEM33 is a binding partner of PERK and TMEM33 overexpression correlates with increased levels of basal and ER stress-induced p-eIF2α and ATF4 expression. 

**a** TMEM33 interacts with PERK in the presence of ER stress. HEK293T cells were transiently transfected with Myc-TMEM33 for 48 h at 37 °C, followed by treatment with 1 μM TG for the times indicated. The whole cell lysates were subjected to immunoprecipitation using anti-Myc antibody or anti-PERK antibody-conjugated Protein A/G and the immunoprecipitates were analyzed by Western blotting as indicated. Representative data from two independent experiments are shown. IP immunoprecipitation; IB immunoblotting; WCE whole cell extracts. UT untreated.

**b** Increased basal and ER stress-induced expression of p-eIF2α in Myc-TMEM33 transfected HEK293T cells. HEK293T cells were transiently transfected with Myc-TMEM33 or pCR3.1 (empty vector) and incubated for 48 h at 37 °C, followed by treatment with 1 μM TG as indicated. Whole cell lysates were analyzed by Western blotting using anti-p-eIF2α antibody. Blots were reprobed with anti-eIF2α and anti-α-Tubulin antibodies (top panel). For quantification of relative p-eIF2α levels in Myc-TMEM transfectants versus control vector at various time points, first total eIF2α levels were normalized against α-Tubulin in corresponding lanes. Expression levels of p-eIF2α were then normalized against α-Tubulin-normalized eIF2α levels in corresponding lanes. The fold Δ in p-eIF2α level was calculated by dividing normalized p-eIF2α level in Myc-TMEM transfectants by normalized p-eIF2α level in pCR3.1 transfectants in the corresponding treatment group. Representative data from three independent experiments are shown. UT, untreated.

**c** Increased expression of ATF4 in Myc-TMEM33 transfected HEK293T cells. Whole cell extracts of empty vector (pCR3.1) or Myc-TMEM33 transfected cells were prepared after thapsigargin treatment (TG) as described in Materials and methods. Cell lysates were sequentially immunoblotted with anti-ATF4 and anti-α-Tubulin antibodies (top panel). Cell lysates were also probed with anti-Myc and anti-α-Tubulin antibodies (bottom panel). For quantification of relative ATF4 levels in Myc-TMEM transfectants versus control vector at various time points, expression levels of ATF4 were normalized against α-Tubulin in corresponding lanes. The fold Δ in ATF4 level was calculated by dividing normalized ATF4 level in Myc-TMEM33 transfectants by normalized ATF4 level in pCR3.1 transfectants in the corresponding treatment group. Representative data from two independent experiments are shown. UT untreated.
MB-231 breast cancer cells (Supplementary Fig. 6). Hence, TMEM33 overexpression is associated with constitutive activation of PERK-p-eIF2α-ATF4 and IRE1α-XBP1-S signaling and autophagy in breast cancer cells.

**Discussion**

This study is the first report of TMEM33 as a new ER stress-inducible and ER transmembrane resident molecule. PERK and IRE1α axes of the UPR play prominent roles in regulation of adaptation and cell death signals, yet how these two receptors are regulated remains unclear. Here, we demonstrate: (1) TMEM33 is a novel binding partner of PERK and that TMEM33 overexpression correlates with increased expression of downstream effectors of PERK, p-eIF2α, and ATF4, and cleaved caspase 7 in cells challenged with thapsigargin, (2) association of enhanced expression of TMEM33 with increases in components of the activated PERK and IRE1α signaling pathways (p-eIF2α, ATF4, CHOP, cleaved caspase 7, XBP1-S) in breast cancer cells, and (3) association of enhanced
Expression of TMEM33 with increased autophagy in breast cancer cells. Autophagy has been associated with tumor cell resistance to various therapies. In pilot studies, we also tested whether TMEM33 may serve as a new prognostic marker in certain estrogen receptor-positive breast cancers. Gene expression microarrays were used to measure TMEM33 expression in endocrine-resistant (LCC9, MCF7RR) and sensitive breast cancer cells (LCC1 and MCF7) [15–17], and in clinical specimens [34–37]. TMEM33 mRNA expression was found to be higher in endocrine-resistant breast cancer cells as compared with their matched sensitive control cells. In addition, early recurrent breast cancer specimens showed high TMEM33 expression when compared with non-recurrent breast tumors (Supplementary Fig. 7). Taken together, our current observations suggest a working model where TMEM33 may function as a critical determinant of the balance between PERK and IRE1α-mediated apoptotic, adaptive and neoplastic transformation events in cancer cells (Fig. 8).

How TMEM33 expression is regulated remains unknown. The proximal promoter region of the TMEM33 gene shows putative binding site for NRF-2, a prosurvival transcription factor downstream of the activated PERK (Supplementary Fig. 1c). It is tempting to speculate that an expression of TMEM33 with increased autophagy in breast cancer cells. Autophagy has been associated with tumor cell resistance to various therapies. In pilot studies, we also tested whether TMEM33 may serve as a new prognostic marker in certain estrogen receptor-positive breast cancers. Gene expression microarrays were used to measure TMEM33 expression in endocrine-resistant (LCC9, MCF7RR) and sensitive breast cancer cells (LCC1 and MCF7) [15–17], and in clinical specimens [34–37]. TMEM33 mRNA expression was found to be higher in endocrine-resistant breast cancer cells as compared with their matched sensitive control cells. In addition, early recurrent breast cancer specimens showed high TMEM33 expression when compared with non-recurrent breast tumors (Supplementary Fig. 7). Taken together, our current observations suggest a working model where TMEM33 may function as a critical determinant of the balance between PERK and IRE1α-mediated apoptotic, adaptive and neoplastic transformation events in cancer cells (Fig. 8).

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Compliance with ethical standards
Conflict of interest TMEM33 (alias SHINC-3) is a Georgetown University patented technology. “Gene SHINC-3 and Diagnostic and Therapeutic Uses Thereof,” US Patent # 7244565. IS and UK are co-inventors of this technology. RH, LJ and RC declare that they have no conflict of interest.

Fig. 8 Proposed model of a role of TMEM33 in regulation of the PERK and IRE1α axes of the unfolded protein response signaling. ER stress leads to increased expression of TMEM33 and activation of PERK resulting in enhanced levels of p-eIF2α and ATF4. Depending of the cell type, overexpression of TMEM33 may also increase expression of p-IRE1α and its effector XBP1-S. Additional downstream effectors include increased expression of CHOP, cleaved caspase-7, cleaved PARP, and autophagosome marker LC3II, and reduced expression of p62. Dashed arrows indicate as yet unknown pathways.

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Ethical standards  The authors declare that all experiments reported in this manuscript were performed in compliance with all current laws and regulations of the United States of America.

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