Supplementary material

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

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Supplementary Materials and methods

Transient cDNA transfections

COS-1 cells were transiently transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacture's protocol. Briefly, $2x10^6$ cells were seeded into T-75 tissue culture flask. The following day, cells were transfected with 10 µg of pCR3.1 plasmid containing Myc-tagged *TMEM33* cDNA or pCR3.1 empty vector. At 48 hours post-transfection, cells were lysed for Western blotting. For transient transfection of HEK-293T cells, 60-mm tissue culture dishes were pretreated with 0.1 mg/mL poly-L-Lysine (Sigma) for 8 hours to overnight at 37^oC. Culture dishes were washed twice with 1x PBS. Approximately $5x10^5$ cells were seeded per dish containing DMEM supplemented with 10% FBS. Twenty-four hours later, cells were transiently transfected with 5µg of pCR3.1 (empty vector) or *Myc-TMEM33* using 25 µL of Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. At 48 hours post-transfection, cells were washed twice with ice-cold 1x PBS and lysed for Western blotting. PC-3 cells were transiently transfected in 60-mm tissue culture dishes without prior treatment with poly-L-Lysine using Lipofectamine 2000 as above. For transient transfection of HeLa cells, approximately $5x10^5$ cells were seeded per 60-mm tissue culture dish containing DMEM supplemented with 10% FBS. Twenty-four hours later, cells were transiently transfected with 4 µg of pCR3.1 (empty vector) or *Myc-TMEM33* using 10 µL of FuGene HD (Roche) according to the manufacturer's protocol. At 48 h post-transfection, cells were washed twice with ice-cold 1x PBS and lysed for Western blotting. MCF-7 and MDA-MB231 breast cancer cells were transiently transfected using Lipofectamine LTX (Invitrogen). Briefly, $2x10^5$ breast cancer cells were seeded per well in six-well plates containing Improved Minimal Essential Medium (IMEM) supplemented with 5% fetal bovine serum. Next day, cells were transfected using 1.5 µg *Myc-TMEM33* plasmid or

empty vector pCR3.1 per well. Twenty-four hours post-transfection, the adherent and floating cells were collected and lysed in RIPA buffer containing protease inhibitor, 10 mM glycerophosphate, 1 mM sodium orthovanadate, 5 mM pyrophosphate, and 1mM PMSF, followed by immunoblotting.

Immunofluorescence and immunostaining

COS-1 cells were grown overnight on coverslips placed in a six well plate, one coverslip/well. Approximately, 3 x 10^4 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 in 1x PBS for 10 minutes, and rinsed with 1x PBS at room temperature (RT) three times. Cells were permeabilized with 0.1% Triton X-100 in 1x PBS for 5 minutes, followed by three washes in 1x PBS. This was followed by a blocking step using 2% BSA in 1x PBS for 30 minutes, and two washes in 1x PBS. Subsequent treatments were as below. In group 1, cells were stained with anti-Myc antibody in blocking buffer (1:500 dilution, 1 hour, RT) followed by three washes in 1x PBS, and incubation with a mixture of 0.5 µg/mL 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes) and donkey anti-mouse IgG conjugated to Alexa Fluor 488 (green) (Molecular Probes) (1:1,000 dilution in blocking buffer,1 hour, RT). Cells were washed four times with 1x PBS, and coverslips were mounted in Fluoromount-G (Southern Biotechnology Associates, Inc, Birmingham, AL).

In group 2, cells were immunostained with a combination of fluorescein (FITC)-conjugated anti-Calnexin antibody (BD Transduction Laboratories) in blocking buffer (1:500 dilution) and rhodamine (TRITC)-conjugated anti-Myc antibody (Santa Cruz Biotechnologies) in blocking

buffer (1:500 dilution), for 1 hour (RT), followed by three washes in 1x PBS and mounting of the coverslips on slides as above.

In group 3, forty-eight after transfection, medium was changed to fresh DMEM containing 10% FBS and 100 nM ER-Tracker Blue-White DPX (Molecular Probes) for 30 minutes at 37^0C. Cell culture medium was removed and cells were fixed in 3.7% paraformaldehyde, followed by permeabilization and blocking as above. Fixed cells were stained with rhodamine (TRITC)-conjugated anti-Myc antibody and coverslips were mounted as above.

In group 4, forty-eight after transfection, media was changed to fresh DMEM containing 10% FBS and 100 nM MitoTracker Green FM (Molecular Probes) for 30 minutes at 37^0C, followed by fixation and immunostaining with rhodamine (TRITC)-conjugated anti-Myc antibody as in group 3.

In group 5, cells were stained with a combination of rhodamine (TRITC)-conjugated anti-Myc antibody in blocking buffer (1:500 dilution) and Alexa Fluor 488-conjugated wheat germ agglutinin (Molecular Probes) (1 μg/mL in blocking buffer) for 1 hour (RT), followed by three washes in 1x PBS and mounting of the coverslips on slides as above. Images were acquired with a camera mounted on a Nikon Eclipse E600 fluorescence microscope.

Subcellular fractionation

Approximately 5 x 10^6 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). Buffer A containing 25 mM Tris-HCl, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$ 2 mM dithiothreitol, complete protease inhibitors (Roche) was added (4 ml buffer A/g wet pellet). An equal volume of buffer B (buffer A containing 0.5 M sucrose) was added, and the cells were

homogenized either by 40 strokes with a Dounce homogenizer equipped with a tight-fitting pestle B or by passing 25-30 times through a 26-G needle. The homogenate was centrifuged twice at 600 x *g* for 5 minutes. The pellet represented the initial nuclear fraction. The supernatant was centrifuged at 10,000 x *g* for 10-20 minutes at 4^{o}C, resulting in the supernatant and the mitochondria-enriched or heavy membrane (HM) fraction (pellet). The supernatant was centrifuged at 120,000 x *g* for 1 hour to collect the microsomal fraction (pellet) and the cytosolic fraction (supernatant). The mitochondria-enriched fraction was resuspended twice in isotonic homogenization buffer C (buffer A containing 0.25 M sucrose) and centrifuged twice at 10,000 x *g* for 15 minutes each time. Mitochondrial proteins in the pellet were extracted in buffer D (buffer C containing 1% NP-40), incubated with mild agitation at 4 °C for 1 hour, and centrifuged at 16,000 x *g* for 1 hour to remove residual pellet. The supernant represented the mitochondrial fraction. Proteins in the microsomal fraction were extracted by resuspending the pellet in buffer D, incubating with mild agitation at 4 °C for 1 hour, and centrifugation at 16,000 x *g* for 1 hour. The initial nuclear fraction was washed twice in buffer A, and the proteins were extracted by resuspending the pellet in buffer E (buffer A containing 750 mM KCl), incubation with mild agitation at 4 °C for 1 hour, and centrifugation at 16,000 x *g* for 1 hour. The cytosolic, mitochondrial (heavy membrane), microsomal (light membrane), and nuclear fractions were stored at -20 °C until use.

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^{o}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. The medium was removed and cells were rinsed twice with 1x PBS, and 500 µL of lysis buffer was added for 10 minutes at 4^0C. Lysed cells were collected by scraping and lysis continued on ice for additional 30 minutes with vortexing every 10 minutes. The supernant was used for Western blotting. For Northern blotting, the cells were detached by trypsinization at 37^{o}C for 5 minutes and the pellet was washed with 1x PBS twice, followed by RNA isolation and Northern blotting.

Western blotting

Cells were lysed in lysis buffer (100 mM HEPES, 150 mM sodium chloride, 1 % NP-40, 10 % glycerol supplemented with proteinase inhibitor cocktail) and protein concentration was determined by Bradford's method (Coomassie Protein Assay Reagent Kit, Pierce Biotechnology, Rockford, IL). Whole cell lysates (25 µg to 100 µg protein) were size fractionated on 12% SDS-PAG using Tris-Glycine as running buffer or NuPAGE 4-12% Bis-Tris gel and MES or MOPS as running buffer, followed by transfer to PVDF membrane (0.45 µm pore size) and immunoblotting. In brief, the blot was washed once in washing buffer (10mM Tris-HCl, pH 7.5, 155 mM NaCl, 0.1% Tween-20), followed by blocking for 1 hour (RT) in washing buffer containing 5% non-fat milk (Bio-Rad), and incubation in fresh blocking buffer containing the indicated dilution of the primary antibody overnight at 4^{o}C on a shaking platform. The immunoblot was washed three times in washing buffer followed by incubation for 2 hour (RT) in blocking buffer containing secondary anti-rabbit or anti-mouse antibody (1:2000, Amersham Biosciences Corp, Piscataway, NJ) or anti-goat antibody (1:2000, Santa Cruz). The blot was washed three times in washing buffer and proteins were detected using the ECL Plus Western

blotting detection system (Amersham Biosciences). For anti-Myc antibody conjugated to horse-radish peroxidase (anti-Myc (9E10) HRP), after incubation with the conjugated antibody the blot was washed in washing buffer as above, followed by detection of proteins with the ECL Plus system. For reprobing, the blot was stripped using Re-Blot plus mild antibody stripping solution as recommended by the manufacturer.

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^{o}C overnight. The pellet was washed twice in ice cold lysis buffer and twice in 1x PBS, and resuspended in 30 μL of Laemmli sample buffer (Bio-Rad Laboratories) containing 5% of 14.2M 2-Mercaptoethanol (Bio-Rad). The sample was boiled for 5 minutes, chilled on ice, and microcentrifuged for 3 minutes at 4^{o}C. The supernatant was electrophoresed using 12% SDS-PAG or NuPAGE 4-12%, followed by immunoblotting as described above. For immunoprecipitation of endogenous TMEM33, PERK, IRE1α, or ATF-6α, custom-generated antibody (TMEM33, 2 μL) or a commercially available antibody (PERK, IRE1, ATF-6α; 12.5 μL (0.2 μg/μL IgG)) was incubated with 25 μL of Protein A/G agarose beads (Santa Cruz) for 1 hour at 4^{o}C on a rotator. The antibody-conjugated agarose beads were washed 1x in cell lysis buffer and used for Western blotting as described above.

Northern blotting

Total RNA was extracted using RNAzol B solution (Tel-Test Inc., Texas) according to the manufacturer's instructions. RNA was size fractionated in a 1.0% formaldehyde agarose gel, transferred onto nylon membrane (Qiagen), and fixed by UV cross-linking. Full length human

TMEM33 cDNA insert and human *GAPDH* cDNA probes were radiolabeled with ^{32}P-dCTP using a random primer DNA labeling kit (Pharmacia Biotech, Piscataway, NJ). Blots were sequentially hybridized first to a radiolabeled *TMEM33* cDNA probe, followed by *GAPD*H cDNA probe at 68^{o}C in ExpressHyb hybridization solution (Clontech, Palo Alto, CA). Blots were washed three times in 2xSSC and 0.05% SDS at 68^{o}C, twice in 0.1x SSC and 0.1% SDS at 50^{o}C. Autoradiographs of the air-dried blots were computer-scanned using the Image-Quant software, version 5.1 (Molecular Dynamics, Sunnyvale, CA). Expression of *TMEM33* mRNA was also examined in multiple human tissue and human cancer cell lines using commercially available poly $(A)^{+}$ mRNA blots (Clontech). These blots were sequentially hybridized with radiolabeled *TMEM33* and *β-actin* cDNA probes in ExpressHyb solution as above.

Supplementary Figure legends

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Supplementary Fig. 2 Expression analysis of endogenous TMEM33 protein in human cancer cells and COS-1 cells

Supplementary Fig. 3 TMEM33 overexpression correlates with increased expression of p-eIF2α in HEK293T cells

Supplementary Fig. 4 ER stress-induced levels of apoptotic molecules in *TMEM33* transfectants as compared to empty vector (pCR3.1) transfectants

Supplementary Fig. 5 Representative publicly available datasets (ONCOMINE) showing increased TNFAIP8 mRNA expression in tumor tissues as compared with benign tissues (adapted from https://www.oncomine.org)

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Supplementary Fig. 8 TMEM33-centric interactive pathways (modified from STRING database)
(http://string905.embl.de/newstring_cgi/show_network_section.pl?taskId=ICWNuYf_Ewcx&interactive=no&advanced_menu=yes&network_flavor=evidence)

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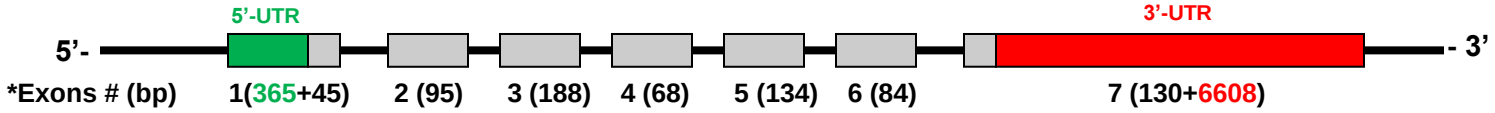
a Genomic *TMEM33 (SHINC3*, 25688 bp): LOCUS, 4p13, Chromosome 4; NC_000004.11 (41937137..41962824) mRNA accession no: NM_018126.2, predicted largest transcript 7717 bp; ORF, 744 bp (366 bp-1109 bp)

UniProt: P57088. TMEM33 gene sequence is conserved in 17 species including mouse, rat, C. elgans, and Drosophila (http://www.genome.ucsc.edu)



*http://genome.ucsc.edu/cgi-bin/hgBlat

b TMEM33 protein (247 aa; 27.978 kDa, pI 9.76): Amino acids are shown below in alternate blocks of black and blue colors corresponding to coding exons, and red amino acids encoded at junctions; phosphorylation site Thr 65; ubiquitination sites Lys 148 and Lys 221; three transmembrane helices (32 -52; 101-121; 156-176); belongs to PER33/POM33 family of conserved proteins (http://www.uniprot.org/uniprot/P57088; http://www.phosphosite.org; http:web.expasy.org/cgi-bin/protparam/protparam?P57088; http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/av.cgi?db=human&l=TMEM33) (underlined, epitope used for generating anti TMEM33 antibody)

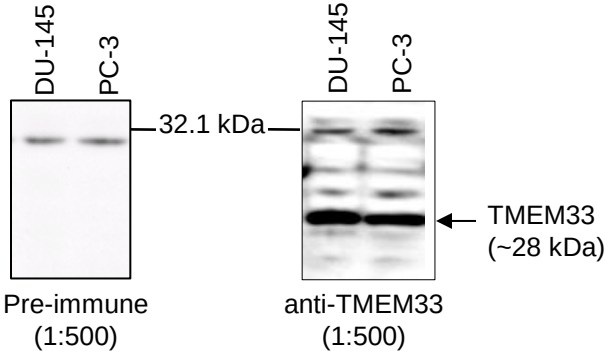
MADTTPNGPQGAGAVQFMMTNKLDTAMWLSRLFTVYCSALFVLPLLGLHEAASFYQRALLANALTSALRLHQRLPHFQLSRAFLA
QALLEDSCHYLLYSLIFVNSYPVTMSIFPVLLFSLLHAATY**TKKVLDARGSNSLPLLR**SVLDKLSANQQNILKFIACNEIFLMPA
TVFMLFSGQGSLLQPFIYYRFLTLRYSSRRNPYCRTLFNELRIVVEHIIMKPACPLFVRRLCLQSIAFISRLAPTVP.*

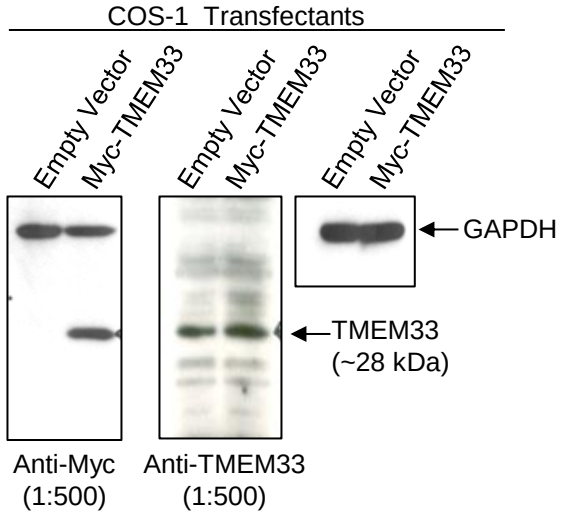
c Putative promoter region of *TMEM33* showing potential transcription factor binding sites (http://www.sabiosciences.com/chipqpcrsearch.php?gene=TMEm33&factor=Over+200+TF&species_id=0&ninfo=n&ngene=n&nfactor=y http://www.genecards.org/cgi-bin/carddisp.pl?gene=TMEM33

Supplementary Fig. 2 Expression analysis of endogenous TMEM33 protein in human cancer cells and COS-1 cells

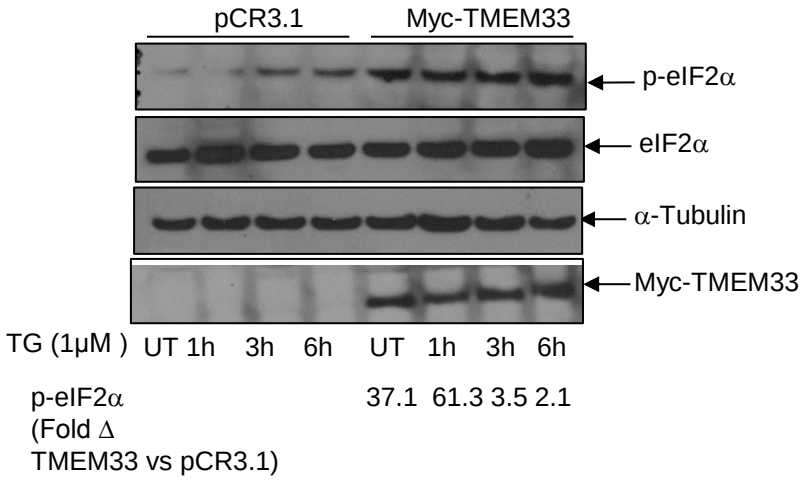
a Immunoblot analysis of endogenous TMEM33 in human prostate cancer cells using anti-TMEM33 antibody. The blot was sequentially probed with anti-TMEM33 antibody and pre-immune serum

b Immunoblot analysis of TMEM33 in COS-1 cells transiently transfected with *Myc-TMEM33* expression vector. The blot was sequentially probed with anti-Myc and anti-TMEM33 antibodies



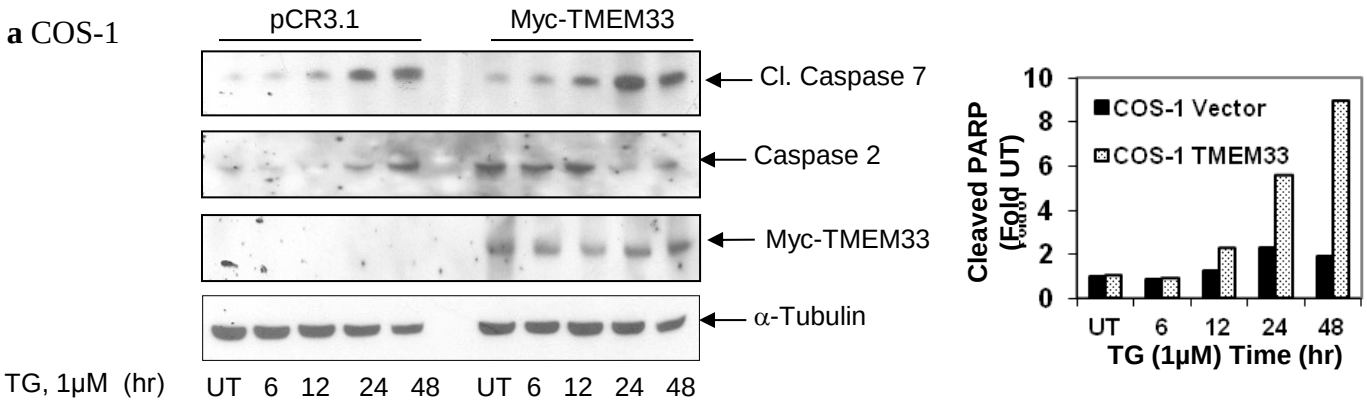


Supplementary Fig. 3 TMEM33 overexpression correlates with increased expression of p-eIF2α in HEK293T cells. Total eIF2 α levels were normalized against α-Tubulin in corresponding lanes. P-eIF2α expression levels were then normalized against α-Tubulin-normalized eIF2α levels in corresponding lanes. Higher levels of p-eIF2α expression, constitutive and in the presence of ER-stress, were seen in Myc-TMEM33 transfected cells as compared to control empty vector (pCR3.1). UT, untreated

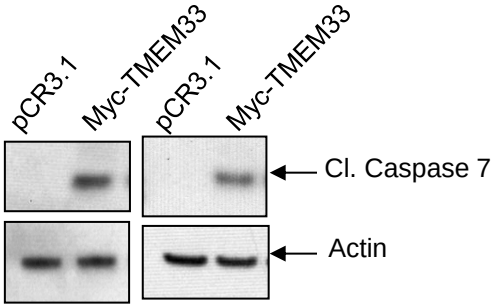


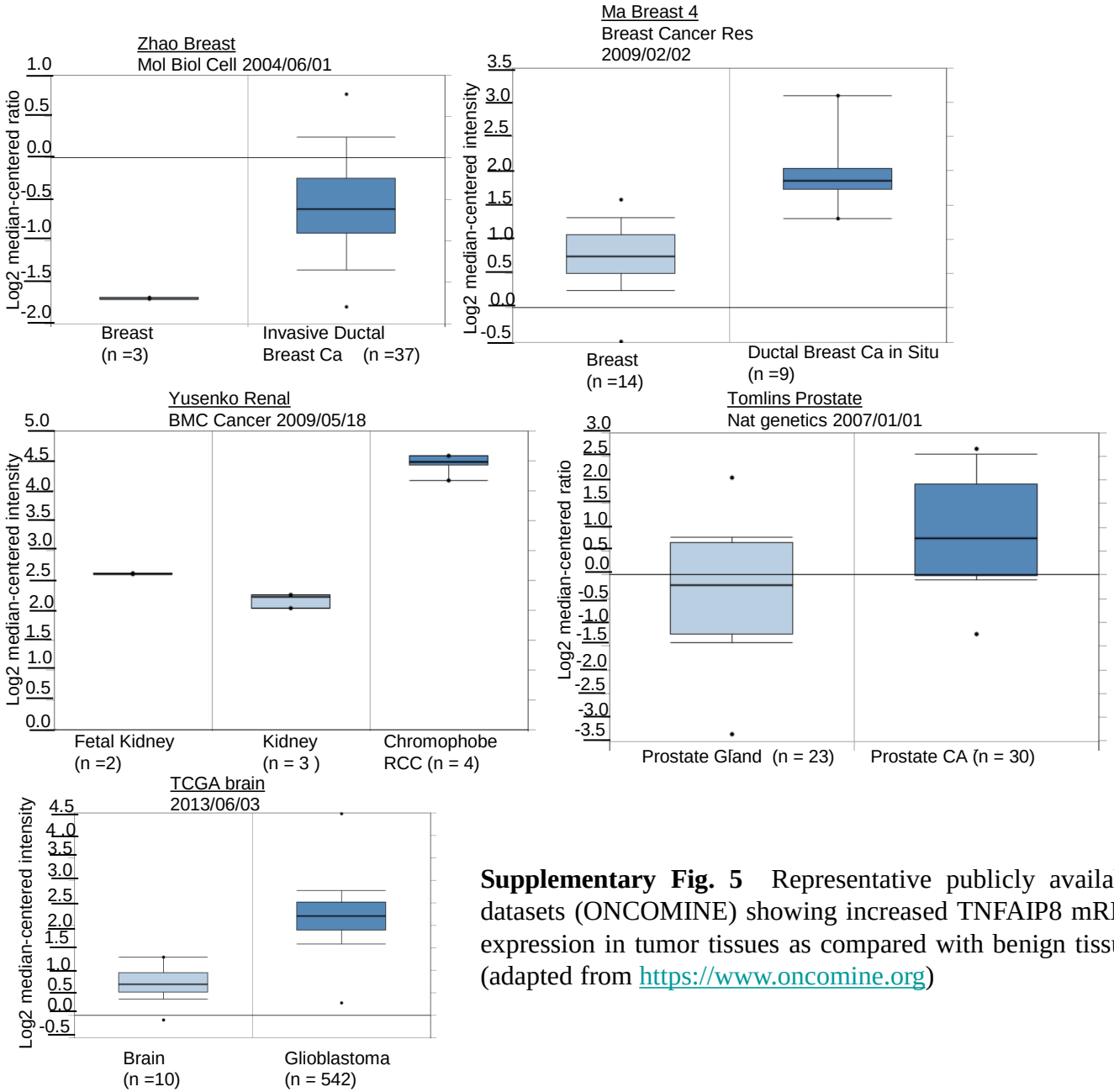
Supplementary Fig. 4 ER stress-induced levels of apoptotic molecules in *TMEM33* transfectants as compared to empty vector (pCR3.1) transfectants

a COS-1



b MCF-7





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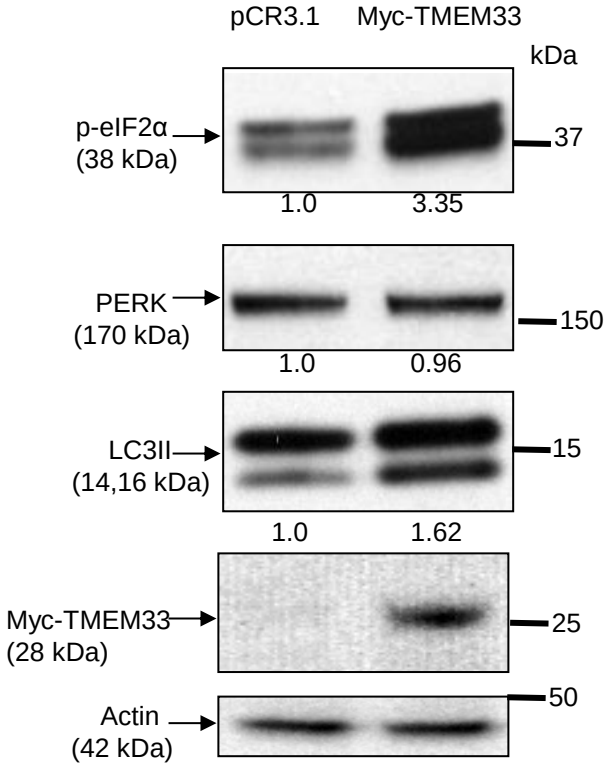

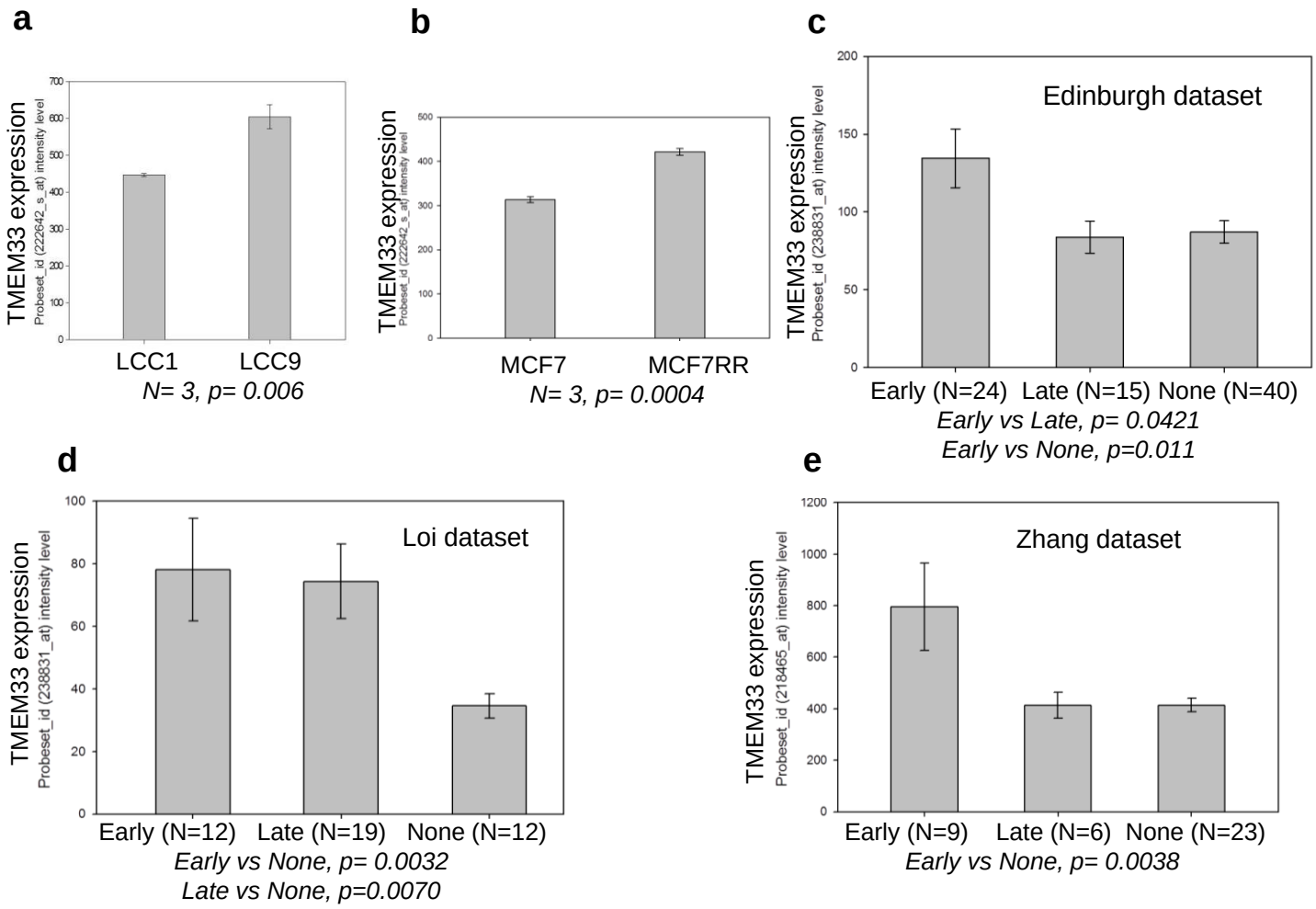

Supplementary Fig. 7 RNA array (HG-U133Plus2.0) profiling showed increased TMEM33 expression in endocrine-resistant breast cancer cells and in early recurrence breast cancer tissues. *TMEM33* mRNA expression profiles were analyzed in sensitive (LCC1) and endocrine-resistant breast cancer cells (LCC9), and sensitive MCF7 and antiestrogen-resistant MCF-7 cells (MCF7RR). We also interrogated three breast cancer datasets for potential correlation between *TMEM33* expression and breast cancer recurrence. Early, late, and non-recurrent groups were categorized according to their time to recurrence, < 3 years (early), > 5 years (late) and non-recurrence (no recurrence ≥ 10 years). *TMEM33* mRNA expression was found to be high in early recurrent versus non-recurrent breast cancers. Fold increase (Probeset_id): **a** 1.352 (222642_s_at); **b** 1.344 ((222642_s_at); **c** early vs. late, 1.604 (238831_at) ; **d** early vs. none, 2.260 (238831_at) ; **e** early vs. none, 1.923 (218465_at)

Supplementary Fig. 8 TMEM33-centric interactive pathways (modified from STRING database) (http://string905.embl.de/newstring_cgi/show_network_section.pl?taskId=ICWNuYf_Ewcx&interactive=no&advanced_menu=yes&network_flavor=evidence)