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Characterization of the cellular localization of C4orf34 as a novel endoplasmic reticulum resident protein

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Human genome projects have enabled whole genome mapping and improved our understanding of the genes in humans. However, many unknown genes remain to be functionally characterized. In this study, we characterized human chromosome 4 open reading frame 34 gene (hC4orf34). hC4orf34 was highly conserved from invertebrate to mammalian cells and ubiquitously expressed in the organs of mice, including the heart and brain. Interestingly, hC4orf34 is a novel ER-resident, type I transmembrane protein. Mutant analysis showed that the transmembrane domain (TMD) of hC4orf34 was involved in ER retention. Overall, our results indicate that hC4orf34 is an ER-resident type I transmembrane protein, and might play a role in ER functions including Ca^{2+} homeostasis and ER stress. [BMB Reports 2014; 47(10): 563-568]

INTRODUCTION

It is estimated that the human genome contains 20,000-25,000 genes, among which chromosome 4 is the fourth largest chromosome, containing 757 protein-encoding genes (1), many of which are related to diseases such as cancer, Huntington's disease, and Parkinson's disease (2). However, many genes discovered in the human genome still have unknown function and are denoted as chromosome 4 putative open-reading frame genes (C4orf genes).

Membrane proteins play key roles in various cellular functions in different membranous organelles within cells. Specifically, they function as receptors, cell adhesion molecules, and adaptor proteins involved in cell to cell recognition, signal transduction for cell growth and survival. Accordingly, their cellular localizations within various organelles are essential for their proper functions. Indeed, the signal sequences or specific domain structure for their localization and topology are well organized and conserved between species (3). Transmembrane proteins can be classified into type I, II and multi-spanning membrane proteins (4). The N-terminus of the type I transmembrane protein faces the extracellular side in the plasma membrane or luminal side of intracellular organelles. In type II transmembrane protein, the C-terminus of the protein faces the extracellular side in the plasma membrane or the luminal side of the intracellular organelles (5).

Many signaling mechanisms are involved in the intracellular targeting of proteins localized to specific organelles. ER resident type I transmembrane proteins usually contain the lysine motif (K(X)KXX) domain in C-terminal cytoplasmic tails of the proteins, enhancing their interaction with COPI vesicle machinery. Similarly, ER resident type II transmembrane proteins contain di-arginine residue (RRXX) domain within the N-terminus of the proteins (6). Another less known mechanism of ER retention/retrieval is that transmembrane domain (TMD) itself plays an important role in ER retention of the membrane proteins (7, 8). The length and composition of TMD is important for the ER retention. For example, ER targeting of Ufe1p is determined by the length of Ufe1p TMD and composition of TMD, but not by its specific sequences (7). However, it is still not clear how TMD is involved in ER retention.

It is known that 271 proteins are probably annotated to single transmembrane proteins in chromosome 4, and that their functions need to be elucidated (1). In this study, we tried to identify novel transmembrane proteins in human C4orf genes that had a single TMD and higher homology with other species. Based on these criteria, we intensively searched human C4orf genes in the National Center for Biotechnology Information (NCBI), applied two transmembrane helix predictors (TMHMM (9) and Scampi (10)), and identified three novel potential transmembrane proteins, C4orf32, C4orf34, and C4orf52. C4orf32, C4orf34, and C4orf52 were cloned by reverse transcriptase-polymerase chain reaction (RT-PCR) of HeLa cDNA, indicating that three C4orf genes were expressed in HeLa cells. Among these, we further characterized the ex-

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pression and cellular localization of C4orf34. We found that it was ubiquitously expressed in the organs of mice, including the heart and brain. hC4orf34 was localized to the ER within the cells. Taken together, our results suggest that C4orf34 is a novel ER resident, type I transmembrane protein that might play a role in ER functions.

RESULTS AND DISCUSSION

Identification of single transmembrane proteins among C4orf genes

To identify novel transmembrane proteins in human C4orf genes, we intensively searched human C4orf genes in NCBI database using two transmembrane helix predictors (TMHMM and Scampi). In addition, we conducted a BLAST search of the NCBI with the following criteria: (1) putative genes have a single TMD and (2) putative genes have higher homology with other species. Based on these criteria, we identified three novel potential transmembrane proteins, C4orf32, C4orf34, and C4orf52 (Fig. 1A). Three C4orf genes were cloned by RT-PCR of the HeLa cDNA, indicating that these putative mRNAs were expressed in HeLa cells (Fig. 1C). Among them, Human C4orf34 (hC4orf34) has 99 putative amino acids and one potential TMD. The similarity of amino acid sequences between hC4orf34 and C4orf34 was 89.7% for mouse, 69.7% for Zebrafish (Danio), 70.7% for *Xenopus* C4orf34, indicating high conservation from invertebrate to mammalian cells (Fig. 1A). Therefore, in this study, we focused on the characterization of human C4orf34 in mammalian cells. Sequence analysis suggests that the C-terminus of hC4orf34 has higher portions of proline residues (9 out of 29) with several proline-rich sequences (PXXP). The proline-rich domain is usually important for protein-protein interactions including the SH3 domain (17); therefore, the C-terminus of C4orf34 might be involved in protein-protein interactions.

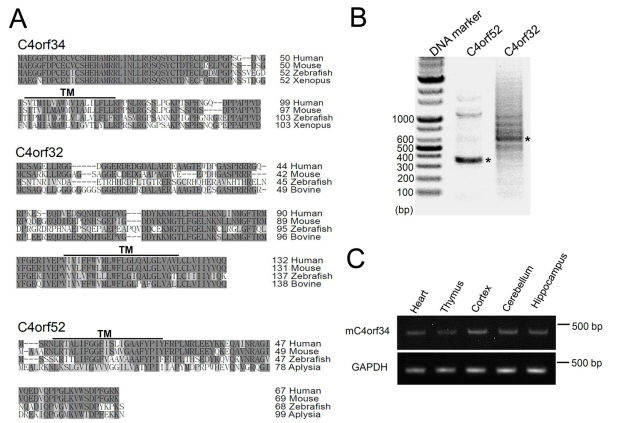

Fig. 1. Analysis of C4orf34, C4orf32, and C4orf52 genes. (A) Alignment of amino acid sequences of C4orf34, C4orf32, and C4orf52 from various species including *Aplysia* and human. Sequence comparison clearly indicates that amino acid sequences of C4orf34, C4orf32 or C4orf52 are highly conserved from invertebrates to mammals. Each gene has a potential TMD. C4orf34: Human, *Homo sapience* (NCBI Accession Number: NP_777581); Mouse, *Mus musculus* (NCBI Accession Number: NP_598458); Zebrafish, *Danio rerio* (NCBI Accession Number: AAI54300); Xenopus, *Xenopus laevis* (NCBI Accession Number: NP_001084560). C4orf52: Human, *Homo sapience* (NCBI Accession Number: NP_001138904); Mouse, *Mus musculus* (NCBI Accession Number: NP_001138905); Zebrafish, *Danio rerio* (NCBI Accession Number: XP_002663002); Aplysia, *Aplysia californica* (NCBI Accession Number: XP_005096219). C4orf32: Human, *Homo sapience* (NCBI Accession Number: NP_689613); Mouse, *Mus musculus* (NCBI Accession Number: EDL12251); Zebrafish, *Danio rerio* (NCBI Accession Number: NP_001018611); Bovine, *Bos taurus* (NCBI Accession Number: NP_001070429). (B) The expression of human C4orf32 and C4orf52 transcript in HeLa cells. mRNA expression of C4orf32 and C4orf52 were examined by RT-PCR in HeLa cells. Each PCR band (*) was confirmed as a C4orf52 (an expected size: 368bp) and C4orf32 (an expected size: 585bp) respectively, using T-A cloning and sequencing. (C) Tissue expression of mouse C4orf34 transcript. RT-PCR revealed that mRNA was ubiquitously expressed in mouse tissues including heart, thymus, cortex, hippocampus and cerebellum.

To check the expression and tissue distribution of the C4orf34 gene, we conducted RT-PCR with specific primer sets using cDNA obtained from various tissues in mouse. To discriminate PCR products of cDNA from those of genomic DNA contaminants, we selected sense and anti-sense primers from different exons, respectively. The mouse C4orf34 (mC4orf34) gene was ubiquitously expressed in mouse tissues including the heart, thymus and hippocampus (Fig. 1B). Taken together, these results suggest that mC4orf34 is ubiquitously expressed in various tissues, including the brain.

ER localization of hC4orf34

To examine the cellular localization, hC4orf34-EGFP was expressed in HeLa cells. The expression of hC4orf34-EGFP showed a network-like pattern including nuclear envelop in HeLa cells (Fig. 2A). The ER structure usually shows an intracellular network pattern within cells (18); therefore, to examine this possibility, we performed immunocytochemistry using the endogenous ER marker, calnexin, in hC4orf34-EGFP expressing cells. hC4orf34-EGFP was co-localized with endogenous calnexin (Fig. 2B), suggesting ER targeting of hC4orf34-EGFP. To further confirm ER targeting of hC4orf34, we expressed hC4orf34-3xFLAG together with Sec61-EGFP, another ER marker, in HeLa cells. Human hC4orf34-3xFLAG was co-localized with Sec61-EGFP (Fig. 2B), further confirming the ER localization of hC4orf34-3xFLAG. Overall, our data suggest that C4orf34 targeted ER in HeLa cells.

ER retention sites of hC4orf34

There is no conventional ER retention signal within hC4orf34 coding sequences (Fig. 1A). To identify ER retention sites within hC4orf34, we generated several deletion constructs as described in Fig. 2C. C-terminal (hC4orf34(ΔC)-EGFP) or N-terminal deletion of hC4orf34 (hC4orf34(ΔN)-EGFP) fused to EGFP

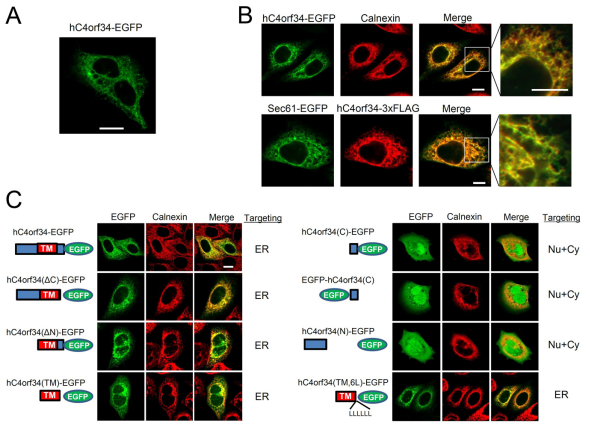

Fig. 2. ER localization of hC4orf34 in HEK293T cells. (A) Cellular localization of hC4orf34-EGFP in HeLa cells. hC4orf34-EGFP was expressed in the network structure within the cytoplasm. Scale bar, 20 μm. (B) Co-localization of hC4orf34 with ER markers in HeLa cells. Immunocytochemistry clearly showed that an ER marker, calnexin, was co-localized with hC4orf34-EGFP in HeLa cells. Another ER marker, sec61-EGFP, was also co-localized with hC4orf34-3xFLAG in HEK293T cells. Scale bar, 20 μm. (C) Identification of ER retention sites within hC4orf34. hC4orf34 and mutants were transfected into HEK 293T cells. Several hC4orf34 constructs were co-localized with calnexin, but hC4orf34(C)-GFP and hC4orf34(N) were not, suggesting that TMD of hC4orf34 might be involved in ER targeting. ER, endoplasmic reticulum; Cy, cytosol; Nu, nuclear. Scale bar, 20 μm.

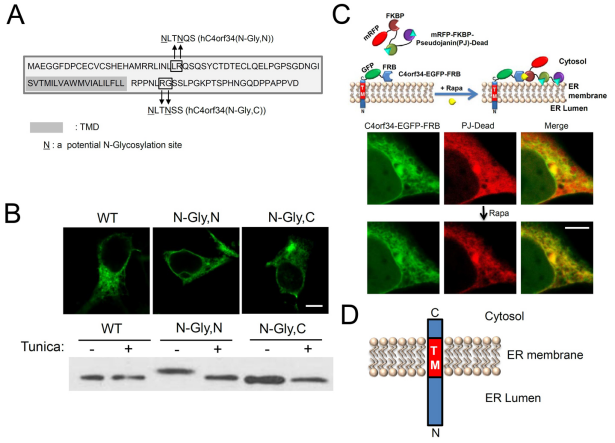

Fig. 3. Identification of topology of hC4orf34. (A) Schematic diagrams showing N-terminal or C-terminal N-glycosylation mutation sites within hC4orf34. To investigate the topology of hC4orf34, we artificially generated N-linked glycosylation within the hC4orf34 N-terminal and C-terminal region. (B) Cellular localization (upper) or Western blotting of hC4orf34 wild type or mutants in the presence or absence of tunicamycin (lower). hC4orf34-GFP, hC4orf34 (N-Gly,N)-GFP or hC4orf34(N-Gly,C)-GFP was transfected into HEK 293T cells. At 24 hr after transfection, cells were treated with 10 μg/ml tunicamycin for 4 hr. All mutants were expressed in ER (upper). Western blotting analysis showed that only hC4orf34 (N-Gly,N) was shifted, and that it was shifted down in response to tunicamycin treatment (lower), suggesting that the N-terminus of C4orf34 faces the luminal side of the ER, while mutated potential N-glycosylation sites were glycosylated. Tunica, 10 μg/ml tunicamycin. (C) A schematic diagram of experimental models of hC4orf34-EGFP-FRB/rapamycin/PJ-DEAD system (upper) and cellular localization of hC4orf34-EGFP-FRB and PJ-DEAD in the absence or presence of rapamycin in HEK293T cells (lower). The localization of PJ-DEAD was shifted from cytoplasm to ER by adding rapamycin. Rapa, 1 mM rapamycin. Scale bar, 20 μm. (D) Schematic diagram showing that hC4orf34 is a type I membrane protein. hC4orf34 has a single TMD, and the N-terminus of hC4orf34 is localized to the luminal side of ER. Tunica, tunicamycin; TM, transmembrane. Scale bar, 20 μm.

was still targeted to ER, similar to that observed for the hC4orf34 wild-type. These results suggest that the TMD might mediate ER retention. To test this, we generated N- and C-terminal deletion mutant (hC4orf34(TM)-EGFP) and expressed it in HEK293T cells. As shown in Fig. 2C, hC4orf34(TM)-EGFP was localized to the ER, suggesting that TMD might mediate ER retention. Expression of the N-terminal (hC4orf34(N)-EGFP) or C-terminal region (hC4orf34(C)-EGFP) of hC4orf34 fused to EGFP showed cytoplasmic expression, suggesting that the N-terminus and C-terminus of hC4orf34 has no ER targeting motif. Accordingly, it is possible that C-terminal fusion of EGFP to hC4orf34 interferes with the proper targeting of the hC4ORF34 C-terminus. To examine this possibility, EGFP was fused to the N-terminus of the C-terminal domain of hC4orf34, generating EGFP-hC4orf34(C). However, this mutant was also localized at the cytoplasm. Although we could not exclude the possibility that the N- or C-terminal domain contains novel ER retention signals, our results suggest that TMD of hC4orf34 might have an ER retention signal.

To examine whether the length of TMD was involved in ER localization, we add 6 leucine within C-terminal region of TMD, and generated hC4orf34(TM,6L)-EGFP which has a 24-25 aa length of TMD. However, hC4orf34(TM,6L)-EGFP was still localized and restricted at ER (Fig. 2C), indicating that the length of TMD was not essential for the ER retention. Instead, it is plausible that hC4orf34 might have ER retention signals within the composition of TMD as reported previously (7). However, further analysis is required for clarifying this.

Determination of topology of hC4orf34

To clarify the topology of hC4orf34, we used asparagine-linked glycosylation (N-glycosylaion) modification, because N-glycosylation only occurred in the luminal side of ER and induced a band shift in the SDS page gel (20). N-glycosylation usually has a potential consensus sequence (NX(S/T), X is not proline). Unfortunately, hC4orf34 had no potential N-glycosylation sites; therefore, two potential N-glycosylation sites within the hC4orf34 N-terminus were generated by replacing $N_{(24)}LL_{(26)}R_{(27)}QS$ with $\underline{N}_{(24)}LT_{(26)}\underline{N}_{(27)}QS$ (hC4orf34(N-Gly,N) or $NLR_{(76)}G_{(77)}SS$ with $\underline{N}LT_{(76)}\underline{N}_{(77)}SS$ (hC4orf34(N-Gly,C) (underline = potential N-glycosylation site) (Fig. 3A). Each mutant of hC4orf34 has two potential N-glycosylation sites within the N-terminus or C-terminus of C4orf34, respectively,

and each mutant was transfected into HEK293T cells. Cellular localization of each mutant was not changed (Fig. 3B, upper), indicating proper ER targeting. However, hC4orf34(N-Gly,N) was shifted in SDS page gels compared to wild type (Fig. 3B, lower). Conversely, hC4orf34(N-Gly,C) showed no shift when compared to wild type. These results suggested that the N-terminus of hC4orf34 might be localized at the luminal side of the ER (Fig. 3D) and N-glycosylation within the N-terminus of hC4orf34(N-Gly,N) may have occurred.

To confirm that this shift was due to N-glycosylation, we pretreated wild type- and mutant-expressing cells with tunicamycin, an N-glycosylation inhibitor in hC4orf34. The treatment of tunicamycin induced a down-shift of hC4orf34(N-Gly,N) to the control level (Fig. 3B, lower), suggesting that the band shift hC4orf34(N-Gly,N) is primarily mediated by N-glycosylation. In wild type, there is no down-shift by tunicamycin treatment, indicating no N-glycosylation site within the hC4orf34 wild type. Overall, these results confirm that hC4orf34 belongs to a type I transmembrane protein and contains a C-terminus located on the cytoplasmic surface (Fig. 3D).

To further confirm the topology of hC4orf34, we used inducible system of the ternary complex between FKBP, rapamycin, and rapamycin-binding domain of mTOR (FRB) (21). First of all, we fused FRB into C-terminus of hC4ORF34-EGFP, generating hC4orf34-EGFP-FRB as described in material and method sections. We cotransfected hC4orf34-EGFP-FRB with Pseudojanin-DEAD (PJ-DEAD), which contains mRFP, inactive sac and inactive INPP5E (12). As shown in Fig. 3C (lower), PJ-DEAD and hC4orf34-EGFP-FRB were expressed in cytoplasm and ER in the absence of rapamycin, respectively. However, in the presence of rapamycin, PJ-DEAD was shifted from cytoplasm to ER (Fig. 3C, lower). This result indicates that FRB is localized in cytoplasmic surface of ER, in which cytoplasmically expressed PJ-DEAD can be associated with hC4orf34-EGFP-FRB in the presence of rapamycin (Fig. 3C (upper)). Overall, our results suggest that the N-terminus of hC4orf34 is located within the luminal side of the ER and that the C-terminus of hC4orf34 is located in the cytoplasmic side (Fig. 3D).

Interestingly, the C-terminus of hC4orf34 has proline-rich sequences, suggesting that it might interact with cytoplasmic proteins. Transmembrane adaptor proteins (TRAPs) are known as integral membrane proteins that provide multiple docking sites for the cytoplasmic proteins containing SH2-domain, plays key roles in immune responses in hematopoietic cells (22). Therefore, it is plausible that hCorf34 might play roles in ER functions via recruitment of cytoplasmic proteins.

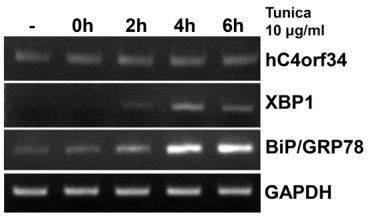

Fig. 4. Effects of ER stress by tunicamycin on endogenous hC4orf34 expression. Total RNA was extracted from tunicamycin-treated Hela cells. Sp-XBP1 and BiP/GRP78 RNA expression levels at 2 h and 4 h were increased in comparison with the control (non-treated and 0 hr), while hC4orf34 RNA expression levels were unchanged. RNA expressions of hC4orf34, Sp-XBP1 and BiP/GRP78 were investigated in the absence or presence of tunicamycin. ER stress by tunicamycin induced the expression of ER-stress response genes including Sp-XBP1 and BiP/GRP78. Sp-XBP1; spliced XBP1, Tunica; tunicamycin.

Effects of ER stress on endogenous hC4orf34 expression

To further examine the possible function of hC4orf34 in ER, we examined whether the expression of hC4orf34 itself could be altered during ER stress-induced response. As shown in Fig. 4, spliced Xbp1 (Sp-XBP1) and BiP/GRP78 RNA expression levels at 2 h and 4 h were increased in comparison with the control (non-treated and 0 hr). In this condition, hC4orf34 RNA expression levels were unchanged. This result indicates that gene expression of hC4orf34 is not changed by ER stress (Fig. 4). Further molecular and cellular mechanistic studies are required to elucidate the exact roles of C4orf34 in the ER.

MATERIALS AND METHODS

DNA constructs

hC4orf32, hC4orf34, and hC4orf52 were cloned by RT-PCR from cDNA of HeLa cells using nested polymerase chain reaction (PCR) by the following specific primer sets: hC4orf32, hC4orf32-S/hC4orf32-A1 (first-round PCR) and hC4orf32-S/hC4orf32-A2 (second-round PCR); hC4orf34, hC4orf34-S/hC4orf34-A1 (first-round PCR) and hC4orf34-S/hC4orf34-A2 (second-round PCR); hC4orf52, hC4orf52-S/hC4orf52-A1 (first-round PCR) and hC4orf52-S/hC4orf52-A2 (second-round PCR) (Supplement table 1). Each PCR band was cloned using TA cloning and confirmed using sequencing. hC4orf34 constructs were cloned by PCR into an expression vector containing the C-terminal EGFP-tag (Invitrogen, pEGFP-N3) and pcDNA 3.1 vector containing C-terminal 3xFLAG into the Hind III-EcoRI site.

The wild type and deletion mutants of hC4orf34 were obtained by performing PCR using the following specific primer sets: hC4orf34, hC4orf34-D3-S/hC4orf34-EcoRI-A; hC4orf34 (PPN), hC4orf34-D3-S/hC4orf34(PPN)-EcoRI-A; hC4orf34(NGI), hC4orf34(NGI)-D3-S/hC4orf34-EcoRI-A; hC4orf34(NGI/PPN), hC4orf34(NGI)-D3-S/ hC4orf34(PPN)-EcoRI-A; hC4orf34(C,PPN), hC4orf34(PPN)-D3-S/hC4orf34-EcoRI-A; hC4orf34(N,NGI), hC4orf34-D3-S/hC4orf34(NGI)-EcoRI-A; hC4orf34(N-Gly,N), hC4orf34 (N-Gly,N)-S/hC4orf34-EcoRI-A and hC4orf34-D3-S/hC4orf34 (N-Gly,N)-A (first-round PCR), and hC4orf34-D3-S/hC4orf34-EcoRI-A (second-round PCR); hC4orf34(N-Gly,C), hC4orf34(N-Gly,C)-S/hC4orf34-EcoRI-A and hC4orf34-D3-S/hC4orf34(N-Gly,C)-A (first-round PCR), and hC4orf34-D3-S/hC4orf34-EcoRI-A (second-

round PCR); hC4orf34(NGI/PPN,6XLeu), hC4orf34(NGI)-D3-S/hC4orf34(6XLeu)-A (first-round PCR), and hC4orf34(NGI)-D3-S/hC4orf34(PPN)-EcoRI-A (second-round PCR) (Supplement table 1). Each PCR band was cloned using pEGFP-N3 or pcDNA 3.1 vector containing C-terminal 3xFLAG into the Hind III-EcoRI site.

pcDNA3.1-FRB was generated by performing recombinant PCR using FRB-XbaI-S and FRB-ApaI-A (PCR template: Lyn11-FRB-CFP). hC4orf34-EGFP was obtained by based on by performing recombinant PCR using the following specific primer sets, hC4orf34-D3-S/EGFP-XhoI-A, and subcloned into pcDNA3.1-FRB, generating pcDNA3.1-hC4orf34-EGFP-FRB. GFPC1-Sec61beta (plasmid 15108) (11), PJ-DEAD (plasmid 37999) (12) and Lyn11-FRB-CFP (plasmid 38003) (13) were obtained from Addgene.

Immunocytochemistry

Cell culture and Western blot were performed as described previously (14, 15). For immunocytochemistry, transfected HeLa cells were fixed with 4% paraformaldehyde (Noble biosciences) for 10 min and then 0.1% TritonX-100 for 5 min, after which they were blocked with 3% bovine serum albumin (BSA, Sigma) in PBS for 1 hr. Primary antibody (anti-flag M2 antibody, Sigma, 1 : 100; anti-calnexin, Abcam, 1 : 100) and secondary antibody (Cy3-conjugated affinipure donkey anti-mouse, Jackson Immunoresearch, 1 : 200) were incubated for 1hr each. All steps were performed at room temperature (RT) and images were captured using a confocal laser scanning microscope (Cal Zeiss LSM 510).

RT-PCR analysis

Total RNA was extracted from HeLa cells and several mouse tissues including the heart, thymus, cortex, hippocampus and cerebellum using Trizol Reagent II. The cDNA was synthesized using Superscript III reverse transcriptase with oligo-dT as the primers. These cDNA samples were used as templates for PCR reactions for GAPDH and mC4orf34-RT-S/mC4orf34-RT-A. Amplification was accomplished by subjecting the samples to 27 to 34 cycles of 94^{o}C for 15 s, 60^{o}C for 15 s and 72^{o}C for 30 s. As a control, mouse GAPDH gene was amplified using mouse GAPDH primers (mGAPDH-RT-S/mGAPDH-RT-A). The PCR products were visualized on 1.2% agarose gel.

For ER stress, total RNA was extracted from treated tunicamycin (10 μg/ml) HeLa cells using Trizol (Molecular research center, inc). These total RNA samples were normalized to 1 μg, and generated cDNA. As a template using 50 ng/μl cDNA, reverse transcription PCR was reacted for 25～32 cycles using the Superscript III kit (Invitrogen). Primers used by this reaction are as follows: hC4orf34-RT-S/hC4orf34-RT-A, hGAPDH-S/hGAPDH-A, XBPI-spl-S/XBPI-spl-A, hBiP/GRPreal-S/hBiP/GRPreal-A (Supplement table 1) (16).

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