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Expression and characterization of transmembrane and coiled-coil domain family 3

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Running title: Expression and characterization of TMCC3

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ABSTRACT

Transmembrane and coiled-coil domain family 3 (TMCC3) has been reported to be expressed in the human brain; however, its function is still unknown. Here, we found that expression of TMCC3 is higher in human whole brain, testis and spinal cord compared to other human tissues. TMCC3 was expressed in mouse developing hind brain, lung, kidney and somites, with strongest expression in the mesenchyme of developing tongue. By expression of recombinant TMCC3 and its deletion mutants, we found that TMCC3 proteins self-assemble to oligomerize. Immunostaining and confocal microscopy data revealed that TMCC3 proteins are localized in endoplasmic reticulum through transmembrane domains. Based on immunoprecipitation and mass spectroscopy data, TMCC3 proteins associate with TMCC3 and 14-3-3 proteins. This supports the idea that TMCC3 proteins form oligomers and that 14-3-3 may be involved in the function of TMCC3. Taken together, these results may be useful for better understanding of uncharacterized function of TMCC3.

INTRODUCTION

The transmembrane and coiled-coil domain (TMCC) family of proteins share common structural motifs (two transmembrane domains and two coiled-coil domains), and are composed of three protein members: TMCC1, TMCC2 and TMCC3. Based on genetic linkage analysis, TMCC1 was reported to be associated with hereditary congenital facial palsy, even though TMCC1 is apparently not the causative gene (1). Recently, TMCC1 was reported to be an endoplasmic reticulum (ER) protein which targets ER membrane through transmembrane domains (2). Both N-terminal and C-terminal regions are predicted to reside in the cytoplasm. TMCC1 interacts with ribosomal proteins through cytoplasmic tails. TMCC1 seems to be involved in ER organization because ER morphology was changed by overexpression of TMCC1 (2). However, the detailed function of TMCC1 is still unclear. It is the same for TMCC2 and TMCC3.

TMCC3 (KIAA1145) was isolated as a novel gene isolated from human brain, and later became known as a novel gene up-regulated in the developing brain, especially in the ventral tegmentum (3-5). There is no resolved structure or defined function. The gene and protein were identified in screening studies (6-8); however, no key information about this gene was added. Here, we report on our examination of the expression of TMCC3 mRNA in various human tissues and developing mouse embryos. We expressed recombinant TMCC3 proteins and identified the contribution of domains to localization and oligomerization.

RESULTS

Expression of TMCC3 in human tissues

To explore the expression of TMCC3 in human tissues, we synthesized cDNA from various human tissues and performed real-time PCR. As shown in Fig. 1, the expression level of TMCC3 was higher in whole brain and spinal cord, which is in accord with previously reported expression in brain tissues (3, 4). We also found the expression of TMCC3 in a wide variety of non-neuronal tissues such as testis, bone marrow, cerebellum of brain, kidney, lung, placenta, thyroid gland and trachea.

Expression of TMCC3 in developing mouse embryo

A broad and weak expression pattern of TMCC3 was examined in the developing hind brain (hb) and lung (lu) forming tissues at E12 (Fig. 2A and B). Especially, mesenchyme of developing tongue (tm) showed strong positive expression of TMCC3 (Fig. 2A). At E14, strong expression of TMCC3 was detected in the trigeminal ganglion (tg), oral epithelium (oe) and hindbrain-forming tissues (Fig. 2E). In addition, developing lung, kidney (kd) and somite (sm) showed the obvious positive expression of TMCC3 (Fig. 2F). Sense probes showed a negative reaction in all tissue sections after *in situ* hybridization (Fig. 2C, D, G, and H).

Expression and characterization of recombinant TMCC3 and its deletion mutants.

To directly examine the physical properties of TMCC3, we constructed plasmids for the expression of full length TMCC3 and its deletion mutant constructs, as shown in Fig. 3A. Full length TMCC3 protein is composed of 477 amino acids, including two coiled-coil domains and two transmembrane domains. The Mutant TMCC3-Δ1 protein lacks N-terminal 149 amino acids; therefore, it loses one of the coiled-coil domains. The Mutant TMCC3-Δ2

proteins start from the 366th residue; therefore, they have only transmembrane domains without any coiled-coil domains. The TMCC3 gene was expressed in HEK293 cells and the recombinant proteins were observed in Western blotting results with anti-Myc antibody. As shown in Fig. 3B, apparent molecular weights of TMCC3 and the deletion mutant proteins were similar to the predicted molecular weights (53 kDa, 36 kDa, and 13 kDa). In the case of TMCC3 and TMCC3-Δ1, there were additional TMCC3 protein bands with molecular weight approximately three-fold greater than predicted. In contrast, there was found a protein band with two-fold greater molecular weight for TMCC3-Δ2. Therefore, it is likely that full length TMCC3 and TMCC3-Δ1 proteins form trimers, while TMCC3-Δ2 forms dimers. We further identified location of the proteins in HEK 293 cells by confocal microscopy images and found that the localization pattern of TMCC3 protein is very similar to that of TMCC1 (2) suggesting that proteins are located in the endoplasmic reticulum (Fig. 3C). Through analysis after fractionation of the cell lysates to cytosol and nuclear fractions, we found that all of the TMCC3 proteins were present in the nuclear membrane fraction. In the case of full length TMCC3, it was also found in the cytosolic fraction (Fig. 3D). Considering that three kinds of TMCC3 recombinant proteins commonly include transmembrane domain, localization of TMCC3 in the membrane fraction including the endoplasmic reticulum is mediated through transmembrane regions, which is also in agreement with a previous report for TMCC1 (2).

Oligomerization of TMCC3 and association of TMCC3 with 14-3-3.

We immuno-precipitated TMCC3 proteins and analyzed co-immuno-precipitated proteins by mass spectroscopy, to identify TMCC3-interacting proteins. In addition to the two TMCC3 protein bands shown in Fig. 3, there was another large TMCC3 protein band with molecular weight much greater than 250 kDa, suggesting the presence of very large TMCC3 oligomers (Fig. 4). Importantly, we found that two bands of 14-3-3 proteins were associated with

TMCC3.

DISCUSSION

The transmembrane and coiled-coil domain (TMCC) family proteins were identified about two decades ago, however their function is still unclear. Among three TMCC members, TMCC1 was recently studied in detail and a functional contribution of TMCC1 to ER membrane organization was suggested. However, other members were never studied in detail. In this study, we tried to understand basal information regarding expression of TMCC3 and function of its main domains.

Based on RT-PCR analysis, TMCC3 has a wide spectrum of expression, with highest expression in brain, spinal cord and testis (Fig. 1). According to the accumulated information regarding the TMCC3 gene in the human genome database GeneCards (http://www.genecards.org), TMCC3 RNA is expressed widely with subtle difference depending on the analysis methods used (e.g., microarray, RNAseq or serial analysis of gene expression: SAGE). The protein expression profile previously obtained using the proteomics approach showed also showed wide expression of TMCC3 with higher expression of TMCC3 in pancreatic juice, lung, testis, placenta and stomach (6). The expression profile of TMCC3 in mouse embryos also showed that TMCC3 is expressed in various tissues including mesenchyme of developing tongue, hind brain forming tissues and developing lung (Fig. 2). Taken together, the function of TMCC3 may be required for normal function of many tissues.

There are two coiled-coil domains and two transmembrane domains in TMCC3 protein. Coiled-coil regions are known to be involved in protein-protein interactions and oligomerization. To characterize the contributions of each coiled-coil region and two transmembrane domains, we produced two deletion mutant constructs that involved deletion

of the first coiled-coil region (TMCC3-Δ1), or of both coiled-coil regions (TMCC3-Δ2). Analysis of the recombinant full length TMCC3 and mutant proteins by SDS-PAGE and immunoblotting, suggested that the second coiled-coil region is required for trimer formation (Fig. 3). Additional deletion of the coiled-coil region resulted in different oligomerization patterns; therefore, we can speculate that the coiled-coil regions are important in normal oligomerization. It also suggests that the transmembrane regions alone can induce protein-protein interaction between TMCC3 proteins, but that the interaction feature can be different from normal situations. In the case of TMCC1, dimer formation through the second coiled-coil domain was suggested. Therefore, there could be some difference in the oligomerization patterns of different TMCC proteins. The location of TMCC2 and TMCC3 in ER was suggested before in the previous study of TMCC1 (2). However, there was no detailed follow-up study regarding TMCC3. Our data confirmed the intracellular location of TMCC3 in ER. Furthermore, we first found by investigation of deletion mutant proteins that the transmembrane domains contribute to the localization of TMCC3 (Fig. 3).

Previously, TMCC1 was reported to interact with TMCC1, TMCC2 and TMCC3; resulting in homo- or hetero- dimerization and oligomerization. Furthermore, TMCC1 interacts with ribosomal proteins such as L4, L6 and P0. When we analyzed the protein bands co-immuno-precipitated with TMCC3 using mass spectroscopy, we found that TMCC3 interacts mostly with other TMCC3 proteins to form trimers, and possibly hexamers, based on the molecular weights of the identified protein bands (Fig. 4). In addition, we found that two 14-3-3 protein bands were associated with TMCC3. These 14-3-3 proteins are known to regulate intracellular signaling by interaction with various other proteins (9). Here, we propose a possibility that 14-3-3 may regulate TMCC3 function or vice versa. Differently from TMCC1, we could not find direct evidence that TMCC3 interacts with ribosomal proteins. We speculate that TMCC1, but not TMCC3, contributes primarily to recruitment of ribosomal

proteins. The information we obtained in this study may be useful for further study on TMCC3.

MATERIALS AND METHODS

Antibodies

The antibody to Myc-tag (mouse anti-Myc-tag IgG antibody) and β-actin antibody (mouse anti- β-actin IgG antibody) were purchased from Cell Signaling Technology (Beverly, MA, USA) and Sigma Aldrich (St Louis, MO, USA), respectively. The FITC-conjugated secondary antibody (donkey anti-mouse IgG antibody) and HRP-conjugated secondary antibody (donkey anti-mouse IgG antibody) were obtained from Jackson ImmunoResearch (West Grove, PA, USA).

Mice

Male and female ICR strain (8 weeks old) was purchased from OrientBio, Inc. (Seoul, Korea). Animal studies were approved by the Institutional Animal Care and Use Committee of Kyungpook National University (KNU 2012-42), and the ICR mice were maintained under specific-pathogen-free conditions. To investigate the TMCC3 expression pattern in the developing mouse embryo, we used time-mated pregnant mice. When the formation of a mating plug was confirmed, this day was taken as embryonic day 0 (E0). In this study, E12 and E14 embryos were harvested after the cervical dislocation of pregnant mice.

Reverse-transcription PCR analysis

To identify the tissue distribution of TMCC3 expression, we obtained several human tissue RNAs (Human Total RNA Master Panel II) from Clontech (Mountain View, CA, USA). We

synthesized cDNA to perform a real-time PCR using the CYBR Green Master Mix. For quantitative analysis of TMCC3 expression, we used the following primers: human TMCC3, 5'-CCACAAGGTCAAACTCACTGC-3', 5'-CAAAGACTTGCTTGATACGTCC-3'; human glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5'-AATCCCATCACCATCTTCCA-3', 5'-TGGACTCCACGACGTACTCA-3'. The PCR products were analyzed with the ABI 7000 machine (Applied Biosystems, Foster City, CA, USA).

In situ hybridization

Section *in situ* hybridization was performed with wax sections according to the standard protocols previously described (10). Briefly, wax embedded embryos were sectioned in 7-μm slices, and treated with proteinase K (20 μg/mL). Digoxigenin (DIG)-labeled RNA probes for TMCC3 were hybridized overnight at 70 °C. Antisense and sense probes were synthesized using mouse TMCC3 plasmid as a template. Positive reactions were identified using NBT/BCIP treatment.

Plasmids for recombinant TMCC3 expression

The TMCC3 cDNA was obtained from human fetal liver cDNA by RT-PCR. The following primer sets were used: TMCC3-WT 5' primer, 5'-CCAGAAGACGGGAAAGTTCG-3'; and TMCC3-WT 3' primer, 5'-AAGCTTCTTGGTATTATCATCCTTTCTATG-3'. The TMCC3 coiled-coil domain deletion mutants were amplified by PCR with the following primers: TMCC3-WT 3' primer: 5'-CAAAGACTTGCTTGATACGTCC-3'; TMCC3-Δ1 5' primer, 5'-GAATTCATGAGGAGCTCAAAGGACATTTC-3'; and TMCC3-Δ2 5' primer, 5'-GAATTCATGGCCTACCAGGCCTACGAGCG-3'. The PCR products were ligated into pcDNA-3.1/Myc-His(-)B vector (Invitrogen, Carlsbad, CA, USA).

Cell culture and establishment of stable cell lines for TMCC3 expression

To express the TMCC3 and its deletion mutants, we obtained the human embryonic kidney cell line, HEK 293, from the American Type Culture Collection (ATCC, Manassas, VA, USA). The HEK 293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, GIBCO, Rockville, MD, USA) with 10% fetal bovine serum (FBS, GIBCO at 37 °C in a CO_2 incubator. For the generation of TMCC3-WT, TMCC3-Δ1, and TMCC3-Δ2 expressing stable cell lines, we transfected TMCC3-WT and TMCC3 coiled-coil domain deletion mutants in HEK 293 cells with FuGENE 6 (Roche, Indianapolis, IN, USA). To establish stable cell lines, the protein-expressing cells were selected in 500 μg/mL G418 (Calbiochem, Darmstadt, Germany) for 14 days.

Western blotting

Expression of TMCC3 and its deletion mutants was determined by Western blotting according to the method previously described (11). To lyse the cells, we used cell lysis buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 150 mM NaCl, 5 mM ethylene-amine-tetra-acetic acid (EDTA), 100 mM NaF, 2 mM Na_3VO_4, and complete protease inhibitor cocktail (Roche) supplemented with 1.0% nonyl-phenoxyl-poly-ethoxyl-ethanol (NP-40). After removal of the cell debris by centrifugation, the cell lysates were resolved on 12.5% SDS-PAGE and the expression of Myc-tagged TMCC3-WT, TMCC3-Δ1, and TMCC3-Δ2 was verified using Western blotting with the anti-Myc-tag antibody (1:1,000 dilution) and HRP-conjugated secondary antibody (1:2,000 dilution).

Confocal images

Immunofluorescence staining and confocal microscopy was performed as previously described (12). After TMCC3-WT, TMCC3-Δ1, and TMCC3-Δ2 expressing stable cell lines

were cultured on glass coverslips in 24-well plates for 24 h, the cells were fixed with 4% paraformaldehyde for 10 min. The fixed cells were permeabilized with PBS containing 3% BSA and 0.1% Triton X-100 (PBS-BT) for 30 min. Then, the permeabilized cells were treated with antibody against Myc-tag for 2 h and incubated with FITC-conjugated goat anti-mouse IgG for 1 h. We used Hoechst no. 33258 (Molecular Probes, Eugene, OR, USA) for nuclei staining. After mounting the samples, the cells were scanned with an LSM 710 microscope (Carl Zeiss, Jena, Germany).

Cytosol and membrane fractionation

The cells were re-suspended with hypotonic lysis buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 0.5% NP-40, 5 mM EDTA and complete protease inhibitor cocktail) and centrifuged at 5000 r/min for 5 min at 4 °C. The supernatants containing the cytosolic fraction were transferred to new tubes and the pellets containing crude nuclei were lysed with membrane lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1% SDS, 700 U/mL DNase I, 5 mM EDTA, and complete protease inhibitor cocktail) for 1 h on ice. The solubilized membrane proteins were collected by centrifugation at 14,000 rpm for 10 min. The protein concentrations were measured using the BCA assay (Pierce, Rockford, IL, USA). Expression of Myc-tagged TMCC3-WT, TMCC3-Δ1, and TMCC3-Δ2 was observed by Western blotting with the anti-Myc-tag antibody.

Immunoprecipitation

Cells of a TMCC3-WT-expressing stable cell line were lysed for 30 min at 4 °C in a cell lysis buffer. After removal of the cell debris by centrifugation, the cell lysates were mixed with the anti-Myc-tag antibody for 2 h at 4 °C. Immuno-complexes containing TMCC3-WT and anti-Myc-tag antibody was collected by Protein A-Sepharose CL-4B (10% (v/v) slurry, Amersham

Pharmacia Biotech) as previously described (13).

Protein identification by mass spectrometry

To identify the TMCC3 and its binding proteins, immune-complexes containing TMCC3-WT and anti-Myc-tag antibody were separated by 12.5% SDS-PAGE. After staining with Coomassie brilliant blue G-250, the protein bands were digested in gel with trypsin. The digested peptides were separated with POROS R2 column (Applied Biosystems) for concentration and desalting. The resulting peptides were analyzed using electrospray ionization-time of flight mass spectrometry/mass spectrometry (ESI-TOF MS/MS). This was done using a Micromass Q-TOF MA equipped with a nano-spray source (Waters, Milford, MA, USA) in an In2gen facility (Seoul, Korea), as previously described (13). The amino acid sequences of the resulting peptides were analyzed using the database from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov).

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FIGURE LEGENDS

Fig. 1. Expression of TMCC3 in human tissues: The human tissue cDNA was analyzed using standard real-time PCR to check TMCC3 expression. The β-actin expression level was used as a control to normalize the amount of template cDNA.

Fig. 2. Expression pattern of TMCC3 in developing embryo: Mouse embryo sections were prepared at embryo day 12 (E12) and at embryo day 14 (E14), and analyzed by *in situ* hybridization using antisense and sense probes of TMCC3. tm: tongue mesenchyme, hb: hind brain, lu: lung, oe: oral epithelium, tg: trigeminal ganglion, kd: kidney, sm: somites.

Fig. 3. Expression and characterization of TMCC3 and its deletion mutant proteins: (**A**) Map of full length TMCC3 and deletion mutant expression vectors. (**B**) Expression of recombinant proteins in HEK293 cells. Cell lysates were prepared from HEK293 cells transfected with TMCC3 and its deletion mutant expression vectors. The cell lysates were separated by SDS-PAGE and analyzed using Western blotting with anti-Myc antibody. The amount of β-actin was used as a control. (**C**) Localization of TMCC3. HEK cells expressing TMCC3 recombinant proteins were visualized by immunofluorescence staining using anti-Myc antibody and confocal microscopy. (**D**) Differential localization of TMCC3 recombinant proteins in cytosolic and membrane fraction. Protein fractions were resolved by SDS-PAGE

and analyzed by Western blotting with anti-Myc antibody.

Fig. 4. Oligomerization of TMCC3 and association of TMCC3 with 14-3-3: Immuno-complexes containing TMCC3-WT and anti-Myc-tag antibody were separated by 12.5% SDS-PAGE. After staining with Coomassie brilliant blue G-250, the co-immuno-precipitated protein bands were examined by mass spectrometry. The protein bands identified were indicated with arrows.

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Figure 1

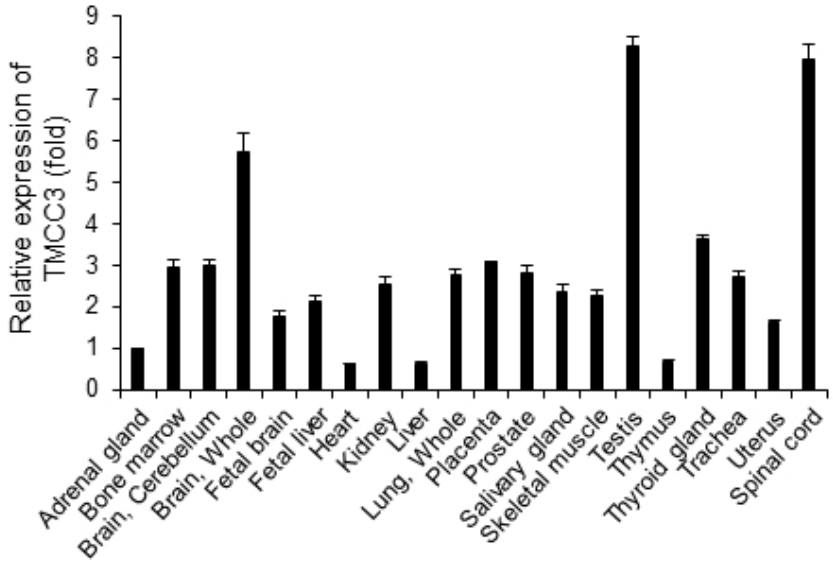

Fig. 1 Figure 1

Figure 2

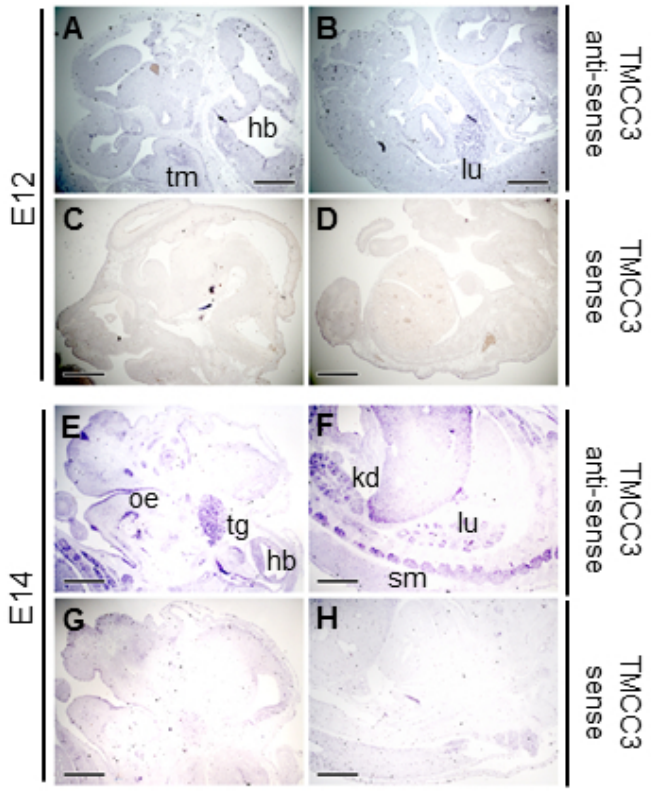

Fig. 2 Figure 2

Figure 3

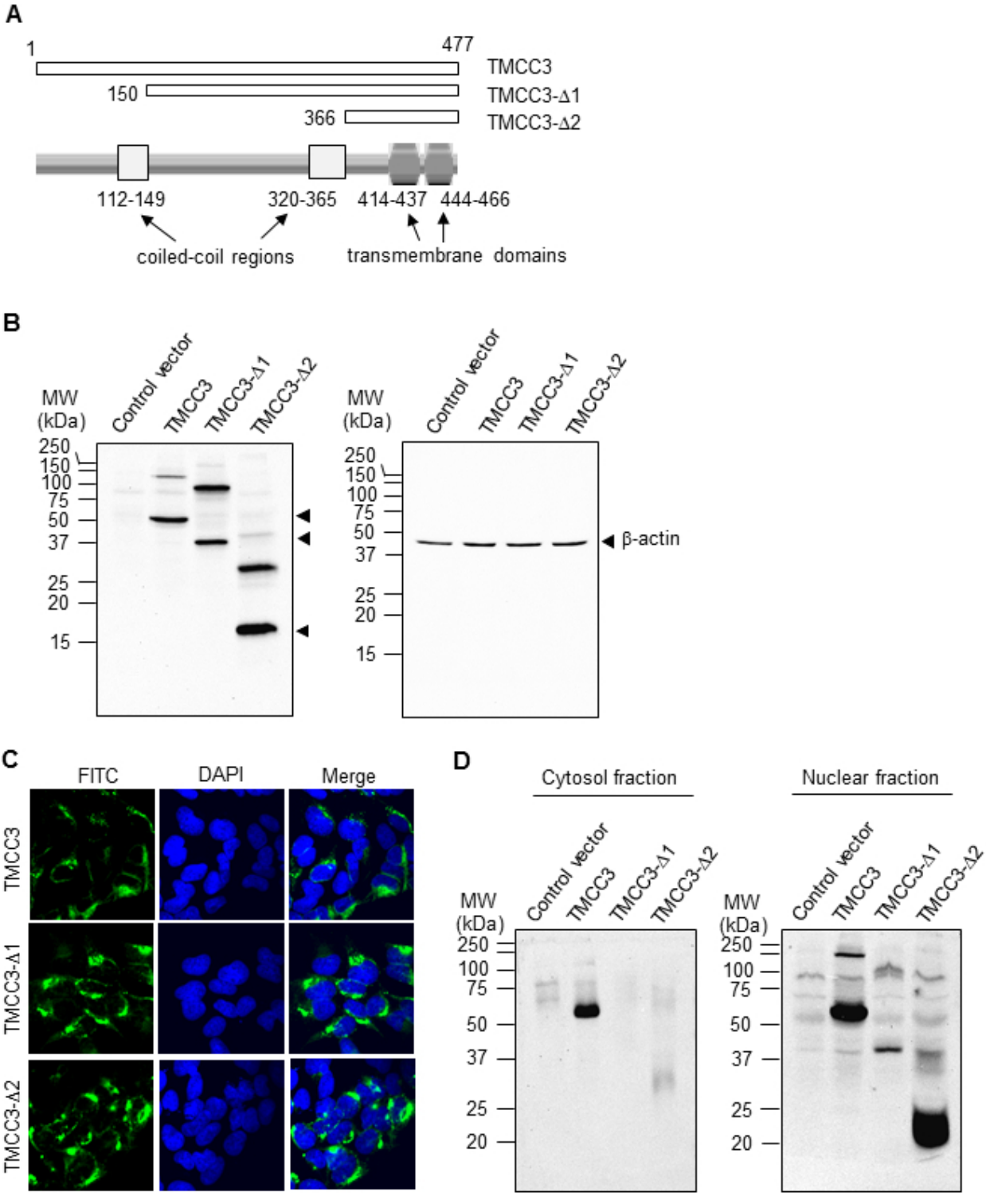

Fig. 3 Figure 3

Figure 4

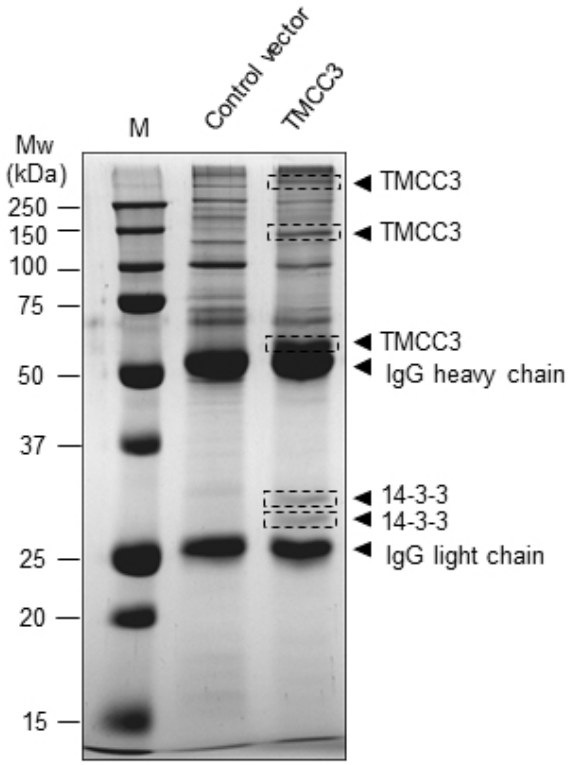

Fig. 4 Figure 4