Transmembrane 163 (TMEM163) protein interacts with specific mammalian SLC30 zinc efflux transporter family members

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SUPPORTING INFORMATION

Supplementary Methods

Co-Immunoprecipitation (co-IP)

HEK-293T cells were seeded at 1.5 × 10^6 cells in 10-cm poly-D-lysine (PDL)-coated tissue culture dishes. Cells were transfected for 24 hours with Turbofect reagent (Thermo Scientific) at a concentration of 2 μg for single plasmid transfections and 2 μg each of pCMV6 construct for co-transfections of TMEM163 and individual ZNT proteins tagged with either Myc-DDK or HA peptide (to equalize the plasmid concentrations across experiments). Twenty-four hours post-transfection, the cells were lysed with 1 mL of the Pierce Co-IP lysis buffer containing 1X protease inhibitor cocktail (PIC; Thermo Scientific) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich). The lysates were centrifuged at 14,000 rpm for 10 minutes at 4°C to pellet and remove cellular debris. We then performed dot blots of lysates to check for expression of the peptide tagged protein and qualitatively determine protein expression before performing co-IP assays. After determining the relative concentrations of total proteins in each sample, the cell lysates were diluted with lysis buffer to a volume of 500 μL and incubated with control agarose resin for 1 hour at 4°C on a rotator to remove non-specific proteins binding to the resin. After lysate pre-clearance, equal volumes of either DDK- or HA-coupled agarose beads were added to the lysates for overnight incubation at 4°C. Protein-bound beads were washed with the lysis buffer three times and eluted with the Pierce co-IP elution buffer. To validate initial co-IP results for specificity, we repeated all experiments with a modified the wash buffer by adding 500 mM of NaCl plus 1% Tween-20 to increase the stringency of the co-IP [28] to possibly reveal the relative strength of the interaction between the monomers. all protein eluates were reduced with 1X Bolt sample reducing agent (Thermo Scientific) and denatured with 1X NuPAGE LDS sample buffer (Thermo Scientific). For SDS-PAGE, the samples were loaded (15 μl) in two identically arranged Bolt 4-12% gradient Bis-Tris PAGE gels (Thermo Scientific) – one gel was used for downstream visualization of DDK-tagged proteins while the other gel is for HA-tagged proteins. Resolved protein samples were then transferred to nitrocellulose membranes. One blot was probed with primary anti-DDK mAb (1:2000) while the other blot with primary anti-HA pAb (1:5000). After a series of washes with Tris-buffered solution plus 0.1% Tween-20 (TBST), all blots were incubated for 1 hour at room temperature with secondary anti-mouse or anti-rabbit IR-Dye™ 800CW (1:15000). All immunoblots were subjected to a series of washes with TBST before scanning them using the LI-COR Odyssey SA™ or DLX™ IR imaging system (LI-COR Biosciences).

To analyze endogenous protein-protein interaction, we used postmortem mouse tissues (pancreas and testis) where mouse Tmem163 protein expression overlapped with Znt1, Znt2, Znt3, and Znt4 protein (Human Protein Atlas; www.proteinatlas.org). Before setting up the co-IP trials, we conjugated the anti-TMEM163 pAb with the agarose resin using the Pierce co-IP kit (Thermo Scientific) according to the manufacturer's recommendations. We used primary antibody conjugation as a way to minimize co-elution of immunoglobulins whose molecular weight is relatively similar to the target protein. The frozen tissues were pulverized using mortar and pestle situated on dry ice or liquid nitrogen and homogenized with T-Per lysis buffer containing 1 mM PMSF and 2X PIC. The homogenates were centrifuged at 13,000 rpm for 15 minutes at 4°C. Except for the pancreas, we added 0.5% DDM detergent in the T-Per lysis buffer to

homogenize the testis tissue to help improve membrane protein solubilization. The respective tissue homogenates were pre-cleared with control agarose resin for 30 minutes at 4°C on a rotator and then incubated overnight at 4°C with anti-TMEM163 pAb coupled to agarose beads. The following day, all samples were washed three times using the Pierce co-IP lysis buffer and the samples were recovered using the Pierce co-IP elution buffer. In cases where we observed low target protein recovery, we used the Pierce protein concentrator (10 kDa MWCO) according to the manufacturer's protocol before performing SDS-PAGE. The samples were loaded (20 μl) into a Bolt 4-12% Bis-Tris PAGE gels and the resolved proteins were blotted. Each blot was probed with the respective primary antibody as follows: anti-TMEM163 pAb (1:500), anti-ZNT1 pAb (1:500), anti-ZNT2 pAb (1:400), anti-ZNT3 mAb (1:500), and anti-ZNT4 pAb (1:500). The antibodies against ZNT1, ZNT2, and ZNT4 were conjugated with Alexa-Fluor-750 using the Zenon Rabbit IgG Labelling Kit (Thermo Scientific) according to the manufacturer's recommendation. Validation of species cross-reactivity for the commercial antibodies showed that only anti-TMEM163 pAb, anti-ZNT3 mAb and anti-ZNT4 pAb were able to detect over-expressed mouse and human proteins from cell lysates. Thus, we used these primary antibodies for WB assay. Following a series of washes with TBST, the blots were incubated with anti-rabbit IR-Dye™ 800CW (1:15000) secondary antibody and washed with TBST several times before analyzing them using the Odyssey SA™ or DLX™ IR scanner.

Immuno-cytochemistry (ICC) and Confocal Microscopy

HEK-293T cells were seeded at 50,000 cells per well in 12-well tissue culture plates containing PDL-coated coverslips. Cells were then transfected with pCMV6 expression constructs of TMEM163 and ZNT proteins tagged with HA or DDK. We used 1 μg of plasmid for single transfections and also 1 μg of each plasmid for co-transfections. Twenty-four hours post-transfection, the samples were washed once with 1X phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA) for 15 minutes. The cells were washed three times with 1X PBS for 5 minutes and washed once with PBT1 (1X PBS, 0.1% BSA, 5% heat-inactivated goat serum, and 0.1% Triton X-100) and then permeabilized with PBT1 for 15 minutes. The samples were incubated with either anti-DDK mouse mAb (1:800) (Millipore Sigma) or anti-HA rabbit pAb (8 μg/mL) (Millipore Sigma) overnight at 4°C. The samples were washed four times with 1X PBS and twice with PBT2 (1X PBS, 0.1% BSA, and 0.1% TritonX-100), then incubated with secondary antibodies diluted in PBT2 for 1 hour at room temperature. For anti-DDK mouse mAb, we used anti-mouse Alexa Fluor-488 (1:500; ex = 490 nm, em = 525 nm). For anti-HA rabbit pAb, we used anti-rabbit Alexa Fluor-568 (1:500; ex = 579 nm, em = 603 nm). The coverslips were then washed six times with 1X PBS. Each coverslip was mounted on a glass slide containing a drop of Prolong Diamond anti-fade reagent plus DAPI (Thermo Scientific), and the edges were sealed with a clear nail polish. Fluorescent images were captured using an Olympus FV3000 laser scanning confocal microscope. The monochromatic images were processed using Adobe Photoshop 2022 to change grayscale images to green for Alexa-488 fluorescence, red for Alexa-568 fluorescence and blue for DAPI fluorescence. All images were cropped and auto-contrasted before the three channels were merged as a single image using Photoshop. We used Adobe Illustrator to compile and label images.

TABLE S1. PCR primer sets. List of In-Fusion (IF) cloning and restriction enzyme (RE) subcloning primers used in this study. In-Fusion primers were designed using the Takara Bio online In-Fusion cloning primer design tools for all pBI constructs and two pCMV6 vectors (ZNT2-HA and ZNT4-HA). For the IF primers designed for pCMV6, we included restriction sites (*Sgf I* and *Mlu I*) for subcloning purpose in case that IF cloning fails. Primers with restriction enzyme sites were used to clone ZNT1 and 3 into the pCMV6 vector with HA or DDK tag using *Sgf I* and *Mlu I* restriction enzymes.

Gene/Plasmid	Primer Sequence
Human *TMEM163* pBI construct (MCS1)	IF Forward: 5'-TCT AGA GAC CAT GGA GCC GGC CGC GGG C-3' IF Reverse: 5'-GAA TTC TCT CAA ACA TCT CGT AGT G-3'
Human *ZNT1* pBI construct (MCS1)	IF Forward: 5'-TAG TCA GCT GAC GCG ACC ATG GGG TGT TGG GGT CGG-3' IF Reverse: 5'-CCG CGC TAG CAC GCG TCA CAA AGA TGA TTC AGG TTG-3'
pCMV6 construct	RE Forward: 5'-AAA AAA GCG ATC GCA CCA TGG GGT GTT GGG GTC GGA AC-3' RE Reverse: 5'-AAA AAA ACG CGT CAA AGA TGA TTC AGG TTG TTT GTT TG-3'
Human *ZNT2* pBI construct (MCS1)	IF Forward: 5'-TAG TCA GCT GAC GCG ACC ATG GAG GCC AAG GAG AAG CAG-3' IF Reverse: 5'-CCG CGC TAG CAC GCG TCA GTC TGA GGG GCC CTG-3'
pCMV6 construct	IF Forward: 5'-GGA GAT CTG CCG CCG CGA TCG CAC CAT GGA GGC CAA GGA GAA GCA GCA T-3' IF Reverse: 5'-GAG CGG CCG CGT ACG CGT GTC TGA GGG GCC CTG GCA TGC-3'
Human *ZNT3* pBI construct (MCS1)	IF Forward: 5'-TAG TCA GCT GAC GCG ACC ATG GAG CCC TCT CCA GCC G-3' IF Reverse: 5'-CCG CGC TAG CAC GCG TCA GGC TTG GGG GGG TTC-3'
pCMV6 construct	RE Forward: 5'-AAA AAA GCG ATC GCA CCA TGG AGC CCT CTC CAG CCG-3' RE Reverse: 5'-AAA AAA ACG CGT GGC TTG GGG GGG TTC CTG-3'

Human *ZNT4* pBI construct (MCS1)	IF Forward: 5'-TAG TCA GCT GAC GCG ACC ATG GCC GGC TCT GGC GCG-3' IF Reverse: 5'-CCG CGC TAG CAC GCG TTA GGG ACT AGA ACT CTG AC-3'
pCMV6 construct	IF Forward: 5'-GGA GAT CTG CCG CCG CGA TCG CAC CAT GGC CGG CTC TGG CGC GTG GAA G-3' IF Reverse: 5'-GAG CGG CCG CGT ACG CGT GGG ACT AGA ACT CTG ACA ATT TG-3'

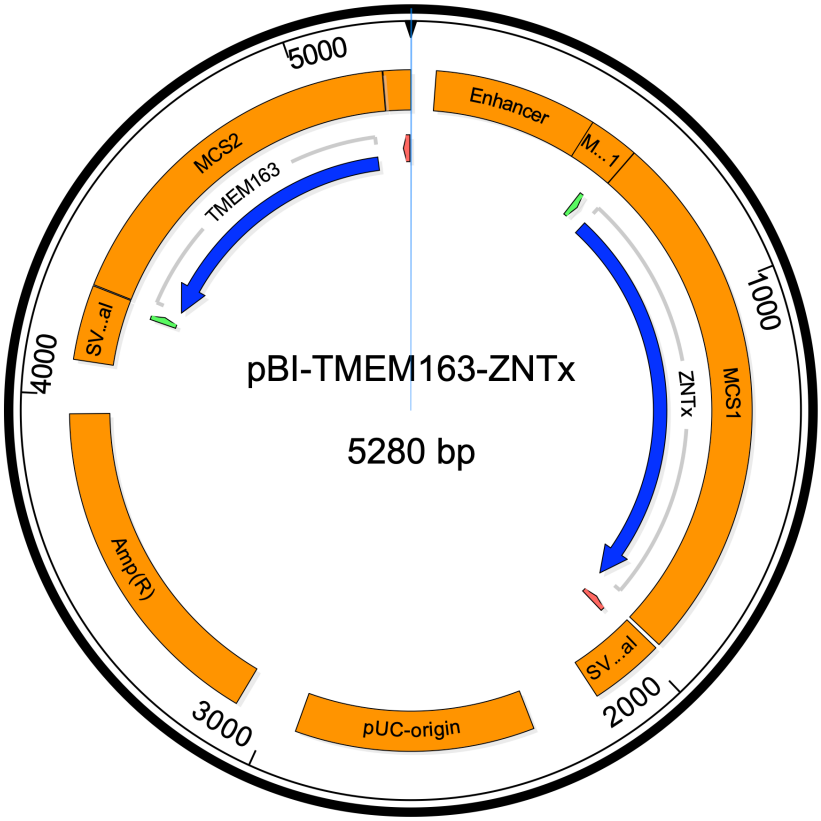

Figure S1. **Schematic diagram of the pBI dual expression vector.** Open reading frame (ORF) of TMEM163 was cloned into the MCS2 while ORF of each ZNTx (where x denotes 1, 2, 3, or 4) was cloned into MCS1 as described in the Materials and Methods section. Single expression constructs use the same configuration but leaving one of the other MCS empty. The pBI vector was purchased from Takara Bio, USA (Mountain View, CA).

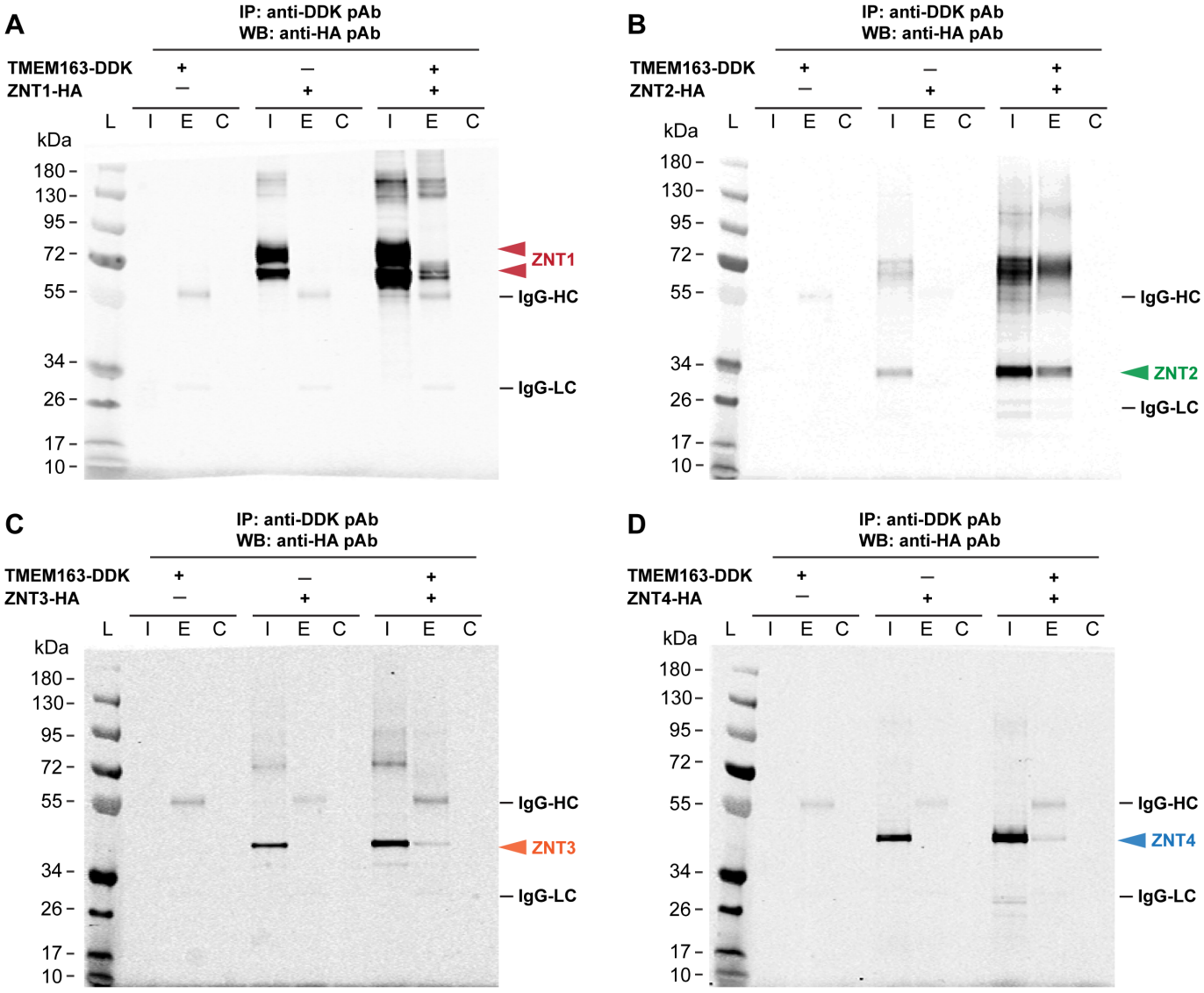

Figure S2. **Validations of co-immunoprecipitation experiments reveal specificity of protein-protein interaction.** To ensure that the proteins do not bind non-specifically to the agarose beads, all co-IP trials included control agarose resin. The resulting control lanes did not show discernible bands for all trials indicating specificity. Representative Western blot (WB) shows TMEM163-DDK co-immunoprecipitated (IP) with **A**) ZNT1-HA, **B**) ZNT2-HA, **C**) ZNT3-HA, and **D**) ZNT4-HA. Each respective ZNT protein co-elutes with TMEM163. Predicted molecular weight (MW) of human proteins: ZNT1 = 55.3-63.0 kDa (*arrowhead*); ZNT2 variant 2 = 35.0 kDa (*arrowhead*); ZNT3 = 41.3 kDa (*arrowhead*); ZNT4 = 47.4 kDa (*arrowhead*); IgG-HC, immunoglobulin-G heavy chain (MW ≅ 55 kDa); IgG-LC, immunoglobulin-G light chain (MW ≅ 23 kDa). L, protein ladder; I, input/cell lysate; C, control resin; E, elution; mAb, monoclonal antibody; pAb, polyclonal antibody. The representative images come from N ≥ 3 independent trials.

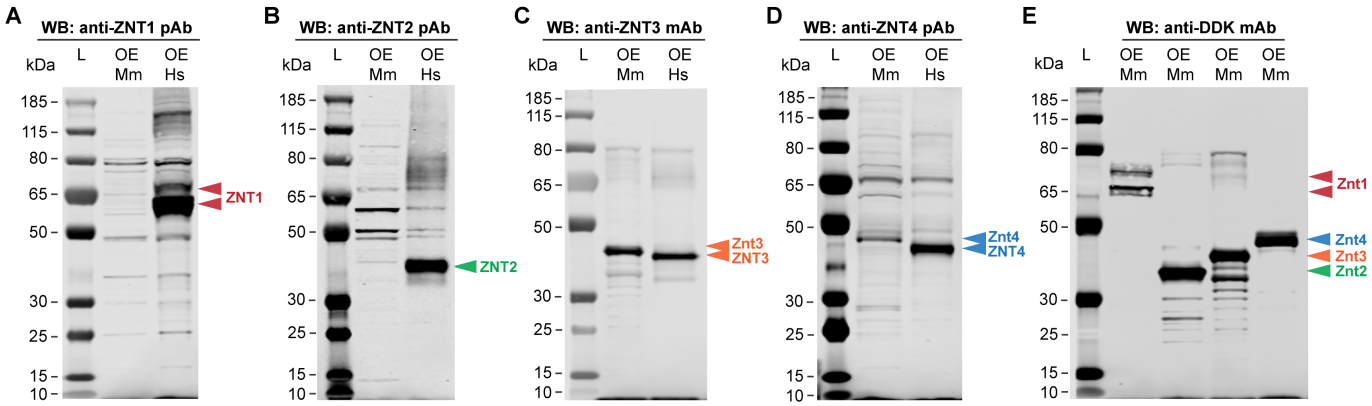

Figure S3. Species cross-reactivity validation of anti-ZNT1 and anti-ZNT2 antibodies. A) Western blot (WB) of mouse Znt1 and human ZNT1 proteins from cell lysates of each respective over-expressed protein (OE Mm and OE Hs). The anti-ZNT1 pAb only recognizes the human ZNT1 protein. **B**) WB of mouse Znt2 and human ZNT2 proteins from cell lysates of each respective over-expressed protein (OE Mm and OE Hs). The anti-ZNT2 pAb only detects the human protein. **C**) WB of mouse Znt3 and human ZNT3 proteins from cell lysates of each respective over-expressed protein (OE Mm and OE Hs). The anti-ZNT3 mAb detects both the mouse and human proteins. **D**) WB of mouse Znt4 and human ZNT4 proteins from cell lysates of each respective over-expressed protein (OE Mm and OE Hs). The anti-ZNT4 pAb detects both the mouse and human proteins. **E**) WB of cell lysates over-expressing mouse (OE Mm) Znt1, Znt2, Znt3, and Znt4 proteins tagged with DDK peptide. Protein expression was detected using anti-DDK mAb. Predicted molecular weight (MW) of mouse proteins: Znt1 = 54.6-62.3 kDa; Znt2 = 31.6 kDa; Znt3 = 41.7 kDa; and Znt4 = 47.7 kDa. Predicted MW of human proteins: ZNT1 = 55.3-63.0 kDa; ZNT2 variant 1 = 40.4 kDa; ZNT3 = 41.8 kDa; and ZNT4 = 47.4 kDa. L, protein ladder; mAb, monoclonal antibody; pAb, polyclonal antibody.

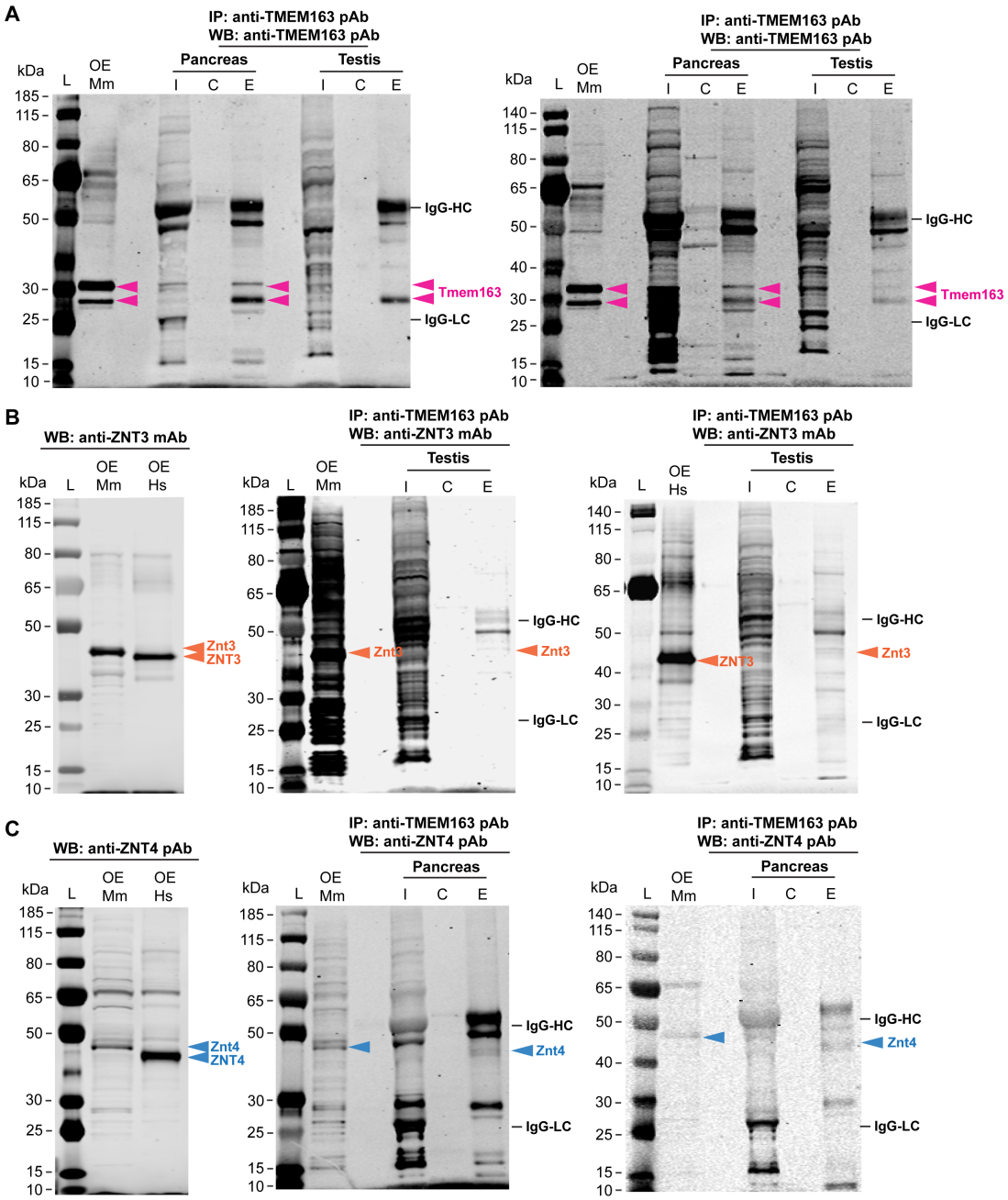

Figure S4. Endogenous mouse Tmem163 protein dimerizes with itself, Znt3, and Znt4 proteins. A) Native co-IP Western blot (WB) images of mouse Tmem163 protein from pancreas and testis show co-elution with itself using anti-TMEM163 pAb. Cell lysates of over-expressed mouse (OE Mm) Tmem163 protein served as positive expression control while the agarose control beads served as negative control. **B**) The leftmost WB shows anti-ZNT3 mAb cross-reactivity against mouse Znt3 and human ZNT3 proteins from cell lysates over-expressing mouse (OE Mm) or human (OE Hs) proteins. The images on the right panel are native co-IP WB of mouse Znt3 protein from testis co-eluting with Tmem163 protein. **C**) The leftmost WB shows anti-ZNT4 pAb cross-reactivity against mouse Znt4 and human ZNT4 proteins from cell lysates over-expressing mouse (OE Mm) or human (OE Hs) proteins. The images on the right panel are native co-IP WB of mouse Znt4 protein from pancreas co-eluting with Tmem163 protein. Predicted molecular weight (MW) of mouse proteins: Tmem163 = 31.5 kDa, Znt3 = 41.7 kDa, and Znt4 = 47.7 kDa. Predicted MW of human proteins: ZNT3 = 41.8 kDa and ZNT4 = 47.4 kDa. IgG-HC, immunoglobulin-G heavy chain (MW ≅ 55 kDa); IgG-LC, immunoglobulin-G light chain (MW ≅ 23 kDa). L, protein ladder; I, input/cell lysate; C, control resin; E, elution; mAb, monoclonal antibody; pAb, polyclonal antibody. The images are representative of N ≥ 4 independent experiments.

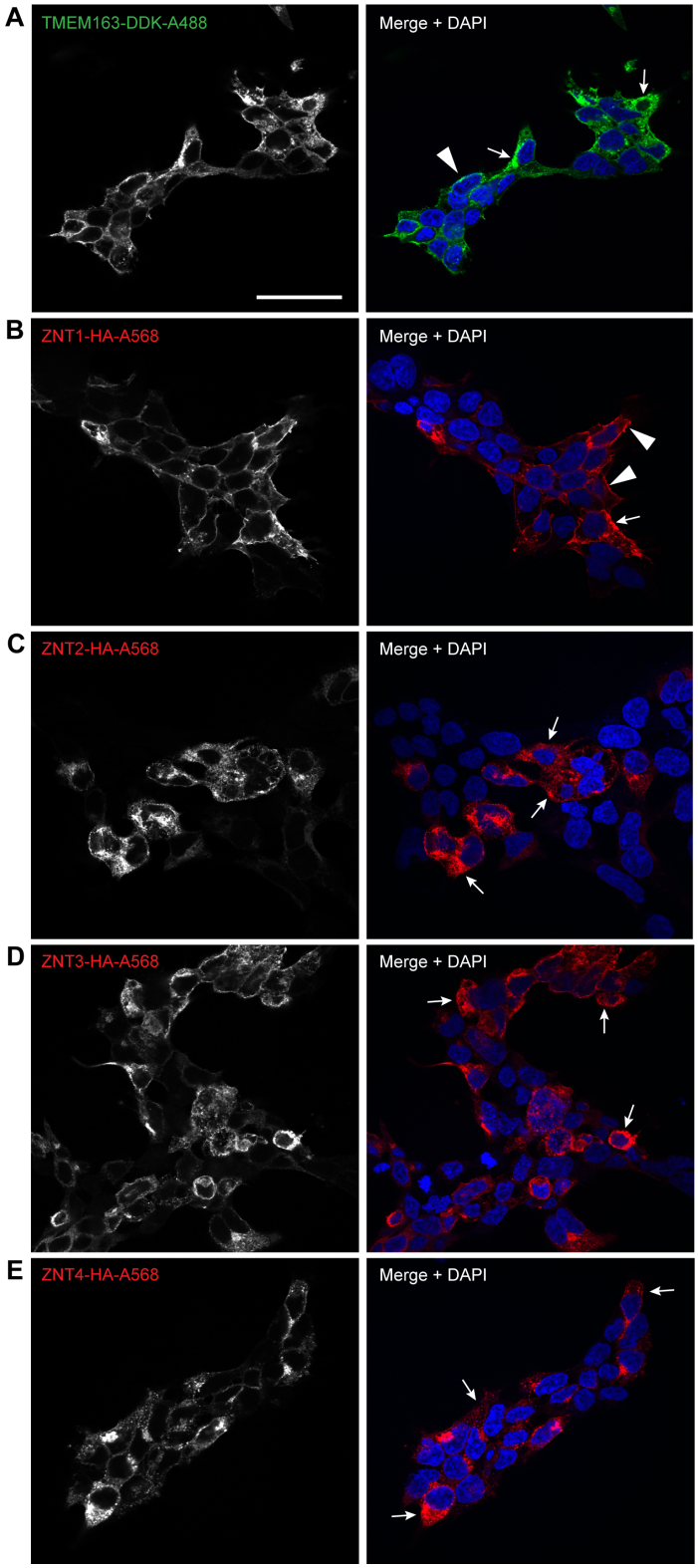

Figure S5. Immunocytochemistry of heterologously expressed TMEM163 and ZNT proteins. Representative confocal images of cells expressing **A**) TMEM163-DDK, **B**) ZNT1-HA, **C**) ZNT2-HA, **D**) ZNT3-HA, and **E**) ZNT4-HA. The DDK peptide tag was detected by anti-DDK mAb and visualized with Alexa-488 (green) anti-mouse secondary antibody. The HA peptide was detected by anti-HA pAb and visualized with Alexa-568 (red) anti-rabbit secondary antibody. DAPI stains the nuclei blue. TMEM163 and ZNT1 are detected within the plasma membrane (arrowhead) and intracellular compartments (arrow). Meanwhile, ZNT2, ZNT3, and ZNT4 proteins exhibit a punctate distribution pattern indicating localized in compartments (arrow). Scale bar: 100 μm.

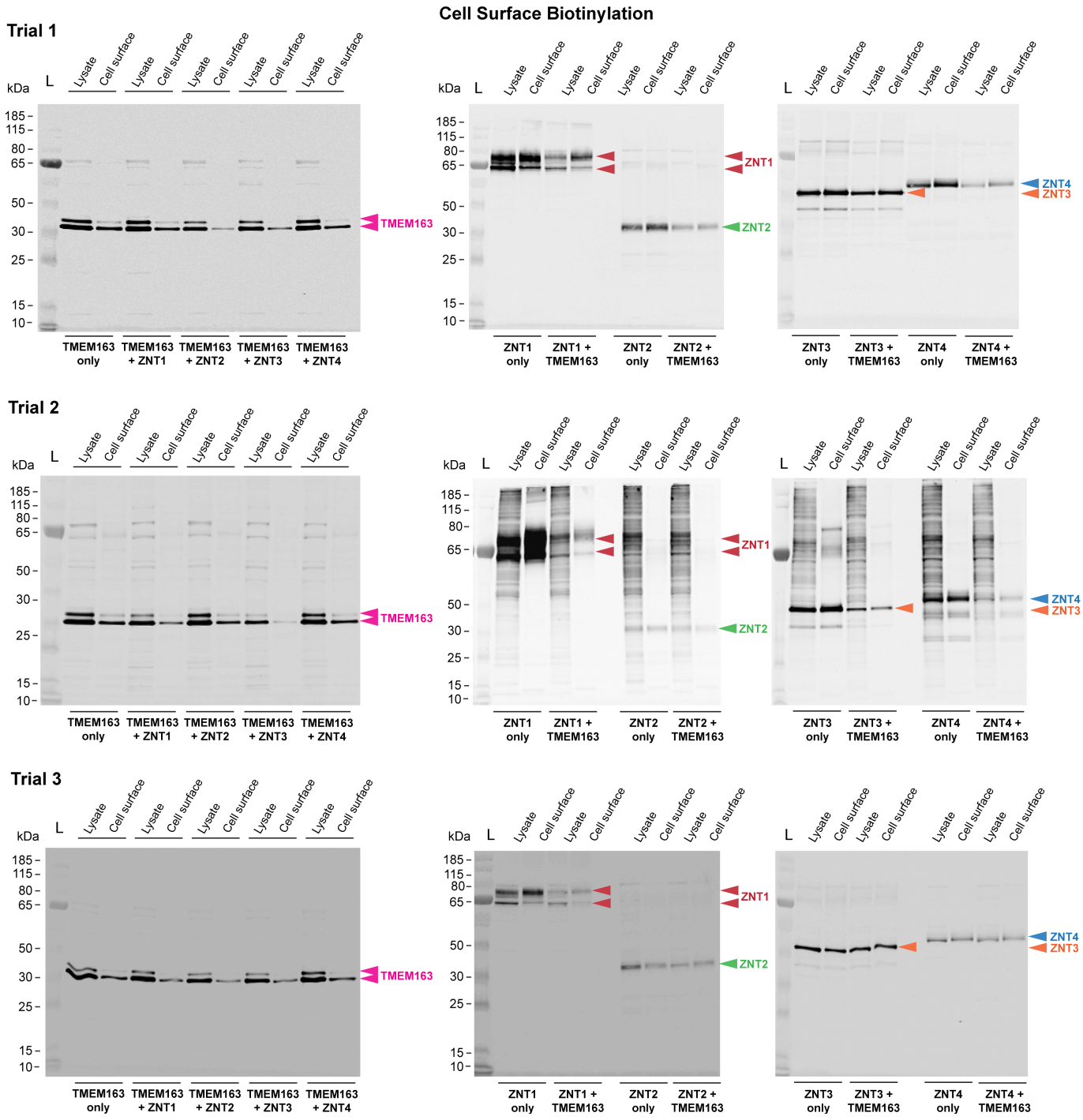

Figure S6. Cell surface biotinylation of heterologously expressed TMEM163 and ZNT proteins. A-C) WB images of TMEM163-DDK co-expressed with ZNT1-HA, ZNT2-HA, ZNT3-HA, and ZNT4-HA proteins. TMEM163 was detected using anti-DDK mAb, while the ZNT proteins were probed with anti-HA pAb. Predicted MW of human proteins: TMEM163 = 31.5 kDa, ZNT1 = 55.3-63.0 kDa, ZNT2 variant 2 = 35 kDa, ZNT3 = 41.8 kDa, and ZNT4 = 47.4 kDa. L, protein ladder.

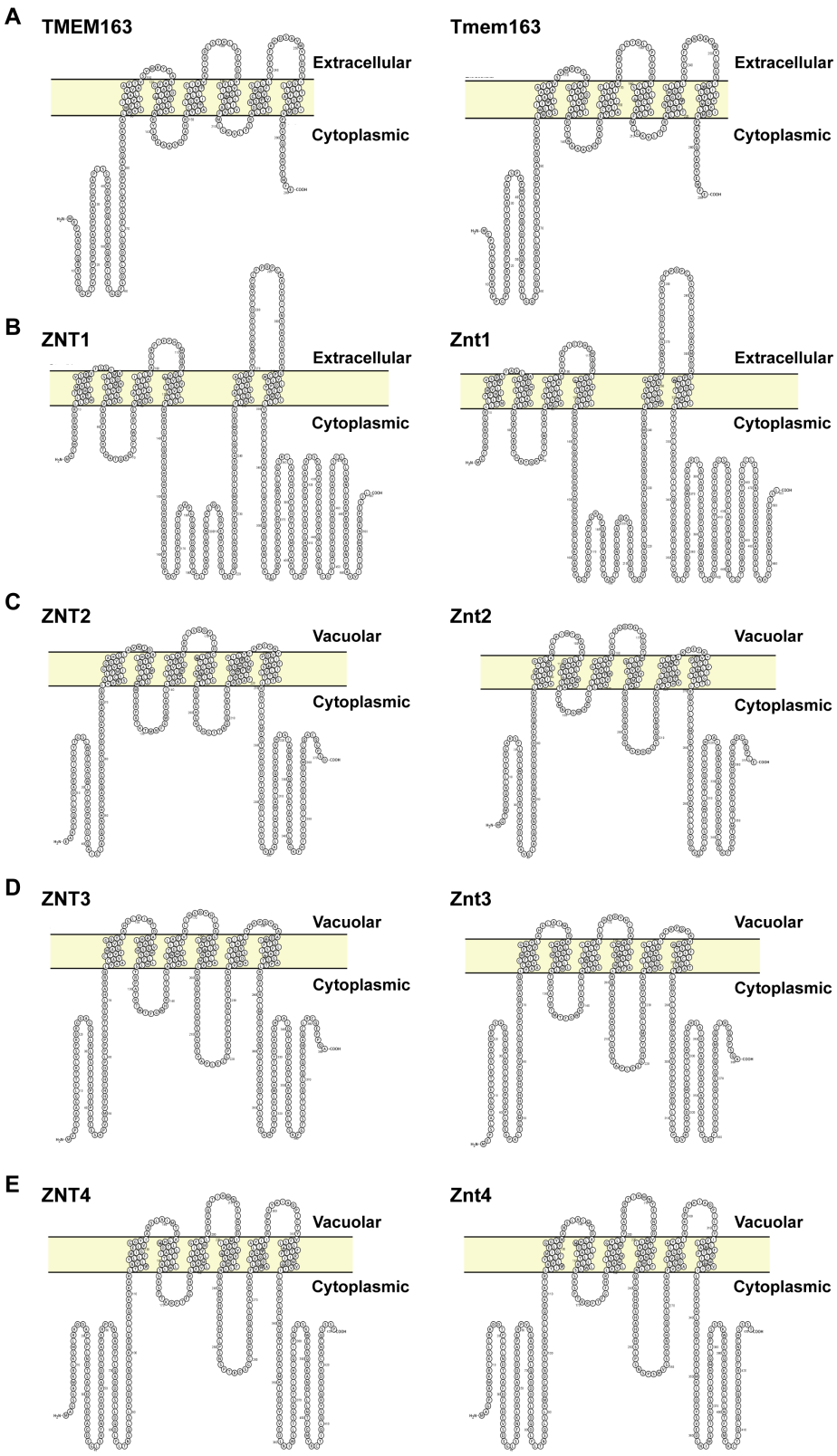

Figure S7. Predicted protein secondary structures. Human (left panel) and mouse (right panel) protein. **A**) TMEM163, **B**) ZNT1, **C**) ZNT2, **D**) ZNT3, and **E**) ZNT4. The diagrams were generated using the online Protter software (https://wlab.ethz.ch/protter/start/). Omasits U, Ahrens CH, Müller S, Wollscheid B. (2014). Bioinformatics, 30(6), 884-886. doi: 10.1093/bioinformatics/btt607.