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Secretory and membrane bound, zinc-requiring enzymes are thought to be activated by binding zinc in the early secretory pathway. One such enzyme, tissue-nonspecific alkaline phosphatase (TNAP), is activated through a two-step mechanism, via protein stabilization and subsequent enzyme activation through metalation, by ZnT5-ZnT6 heterodimers or ZnT7 homodimers. However, little is known about the molecular basis underlying the activation process. Here, we found that the di-proline motif (PP-motif) in luminal loop 2 of ZnT5 and ZnT7 is important for TNAP activation. TNAP activity was significantly reduced in cells lacking ZnT5-ZnT6 heterodimers and ZnT7 homodimers (triple knockout (TKO) cells). The decreased TNAP activity was restored by expressing hZnT5 with hZnT6 or hZnT7, but significantly less so (almost 90% less) by expressing mutants thereof in which the PP-motif was mutated to alanine (PP-AA). In TKO cells, over-expressed hTNAP was not completely activated, and it was converted less efficiently to the holo form by expressing a PP-AA mutant of hZnT5 with hZnT6, whose defects were not restored by zinc supplementation. The zinc transport activity of hZnT7 was not significantly impaired by the PP-AA mutation, indicating that the PP-motif is involved in the TNAP maturation process, although it does not control zinc transport activity. The PP-motif is highly conserved in ZnT5 and ZnT7 orthologs, and its importance for TNAP activation is conserved in the *Caenorhabditis elegans* hZnT5 ortholog CDF5. These results provide novel molecular insights into the TNAP activation process in the early secretory pathway.

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The PP-motif in luminal loop 2 of ZnT transporters plays a pivotal role in TNAP activation

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Short title: The pivotal role of the luminal PP-motif of ZnT transporters

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ABSTRACT

Secretory and membrane bound, zinc-requiring enzymes are thought to be activated by binding zinc in the early secretory pathway. One such enzyme, tissue-nonspecific alkaline phosphatase (TNAP), is activated through a two-step mechanism, via protein stabilization and subsequent enzyme activation through metalation, by ZnT5-ZnT6 heterodimers or ZnT7 homodimers. However, little is known about the molecular basis underlying the activation process. Here, we found that the di-proline motif (PP-motif) in luminal loop 2 of ZnT5 and ZnT7 is important for TNAP activation. TNAP activity was significantly reduced in cells lacking ZnT5-ZnT6 heterodimers and ZnT7 homodimers (triple knockout (TKO) cells). The decreased TNAP activity was restored by expressing hZnT5 with hZnT6 or hZnT7, but significantly less so (almost 90% less) by expressing mutants thereof in which the PP-motif was mutated to alanine (PP-AA). In TKO cells, over-expressed hTNAP was not completely activated, and it was converted less efficiently to the *holo* form by expressing a PP-AA mutant of hZnT5 with hZnT6, whose defects were not restored by zinc supplementation. The zinc transport activity of hZnT7 was not significantly impaired by the PP-AA mutation, indicating that the PP-motif is involved in the TNAP maturation process, although it does not control zinc transport activity. The PP-motif is highly conserved in ZnT5 and ZnT7 orthologs, and its importance for TNAP activation is conserved in the *Caenorhabditis elegans* hZnT5 ortholog CDF5. These results provide novel molecular insights into the TNAP activation process in the early secretory pathway.

Summary statement

Zinc activates TNAP through a two-step mechanism involving ZnT5-ZnT6 heterodimers and ZnT7 homodimers. We show that the PP-motif in luminal loop 2 of hZnT5, hZnT7, and hZnT5 ortholog CDF5 plays a role in TNAP activation in the early secretory pathway.

Keywords: early secretory pathway, PP-motif, TNAP, zinc-requiring enzyme, zinc transport, ZnT.

ABBREVIATIONS FOOTNOTE

HA, hemagglutinin; MT, metallothionein; TKO, triple knockout; TMD, transmembrane domain; TNAP, tissue-nonspecific alkaline phosphatase; WT, wild-type.

INTRODUCTION

Zinc plays crucial roles in many biological processes, in which it is indispensable as a structural component, cofactor, and signaling mediator in protein functions [1–4]. Recent *in silico* analyses indicate that approximately 3,000 proteins have a putative zinc-binding motif [5]. Thus, zinc homeostasis and metabolism are strictly regulated by a number of proteins at systemic and cellular levels [4,6]. Two zinc transporter families, Zn transporter (ZnT, solute carrier 30A (SLC30A)) and Zrt, Irt-like protein (ZIP, SLC39A), operate as essential components to control these processes by transporting zinc across cellular membranes [3,4,7–13]. ZnT transporter functions have been more extensively investigated at a molecular level [9,11] because the three-dimensional structure and the zinc/proton exchange transport mechanism have been resolved in the bacterial ZnT homolog YiiP [14–17]; thus, many mutagenesis studies have been performed in ZnT transporters and their homologs [18–26]. In these studies, many amino acid residues have been shown to be involved in zinc transport and determining metal specificity [18–22]. However, little is known about the amino acid residues or motifs that are associated with other functions of ZnT transporters.

Most ZnT transporters have crucial functions in transporting zinc into intracellular organelles and vesicles [9,27–29]. Among them, ZnT5, ZnT6, and ZnT7 are thought to play key roles in transporting zinc into the early secretory pathway to activate zinc-requiring enzymes that are secreted out of cells or that reside within intracellular organelles, as well as in maintaining the homeostasis of secretory pathway functions [9,27,30]. Thus far, we have shown that ZnT5 and ZnT6 form heterodimers (in which ZnT6 operates as an auxiliary subunit) and ZnT7 forms homodimers [19,31,32], and that these two ZnT transport complexes activate zinc-requiring enzymes such as tissue-nonspecific alkaline phosphatase (TNAP) [27,31,33]. The TNAP activation process is intricately regulated. Cytosolic zinc is likely transferred to ZnT5-ZnT6 heterodimers and ZnT7 homodimers, in which ZnT1, ZnT4, and metallothionein (MT) probably play important, cooperative roles [6,21,34]. Moreover, TNAP is activated by ZnT5-ZnT6 heterodimers or ZnT7 homodimers in an elaborate two-step mechanism in which TNAP is first stabilized by either complex, and then is subsequently activated via metalation by zinc, which is supplied by either complex [6,34,35]. However, the molecular basis of these processes is unknown. In this study, we document that the di-proline motif (PP-motif) in luminal loop 2 of hZnT5 and

hZnT7 (note that luminal loop 6 in hZnT5, which has 15 putative transmembrane domains (TMDs), corresponds to luminal loop 2 in other ZnT transporters, including hZnT7, which have six TMDs that constitute a cation efflux domain [36], but “luminal loop 2” is used for hZnT5 and its orthologs as a matter of practical convenience in this paper), which is highly conserved in ZnT5 and ZnT7 orthologs, is important for TNAP activation. Our results provide molecular insights into how and why ZnT5-ZnT6 heterodimers and ZnT7 homodimers can fully activate TNAP in a specific manner. Thus, they suggest that the luminal motif of ZnTs makes an important contribution to their own unique functions.

EXPERIMENTAL

Cell culture and transfection

Chicken B lymphocyte-derived DT40 cells, DT40 cells deficient in the *ZnT5*, *ZnT6*, and *ZnT7* genes (*ZnT5*^{-/-}*ZnT6*^{-/-}*ZnT7*^{-/-} triple knockout (TKO) cells), and DT40 cells deficient in the *ZnT1*, *MT*, and *ZnT4* genes (*ZnT1*^{-/-}*MT*^{-/-}*ZnT4*^{-/-} cells) were maintained as described previously [33]. DNA transfection into DT40 cells was conducted as previously described [37]. More than three independent clones were established per transfectant in all experiments. Cell viability in high extracellular zinc concentrations was evaluated using *ZnT1*^{-/-}*MT*^{-/-}*ZnT4*^{-/-} cells [21,38]. The cells were cultured in the presence of 30–70 μM ZnSO₄ for 72 h, and viable cells were measured by the Alamar Blue assay (Trek Diagnostic Systems, Westlake, OH, USA) as described previously [39].

Plasmid construction

Plasmids used for the expression of FLAG-tagged human ZnT5 (FLAG-hZnT5) and hemagglutinin (HA)-tagged human ZnT6 (HA-hZnT6), HA-hZnT7, hZnT4-HA, and hTNAP were described previously [19,21,35]. Plasmids used to express FLAG-tagged CDF5 (FLAG-CDF5) and HA-tagged TOC1 (HA-TOC1) were constructed by inserting each cDNA into the pA-Puro and pA-EcoGpt vectors, respectively. Fragments containing full-length cDNAs of CDF5 and TOC1 were amplified by reverse transcription–polymerase chain reaction using total RNA prepared from *Caenorhabditis elegans* as a template. Amplified cDNAs were sequenced in both directions. The

plasmids used to express hZnT5, hZnT7, CDF5, and hZnT4 mutants were constructed by a two-step polymerase chain reaction method, followed by insertion into the pA-Puro vector. All plasmids were linearized with appropriate restriction enzymes prior to electroporation [37]. In growth assays of *Saccharomyces cerevisiae* mutants, hZnT7-HA and its mutants were inserted into the pYES2 vector (Invitrogen, Carlsbad, CA, USA). The pYES2 vector containing *S. cerevisiae* MSC2 (*ScMSC2*) was described previously [40].

Measurement of TNAP activity

Membrane proteins or total cellular protein extracts were prepared from cells that were lysed in ALP lysis buffer. Two to five micrograms of membrane proteins or whole cell lysates was used to measure TNAP activity as described previously [37]. Calf intestine alkaline phosphatase (Promega, Madison, WI, USA) was used to make a standard curve [21].

Immunoblotting and immunoprecipitation experiments

Immunoblotting and immunoprecipitation were performed as described previously [37]. Briefly, blotted polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA) were blocked with a solution of 4% (w/v) skimmed milk and 0.1% Tween-20 (v/v) in phosphate-buffered saline prior to incubation with anti-FLAG-tag (anti-DDDDK; MBL, Nagoya, Japan; 1:3,000 dilution), anti-HA HA-11 (Covance, Princeton, NJ, USA; 1:3,000 dilution), or anti-TNAP (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:3000 dilution) antibodies in blocking solution. To control for loading, *S. cerevisiae* proteins that were electrophoresed on sodium dodecyl sulfate–polyacrylamide gels were silver-stained with 2D-Silver Stain Reagent II (Cosmo Bio, Tokyo, Japan). For immunoprecipitations, membrane proteins from cells that were lysed in NP-40 buffer were incubated with anti-FLAG M2 (Sigma-Aldrich, St. Louis, MO, USA; 1:200 dilution) or anti-HA HA-11 (1:200 dilution) antibodies for 1 h at 4°C prior to the addition of 10 µl of protein G-Sepharose beads (GE Healthcare, Milwaukee, WI, USA) [38]. After an additional incubation for 12 h at 4°C, immunoprecipitates were subjected to immunoblotting. Horseradish peroxidase-conjugated rabbit anti-mouse secondary antibodies (GE Healthcare) or TrueBlot anti-mouse IgG (eBioscience, San Diego, CA, USA) were added at a 1:3,000 dilution for protein detection. A fluoro-image was

obtained using an LAS1000 Plus (Fujifilm, Tokyo, Japan) or LAS500 (GE Healthcare) gel documentation system.

Immunofluorescence staining

Immunostaining for wild-type (WT) hZnT5, hZnT6, CDF5, and TOC, as well as mutants thereof, was performed as described previously [37]. Briefly, cells were stained with an anti-FLAG antibody (anti-DDDDK; MBL; 1:2,000 dilution), followed by Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA; 1:200 dilution), or with anti-HA HA11 (Covance; 1:2,000 dilution), followed by Alexa 594-conjugated goat anti-mouse IgG (Molecular Probes; 1:200 dilution). The stained cells were observed using a Zeiss Axioplan 2 microscope (Carl Zeiss AG, Oberkochen, Germany) that was equipped with a Metamorph digital camera (Olympus, Tokyo, Japan), or a fluorescent microscope FSX100 (Olympus). Images were analyzed using Adobe Photoshop CS.

Model structures of hZnT5, hZnT6, and hZnT7, and sequence similarity

Homology models of hZnT5, hZnT6, and hZnT7 were obtained using a Multiple Mapping Method [41]. The crystal structure of YiiP (PDB ID code 3H90) served as a structural template for modeling homologous mammalian targets. Manual inspection of structurally aligned protein pairs was used to discriminate among alternative alignment choices, and models were built by optimizing the positions of target residues that fold into the known YiiP three-dimensional structural template. All graphic presentations were prepared using the program PYMOL (DeLano Scientific, South San Francisco, CA, USA). The identity and similarity of CDF5 to hZnT5, hZnT7, and hZnT4 were evaluated using the National Center for Biotechnology Information Basic Local Alignment Search Tool server (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Growth assay of an *S. cerevisiae* mutant

The *S. cerevisiae* mutant DY150 *msc2* (*MATa ade2 can1 his3 leu2 trp1 ura3 msc2::HIS3*) was transformed with the indicated plasmids using a lithium acetate/single-stranded carrier DNA/polyethylene glycol method [42]. The transformed yeast cells were pre-cultured in S Raf medium containing the appropriate amino acids for auxotrophy for 18 h at 30°C, and diluted to an optical density at 600 nm of 0.1 in

SGal medium containing appropriate amino acids. After incubation for 1 h at 30°C, aliquots (5 µL) of the suspension culture were spotted onto solid YPG_Egal medium as described previously [40]. Plates were incubated for 3 d at 30 and 37°C.

RESULTS

Analyses of the amino acid residues in the luminal loops of hZnT5 that are associated with TNAP activation

TNAP activity in TKO cells was not restored when human ZnT (hZnT) transporters, such as hZnT2, hZnT3, and hZnT8, were ectopically expressed, or when hZnT1 and hZnT4 were overexpressed, even if they partially (transiently) localized to the early secretory pathway [35]. Moreover, TNAP was stabilized by co-expressing hZnT5 and hZnT6, or by expressing hZnT7 prior to its activation [35]. Intriguingly, we found that a zinc transport-competent mutant hZnT5, in which the HDHD core motif of the intramembranous zinc-binding site is mutated to DDHD or HDHE, failed to activate TNAP in TKO cells when co-expressed with hZnT6 (Supplementary Figure S1). Considering these results, we speculated that hZnT5 and hZnT7 may have a specific sequence (motif) in their luminal loops that is associated with TNAP activation. To investigate this possibility, we used a site-directed mutagenesis strategy to modify luminal loops 1, 2, and 3 of hZnT5 (note that luminal loops 1, 2, and 3 in most ZnT transporters that contain six TMDs correspond to luminal loops 5, 6, and 7 in ZnT5 that contains 15 TMDs, but luminal loops 1, 2, and 3 are used as a matter of practical convenience). We mutated amino acid residues in luminal loops 1, 2, and 3 of hZnT5 that are completely conserved among hZnT5, hZnT7, and the *C. elegans* hZnT5 ortholog (CDF5, see below) (Fig. 1A). Specifically, alanine-scanning mutagenesis was used to modify residues Y436, N441, G444, L445, and I446 in luminal loop 1, P514, P515, and L522 in luminal loop 2, and I607, S609, F616, and G617 in luminal loop 3 of hZnT5. T440 in luminal loop 1 was also mutated to Ala because a Ser residue is present at this position in hZnT7. No mutants showed any extreme reduction in TNAP activity, except for the L445A, P514A, and P515A mutants (approximately 30, 30, and 50% reductions, respectively), when they were stably co-expressed with hZnT6 in TKO cells, compared with that in TKO cells stably co-expressing WT hZnT5 and hZnT6 (Fig. 1B, C, and D). Moreover, we mutated F435 in luminal loop 1, and Q615, F619, and I620 in luminal loop 3 of hZnT5, because the corresponding amino acid residues differ among hZnT7 and CDF5, and they are moderately conserved as Val, Phe, Tyr, and Lys residues, respectively, in other ZnT transporters (Fig. 1A). We examined the possibility that these residues might impair TNAP activation in other ZnT transporters, but TNAP activity was not significantly reduced in TKO cells when these mutants were stably

co-expressed with hZnT6 (Fig. 1B and D), which excludes this possibility. The protein expression levels of all of the hZnT5 mutants were almost the same as that of WT hZnT5 in TKO cells (Fig. 1B, C, and D). Taken together, L445, P514, and P515 in the luminal loops may play pivotal roles in the activation of TNAP in the early secretory pathway.

The PP-motif in luminal loop 2 of hZnT5 is crucial for TNAP activation

We next examined the roles of two Pro residues, P514 and P515 (a PP-motif), in luminal loop 2 of hZnT5, because the motif is uniquely conserved among hZnT5, hZnT7, and CDF5, as opposed to three other amino acids that occupy these positions in most other ZnT transporters (Fig. 1A). To closely examine whether the PP-motif is crucial for TNAP activation, we again used a site-directed mutagenesis strategy to replace one or both Pro residues with Ala in hZnT5. The hZnT5_{PP-AA} mutant, in which the PP sequence was substituted with AA, co-localized with hZnT6 (Fig. 2A, *upper* panels) and formed a heterodimer with hZnT6, as did WT hZnT5 (Fig. 2A, *lower* panels). Interestingly, TNAP activity was approximately 90% lower in TKO cells that stably co-expressed the hZnT5_{PP-AA} mutant and hZnT6, compared with that in cells that stably co-expressed WT hZnT5 and hZnT6 (Fig. 2B). The protein expression levels were almost the same among the WT and mutants hZnT5 proteins, which excludes the possibility that the reduced TNAP activity in the cells resulted from a lower hZnT5 expression level in each mutant.

To investigate TNAP protein expression levels in TKO cells that stably co-express hZnT5_{PP-AA} and hZnT6, we stably expressed hTNAP in these cells. The activity of hTNAP was moderately reduced (approximately 60% less than that of TKO cells co-expressing WT hZnT5 and hZnT6) (Fig. 2C) compared with the endogenous TNAP activity, which was approximately 90% less in TKO cells than in WT cells, as described in Fig. 2B. hTNAP proteins were detected as one major high-molecular-weight band corresponding to *holo*-hTNAP in TKO cells co-expressing WT hZnT5 and hZnT6, while they were detected as one major high-molecular-weight and several minor lower-molecular-weight bands in TKO cells stably co-expressing hZnT5_{PP-AA} and hZnT6. The low-molecular-weight bands probably correspond to *apo*-hTNAP, considering our previous results [30,35] (In our previous report [30], the sizes of the major high-molecular-weight band and the minor lower-molecular-weight

bands were calculated to be ~80-kDa and ~60-kDa, but they were detected as ~75-kDa and ~65-kDa bands, respectively, because a different protein size marker was used and the percentage of polyacrylamide in the gel differed.). The amounts of the low-molecular-weight bands were not significantly changed when protein degradation via the proteasome and lysosome was inhibited by treatment with MG132 and bafilomycin A1 (Fig. 2C). Moreover, zinc supplementation did not fully restore hTNAP activity, and it did not alter the appearance of the low-molecular-weight bands (Fig. 2D). These results suggest that the low-molecular-weight bands were unlikely to be derived from insufficient zinc transport by a heterodimer of hZnT5_{PP-AA} and hZnT6, but rather that they resulted from defects of hTNAP protein maturation during the two-step mechanism, which were caused by mutating the two Pro residues to Ala.

The PP-motif in luminal loop 2 of ZnT7 is crucial for TNAP activation, but not for its zinc transport activity

Next, we examined the effects of the PP-AA mutation on the zinc transport activity of hZnT5 using the yeast *msc2* mutant [43,44], which was recently used to evaluate the zinc transport activity of *Arabidopsis thaliana* ZnT5 and ZnT6 orthologs (MTP12 and MTP5, respectively) [40]. However, we failed to detect any zinc transport activity of the hZnT5_{PP-AA} mutant using the *msc2* mutant under our growth conditions, because co-expression of WT hZnT5 and hZnT6 did not restore the growth of the *msc2* mutant (data not shown). Instead, we examined the effects of the PP-AA mutation on the zinc transport activity of hZnT7. As described above, hZnT7 is highly homologous to hZnT5; thus, the model structures of hZnT5 and hZnT7, which were generated based on the crystal structure of the *Escherichia coli* ZnT homolog YiiP [14,15,17], were well merged. We speculated that the PP-motif in both proteins protrudes into the luminal side of the membrane (Fig. 3A and B). Then, we examined the significance of the PP-motif in hZnT7 for TNAP activation using the same mutagenesis strategy. In a similar manner to the hZnT5 mutants, the TNAP activity in TKO cells stably expressing either single substitution mutants of hZnT7 (hZnT7_{P133A} or hZnT7_{P134A}) was moderately reduced, and it was markedly reduced in cells stably expressing the hZnT7_{PP-AA} mutant (Fig. 3C). Importantly, we confirmed that the expression of the hZnT7_{PP-AA} mutant, but not the hZnT7_{H70A} mutant (a zinc transport-incompetent mutant), restored the growth of the *msc2* mutant in a manner that was comparable to that of WT hZnT7 (Fig. 3D),

indicating that zinc transport activity is not significantly impaired in the hZnT7_{PP-AA} mutant. These results suggest that hZnT5_{PP-AA} probably retains its zinc transport activity, and that the PP-motifs of hZnT5 and hZnT7 have important functions, except in zinc transport, during TNAP activation in the early secretory pathway.

The necessity and sufficiency of the PP-motif in ZnT5 orthologs and other ZnT transporters

The PP-motif is highly conserved in hZnT5 and hZnT7 orthologs (Tables 1 and 2), suggesting that it has a specific and important function in secretory and membrane bound, zinc-requiring enzyme activation. Therefore, we investigated whether the PP-motif is necessary and sufficient for TNAP activation. First, we examined the necessity of the PP-motif in CDF5, which is the *C. elegans* ZnT5 ortholog, because it has 15 putative TMDs [27,36], high overall sequence homology (41% identity and 57% similarity with hZnT5) [45], and, importantly, because its carboxyl terminal portion (the cation efflux domain (pfam01545)) is homologous to hZnT5 (identity, 47%; similarity, 63%), and hZnT7 (identity, 37%; similarity, 53%) (note that *C. elegans* does not have a ZnT7 ortholog). Stable expression of CDF5 alone failed to restore TNAP activity in TKO cells (Fig. 4A), indicating that CDF5 needs an auxiliary subunit. Therefore, we stably co-expressed CDF5 and TOC1, which was speculated to be the *C. elegans* ZnT6 ortholog in our previous studies [30,46], in TKO cells, and examined the TNAP activity. Co-expression of CDF5 and TOC1 restored TNAP activity in TKO cells to approximately 75% of that of the case in which hZnT5 and hZnT6 were co-expressed (Fig. 4A). Consistent with this, CDF5 and TOC1 co-localized to Golgi-like compartments (Fig. 4B), and they formed heterodimers in a similar manner to their human counterparts (Fig. 4C). TNAP activity in TKO cells stably expressing a double substitution mutant (CDF5_{PP-AA}) with TOC-1 was markedly reduced. However, almost no effects were found in cells expressing either of the single Ala substitution mutants (CDF5_{P499A} and CDF5_{P500A}) (Fig. 4D). Hence, these results confirm that the PP-motif of a ZnT5 ortholog is indispensable for appropriate TNAP activation.

Next, we investigated whether the PP-motif is sufficient to confer the ability to activate TNAP to other ZnT transporters. For this purpose, we constructed swapping mutants in which the MNY sequence of hZnT4 was mutated to the PP sequence (hZnT4_{MNY-PP}) or the PP sequence of hZnT5 was mutated to the MNY sequence

(hZnT5_{PP-MNY}) (see Fig. 1A). The zinc transport activity of the hZnT4_{MNY-PP} mutant was confirmed using *ZnT1*^{-/-}*MT*^{-/-}*ZnT4*^{-/-} cells, which are highly sensitive to high zinc concentrations [38,39] and which is reversed by expressing WT hZnT4 [21]. Expressing the hZnT4_{MNY-PP} mutant reversed the zinc sensitive phenotype of the *ZnT1*^{-/-}*MT*^{-/-}*ZnT4*^{-/-} cells to a similar extent to that of cells expressing WT hZnT4 (Fig. 5A), indicating that the MNY-PP mutation did not impair the zinc transport activity of hZnT4. Expressing hZnT4_{MNY-PP} in TKO cells, however, failed to restore TNAP activity (Fig. 5B), whereas co-expressing the hZnT5_{PP-MNY} mutant with hZnT6 resulted in a significant reduction of TNAP activity (Fig. 5C), which is consistent with the results obtained using the hZnT5_{PP-AA} mutant (see Fig. 2B). These results reveal that the PP-motif is important for hZnT5, hZnT7, and their orthologs, as it is a specific motif that is associated with TNAP activation, but it is not sufficient to confer the ability to activate TNAP to other ZnT transporters.

DISCUSSION

Many mutagenesis studies of cation diffusion facilitator proteins, including ZnT transporters, have been performed. For example, extensive analyses have been explored in MTP1 in plants and Zrc1 in yeast, and they have identified a number of amino acid residues that are essential for zinc transport activity and for determining metal substrate specificity [23–26]. In vertebrate ZnTs, a few similar studies have been performed, and they confirmed the importance of the HDHD core motif in zinc transport activity and metal substrate specificity [18–22]. However, investigations of the contribution of other amino acid residues to the functions of ZnTs have not been explored. In this study, we demonstrated that the PP-motif in luminal loop 2 of hZnT5, hZnT7, and the hZnT5 ortholog CDF5 is a specific motif that is associated with TNAP activation. To our knowledge, this is the first evidence of the importance of the conserved luminal motif of ZnTs at a molecular level, and it provides novel molecular insights into enzyme activation in the early secretory pathway.

The PP-motif of luminal loop 2 is occupied by two amino acids in hZnT5, hZnT7, and CDF5, while three amino acids are present in other ZnT transporters (see Fig. 1A). Our results for the hZnT7_{PP-AA} (see Fig. 3D) and hZnT4_{MNY-PP} mutants (see Fig. 5A) indicate that these differences do not seem to cause significant changes in zinc transport activity, suggesting that the PP-motif is uniquely equipped to maintain a

specific conformation, which leads to questions regarding its function in TNAP activation. One possibility is that the PP-motif functions as a protein-binding motif, because the Pro-rich motif has an unusual secondary structure that is known to be involved in protein binding [47,48]. Our results showed that low-molecular-weight bands of hTNAP, which probably correspond to *apo*-hTNAP [30,35], appeared in TKO cells stably co-expressing hZnT5_{PP-AA} and hZnT6 (see Figs. 2C and D), which suggests that the PP-motif may be important for protein-protein interactions in the TNAP maturation process during the two-step mechanism. We hypothesized that hZnT5 or hZnT7 may interact with hTNAP through the PP-motif, but we failed to detect a direct interaction between them (data not shown). The PP-motif may contribute to functional interactions with unknown proteins that are involved in transferring luminal zinc to TNAP for its metalation (and/or stability). If the PP-motif functions in protein-protein interactions, other regions in hZnT5 and hZnT7 would also be crucial, because the PP-motif was not sufficient to confer the ability to activate TNAP to other ZnT transporters such as hZnT4 (see Fig. 5B), and because TNAP activity was not completely lost in TKO cells stably expressing the hZnT5_{PP-AA} mutant and hZnT6 (Fig. 2B and D), their orthologs (Fig. 4D), and the hZnT7_{PP-AA} mutant (Fig. 3C).

Another possibility is that Pro residues in the PP-motif may be hydroxylated and that the hydroxy-Pro residues might function as zinc-coordinating ligands or they might facilitate zinc coordination during TNAP activation, thus reducing TNAP activity in cells expressing the PP-AA mutants. However, this possibility was excluded because TNAP activity was not changed in WT DT40 cells that were cultured in the presence of dimethyloxalylglycine, an inhibitor of prolyl 4-hydroxylase [49] (data not shown), which mainly mediates the hydroxylation of Pro in collagen [50]. This exclusion is also supported by the fact that the PP-motif in hZnT5, hZnT7, and CDF5 differs from the (XPG)_n motif in collagen, which is highly hydroxylated [50].

The PP-motif in luminal loop 2 is highly conserved among ZnT5 and ZnT7 and their orthologs (Tables 1 and 2), but, interestingly, the *A. thaliana* and *S. cerevisiae* ZnT5 orthologs (MTP12 and Msc2, respectively) [40, 43, 51], as well as the *Xenopus tropicalis* ZnT7 ortholog, lack one of the Pro residues. In these species, ZnT5 and ZnT7 orthologs with only one Pro residue may be functional, as was demonstrated for CDF5_{P499A} and CDF5_{P500A} (see Fig. 4D). Moreover, the PP-motif is found in several ZnT transporter homologs that are not assigned as ZnT5 and ZnT7 orthologs in some

species (Table 3). Among them, it is of interest that *S. cerevisiae* Cot1 has the PP-motif, because the *S. cerevisiae* alkaline phosphatase Pho8 is activated by Cot1 (and Zrc1) (both of which are highly homologous to ZnT4) in vacuoles, but not by Msc2 and Zrg17 (a ZnT6 homolog) [52]. The PP-motif of Cot1 may be uniquely important in the activation of Pho8 in vacuoles in *S. cerevisiae*.

The mutation of the HDHD core motif of the intramembranous zinc-binding site to DDHD or HDHE in hZnT5 impaired its TNAP activation property (see Supplementary Figure S1), although neither mutation impairs its zinc transport activity [18,20]. These results indicate that the HDHD core motif is important for ZnT transporters to mediate enzyme activation. Considering that the HDHD core motif is located just below the PP-motif in the model structures of hZnT5 and hZnT7, a unique, cooperative mechanism may be responsible for proper zinc transfer from the HDHD core motif to TNAP through the PP-motif, although future investigations are needed to clarify this.

In conclusion, this is the first report describing the importance of the PP-motif and the HDHD core motif for TNAP activation in the early secretory pathway. During the activation of secretory/membrane-bound metalloenzymes, compartmentalization has been shown to be one important means of regulation [53,54]. Additionally, the present study reveals that there is another form of regulation, which is under the control of the motifs that are embedded in metal transporter sequences, such as the PP-motif and HDHD core motif. A number of physiopathologically important proteins, such as matrix metalloproteinases, angiotensin- or endothelin-converting enzymes, a disintegrin, and metalloproteinase family proteins, are all secretory/membrane-bound zinc-requiring enzymes [27,34]. Thus, clarifying the molecular basis underlying TNAP activation may give us important insights into clinical disorders that are associated with these enzymes.

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DECLARATION OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTIONS

S.F., T. Tsuji, and T.K. designed the study; S.F., T. Tsuji, T.F., T. Takeda, C.M., A.F., Y.N., D.F., H.E., I.S., K.F., Y.M., S.M., M.N., and T.K. collected, analyzed, and interpreted data; S.F., T. Tsuji, and T.K. wrote the manuscript; and S.F. and T.K. submitted the manuscript for publication.

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Table 1. Conservation of the PP motif among ZnT5 orthologs.

ZnT5 ortholog		amino acid sequence		species
ZnT5	510	ARLID PP ELDTHML	523	<i>Homo sapiens</i>
ZnT5	507	ARLID PP ELDTNML	520	<i>Mus musculus</i>
ZnT5	506	ARLVD PP DIDTNML	519	<i>Gallus gallus</i>
ZnT5	505	ARIYD PP DINTDML	518	<i>X. tropicalis</i>
ZnT5	505	TRLVD PP NINTDML	518	<i>Danio rerio</i>
CDF5	494	ERLFD PP NINTDRL	407	<i>C. elegans</i>
Cis4	440	YRLFH PP QMNTDQL	453	<i>Schizosaccharomyces pombe</i>
Msc2	478	ERIFN P IHLHATNE	491	<i>S. cerevisiae</i>
MTP12	510	ERILD P QEISTNSL	523	<i>A. thaliana</i>

The accession nos. of the sequences used are shown in the supplementary material. Pro residues in the PP-motif are shown in bold.

Table 2. Conservation of the PP motif among ZnT7 orthologs.

ZnT7 ortholog		amino acid sequence		species
ZnT7	128	ERALA PP PDVHHERL	141	<i>H. sapiens</i>
ZnT7	128	ERALA PP PDVHHERL	141	<i>M. musculus</i>
ZnT7	128	ERALE PP PDVHHERL	141	<i>G. gallus</i>
ZnT7	128	ERALDT P EVHHERL	141	<i>X. tropicalis</i>
ZnT7	128	ERALE PP PDVHHDRL	141	<i>D. rerio</i>
ZnT86D	126	ERLIE PP EVKHERL	139	<i>Drosophila melanogaster</i>

The accession nos. of the sequences used are shown in the supplementary material. Pro residues in the PP-motif are shown in bold.

Table 3. Distribution of the PP motif in luminal loop 2 in ZnT homologs other than ZnT5 and ZnT7

CDF protein	amino acid sequence	species
Cot1	101 QRIIA PP VIENPKF 114	<i>S. cerevisiae</i>
Zhf1	101 ERFIE PP SVSNPTL 114	<i>S. pombe</i>
CzcD	104 ERF SNPP KVATTGM 117	<i>Bacillus subtilis</i>

The accession nos. of the sequences used are shown in the supplementary material. Pro residues in the PP-motif are shown in bold.

FIGURE LEGENDS

Fig. 1. Evaluation of the involvement of amino acids in the luminal loops of hZnT5 in TNAP activation. (A) Sequence alignment of luminal loops among CDF5, hZnT5, hZnT7, and other human ZnT transporters. The sequences used for alignment are shown in the supplementary material. In hZnT5 and CDF5, luminal loops 5, 6, and 7 correspond to luminal loops 1, 2, and 3 in the other transporters. The PP-motif is boxed in red. Conserved amino acids among hZnT5, hZnT7 and CDF5, which were subjected to alanine-scanning mutagenesis, are indicated in orange. The amino acid residues in hZnT5, which were subjected to the substitution mutagenesis, are shown in blue (substituted amino acids are also shown in blue for ZnT transporters other than hZnT5, hZnT7, and CDF5 for easy comparison.). The PP-motif is boxed in red. LL, luminal loop. (B) Effects of alanine-scanning and substitution mutagenesis of amino acid residues in luminal loop 1 of hZnT5 on TNAP activation. (C) Effects of alanine-scanning mutagenesis of amino acid residues in luminal loop 2 of hZnT5 on TNAP activation. (D) Effects of alanine-scanning and substitution mutagenesis of amino acid residues in luminal loop 3 of hZnT5 on TNAP activation. In (B)–(D), the amino acids of hZnT5 that are indicated in (A) were mutated (see text). TNAP activity in TKO cells stably co-expressing each hZnT5 mutant with hZnT6 was measured, and it is expressed as the mean $\pm$ SD of three independent experiments. Expression of all of the hZnT5 mutants and hZnT6 was confirmed by immunoblotting (*lower panels*). Calnexin (CNX) is shown as a loading control. N.S., non-specific.

Fig. 2. The PP-motif in luminal loop 2 of hZnT5 is crucial for TNAP activation. (A) The hZnT5_{PP-AA} mutant co-localizes with hZnT6 and forms heterodimers with hZnT6 just like WT hZnT5. Stably expressed FLAG-hZnT5 or the FLAG-hZnT5_{PP-AA} mutant and HA-hZnT6 were double-stained (*upper panels*). Membrane proteins were prepared from TKO cells, and FLAG-hZnT5 and HA-hZnT6 were immunoprecipitated with antibodies against the FLAG and HA tags, respectively (*lower panels*). 12% of each aliquot was subjected to an immunoblot analysis (*input*). (B) The PP-motif in luminal loop 2 of hZnT5 is crucial for TNAP activation. TNAP activity in the indicated cells was measured as in Fig. 1B, and it is expressed as the mean $\pm$ SD of three independent experiments. Expression of each protein was confirmed by immunoblotting (*lower panels*). Calnexin (CNX) is shown as a loading control. (C) A minor

low-molecular-weight band of the hTNAP protein appears in TKO cells stably expressing ZnT5_{PP-AA} with hZnT6, but not WT hZnT5. The TNAP activity (the sum of the exogenously expressed hTNAP plus endogenous TNAP) was measured using total cellular proteins that were prepared from the indicated cells (*upper* panel). hTNAP was detected as one high-molecular-weight band of ~75-kDa (in the case of WT hZnT5) or one major band (~75-kDa) and minor ~65-kDa bands (in the case of ZnT5_{PP-AA}). The ~65-kDa bands were not significantly changed by the treatment with both MG132 (30 μ M) and bafilomycin A1 (30 nM) for 2 h (*lower* panel). (D) Zinc supplementation did not significantly restore hTNAP activity, and it did not cause the ~65-kDa bands to disappear. TNAP activity was measured using total cellular proteins that were prepared from cells cultured with 50 or 100 μ M ZnSO₄ for 24 h, as in (C) (*upper* panel). In (C) and (D), the TNAP activity is expressed as the mean $\pm$ SD of triplicate experiments. Twenty micrograms of total cellular protein was loaded in each lane. Tubulin is shown as a loading control. The positions of the protein size markers are indicated on the right.

Fig. 3. The PP-motif in luminal loop 2 of hZnT7 is crucial for TNAP activation.

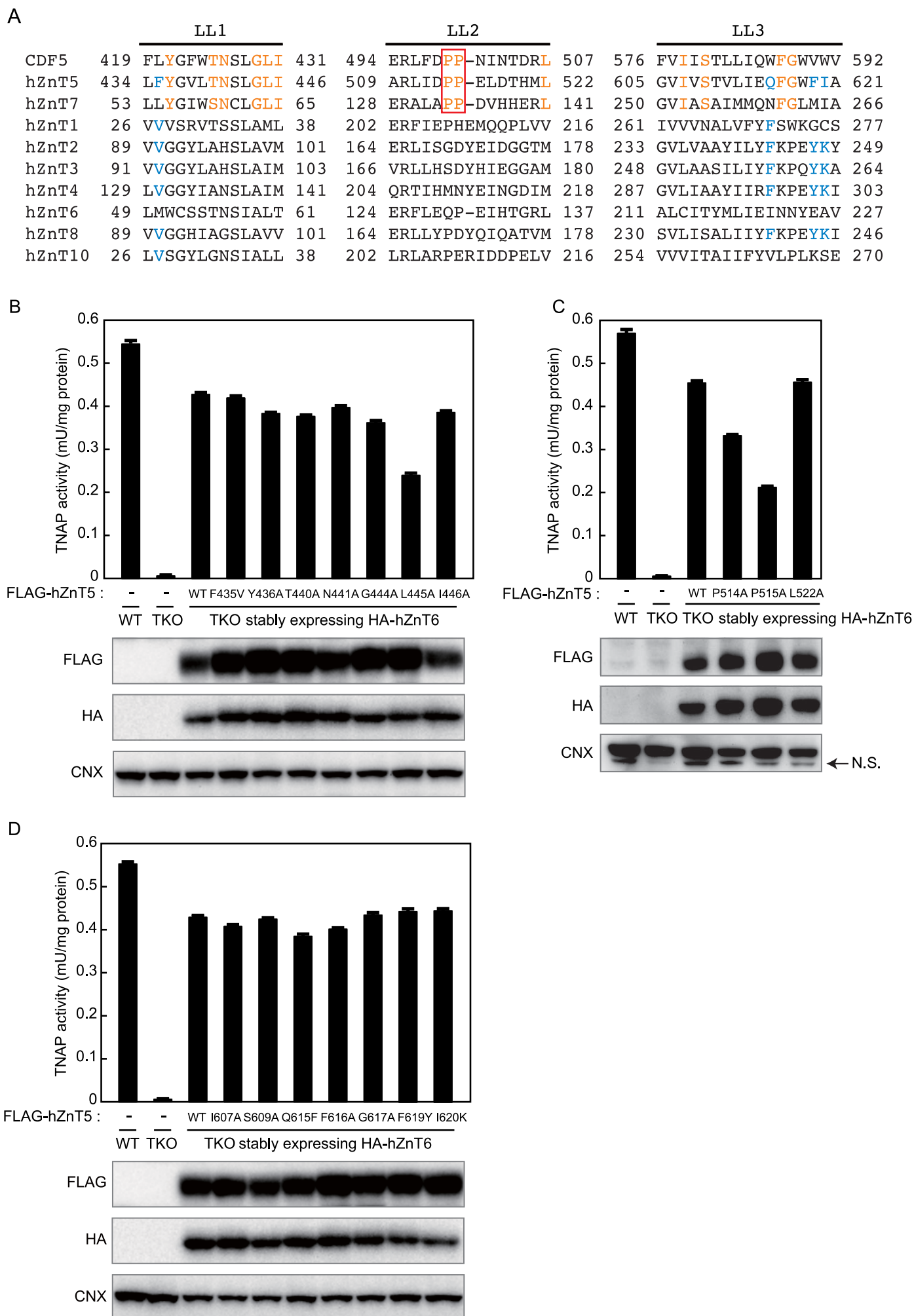
(A) Ribbon representation of the three-dimensional model of hZnT5-hZnT6 heterodimer (left, view from the membrane plane; right, view from the extracellular side). hZnT5 (only the cation efflux domain) and hZnT6 are in yellow and salmon, respectively. The PP-motif and the intramembranous zinc-binding site are shown in red and green, respectively. The corresponding position of hZnT6 is shown in aubergine. Purple circle, zinc ion. (B) Ribbon representation of the three-dimensional model of hZnT5 and hZnT7. The hZnT5 monomer (only the cation efflux domain) is overlaid on the hZnT7 monomer, which are in yellow and gray, respectively. The PP-motifs are shown in red (for hZnT5) and orange (for hZnT7), and the intramembranous zinc-binding sites are shown in green. Purple circle, zinc ion. (C) The PP-motif in luminal loop 2 of hZnT7 is crucial for TNAP activation. TNAP activity in the indicated cells was measured as in Fig. 1B. Expression of the hZnT7 mutants was confirmed by immunoblotting (*lower* panels). Tubulin is shown as a loading control. (D) Complementation analysis of the hZnT7_{PP-AA} mutant using the *S. cerevisiae* mutant DY150 *msc2*. The *msc2* mutant was transformed with pYES2 vectors containing the *ScMSC2*, *hZnT7-HA*, *hZnT7_{H70A}-HA*, or *hZnT7_{PP-AA}-HA* genes. The YPG₂ plates, on which transformed yeast cells were plated, were incubated for 3 d at 30 and 37°C

(upper panel). The expression of each ZnT7 mutant was confirmed by immunoblotting (lower panel). A silver-stained gel is shown as a loading control. One microgram of membrane protein was loaded in each lane for the immunoblotting and silver staining.

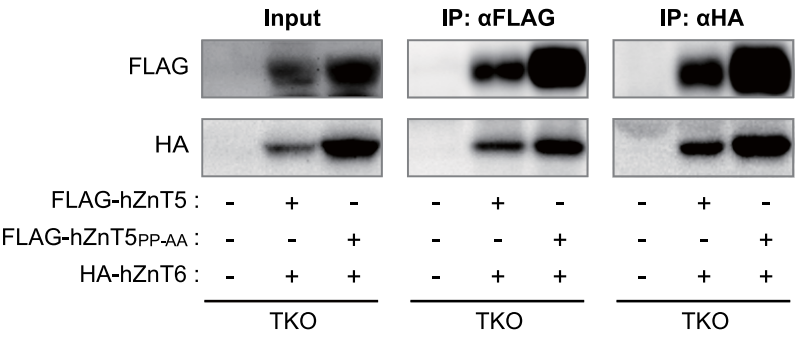
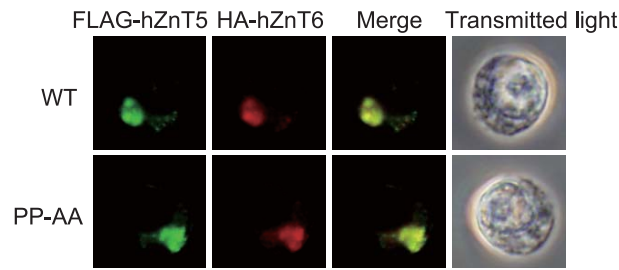
Fig. 4. *C. elegans* CDF5 and TOC1 restore TNAP activity in TKO cells, but the PP-AA mutant of CDF5 does not. (A) Co-expression of CDF5 and TOC1, but not the expression of CDF5 or TOC1 alone, restores TNAP activity in TKO cells (upper panel). The TNAP activity of membrane protein fractions that were prepared from the indicated cells is expressed as the mean $\pm$ SD of three independent experiments. The expression of FLAG-CDF5 and HA-TOC1 was confirmed by immunoblot analysis using membrane proteins that were prepared from the indicated cells (lower panels). TKO cells co-expressing FLAG-hZnT5 and HA-hZnT6 were used as a positive control. Calnexin (CNX) is shown as a loading control. (B) The CDF5_{PP-AA} mutant co-localizes with TOC-1, as do WT hZnT5 and hZnT6. Stably expressed FLAG-CDF5 (upper panels) or the FLAG-CDF5_{PP-AA} mutant (lower panels) and HA-TOC-1 were double-stained. (C) CDF5 and TOC1 physically interact with each other. FLAG-CDF5 and HA-TOC1 were immunoprecipitated with antibodies against the FLAG and HA epitopes, respectively. 12% of each aliquot was subjected to an immunoblot analysis (input). Any specific bands were not detected without using the first (primary) antibody (lanes of “-1st Ab”). TKO cells co-expressing FLAG-hZnT5 and HA-hZnT6 were used as a positive control. (D) The PP-motif in luminal loop 2 of CDF5 is crucial for TNAP activation. TNAP activity in the indicated cells was measured as in (A). Expression of CDF5 mutants and TOC1 was confirmed by immunoblotting (lower panels). Tubulin is shown as a loading control.

Fig. 5. The PP-motif is not sufficient to confer the ability to activate TNAP to other ZnT transporters. (A) Evaluation of the zinc transport activity of hZnT4 mutants using *ZnT1*^{-/-}*MT*^{-/-}*ZnT4*^{-/-} cells. *ZnT1*^{-/-}*MT*^{-/-}*ZnT4*^{-/-} cells stably expressing WT hZnT4 or the hZnT4_{MNY-PP} mutant were grown in the presence of the indicated concentrations of ZnSO₄ for 72 h, and the number of living cells was measured by the Alamar blue assay (and plotted as a percentage of living cells at 30 μ M ZnSO₄ for each group of cells). *ZnT1*^{-/-}*MT*^{-/-}*ZnT4*^{-/-} cells (●), and *ZnT1*^{-/-}*MT*^{-/-}*ZnT4*^{-/-} cells stably expressing WT

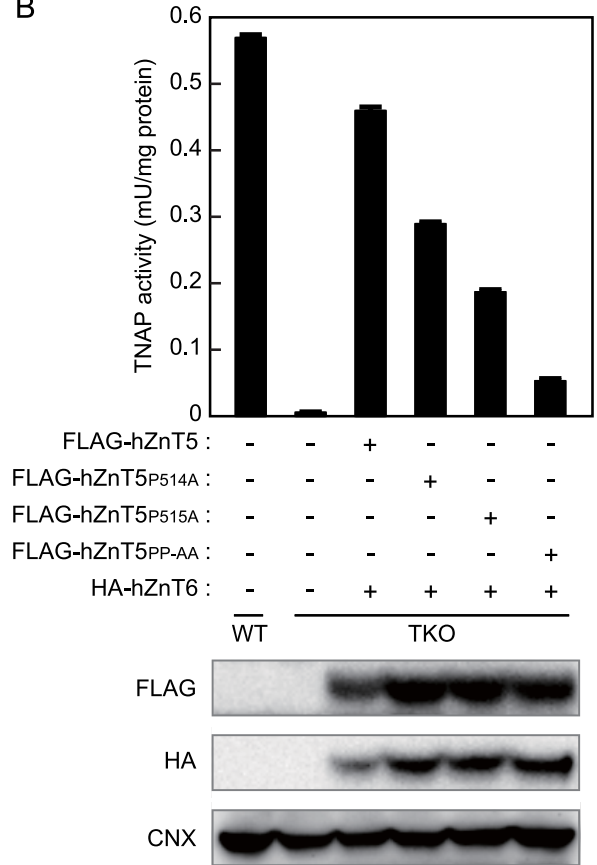
hZnT4 (■) or hZnT4_{MNY-PP} (▲). Each value is the mean $\pm$ SD of three independent experiments. (B) TNAP activity in TKO cells is not restored by expressing the hZnT4_{MNY-PP} mutant. (C) TNAP activity is significantly reduced in TKO cells co-expressing the hZnT5_{PP-MNY} mutant with hZnT6. In (B) and (C), TNAP activity is expressed as the mean $\pm$ SD of three independent experiments. The expression of each ZnT protein was confirmed by immunoblotting (*lower* panels). Tubulin and calnexin (CNX) are shown as loading controls.



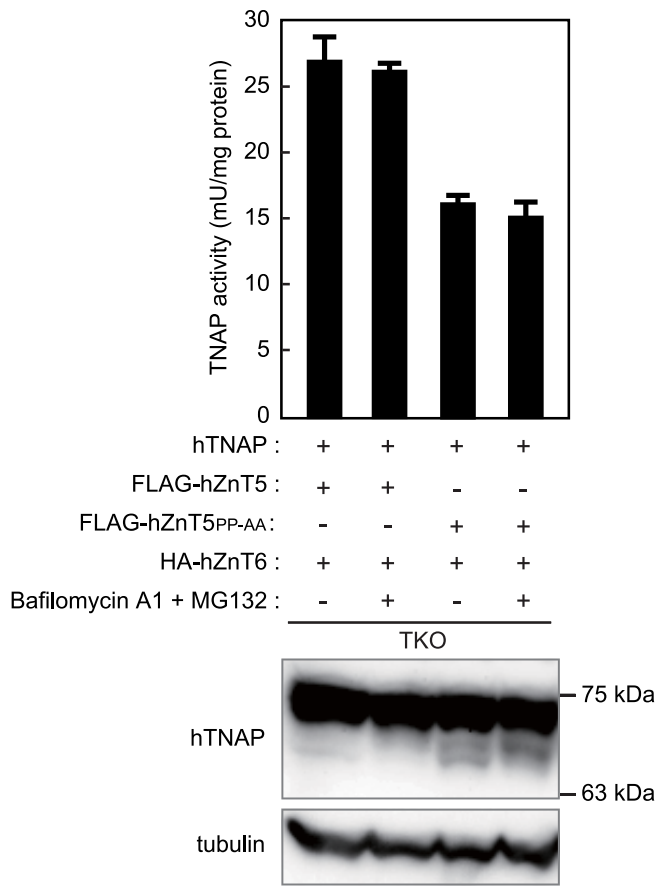
A



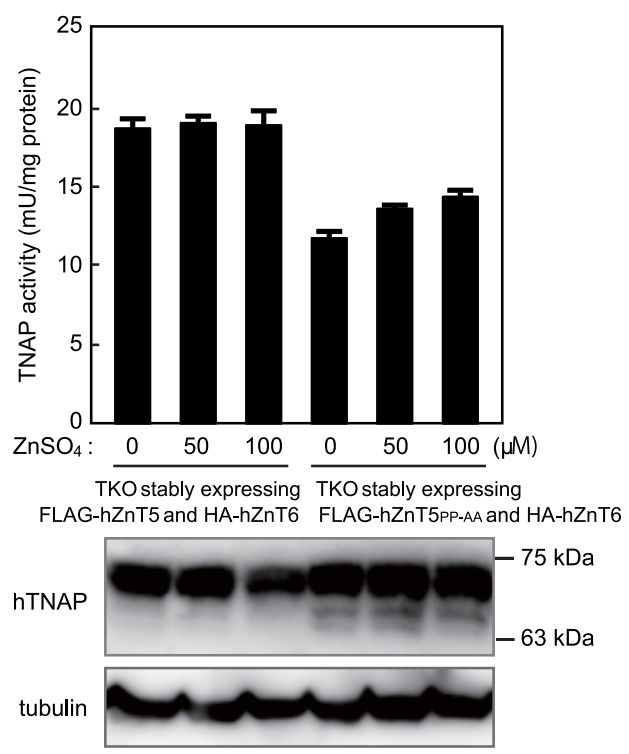
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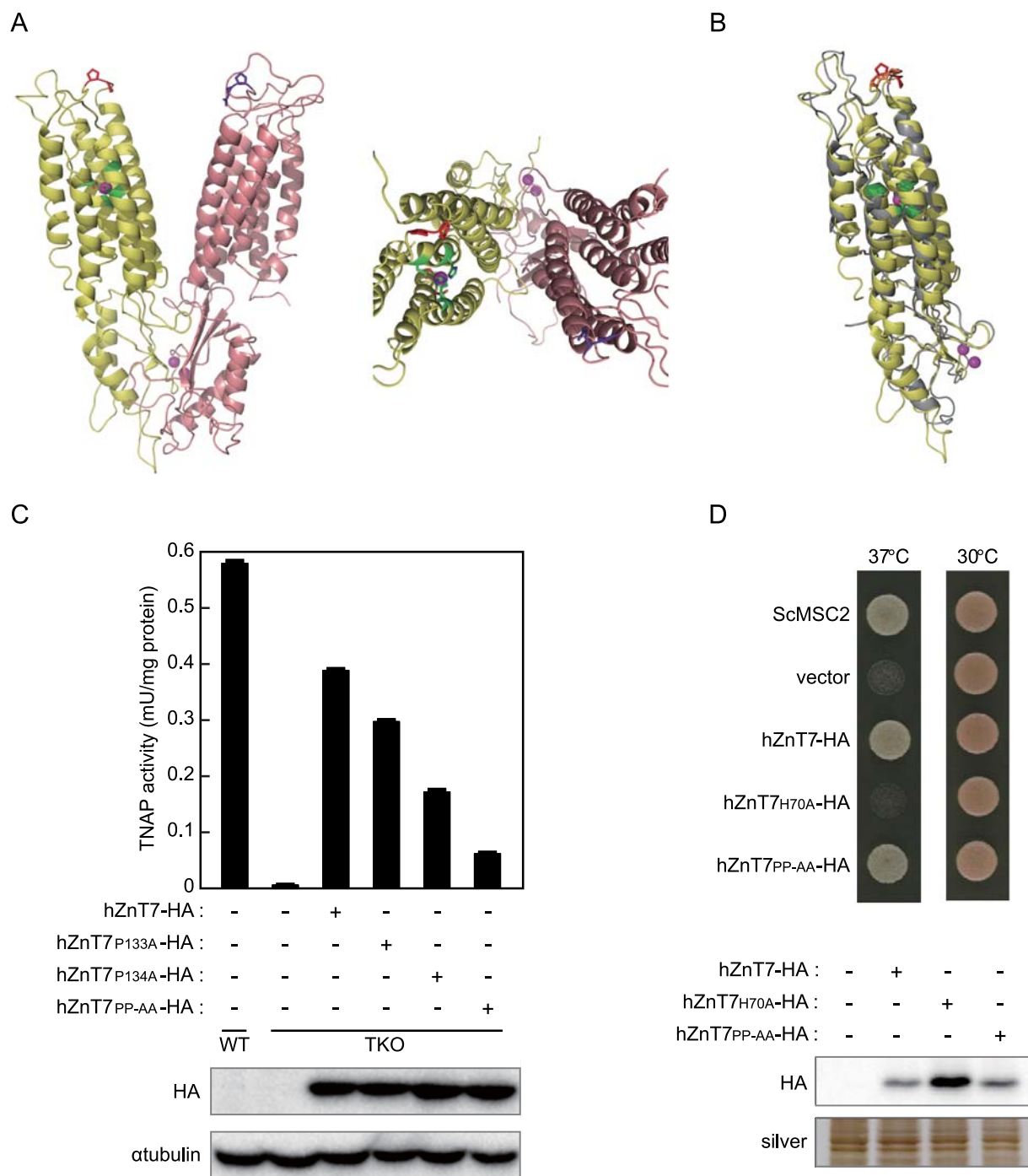


C

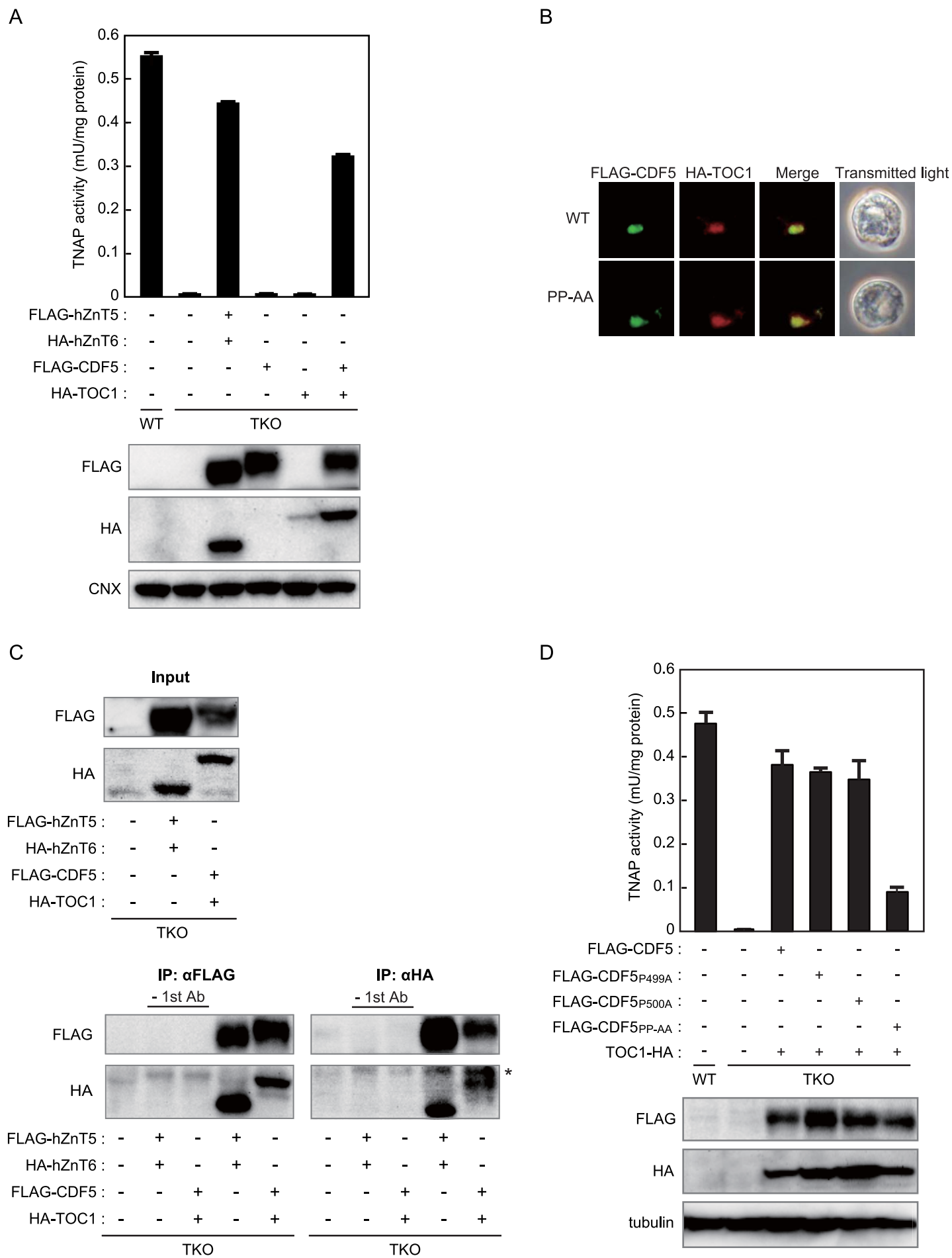


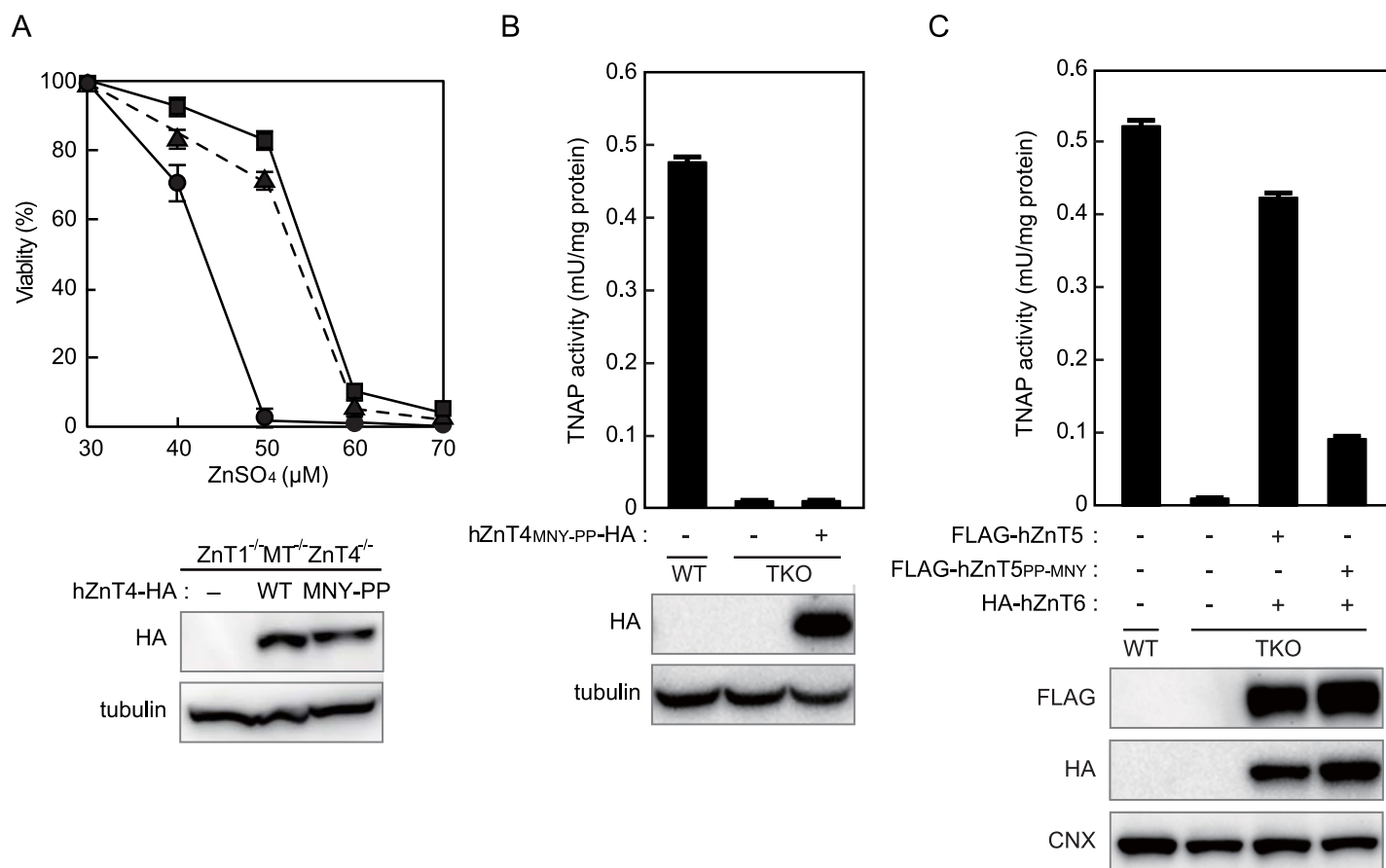
D





Fujimoto et al. Fig. 3





Fujimoto et al. Fig. 5