

Supplementary material

Sequence information

The accession nos. for the sequences used in Fig. 1 and Tables 1–3 are as follows: NP_067017.2 for hZnT1, NP_001004434.1 for hZnT2, NP_003450.2 for hZnT3, NP_037441.2 for hZnT4, NP_075053.2 for hZnT5, NP_060434.2 for hZnT6, AAM21969.1 for hZnT7, NP_776250.2 for hZnT8, NP_061183.2 for hZnT10, NP_740931.2 for CDF5, NP_075023.2 for ZnT5 (*M. musculus*), AAV98201.1 for ZnT5 (*G. gallus*), NP_001015911.1 for ZnT5 (*X. tropicalis*), NP_001002322.1 for ZnT5 (*D. rerio*), NP_594694.1 for Cis4 (*S. pombe*), NP_010491.4 for Msc2 (*S. cerevisiae*), NP_178539.2 for MTP12 (*A. thaliana*), AAO17323.1 for ZnT7 (*M. musculus*), NP_001008788.1 for ZnT7 (*G. gallus*), NP_989256.1 for ZnT7 (*X. tropicalis*), NP_001093556.1 for ZnT7 (*D. rerio*), NP_650049.1 for ZnT86D (*D. melanogaster*), NP_014961.3 for Cot1 (*S. cerevisiae*), NP_593645.1 for Zhf1 (*S. pombe*), and WP_003229873.1 for CzcD (*B. subtilis*).

The canonical HDHD core motif in the intramembranous zinc-binding site of hZnT5 is essential for TNAP activation

Two mutants in the HDHD core motif in the intramembranous zinc-binding site of hZnT5, specifically hZnT5_{H451A} and hZnT5_{D599A}, lacked zinc transport activity and, thus, the ability to activate TNAP [1,2]. Because a previous study reported that other hZnT5 mutants, specifically hZnT5_{H451D} and hZnT5_{D599E}, retained zinc transport activity [1,3], we examined whether they activate TNAP as well as WT hZnT5. Consistent with their zinc transport activity, the two mutant proteins co-localized with hZnT6 (Supplemental Fig. 1A) and formed heterodimers with hZnT6 in TKO cells (Supplemental Fig. 1B). However, unexpectedly, both mutants failed to activate TNAP, as did zinc-transport incompetent mutants, such as hZnT5_{H451A} and hZnT5_{D599A} [4] (Supplemental Fig. 1C), indicating that the HDHD core motif in the intramembranous zinc-binding site of hZnT5 is closely associated with TNAP activation.

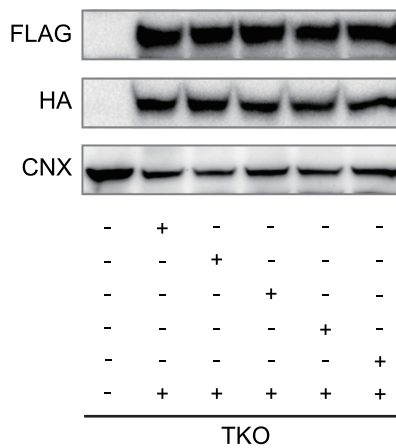
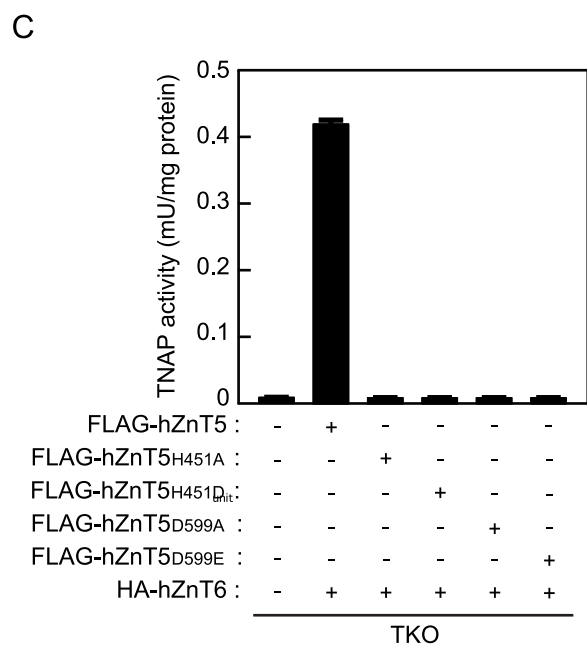
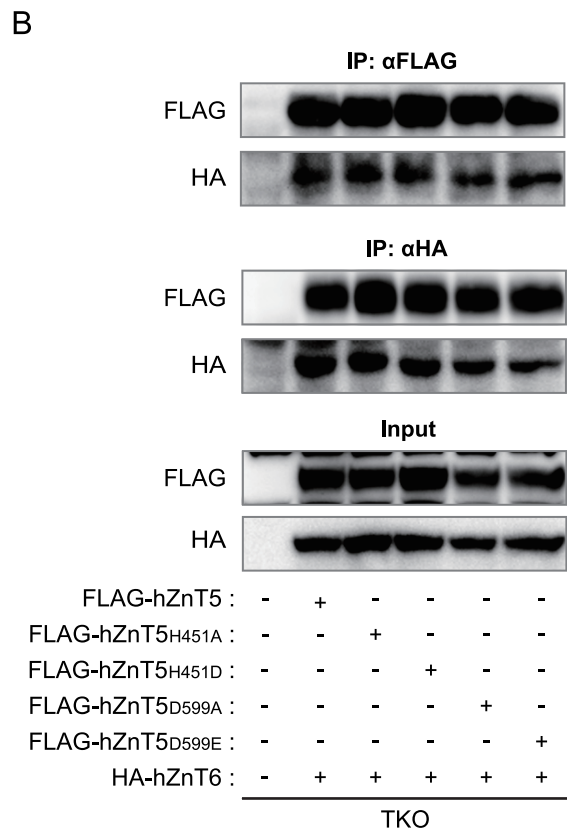
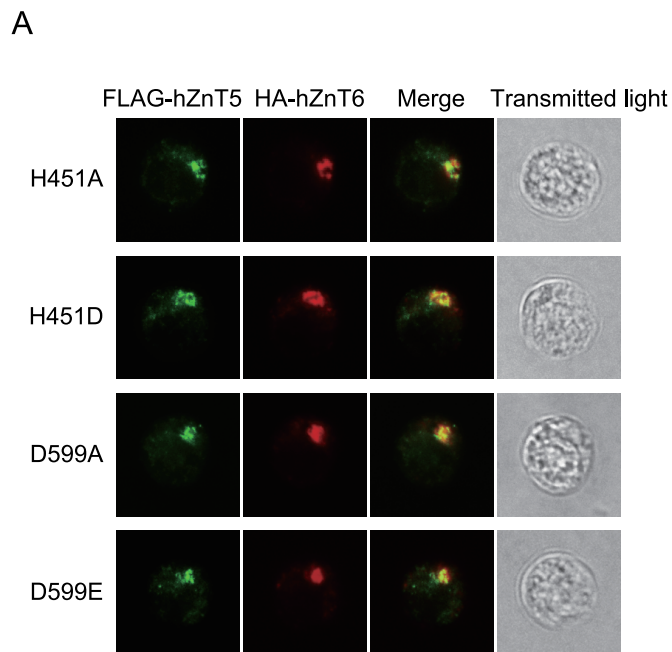
REFERENCES

- 1 Ohana, E., Hoch, E., Keasar, C., Kambe, T., Yifrach, O., Hershfinkel, M. and Sekler, I. (2009) Identification of the Zn²⁺ binding site and mode of operation of a mammalian Zn²⁺ transporter. *J. Biol. Chem.* **284**, 17677–17686.
- 2 Fukunaka, A., Suzuki, T., Kurokawa, Y., Yamazaki, T., Fujiwara, N., Ishihara, K., Migaki, H., Okumura, K., Masuda, S., Yamaguchi-Iwai, Y., Nagao, M. and Kambe, T. (2009) Demonstration and characterization of the heterodimerization of ZnT5 and ZnT6 in the early secretory pathway. *J. Biol. Chem.* **284**, 30798–30806.
- 3 Hoch, E., Lin, W., Chai, J., Hershfinkel, M., Fu, D. and Sekler, I. (2012) Histidine pairing at the metal transport site of mammalian ZnT transporters controls Zn²⁺ over Cd²⁺ selectivity. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 7202–7207.
- 4 Fukunaka, A., Kurokawa, Y., Teranishi, F., Sekler, I., Oda, K., Ackland, M. L., Faundez, V., Hiromura, M., Masuda, S., Nagao, M., Enomoto, S. and Kambe, T. (2011) Tissue nonspecific alkaline phosphatase is activated via a two-step mechanism by zinc transport complexes in the early secretory pathway. *J. Biol. Chem.* **286**, 16363–16373

FIGURE LEGENDS

Figure S1. The canonical HDHD core motif of intramembranous zinc-binding site of hZnT5 is essential for TNAP activation. (A) Human ZnT5 mutants (hZnT5_{H451A}, hZnT5_{H451D}, hZnT5_{D599A}, and hZnT5_{D599E}) co-localized with hZnT6 in TKO cells. Stably expressed FLAG-hZnT5 mutants and HA-hZnT6 were double-stained using antibodies against either the FLAG and HA epitopes, respectively, followed by secondary antibodies conjugated with Alexa 488 and Alexa 594, respectively. (B) Human ZnT5 mutants (hZnT5_{H451A}, hZnT5_{H451D}, hZnT5_{D599A}, and hZnT5_{D599E}) form heterodimers with hZnT6. Membrane proteins were prepared from the indicated cells, and they were subjected to immunoprecipitation with antibodies against either the FLAG or HA epitopes. To estimate the amounts of the FLAG-hZnT5 mutants and HA-hZnT6 in the membrane protein fractions, 12% of each aliquot was subjected to an immunoblot analysis (*input* panels). (C) The HDHD core motif of the intramembranous zinc-binding site is essential for TNAP activation. TNAP activity of membrane protein fractions that were prepared from the indicated cells is expressed as the mean ± SD of three independent experiments (milliunits (mU)/mg protein, *left* graph). Expression of hZnT5 mutants was confirmed by immunoblotting (*right* panels). Calnexin (CNX) is shown as a loading control. Measurement of TNAP activity, immunoblotting and

immunoprecipitation experiments, and immunofluorescence staining were performed as described in the main text.



Supplemental Fig. 1