Metalation and activation of Zn^{2+} enzymes via early secretory pathway-resident ZNT proteins

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Abstract

Zinc (Zn^{2+}), an essential trace element, binds to various proteins, including enzymes, transcription factors, channels, and signaling molecules and their receptors, to regulate their activities in a wide range of physiological functions. Zn^{2+} proteome analyses have indicated that approximately 10% of the proteins encoded by the human genome have potential Zn^{2+} binding sites. Zn^{2+} binding to the functional site of a protein (for enzymes, the active site) is termed Zn^{2+} metalation. In eukaryotic cells, approximately one-third of proteins are targeted to the endoplasmic reticulum; therefore, a considerable number of proteins mature by Zn^{2+} metalation in the early secretory pathway compartments. Failure to capture Zn^{2+} in these compartments results in not only the inactivation of enzymes (*apo*-Zn^{2+} enzymes), but also their elimination via degradation. This process deserves attention because many Zn^{2+} enzymes that mature during the secretory process are associated with disease pathogenesis. However, how Zn^{2+} is mobilized via Zn^{2+} transporters, particularly ZNTs, and incorporated in enzymes has not been fully elucidated from the cellular perspective and much less from the biophysical perspective. This review focuses on Zn^{2+} enzymes that are activated by Zn^{2+} metalation via Zn^{2+} transporters during the secretory process. Further, we describe the importance of Zn^{2+} metalation from the physiopathological perspective, helping to reveal the importance of understanding Zn^{2+} enzymes from a biophysical perspective.

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INTRODUCTION

Zinc (Zn^{2+}) plays essential roles in various biological processes, including cell growth and migration, development, and fertilization, and in the immune and central nervous systems by associating and binding (metalation) to target proteins/enzymes.[1] Under physiological conditions, Zn^{2+} is a redox-inert metal ion that exists solely as a divalent cation, which is a very unique property compared to the redox-active metal ions, iron ($Fe^{2+}\rightleftarrows Fe^{3+}$) and copper ($Cu^{+}\rightleftarrows Cu^{2+}$).[2] Therefore, Zn^{2+} functions as an essential component in numerous proteins/enzymes. Approximately 10% of the proteins encoded by the human genome can bind to Zn^{2+}, suggesting that it is involved in a wide range of physiological functions.[3]

In humans, numerous Zn^{2+} enzymes are folded and activated in early secretory pathway compartments, such as the endoplasmic reticulum (ER) and Golgi apparatus, before reaching their final destination, including the organelles, cell surface, and extracellular space. During this process, the enzyme active site is metalated with Zn^{2+}; however, the underlying molecular mechanism has not been well elucidated. Recently, we published a number of articles showing that the metalation of secretory, membrane-bound, and organelle-resident Zn^{2+} enzymes is regulated by Zn^{2+} transporters belonging to the Zn transporter (ZNT) family located in early secretory pathway compartments. In this review, we summarize the Zn^{2+} enzymes that require Zn^{2+} supplied by ZNT5-ZNT6 and ZNT7 (Table 1) and discuss their

functions from the cellular and physiological perspectives. Finally, we provide future research directions from a biophysical perspective.

Zn^{2+} TRANSPORTERS

The influx and efflux of Zn^{2+} into cells are cooperatively and intricately controlled by 24 Zn^{2+} transporters, which comprise two families: the Zn transporter (ZNT) and ZRT- and IRT-like protein (ZIP) families (Figure 1A).[4,5] ZNTs, which belong to the cation diffusion facilitator superfamily, are efflux transporters that reduce cytosolic Zn^{2+} levels by transporting Zn^{2+} into intracellular compartments or the extracellular space, whereas ZIPs are influx transporters that elevate cytosolic Zn^{2+} levels by mobilizing Zn^{2+} into the cytosol from the extracellular space or intracellular compartments. In humans, 14 ZIPs and 10 ZNTs function in systemic, cellular, and subcellular Zn^{2+} homeostasis (although recent studies have shown that ZNT10 functions as a Mn^{2+} transporter), and mutations in half of these have been implicated in genetic disorders, the various phenotypes of which reflect the multifarious essential functions of Zn^{2+} mediated by both transporter families.[6,7]

Both ZNT and ZIP family members are grouped into solute carriers (SLC) (ZNTs: SLC30A, ZIPs: SLC39A) and thus function in secondary active transport, which does not directly utilize ATP hydrolysis. Studies have indicated that ZNTs function as Zn^{2+}/H^{+} exchangers that operate as an alternating access mechanism mediated by conformational changes between inward- and outward-facing conformations, in a similar manner as the rocker switch (Figure 1B).[8-15] Studies using the prokaryotic ZNT homolog, YiiP, from *Escherichia coli*[8,16,17] or *Shewanella oneidensis*[12] have shown that the stoichiometry of Zn^{2+}/H^{+} antiport is 1:1.[12,16] In contrast, that of ZNT2 is 2:1.[10] This discrepancy remains to be clarified but may be explained by differences in the amino acid constitution in the transmembrane Zn^{2+}-binding site (YiiP has one His and three Asp residues in a DDHD motif, whereas ZNT2 has two His and two Asp residues in a HDHD motif). Recent studies have revealed that ZIPs transport Zn^{2+} by an alternating access mechanism based on a two-domain elevator-type mechanism (Figure 1B).[18-20] They harbor two Zn^{2+} binding sites (M1 and M2) in their transmembrane domains; M1 is essential for Zn^{2+} transport, whereas M2 is auxiliary.[21,22] In contrast to that of ZNTs, the molecular mechanism of ZIPs has not yet been clarified, although Zn^{2+}/HCO_3^{-}[23,24] and Zn^{2+}/H^{+} symport[25] or water-mediated transport[26] mechanisms have been proposed. ZIPs may have diverse Zn^{2+} transport mechanisms determined by the local charge around Zn^{2+}-binding sites and the protonation state dictated by the extracellular pH.[18] This needs to be elucidated in future studies.

ZNTs LOCATED IN THE EARLY SECRETORY PATHWAY COMPARTMENTS

Within mammalian cells, more than 4% of total proteins are loaded with Zn^{2+} in the early secretory pathway,[5] an estimation based on the zinc proteome in *S. cerevisiae.*[27,28]

Therefore, a certain proportion is believed to comprise Zn^{2+} enzymes undergoing activation and metalation within these compartments.

Zn^{2+} is taken up into the lumen of the early secretory compartments by ZNTs, particularly, the ZNT5-ZNT6 heterocomplex and the ZNT7 homocomplex (hereafter referred to as ZNT5-6 and ZNT7).[29,30] Vertebrate cells possess both ZNT5-6 and ZNT7, whereas *Drosophila melanogaster* and yeast express only ZNT7 and ZNT5-6, respectively.[31-34] It remains unknown why ZNT expression differs among species, but it can be suggested that vertebrate cells require more complex regulation via Zn^{2+} supply to Zn^{2+} proteins/enzymes in the early secretory pathway. Recent studies have shown that ZNT5-6 and ZNT7 transport Zn^{2+} into the lumen of the early secretory pathway compartments in different manners (Figure 2). Specifically, ZNT5-6 transports Zn^{2+} into the lumen of the medial Golgi, whereas ZNT7 transports Zn^{2+} into the lumen of the *cis* Golgi. Both functions are required for Zn^{2+} concentrations of ~60 nM in the medial Golgi and ~100 nM in the pre-*cis* Golgi.[35,36] These differences may explain why ZNT5-6 and ZNT7 have unique functional roles, which are reflected in the associated human genetic disorders,[37-39] although they are similar in terms of being required for enzyme maturation.

The Zn^{2+} concentrations in different cellular compartments in the cells are shown in Figure 3, allowing for comparison between those in the early secretory pathway and those in other compartments. This visualization aids in grasping Zn^{2+} dynamics within the cells at a glance.

ROLE OF Zn^{2+} IN EARLY SECRETORY PATHWAY HOMEOSTASIS

Before describing the direct critical roles of ZNT5-6 and ZNT7 in Zn^{2+} enzyme maturation in the next section, we summarize their roles in protein quality control in the early secretory pathway. ZNT5-6 and ZNT7 regulate the Golgi-to-ER retrograde transport of the ER chaperone ERp44 via dynamic conformational changes in its carboxyl terminal portion by Zn^{2+} binding.[40] Accordingly, loss of function of the ZNT complexes results in ERp44 retention in the Golgi[40] and exacerbation of the ER stress response, which is consistent with the fact that some chaperon protein functions are regulated by Zn^{2+}.[40, 41]

In addition to ZNTs, the ER-located ZIP7, which transports Zn^{2+} from the early secretory pathway to the cytoplasm in the opposite direction of ZNT5-6 and ZNT7, plays a critical role in the maintenance of secretory pathway homeostasis.[42,43] Loss of ZIP7 function leads to an increase in the amount of Zn^{2+} in the ER lumen,[44,45] which results in Zn^{2+}-dependent aggregation and inhibition of protein disulfide isomerase,[43] which in turn causes ER stress. Considering that ER stress increases *ZNT5* and *ZIP7* expression,[42,46] both Zn^{2+} deficiency and Zn^{2+}excess in the early secretory pathway trigger the unfolded protein response, indicating that Zn^{2+} homeostasis maintenance in the early secretory pathway is essential for cells.

SPECIFIC Zn^{2+} ENZYMES METALATED AND ACTIVATED BY ZNT5-6 AND ZNT7

ER-located Zn^{2+} enzymes, PIGN, PIGO, and PIGG

Proteins biosynthesized in the ER are trafficked to where they function. During this process, they undergo various post-translational modifications. Glycosylphosphatidylinositol (GPI) anchoring is one of the essential post-translational modifications. In humans, more than 150 GPI-anchored proteins are expressed.[47] GPI-anchored biosynthesis requires more than 20 phosphatidylinositol-glycan anchor biosynthesis (PIG) proteins that sequentially add sugars and other components to phosphatidylinositol in a multi-step reaction process. Therefore, *PIG* gene mutations cause reduced cell-surface expression of GPI-anchored proteins, resulting in congenital GPI deficiency. Using cells lacking certain *PIG* genes, we found that ZNT5-6 and ZNT7 functions are required for the expression of GPI-anchored proteins.[48] Comparative analysis using cells lacking functional ZNT5-6 and ZNT7 as well as multiple PIG proteins suggested that the severe effects on GPI anchor proteins are responsible for the loss of function of three PIG proteins (PIGN, PIGO, and PIGG, which are GPI ethanolamine phosphate transferases), all of which have Zn^{2+} in their active sites.[49,50]

In addition to the above *in vitro* studies, the importance of ZNT5-6 and ZNT7 for PIGN, PIGO, and PIGG functions has been confirmed in an *in vivo* study using medaka fish (*Oryzias latipes*). Medaka fish with impaired Znt5-6 and Znt7 exhibited an abnormal tactile phenotype in that they did not make escape movements when poked with a needle, similarly to zebrafish lacking a certain Pig protein (Figure 4A).[48,51] How Zn^{2+} is correctly supplied to PIG proteins located in the ER by ZNT5-6 and ZNT7 remains to be clarified.

Lysosomal Zn^{2+} enzyme, SMPD1

Sphingomyelin phosphodiesterase 1 (SMPD1), an acid sphingomyelinase, degrades sphingomyelin to ceramide and phosphocholine in lysosomes. *SMPD1* mutations cause abnormal lysosomal sphingomyelin accumulation, resulting in Niemann–Pick disease (types A and B).[52] The activity of SMPD1 depends on two Zn^{2+} ions coordinated to the active site. Zn^{2+} acquisition by SMPD1 was initially speculated to occur in the lysosomes, where it functions. However, we recently showed that SMPD1 is activated by metalation with Zn^{2+} by ZNT5-6 and ZNT7, and thus, that Zn^{2+} acquisition occurs in the early secretory pathway.[53] In cells lacking functional ZNT5-6 and ZNT7, the ceramide and sphingomyelin content ratio was decreased, which was accompanied by the appearance of multilamellar body-like structures due to membrane stacking and accumulation. These phenomena were very similar to those in cells lacking SMPD1, indicating that ZNT5-6 and ZNT7 are involved in sphingolipid metabolism via regulating SMPD1 metalation.

Melanosomal Zn^{2+} enzyme, TYRP1

Melanin, which is the main pigment responsible for skin, hair, and eye color, plays an

important role in protecting against ultraviolet radiation.[54,55] Two types of melanin are synthesized in the body: eumelanin, which is dark brown to black, and pheomelanin, which is reddish orange,[56] both of which are biosynthesized in melanosomes in melanocytes. Melanin biosynthesis starts from tyrosine and involves three melanogenic enzymes: tyrosinase (TYR) and tyrosinase-related proteins 1 and 2 (TYRP1 and TYRP2).[55,57,58] Cu^{+} has long been thought to be the only metal ion critical for melanin synthesis because the rate-limiting enzyme in melanin pigmentation, TYR, requires Cu^{+} for its activity.[59] However, we recently reported that Zn^{2+} is essential for TYRP1 stabilization, which is mediated by ZNT5-6 and ZNT7, and thus, for melanin biosynthesis.[60] Pre-hatched medaka fish deficient in Znt5-6 and Znt7 had a lighter color because of immature melanosomes (brownish) in contrast to the densely packed mature melanosomes with higher melanin contents in wild-type medaka (black) (Figure 4B). The color alteration was consistent with that observed in other animals lacking TYRP1, including mice,[61] bears,[62] chickens,[63] zebrafish,[64] and snakes.[65] Similarly, reduced color tone of cell pellets and melanin content were observed in human melanoma cells deficient in ZNT5-6 and ZNT7, which could be recovered by exogenous ZNT5-6 and ZNT7 expression. These results indicate that both ZNT complexes contribute to melanin biosynthesis through regulating TYRP1 expression by supplying Zn^{2+} ions, which are likely coordinated at the active site.[66] Our study was the first to clearly demonstrate that Zn^{2+} plays an important role in melanin biosynthesis, after Cu^{+} had been thought to be the only important cofactor in this process for more than 70 years.[67,68]

Cell-surface Zn^{2+} enzyme, CAIX, and secretory Zn^{2+} enzymes, MMP2, MMP9, and ATX

Carbonic anhydrase (CA) plays important roles in respiration, photosynthesis, and pH regulation in living organisms by catalyzing the interconversion of carbon dioxide and bicarbonate ions.[69] CA is the first enzyme identified as a Zn^{2+} enzyme.[70] Among CAs, CAIX, an ectoenzyme that is functional on the cell surface, is known as a tumor marker enzyme that regulates the pH environment of cancer cells and contributes to cancer migration, invasion, and poor prognosis.[71,72] CAIX is activated by Zn^{2+} metalation via ZNT5-6 and ZNT7 and, uniquely, via ZNT4 in the early secretory pathway.[73] The detailed mechanisms underlying Zn^{2+} acquisition, including how ZNT5-6, ZNT7, and ZNT4 are used differently, have not yet been clarified.

In addition to CAIX, the Zn^{2+} enzymes matrix metalloproteinases 2 and 9 (MMP2 and MMP9) and autotaxin (ATX) are activated by metalation via ZNT5-6 and ZNT7.[73] However, MMP2 and MMP9 likely also acquire Zn^{2+} via routes other than the ZNT complexes. As MMP2, MMP9, and ATX are involved in tumor malignancy, Zn^{2+} supply via both complexes may serve as a therapeutic target for anticancer drugs.

Zn^{2+} enzymes involved in extracellular ATP metabolism

In addition to CAIX, other cell surface-located Zn^{2+} enzymes are activated by Zn^{2+} metalation via ZNT5-6 and ZNT7.[74,75] Particularly, TNAP and NT5E/CD73, which are GPI-anchored enzymes, failed to be expressed in cells lacking ZNT5-6 and ZNT7.[48] Moreover, their secretory form failed to be activated; therefore, their activity requires ZNT5-6 and ZNT7.

TNAP, NT5E/CD73, and ectonucleotide pyrophosphatase/phosphodiesterases 1 and 3 (ENPP1 and ENPP3, please see below) are of physiopathological significance because they are involved in extracellular ATP metabolism (TNAP releases the terminal phosphates of ATP, ADP, and AMP; NT5E/CD73 converts AMP to adenosine; ENPP1 and ENPP3 convert ATP to AMP).[76] Extracellular ATP metabolism has been suggested to be associated with Zn^{2+} deficiency symptoms. Zn^{2+} deficiency causes various symptoms, including growth retardation, taste disorders, dermatitis, impaired wound healing, and diarrhea, some of which are associated with inflammation.[4,77] Indeed, severe dermatitis has been demonstrated to occur in inherited Zn^{2+} deficiency known as acrodermatitis enteropathica due to inflammation caused by delayed extracellular ATP metabolism.[78] Extracellular ATP induces inflammation by binding to cell-surface receptors (P2X and P2Y), whereas adenosine, the ATP hydrolysate, is related to anti-inflammation via binding to another cell-surface receptor (P1).[79,80] Therefore, Zn^{2+} deficiency is likely associated with inflammation via delaying extracellular ATP clearance and adenosine production (Figure 5).[76]

Zn^{2+} ENZYMES ACTIVATED INDEPENDENTLY OF ZNT5-6 AND ZNT7

Within the plasma membrane, there exist Zn^{2+} enzymes, whose activation in the early secretory pathway does not depend on Zn^{2+} mediated by ZNT5-6 and ZNT7. The representatives are ENPP1 and ENPP3, somatic angiotensin converting enzyme (ACE), germline-specific variant ACE, and ACE2. The independence of their activation has been confirmed.[48,76] Similarly, their homologues, such as ENPP4, 5 and 7, and endothelin converting enzymes, are also likely to be activated independently of ZNT5-6 and ZNT7. The molecular mechanism underlying their Zn^{2+} capture, and the entry of Zn^{2+} into the early secretory pathway remain elusive. Entirely distinct activation mechanisms may exist, which is unlikely considering current knowledge.

CONCLUSIONS AND PERSPECTIVES

Zn^{2+} transported by ZNT5-6 and ZNT7 is involved in the activation of a number of Zn^{2+} enzymes that mature during the secretory process (Table 1). Most of these enzymes have received attention because of their involvement in disease pathogenesis, and Zn^{2+} metalation of these enzymes may be a therapeutic target for clinical drugs. The recent development of inhibitors of ZIP7[81] and ZIP8[82] increases this possibility. As shown in this review, the Zn^{2+} dynamics in the early secretory pathway are beginning to be elucidated. However, there are still many missing pieces. Further studies are required to elucidate the molecular mechanisms

through which the Zn^{2+} enzymes described above (listed in Table 1) exhibit a specific dependence on Zn^{2+} through ZNT5-6 and ZNT7. Additionally, investigating how particular Zn^{2+} enzymes capture Zn^{2+} in cells lacking ZNT5-6 and ZNT7 is essential for a comprehensive understanding of these processes. In recent years, the biological regulatory effects of Zn^{2+} have been intensively studied worldwide, and it is expected that novel physiological effects of Zn^{2+} will be revealed at the molecular level. More than 80 years have passed since the first Zn^{2+} enzyme was identified;[70] therefore, the time is ripe to improve our understanding of Zn^{2+} enzymes from a biophysical perspective.

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AUTHOR DECLARATIONS

Conflict of Interest

The authors have no conflicts to disclose.

Author Contributions

Taiho Kambe: Conceptualization (lead); Data curation (lead); Formal analysis (lead); Funding acquisition (lead); Investigation (lead); Resources (lead); Visualization (equal); Writing – original draft (lead); Writing – review & editing (lead). Takumi Wagatsuma: Visualization (equal); Writing – review & editing (supporting).

DATA AVAILABILITY

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

Table 1. Zinc enzymes that require Zn^{2+} supplied by ZNT5-ZNT6 and ZNT7

Enzymes	Subcellular localization	Number of Zn^{2+} ions in active site	Biological function
Alkaline phosphatases	Cell surface (GPI-anchored)	2	Hydrolysis of a broad range of phosphate monoesters
SMPD1	Lysosomes	2	Hydrolysis of sphingomyelin
CAIX	Cell surface (membrane-spanning)	1	Carbon dioxide–bicarbonate ion interconversion
PIGO, PIGG, PIGN*	ER	2	Synthesis for GPI anchor
NT5E/CD73	Cell surface (GPI-anchored)	2	Hydrolysis of AMP to adenosine
ATX**	Extracellular	2	Hydrolysis of lysophosphatidylcholine to lysophosphatidic acid
TYRP1	Melanosomes	2	Putative DHICA oxidase activity for pigmentation
Matrix metalloproteinases	Extracellular	1	Hydrolysis of collagen

* GPI ethanolamine phosphate transferases, ** can also be activated by Zn^{2+} supplied by ZNT4.

References

1 Fukada, T. & Kambe, T. Molecular and genetic features of zinc transporters in physiology and pathogenesis. *Metallomics* **3**, 662-674 (2011).

2 Kambe, T. Metalation and maturation of zinc ectoenzymes: A perspective. *Biochemistry* **59**, 74-79 (2020).

3 Andreini, C., Banci, L., Bertini, I. & Rosato, A. Counting the zinc-proteins encoded in the human genome. *J Proteome Res* **5**, 196-201 (2006).

4 Kambe, T., Tsuji, T., Hashimoto, A. & Itsumura, N. The physiological, biochemical, and molecular roles of zinc transporters in zinc homeostasis and metabolism. *Physiol Rev* **95**, 749-784 (2015).

5 Kambe, T., Taylor, K. M. & Fu, D. Zinc transporters and their functional integration in mammalian cells. *J Biol Chem* **296**, 100320 (2021).

6 Kambe, T., Hashimoto, A. & Fujimoto, S. Current understanding of ZIP and ZnT zinc transporters in human health and diseases. *Cell Mol Life Sci* **71**, 3281-3295 (2014).

7 Hara, T., Yoshigai, E., Ohashi, T. & Fukada, T. Zinc transporters as potential therapeutic targets: An updated review. *J Pharmacol Sci* **148**, 221-228 (2022).

8 Gupta, S. *et al.* Visualizing the kinetic power stroke that drives proton-coupled zinc(II) transport. *Nature* **512**, 101-104 (2014).

9 Lopez-Redondo, M. L., Coudray, N., Zhang, Z., Alexopoulos, J. & Stokes, D. L. Structural basis for the alternating access mechanism of the cation diffusion facilitator YiiP. *Proc Natl Acad Sci U S A* **115**, 3042-3047 (2018).

10 Golan, Y., Alhadeff, R., Warshel, A. & Assaraf, Y. G. ZnT2 is an electroneutral proton-coupled vesicular antiporter displaying an apparent stoichiometry of two protons per zinc ion. *PLoS Comput Biol* **15**, e1006882 (2019).

11 Ohana, E. *et al.* Identification of the Zn2+ binding site and mode of operation of a mammalian Zn2+ transporter. *J Biol Chem* **284**, 17677-17686 (2009).

12 Coudray, N. *et al.* Inward-facing conformation of the zinc transporter YiiP revealed by cryoelectron microscopy. *Proc Natl Acad Sci U S A* **110**, 2140-2145 (2013).

13 Xue, J., Xie, T., Zeng, W., Jiang, Y. & Bai, X.-C. Cryo-EM structures of human ZnT8 in both outward- and inward-facing conformations. *eLife* **9**, e58823 (2020).

14 Zhang, S. *et al.* Cryo-EM structure of a eukaryotic zinc transporter at a low pH suggests its Zn(2+)-releasing mechanism. *J Struct Biol* **215**, 107926 (2023).

15 Bui, H. B. *et al.* Cryo-EM structures of human zinc transporter ZnT7 reveal the mechanism of Zn(2+) uptake into the Golgi apparatus. *Nat Commun* **14**, 4770 (2023).

16 Lu, M. & Fu, D. Structure of the zinc transporter YiiP. *Science* **317**, 1746-1748 (2007).

17 Lu, Y. J., Liu, Y. C., Lin, M. C., Chen, Y. T. & Lin, L. Y. Coordinative modulation of human zinc transporter 2 gene expression through active and suppressive regulators. *J*

Nutr Biochem **26**, 351-359 (2015).

18 Wiuf, A. *et al.* The two-domain elevator-type mechanism of zinc-transporting ZIP proteins. *Sci Adv* **8**, eabn4331 (2022).

19 Zhang, Y. *et al.* Structural insights into the elevator-type transport mechanism of a bacterial ZIP metal transporter. *Nat Commun* **14**, 385 (2023).

20 Pang, C., Chai, J., Zhu, P., Shanklin, J. & Liu, Q. Structural mechanism of intracellular autoregulation of zinc uptake in ZIP transporters. *Nat Commun* **14**, 3404 (2023).

21 Zhang, T. *et al.* Crystal structures of a ZIP zinc transporter reveal a binuclear metal center in the transport pathway. *Sci Adv* **3**, e1700344 (2017).

22 Zhang, T., Sui, D., Zhang, C., Cole, L. & Hu, J. Asymmetric functions of a binuclear metal center within the transport pathway of a human zinc transporter ZIP4. *FASEB J* **34**, 237-247 (2020).

23 Gaither, L. A. & Eide, D. J. Functional expression of the human hZIP2 zinc transporter. *J Biol Chem* **275**, 5560-5564 (2000).

24 Girijashanker, K. *et al.* Slc39a14 gene encodes ZIP14, a metal/bicarbonate symporter: similarities to the ZIP8 transporter. *Mol Pharmacol* **73**, 1413-1423 (2008).

25 Hoch, E., Levy, M., Hershfinkel, M. & Sekler, I. Elucidating the H(+) coupled Zn(2+) transport mechanism of ZIP4; implications in acrodermatitis enteropathica. *Int J Mol Sci* **21**, 734 (2020).

26 Gupta, S., Merriman, C., Petzold, C. J., Ralston, C. Y. & Fu, D. Water molecules mediate zinc mobility in the bacterial zinc diffusion channel ZIPB. *J Biol Chem* **294**, 13327-13335 (2019).

27. Wang, Y. *et al.* The cellular economy of the Saccharomyces cerevisiae zinc proteome. *Metallomics* **10**, 1755-1776 (2018).

28. Bird, A. J. & Wilson, S. Zinc homeostasis in the secretory pathway in yeast. *Curr Opin Chem Biol* **55**, 145-150 (2020).

29 Suzuki, T. *et al.* Two different zinc transport complexes of cation diffusion facilitator proteins localized in the secretory pathway operate to activate alkaline phosphatases in vertebrate cells. *J Biol Chem* **280**, 30956-30962 (2005).

30 Fukunaka, A. *et al.* Demonstration and characterization of the heterodimerization of ZnT5 and ZnT6 in the early secretory pathway. *J Biol Chem* **284**, 30798-30806 (2009).

31 Ellis, C. D. *et al.* Zinc and the Msc2 zinc transporter protein are required for endoplasmic reticulum function. *J Cell Biol* **166**, 325-335 (2004).

32 Ellis, C. D., Macdiarmid, C. W. & Eide, D. J. Heteromeric protein complexes mediate zinc transport into the secretory pathway of eukaryotic cells. *J Biol Chem* **280**, 28811-28818 (2005).

33 Fang, Y. *et al.* Cation diffusion facilitator Cis4 is implicated in Golgi membrane trafficking via regulating zinc homeostasis in fission yeast. *Mol Biol Cell* **19**, 1295-1303 (2008).

34 Choi, S., Hu, Y. M., Corkins, M. E., Palmer, A. E. & Bird, A. J. Zinc transporters belonging to the Cation Diffusion Facilitator (CDF) family have complementary roles in transporting zinc out of the cytosol. *PLoS Genet* **14**, e1007262 (2018).

35 Kowada, T. *et al.* Quantitative imaging of Labile Zn(2+) in the Golgi apparatus using a localizable small-molecule fluorescent probe. *Cell Chem Biol* **27**, 1521-1531 e1528 (2020).

36 Amagai, Y. *et al.* Zinc homeostasis governed by Golgi-resident ZnT family members regulates ERp44-mediated proteostasis at the ER-Golgi interface. *Nat Commun* **14**, 2683 (2023).

37 Lieberwirth, J. K. *et al.* Bi-allelic loss of function variants in SLC30A5 as cause of perinatal lethal cardiomyopathy. *Eur J Hum Genet* **29**, 808-815 (2021).

38 Penon-Portmann, M. *et al.* De novo heterozygous variants in SLC30A7 are a candidate cause for Joubert syndrome. *Am J Med Genet A* **188**, 2360-2366 (2022).

39 Huang, L. *et al.* Identification of novel compound heterozygous variants in the SLC30A7 (ZNT7) gene in two French brothers with stunted growth, testicular hypoplasia, and bone marrow failure. *Hum Mol Genet* 2016-2031 (2023).

40 Watanabe, S. *et al.* Zinc regulates ERp44-dependent protein quality control in the early secretory pathway. *Nat Commun* **10**, 603 (2019).

41 Saito, Y., Ihara, Y., Leach, M. R., Cohen-Doyle, M. F. & Williams, D. B. Calreticulin functions in vitro as a molecular chaperone for both glycosylated and non-glycosylated proteins. *Embo J* **18**, 6718-6729 (1999).

42 Ohashi, W. *et al.* Zinc transporter SLC39A7/ZIP7 promotes intestinal epithelial self-renewal by resolving ER stress. *PLoS Genet* **12**, e1006349 (2016).

43 Bin, B. H. *et al.* Requirement of Zinc transporter SLC39A7/ZIP7 for dermal development to fine-tune endoplasmic reticulum function by regulating protein disulfide isomerase. *J Invest Dermatol* **137**, 1682-1691 (2017).

44 Woodruff, G. *et al.* The zinc transporter SLC39A7 (ZIP7) is essential for regulation of cytosolic zinc levels. *Mol Pharmacol* **94**, 1092-1100 (2018).

45 Anzilotti, C. *et al.* An essential role for the Zn(2+) transporter ZIP7 in B cell development. *Nat Immunol* **20**, 350-361 (2019).

46 Ishihara, K. *et al.* Zinc transport complexes contribute to the homeostatic maintenance of secretory pathway function in vertebrate cells. *J Biol Chem* **281**, 17743-17750 (2006).

47 Kinoshita, T. Biosynthesis and biology of mammalian GPI-anchored proteins. *Open Biol* **10**, 190290 (2020).

48 Wagatsuma, T. *et al.* Zinc transport via ZNT5-6 and ZNT7 is critical for cell surface glycosylphosphatidylinositol-anchored protein expression. *J Biol Chem* **298**, 102011 (2022).

49 Galperin, M. Y. & Jedrzejas, M. J. Conserved core structure and active site residues in alkaline phosphatase superfamily enzymes. *Proteins* **45**, 318-324 (2001).

50 Mann, K. J. & Sevlever, D. 1,10-Phenanthroline inhibits glycosylphosphatidylinositol anchoring by preventing phosphoethanolamine addition to glycosylphosphatidylinositol anchor precursors. *Biochemistry* **40**, 1205-1213 (2001).

51 Nakano, Y. *et al.* Biogenesis of GPI-anchored proteins is essential for surface expression of sodium channels in zebrafish Rohon-Beard neurons to respond to mechanosensory stimulation. *Development* **137**, 1689-1698 (2010).

52 Schuchman, E. H. Acid sphingomyelinase, cell membranes and human disease: lessons from Niemann-Pick disease. *FEBS Lett* **584**, 1895-1900 (2010).

53 Ueda, S. *et al.* Early secretory pathway-resident Zn transporter proteins contribute to cellular sphingolipid metabolism through activation of sphingomyelin phosphodiesterase 1. *Am J Physiol Cell Physiol* **322**, C948-C959 (2022).

54 Yamaguchi, Y. & Hearing, V. J. Melanocytes and their diseases. *Cold Spring Harb Perspect Med* **4**, a017046 (2014).

55 Solano, F. On the metal cofactor in the tyrosinase family. *Int J Mol Sci* **19**, 633 (2018).

56 Lai, X., Wichers, H. J., Soler-Lopez, M. & Dijkstra, B. W. Structure and function of human tyrosinase and tyrosinase-related proteins. *Chemistry* **24**, 47-55 (2018).

57 Aguilera, F., McDougall, C. & Degnan, B. M. Origin, evolution and classification of type-3 copper proteins: lineage-specific gene expansions and losses across the Metazoa. *BMC Evol Biol* **13**, 96 (2013).

58 Decker, H. & Tuczek, F. The recent crystal structure of human tyrosinase related protein 1 (HsTYRP1) solves an old problem and poses a new one. *Angew Chem Int Ed Engl* **56**, 14352-14354 (2017).

59 Petris, M. J., Strausak, D. & Mercer, J. F. The Menkes copper transporter is required for the activation of tyrosinase. *Hum Mol Genet* **9**, 2845-2851 (2000).

60 Wagatsuma, T. *et al.* Pigmentation and TYRP1 expression are mediated by zinc through the early secretory pathway-resident ZNT proteins. *Commun Biol* **6**, 403 (2023).

61 Murisier, F. & Beermann, F. Genetics of pigment cells: lessons from the tyrosinase gene family. *Histol Histopathol* **21**, 567-578 (2006).

62 Puckett, E. E. *et al.* Genetic architecture and evolution of color variation in American black bears. *Curr Biol* **33**, 86-97 e10 (2023).

63 Li, J. *et al.* A missense mutation in TYRP1 causes the chocolate plumage color in

chicken and alters melanosome structure. *Pigment Cell Melanoma Res* **32**, 381-390 (2019).

64 Braasch, I., Liedtke, D., Volff, J. N. & Schartl, M. Pigmentary function and evolution of tyrp1 gene duplicates in fish. *Pigment Cell Melanoma Res* **22**, 839-850 (2009).

65 Brown, A. R. *et al.* A community-science approach identifies genetic variants associated with three color morphs in ball pythons (Python regius). *PLoS One* **17**, e0276376 (2022).

66 Lai, X., Wichers, H. J., Soler-Lopez, M. & Dijkstra, B. W. Structure of human tyrosinase related protein 1 reveals a binuclear zinc active site important for melanogenesis. *Angew Chem Int Ed Engl* **56**, 9812-9815 (2017).

67 Lerner, A. B., Fitzpatrick, T. B., Calkins, E. & Summerson, W. H. Mammalian tyrosinase; the relationship of copper to enzymatic activity. *J Biol Chem* **187**, 793-802 (1950).

68 Allen, T. H. & Bodine, J. H. Enzymes in ontogenesis (Orthoptera). Xvii. The importance of copper for protyrosinase. *Science* **94**, 443-444 (1941).

69 Supuran, C. T. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. *Nat Rev Drug Discov* **7**, 168-181 (2008).

70 Keilin, D. & Mann, T. Carbonic anhydrase. *Nature* **144**, 442-443 (1939).

71 Swietach, P., Hulikova, A., Vaughan-Jones, R. D. & Harris, A. L. New insights into the physiological role of carbonic anhydrase IX in tumour pH regulation. *Oncogene* **29**, 6509-6521 (2010).

72 Neri, D. & Supuran, C. T. Interfering with pH regulation in tumours as a therapeutic strategy. *Nat Rev Drug Discov* **10**, 767-777 (2011).

73 Tsuji, T. *et al.* Dissecting the process of activation of cancer-promoting zinc-requiring ectoenzymes by zinc metalation mediated by ZNT transporters. *J Biol Chem* **292**, 2159-2173 (2017).

74 Fukunaka, A. *et al.* 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 (2011).

75 Suzuki, E. *et al.* Detailed analyses of the crucial functions of Zn transporter proteins in alkaline phosphatase activation. *J Biol Chem* **295**, 5669-5684 (2020).

76 Takeda, T. A. *et al.* Zinc deficiency causes delayed ATP clearance and adenosine generation in rats and cell culture models. *Commun Biol* **1**, 113 (2018).

77 Haase, H. & Rink, L. Zinc signals and immune function. *Biofactors* **40**, 27-40 (2014).

78 Kawamura, T. *et al.* Severe dermatitis with loss of epidermal Langerhans cells in human and mouse zinc deficiency. *J Clin Invest* **122**, 722-732 (2012).

79 Antonioli, L., Blandizzi, C., Pacher, P. & Hasko, G. Immunity, inflammation and cancer: a leading role for adenosine. *Nat Rev Cancer* **13**, 842-857 (2013).

80 Cekic, C. & Linden, J. Purinergic regulation of the immune system. *Nat Rev Immunol* **16**, 177-192 (2016).

81 Nolin, E. *et al.* Discovery of a ZIP7 inhibitor from a Notch pathway screen. *Nat Chem Biol* **15**, 179-188 (2019).

82 Pujol-Gimenez, J. *et al.* Inhibitors of human divalent metal transporters DMT1 (SLC11A2) and ZIP8 (SLC39A8) from a GDB-17 fragment library. *ChemMedChem* **16**, 3306-3314 (2021).

83. Hessels, A. M. *et al.* eZinCh-2: A Versatile, Genetically Encoded FRET Sensor for Cytosolic and Intraorganelle Zn(2+) Imaging. *ACS Chem Biol* **10**, 2126-2134 (2015).

84. Hessels, A. M., Taylor, K. M. & Merkx, M. Monitoring cytosolic and ER Zn(2+) in stimulated breast cancer cells using genetically encoded FRET sensors. *Metallomics* **8**, 211-217 (2016).

85. Liu, R. *et al.* Organelle-Level Labile Zn(2+) Mapping Based on Targetable Fluorescent Sensors. *ACS Sens* **7**, 748-757 (2022).

Figure legends

Figure 1. Subcellular localization of ZNT and ZIP proteins. A. Most ZIPs function to transport extracellular Zn^{2+} into the cytosol, whereas most ZNTs function to mobilize cytosolic Zn^{2+} into the lumen of intracellular compartments, including the endoplasmic reticulum (ER), Golgi apparatus, trans-Golgi network (TGN), and endosomes, as well as synaptic vesicles, secretory vesicles, and insulin granules in specialized cells. ZNT10 is not shown because it functions as a Mn^{2+} transporter. **B.** ZNTs transport Zn^{2+} in a rocker-switch manner, whereas ZIPs use an elevator-type mechanism.

Figure 2. ZNT5-6 and ZNT7 contribute to the stabilization and metalation of Zn^{2+} enzymes in the early secretory pathway. ZNT5-6 and ZNT7 function to transport Zn^{2+} into the lumen of the early secretory pathway compartments (ER and Golgi) to supply Zn^{2+} to *apo*-enzymes. *Apo*-enzymes are thus converted into *holo*-enzymes. This conversion is required for the activation and stabilization of a number of enzymes (listed in Table 1).

Figure 3. Proposed labile Zn^{2+} concentrations in subcellular compartments in cells. The labile Zn^{2+} concentration is maintained at a very low level within cells, although it may vary among different cell types and fluctuate in response to various stimuli. The measured concentrations, determined using a FRET-based sensor (eZinCh-2, highlighted in light green)[83,84] and a fluorescent type probe (ZnDA, highlighted in light yellow)[36,85], are indicated

as representative examples. Notably, vesicular Zn^{2+} concentrations, such as those found in synaptic vesicles and insulin granules (refer to Figure 1), known to accumulate high amounts of labile Zn^{2+}, are not shown. The omission is due to the ongoing need for further investigation to accurately determine their concentrations.

Figure 4. Phenotypes of medaka fish (*Oryzias latipes*) with disrupted *Znt5* and/or *Znt7*. A. Abnormal tactile phenotype in medaka fish. A mechanosensory stimulation induced swimming away in WT, *Znt5*$^{+/-}$;*Znt7*$^{+/-}$, and *Znt5*$^{+/-}$;*Znt7*$^{-/-}$ (three upper rows), but not in *Znt5*$^{-/-}$;*Znt7*$^{+/-}$ medaka. *Znt5*$^{-/-}$;*Znt7*$^{+/-}$ medaka did not respond to touch (for 0–3 s). The figure shown in this panel was taken from reference (48). **B.** Znt5-6 and Znt7 are required for melanogenesis in medaka fish. Lateral views of *Znt5*$^{-/-}$;*Znt7*$^{-/-}$ embryos at 8–9 days post-fertilization (left) and of *Znt5*$^{+/-}$;*Znt7*$^{-/-}$ (right) embryos before hatching (upper panels). Melanin content was decreased in *Znt5*$^{-/-}$;*Znt7*$^{-/-}$medaka compared to that in *Znt5*$^{+/-}$;*Znt7*$^{-/-}$ littermates (lower panel). The images in these panels were taken from reference (60).

Figure 5. Zn^{2+} enzymes involved in extracellular adenine nucleotide metabolism. ATP released extracellularly is hydrolyzed to ADP, AMP, and adenosine by several enzymes. Among them, TNAP, CD73/NT5E, and ENPPs (ENPP1 and ENPP3) are Zn^{2+} enzymes. Extracellular ATP and ADP bind to ionotropic P2X and metabotropic P2Y receptors and adenosine binds to P1 receptors to transmit signals that have opposing effects (inflammation *vs.* anti-inflammation).

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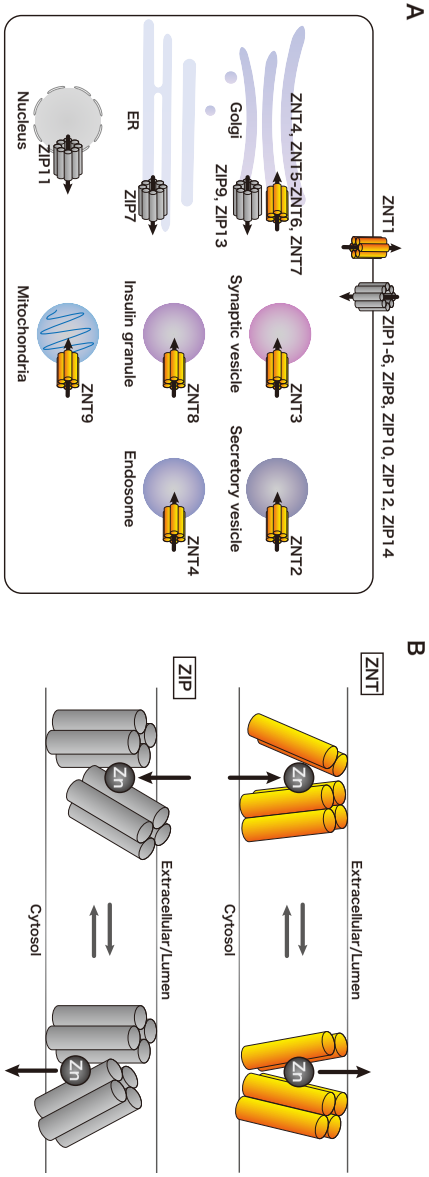
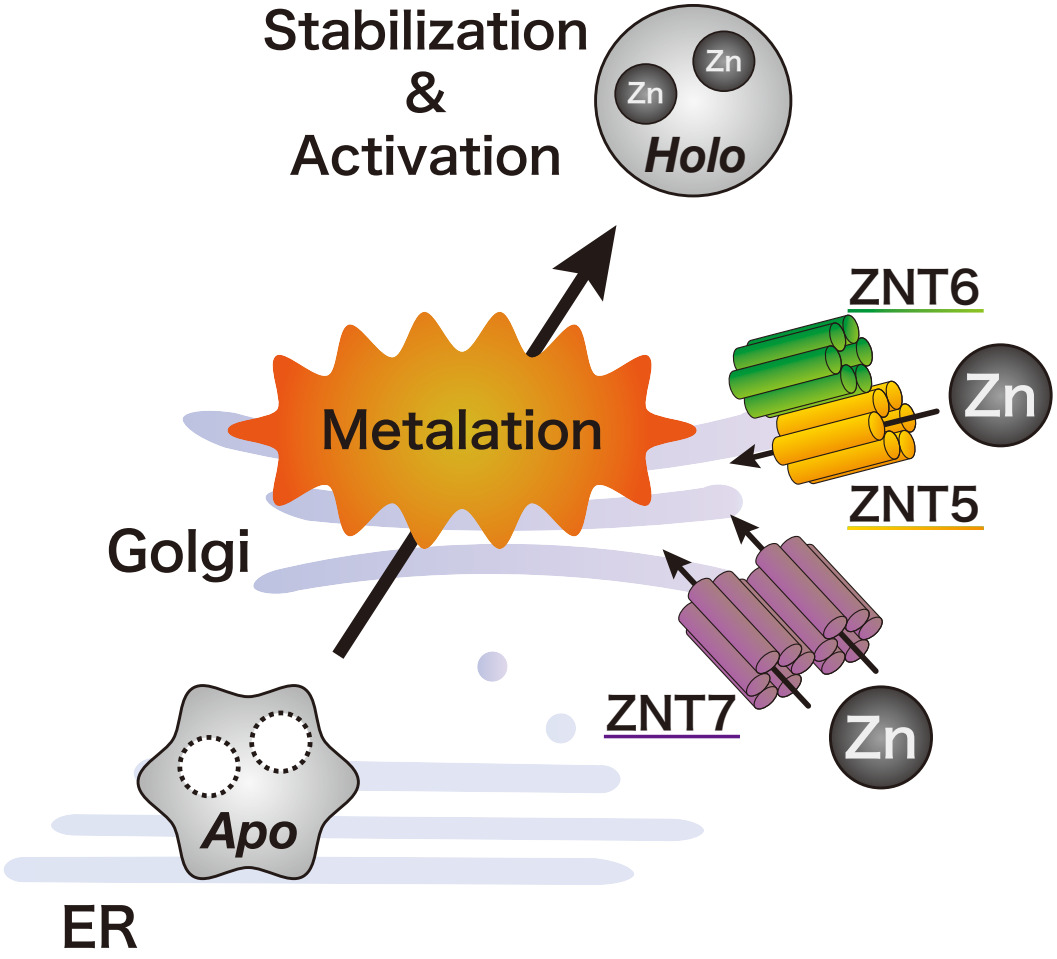


Fig. 1



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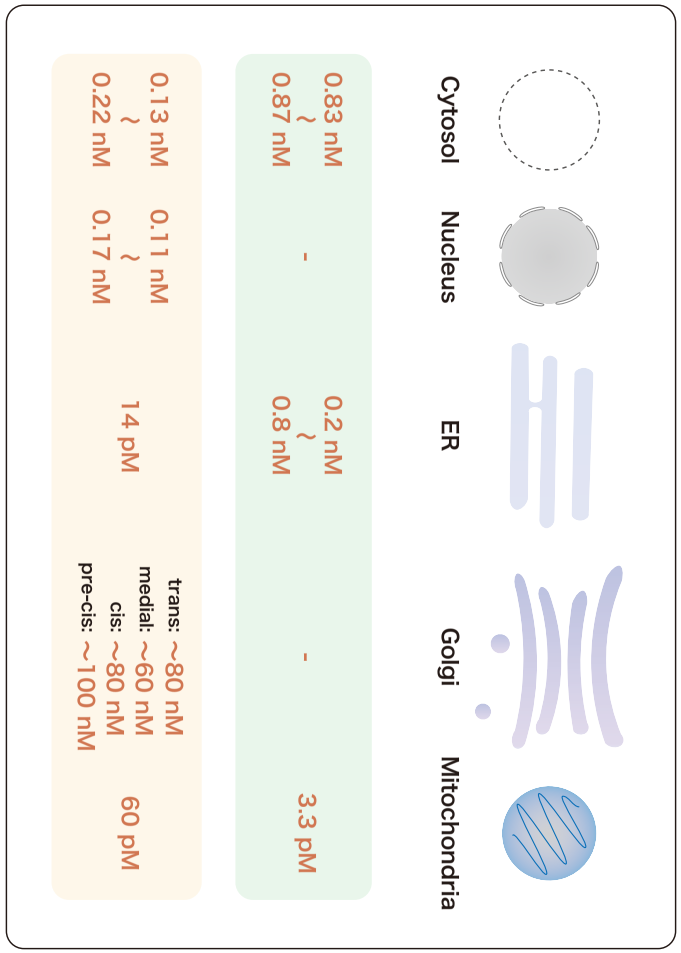

Fig. 3

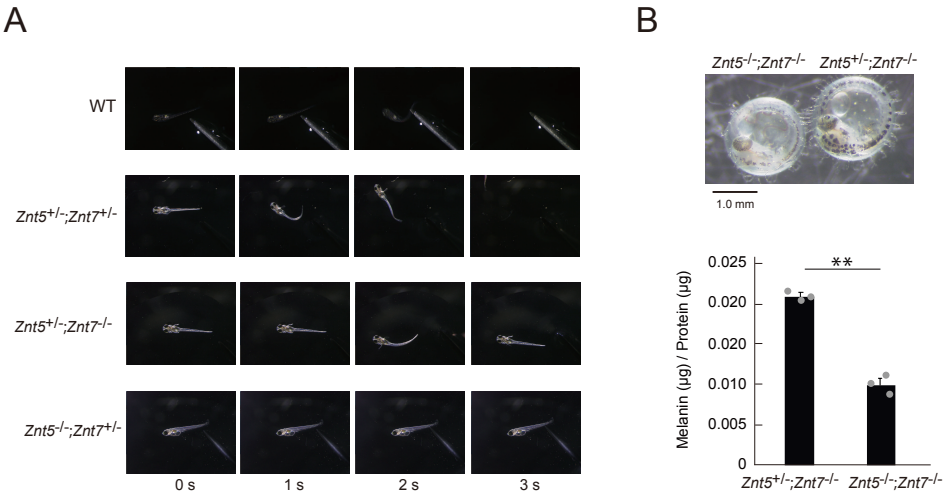

Fig. 4

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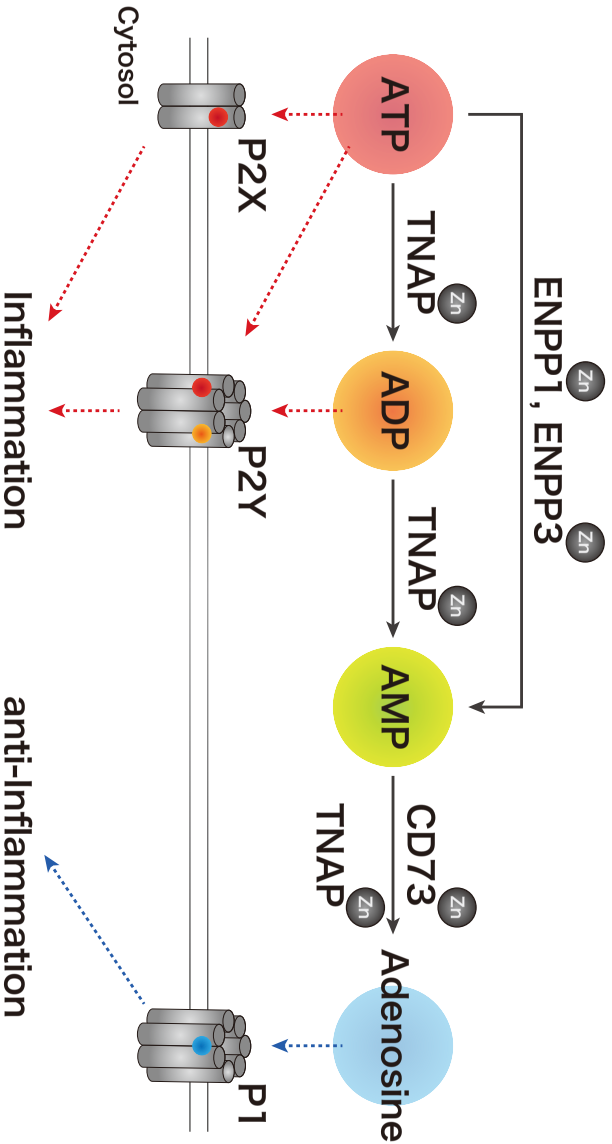

Fig. 5