

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

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Running title: Metalation and activation of Zn^{2+} enzymes via ZNTs

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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 (Zn²⁺) 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, Zn²⁺ 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 (Fe²⁺↔Fe³⁺) and copper (Cu⁺↔Cu²⁺).² Therefore, Zn²⁺ functions as an essential component in numerous proteins/enzymes. Approximately 10% of the proteins encoded by the human genome can bind to Zn²⁺, suggesting that it is involved in a wide range of physiological functions.³

In humans, numerous Zn²⁺ 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 Zn²⁺; 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 Zn²⁺ enzymes is regulated by Zn²⁺ transporters belonging to the Zn transporter (ZNT) family located in early secretory pathway compartments. In this review, we summarize the Zn²⁺ enzymes that require Zn²⁺ 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.

Zn²⁺ TRANSPORTERS

The influx and efflux of Zn²⁺ into cells are cooperatively and intricately controlled by 24 Zn²⁺ 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 Zn²⁺ levels by transporting Zn²⁺ into intracellular compartments or the extracellular space, whereas ZIPs are influx transporters that elevate cytosolic Zn²⁺ levels by mobilizing Zn²⁺ into the cytosol from the extracellular space or intracellular compartments. In humans, 14 ZIPs and 10 ZNTs function in systemic, cellular, and subcellular Zn²⁺ homeostasis (although recent studies have shown that ZNT10 functions as a Mn²⁺ transporter), and mutations in half of these have been implicated in genetic disorders, the various phenotypes of which reflect the multifarious essential functions of Zn²⁺ 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 Zn²⁺/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 Zn²⁺/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 Zn²⁺-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 Zn²⁺ by an alternating access mechanism based on a two-domain elevator-type mechanism (Figure 1B).¹⁸⁻²⁰ They harbor two Zn²⁺ binding sites (M1 and M2) in their transmembrane domains; M1 is essential for Zn²⁺ transport, whereas M2 is auxiliary.^{21,22} In contrast to that of ZNTs, the molecular mechanism of ZIPs has not yet been clarified, although Zn²⁺/HCO₃⁻^{23,24} and Zn²⁺/H⁺ symport²⁵ or water-mediated transport²⁶ mechanisms have been proposed. ZIPs may have diverse Zn²⁺ transport mechanisms determined by the local charge around Zn²⁺-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 Zn²⁺ 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 Golgi⁴⁰ 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 Zn²⁺ 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 Zn²⁺ ions, which are likely coordinated at the active site.⁶⁶ Our study was the first to clearly demonstrate that Zn²⁺ 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 Zn²⁺ enzyme, CAIX, and secretory Zn²⁺ 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 Zn²⁺ 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 Zn²⁺ metalation via ZNT5-6 and ZNT7 and, uniquely, via ZNT4 in the early secretory pathway.⁷³ The detailed mechanisms underlying Zn²⁺ acquisition, including how ZNT5-6, ZNT7, and ZNT4 are used differently, have not yet been clarified.

In addition to CAIX, the Zn²⁺ 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 Zn²⁺ via routes other than the ZNT complexes. As MMP2, MMP9, and ATX are involved in tumor malignancy, Zn²⁺ supply via both complexes may serve as a therapeutic target for anticancer drugs.

Zn²⁺ 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 ZIP7⁸¹ and ZIP8⁸² 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.

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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.

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Figure legends

Figure 1. Subcellular localization of ZNT and ZIP proteins. A. Most ZIPs function to transport extracellular Zn²⁺ into the cytosol, whereas most ZNTs function to mobilize cytosolic Zn²⁺ 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 Mn²⁺ transporter. B. ZNTs transport Zn²⁺ 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 Zn²⁺ enzymes in the early secretory pathway. ZNT5-6 and ZNT7 function to transport Zn²⁺ into the lumen of the early secretory pathway compartments (ER and Golgi) to supply Zn²⁺ 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 Zn²⁺ concentrations in subcellular compartments in cells. The labile Zn²⁺ 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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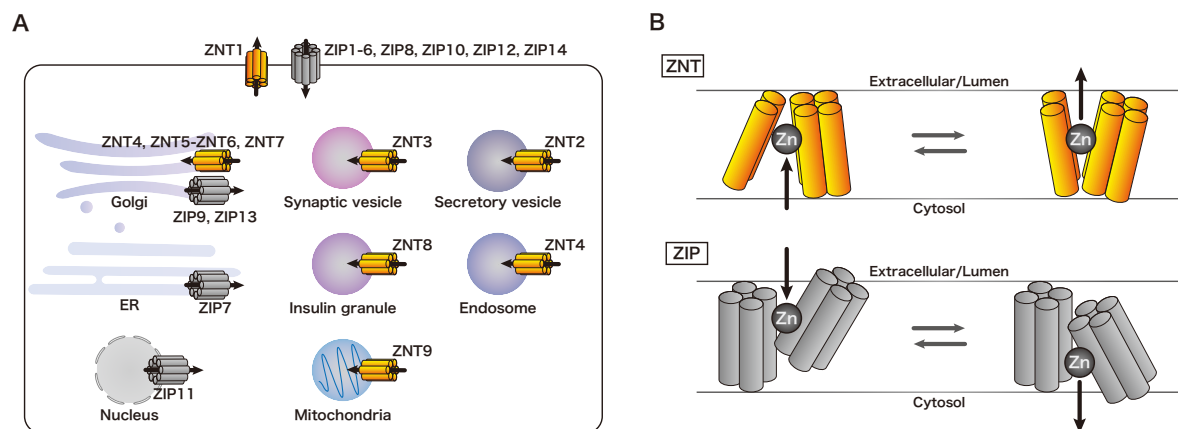


Fig. 1

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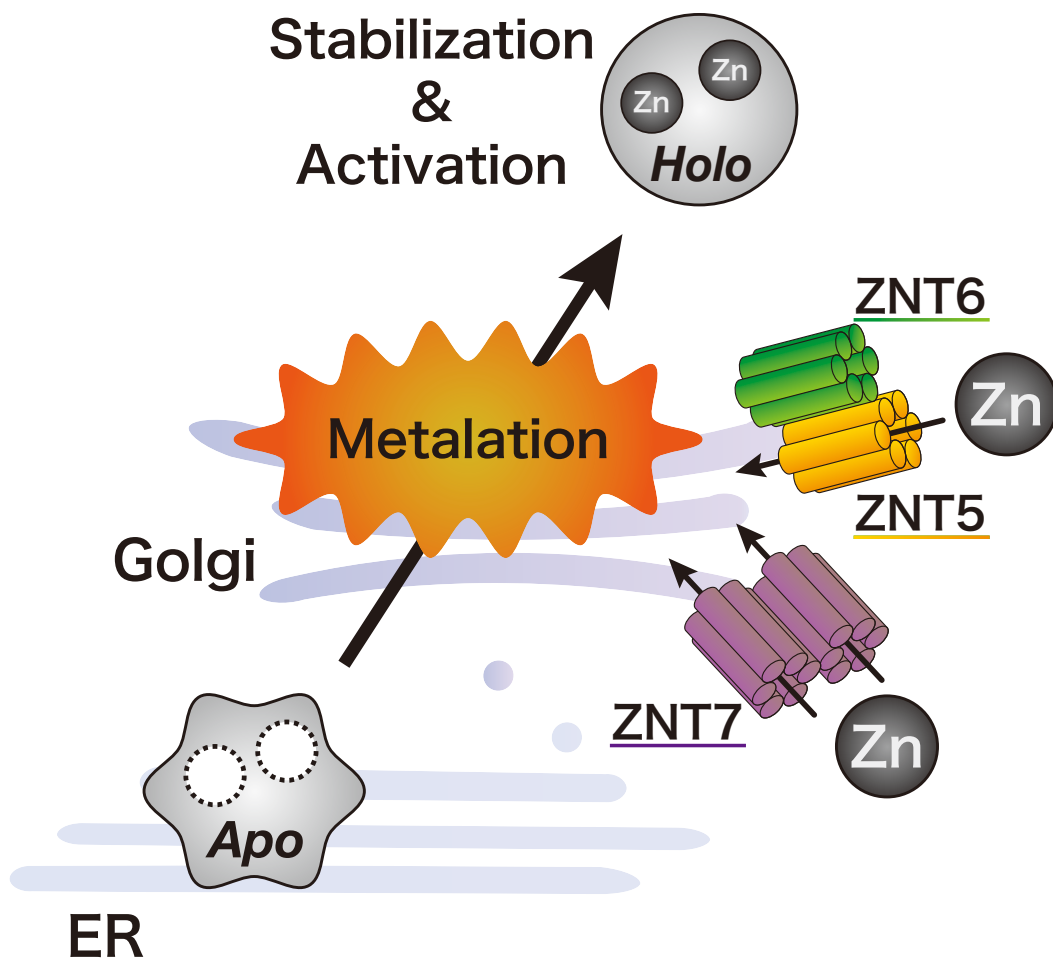


Fig. 2

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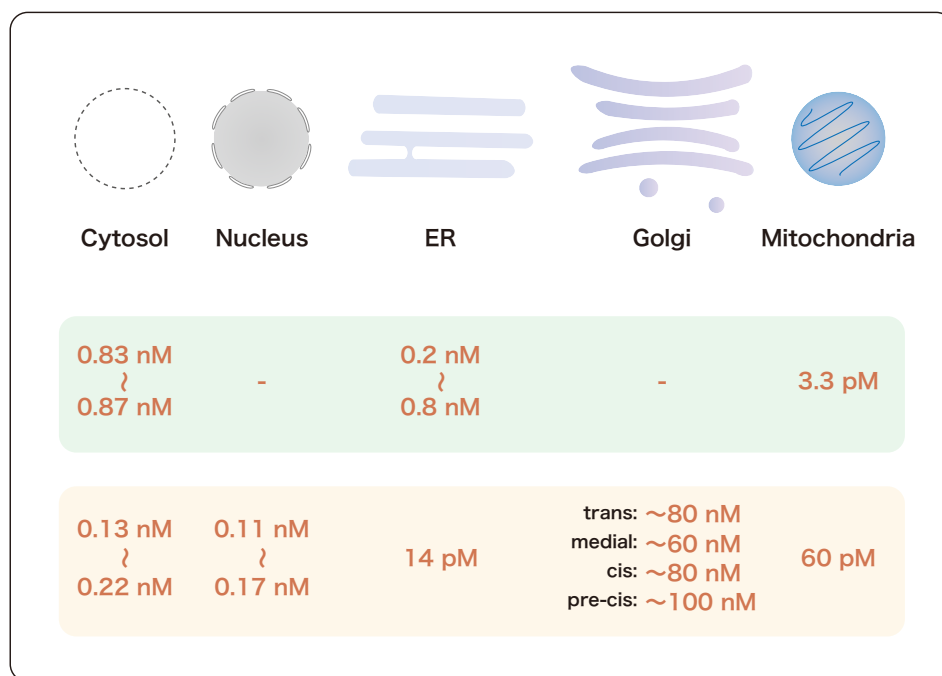


Fig. 3

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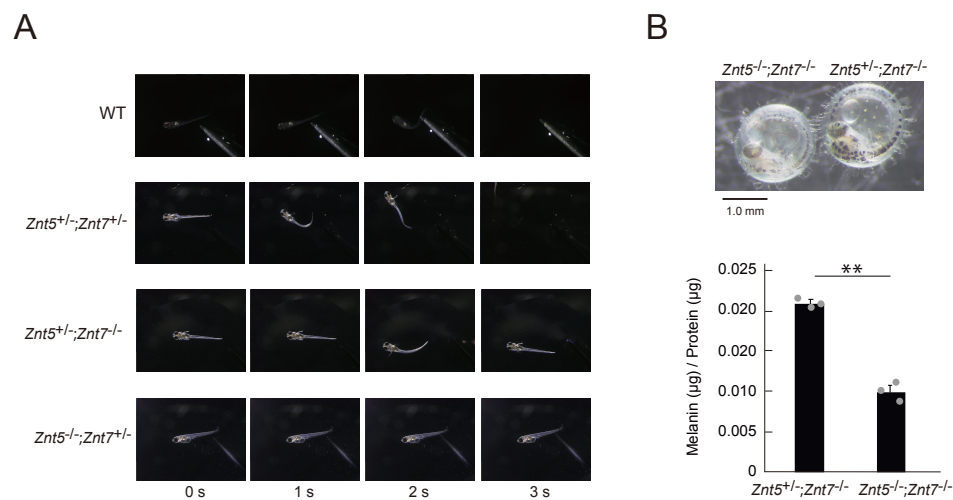


Fig. 4

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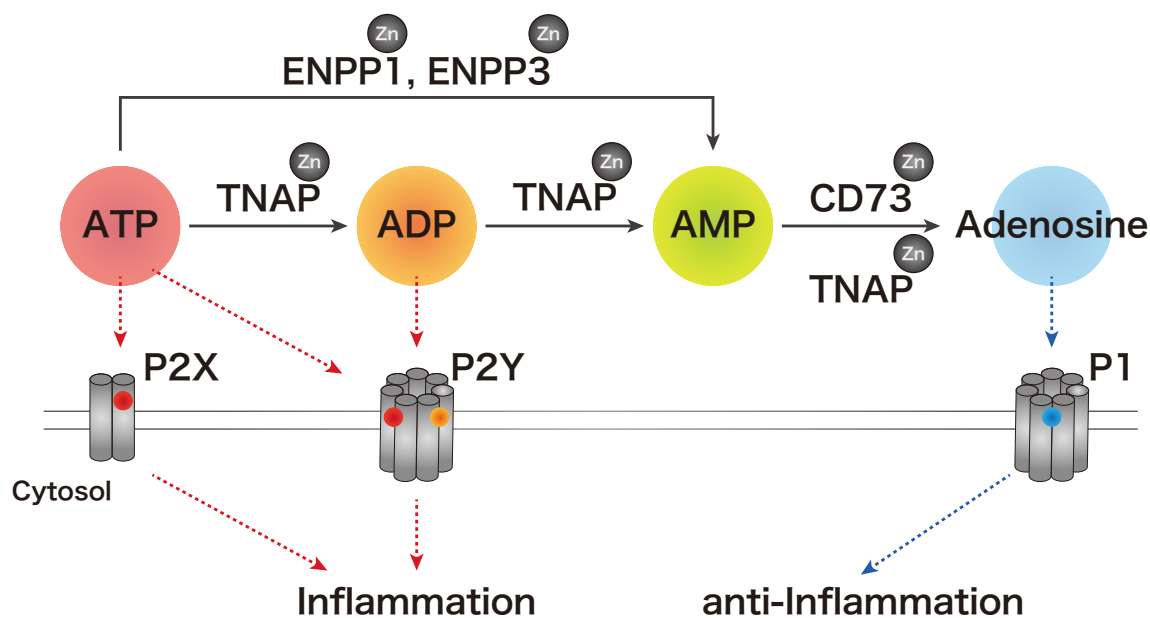


Fig. 5