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Metalation and maturation of zinc ectoenzymes: A perspective

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Abstract

Numerous zinc ectoenzymes are folded and activated in the compartments of the early secretory pathway, such as the ER and the Golgi apparatus, before reaching their final destination. During this process, zinc must be incorporated into the active site; therefore, metalation of the nascent protein is indispensable for the expression of the active enzyme. However, to date, the molecular mechanism underlying this process has been poorly investigated. This is in sharp contrast to the physiological and pathophysiological roles of zinc ectoenzymes, which have been extensively investigated over the past decades. This manuscript concisely outlines the present understanding of zinc ectoenzyme activation through metalation by zinc and compares this with copper ectoenzyme activation in which elaborate copper metalation mechanisms are known. Moreover, based on the comparison, several hypotheses are discussed. Approximately 80 years have passed since the first zinc enzyme was identified; therefore, it is necessary to improve our

understanding of zinc ectoenzymes from a biochemical perspective, which will further our understanding of their biological roles.

Introduction

It has been approximately 80 years since carbonic anhydrase was first identified as a zinc enzyme [1]. Since then, significant progress has been made in understanding the physiological roles of zinc enzymes and their involvement in pathogenesis. About 10% of the human proteome is estimated to contain the zinc-binding motif [2, 3], with approximately one-third of cellular proteins targeted to the ER and thus to the Golgi apparatus in eukaryotic cells [4, 5]. Therefore, a considerable number of proteins are maturated by incorporation of zinc during the secretory process before they reach their final destination. Significant parts of these proteins are zinc ectoenzymes [6-8], which need the zinc ion as an essential catalytic component, and include compartment-resident, membrane-bound, and secretory enzymes, which have recently received much attention owing to their important physiological roles and their potential as therapeutic targets [7, 9-11]. Zinc ectoenzyme representatives are alkaline phosphatases (ALPs) [12, 13], matrix metalloproteinases (MMPs) [7], angiotensin-converting enzymes [14], endothelin-converting enzymes [15], ADAM (A Disintegrin and Metallo-proteinase) family proteins [16], ecto-5′ -nucleotidase (NT5E, also known as CD73) [17, 18], ectonucleotide pyrophosphatase/phosphodiesterases (ENPPs) including Autotaxin [19-21], and secretory/membrane-bound carbonic anhydrases (CAs) [22], which have one or two zinc ions at their active sites (Table 1). An important question regarding zinc ectoenzymes remains, and that is how is their activation, which is achieved by zinc metalation, regulated? Here, an overview of the molecular evidence and hypothesis regarding this fundamental question is provided in a bid to understand zinc ectoenzyme regulation.

Cellular copper homeostasis and copper ectoenzyme activation

Before discussing their zinc counterparts and to allow comparison, the activation and metalation of copper ectoenzymes are briefly overviewed, as the molecular basis of copper metalation is better understood than that of zinc [23, 24].

Copper taken up into the cytosol from the extracellular space, which is mediated by the copper importer CTR1 (copper transporter 1), is transferred to copper chaperone proteins, such as CCS (copper chaperone for superoxide dismutase 1 (SOD1)), COX17 (copper chaperone for cytochrome c oxidase), and ATOX1 (antioxidant-1), that deliver cytosolic copper to the target proteins [23, 25] (Fig. 1). ATOX1 is pivotal for the delivery of copper to ectoenzymes as it transfers cytosolic copper to two ATPases, ATP7A and ATP7B, both of which transport it into the lumen of the *trans*-Golgi network [26]. ATP7A is ubiquitously expressed, while ATP7B is mostly expressed in hepatocytes. Both ATP7A and ATP7B are essential in supplying copper to a number of copper ectoenzymes involved in various physiological responses and pathogenesis. The representative copper ectoenzymes are lysyl oxidase (LOX) [27, 28], peptidylglycine-alpha-hydroxylating monooxygenase (PAM) [29, 30], tyrosinase [31-33], dopamine beta hydroxylase (DBH) [34, 35], and extracellular superoxide dismutase (ecSOD) [36]. During the transfer of copper from cytosolic ATOX1 to the luminal copper ectoenzymes via ATP7A and ATP7B, a copper-mediated intermolecular interaction is formed between ATOX1 and ATP7A/ATP7B [24, 37, 38], and between ATP7A/ATP7B and target copper ectoenzymes such as PAM [30] (Fig. 2A). However, indirect (non-ligand exchanged) copper transfer can be operative in the process of copper ectoenzyme metalation during direct association between them [36, 39]. The ligand exchange through the copper-mediated

intermolecular interaction imparts specificity to both copper and the target protein and also enables a faster exchange than a diffusion mechanism [24], thus ensuring the correct and defined coordination of copper to the transporters/proteins.

Cellular zinc homeostasis and zinc ectoenzyme activation

The size of the copper proteome is generally less than 0.5% [40]. In contrast, the zinc proteome is much larger and known to be about 10%, as described above. The following sections overview and discuss the activation and maturation of zinc ectoenzymes by dissecting the processes described above for copper ectoenzymes: 1) zinc mobilization across the membrane by zinc transporters, and 2) metalation within the ectoenzyme.

There are 23 zinc transporter proteins that maintain subcellular, cellular, and systemic zinc homeostasis by transporting zinc across biological membranes (reviewed in [41-44]), thereby contributing to many pivotal roles in various biological processes crucial to life. Transport of zinc ions (Zn^{2+}) across the membranes, in contrast to copper and iron ions, does not require a redox reaction due to its fully occupied d shell [45]. In copper transporters, solute carriers (SLCs) are responsible for copper uptake (CTR1 and CTR2), while ATPases function in its export (ATP7A and ATP7B). In contrast, both the zinc transporters, ZNT and ZIP, function as SLCs. ZNT proteins operate as H^+/Zn^{2+} antiporters for zinc efflux from the cytosol into the extracellular space or into the lumen of intracellular compartments [46, 47], while ZIP proteins transport zinc in the opposite direction, although their zinc transport mode has not been clearly determined. The H^+/Zn^{2+}

antiporter function of ZNT proteins is useful for zinc transport into the acidic environment of the lumen of intracellular compartments [48, 49].

Zinc taken up into the cytosol by ZIP proteins must be transferred to the lumen of the compartments of the early secretory pathway, such as the ER and Golgi apparatus, for zinc ectoenzyme activation. Therefore, a specific zinc entry route is required to cross biological membranes (Fig. 3). In vertebrate cells, two routes are present, consisting of ZNT5-ZNT6 heterodimers and ZNT7 homodimers. At present, the fundamental question of how cytosolic zinc is derivered to both ZNT complexes has not yet been answered. In copper metabolism, ATOX1 plays a pivotal role in cytosolic copper delivery to copper-transporting ATPases located in the *trans*-Golgi network, as described above. This kind of chaperone protein might operate for zinc delivery, because such a chaperone would enable specific and timely zinc delivery to ZNT complexes, as in the case of ATOX1. From this perspective, zinc may be transferred via ligand exchange through zinc-mediated protein-protein interactions without the release of zinc [50, 51] (Fig. 2B and C). Actually, zinc-mediated intermolecular interactions are formed within a number of protein complexes in cells [52-54].

Addition of excess zinc to the spent medium fails to activate the a*po*-forms of zinc ectoenzymes secreted from cells cultured under zinc-deficient conditions [55], indicating that zinc metalation in the compartments of the early secretory pathway is essential for zinc ectoenzyme activity. In the cell, cytosolic zinc is transported across the membrane to the lumen of these compartments by ZNT5-ZNT6 heterodimers and ZNT7 homodimers [56-58] (Fig. 3). Therefore, both ZNT transport complexes are pivotal for metalation and activation of zinc ectoenzymes [55, 59-61], although the direct comparison of the zinc content of zinc ectoenzymes biosynthesized in wild-type cells with that in the cells lacking both complexes has not been made. However, some insight

into this process is available. In cells lacking both ZNT complexes, the activities of TNAP, placental ALP (PLAP), NT5E, Autotaxin, and MMP9 decrease dramatically, as is the case in cells expressing the ectoenzymes under zinc-deficient conditions [55, 59-61]. However, there are significant differences between the two cases; ectoenzymes, except for Autotaxin, expressed in the former case are destabilized and degraded, but this does not occur in the latter (Table 1) [55, 59-61]. In contrast, CAIX, ENPP1 and ENPP3 retain their activity even when expressed in cells lacking both ZNT complexes (Table 1) [55, 61]. However, CAIX activity moderately decreases when ZNT4 homodimers are also absent (Table 1) [61]. ZNT4 is shown to be involved in the abundance of CAVI [62], suggesting its roles in the activation process in some zinc ectoenzymes. The differences in the dependence of ZNT proteins on the activation are not attributed to the number of zinc ions at their active sites (Table 1), suggesting that a sophisticated mechanism underlies zinc-mediated activation of zinc ectoenzymes.

Exactly how and when do zinc ectoenzymes acquire zinc at their active sites in the lumen of the compartments of the early secretory pathway? As described above, a number of zinc ectoenzymes are destabilized when expressed in cells lacking ZNT5-ZNT6 heterodimers and ZNT7 homodimers, which was restored by the expression of either complex, and also by the expression of their zinc transport incompetent mutants, suggesting that both ZNT complexes can stabilize several zinc ectoenzymes, in addition to their primary functions in zinc metalation. TNAP is rapidly degraded as an endo-H-sensitive form through ubiquitin-proteasomal and lysosomal degradation pathways in cells lacking ZNT5-ZNT6 heterodimers and ZNT7 homodimers [60, 63], which indicates that the TNAP activation process by ZNT complexes may be separated into two steps, the first being a stabilization step and the second a zinc-metalation step (Fig. 4). The molecular mechanism underlying this process remains almost entirely unknown; however, the

highly conserved Pro-Pro motif in luminal loop 2 between transmembrane helices III and IV of ZNT5 and ZNT7 (as ZNT5 has nine extra N-terminal transmembrane helices, the luminal loop 2 actually corresponds to its luminal loop 6 [64]) may be involved. This motif is not found in other ZNT proteins, which failed to activate TNAP even when overexpressed [65], and mutants of ZNT5 and ZNT7 in which Pro-Pro is substituted with Ala-Ala exhibit significantly decreased ability to activate TNAP, despite retaining their zinc transport activity [65]. Thus, a unique structural feature of the Pro-Pro motif may contribute to the activation of TNAP, potentially via a unique interaction. Interestingly, the situation is somewhat different in Autotaxin. Autotaxin is not degraded in cells lacking ZNT5-ZNT6 heterodimers and ZNT7 homodimers, although it is not active [61]. The Pro-Pro motif ZNT5 mutant moderately activates Autotaxin, which may suggest that motifs other than the Pro-Pro motif in ZNT5-ZNT6 heterodimer are involved in Autotaxin activation [61]. The stabilization of Autotaxin may be reminiscent of copper ectoenzymes such as PAM, ecSOD, and DBH [30, 36, 39], which are associated with ATP7A as described above. If applying the metalation mechanism of PAM by ATP7A [30], a challenging but attractive hypothesis is that zinc-mediated intermolecular interactions [50, 51] are formed between ZNT complexes and Autotaxin (Fig. 2B). Further investigation is required to determine this.

The absence of ZNT complexes exacerbates the unfolded protein response [63], thus disturbing the quality control mechanisms of the early secretory pathway such as the ER. This unfolded protein response could be due to an increase in misfolded *apo*-zinc ectoenzyme, or a decrease in the chaperone activity, which is essential for facilitating zinc metalation of nascent zinc ectoenzymes, as a number of chaperones function in the quality control of the ER and the secretory pathway, some of which are regulated by zinc [66, 67]. Consistent with these, zinc deficiency exacerbates unfolded protein response [63, 68].

Conclusions and perspectives

Numerous genetic and biochemical studies have elucidated the physiopathological significance of zinc ectoenzymes [11, 42, 43, 69, 70]. However, as described, numerous questions remain. One reason for this is that it is difficult to investigate zinc coordination using UV-visible absorption, nuclear magnetic resonance, or electron paramagnetic resonance spectroscopies, because of the filled d shell of the zinc ion. The development of zinc fluorescent probes and fluorescence resonance energy transfer sensors has contributed significantly to our understanding of zinc [71-73]; however, a new innovative approach needs to be developed and exploited to provide a new direction for zinc research. For zinc ectoenzyme activation, a detailed 'zinc-ome' analysis, which can distinguish between the *apo*- (not metalated) and *holo*-form (metalated) of zinc ectoenzymes, coupled with a genetic approach may significantly advance our understanding. This may potentially lead to the development of new therapeutic strategies, considering their association with physiopathology.

Table 1. Activation and stability of zinc ectoenzymes when expressed in cells lacking ZNT5-ZNT6 heterodimers and ZNT7 homodimers.

Ectoenzymes	Metalated zinc ions	Enzyme activity	Protein stability	Reference
TNAP	2	Inactive	unstable	57, 59, 60, 65, 74
NT5E	2	Inactive	unstable	55
Autotaxin	2	Inactive	stable	61
MMP9	1	Inactive	unstable	61
PLAP	2	Inactive	unstable	55, 59
ENPP1*	2	Active	stable	55
ENPP3*	2	Active	stable	55
CAIX**	1	Active	stable	61
CAVI***	1	N.A.	N.A	62

*The activity and protein expression do not decrease even when expressed in cells lacking all ZNT4 homodimers, ZNT5-ZNT6 heterodimers, and ZNT7 homodimers. **The activity and protein expression moderately decrease in cells lacking all ZNT4 homodimers, ZNT5-ZNT6 heterodimers, and ZNT7 homodimers. ***The activity moderately decreases in cells with reduced ZNT4 expression.

FIGURE LEGENDS

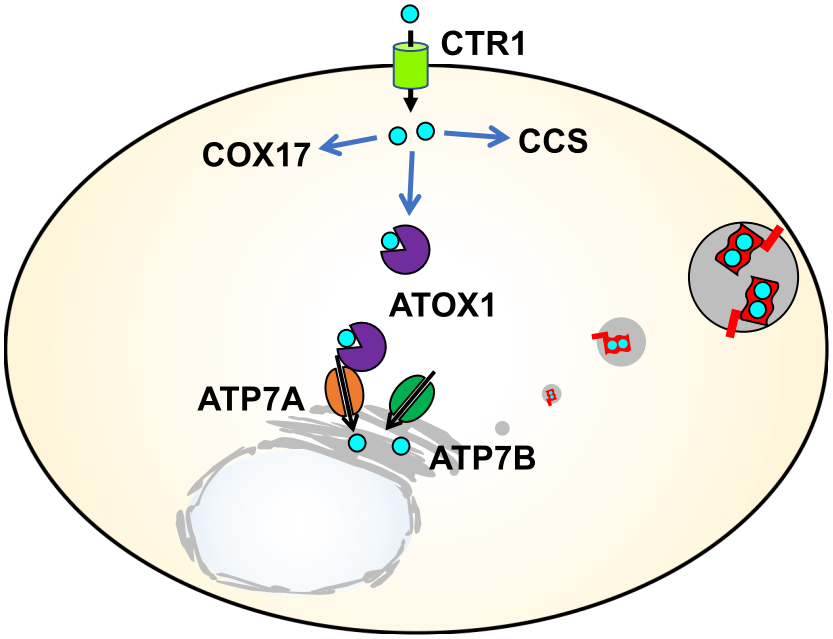

Figure 1. Simple model of cellular copper metabolism for copper ectoenzyme metalation and activation

Copper is taken up into the cytosol by CTR1 and then distributed to three cytosolic copper chaperones, COX17, CCS, and ATOX1. COX17 delivers copper to the mitochondrial inner membrane, and CCS delivers it to cytosolic SOD1 (not shown). ATOX1 delivers cytosolic copper to ATP7A and ATP7B, located in the *trans*-Golgi network, which then transports it to the lumen of the *trans*-Golgi network to metalate and activate copper ectoenzymes. In this cartoon, the *holo*-form of the copper ectoenzyme is trafficked and localized to the intracellular compartments as a monomer. Cu^{+} is represented by a light blue sphere.

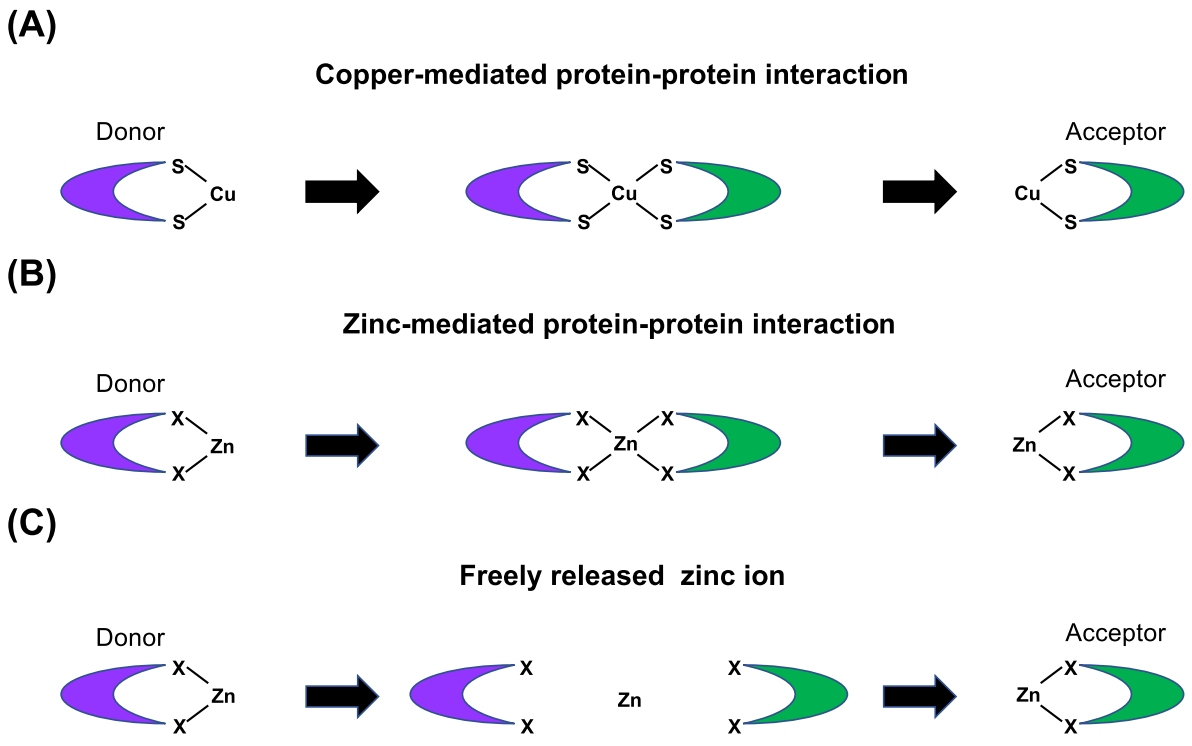

Figure 2. Model of copper transfer and hypothesis of zinc transfer from the donor to the acceptor proteins

(A). The ligand exchange mechanism in copper transfer. An intermolecular interaction via the copper ion (Cu^{+}) is formed during delivery. The combination of donor and acceptor proteins corresponds to that of ATOX1 and ATP7A/ATP7B, or that of ATP7A/ ATP7B and the copper ectoenzyme. Sulfur from the sulfhydryl group of cysteine is functional as a ligand for the copper ion in donor and acceptor proteins. (B). A hypothesis of the ligand exchange mechanism in zinc transfer. An intermolecular interaction via the zinc ion is formed between donor and acceptor proteins during delivery as in the case of copper. (C). Non-ligand-mediated zinc transfer. The zinc ion (Zn^{2+}) is freely released from the donor protein for delivery to the acceptor protein, in which an intermolecular interaction is not formed. In (B) and (C), X usually corresponds to sulfur from the sulfhydryl group of cysteine and nitrogen from the imidazole ring of histidine.

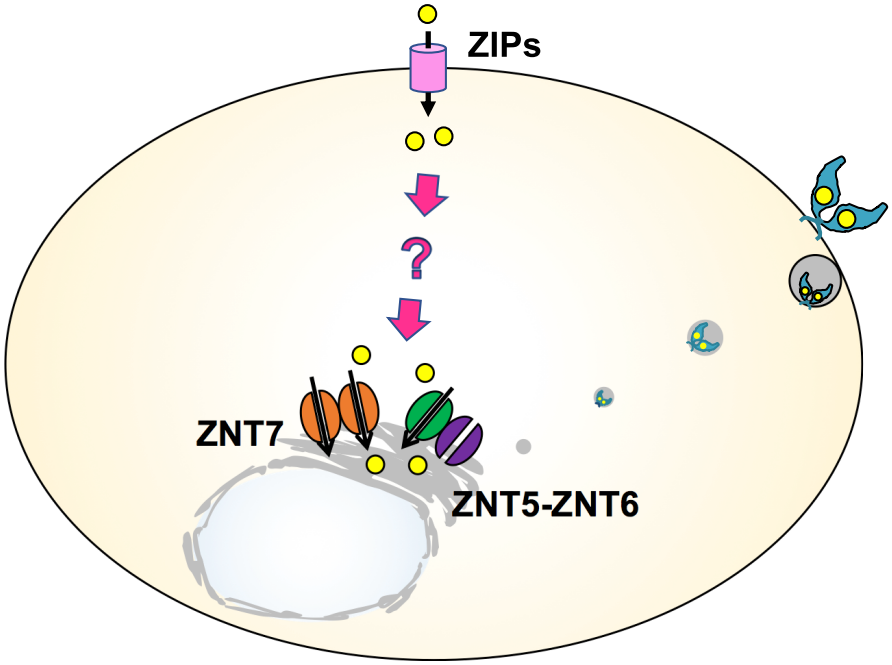

Figure 3. Simple model of cellular zinc metabolism for zinc ectoenzyme metalation and activation

Zinc is taken up by ZIP transporter proteins into the cytosol and then derivered to ZNT5-ZNT6 heterodimers and ZNT7 homodimers, both of which are located close to the compartments of the early secretory pathway, such as the ER and Golgi apparatus. Both ZNT complexes transport cytosolic zinc to the lumen of these compartments to metalate and activate zinc ectoenzymes. In ZNT5-ZNT6 heterodimers, ZNT6 does not possess zinc transport activity. Zinc transport activity is depicted by an arrow. Information regarding the molecular mechanism by which cytosolic zinc is derivered to both ZNT complexes is lacking. In this cartoon, the *holo*-form of a zinc ectoenzyme is trafficked and localized to the plasma membrane as a homodimer. Zn^{2+} is represented by a yellow sphere.

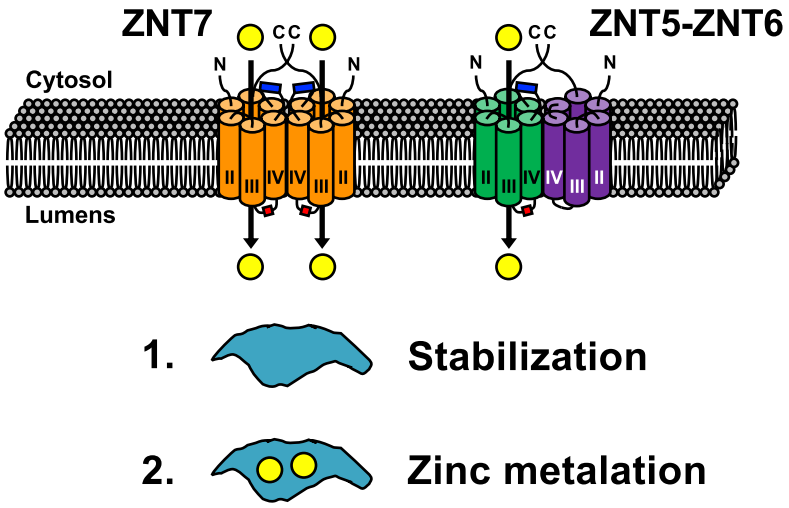

Figure 4. ZNT complexes contribute to both stabilization and metalation of zinc ectoenzymes in the compartments of the early secretory pathway

ZNT5-ZNT6 heterodimers and ZNT7 homodimers activate zinc ectoenzymes via two steps in the compartments of the early secretory pathway; first, stabilizing the ectoenzymes *apo*-form, and second, zinc metalation by transporting cytosolic zinc to the lumen, converting the enzyme to its *holo*-form. The Pro-Pro motif in luminal loop 2 between transmembrane helices III and IV is shown as a red square. The cytosolic histidine-rich loop is shown in blue square to discriminate the position of the Pro-Pro motif from it. The detailed molecular mechanism of this process remains unknown. Luminal loop 2 of ZNT5 actually corresponds to luminal loop 6, because of its long N-terminal portion with nine extra transmembrane helices (not shown). Zn^{2+} is represented by a yellow sphere.

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Notes

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Abbreviations

SLCs, solute carriers; ALPs, alkaline phosphatases; MMPs, matrix metalloproteinases; NT5E, ecto-5'-nucleotidase; ENPPs, ectonucleotide pyrophosphatase/phosphodiesterases; CAs, carbonic anhydrases; TNAP, tissue non-specific ALP; PLAP, placental ALP; CTR1, copper

transporter 1; SOD1; superoxide dismutase 1; CCS, copper chaperone for SOD1; COX17, copper chaperone for cytochrome c oxidase; ATOX1, antioxidant-1; ecSOD, extracellular superoxide dismutase; LOX, lysyl oxidase; PAM, peptidylglycine-alpha-hydroxylating monooxygenase.

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