Dissecting the Process of Activation of Cancer-promoting Zinc-requiring Ectoenzymes by Zinc Metalation Mediated by ZNT Transporters

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Zinc-binding proteins constitute ~10% of the human proteome (1). Zinc has been estimated to be bound by roughly 1000 enzymes (1), in which zinc plays indispensable roles as a structural component and a cofactor at the active site (2–4). Zinc-requiring enzymes are involved in a myriad of critical biological functions in the cytosol, the lumen of various organelles, and the extracellular space.

As compared with cytosolic zinc-requiring enzymes, secreted, membrane-bound, and organelle-resident zinc-requiring enzymes (hereafter “ectoenzymes”) require a distinct regulatory mechanism for activation because ectoenzymes are considered to capture zinc in the early secretory pathway, including in the endoplasmic reticulum and the Golgi apparatus, before trafficking to their final destination. Based on studies conducted using tissue-nonspecific alkaline phosphatase (TNAP), we have proposed a two-step mechanism of activation, in which TNAP is converted from the apo- to the holo-form in the early secretory pathway, through protein stabilization followed by enzyme activation by zinc. In this process, zinc must be mobilized into the lumen of the early secretory pathway organelles by zinc transporter 5 (ZNT5)-ZNT6 heterodimer or ZNT7 homodimers (5–8). However, the molecular basis of the activation process of other zinc-requiring ectoenzymes remains almost entirely unknown.

In addition to being studied for their crucial physiological functions, several zinc-requiring ectoenzymes have attracted research attention based on their pathological roles. Specifically, the following three ectoenzymes related to cancer progression and metastasis have received considerable attention as attractive therapeutic targets: autotaxin (ATX), matrix metalloproteinase 9 (MMP9), and carbonic anhydrase IX (CAIX). Our results provide pivotal insights into the activation processes of zinc-requiring ectoenzymes, and furthermore, they offer novel insights for potential cancer therapy applications given the cancer-promoting potencies of ATX, MMP9, and CAIX.

Zinc-requiring ectoenzymes, including both secreted and membrane-bound enzymes, are considered to capture zinc in their active site for their activation in the early secretory pathway. This idea has been confirmed by our studies conducted using tissue-nonspecific alkaline phosphatase (TNAP), which is elaborately activated by means of a two-step mechanism by zinc transporter 5 (ZNT5)-ZNT6 heterodimers and ZNT7 homodimers, through protein stabilization followed by enzyme activation with zinc in the early secretory pathway. However, the molecular basis of the activation process in other zinc-requiring ectoenzymes remains largely unknown. In this study, we investigated this activation process by using three cancer-promoting zinc-requiring ectoenzymes, autotaxin (ATX), matrix metalloproteinase 9 (MMP9), and carbonic anhydrase IX (CAIX), and the chicken DT40 cell mutants that we generated; we specifically focused on clarifying whether the same or a similar activation mechanism operates in these ectoenzymes. ATX activation required ZNT5-ZNT6 heterodimers and ZNT7 homodimers in a manner similar to TNAP activation, although the protein stability of ATX was differently regulated from that of TNAP. MMP9 required ZNT5-ZNT6 heterodimers and ZNT7 homodimers for its activation as well as secretion; MMP9 was not secreted into the spent medium unless both zinc-transport complexes were present. Finally, CAIX activation by zinc was mediated not only by ZNT5-ZNT6 heterodimers and ZNT7 homodimers but also by ZNT4 homodimers; thus, these three zinc-transport complexes redundantly contribute to CAIX activation. Our results provide pivotal insights into the activation processes of zinc-requiring ectoenzymes, and furthermore, they

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3 The abbreviations used are: TNAP, tissue-nonspecific alkaline phosphatase; ZNT, zinc transporter; ALP, alkaline phosphatase; ATX, autotaxin; CA, carbonic anhydrase; MMP, matrix metalloproteinase; TKO, triple knockout; QKO, quadruple knockout; CNX, calnexin; LPC, lysophosphatidylcholine; LPA, lysophosphatic acid; ACTZ, acetazolamide; PP, di-proline.

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Dissecting Cancer-promoting Ectoenzyme Activation by ZNTs

loproteinas (MMPs), and membrane-bound carbonic anhydrases (CAs) (9–14). ATX is a secreted lysophospholipase D that was originally identified as a tumor cell autocrine motility factor (15). ATX produces lysophosphatic acid (LPA) by hydrolyzing extracellular lysophosphatidylcholine (LPC) (16, 17). LPA is a lipid mediator that activates G protein-coupled receptors to evoke various cellular responses, and thus elevated ATX expression is involved in cancer cell invasion, metastasis, and tumor formation and malignancy (18, 19). MMPs can degrade all the major components of the extracellular matrix, and therefore abnormal MMP expression is associated with cancer dissemination, invasion, and metastasis (9, 20). MMP9 and MMP2 are the most widely studied MMPs because of its involvement in these pathological processes as type IV gelatinases (9, 20). CAs catalyze the interconversion of carbon dioxide and bicarbonate and thus maintain the required pH in biological fluids, and these enzymes are consequently involved in numerous physiological and pathological processes both intracellularly and extracellularly (10, 11). Among CAs, CAIX is a well-established tumor-marker ectoenzyme involved in the control of pH environment of cancer cells (10, 21) and therefore contributes to tumor-associated cell migration, invasion, and poor prognosis (22–24).

In this study, we investigated, in comparison with TNAp, whether the activation processes of ATX, MMP9, and CAIX are governed by zinc metalation mediated by ZNT transporters by using chicken DT40 cell mutants (25). We determined that ZNT5-ZNT6 heterodimers or ZNT7 homodimers were involved in the activation and/or maturation of all three ectoenzymes, although the transporters were distinctly involved, and their involvement in the activation of these enzymes also differed from their role in TNAp activation. We further showed that in addition to ZNT5-ZNT6 heterodimers and ZNT7 homodimers, ZNT4 homodimers contributed to the activation of one of these zinc-requiring ectoenzymes (CAIX). Our results describe novel processes by which ZNT transporters facilitate the activation of zinc-requiring ectoenzymes, and these data could also offer new insights into potential therapeutic strategies for the treatment of cancer.

Results

ATX Requires, like TNAp, ZNT5-ZNT6 Heterodimers or ZNT7 Homodimers for Its Activation—ATX is a two zinc-centered ectoenzyme, and thus the active site geometry of ATX is similar to that of alkaline phosphatases such as TNAp (16, 17). Therefore, zinc-dependent activation of ATX is probably mediated by ZNT5-ZNT6 heterodimers and ZNT7 homodimers. Because the mRNA of atx was not detected in DT40 cells by using RT-PCR analysis (data not shown), we exogenously expressed human ATX in DT40 cells (wild-type (WT) cells) and DT40 cells deficient in znt5, znt6, and/or znt7 (znt5−/−znt6−/− cells, ΔzΔ6 cells, and znt5−/−znt6−/−znt7−/− cells, triple knockout (TKO) cells), and we examined ATX activity by measuring the rate of conversion of LPC to LPA. ATX activity was clearly detected in WT cells (Fig. 1A, left graph), and the activity could be readily distinguished from TNAp activity because the ATX substrate was not hydrolyzed even when human TNAp was overexpressed in the cells (Fig. 1A, right graph). ATX activity was dependent on ZNT5-ZNT6 heterodimers (re-expression of ZNT5 alone, or ZNT6 alone, failed to activate ATX, data not shown) and ZNT7 homodimers. The activity in DT40 cells lacking Znt5-Znt6 heterodimers was decreased to 30% of that in WT cells and that in TKO cells was decreased to <5%, which paralleled what was observed with Tnap activity in the same cells (Fig. 1B). Similar to what we previously reported regarding TNAp activity (5, 6, 26), the decreased ATX activity in the TKO cells was restored following concurrent re-expression of ZNT5-ZNT6 or the re-expression of ZNT7 (Fig. 1C), but it was not reversed by the addition of ZnSO4 into the culture medium (Fig. 1D). However, ATX activity was restored only partially by the simultaneous re-expression of human ZNT5-ZNT6 or ZNT7 (Fig. 1C).

The activation of ATX by ZNT5-ZNT6 heterodimers and ZNT7 homodimers is highly similar to the activation of TNAp, but the stability of ATX and TNAp in the TKO cells was found to differ considerably; although the ATX protein was not degraded (Fig. 1B), TNAp protein was markedly destabilized and was degraded (8, 26). We recently reported that the di-proline motif (PP motif) in luminal loop 2 of ZNT5 and ZNT7 plays a pivotal role in the appropriate activation of TNAp (27), and thus we hypothesized that the aforementioned difference might be explained by differences in the contribution of the PP motif. We investigated this point by using an alanine-substituted mutant of the PP motif of ZNT5 (ZNT5PP-AA) (27). ATX activity was partially but not fully restored (~50% reduction) by the coexpression of ZNT5PP-AA and ZNT6 as compared with the activity following the coexpression of ZNT5 and ZNT6 (Fig. 1E, left graph); by contrast, TNAp activity was reduced to <10% after the coexpression of ZNT5PP-AA and ZNT6 as compared with that after coexpression of ZNT5 and ZNT6 (Fig. 1E, right graph). The ATX protein in TKO cells stably coexpressing ZNT5PP-AA and ZNT6 and in TKO cells stably coexpressing WT ZNT5 and ZNT6 appeared almost identical (Fig. 1E, lower panel), which suggested that the PP motif affects ATX activity less markedly than it affects TNAp activity. Thus, these results indicate that ATX activation requires ZNT5-ZNT6 heterodimers or ZNT7 homodimers in a manner almost identical to TNAp activation but that certain differences exist in the metalation process.

MMP9 Requires ZNT5-ZNT6 Heterodimers or ZNT7 Homodimers for Its Proper Activation and Secretion—We next investigated whether MMP9 and MMP2 gelatinase activity was also regulated by ZNT5-ZNT6 heterodimers or ZNT7 homodimers. We expressed human MMP9 or MMP2 in WT and TKO cells and performed gelatin zymography to compare the activity of MMP9 or MMP2 secreted from these cells. The spent media collected from cultures of WT cells stably expressing MMP9 showed a single clear band (~88 kDa following electrophoresis under non-reducing conditions), but the intensity of this band was substantially diminished in the case of media from cultures of TKO cells stably expressing MMP9 (Fig. 2A). Consistent with MMP9 activity, MMP9 protein level in the spent medium from TKO cells was markedly lower than that from WT cells (Fig. 2A). Similar results were observed in WT or TKO cells stably expressing MMP2. In the gelatin zymograph, the band intensity of MMP2 (seen as an ~61-kDa single clear
band, under non-reducing conditions) and the levels of MMP2 protein in the immunoblot were decreased in the spent media from cultures of TKO cells, compared with those of WT cells (Fig. 2B). In contrast, lack of both Znt5-Znt6 heterodimers and Znt7 homodimers did not cause any defect in ATX secretion because ATX levels, both secreted and intracellular, were almost the same in WT and TKO cells stably expressing ATX (Fig. 2C). These results suggested that ZNT5-ZNT6 heterodimers or ZNT7 homodimers are crucial for both the proper activation and the secretion of MMP9 and MMP2. To further examine this point, we chose to focus on MMP9 because the magnitude of decrease in MMP9 activity and expression in TKO cells was much larger than that seen for MMP2 (Fig. 2A and B). Re-expression of ZNT5 and ZNT6 together, or re-expression of ZNT7 alone, restored both the decreased activity and protein levels of MMP9 in TKO cells back to a level comparable with that in WT cells. Re-expression of ZNT5 alone, or ZNT6 alone, failed to restore MMP9 activity and expression...
FIGURE 2. MMP9 requires ZNT5-ZNT6 heterodimers or ZNT7 homodimer for its activation and secretion into the medium. A, MMP9 activity measured in the spent medium was markedly decreased in the case of TKO cells. The spent medium was prepared by culturing cells for 4 h in medium lacking both FCS and chicken serum. MMP9 activity was examined using gelatin zymography, in which electrophoresis was performed under non-reducing conditions. B, similar to MMP9, MMP2 activity measured in spent medium was also decreased in TKO cells. Gelatin zymography was performed as in A with some minor modifications (see “Experimental Procedures”). A and B, for both WT and TKO cells, the band intensities of MMP9 or MMP2 in the zymography assays and the immunoblots were quantified by densitometric analysis, and the values are shown relative to the WT cells, which were set at 1.0, below each lane. C, secreted ATX protein levels were not significantly different between WT and TKO cells. The serum-free spent media obtained from WT or TKO cells (2 × 10⁶ cells cultured in 500 μl of medium) stably expressing ATX were collected after 4 h of incubation. Total cellular proteins were also prepared from both cells. ATX secreted from the cells (Sec-ATX, upper panel) and present within the cells (Intra-ATX, middle panel) was detected by immunoblotting. D, re-expression of either ZNT5-ZNT6 heterodimers or ZNT7 homodimers in TKO cells restored MMP9 activity and its protein level in the spent medium (top two left panels). In parallel with MMP9 restoration, Tnap activity was restored by re-expression of either ZNT5-ZNT6 heterodimers or ZNT7 homodimers in the same cells (right graph). The band intensities of MMP9 in both the zymography and immunoblots were quantified by densitometric analysis, and the values are shown relative to the WT cells, which were set at 1.0, below each lane. Tnap activity was measured prior to the 4-h incubation of the cells in serum-free medium. E, MMP9 activity (zymograph, upper panel) and protein levels (immunoblot, lower panel) in the serum-free spent media from either WT or TKO cells were increased following zinc supplementation. Cells were cultured for 48 h in normal medium in the presence of 0, 25, or 50 μM ZnSO₄ and then cultured for 4 h in serum-free medium containing the same concentrations of ZnSO₄. The band intensities of MMP9 in both the zymograph and immunoblot were quantified by densitometric analysis, and the values are shown relative to the WT cells, which were set at 1.0, below each lane. At 50 μM ZnSO₄, the MMP9 activity and protein levels in TKO cells were restored to the levels in the WT cells (upper panel). In contrast to MMP9 restoration, Tnap activity was not restored by increasing zinc levels in the same cells (lower graph). F, intracellular MMP9 (intra-MMP9) had a distinct form in TKO cells compared with WT cells. MMP9 was detected at ~90 kDa in TKO cells and ~80 kDa in WT cells. Re-expression of the ZNT5-ZNT6 heterodimer or re-expression of the ZNT7 homodimer in TKO cells shifted the MMP9 band from ~90 to ~80 kDa. G, treatment with bafilomycin A1 (Baf. A1), but not MG132, changed the MMP9 band size in WT cells from ~80 to ~90 kDa. WT and TKO cells were cultured with bafilomycin A1 or MG132 for 4 h. A–G, stable expression of MMP9, MMP2, ATX, FLAG-ZNT5, ZNT5-Myc, and ZNT7-HA was confirmed through immunoblotting. Representative results from three independent experiments are shown. Tubulin and Cnx were used as loading controls. mb. protein, membrane protein.
Dissecting Cancer-promoting Ectoenzyme Activation by ZNTs

(data not shown). Similar restoration was seen in the case with Tnap (Fig. 2D, right graph). Interestingly, both the activity and the protein level of MMP9 in the spent medium from TKO cells increased after zinc supplementation and were restored to almost WT levels at 50 μM ZnSO₄ (Fig. 2E, upper panel). This contrasts sharply with the cases of ATX (Fig. 1D) and Tnap (Fig. 2E, lower graph), which could not be restored simply by increasing zinc levels. These results suggest that MMP9 can capture zinc independently of ZNT5-ZNT6 heterodimers or ZNT7 homodimers if zinc is present in excess but that ZNT5-ZNT6 heterodimers and ZNT7 homodimers play a pivotal role in the proper activation and secretion of MMP9 under normal culture conditions. To investigate in detail how MMP9 activity and expression in the spent medium from TKO cells were impaired, we examined the MMP9 maturation process in the early secretory pathway in TKO cells. Unexpectedly, intracellular MMP9 protein was detected as an ~80-kDa band in WT cells, whereas it appeared as a major band of ~90 kDa in TKO cells (after electrophoresis under reducing conditions) (Fig. 2F). However, coexpression of ZNT5 and ZNT6 or re-expression of ZNT7 in the TKO cells shifted the ~90-kDa band to ~80 kDa, which indicated that a lack of both ZNT zinc-transport complexes led to an increase in the size of the intracellular MMP9 protein (Fig. 2F). Moreover, treatment of WT cells with bafilomycin A1, but not MG132, led to the intracellular MMP9 protein band shifting from ~80 to ~90 kDa (Fig. 2G), a size similar to that in TKO cells. These results indicate that intracellular MMP9 in WT cells, which was detected as an ~90-kDa band in immunoblotting, was proteolytically cleaved in lysosomal compartments and that this did not occur in the TKO cells. Collectively, these findings suggest that proteolytic cleavage ability of MMP9 is likely associated with MMP9 activation and secretion into the spent medium. Similar to TNAP and ATX, MMP9 requires zinc metalation mediated by ZNT5-ZNT6 heterodimers or ZNT7 homodimers for its activation, although the processes by which the transporters facilitate the activation differ.

CAIX Can Acquire Zinc Independently of ZNT5-ZNT6 Heterodimers and ZNT7 Homodimers—Finally, we examined whether ZNT5-ZNT6 heterodimers and ZNT7 homodimers contribute to the process of CAIX activation. The mRNA of calIX was expressed at a moderate level in DT40 cells (Fig. 3A), but the activity of endogenous CalIX was extremely weak using the ΔpH/Δt assay (see “Experimental Procedures”); no significant difference in CalIX activity was seen in cells treated with or without the CA inhibitor acetazolamide (Fig. 3, B and C). Thus, we could not compare the CalIX activity in TKO cells with that in WT cells. Therefore, we established WT and TKO cells stably expressing human CAIX as in the case of the aforementioned ectoenzymes. The activity of exogenously expressed CAIX in WT cells was clearly detected (Fig. 3, B and C), and the activity was completely inhibited by acetazolamide (Fig. 3, B and C), which indicated that this assay was suitable for investigating the features of the CAIX activation process. Our immunoblotting results revealed that CAIX expressed in WT DT40 cells appeared as several bands that included broad upper bands at ~70 kDa and a comparatively sharper lower band at ~60 kDa (Fig. 3C), as reported elsewhere (28, 29). Under zinc-deficient conditions, the broad upper bands gradually disappeared over time (Fig. 3D, lower panel), and this almost paralleled the reduction in CAIX activity, which was decreased by 30% at 24 h and by 60% at >48 h (Fig. 3D, left graph); this result also indicated that CAIX was less sensitive than Tnap to zinc deficiency (Fig. 3D, right graph). The decrease in CAIX activity was reversed by zinc supplementation as in the case of Tnap (Fig. 3E, upper graphs), and this was accompanied by the restoration of the broad upper bands in immunoblots (Fig. 3E, lower panel). These results suggest that the CAIX protein corresponding to the broad upper bands was zinc-responsive.

We then compared CAIX activity between TKO cells and WT cells. Unexpectedly, in TKO cells, CAIX activity was not markedly decreased, and the zinc-responsive broad upper bands in immunoblots did not disappear (Fig. 3F, left graph), although Tnap activity was decreased to <5% in the same cells (Fig. 3F, right graph). These results indicate that CAIX can acquire zinc through an additional pathway that is independent of ZNT5-ZNT6 heterodimers and ZNT7 homodimers even under normal culture conditions.

ZNT4 Homodimers, in Addition to ZNT5-ZNT6 Heterodimers and ZNT7 Homodimers, Redundantly Contribute to CAIX Activation—DT40 cells expressed znt4 mRNA at a high level (Fig. 4A), and ZNT4 partially localized to the Golgi apparatus and formed homodimers (Fig. 4, B and C). Thus, we examined the possibility that ZNT4 functions as a zinc entry route into the early secretory pathway and supplies zinc to CAIX. To test whether CAIX is redundantly activated by ZNT4 homodimers, we disrupted Znt4 in the TKO cells and established quadruple KO DT40 cells (znt4−/− znt5−/− znt6−/− znt7−/− cells, QKO cells) (Fig. 4D). The cell surface protein expression was not altered in QKO cells, compared with either WT or TKO cells (Fig. 4E), indicating that the secretory pathway is not significantly impaired in QKO cells.

In QKO cells, CAIX activity was decreased substantially (by >60%), and this was accompanied by a loss of the zinc-responsive broad upper bands in immunoblots (Fig. 4F). However, CAIX activity and the zinc-responsive bands of CAIX in znt4−/− (Δ4) cells were almost the same as those in WT cells (Fig. 4F), which excludes the possibility that ZNT4 functions as the only route to supply zinc to CAIX. Notably, both the reduction in CAIX activity and the disappearance of the zinc-responsive broad upper bands of CAIX in QKO cells were reversed following the re-expression of ZNT4 or Znt7 or the coexpression of ZNT5 and ZNT6. Re-expression of ZNT5 alone, or ZNT6 alone, failed to activate CAIX (data not shown). Expression of the zinc transport-incompetent mutants of these transporters (ZNT4H146A, ZNT5H451A, and ZNT7H70A) (8, 27, 30) also failed to restore CAIX activity (Fig. 4, G–I). Moreover, the addition of ZnSO₄ to QKO cells neither restored CAIX activity nor reversed the disappearance of the zinc-responsive broad upper bands (Fig. 4F), which indicated that zinc metalation mediated by at least one of these three ZNT complexes is essential for CAIX activity. Because ZNT4 is generally thought to mobilize zinc into lysosomal and endosomal compartments (30–32), in addition to the secretory compartments (33), we also investigated the possibility that ZNT4 homodimers contribute to CAIX activation in a manner that differs from ZNT5-ZNT6 heterodimers and ZNT7 homodimers. To explore this
Dissecting Cancer-promoting Ectoenzyme Activation by ZNTs

A

B

C

D

E

F

WT

WT + ACTZ

WTCAIX

WTCAIX + ACTZ

CAIX activity

[Umol/min/mg protein]

CAIX : - - + +
ACTZ : - + + +

CAIX activity

[Umol/min/mg protein]

CAIX : - + + +
CX : 0 24 48 72 (h)

CAIX activity

[Umol/min/mg protein]

CAIX : + + + +
CX : 0 72 (h)

CAIX activity

[Umol/min/mg protein]

CAIX : + + +
ZnSO4 : 0 0 50 (µM)

CAIX activity

[Umol/min/mg protein]

CAIX : + +
CX : 0 72 (h)

CAIX activity

[Umol/min/mg protein]

CAIX : + +
ZnSO4 : 0 0 50 (µM)

WT

WT + TKO

WT

CAIX

Cnx

CAIX

Cnx

CAIX

Cnx
point, we first compared vesicular zinc levels among WT, TKO, and QKO cells using Zinpyr-1 fluorescence staining (34, 35). The fluorescence intensity, analyzed by flow cytometry, revealed that the vesicular zinc levels were not significantly different in QKO cells, compared with WT and TKO cells (Fig. 5A) (6, 8). Thus, the vesicular zinc content was unlikely to be altered in QKO cells. Moreover, we examined whether ZNT4 homodimers are involved in CAIX activation through cooperative cytosolic zinc handling with ZNT1 and metallothionein, because this cooperative zinc handling is required for proper activation of TNAP (30). However, neither CAIX activity nor the zinc-responsive CAIX bands showed any differences between WT and znt1Δ/Δmt−/−znt4Δ/Δ (Δ1M4) cells (Fig. 5B), which excludes the possibility that ZNT4 is involved in CAIX activation through cooperative cytosolic zinc handling. Furthermore, we examined whether the lack of the PP motif in the luminal loop of ZNT4 (27) could cause the differences in the process of CAIX activation mediated by ZNT4 transport complexes and by ZNT5-ZNT6 or ZNT7 complexes. We coexpressed the ZNT5PP-AA mutant and ZNT6 in QKO cells and examined whether CAIX activity was fully restored. Coexpression of ZNT5PP-AA and ZNT6 restored the decreased CAIX activity to a level comparable with that restored by the coexpression of WT ZNT5 and ZNT6 (Fig. 5C, left graph), which is in contrast to the large difference in Tnap activity observed between the respective cells (Fig. 5C, right graph). These results indicate that the PP motif is not required for CAIX activation and thus that ZNT4, which lacks the motif, can still contribute to the process of CAIX activation. Taken together, our findings suggest that in the activation of CAIX, ZNT4 homodimers are functionally equivalent to ZNT5-ZNT6 heterodimers or ZNT7 homodimers and that zinc metalation mediated by one of these ZNT transport complexes ensures proper CAIX activation in the early secretory pathway.

Discussion

Previously, we have used the DT40 cell system to explore zinc homeostasis, zinc transport protein functions, and the processes involved in activation of zinc-requiring ectoenzymes (5–8, 25–27, 30, 34, 36). In particular, this cell system is useful for investigating the activation process of zinc-requiring ectoenzymes, because the interactions between zinc-requiring ectoenzymes and ZNT transporters are strictly maintained in the system even when gene disruption/re-expression and overexpression strategies are applied. Previously, we have shown that TNAP is specifically activated by concurrent expression of ZNT5 and ZNT6 or expression of ZNT7 using both endogenous Tnap as well as overexpressed TNAP (e.g. human TNAP) (6–8, 27, 28). Importantly, the overexpressed TNAP undergoes the same specific activation process as the endogenous Tnap. As stated above, ZNT5-ZNT6 heterodimers or ZNT7 homodimers are required for activation of TNAP, whereas exogenous expression of ZNT2, ZNT3, or ZNT8 and overexpression of ZNT1 or ZNT4 are not required (8), demonstrating the fidelity of this system. We have also shown the utility of the DT40 system by characterizing the activation of exogenously expressed human placenta alkaline phosphatase in a previous study (5), and used this same approach to explore the unique interaction between three different zinc-requiring ectoenzymes and ZNT transporters in this study.

Based on the TNAP activation process where zinc metalation is mediated by ZNT5-ZNT6 heterodimers and ZNT7 homodimers in the early secretory pathway (37, 38), it was thought that other zinc-requiring ectoenzymes would also be activated in a similar manner. However, with exception of TNAP, the molecular basis of the process used to metalate and activate these enzymes has been poorly investigated and remains largely unknown. In this regard, only a few experiments have been reported on ectoenzymes other than TNAP (33, 39). In this study, we obtained novel and detailed molecular evidence describing how ZNT5-ZNT6 heterodimers and ZNT7 homodimers contribute to the activation of three different zinc-requiring ATX, MMP9, and CAIX. The results we have presented here are important in that they revealed that each ectoenzyme interacts in a unique way with ZNT transporters (Fig. 6). Therefore, this study not only enhances our understanding of the process of ectoenzyme activation but also provides new insights into potential therapeutic strategies against cancers, given the involvement of ATX, MMP9, and CAIX in cancer promotion.

The active site geometry of ATX is homologous to that of TNAP, in which two zinc ions are coordinated by the same amino acid residues (one zinc is coordinated by two His and one Asp and the other by one His and two Asp) (38). This is consistent with our results showing that ZNT5-ZNT6 heterodimers and ZNT7 homodimers are required for ATX activation (much as they are for TNAP activation), although minor differences

FIGURE 3. CAIX can acquire zinc from a pathway independent of ZNT5-ZNT6 heterodimers and ZNT7 homodimers. A, confirmation of endogenous CAIX mRNA expression in DT40 cells. Expression of the indicated ca genes was examined by performing RT-PCR with appropriate primers. B, example of the raw data from the ΔpH/Δt assay used for measuring CAIX activity. Membrane proteins prepared from WT cells and WT cells stably expressing CAIX were used for activity measurement, as described under “Experimental Procedures.” CAIX activity in the membrane proteins isolated from WT cells stably expressing CAIX was completely inhibited upon treatment with the CA inhibitor acetazolamide (ACTZ, 100 μM). The graph also shows endogenous CAIX activity in membrane proteins prepared from parental DT40 cells and ACTZ-treated parental DT40 cells. C, graph showing the calculated CAIX activity (measured as in B). The representative results from three independent experiments are displayed (*, p < 0.01). CAIX protein was detected as broad upper bands at ~70 kDa and a relatively sharper lower band at ~60 kDa (lower panel). D, CAIX activity was decreased up to 60% when cells were cultured in zinc-deficient medium (left graph), which is in contrast to the Tnap activity measured in the same cells (right graph). WT cells stably expressing CAIX were cultured in zinc-deficient medium containing Chelex-treated FCS and chicken serum (CX medium) for 24, 48, and 72 h, and CAIX activity and protein were examined. E, decrease in CAIX activity in DT40 cells cultured in zinc-deficient medium was rescued following supplementation with 50 μM ZnSO4 for 48 h (left graph); a similar response was obtained with Tnap activity in the same cells (right graph). D and E show that CAIX protein detected as the broad upper bands at ~70 kDa were decreased together with the reduction in CAIX activity (lower panels). F, CAIX activity was not markedly altered in TKO cells (left graph), which is in contrast to the large reduction observed in Tnap activity in the same cells (right graph). Consistent with CAIX activity, the broad upper bands at ~70 kDa were retained (lower panel), C–F, CAIX activity was examined in membrane proteins (200 μg) prepared from the indicated cells. Tnap activity was measured as described in Fig. 1. Representative results from three independent experiments are displayed. Cnx was used as the loading control. mb. protein, membrane protein.
between TNAP and ATX activation processes were observed. Currently, these differences cannot be readily explained, although specific regulation by local environmental differences might be operative in these enzymes before zinc coordination in their active site, because ATX and TNAP are glycosylated to considerably distinct levels; TNAP is highly glycosylated, whereas ATX is (relatively) poorly glycosylated.

MMP9 (and probably also MMP2) also requires ZNT5-ZNT6 heterodimers and ZNT7 homodimers for its proper activation and secretion. However, the requirements for these two
ZNT transport complexes differ markedly between MMP9 and ATX. In the case of MMP9, ZNT5-ZNT6 heterodimers and ZNT7 homodimers are probably critical under normal culture conditions, but MMP9 can capture zinc through additional pathways independently of both ZNT transport complexes when zinc is present in excess. Because cellular zinc levels are increased in several cancer and tumor cells, and this is paralleled by elevated expression of ZIP transporters (40–46), our
**FIGURE 5.** Biochemical evidence of the functional equivalency of ZNT4 homodimers and ZNT5-ZNT6 heterodimers or ZNT7 homodimers in CAIX activation. A, vesicular zinc levels were not significantly different in QKO cells, compared with WT and TKO cells. Cells were grown in zinc-deficient, normal, or zinc-supplemented (50 μM ZnSO₄) medium for 48 h, and then loaded with 5 μM Zinpyr-1. After washing, the cells were analyzed by flow cytometry. Each histogram represents ~20,000 cells and shows the zinc levels detected by Zinpyr-1 fluorescence. The upper three histograms (left to right) are from WT, TKO, and QKO cells, respectively, and the lower three histograms (left to right) are from the zinc-deficient, normal, and zinc supplemented conditions, respectively. Control, not loaded with Zinpyr-1. B, CAIX activity was not markedly impaired in znt1/mt/znt4/ (ΔM4) cells (left graph), which is in contrast to the large reduction in Tnap activity in the same cells (right graph). The zinc-responsive broad upper bands in immunoblots requires either ZNT5-ZNT6 heterodimers, ZNT7 homodimers, or ZNT4 homodimers for activation, although the CAIX form that corresponds to the sharper lower band in immunoblots does not. Another unknown pathway(s) may be operative as a zinc entry route. A–D, zinc ion is indicated using a yellow dot.

**FIGURE 6.** Model for the activation process of TNAP, ATX, MMP9, and CAIX by ZNT transporters in the early secretory pathway. Three ZNT transport complexes, ZNT5-ZNT6 heterodimers, ZNT7 homodimers, and ZNT4 homodimers are localized to the early secretory pathway. The ZNT transport complexes interact with each zinc-requiring ectoenzyme in a unique way. A, TNAP activation process is shown for comparison with ATX, MMP9, and CAIX. ZNT5-ZNT6 heterodimers and ZNT7 homodimers are essential for TNAP activation. TNAP is activated in a two-step mechanism and non-metalated TNAP (apo-TNAP) is degraded intracellularly (8). ZNT4 homodimers do not contribute to its activation (data not shown). B, similar to TNAP, ATX activation requires ZNT5-ZNT6 heterodimers and ZNT7 homodimers. However, in contrast to TNAP, apo-ATX is stable and can be secreted into the extracellular space. ZNT4 homodimers do not contribute to its activation (data not shown). C, MMP9 requires ZNT5-ZNT6 heterodimers and ZNT7 homodimers for its proper maturation and secretion, but this may be complemented by excess zinc supplementation. In this case, other unidentified pathways, including through ZNT4 homodimers, might contribute to the zinc entry route. MMP2 may be regulated in a similar manner. D, CAIX is activated in two manners. The CAIX form that corresponds to the zinc-responsive broad upper bands in immunoblots requires either ZNT5-ZNT6 heterodimers, ZNT7 homodimers, or ZNT4 homodimers for activation, although the CAIX form that corresponds to the sharper lower band in immunoblots does not. Another unknown pathway(s) may be operative as a zinc entry route. A–D, zinc ion is indicated using a yellow dot.
results potentially suggest that MMP9 activity is increased in these cells. Although this study has not clarified the pathways that supply MMP9 with zinc when zinc is in excess, one possibility is that ZNT4 functions in this supply pathway as it does in the case of CAIX. However, we have not yet examined this possibility in detail, because we were unable to establish QKO cells stably expressing MMP9 (data not shown).

An intriguing aspect of MMP9 activation is that ZNT5-ZNT6 heterodimers and ZNT7 homodimers are likely required for the intracellular proteolytic cleavage of MMP9 from an ~90- to 80-kDa protein, and this appears to be associated with MMP9 activation and secretion. The cleavage potentially occurs in lysosomes because it was inhibited by bafilomycin A1 treatment; this finding suggests that immature or malformed MMP9 proteins, which are not secreted but are present in WT cells, might be rapidly cleaved from ~90 to ~80 kDa. Such proteolytic cleavage during protein quality control could ensure the secretion of only properly folded intact MMP9 by eliminating immature or malformed MMP9. This quality-control system might require zinc metalation mediated by ZNT5-ZNT6 heterodimers and ZNT7 homodimers, and thus it might not be operative in TKO cells. In this scenario, unknown zinc-requiring enzymes localized to the early secretory pathway might play a major role in the control system. Clarification of this point in future studies is crucial, because MMP9 is one of the primary therapeutic targets in the treatment of cancers.

As compared with the activation of the aforementioned two zinc-requiring ectoenzymes, CAIX activation is somewhat unique. Approximately 60% of total CAIX protein, which corresponded to the zinc-responsive broad upper bands in immunoblots, was activated by zinc metalation mediated by ZNT5-ZNT6 heterodimers, ZNT7 homodimers, or ZNT4 homodimers, whereas the remaining 40% of CAIX was activated by zinc metalation mediated by another pathway(s). We found that the ratios of the intensities of the zinc-responsive broad upper bands to the sharper lower band were frequently inconsistent relative to the ratio of the reductions in CAIX activities; this finding suggests that these two pathways for supplying zinc to CAIX are completely independent of each other. We have not closely examined how the second of these two pathways contributes to CAIX activation, but zinc-permeable proteins localized to the early secretory pathway could be crucial. Moreover, a satisfactory explanation is not yet available for how the broad upper bands correspond to the zinc metalation that is mediated by the zinc-transport complexes of ZNT4, ZNT5-ZNT6, and ZNT7. However, we excluded the possibility that the bands could be attributed to impaired N- or O-linked glycosylation or to protein modification during proteasome- or lysosome-mediated degradation, because neither peptide:N-glycosidase F and O-glycosidase treatment nor MG132 and bafilomycin A1 treatment altered the sizes and intensities of the bands (data not shown). CAIX protein bands of a similar size as the upper bands were observed in the urine of renal carcinoma patients (28, 29). Therefore, clarifying the zinc-responsive properties of these protein species could yield information that is useful for cancer therapy.

This study yielded the intriguing finding that ZNT4 homodimers are equivalent to ZNT5-ZNT6 heterodimers or ZNT7 homodimers in the activation of CAIX in the early secretory pathway but that this is not the case in TNAP activation (8). How might ZNT4 function in a manner equivalent to ZNT5-ZNT6 or ZNT7 in CAIX activation? Given the equivalent functions of the transporters in the CAIX activation process, their subcellular localization would be expected to be similar, and we confirmed that ZNT4, like ZNT5-ZNT6 or ZNT7, was partially localized to the Golgi apparatus in DT40 cells. ZNT4, which probably functions as a zinc/proton exchanger (47, 48), can mobilize zinc into the lumen of the organelles in the early secretory pathway, mainly through the Golgi apparatus, considering that the Golgi luminal pH is estimated to be 6.0–6.7 (49). We showed that CAIX activation, unlike TNAP activation, does not require the PP motif in the luminal loop of ZNT5; this suggests that CAIX might not require specific regulation, which is operative in TNAP activation. However, zinc supplementation failed to restore CAIX activity in QKO cells, which suggests that CAIX activation must be under the control of an elaborate molecular mechanism. Supporting this notion, CAIX activity in QKO cells was not restored following exogenous expression of ZNT2 or Golgi-resident ZNT2 mutants (50), although ZNT2 and ZNT4 perform similar functions in DT40 cells in certain cases (30). Recent results obtained by us and by others indicate that the change of a single amino acid residue markedly affects ZNT transporter functions (36, 51, 52), which suggests that unique conserved amino acids among ZNT4, ZNT5-ZNT6, and ZNT7 complexes might play critical roles in CAIX activation, as in the case of the PP motif in TNAP activation (30).

Another crucial aspect of this study is that our results indicate that both ZNT5-ZNT6 heterodimers and ZNT7 homodimers are closely associated with the activation of ATX, MMP9, and CAIX, despite the fact that their manner of contribution is different; this has led us to hypothesize that these transporters are potentially involved in cancer promotion in certain cases. Notably, the expression of ZNT5 and ZNT7 mRNAs has been reported to increase in prostate cancer (53), which suggests that these molecules could represent novel targets for developing anticancer drugs. Numerous trials have been conducted to generate anticancer drugs targeting these ectoenzymes, but additional in-depth knowledge regarding these enzymes is required because the disease biology is highly complex (9–14). The results we have presented here should offer novel and potentially valuable information for targeting ZNT transporters as therapeutic targets (although in the case of CAIX, the activity of this enzyme could be inhibited if tumor cells express CAIX but not ZNT4).

In summary, the results of this study on the cancer-promoting ectoenzymes ATX, MMP9, and CAIX indicate all three enzymes, like TNAP, require ZNT5-ZNT6 heterodimers and ZNT7 homodimers for their activation, although each enzyme shows certain specific differences relative to TNAP. Our findings provide key evidence indicating the sophisticated process of activation of zinc-requiring ectoenzymes by zinc metalation mediated by ZNT transporters in the early secretory pathway, which enhances our understanding of the molecular basis of

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4 T. Tsuji and T. Kambe, unpublished data.
Dissecting Cancer-promoting Ectoenzyme Activation by ZNTs

Experimental Procedures

Cell Culture and Transfection—Chicken B lymphocyte-derived DT40 cells were cultured as described previously (25). DNA was transcribed into DT40 cells as described (25), and >3 independent clones were established per transfectant in all experiments. To generate zinc-deficient culture media, we used fetal calf serum (FCS) and chicken serum that were treated with human ATX, MMP9, and CAIX to provide useful information for physiology and pathophysiology, and thus their functional targets. Several zinc-requiring ectoenzymes play pivotal roles in zinc biochemistry. Moreover, we expect these findings related to ATX, MMP9, and CAIX to provide useful information for potential cancer therapy applications, given that all three enzymes have attracted considerable attention as therapeutic targets. Several zinc-requiring ectoenzymes play pivotal roles in zinc biochemistry. Moreover, we expect these findings related to ATX, MMP9, and CAIX to provide useful information for potential cancer therapy applications, given that all three enzymes have attracted considerable attention as therapeutic targets. Several zinc-requiring ectoenzymes play pivotal roles in zinc biochemistry. Moreover, we expect these findings related to ATX, MMP9, and CAIX to provide useful information for potential cancer therapy applications, given that all three enzymes have attracted considerable attention as therapeutic targets. Several zinc-requiring ectoenzymes play pivotal roles in zinc biochemistry. Moreover, we expect these findings related to ATX, MMP9, and CAIX to provide useful information for potential cancer therapy applications, given that all three enzymes have attracted considerable attention as therapeutic targets. Several zinc-requiring ectoenzymes play pivotal roles in zinc biochemistry. Moreover, we expect these findings related to ATX, MMP9, and CAIX to provide useful information for potential cancer therapy applications, given that all three enzymes have attracted considerable attention as therapeutic targets. Several zinc-requiring ectoenzymes play pivotal roles in

Measurement of TNP Activity—Membrane proteins prepared from cells were lysed in ALP lysis buffer, and 5 μg of the membrane proteins were used for measuring TNP activity as described previously (25). Calf intestine ALP (Promega, Madison, WI) was used to generate a standard curve (30).

Measurement of ATX Activity—ATX activity was measured according to the colorimetric method described elsewhere (16, 55, 56). Briefly, 10 μg of membrane proteins prepared from cells were lysed in ATX lysis buffer containing 100 mM Tris–HCl, pH 9.0, 500 mM NaCl, 5 mM MgCl2, and 0.1% Triton X-100, and incubated in 96-well plates for 10 min at room temperature. Next, 100 μl of ATX lysis buffer containing 2 mM LPC was added as the substrate solution and incubated for 6 h at 37 °C to generate LPA and choline. After the incubation, 100 μl of a reaction solution containing 100 mM Tris–HCl, pH 8.0, 10 mM EDTA, 2 mM 4-aminoantipyrine (Sigma), 2 mM TOOS reagent (Dojindo Laboratories, Kumamoto, Japan), 100 milliunits of peroxidase (Sigma), and 100 milliunits of choline oxidase (Wako Pure Chemical, Osaka, Japan) was added to produce quinoneimine dye from choline. After incubation for 5–20 min at 37 °C, the dye released by the sequential reactions was measured based on the absorbance at 555 nm. Phospholipase D from Arachis hypogaea (Sigma) was used to generate a standard curve.

Measurement of MMP9 and MMP2 Activity—MMP9 and MMP2 activity was evaluated using gelatin zymography, as described elsewhere (57). Briefly, for detecting MMP9 activity, 16 μl of the spent medium, which was collected after a 4-h incubation of 2.0 × 106 cells in 500 μl of culture medium without FCS and chicken serum, was mixed with 4 μl of a non-reducing sample buffer (0.35 mM Tris–HCl, pH 6.8, 10% SDS, 30% glycerol, and 0.1% bromphenol blue), and then incubated at 37 °C for 20 min before loading onto 6% SDS-polyacrylamide gels containing 0.12% gelatin. After electrophoresis, gels were washed in washing buffer (50 mM Tris–HCl, pH 7.5, 10 mM NaCl, and 2.5% Triton X-100) for 1 h at room temperature. Zymographic gels were rinsed and incubated for 18 h at 37 °C in renaturing buffer (50 mM Tris–HCl, pH 7.5, 10 mM CaCl2, and 0.02% NaN3), after which the gels were stained (5% acetic acid, 10% ethanol, and 0.25% Coomassie Brilliant Blue R-250) for 1 h at room temperature and then photographed using an LAS1000

TABLE 1

| Gene  | Forward sequence (5’ to 3’) | Reverse sequence (5’ to 3’) | Length | Cycle |
|-------|-----------------------------|-----------------------------|--------|-------|
| znt2  | TCTATCGGTCGTCCTGGCATCTGGCATCTG | AGAGAGAGAGAGGGCTGAGTGGGTCACAT | 570    | 34    |
| znt3  | AGTGAAGAGGAGGGCTGAGTGGGTCACAT | TCTATCGGTCGTCCTGGCATCTGGCATCTG | 288    | 34    |
| znt4  | GCCGGGAGAGGGCTGAGTGGGTCACAT | GGGCTGAGAGGGCTGAGTGGGTCACAT | 954    | 28    |
| znt5  | GCTTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGT
plus (Fujifilm, Tokyo, Japan). MMP9 activity was identified as a clear area in a blue-stained background. To detect MMP2 activity, the protocol was modified as follows: 4.0 × 10⁵ cells were used, and zymographic gels were incubated for 20 h, following electrophoresis in SDS-polyacrylamide gels containing 0.09% gelatin. To inhibit protein degradation, cells were treated with 30 µM MG132 (Peptide Institute, Osaka, Japan) or 30 nM bafilomycin A1 (Sigma) in serum-free media for 4 h before collection of the media.

**Measurement of CAIX Activity**—CAIX activity was determined based on the ΔpH/Δt assay, as described elsewhere (58, 59). Briefly, 200 µg of membrane proteins lysed in 200 µl of CA lysis buffer containing 10 mM Tris-HCl, pH 7.5, and 0.1% Triton X-100 were mixed in a reaction vessel with 3.8 ml of 25 mM barbital sodium buffer (pH 8.6, Wako Pure Chemical) at 4 °C in a water bath. To initiate the hydration of CO₂, 2 ml of CO₂-saturated pure water, which was bubbled with CO₂ gas from dry ice for 2 h in ice, was applied as a CO₂ donor. The time was measured at the start point, pH 8.3, and the end point, pH 7.3, of the reaction, and Δt (time from the start point to the end point) was calculated. To inhibit CAIX activity, membrane proteins were incubated with 100 µM acetazolamide (Sigma) for 30 min on ice before initiating the hydration of CO₂. A standard curve was obtained as a linear line by plotting serially diluted units of purified bovine erythrocyte CA (Sigma) against the absolute value of ΔpH/Δt, and CAIX activity was quantified using the standard curve.

**Immunoblotting and Immunoprecipitation**—Immunoblotting and immunoprecipitation were performed as described previously (8, 25). For immunoprecipitation, we used 200 µg of membrane fractions prepared from cell lysed in Nonidet P-40 buffer (6). For immunoblotting, the blotted PVDF membranes (Millipore, Bedford, MA) were blocked with a solution of 5% skimmed milk and 0.1% Tween 20 in PBS and then incubated with various antibodies as follows: monoclonal anti-HA HA-11 (1:3000; catalog no. MMS-101P, Biolegend, San Diego); monoclonal anti-HA clone 3F10 (1:3000; catalog no. 11867423001, Roche Applied Science, Mannheim, Baden-Württemberg, Germany); monoclonal anti-FLAG M2 (1:3000; catalog no. F3165, Sigma); polyclonal anti-FLAG (anti-DDDK; 1:3000; catalog no. PM020, MBL, Nagoya, Japan);anti-TNAP (1:3000; catalog no. sc-30203, Santa Cruz Biochemistry, Santa Cruz, CA); anti-ATX (1:6000; catalog no. D322-3, MBL); anti-CAIX (1:6000; catalog no. NB100-417, Novus Biologicals, Littleton, CO); anti-MMP9 (1:6000; catalog no. AF911, R&D Systems, Minneapolis, MN); anti-ß-tubulin (1:10,000; catalog no. T7816, Sigma); and anti-calnexin (CNX) (1:6000; catalog no. ADI-SPA-860, Enzo Life Sciences). For detection of immunoreactive bands, we used (at 1:3000 dilution) horseradish peroxidase-conjugated antimouse, -rabbit, or -rat IgG (GE Healthcare, NA931, NA934, or NA935) or anti-goat IgG (catalog no. sc-2020, Santa Cruz Bio-technology) antibodies. Fluoroimages were obtained using an LAS1000 Plus (Fujifilm). Densitometric quantification of the band intensity was performed using ImageQuant TL software (GE Healthcare).

**Immunofluorescence Staining**—Immunostaining for ZNT4-FLAG detection was performed as described (25). Briefly, cells were stained with polyclonal anti-FLAG (1:2000; MBL) followed by Alexa 594-conjugated goat anti-rabbit IgG (1:200; catalog no. A11032, Molecular Probes, Eugene, OR) and with anti-GM130 (1:100; catalog no. G65120, Transduction Laboratories, Lexington, KY) followed by Alexa 488-conjugated goat anti-mouse IgG (1:200; catalog no. A11001, Molecular Probes). The stained cells were examined using a fluorescence microscope (catalog no. FSX100, Olympus, Tokyo, Japan).

**Cell Surface Biotinylation Assay**—The cell surface biotinylation assay was performed as described previously (36). Briefly, cells were washed twice with ice-cold PBS and then incubated with EZ-Link, a sulfo-NHS-SS-biotin reagent (Pierce Protein Biology, Thermo Fisher Scientific, Waltham, MA) to biotinylate lysine residues exposed on the extracellular surface. Biotinylated proteins were recovered from streptavidin-coupled beads in 6× SDS sample buffer and then electrophoresed. The proteins transferred to the PVDF membrane were detected by staining with Coomassie Brilliant Blue.

**Zinc-specific Fluorescence Staining**—Cells were grown in zinc-deficient, normal, or zinc-supplemented (50 µM ZnSO₄) medium for 48 h and washed once in phosphate-buffered saline. Zinpyr-1 staining was performed as described previously (34). Briefly, cells were then treated with 5 µM Zinpyr-1 ester (Santa Cruz Biotechnology) for 30 min at room temperature. After extensive washing with phosphate-buffered saline containing 20 mM EDTA to remove extracellular zinc, the cells were resuspended in phosphate-buffered saline containing 1% bovine serum albumin and were subjected to flow cytometry analysis using a BD Accuri C6 flow cytometer (BD Biosciences, Ann Arbor, MI).

**Statistical Analyses**—All data are presented as means ± S.D. Statistical significance was determined using Student’s t tests and accepted at p < 0.01.

**Author Contributions**—T. T. and T. K. conceived the study. T. T. and T. K. wrote the paper. T. T., Y. K., and T. K. designed, performed, and analyzed the experiments. J. C., J. P., and H. S. cloned genes and contributed to the concept. H. F. and M. N. provided technical assistance in measuring enzyme activity. All authors reviewed the manuscript.

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