REVIEW

A Brief History of Tumor Necrosis Factor α – converting Enzyme: An Overview of Ectodomain Shedding

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Many membrane-bound molecules are cleaved at the cell surface, thereby releasing their extracellular domains. This process, often referred to as ectodomain shedding, has emerged as a critical post-translational mechanism for various membrane-bound ligands, receptors, and adhesion molecules. Tumor necrosis factor α (TNFα)-converting enzyme (TACE/ADAM17) was originally identified as an enzyme responsible for releasing the membrane-bound TNFα precursor. However, subsequent studies found an exceptionally large number of target molecules of TACE, including the ligands for epidermal growth factor receptor, L-selectin, CD44, and vascular growth factor receptor 2. Furthermore, *in vivo* studies using TACE-conditional knockout mice demonstrated the crucial roles of TACE and ectodomain shedding under both physiological and pathological conditions. However, the potential clinical application of the manipulation of TACE activity remains to be investigated. (doi: 10.2302/kjm.2012-0003-RE; Keio J Med 62 (1) : 29–36, March 2013)

***Keywords*: TACE/ADAM17, ectodomain shedding, TNFα**

Introduction

Various membrane-bound proteins are proteolytically cleaved to release their extracellular domain. This proteolytic mechanism, also called ectodomain shedding, may appear inconsiderable, with no significant physiological role. It is therefore not surprising that this phenomenon has been overlooked in the past and has been considered a mere degradation process of membrane-bound proteins. However, as I discuss in this review, studies during the past two decades have revealed that ectodomain shedding is an essential post-translational mechanism with critical roles during development, in homeostasis, and under pathological conditions *in vivo*.[1–3]

As its name suggests, tumor necrosis factor α (TNFα)-converting enzyme (TACE), also known as a disintegrin and metalloprotease 17 (ADAM17), was originally identified as an enzyme responsible for releasing the membrane-bound TNFα precursor to produce biologically active soluble TNFα.[4,5] However, studies over the past decade have identified a wide range of membrane-bound proteins in addition to TNFα as substrates for TACE,[1–3] including the ligands for epidermal growth factor receptor (EGFR),[6] which are also produced as membrane-bound precursors and are cleaved to become fully functional. TACE is, therefore, an essential modulator of the TNFα–TNFα receptor signaling pathway and the EGFR ligand–EGFR signaling pathway *in vivo*.[7] Because TNFα and EGFR are involved in the pathogenesis of many disorders, such as rheumatoid arthritis and breast cancer, respectively, TACE has now emerged as a potential molecular target for treating these conditions.[8,9] In this review, I will outline the history of TACE, beginning with its identification, and summarize some of the recent findings uncovered using TACE mutant mice.

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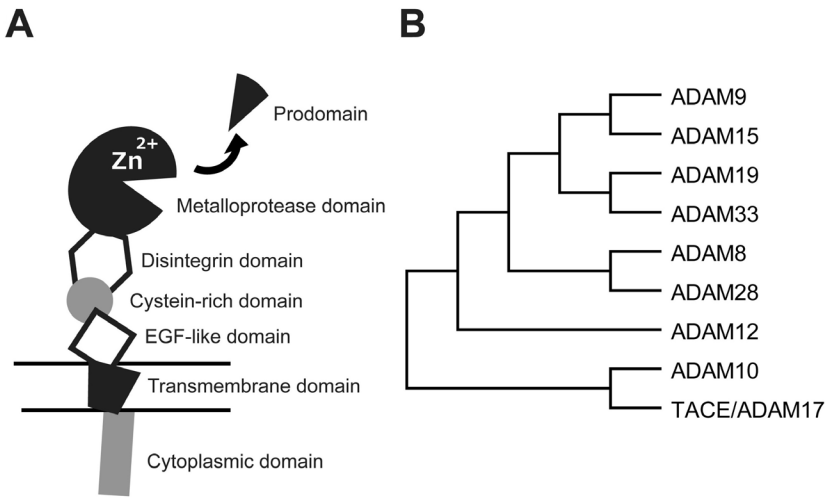

Fig. 1 ADAM gene family and structure.
(A) ADAM proteins contain a metalloprotease domain, a disintegrin domain, a cysteine-rich domain, an EGF-like domain, a transmembrane domain, and a cytoplasmic tail. The prodomain maintains the proteolytic activity in an inactive state until it is cleaved by a furin protease in the trans-Golgi network before delivery of the ADAM protein to the cell surface. The metalloprotease domain contains a zinc (Zn^{2+})-binding domain that is highly conserved among the metalloprotease family. (B) Dendrogram depicting relative amino acid sequence homologies among catalytically active and widely expressed ADAM proteins.

The TNFα Precursor is a Membrane-bound Protein and Must be Shed from the Cell Surface to Become an Active Cytokine

Before discussing the major topic of this review, I will introduce some of the early work on the proteolytic cleavage of pro-TNFα that ultimately led to the identification of TACE. TNFα is a central component of the immune system and also plays critical roles in the pathogenesis of inflammatory diseases such as rheumatoid arthritis, Crohn's disease, and asthma. Because TNFα was initially found in the serum as a soluble protein, TNFα was originally thought to be produced as a soluble cytokine.[10] However, the determination of its cDNA nucleotide sequence unexpectedly revealed that TNFα is initially produced as a type-2 membrane-bound protein that has to be proteolytically cleaved to become soluble.[11,12] This discovery led to the idea that the activity of TNFα could be suppressed by inhibiting this proteolytic activity, thus opening up a potential approach to treating patients with TNFα-related diseases.

Therefore, the mechanism underlying the processing of the TNFα precursor was intensively investigated by pharmaceutical companies in the 1990s. In 1994, it was shown that the production of soluble TNFα can be greatly suppressed by ethylenediaminetetraacetic acid (a chelator used for depleting metal ions) and metalloprotease inhibitors, indicating that the cleavage of TNFα is metalloprotease dependent.[13–15] In addition, Mohler et al.[15] showed that treatment with a metalloprotease inhibitor provides strong protection against lipopolysaccharide (LPS)-induced endotoxin shock in mice. That study showed for the first time that ectodomain shedding is involved in the functional modification of membrane-bound proteins *in vivo* and that the bioactivity of TNFα can be manipulated by inhibiting TNFα cleavage. The results also indicated that the pharmaceutical suppression of TNFα shedding could be beneficial for treating TNFα-related disorders. Later, the importance of ectodomain shedding in the regulation of TNFα function was also confirmed by a study of a mutant mouse line in which pro-TNFα was genetically modified to be resistant to proteolytic cleavage.[16] These cleavage-resistant TNFα mutant mice exhibited very similar immune defects to those found in *Tnfα*-null mice, demonstrating that the soluble form, not the membrane-bound pro-form, is responsible for the major functions of TNFα. Taken together, these observations confirmed that ectodomain shedding is an indispensable mechanism for the functional activation of TNFα *in vivo*.

Identification of TNFα-converting Enzyme, TACE

Three years after publication of the reports establishing that the enzyme involved in TNFα cleavage was metalloprotease dependent,[13–15] two independent groups identified an enzyme capable of cleaving pro-TNFα *in vitro*.[4,5] The enzyme, TACE, was a membrane-bound protein and, as predicted by previous research, had a characteristic Zn^{2+}-binding motif (HExxHxxGxxH), a highly conserved motif among metalloproteases. On the basis

of amino acid homology, TACE was found to belong to the ADAM gene family (**Fig. 1**); therefore, TACE is also called ADAM17.

The identification of TACE marked the first time that a potential link between a sheddase and its target molecule (in this case TACE and TNFα, respectively) had been demonstrated and is undoubtedly one of the most important discoveries in the field of ectodomain shedding. However, it remains elusive why TNFα is produced as a membrane-bound protein and not as a soluble protein, as other cytokines are. It may be that this machinery has evolved to release emergency signaling molecules as rapidly as possible (the cleavage of membrane-bound proteins is generally more rapid than synthesizing them). Nevertheless, because of two important studies published in 1997,[4,5] the newly identified TACE gene emerged as one of the most promising candidate genes responsible for ectodomain shedding of the TNFα precursor. The next step was to generate TACE-deficient mice and to confirm whether TACE really is the relevant sheddase for TNFα *in vivo*. However, as described in the next section, the abrogation of the *Tace* gene in mice unexpectedly resulted in early lethality, and the mice could not be used to analyze TNFα activity *in vivo*.

TACE Proved to Be an Enzyme with Many Substrates

Although TACE had emerged as a candidate enzyme responsible for the cleavage of pro-TNFα, other proteases, including matrix metalloprotease (MMP) 7,[17] ADAM10,[18] ADAM19,[19] and protease 3,[20] had also been implicated in releasing TNFα, raising questions about the identity of the relevant TNFα convertase *in vivo*. Because both the MMP gene family and the ADAM gene family contain a large number of members, it seemed likely that several different proteases could be involved in the processing of TNFα, and the generation and analysis of TACE knockout mice was expected to clarify this issue. Mice lacking TNFα or its receptors (TNF receptor 1 and 2) had been generated and were shown to be viable and fertile, even though they all showed similar immune system defects.[21,22] Therefore, it was assumed that TACE-null mice would be viable without any overt defects. Contrary to this hypothesis, a study published in 1998 showed that the disruption of TACE results in perinatal lethality and a highly complex phenotype.[23]

Among the defects found in the TACE-null mice, the most striking and unexpected were open eyelids (mice are normally born with their eyelids closed) and abnormal skin and fur, which were nearly identical to the phenotypes of EGFR-null mice.[24,25] Seven different ligands had been identified for EGFR, and all of these ligands were produced as membrane-bound precursors that must be cleaved to become soluble growth factors, as is also the case for TNFα.[26] However, the enzyme responsible for the cleavage of the membrane-bound precursors of EGFR ligands had remained unidentified. On the basis of the similarity of the skin and fur defects among the TACE-, EGFR-, and transforming growth factor α (TGFα; one of the EGFR ligands)[27]-deficient mice, Peschon et al.[23] examined whether proteolytic activity against pro-TGFα was inhibited in fibroblasts lacking TACE and found that this was indeed the case. Similarly, the shedding activity of another EGFR ligand, heparin-binding EGF-like growth factor (HB-EGF), was also found to be abrogated in TACE-deficient fibroblasts; in addition, nearly identical defects in the heart valves were found in TACE-, HB-EGF-, and EGFR-deficient mice.[6,28] In short, the analysis of TACE-deficient mice had serendipitously revealed TACE to be the enzyme responsible for processing EGFR ligands. The importance of this study using TACE-deficient mice cannot be overstated: it proved ectodomain shedding to be an essential post-translational mechanism involved in the functional regulation of membrane-bound proteins *in vivo*. Of note, in addition to the TNFα and EGFR ligands, numerous membrane-bound proteins have been identified as TACE substrates (for a list of selected TACE substrates, please refer to Dreymueller et al.[3] and Murphy[1]), indicating that TACE is a critical enzyme involved in ectodomain shedding *in vivo*. However, because of the early lethality of TACE-deficient mice, analysis of the physiological functions of TACE in adult animals remained unaddressed.

TACE Is the Principal Enzyme for Releasing Soluble TNFα *in Vivo*

As described above, the early lethality of TACE-deficient mice was an obstacle in understanding the roles of TACE *in vivo*, and the relevance of TACE as a TNFα sheddase *in vivo* remained to be clarified. To circumvent this issue, I generated a conditional knockout mice using the *Cre*-LoxP system ($Tace^{flox/flox}$ mice).[29] Using $Tace^{flox/flox}$ mice, I first generated conventional knockout mice ($Tace^{-/-}$) and found that they suffered early lethality, with multiple defects in the skin, heart valves, and fur, as previously observed in TACE mutant mice.[23] In a strict sense, the TACE mutant mice reported by Peschon et al.[23] were not TACE null ($Tace^{-/-}$). The mice were generated by targeting the exon that encodes the Zn^{2+}-binding motif, and because the expression of a truncated non-functional TACE protein was detected, the mice are often referred to as $Tace^{\Delta Zn/\Delta Zn}$. Because monocyte/macrophage lineage cells are the major source of TNFα *in vivo*,[30] I crossed $Tace^{flox/flox}$ mice with *LysM-Cre* transgenic mice,[31] in which *Cre* is expressed under the control of the promoter of monocyte/macrophage-specific lysozyme, to specifically disrupt TACE in the monocyte/macrophage lineage. The macrophages obtained from $Tace^{flox/flox}$/ *LysM-Cre* mice expressed low levels of TACE and released lower levels of TNFα on stimulation with LPS *in vitro* com-

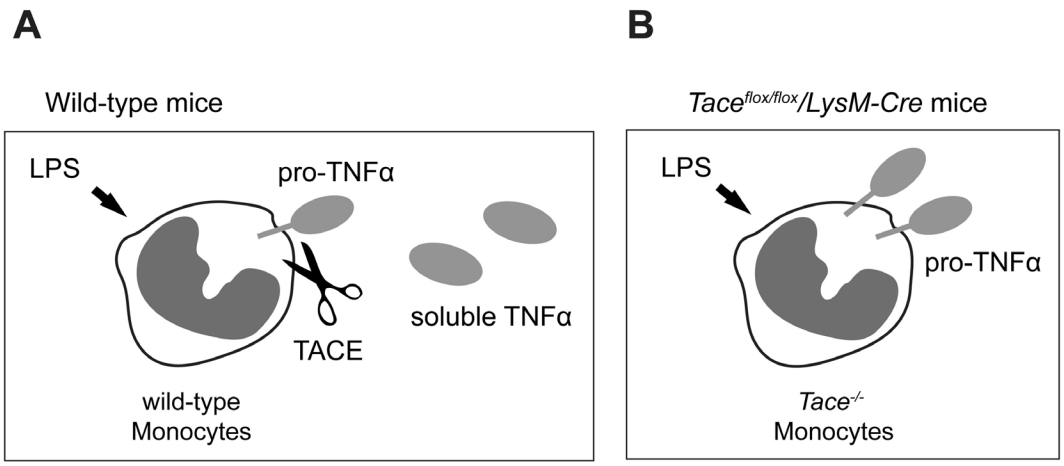

Fig. 2 TACE is essential for releasing TNFα from monocytes/macrophages.
(A) On stimulation with LPS, monocytes/macrophages produce membrane-bound pro-TNFα. Pro-TNFα is then delivered to the cell surface and cleaved by TACE to become soluble and active. (B) In the absence of TACE, pro-TNFα remains on the cell surface and is not fully functional.

pared with wild-type cells. Otherwise, the mutant mice were indistinguishable from their wild-type littermates. To determine whether TACE was responsible for the processing of pro-TNFα *in vivo*, I subjected the mutant mice to a mouse model of LPS-induced endotoxin shock that depends on soluble TNFα. As expected, the *Tace*$^{flox/flox}$/*LysM-Cre* mice were highly resistant to endotoxin shock, whereas most of the control littermates died within the first 16 h after treatment.[29] The serum TNFα levels after LPS injection were also significantly lower in the *Tace*$^{flox/flox}$/*LysM-Cre* mice compared with the wild-type controls (**Fig. 2**).

Taken together, these observations showed that (1) TACE is the principal sheddase for TNFα *in vivo*, (2) TACE expressed in monocyte/macrophage lineage cells plays a critical role in the pathogenesis of endotoxin shock, and (3) the disruption of TACE in these cells confers strong protection against LPS-induced endotoxin shock. The results of this study, I believe, finally provided a conclusive answer regarding which enzyme is primarily responsible for the activation of TNFα *in vivo*.

TACE Is Indispensable for Normal Growth and Adult Homeostasis

The first study using *Tace*$^{flox/flox}$ mice focused on the function of TACE expressed in monocyte/macrophage lineage cells, especially with regard to its relevance as the sheddase for TNFα *in vivo*.[29] However, the roles of TACE in normal growth and adult homeostasis remained to be elucidated. To address this question, I next crossed the *Tace*$^{flox/flox}$ mice with *Sox9-Cre* transgenic mice, in which *Cre* is expressed under the control of the *Sox9* promoter.[32] SOX9 is an essential transcription factor for skeletal development; however, it is also expressed in a wide range of tissues, including the skin, brain, and digestive tract,[33,34] whereas there is little expression in hematopoietic cells and their progenitors. The *Tace*$^{flox/flox}$/*Sox9-Cre* mice (referred to as *Tace/Sox9* mice) were born with open eyes, as observed in the *Tace*$^{-/-}$ mice, but they did not show any defects in their heart valves, and most survived to adulthood. Except for the eyelid defect, *Tace/Sox9* mice appear almost indistinguishable from their wild-type littermates up to approximately 1 week old. However, as they grow older, defects in the skin and hair and growth retardation become conspicuous. The *Tace/Sox9* mice exhibit a highly complex phenotype that includes defects in the skeletal system (bone mass loss and shorter long bones), hematopoietic system (splenomegaly, extramedullary hematopoiesis, and an increase in the number of hematopoietic stem cells), skin (atopic dermatitis-like inflammation), and hair (short, wavy fur). Presumably because of these defects, few mice survive longer than 8 months. Interestingly, I found that the serum levels of granulocyte colony-stimulating factor (G-CSF) were significantly increased in the *Tace/Sox9* mice. Because G-CSF is a potent stimulator of both granulopoiesis and bone resorption, it was assumed that the lack of TACE activity in non-hematopoietic cells (via poorly understood mechanisms) led to the aberrant production of G-CSF and, ultimately, resulted in the hematopoietic defects and bone mass loss observed in *Tace/Sox9* mice.

Because of the severity and complexity of the phenotype of *Tace/Sox9* mice, the existence of a TACE loss-of-function genetic disorder in humans appeared unlikely, and no such case has been reported in the literature. However, surprisingly, a recent study using high-throughput sequencing technology revealed a 4-bp deletion muta-

Table 1 Summary of the phenotypes of TACE mutant mice

Mouse	Phenotype	References
Tace$^{-/-}$ *Tace*$^{\Delta Zn/\Delta Zn}$	Perinatal/embryonic lethality; open eyelids; misshaped heart valves	23, 28, 29
Tace$^{flox/flox}$*/Mx1-Cre*	Resistance to LPS-induced shock; less soluble TNFα production on LPS stimulation; improved insulin resistance and energy homeostasis on high-fat diet; resistance to abdominal aortic aneurysm development	29, 55, 56
Tace$^{flox/flox}$*/LysM-Cre*	Resistance to LPS-induced shock lethality; less soluble TNFα production on LPS stimulation	29
Tace$^{flox/flox}$*/Sox9-Cre*	Open eyelids; growth retardation; elongated growth plates; skin inflammation; reduced bone mass; short, wavy hair; splenomegaly; increased myelopoiesis; early death	32
Tace$^{flox/flox}$*/Tie2-Cre*	Reduced pathological neoangiogenesis; reduced tissue damage in acute lung inflammation	36, 57
Tace$^{flox/flox}$*/HB9-Cre*	Increased Schwann cell myelination	58
Tace$^{flox/flox}$*/Vav-Cre*	Decreased inflammation and alveolar neutrophil recruitment following LPS inhalation; greater resistance to *E. coli* infection	59, 60

tion in the *TACE* gene in siblings (a girl and a boy) born to consanguineous parents.[35] The deletion introduces a frame shift and a premature stop codon, consequently resulting in knockout of the *TACE* gene. The surviving boy (the sister died at 12 years old) exhibited inflammatory lesions in the skin and intestine, left ventricular dilatation, and wiry and disorganized hair. The apparent similarities in the phenotype between the affected boy and *Tace/Sox9* mice indicate that the roles of TACE are highly similar in humans and mice and may further validate TACE mutant mice as a relevant model for studying the functions of TACE in humans. Nevertheless, the striking and complex phenotype observed in the *Tace/Sox9* mice unequivocally shows that ectodomain shedding catalyzed by TACE is indispensable for normal growth and adult homeostasis and that a lack of its activity would lead to a disastrous condition, as illustrated by *Tace/Sox9* mice and the boy with the *TACE* mutation.

Unresolved Issues and the Future Direction of Studies on TACE

Studies in the past decade have established the importance of TACE under physiological conditions, during development, and under many pathological conditions, including cancer,[9] endotoxin shock,[29] and pathological neoangiogenesis[36] (see **Table 1** for a summary of the phenotypes of different TACE mutant mice). These studies suggest that TACE may potentially be a target molecule for the treatment of these disorders; however, considering its numerous substrates and diverse functions *in vivo*, further studies are required to define the role of ectodomain shedding *in vivo* and to understand the biochemical properties of TACE. Some of the fundamental but poorly understood issues regarding the biology of TACE are described below.

How does TACE recognize its substrates?

The specificity of TACE for its substrates appears to be partly dependent on the structure of the extracellular domain and the amino acid sequence of the juxta-membrane domain of the target molecule.[37] TACE cleaves its target molecules approximately 12–16 amino acids from the transmembrane domain; however, no consensus amino acid sequences have been identified for the cleavage site in TACE substrates. Therefore, the substrates for TACE cannot be determined by the amino acid sequence of potential candidate genes, and the identification of the substrates primarily relies on biochemical analyses and cell-based assays (see Overall and Blobel[38] for further discussion). If inhibition of the ectodomain shedding of a given molecule is to be used clinically, it will be necessary to devise a method to selectively inhibit the shedding of the target molecule to avoid potential side effects. An understanding of the mechanisms of substrate recognition for TACE will facilitate the pharmaceutical design of such an agent.

How is the activity of TACE regulated in vivo?

Before delivery to the cell surface, the prodomain of TACE is cleaved by furin protease to allow TACE to become proteolytically active (**Fig. 1A**). Interestingly, recent studies have revealed that iRhom2, a proteolytically inactive member of the rhomboid protease family, is required to promote the trafficking of TACE in immune cells,[39,40] suggesting that iRhom2 is a critical regulator involved in the maturation of TACE, at least in immune cells. In addition, it is widely accepted that the shedding activity of TACE can be rapidly up-regulated by various stimuli and intracellular signaling, including a protein kinase C stimulator (phorbol ester), ultraviolet light, osmotic pressure, and MAP kinases, without increasing the amount of mature TACE protein on the cell surface.[37,41,42]

These observations indicate that TACE can be rapidly activated by various stimuli from the surrounding milieu, presumably by changing its protein conformation. Of note, a recent study showed that the transmembrane domain, but not the cytoplasmic domain, of TACE is required for this activation, suggesting that the activation of TACE is not dependent on intracellular signaling via the phosphorylation of its cytoplasmic domain.[43] Regardless, the mechanism by which TACE recognizes stimuli and becomes catalytically active remains poorly understood. Because suppression of the stimulated shedding of TACE and inhibition of the maturation of TACE are potential therapeutic targets, it will be necessary to understand how and under what circumstances TACE is activated and processed under both physiological and pathological conditions.

What is the function of ectodomain shedding in general?

For TNFα and EGFR ligands (such as TGFα and HB-EGF), the consequence of ectodomain shedding by TACE is relatively clear. These proteins are, in principle, inactive in their membrane-bound pro-from and are activated upon shedding. However, there are several cytokines (such as the cell surface isoform of colony stimulating factor-1) that are active in both their membrane-bound form and their soluble form.[44,45] Some receptors, including vascular growth factor receptor 2 and *c-Kit*, are also subjected to ectodomain shedding,[46,47] and in theory, the cleavage of these receptors results in the down-regulation of the availability of these receptors and, consequently, signaling through these receptors. In contrast, the interleukin 6 receptor, which has also been shown to be released by TACE, acts as a functional soluble receptor via binding to interleukin 6 and gp130.[48] Moreover, there are several cases [e.g., TNFα and its receptors (TNFR1 and TNFR 2)] in which both the membrane-bound ligand and its receptor are cleaved by TACE. Thus, the consequences of ectodomain shedding are highly context dependent and can be different or even opposite when different targets are involved. Therefore, the outcome of TACE inhibition in disorders suspected to be causally related to aberrant TACE activity may not always be as predicted theoretically. For these reasons, it will be essential to understand the roles of TACE in such disorders in as much detail as possible before attempting any clinical trial.

Concluding Remarks

TACE, an enzyme once thought to be solely involved in the processing of pro-TNF, is now considered to be one of the central components of ectodomain shedding *in vivo*, with many identified target molecules, and most likely even more unidentified target molecules. In addition, ADAM10, one of the ADAM gene family members most closely related to TACE (**Fig. 1B**), has also emerged as a major player in the field of ectodomain shedding.[3,49–51] Although these two ADAM proteins appear to have common target molecules, it is becoming clear that each has specific substrates and functions. Emerging data suggest that ADAM10 plays central roles in the activation of Notch signaling and the processing of amyloid precursor protein and cadherins.[50–54]

As described in this review, studies during the past two decades have launched the field of ectodomain shedding; however, we are left with more questions than answers. Further studies are required to address the unresolved issues in this rapidly evolving field. Considering the diverse and fundamental functions of TACE and ADAM10 and their target molecules (e.g., EGFR ligand, TNFα, Notch, and amyloid precursor protein), it is certain that ectodomain shedding is involved in development and homeostasis and also in the pathology of many disorders. Therefore, further investigation and a deeper understating of the roles of ectodomain shedding will be of great importance and relevance in both basic and biomedical research in the coming years.

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