Functional Role of Asparaginyl Endopeptidase Ubiquitination by TRAF6 in Tumor Invasion and Metastasis

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Background
Asparaginyl endopeptidase (AEP) has been implicated in human cancer development. However, the molecular mechanisms underlying AEP regulation, including the role of pro-AEP activation, remain elusive.

Methods
We investigated the regulation of AEP by TRAF6 and its effects on tumor progression and metastasis in cancer cell lines, murine models, and specimens from patients using biochemical analyses, confocal microscopy, immunoelectron microscopy, and migration-invasion assays. The sera of healthy donors and breast cancer patients were examined by enzyme-linked immunosorbent assay, and a tissue array of 314 breast cancer specimens was assessed for AEP and TRAF6 by immunohistochemistry. Furthermore, the effects of AEP inhibitors or monoclonal antibodies on pulmonary metastasis were evaluated in murine models. The statistical significance between groups was determined using two-tailed Student t tests.

Results
We demonstrate that TRAF6 ubiquitinates the proform of AEP through K63-linked polyubiquitin, reversible by USP17, and forms a complex with HSP90α to subsequently promote pro-AEP intracellular stability as well as secretion. Disrupting the interaction between pro-AEP and TRAF6 or inhibiting HSP90α reduced pro-AEP secretion and consequently reduced tumor metastasis. Higher circulating AEP levels were detected in the sera of breast cancer patients, and AEP inhibitors or neutralizing antibodies remarkably decreased tumor metastasis in murine models. Notably, TRAF6 and AEP were overexpressed in human breast neoplasms and correlated with poor prognosis. Patients with low AEP/TRAF6 expression survived for a mean of 111 months (95% confidence interval [CI] = 108 to 115 months), whereas those with high AEP/TRAF6 expression survived for a mean of only 61 months (95% CI = 42 to 79 months; P < .001).

Conclusions
Our study elucidates a novel mechanism of AEP regulation and an alternative oncogenic pathway for TRAF6 in breast cancer, which suggests that AEP and TRAF6 protein levels may have prognostic implications in breast cancer patients. Thus, AEP may serve as a biomarker as well as new therapeutic target.


Proteases perform essential functions in living organisms and excessive proteolysis contributes to cancerogenesis, including tumor growth, angiogenesis, and metastasis (1–5). However, clinical studies of protease inhibitors to treat cancer invariably failed because of severe side effects or lack of statistically significant clinical benefits (2,6). Therefore, better understanding of the intricate mechanisms governing protease regulation and function will be extremely valuable.

AEP, the only known asparaginyl endopeptidase in mammalian genomes, is a unique member of the C13 family peptidases with strict specificity for asparagine bond cleavage (7.8) and has been shown to play crucial roles in kidney physiology, immunity, atherogenesis, and bone metabolism (9–15). Recently, AEP overexpression was observed in solid tumors and acute lymphoblastic leukemia (16–21). Cancer cells with increased AEP expression exhibit enhanced migratory and invasive capabilities through activation of pro-MMP2 and cathepsins (17,22,23), but underlying mechanisms for post-translational regulation augmenting AEP’s role remain elusive. Like all endosomal proteases, AEP is synthesized as an inactive zymogen, and activation is autocatalytic requiring sequential removal of C- and N-terminal propeptidase from pro-AEP. Cellular processing involves at least one additional cleavage event to yield the final mature lysosomal enzyme, active-AEP (24).

Conjugation of ubiquitin monomers to cellular proteins by ubiquitination enzymes E1, E2, and E3 has been recognized as important post-translational modification with profound effects on protein stability, trafficking, and interaction (25,26). Of these three enzymes, E3 ligases confer substrate specificity (27,28). Tumor necrosis factor receptor-associated factor 6 (TRAF6) is such an E3 ligase that mediates conjugation of Lysine-63 (K63)–linked polyubiquitin chains to proteins (29–31). In recent years, TRAF6 has been suggested as an
oncogene based on its role in Akt ubiquitination and other pathways (32,33). In addition, amplification of the TRAF6 locus is a frequent event in several human cancer types (34). However, substrates of TRAF6 in neoplasia are poorly characterized.

Heat shock proteins (HSPs) are stress-inducible, molecular chaperones aiding protein structure and stability (35). Moreover, HSP90α is expressed extracellularly, where it interacts with proteases and promotes tumor invasiveness and metastasis (36,37). We now present evidence that AEP is a substrate for TRAF6 ubiquitination, resulting in AEP/TRAF6/HSP90α complex formation. In addition, our findings show that secreted AEP is involved in metastasis in AEP-overexpressing cancers. Our data also suggest that AEP might serve as a new biomarker and therapeutic target for breast cancer.

**Methods**

**Patient Data**

Breast cancer tissues and peripheral blood were collected from patients and healthy volunteers at Shanghai Ruijin Hospital, Shanghai Jiao Tong University, School of Medicine after obtaining the subjects’ informed consent and institutional review board approval. A total of 314 surgical tissue samples of breast cancer were collected at the Department of Radiation Oncology, Ruijin Hospital. The patient’s age, American Joint Committee on Cancer stage, molecular classification, and so on were obtained from the hospital (Supplementary Table 1, available online). The median age was 53 years (range = 31–85).

**Cell Lines**

HEK293T, MCF10A, and breast carcinoma cell lines MCF7 and MDA-MB-231 were cultured in Dulbecco’s modified Eagle medium (HyClone, Logan, UT) supplemented with 10% fetal bovine serum in 5% carbon dioxide–humidified atmosphere at 37°C.

**Plasmids and Antibodies**

Hemagglutinin (HA)-tagged pro/active-AEP, FLAG-tagged TRAF6, or respective mutants were cloned into pcDNA3.1, pET28a, or pMSCV. Antibodies used in the study include the following: goat antihuman AEP, sheep antimouse AEP, goat anti-HSP90, rabbit anti-HSP70 (R&D, Minneapolis, MN); mouse anti-TRAF6, mouse anti-FLAG, rabbit anti-FLAG, mouse anti-HA, rabbit anti-HA, and mouse anti-ubiquitin (Santa Cruz Biotechnology, Santa Cruz, CA); and mouse anti-ubiquitin and rabbit anti-K63-ubiquitin (Millipore, Bedford, MA).

**Coimmunoprecipitation and Immunoblotting Analysis**

Protein extraction was followed by immunoprecipitation and immunoblotting with antibodies as described (see the Supplementary Methods, available online, for more detail) (32).

**In Vitro Ubiquitination Assays**

In vitro ubiquitination assays were performed as described (see the Supplementary Methods, available online, for more detail) (32).

**In Vitro Deubiquitination Assays.** Immunoprecipitated pro-AEP was prepared from HEK293T cells overexpressing pro-AEP. FLAG-tagged USP17 or DUB3 was purified by immunoprecipitation with FLAG beads. Pro-AEP protein was mixed with USP17 or DUB3 samples in 20-μL reaction (50mM Hepes [pH 8.0], 3 mM dithiothreitol). Reactions were performed at 30°C for 60 minutes with agitation.

**In Vitro Invasion Assays.** In vitro invasion assays were performed as described (38). AEP inhibitors were preincubated with conditioned medium for 30 minutes before addition to cell culture (see the Supplementary Methods, available online, for more detail).

**In Vivo Treatments and Analysis of Tumor Metastasis**

**Formation**

Six-week-old female nude mice were purchased from Slaccas Company (Shanghai, China). To experimentally simulate metastasis, cells were injected into tail veins, and mice were randomized and treated with saline, purified pro-AEP (4 μg/mouse/injection), mouse antihuman AEP antibody (2 μg/mouse/injection; R&D, MAB2199) or AEP inhibitors twice a week for 10 weeks. All mice were killed using carbon dioxide, and lungs were removed and fixed in Bouin’s solution. Lung micrometastases larger than 0.5 mm in diameter in five lobes were counted using an anatomy microscope. Every group included six to eight mice, and the experiment was repeated three times. All animal studies were approved by the Institutional Animal Care and Use Committee of the Shanghai Institutes for Biological Sciences.

**Enzyme-Linked Immunosorbent Assay**

AEP concentrations in the conditioned medium or serum were measured using enzyme-linked immunosorbent assay as previously described (see the Supplementary Methods, available online, for more detail) (39).

**Histological and Immunohistochemical Analysis**

Histological and immunohistochemical analyses were performed as previously described (17). Goat antihuman AEP antibody (R&D) or mouse anti-TRAF6 antibody (Santa Cruz Biotechnology) diluted 1:50 in blocking buffer were used as primary antibodies. Normal goat or mouse immunoglobulin G (R&D) were included as negative controls. Three pathologists from Ruijin Hospital individually scored blinded samples before drawing conclusions.

**Statistics**

Log-rank tests were performed to identify each risk factor associated with patient outcome. Multivariable analyses were performed to assess patient outcome, and the covariables included tumor/node metastasis/distant metastasis, American Joint Committee on Cancer stage, age, menopause, estrogen receptor, progesterone receptor, Her2, and molecular classification. Kaplan–Meier methodology was used to estimate survival curves for human patients. The Kaplan–Meier survival analysis was generated using SPSS version 13.0 (SPSS Inc., Chicago, IL). Differences in AEP protein levels in breast cancer patient sera and healthy volunteers were analyzed using Mann–Whitney U test.

Two-tailed Student t tests were used to analyze differences between protein overexpression and knockdown groups from in vitro and in vivo experiments. One-way analysis of variance was initially performed to determine whether overall statistically significant changes occurred before using two-tailed paired or unpaired Student t tests. Multiple test-adjusted P values of less than .05 were considered statistically significant. All statistical tests were two-sided.
Figure 1. Analysis of the interaction between asparaginyl endopeptidase (AEP) and TRAF6 in cancer cells. **A** Immunoblot (IB) analysis of conditioned medium and lysates of a panel of breast cancer cell lines and immortalized breast epithelial cell line (MCF10A), probed with antibodies to AEP and actin. **B** Presence of a TRAF6-binding motif (XXPXEXXAr/Ac) in human and mouse AEP. **C** Two-way co-immunoprecipitation (Co-IP) of hemagglutinin-tagged pro-AEP (HA-pro-AEP) and FLAG-tagged TRAF6 (FLAG-TRAF6) expressed in HEK293T cells. **D** Co-IP of hemagglutinin (HA)-tagged pro-AEP (HA-pro-AEP) or HA-tagged active AEP and FLAG-tagged TRAF6 (FLAG-TRAF6) expressed in HEK293T cells. **E** Co-IP of endogenous AEP with TRAF6 in MDA-MB-231 cells. IgG = immunoglobulin G. **F** Coelution of AEP and TRAF6 in lysates of MDA-MB-231 cells by size exclusion chromatography. **G** Representative confocal images of the colocalization between endogenous AEP (green) and TRAF6 (red). Scale bar = 10 μm. **H** Electron microscopy showing immunogold-labeled AEP (5-nm gold particle; arrowhead) and TRAF6 (20-nm gold particle; arrow) localized closely in MDA-MB-231 cells. Scale bar = 50 nm. **I** Mapping of the interaction domain of TRAF6 with AEP by GST pull-down. Purification of GST or GST fusion proteins was confirmed by Coomassie staining. **J** Co-IP of FLAG-TRAF6 with wild-type AEP but not TRAF6 binding mutant AEP (E214A). Data are representative of three independent experiments.
Results

Analysis of the Interaction Between AEP and TRAF6 in Cancer Cells

AEP is synthesized as inactive zymogen (pro-AEP; 56 kDa) and undergoes multistep activation resulting in mature enzyme (active AEP; 36 kDa) (40). Although AEP mainly exists in the active form in normal kidney tissue (41) and macrophages (Supplementary Figure 1A, available online), the majority of AEP detected in breast cancer cells was the proform (Figure 1A). Quantification established that active AEP was less than 10% of the proform amount (Supplementary Figure 1B, available online). Sequence analyses of both human and mouse AEP proteins identified a potential TRAF6-binding motif, P-X-E-X-X-(Aromatic/Acidic) (Figure 1B) (42). Coimmunoprecipitation analysis confirmed that HA-pro-AEP interacted with TRAF6 (Figure 1C). Notably, AEP was present in both forms when overexpressed in HEK293T cells (Supplementary Figure 1C, available online).

Figure 2. K63-linked pro-asparaginyl endopeptidase (AEP) polyubiquitination by TRAF6 in cancer cells. A) K63-linked ubiquitination of AEP in MDA-MB-231 cells. Shown is immunoblot (IB) analysis of immunoprecipitated (IP) pro-AEP for the presence of ubiquitin (Ub; left panel), K63-linked ubiquitin (Ub K63; middle panel), and AEP (right panel). Asterisk (*) indicates nonspecific band of immunoglobulin G (IgG) heavy chain. B) In vitro ubiquitination assay with purified wild-type or mutant AEP proteins along with purified TRAF6 incubated with the indicated reaction components in the ubiquitin reaction. In vivo ubiquitination assay (C and D). IB analysis of ubiquitination of AEP in HEK293T cells transfected with HA-Ub, AEP along with FLAG-TRAF6 (C). IB analysis of K63-linked ubiquitin modification of AEP in HEK293T cells transfected with HA-Ub along with wild type AEP or mutant AEP E214A (D). E) IB analysis of K63-linked ubiquitination of AEP in MDA-MB-231 cells with TRAF6 knockdown, AEP knockdown or control.

H) Co-immunoprecipitation of AEP-HA with FLAG-tagged USP17 in HEK293T cells.

G) Coelution of AEP and USP17 in lysates of MDA-MB-231 cells.

I) USP17 deubiquitinates AEP in vitro.

J) USP17 deubiquitinates AEP in vivo. IB analysis of K63-linked ubiquitination of AEP in HEK293T cells transfected with AEP along with TRAF6, USP17, USP17 C89S, or DUB3. Data are representative of three independent experiments.
but TRAF6 was found to only interact with pro-AEP (Figure 1D; Supplementary Figure 1D, available online).

Screening of breast cancer cell lines revealed that both pro-AEP and TRAF6 were highly expressed in MDA-MB-231 cells (Supplementary Figure 1E, available online). Although AEP is usually found in lysosomes/acidic compartments, it displays three different staining patterns in breast cancers: diffuse cytoplasmic positivity, tiny dots within the cytoplasm, and vesicles in cytoplasm (19). Immunelectron microscopy confirmed these findings (Supplementary Figure 1F, available online). Subcellular fractionation results further indicated that pro-AEP was present in the endosome/lysosome/mitochondria and cytosol fractions with TRAF6 (Supplementary Figure 1G, available online). Endogenous interaction between pro-AEP and TRAF6 was detected in MDA-MB-231 cells by coimmunoprecipitation analysis and size exclusion chromatography (Figure 1, E and F). In addition, immunofluorescent staining and immunelectron microscopy microscopy that endogenous AEP and TRAF6 colocalized in distinct cytoplasmic regions in neoplastic cells (Figure 1, G and H).

TRAF6 contains three functional domains—namely, the TRAF-C, RING, and coiled-coil domains (43). Glutathione S-transferase (GST) pull-down assays revealed that the TRAF-C domain was the primary binding domain for AEP (Figure 1I). In contrast with wild-type (WT) pro-AEP, the TRAF6-binding motif mutant pro-AEP[E214A] exhibited reduced binding to TRAF6 (Figure 1J). Taken together, these results indicate that intracellular pro-AEP physically interacts with TRAF6 in cancer cells.

K63-Linked Pro-AEP Polyubiquitination by TRAF6 in Cancer Cells

Interestingly, endogenous pro-AEP was found to be ubiquitinated in a K63-linked manner in MDA-MB-231 cells (Figure 2A), whereas its secreted form was sparsely ubiquitinated (Supplementary Figure 2, available online). As expected, extracellular TRAF6 was not detected (Supplementary Figure 1D, available online). Indeed, purified TRAF6 directly catalyzed K63-linked poly-ubiquitination of pro-AEP but not of the pro-AEP[E214A] mutant in vitro (Figure 2B). Using mutagenesis scanning of the 13 conserved lysine residues in pro-AEP, lysine-318(K318) was identified as the major ubiquitination residue because the pro-AEP[K318R] mutant was not ubiquitinated in vitro (Figure 2B). These in vitro results were confirmed in overexpression experiments in HEK293T cells (Figure 2, C and D), whereas TRAF6 depletion using shRNA blocked pro-AEP ubiquitination (Figure 2E).

USP17 was predicted as deubiquitinating enzyme reversing AEP ubiquitination (44). We validated the interaction between USP17 and pro-AEP by immunoprecipitation analyses and size exclusion chromatography (Figure 2, F and G). To test whether ubiquitinated pro-AEP was a substrate of USP17, we performed in vitro deubiquitination assays, which showed that purified USP17, but not DUB3, attenuated pro-AEP ubiquitination (Figure 2H). Accordingly, WT USP17, but not deubiquitase inactive mutant USP17C99S, remarkably attenuated K63-linked pro-AEP ubiquitination in cells (Figure 2I). Taken together, these results indicate that TRAF6 is an E3 ligase of pro-AEP polyubiquitination, which can be reversed by USP17.

Figure 3. Protein levels of intracellular and extracellular pro–aparaginyl endopeptidase (AEP). A) Immunoblot (IB) analysis of AEP protein levels in conditioned media (CM) or lysates of MDA-MB-231 cells overexpressing TRAF6 or control. B) IB analysis of AEP protein levels in CM or lysates of MDA-MB-231 cells with TRAF6 knockdown, AEP knockdown or control. C) IB analysis of AEP protein levels in CM or lysates of MDA-MB-231 cells overexpressing FLAG-tagged wild-type TRAF6, TRAF6 C89S, or TRAF6 K124R mutant. D) IB analysis of AEP protein levels in CM or lysates of HEK293T cells overexpressing wild-type AEP, AEP E214A, or AEP K318R with or without FLAG-taggedTRAF6. E) IB analysis of CM and lysates of MDA-MB-231 cells overexpressing wild-type ubiquitin, lysine single mutation K48R, or K63R. F) IB analysis of CM and lysates of HEK293T cells transfectedTRAF6 along with USP17, USP17 functional mutant C89S, or control. DUB3 served as a DUB control. Data are representative of three independent experiments.
Protein Levels of Intracellular and Extracellular Pro-AEP

Intriguingly, increased intracellular and extracellular pro-AEP levels were observed when TRAF6 was overexpressed in MDA-MB-231 cells (Figure 3A; Supplementary Figure 3A, available online), whereas TRAF6 depletion substantially reduced pro-AEP levels (Figure 3B; Supplementary Figure 3B, available online). However, TRAF6 did not alter AEP mRNA levels (Supplementary Figure 3C and D, available online), suggesting that the observed increase in pro-AEP abundance was mainly due to post-transcriptional mechanisms. Compared with WT TRAF6 or the auto-ubiquitination inactive TRAF6K12R mutant (43), the RING domain-truncated form of TRAF6 (TRAF6NR), TRAF domain alone (TRAF6-C), or E3 ligase inactive TRAF6C70A mutant did not increase intracellular or extracellular pro-AEP protein levels, indicating that E3 ligase activity was required for TRAF6 to increase pro-AEP levels (Figure 3C; Supplementary Figure 3E, available online). In accordance with the observed reduced ubiquitination (Figure 2B), both pro-AEPE214A and pro-AEPK318R mutants resulted in lower pro-AEP levels (Figure 3D), further suggesting that the K63-linked-ubiquitination was involved in pro-AEP stabilization. Additionally, USP17 expression blocked the TRAF6-enhanced protein levels of pro-AEP (Figure 3F). Taken together, these results indicate that TRAF6-mediated ubiquitination elevates pro-AEP protein levels.

Functional Studies of Interactions Between Ubiquitinated Pro-AEP and HSP90α.

To further understand how TRAF6-mediated pro-AEP ubiquitination stabilized intracellular pro-AEP and promoted secretion, we identified additional proteins that associated with the pro-AEP/TRAF6 complex. Mass spectrometry analysis revealed that HSP90α interacts with both pro-AEP and TRAF6 (Supplementary Tables 4 and 5, available online). Indeed, endogenous or overexpressed HSP90α was found to form a complex with AEP/TRAF6 (Figure 4, A and E). Suppressing HSP90α activity using siRNA or inhibitors led to reduced pro-AEP stability and secretion (Figure 4B and C), whereas modulation of HSP70 had no effect (Figure 4D). However, TRAF6C70A failed to promote complex formation, indicating that recruitment of HSP90α to pro-AEP requires TRAF6 E3 activity (Figure 4E). Taken together, these data suggest that ubiquitination of pro-AEP by TRAF6 recruits HSP90α, which increases intracellular stability and secretion of pro-AEP.

Figure 4. Functional studies of interactions between ubiquitinated pro-asparaginyl endopeptidase (AEP) and HSP90α. A) Co-immunoprecipitation (Co-IP) of endogenous AEP with TRAF6 and HSP90α in MDA-MB-231 cells. IgG = immunoglobulin G. B) Immunoblot (IB) analysis of AEP protein levels in conditioned media (CM) or lysates of MDA-MB-231 cells with HSP90α knockdown or control. C) IB analysis of AEP protein levels in CM or lysates of MDA-MB-231 cells treated with HSP90α inhibitors (17-AAG). Data are representative of three independent experiments.
Role of Pro-AEP Ubiquitination by TRAF6 for Tumor Progression

Overexpression of WT AEP statistically significantly enhanced migration of MDA-MB-231 cells (WT: mean migration rate = 43.17, standard deviation [SD] = 5.26%; Overexpression: mean migration rate = 75.07, SD = 3.48%; P < .001), whereas AEP\textsuperscript{E214A} or AEP\textsuperscript{K318R} mutants did not (Figure 5A, top panel). Similar results were seen for migration (Figure 5A, bottom panel). In addition, knockdown of AEP or TRAF6 inhibited migration and invasion (Supplementary Figures 4 and 5, available online). To further examine the role of AEP in tumor metastasis in vivo, we injected MDA-MB-231 cells into nude mice, resulting in a substantial number of metastatic pulmonary nodules (Figure 5, B–D; Supplementary Figure 6A, available online). However, knockdown of AEP reduced the number of metastatic pulmonary nodules more than 10-fold, which could be rescued by the restoration of WT AEP, but not by mutant AEP\textsuperscript{E214A} or AEP\textsuperscript{K318R} (WT: mean number of lung metastases = 45.33, SD = 7.28; Knockdown: mean number of lung metastases = 3, SD = 1.46; Rescue: mean number of lung metastases = 42.33, SD = 6.26; AEP\textsuperscript{E214A}: mean number of lung metastases = 3.8, SD = 1.70; AEP\textsuperscript{K318R}: mean number of lung metastases = 2.86, SD = 1.06; P < .001) (Figure 5, C and D; Supplementary Figure 6A, available online). Notably, TRAF6 depletion completely inhibited metastatic pulmonary nodule formation, which could be restored by restoring TRAF6 expression (Figure 5, C and D; Supplementary Figure 6A, available online). Taken together, both the in vitro and in vivo results indicate that polyubiquitination of AEP by TRAF6 promotes tumor progression.

Overexpression of enzymatic inactive AEP\textsuperscript{C189S} in cells and rescue of AEP knockdown cells with AEP\textsuperscript{C189S} failed to promote cell migration and invasion (Supplementary Figure 7A, available online).

![Figure 5](https://academic.oup.com/jnci/article-abstract/106/4/dju012/913989/fig5)

**Figure 5.** Role of pro-asparaginyl endopeptidase (AEP) ubiquitination by TRAF6 for tumor progression. A) Cell migration (upper panel) and Matrigel-transwell invasion (lower panel) analysis of MDA-MB-231 cells stably expressing wild-type (WT) AEP or AEP\textsuperscript{E214A}, AEP\textsuperscript{K318R}, B–D) AEP and TRAF6 knocked down MDA-MB-231 cells (lung metastasis subclone; LM2), which were subsequently rescued with AEP, TRAF6, or various AEP mutants. Original nucleotide sequence of AEP containing targeted knockdown sequence served as rescue control. Lung metastatic nodules from each group were dissected and analyzed by immunoblot (IB) with the indicated antibodies. Representative Western blot analysis was shown B. Lung metastases were counted 8 to 9 weeks after inoculation of indicated cells through tail vain. Representative hematoxylin and eosin sections and photos re shown (C), and metastasis nodules were counted under anatomy microscope (n = 6–8 per group). Scale bar = 1 mm. (D) Values are mean ± standard deviation. P < .001 (two-sided Student t test).
online), while also failing to rescue wild-type AEP function in lung metastatic models (Supplementary Figure 7B, available online). These results strongly suggest that the enzymatic activity of AEP is essential for its role in metastasis.

Pro-AEP has been demonstrated to undergo a multistep autoactivation process under acidic conditions (40). However, ubiquitinated pro-AEP prevented cellular autoactivation (Supplementary Figure 2A, available online) possibly through occlusion of the autoactivation site N323 by the proximate polyubiquitin chain at K318. Once secreted, pro-AEP was sparsely ubiquitinated (Supplementary Figure 2, B and C, available online), indicating that ubiquitinated AEP is an intermediate regulatory form. Pro-AEP was secreted under stress conditions, such as starvation and hydrogen peroxide stimulation (Supplementary Figure 10A, available online). However, detailed understanding of the pro-AEP secretion process will require further investigation.

Figure 6. Functional role of secreted asparaginyl endopeptidase (AEP) in tumor progression. A) Cell migration analysis of MDA-MB-231 cells cultured with gradually increased amount of conditioned medium (CM; 0%, 2.5%, and 25% v/v) from AEP-overexpressing cells with or without AEP inhibitor (AEPI). B) Matrigel-transwell invasion analysis of MDA-MB-231 cells cultured with CM (25% v/v) from AEP overexpressing cells with or without AEPI. C) Matrigel-transwell invasion analysis of MDA-MB-231 cells cultured with recombinant and purified AEp. 0.1% fetal bovine serum (FBS) served as a positive control. D) Lung metastatic models were established as shown in Figure 5B. Mice were given 100 μg AEPI/100 μL saline, AEP protein, or monoclonal antibody (n = 6–8 per group) by intravenous injection with 2-day intervals for 4 weeks since day 4 after cell injection. E) Representative hematoxylin and eosin staining analysis and photos of lung metastasis are shown. Scale bar = 1 mm. The numbers of lung nodules were counted under anatomy microscope. Values are mean ± standard deviation. P < .001 (two-sided Student t test).
Functional Role of Secreted AEP in Tumor Progression

Next, the role of circulating AEP in tumor progression was examined. Analyzing AEP levels in sera from mice with experimental lung metastases revealed that circulating AEP levels associated with the number of metastatic pulmonary nodules (Figure 5D, Supplementary Figure 6B, available online). Also, when cultured in conditioned medium from cells overexpressing AEP, cancer cells showed dose-dependent enhanced migration (conditioned medium containing 25% empty vector vs conditioned medium containing 2.5% or 25% circulating AEP; \( P < .001 \) for both) and invasion (conditioned medium containing 25% empty vector vs conditioned medium containing 25% circulating AEP; \( P < 0.001 \) (Figure 6, A and B). Additionally, purified recombinant AEP also promoted cancer cell invasion (Figure 6C) and lung metastasis in vivo (Figure 6, D and E). Preincubation of conditioned medium with hydrophilic, poorly membrane permeable AEP-specific inhibitors (Supplementary Figure 8A, available online) (44,45) diminished cancer cell migration and invasion capability (Figure 6, A and B). In addition, blocking circulating AEP by an AEP inhibitor or anti-AEP antibody statistically significantly inhibited tumor progression and metastasis (AEP: mean number of lung metastases = 44, SD = 14.32; AEP inhibitor: mean number of lung metastases = 7.2, SD = 2.1; anti-AEP antibody: mean number of lung metastases = 7.07, SD = 2.01; \( P < .001 \)) (Figure 6, D and E; Supplementary Figure 8B, available online). These results strongly suggest that secreted AEP promotes tumor progression.

Relationship Between AEP and TRAF6 and Prognosis in Human Breast Cancer

To evaluate the potential clinical implications of AEP and TRAF6 in cancer prognosis and diagnosis, we compared AEP and TRAF6 expression in breast cancer tissues and adjacent normal tissues. Concordant with published reports (19,38), AEP was overexpressed in breast cancer tissues (Figure 7A; Supplementary Figure 9, available online). Whereas TRAF6 mRNA expression was upregulated in breast cancer according to the Cancer Genome Atlas database (Supplementary Figure 9D, available online), we confirmed that TRAF6 protein was overexpressed (Figure 7A) and that AEP interacted with TRAF6 and was ubiquitinated by K63-linked polyubiquitin chains in breast tumor tissue (Figure 7, B and C). AEP levels were also significantly higher in sera of cancer patients compared with those of healthy volunteers and benign fibroadenoma patients (Figure 7D).

Immunohistochemical analyses of 313 human breast cancer specimens showed that protein levels of AEP and TRAF6 were correlated (AEP Spearman correlation coefficient = 0.414; \( P < .001 \)) (Figure 8A; Supplementary Figure 9, available online). Furthermore, TRAF6 or AEP expression levels were statistically significantly associated with patient survival when patients were grouped by low or high expression of TRAF6 or AEP (TRAF6: hazard ratio \([\text{HR}]=2.26,95\%\text{ confidence interval }[\text{CI}]=1.23 \text{ to } 3.30, P < .001; \) AEP: \([\text{HR}]=3.6,95\%\text{ CI }=2.32 \text{ to } 4.88, P < .001\) (Figure 8, B and C; Supplementary Tables 6 and 7, available online). The mean survival

Figure 7. Functional role of secreted asparaginyl endopeptidase (AEP) in tumor progression. A) Immunoblot (IB) analysis of pro-AEP and TRAF6 expression in paired breast tumors (T) and adjacent normal tissues (N) \((n = 4)\). B) Co-immunoprecipitation (Co-IP) analysis of the endogenous interaction between AEP and TRAF6 in breast tumors (T). Asterisk (*) indicates nonspecific band of immunoglobulin G (IgG) heavy chain. C) Immunoprecipitation (IP) analysis of K63-linked ubiquitination of AEP in breast tumors (T). D) AEP protein levels in serum of healthy volunteers \((n = 28)\), benign fibroadenoma patients \((n = 26)\), or malignant breast cancer patients \((n = 64)\) were determined by enzyme-linked immunosorbent assay \((\text{upper panel})\) or IB \((\text{lower panel})\). Values are mean ± standard deviation. \( P < .001 \) (two-sided Student \( t \) test). Ub = ubiquitin.
of patients decreased when tumors had high AEP and/or TRAF6 levels (Figure 8, B–D). Patients with low AEP/TRAF6 expression survived for a mean of 111 months (95% CI = 108 to 115 months), whereas those with high AEP/TRAF6 expression survived for a mean of only 61 months (95% CI = 42 to 79 months; \( P < .001 \)). These results demonstrate that AEP and TRAF6 are overexpressed in breast cancer and may have prognostic value.

### Functional Role of TRAF6 During Stress Responses

Upregulation of TRAF6 during stress conditions (Supplementary Figure 10, A and B, available online) enhances K63-linked pro-AEP polyubiquitination, which in turn recruited HSP90\(\alpha\) to stabilize pro-AEP. TRAF6 overexpression also increased endosomal and decreased lysosomal AEP localization (Supplementary Figure 10, D–H, available online). Moreover, proteasome inhibition using MG132 did not affect AEP protein levels, whereas lysosomal acidification inhibitors (ammonium chloride and chloroquine), which inhibited AEP maturation/turnover in the lysosomes, only increased intracellular but not secreted AEP levels (Supplementary Figure 10I, available online). We postulate that K63-linked polyubiquitination decreased pro-AEP lysosomal degradation and promoted secretion through microvesicles because more than 80% of pro-AEP released by cancer cells was found in microvesicles (Supplementary Figure 11, available online). Moreover, HSP90\(\alpha\) was found in complex with pro-AEP in patient sera (Supplementary Figure 11D, available online). However, the precise mechanism of AEP secretion requires further investigation.

### Discussion

Although AEP is usually found in the late endosomal/lysosomal compartments (16,41), it has also been implicated in cancer cell proliferation (21,47,48). Our findings confirm that AEP is distributed in both the endosome/lysosome and cytoplasm of cancer cells, in accordance with previous reports (19). More importantly, we found that pro-AEP coexists with TRAF6 in the cytoplasmic fraction. We further demonstrate that pro-AEP is polyubiquitinated by TRAF6 in the cytosol (Figure 9). Although lysosomal proteases have been reported to enter the cytosol during stress conditions, the mechanism of this rerouting remains elusive (49). We hypothesize that pro-AEP likely enters the cytosol after its synthesis in the endoplasmic reticulum (ER) because pro-AEP is modified by glycosylation, which occurs in route through the lysosomal pathway during biosynthesis. Mass spectrometry results showed that AEP can interact with transport complex members, such as Sec23, which may help translocation from the ER/endosomes to the cytosol. Moreover, AEP has been reported to oscillate between lysosomal and secretory pathways (50). Therefore, we hypothesize that pro-AEP may escape from the endocytic/secretory pathway to the cytosol at a later stage.
Higher levels of AEP were found in sera from breast cancer patients compared with healthy control subjects. Also, serum AEP was found to promote tumor invasion and metastasis whereas abolishing AEP activity by inhibitors or antibodies diminished tumor metastasis in mice.

Previous reports indicated that AEP expression positively correlates with clinicopathologic and biological variables in colorectal cancer and that TRAF6 is a novel oncogene (18). Concordantly, we find that both AEP and TRAF6 were overexpressed in malignant breast cancer and associated with poor prognosis. These effects were even more pronounced when both proteins were present, indicating that AEP and TRAF6 might possess prognostic value in patients.

Despite these new findings, elucidation of the exact role and underlying mechanism of action for AEP in development and progression of cancer will require additional investigations. Previous studies have shown that AEP is localized at the apex of invading cells, forming a complex with integrins expressed on lamellipodia and invadopodia (17). The binding of AEP to these integrins significantly enhances AEP’s ability to proteolytically activate pro-MMP2 and cathepsin L (data not shown). However, additional pathophysiologically relevant AEP substrates still need to be identified to further clarify its function and overall role in tumor development and metastasis. Molecular mechanisms regulating pro-AEP activation process and subcellular localization also remain elusive.

In summary, our study uncovered a novel mechanism of AEP regulation and investigated its role in breast cancer tumor progression. We put forth that AEP might be of prognostic value and a potential therapeutic target. We have also identified an alternative oncogenic pathway for TRAF6 that uses AEP as its substrate.

Figure 9. A schematic diagram of asparaginyl endopeptidase (AEP) regulation by TRAF6 in promoting tumor metastases. Various stress stimuli in the tumor microenvironment induce TRAF6 ubiquitination of pro-AEP by K63-linked polyubiquitin, which is reversible by USP17. This leads to formation of a complex with HSP90α and subsequent enhancement of AEP intracellular stability and secretion. Secreted AEP promotes tumor progression through degradation of tumor matrix.

References


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Notes

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