



**EMORY**  
LIBRARIES &  
INFORMATION  
TECHNOLOGY

**OpenEmory**

## **Asparagine endopeptidase is an innovative therapeutic target for neurodegenerative diseases**

Zhentao Zhang, *Emory University*

Manling Xie, *Emory University*

[Keqiang Ye](#), *Emory University*

---

**Journal Title:** Expert Opinion on Therapeutic Targets

**Volume:** Volume 20, Number 10

**Publisher:** Taylor & Francis: STM, Behavioural Science and Public Health  
Titles | 2016-10-01, Pages 1237-1245

**Type of Work:** Article | Post-print: After Peer Review

**Publisher DOI:** 10.1080/14728222.2016.1182990

**Permanent URL:** <https://pid.emory.edu/ark:/25593/s59vh>

---

Final published version: <http://dx.doi.org/10.1080/14728222.2016.1182990>

### **Copyright information:**

© 2016 Informa UK Limited, trading as Taylor & Francis Group.

*Accessed October 19, 2019 4:22 AM EDT*



# HHS Public Access

Author manuscript

*Expert Opin Ther Targets*. Author manuscript; available in PMC 2017 October 01.

Published in final edited form as:

*Expert Opin Ther Targets*. 2016 October ; 20(10): 1237–1245. doi:10.1080/14728222.2016.1182990.

## Asparagine endopeptidase is an innovative therapeutic target for neurodegenerative diseases

Zhentaο Zhang<sup>1,2</sup>, Manling Xie<sup>2</sup>, and Keqiang Ye<sup>2,\*</sup>

<sup>1</sup>Department of Neurology, Renmin Hospital of Wuhan University, Wuhan 430060, China

<sup>2</sup>Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA 30322, USA

### Abstract

**Introduction**—Asparagine endopeptidase (AEP) is a pH-dependent endolysosomal cysteine protease that cleaves its substrates after asparagine residues. Our most recent study identifies that it possesses the delta-secretase activity, and that it is implicated in numerous neurological diseases such as Alzheimer’s disease (AD) and stroke. Accumulating evidence supports that the inhibition of AEP exhibits beneficial effects for treating these devastating diseases.

**Areas covered**—Based on recent evidence, it is clear that AEP cleaves its substrate, such as amyloid precursor protein (APP), tau and SET, and plays a critical role in neuronal cell death in various neurodegenerative diseases and stroke. In this article, the basic biology of AEP, its knockout phenotypes in mouse models, its substrates in neurodegenerative diseases, and its small peptidyl inhibitors and prodrugs are discussed. In addition, we discuss the potential of AEP as a novel therapeutic target for neurodegenerative diseases.

**Expert opinion**—AEP plays a unique role in numerous biological processes, depending on both pH and context. Most striking is our most recent finding; that AEP is activated in an age-dependent manner and simultaneously cleaves both APP and tau, thereby unifying both major pathological events in AD. Thus, AEP acts as an innovative trigger for neurodegenerative diseases. Inhibition of AEP will provide a disease-modifying treatment for neurodegenerative diseases including AD.

### Keywords

Asparagine endopeptidase; cysteine protease; legumain; neurodegenerative diseases

---

\*Corresponding author: Keqiang Ye, Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA 30322, USA, kye@emory.edu.

#### Declaration of interest

The work was supported by a grant from the National Institute of Health (RO1, NS045627) to K Ye, and a grant from the National Natural Science Foundation of China (No. 81571249) to Z Zhang. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

## 1. Introduction

### 1.1 AEP original identification as legumain in plants

Asparagine endopeptidase (AEP), also called mammalian legumain, is a cysteine protease that hydrolyzes substrates at the C-terminus of asparagine residues <sup>1,2</sup>. AEP was originally identified in plants as the vacuolar processing enzyme, legumain. The mammalian legumain was initially cloned by Barrett and his colleagues <sup>3,4</sup>. AEP belongs to peptidase family C13, and is thus unrelated to the better known cysteine peptidases of the papain family, C1 <sup>5</sup>. It shares homology with a family of proteases that includes caspases and separase <sup>6</sup>, but not with the papain-fold lysosomal proteases. The human and mouse legumain cDNA amino acid sequences share approximately 83% identity with 433 amino acids. It is particularly abundant in kidney and placenta with main lysosome distribution. Mammalian legumain appears to be restricted to the hydrolysis of asparaginyl bonds in substrates of all kinds but occasionally cleaves after aspartic acid residues. There seem to be no strong preferences for particular amino acids in other subsites, and glycosylation of asparagine totally prevents the hydrolysis by legumain <sup>7</sup>. AEP activation is autocatalytic, requires sequential removal of C- and N-terminal pro-peptides at different pH thresholds. Removal of the C-terminal and N-terminal propeptide requires cleavage after N323 and D25, respectively, which will be further trimmed to yield the mature and fully active 36 kDa enzyme <sup>8</sup>. The maximal endopeptidase enzymatic activity is found at pH 5.8 under normal assay conditions, and the enzyme is irreversibly denatured at pH 7 and above <sup>3</sup>.

### 1.2 Endogenous AEP substrates

Although mounting evidence indicates that AEP plays a crucial role in immunity, cancers and neurological diseases, only a few AEP substrates have been identified to date. In the absence of AEP, only the single-chain form of cathepsins B, H, and L were detected in the kidney, while these cathepsins were detected as both the single-chain and two-chain forms in wild-type mice <sup>9</sup>. These observations suggest that these cathepsins are AEP substrates. Proteolysis of invariant chain influences the timing and location of peptide loading onto class II major histocompatibility complex (MHC) molecules and therefore may affect initiation of an immune response <sup>10, 11, 12</sup>. Intracellular toll-like receptor 3 (TLR3), TLR7, and TLR9 localize in endosomes and recognize single-stranded RNA and nucleotides from viruses and bacteria. TLR9 requires a proteolytic cleavage for its signaling. AEP cleaves TLR9 and plays a critical role in TLR processing and signaling in dendritic cells <sup>13</sup>. AEP has been linked to progelatinase A processing, mediating cancer metastasis <sup>14</sup>. In addition, AEP also processes prothymosin  $\alpha$ , a protein involved in chromatin remodeling, into thymosin  $\alpha.1$  and  $\alpha.11$  <sup>15</sup>. Moreover, the substrates for AEP include L-asparaginase used as part of the therapeutic regimen in childhood acute lymphoblastic leukemia <sup>16</sup>, fibronectin <sup>17</sup>, and the nuclear phosphoprotein SET, which inhibits DNA nicking and neuronal cell death <sup>18</sup>. We will discuss the reported literatures regarding the phenotypes of AEP knockout mice, revealing the interesting physiological functions of this exciting protease by shedding numerous biological substrates and disclosing the potential pathological roles in various human disorders.

### 1.3 AEP activation in brain disorders

Though AEP is involved in many physiological and pathological processes including immunity and cancer progression, its biological role in the nervous system was first elucidated by our group in 2008<sup>18</sup>. We reported that AEP is activated during brain acidosis induced by brain ischemia and epileptic seizure. The activated AEP cuts SET, an inhibitor of DNase, and triggers DNA damage in the brain. The cleavage of SEP by AEP is inhibited by PIKE-L, a pro-survival protein distributed in the nucleus and associated with plasma membrane<sup>18–20</sup>. Thus, AEP might be one of the major proteinases activated by acidosis and triggering neuronal injury during neuro-excitotoxicity or ischemia. Most recently, we found substantial AEP protein levels in the brain, where it cleaves both amyloid precursor protein (APP) and tau in an age-dependent manner, indicating that it possesses the innovative delta-secretase activity. In addition, AEP activity is greater in brain tissues from human Alzheimer's disease (AD) patients than in healthy controls, and this activity mediates AD onset and progression<sup>21, 22</sup>. Hence, the central role of AEP activity in APP and tau pathology makes this enzyme an attractive therapeutic target for treating AD and other neurodegenerative disorders associated with neurofibrillary tangles and senile plaques<sup>21–23</sup>. In alignment with its role in neurodegenerative diseases, AEP was reported to play an important role in axonal regrowth after spinal cord injury in zebrafish. The expression of AEP was increased in neurons of regenerative nuclei during the phase of axon regrowth/sprouting. Reducing the expression of AEP impaired axonal regeneration and locomotor recovery<sup>24</sup>.

### 1.4 AEP in immunity and cancer

AEP plays an important role in numerous physiological and pathological processes including immunity and cancer progression. The role of AEP in antigen presentation was initially inferred from the ability of purified AEP to execute the early cleavages of tetanus toxin antigen<sup>7</sup>. Additionally, AEP regulates the presentation of the myelin basic protein epitope, which is a candidate autoantigen in the inflammatory demyelinating disease multiple sclerosis<sup>10</sup>. More recently, the results of experiments using a cell-permeable AEP inhibitor are consistent with the involvement of AEP in class II MHC maturation through proteolysis of the invariant chain (Ii)<sup>11</sup>. Employing AEP null mice, Ploegh et al., demonstrated that AEP is essential for the processing of cathepsin L but not for class II MHC-restricted antigen presentation in mice<sup>25</sup>.

In addition to its important roles in immunity, AEP is also implicated in tumor progression. The expression of AEP is highly upregulated in several cancer types such as colon, prostate and breast cancer<sup>26</sup>. AEP has been found to promote cancer cell invasiveness both *in vitro* and *in vivo*<sup>27</sup>. The effect of AEP on cancer metastasis is possibly mediated by its proteolytic processing of progelatinase A, a member of matrix metalloproteinase family involved in the turnover of extracellular matrix<sup>14, 27</sup>. Moreover, AEP is also implicated in osteoclast formation and bone resorption<sup>28</sup>.

## 2. AEP functions in physiology and human brain diseases

### 2.1 Phenotypes in AEP deficient mice

**2.1.1 Kidney function and lysosomal proteases**—AEP is highly expressed in the proximal tubular cells (PTCs) of kidney<sup>29</sup>. The PTCs is responsible for the uptake of proteins from the crude urine. The protein will be further degraded in the lysosomes of PTCs. Interestingly, AEP knockout mice show accumulated proteins in their PTC endosomes and lysosomes, indicating AEP is required for the normal processing of these proteins. As a result, the AEP knockout mice develop hyperplasia of PTCs, interstitial fibrosis, glomerular cysts, proteinuria and decreased glomerular filtration<sup>29</sup>. These findings are consistent with the view that AEP is required for normal protein processing by PTCs.

In the PTCs of wild-type mice, AEP is mainly expressed in the late endosomes and lysosomes. In AEP knockout mice, the lysosomes and late endosomes are enlarged and contain electron-dense and membranous materials. The lysosomal proteases such as cathepsin B, H, and L are synthesized as proforms or zymogens. They transport into the endocytic compartments where their prodomains are removed by proteolysis. The resulting single-chain form is then cleaved into the two-chain form<sup>30</sup>. AEP mediates this latter cleavage event in kidney cells<sup>9</sup>. The processing of cathepsins B, H, and L is altered in AEP deficient mice<sup>9</sup>.

**2.1.2 Hematologic system**—AEP also plays an important role in the hematologic system. We found that AEP knockout mice develop fever, cytopenia, hepatosplenomegaly, and hemophagocytosis. Furthermore, AEP knockout mice also show severe anemia and extramedullary hematopoiesis. Some plasma membrane components are altered in red blood cells from AEP-null mice. The activity of natural killer cells is also affected in AEP knockout mice. These symptoms are similar to hemophagocytic syndrome/hemophagocytic lymphohistiocytosis (HLH)<sup>31</sup>. HLH is a life-threatening condition caused by overstimulation and over activity of the immune system. Our results indicate that AEP might participate in the development of HLH. It has been proposed that HLH is caused by persistent antigen presentation, leading to the excessive cytokine production and systemic inflammation<sup>32</sup>. Given the fact that AEP is required for microbial tetanus toxin antigen presentation<sup>7</sup>, the hyperimmune response in HLH might be caused by defective antigen presentation in AEP knockout mice.

**2.1.3 Antigen processing**—Foreign protein antigens are degraded to generate antigenic peptides, which then load onto class II MHC molecules for presentation to T cells. It has been suggested that AEP processes a microbial antigen for Class II MHC presentation<sup>7</sup>. However, no differences in processing of the invariant chain or maturation of class II MHC products are found in AEP-deficient mice, compared with wild-type controls. In AEP-deficient mice, the presentation of OVA and myelin oligodendrocyte glycoprotein, two antigens that contain asparagine residues within or in proximity to the relevant epitopes was unimpaired<sup>25</sup>. Recently it was reported that a reduction in the secretion of proinflammatory cytokines in response to TLR9 stimulation was found in myeloid and plasmacytoid dendritic cells (DCs) deficient for the AEP. Upon stimulation, full-length TLR9 is fragmented into a

72 kDa piece and this processing is strongly decreased in AEP deficient DCs<sup>13</sup>. AEP is also critical for TLR7 processing and anti-influenza virus immune responses<sup>33</sup>. Hence, AEP plays a critical role in TLR processing and signaling in DCs. Based on these results from genetic knockout mice, it is clear that AEP plays a critical role in immunity and normal kidney physiology and homeostasis. The phenotype of AEP knockout mice are summarized in table 1.

## 2.2 Emerging role of AEP in neurological diseases of stroke and ALS

**2.2.1 Stroke and AEP**—Stroke elicits acidosis in the brain<sup>34</sup>. Since AEP is activated under conditions of acidosis, we investigated the potential role of AEP in ischemic stroke using a transient middle cerebral artery occlusion (MCAO) model. The expression of AEP in the ischemic core was upregulated 48 h following artery occlusion. AEP activation was also found in the surrounding tissues adjacent to ischemia core. Activated AEP cleaves its substrate SET, and mediates neuronal cell death. SET remained intact in the AEP knockout mice. As a result, neuronal cell death was attenuated in AEP knockout mice<sup>18</sup>, indicating AEP-mediated proteolytic processing of SET is required for neuronal cell death caused by ischemia. We suggest that blockade of AEP-mediated SET cleavage may attenuate neuronal cell death induced by brain ischemia, independent of caspases.

Fitting with these observations, Ishizaki et al., found that both protein and mRNA levels of AEP are increased in the peri-infarct area in a rat transient MCAO model. AEP mRNA was increased 3 h after occlusion, with a maximum expression at 24 and 48 h after MCAO<sup>35</sup>. This time pattern is similar to that of cathepsin B<sup>36</sup>. In addition, they show that in the peri-infarct area, AEP is processed into its active form. AEP was mainly found in branches of astroglial cells and microglia, suggesting AEP may be secreted<sup>37</sup> and may function as a chemo-attractant for invading inflammatory cells after stroke<sup>35</sup>. However, They found that the infarct volumes between wild-type and AEP knockout mice were similar, suggesting that AEP might be not involved in the acute stage of neurodegeneration but may play a role in neuroinflammation during stroke<sup>35</sup>. These observations are different from our findings. We found that DNA damage is decreased in AEP knockout mice compared to wild-type mice, indicating that AEP activation is an early event in neurodegeneration<sup>18</sup>. The discrepancy might be resulted from mice age differences. In our study, we employed 2–3 months old young mice, whereas more than 1 year-old mice were included in Ishizaki's experiments. Since AEP mediates numerous age-dependent physiological processes including bone marrow development, immunity and kidney functions<sup>29, 31, 38</sup>, which may implicate in stroke-triggered neuronal cell death.

**2.2.2 ALS and AEP**—Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease characterized by progressive muscle weakness due to degeneration of the motor neurons. Mutations in four genes (C9ORF72, SOD1, TARDBP, and FUS/TLS) account for over 50% of the familial cases. Dysfunction in RNA processing and protein homeostasis is an emerging theme in the pathogenesis of ALS<sup>39</sup>. TDP-43 is a ubiquitously expressed DNA-/RNA-binding protein. It plays a critical role in regulating RNA splicing. Aggregation of TAR DNA-binding protein 43 (TDP-43) is a pathological hallmark of several neurodegenerative diseases including ALS and frontotemporal lobar

degeneration (FTLD) <sup>40</sup>. Normally, TDP-43 is a nuclear protein. It redistributes to the cytoplasm under pathological conditions and form aggregates. TDP-43 is a major protein component in ubiquitin-positive, tau-negative inclusions of FTLD and ALS <sup>41</sup>. However, the pathogenicity of TDP-43 aggregates and the accompanying protein modifications, including hyper-phosphorylation, ubiquitination and cleavage into C-terminal fragments (CTFs), remain poorly understood <sup>40</sup>.

Studies comparing frontal cortex and spinal cord from FTLD and ALS cases, respectively, indicate that TDP-43 CTFs are enriched selectively in brain <sup>42, 43</sup>. However, the proteases responsible for their generation have not been illustrated. Recently, we identified two truncated TDP-43 peptides, terminating C-terminal to asparagines 291 (N291) and 306 (N306) in human FTLD brains. In brains from AEP wild-type and AEP knockout mice, we showed that TDP-43 proteolytic fragments were substantially reduced in the absence of AEP. These results indicate that TDP-43 is a substrate of AEP during the pathogenesis of ALS and FTLD <sup>44</sup>. The role of AEP-mediated TDP-43 cleavage in neurodegeneration remains unclear. However, almost all of the TDP-43 mutations associated with familial ALS and FTLD are localized in the C-terminus of the protein, suggesting that cleavage of TDP-43 by AEP may cause the loss of physiological functions or gain of pathological functions <sup>45</sup>. Furthermore, disease-associated mutations of TDP-43 may affect its proteolysis by AEP. The exact pathological role of AEP-cleaved TDP-43 in ALS and FTLD progression remains unknown. Conceivably, specific expression of these fragments in the CNS may shed light into the potential effect by these events in these neurodegenerative diseases.

### 2.3 AEP mediates the neurofibrillary pathology in Alzheimer's disease

**2.3.1 Tau and AEP**—Pathologically, AD is characterized by the accumulation of the  $\beta$ -amyloid (A $\beta$ ) and tau. The dysfunction of APP proteolysis and the abnormal phosphorylation of tau lead to the formation of neuritic plaques and neurofibrillary tangles (NFTs), respectively. Abnormal A $\beta$  and tau aggregation cause neuronal degeneration and dementia. The proteolytic processing of tau regulates its aggregation and neurotoxic effects. To explore whether tau is a substrate of AEP, we incubated recombinant tau with kidney lysates prepared from wild-type and AEP knockout mice, respectively. We found that tau was cleaved in wild-type kidney lysates but not in AEP knockout kidney lysates. In addition, we also employed AEP mutants that abolish the cysteine protease activity of AEP (C189S) and the zymogen autocleavage required for its activation (N323A) to further demonstrate the cleavage specificity for AEP. The cleavage of tau by AEP was further investigated by anti-AEP antibody and an AEP inhibitor, AENK. Using mass spectrometry, we found that tau from human AD patients is cleaved by AEP after the N368 residue <sup>21</sup>.

Tau is also a substrate of several endogenous proteases. Among them, caspases and calpain have been intensively investigated <sup>46–48</sup>. We found that AEP cleaves tau independent of caspases or calpains and that hyperphosphorylation of tau does not affect its fragmentation by AEP. Conversely, overexpression of the AEP-truncated tau<sub>1–368</sub> fragment in primary neurons elicits tau hyperphosphorylation. Phosphorylation of tau regulates its functions in regulating microtubule dynamics. Notably, the biological effect of promoting microtubule

polymerization is lost in the AEP-cleaved tau fragment. Remarkably, the truncated tau<sub>1-368</sub> was strongly neurotoxic when expressed in cultured neurons.

To investigate the effect of AEP cleavage on filament formation, we monitored accumulation of PHFs using purified tau recombinant proteins. As expected, the cleaved fragment tau<sub>1-368</sub> is prone to aggregate<sup>21</sup>. These findings are consistent with a previous report that in the brains of AD patients, the level of activated AEP is significantly increased and translocates from neuronal lysosomes to the cytoplasm, where it is associated with hyperphosphorylated tau<sup>49</sup>. It has been reported that AD-related factors such as A $\beta$  and apolipoprotein E4 induce lysosomal membrane damage. AEP may translocate from lysosomes to the cytosol when lysosomal permeability increases in the vulnerable neuron<sup>50-52</sup>. Furthermore, it is well established that Cystatin C levels in the CSF of AD patients are lower compared to non-demented individuals<sup>53,54</sup>, leading to AEP activation. It has been reported that brain pH is decreased in AD patients compared to controls<sup>55-57</sup>. We found that pH in the tau P301S transgenic mice brain cortex and hippocampus was lower compared to in nontransgenic mice<sup>21</sup>. Furthermore, age is the major risk factor for AD and the pH in the brain gradually decreases during aging<sup>58</sup>. These results indicate AEP might be activated by acidosis during ageing and in AD brain. It should be noted that there are also reports that pH in the left hippocampus is increased towards alkaline side compared to MCI but this difference did not reach statistical significance<sup>59</sup>. The relationship between brain acidosis, AEP activation, and AD pathology needs further investigation.

**2.3.2 Protein phosphatase-2A in AD related to AEP**—The activity of protein phosphatase-2A (PP2A), which regulates the phosphorylation of tau, is negatively regulated in human AD brains by the phosphoprotein SET. SET is also known as inhibitor-2 of PP2A, I2(PP2A)<sup>60</sup>. In AD brain, PP2A activity is compromised, and SET is overexpressed<sup>61</sup>. We have previously shown that SET is selectively cleaved at N175<sup>18</sup> into an N-terminal fragment, I2NTF, and a C-terminal fragment, I2CTF, and both fragments inhibit PP2A<sup>62, 63</sup>. Overexpression of the CTF of SET elicits AD pathology and cognitive impairment, indicating cleavage of SET could initiate AD in animal model<sup>62</sup>. On the other hand, it has also been shown that SET in the neuronal cytoplasm is sufficient to impair PP2A methylation and activity, leading to tau hyperphosphorylation<sup>64</sup>. By analyzing the spinal cords from ALS and control cases, Iqbal and his colleagues found a selective increase in the cleavage of SET and inhibition of PP2A activity in the spinal cords of ALS, similar to what has been reported in AD cases. Intracerebroventricular injection of AAV1 encoding AEP-generated I2CTF fragment caused cognitive impairments and motor deficits in rats. Furthermore, the rats show tau and TDP-43 pathologies, accumulation of intraneuronal A $\beta$ , and degeneration of motor neurons in the spinal cord<sup>61, 62</sup>. These findings indicate that AEP-mediated cleavage of SET participates in the pathogenesis of both AD and ALS. The role of AEP in the deposition of tau is summarized in Figure 1.

## 2.4 AEP as an age-regulated $\delta$ -secretase in Alzheimer's disease

**2.4.1 APP processing involves AEP**—Genetic, biochemical, and behavioral research suggest that physiologic generation of the neurotoxic A $\beta$  peptide from sequential APP proteolysis is the crucial step in the development of AD. APP is metabolized in a rapid and

highly complex fashion by a series of sequential secretases, including  $\beta$ -secretases (BACE1),  $\gamma$ -secretase and the ADAM family as  $\alpha$ -secretases. We provided a variety of biochemical evidence that APP is cleaved by active AEP in human AD brain at N373 and N585 residues<sup>22</sup>. Interestingly, we also found that AEP expression levels are escalated in an age-dependent manner, tightly coupled to APP fragmentation in the aged brains. It is worth noting that APP is cleaved in human AD brains but not in healthy controls. Accordingly, AEP enzymatic activity is elevated in 5XFAD mouse models. We found that the AEP-generated APP fragment APP<sub>586-695</sub> is more readily cleaved by  $\beta$ -secretase. Conceivably, removal of APP C-terminal fragment by AEP may relieve the steric hindrance and promote APP processing by BACE1, accelerating the production of A $\beta$ . We tested this hypothesis in cultured neurons and HEK293 cells. We found that depletion of AEP significantly reduces A $\beta$  production. On the other hand, over-expression of the C-terminal fragment APP<sub>586-695</sub> markedly elevated A $\beta$  production when compared with full-length APP. Furthermore, blockade of AEP cleavage of APP at N585 reduced A $\beta$  production. Hence, AEP cleavage of APP at N585 produce an APP C-terminal fragments that is more readily processed by BACE1 than full-length APP (Figure 2). Furthermore, AEP-generated APP<sub>1-373</sub> but not other AEP-cleaved APP fragments are neurotoxic. The proportion of positive neurons with AEP-derived APP<sub>586-695</sub> fragment immunoreactivity is much higher in brain sections from AD patients than control<sup>22</sup>.

**2.4.2 AEP mediates AD pathology**—Synaptic loss is believed to be the basis of cognitive impairment in the early phase of AD<sup>65</sup>. The A $\beta$  peptide, which plays a crucial role in the pathogenesis of AD, alters hippocampal-dependent synaptic plasticity and memory and mediates synapse loss. As expected, deletion of AEP from the 5XFAD transgenic mouse model rescues the synaptic loss. Long-term potentiation (LTP), a measure of synaptic plasticity that underlies learning and memory, is ameliorated when AEP is deficient from 5XFAD mice. In addition, we also found approximately 30% reduction in A $\beta$  peptide in 5XFAD/AEP<sup>-/-</sup> mice compared to 5XFAD mice at 6 months of age. The spatial memory impairment of 5XFAD mice was also partially reversed when AEP was deleted. Deletion of AEP also attenuated the memory impairment in the APP/PS1 mouse model<sup>22</sup>. To assess the physiological role of AEP cleavage of tau in synaptic function and behavior, we bred AEP knockout mice with tau P301S transgenic mice to knock out AEP in tau P301S mice. In the absence of AEP, tau<sub>1-368</sub> fragment is eradicated from tau P301S mice. The defects in synaptic loss, dendritic spine structure, and LTP are rescued when AEP is deleted from tau P301S mice. Both Morris Water Maze and contextual and cued fear conditioning tests demonstrate that eradication of AEP reverses the memory deficits in tau P301S mice<sup>21</sup>. To evaluate whether the effects of AEP are mediated via cleavage of tau, we injected AAVs encoding human tau P301S or AEP non-cleavable tau P301S (tau P301SN255AN368A) into the hippocampus of wild-type mice. Immunohistochemical characterization of NFT and electrophysiology for LTP analysis and memory behavioral tests are all consistent with an interpretation that cleavage of tau by AEP is required for the AD pathogenesis<sup>21</sup>. Collectively, these innovative findings provide novel insight into the molecular mechanisms of how AEP cross-talks with the well-characterized secretases fragmenting APP and proteinases degrading tau, contributing to the cognitive impairment.

We propose a cellular model that reflects our current view about the biological processes (Figures 1, 2). During ageing and neurodegenerative process, AEP may translocate from the endolysosome into the cytoplasmic space, where it cleaves tau, resulting in hyperphosphorylation of the truncated neurotoxic fragments and neurofibrillar tangle formation. Moreover, AEP cuts SET, leading to PP2A inhibition and consequent tau hyperphosphorylation. Additionally, AEP cleaves APP in the endolysosomal organelles. The resultant APP<sub>585-695</sub> fragment may be further processed by BACE1 to produce A $\beta$ . Furthermore, the cleavage of TDP-43 by AEP may interfere with its normal function or generate toxic fragments that promote the pathogenesis of ALS and FTLN (Table 2). The potential impact of these discoveries is substantial, because it will address the fundamental question of how the aging process initiates the decomposing protease that regulates APP and tau degradation, leading to AD onset and progression. To understand how APP and tau are processed beyond  $\alpha$ -,  $\beta$ -, and  $\gamma$ -secretases and conventional proteinases including caspases and calpain are groundbreaking findings for AD research. In addition to leading to a better understanding of the physiological functions of AEP in cellular and molecular levels, this knowledge will provide the innovative drug target for developing new treatment of neurodegenerative diseases.

## 2.5 Development of small molecular inhibitors of AEP for AD and neurological disease therapeutics

AEP is implicated in a number of pathological conditions including cancer and neurodegenerative diseases. Highly potent and selective inhibitors of AEP are needed for studying the functional roles of AEP as well as for the development of AEP-based therapeutics<sup>66</sup>. It has been reported that Michael acceptor inhibitors based on the backbone Cbz-L-Ala-L-Ala-L-Asn (Cbz = benzyloxycarbonyl) show irreversible inhibition of AEP. Integrated in halomethylketone inhibitors, aza-asparagine is accepted by legumain in the P1-position. The most potent and selective inhibitor of this series is Cbz-L-Ala-L-Ala-AzaAsn-chloromethylketone. Papain and cathepsin B are not inhibited by this compound at concentrations up to 100 mM<sup>67</sup>. Later, Powers and his colleagues synthesized a new class of benzylcarbamate-aza-peptidyl Michael acceptors as selective AEP inhibitors. Aza-asparaginyll Michael acceptors react with thiols, which provides insight into the mechanism of their inhibition of AEP<sup>68</sup>. Lee et al. developed aza-peptidyl Asn epoxides, which are highly selective and potent AEP inhibitors. Based on aza-peptidyl Asn epoxides, they further developed near-infrared fluorophore-labeled activity-based probes (ABPs), which can be used for noninvasive *in vivo* imaging. Using these probes, they specifically labeled AEP in various normal tissues as well as in solid tumors<sup>69</sup>. The development of the ABPs provides useful tool to study the physiological and pathological role of AEP *in vivo*.

Most recently, the selective AEP inhibitors based on the aza-asparaginyll scaffold have been generated. Lee et al., synthesized a library of aza-peptidyl AEP inhibitors. These inhibitors are highly selective and specific to AEP. The IC<sub>50</sub> values against recombinant AEP were as low as 4 nM. Furthermore, the inhibitors have little or no cross-reactivity with cathepsins. These new AEP inhibitors can be used to study AEP functions in multiple disease models<sup>66</sup>. Most recently, Higgins et al. conducted extensive SAR by synthesizing numerous Asn scaffold peptidyl derivatives to optimize the legumain inhibitor. They have also identified a

sub-nanomolar biphenyl carbamate AEP inhibitor with cyano warhead<sup>70</sup>. Nevertheless, it remains unclear whether these optimized small peptidyl skeletal inhibitors own any appropriate druggability toward human disorders including cancer, stroke, and AD. Moreover, whether these compounds are stable in the circulatory system or possess acceptable systemic toxicities remain unknown. Usually, due to the intrinsic shortcomings, the peptidyl compounds possess poor pharmacokinetic profiles, hurdling them from transforming into promising therapeutic clinical agents. More translational research is necessary to assess these interesting small molecular AEP inhibitors toward human disorders in various animal models.

### 3. AEP as a potential therapeutic target for brain diseases

Recently, we found that AEP cleaves both APP and tau, contributing to both amyloid and tau pathology in AD. We have also identified APP and tau fragmentation by AEP in human AD brains, and that AEP expression levels and activity are escalated in aged mice and AD brain compared to young mice or control human brain. These findings indicate that AEP acts as a novel age-dependent protease in AD progression. Certainly, identifying the physiological roles of AEP in cleaving APP and tau during aging process and delineating their biological functions in mediating neuronal cell death directly impacts on the diagnosis, prevention and treatment of neurodegenerative diseases. Clearly, these exciting discoveries strongly support that AEP is a novel drug target for suppressing both A $\beta$  formation and tau aggregation. Since it is also involved in neuronal cell death during stroke and other excitotoxicities, the pharmacological agents inhibiting AEP will be powerful therapeutic tool for treating many other neurological diseases and human cancers as well.

In addition to the substrate asparagine-based competitive or covalent peptidyl inhibitors targeting the active thiol site, the development of ultra-high throughput (uHTS) drug screens, incorporating large numbers of druggable chemicals, a fluorescent substrate and recombinant pure and active AEP enzyme will be a feasible alternative approach to identify much more promising small AEP inhibitors. Conceivably, the positive outcomes from such a screening, after specificity validation against numerous cysteine proteases, *in vitro* ADMET triage, and *in vivo* pharmacokinetic profiling, will yield much more potent and selective small molecular AEP inhibitors with druggable features for examining the *in vivo* therapeutic efficacy in disease models. Furthermore, it is crucial to determine the ability of the compounds to cross the blood-brain barrier (BBB). Only the compounds that can cross the blood-brain barrier should be pursued for drug development. Currently there is no evidence regarding the brain penetrance of compounds targeting AEP. Usually, certain small molecules with a molecular weight of less than 400 Da and form less than 8 hydrogen bonds can cross the BBB via lipid-mediated free diffusion. However, most of the druggable chemicals do not meet this criterion. To cross the BBB, some small compounds can be reengineered that access carrier-mediated transport (CMT) systems within the BBB. Large molecules can also be reengineered with molecular Trojan horse delivery systems to cross the BBB via receptor-mediated transport systems<sup>71</sup>. The unbiased drug discovery will allow us one step closer to the ideal therapeutic candidate for the clinical trials against various human disorders including AD.

## 4. Expert Opinion

Recently, converging evidence suggests that AEP plays a role in the pathogenesis of CNS diseases, and may serve as a potential therapeutic target. AEP is upregulated during ageing and pathological conditions such as AD<sup>21, 22</sup>. As an age- and pH-dependent protease, AEP mediates the proteolytic processing of its substrates. Some of the AEP substrates in the CNS have been identified recently, including SET, TDP-43, APP, and tau<sup>18, 21, 22, 44</sup>. However, it remains unclear whether it cleaves other substrates under physiological and pathological conditions. Since AEP is the only known protease that specifically cleaves after asparagine residues, the fragments generated by AEP cleavage can be identified using techniques like mass spectrometric analysis. Those fragments that end with asparagine residues should be further verified using *in vitro* AEP cleavage assay. It should be kept in mind that AEP is only activated under acidic condition. The pH of the reaction is critical for a successful cleavage assay. Moreover, AEP knockout tissue should be used as a negative control to confirm whether a protein is a real AEP substrate.

AD's physiopathology is not yet fully understood. It has been shown that AEP mediates the proteolytic processing of several important players in AD. For example, truncation of SET by AEP elicits tau phosphorylation, while truncation of APP and tau promotes the deposition of A $\beta$  and tau, respectively<sup>18,21,22</sup>. Presumably, activation of AEP is an early event in the pathogenesis of AD. Furthermore, AEP cleaves TDP-43 in FTLN brain<sup>44</sup>. Although the consequence of this cleavage has not been illustrated, it is possible that the abnormally cleaved fragments may affect the normal functions of TDP-43, or this cleavage may produce fragments that are prone to aggregate, or toxic to vulnerable neurons. In fact, it has been reported that some of the TDP-43 fragments can trigger pathological features of TDP-43 proteinopathies<sup>43</sup>. It should be noted that other post-translational modifications such as ubiquitination and phosphorylation also regulate the physiological and pathological functions of tau and TDP-43. The relationship between truncation and other post-translational modifications should be further studied to illustrate the mechanisms of protein aggregation and gain of toxic functions in neurodegenerative diseases.

We found that deletion of AEP from several AD models ameliorates the synaptic dysfunction and behavioral impairments, strongly supporting that AEP inhibitors might be useful for treating AD<sup>21, 22</sup>. If we could successfully establish that AEP is pathologically implicated in processing APP and tau during human AD onset and progression, this knowledge may be extended to other age-related neurodegenerative diseases including Parkinson's disease (PD), FTLN, etc. To determine AEP's biological roles in AD development will certainly expand the preclinical AD pathology horizon. In the past two decades, tremendous efforts have been spent over A $\beta$  or tau-targeted treatment by blocking the activity of  $\beta$ - and  $\gamma$ -secretase or kinases phosphorylating tau, or promoting their clearance. Compounds claiming disease-modifying abilities in AD have thus far failed to produce effects that are clinically significant. Since AD is a complex and multi-factorial disorder, targeting one protease, AEP, that simultaneously regulates both APP and tau cleavage will provide the unprecedented advantage over the strategy pertinent to either APP or tau alone.

## Bibliography

1. Hara-Hishimura I, Takeuchi Y, Inoue K, et al. Vesicle transport and processing of the precursor to 2S albumin in pumpkin. *Plant J.* 1993; 4(5):793–800. [PubMed: 8275099]
2. Dando PM, Fortunato M, Smith L, et al. Pig kidney legumain: an asparaginyl endopeptidase with restricted specificity. *Biochem J.* 1999; 339(Pt 3):743–9. [PubMed: 10215615]
3. Chen JM, Dando PM, Rawlings ND, et al. Cloning, isolation, and characterization of mammalian legumain, an asparaginyl endopeptidase. *J Biol Chem.* 1997; 272(12):8090–8. This article reports for the first time the cloning and characterization of mammalian legumain. [PubMed: 9065484]
4. Chen JM, Dando PM, Stevens RA, et al. Cloning and expression of mouse legumain, a lysosomal endopeptidase. *Biochem J.* 1998; 335(Pt 1):111–7. [PubMed: 9742219]
5. Rawlings ND, Barrett AJ. Families of cysteine peptidases. *Methods in enzymology.* 1993; 244:461–86.
6. Chen JM, Rawlings ND, Stevens RA, et al. Identification of the active site of legumain links it to caspases, clostripain and gingipains in a new clan of cysteine endopeptidases. *FEBS Lett.* 1998; 441(3):361–5. [PubMed: 9891971]
7. Manoury B, Hewitt EW, Morrice N, et al. An asparaginyl endopeptidase processes a microbial antigen for class II MHC presentation. *Nature.* 1998; 396(6712):695–9. [PubMed: 9872320]
8. Li DN, Matthews SP, Antoniou AN, et al. Multistep autoactivation of asparaginyl endopeptidase in vitro and in vivo. *Journal of Biological Chemistry.* 2003; 278(40):38980–90. [PubMed: 12860980]
9. Shirahama-Noda K, Yamamoto A, Sugihara K, et al. Biosynthetic processing of cathepsins and lysosomal degradation are abolished in asparaginyl endopeptidase-deficient mice. *J Biol Chem.* 2003; 278(35):33194–9. [PubMed: 12775715]
10. Manoury B, Mazzeo D, Fugger L, et al. Destructive processing by asparagine endopeptidase limits presentation of a dominant T cell epitope in MBP. *Nat Immunol.* 2002; 3(2):169–74. [PubMed: 11812994]
11. Manoury B, Mazzeo D, Li DN, et al. Asparagine endopeptidase can initiate the removal of the MHC class II invariant chain chaperone. *Immunity.* 2003; 18(4):489–98. [PubMed: 12705852]
12. Beck H, Schwarz G, Schroter CJ, et al. Cathepsin S and an asparagine-specific endoprotease dominate the proteolytic processing of human myelin basic protein in vitro. *Eur J Immunol.* 2001; 31(12):3726–36. [PubMed: 11745393]
13. Sepulveda FE, Maschalidi S, Colisson R, et al. Critical role for asparagine endopeptidase in endocytic Toll-like receptor signaling in dendritic cells. *Immunity.* 2009; 31(5):737–48. [PubMed: 19879164]
14. Chen JM, Fortunato M, Stevens RA, et al. Activation of progelatinase A by mammalian legumain, a recently discovered cysteine proteinase. *Biol Chem.* 2001; 382(5):777–83. [PubMed: 11517930]
15. Sarandeses CS, Covelo G, Diaz-Jullien C, et al. Prothymosin alpha is processed to thymosin alpha 1 and thymosin alpha 11 by a lysosomal asparaginyl endopeptidase. *J Biol Chem.* 2003; 278(15):13286–93. [PubMed: 12554742]
16. Patel N, Krishnan S, Offman MN, et al. A dyad of lymphoblastic lysosomal cysteine proteases degrades the antileukemic drug L-asparaginase. *J Clin Invest.* 2009; 119(7):1964–73. [PubMed: 19509471]
17. Morita Y, Araki H, Sugimoto T, et al. Legumain/asparaginyl endopeptidase controls extracellular matrix remodeling through the degradation of fibronectin in mouse renal proximal tubular cells. *FEBS Lett.* 2007; 581(7):1417–24. [PubMed: 17350006]
18. Liu Z, Jang SW, Liu X, et al. Neuroprotective actions of PIKE-L by inhibition of SET proteolytic degradation by asparagine endopeptidase. *Mol Cell.* 2008; 29(6):665–78. This article reports that the cleavage of SET by AEP mediates neuronal cell death induced by MCAO. [PubMed: 18374643]
19. Ye K, Snyder SH. PIKE GTPase: a novel mediator of phosphoinositide signaling. *J Cell Sci.* 2004; 117(Pt 2):155–61. [PubMed: 14676271]
20. Ye K. PIKE/nuclear PI 3-kinase signaling in preventing programmed cell death. *J Cell Biochem.* 2005; 96(3):463–72. [PubMed: 16088938]

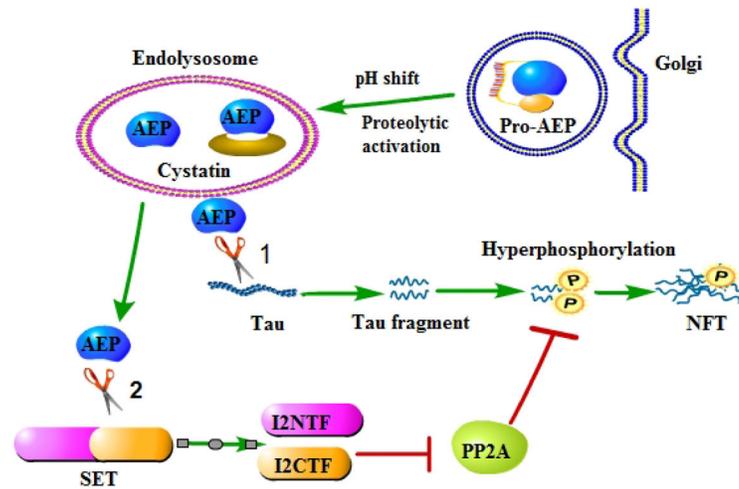
- 21••. Zhang Z, Song M, Liu X, et al. Cleavage of tau by asparagine endopeptidase mediates the neurofibrillary pathology in Alzheimer's disease. *Nat Med.* 2014; 20(11):1254–62. This article reports that AEP cleaves tau in an age-dependent manner, promotes its phosphorylation and aggregation. Deletion of AEP reverses the tau pathology in tau P301S transgenic mice. [PubMed: 25326800]
- 22••. Zhang Z, Song M, Liu X, et al. Delta-secretase cleaves amyloid precursor protein and regulates the pathogenesis in Alzheimer's disease. *Nat Commun.* 2015; 6:8762. This article reports that AEP acts as a delta-secretase that cleaves APP after N373 and N585. The resultant APP<sub>586-695</sub> fragment is a better substrate for BACE1 than full-length APP. Thus the proteolysis of APP by AEP promotes A $\beta$  production. Deletion of AEP from 5XFAD and APP/PS1 mouse model alleviates A $\beta$  deposition and cognitive impairments. [PubMed: 26549211]
23. Rosenmann H. Asparagine endopeptidase cleaves tau and promotes neurodegeneration. *Nat Med.* 2014; 20(11):1236–8. [PubMed: 25375922]
24. Ma L, Shen YQ, Khatri HP, et al. The asparaginyl endopeptidase legumain is essential for functional recovery after spinal cord injury in adult zebrafish. *PLoS One.* 2014; 9(4):e95098. [PubMed: 24747977]
25. Maehr R, Hang HC, Mintern JD, et al. Asparagine endopeptidase is not essential for class II MHC antigen presentation but is required for processing of cathepsin L in mice. *J Immunol.* 2005; 174(11):7066–74. [PubMed: 15905550]
26. Murthy RV, Arbman G, Gao J, et al. Legumain expression in relation to clinicopathologic and biological variables in colorectal cancer. *Clin Cancer Res.* 2005; 11(6):2293–9. [PubMed: 15788679]
27. Liu C, Sun C, Huang H, et al. Overexpression of legumain in tumors is significant for invasion/metastasis and a candidate enzymatic target for prodrug therapy. *Cancer Res.* 2003; 63(11):2957–64. [PubMed: 12782603]
28. Choi SJ, Reddy SV, Devlin RD, et al. Identification of human asparaginyl endopeptidase (legumain) as an inhibitor of osteoclast formation and bone resorption. *J Biol Chem.* 1999; 274(39):27747–53. [PubMed: 10488118]
- 29•. Miller G, Matthews SP, Reinheckel T, et al. Asparagine endopeptidase is required for normal kidney physiology and homeostasis. *FASEB J.* 2011; 25(5):1606–17. This article reports the phenotypes of AEP knockout mice. [PubMed: 21292981]
30. Erickson AH. Biosynthesis of lysosomal endopeptidases. *J Cell Biochem.* 1989; 40(1):31–41. [PubMed: 2663888]
- 31•. Chan CB, Abe M, Hashimoto N, et al. Mice lacking asparaginyl endopeptidase develop disorders resembling hemophagocytic syndrome. *Proc Natl Acad Sci U S A.* 2009; 106(2):468–73. This article reports the phenotypes of AEP knockout mice. [PubMed: 19106291]
32. Jordan MB, Hildeman D, Kappler J, et al. An animal model of hemophagocytic lymphohistiocytosis (HLH): CD8+ T cells and interferon gamma are essential for the disorder. *Blood.* 2004; 104(3):735–43. [PubMed: 15069016]
33. Maschalidi S, Hässler S, Blanc F, et al. Asparagine endopeptidase controls anti-influenza virus immune responses through TLR7 activation. *PLoS Pathog.* 2012; 8(8):e1002841–e41. [PubMed: 22916010]
34. Back T, Hoehn M, Mies G, et al. Penumbra tissue alkalosis in focal cerebral ischemia: relationship to energy metabolism, blood flow, and steady potential. *Ann Neurol.* 2000; 47(4):485–92. [PubMed: 10762160]
35. Ishizaki T, Erickson A, Kuric E, et al. The asparaginyl endopeptidase legumain after experimental stroke. *J Cereb Blood Flow Metab.* 2010; 30(10):1756–66. [PubMed: 20234379]
36. Rickhag M, Wieloch T, Gido G, et al. Comprehensive regional and temporal gene expression profiling of the rat brain during the first 24 h after experimental stroke identifies dynamic ischemia-induced gene expression patterns, and reveals a biphasic activation of genes in surviving tissue. *J Neurochem.* 2006; 96(1):14–29. [PubMed: 16300643]
37. Clerin V, Shih HH, Deng N, et al. Expression of the cysteine protease legumain in vascular lesions and functional implications in atherosclerosis. *Atherosclerosis.* 2008; 201(1):53–66. [PubMed: 18377911]

38. Moss CX, Matthews SP, Lamont DJ, et al. Asparagine deamidation perturbs antigen presentation on class II major histocompatibility complex molecules. *J Biol Chem*. 2005; 280(18):18498–503. [PubMed: 15749706]
39. Ling SC, Polymenidou M, Cleveland DW. Converging mechanisms in ALS and FTD: disrupted RNA and protein homeostasis. *Neuron*. 2013; 79(3):416–38. [PubMed: 23931993]
40. Janssens J, Van Broeckhoven C. Pathological mechanisms underlying TDP-43 driven neurodegeneration in FTL-ALS spectrum disorders. *Hum Mol Genet*. 2013; 22(R1):R77–87. [PubMed: 23900071]
41. Arai T, Hasegawa M, Akiyama H, et al. TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Biochem Biophys Res Commun*. 2006; 351(3):602–11. [PubMed: 17084815]
42. Igaz LM, Kwong LK, Xu Y, et al. Enrichment of C-terminal fragments in TAR DNA-binding protein-43 cytoplasmic inclusions in brain but not in spinal cord of frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Am J Pathol*. 2008; 173(1):182–94. [PubMed: 18535185]
43. Igaz LM, Kwong LK, Chen-Plotkin A, et al. Expression of TDP-43 C-terminal Fragments in Vitro Recapitulates Pathological Features of TDP-43 Proteinopathies. *J Biol Chem*. 2009; 284(13): 8516–24. [PubMed: 19164285]
44. Herskowitz JH, Gozal YM, Duong DM, et al. Asparaginyl endopeptidase cleaves TDP-43 in brain. *Proteomics*. 2012; 12(15–16):2455–63. [PubMed: 22718532]
45. Lagier-Tourenne C, Polymenidou M, Cleveland DW. TDP-43 and FUS/TLS: emerging roles in RNA processing and neurodegeneration. *Hum Mol Genet*. 2010; 19(R1):R46–64. [PubMed: 20400460]
46. Gamblin TC, Chen F, Zambrano A, et al. Caspase cleavage of tau: linking amyloid and neurofibrillary tangles in Alzheimer's disease. *Proc Natl Acad Sci U S A*. 2003; 100(17):10032–7. [PubMed: 12888622]
47. Johnson GV, Jope RS, Binder LI. Proteolysis of tau by calpain. *Biochem Biophys Res Commun*. 1989; 163(3):1505–11. [PubMed: 2551295]
48. Litersky JM, Scott CW, Johnson GV. Phosphorylation, calpain proteolysis and tubulin binding of recombinant human tau isoforms. *Brain Res*. 1993; 604(1–2):32–40. [PubMed: 8384512]
49. Basurto-Islas G, Grundke-Iqbal I, Tung YC, et al. Activation of asparaginyl endopeptidase leads to Tau hyperphosphorylation in Alzheimer disease. *J Biol Chem*. 2013; 288(24):17495–507. This article reported that AEP-mediated cleavage of SET promotes tau phosphorylation in AD. [PubMed: 23640887]
50. Ji ZS, Miranda RD, Newhouse YM, et al. Apolipoprotein E4 potentiates amyloid  $\beta$  peptide-induced lysosomal leakage and apoptosis in neuronal cells. *J Biol Chem*. 2002; 277(24):21821–8. [PubMed: 11912196]
51. Ditaranto K, Tekirian TL, Yang AJ. Lysosomal membrane damage in soluble A $\beta$ -mediated cell death in Alzheimer's disease. *Neurobiol Dis*. 2001; 8(1):19–31. [PubMed: 11162237]
52. Yang AJ, Chandswangbhuvana D, Margol L, et al. Loss of endosomal/lysosomal membrane impermeability is an early event in amyloid A $\beta$ 1-42 pathogenesis. *J Neurosci Res*. 1998; 52(6): 691–8. [PubMed: 9669318]
53. Simonsen A, McGuire J, Podust V, et al. A novel panel of cerebrospinal fluid biomarkers for the differential diagnosis of Alzheimer's disease versus normal aging and frontotemporal dementia. *Dement Geriatr Cogn Disord*. 2007; 24(6):434–40. [PubMed: 17971664]
54. Hansson SF, Andréasson U, Wall M, et al. Reduced levels of amyloid-beta-binding proteins in cerebrospinal fluid from Alzheimer's disease patients. *J Alzheimers Dis*. 2008; 16(2):389–97.
55. Yates CM, Butterworth J, Tennant MC, et al. Enzyme activities in relation to pH and lactate in postmortem brain in Alzheimer-type and other dementias. *Journal of neurochemistry*. 1990; 55(5): 1624–30. [PubMed: 2213015]
56. Fang B, Wang D, Huang M, et al. Hypothesis on the relationship between the change in intracellular pH and incidence of sporadic Alzheimer's disease or vascular dementia. *International Journal of Neuroscience*. 2010; 120(9):591–95. [PubMed: 20707633]

57. Pirchl M, Humpel C. Does acidosis in brain play a role in Alzheimer's disease? *Neuropsychiatrie: Klinik, Diagnostik, Therapie und Rehabilitation: Organ der Gesellschaft Osterreichischer Nervenärzte und Psychiater*. 2008; 23(3):187–92.
58. Forester BP, Berlow YA, Harper DG, et al. Age-related changes in brain energetics and phospholipid metabolism. *NMR in Biomedicine*. 2010; 23(3):242–50. [PubMed: 19908224]
59. Mandal PK, Akolkar H, Tripathi M. Mapping of hippocampal pH and neurochemicals from in vivo multi-voxel 31P study in healthy normal young male/female, mild cognitive impairment, and Alzheimer's disease. *Journal of Alzheimer's Disease*. 2012; 31(S3):S75–86.
60. Chohan MO, Khatoon S, Iqbal IG, et al. Involvement of I2PP2A in the abnormal hyperphosphorylation of tau and its reversal by Memantine. *FEBS Lett*. 2006; 580(16):3973–9. [PubMed: 16806196]
61. Wang X, Blanchard J, Grundke-Iqbal I, et al. Alzheimer disease and amyotrophic lateral sclerosis: an etiopathogenic connection. *Acta Neuropathol*. 2014; 127(2):243–56. [PubMed: 24136402]
62. Wang X, Blanchard J, Kohlbrenner E, et al. The carboxy-terminal fragment of inhibitor-2 of protein phosphatase-2A induces Alzheimer disease pathology and cognitive impairment. *FASEB J*. 2010; 24(11):4420–32. [PubMed: 20651003]
63. Arnaud L, Chen S, Liu F, et al. Mechanism of inhibition of PP2A activity and abnormal hyperphosphorylation of tau by I2(PP2A)/SET. *FEBS Lett*. 2011; 585(17):2653–9. [PubMed: 21806989]
64. Chasseigneaux S, Clamagirand C, Huguet L, et al. Cytoplasmic SET induces tau hyperphosphorylation through a decrease of methylated phosphatase 2A. *BMC Neurosci*. 2014; 15:82. [PubMed: 24981783]
65. Oddo S, Caccamo A, Shepherd JD, et al. Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular A $\beta$  and synaptic dysfunction. *Neuron*. 2003; 39(3):409–21. [PubMed: 12895417]
66. Lee J, Bogyo M. Synthesis and evaluation of aza-peptidyl inhibitors of the lysosomal asparaginyl endopeptidase, legumain. *Bioorg Med Chem Lett*. 2012; 22(3):1340–3. [PubMed: 22243962]
67. Niestroj AJ, Feussner K, Heiser U, et al. Inhibition of mammalian legumain by Michael acceptors and AzaAsn-halomethylketones. *Biol Chem*. 2002; 383(7–8):1205–14. [PubMed: 12437107]
68. Gotz MG, James KE, Hansell E, et al. Aza-peptidyl Michael acceptors. A new class of potent and selective inhibitors of asparaginyl endopeptidases (legumains) from evolutionarily diverse pathogens. *J Med Chem*. 2008; 51(9):2816–32. [PubMed: 18416543]
69. Lee J, Bogyo M. Development of near-infrared fluorophore (NIRF)-labeled activity-based probes for in vivo imaging of legumain. *ACS Chem Biol*. 2010; 5(2):233–43. [PubMed: 20017516]
70. Higgins C, Bouazzaoui S, Gaddale K, et al. P3 SAR exploration of biphenyl carbamate based Legumain inhibitors. *Bioorg Med Chem Lett*. 2014; 24(11):2521–4. [PubMed: 24775305]
71. Pardridge WM. Drug transport across the blood-brain barrier. *J Cerebr Blood F Met*. 2012; 32(11):338–48.

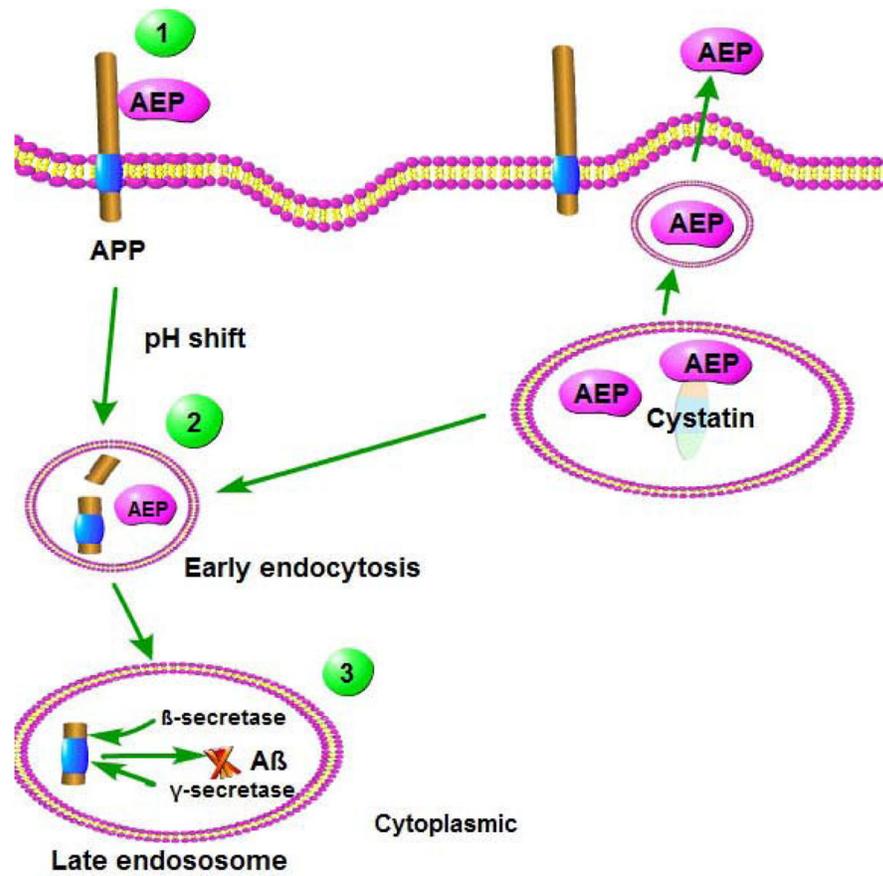
### Article Highlights

- AEP is implicated in numerous human diseases including immune disorder, cancer, and neurological diseases.
- Under pathological conditions such as AD, ALS and FTLD, AEP participates in neurodegeneration through the cleavage of its substrates such as SET, APP, tau, and TDP-43. In AD, AEP is activated in an age-dependent manner, and mediates both the tau and A $\beta$  pathology.
- Inhibition of AEP activity is a promising therapeutic strategy for cancer and neurological diseases.
- A series of substrate asparagine-based competitive or covalent peptidyl inhibited has been developed recently. However, ultra-high throughput drug screen with a large number of chemical library might help to identify much more promising small AEP inhibitors.



**Figure 1. AEP promotes tau aggregation in AD**

1) AEP might translocate from the endolysosome into the cytoplasmic space, where it cleaves tau. 2) Intracellular AEP cuts SET, leading to PP2A inhibition and consequent tau hyperphosphorylation.



**Figure 2. AEP cuts APP and promotes the production of A $\beta$**   
 AEP cleaves APP extracellularly (1) and/or in the endolysosome (2) and promotes the production of A $\beta$  (3).

**Table 1**Phenotype of AEP<sup>-/-</sup> mice

System	Phenotype	Ref.
Kidney	The mice develop progressive kidney pathology and decreased glomerular filtration rate	[29]
Lysosomal proteases	The processing of cathepsins B, H, and L is altered	[9]
Hematologic system	Fever, cytopenia, hepatosplenomegaly, hemophagocytosis, extramedullary hematopoiesis, lower natural killer cell activity	[31]
Antigen processing	Dendritic cells (DCs) show reduction in the secretion of proinflammatory cytokines in response to TLR9 stimulation	[13]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

**Table 2**

AEP substrates in the development of neurodegenerative diseases

AEP Substrates	Cleavage site (a.a.)	Pathological function	Ref.
APP	373 and 585	APP <sub>1-373</sub> fragment is toxic to cultured neurons. APP <sub>586-695</sub> fragment promotes the production of A $\beta$	[22]
Tau	255 and 368	Tau 1-368 fragment is more prone to aggregate, and is toxic to neurons	[21]
SET	175	The AEP-derived SET fragments lost the DNase inhibitor activity. Overexpression of SET fragments in rat brain decreases PP2A activity, causes abnormal hyperphosphorylation of tau and neurodegeneration	[18, 61]
TDP 43	291 and 306	Unknown	[44]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript