

ARTICLE OPEN



ADAMTS4 is involved in the production of the Alzheimer disease amyloid biomarker APP669-711

Masaya Matsuzaki¹, Miyabishara Yokoyama¹, Yota Yoshizawa¹, Naoki Kaneko², Hiroki Naito², Honoka Kobayashi¹, Akihito Korenaga², Sadanori Sekiya², Kentaro Ikemura³, Gabriel Opoku³, Satoshi Hirohata³, Shinichi Iwamoto², Koichi Tanaka² and Taisuke Tomita¹✉

© The Author(s) 2023

Amyloid- β (A β) deposition in the brain parenchyma is one of the pathological hallmarks of Alzheimer disease (AD). We have previously identified amyloid precursor protein (APP)669-711 (a.k.a. A β (-3)-40) in human plasma using immunoprecipitation combined with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (IP-MALDI-MS). Furthermore, we found that the level of a composite biomarker, i.e., a combination of APP669-711/A β 1-42 ratio and A β 1-40/A β 1-42 ratio in human plasma, correlates with the amyloid PET status of AD patients. However, the production mechanism of APP669-711 has remained unclear. Using in vitro and in vivo assays, we identified A Disintegrin and Metalloproteinase with a Thrombospondin type 1 motif, type 4 (ADAMTS4) as a responsible enzyme for APP669-711 production. ADAMTS4 cleaves APP directly to generate the C-terminal stub c102, which is subsequently proteolyzed by γ -secretase to release APP669-711. Genetic knockout of *ADAMTS4* reduced the production of endogenous APP669-711 by 30% to 40% in cultured cells as well as mouse plasma, irrespectively of A β levels. Finally, we found that the endogenous murine APP669-711/A β 1-42 ratio was increased in aged AD model mice, which shows A β deposition as observed in human patients. These data suggest that ADAMTS4 is involved in the production of APP669-711, and a plasma biomarker determined by IP-MALDI-MS can be used to estimate the level of A β deposition in the brain of mouse models.

Molecular Psychiatry (2023) 28:1802–1812; <https://doi.org/10.1038/s41380-023-01946-y>

INTRODUCTION

Alzheimer disease (AD) in particular accounts for a large population of the causes of dementia [1]. Senile plaques, which are formed by the aggregation and deposition of amyloid- β (A β), and neurofibrillary tangles composed of tau protein, are the pathological features observed in the brains of patients with AD. A β is produced by the proteolytic cleavage of amyloid precursor protein (APP) by β - and γ -secretases [1–3]. Several genetic mutations linked to familial AD increase the production or aggregation of A β . In contrast, patients with sporadic AD have been reported to have a decreased clearance rate of brain A β , rather than its increased production rate [4]. These data strongly indicate that the accumulation of A β triggers the onset of AD. However, the deposition of A β in the brain starts 10–20 years before the appearance of the clinical symptoms of AD. Moreover, recent results of clinical trials have implicated that anti-A β treatment should be performed on patients in the mild cognitive impairment or prodromal AD stage for beneficial effects [5]. However, cognitive function tests such as Mini-Mental State Examination (MMSE) and Clinical Dementia Rating (CDR) are difficult to diagnose the amyloid deposition in the asymptomatic stage. Amyloid imaging using positron emission tomography (PET) and quantification of A β 42 and tau levels in the cerebrospinal fluid (CSF) is utilized to estimate the brain A β deposition. However, it is difficult to identify amyloid deposition in subjects in the

asymptomatic stage using cognitive function tests, such as MMSE and CDR. Amyloid imaging using PET, and the quantification of A β 42 and tau levels in the CSF are utilized to estimate brain A β deposition levels. However, these methods are expensive or highly invasive. Thus, determination of the pathological stage of brain amyloid deposition, and estimation of AD risk by a non-invasive and cost-effective method is essential for the development of anti-A β therapeutics.

The N- and C-terminal lengths of A β are heterogeneous. The dominant A β species in senile plaques start with a pyroglutamate residue at the 3rd position and end with an alanine at the 42nd position [6–8]. However, the major secreted form of A β in the conditioned medium of cultured cells is A β 1-40 [9, 10]. These heterogeneities are due to proteolytic modifications after A β deposition and multiple cleavage mechanisms during the A β -generating process [2]. In particular, γ -secretase cleaves APP at multiple sites, resulting in the generation of A β ending at the 37th, 38th, 40th, 42nd, and 43rd positions. For the N-terminal side, the β -secretase BACE1 cleaves A β mainly at the 1st and 11th positions. In addition, other enzymes have been implicated in the generation of N-terminally truncated A β species, which have been identified from the conditioned medium of cultured cells. However, N-terminally elongated A β species have not been investigated to date. In recent years, we identified a novel N-terminally elongated A β , APP669-711 (a.k.a. A β (-3)-40), in

¹Laboratory of Neuropathology and Neuroscience, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo 113-0033, Japan. ²Koichi Tanaka Mass Spectrometry Research Laboratory, Shimadzu Corporation, Kyoto 604-8511, Japan. ³Department of Medical Technology, Graduate School of Health Sciences, Okayama University, Okayama 700-8558, Japan. ✉email: taisuke@mol.f.u-tokyo.ac.jp

Received: 13 March 2022 Revised: 22 December 2022 Accepted: 6 January 2023

Published online: 1 February 2023

human plasma using an ultrasensitive immunoprecipitation (IP) method combined with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (IP-MALDI-MS) [11]. Importantly, the plasma APP669-711/A β 1-42 ratio is increased in amyloid PET-positive AD patients. Furthermore, a composite plasma biomarker combining APP669-711/A β 1-42 ratio and plasma A β 1-40/A β 1-42 ratio was shown to enable the estimation of brain A β accumulation in amyloid PET-positive individuals with 90% accuracy using 0.5 ml of blood [12]. Its sensitivity and specificity were 91.7% to 100% and 84.5%, respectively, indicating a separability comparable to that of amyloid PET. Head-to-head comparison analysis demonstrated that this composite biomarker showed the highest correlation with CSF A β 42/40 ratio [13]. However, how APP669-711 is produced, and the mechanisms that regulate APP669-711 levels remain unclear. In this study, we found that the secreted metalloprotease, a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS4), and γ -secretase are involved in the generation of APP669-711, both in vitro and in vivo.

MATERIALS AND METHODS

Animals

All experiments using animals were performed according to the guidelines provided by the Institutional Animal Care Committee of Graduate School of Pharmaceutical Sciences, The University of Tokyo (Protocol no. P29-30 and P30-3), and Graduate School of Health Sciences, Okayama University (#OKU2022-413). APP/PS1 mice (B6.Cg-Tg(APPswe, PSEN1dE9)85Dbo/Mmjax, Jackson Laboratory, JAX mouse #005864) and *Adamts4*^{-/-} mice (B6.129P2-Adamts4tm1Dgen/J, Jackson Laboratory, JAX mouse #005770) were used. Detailed experimental methods are described in the Supplementary Information.

Antibodies, chemicals, plasmids, cells, and immunological methods

For the generation of CRISPR knockout cells, we used Cas9 nickase (D10A) which requires two adjacent guide RNAs for cleaving target regions [14–16]. cDNAs encoding gRNA sequences (Supplementary Information) were inserted into pX335-U6-Chimeric_BB-CBh-hSpCas9n(D10A) (a gift from Dr. Feng Zhang, Addgene plasmid #42335; <http://n2t.net/addgene:42335>; RRID: Addgene_42335) and pBabe Puro U6 BbsI (kindly provided by Dr. Dario Alessi (University of Dundee). Transfection into HEK293A and A549 cells using polyethylenimine (Polysciences) and lipofectAMINE LTX (Invitrogen), respectively, were described previously [14, 15]. A549 cells have 5.8 ± 1.40 copies of chromosome 1 per karyotype [17], in which the *ADAMTS4* gene locates. Thus, we had chosen the monoclonal cell line that harbors three mutations within the target genomic sequence. Materials, detailed protocol for cultured cells, molecular biology, and immunological methods are described in the Supplementary Information.

In vitro cleavage assay

Plasmids encoding APP81 are transformed into *E. coli* BL21(DE3) (Novagen), and the substrate were purified as described in the Supplementary Information. Same volume (80 μ l) of 17 μ M APP81 and 100 nM ADAMTS4 protein (diluted in assay buffer (50 mM HEPES, 50 mM NaCl, 1 mM CaCl₂, 0.05% Brij-35, pH 7.5)) were mixed and incubated at 37 °C for indicated hours. For immunoblotting, the reaction was halted by the addition of 2x Laemmli sample buffer. For MALDI-TOF MS, the mixture was stored at -80 °C until use.

MALDI-TOF MS

Mass spectra were acquired using a MALDI-linear TOF mass spectrometer (AXIMA Performance, Shimadzu/KRATOS, Manchester, UK) in the positive ion mode. MS/MS analysis for the identification of the proteolyzed peptides was performed using a MALDI-QIT reflectron TOF mass spectrometer (AXIMA Resonance, Shimadzu/KRATOS, Manchester, UK) in the positive ion mode and MS/MS product ions were generated by collision-induced dissociation with argon gas. The *m/z* reported in the linear TOF and the QIT-reflectron TOF represent the average and monoisotopic peak of the protonated signal [M + H]⁺, respectively. The *m/z* value was calibrated with human angiotensin II, human ACTH fragment 18-39, bovine

insulin oxidized beta-chain, and bovine insulin. Detailed protocol for solid-phase extraction, IP-MALDI-MS, and MS/MS analyses are described in the Supplementary Information.

Statistical analysis

All samples were analyzed in a randomized manner. For quantitative analyses, Student's *t* test was used for comparisons between two-group data, and Tukey's test was used for multiple group comparisons. Statistical analyses were performed by KyPlot or Excel software. In the figures, statistical results were indicated by absolute *p* values. A *p* value <0.05 was considered to have a significant difference.

Experimental details are described in the Supplementary Information.

RESULTS

APP669-711 is proteolytically generated from various cells under physiological conditions

Although γ -secretase-mediated C-terminal variations of A β have been extensively analyzed, the N-terminal variations of secreted A β have not been investigated to date. Importantly, several analyses of cell-based models and genetically modified animals harboring the APP mutant carrying the Swedish mutation (APPswe) located at the (-1st) and (-2nd) positions of A β , which significantly increases the β -secretase-mediated cleavage at the 1st position, to induce the overproduction of A β have been performed [18, 19]. However, the profiles of secreted A β from endogenous APP and overexpressed APPswe are distinct in mouse neuroblastoma Neuro2a cells [20]. We previously demonstrated using IP-MALDI-MS and the 6E10 antibody that human neuroblastoma BE(2)-C cells secrete endogenous APP669-711 as well as A β [12]. We further analyzed the conditioned media from several human-derived cell lines. We found that APP669-711 was secreted from A549 adenocarcinoma cells as well as HEK293A cells expressing wild-type APP (APPwt), but not CCF-STTG1 astrocytoma, H4 neuroglioma, nor naïve HEK293A cells (Fig. 1C), suggesting that APP669-711 is produced from only some cultured cell lines under physiological conditions. To investigate the mechanism of the generation of APP669-711, we analyzed the effects of several inhibitors that affect APP metabolism in BE(2)-C cells (Fig. 1D, E). The γ -secretase inhibitor DAPT abolished the production of APP669-711. In contrast, treatment with the specific and potent BACE1 inhibitor MBSI increased APP669-711 production. Notably, GM6001, a pan metalloprotease inhibitor, partially inhibited APP669-711 production, although the ADAM10/17-specific inhibitor INCB3619 did not affect APP669-711 levels. These data suggested that an unknown metalloprotease-mediated cleavage at the APP669 site (a.k.a. (-3rd) position in A β) is involved in the generation of APP669-711.

In the A β production pathway, APP is cleaved by BACE1 at the 1st position of A β to generate c99, which is a direct substrate for γ -secretase. According to the proteolytic model above, the APP669-site cleavage of APP results in the production of the C-terminal stub c102, which is c99 with 3 extra N-terminal amino acid residues. To detect endogenous c102, we generated N-terminal end-specific anti-c102 antibodies (anti-c102#1 for immunoblotting, and anti-c102#3 for immunohistochemistry). The specificity of the antibodies was confirmed by immunoblotting of HEK293A cell lysates expressing recombinant c99 or c102, the latter being a sequence with 3 extra amino acid residues between the signal peptide and A β sequence in the c99 expression vector (Fig. 2A). By IP using the anti-c102 antibody, we confirmed the appearance of endogenous c102 in the lysates of A549 cells upon DAPT treatment (Fig. 2B, C). Furthermore, cotreatment of GM6001 reduced c102 levels in A549 cell lysates, supporting our notion that APP669-site cleavage of APP is mediated by an unknown metalloprotease. To further confirm that c102 is a direct substrate for APP669-711, we analyzed the conditioned medium of HEK293A cells expressing APPwt, c99,

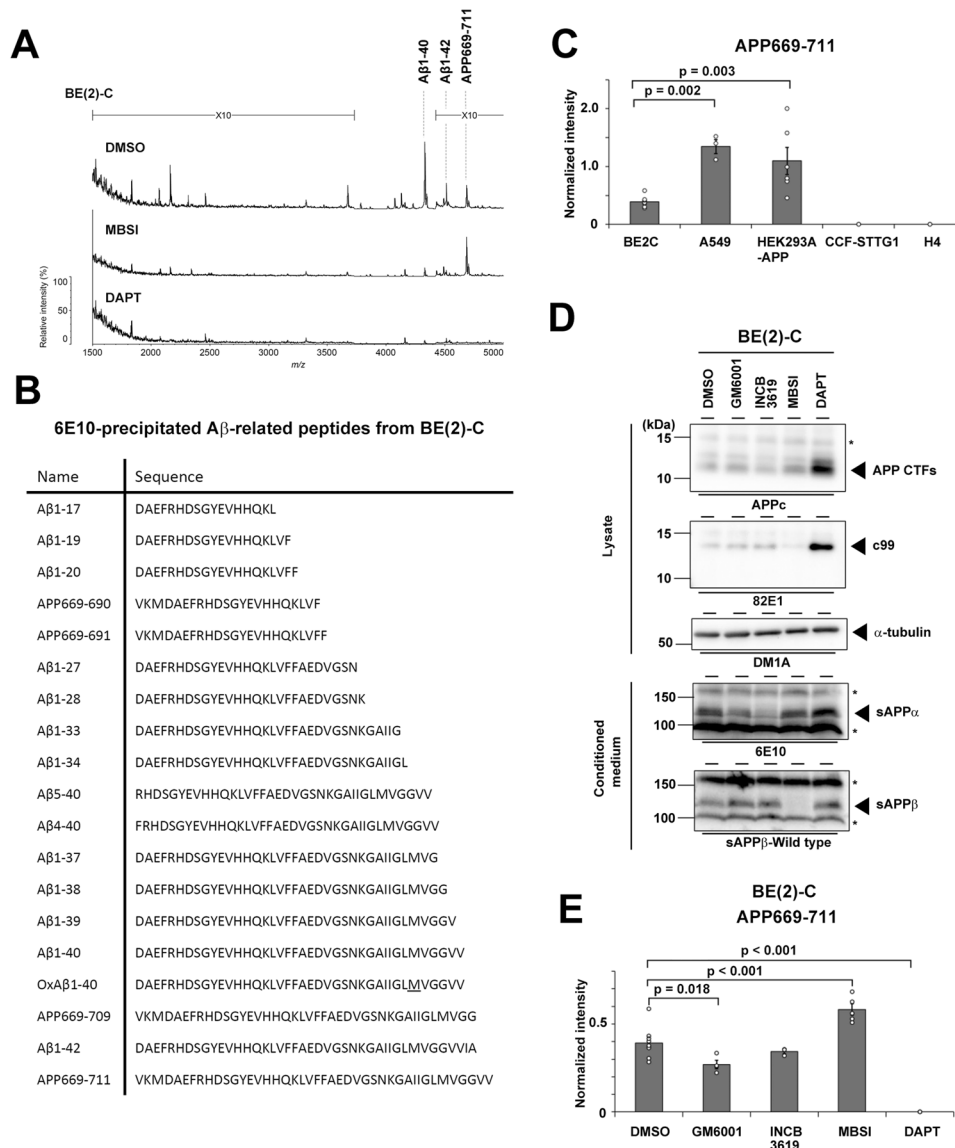


Fig. 1 Detection of APP669-711 in the conditioned medium of cultured cell lines. **A** IP-MALDI-MS spectrum of the 6E10 antibody-precipitated endogenous A β peptide variants in the conditioned medium from BE(2)-C cells. Various A β peptides, which are diminished by either MBSI or DAPT treatment, were identified. **B** The identified A β peptides secreted in **A**. Ox β 1-40 contained an oxidized methionine at the A β 35th residue. **C** Comparison of the level of endogenous APP669-711 in the conditioned medium of human-derived cultured cells ($n = 4-6$, mean \pm s.e.m. Tukey test). **D** Pharmacological effects of secretase inhibitors on APP metabolism in BE(2)-C cells. Asterisks indicate non-specific bands. **E** Pharmacological effects of secretase inhibitors on the production of APP669-711 from BE(2)-C cells ($n = 4$ or 5, mean \pm s.e.m. Tukey test).

and c102 by IP-MALDI-MS (Fig. 2D, E). As expected, we detected the A β 1-37, A β 1-38, A β 1-39, A β 1-40, A β 1-42, and APP669-711 (A β (-3)-40) in the media of APPwt-expressing cells. From recombinant c99, various A β species starting at the 1st position were identified. In contrast, the conditioned medium of cells expressing c102 contained various A β species starting at APP669 position (i.e., APP669-708 (A β (-3)-37), APP669-709 (A β (-3)-38), APP669-710 (A β (-3)-39), APP669-711 (A β (-3)-40), APP669-713 (A β (-3)-42)). DAPT treatment completely abolished the production of all A β species derived from APPwt and c102 (Fig. 2F, G). These data strongly indicate that APP669-711 is also proteolytically produced from APP by sequential cleavage by a yet-unknown metalloprotease at the APP669 site, and γ -secretase within the transmembrane domain.

We then comprehensively analyzed the endogenous A β species secreted from mouse neuroblastoma Neuro2a cells by IP-MALDI-

MS. For IP, instead of the 6E10 antibody, which is a human A β -specific antibody, we utilized the 4G8 antibody targeting the common sequence in the middle of human and murine A β (Fig. 3A, B). We successfully detected 34 types of A β -associated peptides in the cultured medium (Fig. 3C). As observed in human-derived cultured cells, GM6001 treatment of neuro2A cells significantly reduced the production of endogenous APP669-711, whereas MBSI did not affect APP669-711 levels. Intriguingly, treatment with MBSI, but not GM6001, abolished the production of A β species starting at the 1st, 2nd, and 11th positions, suggesting that BACE1 activity is specifically required for the proteolysis of APP at these sites. In contrast, the production of A β species starting at the APP669 and A β 12th positions was significantly reduced by GM6001, but not MBSI. Furthermore, we analyzed the effects of the Tissue inhibitor of metalloproteinase 3 (TIMP3), which is an endogenous potent metalloprotease inhibitor

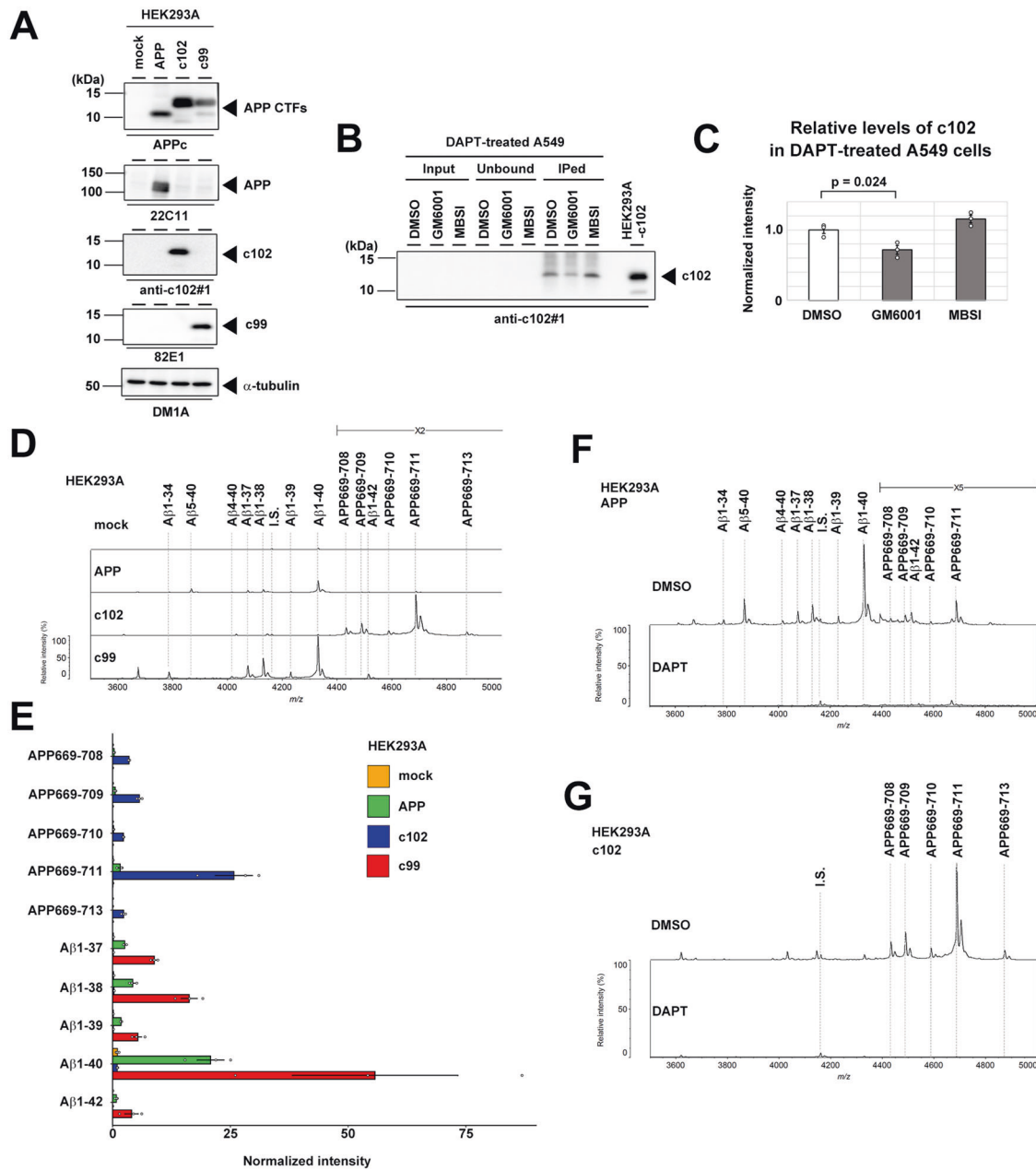


Fig. 2 Processing of c102, a direct substrate for APP669-711. **A** Immunoblot analysis of HEK293A cells expressing APP, c102, and c99. Note that the N-terminal end-specific antibodies anti-c102 and 82E1 detected c102 and c99, respectively. **B** Detection of endogenous c102 in the lysate of A549 cells by IP using an anti-c102 antibody. Cells were treated with DAPT to increase c102 levels. **C** Changes in c102 levels by GM6001 or MBSI in A549 cells co-treated with DAPT ($n = 3$, mean \pm s.e.m. Tukey test). **D** IP-MALD-MS spectrum of the 6E10 antibody-precipitated A β peptides in the conditioned medium from HEK293 cells expressing APP, c102, or c99. I.S. internal standard. **E** Levels of A β peptides secreted from HEK293A cells transfected with APP, c102, or c99 ($n = 4$ or 5 or 4, mean \pm s.e.m.). **F** Effects of DAPT on the production of A β peptides of APP-expressing HEK293A cells. **G** Effects of DAPT on the production of A β peptides of c102-expressing HEK293A cells.

implicated in AD pathogenesis [21, 22], on secreted APP669-711 levels from Neuro2a cells (Fig. 3D). Notably, TIMP3 treatment completely inhibited the production of endogenous APP669-711, whereas secreted A β levels were unaltered. A comparison of absolute levels of A β 1-40 and APP669-711 indicated that the processing ratio of BACE1 and ADAMTS4 on APP is 1:0.15. We then estimated the proportion of APP undergoing processing by BACE1 and α -secretase by end-specific ELISAs for sAPP β and sAPP α , respectively, as endogenous A β 17-40 was not detected in IP-MALDI-MS spectra. A comparison of absolute levels of sAPP β and sAPP α was 1:4.1. Thus, we speculate that the proportion of endogenous APP substrates undergoing processing by the BACE1,

α -secretase, and ADAMTS4 is 1:4.1:0.15. These data support our notion that the metalloprotease-mediated proteolytic production pathway for APP669-711 is independent of the A β -generating pathway, and is conserved in murine cells.

Identification of ADAMTS4 as an APP669 site-cleaving enzyme

A comprehensive analysis of various A β peptides from GM6001-treated cells suggests that the same metalloprotease is involved in cleaving APP669 and the A β 12th positions of A β . Recently, it was reported that the A β 12th position is cleaved by the secreted metalloprotease ADAMTS4 [23]. ADAMTS4 is identified as aggrecanase-1, which is a major aggrecan-degrading enzyme and is

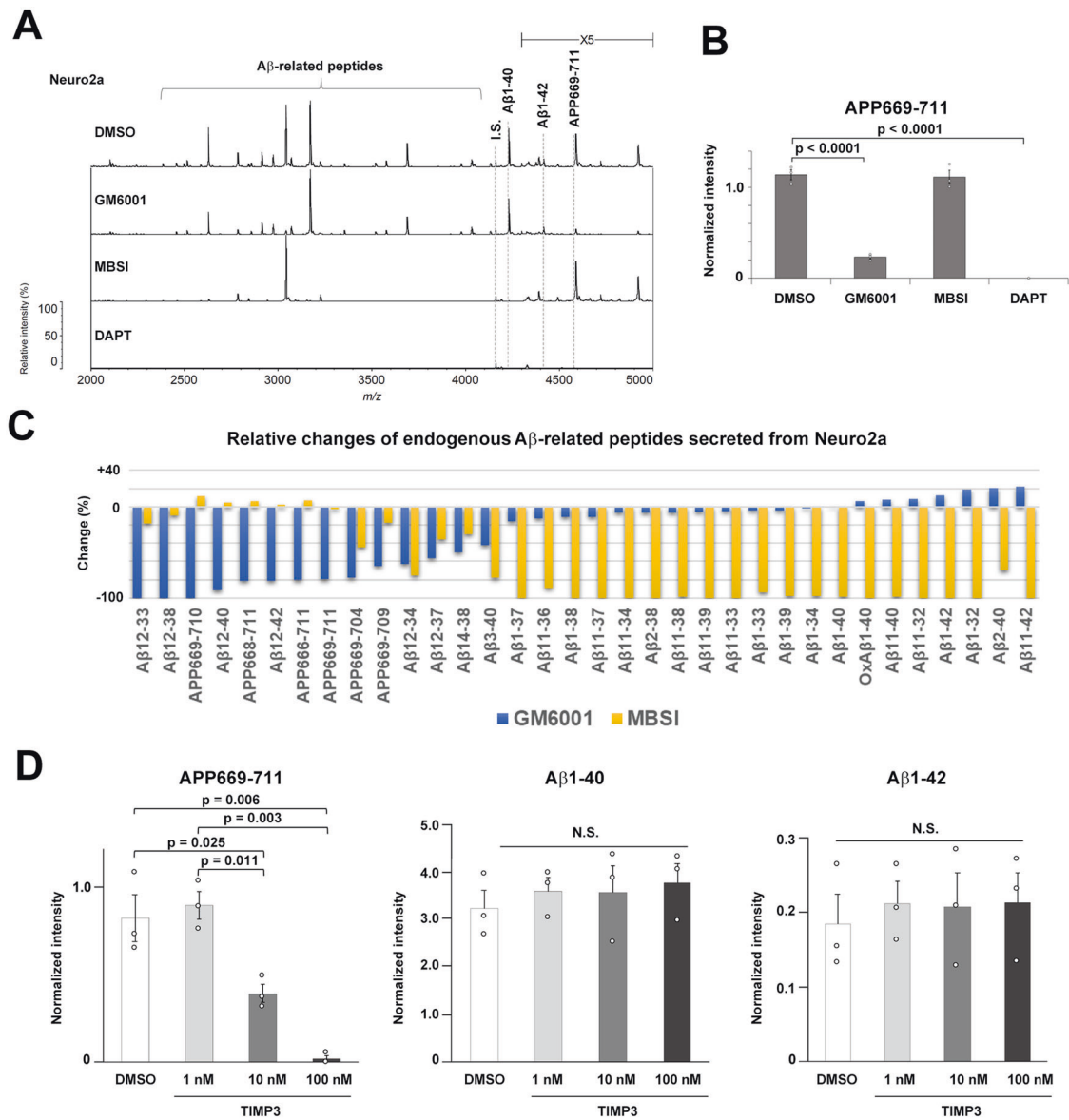


Fig. 3 Metalloprotease-dependent production of APP669-711 from Neuro2a cells. **A** IP-MALD-MS spectrum of the 4G8 antibody-precipitated endogenous Aβ peptides in the conditioned medium from Neuro2a cells. Various Aβ peptides were identified. I.S. internal standard. **B** The pharmacological effect of secrete inhibitors on the production of APP669-711 from Neuro2a cells ($n = 3$, mean \pm s.e.m. Tukey test). **C** Comparison of the effects of GM6001 and MBSI on the production of endogenous Aβ peptides in Neuro2a cells. **D** Effects of TIMP3 treatment of on the APP669-711 and Aβ production from Neuro2a cells ($n = 3$, mean \pm s.e.m. Tukey test. N.S. not significant).

implicated in the development of osteoarthritis [24, 25]. The proteolytic activity of ADAMTS4 is inhibited by TIMP3 [26, 27]. The recognition motif for ADAMTS4 (i.e., E-[AFVLMY]-X[0,1]-[RK]-X[2,3]-[ST]-[VYIFWMLA]) contains Glu at the P1 position and partially corresponds to the (−4th) position in the human Aβ sequence, in addition to the Aβ 3rd and Aβ 11th positions [28]. Of note, the Aβ 3rd position in the murine Aβ sequence does not match with the motif, as the Aβ 5th position is Gly instead of Arg in humans. Walter et al. reported that the overexpression of ADAMTS4 in HEK293 cells expressing APPsw resulted in the overproduction of Aβ4-40 and Aβ12-40 [23]. They also showed that the Aβ peptide starting at the 1st position is also a direct substrate for ADAMTS4. However, they did not investigate the effects of ADAMTS4 on APP669-711 production.

To test whether ADAMTS4 can cleave APP directly at the APP669 site, we first performed the *in vitro* digestion assay using a novel recombinant APP substrate, APP81 (Fig. 4A). APP81 encodes

the APP619-699 sequence tagged with N-terminal FLAG and C-terminal V5-His tags. Coincubation of purified APP81 with recombinant ADAMTS4 resulted in the appearance of low-molecular-weight peptides that were recognized by anti-His tag and anti-Aβ (6E10) antibodies (Fig. 4B). This reaction was specifically inhibited by the addition of metal chelator ethylenediaminetetraacetic acid. We then analyzed the proteolyzed peptides by MALDI-TOF-MS (Fig. 4C, D). All N- and C-terminal fragments generated by APP669, Aβ 4th, and Aβ 12th-site cleavages (FLAG-APP668 and APP669-His; FLAG-Aβ3 and Aβ4-His; and FLAG-Aβ11 and Aβ12-His, respectively) were detected. Moreover, a peptide derived from double digestion by APP669 and Aβ 12th-site cleavages (i.e., APP669-Aβ11) was also identified by MS/MS analysis. These data indicate that ADAMTS4 can directly cleave APP at APP669, the 4th and 12th positions.

Next, we analyzed the conditioned medium of HEK293A cells expressing APPwt and ADAMTS4 (Figs. 5A, B and S1). Consistent

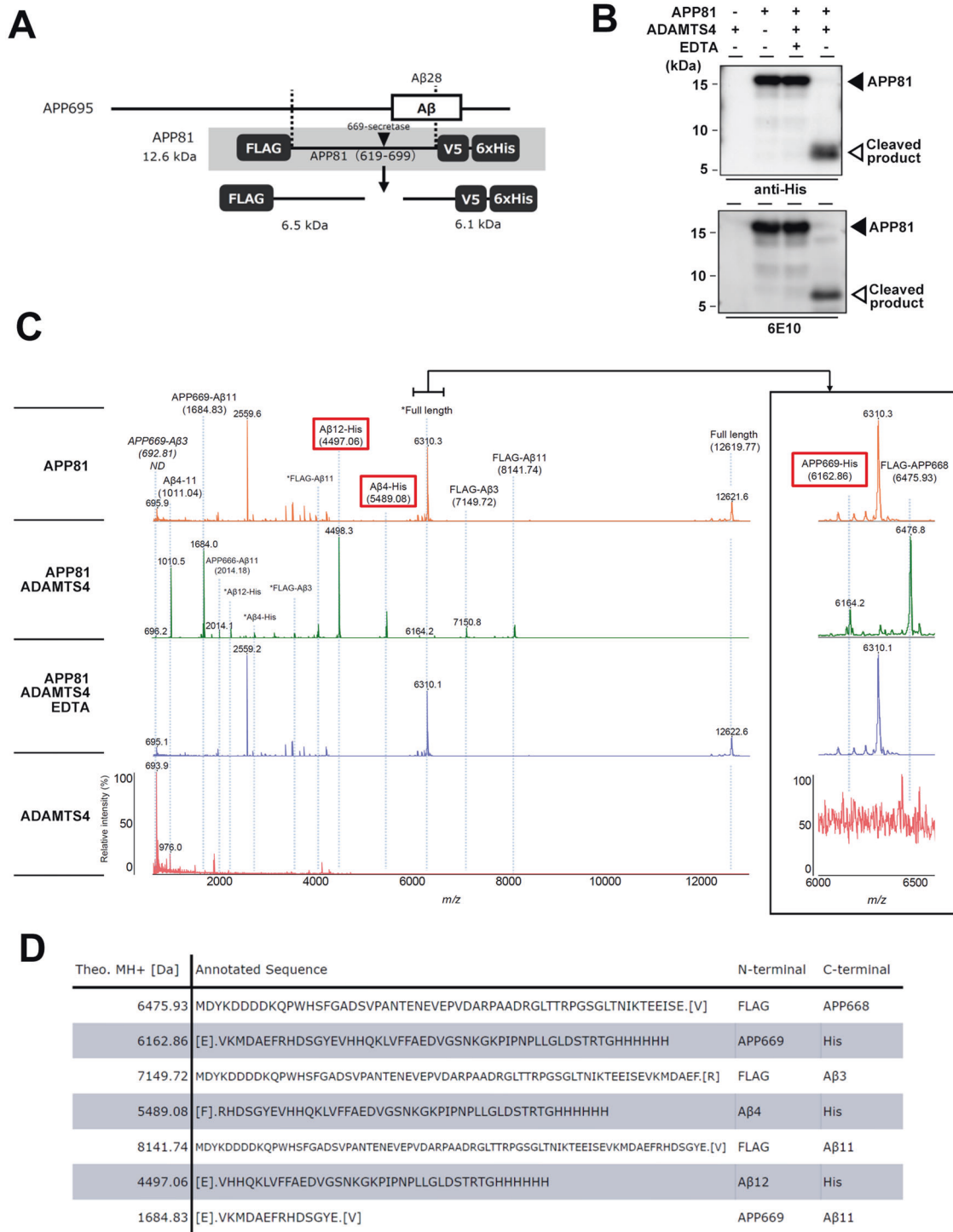


Fig. 4 **In vitro** cleavage assay using recombinant APP81 substrate. **A** Schematic depiction of the recombinant APP81 substrate and the *in vitro* assay. **B** Immunoblot analysis of APP81 (black triangles) and cleaved products (white triangles) generated by recombinant ADAMTS4. Note that the ADAMTS4-mediated cleavage was inhibited by preincubation with EDTA. **C** MALDI-MS spectrum of the reaction mixture of APP81 and ADAMTS4 (green). Several proteolytic fragments, which were not identified in the substrate only (orange), enzyme only (red), nor EDTA coincubated (blue) samples, were detected. The inset indicates the enlarged spectrum around m/z 6000 to 6600. Asterisks indicate double protonated signals. **D** Theoretical molecular weights of peptides specifically identified from the reaction mixture, and their annotated sequences are shown.

with a previous report, the level of Aβ₄₋₄₀ was significantly increased by ADAMTS4 expression [23]. The level of APP669-711 was also slightly, but significantly upregulated by ADAMTS4, and was reduced by treatment with GM6001 or TIMP3. The production of Aβ₁₋₄₀ and Aβ₁₋₄₂ was not altered by ADAMTS4

overexpression, GM6001, or TIMP3 treatment. Furthermore, co-expression of c102 with ADAMTS4 did not increase Aβ₄₋₄₀ levels (Fig. 5C). Notably, the production of APP669-711 from APPwt expressed in HEK293A cells was unaltered by the addition of the conditioned medium of ADAMTS4-expressing HEK293A cells,

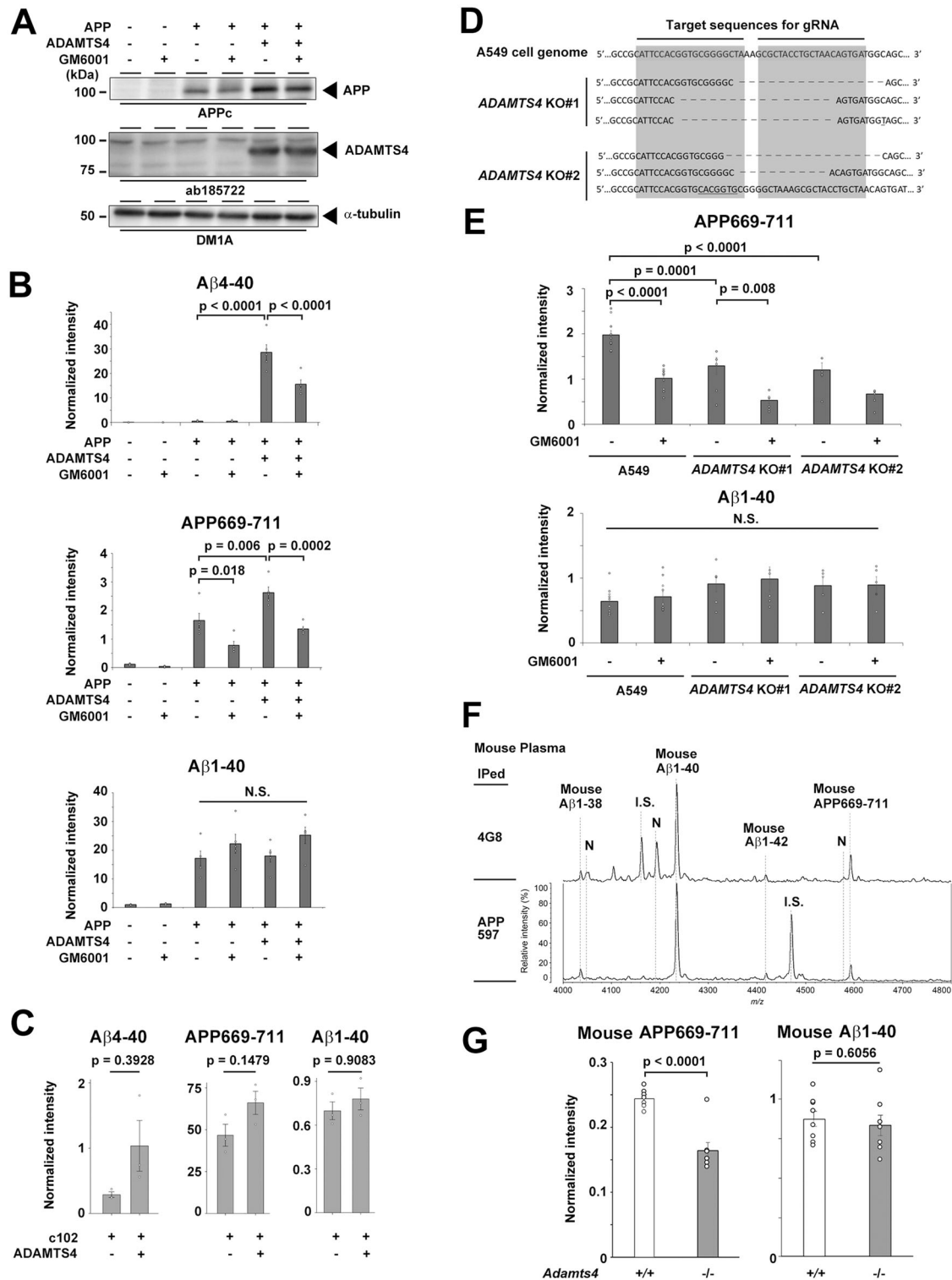


Fig. 5 Effects of ADAMTS4 on APP669-711 production. **A** Immunoblot analysis of HEK293A cell lysates expressing APP and ADAMTS4. **B** Levels of A β peptides in the conditioned medium of HEK293A cells in **A** ($n = 4$ or 5 , mean \pm s.e.m. Tukey test. N.S. not significant). **C** Levels of A β peptides in the conditioned medium of HEK293A cells expressing c102 and ADAMTS4 ($n = 3$, mean \pm s.e.m. Tukey test). **D** Target sequence of the ADAMTS4 locus in A549 cells and genomic sequences of the monoclonal A549 cell lines. **E** Levels of endogenous A β peptides in the conditioned medium of A549 cells in **D** ($n = 4$ or 5 , mean \pm s.e.m. Tukey test. N.S. not significant). **F** IP-MALDI-MS spectrum of the 4G8 or APP597 antibody-precipitated endogenous A β peptides in the plasma of wt mice (100 μ l for APP597). I.S. internal standard (distinct peptides were used for 4G8 and APP597). N non-specific peak. **G** Levels of endogenous APP669-711 and A β 1-40 in the plasma of wt and *Adams4* knockout mice ($n = 7$, mean \pm s.e.m. Student's *t* test).

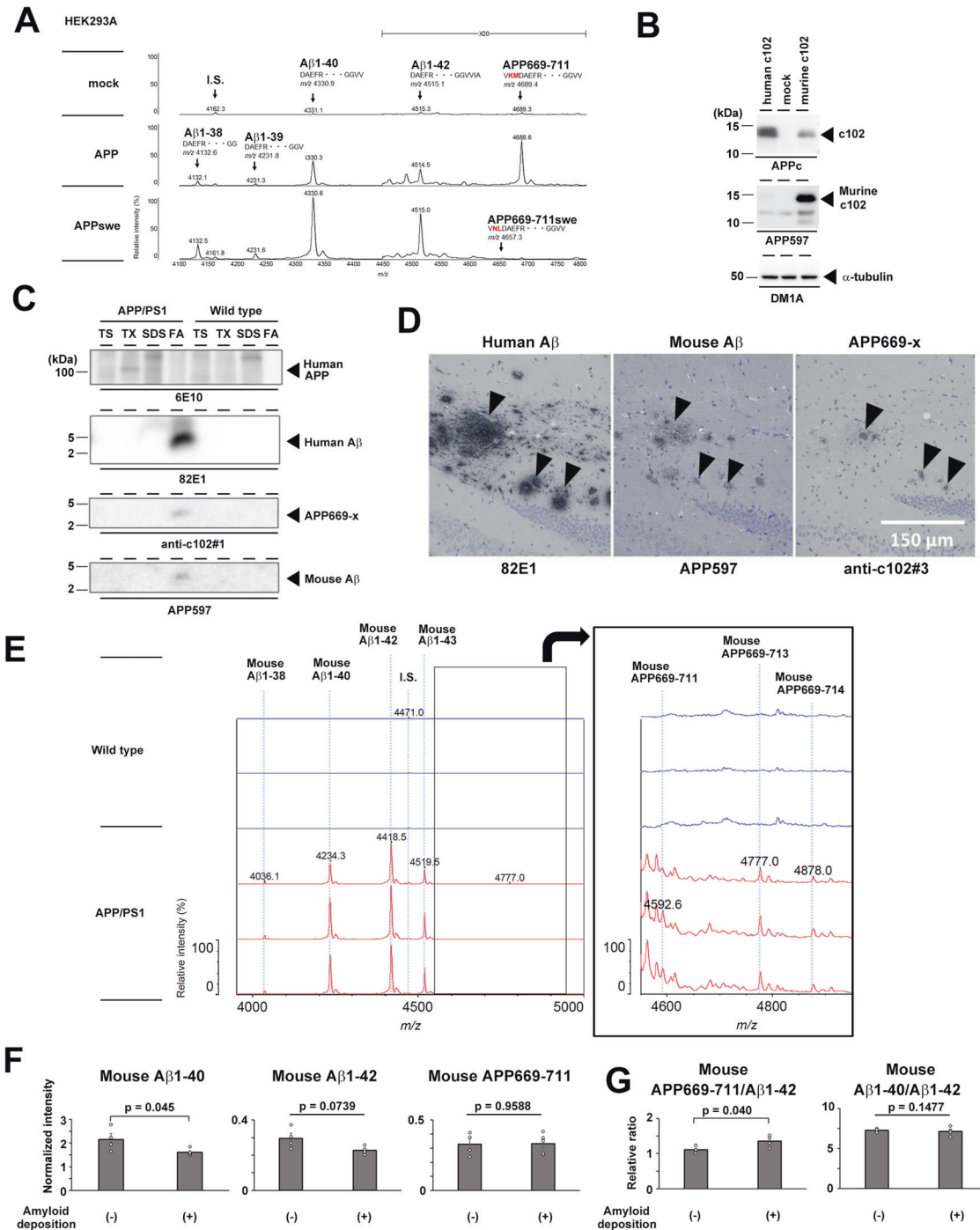


Fig. 6 APP669-711 in the A β plaque-laden AD model mice. **A** Effects of the Swedish mutation in APP on the production of APP669-711. Levels of A β peptides in the conditioned medium of HEK293A cells expressing APPwt and APPswe are shown. **B** The specificity of the APP597 antibody against the murine A β sequence was analyzed by immunoblot analysis of HEK293A cells expressing human c102, or murine c102. **C** Immunoblot analysis of brain fractions of aged wt and APP/PS1 mice. TS Tris buffer-soluble fraction, TX 1% Triton-X-soluble fraction, SDS SDS-soluble fraction, FA formic acid-soluble fraction. **D** Immunohistochemical analysis of the brains of aged APP/PS1 mice. Arrowheads indicate A β plaques. **E** IP-MALDI-MS analysis using APP597 antibody of FA fraction of APP/PS1 mouse ($n = 3$, 18–22 months old). Wild-type mice (22 months old) were used as a control. Enlarged spectrum in the red box were shown in the inset (black box). **F** Levels of endogenous mouse A β peptides in the plasma of young (2 months old, (-)) and aged (23–25 months old, (+)) APP/PS1 mice. The plasma was analyzed by IP-MALDI-MS using the APP597 antibody ($n = 4$ or 5, mean \pm s.e.m. Student's t test). **G** Relative ratio of mouse A β peptides in the plasma ($n = 4$ or 5, mean \pm s.e.m. Student's t test).

suggesting that ADAMTS4 cleavage at the APP669 site occurred on APP in the secretory pathway, rather than the cell surface APP (Fig. S2). We then analyzed the effects of the genetic knockout of ADAMTS4 in A549 cells, which secrete endogenous A β and

APP669-711. IP-MALDI-MS analysis of the conditioned medium of A549 cells using 6E10 antibody revealed that endogenous A β 4-40 is hardly produced from this cell line unless ADAMTS4 is overexpressed (Fig. S3). Two monoclonal A549 cell lines that

harbor deletion/substitution mutations at the *ADAMTS4* locus by the CRISPR/Cas9 system were selected (Fig. 5D). Both cell lines showed a 30–40% reduction in secreted APP669-711 levels, whereas A β production was unaltered (Fig. 5E). Notably, GM6001 treatment further decreased the production of APP669-711 from *ADAMTS4*-knockout A549 cells. Finally, we analyzed the effects of the genetic ablation of *Adamts4* on the level of endogenous APP669-711 in vivo. Human A β and mouse A β differ by three amino acids (R5/Y10/H13 in human A β are G5/H10/R13 in mouse A β), and the antibody APP597 was raised against a synthetic peptide encoding murine A β (see Fig. 6B, E) [29]. IP-MALDI-MS using the 4G8 antibody or the murine A β -specific APP597 antibody enabled us to detect endogenous murine A β -associated species, such as A β 1-40, A β 1-42, and APP669-711 in wt mouse plasma (Fig. 5F). We then applied this method to the plasma samples obtained from *Adamts4*^{-/-} mice [30]. Consistent with the data of *ADAMTS4*-knockout A549 cells, the plasma levels of murine APP669-711 in *Adamts4*^{-/-} mice were 33% lower than those of wt mouse plasma (Fig. 5G). Collectively, these data strongly suggest that *ADAMTS4* is involved in the generation of approximately one-third of the APP669-711 in plasma.

Endogenous murine APP669-711 in the brains of A β plaque-laden Alzheimer disease model mice

We and others previously reported that the APP669-711 peptide has the ability to form amyloid fibrils [12, 31]. However, the existence of N-terminally elongated A β species in amyloid plaques has not been investigated to date. *ADAMTS4* is expressed in the central nervous system [24], and our cell-based assay revealed that neuroblastoma cell secretes APP669-711. Furthermore, APP669-711 was detected in the human CSF [32]. Notably, the intergenic variant located near the *ADAMTS4* gene has been identified as one of the genetic risk factors for sporadic AD by genome-wide meta analysis [33]. To analyze the pathological effects and the diagnostic value of APP669-711 in the brains of A β plaque-laden AD model mice, we analyzed aged mice that express human APP^{sw} and mutant *Psen1* (APP/PS1 mice) [34]. Of note, HEK293A cells expressing APP^{sw} produced no APP669-711 carrying the Swedish mutation, suggesting that amino acid substitutions at A β (-2nd) and (-1st) sites (i.e., KM670/671NL) abolish the production of APP669-711 from human APP (Fig. 6A). Thus, using the mouse A β sequence-specific antibody APP597, we analyzed the effects of the brain deposition of amyloid comprised of human A β on the production of APP669-711 from mouse APP, separately from the processing of human APP encoded by the transgene. The specificity of APP597 was confirmed by immunoblot analysis of cell lysate expressing murine c102 (Fig. 6B). For human A β , we utilized 82E1 antibody that specifically reacts with N terminus of human A β 1-x (Fig. 2A). As expected, we detected the deposition of 82E1-positive human A β (i.e., human A β 1-x) in the brain parenchyma and substantial enrichment of human A β in the formic acid-soluble fraction. Notably, APP597 reacted with a 4-kDa band corresponding to human A β in the formic acid-soluble fraction (Fig. 6C). Furthermore, a similar 4-kDa band was detected by the anti-c102#1 antibody. Consistent with the biochemical analysis, immunohistochemical analysis demonstrated that the plaques in the cortex were positive for APP597 and anti-c102#3 antibodies, suggesting that endogenous murine A β peptides including APP669-x peptides formed insoluble aggregates and were deposited in human A β plaques. To characterize the deposited endogenous murine A β species in the aged APP/PS1 mice, we performed IP-MALDI-MS analysis using APP597. We confirmed that various mouse A β peptides, such as A β 1-38, A β 1-40, A β 1-42, and A β 1-43 were recovered from the formic acid fraction of APP/PS1 mouse brains. In addition, murine APP669-711 (A β (-3)-40), APP669-713 (A β (-3)-42), and APP669-714 (A β (-3)-43) were detected (Fig. 6E). Finally, we analyzed the level of endogenous murine A β species in the plasma of aged APP/PS1

mice, which contained a substantial amount of human A β , by IP-MALDI-MS using the APP597 antibody. Comparing the peptide levels of various A β species in the plasma of young and aged APP/PS1 mice, we found a significant increase in murine APP669-711/murine A β 1-42 ratio in the plasma, as observed in the plasma of humans with A β plaques in the brain (Fig. 6F, G). However, the plasma murine A β 1-40/murine A β 1-42 ratio was unaltered. These results suggest that, in AD model mice with A β plaque development, APP669-711 is produced in the brain and deposited together with human A β . In addition, the present data also indicate that the levels of mouse A β species in plasma are useful as surrogate biomarkers of amyloid deposition in the brains of AD model mice.

DISCUSSION

Genetic evidence has highlighted the importance of the proteolytic processing pathway of APP in the pathogenesis of AD [1–3]. In addition to BACE1, which generates the A β starting at 1st and 11th positions, several enzymes (e.g., cathepsin B, meprin, and *ADAMTS4*) were reported as enzymes responsible for producing N-terminally truncated A β [35]. In this study, we identified that *ADAMTS4* is a key enzyme in the production of the N-terminally elongated A β species APP669-711, which is a component of a plasma biomarker of brain amyloid deposition [11, 12]. Our biochemical and cell biological results indicated that APP669-711 is proteolytically produced by the sequential cleavage of APP, similarly to that of A β ; *ADAMTS4* cleaves APP at the APP669 site to generate the C-terminal stub c102, which is a direct substrate for γ -secretase. Notably, the production of APP669-711 increased upon treatment with a BACE1 inhibitor, and the Swedish mutation that enhances BACE1-mediated cleavage abolished the production of APP669-711. Consistent with these results, the Swedish mutation at the (-2nd)(-1st) residues of A β (KM to NL) causes a mismatch with the recognition motif for *ADAMTS4*. Furthermore, APP^{sw} might hinder this processing mechanism both in vitro and in vivo, as suggested previously [35].

Our results also clarified the complex mechanism of APP669-711 production. *ADAMTS4* is responsible for 30% to 40% of the secreted APP669-711 in the conditioned medium of cultured cells as well as in mouse plasma in vivo. GM6001 treatment further reduced APP669-711 secretion from *ADAMTS4*-knockout A549 cells. Moreover, the treatment of cells with the protease inhibitor TIMP3 abolished the production of APP669-711. Thus, a TIMP3-sensitive metalloprotease, in addition to *ADAMTS4*, may also be involved in the APP669-711 production pathway. We are currently analyzing the activities of other proteases that have been reported as TIMP3-sensitive metalloproteases. Another intriguing feature is that although all known APP secretases to date are transmembrane proteins, *ADAMTS4* is a secreted protease. This raises the possibility that cells secreting *ADAMTS4* are distinct from the substrate-expressing cells. Thus, further cell biological studies are required to demonstrate which cell type(s) is involved, and where the cleavage actually occurs at the subcellular level. However, the generation of APP669-711 was found to be dependent on the γ -secretase activity, indicating that *ADAMTS4* cleaves membrane-tethered APP, rather than the secreted A β species, in the APP669-711 production pathway. Supporting this hypothesis, almost no A β starting at 4th position was secreted from HEK293A cells expressing recombinant c102 or c99, indicating that the C-terminal stubs of APP are not direct substrates for the A β 4th site cleavage. Robust *ADAMTS4* activity might result in additional cleavage of APP at the A β 4th site to generate c96, which is a direct substrate of A β 4-x. Nevertheless, identification of the APP669-cleaving enzyme(s) and clarifying the precise mechanistic crosstalk in the APP669-711 production pathway is crucial for understanding the biological mechanism of surrogate biomarkers of brain amyloid deposition.

Our study also identified the presence of endogenous murine APP669-711 in mouse plasma. Furthermore, we found an increase in the murine APP669-711/murine A β 1-42 ratio in the plasma of AD model mice with human A β deposition similar to that observed in humans. However, the absolute levels of murine APP669-711, as well as murine A β 1-42 in plasma were not significantly altered in aged AD model mice, although we observed a reduction in murine A β 1-40 levels. To date, the origin of the plasma A β species remains unclear. Several studies have suggested that a proportion of brain A β is effluxed into the blood via the blood-brain barrier, and is then cleared from the liver or kidneys [36–39]. Although ADAMTS4 and APP are expressed in various cells of peripheral tissues, we found that endogenous murine APP669-x as well as A β peptides were co-deposited together with human A β in the APP/PS1 mouse brains. Thus one possibility is that plasma APP669-711 is originally derived from the brain. Supporting this notion, IP-MALDI-MS analysis revealed the existence of APP669-711 in the human CSF [32]. Pharmacokinetic studies, and understanding the metabolism of APP669-711 as well as murine A β are required. Nevertheless, our findings indicate that the murine APP669-711/murine A β 1-42 ratio in the plasma may be useful for the evaluation of drug efficacy/target engagement in the development of anti-A β drugs using AD model mice.

Identification of ADAMTS4 as a responsible enzyme for APP669-site cleavage raises the important possibility of the pathological/diagnostic importance of APP669-711 in humans. Recent genome-wide association studies demonstrated that the intergenic variant *rs4575098* located near the *ADAMTS4* gene is associated with AD [33, 40]. Pathological and biochemical analyses demonstrated that A β 4-x species, which are generated by ADAMTS4-mediated processing, are deposited in AD brains [23, 41, 42]. Our study also suggested the possibility that APP669-711 is a component of senile plaques. Thus, changes in the expression of *ADAMTS4* might affect the pathological process of AD, possibly by increasing the production of A β -related species including APP669-711 and A β 4-x. Although the average level of plasma APP669-711 in A β PET-positive individuals was comparable to that in A β -negative individuals, some people showed significantly higher/lower levels of APP669-711 [12]. The balance of ADAMTS4 and TIMP3 levels is crucial in the development of osteoarthritis, in which the overactivation of ADAMTS4 is implicated in its pathogenesis [25]. In addition, some studies showed changes in plasma ADAMTS4 levels in other inflammatory diseases, such as acute coronary syndrome [43]. Although it remains unclear whether plasma ADAMTS4 is directly involved in APP processing in peripheral tissues, these studies suggest the possibility that variations in APP669-711 levels might reflect disease conditions.

In conclusion, we identified ADAMTS4 as an important enzyme involved in APP processing. Further cell biological and genetic analyses are expected to clarify the importance of ADAMTS4, not only in the diagnosis of brain amyloid deposition but also in the pathogenesis of AD.

REFERENCES

- Knopman DS, Amieva H, Petersen RC, Chételat G, Holtzman DM, Hyman BT, et al. Alzheimer disease. *Nat Rev Dis Prim*. 2021;7:33.
- Kikuchi K, Kidana K, Tatebe T, Tomita T. Dysregulated metabolism of the amyloid- β protein and therapeutic approaches in Alzheimer disease. *J Cell Biochem*. 2017;118:4183–90.
- Long JM, Holtzman DM. Alzheimer disease: an update on pathobiology and treatment strategies. *Cell*. 2019;179:312–39.
- Mawuenyega KG, Sigurdson W, Ovod V, Munsell L, Kasten T, Morris JC, et al. Decreased clearance of CNS β -amyloid in Alzheimer's disease. *Science*. 2010;330:1774.
- Cummings J, Aisen P, Apostolova LG, Atri A, Salloway S, Weiner M. Aducanumab: appropriate use recommendations. *J Prev Alzheimer's Dis*. 2021;8:398–410.
- Iwatsubo T, Odaka A, Suzuki N, Mizusawa H, Nukina N, Ihara Y. Visualization of A β 42(43) and A β 40 in senile plaques with end-specific A β monoclonals:

- evidence that an initially deposited species is A β 42(43). *Neuron*. 1994;13:45–53.
- Iwatsubo T, Mann DMA, Odaka A, Suzuki N, Ihara Y. Amyloid β protein (A β) deposition: A β 42(43) precedes A β 40 in down syndrome. *Ann Neurol*. 1995;37:294–9.
 - Saido TC, Iwatsubo T, Mann DMA, Shimada H, Ihara Y, Kawashima S. Dominant and differential deposition of distinct beta-amyloid peptide species, A beta N3(pE), in senile plaques. *Neuron*. 1995;14:457–66.
 - Suzuki N, Cheung TT, Cai XD, Odaka A, Otvos L, Eckman C, et al. An increased percentage of long amyloid β protein secreted by familial amyloid β protein precursor (β APP717) mutants. *Science*. 1994;264:1336–40.
 - Asami-Odaka A, Ishibashi Y, Kikuchi T, Kitada C, Suzuki N. Long amyloid β -protein secreted from wild-type human neuroblastoma IMR-32 cells. *Biochemistry*. 1995;34:10272–8.
 - Kaneko N, Nakamura A, Washimi Y, Kato T, Sakurai T, Arahata Y, et al. Novel plasma biomarker surrogating cerebral amyloid deposition. *Proc Jpn Acad Ser B Phys Biol Sci*. 2014;90:353–64.
 - Nakamura A, Kaneko N, Villemagne VL, Kato T, Doecke J, Doré V, et al. High performance plasma amyloid- β biomarkers for Alzheimer's disease. *Nature*. 2018;554:249–54.
 - Janelidze S, Teunissen CE, Zetterberg H, Allué JA, Sarasa L, Eichenlaub U, et al. Head-to-head comparison of 8 plasma amyloid- β 42/40 assays in Alzheimer disease. *JAMA Neurol*. 2021;78:1375–82.
 - Araki M, Ito K, Takatori S, Ito G, Tomita T. BORCS6 is involved in the enlargement of lung lamellar bodies in *Lrrk2* knockout mice. *Hum Mol Genet*. 2021;30:1618–31.
 - Chiu YW, Hori Y, Ebinuma I, Sato H, Hara N, Ikeuchi T, et al. Identification of calcium and integrin-binding protein 1 as a novel regulator of production of amyloid β peptide using CRISPR/Cas9-based screening system. *FASEB J*. 2020;34:7661–74.
 - Gopalappa R, Suresh B, Ramakrishna S, Kim HH. Paired D10A Cas9 nickases are sometimes more efficient than individual nucleases for gene disruption. *Nucleic Acids Res*. 2018;46:e71.
 - Isaka T, Nestor AL, Takada T, Allison DC. Chromosomal variations within aneuploid cancer lines. *J Histochem Cytochem*. 2003;51:1343–53.
 - Citron M, Oltersdorf T, Haass C, McConlogue L, Hung AY, Seubert P, et al. Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. *Nature*. 1992;360:672–4.
 - Cai X-D, Golde TE, Younkin SG. Release of excess amyloid β protein from a mutant amyloid β protein precursor. *Science*. 1993;259:514–6.
 - Wang R, Sweeney D, Gandy SE, Sisodia SS. The profile of soluble amyloid β protein in cultured cell media. *J Biol Chem*. 1996;271:31894–902.
 - Kikuchi K, Tatebe T, Sudo Y, Yokoyama M, Kidana K, Chiu YW, et al. GPR120 signaling controls amyloid- β degrading activity of matrix metalloproteinases. *J Neurosci*. 2021;41:6173–85.
 - Hoe HS, Cooper MJ, Burns MP, Lewis PA, Van Der Brug M, Chakraborty G, et al. The metalloprotease inhibitor TIMP-3 regulates amyloid precursor protein and apolipoprotein E receptor proteolysis. *J Neurosci*. 2007;27:10895–905.
 - Walter S, Jumpertz T, Hüttenrauch M, Ogorek I, Gerber H, Storck SE, et al. The metalloprotease ADAMTS4 generates N-truncated A β 4-x species and marks oligodendrocytes as a source of amyloidogenic peptides in Alzheimer's disease. *Acta Neuropathol*. 2019;137:239–57.
 - Lemarchant S, Pruvost M, Montaner J, Emery E, Vivien D, Kanninen K, et al. ADAMTS proteoglycanases in the physiological and pathological central nervous system. *J Neuroinflammation*. 2013;10:1–8.
 - Verma P, Dalal K. ADAMTS-4 and ADAMTS-5: key enzymes in osteoarthritis. *J Cell Biochem*. 2011;112:3507–14.
 - Kashiwagi M, Tortorella M, Nagase H, Brew K. TIMP-3 is a potent inhibitor of aggrecanase 1 (ADAM-TS4) and aggrecanase 2 (ADAM-TS5). *J Biol Chem*. 2001;276:12501–4.
 - Hashimoto G, Aoki T, Nakamura H, Tanzawa K, Okada Y. Inhibition of ADAMTS4 (aggrecanase-1) by tissue inhibitors of metalloproteinases (TIMP-1, 2, 3 and 4). *FEBS Lett*. 2001;494:192–5.
 - Hills R, Mazarella R, Fok K, Liu M, Nemirovskiy O, Leone J, et al. Identification of an ADAMTS-4 cleavage motif using phage display leads to the development of fluorogenic peptide substrates and reveals matrilin-3 as a novel substrate. *J Biol Chem*. 2007;282:11101–9.
 - Takasugi N, Sasaki T, Suzuki K, Osawa S, Isshiki H, Hori Y, et al. BACE1 activity is modulated by cell-associated sphingosine-1-phosphate. *J Neurosci*. 2011;31:6850–7.
 - Demircan K, Topcu V, Takigawa T, Akyol S, Yonezawa T, Ozturk G, et al. ADAMTS4 and ADAMTS5 knockout mice are protected from versican but not aggrecan or brevican proteolysis during spinal cord injury. *Biomed Res Int*. 2014;2014:1–8.
 - Beyer I, Rezaei-Ghaleh N, Klafki HW, Jahn O, Haußmann U, Wiltfang J, et al. Solid-phase synthesis and characterization of N-terminally elongated A β 3-x-peptides. *Chem A Eur J* 2016;22:8685–93.

32. Klafki HW, Wirths O, Mollenhauer B, Liepold T, Rieper P, Esselmann H, et al. Detection and quantification of A β –3–40 (APP669-711) in cerebrospinal fluid. *J Neurochem*. 2022;00:1–12.
33. Jansen IE, Savage JE, Watanabe K, Bryois J, Williams DM, Steinberg S, et al. Genome-wide meta-analysis identifies new loci and functional pathways influencing Alzheimer's disease risk. *Nat Genet*. 2019;51:404–13.
34. Jankowsky JL, Slunt HH, Ratovitski T, Jenkins NA, Copeland NG, Borchelt DR. Co-expression of multiple transgenes in mouse CNS: a comparison of strategies. *Biomol Eng*. 2001;17:157–65.
35. Armbrust F, Bickenbach K, Marengo L, Pietrzik C, Becker-Pauly C. The Swedish dilemma—the almost exclusive use of APP^{Sw}-based mouse models impedes adequate evaluation of alternative β -secretases. *Biochim Biophys Acta Mol Cell Res*. 2022;1869:119164.
36. DeMattos RB, Bales KR, Cummins DJ, Paul SM, Holtzman DM. Brain to plasma amyloid- β efflux: a measure of brain amyloid burden in a mouse model of Alzheimer's disease. *Science*. 2002;295:2264–7.
37. Shibata M, Yamada S, Ram Kumar S, Calero M, Bading J, Frangione B, et al. Clearance of Alzheimer's amyloid- β 1-40 peptide from brain by LDL receptor-related protein-1 at the blood-brain barrier. *J Clin Invest*. 2000;106:1489–99.
38. Shiiki T, Ohtsuki S, Kurihara A, Naganuma H, Nishimura K, Tachikawa M, et al. Brain insulin impairs amyloid- β (1-40) clearance from the brain. *J Neurosci*. 2004;24:9632–7.
39. Yamada K, Yabuki C, Seubert P, Schenk D, Hori Y, Ohtsuki S, et al. A β immunotherapy: Intracerebral sequestration of A β by an anti-A β monoclonal antibody 266 with high affinity to soluble A β . *J Neurosci*. 2009;29:11393–8.
40. Marioni RE, Harris SE, Zhang Q, McRae AF, Hagenaars SP, Hill WD, et al. GWAS on family history of Alzheimer's disease. *Transl Psychiatry*. 2018;8:99.
41. Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL, Beyreuther K. Amyloid plaque core protein in Alzheimer disease and Down syndrome. *Proc Natl Acad Sci USA*. 1985;82:4245–9.
42. Portelius E, Bogdanovic N, Gustavsson MK, Volkman I, Brinkmalm G, Zetterberg H, et al. Mass spectrometric characterization of brain amyloid beta isoform signatures in familial and sporadic Alzheimer's disease. *Acta Neuropathol*. 2010;120:185–93.
43. Zha Y, Chen Y, Xu F, Li T, Zhao C, Cui L. ADAMTS4 level in patients with stable coronary artery disease and acute coronary syndromes. *Biomed Pharmacother*. 2010;64:160–4.

ACKNOWLEDGEMENTS

The authors are grateful to Drs. Kei Maruyama (Saitama Medical University), Tohru Fukuyama and Satoshi Yokoshima (Nagoya University), Takeshi Iwatsubo and Hidenori Ichijo (The University of Tokyo), and Dario Alessi (University of Dundee) for valuable reagents, and our current and previous laboratory members for helpful discussions. This work was supported in part by Shimadzu Corporation, a Grant-in-Aid for Scientific Research (A) from the Japan Society for the Promotion of Science (JSPS) [17H04313, 20H00548 to SH and 15H02492, 19H01015 to TT]; Strategic Research Program for Brain Sciences from the Japan Agency for Medical Research and Development [JP22dm0207073 to TT]; Moonshot R&D from Japan Science and Technology Agency [JPMJMS2024 to TT]. MY receives a research fellowship for young

scientists from the JSPS [22J14778] and Sadako O. Hirai Ban Award for Young Researchers.

AUTHOR CONTRIBUTIONS

MM, MY, NK, and TT designed experiments. MM, MY, YY, NK, and HK performed enzymological, cell biological, and animal experiments. NK, HN, AK, SS, SI, and KT established and performed the IP-MALDI-MS experiments. KI, GO, and SH performed experiments using ADAMTS4 knockout animals. MM, MY, YY, NK, SH, SI, and TT performed analysis on all experiments. MM, MY, NK, SH, SI, and TT wrote the manuscript. MM, MY, and YY contributed equally to this work.

FUNDING

Open access funding provided by University of Tokyo.

COMPETING INTERESTS

NK, HN, AK, SS, SI, and KT are full-time employees of Shimadzu Corporation, Japan. Other authors have no other conflicts of interest in association with this manuscript.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41380-023-01946-y>.

Correspondence and requests for materials should be addressed to Taisuke Tomita.

Reprints and permission information is available at <http://www.nature.com/reprints>

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2023