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BACE1 Cleavage Site Selection Critical for Amyloidogenesis and Alzheimer's Pathogenesis

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Mutations in amyloid β precursor protein (APP) gene alter APP processing, either causing familial Alzheimer's disease (AD) or protecting against dementia. Under normal conditions, β-site APP cleaving enzyme 1 (BACE1) cleaves APP at minor Asp^{1} site to generate C99 for amyloid β protein (Aβ) production, and predominantly at major Glu^{11} site to generate C89, resulting in truncated Aβ production. We discovered that A673V mutation, the only recessive AD-associated APP mutation, shifted the preferential β-cleavage site of BACE1 in APP from the Glu^{11} site to the Asp^{1} site both in male and female transgenic mice *in vivo* and in cell lines and primary neuronal culture derived from timed pregnant rats *in vitro*, resulting in a much higher C99 level and C99/C89 ratio. All other mutations at this site, including the protective Icelandic A673T mutation, reduced C99 generation, and decreased the C99/C89 ratio. Furthermore, A673V mutation caused stronger dimerization between mutant and wild-type APP, enhanced the lysosomal degradation of the mutant APP, and inhibited γ-secretase cleavage of the mutant C99 to generate Aβ, leading to recessively inherited AD. The results demonstrate that APP673 regulates APP processing and the BACE1 cleavage site selection is critical for amyloidogenesis in AD pathogenesis, and implicate a pharmaceutical potential for targeting the APP673 site for AD drug development.

Key words: Alzheimer's disease; BACE1

Significance Statement

β-site APP cleaving enzyme 1 (BACE1) is essential for amyloid β protein production. We discovered that A673V mutation shifted the BACE1 cleavage site from the Glu^{11} to the Asp^{1} site, resulting in much higher C99 level and C99/C89 ratio. All other mutations at this site of amyloid β precursor protein (APP) reduced C99 generation and decreased the C99/C89 ratio. Furthermore, A673V mutation resulted in stronger dimerization between mutant and wild-type APP, enhanced the lysosomal degradation of the mutant APP, and inhibited γ-secretase cleavage of the mutant C99 to generate amyloid β protein, leading to recessively inherited Alzheimer's disease (AD). The results demonstrate that APP673 regulates APP processing, and the BACE1 cleavage site selection is critical for amyloidogenesis in AD pathogenesis, and implicate a pharmaceutical potential for targeting the APP673 site for AD drug development.

Introduction

Deposition of amyloid β protein (Aβ) to form neuritic plaques is the unique neuropathological feature of Alzheimer's disease (AD). Aβ is derived from amyloid β precursor protein (APP). Under normal conditions, the majority of APP proteins are processed in the nonamyloidogenic pathway: cleaved first by α-secretase at Leu^{17} site of Aβ domain to generate the secreted sAPPα and the membrane-bound C83 (Esch et al., 1990). C83 is subsequently cleaved by γ-secretase to generate CTFγ and p3 fragment, precluding Aβ production (Song et al., 1999; Zhang et al., 2000; Qing et al., 2008). In the amyloidogenic pathway, APP is cleaved first by β-secretase at Asp^{1} site to generate the secreted sAPPβ and the membrane-bound fragment C99. C99 is further cleaved by γ-secretase to generate CTFγ and Aβ. β-cleavage is a rate-limiting step and the β-secretase cleavage product C99 is directly associated with the overall production of heterogeneous Aβ species (Li et al., 2006; Sun et al., 2012).

β-site APP cleaving enzyme 1 (BACE1) cleaves APP at two β-secretase sites. Under physiological conditions, BACE1 predominantly cleaves APP at the major Glu^{11} site within Aβ, yielding the nonamyloidogenic C89 and resulting in truncated Aβ production; whereas it cleaves APP at the minor Asp^{1} site, generating C99 and leading to Aβ generation (Deng et al., 2013).

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Enhancing BACE1 cleavage of APP or shifting its preferential cleavage site from Glu^{11} to Asp^{1} resulted in higher C99 and Aβ production and hence facilitated neuritic plaque formation under pathological conditions (Mullan et al., 1992; Sun et al., 2006a, b; Deng et al., 2013; Ly et al., 2013; Zhang et al., 2014).

Whereas most AD cases are sporadic with late onset at the age of 65 years and older, <1% are early-onset familial AD (FAD) cases. The FAD cases are caused by mutations in *APP*, *presenilin* 1 (*PSEN*1), or *presenilin* 2 (*PSEN*2) genes. More than 30 FAD-associated APP point mutations have been discovered on 17 residues, which are in close vicinity of secretase cleavage sites. These mutations have been shown to cause FAD by altering APP processing, including enhancing C99 production (Swedish mutation KM670/671NL) (Mullan et al., 1992; Felsenstein et al., 1994; Perez et al., 1996), inhibiting nonamyloidogenic α-cleavage of APP (Arctic mutation E693G) (Nilsberth et al., 2001; Stenh et al., 2002; Sahlin et al., 2007), increasing $A\beta_{42}$ level or the ratio of $A\beta_{42}$ to $A\beta_{40}$ (London mutation V717I and Florida mutation I716V) (Goate et al., 1991; Eckman et al., 1997; De Jonghe et al., 2001), or accelerating $A\beta_{40}$ fibril formation (Dutch mutation E693Q and Iowa mutation D694N) (Wisniewski et al., 1991; Van Nostrand et al., 2001). Our laboratory further demonstrated that APP Swedish mutation strongly shifted the BACE1 cleavage site from the Glu^{11} site to the Asp^{1}, resulting in a higher C99/C89 ratio and promoting Aβ generation (Deng et al., 2013).

All the known pathogenic mutations in the APP gene are dominantly inherited, except an A673V mutation (APP_{rIta}) identified in an Italian family (Di Fede et al., 2009). This alanine to valine missense mutation occurs at the 673rd amino acid position of APP770 (i.e., the second amino acid of Aβ) and acts in a recessively inherited manner in AD pathogenesis. APP_{rIta} mutation increased Aβ production, and the resulting mutant $A\beta_{A2V}$ displayed altered oligomerization, aggregation, and toxicity (Di Fede et al., 2009; Benilova et al., 2014; Maloney et al., 2014). However, the mechanism underlying the recessive inheritance of this mutant remains elusive. The unique recessiveness of this *APP* mutation might provide insights into how altered APP processing and possible quantitative change of Aβ accumulation lead to Alzheimer's development. Intriguingly, a recently identified mutation at the same site changing alanine into threonine (APP_{Ice}) was shown to be protective against AD in the Icelandic population (Peacock et al., 1993; Jonsson et al., 2012), highlighting the close involvement of altered APP processing in AD pathogenesis. In this study, we demonstrated that the alanine at the 673rd amino acid position of APP770 was critical for regulating β-secretase cleavage site selection and the BACE1-mediated amyloidogenesis in AD pathogenesis.

Materials and Methods

Plasmid constructs. Mutations of APP were introduced into the cDNA coding sequence of APP695 by PCR-based site-directed mutagenesis, and the mutated cDNAs were cloned into expression vector pcDNA4-mycHis (Invitrogen). Human APP_{Wt}, APP_{rIta}, or APP_{Swe} cDNAs were also cloned into an inducible mammalian expression vector pIND (SP1)/Hygro to generate pIND-APP_{Wt}, pIND-APP_{rIta}, and pIND-APP_{Swe}. pIND (SP1)/Hygro vector contains modified ecdysone response elements and SP1 enhancers. Plasmid pVgRXR expresses the heterodimeric ecdysone receptor (VgEcR) and the retinoid X receptor (RXR) (Invitrogen). Endoplasmic reticulum (ER) retention signal was introduced into pcDNA4-$APP_{Wt\text{-}ER}$ and pcDNA4-$APP_{rIta\text{-}ER}$ by mutating QMQN in the C-terminal tail of APP to KKQN. To generate plasmid pFlag-APP, the coding sequence of the FLAG tag was inserted between the signal peptide and the luminal/extracellular domain of APP695, and the resulting DNA was cloned into pcDNA4-myc-His. For pulse-chase assay, APP_{Wt} and APP_{rIta} were cloned into pSNAP (New England Biolabs). cDNAs coding for C99, C89, and C83 with a methionine in the N termini were cloned into pcDNA4, confirmed by DNA sequencing and expressed in HEK293 cells as CTF markers. CTFs derived from APP processing can be resolved by the comigration with these markers.

Immunoblot analysis. Cells were lysed in RIPA-DOC lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.4% SDS, 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.2) supplemented with complete mini protease inhibitor mixture tablet (Roche Diagnostics). The samples were resolved by SDS-PAGE on 8% Tris-glycine or 16% Tris-tricine gels, then transferred to Immobilon-FL PVDF membranes (Millipore). C20, a rabbit antibody against the last 20 amino acids of APP C terminus, was used to detect APP and its CTF products (Ly et al., 2013). β-actin was detected using monoclonal antibody AC-15 (Sigma). The membranes were incubated with IRDye 800CW-labeled goat anti-mouse or anti-rabbit antibodies in PBS with 0.1% Tween 20 at 22°C for 1 h, and visualized on the Odyssey system (LI-COR Biosciences). All quantifications were performed using the LI-COR Odyssey system or ImageJ. To perform coimmunoprecipitation, plasmids coding for FLAG-APP and $C99_{Wt}$ or $C99_{A2V}$ were cotransfected into HEK293 cells, and the detailed procedure was performed as described previously (Eggert et al., 2009).

Intracranial injection and immunoprecipitation. All the protocols of animal procedure were approved by University of British Columbia Animal Care Center. Adeno-associated virus-9 viruses expressing human APP_{Wt} or human APP_{rIta} at the titer of $\sim 2 \times 10^{12}$ genome copies/ml were generated by Vector Core, University of Pennsylvania. The brains of 3 neonatal male and 3 female mice within 12 h were intracranially injected with 1 μl of the virus into the ventricle. Two weeks after injection, pups were killed. Half brains were homogenized in lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA) supplemented with complete protease inhibitors (Roche). After centrifugation, the supernatant was added to the C20 antibody immobilized on protein-G agarose bead, and incubated at 4°C for overnight. Proteins were eluted from the beads by boiling in SDS sample buffer.

Cell culture and transfection. HEK293 cells (RRID:CVCL_0045) were cultured in DMEM containing 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 U/ml penicillin G sodium, and 50 μg/ml streptomycin sulfate (Invitrogen). PC12 cells (RRID: CVCL_0481) were cultured in DMEM supplemented with 15% FBS. Stable cell lines were maintained in media containing zeocin (50 μg/ml). All cells were maintained at 37°C in an incubator containing 5% CO_2. For transfection, cells were grown to 70% confluence in a 35 mm plate and transfected with 2 μg plasmid DNA with 4*u*L of Lipofectamine 2000 Reagent (Invitrogen). To establish APP-inducible cell lines, BACE1-stable cell line 293B2 cells were cotransfected with pVgRXR and pIND-APP_{Wt}, pIND-APP_{rIta}, or pIND-APP_{Swe}. Activation of the exogenous APP gene transcription in the transfected cells was dependent on the binding of a heterodimer of VgEcR and RXR receptors in the presence of a ligand, such as the ecdysone analog ponasterone A (Invitrogen). The expression level of APP variants was controlled by the different dosages of ponasterone A treatment (Li et al., 2006).

Primary neurons and virus transduction. E18 cortical neurons derived from pregnant rats were maintained in neurobasal medium supplemented with B27 in poly-D-lysine-coated plates. For each 35 mm dish, 4 μl of viruses expressing either human APP_{Wt} or human APP_{rIta} at the titer of $\sim 2 \times 10^{12}$ genome copies/ml was added to the culture on DIV 3. Half of the medium was replaced with fresh medium every 3 d. Chloroquine (CHL, from Sigma) (100 μg/ml) or solvent control (H_2O) was added to the neurons on DIV 9, and cells were harvested for immunoblot assay after overnight CHL treatment.

Pharmacological treatments of cells. L-685,458 (Sigma), a potent γ-secretase inhibitor, was dissolved in DMSO and applied to cell culture medium at 1 μM final concentration for 3 h. Cycloheximide (Sigma), an inhibitor for protein translation, was used at 100 μg/ml for the indicated times to inhibit APP synthesis. CHL (Sigma) is a lysosomal degradation inhibitor. MG132 (from Millipore) is a potent, reversible, and cell-permeable proteasome inhibitor. To determine the effect of APP mutations on APP degradation, HEK293 were treated with CHL at 100 μM or MG132 at 10 μM.

Aβ ELISA. Conditioned medium from cell culture was collected, and protease inhibitors and 4-(2-aminoethyl)-benzenesulfonyl fluoride

(Roche) were added to prevent Aβ degradation. The concentration of $A\beta_{42}$ was detected using β-amyloid 1–42 colorimetric ELISA kit (Invitrogen) according to the manufacturer's instructions. For enrichment of Aβ immobilized on the ELISA plate, 300 μl of the total ~1 ml conditioned medium was added to the plate. After incubation at room temperature for 3 h, the medium was replaced by another 300 μl of the same conditioned medium to repeat the 3 h reaction. After all the medium was applied to the plate, secondary antibody was added. Conditioned media from PC12 cells without any overexpression were used as control to avoid Aβ contamination.

Statistical analysis. The numbers of replication for each experiment were at least three times as indicated in the figure legends. All results are expressed as mean ± SEM. Two-tailed Student's *t* test was used to analyze the difference between two groups; and multiple-comparison test was analyzed by ANOVA followed by *post hoc* Newman–Keuls test, unless otherwise specified in figure legends.

Results

APP_{rIta} mutation shifted BACE1 cleavage site and increased Aβ production

To investigate the effect of the Italian APP A673V mutation (hereafter referred as APP_{rIta}) on APP processing, adeno-associated viruses carrying human APP_{Wt} and APP_{rIta} were injected into neonatal mouse brains. After immunoprecipitation, APP_{rIta} displayed clear C99 overexpression but not C89, and APP_{Wt} apparently produced more C89 than C99 (Fig. 1*A*), suggesting that A673V mutation shifted the major BACE1 cleaving site in APP from the Glu^{11} site to Asp^{1} site. A similar result was also reported previously (Kimura et al., 2016).

To confirm the *in vivo* results *in vitro* and to further investigate the mechanism, we transfected APP_{Wt}, APP_{rIta}, or APP_{Swe} into PC12 cells, a rat pheochromocytoma cell line expressing readily detectable endogenous BACE1. APP and its CTFs, including the α-secretase product C83 and BACE1 products C89 and C99, were examined by Western blot with C20 antibody. APP_{rIta} and APP_{Swe} significantly increased C99 levels to 228.8 ± 6.9% and 209.1 ± 9.8%, respectively, compared with APP_{Wt} ($p < 0.001$) (Fig. 1*B*). Additionally, APP_{rIta} also exhibited much weaker C83, suggesting the reduction of α-cleavage of APP (Fig. 1*B*). To examine the effect of A673V mutation on Aβ production, APP variants were overexpressed in PC12 cells, and conditioned media were analyzed using Aβ ELISA assay. APP_{rIta} and APP_{Swe} increased the Aβ40 levels to 12.8 ± 0.2-fold and 56.4 ± 0.5-fold, respectively ($p < 0.05$) (Fig. 1*C*), and enhanced Aβ42 levels to 2.96 ± 0.09-fold and 6.73 ± 0.10-fold, respectively ($p < 0.05$) (Fig. 1*D*). The data suggested that the A673V mutation enhanced APP processing at the Asp^{1} β-site cleavage and promoted subsequent Aβ generation compared with APP_{Wt}.

Under physiological conditions, the majority of APP undergoes α-secretase pathway, with C83 (CTFα) being the predominant form of APP CTFs. Increasing BACE1 expression causes the majority of APP undergoing β-secretase pathway, resulting in CTFβ (C89 and C99) being predominant forms of APP CTFs (Deng et al., 2013). C89 and C99 are generated by BACE1 cleavage of APP at two β-secretase sites, Glu^{11}(also known β′-site) and Asp^{1} (also known as β-site), respectively, and the Glu^{11} is the major β-secretase site to processing wild-type APP under normal conditions (Deng et al., 2013). To specifically assess BACE1 cleavage of APP_{rIta}, APP plasmids were transfected into a BACE1-stable cell line 293B2. Consistent with our previous report, the major CTFs in APP_{Wt}-expressing cells was C89 derived by BACE1 cleavage at Glu^{11} site (Fig. 1*E*). The Swedish mutation APP_{Swe} (APP K670N/M671L) altered BACE1-cleavage site selection, resulting in the Asp^{1} site as the preferential β-secretase cleavage site and C99 as the major product (Fig. 1*E*). Similar to the Swedish mutation, the A673V mutation in APP_{rIta} also shifted β-secretase cleavage site from the normally predominant Glu^{11} site to Asp^{1} site, leading to C99 as the predominant β-secretase product (Fig. 1*E*). More strikingly, the A673V mutation was more potent than the Swedish mutation on shifting preferential β-secretase site from the Glu^{11} to Asp^{1}, resulting in even higher C99/C89 ratio, 7.51 ± 0.17-fold for $APP695_{rIta}$ and 3.15 ± 0.06-fold for $APP695_{Swe}$ ($p < 0.05$) (Fig. 1*E*). These data unequivocally demonstrate that the A673Vmutation shifts the preferential β-secretase site from the Glu^{11} to Asp^{1}, and the effect on the β-secretase site selection is even stronger than the Swedish mutation.

To confirm the enhanced C99 production from APP_{rIta} by BACE1 was due to the higher cleavage efficiency at this site, but not the weak C89 and C83 production which may spare more full-length APP_{rIta} for BACE1 to cleave at the Asp^{1} site, *in vitro* kinetics assay was performed by recombinant BACE1 and an artificial substrate spanning from residue 639–681 (with Asp^{1} site between 671 and 672) (Fig. 1*F*). As expected, the recombinant BACE1 did not cleave either the wild-type or the rIta substrate at neutral pH. At pH 4, which was an optimal pH for recombinant BACE1 activity, the rIta substrate showed much faster reduction than the wild-type substrate, suggesting that the rIta mutant was a better substrate of BACE1. Therefore, this mutation in full-length APP may directly enhance BACE1 cleavage at the Asp^{1} site. However, it should be noted that we did not detect the cleaved products from these artificial substrates by using anti-GFP antibody. This could be because the cleaved fragments were not stable *in vitro* at lower pH, or the fragments were further degraded by the recombinant BACE1 when they were mixed with BACE1 in the solution. The latter circumstance, if true, is unlikely to happen in cells because intracellular BACE1 is anchored at the membrane, and the N-terminal fragments of APP are free in the lumen of organelles and quickly secreted out of the cells, which is the reason why secreted APPβ is used as an indicator of β-cleavage.

As BACE1 cleaves full-length APP at Glu^{11} site within C99 region, it may also cleave C99 at the same site, which could alter the C99/C89 ratio. To examine whether such effects contribute to C99/C89 ratio in our cell-based system, several APP mutants were generated to abolish C99 (M671V) (Vetrivel et al., 2011) and/or C89 (Y681A) from APP_{WT}, APP_{Swe}, and APP_{rIta}. In APP_{WT} (Fig. 1*G*), M671V did not reduce C89, and Y681A only slightly increased C99, which may be derived from full-length APP spared from Glu^{11} cleavage. Another APP mutation (E682V) inhibiting Glu^{11} cleavage displayed a similar effect on CTF pattern (Vetrivel et al., 2011). For APP_{rIta} and APP_{Swe} (Fig. 1*H*), the Y681A mutation did not cause significant increase of C99, presumably because APP_{rIta} and APP_{Swe} (producing wild-type C99) do not produce much of C89 by BACE1, and there was not much of full-length APP being spared from Glu^{11} cleavage. Together, sequential cleavage of APP and C99 by BACE1 might happen, but it does not essentially alter the C99/C89 ratio. Therefore, the C99/C89 ratio indicates the portions of an APP variant being cleaved at Asp^{1} site and at Glu^{11} site. The cleavage preference may reflect the energy barrier for BACE1 cleavage. The lower barrier, the faster cleavage, and the more of CTF production; and inhibiting one of the two sites (Asp^{1} and Glu^{11}) does not essentially alter the cleavage kinetics at the other one.

To determine whether APP_{rIta} expression levels affect β-cleavage site selection and Aβ generation, an inducible APP expression system (Li et al., 2006) was introduced to 293B2 cells to control

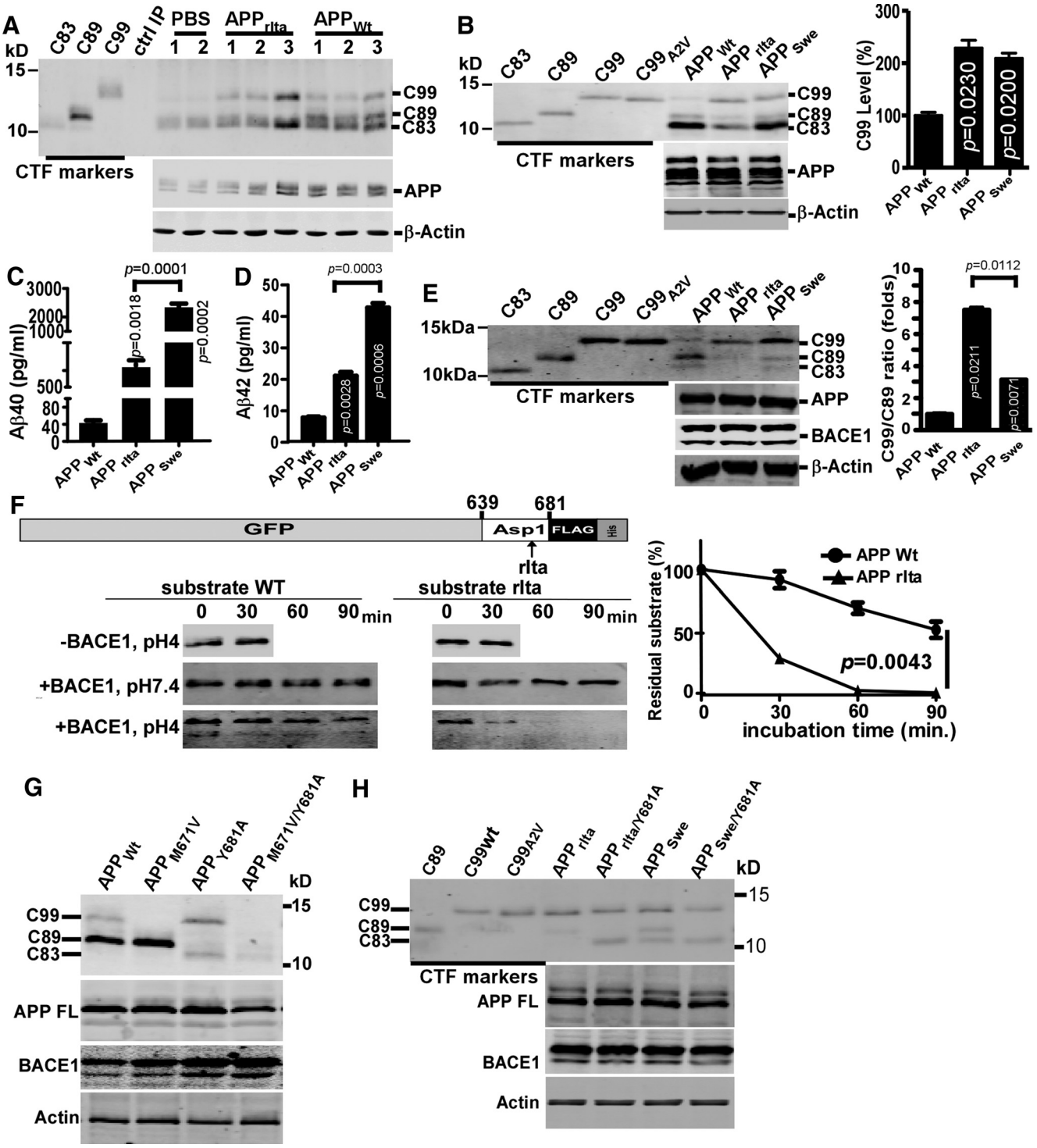

Figure 1. Effects of APP A673V (rIta) mutation on APP processing and Aβ generation. ***A***, APP_{Wt} and APP_{rIta} adeno-associated viruses were transduced into the brains of 3 neonatal mice, and CTFs were analyzed after immunoprecipitation. PBS was injected into 2 mice as control. In the "control IP," only lysis buffer, but not brain lysate, was added to the C20 antibody immobilized on protein-G beads. cDNAs coding for C99, C89, and C83 with a methionine in the N termini were cloned into pcDNA4, confirmed by DNA sequencing, and expressed in HEK293 cells as CTF markers. ***B***, $APP695_{WT}$, $APP695r_{Ita}$, and $APP695_{Swe}$ plasmids were transfected into PC12 cells. APP and its CTFs were detected by C20 antibody. C99 levels in APP variants were normalized to that in APP_{WT}. ***C***, ***D***, Aβ40 and Aβ42 in the conditioned media of PC12 cells expressing APP_{WT}, APP_{rIta} and APP_{Swe} were quantified by ELISA and expressed as pg/ml. ***E***, 293B2 were transfected with $APP695_{Wt}$, $APP695r_{Ita}$, or $APP695_{Swe}$ plasmids, and CTF markers and cell lysates were blotted for APP, BACE1, and CTFs. C99/C89 ratios in APP mutants were normalized to that of APP_{Wt}. ***F***, *In vitro* BACE1 cleavage kinetics. APP fragments spanning from 639 to 681 (β-site between M671–D672) with or without the rIta mutation at A673 were inserted between GFP and FLAG-His tags and expressed in bacteria. The recombinant proteins were purified with His tag beads. The 0.35 μg of recombinant proteins in 35 μl buffered solutions were incubated at 37°C with or without 2.1 μl recombinant BACE1 (R&D Systems, 500 μg/ml) at different pH values for the indicated times. The Western blot bands by anti-FLAG antibody were quantified and plotted. ***G***, APP_{Wt} and APP_{Wt} carrying M671V (to inhibit Asp-1-cleavage) and/or Y681A (to inhibit Glu-11-cleavage) mutations were expressed in 293B2 cell, and the whole-cell lysates were blotted for CTFs, full-length APP, and BACE1. ***H***, APP_{rIta} with or without the Y681A mutation and APP_{Swe} with or without the Y681 mutation were expressed in 293B2 cells, and whole-cell lysates were blotted for CTFs, APP, and BACE1. Data are mean ± SEM; $n = 3$ technical replicates. Statistical analysis: one-way ANOVA with *post hoc* Newman–Keuls tests.

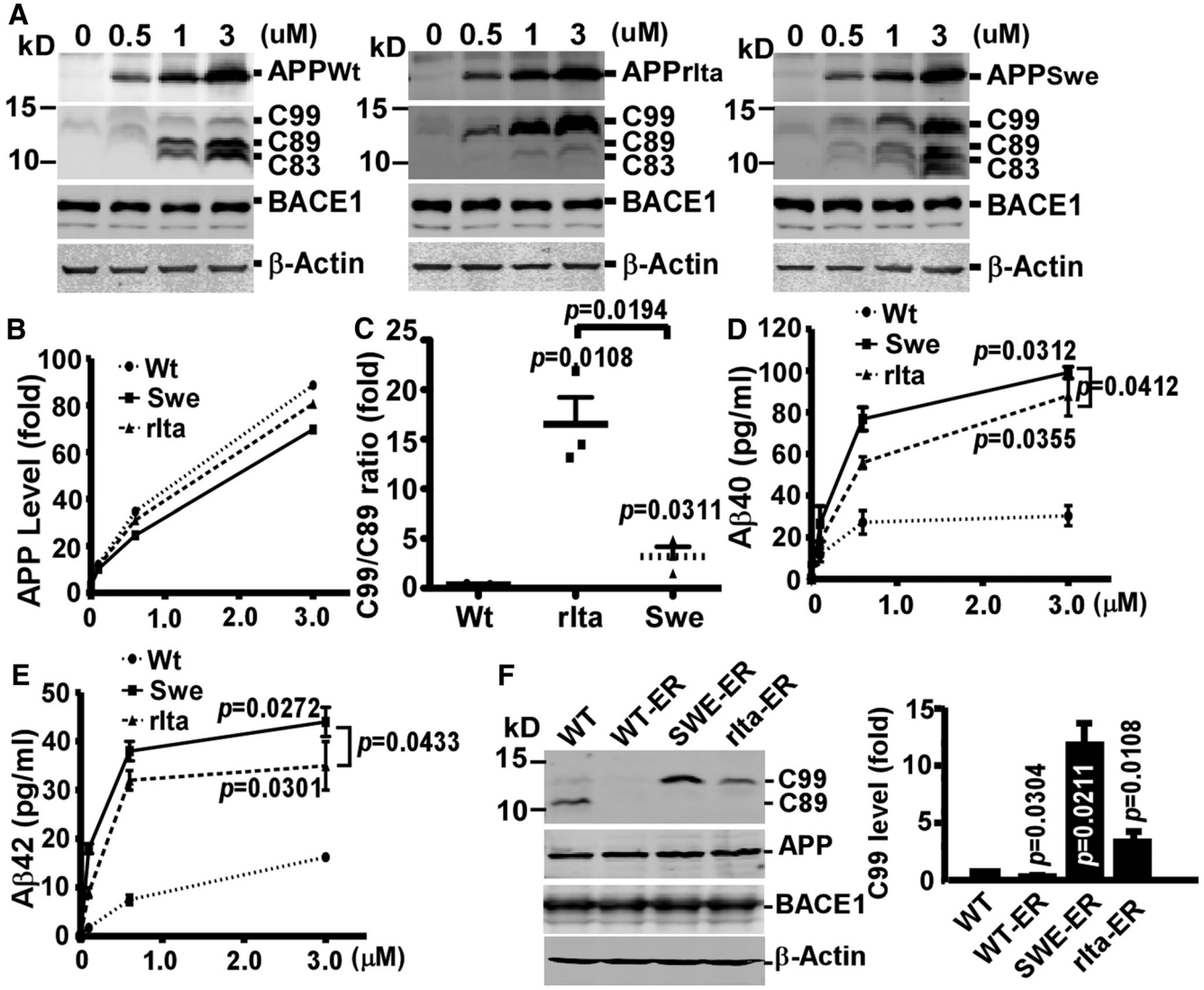

Figure 2. The A673V mutation altered APP processing independently of expression level and enabled BACE1 to cleave nascent APP. To determine whether APP_{rIta} expression levels affect β-cleavage site selection and Aβ generation, an inducible APP expression system (Li et al., 2006) was introduced to 293B2 cells to control APP expression at different levels. ***A***, pIND-APP_{Wt}, pIND-APP_{rIta}, and pIND-APP_{Swe} were transfected into 293B2 cells. After ponasterone A treatment at different concentrations, cell lysates were blotted for full-length APP, CTFs, and BACE1. Full-length APPs (***B***), CTFs (***C***), Aβ40 (***D***), and Aβ42 (***E***) were quantified. ***F***, APP_{Wt}, APP_{Swe}, and APP_{rIta} bearing the KKQN ER retention signal were expressed in 293B2 cells. C99s were quantified. Data are mean ± SEM; $n = 3$ technical replicates. Statistical analysis: one-way ANOVA with *post hoc* Newman–Keuls tests.

APP expression at different levels. While the expression levels of APP variants are similar ($p > 0.05$), APP_{rIta} consistently displayed the highest C99/C89 ratio (16.7 ± 2.8-fold) compared with 3.2 ± 0.2-fold of APP_{Swe} relative to APP_{WT} (Fig. 2*A–C*). These data indicated that the effect of A673V mutation on β-cleavage shift from Glu^{11} to Asp^{1} was intrinsic and independent of the expression level of APP. Meanwhile, although APP levels followed a linear pattern upon the induction of expression, Aβ40 and Aβ42 levels reached plateau for all the APP variants (Fig. 2*D*,*E*). Consistent with the result in PC12 cells, Aβ40 and Aβ42 levels in APP_{rIta} were significantly lower than APP_{Swe}, but higher than APP_{Wt}, whether Aβ levels reached plateau or not ($p < 0.05$). The data suggested the intrinsic nature of A673V mutation on Aβ generation.

To investigate the mechanism underlying the altered β-cleavage site selection by the A673V mutation, we generated APP-ER retention plasmids by mutating APP C-terminal sequence QMQN to KKQN in APP_{Wt}, APP_{rIta}, and APP_{Swe} plasmids. The KKXX-COOH motif (where X refers to any amino acids) retains nascent APP in the ER in the default structure, excluding the influence from possible intracellular trafficking and further modification of these APP variants upon exit from the ER. When expressed in 293B2 cells, both $APP_{Swe\text{-}ER}$ and $APP_{rIta\text{-}ER}$ generated a remarkable amount of C99, with 11.8 ± 2.7-fold for $APP_{Swe\text{-}ER}$ and 4.0 ± 0.9-fold for $APP_{rIta\text{-}ER}$ compared with APP_{Wt}, whereas $APP_{Wt\text{-}ER}$ produced a trace amount, if any, of C99. C89 and C83 were hardly detected. (Fig. 2*F*). These results suggest that the nascent APP_{Swe} and APP_{rIta} are structurally more susceptible to β-cleavage at Asp^{1} site. Although it remains unclear at this stage whether later trafficking and modifications alter this susceptibility, the higher C99 generation from the nascent mutants in the ER may partly contribute to the higher total C99 and Aβ production of APP_{rIta} and APP_{Swe}.

Wild-type APP suppressed amyloidogenic processing of APP_{rIta}

To examine the β-cleavage under the $APP_{Wt/rIta}$ heterozygous state, we coexpressed nontagged APP_{Wt} or APP_{rIta} (APP_{Wt}-NT

and APP_{rIta}-NT) with APP_{Wt}-GFP or APP_{rIta}-GFP in 293B2 cells. This way, CTF-GFP from APP-GFP variants were easily separated with the CTF-NT from APP-NT variants, and the effects of one of the two variants on another could be unambiguously demonstrated. Surprisingly, APP_{rIta}-GFP significantly increased C99/C89 ratios of APP_{Wt}-NT and APP_{rIta}-NT. By contrast, APP_{Wt}-GFP overexpression only reduced C99/C89 ratio of APP_{rIta}-NT but not that of APP_{Wt}-NT (Fig. 3*A*,*B*). APP_{Wt}-GFP also proportionally reduced C99 and C89 from APP_{Wt}-NT, which could be due to competition for BACE1 cleavage. A dosage course further demonstrated that APP_{Wt}-GFP gradually downregulated C99 and decreased C99 to C89 ratio of APP_{rIta}-NT. In contrast, APP_{rIta}-GFP increased both C99 and C99/C89 ratio of APP_{rIta}-NT Figure 3*C*).

The enhanced coimmunoprecipitation between APP_{Wt} and APP_{rIta} (Fig. 3*D*) suggested that APP_{Wt} was more likely to form oligomers with APP_{rIta} than APP_{Wt} itself, and the APP_{Wt}/APP_{rIta} hetero-oligomerization may account for the altered BACE1 cleavage preference in APP_{rIta} (Kaden et al., 2008). Moreover, $C99_{rIta}$ also displayed stronger coimmunoprecipitation with APP_{Wt} than $C99_{Wt}$ (Fig. 3*E*), implicating that A2V mutation in C99 promoted the oligomerization through the Aβ region, but not allosterically through the E1 or E2 dimerization domains of APP.

Given that the APP dimers and C99 dimers inhibit γ-cleavage and Aβ production (Eggert et al., 2009) and the production of Aβ was lower in APP_{rIta} than APP_{Swe}, despite similar C99 levels between the two mutants (Fig. 3*B*--*E*), we examined whether the γ-secretase cleavage of C99 was affected by this mutation. $C99_{Wt}$ (also produced from APP_{Swe}) and $C99_{A2V}$ were expressed in HEK293 cells, and the cells were treated with each of γ-secretase inhibitor L-685,458, lysosome inhibitor CHL, and proteasome inhibitor MG132 for 3 h (Fig. 3*F*). To equalize $C99_{Wt}$ and $C99_{rIta}$ levels without inhibitor treatment, we transfected ~10 times more of $C99_{rIta}$ expression vector, which suggests that the catabolism of $C99_{rIta}$ was much stronger than that of $C99_{Wt}$. Regarding γ-secretase inhibition, $C99_{Wt}$ level was increased by 183.6 ± 11.3%, but $C99_{rIta}$ was only increased by 53 ± 4.3%. By contrast, MG132 hugely increased $C99_{rIta}$ by ~25-fold and increased $C99_{Wt}$ by a much milder ~8-fold. Hence, compared with $C99_{Wt}$, $C99_{rIta}$ was more efficiently degraded by proteasome, and only a relatively small portion was cleaved by γ-secretase (Fig. 2*D*). Consistently, when the same amount of basal C99 proteins was expressed in HEK293 cells, $A\beta_{40}$ secretion from $C99_{rIta}$-expressing cells was only 36.0 ± 3.5% of that from $C99_{Wt}$-expressing cells ($p < 0.05$; Fig. 3*G*). Both changes of C99 level upon γ-secretase inhibition and Aβ production revealed a weaker γ-cleavage of $C99_{rIta}$ compared with $C99_{Wt}$. The weaker γ-cleavage of $C99_{A2V}$, which is at least partially due to potent proteasome degradation, is presumably the most important mechanism underlying the disproportional Aβ and C99 productions from APP_{rIta}, compared with APP_{Swe}.

APP_{rIta} mutation directed mature APP to its lysosome-dependent degradation

The maturation of APP is required for APP processing and Aβ generation (Perez et al., 1999; Steinhilb et al., 2001). We found that APP_{rIta} proteins displayed significantly less mature form (53 ± 5%) than APP_{Wt} ($p < 0.05$) (Fig. 4*A*,*B*). To examine whether the A673V mutation affected APP maturation, pulse chase assay was performed for overexpressed APP_{Wt} and APP_{rIta} in HEK293 cells (Fig. 4*C*,*D*). The reduction of APP_{rIta} did not show apparent difference from that of APP_{Wt}, suggesting that the A673V mutation did not affect the maturation or the ER exportation of the nascent immature APP.

Next, we examined whether the weak mature form of APP_{rIta} was due to faster degradation. HEK293 cells were transfected with APP_{Wt} or APP_{rIta} and then treated with proteasome inhibitor MG132 or lysosome inhibitor CHL (Fig. 4*E*). The mature forms of both APP_{Wt} and APP_{rIta} were accumulated upon acute lysosomal inhibition by CHL but not the proteasomal inhibition by MG132 (Fig. 4*E*,*F*). Moreover, the accumulation of mature APP_{rIta} was stronger than that of mature APP_{Wt} (1.31 ± 0.09-fold for APP_{Wt} vs 3.38 ± 0.28-fold for APP_{rIta}) (Fig. 3*F*), indicating that mature APP_{rIta} underwent faster lysosome-dependent degradation. The weaker mature APP_{rIta} and the CHL-induced rescue of mature APP_{rIta} were also observed in primary rat neurons transduced with viruses expressing APP_{Wt} or APP_{rIta} (Fig. 4*G*). While APP_{rIta} arrested in trans-Golgi network (APP_{rIta}-TGN) displayed similar amounts of mature form with APP_{Wt}-TGN, the mature form of APP_{rIta} arrested at the plasma membrane (APP_{rIta}-PM) appeared to be much weaker compared with APP_{Wt}-PM (Fig. 4*H*,*I*). It is very likely that a significant amount of mature APP_{rIta} is directly transported to the lysosome from the trans-Golgi network for degradation instead of being targeted to the plasma membrane.

Residue 673 of APP was critical for BACE1 cleavage of APP

Our data had shown that the recessive Italian mutation A673V altered the preferential BACE1 cleavage site, resulting in higher C99/C89 ratio and promoting C99 production. Meanwhile, a newly identified missense mutation of alanine to threonine on the same residue (A673T) in the Icelandic population, in contrast, was protective against AD development (Jonsson et al., 2012), suggesting that the amino acid at position 673 of APP might be critical for BACE1 cleavage site selection for APP processing. To examine this issue, we generated additional APP expression plasmids carrying different missense mutations at 673rd amino acid position of APP770, replacing the alanine at 673 site with negatively charged aspartic acid (D), positively charged arginine (R), hydrophobic leucine (L), hydrophobic and structurally rigid proline (P), hydrophilic glutamine (Q), or serine (S). It was worth mentioning that hydrophilic glutamine (Q) and serine (S) were selected for their structural similarity to hydrophobic alanine. Some of the amino acid substitutions altered migration rate of C99 markers by SDS-PAGE, implying an SDS-resistant structural change (Fig. 5*A*). Substitution of the alanine at 673 with D, P, Q, R, or S almost abolished C99 generation (Fig. 5*B*). In APP_{A673L}, BACE1 produced weak but clearly detectable $C99_{A2L}$ (A2 in C99 was A673 in APP770) (Fig. 5*B*) that comigrated with $C99_{A2L}$ marker (data not shown). Meanwhile, C89 (without the mutation site) from the APP_{A673L} mutant was hugely decreased compared with wild-type and other artificial mutants, resulting in high $C99_{A2L}$/C89 ratio (Fig. 5*B*), which was similar to A673V. L is the only residue structurally similar to V among the six substituting amino acids. Presumably, short alkane side chains at the position 673 of APP suppress BACE1 cleavage of APP at the Glu^{11} site. Therefore, with the exception of the pathogenic A673V mutation that enhances C99 production, all other artificial mutations inhibited, instead of enhanced, BACE1 cleavage at Asp^{1} site and suppressed C99 production.

Next, we examined the β-cleavage of the Icelandic mutant A673T in 293B2 cells (Fig. 5*C*) and PC12 cells (Fig. 5*D*). Interestingly, this mutation only mildly reduced C99 and C99/C89 ratio in both cell lines and suppressed $A\beta_{40}$ and $A\beta_{42}$ secretion from PC12 cells by ~60% and ~40%, respectively (Fig. 5*D*–*F*). Thus, APP amino acid at position 673 is critical for BACE1 cleavage and a single amino acid change at residue 673 can change BACE1

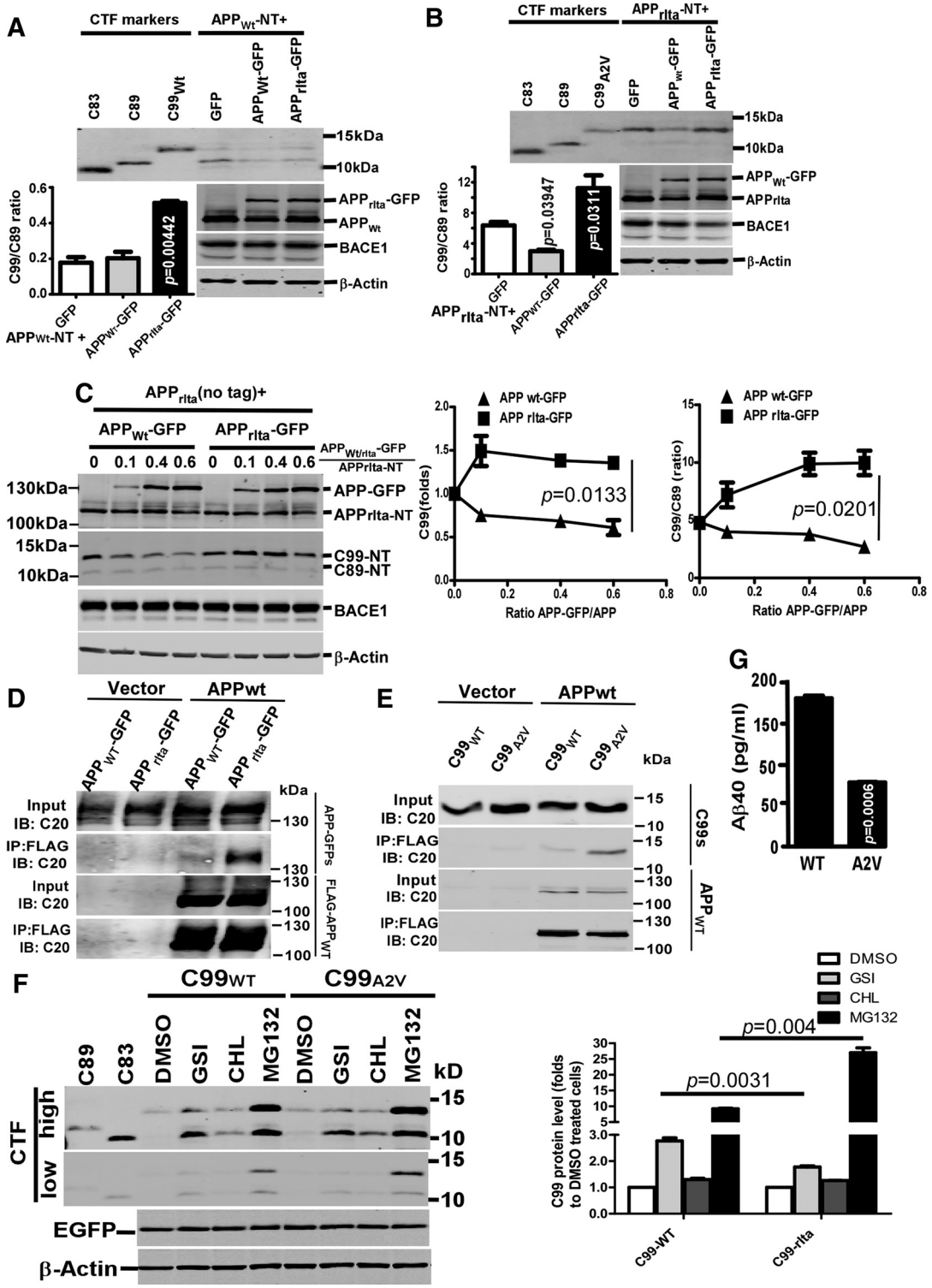

Figure 3. Enhanced dimerization suppressed C99 production from APP_{rlta} and γ-cleavage of $C99_{rlta}$. Nontagged APPWt (APPWt-NT) (***A***) or APPrlta (APPrlta-NT) (***B***) were coexpressed with GFP, or APPWt-GFP, or APPrlta-GFP in 293B2 cells, and the cell lysates were blotted for CTFs-NT, APPs, and BACE1. The ratios of C99-NT/C89-NT were plotted. ***C***, APP_{rlta}-NT (nontagged APP_{rlta}) plasmid was cotransfected into 293B2 cells with increasing amount of either APP_{Wt}-GFP or APP_{rlta}-GFP plasmid. C20 antibody was used to detect APPs and CTFs derived from AP_{rlta}-NT, and 9E10 for BACE1. Protein bands for full-length $APP_{Wt/rlta}$-GFP and full-length APP_{rlta}-NT were quantified, and the ratios of $APP_{Wt/rlta}$-GFP/APP_{rlta}-NT were indicated. The ratios of $C99_{rlta}$ in APP-GFP expressing cells to $C99_{rlta}$ in cells without APP-GFP expression were plotted. The ratios of $C99_{rlta}$/C89 over the $APP_{Wt/rlta}$-GFP dosage courses were plotted as well. ***D***, Flag-APP_{Wt} plasmid and empty vector were transfected with APP_{Wt}-GFP or APP_{rlta}-GFP. The immunoprecipitated Flag-APP_{Wt} by the FLAG-beads and the coprecipitated APP-GFPs were both detected with C20 antibody. ***E***, Flag-APP_{Wt} and empty vector were transfected with $C99_{Wt}$ or $C99_{A2V}$. The immunoprecipitated FLAG-APP_{Wt} by the FLAG-beads and the coprecipitated C99s were detected with C20 antibody. ***F***, pcDNA4 –$C99_{WT}$ or -$C99_{A2V}$ with a signal peptide were cotransfected with pEGFP plasmid (as an internal control for transfection) into HEK293 and then treated with indicated catabolism inhibitors overnight. DMSO, control solvent; GSI, γ-secretase inhibitor L-685,458; MG132, proteasome inhibitor. GFP was used for transfection efficiency control, and β-actin was used as for protein loading control. C99 level was quantified and compared between $C99_{WT}$ and $C99_{A2V}$. ***G***, Conditioned medium was collected to determine the amount of $A\beta_{40}$ in HEK293 with $C99_{WT}$ (WT) and $C99_{A2V}$ (A673V) overexpression. Data are mean ± SEM; $n = 3$ technical replicates. Statistical analysis: one-way ANOVA with *post hoc* Newman–Keuls tests.

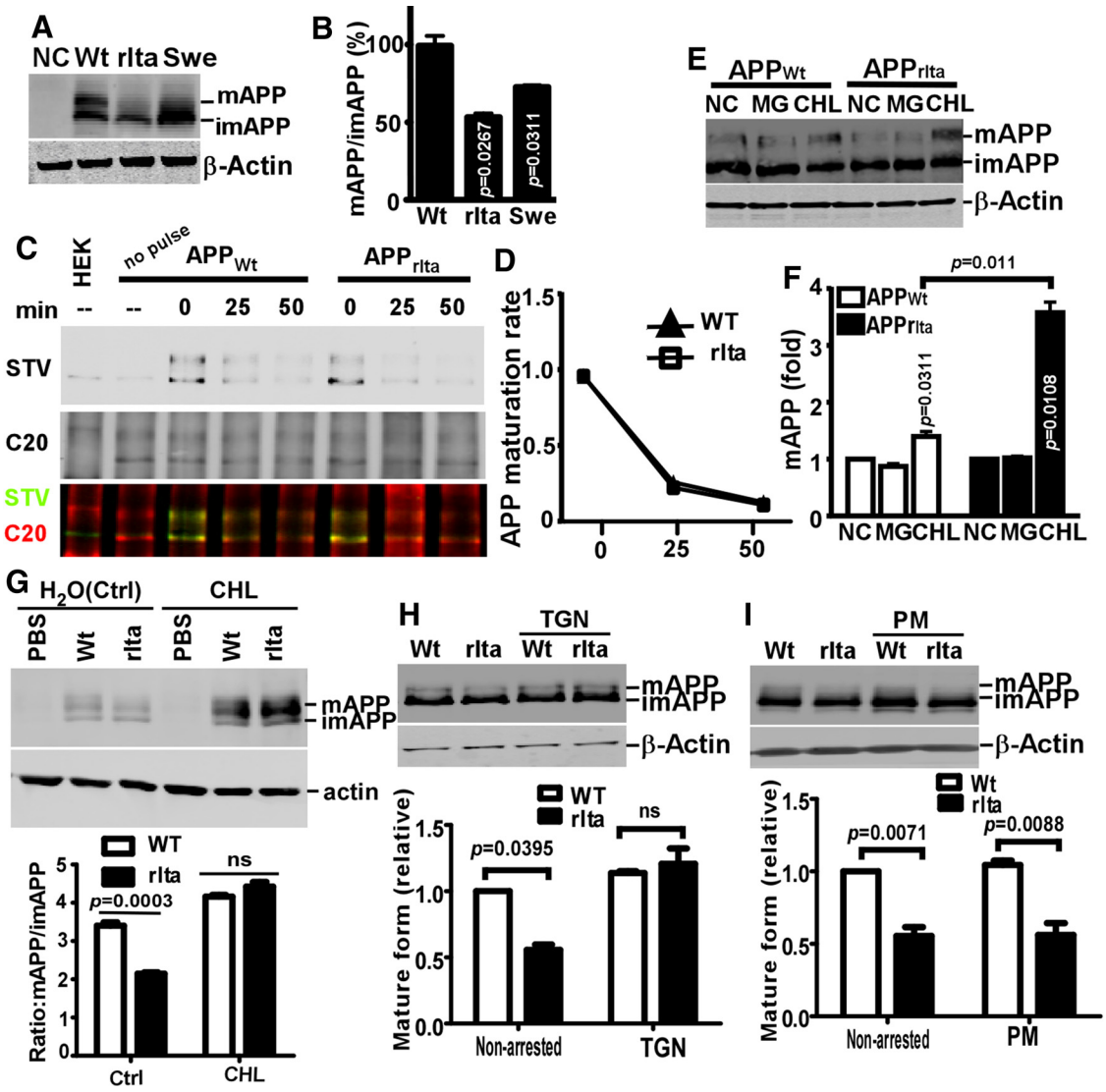

Figure 4. Lysosome-dependent degradation of mature APP_{A673V}. ***A***, Plasmid pcDNA4-APP_{Wt}, pcDNA4-APP_{rIta}, or pcDNA4-APP_{Swe} were transfected into HEK293 cells. The nascent APP (imAPP) and modified APP (mAPP) in cell lysate were resolved on 8% glycine SDS-PAGE gel and detected by C20 antibody. ***B***, The ratios of mAPP/imAPP in ***A*** were quantified and normalized to that in APP_{Wt}. ***C***, ***D***, APP_{Wt}-SNAP and APP_{rIta}-SNAP were overexpressed in HEK293 cells and pulse-labeled. After indicated chasing time, cells lysate was blotted for pulse-labeled APP with streptavidin (green) and total APP with C20 (red). The maturation rate (as indicated by the reduction of pulse-labeled immature APP) of APP_{Wt} and APP_{rIta} were plotted after normalization with total APP. ***E***, pcDNA4-APP_{Wt} and pcDNA4-APP_{rIta} were introduced into HEK293 cells and then treated with10 μM MG132 or 100 μM CHL for 45 min. ***F***, Mature APP protein levels were quantified. Data are mean ± SEM; $n = 3$ technical repeats. Statistical analysis: ANOVA with *post hoc* Newman–Keuls test. ***G***, Viruses expressing human APP_{Wt} or APPrIta were tranduced into primary rat neurons on DIV 3, and neuronal cells with overnight treatment of CHL orsolvent H_2O (Ctrl) were harvested for blotting of full-length APP on DIV 10. Ratios of mature APP to immature APP were plotted. ***H***, The SDYQRL trans-Golgi targeting motif (Wong and Hong, 1993) was fused to APP_{Wt} or APP_{rIta}, and the fusion proteins were expressed in HEK293 cells, full-length APPs were blotted with C20 antibody, and the levels of mature APP variants after normalization with immature APP variants were plotted. ***I***, The NPTY internalization signal (Perez et al., 1999) was deleted from APP_{Wt} and APP_{rIta} to retain them on the plasma membrane (PM). The resulting APP variants with a FLAG tag fused to the N termini after signal peptide removal were overexpressed in HEK293 cells. Full-length APPs were blotted with anti-FLAG antibody as the NPTY motif was within the epitope of C20 antibody. Levels of mature APP variants after normalization with immature APP variants were plotted. ***G–I***, Data are mean ± SEM; $n = 2$ technical repeats. Statistical analysis: two-way ANOVA with Newman–Keuls tests.

cleavage site preference to Asp^{1} or Glu^{11}, resulting in either increasing or decreasing C99 and Aβ generation.

Discussion

The A673V mutation in *APP* gene is the only known recessive mutation causing AD. Both parental linkages of the proband were heterozygous carriers and the heterozygous family members did not show any AD symptoms by the age of 88. By contrast, the homozygous proband developed dementia symptoms by the age of 36 and died at 46, and his homozygous sister also showed mild cognitive impairment (Giaccone et al., 2010). All other FAD-associated mutations in *APP* are dominant, and the majority result in increasing Aβ level. However, how the mutations affect APP processing leading to AD pathogenesis remains elusive. A recently identified APP mutation (A673T) was shown to be inversely correlated to AD incidence, which implicated a protective role of this mutation. Thus, different mutations on the same site could have opposite effects on AD occurrence due to its differential effect on APP processing. Under physiological conditions, the wild-type APP protein is preferentially cleaved by BACE1 at the Glu^{11} β-secretase site to generate C89 and truncated Aβ. In this report, we clearly showed that the recessive Italian A673V mutation altered the β-secretase cleavage site selection, resulting in

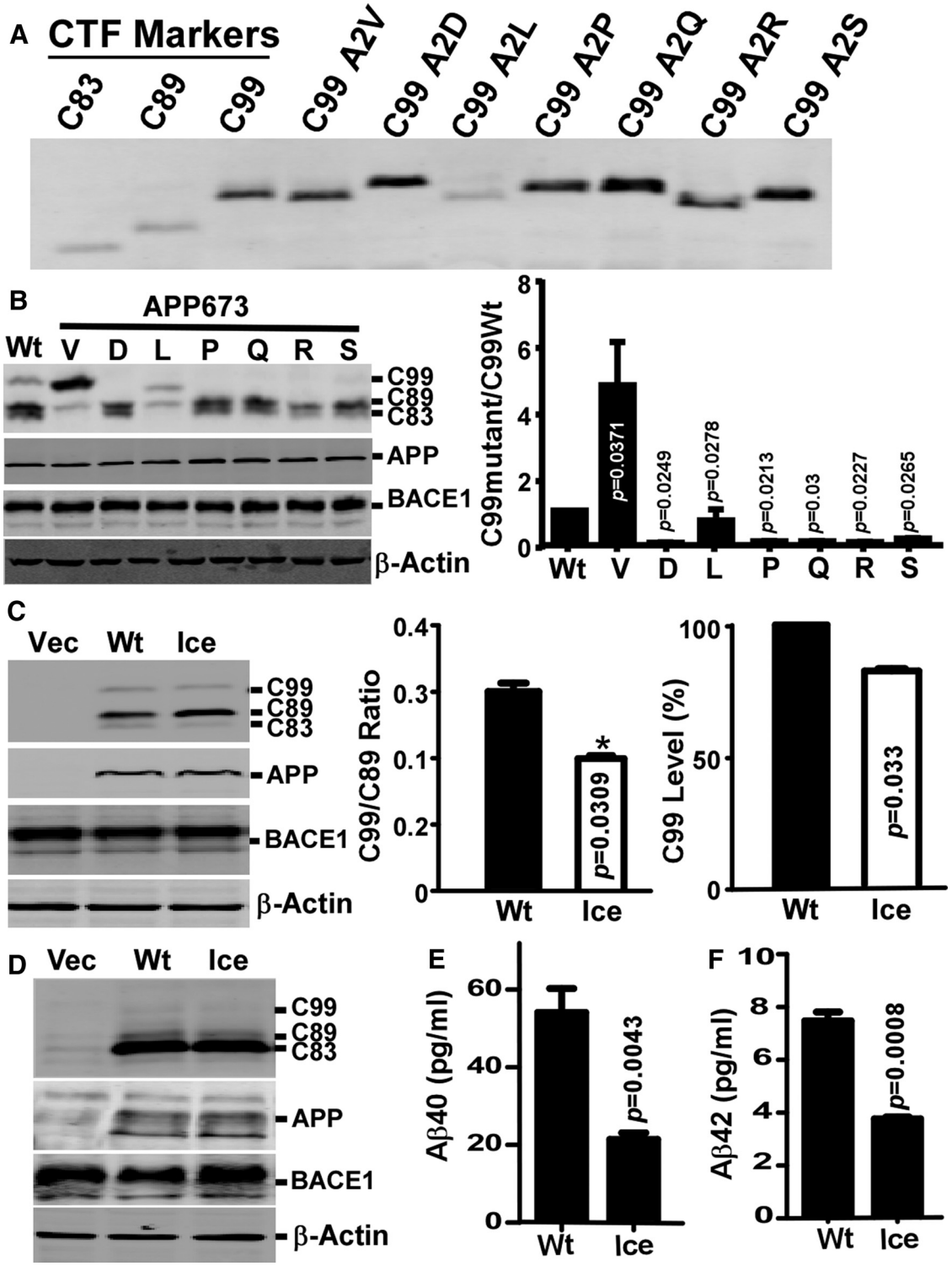

Figure 5. Effects of amino acid substitution of A673 on β-cleavage. ***A***, C99 with the indicated mutations at position 2 (position 673 in full-length APP) displayed altered gel mobility by SDS-PAGE. ***B***, A673 in APP were substituted with V, D, L, P-R, or S, and mutants were expressed in 293B2 cells. C99 levels were quantified. ***C***, APP_{Wt} and APP_{Ice} were expressed in 293B2 cells, and CTFs were quantified. ***D***, APP_{Wt} and APP_{Ice} were expressed in PC12 cells, and C99s and C89s were quantified. Secreted Aβ40 (***E***) and Aβ42 (***F***) were measured by ELISA. Data are mean $\pm$ SEM; $n = 3$ technical repeats. Statistical analysis: one-way ANOVA with *post hoc* Newman–Keuls tests.

Asp^{1} site as the preferential site for the mutant APP_{rIta} protein. By facilitating BACE1 cleavage at Asp^{1} site, this A673V mutation produced more Aβ substrate C99. The effect on BACE1 cleavage site shifting by A673V mutation is even stronger than that by the Swedish mutation, resulting in much higher C99/C89 ratio. On the other hand, BACE1 cleavage at the Asp^{1} site was compromised by the protective Icelandic A673T mutation. Therefore, the difference in β-cleavage is in perfect agreement with the opposite effects of these two mutants on AD pathogenesis. It should be noted that the ratios of C99/C89 presented do not necessarily reflect the real relative amount of C99 to C89 generated by BACE1 because the catabolism of CTFs was not blocked. CTFs

could be degraded or further processed by proteasome, lysosome, γ-secretase, α-secretases, etc. If these catabolism pathways have a preference to one of C99 and C89 over the other, the ratio of C99/C89 can no longer indicate the BACE1 cleavage preference. Nonetheless, our conclusion that the A673V mutation enhances C99 and suppresses C89 productions is based on the comparison with the C99 and C89 from APP_{Wt}, and is therefore not affected by the preferential catabolism of different CTFs.

The cleavages of BACE1 at the Asp^{1} and Glu^{11} sites in a certain APP variant are very likely independent of each other. This is evident concerning the fact that inhibition of C99 did not affect C89 and vice versa. Hence, the ratio of C99 to C89 is not necessarily a consequence of the "competition" of the two sites for BACE1 cleavage. It may rather reflect the relative energy barriers for BACE1 to cleave APP at the two sites that are preset for each APP variant. The higher energy barrier results in lower cleavage efficiency, and lower C99 or C89 generation.

While the A673V mutation markedly increased C99 production and significantly suppressed C89, it was pathogenic only in homozygotes. By contrast, the APP mutation E682K having a similar effect as A673V but with milder C99 increase is autosomal-dominant (Zhou et al., 2011). The decreased pathogenic effect of A673V mutation might be due to the impaired processing of $C99_{rIta}$ into Aβ compared with $C99_{Wt}$; the higher tendency of oligomerization suppressing β-cleavage of full-length APP_{rIta} and γ-cleavage of $C99_{rIta}$ (Eggert et al., 2009), the faster degradation of mature APP_{rIta} reducing the amount of APP as BACE1 substrate or the hugely reduced C89 diminishing Aβ 11-x. Aβ 11-x was also suggested to be toxic Aβ species (Pike et al., 1995; Cai et al., 2001; Barritt and Viles, 2015). In the heterozygotes, the overall effect of all the above mechanisms caused by A673V mutation may suppress Aβ production; however, both alleles at this site need to be mutated to increase Aβ production to trigger AD pathogenesis.

Our data demonstrate that the amino acids adjacent to the Asp^{1} site are critical for directing BACE1's preferential cleavage site selection. A single amino acid change at residue 673 can affect β-cleavage of APP and γ-cleavage of C99. Valine substitution (recessive Italian mutation) enhances C99 production, whereas threonine (Icelandic mutation), aspartic acid, proline, glutamine, arginine, or serine substitution inhibits C99 generation. These data indicate that the position of APP 673 appears to serve as a "switch" governing the BACE1-mediated amyloidogenesis. The differential effects of the pathogenic Italian A673V mutation and the protective Icelandic A673T mutation on β-cleavage site selection further highlight the key roles of β-cleavage in AD pathogenesis. Given the importance of position 673 in controlling BACE1-mediated amyloidogenesis, targeting this site to direct BACE1 cleavage away from Aβ-generating Asp^{1} site without affecting other functions of BACE1 could be promising strategy for AD drug development.

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