

Suppressor Mutations for Presenilin 1 Familial Alzheimer Disease Mutants Modulate γ -Secretase Activities*

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Eugene Futai^{†§1}, Satoko Osawa[¶], Tetsuo Cai^{||}, Tomoya Fujisawa[‡], Shoichi Ishiura[§], and Taisuke Tomita^{||}

From the [‡]Department of Molecular and Cell Biology, Graduate School of Agricultural Sciences, Tohoku University, Sendai, Miyagi 981-8555, the [§]Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, Meguro-ku, Tokyo 153-8902, the [¶]Laboratory of Neuropathology and Neuroscience, Graduate School of Pharmaceutical Sciences and ^{||}Laboratory of Neuropathology and Neuroscience, Faculty of Pharmaceutical Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan

γ -Secretase is a multisubunit membrane protein complex containing presenilin (PS1) as a catalytic subunit. Familial Alzheimer disease (FAD) mutations within PS1 were analyzed in yeast cells artificially expressing membrane-bound substrate, amyloid precursor protein, or Notch fused to Gal4 transcriptional activator. The FAD mutations, L166P and G384A (Leu-166 to Pro and Gly-384 to Ala substitution, respectively), were loss-of-function in yeast. We identified five amino acid substitutions that suppress the FAD mutations. The cleavage of amyloid precursor protein or Notch was recovered by the secondary mutations. We also found that secondary mutations alone activated the γ -secretase activity. FAD mutants with suppressor mutations, L432M or S438P within TMD9 together with a missense mutation in the second or sixth loops, regained γ -secretase activity when introduced into presenilin null mouse fibroblasts. Notably, the cells with suppressor mutants produced a decreased amount of A β 42, which is responsible for Alzheimer disease. These results indicate that the yeast system is useful to screen for mutations and chemicals that modulate γ -secretase activity.

The γ -secretase is closely associated with familial Alzheimer disease (FAD)² (1). Presenilin is the catalytic subunit of γ -secretase, which cleaves the intramembrane domain of amyloid precursor protein (APP), thus generating amyloid β (A β) peptides. More than 150 missense mutations within the two isoforms (PS1 or PS2) of presenilin are identified for the disease. These mutations decrease the generation of A β but increase the ratio between A β 42 and A β 40 (2–4), which contain 42 and 40 amino acid residues, respectively. The increased ratio leads to forma-

tion of senile plaques in the brains of patients and is responsible for the disease (1). γ -Secretase modulators also alter the ratios among A β species and are classified as A β 42-lowering or -raising compounds (5). The former could represent promising therapeutic agents for the treatment of Alzheimer disease.

γ -Secretase, a member of the intramembrane-cleaving protease family, is composed of four membrane proteins as follows: presenilin (PS1 or PS2), nicastrin (NCT), Aph-1 (anterior pharynx-1), and Pen2 (6, 7). Crystal structures of the protease family members, including rhomboid, site-2 protease, and signal peptide peptidases PSH (8–10), have a membrane-embedded chamber structure, which harbors catalytic residues accessible for water molecules. Although the crystal structure of γ -secretase is currently unavailable, similar chamber-like structures have been observed by electron microscopy (11, 12).

Structural analysis, including cysteine-scanning assays, have suggested that PS1 contains nine transmembrane domains (TMD1–9) (13–15), and a hydrophilic “catalytic pore” is formed by TMD1, -6, -7, and -9 (16–20). The catalytic residues Asp-257 and Asp-385, within TMD6 and -7, respectively, are located in the chamber-like structure (16, 17). The catalytic pore resides in the convex side of a horseshoe-like transmembrane arrangement of γ -secretase, identified by cryo-electron microscopy (21).

The interactions between PS1 and other subunits have also been evaluated. Aph-1, Pen2, and NCT, which contain seven, three, and one transmembrane domains, respectively, interact with PS1. Pen2 associates directly with the WNF motif within the TMD4 of PS1 (22, 23). The NCT-Aph-1 subcomplex interacts with the carboxyl-terminal region of PS1, including TMD8 and TMD9 (24–26). Prior to entry into the catalytic site, the substrate interacts with the extracellular domain of NCT (27), as well as the recognition site of PS1, which is formed by TMD1 and the carboxyl terminus, including TMD9 (28, 29).

It is difficult to study the catalytic mechanism or interaction between subunits in γ -secretase, which is a transmembrane protease with multiple subunits. However, the assay system could be constructed in the yeast cell, which does not have functional γ -secretase homologue or APP. Through a yeast transcriptional activator Gal4 system using an artificial substrate containing APP segments, Edbauer *et al.* (7) demonstrated that the four subunits of γ -secretase are essential for its protease activity. We also established a system to evaluate the specificity of γ -secretase and to screen mutations (30–32). We have isolated constitutively active PS1 mutants that do not

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¹ To whom correspondence should be addressed: 1-1 Amamiya, Tsutsumidori, Aoba-ku, Sendai, Miyagi 981-8555, Japan. Tel./Fax: 81-22717-8892; E-mail: futai@biochem.tohoku.ac.jp.

² The abbreviations used are: FAD, familial Alzheimer disease; A β , amyloid β peptide; APP, amyloid precursor protein; CTF, carboxyl-terminal fragment; NCT, nicastrin; NTF, amino-terminal fragment; PS1, presenilin 1; PS2, presenilin 2; Pen2, presenilin enhancer 2; NSAID, nonsteroidal anti-inflammatory drug; TMD, transmembrane domain; DKO, double knock-out; DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine *t*-butyl ester.

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require NCT for proteolysis (30). We also observed a difference between PS1 and PS2 activities (32), and we detected the release of different A β species (A β 40, A β 42, and A β 43) after cleavage in yeast microsomes (31). Thus, yeast cells are a useful model system to analyze γ -secretase.

In this study, the properties of PS1 FAD mutations were analyzed using the yeast system. When FAD mutations, L166P or G384A (Leu-166 to Pro or Gly-384 to Ala, respectively), were introduced into PS1, yeast cells could not grow by lack of APP cleavage. We identified amino acid substitutions in PS1 that suppress the original mutations. Critical secondary mutations were in a catalytic pore as follows: K380E, S384P, or L432M. Using PS1/PS2 double knock-out mouse embryonic fibroblasts (33), we could show that the secondary mutations increased the level of A β and the intracellular domain but decreased A β 42. Furthermore, we studied individual secondary mutations, and we discuss their modulatory effects.

Experimental Procedures

γ -Secretase Reconstitution in Yeast—To reconstitute γ -secretase in yeast, human PS1, NCT, FLAG-Pen2, Aph-1aL-HA, APP fragment (C55), Notch-1 fragment (Notch_{TM}), and Gal4 were cloned into respective vectors, as described previously (30). Briefly, PS1 and NCT were cloned into the KpnI and XbaI sites of pBEVY-T (34). FLAG-Pen2 and Aph1aL-HA were cloned into the KpnI and XbaI sites of pBEVY-L (34). APP_{C55}-Gal4p and Notch_{TM}-Gal4p were fused to the *SUC2* signal peptide sequence and cloned into the BamHI and EcoRI sites of p426ADH (35). APP_{C55} and Notch_{TM} represent amino acids 672–726 of human APP770 and 1703–1754 of mouse Notch-1, respectively. These recombinant plasmids were transformed into *Saccharomyces cerevisiae* strain PJ-69–4A (*MATa trp1-901 leu2-3, 112 ura3-52 his3-200 gal4 Δ gal80 Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*) (36). Reporter gene expression was monitored to assess substrate cleavage. The expression of *HIS3* (His) and *ADE2* (Ade) was estimated by colony growth on minimal SD agar medium, lacking Leu, Trp, His, Ura, and SD-LWHUade (30). β -Galactosidase was assayed using *o*-nitrophenyl β -D-galactopyranoside, as described previously (30). Exponentially growing cells (1×10^7 cells) were lysed using glass beads in 30 μ l of lysis buffer (20 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 50 mM KCl, 1 mM EDTA, 5% glycerol, and 1 mM dithiothreitol (DTT)), including a protease inhibitor mixture (30). Cell lysates were centrifuged for 10 min at $15,000 \times g$, and β -galactosidase activities and protein concentrations (Bradford protein assay, Bio-Rad) were estimated in the resulting supernatants.

Random Mutagenesis by PCR—Random mutations were introduced into PS1 FAD mutants, L166P and G384A, by polymerase chain reaction (PCR), as described previously (30). Briefly, the 50- μ l reaction contained 50 ng of template DNA, 0.2 mM dGTP, 1 mM dATP/dTTP/dCTP, 3 mM MgCl₂, 0.5 mM MnCl₂, 1.25 units of rTaq in $1 \times$ rTaq buffer, and 400 nM primers. Primer sequences are as follows: PS1S, 5'-TTCAA-GCTATACCAAGCATACAATCAACTCCCCGGGTACCA-AAAATGACAGAGTTACCTGCACCGTTG-3', and PS1AS, 5'-GATCCGCTTATTTAGAAGTGTCGAATTCGACCTCG-GTACCATGCTAGATATAAAATTGATGGAATGC-3'.

PCRs were performed under the following conditions: 94 °C for 5 min; 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 120 s (30 cycles); and 72 °C for 10 min. The error ratio of these conditions was \sim 2.6 mutations per *PS1* gene. Mutagenized PS1 cDNA fragments (4 μ g) were then cotransformed with 4 μ g of the KpnI fragment from NCTpBEVY-T into PJ69-4A. PS1 primers containing 40-bp regions from pBEVY-T (34) enable homologous recombination between two fragments (37). Approximately 10^6 transformants were screened on the selection medium SD-LWHUade (Table 1). Plasmid DNAs were isolated from yeast colonies, and mutations were identified by DNA sequencing. Site-directed mutations were introduced using a QuikChange mutagenesis kit (Stratagene).

Protein Expression in Mouse Embryonic Fibroblasts—For expression in mouse cells, wild-type or mutant PS1 cDNAs were inserted into the EcoRI site of pMXs-puro (38). A highly efficient retroviral infection was performed as reported previously (23, 38). Briefly, retroviral plasmids were transfected into the packaging cell line PLAT-E using FuGENE 6 (Roche Applied Science). After 48 h, conditioned media were filtered through a 0.45- μ m pore and used as viral stocks. For infection, cells were cultured with the viral stock containing 5 μ g/ml Polybrene. After 6 h, the virus stock was replaced with new medium lacking virus and analyzed further.

To measure the amount of secreted A β , recombinant retroviruses encoding each mutant PS1 were transiently infected into mouse fibroblasts from PS1/PS2 double knock-out (DKO) mice stably expressing APP-NL (KM670/671NL Swedish mutation) (23). After incubation for 24 h, conditioned media were collected and subjected to two-site ELISAs (*i.e.* BNT77/BA27 and BNT77/BC05 for A β 40 and A β 42, respectively) (39) or to immunoblot analysis using the urea/SDS-PAGE system (40–43). Cells were also recovered and subjected to immunoblotting. Media with increasing concentrations (50–150 μ M) of sulindac sulfide (Sigma) were used to analyze the A β 42-lowering effect of NSAIDs.

To measure the amount of the intracellular domains of APP and Notch, retrovirus with mutant PS1 were infected into 1210/DKO-expressing γ -substrates and cognate luciferase reporters (44). For the generation of 1210/DKO cells, retroviral expression vectors containing SPC99gvp-6myc (modified SPA4CT (45) fused with Gal4/VP16 and a hexa-Myc epitope tag) in pMXs-puro, N Δ E-6myc (truncated mouse Notch (46) fused with a hexa-Myc epitope tag) in pLPCX (Clontech), UAS-firefly luciferase in pMXs-EGFP_{II}, and TP1-*Renilla* luciferase in pMXs-II were constructed. pMXs-EGFP_{II} is a derivative-encoding enhanced GFP, rather than the puromycin resistance gene in pMXs-puroII (47). pMXs-II was generated by excising the puromycin resistance gene in pMXs-puroII. 1210/DKO cells express truncated APP (SPC99gvp-6myc) and Notch (N Δ E-6myc), which correspond to different luciferase reporter systems (*i.e.* Gal4/VP16-fused APP intracellular domain-driven UAS-firefly luciferase and Notch intracellular domain-driven TP1-*Renilla* luciferase, respectively). Following infection, luciferases were assayed as described previously (44).

Antibodies—Anti-G1L3, anti-G1Nr3, and PNT3 antibodies against the human PS1 loop, human PS1 amino terminus, and human Pen-2 amino terminus, respectively, were used as

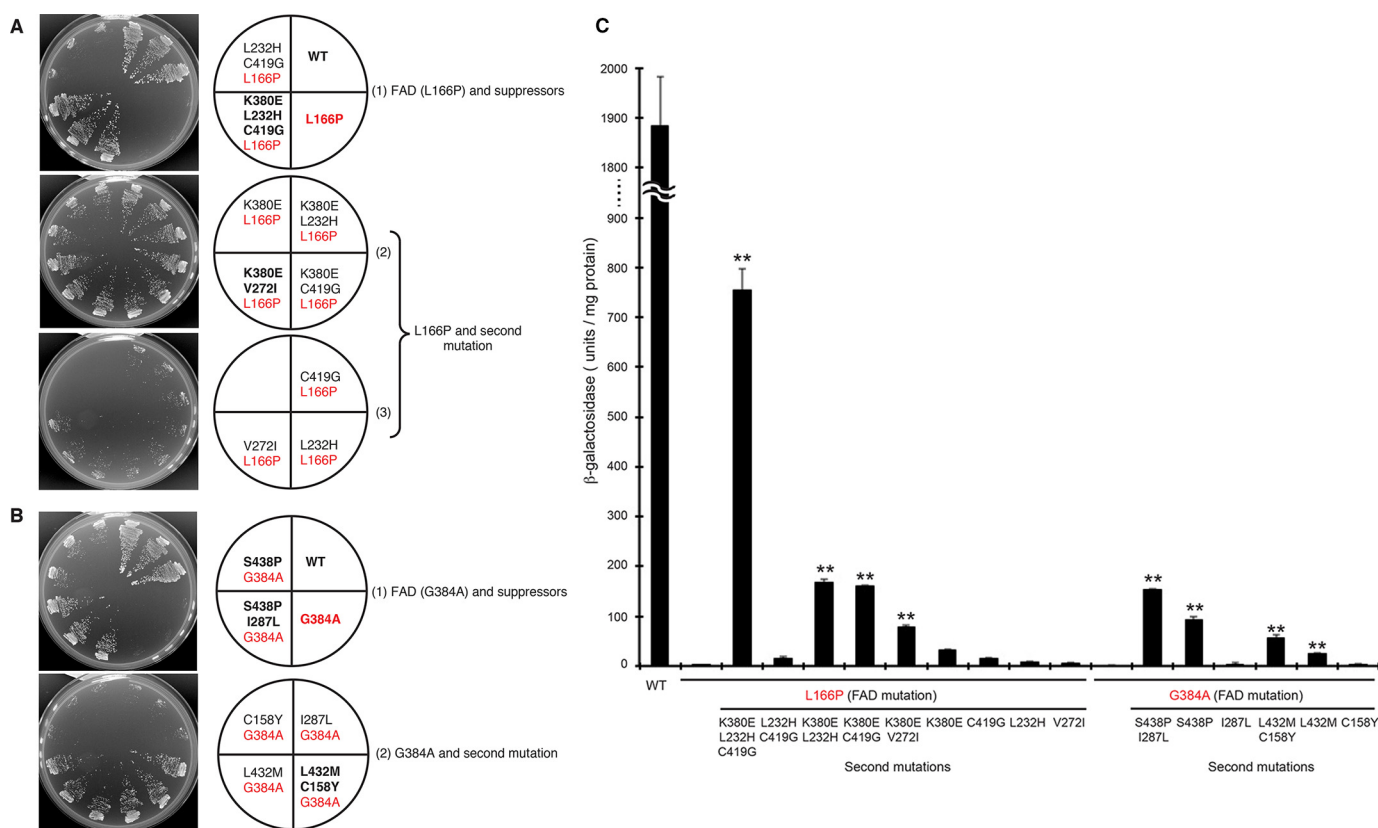


FIGURE 1. Growth and β -galactosidase activity of FAD mutants with secondary suppressor mutations. Growth of FAD mutants with secondary mutations (A and B). Four γ -secretase subunits were reconstituted in yeast with APP_{C55}-Gal4p; Gal4p cleaved from APP_{C55}-Gal4p activates *HIS3* and *ADE2* genes, which leads to growth on selection medium lacking His and adenine. Growth of cells expressing wild-type (WT) PS1, FAD mutants (L166P or G384A), and secondary suppressors was examined after incubation at 30 °C for 3 days in selection media (SD-LWHUAde). Three independent clones were evaluated for each strain. Growth of the mutants in medium containing His and adenine (SD-LWU) was essentially the same as that of WT (data not shown). FAD and secondary mutations are indicated in red and black, respectively. β -Galactosidase activity of the FAD mutants and their suppressors (C). β -Galactosidase activity was assayed for each cell expressing either WT, FAD mutants, or FAD plus secondary suppressor mutations. One unit of β -galactosidase activity corresponds to 1 nmol of *o*-nitrophenyl β -galactoside hydrolyzed per min and is expressed as units/mg lysate protein. Activity was normalized to that of the control cells without nicastrin (35 units/mg protein). Representative results from three independent assays are shown with standard deviations. Statistical analysis was performed by one-way analysis of variance followed by Dunnett's multiple comparison test. Asterisks indicate $p < 0.01$ (**) compared with the FAD mutations (L166P or G384A).

described previously (41, 48). The anti-PS1NT antibody was provided kindly by Dr. G. Thinakaran (49). Anti-Nct (N1660) and anti- α -tubulin (DM1A) were purchased from Sigma. Anti-APP carboxyl terminus (APP(c)) and anti-A β 1-x (82E1) were purchased from IBL.

Results

Secondary Mutations Suppressing PS1 FAD Mutations—Plasmids encoding γ -secretase and APP-based recombinant substrates (APP_{C55}-Gal4p) were introduced into the yeast strain PJ69-4A, which possesses the *HIS3*, *ADE2*, and *lacZ* genes under Gal4p control. After cleavage of the substrate by γ -secretase, Gal4p is released from the APP transmembrane domain (APP_{C55}) to activate the transcription of *HIS3/ADE2* and *lacZ*. Thus, the protease activity could be monitored by cell growth and β -galactosidase activity (30, 32). When FAD mutants (L166P or G384A) of PS1 were introduced into cells with APP_{C55}-Gal4p, severe loss-of-function was observed, indicated by the absence of growth in the media lacking adenine and histidine (Fig. 1, A and B, FAD mutation shown in red). To understand why the specificity of γ -secretase changed, secondary mutations that could suppress FAD mutations were iso-

lated. After PCR-based mutagenesis of PS1 harboring FAD mutations, true revertants and secondary mutants were isolated by screening 1×10^6 cells (Table 1). Two L166P suppressors contained the K380E/L232H/C419G or K380E/V272I replacements (Table 1 and Fig. 1A). L166P in combination with the secondary mutations K380E/L232H and K380E/C419G were also able to grow but that with L232H/C419G (a combination without K380E) could not (Fig. 1A). When each replacement (K380E, C419G, L232H, or V272I) was introduced into L166P, no positive growth was observed, except that L166P/K380E formed small colonies (Fig. 1A). These results indicate the importance of the K380E replacement for suppression.

β -Galactosidase activity semi-quantitatively determined the cleavage of APP_{C55}-Gal4p by the mutants. L166P/K380E/L232H/C419G mutant possessed the highest activity, which was equivalent to 40% that of wild type (Fig. 1C). L166P/K380E/L232H, L166P/K380E/C419G, and L166P/K380E/V272I showed low but significant β -galactosidase activity (9, 8.5 and 4% of wild type, respectively). However, L166P/L232H/C419G (combination of secondary mutations from the original suppressor but without K380E) showed diminished activity (0.8% of wild type).

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TABLE 1

Growth of mutants that suppress FAD mutations of PS1 (L166P and G384A)

Mutants	Growth ^a	No. of clones isolated
L166P, PCR mutation		
Revertants		
L166 (true revertant)	+++	104
L166T	+++	34
L166S	++	60
L166H	++	2
Second mutation		
K380E/L232H/C419G	+++	1
K380E/V272I	++	1
Directed mutation		
L232H/C419G	–	
K380E/L232H	++	
K380E/C419G	++	
K380E	+	
C419G	–	
L232H	–	
V272I	–	
G384A, PCR mutation		
Revertants		
G384 (true revertant)	+++	2
Second mutation		
S438P	+	12
S438P/I287L	++	1
L432M/C158Y	++	1
Directed mutation		
I287L	–	
L432M	+	
C158Y	–	

^a The growth of cells with PS1 mutations (L166P and G384A) were analyzed on SD-LWHUAd medium after 3 days at 30 °C. All cell contains NCT, Aph-1aL, Pen2, and APP_{C55}-Gal4p. +++ represent wild-type growth, formed >1 mm colonies and – represents no growth. ++ or + represents partial growth, formed small colonies (>0.5 or <0.5 mm, respectively). 9.5×10^5 or 1.1×10^6 cells were screened for L166P or G384A suppressors, respectively.

Three G384A suppressors contained the secondary mutations, S438P, S438P/I287L, and L432M/C158Y restored the growth of G384A (Table 1 and Fig. 1B). Effect of S438P/I287L was stronger than S438P alone, whereas I287L showed no effect on the cell growth (Fig. 1B). Similarly, L432M partially restored cell growth but C158Y had no effect (Fig. 1B). These results indicate that two replacements were necessary for suppression, and S438P and L432M are essential.

β -Galactosidase activities of the G384A suppressors correlated with the growth. G384A/S438P/I287L exhibited the highest activity (8% that of wild type, Fig. 1C), followed by G384A/S438P, G384A/L432M/C158Y, and G384A/L432M, which exhibited 5, 2.5, and 1.5% wild-type activity, respectively (Fig. 1C).

These results indicate that multiple secondary mutations are required for suppression of FAD mutants. In contrast to L166P located within TMD3, the suppressor mutations L232H, V272I, K380E, and C419G map to TMD5, TMD7, TMD8, and to the sixth loop, respectively (Fig. 2A). In contrast, G384A is located within TMD7, and the suppressor mutations map to TMD9 (L432M and S438P) and to the second (C158Y) and sixth loops (I287L, Fig. 2B).

Suppressor Mutations Restored Notch Cleavage—To further understand the suppression of PS1 FAD mutations, we tested the cleavage of Notch_{TM}-Gal4p (transmembrane domain of Notch-1 fused to Gal4p) in yeast. The γ -secretase containing the FAD mutation was able to release Notch fragments from Notch_{TM}-Gal4p at reduced efficiency; L166P and G384A mutants exhibited 25 and 50% of the β -galactosidase activity as

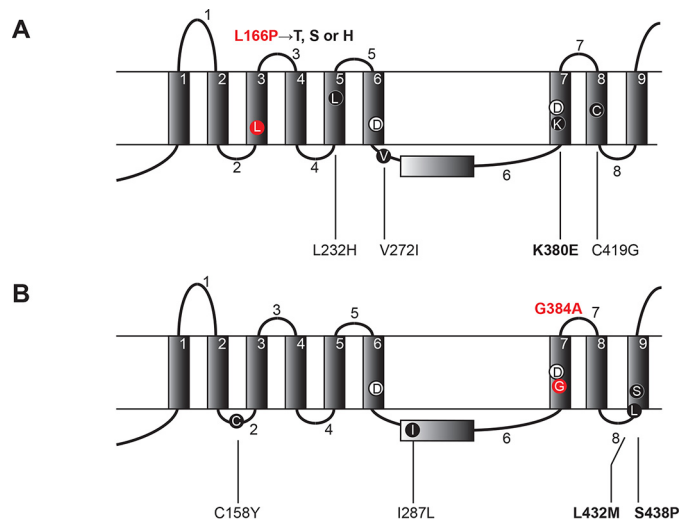


FIGURE 2. FAD mutations and secondary suppressor mutations map to the transmembrane and loop domains of PS1. FAD mutation (L166P or G384A) and secondary suppressors (A and B) are shown. PS1 models with the nine transmembrane domains (TMD) 1–9 and the eight loop domains 1–8 (13–15) are shown. The suppressor mutations for L166P (red) include the primary mutation K380E together with three variable mutations (L232H, V272I and C419G) (A). The suppressor mutations for G384A (red) include the primary mutation L432M or S438P with secondary mutation C158Y or I287L, respectively (B). The critical secondary mutations are indicated in **boldface** and the FAD mutations are in **red**. Catalytic Asp residues in TMD6 and TMD7 (Asp-257 and Asp-385) are also indicated.

that of the wild type, respectively (Fig. 3A). When the suppressor mutants were examined, the activities induced by L166P/K380E/L232H/C419G, G384A/L432M/C158Y and G384A/S438P/I287L become similar to that by the wild type, indicating that the Notch-cleaving activity of FAD mutants was recovered to wild-type levels by the second mutations. Immunoblotting confirmed that the expression of mutant PS1s was at a similar level to that of wild type (Fig. 3B).

Second Mutations Activate γ -Secretase Activities—Next, we tested the cleavage of APP and Notch by PS1 carrying second mutations alone. K380E/L232H/C419G and S438P/I287L showed higher β -galactosidase activities for APP and Notch cleavage (Fig. 3, C and D); APP cleavage by the two mutants was 2.2- and 1.9-fold higher than wild type, respectively (Fig. 3C). In contrast, L432M/C158Y mutant showed wild-type activity. Essentially the same results were obtained for Notch cleavage (Fig. 3D). These results suggest that K380E/L232H/C419G and S438P/I287L enhance protease activity for APP and Notch.

Secondary Mutations Suppressed L166P and G384A in Mouse Fibroblasts—We further analyzed the PS1 FAD mutation in embryonic fibroblasts (DKO cells) from PS1/PS2 double knock-out mice (Fig. 4). Wild-type and mutant PS1 cDNAs were cloned into a transfer vector to generate recombinant retroviruses, which were then infected into DKO cells.

In DKO cells with wild-type or mutant PS1, we detected similar amounts of mature NCT and Pen-2 (Fig. 4A, lanes 2–7). The amounts of PS1, full-length and amino- and carboxyl-terminal fragments (PS1NTF and PS1CTF, respectively), were also similar, except L166P/K380E/L232H/C419G mutant (Fig. 4A, lanes 2–7). NTF bands of L166P/K380E/L232H/C419G were more intense, and PS1 full-length band of L166P/K380E/L232H/C419G mutant migrated faster than the wild type (Fig.

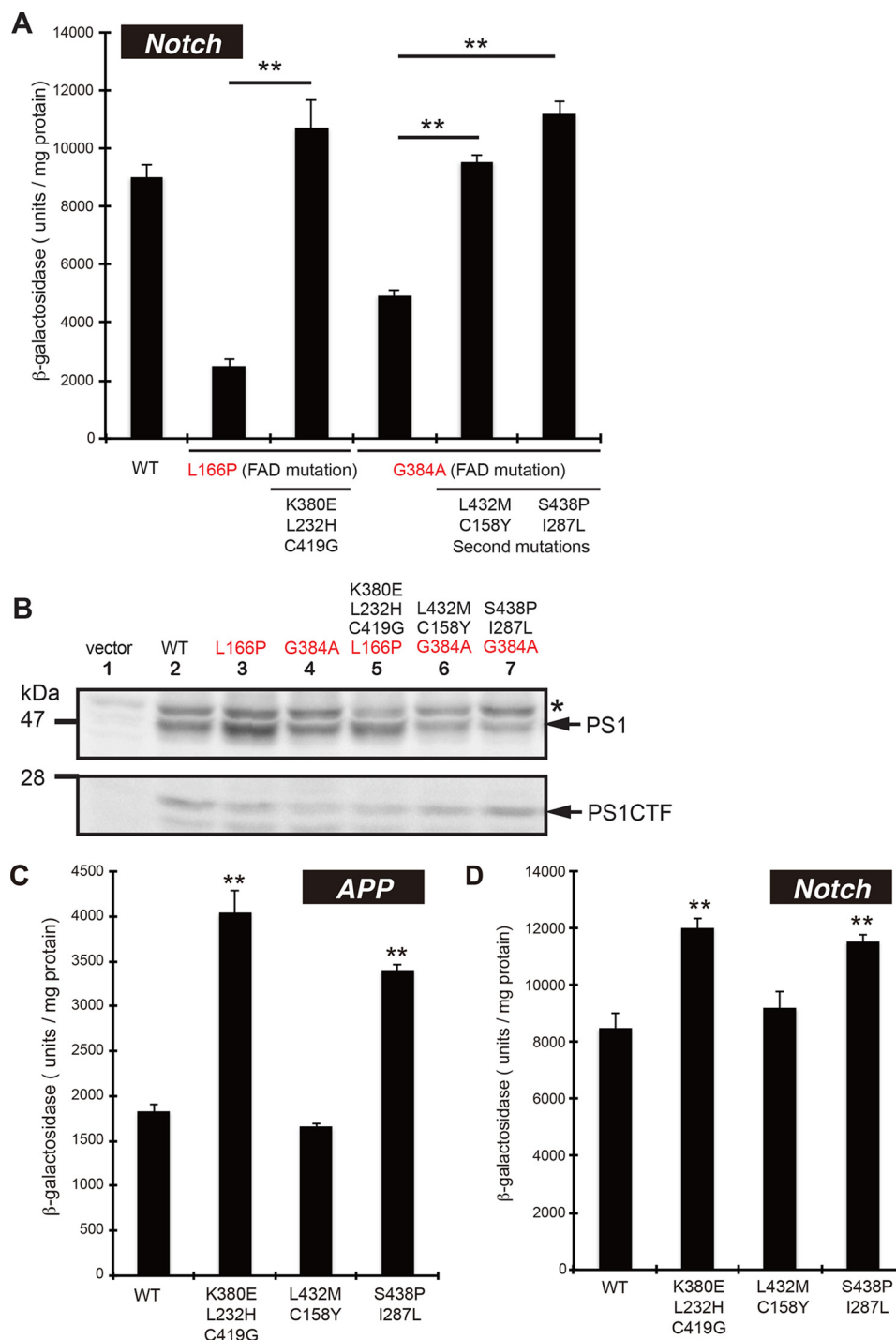


FIGURE 3. β -Galactosidase activity of FAD suppressors or PS1 with the secondary mutations alone. β -Galactosidase activity of wild-type (WT), FAD mutations (L166P, G384A), their suppressor mutants (A), or the secondary mutants (C and D). APP_{C55} -Gal4p or Notch_{TM}-Gal4p was used for each assay and indicated as APP (C) or Notch (A and D), respectively. One unit of β -galactosidase activity corresponds to 1 nmol of *o*-nitrophenyl β -galactoside hydrolyzed per min and is expressed as units/mg lysate protein. Activity was normalized to that of the control cells without nicastrin (35 units/mg protein for APP_{C55} -Gal4p and 95 units/mg protein for Notch_{TM}-Gal4p). Asterisks (**) indicate $p < 0.01$. Expression of WT and mutant PS1 in yeast (B). Cell lysates from WT or mutant PS1s were analyzed by immunoblotting using an antibody against the carboxyl terminus of PS1. Positions of PS1 and its amino terminus (PS1CTF) are indicated. An asterisk in the immunoblot indicates nonspecific band.

4A, lane 5). These results suggest that this mutant has different post-translational modification and stability/turnover.

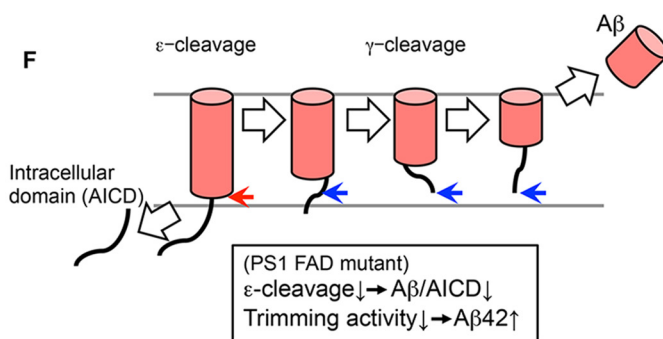
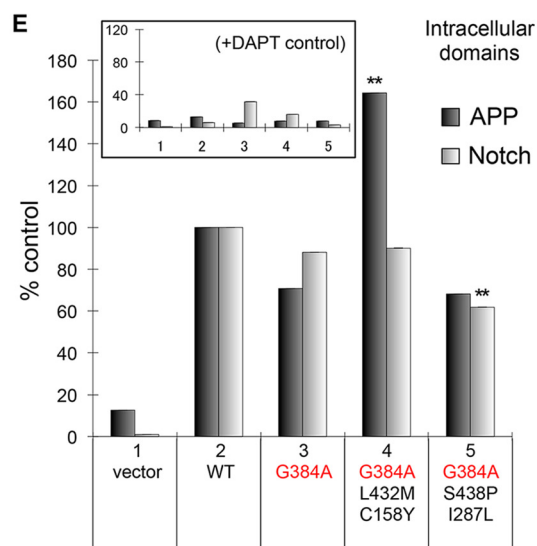
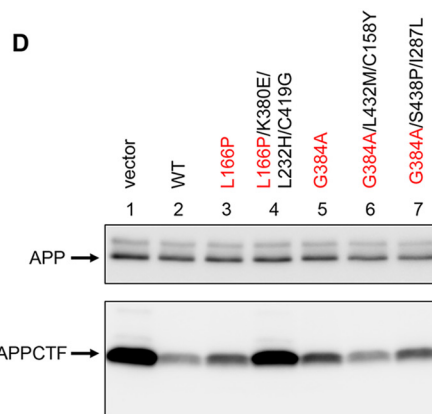
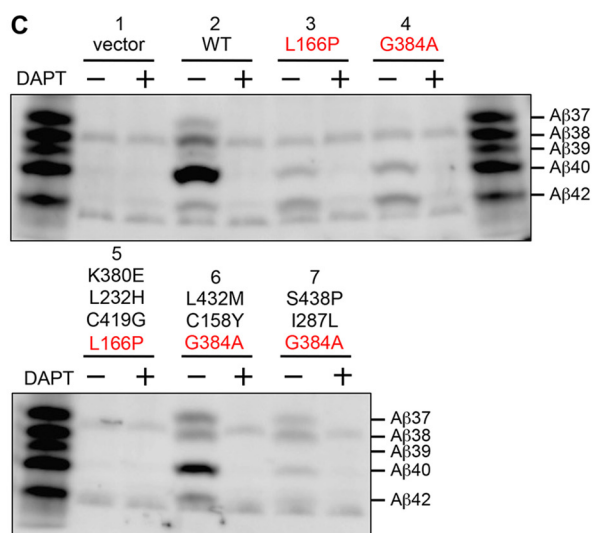
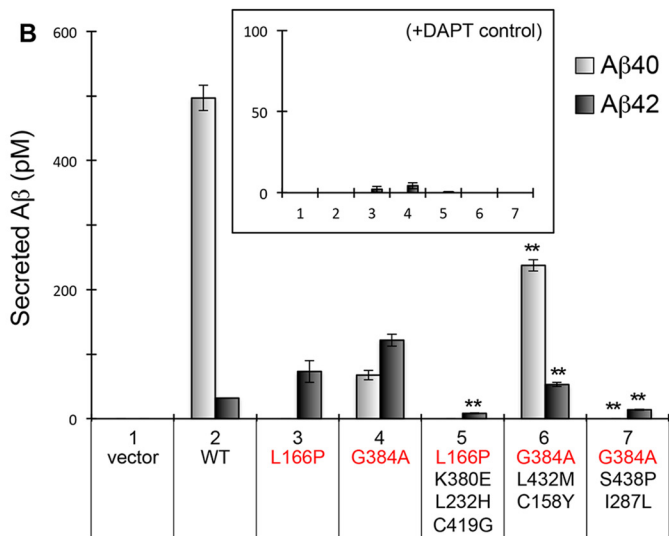
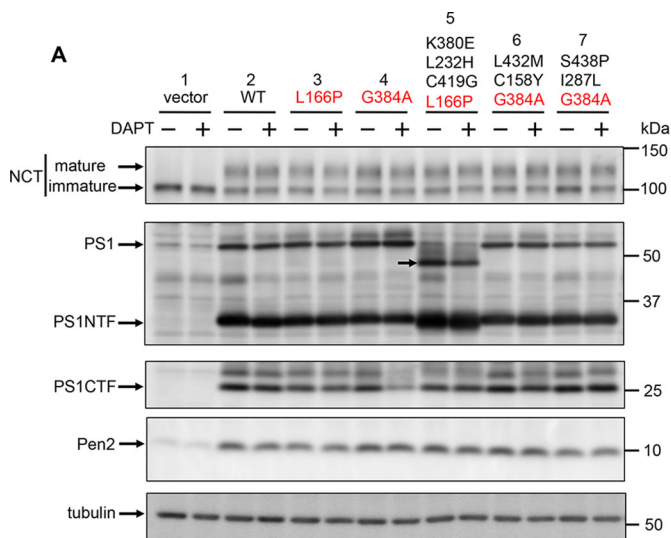
Cells harboring L166P and G384A FAD mutants secreted a significantly increased amount of A β 42 compared with the wild type (2.3- and 3.8-fold higher, respectively) but a decreased amount of total A β (Fig. 4B, lanes 3 and 4). γ -Secretase activity

was confirmed by the addition of *N*-[*N*-(3,5-difluorophenacetyl)-*L*-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT), a specific γ -secretase inhibitor (Fig. 4B, inset). As shown previously, γ -secretase cleaves APP by two different proteolytic activities (Fig. 4F) (5, 50). The initial cleavage is similar to that of endopeptidase (ϵ -cleavage), leading to a separation of the intracellu-

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lar domains and long A β peptides. Next, long A β peptides (A β 48 or A β 49) are processed in a similar manner to that by carboxypeptidase-like γ -cleavage, producing different A β spe-

cies (A β 37 to A β 46). Thus, decreased total A β and increased longer A β 42 species among FAD mutants suggest that ϵ -cleavage and trimming activities are damaged (Fig. 4F).



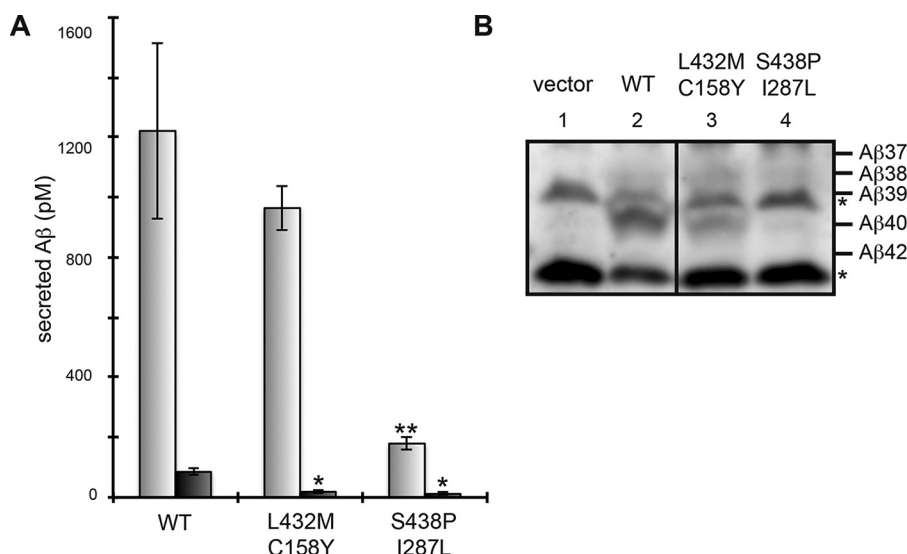


FIGURE 5. A β production by PS1 with secondary mutations alone. Using the retroviral infection system, PS1/PS2 DKO cells, stably expressing APP-NL, were transfected with WT or the second mutants (L432M/C158Y or S438P/I287L) (A and B). After transfection, the secreted A β peptides were quantified by ELISA using antibodies against A β 40 (gray) or A β 42 (black) (A) and analyzed by immunoblotting (B). Asterisks (* or **) indicate $p < 0.05$ or $p < 0.01$, respectively (A). Asterisks in the immunoblot indicate nonspecific bands (B).

In contrast to the FAD mutants, the decreased level of A β 42 was detected with suppressor mutants (Fig. 4B). G384A/L432M/C158Y resulted in a decreased level of A β 42 and an increased level of A β 40 (44 and 350% of G384A levels, respectively; Fig. 4B, lane 6). L166P/K380E/L232H/C419G and G384A/S438P/I287L were associated with a decrease of both A β 40 and A β 42 (Fig. 4B, lanes 5 and 7). Because these suppressor mutant cells may secrete shorter A β peptides, we analyzed the levels of A β using immunoblotting (Fig. 4C). Compared with G384A, G384A/L432M/C158Y caused secretion of increased levels of shorter A β 37, A β 38, and A β 40 peptides (Fig. 4C, lanes 4 and 6), suggesting higher ϵ -cleavage and trimming activity induced by the suppressor FAD mutant. However, G384A/S438P/I287L was associated with increased levels of A β 37 and A β 38 but decreased levels of A β 40 and A β 42 (Fig. 4C, lanes 4 and 7), suggesting that the suppressor mutation G384A/S438P/I287L leads to increased trimming activity of γ -secretase. Essentially no A β peptides were detectable in the media from cells expressing the L166P/K380E/L232H/C419G (Fig. 4C, lane 5). Next, we detected APP carboxyl-terminal fragments in DKO cells (Fig. 4D). In contrast to the FAD mutants, G384A/L432M/C158Y and G384A/S438P/I287L showed significant reduction of APP CTFs (Fig. 4D, lanes 6 and 7), con-

firmed the increased cleavage. However, L166P/K380E/L232H/C419G results in accumulation of APPCTF (Fig. 4D, lanes 5). Considering the somewhat different metabolism and/or the post-translational modification of PS1 (Fig. 4A), we did not pursue the function of L166P/K380E/L232H/C419G further, and instead we focused on the G384A suppressors in this study.

To further confirm the γ -secretase activity of the mutants, we evaluated the release of intracellular APP and Notch domains fused to luciferase reporters (44). In this assay, luciferase activity is correlated to that of ϵ -cleavage of APP and Notch. G384A mutants showed decreased levels of the APP intracellular domain (70% that of wild type), but G384A/L432M/C158Y mutants showed increased APP intracellular domain levels (230% that of G384A; Fig. 4E, lanes 2–4), suggesting that the ϵ -cleaving activity is highly enhanced in the suppressor mutant cells. In contrast, G384A/S438P/I287L mutant cells produced a similar amount of the APP intracellular domain as did G384A cells (Fig. 4E, lanes 3 and 5), indicating that ϵ -cleaving activity was not affected. This result supports the notion that G384A/S438P/I287L specifically affects the trimming activity of A β in DKO cells. Notch intracellular domain produced by G384A and the suppressed cells were sim-

FIGURE 4. Production of A β and the intracellular domains of APP and Notch by PS1 with FAD mutations and secondary suppressors in mouse embryonic fibroblasts. Using the retroviral infection system, PS1/PS2 DKO cells, stably expressing APP-NL, were transfected with WT or mutant PS1, L166P, G384A, L166P/K380E/L232H/C419G, G384A/S438P/I287L, or G384A/L432M/C158Y (A–D). Cells were harvested and analyzed by immunoblotting using antibodies specific to NCT (N1660), PS1 (anti-PS1NT for NTF and G1L3 for CTF), Pen2 (PNT3), or tubulin (DM1A) (A). Secretion of A β peptides including A β 40 and A β 42 (B and C). After transfection, media were recovered, and the secreted A β peptides were quantified by ELISA using antibodies against A β 40 (gray) or A β 42 (black) (B). Values for 10 μ M DAPT (+), N-[N-(3,5-difluorophenacetyl)-L-alanyl]-5-phenylglycine t-butyl ester, are shown as the controls (inset) (B). Media were also analyzed using urea/SDS-PAGE followed by immunoblotting with an antibody specific to A β (82E1). Synthetic A β 37, A β 38, A β 39, A β 40, and A β 42 were loaded as indicated (C). APP and APP CTF in cell lysates were analyzed by immunoblotting (D). Effects of FAD and suppressor mutations on ϵ -cleavage of APP and Notch in a cell-based γ -secretase assay are shown (E). 1210/DKO cells expressing APP and Notch luciferase reporters were infected transiently with recombinant retrovirus encoding PS1 mutants. Relative levels of intracellular domains of APP (black) and Notch (gray) are indicated ($n = 3$; means \pm S.E. of the mean). Values for 10 μ M DAPT (+) are shown as a control (inset) (E). Data represent the mean \pm S.D.; $n = 3$. Statistical analyses were performed by one-way analysis of variance followed by Dunnett's multiple comparison test. Asterisks indicate $p < 0.01$ (**) compared with PS1 L166P or G384A (B and E). FAD and secondary mutations are indicated in red and black, respectively. Schematic model of processive intramembrane cleavage by γ -secretase is shown (F). γ -Secretase cleaves APP in a manner similar to that of endopeptidase (ϵ -cleavage), followed by a mechanism similar to that of carboxypeptidase (γ -cleavage). Decreased total A β /APP intracellular domain (AICD) and increased longer A β peptides by PS1 FAD mutations can be explained by partial loss of both ϵ - and γ -cleavage activities.

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ilar, albeit slightly decreased by G384A/S438P/I287L cells (Fig. 4E), suggesting that the effects of FAD and secondary mutations have little effect on Notch cleavage in DKO cells.

Second Mutations Modulate γ -Secretase Activities—Next, we analyzed PS1 carrying second mutations alone in PS1/PS2 DKO cells. Cells harboring L432M/C158Y and S438P/I287L mutants secreted a significantly low amount of A β 42 (22 and 13% wild type, respectively) (Fig. 5A). S438P/I287L was also associated with decreased A β 40 levels. Notably, immunoblot analysis revealed that the ratio of shorter A β species (*i.e.* A β 37, A β 38, and A β 39) to the A β 40 mutant was increased in DKO cells with L432M/C158Y or S438P/I287L (Fig. 5B, lanes 3 and 4). Thus, L432M/C158Y and S438P/I287L altered trimming activity of γ -secretase activity to reduce A β 42. These results suggest that γ -secretase is affected by the second mutations alone. A β 42/A β 40 ratios of wild type, L432M/C158Y, and S438P/I287L were 0.070, 0.019, 0.065, respectively. Robust reduction of the ratio was observed with the L432M/C158Y mutant.

Effect of Second Mutations on PS1 Mutants and NSAIDs—We tested the effects of G384A suppressors on different mutations at Gly-384 (*i.e.* G384P or G384K) (51). Consistent with previous results, G384P and G384K mutations failed to restore the β -galactosidase activities in yeast (Fig. 6, A and B) and A β production from DKO cells (Fig. 6C) (51). Among the suppressor mutants, G384P/L432M/C158Y and G384P/S438P/I287L had increased activity (13 and 36% wild type, respectively) with Notch_{TM}-Gal4p (Fig. 6B), indicating partial activation of Notch cleavage by the suppressors, although APP cleavage in yeast and A β production in DKO cells were not restored (Fig. 6, A–C).

Next, we tested the effect of G384A suppressor mutation on L166P mutation. L166P/L432M/C158Y and L166P/S438P/I287L showed higher β -galactosidase activities than L166P mutant (Fig. 6, A and B), suggesting that FAD mutations were suppressed. In DKO cells, L166P/L432M/C158Y produced increased levels of A β 40 and decreased levels of A β 42 compared with L166P mutants (Fig. 6C). The level of A β 40 was close to wild type, suggesting that the suppressor mutations are more effective on L166P. In contrast, L166P/S438P/I287L produced decreased levels of both A β 40 and A β 42 (Fig. 6C). These results suggest that the secondary mutations, L432M/C158Y and S438P/I287L, could restore the γ -secretase activities of different FAD mutants effectively.

Finally, we tested the effect of the second mutation on γ -secretase modulator. Consistent with the previous results (52), NSAID sulindac sulfide showed A β 42-lowering effect on cells with wild type PS1, but the effect was diminished with the G384A mutant (Table 2). When the L432M/C158Y mutation was combined with G384A, cells displayed a significant A β 42-lowering effect (Table 2), indicating that the second mutations restored the NSAID insensitivity of the G384A mutant.

Discussion

FAD mutations affecting A β production have been analyzed extensively (1). However, the role of FAD mutations on the function and structure of PS1 is poorly understood. To address this problem, we examined γ -secretase with PS1 FAD mutation in yeast (30–32). Utilizing the advantages of yeast for genetic screening, we identified suppressor mutations for G384A and

L166P FAD mutants. The protease activity of γ -secretase consists of endopeptidase-like cleavage (ϵ -cleavage) and carboxypeptidase-like trimming (γ -cleavage) (Fig. 4F) (5, 50). The FAD mutations severely damaged ϵ -cleavage in yeast, and the suppressor mutations activated this cleavage of FAD mutants or wild-type PS1. In mouse fibroblasts (PS1/PS2 DKO cell), FAD mutation mildly damaged ϵ -cleavage of APP, and the main outcome is reduced trimming (γ -cleavage) and thus increased pathogenic A β species, A β 42. The suppressor mutations activated the trimming and reduce the A β 42 production in fibroblasts. These findings led us to study the effect of each mutation on γ -secretase as discussed below.

With respect to the G384A suppressors, two second mutations, L432M/C158Y and S438P/I287L, showed A β 42-lowering modulatory effects on FAD mutants (L166P and G384A). Unfortunately, however, the L166P suppressor, L166P/K380E/L232H/C419G, suffered post-translational modification and reduction of total A β production in fibroblasts. Further biochemical and cell biological studies are needed to study the structure-function relation of L166P and the secondary mutation. L432M/C158Y and S438P/I287L were found to change the γ -secretase of wild-type PS1 in DKO cells, reducing the amount of A β 42. It is intriguing that the second mutations alone activated γ -secretase and lowered the amount of A β 42. These two mutations are the “A β 42-lowering modulatory mutation” of PS1, which caused the conformational changes of γ -secretase. Such mutations may be included in the rare protective variant of PS1 against Alzheimer disease, similar to the APP A673T mutation in the elderly Icelandic population (53).

We mapped the locations of mutations on the PS1 structure predicted from the crystal structure of signal peptide peptidase PSH (Fig. 7) (10). L432M/C158Y and S438P/I287L possess critical secondary mutations in TMD9 (L432M and S438P, respectively), which are more important than those in the second (C158Y) and sixth loops (I287L) (Fig. 2B). Leu-432 and Ser-438 residues are conserved throughout PS1/PS2 in all species and are located close to the following sequence: ⁴³²LPALPIS⁴³⁸. This sequence includes the PAL motif (Pro-Ala-Leu, underlined), which is completely conserved among PS1, PS2, and signal peptide peptidase. A mutation within PAL motif abolishes γ -secretase activity (26). Leu-432 and Ser-438 are also located close to the catalytic Asp-257 residue in TMD6 (15). Because S438P is an active-state mutant, active in the absence of nicas-trin (30), these mutations may affect the structure of the PAL motif and the catalytic pore. Thus, mutations near the PAL motif may increase the accessibility of the substrate to the catalytic pore.

Comparison of the two substrates (APP or Notch) in yeast showed that the effects of mutations are substrate-specific. APP cleavage was completely lost with L166P or G384A, and APP cleavage by the suppressors was up to 40% of wild type. However, Notch cleavage was weakly damaged (25 or 50% wild type) in FAD mutants and similar to the wild type in suppressors. As shown in Fig. 3, Notch_{TM}-Gal4p cleavage consistently produced 5-fold more β -galactosidase than did APP_{C55}-Gal4p, suggesting that Notch was cleaved at a higher efficiency than that of APP in yeast. It has been shown that γ -secretase easily captures a short extracellular domain of Notch but has

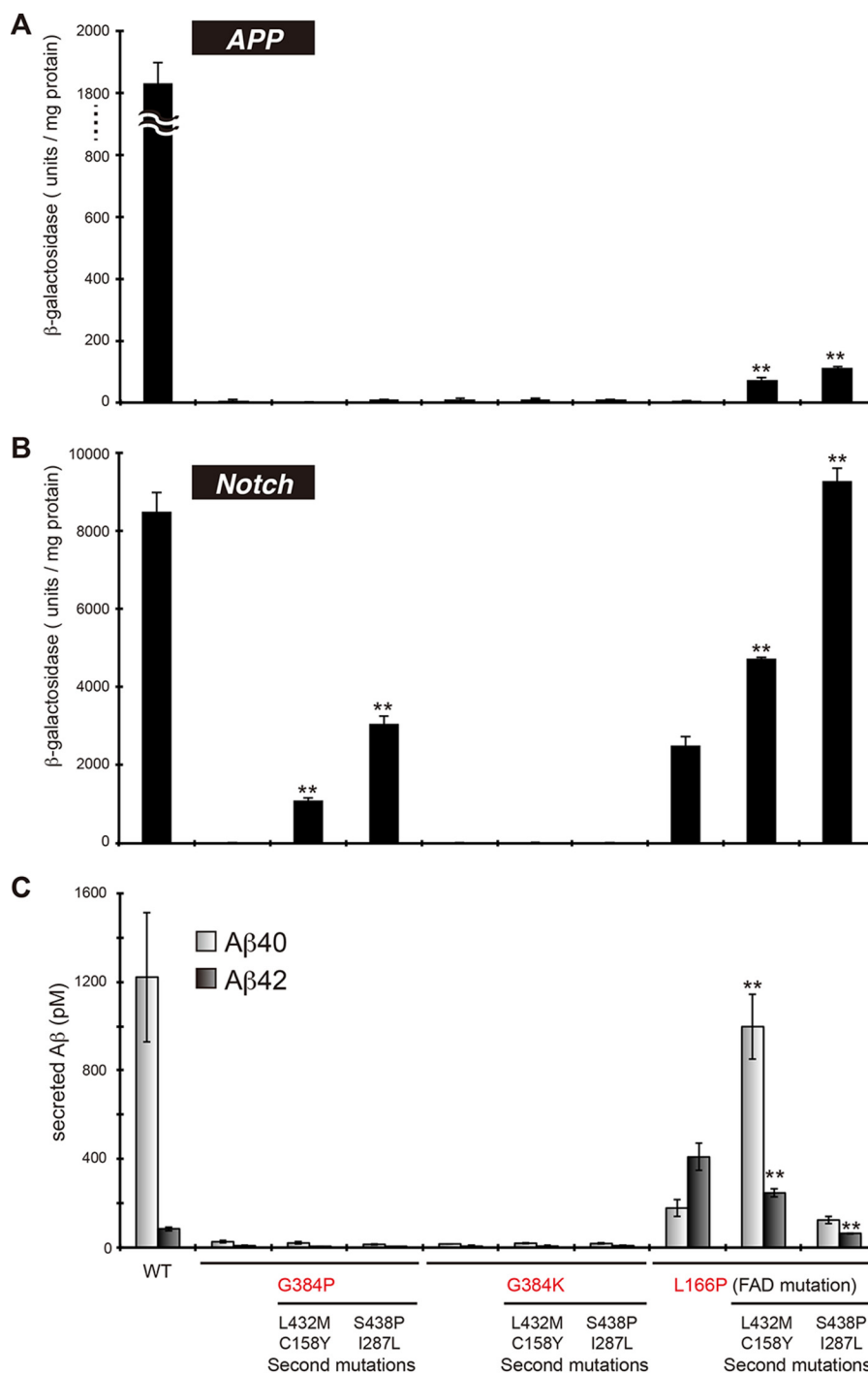


FIGURE 6. **Suppression of G384P, G384K, or L166P mutants by the secondary mutations.** Wild-type (WT) PS1, G384P, G384K, L166P or the suppressor mutants were examined for β -galactosidase activity in yeast (A and B) or A β production in PS1/PS2 DKO cells stably expressing APP-NL (C). β -Galactosidase activity corresponds to APP_{C55}-Gal4p (A) or Notch_{TM}-Gal4p cleavage (B). Secreted A β peptides were quantified by ELISA using antibodies against A β 40 (gray) or A β 42 (black) (C). Asterisks (**) indicate $p < 0.01$.

decreased interaction with the long extracellular domain of APP_{C55} (54). Therefore, a substrate-specific effect of these mutations may be due to substrate preference.

Substitutions of G384, G384P, and G384K significantly reduced A β and Notch intracellular domain generation in a similar manner to that by D385A mutant (51). Consistent with this, neither G384P nor G384K recovered the γ -secretase activity in yeast and DKO cells. The G384A suppressors, L432M/C158Y and S438P/I287L, only restored the Notch cleavage in

the G384P mutation but had no effect on APP cleavage nor A β production and had no effect on G384K. These results support the notion that the G384K mutation caused a loss-of-function effect (51), although G384P severely affected the γ -secretase activity that can be slightly restored by suppressor mutation. The G384A suppressors restored decreased L166P activity. These results indicate that secondary mutations generally activate γ -secretase and that the specific effect to each FAD mutation is small.

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TABLE 2

The diminished A β 42 response of PS1-G384A to sulindac sulfide was restored by the second mutation L432M/C158Y

PS1	A β 42 (% of A β 40 + A β 42)		
	0 μ M	50 μ M	150 μ M
WT	10.15 \pm 1.92	9.91 \pm 3.20	3.37 \pm 0.55 ^a
G384A	35.5 \pm 7.99	33.25 \pm 6.41	47.95 \pm 8.70
G384A/L432M/C158Y	29.05 \pm 4.89	23.59 \pm 6.15	18.42 \pm 3.95 ^a

^a PS1/PS2 DKO cells with PS1-WT or mutant PS1 were treated with increasing concentrations of A β 42-lowering NSAID sulindac sulfide and A β 40 and A β 42 levels in conditioned media were quantified as Fig. 4B ($n = 4$; $p < 0.05$ compared with 0 μ M control).

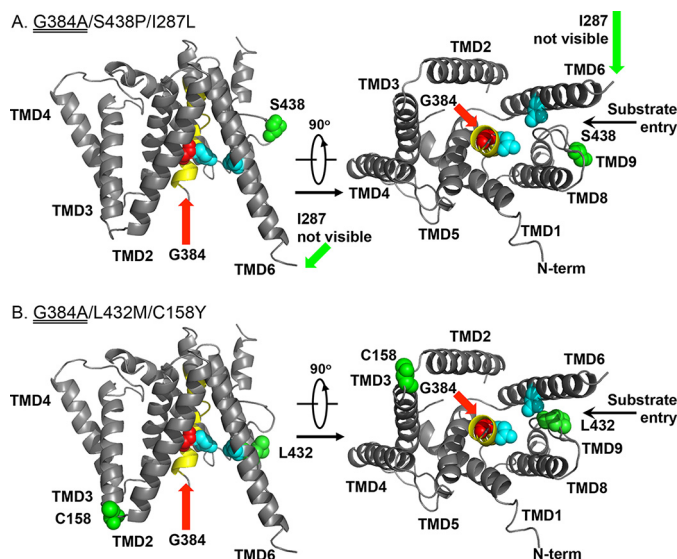


FIGURE 7. Secondary mutations suppressing the FAD mutants in the structure-based PS1 model. A structure-based model of the transmembrane core of human PS1 (10) (Protein Data Bank code 4HYG) is shown with a sphere containing the following residues: two catalytic Asp (blue), the site of FAD mutant (G384A) (red), and suppressor mutations (green). TMD7 containing the FAD mutation is shown in yellow.

γ -Secretase is a prominent drug target for Alzheimer disease. However, a growing list of substrates (55, 56), including developmentally indispensable Notch, has rendered inhibitor design quite challenging. γ -Secretase modulators have been found to reduce A β 42 production without affecting Notch processing (5). The target of GSM-1 was determined to interact with the luminal side of TMD1 of PS1 and modulate γ -secretase activity (40). Similar to these modulators, secondary mutations also modulate the protease function and reduce A β 42 without affecting Notch cleavage in DKO cells. Considering the development of new modulators, TMD9 (Leu-432 and Ser-438) may serve as potential target regions. TMD9 (PAL motif) was previously shown to interact with γ -secretase inhibitors.

In this study, we tested the effect of NSAIDs (sulindac sulfides), which modulate the cleavage reaction lowering A β 42 (Table 2) (57). It has been known that PS1 FAD mutations, including G384A, become insensitive to NSAIDs (52). However, we showed that L432M/C158Y restored the sensitivity to sulindac sulfide. Our results suggest that the second mutations activate PS1 by recovering the conformational flexibility of PS1 that was locked by G384A mutation and act synergistically with NSAIDs to reduce A β 42. Structural analysis of the PS1 protein with the second mutations will provide a novel mechanistic insight of A β 42 generation and trimming

activity of γ -secretase. Taken together, our results indicate that the yeast system can be used to screen for mutations and chemicals that modulate γ -secretase activity and block A β 42 production specifically.

Author Contributions—E. F. and T. T. conceived and coordinated the study and wrote the paper. E. F. designed, performed, and analyzed the experiments shown in Figs. 1–3 and 6 and Table 1. T. T., S. O., and T. C. designed, performed, and analyzed the experiments shown in Figs. 4–6 and Table 2. T. F. performed the experiments in Fig. 3. S. I. provided technical assistance and contributed to the preparation of the paper. All authors reviewed the results and approved the final version of the manuscript.

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