γ-secretase is an intramembrane protease complex consisting of nicastrin, presenilin-1/2, APH-1a/b, and Pen-2. Hydrolysis of the 99-residue transmembrane fragment of amyloid precursor protein (APP-C99) by γ-secretase produces β-amyloid (Aβ) peptides. Pathogenic mutations in PSEN1 and PSEN2, which encode the catalytic subunit presenilin-1/2 of γ-secretase, lead to familial Alzheimer’s disease in an autosomal dominant manner. However, the underlying mechanism of how the mutant PSEN gene may affect the function of the WT allele remains to be elucidated. Here we report that each of the loss-of-function γ-secretase variants that carries a PSEN1 mutation suppresses the protease activity of the WT γ-secretase on Aβ production. Each of these γ-secretase variants forms a stable oligomer with the WT γ-secretase in vitro in the presence of the detergent CHAPSO (3-[3-cholamidopropyl]dimethylammonio)-2-hydroxy-1-propanesulfonate, but not digitonin. Importantly, robust protease activity of γ-secretase is detectable in the presence of CHAPSO, but not digitonin. These experimental observations suggest a dominant negative effect of the γ-secretase, in which the protease activity of WT γ-secretase is suppressed by the loss-of-function γ-secretase variants through hetero-oligomerization. The relevance of this finding to the genesis of Alzheimer’s disease is critically evaluated.

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Significance

The vast majority of familial Alzheimer’s disease (AD) cases are linked to mutations in presenilin-1 (PS1) in an autosomal dominant manner. PS1 is the catalytic component of γ-secretase, which cleaves amyloid precursor protein into Aβ peptides. It remains unclear whether the causal role of PS1 mutations in AD development is effected through γ-secretase, and if yes, whether the dominant negative effect of PS1 mutant allele is effected through the proteolytic activity of γ-secretase. In this study, we provide compelling evidence to prove a dominant negative effect by the loss-of-function γ-secretase mutants on the production of Aβ42/40 by WT γ-secretase through hetero-oligomerization. These data, together with our prior knowledge on the function of γ-secretase, have important ramifications on the two key questions listed here.

See Commentary on page 12635.

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γ-secretase no longer interacts with each other, there is a complete loss of the protease activity. These findings have important ramifications on our understanding of the functional mechanism of γ-secretase and its relationship to AD development.

Results

Enzyme and Substrate Concentrations in the Cleavage Assay. In this manuscript, loss of function strictly refers to the loss of the proteolytic activity of γ-secretase toward the substrate APP-C99. More specifically, because the production of Aβ40 and Aβ42 is closely correlated with the proteolytic activity of γ-secretase (31, 32), it is used as an exclusive indicator throughout this study. We sought to investigate the potential dominant negative effect of AD-derived γ-secretase mutants on the proteolytic activity of WT γ-secretase. Both WT and mutant γ-secretases used in this study were individually overexpressed in HEK293 cells and biochemically purified to homogeneity (33). Dominant negative effect may be achieved by two mutually nonexclusive means: sequestration of substrate by the loss-of-function γ-secretase mutants and hetero-oligomerization between WT and mutant γ-secretases (34, 35).

Discovery and confirmation of the second possibility requires sufficient molar excess of the substrate over the γ-secretase mutant.

First, we determined the working concentrations of γ-secretase at which the proteolytic cleavage assays would be performed. Because γ-secretase exhibits a generally low level of proteolytic activity toward the substrate APP-C99, an incubation time of 3 h was used for all γ-secretase cleavage assays reported in this study. With a fixed substrate concentration of 5 μM, a wide range of WT γ-secretase concentrations from 1 nM to 1.02 μM was examined (Fig. L4). Within the concentration range of 2–64 nM, the combined production of Aβ40 and Aβ42 is approximately linearly proportional to the amount of WT γ-secretase (Fig. 1B).

To ensure sufficient excess of the substrate, we chose 8 or 16 nM for the WT γ-secretase for all subsequent experiments.

Next, with a fixed concentration of 8 nM for the WT γ-secretase, we further examined the requirement of substrate concentration. Within the substrate concentration range of 40–640 nM, the combined production of Aβ40 and Aβ42 steadily increases with increasing APP-C99 concentrations, indicating that the substrate is not yet in sufficient excess over the enzyme (Fig. 1C). At a substrate concentration of 1.25 μM or above, the production of Aβ40 and Aβ42 remains largely the same, suggesting the substrate is no longer the limiting factor. We chose 5 μM for the substrate APP-C99 for all subsequent experiments.

Dominant Negative Effect by Loss-of-Function γ-secretase Mutants.

With both catalytic aspartate residues of PS1 mutated to alanine (Ala), the γ-secretase with PS1-D257A/D385A (γ-secretase-DD) represents a loss-of-function mutant (Fig. S1A). We incubated varying amounts of the catalytic mutant γ-secretase-DD with 8 nM WT enzyme and examined the combined production of Aβ40 and Aβ42. Strikingly, the proteolytic activity of the WT γ-secretase decreases significantly with increasing concentrations of the catalytic mutant (Fig. 1D), with the production of Aβ40 and Aβ42 both decreasing (Fig. S1B and C). The ratio of Aβ42 over Aβ40 remains largely the same (Fig. S1D). The decreased cleavage activity was specifically caused by the mutant γ-secretase-DD, because neither a control membrane protein nor BSA was able to suppress the proteolytic activity of the WT γ-secretase (Fig. 1E). The decreased activity of WT γ-secretase cannot be explained by potential substrate sequestration by the mutant γ-secretase, because the concentration of the substrate APP-C99 was 80-fold higher than the highest concentration of the mutant γ-secretase used. This analysis strongly suggests a dominant negative effect for the catalytic mutant γ-secretase-DD over the proteolytic activity of WT γ-secretase.

The vast majority of AD-derived PS1 mutations give rise to loss-of-function γ-secretase mutants (18). To investigate whether these γ-secretase mutants are dominant negative over the WT enzyme, we chose four such representative PS1 missense mutations: Y115H, L166P, C410Y, and L435F. Each of these four mutations was reconstituted into a distinct γ-secretase mutant. In contrast to the WT γ-secretase, these four loss-of-function mutants exhibit very low levels of the proteolytic activity (18) (Fig. S1A). With a fixed concentration of 16 nM for the WT γ-secretase, the potential dominant negative effect for each of the four mutants was examined (Fig. 2 A–D). In all cases, the total production of Aβ40 and Aβ42 by the WT γ-secretase decreases with increasing concentrations of the mutant enzyme. Thus, all four loss-of-function mutants exhibit dominant negative effect in the in vitro cleavage assays.

A number of the AD-derived PS1 mutations have little effect on the proteolytic activity of the corresponding γ-secretase mutants. To investigate whether these γ-secretase mutants have any effect on the WT enzyme, we examined one such representative PS1 mutation S365A (Fig. S1). In contrast to the loss-of-function γ-secretase mutants, increasing concentrations of the mutant γ-secretase-S365A led to proportionally increased production of Aβ40 and Aβ42 (Fig. 2E). Therefore, for all PS1 mutations examined, only the loss-of-function γ-secretase mutants exhibit a dominant negative effect on the proteolytic activity of the WT γ-secretase (Fig. 2F).

Physical Basis of the Dominant Negative Effect. The dominant negative effect of the loss-of-function γ-secretase mutants on the proteolytic activity of the WT enzyme strongly suggests physical
association. To examine this scenario, we individually overexpressed and biochemically purified a number of γ-secretase variants. First, we investigated potential interactions between HA-tagged WT γ-secretase and Flag-tagged catalytic mutant γ-secretase-DD, using an in vitro pull-down assay (Fig. 3A). The HA or Flag tag is placed at the N terminus of PS1 and can be easily detected by a monoclonal antibody raised against the tag (Fig. 3, lanes 1–3). Using the anti-Flag antibody to perform the pull-down step, the HA-tagged WT γ-secretase was detected in the pellet only in the presence of the Flag-tagged γ-secretase-DD (lane 7), but not in its absence (lane 6). These results demonstrate a direct interaction between the HA-tagged γ-secretase and the Flag-tagged γ-secretase-DD. Swapping of the HA and Flag tags had no effect on this conclusion (Fig. S2A).

Next, we individually examined the potential interactions of the HA-tagged WT γ-secretase with three Flag-tagged γ-secretase mutants: PS1-Y115H, PS1-C410Y, and PS1-ΔE9 (Fig. 3B). Similar amounts of the γ-secretase variants were used in these assays (lanes 1–4). The results are unambiguous: the HA-tagged WT γ-secretase was detectable only in the presence of the Flag-tagged γ-secretase mutants (lanes 6–8), but not in their absence (lane 5). Swapping of the tags on the WT and mutant γ-secretases still allowed the mutual interactions (Fig. S2B). The observed interactions should not be restricted to those between the WT and mutant γ-secretases. The Flag-tagged WT γ-secretase and the Flag-tagged γ-secretase-DD were able to pull-down the HA-tagged WT γ-secretase and the HA-tagged γ-secretase-DD, respectively (Fig. 3C, lanes 8 and 10).

**Effect of Detergents on γ-Secretase Oligomerization.** Our results clearly demonstrate that purified, recombinant γ-secretase oligomerizes in vitro, which explains the dominant negative effect of the loss-of-function γ-secretase mutants over the WT enzyme. We suspected that the proteolytic activity toward the substrate APP-C99 may strictly depend on the oligomerization of γ-secretase. Notably, both the proteolytic activity assays and the pull-down experiments were performed in the presence of the detergent CHAPSO {3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate}. In contrast to CHAPSO, other detergents such as digitonin are known to cripple the proteolytic activity of γ-secretase extracted from cells (22). Regardless of the choice of the extraction detergent for the WT γ-secretase, inclusion of CHAPSO in the assay buffer allowed robust proteolytic activity (Fig. 4A, orange bars). The use of digitonin (Fig. 4A, blue bars) or the inclusion of amphipol, but not detergent (Fig. 4A, pink bars), in the assay buffer led to reduction of the proteolytic activity by at least 90%.

If the oligomerization of γ-secretase is required for its proteolytic activity, then the crippled activity in digitonin might be caused by disruption of γ-secretase oligomerization. To investigate this scenario, we re-examined the interactions between the HA-tagged WT γ-secretase and the Flag-tagged γ-secretase-DD in the presence of digitonin, using the pull-down assay (Fig. 4B). In contrast to the results obtained under CHAPSO, no physical association was detected between these two γ-secretase variants, using the anti-Flag or the anti-HA antibody. Compared with that in CHAPSO, the proteolytic activity of γ-secretase is severely compromised in the presence of amphipol (Fig. 4A, pink bars). Consistently, the HA-tagged WT γ-secretase no longer interacted with the Flag-tagged γ-secretase-DD in the presence of digitonin (Fig. 4C). Swapping of the HA and Flag tags on the WT and mutant γ-secretases had no effect on this conclusion (Fig. S3).

We used analytical ultracentrifugation to investigate the oligomerization status of γ-secretase in the presence of detergents. Consistent with the results of the in vitro pull-down assays, WT γ-secretase is highly poly-disperse in the presence of CHAPSO, appearing in a number of peaks, each with a distinct sedimentation coefficient (Fig. 5A). In contrast, WT γ-secretase is monodisperse in the presence of digitonin (Fig. 5B) and exhibits a low level of poly-dispersion in the presence of amphipol A8-35 (Fig. 5C). The extent of γ-secretase oligomerization under the three detergents correlates well with the proteolytic activity of γ-secretase.

We further examined the oligomerization status of γ-secretase under detergents, using electron microscopy (EM). Consistent with the results of analytical ultracentrifugation, WT γ-secretase appeared to be mostly monomeric in the presence of digitonin by negative staining EM (Fig. 5D). In sharp contrast, WT γ-secretase appeared

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**Fig. 2.** The loss-of-function γ-secretase mutants, each containing an AD-derived mutation in PS1, inhibit the production of Aβ40 and Aβ42 by WT γ-secretase. (A) Dominant negative effect of the γ-secretase mutant (PS1-Y115H) on WT γ-secretase. The concentration of WT γ-secretase is 16 nM in A–E. The total amount of Aβ40 and Aβ42 produced by WT γ-secretase alone is normalized as 1 in all panels. Each experiment was repeated three times, with the SD shown. (B) Dominant negative effect of the γ-secretase mutant (PS1-L166F) on WT γ-secretase. (C) Dominant negative effect of the γ-secretase mutant (PS1-C410Y) on WT γ-secretase. (D) Dominant negative effect of the γ-secretase mutant (PS1-L435F) on WT γ-secretase. (E) The γ-secretase mutant (PS1-ΔE9), which has a proteolytic activity comparable to that of the WT γ-secretase, exhibits no dominant negative effect. In fact, the proteolytic activity increases with increasing amounts of the mutant γ-secretase. (F) A summary of the dominant negative effect by five γ-secretase mutants. The concentrations of WT and mutant γ-secretases are 16 and 40 nM, respectively, in each case.
to form oligomers of several different sizes in the presence of CHAPSO (Fig. 5E). To stabilize the oligomers, we applied a low-concentration range of the crosslinking reagent glutaraldehyde to γ-secretase, using the Grafix protocol (36), and collected EM micrographs (Fig. 5F). 2D classification of the EM particles reveals tantalizing features of γ-secretase (Fig. 5G), which strongly suggest oligomerization.

### Discussion

In this manuscript, we present compelling evidence to support the dominant negative effect of the loss-of-function γ-secretase mutants on the proteolytic activity of the WT γ-secretase. The physical basis for the dominant negative effect appears to be the oligomerization of γ-secretase. Both the proteolytic activity measurements and the pull-down assays were performed in vitro, using highly purified, recombinant γ-secretase. Such an experimental design, which contrasts with previous cell-based studies, allows clear delineation of the experimental logic and clean interpretation of the results. Nonetheless, the observed dominant negative effect in vitro is yet to be thoroughly examined in cells. Preliminary experiments appear to confirm the same dominant negative effect in cells (Fig. S4), which confirms the conclusion of an earlier study (16). Consistently, PS1 homodimers have been previously detected by fluorescent lifetime imaging microscopy in live mammalian cells (37). Intriguingly, the substrate APP-C99 has been reported to dimerize in previous studies (38–40).

![Fig. 3. γ-secretase molecules interact with each other in the presence of the detergent CHAPSO. (A) WT γ-secretase interacts with the catalytic mutant γ-secretase (PS1-D257A, D385A). All pull-down experiments described in this figure were performed using purified recombinant proteins. In the experiments described in A and B, the WT and mutant γ-secretases are differentially tagged and incubated together. Immunoprecipitation using one specific antibody was followed by Western blots using another specific antibody. (B) WT γ-secretase interacts with each of the three mutant γ-secretase proteins (PS1-Y115H, C410Y, and ΔE9). (C) WT or catalytic mutant γ-secretase forms oligomers. In these experiments, the WT (or catalytic mutant) γ-secretase proteins are differentially tagged. The pull-down experiments were performed to assess the WT–WT or mutant–mutant γ-secretase interactions, with WT–mutant interactions as the control. A similar pull-down efficiency is observed in all combinations.](image)

![Fig. 4. The interactions among γ-secretase molecules are strictly dependent on the choice of detergents and correlate with the proteolytic activity. (A) WT γ-secretase exhibits robust proteolytic activity in the reaction buffer containing CHAPSO, but not digitonin or amphipol A8-35. WT γ-secretase was purified in three different detergents: CHAPSO, digitonin, and amphipol A8-35. Then the proteolytic activity assays were performed in three different buffers. Regardless of the original detergent used in purification, WT γ-secretase is highly active, as long as the reaction buffer contains the detergent CHAPSO. Each experiment was repeated three times, with the SD shown. (B) γ-secretase containing catalytic mutations and WT γ-secretase cannot pull down each other in digitonin buffer. (C) γ-secretase containing catalytic mutations and WT γ-secretase in amphipol A8-35 cannot pull down each other.](image)
The physical basis remains to be elucidated. Notably, under our experi-
mantle conditions, only a very small fraction of the Flag-tagged
γ-secretase forms an oligomer, the nature of the
γ-secretase variants in vitro. Under
an equimolar ratio of 1:1 between WT and mutant
γ-secretases, should be carefully examined under in vivo cir-
cumstances, especially in patients’ brains. In our study, the pro-
duction of Ap40 and Ap42 is used as an exclusive indicator of the
proteolytic activity. It remains to be investigated whether such a
mechanism applies to other Aβ species such as Ap43, which is
thought to play a crucial role in amyloidogenesis and AD path-
ology (42, 43). Nevertheless, the dominant negative effect indi-
cated by production of Ap40 and Ap42 had been previously
suggested for the production of the intracellular domain of APP
(16). Cleavage of other type I substrates by γ-secretase may also
follow the same mechanism (16).

Materials and Methods

Protein Purification. γ-secretase for enzymatic assay was expressed and pu-
rified as described (33). For pull-down assay, the proteins were purified
through strep tag on PEN2 by Strep-Tactin Superflow resin (IBA). Either flag
tag or HA tag was fused to the N terminus of PS1. In the case of analytical
ultracentrifugation, the detergents used for γ-secretase were exchanged via
gel filtration in Superose-6 column (GE Healthcare).

Activity Assays. A different amount of purified WT or mutant γ-secretase was
mixed with APP-C99 substrate in 0.5% CHAPSO, 25 mM Hepes at pH 7.0,
Cell-Based Activity Assays. **PSN1-1/PSN2-2 double-knockout** HeLa cell line was generated using CRISPR/Cas9 technology coupled with a CMV/PuroR reporter system, as reported (44). To determine a suitable amount of PS1 plasmid for transfection, a different amount of plasmid-containing PS1 and 500 ng plasmid-containing APP-C99 was cotransfected into 70% confluent HeLa cells in a 12-well plate, using Neofect, and 62.5 ng PS1 plasmid was finally used as total amount to transfect for each reaction. Transfected cells were cultured for 60 h before the cell medium was harvested. **2 μL** medium was detected by the AlphaLIISA assay, as described (18).

Pull-Down Assays. Around 1 μg differently tagged γ-secretase was pre-incubated at room temperature for about 15 min. The γ-secretase was then mixed and incubated with anti-Flag M2 or anti-HA affinity resin for 2 h at 4 °C. The resin was washed by using the corresponding buffer with ~30-fold volume to eliminate the nonspecifically bound protein. γ-secretase was eluted with Flag peptide or HA peptide and analyzed by Western blot.

Analytical Ultracentrifugation Analysis. The peak fractions of WT γ-secretase purified by pull-down were collected and concentrated to 1 mg/mL, as measured by OD280. The samples were applied to analytical ultracentrifugation analysis, using a Beckman XL-I analytical ultracentrifuge (Beckman Coulter) equipped with an eight-cell an-50 Ti rotor. When centrifuge γ-secretase with 35,000 rpm (89, 180 g; 98, 74 g from cell center to cell bottom) for 5 h at 20 °C, the ultraviolet (UV) absorbance was recorded to determine the distribution of protein complex. The data of sedimentation velocity was analyzed using Sedfit (45).

**Grafix of γ-secretase.** Next, 1 mg·mL⁻¹ γ-secretase purified by CHAPOS was prepared in low-salt buffer (50 mM Heps at pH 7.4, 50 mM NaCl, 0.5% CHAPOS), and 200 μL protein sample was added to a gradient consisting of 10-30% (vol/vol) glycerol and 0-0.05% glutaraldehyde in the low-salt buffer. Ultracentrifugation was performed for 20 h at 33,000 rpm(82, 274 g-186, 575 g along the gradient) in a SW41 Ti rotor (Beckman). The fractionated sample was quenched by 50 mM Tris at pH 7.4 and applied to negative stain preparation.

**Negative Staining Electron Microscopy.** Samples of WT or Grafixed γ-secretase (4 μL) were applied to glow-discharged continuous carbon-coated grids (Zhongjingkey Technology) and stained by 2% uranyl acetate. The grids were imaged on a FEI Tecnai F20 microscope at 200 kV. Approximately 140 images were collected for the Grafixed sample. About 7,000 particles were automatically picked to perform 2D classification with RELION (46).

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