Supporting Information

Mechanisms of γ-Secretase Activation and Substrate Processing

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1. Supporting Methods

Restoration of wildtype γ-secretase for molecular dynamics simulations

We performed initial GaMD simulations on the earlier published cryo-EM structure of γ-secretase bound by the Notch substrate (PDB: 6IDF[1]). Residue Ala385 at the active site was mutated back to aspartate, whereas the disulfide bond between the N-terminus of Notch substrate and hydrophobic loop 1 (HL1) loop was kept intact. In aspartyl proteases, proximity between the two active site Asp residues necessitates protonation of one of them, preventing charge repulsion. Testing GaMD simulations were performed to determine which of the two Asp residues was protonated in γ-secretase. We performed multiple 300 ns GaMD simulations **(Table 1)** on three different systems: the original cryo-EM structure, Asp257 protonated, and with Asp385 protonated. The distance time course plots **(Figure S2)** revealed that the system with protonated Asp257 in the N-terminal fragment (NTF) subunit of PS1 facilitated the activation of γ-secretase, as the two active-site aspartates approached each other to a distance of ~6-7 Å between the Cγ atoms. In contrast, simulations of the original cryo-EM structure did not show significant change from the starting Asp257:Cγ-Ala385:Cβ distance of ~10-11 Å. In the system with protonated Asp385 in the C-terminal fragment (CTF) of PS1, the two aspartates maintained a distance of ~10-11 Å between the Cγ atoms of the two aspartates. Therefore, Asp257 was protonated in subsequent GaMD simulations being similar to the setup of a previous computational study[2].

Next, we proceeded to simulate γ-secretase bound by APP (PDB: 6IYC[3]). Residue Ala385 was similarly mutated back to Asp385 in the wildtype γ-secretase, which was compared to the original cryo-EM system in 300 ns GaMD simulations **(Table 1)**. The disulfide bond between substrate and enzyme was still kept. Free energy calculations showed that the active-site residues Asp257 and Ala385 maintained ~10 Å distance between their sidechain terminal C atoms in the cryo-EM system even though water molecules were observed entering the active site **(Figure S3A)**. The substrate remained distant from the active site residues, with ~5-6 Å between the Cγ atom of protonated Asp257 and the carbonyl oxygen of Leu49 in APP. In contrast, activation of γ-secretase was observed during three independent 300 ns GaMD simulations of the computationally restored wildtype enzyme **(Figure S3B, Movie S1)**. The protonated Asp257 formed a hydrogen bond with the carbonyl oxygen in Leu49 of the scissile amide bond in APP. Water molecules entered the PS1 active site. One water molecule was trapped between the two catalytic Asp residues through stable hydrogen bonds. This would induce nucleophilic attack of the carbonyl carbon of Leu49 by the activated water molecule, which is a key step for substrate proteolysis. The enzyme active site was thus well poised for proteolysis of APP for the ε cleavage between residues Leu49 and Val50. The two aspartates were ~7 Å apart between their Cγ atoms **(Figure S3B)**. The distance between the carbonyl carbon of Leu49 and the water oxygen was ~3.8 Å. Therefore, our GaMD simulations successfully captured activation of the APP-bound γ-secretase in the presence of the enzyme-substrate disulfide bond. Next, the artificial disulfide bond between the N-terminus of APP substrate and the HL1 loop of PS1 was also removed by computationally restoring the wildtype residues for further simulations as summarized in **Table 1.**

2. Supporting Figures

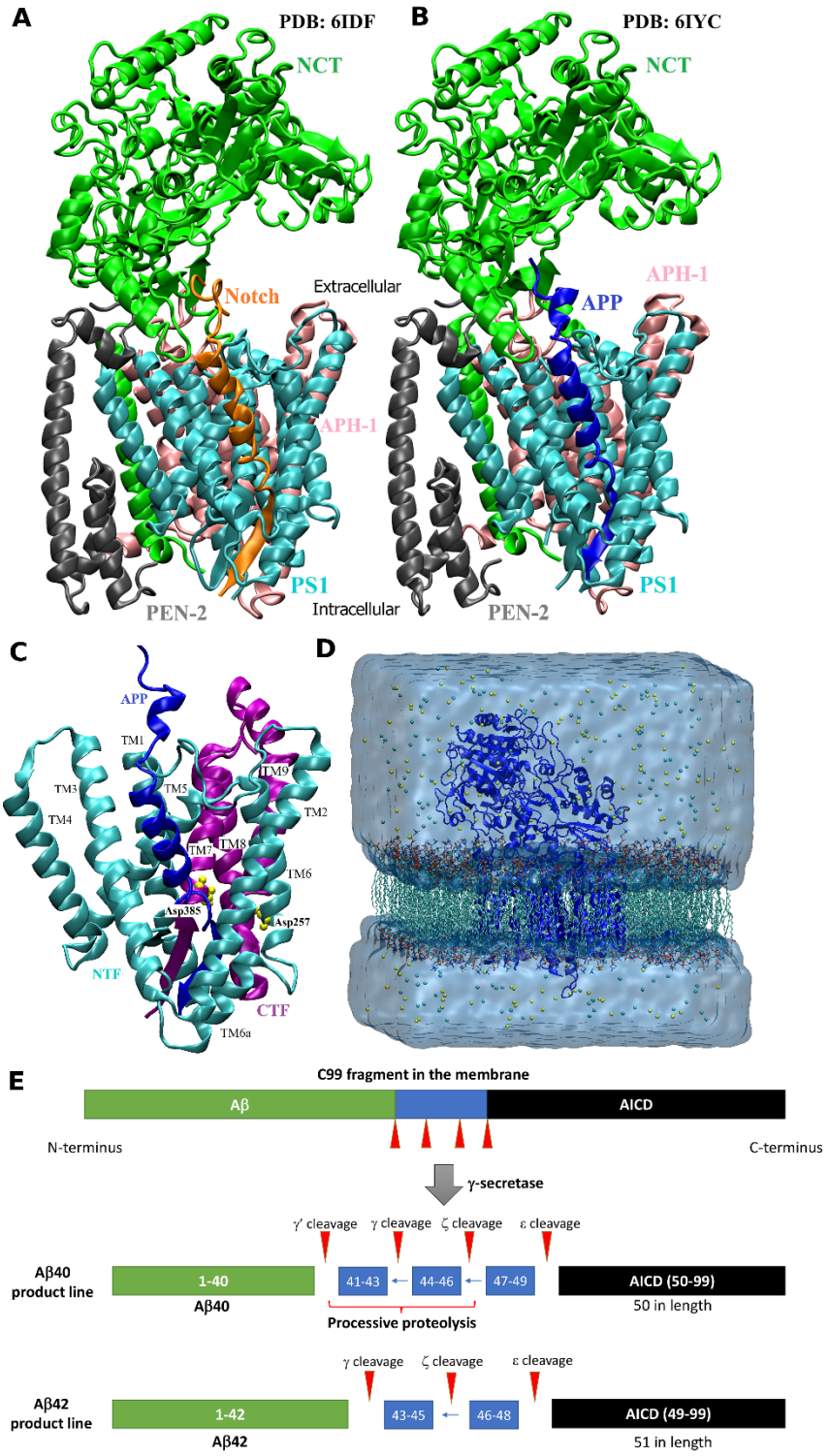

Figure S1: Ribbon representations of the (A) Notch- and (B) APP-bound γ-secretase complexes that include the presenilin (PS1), presenilin enhancer 2 (PEN2), anterior pharynx-defective 1

(APH1) and Nicastrin (NCT) subunits. (C) Representation of the catalytic PS1 domain of APP-bound γ-secretase. The transmembrane (TM) helices and active-site Asp385 and Asp257 residues are labelled. The N-terminal fragment (NTF) is colored in cyan and C-terminal fragment (CTF) is colored in purple (D) Computational model of γ-secretase complex in GaMD simulations. The protein was embedded into a POPC lipid bilayer and solvated in an aqueous medium of 0.15 M NaCl. (E) Schematic representation of APP substrate processing by γ-secretase.

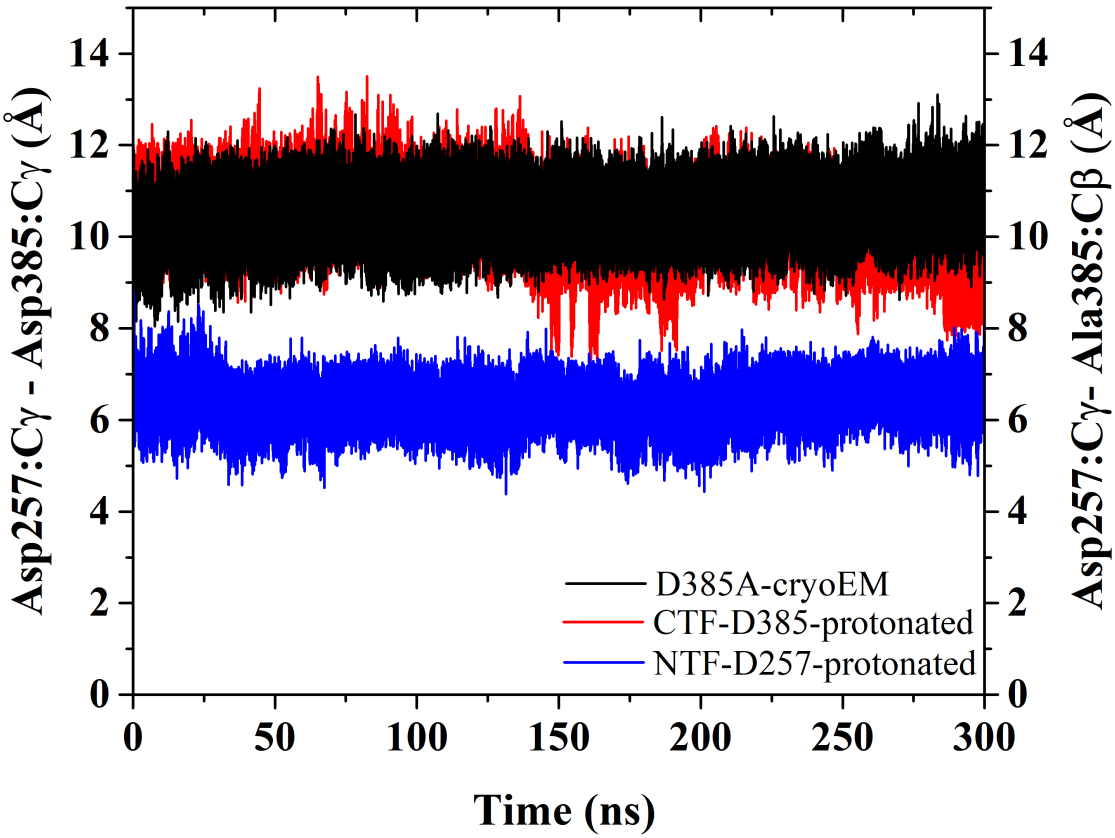

Figure S2: Time course of the Asp257:Cγ - Ala385:Cβ distance calculated from GaMD simulations of the cryo-EM D385A system (black) and Asp257:Cγ - Asp385:Cγ distance calculated from GaMD simulations of D385-protonated (red) and D257-protonated (blue) systems of Notch-bound γ-secretase complex. The disulfide bond between the N-terminus of Notch and PS1 HL1 loop was kept in these simulations.

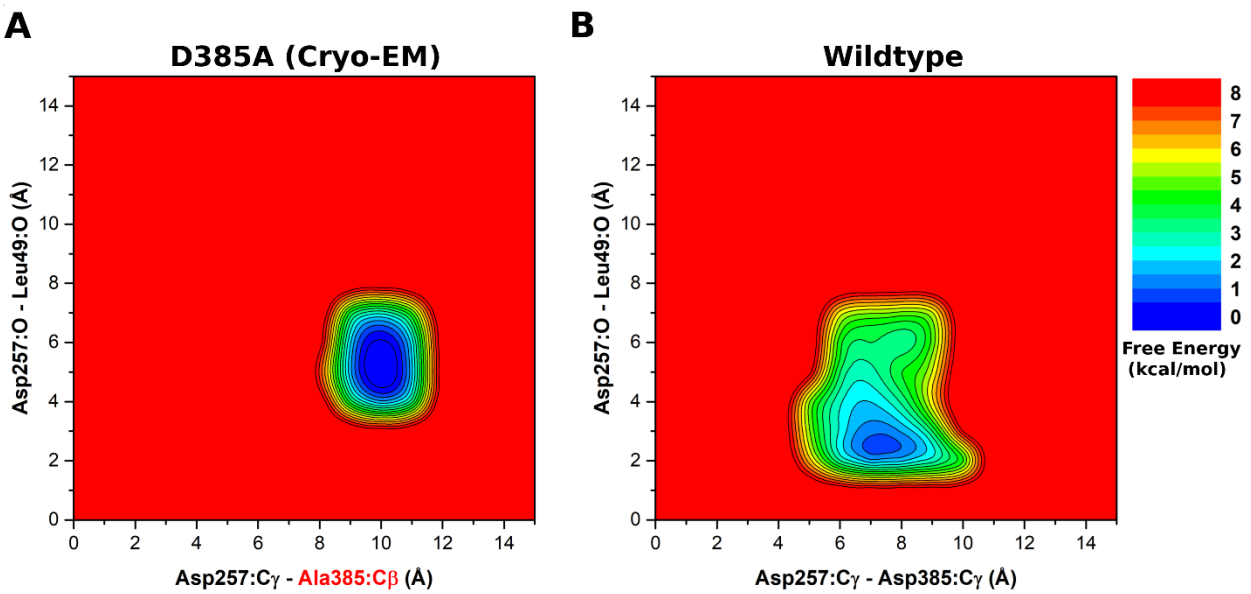

Figure S3: (A) 2D free energy profile of the Asp257:Cγ - Ala385:Cβ and Asp257:sidechain O - Leu49:O distances calculated from GaMD simulations of the D385A cryo-EM structure of γ-secretase bound by APP. (B) 2D free energy profile of the Asp257:Cγ - Asp385:Cγ and Asp257: protonated O - Leu49: O distances calculated from GaMD simulations of the wildtype γ-secretase. The disulfide bond between the N-terminus of APP and PS1 HL1 loop was kept in these simulations.

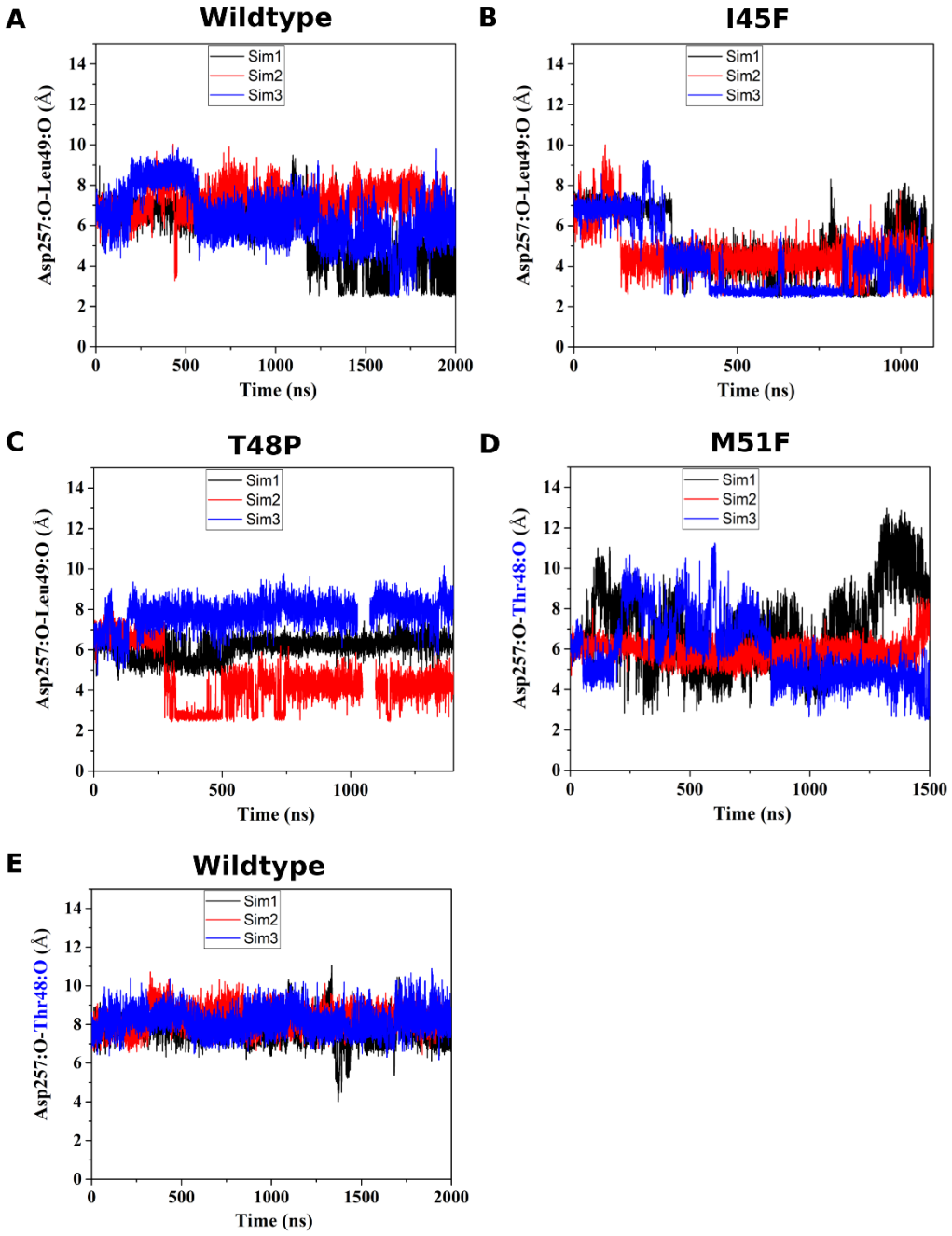

Figure S4: Time courses of the Asp257:protonated O - Leu49:O distance calculated from GaMD simulations of (A) wildtype, (B) I45F, and (C) T48P APP bound γ-secretase, and the Asp257:protonated O – Thr48:O distance calculated from GaMD simulations of the (D) M51F and (E) wildtype APP bound γ-secretase.

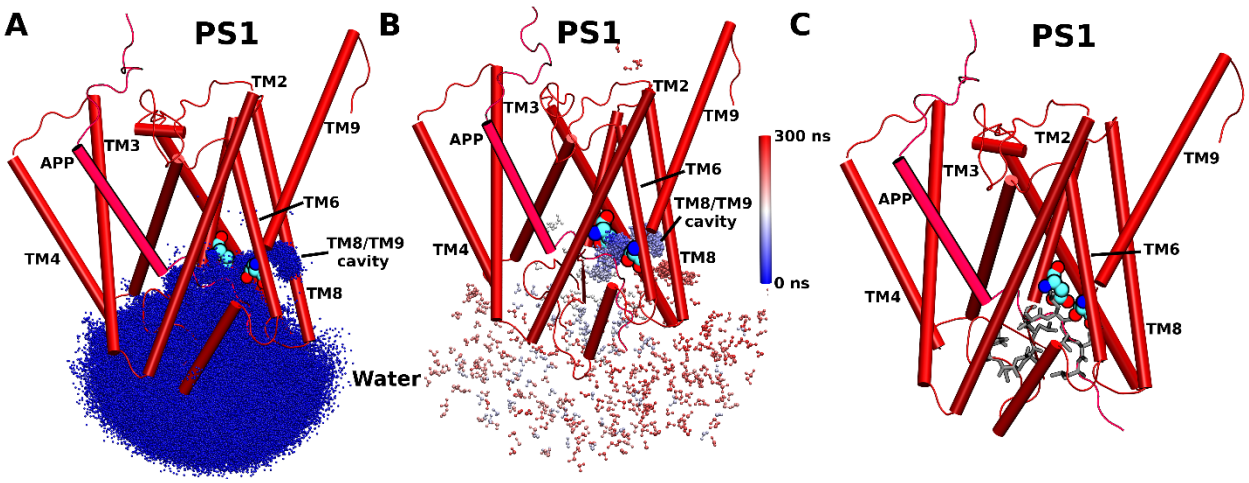

Figure S5: (A) Traces of water molecules (blue) observed surrounding the active site during GaMD simulations of γ-secretase (red). Water molecules entered the enzyme active site through an open channel on intracellular side. The active site is highlighted with the catalytic aspartates shown in spheres. Another water cavity adjacent to the active site was located between the TM8 and TM9 in PS1 near the protein surface. (B) Pathway of the water molecule that made its way out of the active site, visited the adjacent TM8/TM9 cavity and then exited into bulk water through the intracellular water channel. The water molecule is shown in balls-and-sticks and colored according to the simulation time. (C) Protein residues (gray sticks) that comprise of the intracellular water channel. These residues are also listed in **Table S2**.

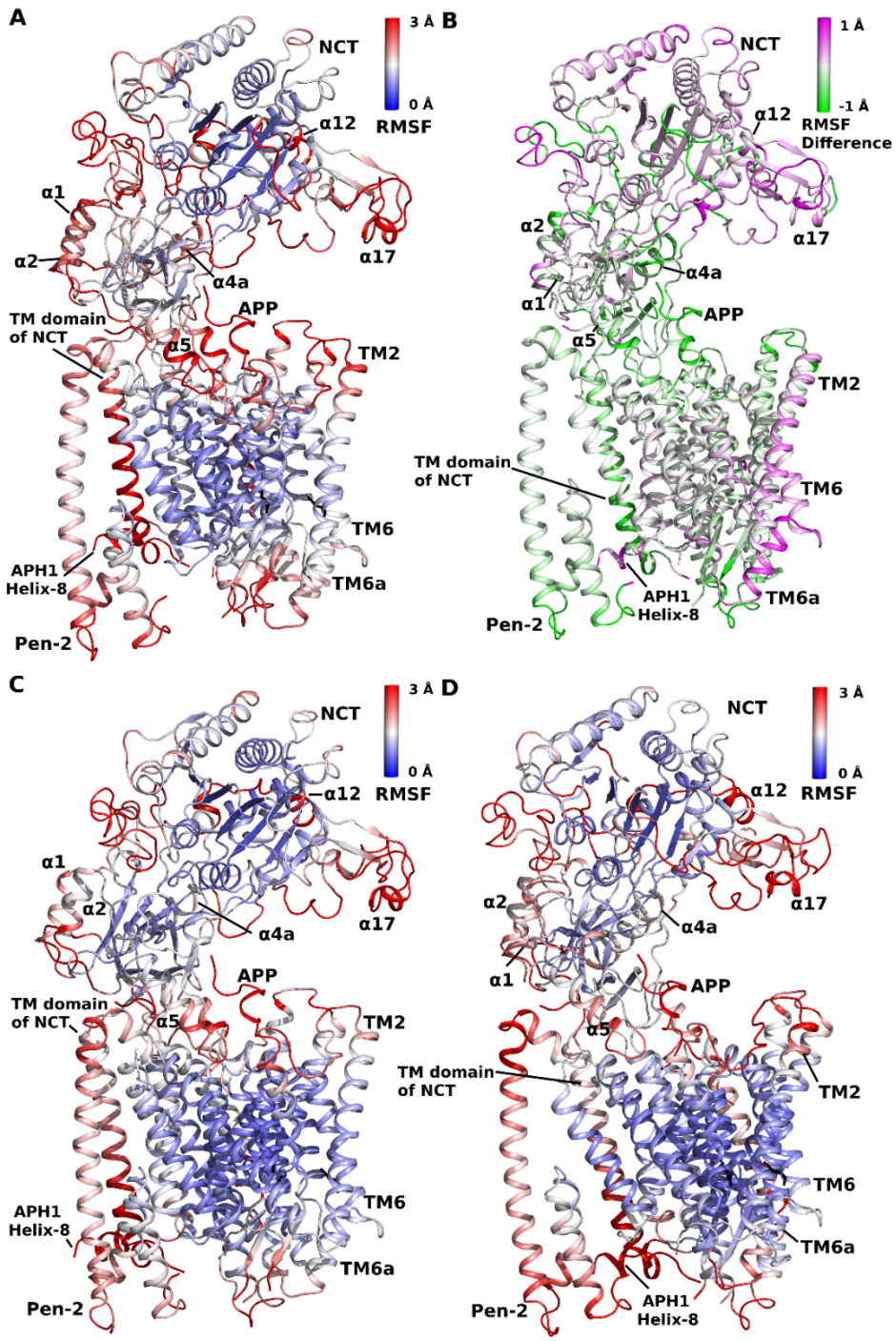

Figure S6: Comparison of structural flexibility of wildtype and mutant APP-bound γ-secretase calculated from GaMD simulations. (A) Root-mean-square fluctuations (RMSFs) of the wildtype APP-bound γ-secretase, (B) RMSF difference between the M51F and wildtype APP bound γ-secretase, (C) RMSFs of the I45F APP bound γ-secretase and (D) RMSFs of the T48P mutant APP-bound γ-secretase.

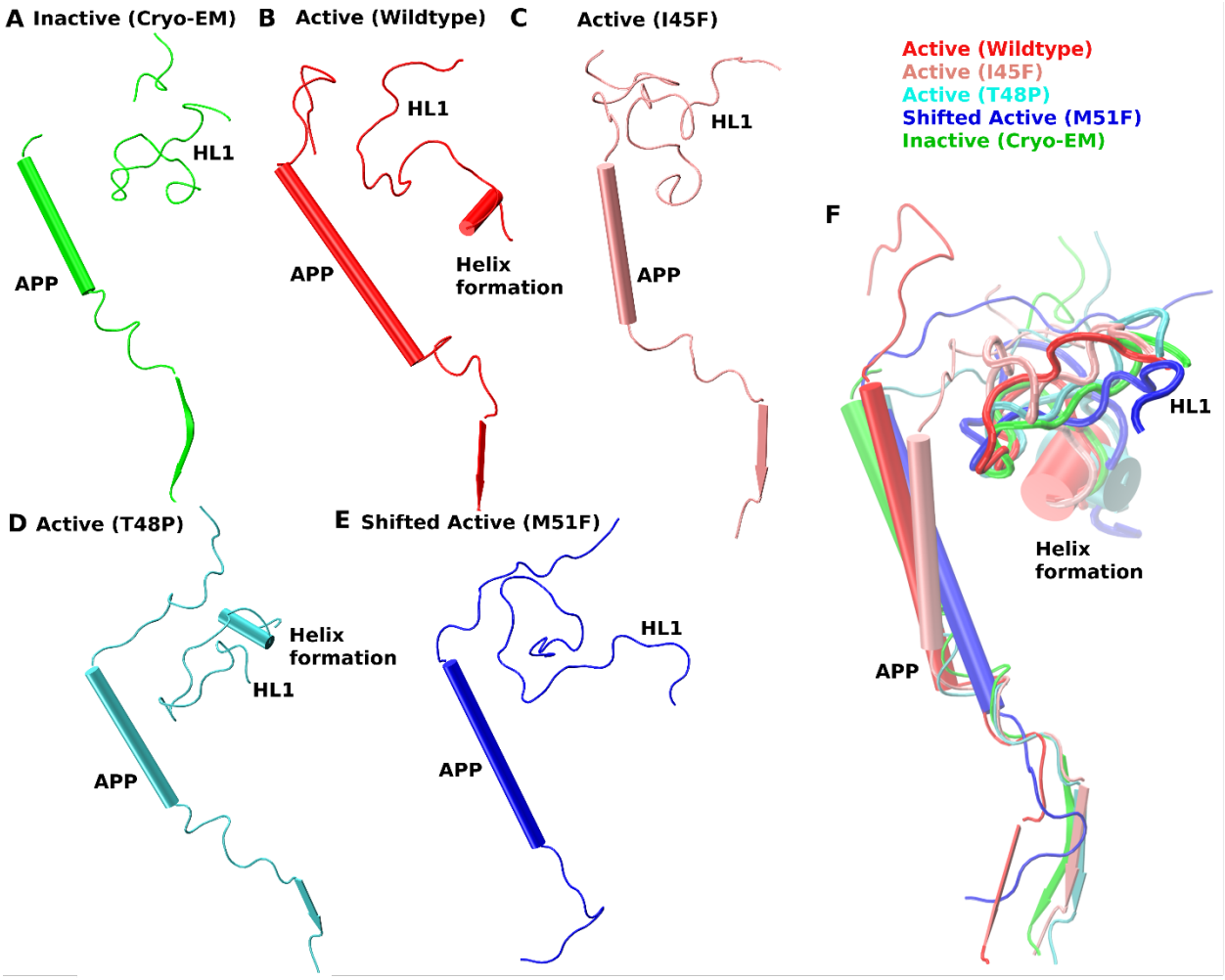

Figure S7: Side view of APP substrate and PS1 HL1 loop in the (A) inactive, representative active conformations of the (B) wildtype, (C) I45F and (D) T48P mutant APP-bound γ-secretase and the (E) Shifted conformation of M51F mutant APP-bound γ-secretase complex obtained from the GaMD simulations. (F) Comparison of APP substrate and PS1 HL1 loop in the different systems of APP-bound γ-secretase complex.

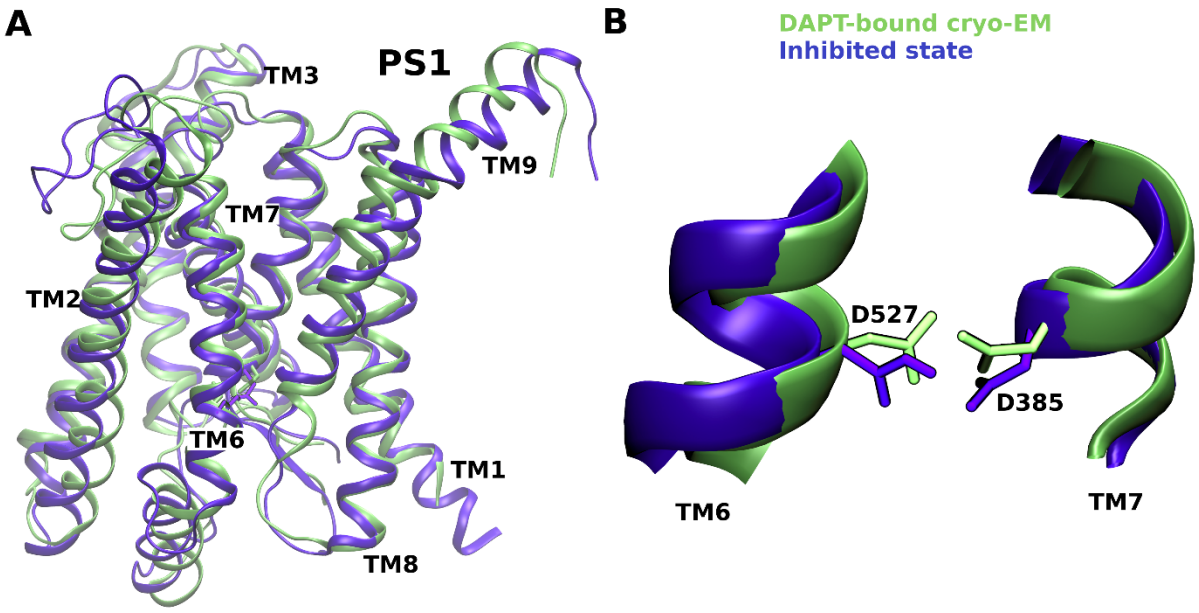

Figure S8: Comparison of the DAPT-bound cryo-EM structure (PDB:5FN2) of γ-secretase (lime green) and the Inhibited low-energy conformational state observed in the GaMD simulation of γ-secretase bound by the wildtype APP (violet). Superimposed structures are shown for (A) the PS1 and (B) active site. The Cγ atom distance between the catalytic aspartates in the DAPT-bound structure is 3.89 Å, whereas this distance in the simulation predicted Inhibited state is closely similar as 3.88 Å.

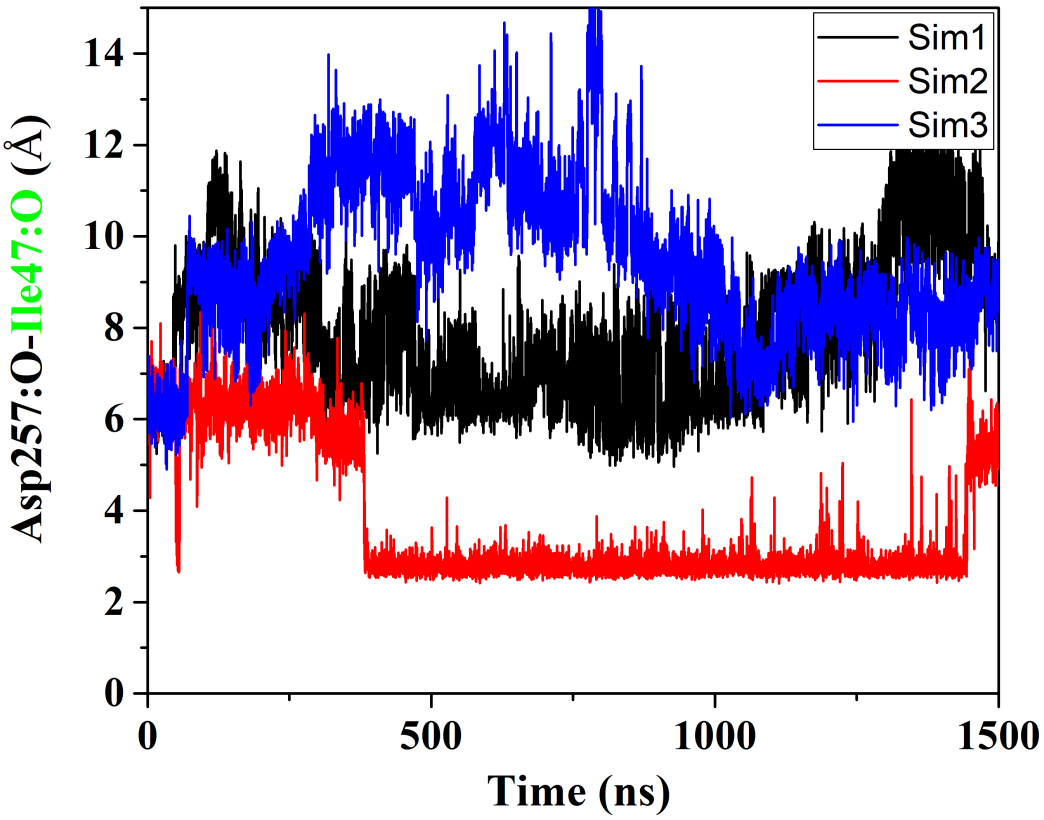

Figure S9: Time course of the Asp257:protonated O - Ile47:O distance calculated from GaMD simulations of the M51F APP bound γ-secretase.

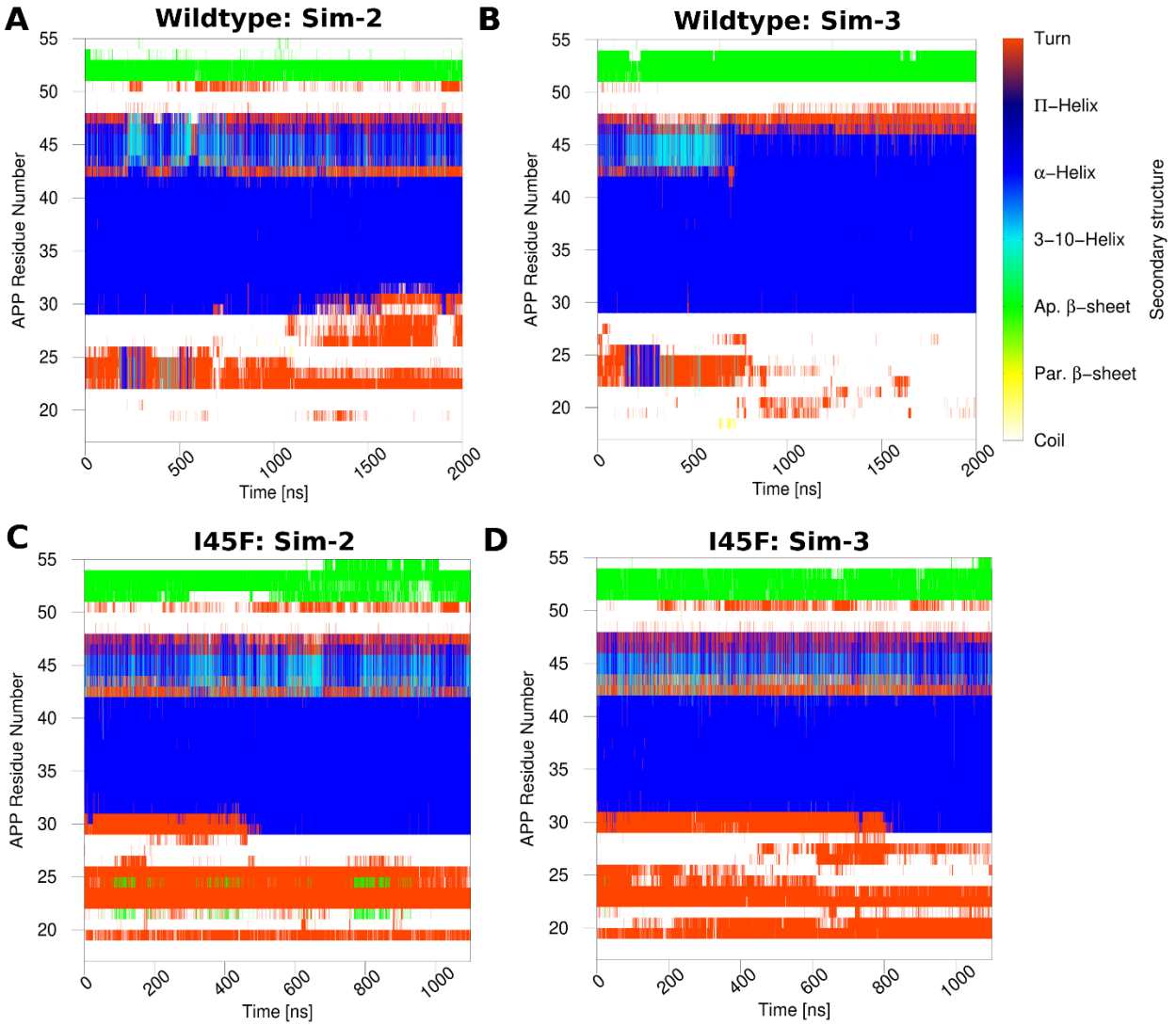

Figure S10: Time courses of the APP secondary structures in the wildtype and I45F forms as bound to γ-secretase calculated from GaMD simulations: (A) Sim-2 and (B) and Sim-3 for the wildtype (Sim-1 in Figure 4A), and (C) Sim-2 and (D) Sim-3 for the I45F mutant (Sim-1 in Figure 4B).

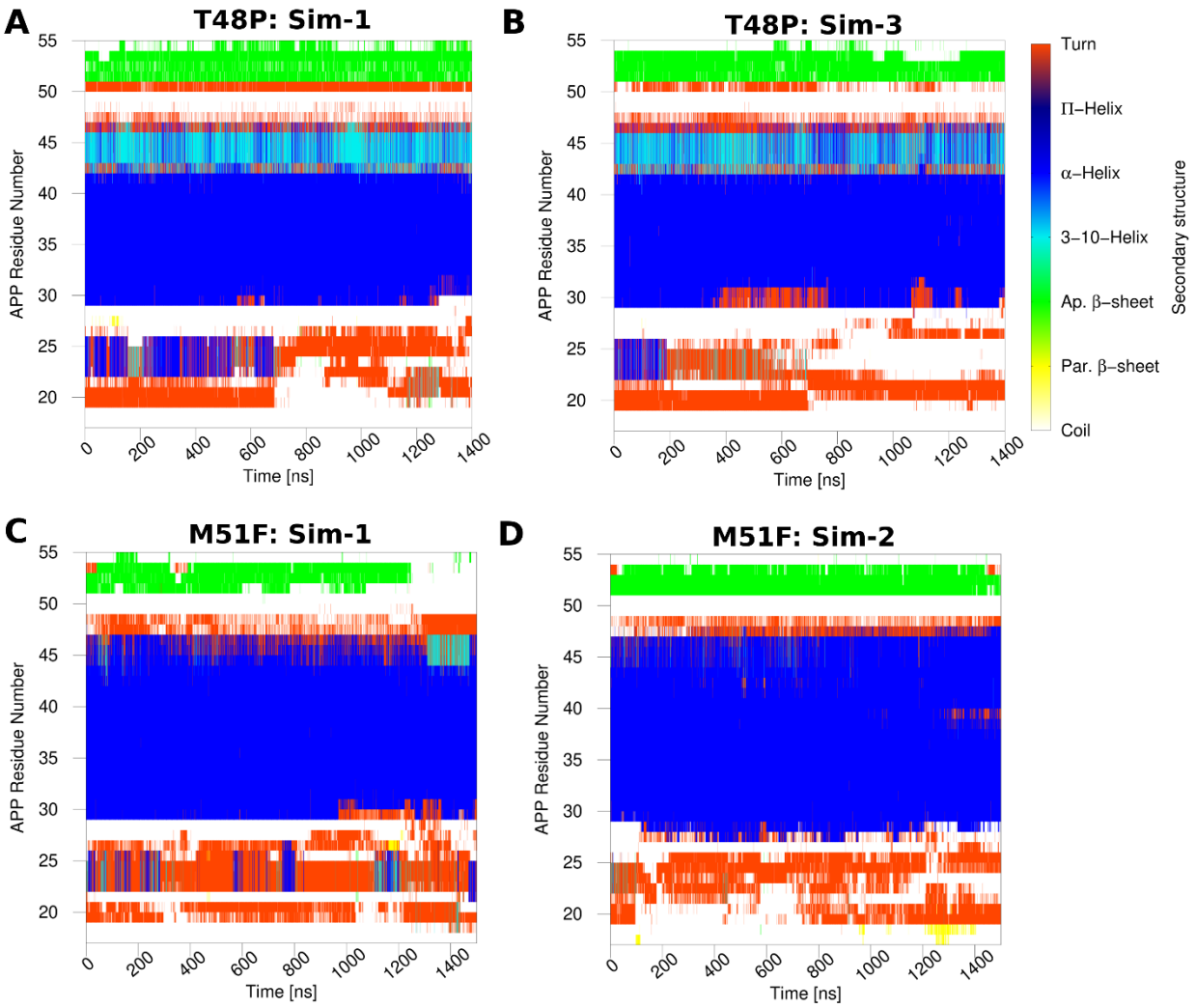

Figure S11: Time courses of the APP secondary structures in the T48P and M51F forms as bound to γ-secretase calculated from GaMD simulations: (A) Sim-1 and (B) and Sim-3 for the T48P mutant (Sim-2 in Figure 4C), and (C) Sim-1 and (D) Sim-2 for the M51F mutant (Sim-3 in 4D).

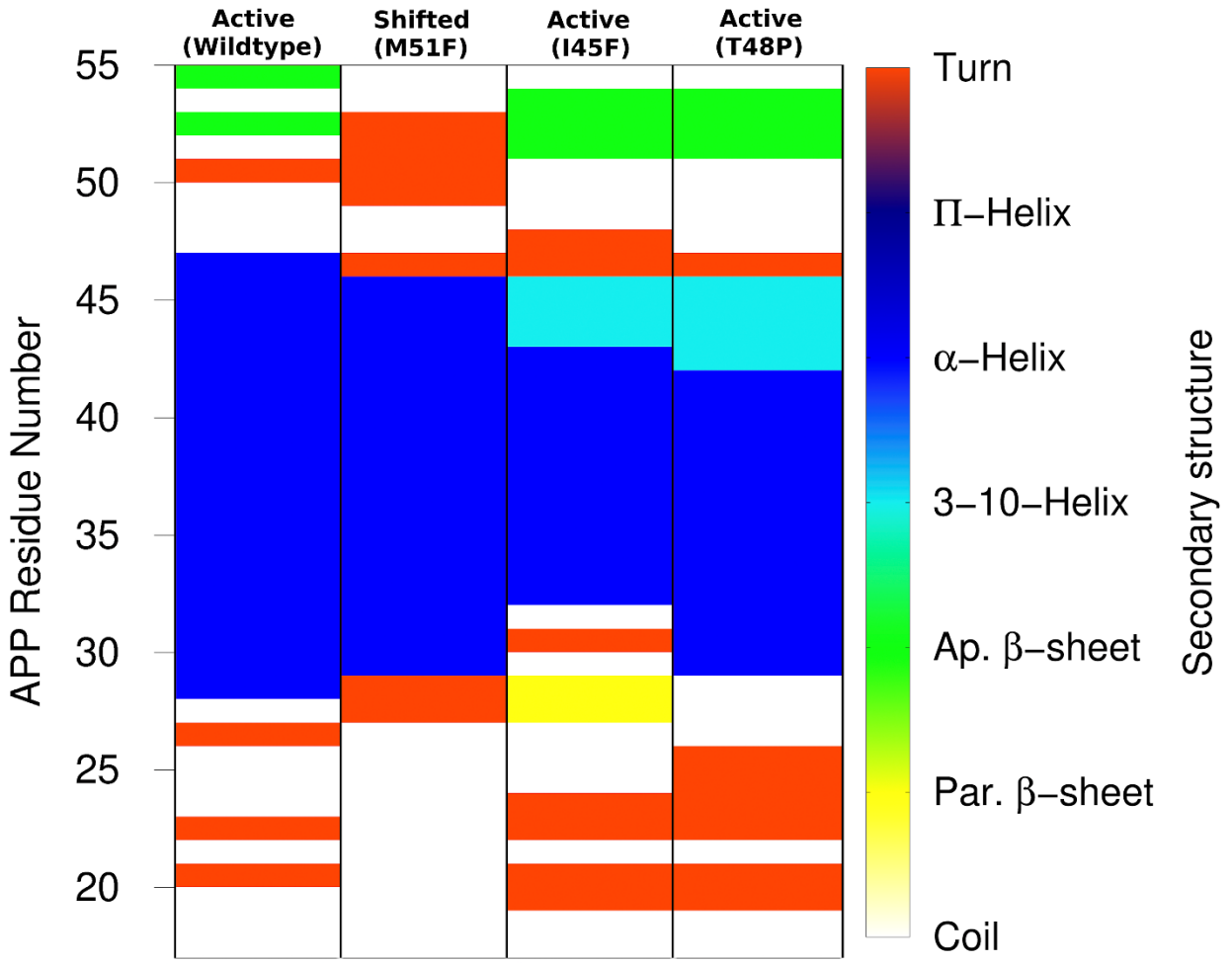

Figure S12: Secondary structures of the APP substrate in the representative active conformations of the wildtype, I45F and T48P mutant APP-bound γ-secretase and the shifted active conformation of M51F mutant APP-bound γ-secretase using the top ranked PS1-APP structural clusters obtained from the corresponding GaMD simulations.

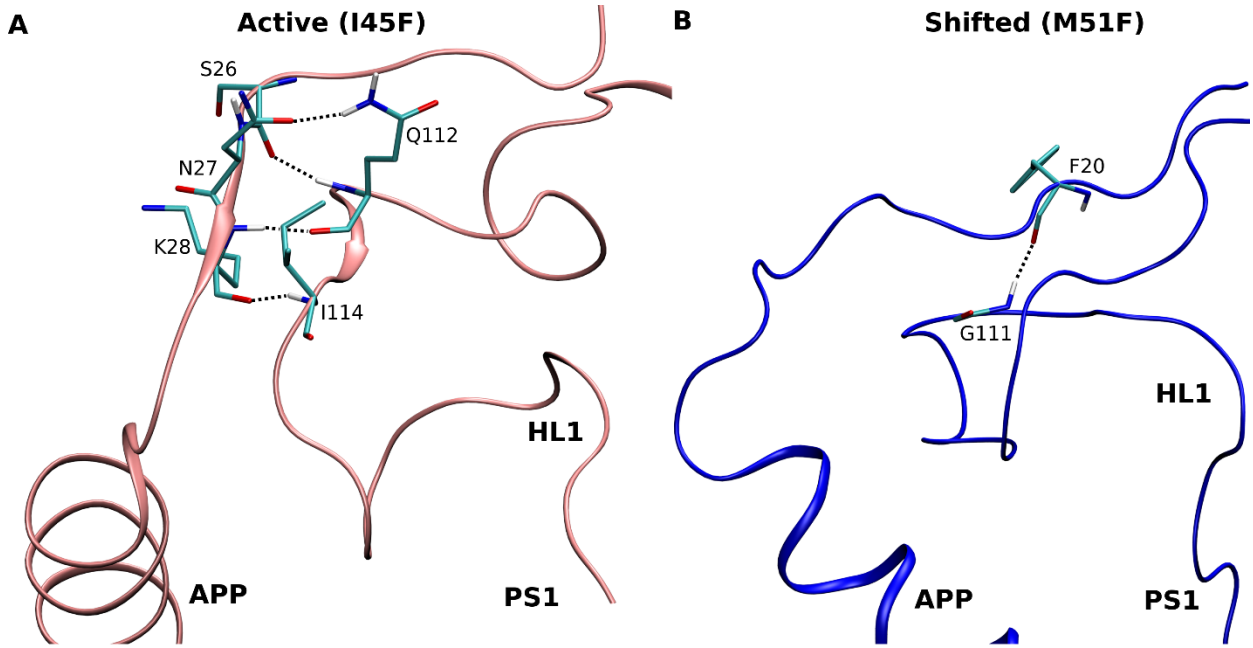

Figure S13: Hydrogen bonds formed between residues G111, Q112 and I114 of the PS1 HL1 loop and the N-terminus of APP substrate in the (A) Active and (B) Shifted conformations of γ-secretase obtained from the GaMD simulations of the I45F and M51F mutant APP systems.

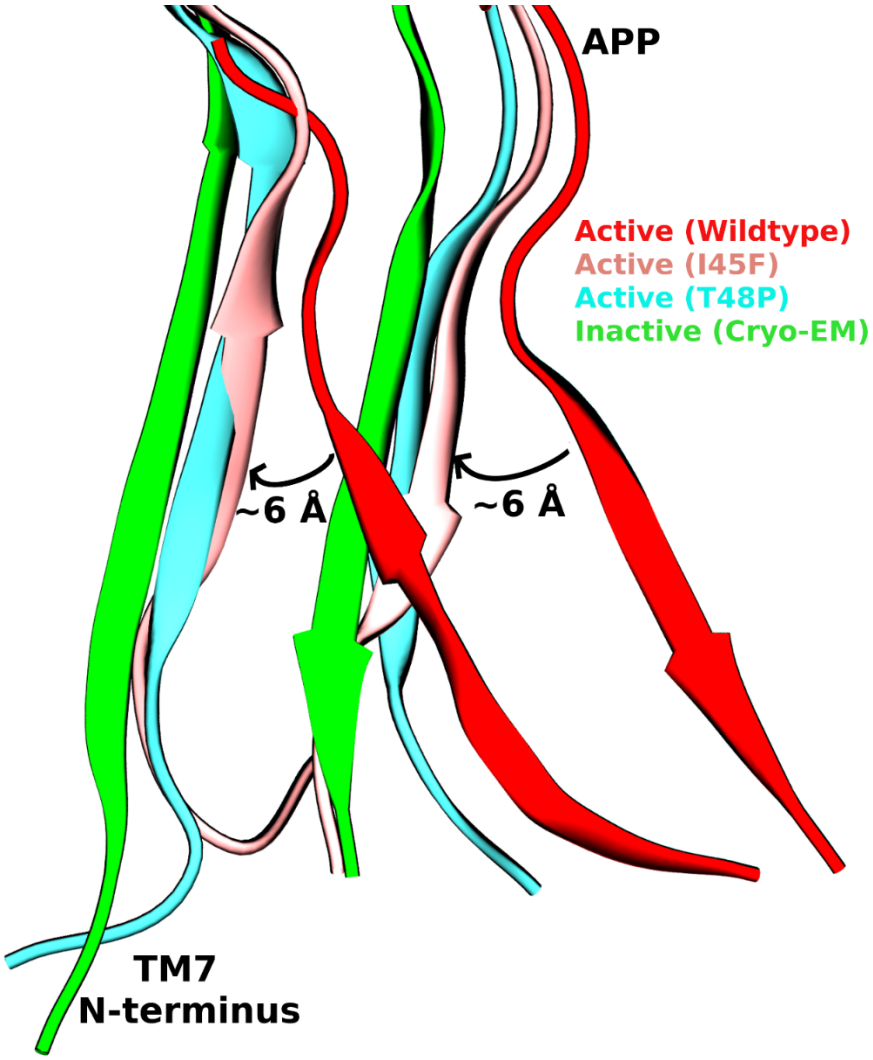

Figure S14: Comparison of the β-sheet conformational changes in the N-terminus of PS1 TM7 and the C-terminus of APP substrate in the inactive (cryo-EM) and active conformations of the wildtype, I45F and T48P mutant APP-bound γ-secretase observed in the GaMD simulations. Relative to the wildtype active conformation, the N-terminus of PS1 TM7 and the C-terminus of APP substrate moved away from the PS1 TM6a by ~6 Å to maintain the β-sheet structure in the I45F and T48P active conformations.

3. Supporting Tables

Table S1: List of amino acid residues constituting the water channel through which the water molecules intracellularly enter the active site of the enzyme. The table also consists of list of amino acid residues constituting the TM8/TM9 water cavity which is adjacent to the active site and visited by water molecules.

S.N.	Channel visited by water	Residues comprising the channel
1	**Intracellular open channel**	L282 F283 I287 K380 L381 G382 T48 (APP) L49 (APP) V50 (APP) M51 (APP) L52 (APP)
2	**PS1 TM8/TM9 cavity**	D385 F386 Y389 G417 L418 T421 L432 P433 A434 L435 P436 I437 S438

Table S2: List of amino acid residues constituting the S1', S2' and S3' subpockets in the representative wildtype active, I45F active, T48P active and M51F shifted active conformations of γ-secretase obtained from the corresponding GaMD simulations. The residues that are within 5 Å of APP substrate residues P1', P2' and P3' are listed in the table.

System	S1'	S2'	S3'
Active (Wildtype)	V261 L268 R269 L271 V272 L381 G382 P433	V272 I287 V379 K380 L381 G382 L425 K430 A431 L432	Y154 L271 V272 A275 T281 L282 F283 I287 G378 V379 K380 L381 G382
Shifted (M51F)	I143 T147 L150 Y256 D257 A260 V261 L271 V272 A275 E276 P433	D257 V261 R269 V272 E273 Q276 E277	V261 V379 K380 L381 Y389 L421 T422 L425 A431 L432 P433 A434 L435
Active (I45F/T48P)	V261 V272 F283 I287 K380 L381 G382 D385 A431 L432 P433 A434 L435	V82 L85 V261 V379 K380 L381 G382 D385 L418 T421 L422 L425 A431 L432 P433 A434 L435 P436	V272 A275 L282 I287 V379 K380 L381 L425 K430 A431 L432

4. References

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(2) Hitzenberger, M.; Zacharias, M. gamma-Secretase Studied by Atomistic Molecular Dynamics Simulations: Global Dynamics, Enzyme Activation, Water Distribution and Lipid Binding. *Front Chem* **2019,** *6*.

(3) Zhou, R.; Yang, G.; Guo, X.; Zhou, Q.; Lei, J.; Shi, Y. Recognition of the amyloid precursor protein by human gamma-secretase. *Science (New York, N.Y.)* **2019,** *363* (6428), eaaw0930