Expanded View Figures

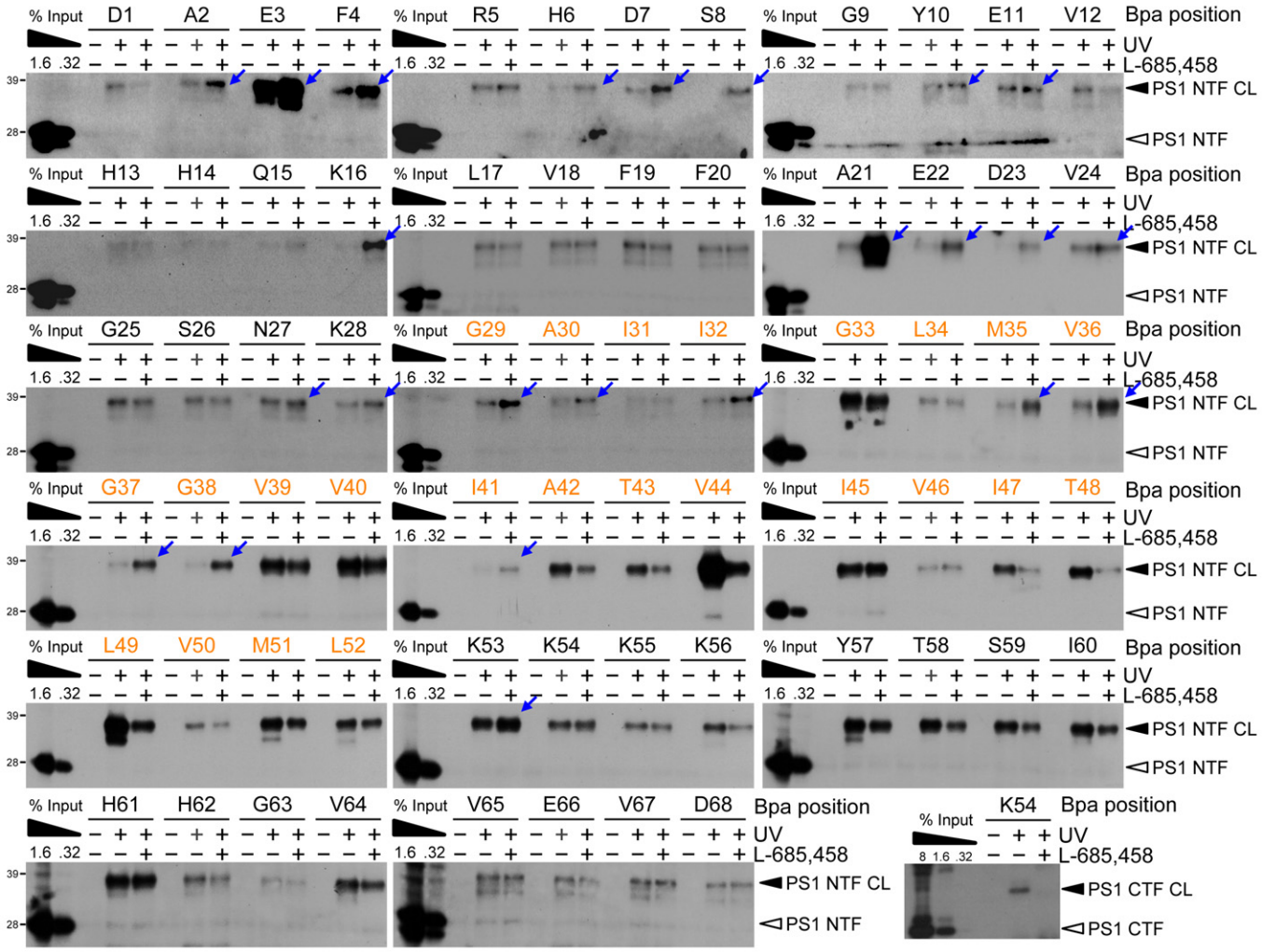

Figure EV1. Additional pharmacological validation of substrate crosslinking to the PS1 NTF and CTF.
Functional relevance of interactions with the PS1 NTF and CTF was validated by the modulation of substrate crosslinking in the presence of L-685,458. For the PS1 NTF, with only a few exceptions, L-685,458 increased the crosslinking efficiency of substrates carrying the Bpa substitutions in the extracellular domain supporting the presence of exosites in the PS1 NTF. Decreased binding to the PS1 NTF of some residues in this region may indicate minor local steric changes caused by L-685,458. For the PS1 CTF, M51 and L52 as well as residues for which crosslinking could not be detected even in the longest exposures on the films are not shown. TMD residues are highlighted in orange. Blue arrows indicate the residues for which increased labeling was observed.

Figure EV2. Additional pharmacological validation of substrate crosslinking to NCT and PEN-2.

A Validation of functional relevance of C99-Bpa interactions with NCT by the modulation of substrate crosslinking in the presence of L-685,458. For a number of substrates binding to mature NCT was increased in the presence of L-685,458, suggesting that the exclusion of substrate from the active site by the GSI caused substrate trapping at exosites in NCT. Residues for which crosslinking could not be detected even in the longest exposures on the films are not shown. Note that the crosslinking to immature NCT that was observed for all substrate residues did not change in the presence of L-685,458, suggesting functionally irrelevant binding. See also (B). Green arrows indicate the residues for which increased labeling was observed.

B Deglycosylation by endoH revealed that the crosslink of H6 to NCT originated from binding to complex glycosylated mature NCT, while that of D1 from binding to immature NCT. Closed and open circles indicate partial endoH resistance of mature NCT (red) and endoH sensitivity of immature NCT (black) for the crosslinked samples or the uncrosslinked input control, respectively.

C Validation of functional relevance of C99-Bpa interactions with PEN-2 by the modulation of substrate crosslinking in the presence of L-685,458. Increased substrate crosslinking in the presence of the active site-targeting GSI is consistent with the presence of exosites in PEN-2 and supports its role in substrate recruitment. Residues for which crosslinking could not be detected even in the longest exposures on the films are not shown. TMD residues are highlighted in orange. Yellow arrows indicate the residues for which increased labeling was observed.

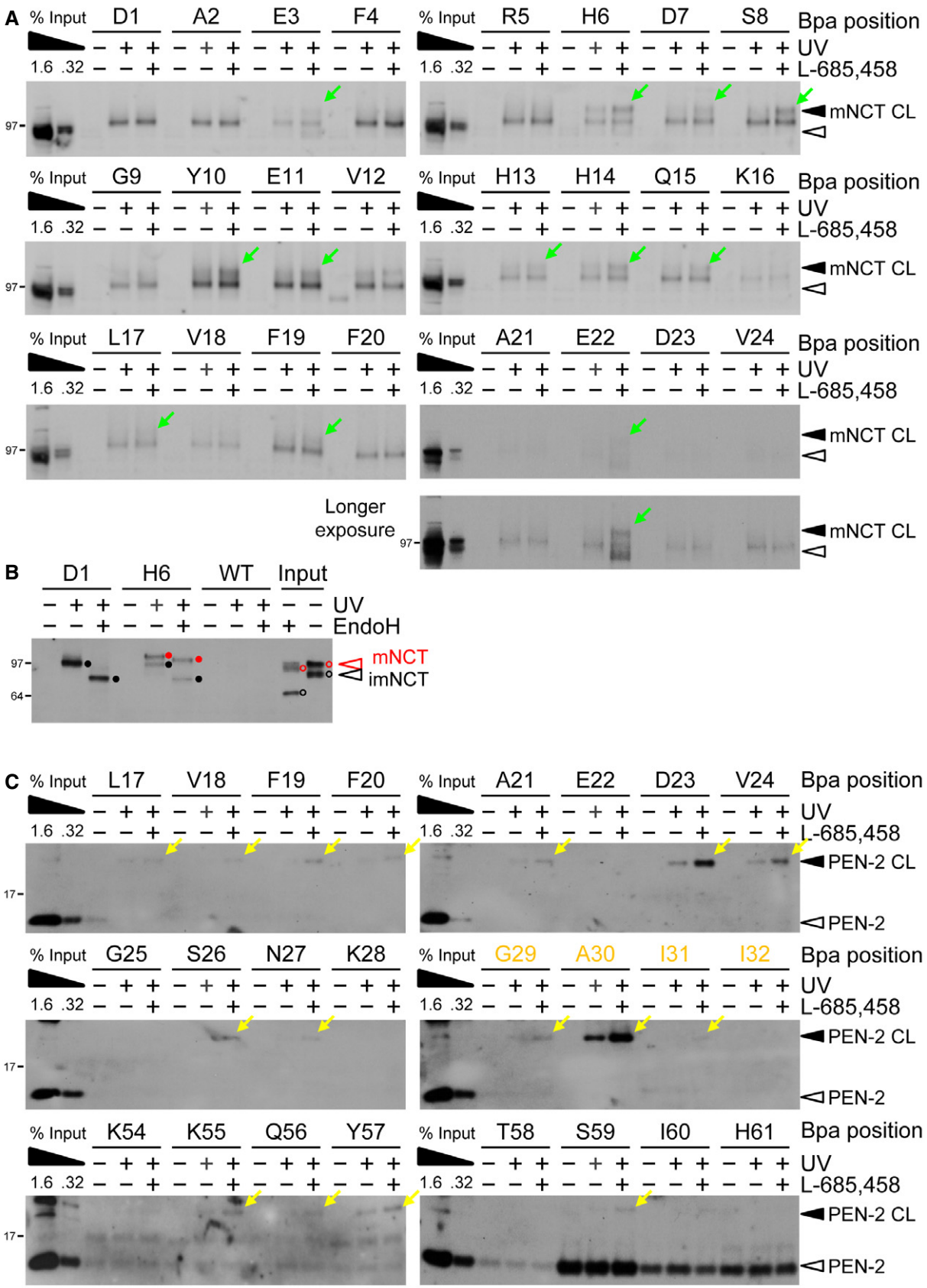

Figure EV2.

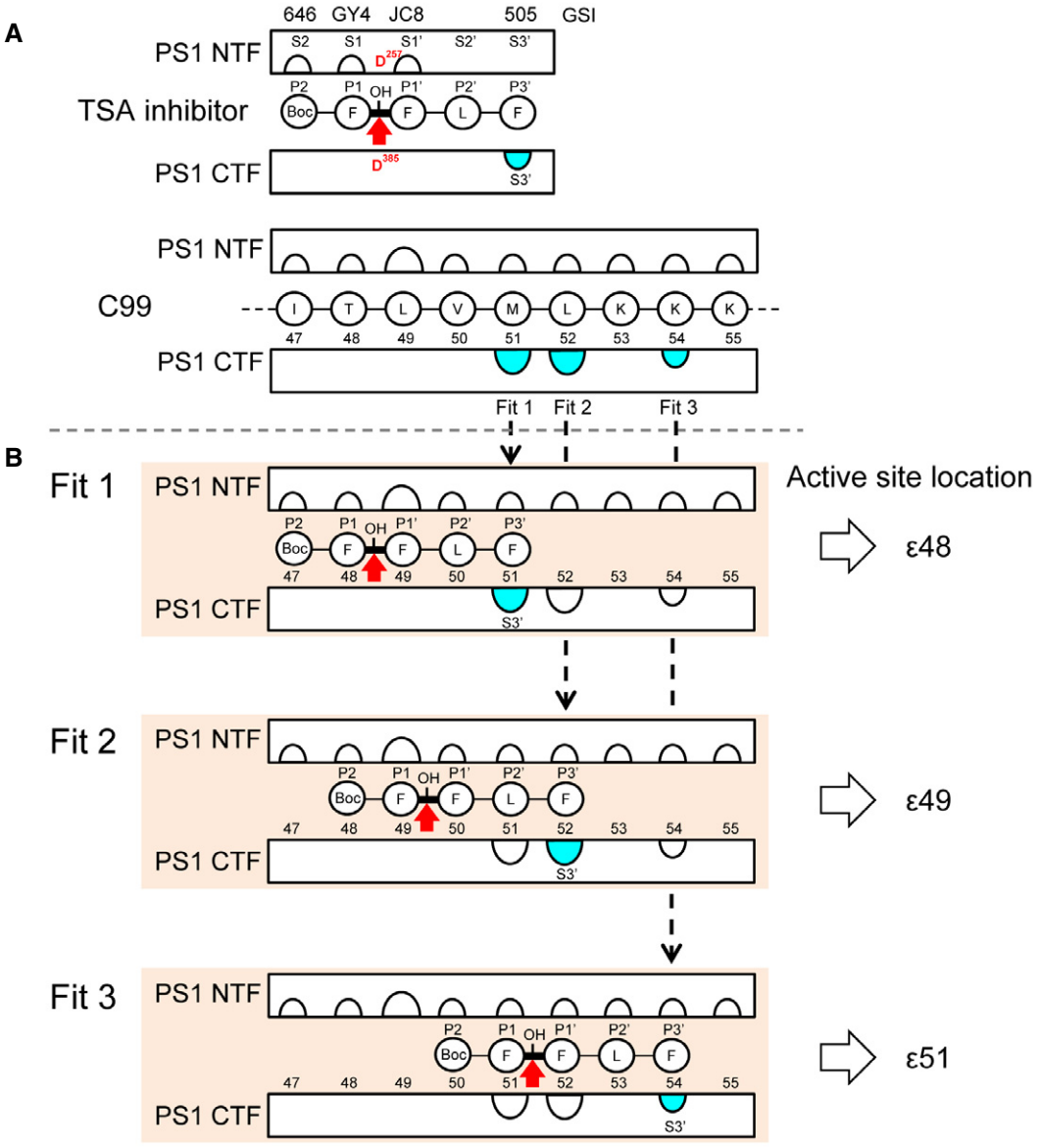

Figure EV3. Graphic representation of C99 interactions with subsites in the γ-secretase active site.

A Comparison of the C99 interaction sites with the known interaction sites of transition state analog GSIs (Li *et al*, 2000b; Shelton *et al*, 2009) suggests that the binding site of these GSIs locates at the ε-cleavage sites of C99. Using differently placed photoreactive benzophenone moieties in the FFLF peptide-based L-685,458 GSI derivatives L-852,646 (646), GY4, JC8, and L-852,505 (505), the S2-S1′ pockets were previously located to the PS1 NTF (Li *et al*, 2000b; Shelton *et al*, 2009). In addition, the S3′ pocket could almost exclusively be located to the PS1 CTF by GSI 505 (Li *et al*, 2000b; Shelton *et al*, 2009). Preferential location of the S3′ pocket to the PS1 CTF was also observed for the related GSI III-63 (Kornilova *et al*, 2005). As determined here, the only sites of C99 that crosslinked to the PS1 CTF were residues M51, L52, and K54.

B Combining the information given in (A), we fitted the S3′ pocket in the PS1 CTF determined by GSI 505 with each of these residues (fit 1, 2, and 3). By this analysis, the hydroxyl group of GSI 505 and thus the γ-secretase active site were found to locate at the ε48, the major ε49, and the very minor ε51 site, respectively.

Data information: Subsite pockets in the PS1 CTF are highlighted in light blue according to the subunit color code used. Red arrow indicates the location of the γ-secretase active site.

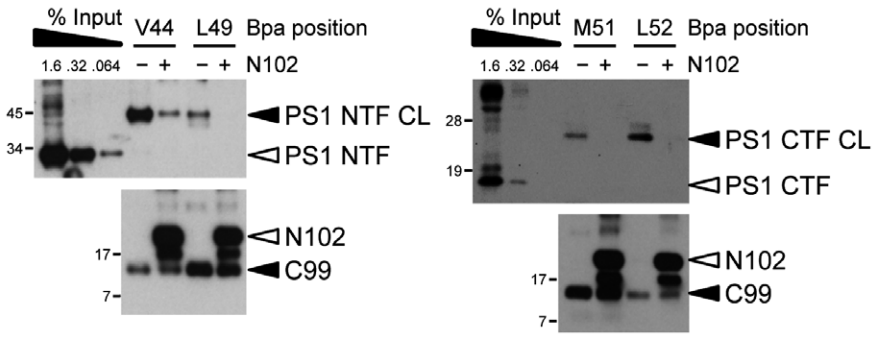

Figure EV4. Competition of C99 binding to the active site region of γ-secretase by Notch1.
Excess amounts of Notch1-based N102-FmH substrate compete with C99-Bpa substrates crosslinking to γ-secretase in the active site region.

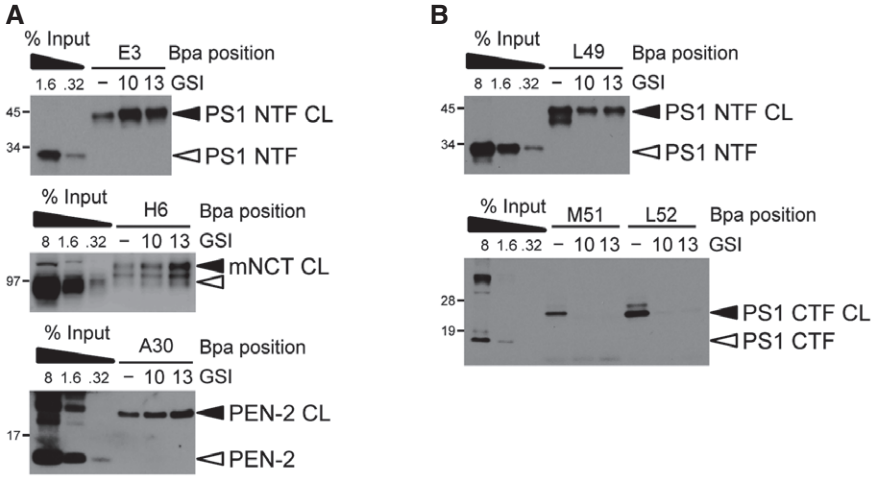

Figure EV5. Helical peptide inhibitors target a substrate-binding site different from the exosites.

A Helical peptide GSIs D-10 and D-13 mimicking TMD segments V36-I45 (D-10) and V36-T48 (D-13) of APP and targeting a putative exosite termed docking site (D-10) or both docking site and active site (D-13) (Kornilova *et al*, 2005) do not compete for substrate binding at exosites, suggesting that docking site and exosites are distinct. 10, D-10; 13, D-13.

B Inhibition of substrate binding in the active site by D-10 and D-13 suggests that these GSIs interfere with substrate binding before or at the active site.