APPENDIX

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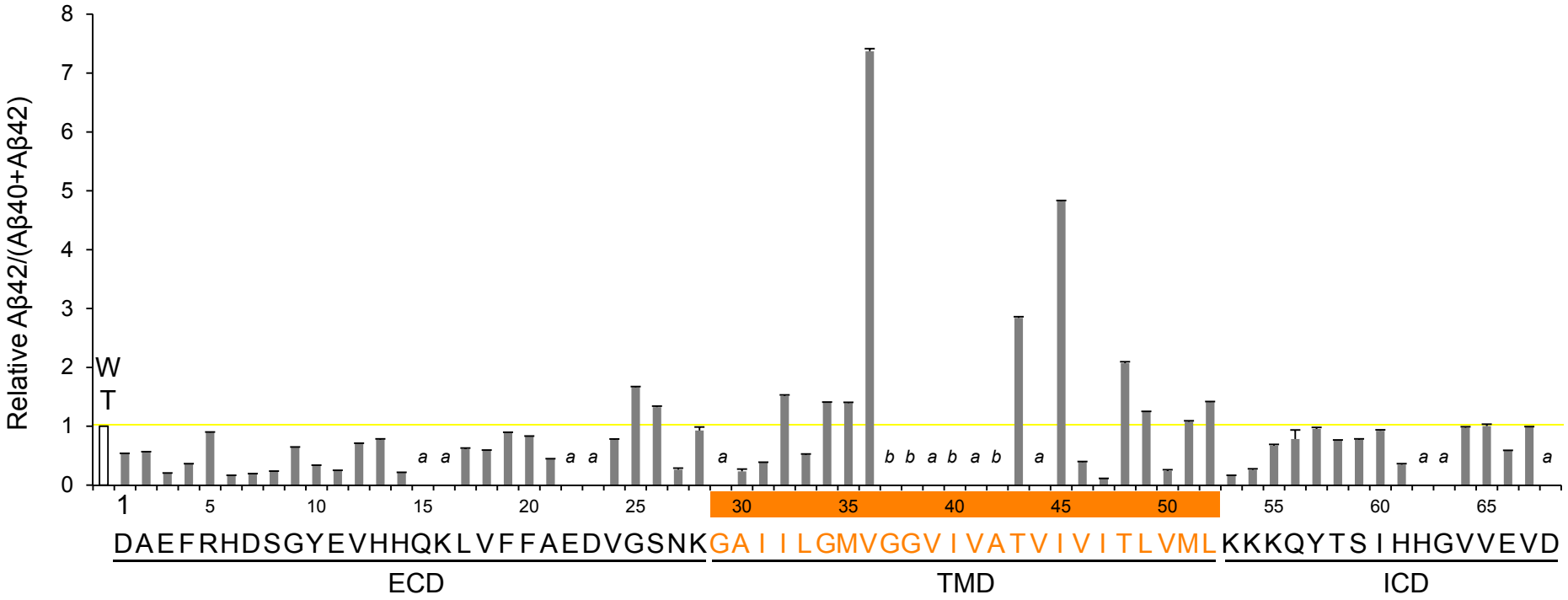
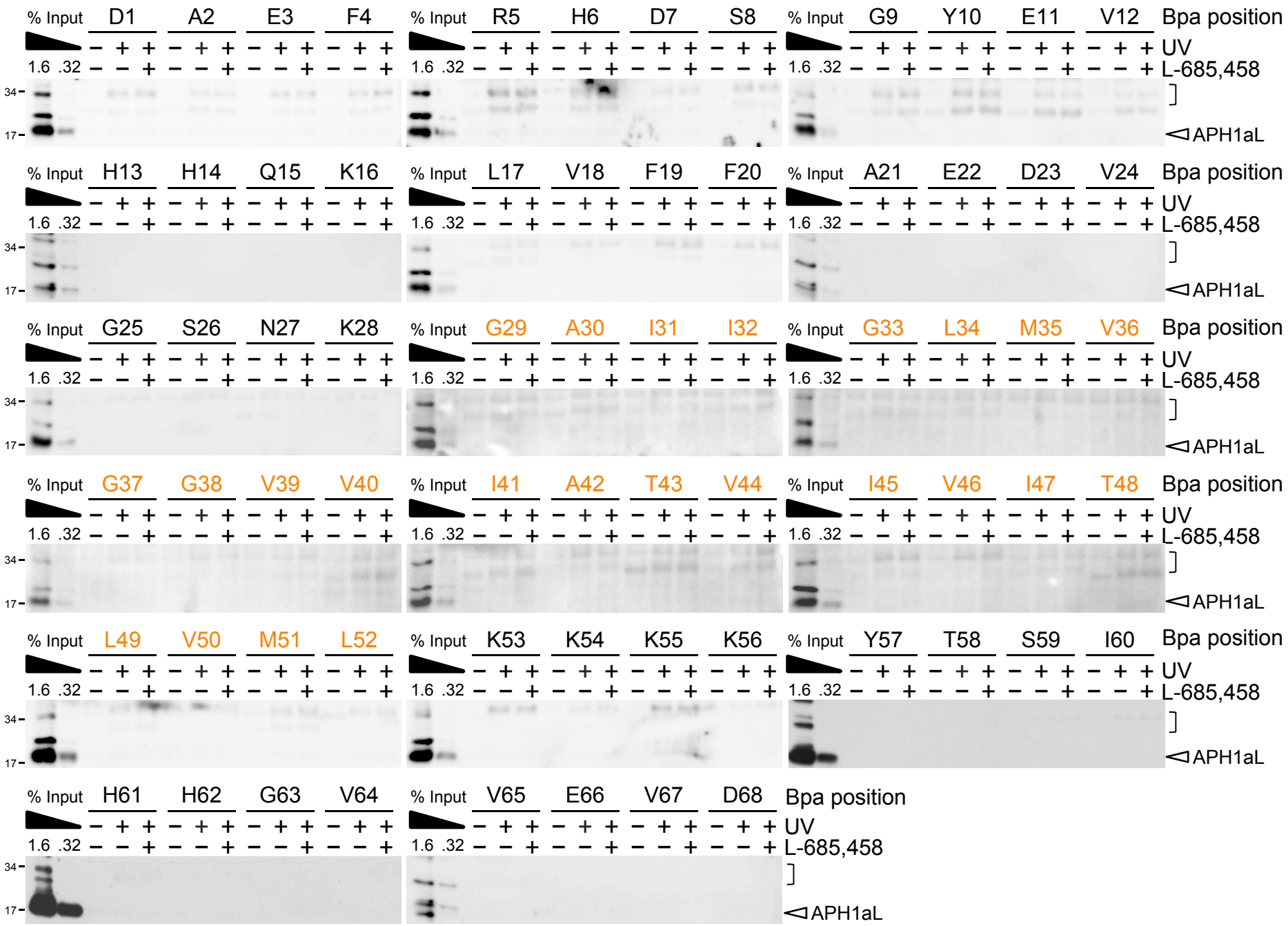


Fukumori and Steiner, Appendix Figure S1



Fukumori and Steiner, Appendix Figure S2

Appendix Figure Legends

Appendix Figure S1 – Aβ42/(Aβ40+Aβ42) ratios of C99-Bpa substrates.

The large majority of the C99-Bpa constructs do not cause pathogenic APP processing. Aβ42/(Aβ40+Aβ42) ratios are expressed relative to that for WT C99 (typically 10-20% in each set of experiments), which was set to 1 (yellow line). Bars denote the mean ± S.E. (n = 3).

a, While only small amounts of Aβ40 were detected (with the exception of C99-V44Bpa, which showed robust amounts of Aβ40), Aβ42 levels were generally below the limit of detection.

b, Aβ40 and Aβ42 levels could both not be detected, most likely because the location of these Bpa mutations in the epitope region of the detection antibodies might have disrupted Aβ recognition.

Appendix Figure S2 - APH-1 is unlikely to provide functionally relevant C99 interaction sites.

Crosslinking to APH-1 was overall very inefficient over the whole range of C99. Unlike observed for all other subunits, potential C99-APH-1 crosslink bands were insensitive to L-685,458 suggesting that APH-1 does not play a functional role in substrate recruitment. Bracket indicates the molecular weight range of putative APH-1aL-C99 crosslink bands. TMD residues are highlighted in orange.

Appendix Table S1

Effect of GSIs of various structural classes on C99 crosslinking to exosites versus the active site of γ-secretase

		GSI			
Bpa position	Binding target	III-31-C	LY-411575	Begacestat	ELN594
E3	PS1 NTF	↑	↑	↑	↑
H6	NCT	↑	↑	↑	↑
A30	PEN-2	↑	↑	↑	↑
L49	PS1 NTF	↓	↓	↓	↓

Appendix Supplementary Methods

Expression and purification of Bpa-containing APP C99 substrates

C99-Bpa constructs in pQE60 (Qiagen) containing the desired amber codon mutations were co-transformed with pDule-*p*Bpa (Farrell et al, 2005) co-expressing a Bpa-specific aminoacyl-tRNA synthetase and the corresponding amber suppressor tRNA (kind gift from Peter G. Schultz, The Scripps Research Institute) into *E. coli* Rosetta (Novagen). For the expression of the C99-K16Bpa construct pSup-BpaRS-6TRN was used (Ryu & Schultz, 2006). Transformants were selected, cultured and maintained in LB media containing 100 μg/ml ampicillin and 5 μg/ml tetracycline. To express the substrate proteins, 100 ml overnight pre-cultures were inoculated into 1 liter LB media containing antibiotics and cultured at 37°C for 1 hr. Following addition of 5 ml of 2 M Tris pH 8.5, 10 ml of 50 mM Bpa (Bachem) in 1 M HCl, and 0.6 ml of 1 M IPTG (Sigma), culturing was continued for 6-7 hr. Cells were harvested by centrifugation at 7000 x *g* for 10 min at 4°C and stored at -20°C until further use. For protein purification, the frozen cell pellets were thawed, resuspended in 50 ml of 20 mM Tris pH 7.5, 1 mM EDTA, and subjected to sonication. The resulting sonicates were centrifuged at 50.000 x *g* for 15 min at 4°C and the pellets solubilized in lysis buffer (20 mM Tris pH 8.5, 6 M urea, 1% Triton-X 100, 1% SDS, 1 mM $CaCl_2$, 100 mM NaCl) with protease inhibitor (PI) mix (Sigma) O/N at 4°C with rotation. After a clarifying centrifugation at 50.000 x *g* for 15 min 4°C, lysates were diluted with 3 volumes of 20 mM Tris pH 7.5, 150 mM NaCl and then rotated with 1 ml of a 50% slurry of Ni-NTA agarose beads (Qiagen) for 1-2 hr at RT in batch mode. Beads were loaded on a column, washed once with 1 ml of 20 mM Tris pH 8.5, 300 mM NaCl, 1% Triton-X 100 buffer and then once with 5 ml of 20 mM Tris pH 8.5, 300 mM NaCl, 0.2% SDS, followed by elution of bound substrate proteins with 20 mM Tris pH 8.5, 300 mM NaCl, 0.2% SDS, 100 mM imidazole. Eluates containing purified substrate proteins were stored at -20°C in aliquots until further use in γ-secretase in vitro cleavage assays or photocrosslinking experiments. Purification of all other APP substrate constructs with Bpa substitutions was performed as described above. Expression and purification of unmodified APP C100-His_6 and Notch1 N102-FmH substrates was carried out likewise but with omission of the respective selection antibiotic and Bpa. To purify N102-FmH, frozen cell pellets were resuspended in 100 mM Tris pH 8.5 with 1x PI mix (Sigma). Following sonication and addition of NaCl to a final concentration of 0.5 M, sonicates were centrifuged at 18.000 x *g* for 15 min at 4°C. The resultant supernatant was further centrifuged at 100,000 x *g* for 1 hr at 4°C and the pellet fraction solubilized in lysis buffer and subjected to purification as above.

γ-Secretase in vitro cleavage assays

Membrane fractions of HEK293 cells were prepared as described previously (Winkler et al, 2009). For subsequent use in γ-secretase in vitro assays, membranes were lysed in 150 mM Na-Citrate pH 6.4, 1% CHAPSO, 1x Complete PI mix (Roche) by incubation on ice for 1 hr. After centrifugation at 100.000 x *g* for 90 min at 4°C, the CHAPSO lysates were diluted to a final assay buffer concentration of 150 mM Na-Citrate pH 6.4, 10 mM DTT, 0.1 mg/ml BSA, 0.5 mg/ml phosphatidylcholine (Sigma, P3556), 0.25% CHAPSO, 1x Complete PI mix, with or without 1 μM L-685,458 in DMSO, mixed with 2 μM substrate and incubated with shaking O/N at 37°C. Samples were mixed with SDS-PAGE sample buffer, electrophoresed on 10-20% Tris-Tricine gels (Life Science), blotted on nitrocellulose membranes and analyzed for AICD generation by immunostaining using penta-His antibody. To measure Aβ40 and Aβ42 species, aliquots of the assays were diluted 50-fold with blocking buffer (PBS supplemented with 1% BSA and 0.05% Tween 20) and analyzed by Aβ sandwich immunoassay (Meso Scale Discovery (MSD)) as described previously (Shirotani et al, 2007). For some substrates (C99-Bpa E3, F4, H6, D7, S8 and Y10), the detection of Aβ40 and Aβ42 species was not possible in the conventional immunoassay presumably due to disruption of the Aβ1-16 epitope of the 2D8 capture antibody by Bpa. For these substrates, the MSD V-PLEX Plus Aβ Peptide Panel 1 (4G8) kit was used.

Appendix Supplementary References

Farrell IS, Toroney R, Hazen JL, Mehl RA, Chin JW (2005) Photo-cross-linking interacting proteins with a genetically encoded benzophenone. *Nat Methods* 2: 377-384

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Winkler E, Hobson S, Fukumori A, Dumpelfeld B, Luebbers T, Baumann K, Haass C, Hopf C, Steiner H (2009) Purification, pharmacological modulation, and biochemical characterization of interactors of endogenous human γ-secretase. *Biochemistry* 48: 1183-1197