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Supplemental Information

Dimeric Transmembrane Orientations of APP/C99 Regulate γ-Secretase Processing Line Impacting Signaling and Oligomerization

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Transparent Methods

cDNA constructs

Wild type C99 with a HA-tag was cloned into the pLVX-CMV bicistronic retroviral vector. cDNAs coding for the ten fusion proteins cc-C99$^{18-99}$ to cc-C99$^{27-99}$ (referred to cc-del0 to cc-del9) and their associated mutants were generated by overlap extension using PCR with synthetic nucleotides. All constructs were sequenced by Macrogen.

Primary neuronal cultures

Primary cultures of cortical neurons were prepared from E18 rat (for western blots and qPCR experiments) or mouse (for confocal experiments) embryos. The cortex was dissected and dissociated in HBSS depleted of calcium and magnesium. HBSS (with calcium and magnesium) was added and the mixture centrifuged through Fetal Bovine Serum (FBS) for 10 min at 1000g to pellet cells. Cells were plated at a density of 1.6 x 10^6 cells/cm² (for western blots and qPCR experiments) or 100,000 cells/cm² (for confocal experiments) in culture dishes pre-treated with 10 µg/ml of poly-L-lysine in phosphate buffered saline (PBS) and cultured for 7 days *in vitro* in Neurobasal® medium supplemented with 2% v/v B-27® supplement medium and 1mM L-glutamine (all from Life Technologies) at 37 °C, 5% CO_2 in a humidified incubator. Half of the medium was renewed every 2-3 days prior to infection.

Cell cultures and transfection

Chinese hamster ovary (CHO) cell lines (ATCC) were grown in Ham's F12 medium (Thermofisher Scientific) and were supplemented with 10% of FBS (Sigma-Aldrich). The MEF (Murine Embryonic Fibroblast) APP-/- cell lines, MEF dPS-/- (presenilin 1 and 2 knock out) and the human neuroblastoma SH-SY5Y cell lines (ATCC) were grown in DMEM/F12 medium (Thermofisher Scientific) and were supplemented with 10% of FBS. SH-SY5Y cells were differentiated into neurons with 10 µM of retinoic acid (Sigma-Aldrich) after 7 days. All cell cultures were maintained at 37 °C in a humidified incubator (5% CO_2). For transient transfection, cells were seeded at a density of 3.10^5 cells/well 24h before transfection (2 µg of pLVX-CMV DNA/well) with lipofectamine 2000™ according to the manufacturer's instruction (Invitrogen). The SH-SY5Y were treated with 300 nM of trichostatin A (TSA) during 24h after differentiation prior to RNA extraction.

Cross-linking studies

The ten cc-del(X) fusion proteins were mutated by directed mutagenesis (Gly29 into Cys) to generate the cc-del(X) G29C constructs. After transfection, CHO cells were washed in PBS and resuspended in 1 ml of PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$. Cross-linking was performed with a final concentration of 100 µM of the crosslinker N, N'-1, 2-phenylenedimaleimide (o-PDM) dissolved in DMSO for 10 min at room temperature. Cells were lysed in NP40 lysis buffer containing 2% of *β-mercaptoethanol to quench the reaction. Lysates were analyzed by Western blotting, with an antibody directed against the C-terminus fragment of APP (Cter).*

Generation of cell lines

MEF and SH-SY5Y cell lines stably expressing the different cc-del(X) fusion proteins were established by viral transduction. Viruses were generated by transient transfection in HEK cells with the vectors psPAX2 and pMD2.G (Clontech) using the calcium phosphate method, according to the manufacturer's instruction (Promega). Viruses were then concentrated with Centricon filter units (Merck Millipore). Infected cells were selected with 20 µM of puromycin for 1 week and maintained in culture with 2 µM of the antibiotic.

Western blotting and blue native gel electrophoresis

For SDS/LDS-PAGE electrophoresis, cells were commonly lysed in Laemmli 2X buffer. For isolation of Coomassie Blue positive bands associated with cc-del7, Laemmli lysis buffer was replaced with NP-40 lysis buffer. LDS sample buffer (ThermoFisher Scientific) was added to the lysates and the mix was boiled 10 minutes at 70°C. Proteins (25 µg) were separated on Bolt™ 4-16% Bis-Tris Protein Gels (Invitrogen) and transferred onto nitrocellulose membrane via iBlot gel transfer stacks (Invitrogen). For Blue Native PAGE, cells were lysed in native blue buffer, DDM 10% (Invitrogen) and 100X protease inhibitor (Pierce). After centrifugation (100,000*g*, 1 h at 4 °C) supernatants were collected (15 µg of proteins) and 5% of sample buffer added. Protein lysates were separated using the Novex® NativePAGE™ Bis-Tris gel system (Invitrogen). The electrophoresis and transfer were performed as per manufacturer's instructions. Membranes were incubated overnight at 4 °C with primary antibody at following dilutions: 1:1,000 anti-HA, [3F10] (Roche); 1:2,500 anti-Amyloid Precursor Protein [*Y188*] (referred to Cter - Abcam); 1:5,000 anti-Amyloid *β,* WO-2 MABN10 (Merck Millipore). Membranes were washed, and further incubated with the secondary antibody (1:5,000 anti-rat [7077S]; anti-rabbit [7074S]; anti-mouse [7076S] (Cell Signaling Technology)) conjugated

to horseradish peroxidase followed by ECL (Thermo Scientific). Detection of membranes was performed with the Vilber Fusion Solo S system.

Immunoprecipitation and immunoblotting

CHO cells were transfected with HA-C99 and cc-del(X) expression plasmids. The supernatant was collected, lyophilized, resuspended in water and pre-cleared with recombinant protein G agarose (Invitrogen). Immunoprecipitation of lysates was performed after lysis in NP-40 lysis buffer. Immunoprecipitation was performed with a monoclonal anti-HA antibody (Roche). Immunoblotting was performed as described above, using a different HA-Tag antibody (C29F4 Rabbit mAb #3724 - Cell Signaling Technology) as primary antibody.

Mass spectrometry

For the identification of peptides, samples were reduced and alkylated before digestion O/N with trypsin at 30 °C in 50 mM NH_4HCO_3 [pH 8.0]. Peptides were dissolved in solvent A (0.1% TFA in 2% ACN), directly loaded onto reversed-phase pre-column (Acclaim PepMap 100, Thermo Scientific) and eluted in backflush mode. Peptide separation was performed using a reversed-phase analytical column (Acclaim PepMap RSLC, 0.075 x 250 mm,Thermo Scientific) with a linear gradient of 4%-36% solvent B (0.1% FA in 98% ACN) for 36 min, 40%-99% solvent B for 10 min and holding at 99% for the last 5 min at a constant flow rate of 300 nl/min on an EASY-nLC 1000 UPLC system. The peptides were analyzed by an Orbitrap Fusion Lumos tribrid mass spectrometer (ThermoFisher Scientific). The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Fusion Lumos coupled online to the UPLC. Intact peptides were detected in the Orbitrap at a resolution of 120,000. Data-dependent ions were selected for MS/MS using HCD setting at 35; ion fragments were detected in the Iontrap. The resulting MS/MS data was processed using Sequest HT search engine within Proteome Discoverer 2.3 against a homemade protein database containing the sequences of HA-C99 and cc-del(X) expression plasmids. Trypsin was specified as cleavage enzyme allowing up to 2 missed cleavages, 2 modifications per peptide and up to 2 charges. Mass error was set to 10 ppm for precursor ions and 0.1 Da for fragment ions. Oxidation on Met was considered as variable modification. Peptide spectral matches (PSM) were filtered using charge-state versus cross-correlation scores (Xcorr) and manually validated. For Parallel Reaction Monitoring (PRM) experiments, a targeted mass list was included for the potential C-term sequences, considering potential oxidation of Met and charge state +2. MS1 spectra were obtained at a resolution of 120.000 with an AGC target of 1E6 ions and a maximum injection time of 50 ms, Targeted-MS2 spectra were acquired with

an AGC target of 5E4 ions and a maximum injection time of 100ms at a resolution of 30.000. Targeted-MS2 scans were analyzed by Skyline 19.1.0.193 and manually validated.

Dual luciferase assays

CHO cells were transfected as described above with the following plasmids: cc-del(X) vectors, Fe65, Gal4Tip60 and Gal4RE-luc as reporter gene, according to the procedure previously described (Huysseune et al., 2007). The firefly luciferase activity was standardized with the Renilla luciferase reporter pRLTK. Both luciferase activities were measured with the Dual Glo™ luciferase assay system (Promega).

AICD detection

MEF APP-/- cc-del(X) cells were grown in 10 cm dishes. Prior to lysis, cells were washed in PBS, collected in cold hypotonic buffer (1 mM MOPS, 1 mM KCl, pH 7.0) and incubated at 25 °C for 25 min. Mechanical lysis was performed by syringe and lysate was clarified at 1,000*g* for 15 min at 4 °C. The post nuclear fraction was centrifuged at 16,000*g* for 40 minutes. Pellets were solubilized in trisodium citrate (150 mM, pH 6.4). Samples were incubated either on ice (considered as time point 0 h) or at 37 °C for 2 h, then analyzed by Western blotting revealed with the APP Cter antibody. Quantification of AICD was performed with Bio-1D software.

Assessment of γ-secretase ability to cleave stable dimers

Cell lines were seeded at a density of 2 x 10^6 per 10 cm dish for 48 hours. They were washed twice with PBS and re-suspended in PBS with 1mM $MgCl_2$ and 0.1 mM $CaCl_2$ with 100 µM of the crosslinker N,N'1,2-phenylenedimaleimide (o-PDM) or DMSO (negative control) for 10 minutes at room temperature. Cells were then washed twice with PBS and collected in cold hypotonic buffer (1 mM MOPS, 1 mM KCl, pH 7.0) with 2% β-mercaptoethanol to quench the reaction and 100X protease inhibitor cocktail (Pierce). Lysates were subsequently processed for AICD detection as described above.

RNA extraction and quantitative real time PCR

Total RNA was purified using RNeasy Mini Kit (Qiagen). Reverse transcription (RT) was performed withthe reverse transcriptase core kit using a T3thermocycler (Westburg). Quantitative (q) real time PCR (q-RT PCR) was performed with the MESA Green qPCR MasterMix Plus for SYBR Assay® (Eurogentec) using the Applied Biosystems™

StepOnePlus™ Real-Time PCR System. The relative amplification of cDNA fragments was calculated by the $2^{-\Delta\Delta Ct}$ method with GAPDH as control.

Confocal microscopy

SH-SY5Y cells and mouse primary neurons expressing cc-del6, cc-del7 and HA-C99 were grown on chamber slides with coverglass (ThermoFischer Scientific) or on pre-coated poly-D-lysine coverslips in 24 well plates, respectively. SH-SY5Y cells were differentiated over 7 days with retinoic acid as described above. Cells were fixed with 4% paraformaldehyde for 15 minutes, permeabilized, and then blocked with 0.5% Triton X-100 in phosphate-buffered saline containing 100 µg/mL of goat γ-globulin (Jackson Immunoresearch Laboratories) for 1 h at room temperature. Cells were incubated overnight at 4 °C with primary antibodies (rat anti-HA antibody, 1:500, Roche; mouse anti-MAP2 antibody, 1:1,000, Sigma-Aldrich) and then incubated for 1h at room temperature with DAPI and secondary antibodies (goat anti-rat IgG antibody coupled to Alexa 568, 1:500, Invitrogen/Life Technologies; goat anti-mouse IgG antibody coupled to Alexa 488, 1:500, Invitrogen/Life Technologies). Slides were examined by confocal microscopy using a confocal server spinning disc Zeiss platform equipped with a ×100 objective.

Statistical analysis

The statistical analyses were performed with JMP pro 13 software. If more than two groups were compared, for parametric test ANOVA with post hoc tests as indicated were used or for non-parametric test Kruskall-Wallis with post hoc tests as indicated were used. (*, $p < 0,05$; **, $p < 0,01$; ***, $p < 0,001$). The number of biological replicate (n) analyzed is indicated in figure legends along with the number of independent experiment (N).

Modeling dimers with different interfaces

Helical wheel diagrams have been drawn from grigoryanlab website (https://grigoryanlab.org/). Modeling of the cc-del6 and cc-del7 were performed with PyMol software and readapted from the structure 2loh.pdb (Nadezhdin et al., 2012).

Supplementary Figures

Supplementary Figure 1. Validation of the coiled-coil C99 fusion proteins.

A. Representation of the cc-del(X) and the corresponding cc-del(X) G29C, G37C and L49C sequences with cc-del0 as an example. Residues deleted in cc-del(X) constructs are in grey. Introduction of cysteine at position 29 (G29C) is highlighted in red. The mutation allows cross-linking with N,N'-1,2-phenylenedimaleimide (o-PDM) if two cysteines residues are close enough. **B.** Alpha helical prediction of the precise position of amino acids residues with the cysteine mutation at Gly29. Cross-linking predictions are indicated by a color code associated to the position Cys29 in the helical diagram. Cys29 is indicated in red, blue or green if it is present at the interface, at the border of the interface or outside the interface, respectively. **C.** Crosslinking study on living cells. CHO cells were transfected with the ten cc-del(X) G29C vectors or four cc-del(X) G37C vectors and treated (+) or not (-) o-PDM. Cell lysates were analyzed by Western Blotting and revealed with APP Cter antibody. **D.** Crosslinking study on living cells. CHO cells were transfected with the ten cc-del(X) L49C vectors and treated (+) or not (-) with o-PDM. Cell lysates were analyzed by Western Blotting and revealed with APP Cter antibody. Top: high exposure, bottom: low exposure. Related to Figure 1.

Supplementary Figure 2. Assessment of γ-secretase ability to cleaved crosslinked dimers.

A. Representative Western Blot of MEFs cells APP-/- stably expressing the cc-del(6) construct with the G29C mutations analyzed by (L)SDS-PAGE followed by immunoblot with 1:1000 anti C-terminal antibody [Y188] (Abcam) to detect crosslinked dimers and cc-del(6)/CTFs (low exposure) and AICD release (long exposure). **B.** Representative Western Blot of MEFs cells stably expressing the cc-del(6) construct without the G29C mutations analyzed by (L)SDS-PAGE followed by immunoblot with 1:1000 anti C-terminal antibody [Y188] (Abcam) to assess the effect of o-PDM on γ-secretase activity. Related to Figure 1.

Supplementary Figure 3. Subcellular localization of cc-del(X) in SH-SY5Y cells

Cells were differentiated by retinoic acid treatment for 7 days, stained with HA antibody (green) and DAPI (blue) and analyzed by confocal microscopy. White arrows indicated localization of cc-del(X) constructs. Related to Figure 3 and 5.

Supplementary Figure 4. Oligomerization properties of $C99^{n-99}$ and cc-$C99^{n-99}$.

A. Deletions of amino acids 18 to 27 in the C99 sequence followed by fusion with HA-tag in the N-terminal part. **B.** Profile of deletion in the N-terminal of C99. CHO cells were transiently transfected with mock vector, HA-C99 and one of the ten truncated C99 (where n is the first amino acid of the sequence). Cells were lysed and analyzed by SDS-PAGE followed by immunoblotting with Cter APP antibody (top) or HA antibody (bottom). **C.** Profile of coiled coil in native condition. CHO cells were transiently transfected with cDNA of mock vector, HA-C99 and the ten coiled coil constructs. Cells were lysed and analyzed on native page followed by immunoblotting with APP Cter antibody (top) or HA antibody (bottom). Related to Figure 4.

Supplementary Figure 5. β-aggregation propensity of cc-del(X) constructs.

A. Beta-aggregation propensity prediction using the Tango algorithm (Fernandez-Escamilla et al., 2004) for cc-del(X) constructs. **B.** Partial sequence of cc-del6/7 constructs. The additional amino acid (Val24) present in cc-del6 compared to cc-del7 is shown in bracket. Mutations are indicated by arrows. Amino acid sequences predicted to have a high propensity for cross-β aggregation are in bold. **C.** β-aggregation propensity prediction of cc-del6 and cc-del7 with and without the V24A and G25V mutations, respectively. Analysis done using the Tango algorithm (Fernandez-Escamilla et al., 2004). **D.** Analysis of the profile of cc-del(X) constructs and Aβ-like peptides in denaturing conditions. CHO cells were transiently transfected with mock vector, HA-C99, the cc-del6 and cc-del7 constructs with or without the indicated mutation or their respective truncated forms at position 42 or 43. Cell lysates were analyzed by Western Blotting and revealed with HA antibody. Related to Figure 4.

Supplementary Figure 6. Effect of pro-ß ALLLV motif in cc-del(x) constructs.

Analysis of the profile of cc-del(X) constructs with or without the pro-ß ALLLV motif in denaturing conditions. CHO cells were transiently transfected with mock vector, HA-C99 or cc-del(x) constructs with or without the pro-ß ALLLV motif. Cell lysates were analyzed by Western Blotting and revealed with HA antibody. Related to Figure 4.

Supplementary Figure 7. Sample preparation for mass spectrometry.

A. Sample preparation for mass spectrometry (MS) analysis of oligomers associated with cc-del6. Conditioned media of CHO cells expressing cc-del6 were immunoprecipitated and analyzed by Western blotting before (1) or after treatment with formic acid (2). Samples treated

with formic acid were further analyzed by tandem mass spectrometry (MS/MS). **B.** Sample preparation for mass spectrometry (MS) analysis for oligomers associated for cc-del6 V44F-I45F and cc-del6 V50F-M51F. Cell lysates of CHO cells expressing cc-del6 were immunoprecipitated as described in (A) and analyzed by Western blotting before (1) or after treatment with formic acid (2). Samples treated with formic acid were further analyzed by tandem mass spectrometry (MS/MS) (3). For each condition, the lysates of three confluent 15cm^2 dishes were pooled. Related to Figure 6.

Supplementary Figure 8. Identification of Aβ C-terminus in cc-del6 by LC-MS/MS.

Identification of C40 and C43 peptides by LC-MS/MS. The LC-MS spectrum shows MS^2 obtained from a +2 charged parent ion of m/z 543.3223 (C40) and 693.902 (C43) respectively, which upon HCD fragmentation gave rise to the y- and b- series of daughter ions allowing confirmation of the identified peptide sequences. Related to Figure 6.

Supplementary Figure 9. Identification of Aβ C-terminus in cc-del6 V44F-I45F by LC-MS/MS.

A. Identification of C45 and C46 peptides by LC-MS/MS. The upper LC-MS/MS spectrum shows MS^2 obtained from a +2 charged parent ion of m/z 832.982 (C45), which upon HCD fragmentation gave rise to the y- and b- series of daughter ions allowing confirmation of the identified peptide sequences. The lower chromatogram shows peak area contribution from a +2 precursor charged parent ion of m/z 882.5069 (C46) (small insert) and the corresponding coeluting transitions for 10 y- and b-daughter ions determined by a PRM experiment. **B.** Relative quantification of C45 and C46 peptides. The panels show the precursor ions intensities from C45 and C46 parent ions determined from the PRM experiment. Related to Figure 6.

Supplementary Figure 10. Identification of Aβ C-terminus in cc-del6 V50F-M51F by LC-MS/MS.

Identification of C40, C43 and C46 peptides by LC-MS/MS. The upper LC-MS/MS spectrum shows MS^2 obtained from a +2 charged parent ion of m/z 543.3228 (C40), which upon HCD fragmentation gave rise to the y- and b- series of daughter ions allowing confirmation of the identified peptide sequences. The lower chromatograms show peak area contribution from a +2 precursor charged parent ion of m/z 693.9118 (C43) or 847.5111 (C46) (small inserts) and

the corresponding coeluting transitions for y- and b-daughter ions determined by a PRM experiment. Related to Figure 6.

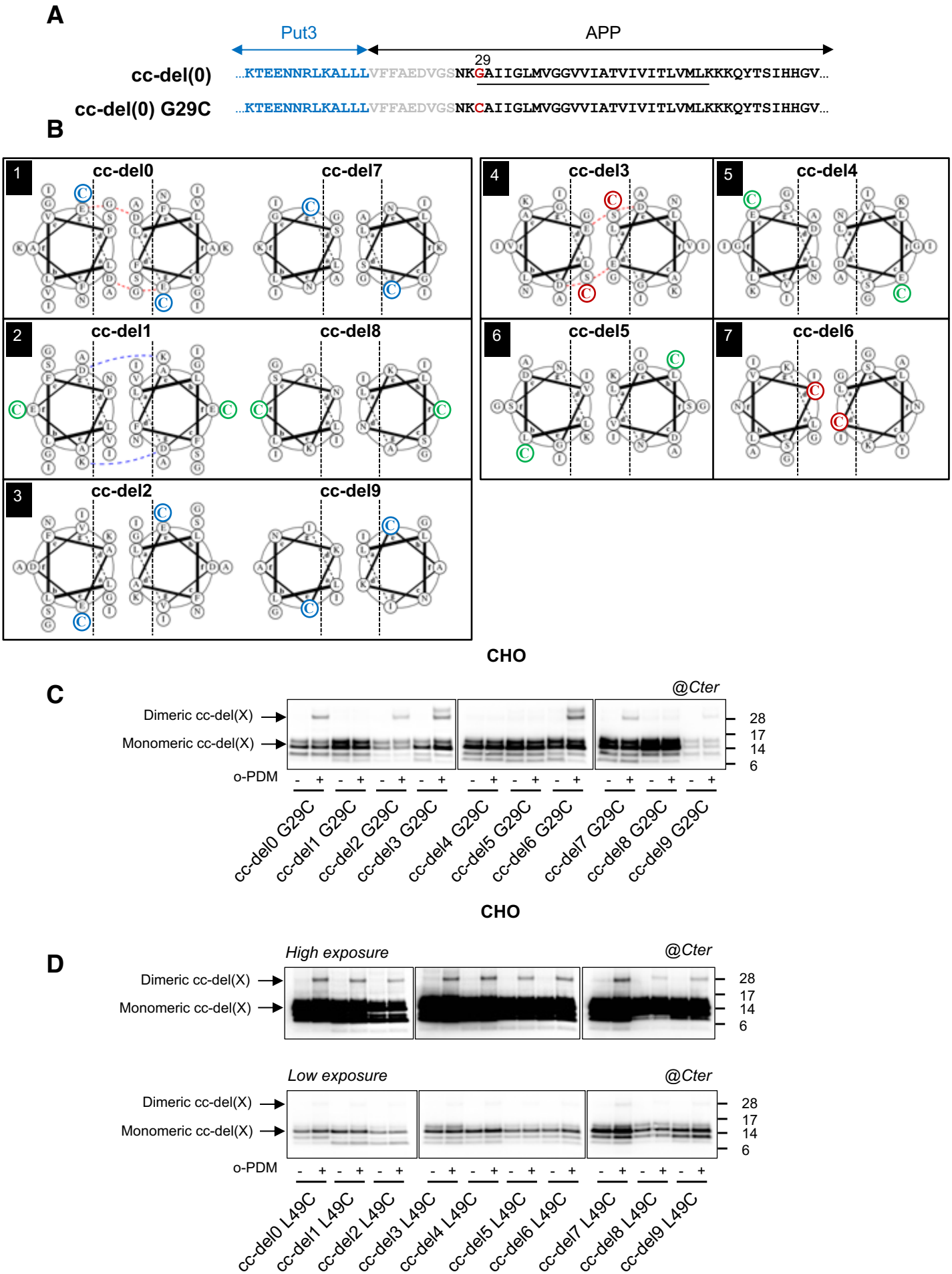
Supplementary Figure 11. Coomassie Blue Staining of MEFs infected with cc-del(x).

Coomassie Blue Staining of MEF cells WT, PSEN1 -/- or PSEN2-/- infected with C99, cc-del6 or cc-del7 in denaturing conditions. Related to Figure 6.

Supplementary Figure 12. Identification of Aβ C-terminus in cc-del7 by LC-MS/MS.

Identification of C39 and C36 peptides by LC-MS/MS. The chromatogram shows peak area contribution from a +2 precursor charged parent ion of m/z 493.7875 (C39) or m/z 387.2322 (C36) (small inserts) and the corresponding coeluting transitions for y- and b-daughter ions determined by a PRM experiment. Related to Figure 6.

Supplementary Figure 1



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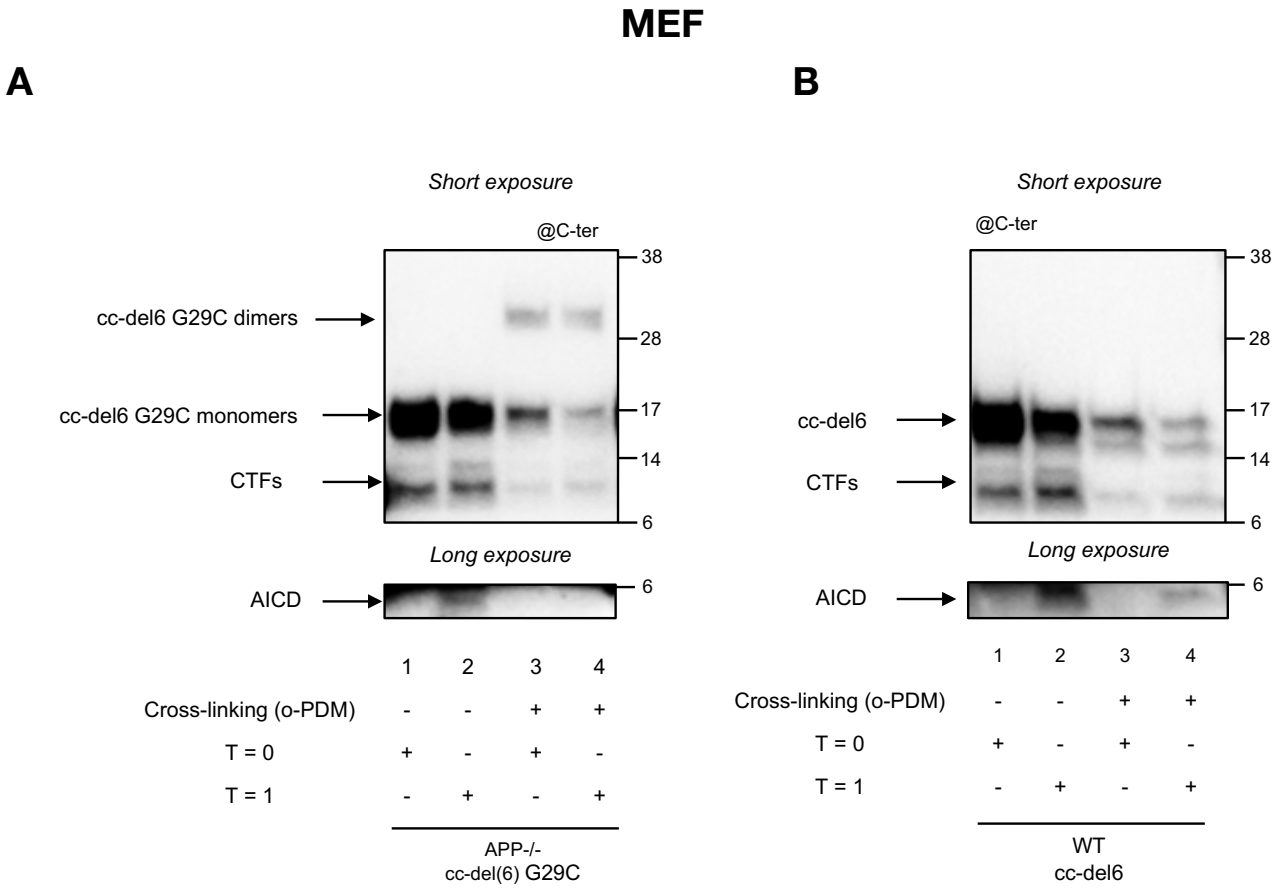

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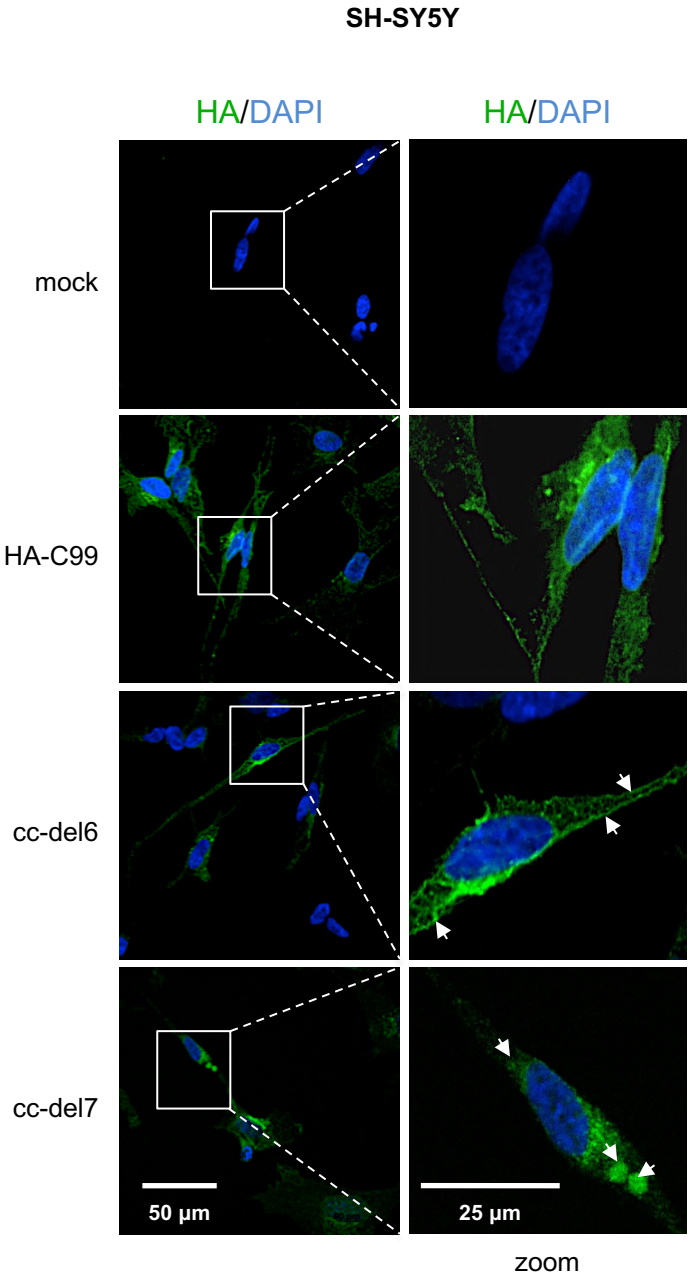

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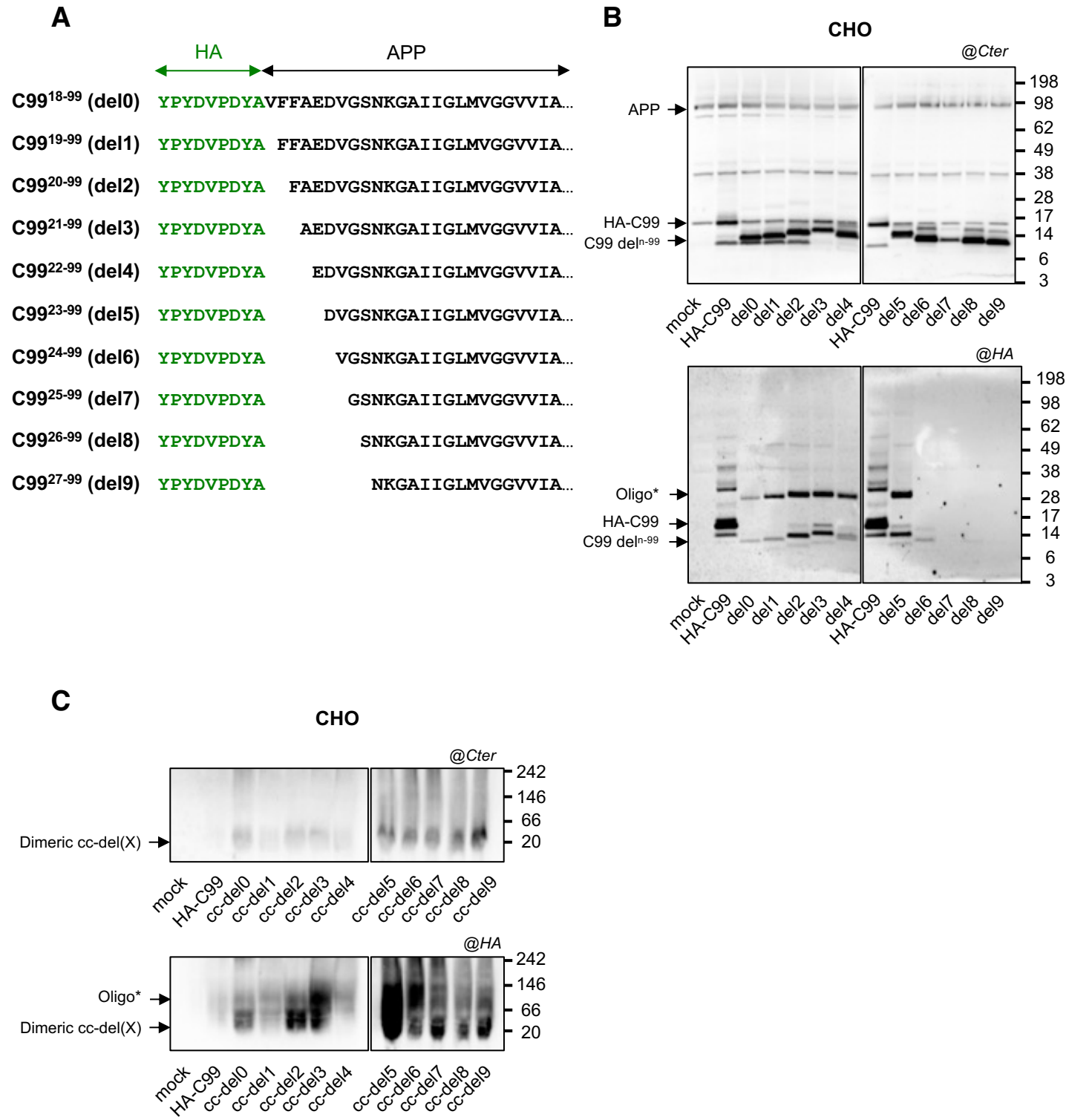

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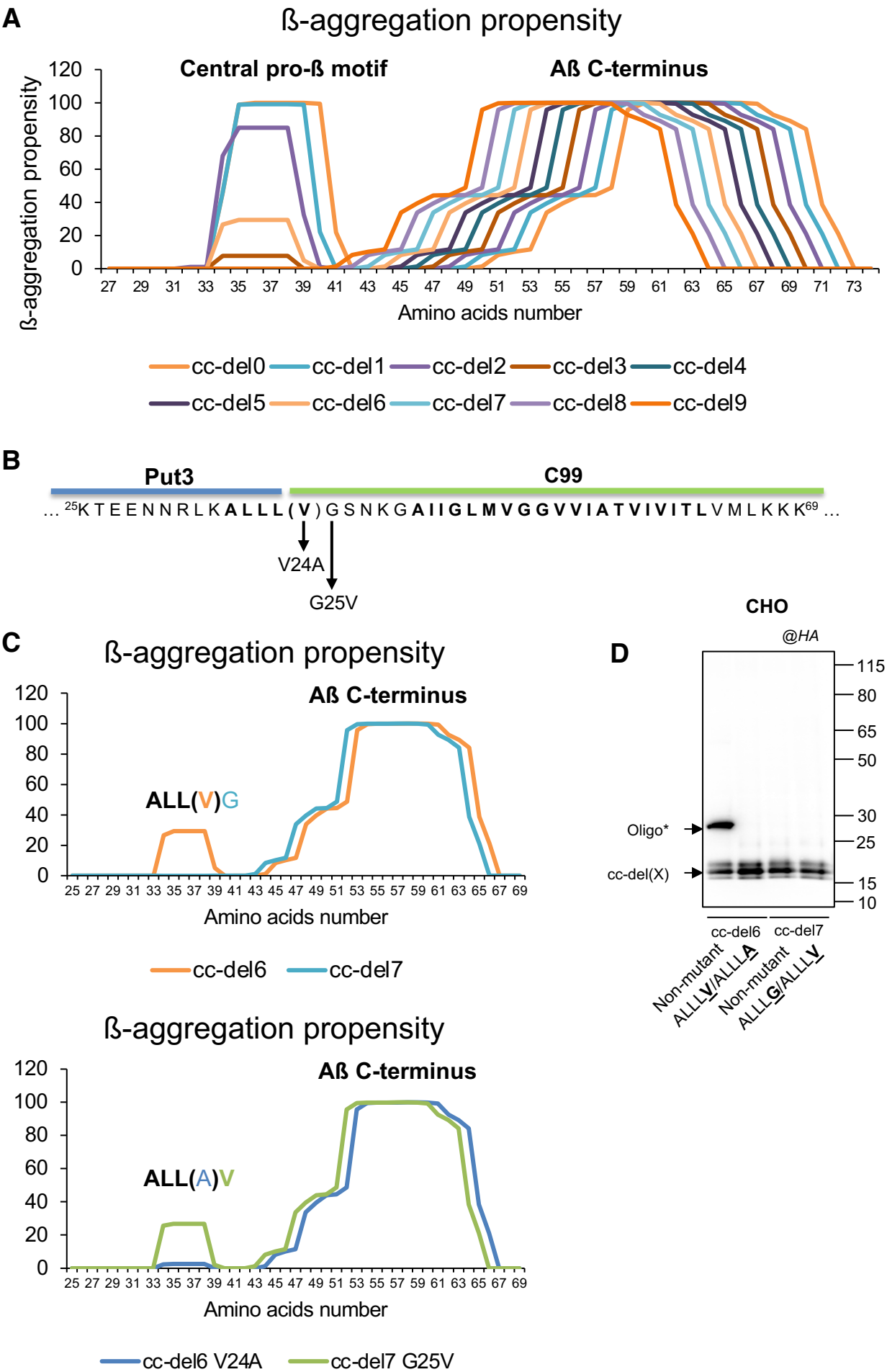

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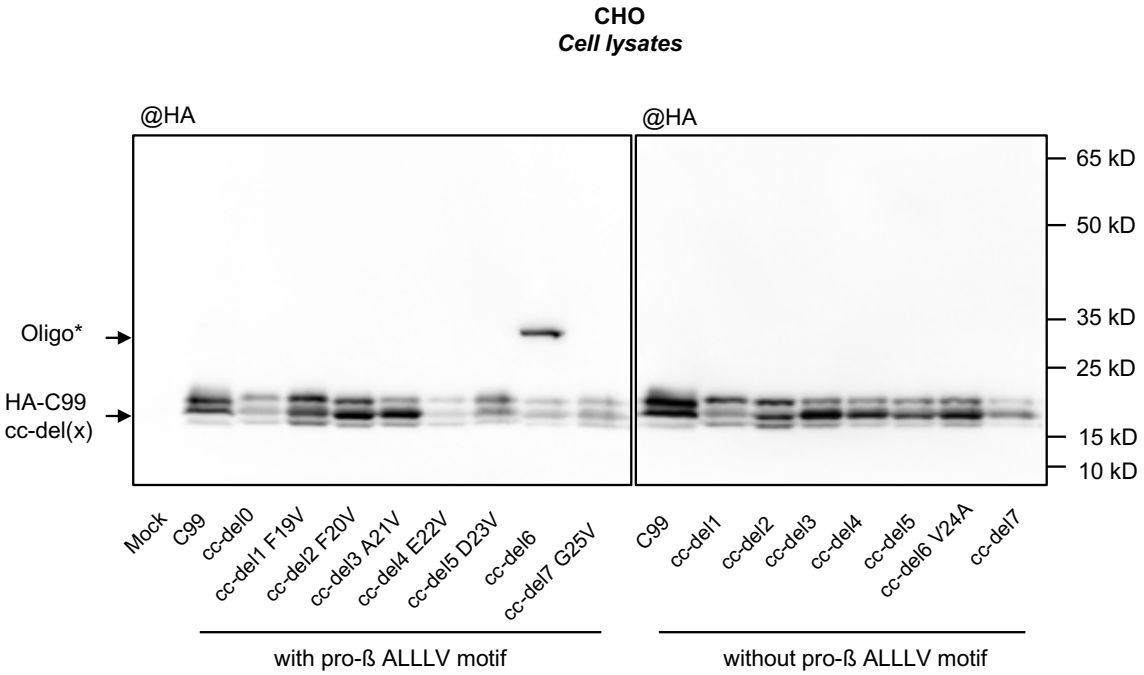

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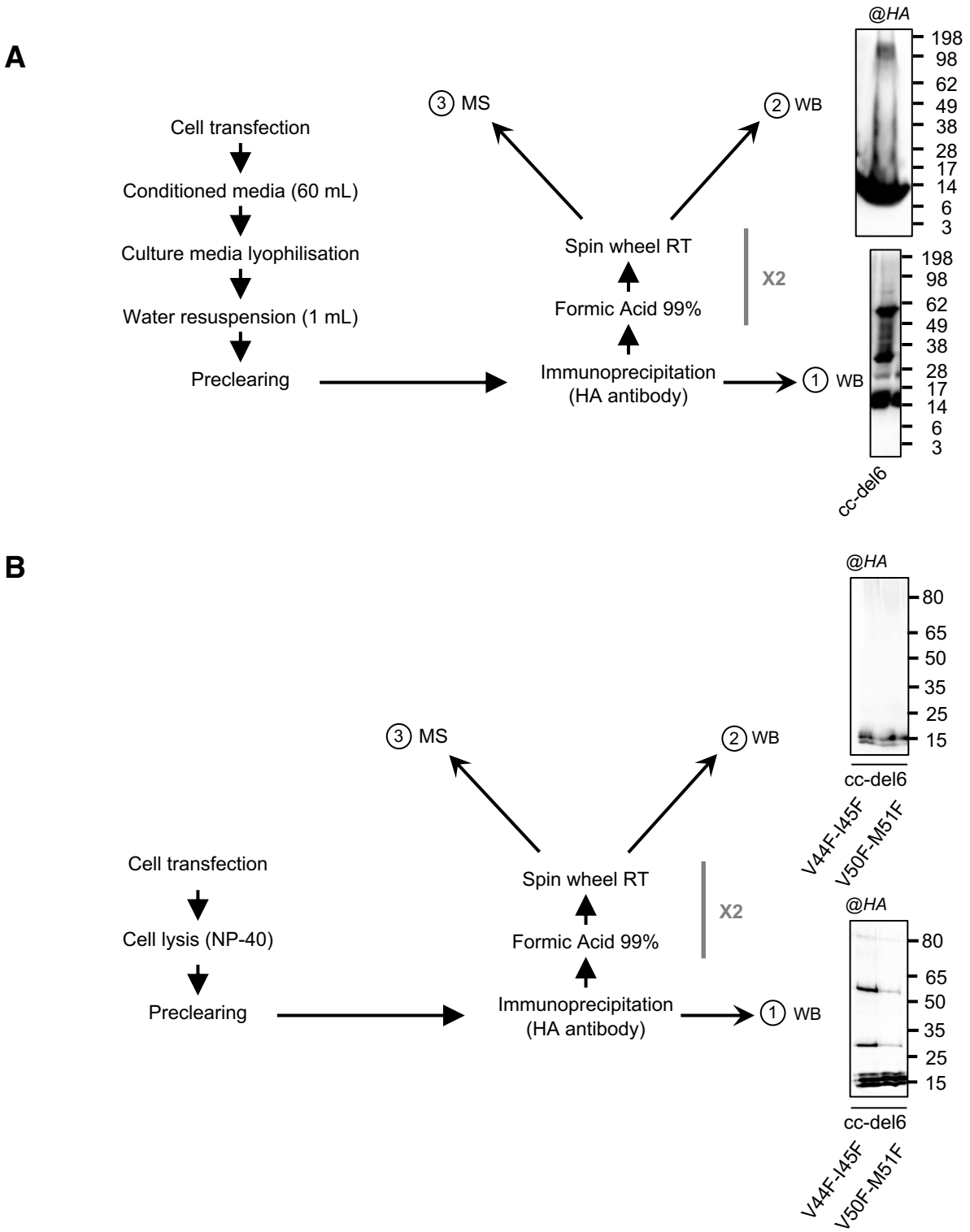


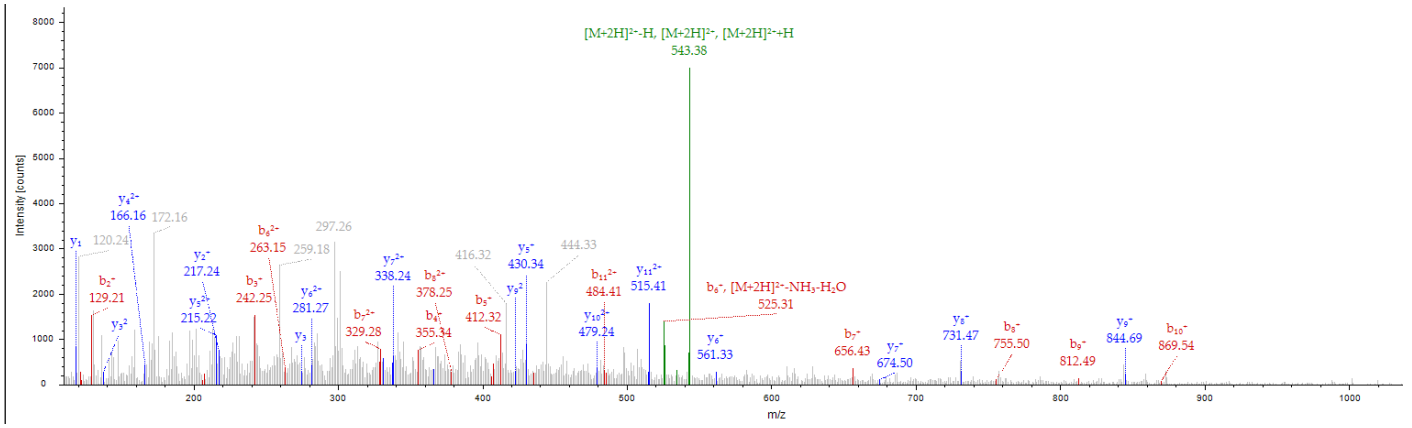
Supplementary Figure 8

CHO

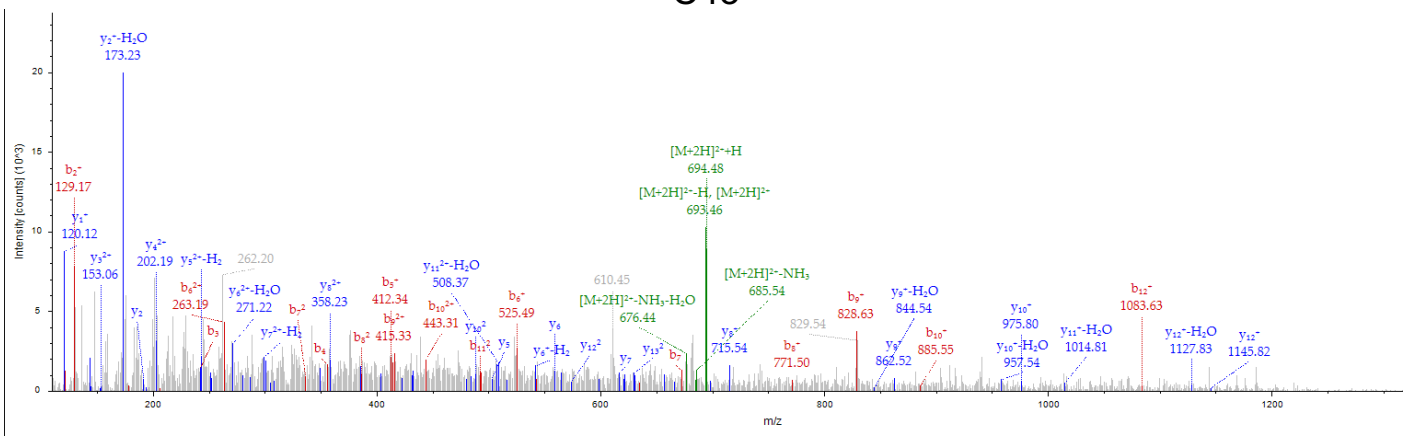
Culture medium

cc-del6

C40



C43

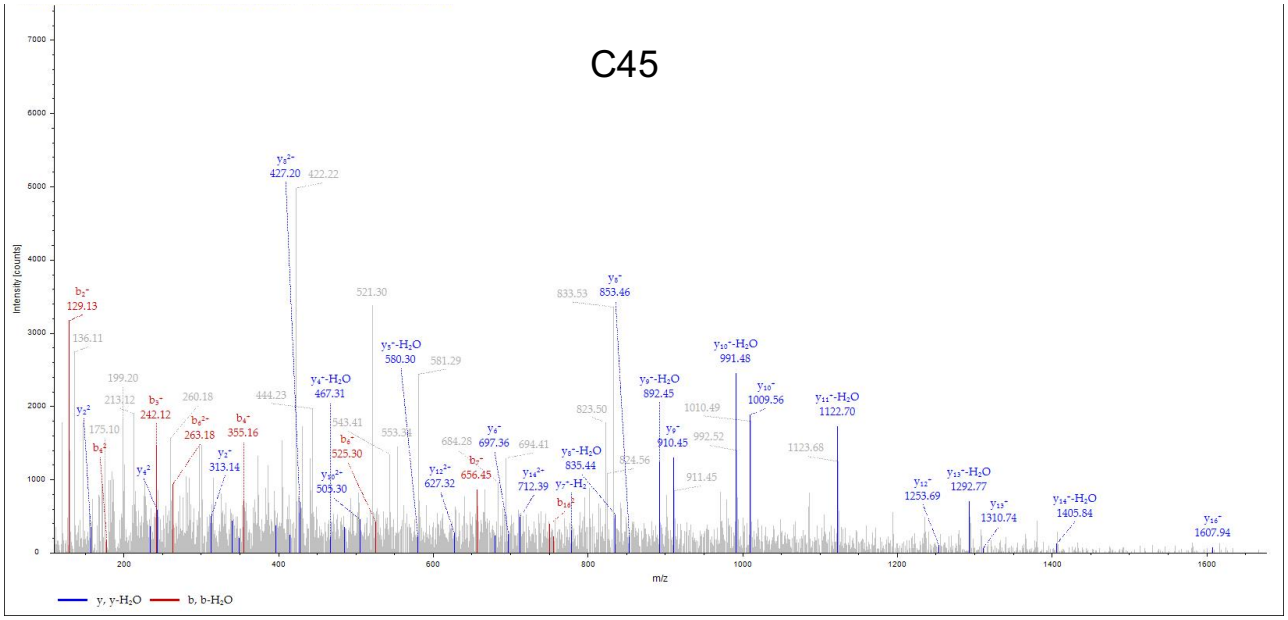


Supplementary Figure 9

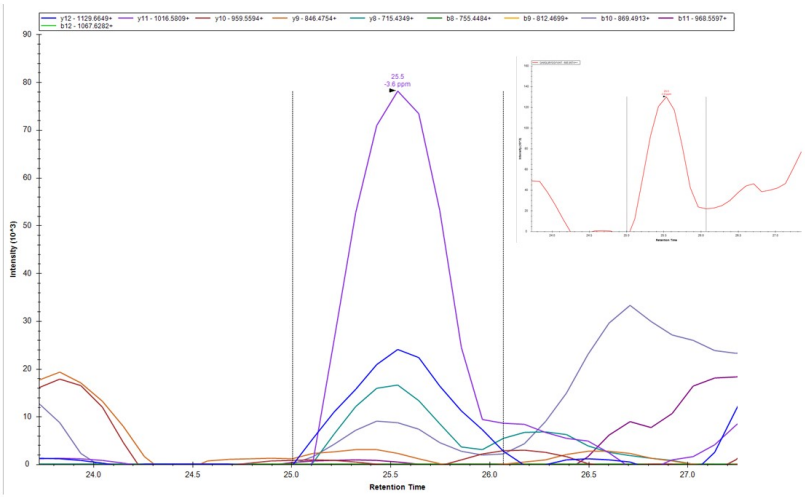
CHO
Cell lysates

cc-del6 V44F-I45F

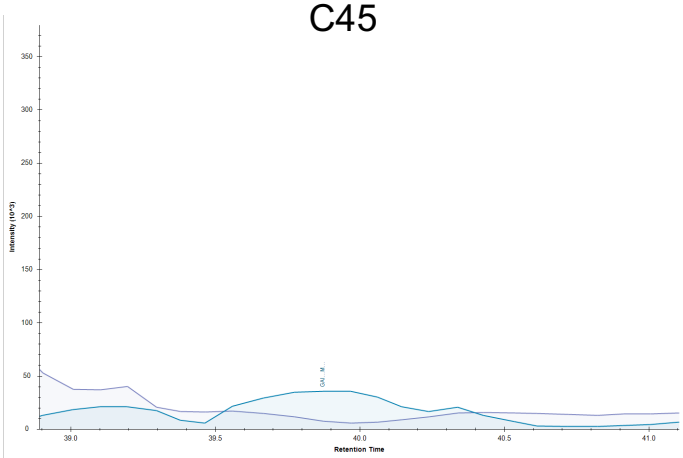
C45



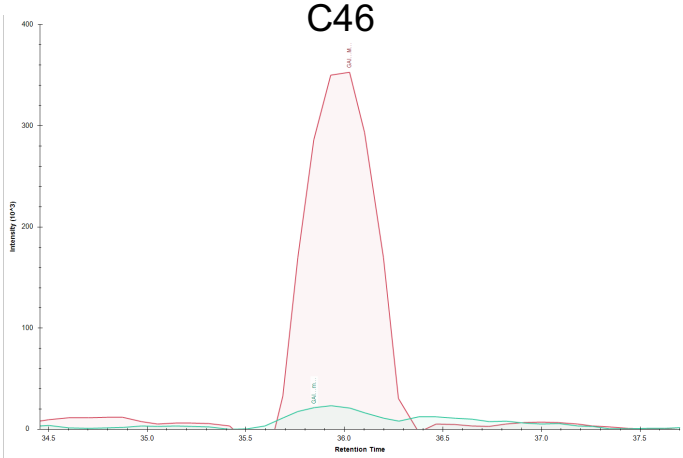
C46



C45



C46



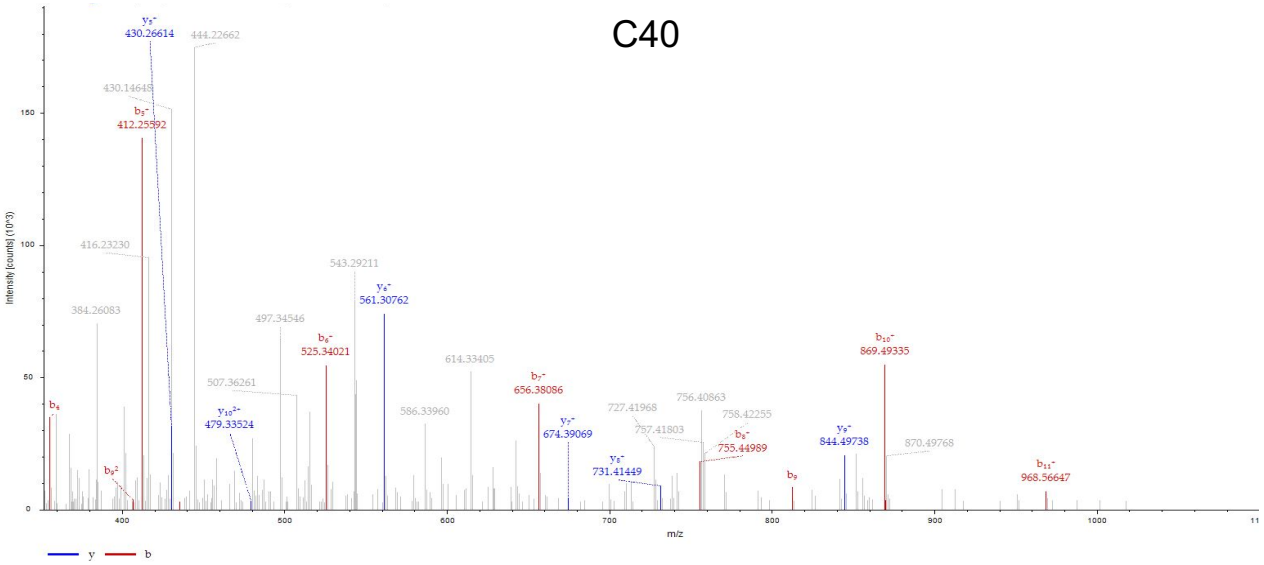
Supplementary Figure 10

CHO

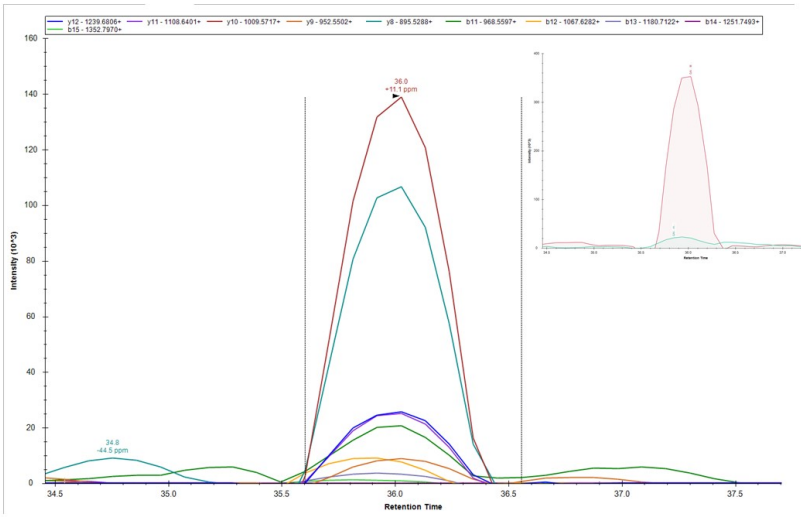
Cell lysates

cc-del6 V50F-M51F

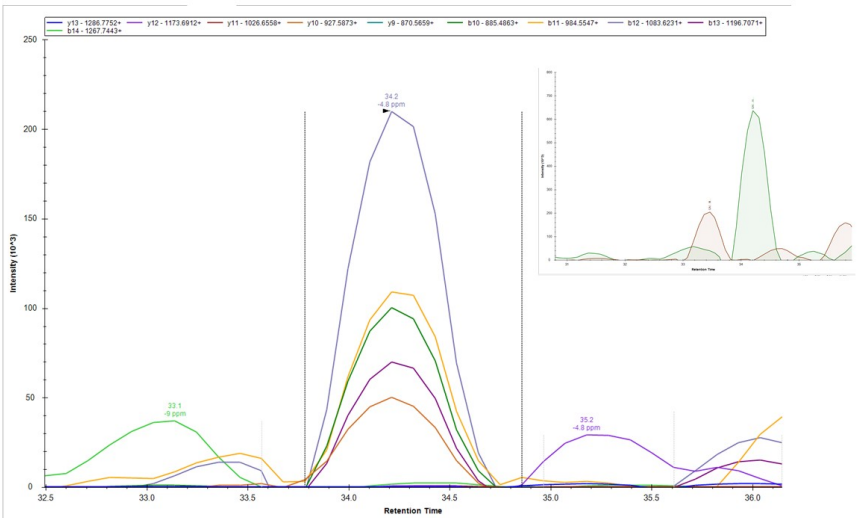
C40



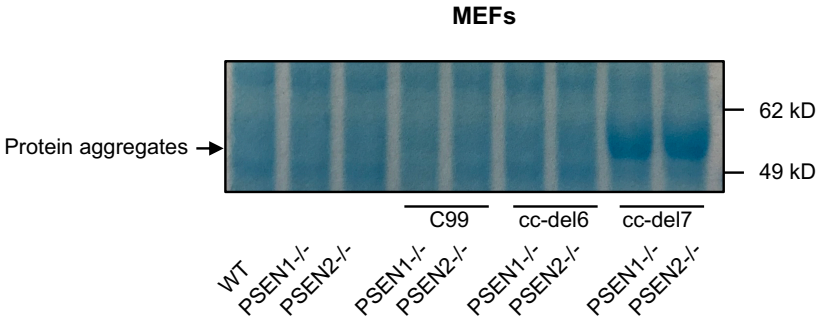
C43



C46



Supplementary Figure 11

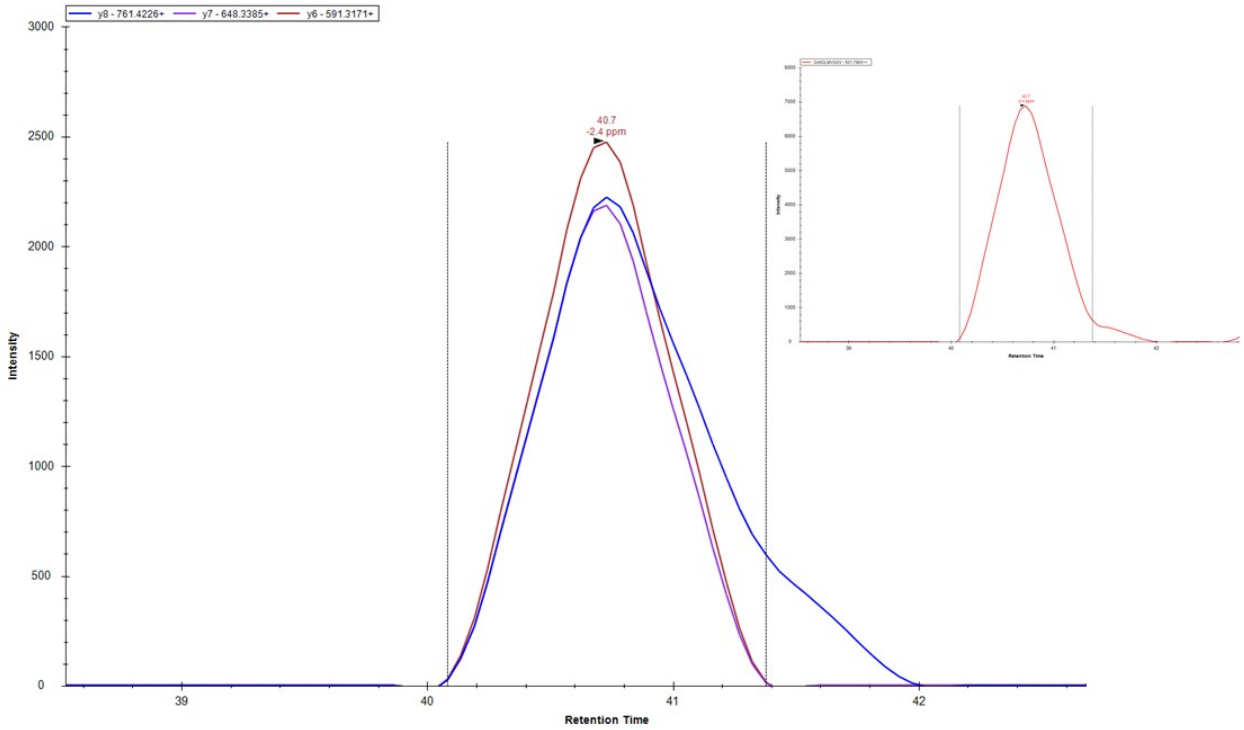


Supplementary Figure 12

MEFs PSEN1-/- and PSEN2-/-
Cell lysates

cc-del7

C39



C36