SUPPLEMENTAL INFORMATION

Spatial Snapshots of Amyloid Precursor Protein Intramembrane Processing via Early Endosome Proteomics

Hankum Park[1,2,^], Frances V. Hundley[1,2*], Qing Yu[1*], Katherine A. Overmyer[3,4], Dain R. Brademan[3,4], Lia Serrano[3], Joao A. Paulo[1], Julia C. Paoli[1,2], Sharan Swarup[1,2,#], Joshua J. Coon[3,4,5], Steven P. Gygi[1], and J. Wade Harper[1,2]

[1]Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA
[2]Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD 20815, USA
[3]Department of Biomolecular Chemistry, University of Wisconsin–Madison, Madison, WI 53706, USA
[4]Morgridge Institute for Research, Madison, WI 53715, USA
[5]Department of Chemistry, University of Wisconsin–Madison, Madison, WI 53706, USA
[^]Current address: Department of Dental Science, School of Dentistry and Dental Research Institute, Seoul National University, Seoul 03080, Republic of Korea
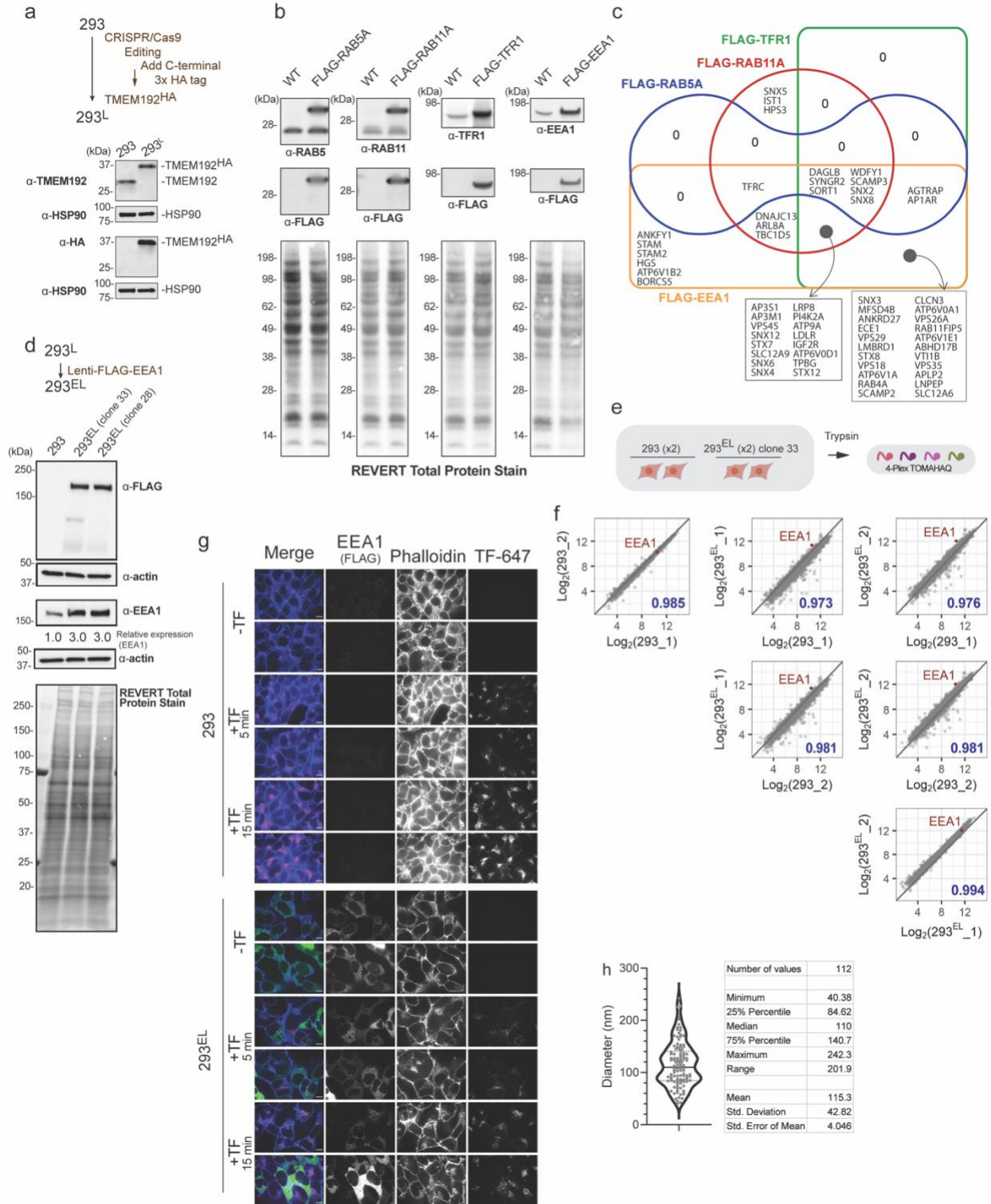[#]Current Address: Casma Therapeutics, Cambridge MA 02139
*, equal contribution

Corresponding author: wade_harper@hms.harvard.edu

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Supplementary Fig. 1. Identification of EEA1 as a candidate affinity reagent for early endosome purification.

a, Scheme summarizing how 293 cells were gene edited to place a C-terminal 3xHA tag on the endogenous TMEM192 gene as described[31] to create 293L cells. Cell extracts from 293 or 293L cells were subjected to immunoblotting with α-TMEM192 and α-HA to demonstrate homozygous targeting of the TMEM192 gene to

introduce the 3xHA tag, with α-HSP90 used as a loading control. α-TMEM192 and α-HA blots were on separate gels with separate α-HSP90 loading control blots.

b, The indicated FLAG-tagged proteins were stably expressed in 293[L] cells using a lentiviral construct. Cell extracts were subjected to immunoblotting with the indicated antibodies. Total proteins were stained with REVERT.

c, Overlap in endosomal proteins found in FLAG-EEA1, FLAG-TFR1, FLAG-RAB11, and FLAG-RAB5A immune complexes from non-detergent extracts.

d, 293[L] cells were transduced with a lentivirus expressing FLAG-EEA1 to create 293[EL] cells. Extracts from parental 293 or 293[EL] cells were subjected to immunoblotting with α-EEA1 or α-FLAG antibodies. Quantification of blots revealed ~3-fold higher levels of FLAG-EEA1 than the endogenous protein. Total proteins were stained with REVERT.

e,f, Total proteome analysis of duplicate cultures of 293 and 293[EL] cells. The relative abundance of individual proteins based on TMT reporter ion intensities are shown for all pair-wise combinations of biological duplicate cells (n=2) employed in the 8-plex TMT experiment. Pearson's coefficients (R) for pairwise comparisons are shown. The position of EEA1 is indicated by the red circle.

g, 293 or 293[EL] cells (clone 28) were serum starved for 1h prior to addition of serum-free media with or without Alexa-647-labeled TF (25 μg/mL). Cells were fixed at the indicated times and subjected to imaging to visualize Alexa-647-labeled TF, immunofluorescence of FLAG-EEA1, and phalloidin. Images are representative of 5 fields from two replicate experiments. Scale bar, 10 μm.

h, Size distribution of vesicles identified in FLAG-EEA1 immune complexes by transmission electron microscopy. The diameter for a total of 112 vesicles was measured.
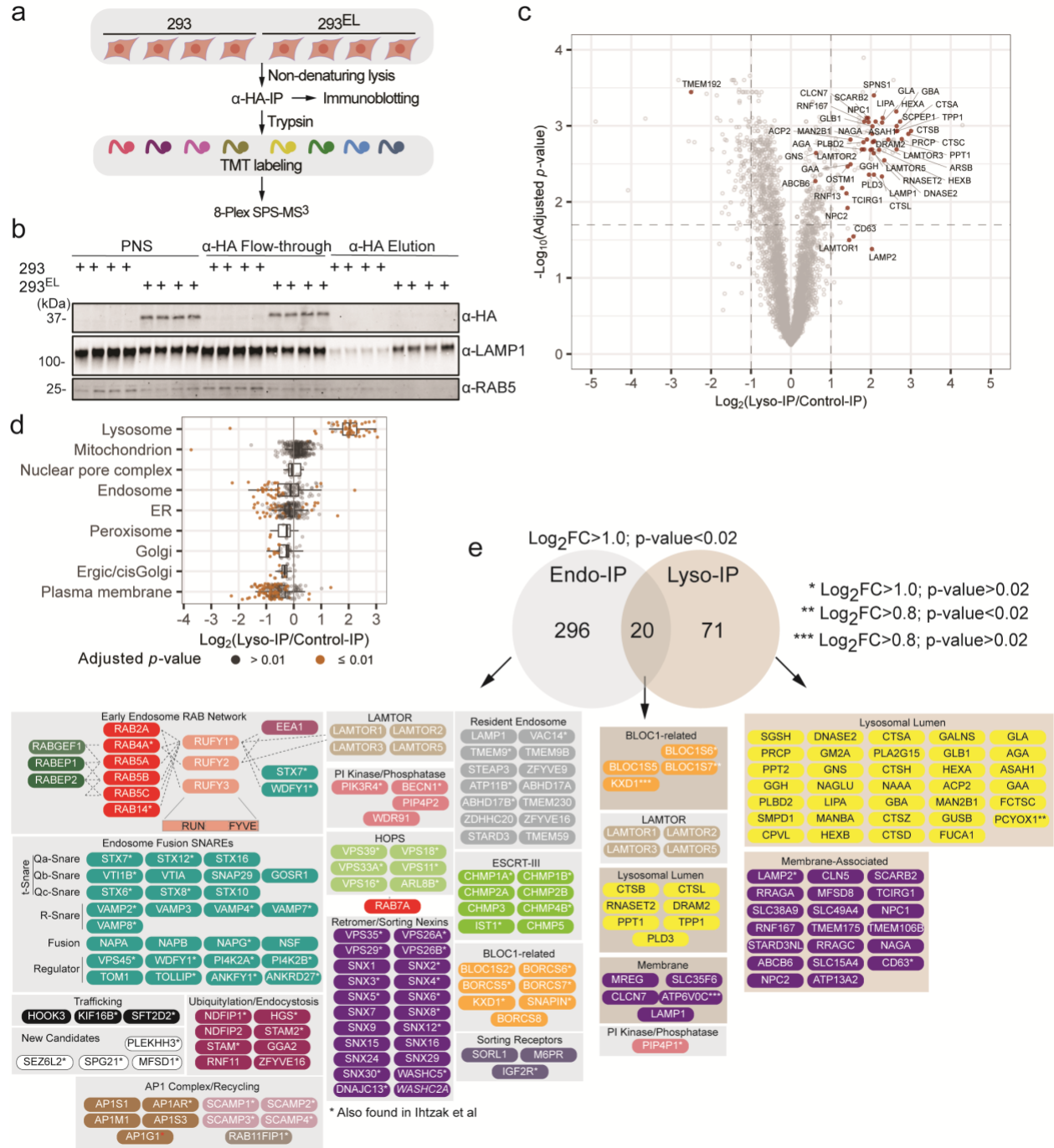
Supplementary Fig. 2. Landscape of the lysosomal proteome and endosomal lipidome.
a, Scheme depicting quantitative proteomics of the Lyso-IP proteome. 293 control cells or 293[EL] cells (clone 33) in biological quadruplicate (n=4) were subjected to the Lyso-IP protocol (α-HA IP) and either subjected to immunoblotting or trypsinized prior to labeling with TMT and analysis by mass spectrometry.
b, Immunoblot of proteins from the experiment outlined in panel a. Note, TMEM192[HA] is not released from the α-HA antibody magnetic beads under the elution conditions employed. For input and flow through, 0.05% of total was subjected to immunoblotting, whereas 6% of the elution was analyzed.
c, Proteomic analysis of lysosomes purified by Lyso-IP. Volcano plot for quadruple Lyso-IP associated proteins relative to control cells lacking TMEM192[HA] is shown. Selected proteins linked with lysosomes are

shown in red. Two-sided Student's t-test was performed and adjusted for multiple comparisons by two-stage Benjamini & Hochberg step-up procedure.

d, Box plots depicting the enrichment of various classes of proteins based on the annotation of Itzhak et al,[32] demonstrating that proteins assigned to lysosomes are the most highly enriched, while endosomal, ER, mitochondrial, and PM proteins are depleted. Orange dots correspond to proteins with an adjusted p-value < 0.01. Left border, interior line, and right border in the box plot represent the 1st quartile, median, and 3rd quartile, respectively.

e, Comparison of proteins associated with Endo- and Lyso-IPs. Proteins with $Log_2FC>1.0$ (p-value<0.02) present in either Endo- or Lyso-IPs were compared in a Venn diagram. Two-sided Student's t-test was performed and adjusted for multiple comparisons by two-stage Benjamini & Hochberg step-up procedure. Proteins that are shared or exclusive to each organelle enrichment method are shown.

a

293

	Input	flow-through	IP eluate	

Replicate: 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2
TF treatment (min): * * 0 0 5 5 15 15 * * 0 0 5 5 15 15 * * 0 0 5 5 15 15

(kDa)
75- α-TF
150-
100- α-LAMP1
25- α-RAB5

b

293[EL]

	Input	flow-through	IP eluate	

Replicate: 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2
TF treatment (min): * * 0 0 5 5 15 15 * * 0 0 5 5 15 15 * * 0 0 5 5 15 15

(kDa)
75- α-TF
100- α-LAMP1
25- α-RAB5

c

DMSO   Hydroxy Dynasore

RAB5

FLAG (EEA1)

Merge

EEA1+ and also RAB5+
RAB5+ and also EEA1+

○ Hydroxy Dynasore
○ DMSO

0   20   40   60   80
Mander's cofficient (%)

d

Class

□ Cer[NS]   ● PE-NMe2
○ CL   ⊠ PG
△ HexCer[NS]   ▣ PI
+ LysoPC   ▣ Plasmanyl-PC
× LysoPE   ▣ Plasmanyl-PE
◇ LysoPG   ▪ Plasmenyl-PE
▽ PA   ● PS
▣ PC   ▲ SM
✳ PE   ✦ SP
✚ PE-NMe   ● TG

$-\text{Log}_{10}$(Adjusted p-value)

Log_2(Endo-IP/Control-IP)

-2.5   0.0   2.5   5.0

e

Endosomal Phosphatidylcholine		Endosomal Plasmanyl-phosphatidylcholine	
Enriched	De-enriched	Enriched	De-enriched
PC 41:0	PC 38:5	O-39:0	O-38:6
PC 17:0_18:0	PC 38:7	O-34:0	O-42:6
PC 43:1	PC 40:7	O-28:0	O-40:6
PC 42:0	PC 34:3	O-16:0_15:0	
PC 36:0	PC 36:4	O-36:0	
PC 46:2	PC 17:1_18:1	O-38:0	
PC 40:0	PC 18:2_18:1	O-35:0	
PC 16:0_18:0	PC 36:4	O-35:0	
PC 38:0		O-34:1	
PC 44:2		O-16:0_16:0	
PC 31:0		O-16:0_15:0	
PC 16:0_16:0		O-39:0	
PC 38:4		O-32:1	
PC 15:0_16:0		O-38:1	
PC 14:0_16:0		O-16:0_17:0	
PC 14:0_15:0		O-38:2	
PC 32:1		O-37:0	
PC 40:1		O-42:2	
PC 42:1		O-38:1	
PC 14:0_14:0		O-30:0	
PC 36:1		O-34:1	
PC 16:0_17:0		O-38:2	
PC 42:2		O-34:1	
PC 16:1_18:1			
PC 14:0_16:1			

f

BMP 18:1_22:6

Log_2(Fold-enrichment)

Endo-IP   Lyso-IP

BMP 22:6_22:6

Log_2(Fold-enrichment)

Endo-IP   Lyso-IP

BMP 36:2

Log_2(Fold-enrichment)

Endo-IP   Lyso-IP

**Supplementary Fig. 3. Analysis of Endo-IP for capture of dynamic cargo and lipidome analysis.**

a-b, Immunoblots of Endo-IPs from the experiment outlined in Fig. 3a. 293 or 293[EL] cells (clone 33) were serum starved for 1h prior to addition of serum-free media supplemented with 25 µg/ml holo-transferrin (TF) in duplicate (n=2). At the indicated times, cells were harvested and extracts subjected to Endo-IP followed by immunoblotting with the indicated antibodies. Control cells not serum starved (*). The input and flow-through after the α-FLAG IP are also shown. For input and flow through, 0.5% of total was subjected to immunoblotting, whereas 6% of the elution was analyzed. Proteomic analysis of these samples is shown in **Fig. 3c,d**.

c, 293[EL] cells (clone 33) were treated with DMSO (as control) or Hydroxy Dynasore (20 µM, 3h) prior to immunofluorescence using α-FLAG (green, to detect FLAG-EEA1) and α-RAB5 (magenta) antibodies, and nuclei were stained with Hoechst 33342 (blue). Image analysis indicates that the Mander's coefficient is ~0.7 for both the overlap of FLAG puncta with RAB5 and RAB5 puncta with FLAG over 10 cells.

d, Volcano plot depicting the enrichment of various lipid classes within Endo-IP as compared with control immune complexes (data from **Supplementary Data 6**) (n=3). Two-sided Student's t-test was performed and adjusted for multiple comparisons by two-stage Benjamini & Hochberg step-up procedure.

e, Individual phosphatidylcholine species found to be enriched or de-enriched in Endo-IPs based on lipidomics (data from **Supplementary Data 6**).

f, Log_2FC for the indicated BMP species identified by lipidomics in either Endo-IP or Lyso-IP samples (n=3) (data from **Supplementary Data 6**).
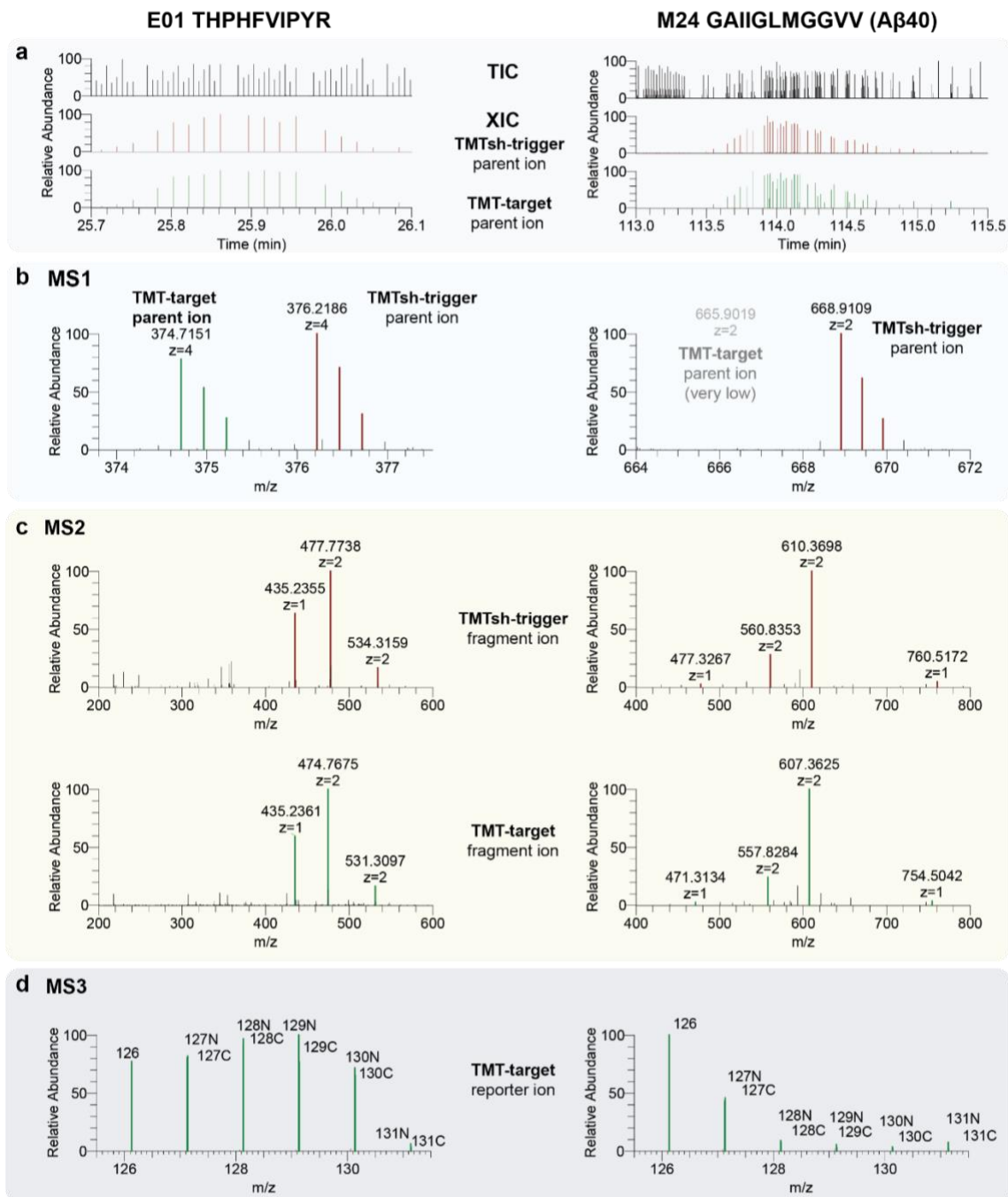
Supplementary Fig. 4. Half-tryptic peptides for quantification of Aβ peptides.
a-d, MS[2] fragmentation patterns for selected APP peptides, indicating fragment ions employed as triggers for TOMAHAQ proteomics.
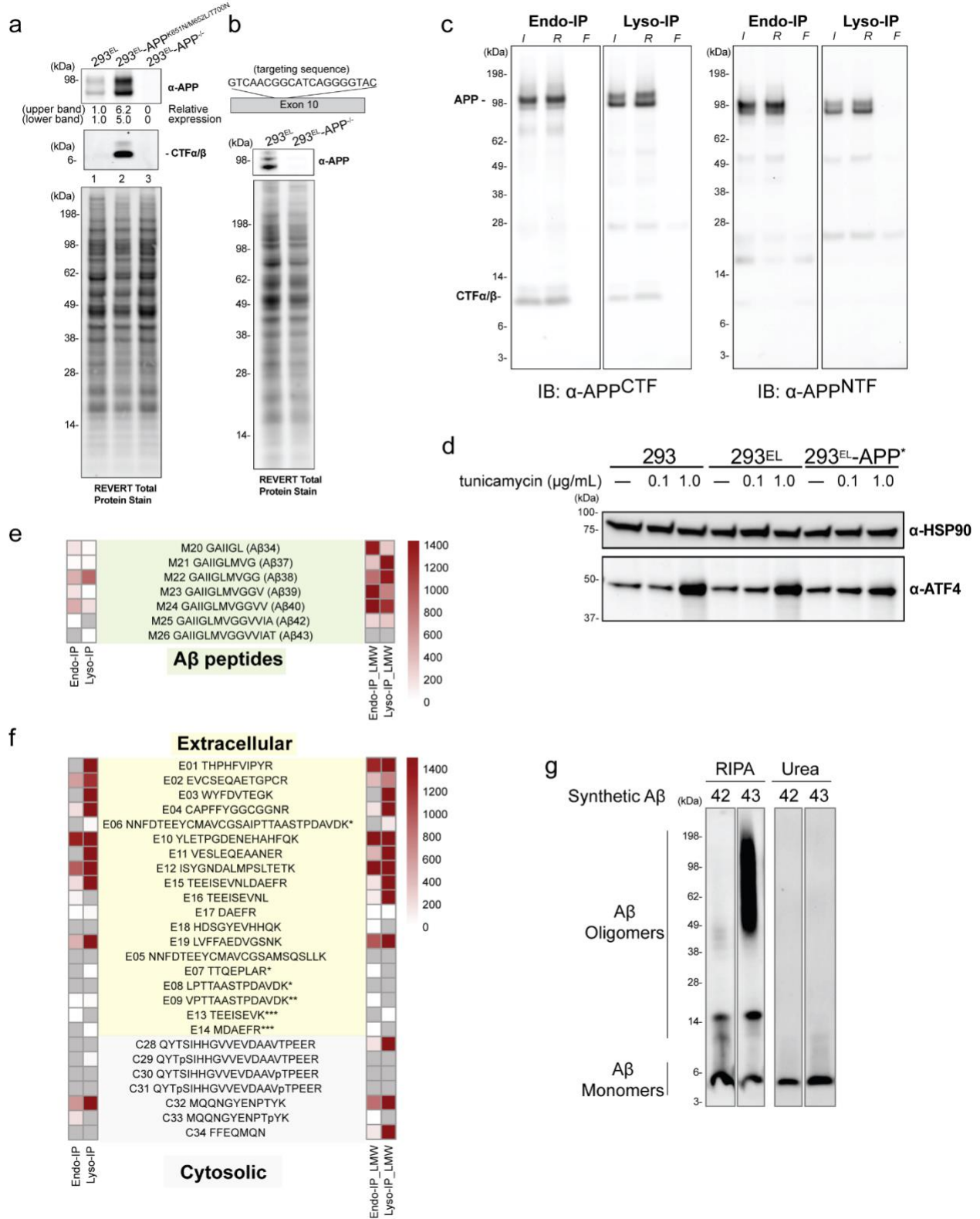
Supplementary Fig. 5. Application of spatial endolysosomal proteomics to analysis of APP processing.
a, 293^{EL}-APP$^{-/-}$ cells (see panel b) were transduced with a lentiviral vector expressing APP$^{Sw;T700N}$ to generate 293^{EL}-APP* and extracts from these cells immunoblotted with the indicated antibodies. APP was expressed at

6-fold higher levels than endogenous APP in parental cells. CTFα/β were not appreciably detected in 293^{EL} cells.

b, Creation of 293^{EL} cells lacking APP. The indicated CRISPR gRNA was used to create a frameshift in exon 14 of APP. Extracts from parental and APP$^{-/-}$ cells were subjected to immunoblotting to demonstrate loss of APP, using total amount of loaded proteins as a loading control.

c, Full immunoblots of data shown in **Fig. 5f** probed with α-C-terminal APP (α-APP^{CTF}) and α-N-terminal APP (α-APP^{NTF}) antibodies.

d, 293^{EL} or 293^{EL}-APP* cells were either left untreated or treated with Tunicamycin (1h) as a positive control for induction of the integrated stress response, and cell extracts probed with α-ATF4.

e,f, Heat maps showing the signal/noise (S/N) ratios for APP peptides in the context of biological duplicate Endo-IP or Lyso-IP, with or without LMW enrichment (n=2). Gray squares indicate cases where the endogenous peptide was below the detection limit, whereas white squares indicate peptide detection but with extremely low S/N.

g, SDS-PAGE analysis of synthetic Aβ42/43 solubilized in either RIPA buffer or 8 M urea. Peptides were detected by α-Aβ (6E10). Samples solubilized in RIPA buffer have detectable oligomers and are observed as monomers when solubilized in urea.
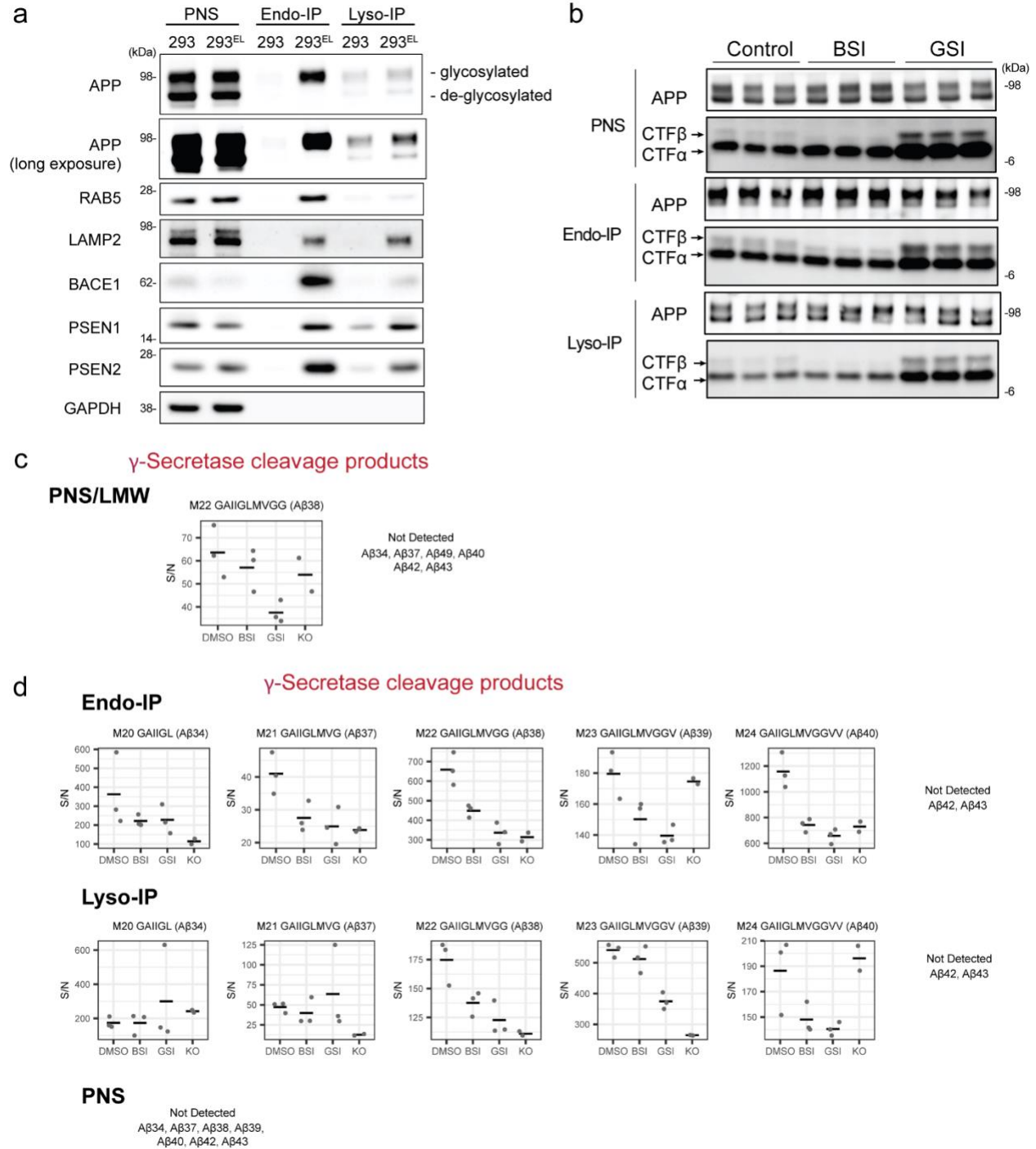
Supplementary Fig. 6. Digital snapshots of APP/Aβ processing in early endosome and lysosomal compartments.

a, 293 or 293[EL] cells were subjected to Endo-IP (α-FLAG) or Lyso-IP (α-HA) and immune complexes lysed in RIPA buffer prior to immunoblotting with the indicated antibodies. PNS fractions were analyzed in parallel. The positions of glycosylated and de-glycosylated APP are indicated.

b, Immunoblots of full-length APP and the CTFα/β fragments in the PNS, Endo-IP or Lyso-IP corresponding to the experiments shown in **Fig. 6b,c** (biological triplicates, n=3). The images for Endo-IP and Lyso-IP are the same images as in **Fig. 6c** but are shown here to allow comparison with the PNS sample.

c, Signal/noise plots for γ-Secretase cleavage products identified in the PNS sample with LMW enrichment corresponding to **Fig. 6a,b** (biological triplicates, n=3).

d, Signal/noise plots for γ-Secretase cleavage products from PNS, Endo-IP, and Lyso-IP without LMW enrichment. Peptides below the detection limit are indicated (biological triplicates, n=3).
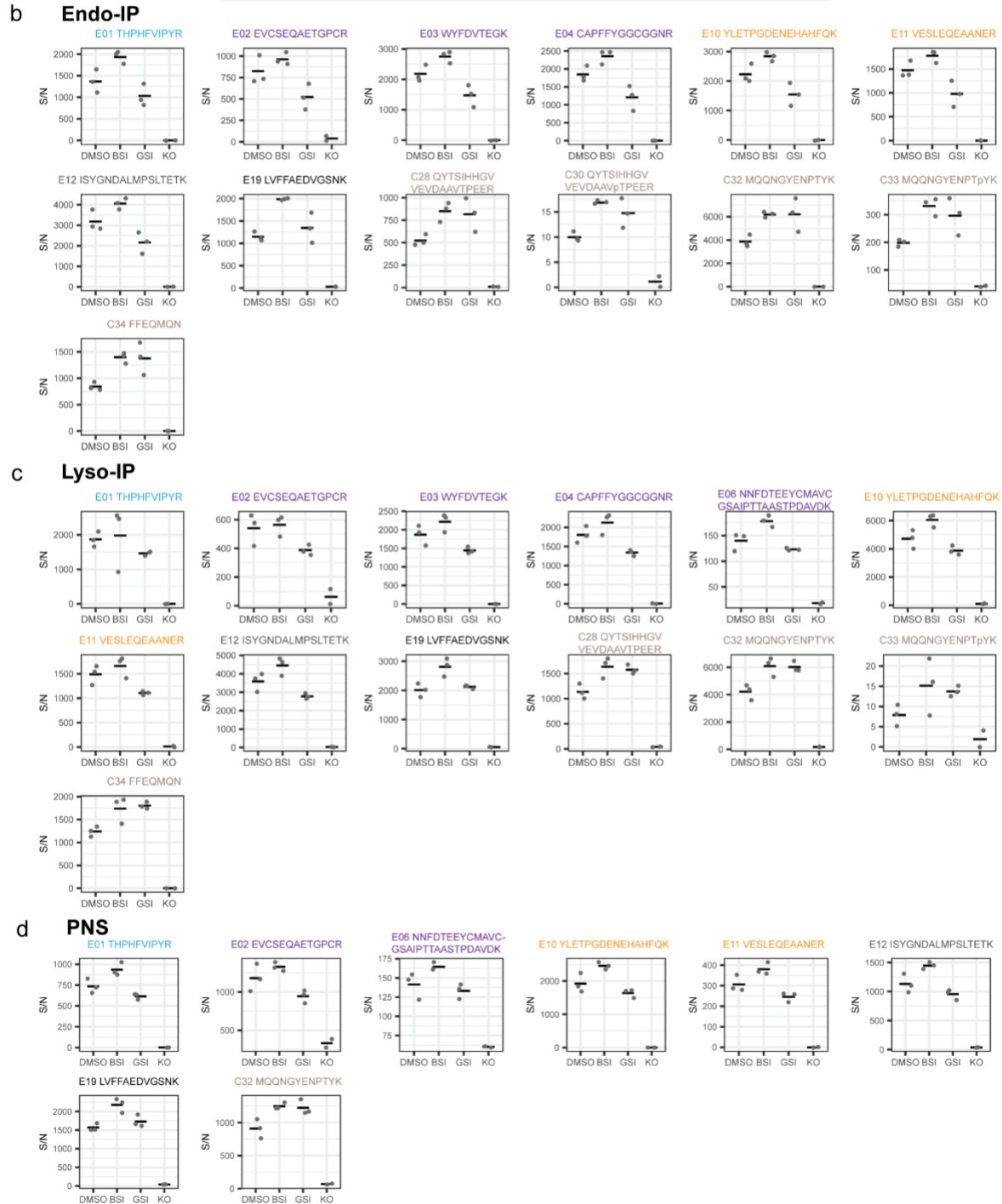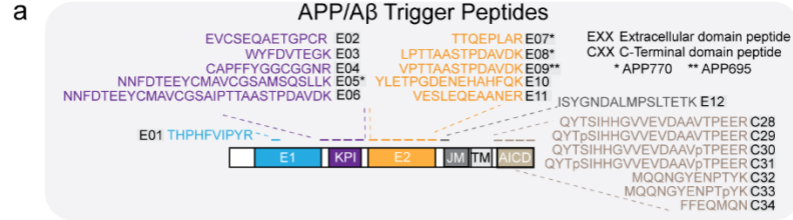
Supplementary Fig. 7. Analysis of peptides derived from extracellular and C-terminal domains in APP present in PNS, Endo-IP and Lyso-IP.

a, Domain schematic of APP indicating the sequences and locations of APP trigger peptides in these regions of the protein.

b-d, Signal/noise plots for peptides derived from the extracellular and C-terminal domains of APP present in the Endo-IP (panel B), Lyso-IP (panel C), and PNS (panel D) without LMW enrichment (corresponding to **Fig. 6a,b**) (biological triplicates, n=3). Color of peptide sequences corresponds to the domain localization in panel A. Peptides not shown were not detected above the signal seen in samples from 293[EL]-APP^{-/-} cells.
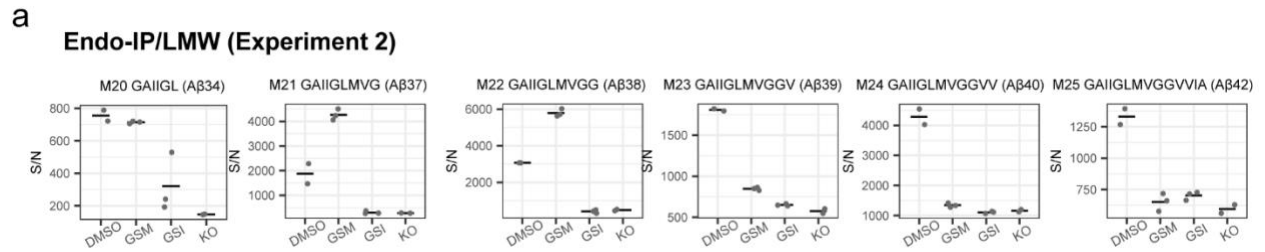
Supplementary Fig. 8. Quantitative assessment of γ-secretase modulator action on APP in early endosomes.

a, Quantitative analysis of Aβ peptides in biological triplicate (n=3) Endo-IP samples after LMW filtration from a fully independent replication of the Endo-IP experiment in **Fig. 7a**. Signal-to-noise for MS^3 intensities (relative to APP$^{-/-}$ cells, n=2) is shown. Asterisks refer to *t*-test of DMSO treated samples versus compound treatment: *n.s.*, not significant; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.