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Supplemental information

Exploring the landscape of ectodomain shedding by quantitative protein terminomics

Kazuya Tsumagari, Chih-Hsiang Chang, and Yasushi Ishihama

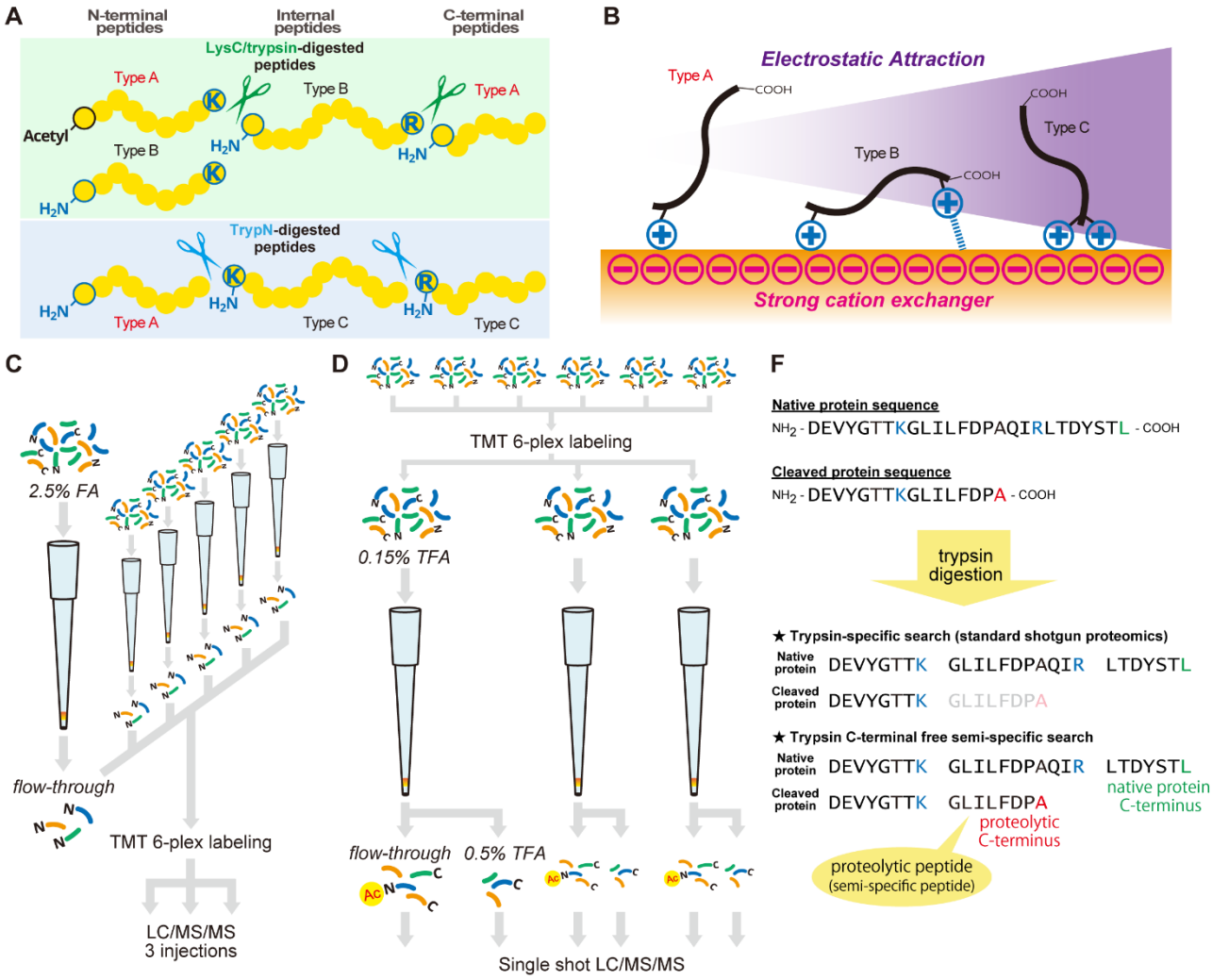
A
N-terminal peptides
Internal peptides
C-terminal peptides
LysC/trypsin-digested peptides
Type A
Type B
Type A
Acetyl
Type B
TrypN-digested peptides
Type A
Type C
Type C
B
Electrostatic Attraction
Type A
COOH
Type B
COOH
Type C
COOH
Strong cation exchanger
C
2.5% FA
flow-through
TMT 6-plex labeling
LC/MS/MS
3 injections
D
TMT 6-plex labeling
0.15% TFA
flow-through
0.5% TFA
Single shot LC/MS/MS
F
Native protein sequence
NH2 - DEVYGTTKGLILFDPAQIRLTDYSTL - COOH
Cleaved protein sequence
NH2 - DEVYGTTKGLILFDPA - COOH
trypsin digestion
★ Trypsin-specific search (standard shotgun proteomics)
Native protein DEVYGTTK GLILFDPAQIR LTDYSTL
Cleaved protein DEVYGTTK GLILFDPA
★ Trypsin C-terminal free semi-specific search
Native protein DEVYGTTK GLILFDPAQIR LTDYSTL
native protein C-terminus
Cleaved protein DEVYGTTK GLILFDPA
proteolytic C-terminus
proteolytic peptide (semi-specific peptide)
E
Acetylated N-terminal peptide
C-terminal peptide
% of peptides
0
25
50
75
A-431
A549
HCT-116
HeLa
Hep G2
MCF-7
PC-3
SH-SY5Y
SK-BR-3
U-251 MG
G
PMA + DMSO
PMA + BB-94
Protein (µg)
0
100
200
300
400
A-431
A549
HCT-116
HeLa
Hep G2
MCF-7
PC-3
SH-SY5Y
SK-BR-3
U-251 MG

Figure S1. Terminal peptide enrichment by SCX, Related to Figure 1.

(A, B) Digestion with TrypN yields peptides with at least a +2 charge with Lys or Arg and an α-amino group at the N-terminus (Type C). On the other hand, peptides derived from protein N-termini have a +1 charge with neither Lys nor Arg, and only His-containing peptides with an unmodified N-terminus have a +2 charge (Type A). In addition, digestion with trypsin yields internal peptides with at least a +2 charge (Type B). In this case, C-terminal peptides without His have a +1 charge and those with His have a +2 charge (Type A). Based on these facts, we isolated N-terminal peptides and C-terminal peptides using SCX at low pH, respectively. Note that acetylated N-terminal peptides generated by trypsin/LysC digest have a 0 or +1 charge (Type A) and are isolated together with C-terminal peptides.

(C) For N-terminal peptide enrichment, TrypN-digested peptides were dissolved in 2.5% formic acid (FA) containing 30% acetonitrile (ACN) and loaded on the SCX-StageTip. The flow-through fraction was collected. Following TMT-labeling, the isolated N-terminal peptides were analyzed in triplicate.

(D) TMT-labeled peptides were divided into three parts and subjected to C-terminal peptide enrichment using three SCX-StageTips. Peptides were dissolved in 0.15% trifluoroacetic acid (TFA) containing 30% ACN and loaded on the SCX-StageTip. The flow-through and the 0.5% TFA-eluted fractions were separately collected and subjected to single shot analysis. (E) Efficiency of C-terminal peptide enrichment. As described above, acetylated N-terminal peptides were isolated together with C-terminal peptides. Remaining % peptides mainly correspond to internal peptides (Type B peptides in Figure S1A).

(F) Example of terminus and cleavage site identification by trypsin semi-specific search. In standard shotgun proteomics, only peptides whose N- and C-termini are both in accord with trypsin specificity are considered. The C-terminus of native protein C-terminal peptide does not match to the specificity of trypsin (cleavage at C-terminal side of K/R, highlighted in blue), but can be identified (highlighted in green). In C-terminal free semi-specific search, the peptides whose C-terminus is not with the specificity of trypsin (highlighted in red) can be additionally identified. We refer to the total of native protein termini and proteolytic termini as “all termini” in Figure 1E.

(G) The protein yields from a biological replicate consisting of three 15 cm dishes are shown for each condition. For each condition, three replicates were prepared, and the data are presented as mean ± standard error of the mean (SEM). The approximate number of cells per 15 cm dish at 90% confluency ranged from 10 million (HeLa) to 30 million (SH-SY5Y).

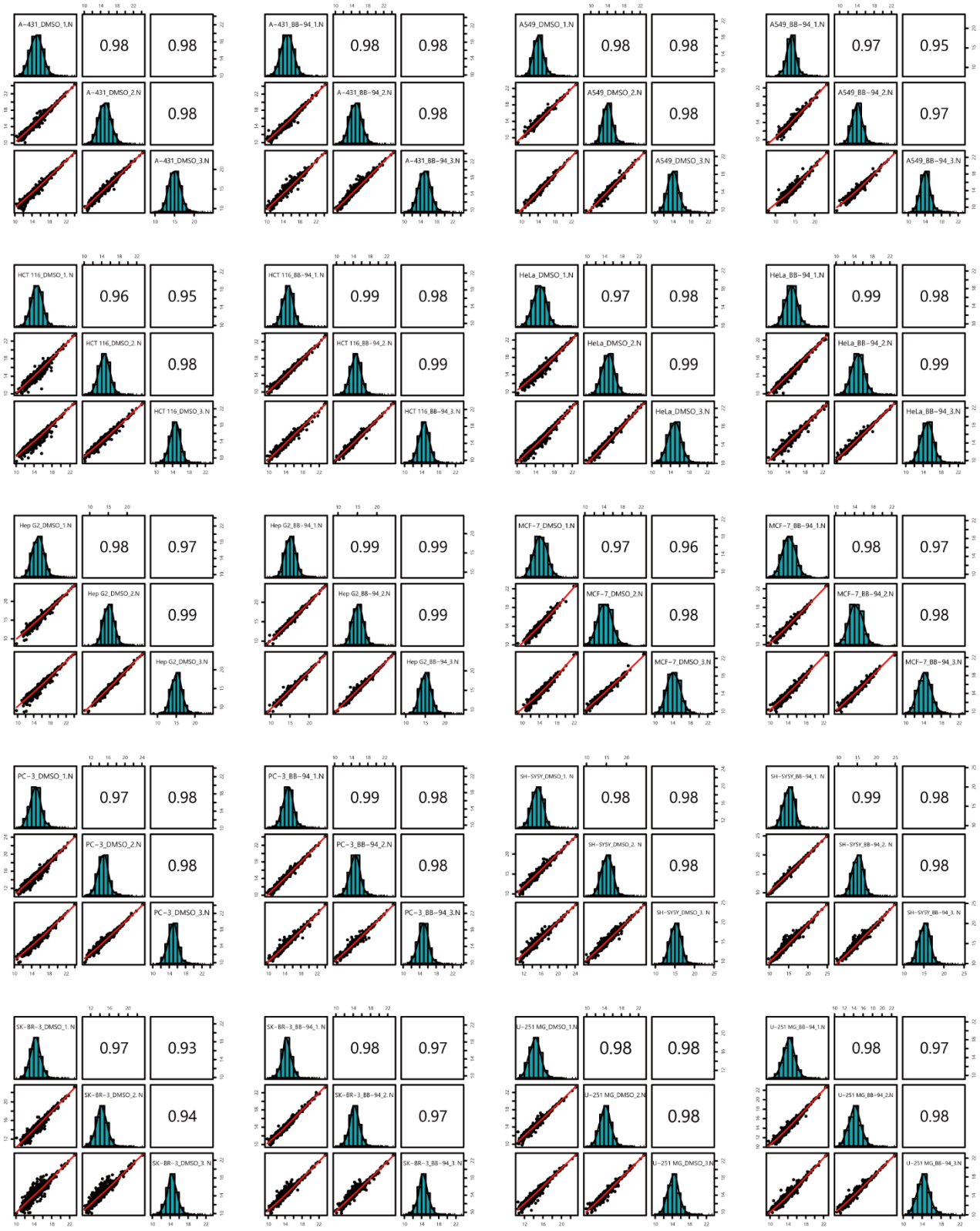

Figure S2. Reproducibility of N-terminomics, Related to Figure 1.

Logarithmized normalized TMT-reporter intensities of N-terminal peptides are plotted against each other for triplicate determinations in each condition, and the corresponding Pearson correlation coefficients are shown.

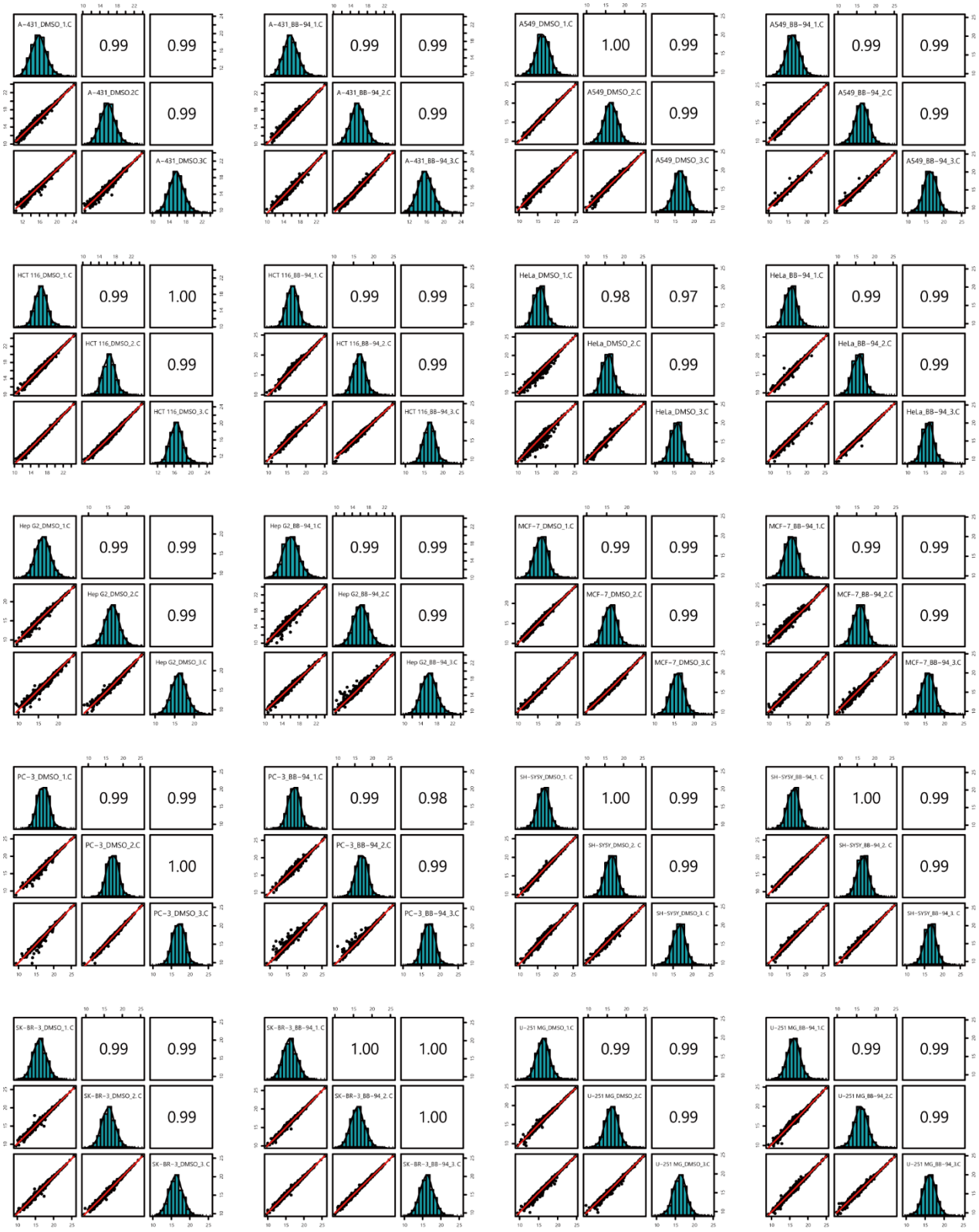

Figure S3. Reproducibility of C-terminomics, Related to Figure 1.

Logarithmized normalized TMT-reporter intensities of C-terminal peptides are plotted for against each other for triplicate determinations in each condition, and the corresponding Pearson correlation coefficients are shown.

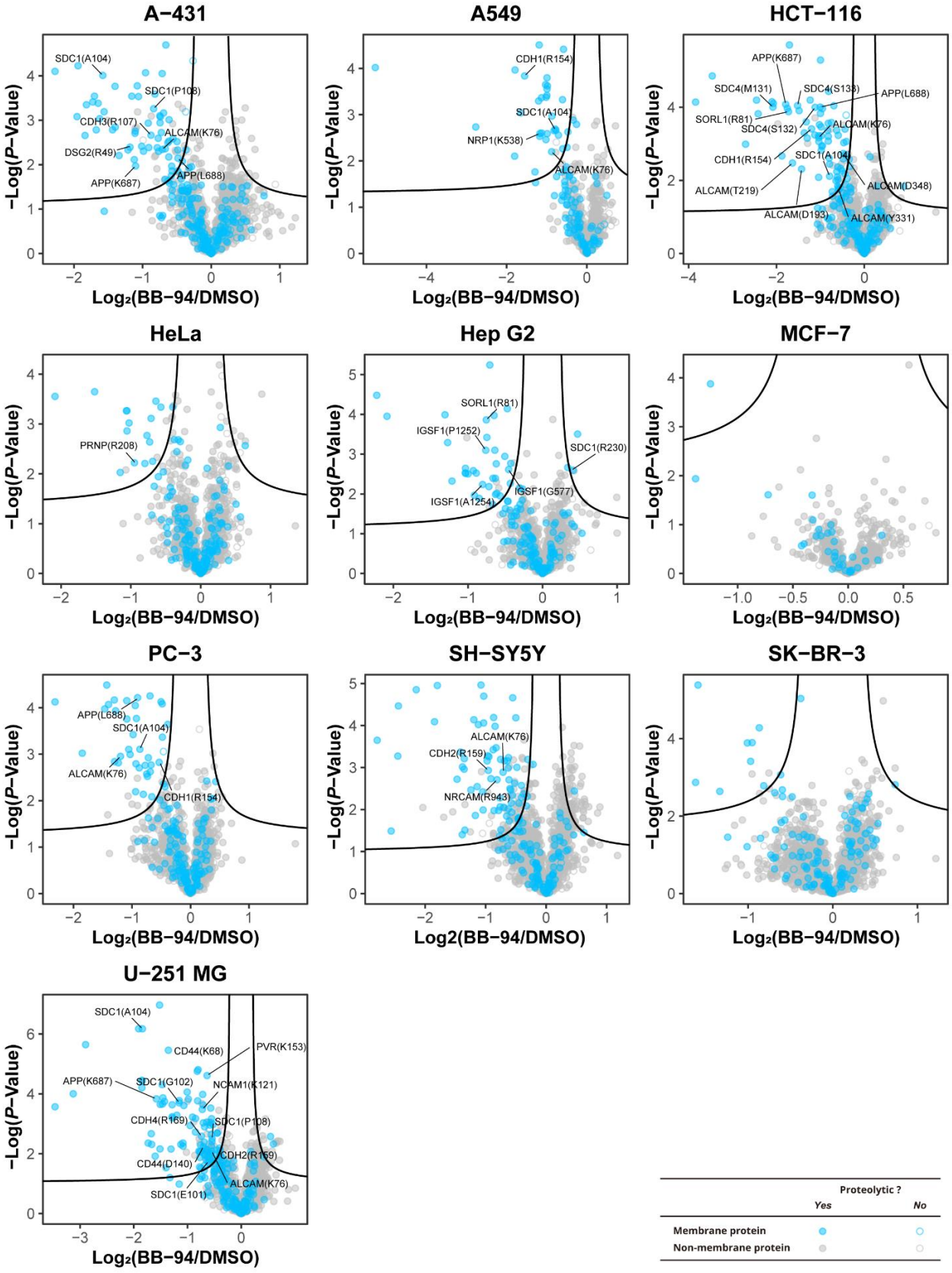
A−431
SDC1(A104)
SDC1(P108)
CDH3(R107)
ALCAM(K76)
DSG2(R49)
APP(L688)
APP(K687)
A549
CDH1(R154)
SDC1(A104)
NRP1(K538)
ALCAM(K76)
HCT−116
APP(K687)
SDC4(M131)
SDC4(S138)
APP(L688)
SORL1(R81)
SDC4(S132)
ALCAM(K76)
CDH1(R154)
SDC1(A104)
ALCAM(T219)
ALCAM(D348)
ALCAM(D193)
ALCAM(Y331)
HeLa
PRNP(R208)
Hep G2
SORL1(R81)
IGSF1(P1252)
SDC1(R230)
IGSF1(G577)
IGSF1(A1254)
MCF−7
PC−3
APP(L688)
SDC1(A104)
ALCAM(K76)
CDH1(R154)
SH−SY5Y
ALCAM(K76)
CDH2(R159)
NRCAM(R943)
SK−BR−3
U−251 MG
SDC1(A104)
CD44(K68)
PVR(K153)
APP(K687)
SDC1(G102)
NCAM1(K121)
CDH4(R169)
SDC1(P108)
CD44(D140)
CDH2(R159)
ALCAM(K76)
SDC1(E101)
−Log(P−Value)
Log₂(BB−94/DMSO)
Log2(BB−94/DMSO)
Proteolytic ?
Yes
No
Membrane protein
Non-membrane protein

Figure S4. Volcano plots for each cell line in N-terminomics, Related to Figure 1.

For quantified N-terminal peptides in the respective cell lines, volcano plots were created using Perseus with truncation at the false discovery rate of 0.05 and an artificial within groups variance (S_0) of 0.1. N-Terminal peptides of membrane proteins are highlighted in color, and proteolytic termini are highlighted with filled circles. The sites mentioned in the main text are labeled with their gene name and the P1 site.

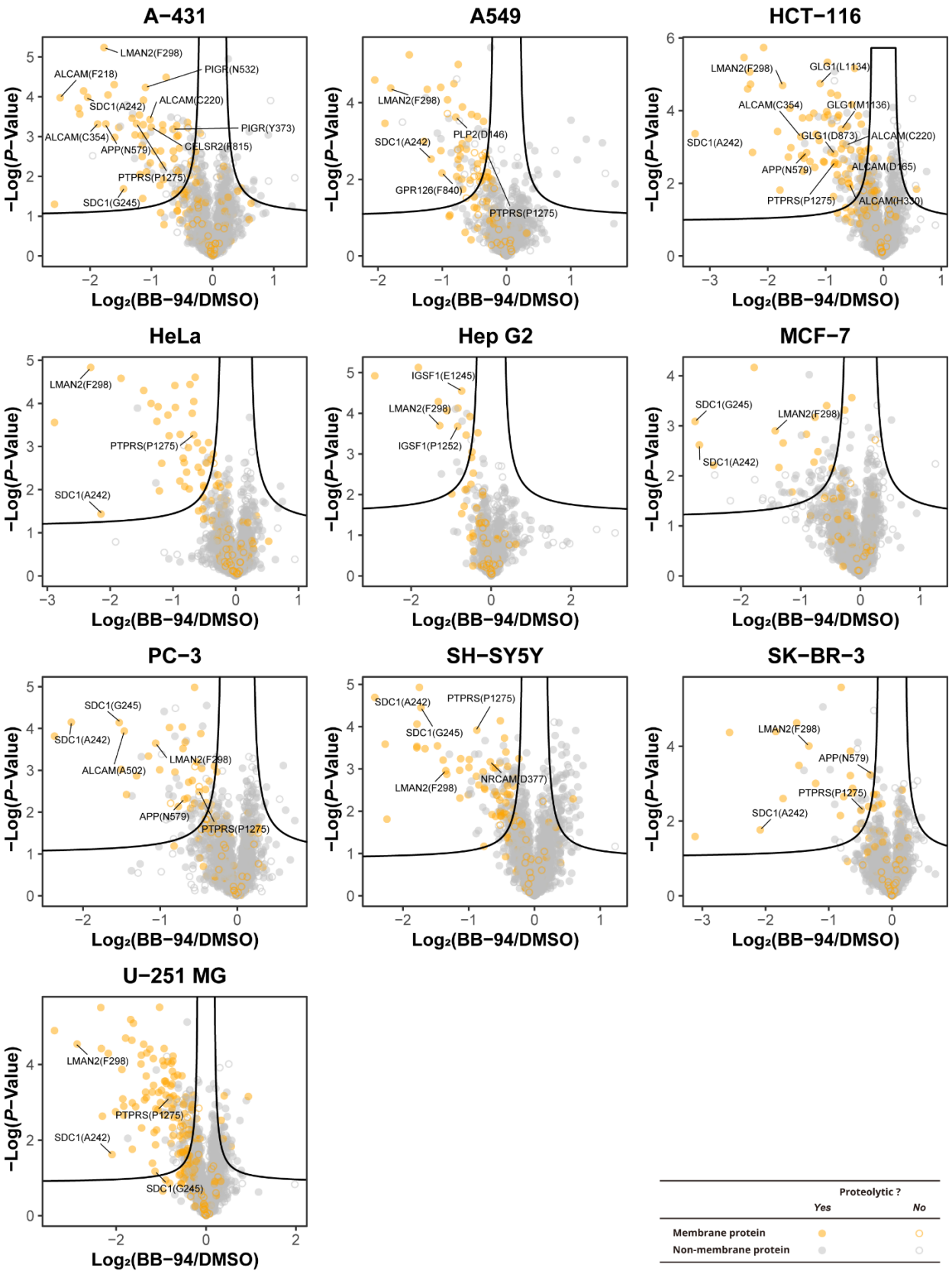
A−431
LMAN2(F298)
ALCAM(F218)
PIGR(N532)
SDC1(A242)
ALCAM(C220)
PIGR(Y373)
ALCAM(C354)
APP(N579)
CELSR2(R815)
PTPRS(P1275)
SDC1(G245)
−Log(P-Value)
Log₂(BB−94/DMSO)
A549
LMAN2(F298)
PLP2(D146)
SDC1(A242)
GPR126(F840)
PTPRS(P1275)
HCT−116
LMAN2(F298)
GLG1(L1134)
ALCAM(C354)
GLG1(M1136)
GLG1(D873)
ALCAM(C220)
SDC1(A242)
APP(N579)
ALCAM(D165)
PTPRS(P1275)
ALCAM(H330)
HeLa
LMAN2(F298)
PTPRS(P1275)
SDC1(A242)
Hep G2
IGSF1(E1245)
LMAN2(F298)
IGSF1(P1252)
MCF−7
SDC1(G245)
LMAN2(F298)
SDC1(A242)
PC−3
SDC1(G245)
SDC1(A242)
LMAN2(F298)
ALCAM(A502)
APP(N579)
PTPRS(P1275)
SH−SY5Y
SDC1(A242)
PTPRS(P1275)
SDC1(G245)
NRCAM(D377)
LMAN2(F298)
SK−BR−3
LMAN2(F298)
APP(N579)
PTPRS(P1275)
SDC1(A242)
U−251 MG
LMAN2(F298)
PTPRS(P1275)
SDC1(A242)
SDC1(G245)
Proteolytic ?
Yes
No
Membrane protein
Non-membrane protein

Figure S5. Volcano plots for each cell line in C-terminomics, Related to Figure 1.

For quantified C-terminal peptides in the respective cell lines, volcano plots were created using Perseus with truncation at the false discovery rate of 0.05 and an artificial within groups variance (S_0) of 0.1. C-Terminal peptides of membrane proteins are highlighted in color, and proteolytic termini are highlighted with filled circles. The sites mentioned in the main text are labeled with their gene name and the P1 site.

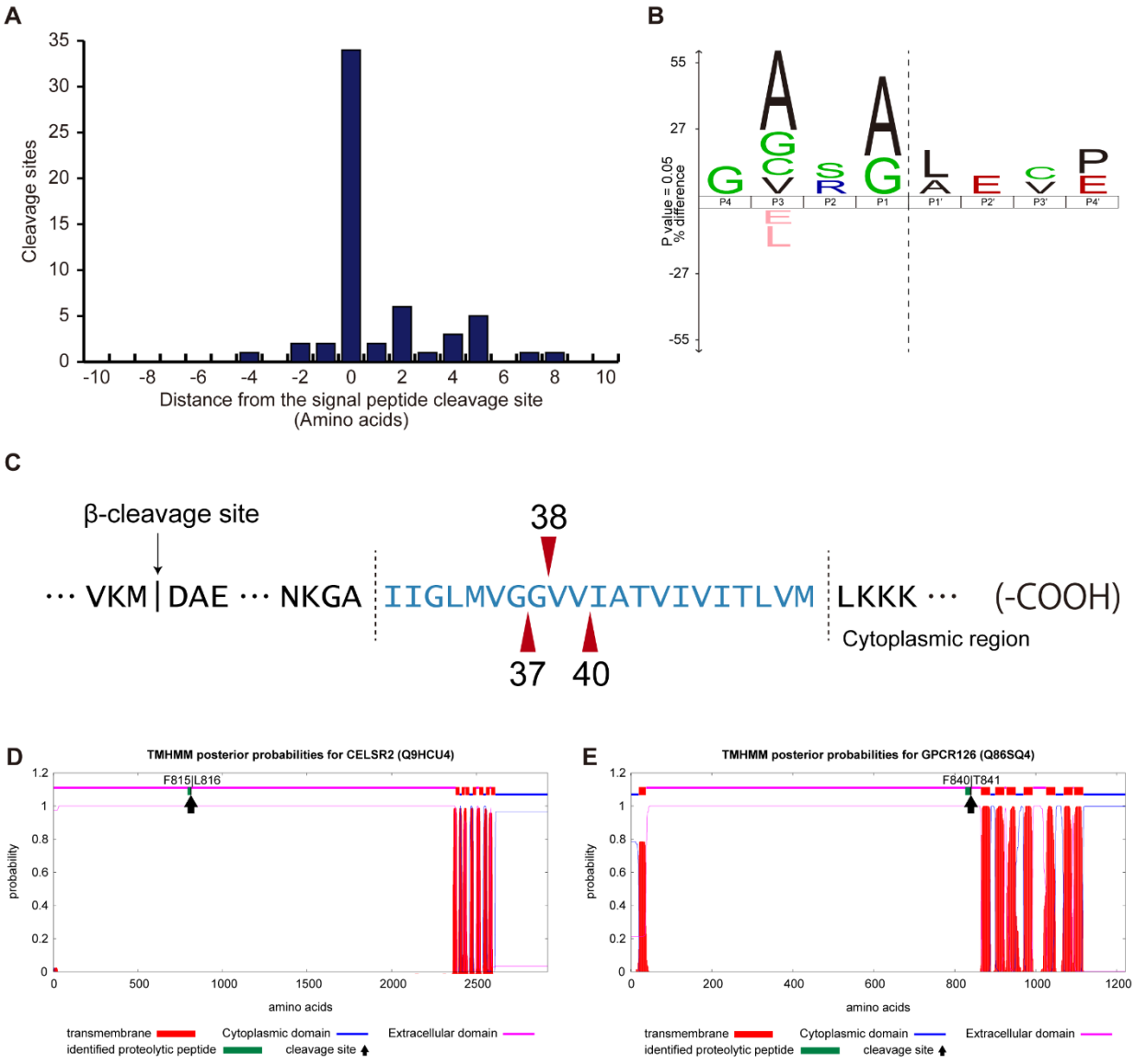

Figure S6. Signal peptide cleavage sites, APP γ-cleavage sites, and multi-pass membrane protein cleavage sites, Related to Figure 3.

(A) Distribution of the distance (number of amino acids) from the predicted signal peptide cleavage site to the identified cleavage site.

(B) Sequence logo of the cleavage sites located in the vicinity of the predicted signal peptide cleavage sites (±4 amino acids) generated by iceLogo. The dashed line shows the cleavage site.

(C) Downregulated γ-cleavage sites within the transmembrane domain of APP. The arrowheads indicate the identified cleavage sites, and the numbers indicate the length (number of amino acids) of generated amyloid β peptides.

(D, E) The extracellular domain and cytoplasmic domain were predicted for CELSR2 and GPCR126 using the TMHMM server. The identified proteolytic peptides and cleavage sites are shown together.


ADAM10
ADAM17
MMP-2
MMP-3
MMP-7
MMP-8
MMP-9
MMP-12
MMP-13
MT−MMP1
Meprin β
Furin
PCSK7
Cathepsin L
Cathepsin S
Legumain
Residue
Position
V Y W T S P F M K L I H G E Q C D N R A
P4 P3 P2 P1 P1′ P2′ P3′ P4′
Scale (log2)
−4 −3 −2 −1 0 1 2 3 4

Figure S7. PWMs for 16 selected sheddases, Related to Figure 6.

For the selected sheddases, PWMs were computed based on the sequences of ±4 amino acids flanking the substrate cleavage sites reported in MEROPS.

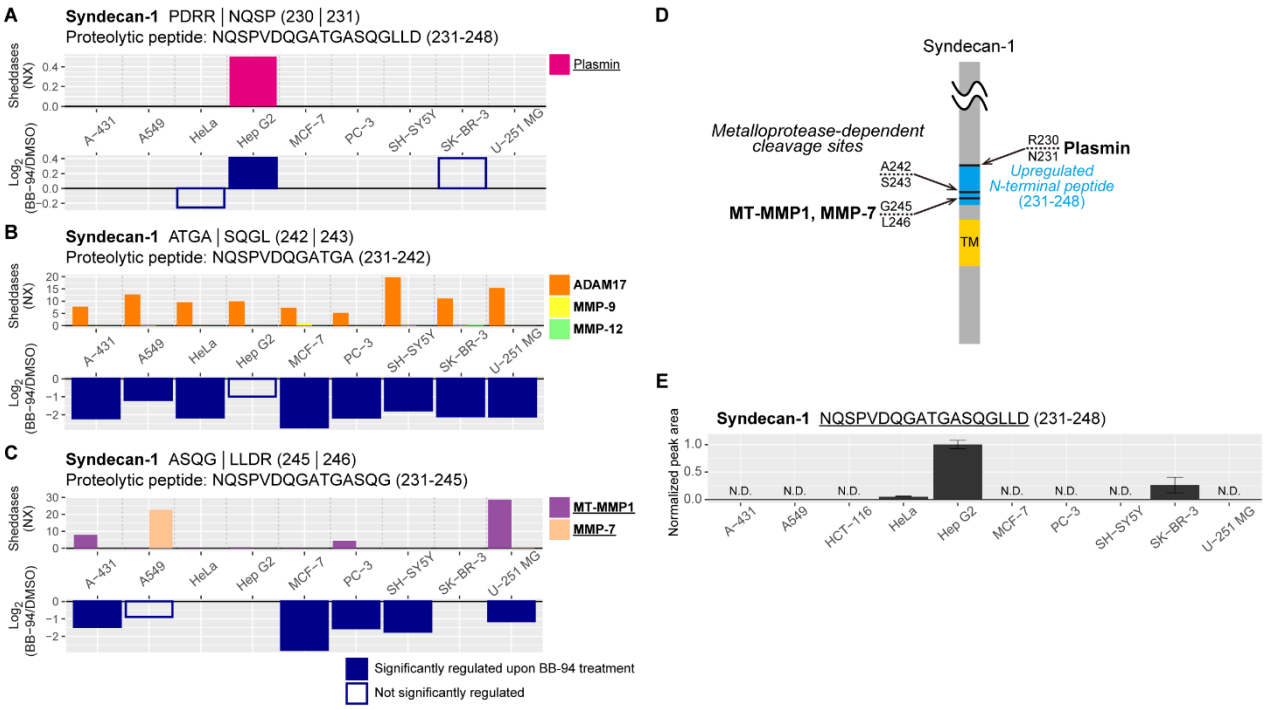

Figure S8. Sheddase expression profiles and proteolytic sites, Related to Figure 6.

(A-C) For syndecan-1 cleavage sites that have been registered with MEROPS, the quantitative results of our terminomics (BB-94/DMSO) and the mRNA expression profiles of the putative responsible sheddases (PWM score >2 in Figure 6B and/or those registered with MEROPS as responsible sheddases) obtained from the Human Protein Atlas are shown. Note that the cleavage at syndecan-1 R230↓N231 was not downregulated but upregulated by BB-94 treatment and that the expression profile in HCT-116 was not available. The sheddases with PWM score >2 are shown in bold, and the sheddases that have been registered as responsible sheddases are underlined. NX, normalized expression.

(D) Summary of syndecan-1 cleavage sites in Hep G2 cells. The identified peptide presenting the upregulated site is marked with blue. According to MEROPS, cleavage at R230↓N231 is mediated by plasmin, while cleavage at R245↓L246 is mediated by MT-MMP1 or MMP-7. The cleavage at R242↓S243 has been reported, but the responsible sheddase is unknown. TM, transmembrane domain.

(E) Peak area of the syndecan-1 proteolytic N-terminal peptide (231-NQSPVDQGATGASQGLLD-248) in each cell line (*N* = 3). The error bars show the standard error of the mean. N.D., not detected.

Transparent Methods

Cells

Basically, cells were cultured in Dulbecco's modified Eagle's medium (DMEM; FUJIFILM Wako) supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin and 100 µg/mL streptomycin (FUJIFILM Wako) on 15 cm culture dish at 37°C in a humidified incubator with 5% CO_2. PC-3 cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI1640; FUJIFILM Wako) with the same supplements as listed above. SH-SY5Y cells and U-251 MG cells were cultured in DMEM/Ham's F-12 (FUJIFILM Wako) including non-essential amino acids (FUJIFILM Wako) and the same supplements as listed above. We did not perform specific authentication of the cell lines used in this study.

Nine dishes of 90% confluent cells per condition were prepared. Supernatants of three dishes were pooled during supernatant collection to make a biological replicate. Protein yields from the supernatants for each cell line are summarized in Figure S1G.

Inhibitor treatment and supernatant preparation

Cells were washed with phosphate-buffered saline (PBS; FUJIFILM Wako) three times and incubated for 1 h with Hanks' balanced salt solution containing Ca^{2+} and Mg^{2+} (HBSS (+); FUJIFILM Wako) together with 10 µM BB-94 (Selleck) or DMSO (FUJIFILM Wako) for inhibitor pretreatment. Then, the buffer was replaced with fresh HBSS (+) containing 1 µM PMA (FUJIFILM Wako) and 20 µM BB-94 or DMSO, and the cells were incubated for 1 h. The supernatants were quickly collected on ice, and centrifuged at 7,180 *g* for 30 min to remove cell debris. Then 2 mM ethylenediaminetetraacetic acid (EDTA; DOJINDO), 2 mM ethyleneglycol bis(2-aminoethyl ether)-*N*,*N*,*N*,*N* tetraacetic acid (EGTA; DOJINDO), 1 mM phenylmethylsulfonyl fluoride (PMSF; FUJIFILM Wako) and 0.1x protease inhibitor cocktail (Sigma-Aldrich) were added to the supernatant to inhibit further proteolytic degradation, and the solution was stored at -80°C until use.

Protein purification and digestion

Culture media were concentrated up to ~50-fold using an Amicon® Ultra filter (3,000 NMWL; Merck), acidified with 2.5% trifluoroacetic acid (TFA; final concentration), and further concentrated using a SpeedVac (Thermo Fisher Scientific). Proteins were reconstituted with 100 µL water and purified by methanol-chloroform precipitation, for which the protein solution was successively mixed with 300 µL methanol, 75 µL chloroform, and 225 µL water and centrifuged at 21,000 *g* for 5 min. The resulting pellet was washed with 225 µL methanol and dissolved in phase-transfer surfactant (PTS) buffer (Masuda et al., 2009) containing 100 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride (Tris-HCl, pH 8.5), 12 mM sodium deoxycholate (SDC; FUJIFILM Wako), 12 mM sodium *N*-lauroylsarcosinate (SLS; FUJIFILM Wako). The protein concentration was determined by means of bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). For each condition, 10 µg of protein was

reduced with 10 mM dithiothreitol (DTT; FUJIFILM Wako) and alkylated with 40 mM 2-chloroacetamide (CAA; Sigma-Aldrich). Digestion was performed according to the PTS protocol (Chang et al., 2021; Masuda et al., 2009). Briefly, for C-terminomics, the protein solution was 5-fold diluted with 50 mM ammonium bicarbonate, and proteins were sequentially digested with LysC (1:50, w/w; FUJIFILM Wako) for 3 h at 37 °C and trypsin (50:1, w/w; Promega) overnight at 37 °C. In the case of N-terminomics, following 10-fold dilution with 10 mM $CaCl_2$, proteins were digested with TrypN (1:50, w/w; Protifi) overnight at 37 °C. Note that TrypN can be replaced with LysargiNase (Merck, Cat#EMS0008). Digestion was stopped with 0.5% TFA (final concentration), and the detergents were removed by liquid-liquid extraction using an equal volume of ethyl acetate. Peptides were purified with a reversed-phase SDB-XC StageTip (Rappsilber et al., 2007).

TMT labeling

Peptides were dissolved in 5 µL of 200 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; DOJINDO)-NaOH (pH 8.5), mixed with 0.1 mg of TMT reagents dissolved in 5 µL of acetonitrile (ACN), and agitated at room temperature for 1 h. Excessive reagents were quenched with 0.33% hydroxylamine (final concentration), and the solution was acidified with 1% TFA (final concentration). Note that N-terminal peptides were TMT-labeled after N-terminal peptide enrichment, and then 6-plexed peptides were mixed. ACN was evaporated using a SpeedVac. Labeled peptides were purified using a reversed-phase SDB-XC StageTip (Rappsilber et al., 2007).

N-Terminal peptide enrichment

The TrypN digest of 10 µg protein was dissolved in 50 µL of N-loading buffer (2.5% formic acid (FA), 30% ACN) and loaded onto a StageTip with 16-gauge double-plug CationSR membranes (GL Sciences), which had been successively preconditioned with 50 µL methanol, 50 µL 80% ACN containing 0.1% TFA, 100 µL 500 mM ammonium acetate containing 30% ACN, and 300 µL N-loading buffer. Following peptide loading, the StageTip membranes were washed with 50 µL N-loading buffer, and the wash solution was collected together with the flow-through fraction as the N-terminal peptides-enriched fraction. This fraction was evaporated using a SpeedVac and TMT-labeled as described above.

C-Terminal peptide enrichment

A mixture of 6-plexed TMT-labeled peptides was dissolved in 150 µL of C-loading buffer (0.15% TFA, 30% ACN), divided into three parts, and subjected to C-terminal peptide enrichment using three StageTips. A solution of peptides in 50 µL C-loading buffer was loaded onto a StageTips with 16-gauge double-plug CationSR membranes (GL Sciences), which had been successively preconditioned with 50 µL methanol, 50 µL 80% ACN containing 0.1% TFA, 100 µL 500 mM ammonium acetate containing 30% ACN, and 300 µL C-loading buffer. Subsequently, peptides were eluted with 0.5% TFA containing 30% ACN (0.5% TFA fraction). The flow-through fraction and the

0.5% TFA fraction were separately collected and evaporated.

Liquid Chromatography/Tandem Mass Spectrometry

For N-terminal peptide analysis, one-third of the peptides was injected, and triplicate analyses were carried out (3 LC/MS/MS runs per cell line). For C-terminal peptide analysis, three sets of flow-through and 0.5% TFA fractions were subjected to single shot analysis (6 LC/MS/MS runs per cell line) (Figure S1C, D).

A nanoLC/MS/MS system comprising an UltiMate 3000RSLCnano pump (Thermo Fisher Scientific) and an Orbitrap Fusion Lumos tribrid mass spectrometer (Thermo Fisher Scientific) was employed. Peptides were injected by an HTC-PAL autosampler (CTC Analytics), loaded on a 15 cm fused-silica emitter self-packed with ReproSil-Pur C18-AQ (3 μm; Dr. Maisch), and separated by a linear gradient, that is, 5% B for 1 min, 5−15% B in 4 min, 15−40% B in 100 min, 40−99% B in 5 min, and 99% B for 10 min (Solvent A, 0.5% acetic acid; solvent B, 0.5% acetic acid in 80% ACN) at the flow rate of 500 nL/min. Peptides were ionized at 2,400 V. All MS1 spectra were acquired over the range of 375–1500 *m/z* in the Orbitrap analyzer (resolution = 120,000, maximum injection time = 50 ms, automatic gain control = 4e5). For the subsequent MS/MS analysis, precursor ions were selected and isolated in top-speed mode (cycle time = 3 sec, isolation window = 1.4 m/z), activated by higher-energy collisional dissociation (HCD; normalized collision energy = 38), and detected in the Orbitrap analyzer (resolution = 50,000, maximum injection time = 105 ms, automatic gain control = 1e5). Dynamic exclusion time was set to 30 sec.

LC/MS/MS raw data processing

LC/MS/MS raw data were processed using MaxQuant (v.1.6.7.0) with the Andromeda search engine (Cox and Mann, 2008; Cox et al., 2011). Database search was implemented against the UniprotKB/SwissProt (2019_3) human database including isoform sequences concatenated with commonly observed contaminant protein sequences set in MaxQuant. Two analysis groups were made in MaxQuant, enabling one combined analysis for TrypN with N-terminal free semi-specificity and trypsin/P with C-terminal free semi-specificity. The following parameters were applied: 10-plexed TMT quantification at the MS2 level, precursor mass tolerance of 4.5 ppm, fragment ion mass tolerance of 20 ppm, and minimal peptide length of 7 amino acids. Cysteine carbamidomethylation was set as a fixed modification, while methionine oxidation and acetylation on the protein N-terminus were allowed as variable modifications. False discovery rates were estimated by searching against a reversed decoy database and filtered for <1% at the peptide-spectrum match and protein level.

Protein inference

In terminomics, isoforms are often indistinguishable due to limited sequence coverage. Thus, we used the canonical isoform for bioinformatics analysis of membrane proteins, if the peptide can be derived from the canonical isoform. If not, we employed the leading protein given by MaxQuant.

Peptide list processing and normalization of TMT-reporter intensities

Firstly, peptides derived from contaminant proteins such as keratins and bovine serum-derived proteins were excluded. We utilized peptides with no missed cleavages for analyses. TMT-reporter intensities were $\log_2$-converted and normalized in individual cell lines by the trimmed mean of M values (TMM) method (Robinson and Oshlack, 2010) on an R framework with the Bioconductor edgeR package (v. 3.28.1) using the default parameters. As mentioned in the main text, N-terminally acetylated N-terminal peptides are never quantified in our N-terminomics workflow, since acetylated N-terminal peptides should not have TMT-reactive sites. Nevertheless, TMT-reporter signals were sometimes observed for such peptides, e.g. due to co-fragmentation. We manually excluded 77 such N-terminally acetylated peptides, reducing the number of total identified N-terminal peptides from 6,189 to 6,181.

Statistical and bioinformatics analysis

Using Perseus (v.1.6.7.0) (Tyanova et al., 2016), we performed two-sample Welch *t*-tests to identify terminal peptides with a significant alteration upon BB-94 treatment employing a 5% permutation-based FDR filter. Proteolytic events were considered significantly altered if at least one of the N- and C-terminal peptides was significantly altered.

Membrane proteins were annotated with the UniProt Knowledgebase (UniProtKB) keywords “transmembrane” or “GPI-anchor”. Membrane protein topologies and positions of the transmembrane domain and GPI-anchor site were retrieved from UniProtKB, and further analyses were performed using Microsoft Excel.

Other statistics analyses were performed in the R framework (v.3.6.1) with the exactRankTests package (v.0.8-31), psych package (v.1.9.12.31) and pheatmap package (v.1.0.12). Bioinformatics analysis was performed using DAVID (v.6.8) (Huang et al., 2009), STRING (v.11.0) (Szklarczyk et al., 2019), and Cytoscape (v.3.8.0) (Shannon et al., 2003). Sequence logos were created using IceLogo (v.1.0) (Colaert et al., 2009).

PWM construction and scoring

Flanking sequences surrounding substrate cleavage sites for sheddases were obtained from the MEROPS database (Rawlings et al., 2018) and the previous study (Tucher et al., 2014). Sequences including non-natural amino acids were excluded. PWM construction and PWM score computation were performed essentially as described previously (Imamura et al., 2017). Briefly, the PWM score was computed by summing $\log_2$ weights for the flanking ±4 residues surrounding each cleavage site.

In vitro sheddase assay

The substrates were designed as the flanking ±4 residues surrounding each cleavage site. Three substrate sequences were concatenated and synthesized as a 24-mer peptide. *In vitro* protease reaction was performed by incubating 10 pmol substrate peptide with 100 ng recombinant sheddase (all from R&D Systems) in 30 µL reaction buffer as instructed by the manufacturer at 37 °C for 3 h. If necessary, proteases were activated just before the reaction according to the manufacturer's instructions. The reaction was halted by acidification of the mixture with TFA. The products were desalted with SDB-XC StageTip, and analyzed using the LC/MS/MS system described above, with a short gradient: 5−40% B in 20 min, 40−99% B in 1 min, and 99% B for 4 min (Solvent A, 0.5% acetic acid; solvent B, 0.5% acetic acid in 80% ACN) at the flow rate of 500 nL/min. Peak area quantification was performed using Skyline (v20.2).

Supplemental References

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