Electronic Supplementary Information

Luminescence Detection of Peptide:*N*-Glycanase Activity Using Engineered Split Inteins

Tsuyoshi Takahashi,[a] Tatsuya Uchibayashi,[a] Nozomi Ishii,[a] Ichiro Matsuo,[a] Yukiko Yoshida,[b] and Tadashi Suzuki[c]

[a]Graduate School of Science and Technology, Gunma University, 1-5-1 Tenjin-cho, Kiryu, Gunma, 376-8515, Japan

[b]Ubiquitin Project, Tokyo Metropolitan Intsitute of Medical Science, 2-1-6 Kamikitazawa, Setagaya-ku, Tokyo 156-8506, Japan

[c]Glycometabolic Biochemistry Laboratory, RIKEN Cluster for Pioneering Research, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

Contents

Experimental Section

Materials and methods

All chemicals and solvents were of reagent or HPLC grade. Amino acid derivatives and reagents for peptide synthesis were purchased from Watanabe Chemical and AAPPTEC. *N*,*N*′-diacetylchitobiose was purchased from Seikagaku Corporation. Other chemicals and reagents were purchased from Tokyo Chemical Industry, Nacalai Tesque, Sigma Aldrich, Wako Chemical, TOYOBO, and QIAGEN. NMR spectrum was recorded on a JEOL JNM-ECX400 spectrometer (400 MHz). Micowave-assisted chemistry was performed on a Biotage Initiator+ synthesizer. Electrospray ionization mass spectrometry was measured on a Shimadzu LCMS-2020 single quadrupole liquid chromatography mass spectrometer. HPLC was performed on Cosmosil C18 AR-II (10 × 250 mm) and Daisopak SP-120 C4-Bio (4.6 × 150 mm and 10 × 250 mm, Daiso Chemical) packed columns by employing Hitachi L-7100 and Shimadzu SCL-10Avp HPLC systems. PCR was performed on a MiniCycler PTC-150 thermal cycler (MJ Research). DNA sequence was analyzed using BigDye Terminator 3.1 cycle sequencing kit. UV-visible absorption spectra were recorded on a Shimadzu UV-2450 spectrophotometer using a quartz cell with 1.0 cm or 0.5 cm pathlength. Histidine-tagged proteins were purified on a Ni-NTA agarose resin (QIAGEN). Size-exclusion chromatography was performed on a HiLoad 16/60 Superdex 200 pg (GE Healthcare) by employing a Pharmacia LKB LCC-501 plus FPLC system. The peptide **5Asp** and IntCm-NLlg were prepared as previously reported (**5Asp** = NLs-IntN, IntCm-NLlg = IntCmut3-NLlg).[1]

Synthesis of *N*-[2-(acetylamino)-4-*O*-[2-(acetylamino)-2-deoxy-β-D-glucopyranosyl]-2-deoxy-β-D-glucopyranosyl]-N^2-[9*H*-fluoren-9-ylmethoxy]-L-asparagine [Fmoc-Asn(GlcNAc$_2$)-OH]:

N,*N*′-diacetylchitobiose (50 mg, 118 μmol) was dissolved with saturated aqueous ammonium hydrogen carbonate solution (1.5 mL) and the mixture was stirred at 40°C for 24 h. Solvent was evaporated and the product was lyophilized. Quantitative yield of the product, 2-(acetylamino)-4-*O*-[2-(acetylamino)-2-deoxy-β-D-glucopyranosyl]-2-deoxy-β-D-glucopyranosylamine, was obtained.

The product was dissolved in dimethylsulfoxide (1.5 mL). Fmoc-Asp(OPfp)-OtBu (136 mg, 236 μmol) and diisopropyl ethylamine (24.7 μL, 142 μmol) were added and the mixture was stored at 4°C for overnight. Water (4.5 mL) was added and the crude product was lyophilized. The crude product was washed with Et_2O, and dried in vacuo. The solid was dissolved and reacted with trifluoroacetic acid (TFA; 1 mL) at 4°C for 1 h. The product was washed with Et_2O and dried in vacuo. The crude product was purified by RP-HPLC on a Cosmosil C18 AR-II column to give Fmoc-Asn(GlcNAc$_2$)-OH (36 mg, 47 μmol, 40% yield from *N*,*N*′-diacetylchitobiose). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.22 (d, J = 9.2 Hz, 1H), 7.86 (d, J = 7.3 Hz, 2H), 7.83 (d, J = 9.2 Hz, 1H), 7.70 (d, J = 8.7 Hz, 1H), 7.69 (d, J = 7.3 Hz, 2H), 7.44 (d, J = 8.7 Hz, 1H), 7.39 (t, J = 7.5 Hz, 2H), 7.29 (t, 7.5 Hz, 2H), 4.81 (t, J = 9.6 Hz, 1H), 4.36 – 4.14 (m, 6H), 3.71 (m, 2H), 3.6 – 3.37 (m, 6H), 3.3 – 3.1 (m, 5H), 2.99 (m, 2H), 1.79 (s, 3H), 1.72 (s, 3H). ESI-MS; *m*/*z* calcd for $C_{35}H_{45}N_4O_{15}$ (exact mass) $[M+H]^+$ 761.29, found 761.02.

Synthesis of peptides (5GN2, 3GN2, 8GN2, 3GN1, 3Asp, 8Asp):

Peptides bearing $GlcNAc_2$ and GlcNAc were synthesised by an Fmoc solid-phase method on a Rink amide PEG resin XV (Watanabe Chemical Industry) using *O*-(7-azabezotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate (HATU) as a coupling reagent. Side-chain protections for amino acids were as follows: Arg(Pbf), Asp(OtBu), Cys(Trt), Glu(OtBu), Gln(Trt), Ser(tBu), Thr(tBu) and Tyr(tBu). Fmoc group was removed with 20% piperidine in NMP at room temperature (1 min × 1, 15 min × 1). The coupling condition of Fmoc amino acids except for Fmoc-Asn($GlcNAc_2$)-OH and N^2-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-*N*- [3,4,6-tri-*O*-acetyl-2-(acetylamino)-2-deoxy-β-D-glucopyranosyl]-L-asparagine [Fmoc-Asn(Ac_3-β-D-GlcNAc)-OH] was as follows: Fmoc-AA-OH (10 eq), HATU (10 eq) and diisopropyl ethylamine (DIEA; 12 eq) in 1-methyl-2-pyrrolidone (NMP) under microwave heating at 60°C for 20 min. Fmoc-Asn($GlcNAc_2$)-OH (1.2 eq), (7-azabenzotriazol-1-yloxy)tripyrrolidino- phosphonium hexafluorophosphate (PyAOP; 3.8 eq), 1-hydroxy-7-azabenzotriazole (HOAt; 3.8 eq), and 2,4,6-trimethylpyridine (collidine; 5 eq) in NMP were used under microwave heating at 60°C for 50 min. Fmoc-Asn(Ac_3-β-D-GlcNAc)-OH (2 eq), PyAOP (3.8 eq), HOAt (3.8 eq), and collidine (6 eq) were used under microwave heating at 60°C for 30 min.

After acetylation using acetic acid (10 eq), HATU (10 eq) and DIEA (12 eq) at the N-terminus, the peptidyl resin was washed with NMP and dichloromethane, and dried in vacuo. The peptidyl resin was treated with 2.5% triisopropylsilane (TIS) and 2.5% water in TFA for 2 h at room temperature. The resin was removed by filtration, and the filtrate was added into Et_2O to precipitate the peptide. The solid was washed with Et_2O, and dried in vacuo. The peptide bearing Ac_3-β-D-GlcNAc moiety was treated with 5% hydrazine in water in the presence of 60 mM dithiothreitol (DTT) for 1 h at room

temperature. The crude peptides were purified with RP-HPLC on a Daisopak SP-120 C4-Bio semi-preparative column (10 × 250 mm) using a linear gradient of acetonitrile/0.1% TFA at a flow rate of 3.0 mL/min under 40°C, and lyophilised. The peptides were identified by ESI-MS; **5GN2**, *m*/*z* calcd for $C_{89}H_{149}N_{23}O_{35}S$ $[M+2H]^{2+}$ 1066.67, found 1066.45. **3GN2**, *m*/*z* calcd for $C_{89}H_{149}N_{23}O_{35}S$ $[M+2H]^{2+}$ 1066.67, found 1066.47. **8GN2**, *m*/*z* calcd for $C_{87}H_{143}N_{23}O_{37}S$ $[M+2H]^{2+}$ 1067.63, found 1067.27. **3GN1**, *m*/*z* calcd for $C_{81}H_{136}N_{22}O_{30}S$ $[M+2H]^{2+}$ 965.07, found 964.94. **3Asp**, *m*/*z* calcd for $C_{73}H_{122}N_{20}O_{26}S$ $[M+2H]^{2+}$ 863.97, found 863.61. **8Asp**, *m*/*z* calcd for $C_{71}H_{116}N_{20}O_{28}S$ $[M+2H]^{2+}$ 864.93, found 864.75. HPLC chromatograms and mass spectra of the purified peptides were shown in Fig. S1 and Fig. S2.

The peptides were dissolved in dimethylsulphoxide (DMSO) to prepare stock solutions. The concentrations of the peptides were determined by amino acid analysis using the phenyl isothiocyanate (PITC) method on a Wakopak WS-PTC column (4.0 × 200 mm).

Synthesis of peptides (3GN2-R9, 3GN1-R9, 3Asp-R9):

To synthesise the peptide bearing R9, the peptide having a sequence of H-RRRRRRRRR-βAla-βAla-C(Npys)-NH_2 (Npys = 3-nitro-2-pyridinesulphenyl) [R9-Cys(Npys)] was synthesised. The peptidyl resin, H-[Arg(Pbf)]$_9$-βAla-βAla-Cys(Trt)-NH-Rink amide MBHA resin, was synthesised on a Rink amide MBHA resin (AAPPTEC) on a ABI 433A peptide synthesizer (Applied Biosystems) using HATU. The peptidyl resin was treated with 2,2′-dithiobis(5-nitropyridine) (5 eq), 2.5% (v/v) triisopropylsilane (TIS), and 2.5% (v/v) water in TFA for 3 h at room temperature.[2] The resin was removed by filtration, and the filtrate was added into Et_2O to precipitate the peptide. The solid was washed with Et_2O, and dried in vacuo.

The purified peptides **3GN2**, **3GN1**, or **3Asp** (1 mM) was dissolved in 100 mM ammonium acetate buffer (pH 6.0). Crude R9-Cys(Npys) (2.4 mM) was dissolved in the buffer, and mixed with **3GN2**, **3GN1**, or **3Asp**, and the mixture was stirred for 90 min at room temperature. After the addition of acetic acid and water, the product peptide **3GN2-R9**, **3GN1-R9**, or **3Asp-R9** was purified with RP-HPLC on the Daisopak SP-120 C4-Bio semi-preparative column, and lyophilised. The peptides were identified by ESI-MS; **3GN2-R9**, *m*/*z* calcd for $C_{152}H_{274}N_{63}O_{47}S_2$ $[M+4H]^{4+}$ 950.08, found 950.15. **3GN1-R9**, *m*/*z* calcd for $C_{144}H_{261}N_{62}O_{42}S_2$ $[M+4H]^{4+}$ 899.28, found 899.18. **3Asp-R9**, *m*/*z* calcd for $C_{136}H_{249}N_{60}O_{38}S_2$ $[M+4H]^{4+}$ 849.24, found 848.75. HPLC chromatograms and mass spectra of the purified peptides bearing R9 were shown in Fig. S3 and Fig. S4. The peptides were dissolved in DMSO and the concentrations were determined by amino acid analysis described above.

Expression of yPNGase

The plasmids encoding yPNGase and yPNGase-mut were prepared as described previously.[3] The plasmids were transformed into BL21(DE3)pLysS. The transformed *E. coli* cells were grown in LB broth at 37°C in the presence of kanamycin (34 μg/mL) and chloramphenicol (34 μg/mL) until OD_{600} = 0.8. IPTG (1 mM) was added to express at 37°C for 3 h. Cells were collected by centrifugation and frozen at -80°C. Cells were resuspended in a bind buffer [50 mM phosphate (pH 8.0), 0.5 M NaCl, 5 mM imidazole], and sonicated on ice. After centrifugation (10,000 × g for 60 min at 4°C), the supernatant was loaded onto Ni-NTA agarose resin. After washing with a wash buffer [50 mM phosphate (pH 8.0), 0.5 M NaCl, 30 mM imidazole], the protein was eluted with an elution buffer [50 mM phosphate (pH 8.0), 0.5 M NaCl, 0.5 M imidazole]. The protein was further purified on a HiLoad 16/60 Superdex 200 pg gel filtration

column with PNGase buffer [20 mM phosphate (pH 7.2), 150 mM NaCl, 5 mM DTT]. The purified yPNGase and yPNGase-mut were concentrated using an Amicon Ultra-15 concentrator. The concentration of yPNGase was determined by the Bradford assay.

PTS assay of peptides with IntCm-NLlg

The peptides **5GN2**, **3GN2**, **8GN2**, **5Asp**, **3Asp**, and **8Asp** were mixed with a buffer and then IntCm-NLlg was added. Final concentrations were as follows: [peptide] = 200 nM and [IntCm-NLlg] = 200 nM in 50 mM phosphate (pH 7.0), 150 mM NaCl, 2 mM DTT and 0.1% (w/v) bovine serum albumin (BSA). The reaction mixtures were incubated at 25°C. After the PTS reactions, 0.5 μL of coelenterazine (250 μM) in methanol was added to 50 μL aliquots of the reaction mixture, and the luminescence was measured on a Glomax Multi Jr Luminometer (Promega).

HPLC analysis of the reactions with yPNGase

5GN2, **3GN2**, and **3GN1** (5 μM each) were reacted with yPNGase (100 nM) in PNGase buffer at 25°C. The reaction was quenched by adding 5% TFA, and analysed by HPLC on the Daisopak SP-120 C4-Bio (4.6 × 150 mm). The peak areas were calculated using the analytical software (Class-VP, Shimadzu). The area of an individual peak was expressed as a fraction of the sum of the substrate and the product peak areas.

PTS-based luciferase assay with yPNGase

5GN2, **3GN2**, and **3GN1** (5 μM each) were reacted with yPNGase (100 nM) in PNGase buffer in the presence of 0.1% (w/v) BSA at 25°C. After the PNGase reactions of **5GN2** and **3GN1**, 2 μL of the reaction solution was mixed

with 46.9 μL of the buffer [50 mM phosphate (pH 7.0), 150 mM NaCl, 2.13 mM DTT, 0.1066% BSA] and 1.1 μL of IntCm-NLlg (9.1 μM). Their final concentrations were as follows: [peptide] = 200 nM, [IntCm-NLlg] = 200 nM, and [yPNGase] = 4 nM in 50 mM phosphate (pH 7.0), 150 mM NaCl, 2 mM DTT and 0.1% (v/v) BSA. The mixtures were incubated at 25°C for 1 h, and then 0.5 μL of coelenterazine (250 μM) in methanol was added and luminescence intensity was measured.

After the PNGase reaction of **3GN2**, 2 μL of the reaction solution was mixed with 3 μL of Z-VAD(OMe)-fmk (Peptide Institute; 83.3 μM), and then mixed with 43.9 μL of a buffer and 1.1 μL of IntCm-NLlg (9.1 μM). The mixtures were incubated at 25°C for 1 h, and then 0.5 μL of coelenterazine (250 μM) in methanol was added and luminescence intensity was measured.

In PNGase reaction in the presence of IntCm-NLlg, **3GN2** (5 μM) and yPNGase (100 nM) or yPNGase-mut (100 nM) were mixed in PNGase buffer, and IntCm-NLlg (200 nM) was added [final buffer condition: 50 mM phosphate (pH 7.0), 150 mM NaCl, 2 mM DTT and 0.1% BSA]. After the reaction, 0.5 μL of coelenterazine (250 μM) in methanol was added to a 50 μL aliquot of the reaction mixture, and the luminescence was measured. The assay using various concentrations of yPNGase (100, 20, 5, 1, 0.2, 0.04, and 0 nM) was also performed.

In ENGase (*endo*-β-*N*-acetylglucosaminidase from *Mucor hiemalis*; Tokyo Kasei Kogyo, A1651) reaction, **5GN2** and **3GN2** (5 μM each) were reacted with ENGase (2 unit/L) at 37°C in PNGase buffer. After the reaction, HPLC analysis and PTS-based luciferase assay were performed.

Preparation of cytosolic extracts from HeLa^{Ngly1}, HeLa^{KO}, and HeLa^{wt} cells

The Ngly1 knockout cell clone (HeLa^{KO}) was constructed by targeting

exon 6 of the Ngly1 gene in $HeLa^{wt}$ using a CRISPR/Cas9 method, and the $HeLa^{Ngly1}$ cell clone was established by a recombinant retrovirus infection method using the $HeLa^{KO}$ cell clone as previously reported.[4] $HeLa^{Ngly1}$, $HeLa^{KO}$, and $HeLa^{wt}$, and 3T3-L1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 unit/mL penicillin/streptomycin, 2 mM L-glutamine, and 1 μg/mL puromycin (for $HeLa^{Ngly1}$ only) at 37°C in humidified 5% CO_2 atmosphere. Confluent cell cultures were harvested by the addition of a trypsin/EDTA solution. The cells were collected by centrifugation, washed with Dulbecco's PBS (dPBS) (× 2), and stored at −80°C.

The cell pellet (1 volume) was resuspended with PNGase buffer (3 volume) containing 1 × complete protease inhibitor cocktail (Roche) and 1 mM phenylmethylsulphonyl fluoride at 4°C. The cells were homogenized with a Dounce homogenizer. The homogenates were centrifuged at 15,000 × g for 10 min at 4°C, and the supernatants were further centrifuged at 100,000 × g for 1 h at 4°C. The supernatants, used as the cytosolic fractions for the NGLY1 activity measurement, were stored at −80°C. The concentrations of total proteins of each fraction were determined by the Bradford method.

HPLC analysis of the reaction using the cytosolic fractions of $HeLa^{Ngly1}$, $HeLa^{KO}$, and $HeLa^{wt}$ cells

In the reactions of **5GN2**, **3GN2**, and **3GN1** with the cytosolic fractions from $HeLa^{Ngly1}$, $HeLa^{KO}$, and $HeLa^{wt}$ cells, 0.5 μL of 0.72 mM each peptide in DMSO was mixed with 9.5 μL of the cell extracts (total protein concentration of each sample is approximately 1.0 mg/mL). The mixtures were incubated at 25°C for 4 h, and then diluted by adding 20 μL of PBS. The sample solutions were analysed by RP-HPLC described above.

PTS-based luciferase assay using the cytosolic fractions of HeLa^{Ngly1}, HeLa^{KO}, and HeLa^{wt} cells

In the reactions of **5GN2**, **3GN2**, and **3GN1** with the cytosolic fractions from HeLa^{Ngly1}, HeLa^{KO}, and HeLa^{wt} cells, 1 μL of 50 μM each peptide in PNGase buffer was mixed with 9 μL of the cell extracts at 25°C. After the reactions, 2 μL aliquots of the sample solution were used for the PTS reaction and luciferase assay described above.

PTS-based luciferase assay in live cells

The plasmid encoding IntCm-NLlg in mammalian cells was constructed using pmCherry-C1 (Clontech). The PCR product was cloned into XhoI/XbaI restriction sites of pmCherry-C1 and the purified plasmid was sequenced. The amino acid sequence was shown in Fig. S1.

HeLa^{Ngly1}, HeLa^{KO}, and HeLa^{wt} cells (8×10^3 cells) and 3T3-L1 cells (1.5×10^4 cells) were cultured onto 96-well plates and were incubated at 37°C for 24 h. The cells were transfected with the plasmid to express IntCm-NLlg using Lipofectamine 3000 and P3000 reagents according to the manufacture's instruction. After 23 h, when cells were treated with Z-VAD(OMe)-fmk, the media were changed with 50 μL of Z-VAD(OMe)-fmk (50 μM) in DMEM supplemented with 10% FBS, and the cells were incubated at 37°C for 1 h. After 24 h of transfection, the cells were washed by dPBS (× 2), and 50 μL aliquots of **3GN2-R9**, **3GN1-R9**, and **Asp-R9** (10 μM each) in dPBS were added and incubated at 37°C for 1 h. After the reaction, 0.5 μL of furimazine (250 μM) in methanol was added and the luminescence was measured on an EnSpire Multimode Plate Reader (PerkinElmer). We also performed the PTS-based luciferase assay in live cells using HeLa^{Ngly1}, HeLa^{KO}, HeLa^{wt}, and 3T3-L1 cells

in the absence and presence of 3Asp-R9 (10 μM) (Fig. S12).

Cell viability assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay was performed to evaluate the toxic effect of the peptides. After the PTS-based luciferase aasay in live cells described above, the solutions were changed into DMEM supplemented with 10% FBS and MTT (0.5 mg/mL), and the cells were further incubated at 37°C for 3.5 h. The precipitated formazan was dissolved by 0.04 M HCl in 2-propanol. Absorbance at 570 nm was then measured. Cell viability was analysed at the ratio of the absorbance at 570 nm of the cells treated with the peptides to the cells without the peptides.

References

1. M. Kawasse, M. Fujioka and T. Takahashi, *ChemBioChem*, 2021, **22**, 577-584.
2. A. K. Ghosh and E. Fan, *Tetrahedron Lett.*, 2000, **41**, 165-168.
3. T. Suzuki, H. Park, N. M. Hollingsworth, R. Sternglanz and W. J. Lennarz, *J. Cell Biol.*, 2000, **149**, 1039-1051; S. Katiyar, T. Suzuki, B. J. Balgobin and W. J. Lennarz, *J. Biol. Chem.*, 2002, **277**, 12953-12959.
4. Y. Yoshida, M. Asahina, A. Murakami, J. Kawawaki, M. Yoshida, R. Fujinawa, K. Iwai, R. Tozawa, N. Matsuda, K. Tanaka and T. Suzuki, *Proc. Natl. Acad. Sci. USA*, 2021, **118**, e2102902118.

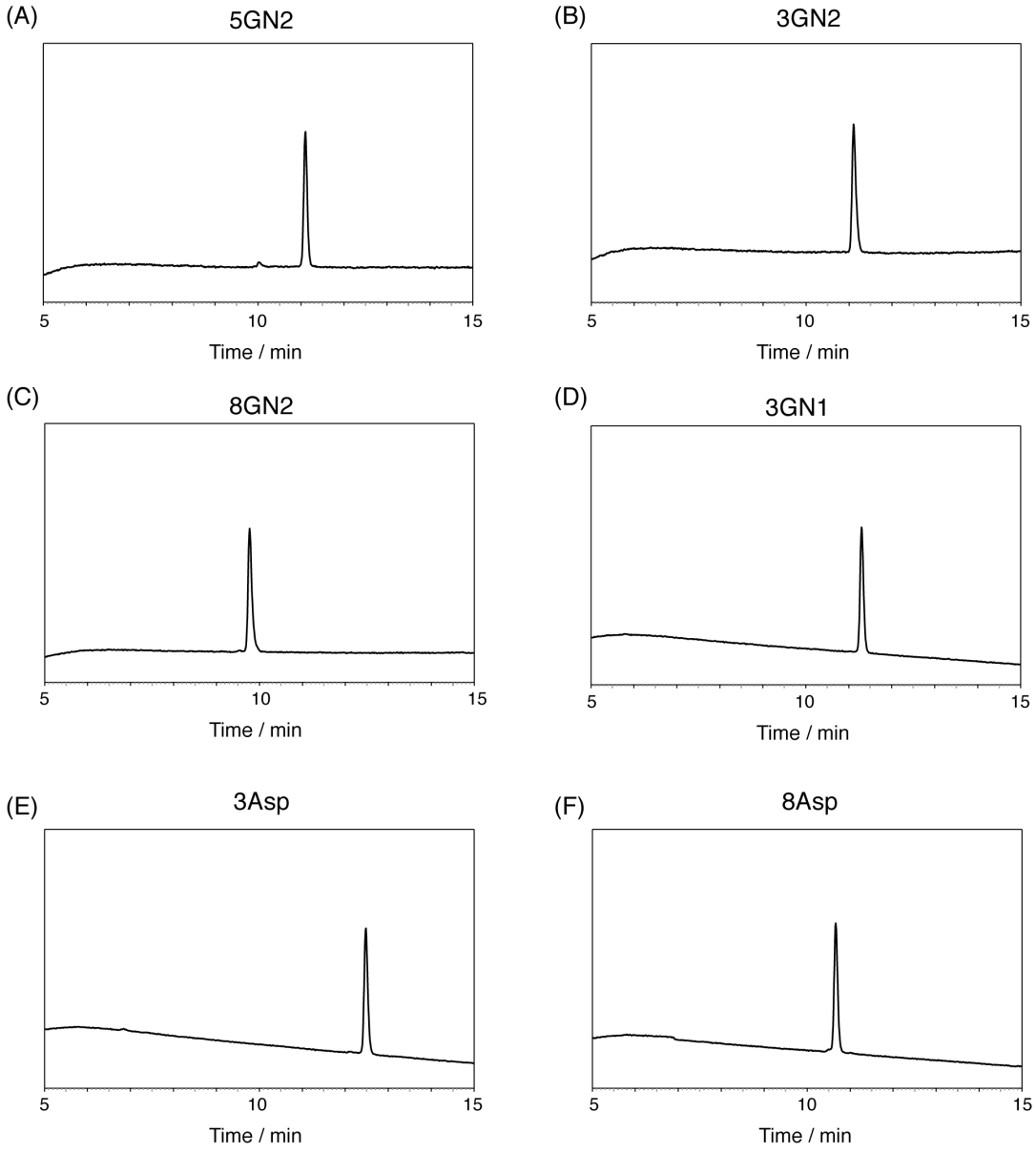

Fig. S1 HPLC chromatograms of (A) 5GN2, (B) 3GN2, (C) 8GN2, (D) 3GN1, (E) 3Asp, and (F) 8Asp peptides. The peptides were analyzed using a 15 min gradient of 15-45% acetonitrile in 0.1% (v/v) TFA at 1 mL/min on a Daisopak SP-120 C4-Bio (4.6 mm x 150 mm).

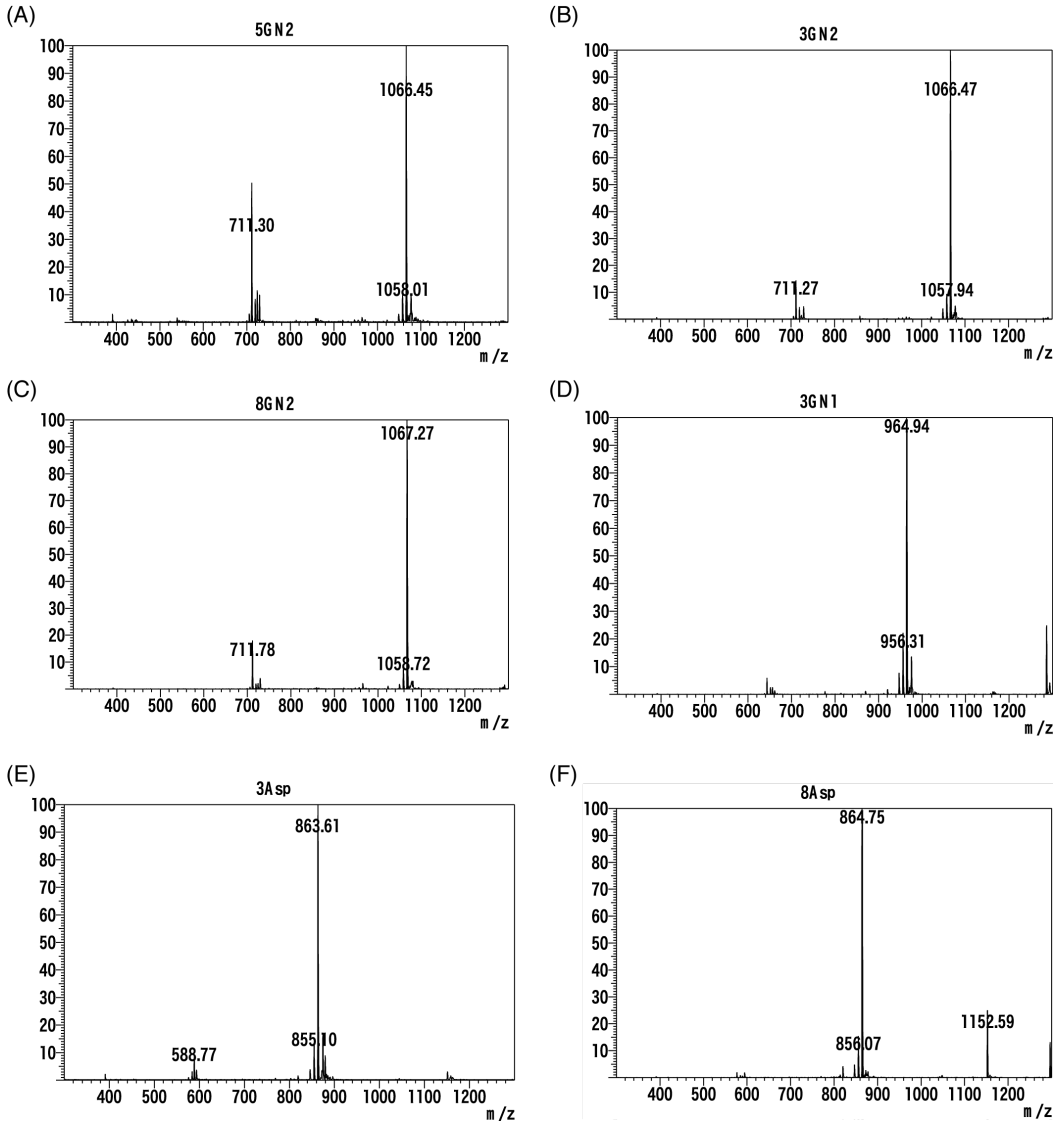

Fig. S2 Mass spectra of (A) 5GN2, (B) 3GN2, (C) 8GN2, (D) 3GN1, (E) 3Asp, and (F) 8Asp peptides.

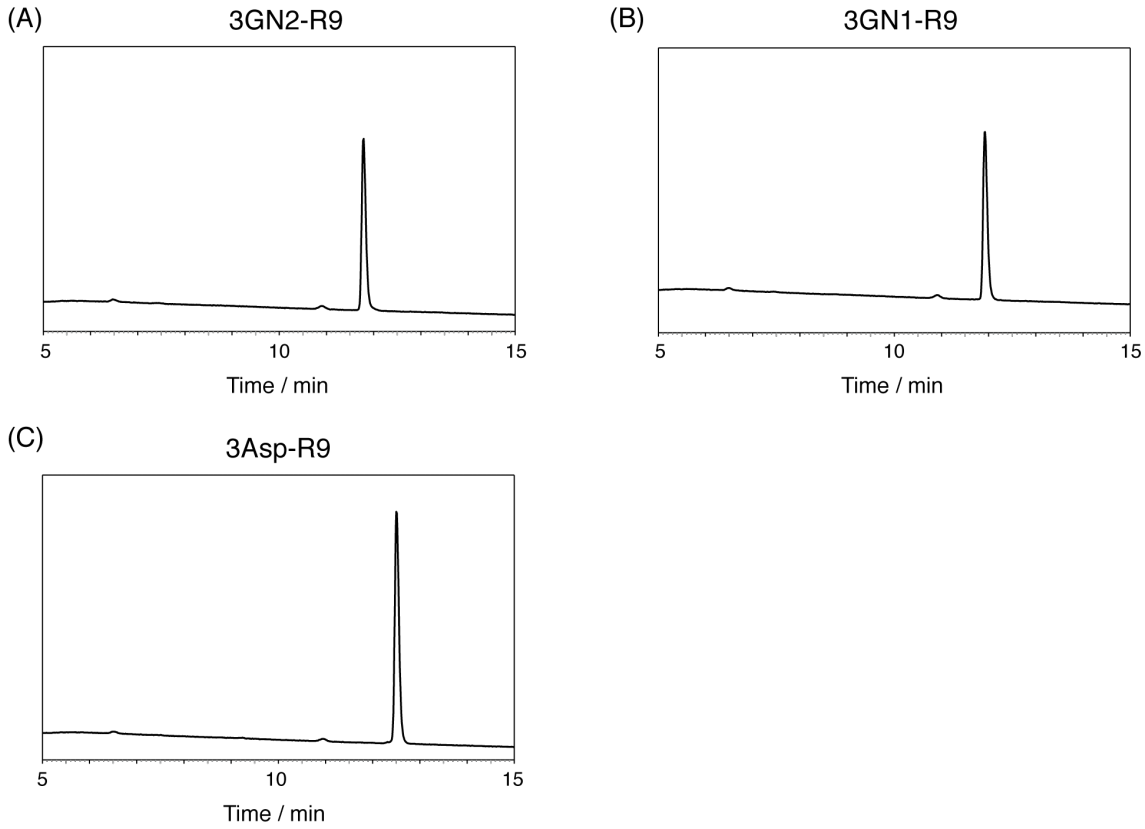

Fig. S3 HPLC chromatograms of (A) 3GN2-R9, (B) 3GN1-R9, and (C) 3Asp-R9 peptides. The peptides were analyzed using a 15 min gradient of 10-40% acetonitrile in 0.1% (v/v) TFA at 1 mL/min on a Daisopak SP-120 C4-Bio (4.6 mm x 150 mm).

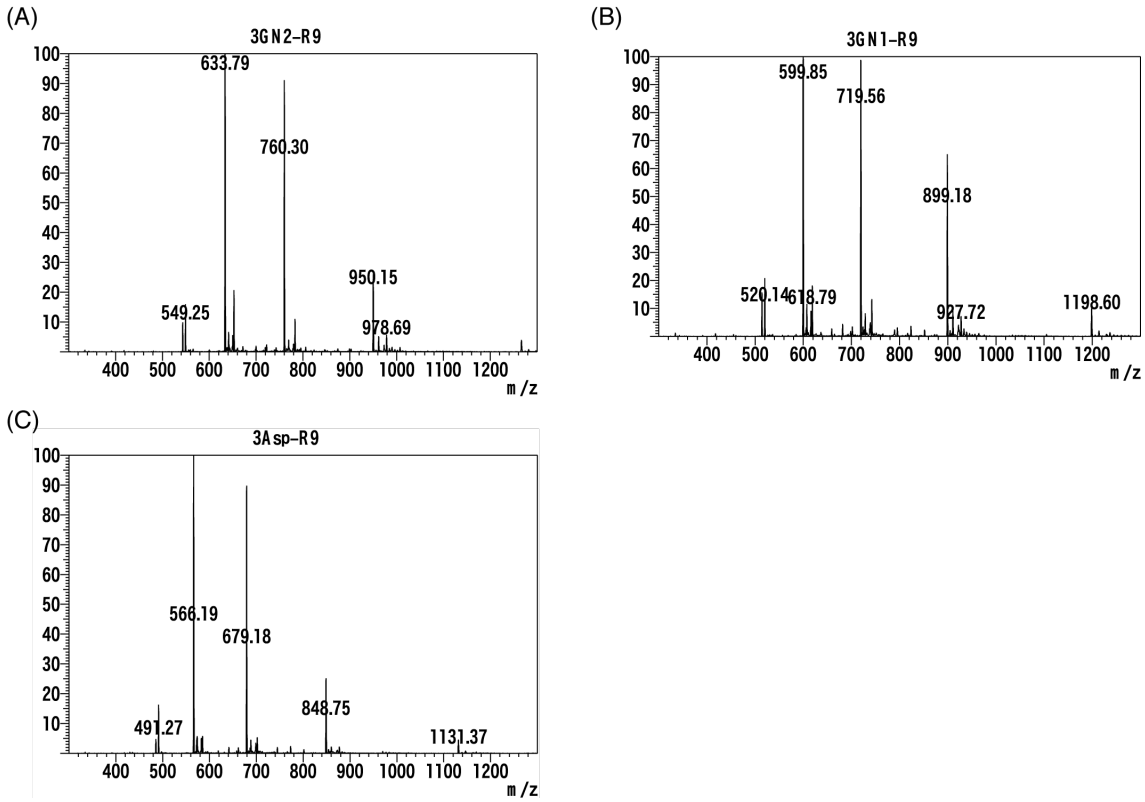

Fig. S4 Mass spectra of (A) 3GN2-R9, (B) 3GN1-R9, and (C) 3Asp-R9 peptides.

MNHKVHHHHHHMELGTGGSGGSGYRLLPIGKIVEERIECSVYSVDNNGNIYT
QPVVQWHDRGEQEVFEYWLEDGSLIRATRDHKFMTVDGQMVPIDEIFERELD
LMRVDNLPNIKIATRKYLGKQKVYDIGVERDHNFALKNGFIASNCFEKISGG
SGGSGGSVFTLEDFVGDWEQTAAYNLDQVLEQGGVSSLLQNLAVSVTPIQRI
VRSGENALKIDIHVIIPYEGLSADQMAQIEEVFKVVYPVDDHHFKVILPYGT
LVIDGVTPNMLNYFGRPYEGIAVFDGKKITVTGTLWNGNKIIDERLITPDGS
MLFRVTINS

MVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKV
TKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFED
GGVVTVTQDSSLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSERMYPED
GALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNED
YTIVEQYERAEGRHSTGGMDELYKSGLRSRGSGYRLLPIGKIVEERIECSVYS
VDNNGNIYTQPVVQWHDRGEQEVFEYWLEDGSLIRATRDHKFMTVDGQMVPID
EIFERELDLMRVDNLPNIKIATRKYLGKQKVYDIGVERDHNFALKNGFIASNC
FEKISGGSGGSGGSVFTLEDFVGDWEQTAAYNLDQVLEQGGVSSLLQNLAVSV
TPIQRIVRSGENALKIDIHVIIPYEGLSADQMAQIEEVFKVVYPVDDHHFKVI
LPYGTLVIDGVTPNMLNYFGRPYEGIAVFDGKKITVTGTLWNGNKIIDERLIT
PDGSMLFRVTINS

Fig. S5 The amino acid sequence of IntCm-NLlg (upper) and a fusion of mCherry and IntCm-NLlg (lower). The sequences corresponding to mCherry and IntCm-NLlg were shown in cherry and blue, respectively.

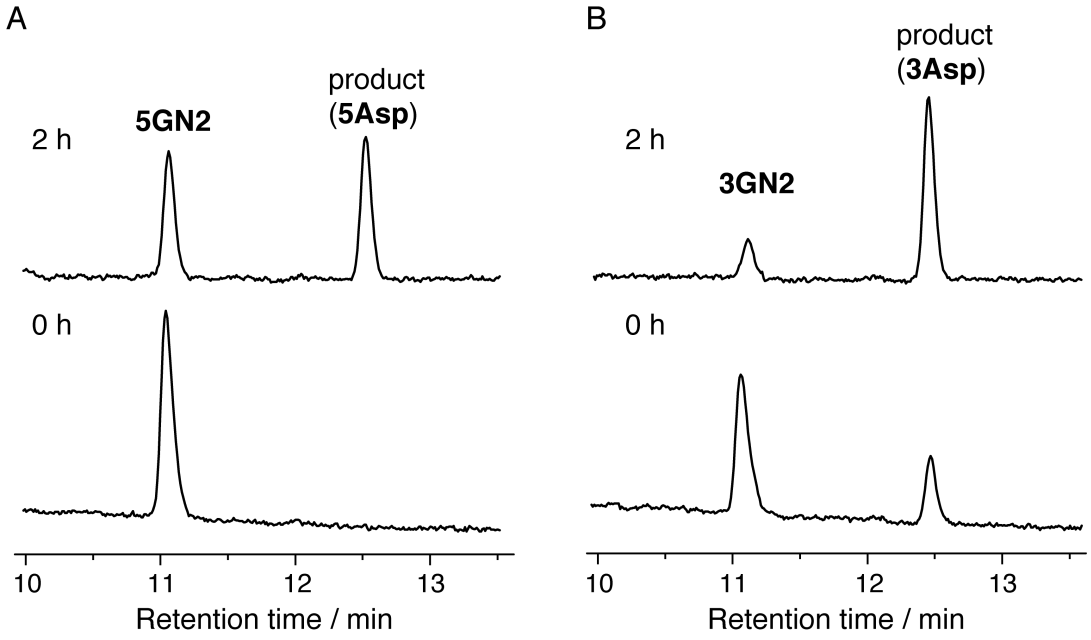

Fig. S6 HPLC profiles of the reactions of (A) peptide **5GN2** (5 μM) and (B) peptide **3GN2** (5 μM) with yPNGase (100 nM) in a buffer at 25°C for 0 and 2 h.

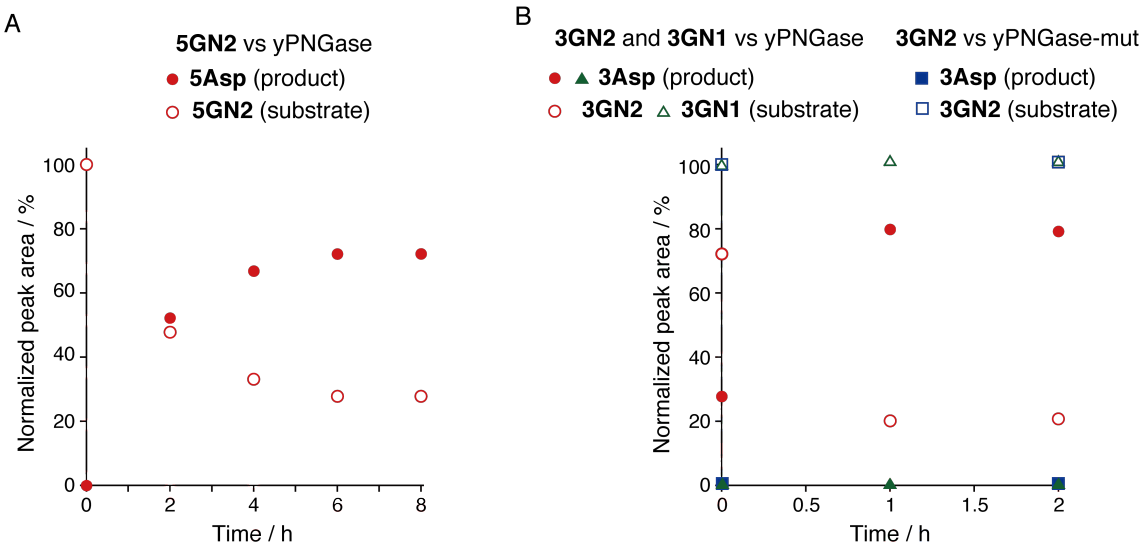

Fig. S7 HPLC analysis of the reactions of (A) **5GN2** and (B) **3GN2** (5 μM each) with yPNGase (100 nM; red circule) and with yPNGase-mut (blue square), and **3GN1** with yPNGase (green triangle) in a buffer at 25°C. Substrates and products of the reactions are shown in open and closed symbols, respectively.

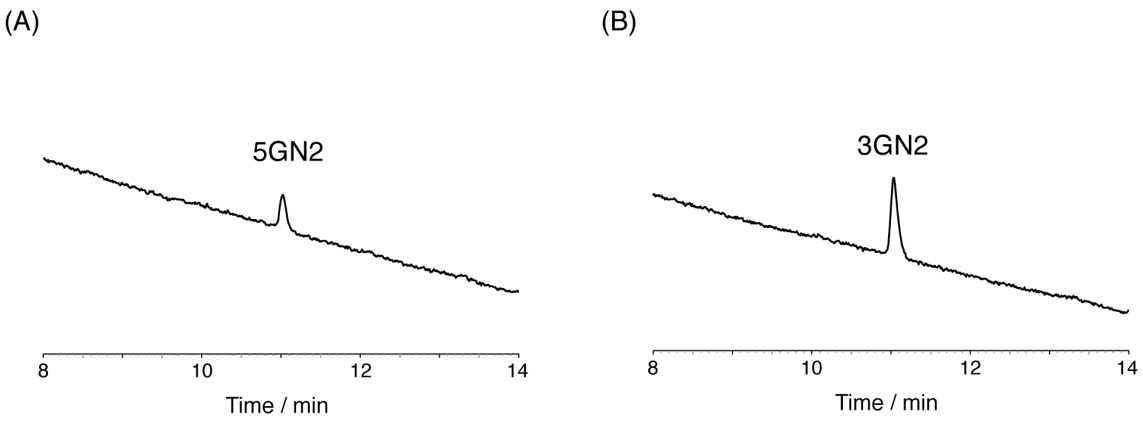

Fig. S8 HPLC profiles of the reaction of (A) **5GN2** (5 μM) and (B) **3GN2** (5 μM) with ENGase at 37°C for 8 h.

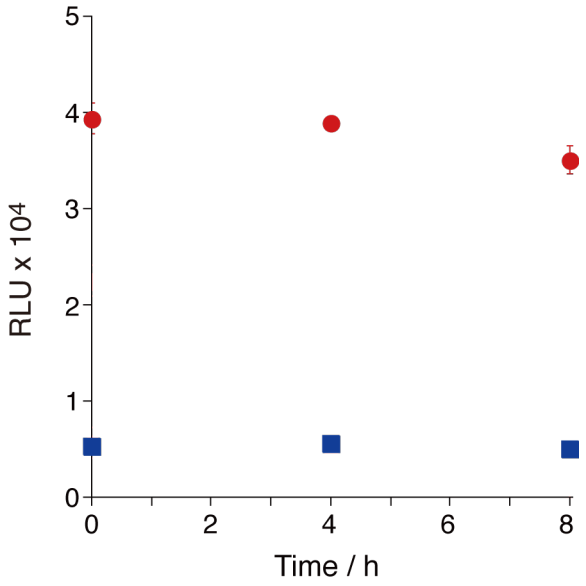

Fig. S9 PTS-based luciferase assay after the reactions of **5GN2** (5 μM; red closed circle) and **3GN2** (5 μM; blue closed square) with ENGase at 37°C.

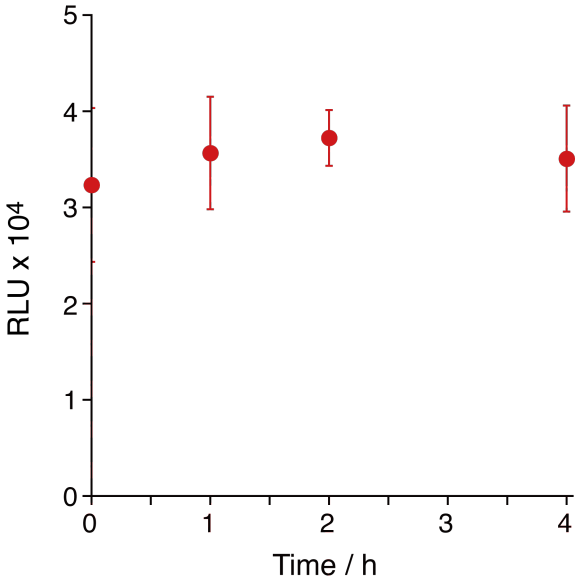

Fig. S10 PTS-based luciferase assay after the reactions of **5GN2** (5 μM) with HeLa^{Ngly1} extracts at 25°C in a buffer.

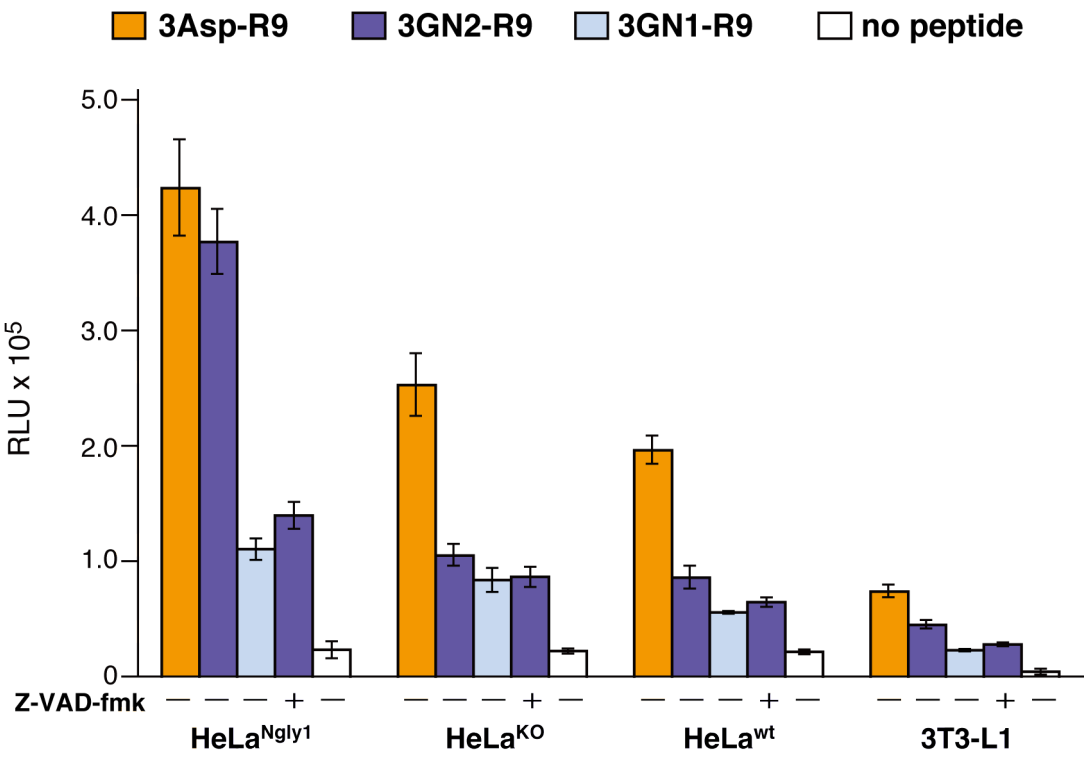

Fig. S11 PTS-based luciferase assay in live cells using peptides **3Asp-R9**, **3GN2-R9**, and **3GN1-R9** (10 μM each) in the presence or absence of Z-VAD(OMe)-fmk in $HeLa^{Ngly1}$, $HeLa^{KO}$, $HeLa^{wt}$, and 3T3-L1 cells. Luminescence was recorded by adding furimazine (2.5 μM).

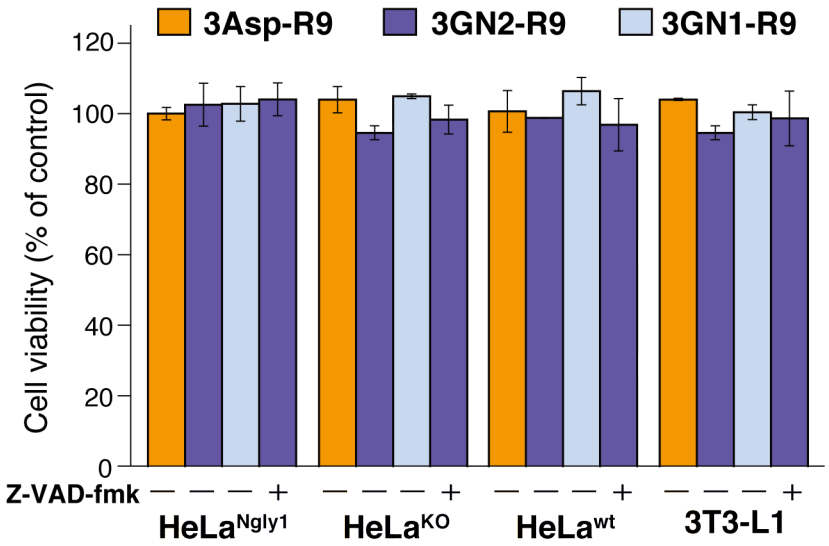

Fig. S12 Viability of HeLa^{Ngly1}, HeLa^{KO}, HeLa^{wt}, and 3T3-L1 cells that express IntCm-NLlg in the absence (100% viable) and presence of **3Asp-R9** (10 μM; orange bar), **3GN2-R9** (10 μM; purple bar), and **3GN1-R9** (10 μM; light blue bar).