Supplemental Methods

Purification and characterization of recombinant modB and mod2B.

Each CHO cell line stably expressing modB or mod2B was cultured in EX-CELL ACF CHO medium (Sigma-Aldrich). The conditioned medium (CM) derived from each cell line was collected. For AF-Blue chromatography, an equivalent amount of distilled water was added and filtered with a Steritop-GP (0.22 μm; Merck Millipore). Then the samples were applied to a TOYOPEARL AF-Blue HC-650 column (Tosoh) equilibrated with 0.1 M Tris-acetate buffer (TAB, pH 7.5). After washing the column with 10 column volumes (CV) of TAB and 150 mM sodium chloride/TAB, the bound proteins were eluted with 0.6 M sodium chloride/TAB. The eluate was applied to Phos-tag agarose (Wako) balanced with TAB containing 20 mM zinc acetate. After washing the column with 40 CV of TAB, the bound proteins were eluted with 0.2 M sodium phosphate buffer (NaPB) (pH 6.0). The eluate was dialyzed against 10 mM NaPB and concentrated with an Amicon ultra device (30,000 MWCO; Merck Millipore). Then, the eluate was applied to a HiTrap SP HP column (GE Healthcare). The elution fractions containing β-Hex activity were collected. Each fraction containing recombinant Hex was subjected to SDS-PAGE on a 10% (w/v) acrylamide gel and silver staining with a Dodeca Silver Stain Kit (Bio-Rad). The molecular weight of each

recombinant Hex was calculated based on that of the APRO marker (APRO Science). Immunoblotting was performed with hHexA-specific polyclonal Ab (1). Briefly, aliquots of HexB fractions were subjected to SDS-PAGE on a 10% (w/v) acrylamide gel. The proteins were transferred to PVDF membranes. After blocking with 50% (v/v) Blocking One (Nacalai Tesque) in TBS (pH 7.4) at room temperature (r.t.) for 1 h, the membrane was treated with hHexA-specific Ab diluted with Blocking One / TBS (1:1,000 dilution) overnight at 4 ^{o}C. After washing with TBS containing 0.1% (v/v) Tween 20, the membrane was treated with biotinylated Ab against rabbit IgG (Vector Laboratories, 1:1,000 dilution) at r.t. for 1 h. After washing, HRP-linked Ab against biotin (Cell Signaling Technology, 1:1,000 dilution) were treated for 1 h at r.t. After washing with same buffer, detection of antibody binding was carried out with ECL (PerkinElmer Inc.) according to the manufacturer's instructions. Lectin blotting to detect the terminal M6P residues of *N*-glycans attached to modified HexBs was performed with the recombinant CI-M6PR domain9 (1) provided by Dr. Chiba, Y. (Glycomedicine Technology Research Center, AIST, Ibaraki, Japan). Briefly, transferred membrane was treated with recombinant CI-M6PR domain9 (5 μg of protein) diluted with Blocking One / TBS overnight at 4 ^{o}C. After washing, the membrane was treated with Ab against penta His (Qiagen, 1:1,000 dilution) at r.t. for 1

h. After washing, HRP-linked Ab against mouse IgG, IgM (Thermo Scientific, 1:1,000 dilution) were treated for 1 h at r.t.. The M6P contents of modified HexBs were measured using three-step analyses including acid hydrolysis, derivatization of M6P, and high performance liquid chromatography (2). The GM2-degradation assay was performed *in vitro* (3). Briefly, the GM2 ganglioside (Enzo Life Sciences) was incubated with modified HexBs (MUGS-degrading activity 2 μmol h^{-1}) in the presence or absence of 5 μg of synthesized GM2AP in 10mM sodium citrate buffer (pH 4.5) containing 0.1% (w/v) BSA at 37 °C for 22 h. Then glycosphingolipids were isolated using a C18 Sep-Pak Cartridge (Waters) and spotted on a HPTLC silica gel 60 plate (Merck). The TLC plate was developed with $CHCl_3$/MeOH/0.2% (w/v) $CaCl_2$ [60/40/9,(v/v)] and sprayed with 11.4% (v/v) H_2SO_4 / 0.05% (w/v) orcinol.

Synthesis and fluorescence properties of HMDER-βGlcNAc.

The procedure to synthesize the new molecule mostly followed the synthesis of HMDER-βGal (4), except for the use of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride instead of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide. Briefly, a mixture of HMDER (4), 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride (Sigma-Aldrich)

and Cs_2CO_3 (Wako) in dry *N,N*-dimethylformamide (DMF; Wako) was stirred overnight at room temperature under an argon atmosphere in the dark. The reaction mixture was filtered through a celite pad, and the solvent was evaporated *in vacuo*. The residue was dissolved in CH_2Cl_2, and the resulting solution was washed with water, dried over Na_2SO_4 and concentrated. The triacetylated HMDER-βGlcNAc was purified by chromatography on silica gel using CH_2Cl_2/methanol (10/1) as the eluent. This intermediate was dissolved in dry methanol, and 28% sodium methoxide in methanol (ca. 50 equiv.) (Wako) was added. The reaction mixture was stirred at r.t. for 1 h, neutralized with 10 mM triethylammonium acetate buffer (pH 7.0), and concentrated. Column chromatography over silica gel using CH_2Cl_2/methanol (10/1) as the eluent afforded HMDER-βGlcNAc as an orange powder (63% yield over two steps). The product was identified using a JNM-LA300 instrument (JEOL) and a JMS-T100LC AccuToF (JEOL) for NMR and mass spectra, respectively. The resulting spectra for ^{1}H NMR (300 MHz, CD_3OD) were: δ 7.37–7.39 (m, 2H), 7.25–7.27 (m, 1H), 6.77–6.86 (m, 3H), 6.67–6.70 (m, 2H), 6.42–6.44 (m, 2H), 5.23 (s, 2H), 5.07 (d, 1H, J = 8.8 Hz), 3.88–3.94 (m, 2H), 3.70–3.74 (m, 1H), 3.57 (t, 1H, J = 9.2 Hz), 3.44–3.49 (m, 2H), 3.37 (q, 4H, J = 7.1 Hz), 1.97 (d, 3H, J = 2.2 Hz), 1.15 (t, 6H, J = 7.1 Hz). Those for ^{13}C NMR (75 MHz, CD_3OD) were: δ 173.9, 159.6, 153.2, 152.9, 150.2, 146.2, 140.3, 131.1,

130.8, 129.4, 129.2, 124.8, 121.9, 120.4, 112.3, 109.6, 104.8, 100.8, 100.7, 98.6, 85.6, 78.3, 75.8, 72.6, 71.8, 62.5, 57.3, 45.4, 23.0, 12.8. Those for high-resolution mass spectrometry (electron spray ionization, positive ion mode) were: calculated for $[M + Na]^+$, 599.23693; found, 599.23328 (Δ-3.66 mmu).

For the analyses of the fluorescence properties, HMDER-βGlcNAc was dissolved in dimethyl sulfoxide (fluorometric grade, Dojindo) to obtain stock solutions. A solution of 5 μl HMDER-βGlcNAc in 0.2 M NaPB (pH 7.4) was stirred at 25 ^{o}C, and one unit of β-*N*-acetylglucosaminidase (Sigma-Aldrich, A2264) was added at 30 s. The fluorescence intensity was monitored with 525 nm excitation and 550 nm emission wavelengths using a FP-6500 fluorescence spectrometer (JASCO). The fluorescent product was identified as HMDER by a high performance liquid chromatography analysis (data not shown).

Delivery of AFO-HexBs to late endosomes/lysosomes in SD fibroblasts.

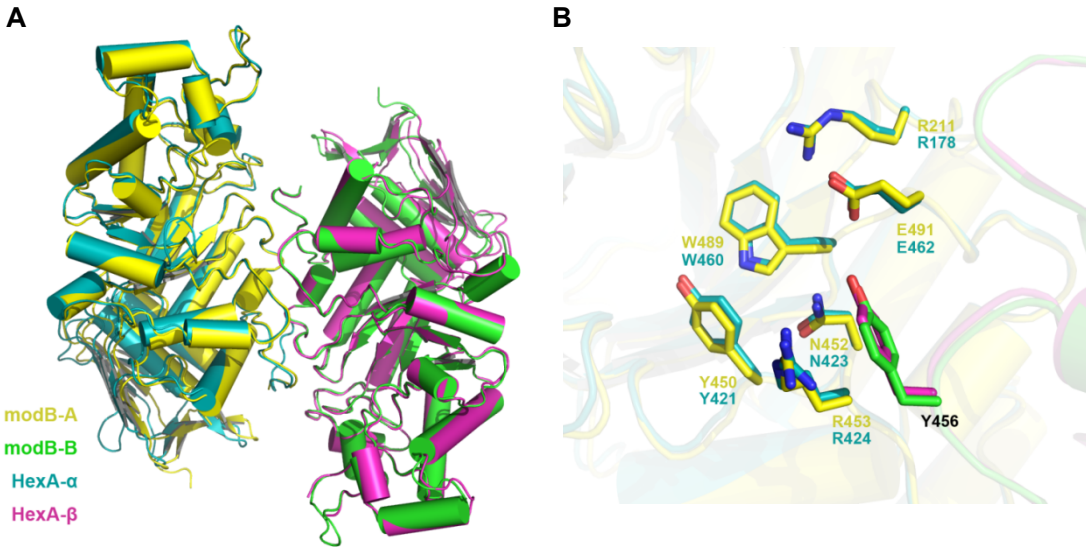
SD fibroblasts were fixed in 4% (w/v) paraformaldehyde (Wako) and immunostained with LAMP-1-specific Ab (H-228, Santa Cruz Biotechnology, 1:200 dilution) to visualize the late endosomal/lysosomal distribution of AFO-HexBs with a LSM700 in acidic sodium acetate buffer (pH 4.5).

Imaging the β-Hex activity with HMDER-βGlcNAc *in situ*.

Brain sections (each 10 μm thick) were incubated for 30 min with HMDER-βGlcNAc (20 μM) at pH 6.0, and then examined with a BIOREVO BZ-9000.

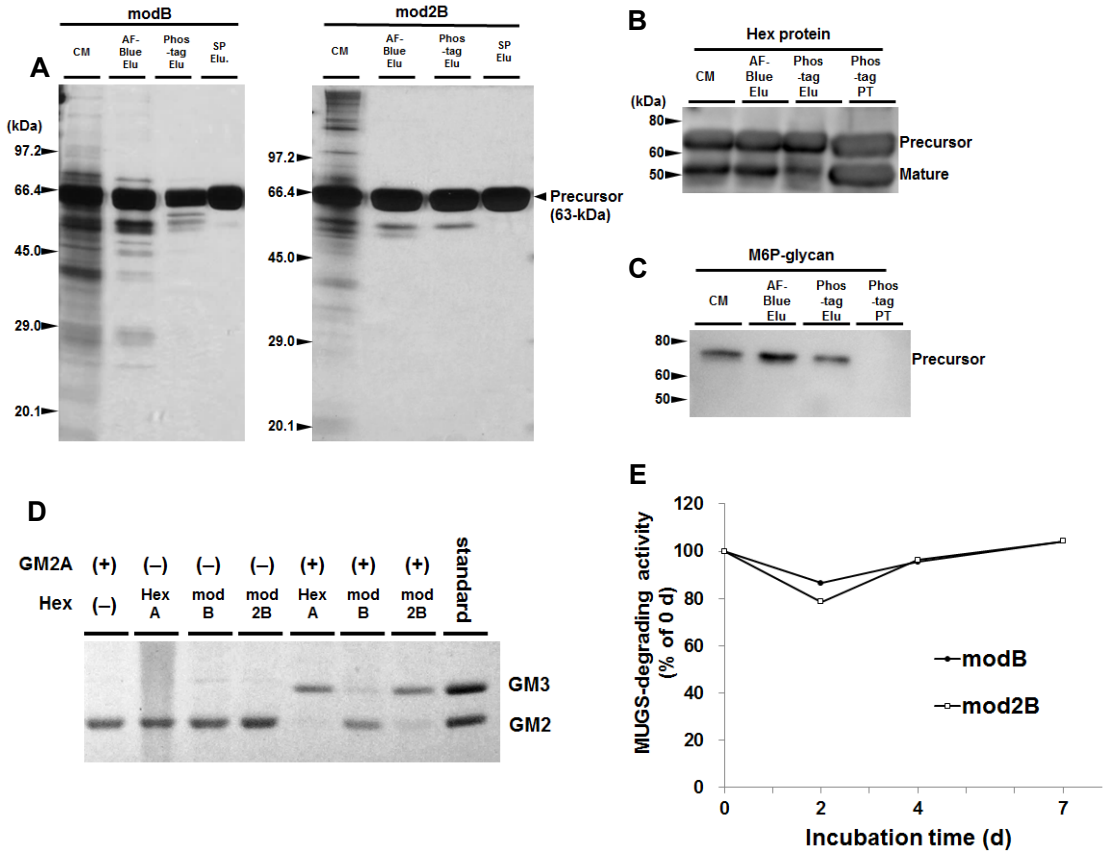
Supplemental Figures and Tables

Supplemental Figure 1. Structural comparison between modB and HexA.



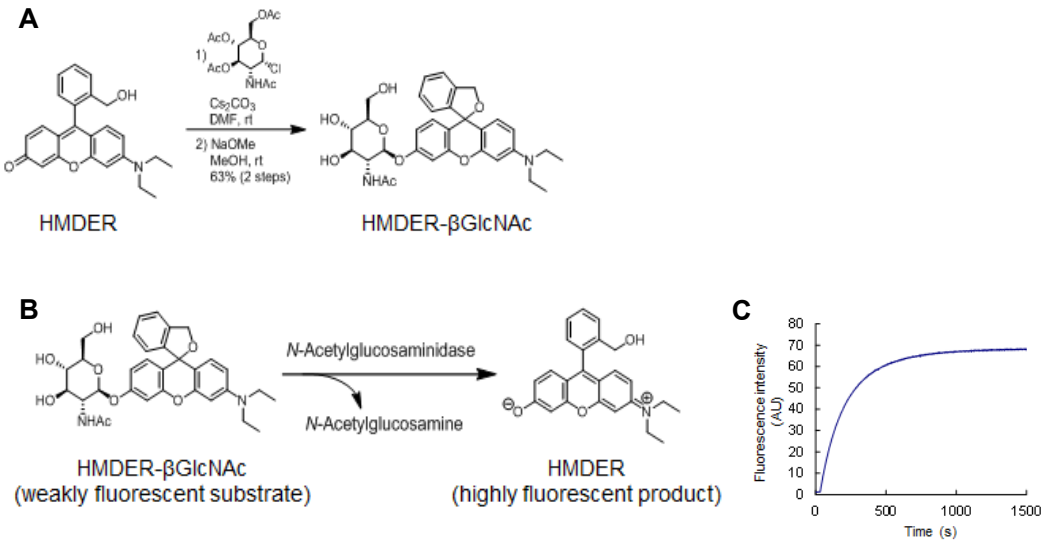
(A) A superposed model of modB (β_1' homodimer, PDB ID: 5BRO) with HexA (α/β heterodimer, PDB ID: 2GJX). The root-mean square difference (RMSD) between the dimers was 0.404 Å. **(B)** Active site residues of the modB-β_1' subunits and HexA-α subunits.

Supplemental Figure 2. Purification and characterization of M6P-carrying modB and mod2B.



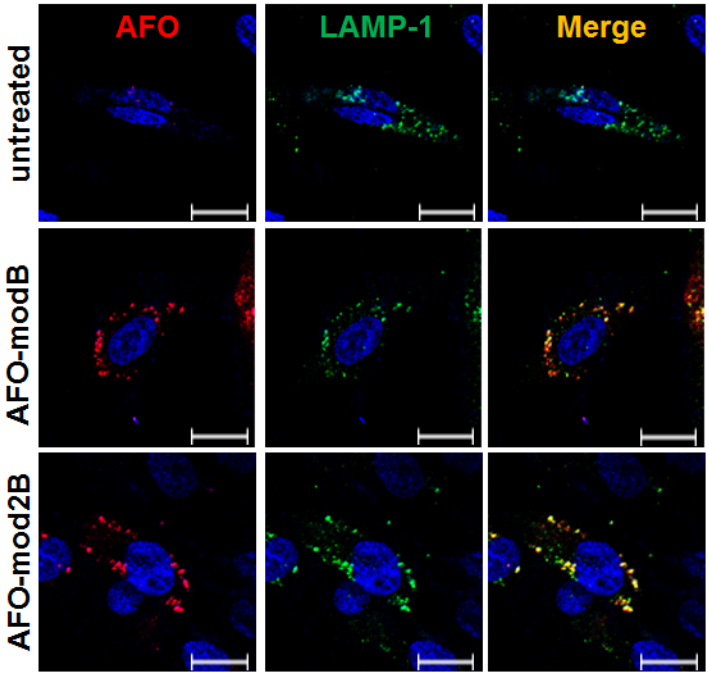
(A) ModB and mod2B were purified by three-step column chromatography utilizing AF-Blue, Phos-tag and SP-resins. Each fraction was separated by SDS-PAGE, and then silver staining was performed. Each lane contained 2 μg of protein. CM: conditioned medium. Elu: elution fraction. **(B, C)** Immunoblotting and Dom9-lectin blotting of M6P-carrying mod2B. Each fraction of mod2B was separated by SDS-PAGE, and then blotting with hHexA-specific Ab **(B)** and recombinant CI-M6PR-Dom9 **(C)**. Each lane contained 100 nmol h^{-1} (MUGS activity). CM: conditioned medium. Elu: elution fraction, PT: pass through fraction. **(D)** *In vitro* degradation of GM2 in the presence of synthetic GM2A. GM2 was incubated with *Om*HexA (5), modB or mod2B (same MUGS-degrading activity) in the presence or absence of chemically synthesized monoglycosylated GM2A. Glycosphingolipids were purified and subjected to TLC. The TLC plates were sprayed with orcinol reagent. **(E)** The thermostability of the MUGS-degrading activities of modB and mod2B. Purified modB and mod2B (each homodimeric modified β-precursor) were incubated at 37 ^{o}C in sodium phosphate buffer (pH 6.0) containing 30% (v/v) of SD mouse plasma.

Supplemental Figure 3. Synthesis and enzymatic reaction of HMDER-βGlcNAc.



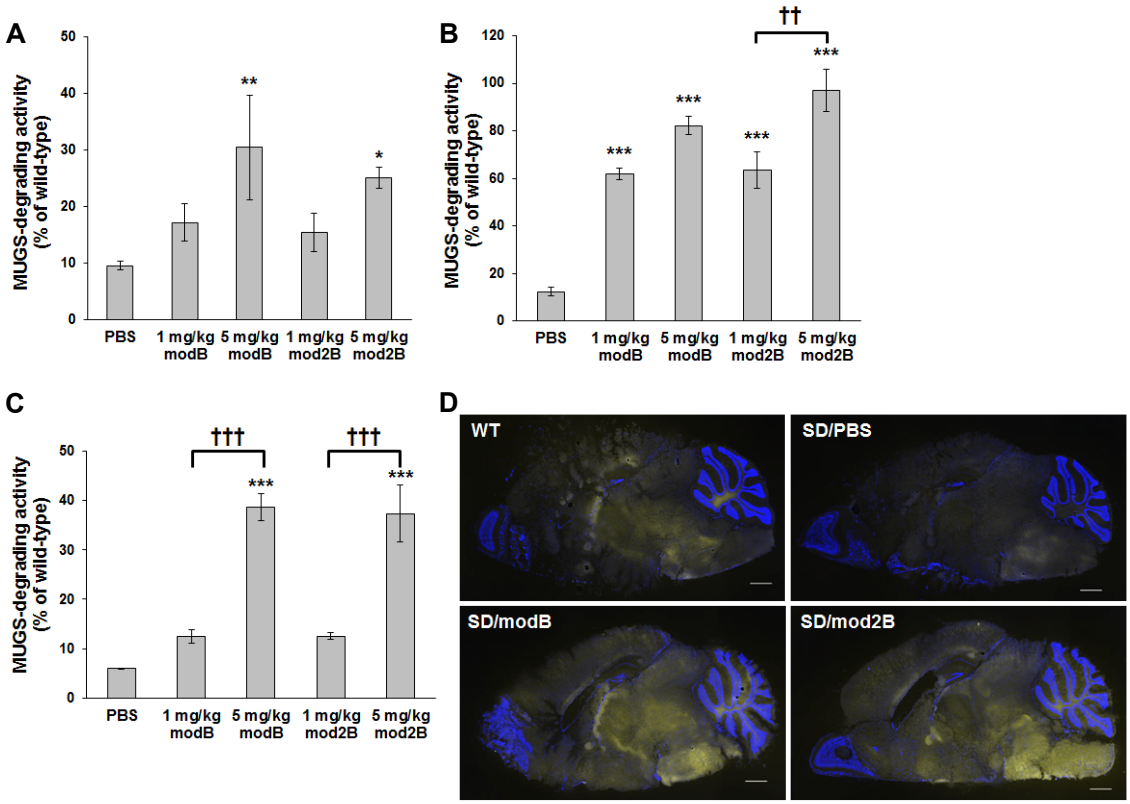
(A) Synthesis of HMDER-βGlcNAc. **(B, C)** Enzymatic reaction of HMDER-βGlcNAc with β-*N*-acetylglucosaminidase. β-*N*-Acetylglucosaminidase enhanced the fluorescence intensity of HMDER-βGlcNAc by 57-fold, which was similar to the previously reported value (76-fold) of HMDER-βGal upon activation by β-galactosidase (4). HMDER-βGlcNAc was thus validated as a fluorescence probe for sensing β-hexosaminidase activity.

Supplemental Figure 4. Delivery of AFO-HexBs to late endosomes/lysosomes in SD fibroblasts.



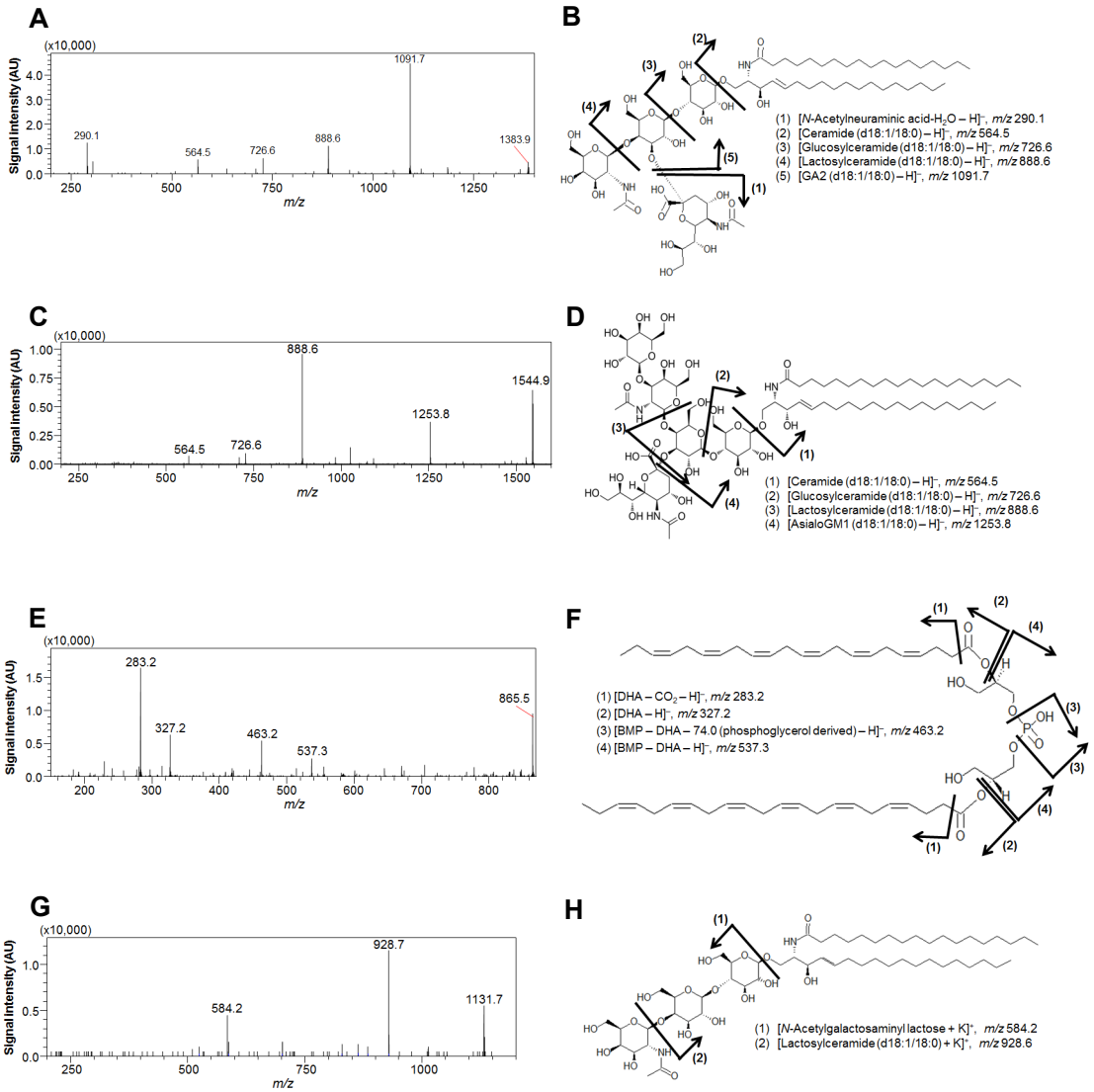
SD fibroblasts were treated with AFO-modB and AFO-mod2B (each 100 pmol). The cells were then fixed and immunostained with hLAMP-1-specific Ab, and examined with a confocal fluorescent microscope in acidic buffer (pH 4.5). Blue: nuclei, Green: LAMP-1, Red: AFO. Scale bars indicate 20 μm.

Supplemental Figure 5. Recovery of the β-Hex activity in SD mice after i.c.v. administration.



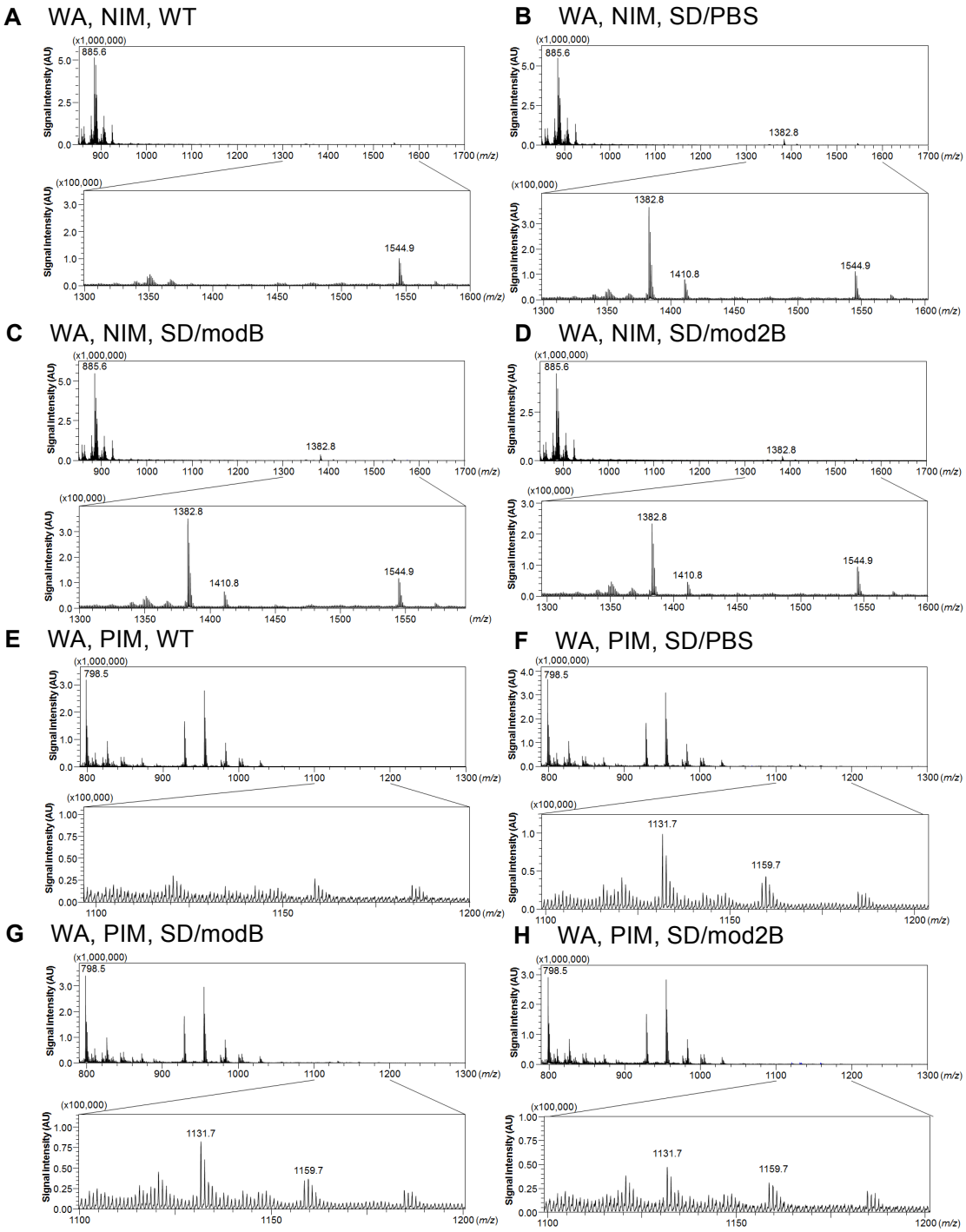
(A) Recovery of the β-Hex activity in SD mouse brains. ModB and mod2B (1 or 5 mg/kg BW dose) were administered to SD mice by i.c.v. injection. Error bars show the means ± SEM (PBS-treated, $n = 10$, 1 mg/kg BW dose, $n = 3$, 5 mg/kg BW dose, $n = 4$). ANOVA with a Tukey *post-hoc* test, $*P < 0.05$, $**P < 0.01$ (vs PBS-treated control). **(B, C)** Recovery of the β-Hex activity in SD mouse livers **(B)** and spleens **(C)**. ModB and mod2B (1 or 5 mg/kg BW dose) were administered to SD mice by i.c.v. injection. Error bars show the means ± SEM (PBS-treated, $n = 8$, 1 mg/kg BW dose, $n = 3$, 5 mg/kg BW dose, $n = 4$). ANOVA with a Tukey *post-hoc* test, $***P < 0.001$ (vs PBS-treated control), $††P < 0.01$, $†††P < 0.001$. **(D)** *In situ* imaging of β-Hex activity with HMDER-βGlcNAc. Brain sections were incubated with HMDER-βGlcNAc, and then were examined with a BIOREVO BZ-9000 device. Yellow: HMDER, Blue: nuclei.

Supplemental Figure 6. MS/MS spectra detected by MALDI-MS/MS directly from the brain sections.



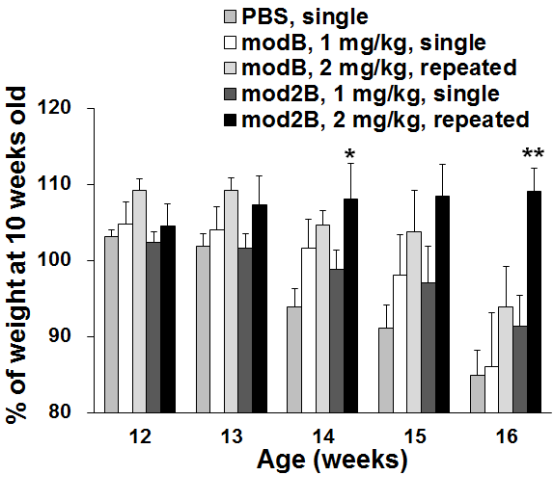
(A, B) GM2 (d18:1/18:0) was fragmented into a main product ion at *m/z* 1091.7, resulting from the loss of the *N*-acetylneuraminic acid. Similar to a previous report (6), we also monitored the product ions at *m/z* 290.1, 564.5, 726.6 and 888.6 by a MS/MS analysis. **(C, D)** GM1 (d18:1/18:0) was fragmented into product ions at *m/z* 564.5, 726.6, 888.6 and 1253.8, similar to a previous report (7). **(E, F)** BMP (22:6/22:6) was fragmented into product ions at *m/z* 283.2, 327.2, 463.2 and 537.3. DHA: docosahexaenoic acid. **(G, H)** GA2 (d18:1/18:0) was fragmented into product ions at *m/z* 584.2 and 928.6.

Supplemental Figure 7. Averaged spectra from the brain slices of WT and SD mice.



Brains from 11-week-old WT and SD mice treated with PBS, modB or mod2B (1 mg/kg BW dose) examined using the negative ion mode (NIM) or positive ion mode (PIM). WA: whole area.

Supplemental Figure 8. Comparison of the changes in body weight.



PBS, modB and mod2B (1 or 2 mg/kg BW dose) were singly or repeatedly administrated to SD mice by *i.c.v.* injection. Error bars show the means ± SEM (PBS-treated, $n = 11$, modB single injection, $n = 4$, modB repeated injection, $n = 4$, mod2B single injection, $n = 6$, mod2B repeated injection, $n = 4$). ANOVA with a Tukey *post-hoc* test, $^{*}P < 0.05$, $^{**}P < 0.01$ (vs PBS-treated control).

Supplemental Table1. Three-step column chromatography for purification of modified HexBs

	modB		mod2B	
	Purification Factor	Yield (%)	Purification Factor	Yield (%)
Conditioned medium	1.0	100	1.0	100
AF-Blue elution	2.1	83	1.9	80
Phos-tag elution	2.7	62	2.1	61
SP elution	3.3	28	2.6	22

Supplemental Table 2. Data collection and refinement statistics.

	modB†
Data collection	
Space group	$P4_22_12$
Cell dimensions	
a, *b*, *c* (Å)	126.47, 126.47, 88.31
α, β, γ (°)	90, 90, 90
Resolution (Å)	40–2.4(2.5–2.4)*
R_{merge}	0.143(0.698)
$I / \sigma I$	12.3(2.6)
Completeness (%)	100(98.8)
Redundancy	12.0(9.2)
Refinement	
Resolution (Å)	40–2.4
No. reflections	27,149
R_{work} / R_{free}	0.187/0.240
No. atoms	
Protein	3,952
Ligand/ion	65
Water	150
B-factors	
Protein	35.9
Ligand/ion	76.2
Water	31.0
R.M.S. deviations	
Bond lengths (Å)	0.013
Bond angles (°)	1.610

† A single crystal was used for data collection.

* Values in parentheses are for the highest-resolution shell.

Supplemental Table 3. The primer sets and polymerase chain reaction (PCR) conditions.

Fragment	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temperature (^{o}C)	Number of Cycles
1	AAAGAATTC CTCGAGCAC CATGCTGCTG GCGCTG	GGTGCCAGAG GGCTCAGACC CACTGTAACA TGGAGTCAG	65	30
2	GAGCCCTCTG GCACCTTTGG ACCTATAAA C	GAGGGAAAAA GATCTTACAT GTTCTCATG	60	30

Supplemental References

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