Supportive Information

The ceramide moiety of disialoganglioside (GD3) is essential for GD3 recognition by the sialic acid-binding lectin SIGLEC7 on the cell surface

Noboru Hashimoto[1,3], Shizuka Ito[1], Akiko Tsuchida[1], Robiul H. Bhuiyan[1,2], Tetsuya Okajima[1], Akihito Yamamoto[3], Keiko Furukawa[2], Yuhsuke Ohmi[2], and Koichi Furukawa[1,2]*

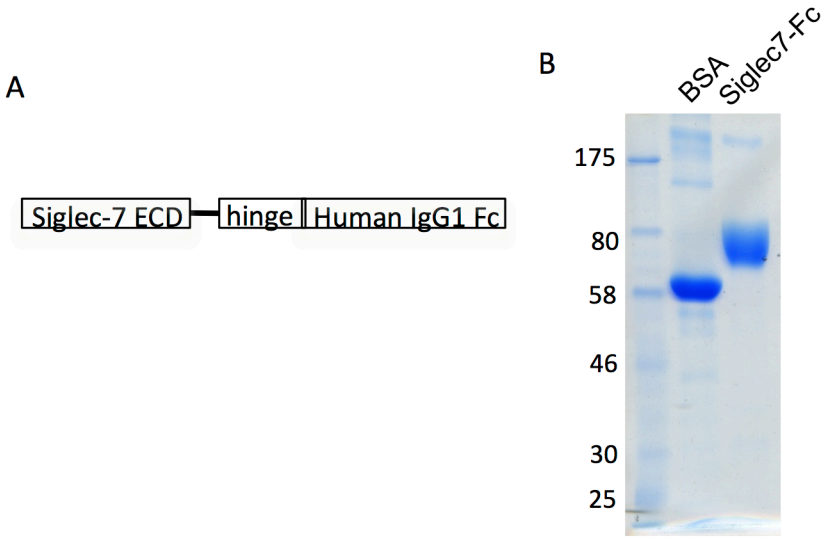


Figure S1. Expression and purification of Siglec-7-Fc.
pEE14 Siglec-7-Fc expression vector (A) was transfected into HEK293T, and cultured in ITS-containing D-MEM for 3 days. Siglec-7-Fc protein secreted in the supernatant was collected and purified using protein A-conjugated beads (B). Purified Siglec-7-Fc protein (5 μg) as well as BSA was applied in SDS-PAGE and stained by Coomassie brilliant blue.

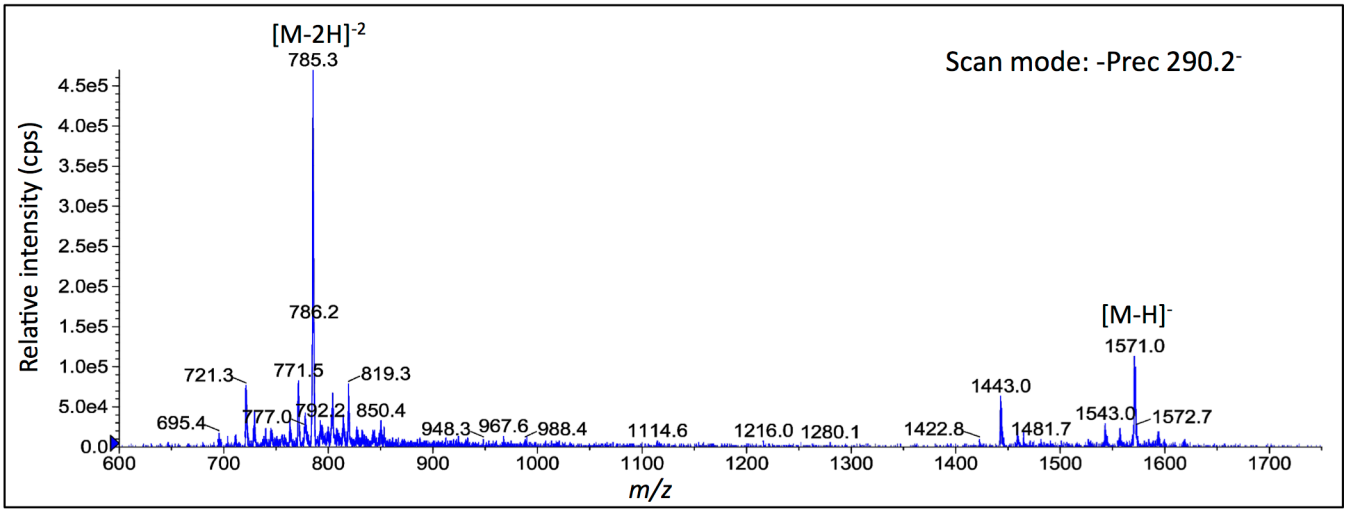
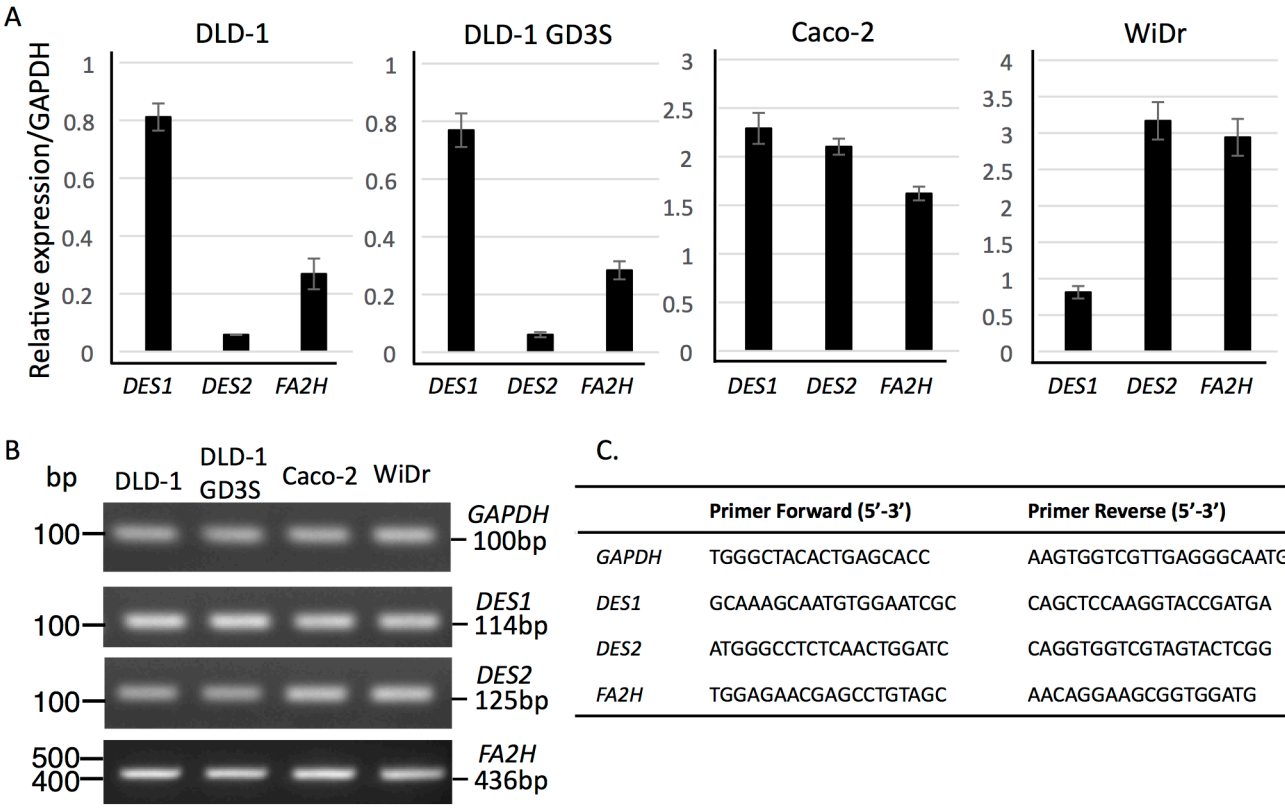


Figure S2. Precursor ion scan using triple quadrupole mass spectrometer against DLD-1 GD3S−derived GD3.

Precursor ions of Neu5Ac (*m/z* 290^-) were analyzed in the mass range of 600-1800 (*m/z*) in negative ion mode. Both of $[M-H]^-$ and $[M-2H]^{2-}$ were detected. In the analysis of GD3, $[M-2H]^{2-}$ was more abundantly detected than $[M-H]^-$.



C.

	Primer Forward (5'-3')	Primer Reverse (5'-3')
GAPDH	TGGGCTACACTGAGCACC	AAGTGGTCGTTGAGGGCAATG
DES1	GCAAAGCAATGTGGAATCGC	CAGCTCCAAGGTACCGATGA
DES2	ATGGGCCTCTCAACTGGATC	CAGGTGGTCGTAGTACTCGG
FA2H	TGGAGAACGAGCCTGTAGC	AACAGGAAGCGGTGGATG

Figure S3. Expression levels of ceramide modification-related enzymes.
A, Expression levels of *DES1*, *DES2* and *FA2H* relative to house keeping gene *Gapdh* in DLD-1, DLD-1 GD3S, Caco-2 and WiDr were analyzed by real time q-PCR. *DES2* and *FA2H* were highly expressed in WiDr. B, PCR products were applied to gel electrophoresis. C, A list of primers used in this experiment.

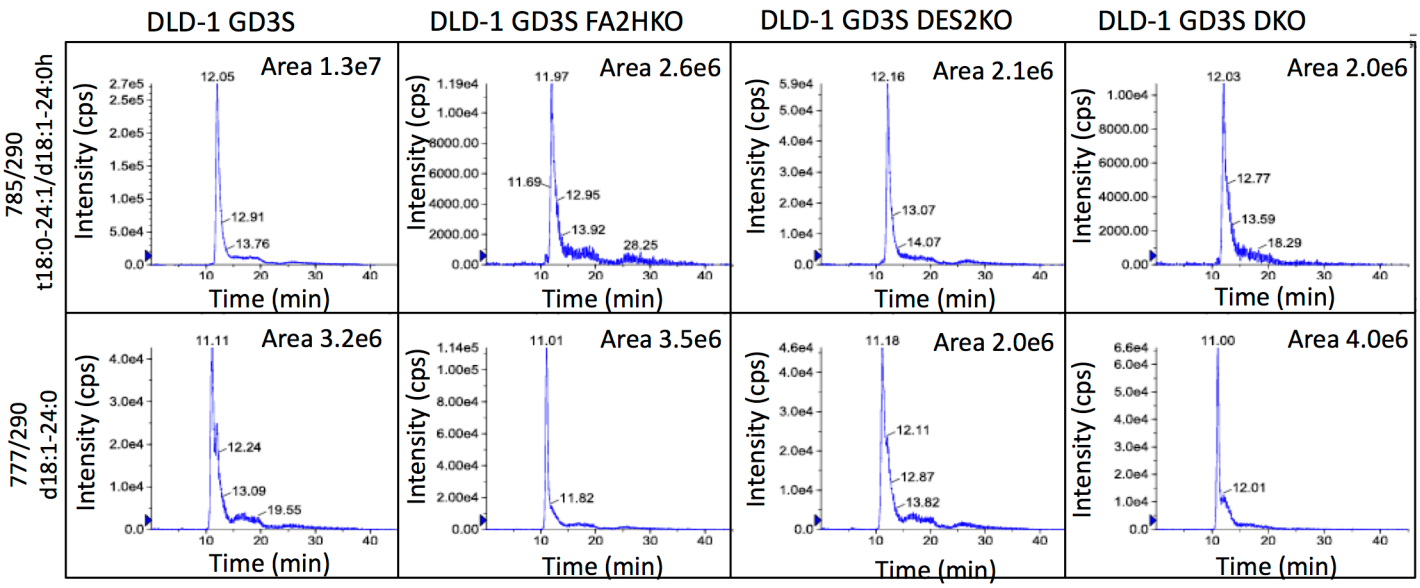
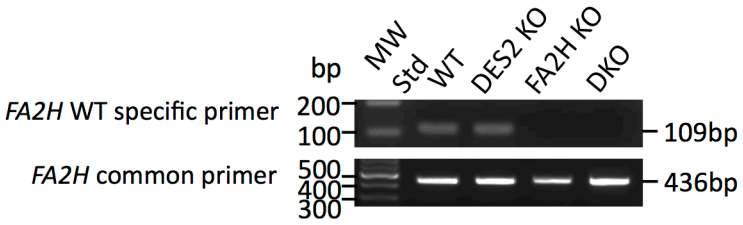


Figure S4. Quantification of major hydroxylated GD3 by HILIC/MRM analysis.

To exclude the effect of solvent condition by eluting at the same retension time, HILIC-MRM analyses were performed. HILIC column can separate molecular species of gangliosides based on differences in glycan structures. To examine the influence of knockout of hydroxylases, DES2 and FA2H, the main GD3 species t18:0-24:1/d18:1-24:0h (*m/z* 785) and the corresponding regular structure d18:1-24:0 (*m/z* 777) were analyzed by MRM. Their area-value means abundance of their ceramide strucure of GD3. Knockout of the hydroxylase genes resulted in the decrease of the ratio of hydroxylated ceramide structures of GD3. This result was summarized in Figure 5C.



	Primer Forward (5'-3')	Primer Reverse (5'-3')
FA2H WT specific	CCAGCTTCGTGCGGCACCA	GCGTTGGCCGAGTGCCTGTG
FA2H common	TGGAGAACGAGCCTGTAGC	AACAGGAAGCGGTGGATG

Figure S5. Confirmation of knockout of FA2H by PCR with WT seq-specific primers.
FA2H WT specific primer recognize 1 base difference of 3' terminal side. PCR with confronting two-pair primers was performed to confirm the complete deletion of genes following a past article (Hamajima N, et al. (Jpn J Cancer Res. 2000, 91:865-8)). Deletion of *FA2H* was confirmed in *FA2H* KO and DKO DLD-1 GD3S. As for *DES2 knockout*, it was hard to detect one base deletion in this system.

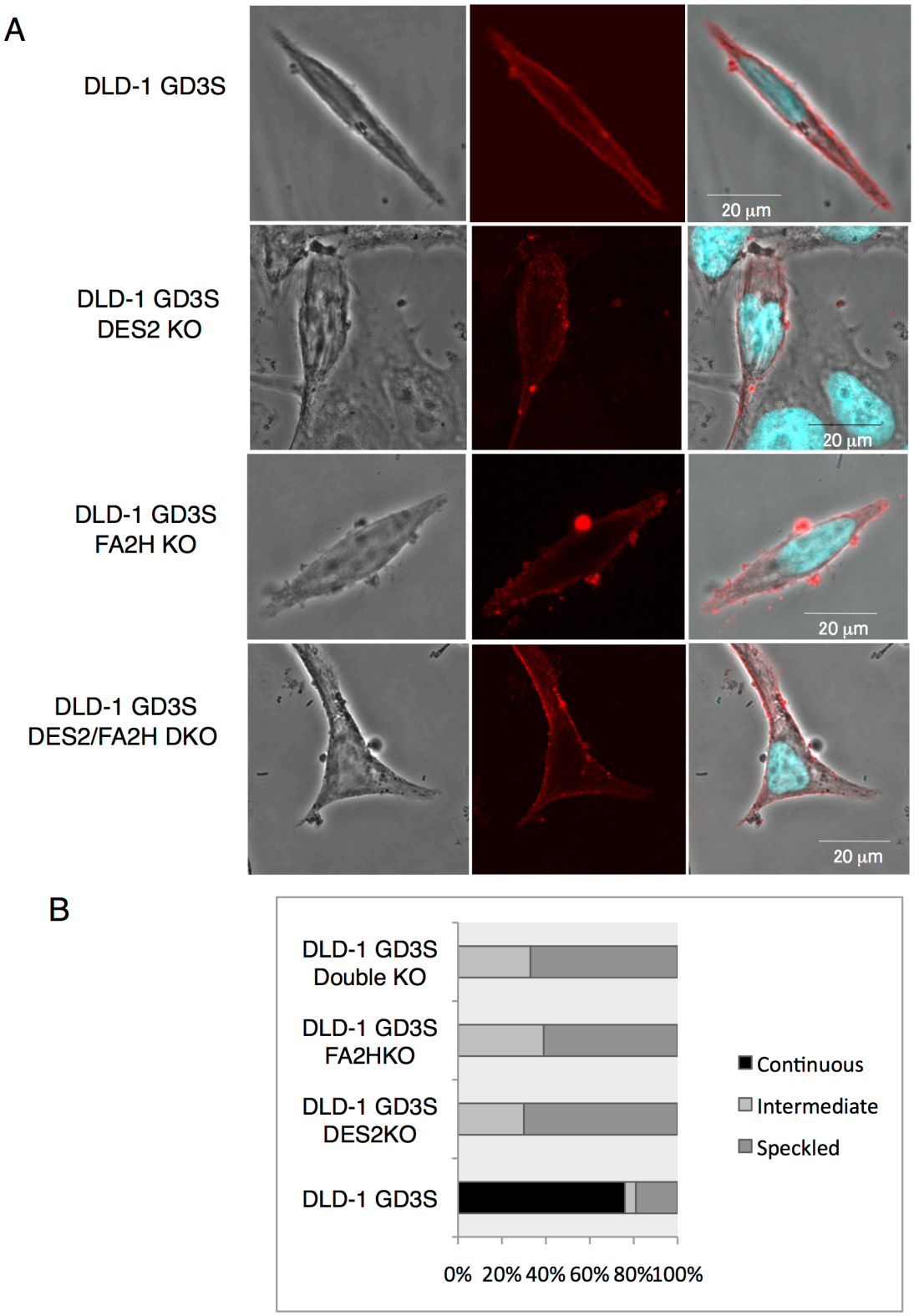


Figure S6. Intracellular distribution of GD3 in DLD-1 GD3S and transfectant cells.
DLD-1 cells were transfected with GD3 synthase cDNA (DLD-1 GD3S). Then, DES2, FA2H or both of them were knocked-out using CRISPR/Cas9 system. A, these cells were cultured in glass-bottom dishes and fixed and stained by anti-GD3 mAb (R24) and Alexa-568-labeled goat anti-mouse IgG (Invitrogen) as described in the legend for Fig. 6. The staining patterns were classified into continuous, speckled, and intermediate types based on the imaging results by confocal microscope (FV10). B, Results were presented as ratio (%) among all stained cells in the individual cell lines.