Supplementary Data

Comprehensive profiling of N-linked glycosylation sties in HeLa cells using hydrazide enrichment

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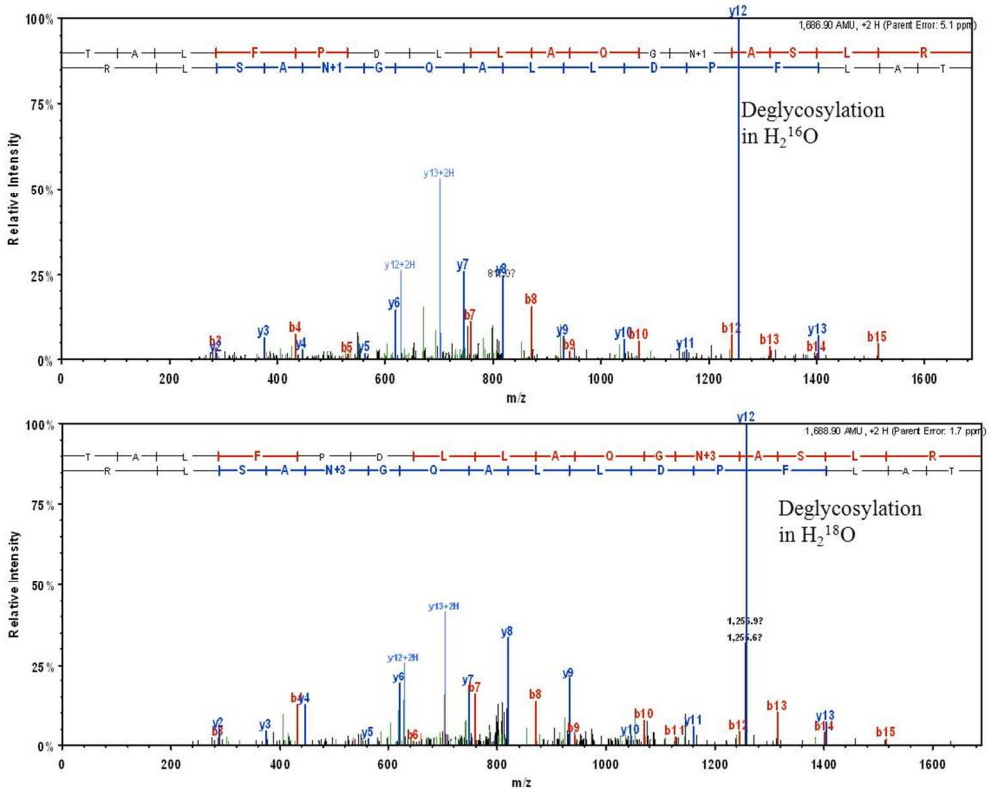
Supplementary Experimental

Digestion *of Flow-through and wash* after conjugation with hydrazide resins

Due to high urea concentration, water was added to the *Flow-through and wash* fractions to reduce the concentration of urea to less than 2 M. Reduction and alkylation were carried out with 50 mM DTT and 500 mM IAM, incubated at room temperature in the dark for 1 hour (reduction) and 30 minutes (alkylation), respectively. The pH was adjusted 1.0 M triethylammonium bicarbonate (pH 8.5) prior to addition of 20 µg sequencing grade modified porcine trypsin in 1 mL of 50 mM ABC. Protein digestion was carried out overnight at room temperature. Formic acid was added to stop the protein digestion.

Supplementary Figures

a



b

Deglycosylation in $H_2{}^{16}O$

B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	102.1			84.0	T	1,687.9	844.5	1,670.9	1,669.9	16
2	173.1			155.1	A	1,586.9	793.9	1,569.8	1,568.8	15
3	286.2			268.2	L	1,515.8	758.4	1,498.8	1,497.8	14
4	433.2			415.2	F	1,402.7	701.9	1,385.7	1,384.7	13
5	530.3			512.3	P	1,255.7	628.3	1,238.6	1,237.7	12
6	645.3	323.2		627.3	D	1,158.6	579.8	1,141.6	1,140.6	11
7	758.4	379.7		740.4	L	1,043.6	522.3	1,026.6	1,025.6	10
8	871.5	436.2		853.5	L	930.5	465.8	913.5	912.5	9
9	942.5	471.8		924.5	A	817.4	409.2	800.4	799.4	8
10	1,070.6	535.8	1,053.6	1,052.6	Q	746.4	373.7	729.4	728.4	7
11	1,127.6	564.3	1,110.6	1,109.6	G	618.3	309.7	601.3	600.3	6
12	1,242.6	621.8	1,225.6	1,224.6	N+1	561.3		544.3	543.3	5
13	1,313.7	657.3	1,296.6	1,295.7	A	446.3		429.2	428.3	4
14	1,400.7	700.9	1,383.7	1,382.7	S	375.2		358.2	357.2	3
15	1,513.8	757.4	1,496.8	1,495.8	L	288.2		271.2		2
16	1,687.9	844.5	1,670.9	1,669.9	R	175.1		158.1		1

Deglycosylation in $H_2{}^{18}O$

B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	102.1			84.0	T	1,689.9	845.5	1,672.9	1,671.9	16
2	173.1			155.1	A	1,588.9	794.9	1,571.8	1,570.8	15
3	286.2			268.2	L	1,517.8	759.4	1,500.8	1,499.8	14
4	433.2			415.2	F	1,404.7	702.9	1,387.7	1,386.7	13
5	530.3			512.3	P	1,257.7	629.3	1,240.6	1,239.7	12
6	645.3	323.2		627.3	D	1,160.6	580.8	1,143.6	1,142.6	11
7	758.4	379.7		740.4	L	1,045.6	523.3	1,028.6	1,027.6	10
8	871.5	436.2		853.5	L	932.5	466.8	915.5	914.5	9
9	942.5	471.8		924.5	A	819.4	410.2	802.4	801.4	8
10	1,070.6	535.8	1,053.6	1,052.6	Q	748.4	374.7	731.4	730.4	7
11	1,127.6	564.3	1,110.6	1,109.6	G	620.3	310.7	603.3	602.3	6
12	1,244.6	622.8	1,227.6	1,226.6	N+3	563.3		546.3	545.3	5
13	1,315.7	658.3	1,298.7	1,297.7	A	446.3		429.2	428.3	4
14	1,402.7	701.9	1,385.7	1,384.7	S	375.2		358.2	357.2	3
15	1,515.8	758.4	1,498.8	1,497.8	L	288.2		271.2		2
16	1,689.9	845.5	1,672.9	1,671.9	R	175.1		158.1		1

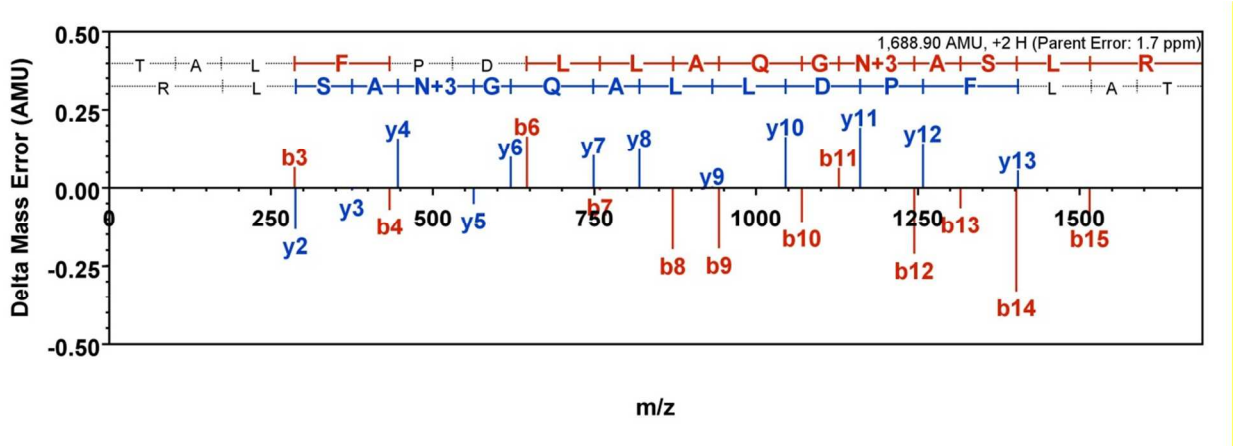


Figure S1. a) MS/MS spectrum and b) MS/MS ion lists and matches of (R)TALFPDLLAQGN*ASLR(L) identified in isoform 1 of CD276 antigen deglycosylated in $H_2{}^{16}O$ (top) and $H_2{}^{18}O$ (bottom).

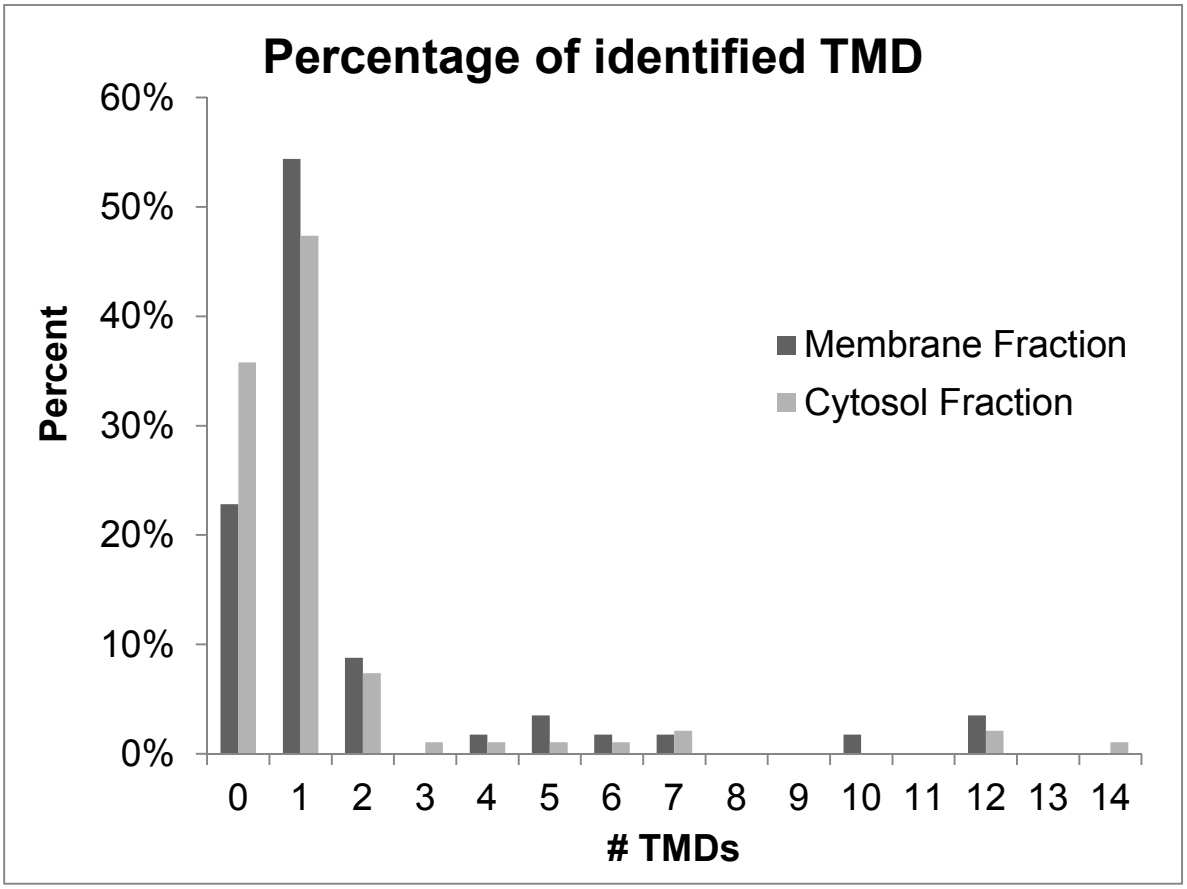


Figure S2. Percentage of identified TM domains in membrane (dark grey) and cytosolic (light grey) fraction.

Table S8. David Functional Annotation Chart: KEGG pathways related to cancer

Term	Count	%	PValue	Fold Enrichment	Bonferroni	Benjamini	FDR
hsa04142:Lysosome	23	20	1.95E-20	14.48717949	1.38E-18	1.38E-18	2.02E-17
hsa04512:ECM-receptor interaction	11	9.565217	1.17E-07	9.650621118	8.31E-06	4.16E-06	1.22E-04
hsa04514:Cell adhesion molecules (CAMs)	10	8.695652	5.49E-05	5.583003953	0.003888	7.79E-04	0.056932
hsa04510:Focal adhesion	11	9.565217	2.91E-04	4.033095393	0.0204811	0.002583	0.302056
hsa05222:Small cell lung cancer	7	6.086957	8.20E-04	6.141304348	0.0565665	0.006449	0.847618
hsa00600:Sphingolipid metabolism	4	3.478261	0.014827196	7.558528428	0.6537567	0.100631	14.36245
hsa04810:Regulation of actin cytoskeleton	8	6.956522	0.023801012	2.742163802	0.8191897	0.132835	22.12165
hsa05200:Pathways in cancer	10	8.695652	0.029302846	2.246818664	0.878955	0.149925	26.55875

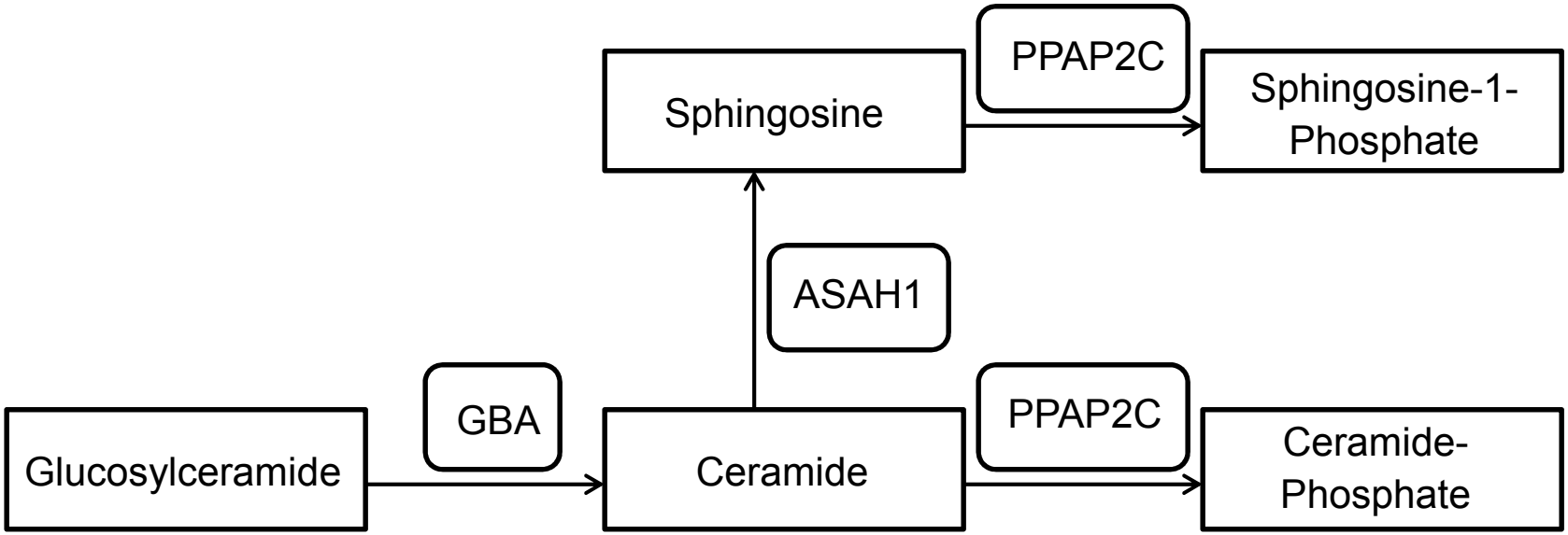


Figure S3. Ceramide pathway. Enzumes: GBA: Glucosylceramidase, ASAH1: acid Ceramidase 1, PPAP2C: Lipid phosphate phosphohydrolase 2.

Sphingolipid metabolism

Sphingolipids play key roles in cell recognition and signal transmission.[1] In the sphingolipid pathway, tumor suppressors like ceramide are found as well tumor promoters such as sphingosine-1-phosphate (S1P). In this pathway, we identified four enzymes, of which three (GBA, ASAH1, and PPAP2C) directly control the synthesis and metabolism of ceramide (**Supplementary Figure S3**). In addition, proactivator polypeptide (prosaposin) was found. Prosaposin cleaves into four proteins, saposins A – D. Each saposin plays a role in glycosphingolipid catabolism.

1. Ogretmen, B.; Hannun, Y. A., Biologically active sphingolipids in cancer pathogenesis and treatment. *Nat. Rev. Cancer* **2004,** 4, (8), 604-616.