#### The Glycosylation Design Space for Recombinant Lysosomal Replacement Enzymes Produced in CHO Cells

Tian et al.

#### **Supplementary Information**

**Supplementary Figure 1.** RNAseq transcriptome profiling of CHO cells showing predicted expression of selected genes.

**Supplementary Figure 2.** Detailed presentation of N-glycans identified by site-specific N-glycan profiling of GLA produced in CHO WT and engineered KO/KI clones as indicated.

**Supplementary Figure 3.** Characterization of growth and yield performance of glycoengineered CHO clones expressing GLA.

**Supplementary Figure 4.** Detailed presentation of N-glycans identified by site-specific N-glycan profiling of GBA produced in CHO WT and engineered KO clones as indicated.

Supplementary Figure 5. Targeted MS/MS manual annotation of the most representative glycopeptides.

Supplementary Figure 6. In vitro assay of enzyme specific activity and plasma stability of GLA glycovariants.

Supplementary Figure 7. SDS-PAGE Western blot analysis of infused GLA glycovariants in mice plasma.

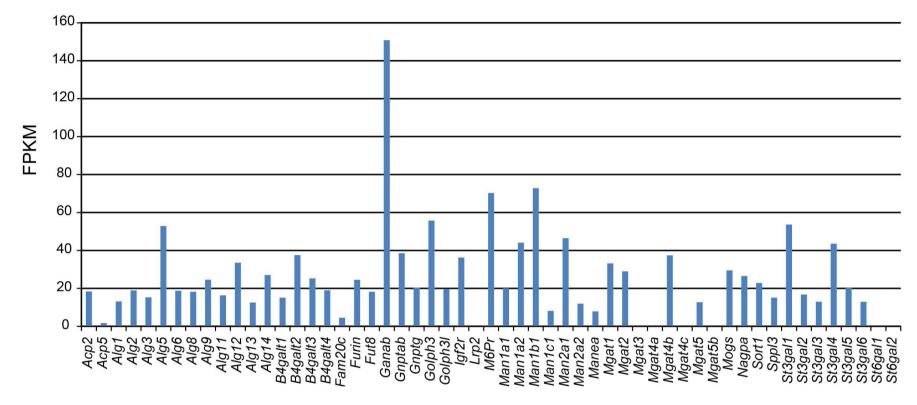
Supplementary Figure 8. Relative distribution of GLA glycovariants among the four major visceral organs.

Supplementary Table 1. CRISPR gRNA design and list of PCR primers used for gRNA target sites.

Supplementary Table 2. Summary of CHO mutant clones stably expressing GLA and cell line ancestry.

Supplementary Table 3. Summary of CHO mutant clones stably expressing GBA and cell line ancestry.

Supplementary Table 4. Sequence analysis of CHO mutant clones stably expressing GBA.



Supplementary Figure 1. RNAseq transcriptome profiling of CHO cells showing predicted expression of select genes studied.

RNAseq analysis was performed with CHO GS<sup>-/-</sup> cells as previously reported<sup>1</sup>. Genes known to function in N-glycosylation and M6P-tagging, including glycosyltransferases, glycosylhydrolases, enzymes involved in dolichol-linked precursor oligosaccharide synthesis, and other related genes, are shown. Source data are provided as a Source Data file.

<sup>1</sup>Yang, Z. et al. Engineered CHO cells for production of diverse, homogeneous glycoproteins. *Nat. Biotechnol.* **33**, 842-844 (2015).

			I	N108			N161						٦	V184		
#1	Fabrazyme	1000 1X 25	86 2 X 20 3 X 14	59	44	26 16	1 X P 100 1 X 11 11	1 X P 84	19	15	13	100 1 XP 14	2 X P 84	1XP 39 1X	1 X P 24 2 X P 11	23
#2	WT (FAB#A7)	100	67	32	22	2 X •	1 X P 100	1XP 57	53	35	20	1 X P 100	1 X • • • • • • • • • • • • • • • • • •	1 <u>X</u> P	2 X P 30	1 X P 16
#3	WT (FAB#H9)	100 16	96	1 X	44	2 X • • • • • • • • • • • • • • • • • •	1 X P 100 1 X P 1 X P 1 X P	<u>1 X P</u> 67	40	25	23	1 X P 100	1 X • • • • • • • • • • • • • • • • • •	39	1 <u>X</u> P 19	2 X P 18
#4	KO <i>Alg3</i> (FAB478F5)	1 X P 100	78	43	1 X • • • • • • • • • • • • • • • • • •	2 X	1 X P 100	70	52	15	14	1 X P 100	1 X			
#5	KO <i>Alg3</i> (FAB478H2)	1XP 100	69 3X	46	40	2 X	1X 100 1XP 24	1 X P 94	49 1XP 24	43 1× 16	26 2 X 11	1 X P 100				
#6	KO <i>Alg9</i> (FAB480C2)	100	64	24	1 X 20	1 X P 12	100	34	1 X 28	1 X P 12		1 X P 100	1 X P 87	1 X • • •	12	

**Supplementary Figure 2.** Detailed presentation of N-glycans identified by site-specific N-glycan profiling of GLA produced in CHO WT and engineered KO/KI clones as indicated.

N-glycan structures and their relative abundances at each of the three N-glycosites (N108, N161, and N184) of GLA are illustrated with their relative abundance adjusted to the most abundant structure. Minor glycoforms identified with relative abundance less than 10% are not shown. Same N-glycan composition may represent isobaric structures.

(Page 1 of 12, continued)

#7 KO <i>Alg12</i> (FAB664B2)	1 XP 100 93 41 32 31 2 X 2 2 X 19 12	$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	1 XP 1 XP 1 XP 1 XP 1 XP 1 XP 1 XP 1 XP
#8 KO <i>Alg5</i> (FAB667E12)	$1 \times 0$ $1 \times $	$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	1XP 1XP 1XP 1XP 1XP 1XP 2XP 2XP 2XP 2XP 2XP 2XP 2XP 2
#9 KO <i>Alg6</i> (FAB479A8)	$\begin{array}{c} 2 \\ 2 \\ 1 \\ 1 \\ 1 \\ 0 \\ 7 \\ 7 \\ 2 \\ 7 \\ 2 \\ 7 \\ 2 \\ 6 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1$	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	$ \begin{array}{c} 1 \times P \\ 1 \times P $
#10 KO <i>Alg8</i> (FAB662F5)	1 X +       2 X +       1 X P         100       44       42       25       25         3 X +       1 X P       1 X P       1 X P         22       14       13       13	1XP 1XP 1XP 1XP 1XP 1XP 1XP 1XP	1XP 100 42 31 18 15
#11 KO <i>Manea</i> (FAB560A4)	1 X 2 X 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	1XP         1XP <th12p< th=""> <th12p< th=""> <th12p< th=""></th12p<></th12p<></th12p<>	1XP 100 23 22 16

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#12 KO <i>Man1a1</i> (FAB442G8)	100	1 X 51	43	2 ×	14	1 X P 100	35	1 X P 14	1 X • • • • • • • • • • • • • • • • • •		1 X P 100	2X P 31	29	1 <u>X</u> P 26	<u>1 X P</u> 14
#13 KO <i>Man1a2</i> (FAB443C5)	1 X 100	72 1X	49 3 X 12	32	2X	1XP 100	73	1 <u>X</u> P 37	1XP 37	1 X	1 <u>XP</u> 100	1 <u>X</u> P 34	<u>2 X P</u> 30	1 X	1XP 12
#14 KO <i>Man1b1</i> (FAB444E4)	100	55	1 X	15	2 X •	1XP 100	<u>1 X P</u> 87	44	1 X P 42	1 X	1 X P 100 2 X P 12	38	33	2XP 15	1 <u>X</u> P 13
#15 KO <i>Man1c1</i> (FAB445H5)	2X• 100	59	1 1 1	20	2 ×	1 X P 100	72	1XP 44	36	21	1 X P 100	<u>2XP</u> 31	27	1 X	1XP 15
#16 KO Man1a1/1a2/1b1/1c1 (FAB725G4)	100	94	1 X P 41	41	39	1 X P 100	<u>1 X P</u> 70	<u>1 X P</u> 39	17	13	1 X P 100	<u>1 X P</u> 89	1 X P 47	<u>1 X P</u> 14	•1 X P •1 3
	25	1 X P 17	16			12	11	1 X P 10			1 X P 12	12	12	11	

#17 KO Man2a1/2 (FAB712D1)	100 35 11	1XP         0	1XP 100 55 37 22 14
#18 KO <i>Mgat1</i> (FAB495B10)	100 45	1 X P 1 X P 1 0 46 46	1XP 1XP 100 56 17
#19 KO <i>Mgat2</i> (FAB496E4)	100 27 22 13	1XP 1XP 100 57 32 14 12	$\begin{array}{c} 1 \times P \\ 1 \times P \\ 1 \times P \\ 1 \times P \\ 2 \times P \\$
#20 KO <i>Mgat4b/5</i> (FAB497G4)	1 X 1 X 1 0 39	$\begin{array}{c c} 1 \underline{X} \underline{P} \\ 1 \underline{X} \underline{P} \\ 1 0 0 \\ 6 4 \\ 1 2 \end{array}$	$\begin{array}{c} 1 \times P \\ 1 \times P \\ 1 \times 0 \end{array} \begin{array}{c} 2 \times P \\ 3 \times 0 \end{array} \begin{array}{c} 1 \times P \\ 1 \times 0 \end{array} \begin{array}{c} 2 \times P \\ 1 \times 0 \end{array} \begin{array}{c} 1 \times P \\ 1 \times 0 \end{array} \begin{array}{c} 1 \times P \\ 1 \times 0 \end{array} \begin{array}{c} 1 \times P \\ 1 \times 0 \end{array}$
#21 KO <i>Mogs</i> (FAB446A2)	100 18	1 <u>XP</u> 100 27	1 <u>x</u> P 100 77

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#22 KO Ganab (FAB572E8)	100	79				100	1 <u>X P</u> 44	26	1 <u>x p</u> 14		100 100	94	65	62	1 <u>X</u> P 22
#23 KO <i>Gnptab</i> (FAB400C7)	100	1 X • • • • • • • • • • • • • • • • • •	15			100	79	1 X • • • • • • • • • • • • • • • • • •	22		2 X • • • • • • • • • • • • • • • • • •	75 1X 30	73 1 X 26		52
#24 KO <i>Gnptg</i> (FAB453C1)	100	54	1 X• 29	20	2X• • • • • 16	100	1 X •	41	34	1 X • • • • • • • • • • • • • • • • • •	1 X 0 1 100	16	1 X •		
#25 KO Gnptab/g (FAB546A2)	100 3 X • 16	57	1 X	2×	26	100	43	1 X			2X 100 3X 13	1 X	41	19	2 X
#26 KO <i>Nagpa</i> (FAB399C3)		53 1 X P 22	44	39	2X	1 X P 1 X P 100	1 X P 1 X P 46	1 X 1 X 1 X 1 X 1 X 1 X 1 X 1 X 1 X 1 X	1 X •	2X 2XP 12	2 X 2 X P 100	1 X P 1 X P 64	1 X P 1 X P 27	2 X P 2 X P 13	1 X P 1 X P 1 2

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#27 KO <i>Acp2</i> (FAB451H7)	1 X • 2 X • 0 • 0 • 0 • 0 • 0 • 0 • 0 • 0 • 0 •		0 78 52 39	$\begin{array}{c} X \\ \hline \\ 1 \\ X \\ 32 \end{array}  \begin{array}{c} 1 \\ X \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\$
#28 KO <i>Acp5</i> (FAB454F2)	1 X 1 X 1 00 26 24		Ϋ́Ϋ́Ύ	1XP 100 26 22
#29 KO <i>Acp2/5</i> (FAB462C1)	100 23 17			$\begin{array}{c} 1 \times P \\ \hline 1 \times P \\ 1 00 \\ \hline 1 \\ 1 1 \end{array}$
#30 KO <i>Acp2/5</i> (FAB462B2)	1 X 1 X 1 00 78 39 1 X 2 X 10 10 10 10 10 10 10 10 10 10	2X 2X 1X 35 16 100		$\begin{array}{c} 1 \\ \hline 1 \hline 1$
#31 KO <i>B4galt1/3</i> (FAB688F7)	$\begin{array}{c} 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 \\ 1 $	1 X 0 1 X 0 1 X 0 1 X 0 20 1 X 20 1 7 19 1 X 11 11	0  60  33  25	$\begin{array}{c} 1 \\ 1 \\ 23 \\ 23 \\ 100 \\ 27 \\ 27 \\ 27 \\ 19 \\ 19 \\ 19 \\ 17 \\ 12 \end{array}$

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t <b>3gal4/6</b> 494B4)	100	35	1 X 20	12	1 X	<u>1 X P</u> 100	1 X P 64	48	1 X		1 X P 100	64	1 <u>X</u> P 41	2XP 18	1 X P 16
		58 <u>1XP</u>	42	1 X • • • • • • • • • • • • • • • • • •	3 X • 36 2 X •	1 X P 100	95	61	1 X •	1 X P 16	16 <u>1XP</u> 100	2 X P 41 2 X P	29	1 X	1 X P 15
<i>lgf2r</i> 441C2)	23	17 1X 41	13 13 38	12 2 X • 21	11 11	14 1 X P 100	12 12 12 70	1 <u>X</u> P 32	1 X •		13 <u>1 x P</u> 100	12 2 X P 45	1 X P	1 X 26	1 X P 14
<b>Мбрг</b> 791F5)	100	72	1 X •	1 <u>X P</u> 17		1 X P 100	1 X P 46	1 X	1 <u>XP</u> 14	14	1 X P 100	1X 89	1 X P 86	1 <u>X</u> P 70	18
Sort1 534F1)	100	1 X • 58	36	14	2 X • • • • • • • • • • • • • • • • • •	1 X P 100	1 X P 52	37	1 X • • • • 32	1 <u>X</u> P 19	1 X P 100	1 X • • • • • • • • • • • • • • • • • •	1 X P 26	1 X P 18	10

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#37	KO <i>Lrp2</i> (FAB535C2)	100	42	1 X • • • • • • • • • • • • • • • • • •	2 X	16	<u>1 X P</u> 100	1XP 51	42	26	1 X •	1 X P 100	1 <u>X</u> P 30	1 X • • • • • • • • • • • • • • • • • •	1 X P 16	<u>2 X P</u> 13
#38	KO <i>Sppl3</i> (FAB499B1)	100	1 X • • • • 19	13			1 X P 100	78	1 <u>X</u> P 70	50		1 X P 100	1 X F 25	15	1 X • • • • • • • • • • • • • • • • • •	1 <u>X</u> P
#39	KO <i>Furin</i> (FAB555E6)	100	89	57	2 Х		1 <u>x</u> 100 1 <u>x</u> 26	$\frac{1 \times P}{66}$	1 X P 61	43	1 X P 27	1 X P 100 1 X P 1 X P 1 1	1X 39 2XP	1 X 1 X P 38	1 <u>X</u> P 19	1 X 2 X P 17
#40	KO <i>Fam20c</i> (FAB604F9)	100	47	1 X	2 %		1 X P 100	<u>1 X P</u> 54	51	1 <u>X</u> P 27	1 X	1 X P 100	1 <u>X</u> P 37	2 X P 22	1 X P	
#41	KO Golph3 (FAB605D1)	100	64	58	2 X	3X <b>•</b> 12	1 X P 100	1 X P 53	1 X P	21		<u>1 X P</u> 100 <u>1 X P</u> 13	47	2XP 24	1 X	13

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#42 KO <i>Golph3l</i> (FAB606E12)	1 X 2 X 2 X 1 X 1 X 1 X 1 X 1 X 1 X 1 X	$\frac{1 \times P}{100}  \frac{1 \times P}{48}  \frac{1 \times P}{22}  \frac{1 \times \Phi}{22}  \frac{1 \times \Phi}{100}$	1XP 1XP 1XP 1XP 1XP 1XP 1XP 1XP
#43 KO <i>Mgat1/Alg3</i> (FAB611H3)	1 <u>XP</u> 100 13 11	$\begin{array}{c} 1 \underline{X} \underline{P} \\ 1 \underline{X} \underline{P} \\ 1 0 0 \end{array} \qquad \begin{array}{c} 1 \underline{X} \underline{P} \\ 1 \underline{X} \underline{P} \\ 1 \underline{X} \underline{P} \\ 1 0 \end{array}$	1 X P 100 36
#44 KO Gnptab/g KO Mgat1 (FAB568A8)	100	100	100
#45 KO Gnptab KO Man2a1/2 (FAB713A7)	100	1 X 1 X 1 X 1 X 1 X 1 X 1 X 1 X	100 17
#46 KO Gnptab/g KO Mgat2 (FAB570B7)		100 97 52 39 28 1X 1X 28 26 15 13	1 X 1 X 1 X 1 X 1 X 1 X 1 X 1 X

(Page 9 of 12, continued)

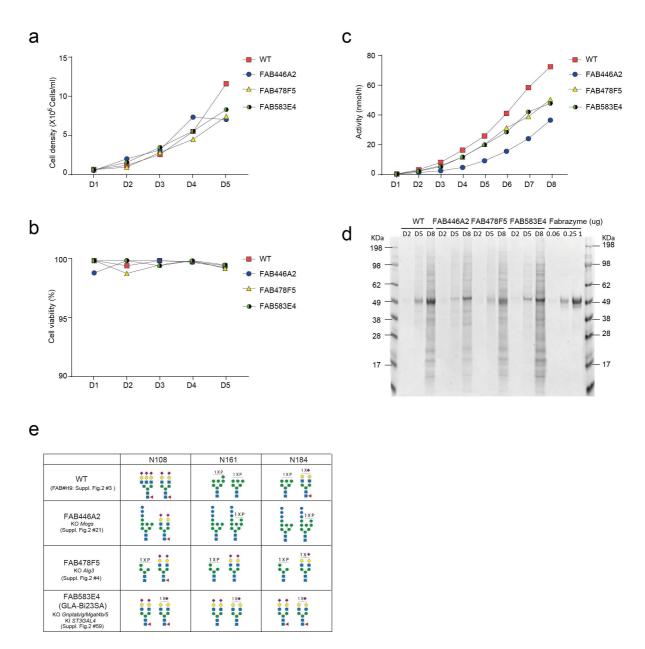
КО	Gnptab/g Mgat4b/5 AB567H3)	100	1 X • 45				100	1 X •	29	27	18	100	1 X	55	1 X • 1 X •	1 X • • • •
	I <i>GNPTG</i> AB677F1)	3X 26	95 1X 11	2X• 52	1 X	33	1 X P 100	1XP 54	1 <u>x</u> P 27	23	1 <u>X</u> P 12	1 X P 100 1 X P 100	<u>2 X P</u> 24	2 X	16	1 <u>x</u> P 15
	I <i>GNPTG</i> AB677C4)	100 12		51	2 X	3 X • • • • • • • • • • • • • • • • • •	1 X P 100	44	1 X P 25	1 X		1 X P 100 2 X • 11	42	2XP	1 X P 16	12
	(I <i>GNPTAB</i> FAB695A8)	100	1 X 51 3 X 15	1XP 49	11 11	1 X P 31	1 X P 100	1 X P 47	1 <u>X</u> P 20	1 <u>X</u> P 13	12	1 X P 100	2 X P 86	1 <u>X</u> P 36	22	1 X P 17
	(1 <i>GNPTAB</i> FAB695G2)	2 X 25	1 X P 57 1 X P 21	46	34	1 XP 34	1 X P 100	<u>1 X P</u> 53	23	12		1 X P 100	91 1XP 91	2XP 42	1 <u>X</u> P	15

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#52	KI GNPTAB KI GNPTG (Fab819F7)	2 X • • • • • • • • • • • • • • • • • •	32 11XP	1 <u>X</u> P 23	1 X	12	1XP 100	1 X P 43	2 X P 29			1 <u>x</u> 100	2XP 56	40	2 X P 38	<u>1XP</u> 32
#53	KI <i>GNPTAB</i> KI GNPTG (Fab819F6)	100 100 1X 35	74 2 X 16	67 2 X • 11	1XP 43	<u>1 X P</u> 35	1 <u>X</u> P 100	1XP 36	1 <u>x</u> 16	P		1 <u>X</u> 100	1 <u>X</u> P 63	2 X P 42	1 <u>X</u> P 24	19
#54	KI <i>GNPTAB</i> KO <i>Alg3</i> (Fab792G6)	1 X P 100					1 X P 100	20	17	1 X • 15		1 X P 100				
#55	KI GNPTAB KO Alg3 (Fab793F10)	1 X P 100					1 X P	15	1 X •	• • •		1 X P 100				
#56	KO B4galt1/3 KO Gnptab/g KO Mgat4b/5 (Fab571C2)	100	1 X 1 X 98	11	23		1 X 1 X 1 X 1 X 1 X 1 X 1 X 1 X 1 X 1 X	94	1 X • 51 1 X • 19	48	1 X 1 X 1 X 37	100 1 X 16	70	1 X • • • •	31	21

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#57 KO St3gal4/6 KI ST6GAL1 (Fab532D2)			1 X P 100	1 X P 41 16	1 <u>XP</u> 13 11	1 <u>XP</u> 100 26	
#58 KO Gnptab/St3gal4/6 KI ST6GAL1 (Fab857D2)	1 X 1 X 1 X 1 X 1 X 1 X 1 X 1 X 1 X 1 X			1 X 65 33 1 X 1 X 1 X 1 X 1 X 1 X 1 X 1 X	25 21		1 X 2 X 3 31 12
#59 KO Gnptab/g/Mgat4b/5 KI ST3GAL4 (Fab583E4)	1 X 100 18			11		1 X 0 0 100 55	
#60 KO Gnptab/St3gal4/6 KO Fut8 KI ST6GAL1 (Fab870B1)		•		52 36	21 19	100 37	20 17
#61 KO Gnptab/g/Mgat4b/5 KO Fut8 (Fab584G10)	1 X 1 X 1 X 1 X 1 X 1 X 1 X 1 X			56 13		1 X 100 53	28



**Supplementary Figure 3.** Characterization of growth and yield performance of glycoengineered CHO clones expressing GLA.

**a** Viable cell density and **b** cell viability as determined by Trypan blue exclusion test. Data from day 1-5 are shown as accurate cell counting after day 6 was complicated by tendency for clumping of cells. **c** Yield of GLA enzyme activity determined in culture medium ( $2.5 \mu$ L) by release of p-nitrophenol per hour with a pNP-Gal enzyme assay. The substrate concentration was reduced to 1.2mM to fit the linear regression of absorbance at 405nm. **d** SDS-PAGE Coomassie analysis of GLA in culture medium (10  $\mu$ L loaded) after two, five and eight days of culture (D2, D5 and D8, respectively). **e** Summary of the glycan features of the glycoengineered CHO clones and gene modification information. The two most abundant N-glycans are illustrated and detailed structures shown in **Supplementary Fig. 2, Panels 3, 21, 4 and 59**). Source data are provided as a Source Data file.

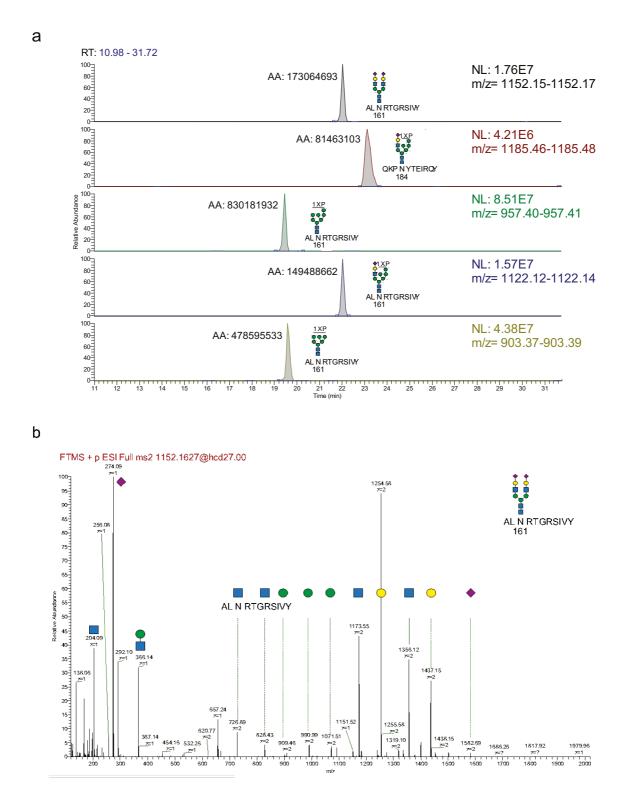
	N19	N59	N146	N270
#1 WT (GBA#A5)	1 X • • • • 100 68		3 X 3 X 100 84 37 36 36 1 X 2 X 2 X 2 X 2 X 100 10 100 100 10 10	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$
#2 KO: <i>Alg3</i>	1 X • 1 00	1X 1X 1X 1X 1X 1X 1X 1X 1X 1X 1X 1X 1X 1	100 58 41 27 23	21 19 18 13 11 1XP 1XP 1XP 1XP 100 46 36 15
(GBA827D2) #3			1XP 2X0 1XP 000 18 13 18 13 10 1X0 000 1X0 000 1X0 1X0 1X0 1X0 1X0 1X0 1X0	<u>1xp</u> <u>1xp</u> <u>1xp</u> <u>1x</u>
KO: <i>Alg9</i> (GBA829F2)	100 30	1 X <b>0 0 0 0 0 0 0 0 0 0</b>	3X0         1X0           100         66         55         26         17           1XP         1X0         1X0         17           1XP         12         12         17	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$
#4 KO: <i>Gnptab</i> (GBA826B1)			3X 3X 2X 3X 3X 3X 3X 3X 3X 3X 3X 3X 3	3X•         1X•           100         45         41         39         35           2X•         2X•         2X•         41         39         35
#5 KO: <i>Mgat1</i> (GBA828D9)	100	100 20 17	21 11 100 32	31         16         13           100         37         22         20

**Supplementary Figure 4.** Detailed presentation of N-glycans identified by site-specific N-glycan profiling of GBA produced in CHO WT and engineered KO clones as indicated.

N-glycan structures and their relative abundances at each of the four N-glycosites (N19, N59, N146, and N270) of GBA are illustrated with their relative abundance adjusted to the most abundant structure. Minor glycoforms identified with relative abundance less than 10% are not shown. Same N-glycan composition may represent isobaric structures.

#6 KO: <i>Mgat1</i> (GBA828E9)	100	100 18	100 16	1 <u>XP</u> 100 33 25 19
#7 KO: <i>Gnptab/Mgat1</i> (GBA831C8)	100	100 20 16	100	100 23
#8 KO: <i>Gnptab/Mgat1</i> (GBA831F10)	100	100 14	100 15	100 21
#9 KO: <i>Gnptab/Man2a1/2</i> (GBA900D6)	100	100	100 21 11	
#10 KO: <i>Gnptab/Mgat2</i> (GBA901D9)	100	100 45 19	100 32	100 93 47 19

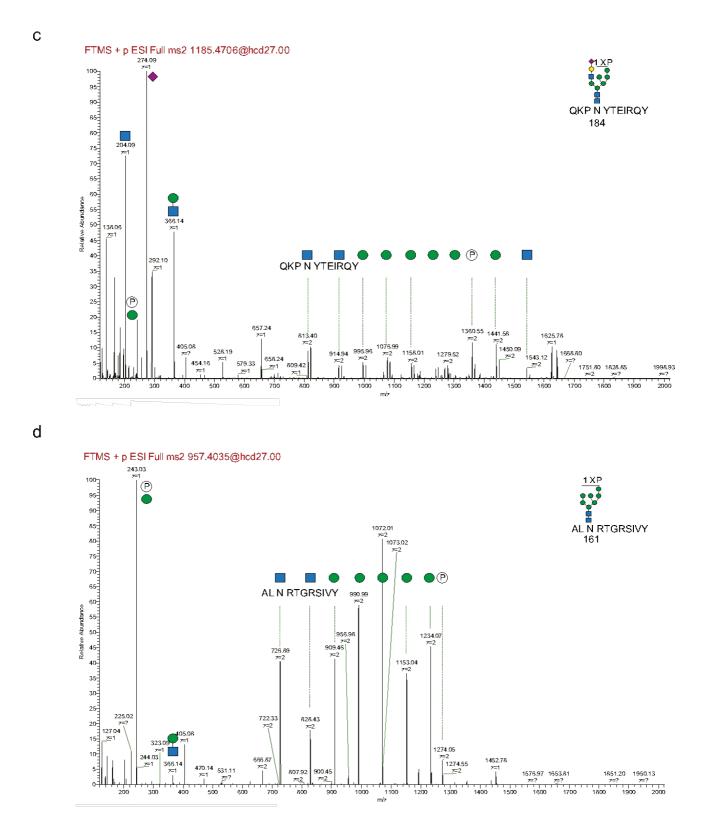
(Page 2 of 2)



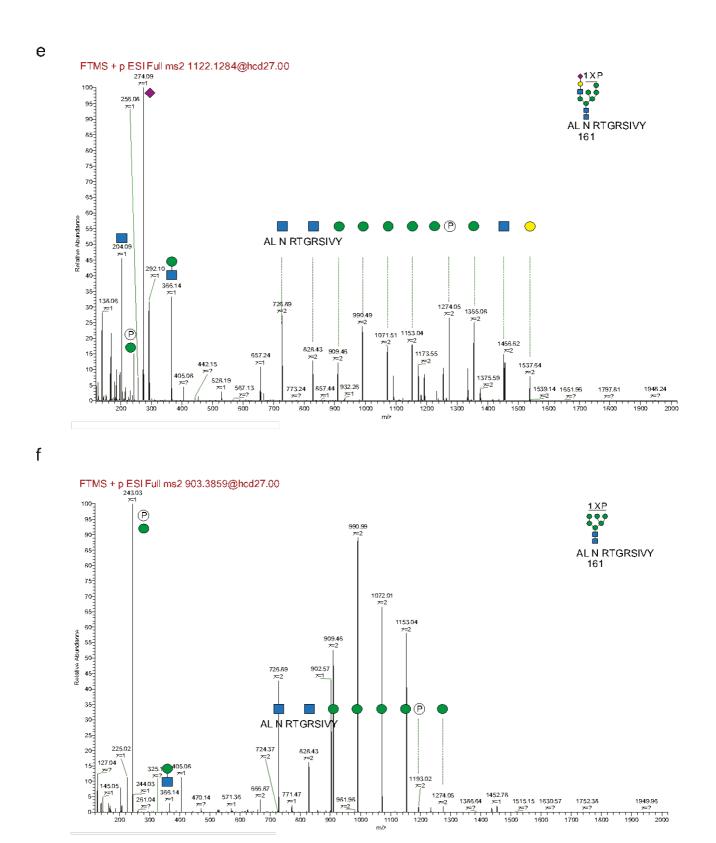
Supplementary Figure 5. Targeted MS/MS manual annotation of the most representative glycopeptides.

**a** Extracted ion chromatograms of 5 representative glycopeptide precursors of GLA produced in CHO WT. **b**-**f** MS2 manual annotation of these precursors.

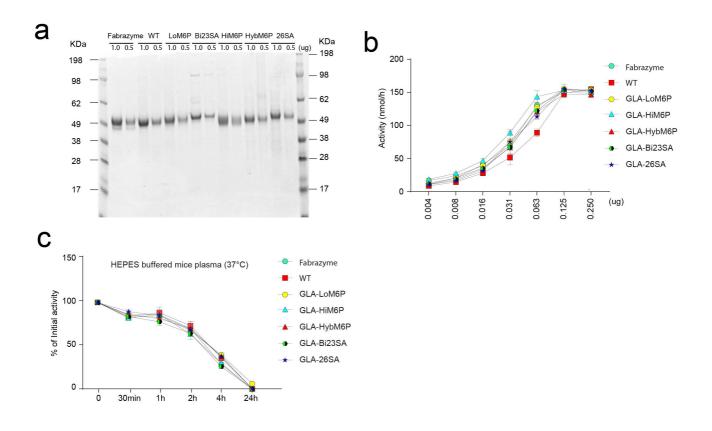
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Supplementary Figure 5 continued. (Page 2 of 3, continued)

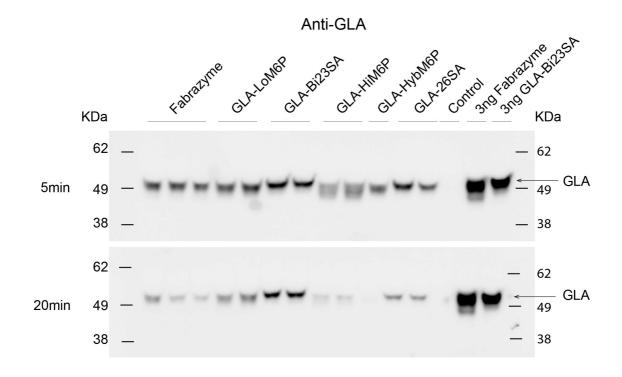


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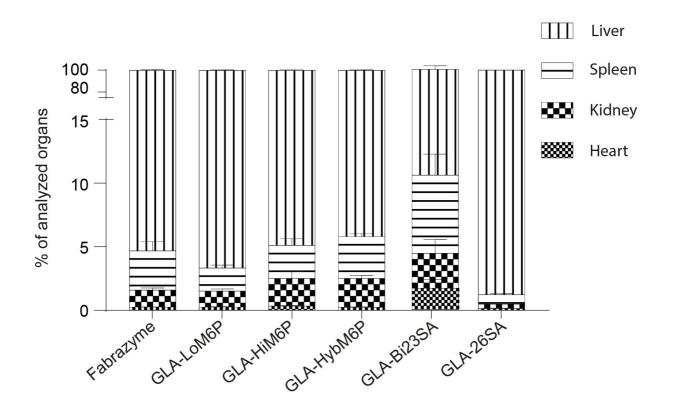
Supplementary Figure 6. In vitro assay of enzyme specific activity and plasma stability of GLA glycovariants.

**a** SDS-PAGE Coomassie analysis of the purified GLA variants used in mouse studies. **b** Specific activity of GLA variants assessed by a pNP-Gal enzyme assay. Release of p-nitrophenol per hour was used to show the enzyme activity. Each data point represents the mean of three replicates  $\pm$  Standard Deviation (SD). **c** In vitro stability of GLA variants (10 µg/ml in HEPES buffered mouse plasma) at 37°C was measured by the remaining activity. Each data point represents the mean of three replicates  $\pm$  SD. Source data are provided as a Source Data file.



Supplementary Figure 7. SDS-PAGE Western blot analysis of infused GLA glycovariants in mouse plasma.

Plasma from mice infused with 1 mg/kg GLA variants used in Figure 5c collected at 5 and 20 min time points were analyzed. Blood samples were collected from tail vein bleeds, and plasma separated by centrifugation and diluted 100-fold in 0.1% BSA. Each lane represent one randomly selected mouse from the indicated group, and 10  $\mu$ l of the diluted plasma was loaded in each lane. Control indicates was plasma collected from an untreated Fabry mouse. 3 ng Fabrazyme and GLA-Bi23SA was loaded respectively as positive controls. A rabbit anti-GLA polyclonal antibody (Sigma) was used 1:1,000 and an HRP-conjugated goat anti-rabbit immunoglobulins 1:3,000 (Dako P0448). Source data are provided as a Source Data file.



Supplementary Figure 8. Relative distribution of GLA glycovariants among the four major visceral organs.

GLA enzyme activity in dissected organs was assayed 24 h after 1 mg/kg infusion (same group as Figure 5e, Exp. #1). Activities in each whole organ and the sum of total recovered activities in the four major visceral organs were calculated. Relative distribution was shown by the % of each organ among the sum of total recovered activities. Error bars are presented with SD. Source data are provided as a Source Data file.

# Supplementary Table 1. CRISPR gRNA design and list of PCR primers used for gRNA target sites.

Gene	gRNA	Forward primer (5'-3')	Reverse primer (5'-3')
Acp2	GCTCTGCGGCAGCGCTATAG	TCGTCTCTTCCCAGACAAGC	TAGGGTCTGTGAGCCATCCC
Acp5	GGATGCACGGACGGTACTGC	GTGCAGTTTTCAGGGGCTTG	CTCCCCAGAGTAAGGTCCCA
Alg1	TTCTGCAAGAGCTCATCTCG	CCCCAGTACACAACTACCCC	AGTACATGCTGGCCTTGAACA
Alg2	CTGTGACGTGAAGATATGGA	CTGCTGCTGGACAGTTCCAA	ATTGCAGAAGCTCGAGCGAA
Alg3	GCTGCTGGGCTGCGGAAACG	TAGCTAGAAACCCTGGTGCC	TAGTGAACTCACATGCCACCC
Alg5	GGACTCTAAGTTCACTTACG	TAGTAGGAGAGAGCCGACCC	CTTGGGTTCCTCCAGCAAGT
Alg6	TCTTAATAGGACTCACAGTG	AAGCAGATGCAGCCCACTCA	CAAGTGACGGACTTAGCAGGA
Alg8	TCGGTGTACTTCAAAATCCG	GTGCAGTGGTCTAAGAACCCA	TCAAGGCCTGGCAGCTTAC
Alg9	GAGCAGACATTTGAAAGCAG	GCCCAAGACCATCGGTTAGAT	TGTCCGGATTTAGTCTTCGCT
Alq11	ACTGGTGACATTAATGTCAG	TGAGTCCCTTTCTTTTTGTGCC	TCAGGAACACGCTGTGTCAG
Alq12	AAATCACCAGGCAAGTCAGG	CAGTGTGACCTTAAGCAGGGT	CAGGTCATGCGTAGCCTGTA
Alg13	GATCTTGTCATCAGCCACGC	AGTTATGAACCACGGAGCCA	TTGGAAGCTTAGCCAACTGGT
Alg14	CTGCGGCAGCTAGAATCAGG	TTTGACCGCCCAACTCATCA	AGCGCTCGTAAAGGTGCTAA
B4galt1	GGGCGGTCGTTATTCCCCCA	CCCAAACCTCACCTGGTTGAT	GCTGGCTAACATCTTCGTTCC
B4galt3	GCAGGACGGTACCGGCCCCC	ATGCCATATGCAAGCTGCTG	GTGGGTCCTGTGTCGGTATC
Fam20c	GGGAAGCCTGACCAGATCGA	ATAGGTCACCGACTCTCCCT	GCCAATAACATCTGCTTCTACGG
Furin	GACCAAGCGGGACGTGTATC	GCCCATCTCGGTCTCATTGC	TGGGGAAGAAGACCAGAACCC
Fut8	GATCCGTCCACAACCTTGGC	AGAGTCCATGGTGATCCTGC	TACTGTTTAAGGGGAGGGGGA
Ganab	GAAGGCTTCGATCCTCTAGC	GTCGTCTTGCCAACCCCAAA	CACACCCAGTCTCTTCCCAA
Gnptab	GTCACATTCATCGCATCGAG	ACCAACGGGCAGATTCCTTC	CTAGGTGCCCACCCATCTTAG
Gnptg	GCGATGGCGGTGCGGGTGGC	CTTCCGGTTTTGAGCGCAG	CAGCCAAGGGCTTTCCTCG
Golph3	GAAAGGCTCAGTGCAACACT	GCACAACTGACTCCAGGATG	GAGCTCTTCAGATGCCATAACC
Golph3l	TGACTTCAGTTCGACGGGTA	CTCTTTCCCATGTTCCTCCA	TGTGTGTGTATAGGTCTTCTGTGG
lgf2r	GACAAAAACCTGTCGATCAG	GCTACACATGGGAGGCTGTT	CAAACCCAAAGCTGCGGAAA
Lrp2	TCACACAAGGAATTCCAGTG	ATCAGTGCCCACTGCCTAAC	AAGGAACCCAGGTCAAGCAA
M6Pr	GCTATAGATTCAGAGTATGC	AAGGGAGGGGTGCAGTTTTT	GACCAGCTGTGGAACTAGGC
Man1a1	GTAAATATACGCTTCGTCGG	TGGGCAAGCACACAGGTTTA	TGACCACCGGAACACGAAAA
Man1a2	GTCTGTGTTCGTCGGGTCCA	CCACAGGGCTACCTTGAGAC	GTCCTTCCAGGCTATGGCTC
Man1b1	GAGTACATACCACCTATCGG	AGCACCAGCACAAAGGGATT	GCTTTCACCCTCTCATTACGC
Man1c1	GCCCCGGGCGAGGACGATCC	TGGAGGTGATGGCCGAAAAC	CAGTGTGCCTGAAGGGTCTC
Man2a1	GAGTGAAGCCTCGATCGGGT	TAATCACAGCTGCGAGGTGG	ACTGCTATGCACCCCCATTC
Man2a2	GCCCAGAGAAAGCGTCGTCG	AGCGGCATATTCAGGGGAAC	GGGACTGCATACATTGGCCT
Manea	ATAGCCAAGAACTATCCACA	CGCCCCTTGGAAAACAAA	CGAACTAATTACCAACCAATTGAGG
Mgat1	GAGGGGGTCGCAGGCACACG	GTGCTTTGGGGTGCTATCCT	TGTGACTGCACTGCCATAGG
Mgat2	GCGACCGGTACCGCAGCGTT	GCGACAAAGGAAGAACGACG	TAGGTCTCTGGGGCAGTCTC
Mgat4b	GAGAGGCAGGCGCTGCGGGA	TAGCCTGTGTGTGTCAACCC	TGGGGAAGGGACAGGTTAGA
Mgat5	GACAATCTCGTCAATGGCAC	ACCTGCAGAGGTTTTCAGTTCT	GCCTTCACAACAATCATGCCA
Mogs	GGTGTCCCTGTTCTTCTACG	TTTAGCTCAGCCCACTCCAG	CTCCCTACCCGTACCACTCT
Nagpa	GGGCTGCAGAACGCGCAGTT	AGAACGGTGGTTTCTTCCGC	GCGTTCAATGACACACGACT
Sort1	TTAACAGCAGAGGTATCTGG	AGGACCATGCCCTGCTCTC	ATAGCCAGATGGGGACAGGTAG
Sppl3	GAGGCTTGGCAGGCGGACAA	ATGTCACCGACAAACGGGAC	CCACACCCAACTGATCCCC
St3gal4	GGTCGAAGTGGGCCGACTCA	AAGAGCGTGTCTGGGTTGTT	GCAGGGTCCACTTCTGGATT
St3gal6	GGAGTTGTGATCATTGTGAG	TCTTGGGTGCTTCTGAGTGTG	GGACACAGAAAATGGGATGTTG

### Supplementary Table 2. Summary of CHO mutant clones stably expressing GLA and cell line ancestry.

Project number	Targeted genes	Parental CHO line
FAB399	KO Nagpa	WT#H9*
FAB400	KO Gnptab	WT#H9
FAB441	KO lgf2r	WT#H9
FAB442	KO Man1a1	WT#H9
FAB443	KO Man1a2	WT#H9
FAB444	KO Man1b1	WT#H9
FAB445	KO Man1c1	WT#H9
FAB446	KO Mogs	WT#H9
FAB451	KO Acp2	WT#H9
FAB453	KO Gnptg	WT#H9
FAB454	KO Acp5	WT#H9
FAB462	KO Acp2/5	WT#H9
FAB478	KO Alg3	WT#H9
FAB479	KO Alg6	WT#H9
FAB480	KO Alg9	WT#H9
FAB494	KO St3gal4/6	WT#H9
FAB495	KO Mgat1	WT#H9
FAB496	KO Mgat2	WT#H9
FAB497	KO Mgat4b/5	WT#H9
FAB499	KO Sppl3	WT#H9
FAB532	KI ST6GAL1/KO St3gal4/6	FAB494 B4
FAB534	KO Sort1	WT#H9
FAB535	KO Lrp2	WT#H9
FAB540	KO Fut8	WT#H9
FAB546	KO Gnptab/g	WT#H9
FAB555	KO Furin	WT#H9
FAB560	KO Manea	WT#H9
FAB567	KO Mgat4b/5/Gnptab/g	FAB546A2
FAB568	KO Mgat1/Gnptab/g	FAB546A2
FAB570	KO Mgat2/Gnptab/g	FAB546A2
FAB571	KO B4galt1/3/Mgat4b/5/Gnptab/g	FAB567H3
FAB572	KO Ganab	WT#H9
FAB583	KI ST3GAL4/ KOMgat4b/5/Gnptab/g	FAB567H3
FAB584	KO Fut8/Mgat4b/5/Gnptab/g	FAB567H3
FAB604	KO Fam20c	WT#H9
FAB605	KO Golph3	WT#H9
FAB606	KO Golph3l	WT#H9
FAB611	KO Alg3/Mgat1	FAB495B10
FAB662	KO Alg8	WT#H9
FAB664	KO Alg12	WT#H9
FAB667	KO Alg5	WT#H9
FAB677	KI GNPTG	WT#H9
FAB688	KO B4galt1/3	WT#H9
FAB695	KI GNPTAB	WT#H9
FAB712	KO Man2a1/2	WT#H9
FAB713	KO Man2a1/2/Gnptab	FAB400C7
FAB725	KO Man1a1/1a2/1b1/1c1	FAB442G8
FAB791	KO M6pr	WT#H9
FAB792	KI GNPTAB /KO Alg3	FAB695G2
FAB793	KI GNPTAB /KO Alg3 KI GNPTAB /KO Alg3	FAB695A8
FAB819	KI GNPTAB/G	FAB695G2
FAB819 FAB857	KI ST6GAL1/KO St3gal4/6/Gnptab	FAB532D2
FAB870	KI ST6GAL1/KO St3gal4/6/Gnptab/Fut8	FAB352D2 FAB857D2
		1,10007.02

\* Clone WT#H9 was used as parental clone for all gene engineering.

### Supplementary Table 3. Summary of CHO mutant clones stably expressing GBA and cell line ancestry.

Project number	Targeted genes	Parental CHO line
GBA826	KO Gnptab	GBA#A5
GBA827	KO Alg3	GBA#A5
GBA828	KO Mgat1	GBA#A5
GBA829	KO Alg9	GBA#A5
GBA831	KO Gnptab/Mgat1	GBA#A5
GBA900	KO Gnptab/Man2a1/2	GBA826B1
GBA901	KO Gnptab/Mgat2	GBA826B1

### Supplementary Table 4. Sequence analysis of CHO mutant clones stably expressing GBA.

<b>Clone</b> GBA826B1	<b>Targeted genes</b> KO <i>Gnptab</i>	InDels	Alignment
	WT .		GTCACATTCATCGCATCGAG <mark>GGG</mark>
GBA827C2	KO KO <i>Alg3</i>	+1bp	GTCACATTCATCGCATCCGAG <mark>GGG</mark>
	WT		GCTGCTGGGCTGCGGAAACG <mark>CGG</mark>
GBA827D2	KO KO <i>Alg3</i>	-1bp	GCTGCTGGGCTGCGGAAACG <mark>CGG</mark>
00/02/02	WT		GCTGCTGGGCTGCGGAAACG <mark>CGG</mark>
GBA828D9	KO KO Mgat1	+1bp	GCTGCTGGGCTGCGGAACACGCGG
	WT		GAGGGGGTCGCAGGCACACG <mark>GGG</mark>
GBA828E9	KO KO Mgat1	+1bp	GAGGGGGTCGCAGGCACCACG <mark>GGG</mark>
00/102020	WT		GAGGGGGTCGCAGGCACACG <mark>GGG</mark>
GBA829F2	KO KO <i>Alg9</i>	+1bp	GAGGGGGTCGCAGGCACCACG <mark>GGG</mark>
	WT		<u>GAGCAGACATTTGAAAGCAG</u> TGG
	KO-alle1	-7bp	GAGCAGACATTTGAAAGCAG <mark>TGG</mark>
GBA831C8	KO-alle2 KO <i>Mgat1</i>	-2bp	GAGCAGACATTTGATTTG <mark>TGG</mark>
	WT		<u>GAGGGGGTCGCAGGCACACG<mark>GGG</mark></u>
	KO KO Gnptab	+1bp	GAGGGGGTCGCAGGCACCACG <mark>GGG</mark>
	WT		<u>GTCACATTCATCGCATCGAG</u> GGG
GBA831F10	KO KO <i>Mgat1</i>	+1bp	GTCACATTCATCGCATCCGAG <mark>GGG</mark>
	WT		<u>GAGGGGGTCGCAGGCACACG<mark>GGG</mark></u>
	KO KO Gnptab	+1bp	GAGGGGGTCGCAGGCACCACG <mark>GGG</mark>
	WT		GTCACATTCATCGCATCGAGGGG
GBA900D6	KO KO Man2a1	+1bp	GTCACATTCATCGCATCCGAG <mark>GGG</mark>
	WT		GAGTGAAGCCTCGATCGGGT <mark>TGG</mark>
	KO KO Man2a2	-4bp	GAGTGAAGCCTCGATCGGGT <mark>TGG</mark>
	WT		<u>GCCCAGAGAAAGCGTCGTCG</u> AGG
	KO KO Gnptab	-1bp	GCCCAGAGAAAGCGTCGTCG <mark>AGG</mark>
	WT		GTCACATTCATCGCATCGAG <mark>GGG</mark>
GBA901D9	KO KO <i>Mgat2</i>	+1bp	GTCACATTCATCGCATCCGAG <mark>GGG</mark>
	WT		GCGACCGGTACCGCAGCGTTAGG
	KO KO Gnptab	+1bp	GCGACCGGTACCGCAGCCGTT <mark>AGG</mark>
	WT		GTCACATTCATCGCATCGAG <mark>GGG</mark>
	КО	+1bp	GTCACATTCATCGCATCCGAG <mark>GGG</mark>

Note: Nucleic acids <u>UNDERLINED</u> are the gRNA targeting sequence;

Nucleic acids in **RED** are the PAM sequence;

Nucleic acids in GREEN are insertions;

Nucleic acids in GRY are deletions.