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(54) **Title:** PRODUCTION OF N-GLYCOPROTEINS FOR ENZYME ASSISTED GLYCOMODIFICATION

(57) **Abstract:** The present invention relates to a cell comprising a gene encoding a polypeptide of interest, wherein the polypeptide of interest is expressed comprising one or more posttranslational modification patterns. These modifications are useful for example in improvement of pharmacokinetic properties, i.e. by attaching PEG chains to proteins. The present invention also relates to methods for producing the antibodies and compositions comprising the antibodies, and their uses.



Production of N-glycoproteins for enzyme assisted glycomodification

Technical field of the invention

The present invention relates to a cell comprising a gene encoding a polypeptide
5 of interest, wherein the polypeptide of interest is expressed comprising one or
more posttranslational modification patterns. These modifications are useful for
example in improvement of pharmacokinetic properties, i.e. by attaching PEG or
HEP chains to proteins. The present invention also relates to methods for
producing glycoproteins and compositions comprising the glycoproteins, and their
10 uses.

Background of the invention

Glycoprotein biologics is the fastest growing class of therapeutics, and most of
these can only be produced recombinantly in mammalian cells with capacity for
15 human-like glycosylation. The Chinese hamster ovary (CHO) cell has gained a
leading role as host cell for recombinant production of glycoprotein therapeutics
mainly because it produces rather simple N-glycans with branching and capping
similar to what is produced in some human cells.

20 Notably, CHO produce complex-type heterogenous N-glycans with bi-, tri-, and
tetraantennary structures with core α 6Fucose (Fuc), a minor amount of poly-N-
Acetyllactosamine (poly-LacNAc) mainly on the α 1,6 arm of tetraantennary
structures, and exclusive capping of LacNAc with α 2,3 linked neuraminic acid
(NeuAc).

25 CHO does not generally produce the non-human and in man immunogenic capping
structures, such as N-glycolylneuraminic acid (NeuGc) or α 3Gal, although the
occurrence of these have been reported perhaps as a result of gene induction. N-
glycosylation may vary for different proteins as well as for different glycosites in
30 individual proteins, and e.g. IgG antibodies are produced with truncated N-glycan
structures at the conserved Asn297 glycosite (biantennary structures with core
 α 6Fuc, limited LacNAc, and NeuAc capping).

A major concern with CHO is the substantial heterogeneity in N-glycan processing, which can be difficult to control during bioprocessing and can pose issues for bioactivity as well as biosafety.

- 5 Thus, a major activity in bioprocessing of therapeutics is devoted to glycan analysis and control of fermentation to achieve consistency.

Substantial efforts in the last two decades have been devoted to genetic glycoengineering of CHO cells with the aims to expand the capacity for
10 glycosylation, reduce heterogeneity, and improve or alter especially sialylation.

These studies have essentially all used random integration of cDNAs encoding glycosyltransferases and experienced problems with stability, consistency, and predictability of the introduced glycosylation capacity. The major obstacle has
15 been the need to rely on overexpression of glycosyltransferases and competition with the endogenous expressed enzymes because of lack of simple methods to knock these out in cell lines.

Thus, only very few such glycoengineered CHO cells have not reached production
20 of clinical therapeutics. One successful glycoengineering strategy has, however, emerged after the discovery that IgGs without core α 6Fuc on the Asn297 N-glycan exhibits markedly higher Antibody-Dependent Cell Cytotoxicity (ADCC).

Thus, using two rounds of homologous recombination both alleles of the fut8 gene
25 encoding the α 6fucosyltransferase controlling core fucosylation was knocked out in CHO, and at least one therapeutic IgG produced in CHO without the fut8 gene is now in clinical use.

More recently, the fut8 gene was knocked out using precise gene editing with Zinc
30 finger nuclease (ZFN) gene targeting with a fraction of time and resources spent.

The emergence of precise gene editing technologies for knockout (KO) and knockin (KI) have opened up for an entirely different level of speed and ease with which stable genetic manipulation of host cell lines to remove and introduce
35 glycosyltransferase genes can be achieved, and this will undoubtedly impact

engineering of mammalian host cell factories for recombinant production of therapeutics.

Thus, there is a need for mammalian, and especially CHO cells, that have specific
5 glycosylation patterns with or without sialylation.

Summary of the invention

The present invention relates to a cell comprising a gene encoding a polypeptide of interest, wherein the polypeptide of interest is expressed comprising one or
10 more posttranslational modification patterns.

One object of the present invention relates to a cell comprising one or more glycosyltransferase genes that have been inactivated, and that has new and/or more homogeneous stable glycosylation capacities, wherein mgat2 has been
15 knocked out (KO), and with and without KO of mgat4A and/or mgat4B and/or mgat5 allowing production of N-glycans with monoantennary structure.

Another object of the present invention relates to a cell comprising one or more glycosyltransferase genes that have been inactivated, and that has new and/or
20 more homogeneous stable glycosylation capacities, wherein mgat2 has been knocked out (KO), and with and without KO of mgat4A and/or mgat4B and/or mgat5 in combination with KO of sialyltransferases allowing production of N-glycans with monoantennary structure and without sialic acid capping.

25 Yet another object of the present invention relates to a cell comprising one or more glycosyltransferase genes that have been inactivated, and that has new and/or more homogeneous stable glycosylation capacities, wherein mgat2 has been knocked out (KO), and with and without KO of mgat4A and/or mgat4B and/or mgat5 in combination with KO of sialyltransferases and B3gnt2 allowing
30 production N-glycans with monoantennary structure and without sialic acid capping and without poly-LacNAc.

In one embodiment of the present invention the cell is a mammalian cell or an insect cell.

In another embodiment of the present invention the cell is derived from Chinese hamster ovary or from human kidney.

5 In a further embodiment of the present invention the cell is selected from the group consisting of CHO, NS0, SP2/0, YB2/0, CHO-K1, CHO-DXB11, CHO-DG44, CHO-S, HEK293, HUVEC, HKB, PER-C6, NS0, or derivatives of any of these cells. In one embodiment of the present invention is the cell a CHO cell.

10 In another embodiment of the present invention the cell furthermore encodes an exogenous protein of interest.

In yet another embodiment of the present invention the protein of interest is an antibody, an antibody fragment, such as a Fab fragment, an Fc domain of an

15 antibody, or a polypeptide.

In yet another embodiment of the present invention the protein of interest is a coagulation factor such as coagulation factor II (FII), coagulation factor V (FV), coagulation factor VII (FVIIa), coagulation factor VIII (FVIII), coagulation factor
20 IX (FIX), coagulation factor X (FX), or coagulation factor XIII (FXIII).

One aspect of the present invention relates to a method for the production of di- and triPEGylated erythropoietin (EPO) glycoproteins, the method comprising the step of enzymatic glycoPEGylation of glycoengineered EPO variants.

25

Another aspect of the present invention relates to a method for the production of recombinant glycoproteins, comprising the step of generating a cell with specific glycosylation properties, wherein specific glycosylation property is the capacity for monoantennary N-glycan synthesis.

30

A further aspect of the present invention relates to a glycoprotein obtainable from a method according to the present invention.

In one embodiment of the present invention has the glycostructure outcome one

35 or more of the following changes selected from the group consisting of simpler

glycan structure, more homogeneous product, more sialic acids per molecule, non-sialylated, non-galactosylated, more homogeneous bi-antennary, more homogeneous monoantennary, more homogeneous triantennary, more homogeneous without poly-LacNAc, higher productivity in cell culture, new stable
5 homogeneous glycosylation capacities, more human glycostructure, more homogeneous glycosylation and improved ADCC targeting of IgG, modified fucose level, no fucose, improved substrate for generating glycoconjugates.

One aspect of the present invention relates to a glycoprotein according to the
10 present invention, which is a homogeneous glycoconjugate produced from a glycoprotein having a simplified glycan profile.

Another aspect of the present invention relates to a glycoprotein according to the present invention, which is a homogeneous glycoprotein conjugate comprising a
15 polymer selected from, PEG, HEP, XTEN, PSA, HES.

A further aspect of the present invention relates to a glycoprotein according to the present invention, which is a homogeneous PEGylated protein conjugate produced from a protein variant according to the invention having a simplified glycan
20 profile.

Another aspect of the present invention relates to a glycoprotein according to the present invention, which is a homogeneous PEGylated EPO conjugate produced from an EPO variant having a simplified glycan profile.

25

One aspect of the present invention relates to a glycoprotein according to the present invention, which is a simplified enzymatic glycoPEGylation process using glycoengineered EPO variants that provides high yield of di- and triPEGylated EPO forms.

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Another aspect of the present invention relates to a glycoprotein according to the present invention, which is a protein conjugate according to the invention comprising FII, FV, FVIIa, FVIII, FIX, FX, FXIII, a Fab fragment of an antibody, or a Fc domain of an antibody.

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A further aspect of the present invention relates to a glycoprotein according to the present invention, which is a protein-protein conjugate produced from a glycoprotein with a simplified glycan profile.

- 5 In one embodiment of the present invention is the glycoprotein according to the present invention a protein-protein conjugate produced from a glycoprotein with a simplified glycan profile comprising a Fab fragment and one of either FII, FV, FVIIa, FVIII, FIX, FX or FXIII.
- 10 In another embodiment of the present invention is the glycoprotein according to the present invention a protein-protein conjugate produced from a glycoprotein with a simplified glycan profile comprising a Fc Domain and one of either FII, FV, FVIIa, FVIII, FIX, FX or FXIII.
- 15 In a further embodiment of the present invention is the glycoprotein according to the present invention a homogeneous PEGylated EPO conjugate produced from an EPO variant having a simplified glycan profile.

- One aspect of the present invention relates to the use of recombinant
- 20 glycoproteins comprising monoantennary N-glycans for enzymatic modification of polypeptides.

Brief description of the figures

- Unless specified differently all glycoprofiling analyses are performed with EPO
- 25 expressed in CHO-GS cells engineered as indicated. Glycoprofiling Figures are MALDI-TOF spectra of PNGase F released permethylated N-glycans.

- FIG 1. A graphic depiction of the human CAZy GT families involved in the biosynthesis of the major mammalian glycoconjugates and their different glycosylation pathways. The common mammalian glycan structures are depicted
- 30 for (A) O-glycans including O-GalNAc, O-GlcNAc, O-Fuc, O-Glc, O-Man, O-Xyl; (B) N-glycans; (C) GPI-anchors; (D) C-glycans; (E) glycosphingolipids; (F) hyaluronan and (G) hydroxy-lysine. Designations for monosaccharides according to the

Consortium for Functional Glycomics are indicated
(<http://www.functionalglycomics.org>).

FIG 2 A graphic representation of the different glycan structures and glycosylation pathways found in CHO cell lines with designations of glycosyltransferase genes expressed in CHO cell lines and their predicted role in biostynthetic steps. The genes are assigned to the major confirmed or putative functions in the O-glycosylation (O-GalNAc, O-Fuc, O-Glc, O-GlcNAc, O-Xyl, and O-Man) and N-glycosylation pathways relevant for recombinant glycoprotein therapeutics today. (A) O-GalNAc glycosylation pathway with extended core 1, core 2, core 3, and core 4 structures; (B) N-glycosylation pathway; (C) glycosphingolipid biosynthetic pathways; (D) C-glycan, and (E) GPI-anchors. The CAZy families of glycosyltransferase (GT genes are notated and GT genes expressed in CHO cell lines are shown. Glycan structures not synthesized in CHO are boxed. Designations for monosaccharides according to the Consortium for Functional Glycomics are indicated.

FIG 3 Graphic depictions of all genes encoding isoenzymes with potential to regulate N-glycosylation branching, elongation, and terminal capping expressed in CHO. The depiction shows the knockout screen of glycosyltransferase genes involved in N-glycosylation in CHO using human EPO as recombinant expressed reporter glycoprotein. (A) The common tetraantennary N-glycan with poly-LacNAc on the β 6-antenna and capping by sialic acids is shown, the knockout genes are boxed, and genes not expressed in the CHO cells are annotated. Note that human ST6Gal-I (ST6GAL1) has been introduced by ZFN-mediated knock-in.

Designations for monosaccharides according to the Consortium for Functional Glycomics (CFG) are indicated. (B) Schematic depiction of the actual nuclease targeted regions relative to the predicted general domain structure of type II glycosyltransferase proteins (upper panel) and the targeted exon for each gene numbering the respective genes from first coding exon (lower panel). The exon structure depiction only includes the first targeted exons and does not include all exons for all genes.

FIG 4 Glycoprofiling of EPO expressed in CHO WT cells. MALDI-TOF spectra of PNGase F released permethylated N-glycans with predicted structures for the four major species.

FIG 5 Glycoprofiling of EPO expressed in CHO cells with KO of genes involved in complex type N-glycan biosynthesis and antennary formation, showing that double KO of mgat4B/5 and triple KO of mgat4A/4B/5 results in homogeneous biantennary N-glycans with a minor amount of poly-LacNAc. Since single KO of mgat4A had minor effects compared to WT it is likely that minor amounts of triantennary structures are present in the double mgat4B/5 KO.

FIG 6 Glycoprofiling with KO of B4galt1/2/3/4 genes involved in LacNAc biosynthesis, showing that only B4galt1 KO alone produced substantial albeit partial loss of galactosylation, while only stacked KO of B4galt1/3 resulted in near complete loss of galactosylation, while stacked KO of B4galt1/3 resulted in near complete loss of galactosylation. Stacked KO of B4galt1/2/3 resulted in essentially complete loss of galactosylation.

FIG 7 Glycoprofiling with KO of three β 3GlcNAc-transferase genes showing that KO of B3gnt2 results in complete loss of poly-LacNAc, whereas KO of B3gnt1 and B3gnt1 had no effects. Lower PANEL shows that KO of B3gnt2 in combination with mgat4A/4B/5 results in complete loss of poly-LacNAc on biantennary structures.

FIG 8 Glycoprofiling with KO of three α 2,3sialyltransferase genes with claimed roles in N-glycosylation, showing that only the double KO of st3gal4/6 resulted in complete loss of sialic acid capping.

FIG 9 Immunofluorescence cytology with ConA and L-PHA lectin staining showing loss of L-PHA with knockout of mgat5.

FIG 10 Immunofluorescence cytology with a monoclonal antibody (clone 1B2) to LacNAc. Panel A shows surprisingly that only stacked KO of B4galt1/3, and not B4galt1/2/4, abolished galactosylation. Panel B shows that KO of B4galt1 in CHO-GS with KO of mgat4A/4B/5 eliminated immunoreactivity for LacNAc while KO of B4galt2, 3, and 4 in the same CHO-GS had no substantial effects.

FIG 11 Recombinant expression of a therapeutic IgG in CHO with KO of B4galt1 resulted in homogenous biantennary N-glycans without galactosylation.

Glycoprofiling of IgG produced in CHO WT and B4galt1 KO showing essentially complete loss of the incomplete galactosylation and sialylation characteristic for

the conserved N-glycan at Asn297. KO of B4galt1 in combination with fut8 resulted in N-glycans without galactosylation and fucose.

FIG 12 Immunofluorescence cytology with LEL lectin shows that B3gnt2 is the key gene controlling the LacNAc initiation in CHO cells.

- 5 FIG 13 Immunofluorescence cytology with monoclonal antibody to LacNAc (1B2). Knockout of St3gal3, St3gal4, and St3gal6 individually using immunocytology to evaluate exposure of LacNAc shows that only KO of St3gal4 produced substantial exposure of LacNAc.

- FIG 14 Glycoprofiling of EPO expressed in CHO with KO of mgat4a/4b/5 and ZFN-mediated knockin of human ST6GAL1. Panel A shows profiling of EPO with homogenous α 2,6NeuAc capping of the complete range of N-glycan antennary structures. Also shown is KI of ST6GAL1 in CHO with KO of mgat4a/4b/5/B3gnt2, which produced more homogeneous tetraantennary structures. Panel B shows profiling with additional KO of mgat4a/4b/5 where EPO is produced with
10 homogenous biantennary N-glycans capped by α 2,6NeuAc. Also shown are KI of human ST3Gal-4 in combination with KO of st3gal4/6/mgat4a/4b/5/B3gnt2 where EPO is produced with homogeneous biantennary N-glycans without poly-LacNAc and capped by α 2,3NeuAc.
15

- FIG 15 Negative ion ESI-MS/MS of the precursor ions at m/z 1183.92 (sialylated biantennary N-glycan with core fucose) from EPO produced in CHO with
20 Mgat4a/4b/5 KO (Top Panels) and with both Mgat4a/4b/5 and st3gal4/6 KO and KI of ST6GAL1 (Bottom Panels). The presence of the diagnostic fragment ions at m/z 306.12 demonstrates the presence of α 2,6 terminal sialylation.

- FIG 16 Analysis of targeted KI of ST6GAL1 by junction PCR. Panel A shows that a
25 modified ObLiGaRe targeted KI strategy utilizing two inverted ZFN binding sites flanking the ST6GAL1 full open reading frame in donor plasmid was used. 5' and 3' junction PCR confirmed targeted integration into the Safeharbor#1 site in the CHO clone with st3gal4/6 KO. (b) 5' and 3' junction PCR confirmed targeted integration in the CHO clone with st3gal4/6/mgat4a/4b/5 KO. The status of allelic
30 copy number of integration was determined by WT allelic PCR. The presence of desired band in WT allelic PCR indicates the presence of Safeharbor#1 site without the integration of targeted KI of gene of interest on at least one of the allele. The

results showed biallelic integration of ST6Gal-I at Safeharbor#1 site in the CHO st3gal4/6 KO clone and monoallelic integration in the mgat4a/4b/5/St3gal4/6 KO clone. The ObLiGaRe KI strategy was highly efficient with approximately 30-50% single cloned cells expressing ST6GAL1 as evaluated by antibody and lectin

5 immunocytology.

FIG 17 Graphic depiction of the genetic deconstruction of N-glycosylation in CHO established using EPO as N-glycoprotein reporter. Several KO CHO lines have potential for production of glycoprotein therapeutics with more homogenous glycosylation (e.g. biantennary α 2,3-sialylated N-glycans for EPO, and

10 biantennary non-galactosylated/sialylated with and without core Fuc for IgG.

Examples of reconstruction based on KI are shown producing homogenous α 2,6-sialylation after KO of α 2,3-sialylation capacities with either WT N-glycan branching heterogeneity or homogenous biantennary structure, homogeneous α 2,3-sialylation after KO of endogeneous sialylation, and homogeneous

15 tetranatennary structures with α 2,6-sialylation after KO of endogeneous branching and sialylation capacities. Reconstruction may address homogeneity as shown, but also branching, poly-LacNAc elongation, and any type of capping as indicated.

FIG 18 Glycoprofiling of EPO expressed in CHO with KO of Cosmc to eliminate O-GalNAc elongation with Gal and NeuAc showing improved capacity for sialic acid

20 capping of N-glycans at all three N-glycosites present in EPO, when compared to WT as shown in FIGURE 4.

FIG 19 Glycoprofiling of EPO expressed in CHO with KO of B4galT7 to eliminate O-Xyl elongation with Gal (PANEL A) and Pomgnt1 to eliminate O-Man elongation with Gal and NeuAc (PANEL B) showing enhanced capacity for sialic acid capping

25 of N-glycans at all three N-glycosites present in EPO. PANEL C illustrates glycoprofiling of EPO expressed in double KO of B4galt7 and pomgnt1, and Panel D illustrates glycoprofiling of EPO expressed in triple KO of B4galt7, pomgnt1, and cosmc showing the same improved capacity for sialic acid capping of N-glycans in EPO.

30 FIG 20 Glycoprofiling of EPO expressed in CHO with KO of B4galT4 to eliminate a galactosyltransferase paralog not utilized for N-glycans and this glycosylation

pathway showing improved capacity for sialic acid capping of N-glycans at all three N-glycosites present in EPO.

FIG 21 Glycoprofiling of EPO expressed in CHO with KO of Mgat2 showing loss of tetra and triantennary N-glycan structures and appearance of mono and
 5 biantennary N-glycans with galactose and NeuAc capping (PANEL B), and without sialic acid capping with KO of st3gal4/6 (PANEL C). Further KO of mgat4A/4B/5 resulted in monoantennary structures with minor amounts of poly-LacNAc (PANELS D and E). Reintroduction of human MGAT4A and MGAT5 in combination with ST6Gal-I resulted in loss of monoantennary N-glycans and restoration of tri
 10 and tetraantennary structures (PANELS H and I).

FIG 22 SDS-PAGE gel analysis of glycoPEGylation reactions of recombinant wildtype EPO-Myc-His6 and recombinant EPO-Myc-His6 with monoantennary N-glycans. Lanes contained: (1) HiMark Standard, (2) ST3Gal3, (3) Sialidase, (4) wt hEPO, wt EPO-Myc-His6 glycoPEGylated with ST3Gal-III and (5) 1x, (6) 5x, (7)
 15 10x, (8) 25x, (9) 50x 10kDa-PSC reagent respectively, (10) EPO-Myc-His6 glycosylation variant and EPO-Myc-His6 glycosylation variant glycoPEGylated with ST3Gal-III and (11) 1x, (12) 5x, (13) 10x, (14) 25x, (15) 50x 10kDa-PSC reagent. Bands above approximately 160 kD indicates GlycoPegylation with more than two PEG chains at one or more N-glycans.

20 FIG 23 Quantification of number of attached PEG chains by densitometry of SDS-PAGE gels. (PANEL A) Product profile of enzymatic 10kDa-GlycoPEGylation reaction with EPO-Myc-His6 produced in ordinary CHO cell lines. (PANEL B) Product profile of enzymatic 10kDa-GlycoPEGylation reaction with EPO-Myc-His6 produced in Mgat2/4a/4b/5 KO CHO cell lines. Profiles A and B were obtained by
 25 densitometry measurements on lane 9 and lane 15 in FIGURE 22, respectively.

The present invention will now be described in more detail in the following.

Detailed description of the invention

Sugar chains of glycoproteins are roughly divided into two types, namely a sugar
 30 chain which binds to asparagine (N-glycoside-linked sugar chain) and a sugar chain which binds to other amino acid such as serine, threonine (O-glycoside-linked sugar chain), based on the binding form to the protein moiety.

The sugar chain terminus which binds to asparagine is called a reducing end, and the opposite side is called a non-reducing end. It is known that the N-glycoside-linked sugar chain includes a high mannose type in which mannose alone binds to
5 the non-reducing end of the core structure; a complex type in which the non-reducing end side of the core structure has at least one parallel branches of galactose-N-acetylglucosamine (hereinafter referred to as "Gal-GlcNAc") and the non-reducing end side of Gal-GlcNAc has a structure of sialic acid, bisecting N-acetylglucosamine or the like; a hybrid type in which the non-reducing end side of
10 the core structure has branches of both of the high mannose type and complex type.

In general, most of the humanized antibodies of which application to medicaments is in consideration are prepared using genetic recombination techniques and
15 produced using Chinese hamster ovary tissue-derived CHO cell as the host cell. As described above, the sugar chain structure play important roles for the structure, function, size, circulatory half-life, and pharmacokinetic behaviour of glycoprotein drugs. Moreover, the sugar structure plays a remarkably important role in the effector function of antibodies and differences are observed in the sugar chain
20 structure of glycoproteins expressed by host cells, development of a host cell which can be used for the production of an antibody having higher effector function is desired.

Thus, an object of the present invention is to provide a cell capable of expressing
25 a gene encoding a polypeptide of interest, wherein the polypeptide of interest is expressed comprising one or more posttranslational modification patterns.

In one embodiment of the present invention is the posttranslational modification pattern a glycosylation.

30

The optimal glycoform of a glycoprotein may be identified by the following process:

(i) producing a plurality of different glycoforms of said glycoprotein by expressing
35 in cell lines harboring at least one novel glycosylation capacity for example by

harboring two or more modifications of GT gene expression levels, and (ii) determination of the activity of the different glyco forms in comparison with a reference glycoprotein in (a) suitable bioassay(s); and (iii) selection of the glycoform with the higher/highest activity and determination of the production cell
5 genotype fingerprint which is correlated with the higher/highest activity level of said glycoprotein.

The above described process allows the identification of the optimal glycoform of a glycoprotein. For those skilled in the art by using the genotype fingerprint
10 identified in (iii) may generate an efficient engineered cell line with the optimal genotype for producing glycoprotein with said glycoform.

The genes involved in this highly complex machinery have been examined by the present inventors (see the examples) and effects have been identified.

15

Thus, in one aspect of the present invention is one or more of the group selected from mgat4A, mgat4B, mgat4C, mgat5, mgat5B, B4galt1, B4galt2, B4galt3, B4galt4, B3gnt1, B3gnt2, B3gnt8, st3gal3, st3gal4, and st3gal6 involved in the posttranslational modification patterns.

20

In another aspect of the present invention are one or more of these genes knocked out or in.

The present invention relates to a cell comprising a gene encoding a polypeptide
25 of interest, wherein the polypeptide of interest is expressed comprising one or more posttranslational modification patterns.

One object of the present invention relates to a cell comprising one or more glycosyltransferase genes that have been inactivated, and that has new and/or
30 more homogeneous stable glycosylation capacities, wherein mgat2 has been knocked out (KO), and with and without KO of mgat4A and/or mgat4B and/or mgat5 allowing production of N-glycans with monoantennary structure.

A preferred embodiment of the present invention relates to a cell comprising one
35 or more glycosyltransferase genes that have been inactivated, and that has new

and/or more homogeneous stable glycosylation capacities, wherein mgat2 has been knocked out (KO), and with or without KO of mgat4A and/or mgat4B and/or mgat5 allowing production of N-glycans with monoantennary structure.

- 5 One embodiment of the present invention relates to a cell comprising one or more glycosyltransferase genes that have been inactivated, and that has new and/or more homogeneous stable glycosylation capacities, wherein mgat2 has been knocked out (KO).
- 10 Another embodiment of the present invention relates to the cell as described herein, wherein mgat2 has been knocked out (KO), and with KO of mgat4A and/or mgat4B and/or mgat5 allowing production of N-glycans with monoantennary structure.
- 15 Yet another embodiment of the present invention relates to the cell as described herein, wherein mgat2 and mgat4A has been knocked out (KO), allowing production of N-glycans with monoantennary structure.

- A further embodiment of the present invention relates to the cell as described
20 herein, wherein mgat2 and mgat4B has been knocked out (KO), allowing production of N-glycans with monoantennary structure.

- An even further embodiment of the present invention relates to the cell as described herein, wherein mgat2 and mgat5 has been knocked out (KO), allowing
25 production of N-glycans with monoantennary structure.

- Another embodiment of the present invention relates to the cell as described herein, wherein mgat2 has been knocked out (KO) together with KO of one or more of the genes selected from the group consisting of mgat4A, mgat4B and
30 mgat5.

- Another object of the present invention relates to a cell comprising one or more glycosyltransferase genes that have been inactivated, and that has new and/or more homogeneous stable glycosylation capacities, wherein mgat2 has been
35 knocked out (KO), and with and without KO of mgat4A and/or mgat4B and/or

mgat5 in combination with KO of sialyltransferases allowing production of N-glycans with monoantennary structure and without sialic acid capping.

A preferred embodiment of the present invention relates to a cell comprising one
5 or more glycosyltransferase genes that have been inactivated, and that has new and/or more homogeneous stable glycosylation capacities, wherein mgat2 has been knocked out (KO), and with or without KO of mgat4A and/or mgat4B and/or mgat5 in combination with KO of sialyltransferases allowing production of N-glycans with monoantennary structure and without sialic acid capping.

10

One embodiment of the present invention relates to a cell comprising one or more glycosyltransferase genes that have been inactivated, and that has new and/or more homogeneous stable glycosylation capacities, wherein mgat2 has been knocked out (KO) in combination with KO of sialyltransferases allowing production
15 of N-glycans with monoantennary structure and without sialic acid capping.

Another embodiment of the present invention relates to the cell as described herein, wherein mgat2 has been knocked out (KO), and with KO of mgat4A and/or mgat4B and/or mgat5 in combination with KO of sialyltransferases allowing
20 production of N-glycans with monoantennary structure and without sialic acid capping.

Yet another embodiment of the present invention relates to the cell as described herein, wherein mgat2 and mgat4A has been knocked out (KO) in combination
25 with KO of sialyltransferases allowing production of N-glycans with monoantennary structure and without sialic acid capping.

A further embodiment of the present invention relates to the cell as described herein, wherein mgat2 and mgat4B has been knocked out (KO), in combination
30 with KO of sialyltransferases allowing production of N-glycans with monoantennary structure and without sialic acid capping.

An even further embodiment of the present invention relates to the cell as described herein, wherein mgat2 and mgat5 has been knocked out (KO), in

combination with KO of sialyltransferases allowing production of N-glycans with monoantennary structure and without sialic acid capping.

Another embodiment of the present invention relates to the cell as described
5 herein, wherein mgat2 has been knocked out (KO) in combination with KO of sialyltransferases and with KO of one or more of the genes selected from the group consisting of mgat4A, mgat4B and mgat5.

Yet another object of the present invention relates to a cell comprising one or
10 more glycosyltransferase genes that have been inactivated, and that has new and/or more homogeneous stable glycosylation capacities, wherein mgat2 has been knocked out (KO), and with and without KO of mgat4A and/or mgat4B and/or mgat5 in combination with KO of sialyltransferases and B3gnt2 allowing production N-glycans with monoantennary structure and without sialic acid
15 capping and without poly-LacNAc.

A cell comprising one or more glycosyltransferase genes that have been inactivated, and that has new and/or more homogeneous stable glycosylation capacities, wherein mgat2 has been knocked out (KO), and with or without KO of
20 mgat4A and/or mgat4B and/or mgat5 in combination with KO of sialyltransferases and B3gnt2 allowing production N-glycans with monoantennary structure and without sialic acid capping and without poly-LacNAc.

One embodiment of the present invention relates to a cell comprising one or more
25 glycosyltransferase genes that have been inactivated, and that has new and/or more homogeneous stable glycosylation capacities, wherein mgat2 has been knocked out (KO) in combination with KO of sialyltransferases and B3gnt2 allowing production N-glycans with monoantennary structure and without sialic acid capping and without poly-LacNAc.

30

Another embodiment of the present invention relates to the cell as described herein, wherein wherein mgat2 has been knocked out (KO), and with KO of mgat4A and/or mgat4B and/or mgat5 in combination with KO of sialyltransferases and B3gnt2 allowing production N-glycans with monoantennary structure and
35 without sialic acid capping and without poly-LacNAc.

Yet another embodiment of the present invention relates to the cell as described herein, wherein mgat2 and mgat4A has been knocked out (KO) in combination with KO of sialyltransferases and B3gnt2 allowing production N-glycans with
5 monoantennary structure and without sialic acid capping and without poly-LacNAc.

A further embodiment of the present invention relates to the cell as described herein, wherein mgat2 and mgat4B has been knocked out (KO), in combination
10 with KO of sialyltransferases and B3gnt2 allowing production N-glycans with monoantennary structure and without sialic acid capping and without poly-LacNAc.

An even further embodiment of the present invention relates to the cell as
15 described herein, wherein mgat2 and mgat5 has been knocked out (KO), in combination with KO of sialyltransferases and B3gnt2 allowing production N-glycans with monoantennary structure and without sialic acid capping and without poly-LacNAc.

20 Another embodiment of the present invention relates to the cell as described herein, wherein mgat2 has been knocked out (KO) in combination with KO of sialyltransferases and B3gnt2 and with KO of one or more of the genes selected from the group consisting of mgat4A, mgat4B and mgat5.

25 In one embodiment of the present invention the cell is a mammalian cell or an insect cell.

In another embodiment of the present invention the cell is derived from Chinese hamster ovary or from human kidney.

30

In a further embodiment of the present invention the cell is selected from the group consisting of CHO, NS0, SP2/0, YB2/0, CHO-K1, CHO-DXB11, CHO-DG44, CHO-S, HEK293, HUVEC, HKB, PER-C6, NS0, or derivatives of any of these cells. In one embodiment of the present invention is the cell a CHO cell.

35

In another embodiment of the present invention the cell furthermore encodes an exogenous protein of interest.

In yet another embodiment of the present invention is the protein of interest an
5 antibody, an antibody fragment, such as a Fab fragment, an Fc domain of an antibody, or a polypeptide.

In yet another embodiment of the present invention the protein of interest is a coagulation factor such as coagulation factor II (FII), coagulation factor V (FV),
10 coagulation factor VII (FVIIa), coagulation factor VIII (FVIII), coagulation factor IX (FIX), coagulation factor X (FX), or coagulation factor XIII (FXIII).

One aspect of the present invention relates to a method for the production of di- and triPEGylated glycoproteins, the method comprising the step of enzymatic
15 glycoPEGylation of glycoengineered variants.

In one embodiment of the present invention is the glycoprotein EPO.

Another aspect of the present invention relates to a method for the production of
20 recombinant glycoproteins, comprising the step of generating a cell with specific glycosylation properties, wherein specific glycosylation property is the capacity for monoantennary N-glycan synthesis.

A further aspect of the present invention relates to a method for producing an
25 enzymatically modified glycoprotein, comprising the step of generating a cell with specific glycosylation properties, and the enzymatically modification of interest.

In one embodiment of the present invention is the enzymatically modification a polymer.
30

In another embodiment of the present invention is the enzymatically modification a conjugation to another protein.

A further aspect of the present invention relates to a glycoprotein obtainable from
35 a method according to the present invention.

In one embodiment of the present invention has the glycostructure outcome one or more of the following changes selected from the group consisting of simpler glycan structure, more homogeneous product, more sialic acids per molecule, non-sialylated, non-galactosylated, more homogeneous bi-antennary, more homogeneous monoantennary, more homogeneous triantennary, more homogeneous without poly-LacNAc, higher productivity in cell culture, new stable homogeneous glycosylation capacities, more human glycostructure, more homogeneous glycosylation and improved ADCC targeting of IgG, modified fucose level, no fucose, improved substrate for generating glycoconjugates.

One aspect of the present invention relates to a glycoprotein according to the present invention, which is a homogeneous glycoconjugate produced from a glycoprotein having a simplified glycan profile.

15

Another aspect of the present invention relates to a glycoprotein according to the present invention, which is a homogeneous glycoprotein conjugate comprising a polymer.

20 The glycoprotein can be enzymatically modified with the polymer, and the glycoprotein can be produced by the cell according to the present invention.

Another aspect of the present invention relates to a glycoprotein according to the present invention, which is a homogeneous glycoprotein conjugate comprising a polymer selected from, PEG, HEP, XTEN, PSA, HES.

25

A further aspect of the present invention relates to a glycoprotein according to the present invention, which is a homogeneous PEGylated protein conjugate produced from a protein variant according to the invention having a simplified glycan profile.

30

Another aspect of the present invention relates to a glycoprotein according to the present invention, which is a homogeneous PEGylated protein conjugate produced from an protein variant having a simplified glycan profile.

35

One aspect of the present invention relates to a glycoprotein according to the present invention, which is a simplified enzymatic glycoPEGylation process using glycoengineered protein variants that provides high yield of di- and triPEGylated protein forms.

5

Another aspect of the present invention relates to a glycoprotein according to the present invention, which is a protein conjugate according to the invention comprising FII, FV, FVIIa, FVIII, FIX, FX, FXIII, a Fab fragment of an antibody, or a Fc domain of an antibody.

10

A further aspect of the present invention relates to a glycoprotein according to the present invention, which is a protein-protein conjugate produced from a glycoprotein with a simplified glycan profile.

- 15 In one embodiment of the present invention is the glycoprotein according to the present invention a protein-protein conjugate produced from a glycoprotein with a simplified glycan profile comprising a Fab fragment and one of either FII, FV, FVIIa, FVIII, FIX, FX or FXIII.
- 20 In another embodiment of the present invention is the glycoprotein according to the present invention a protein-protein conjugate produced from a glycoprotein with a simplified glycan profile comprising a Fc Domain and one of either FII, FV, FVIIa, FVIII, FIX, FX or FXIII.
- 25 In a further embodiment of the present invention is the glycoprotein according to the present invention a homogeneous PEGylated EPO conjugate produced from an EPO variant having a simplified glycan profile.

- One aspect of the present invention relates to the use of recombinant
- 30 glycoproteins comprising monoantennary N-glycans for enzymatic modification of polypeptides.

Knockout means full or partial impairment of the function of the gene of interest.

In one aspect of the present invention is one or more of the above mentioned genes knocked out using zinc finger nucleases ZFN. ZFNs can be used for inactivation of a FUT8 gene or any of the other genes disclosed herein. ZFNs comprise a zinc finger protein (ZFP) and a nuclease (cleavage) domain.

5

In another aspect of the present invention is one or more of the above mentioned genes knocked out using transcription activator-like effector nucleases (TALENs).

TALENs are artificial restriction enzymes generated by fusing a TAL effector DNA
10 binding domain to a DNA cleavage domain.

In yet another aspect of the present invention is one or more of the above mentioned genes knocked out using CRISPRs (clustered regularly interspaced short palindromic repeats).

15

CRISPRs are DNA loci containing short repetitions of base sequences. Each repetition is followed by short segments of "spacer DNA" from previous exposures to a virus.

20 CRISPRs are often associated with cas genes that code for proteins related to CRISPRs. The CRISPR/Cas system is a prokaryotic immune system that confers resistance to foreign genetic elements such as plasmids and phages and provides a form of acquired immunity.

25 CRISPR spacers recognize and cut these exogenous genetic elements in a manner analogous to RNAi in eukaryotic organisms.

The CRISPR/Cas system is used for gene editing (adding, disrupting or changing the sequence of specific genes) and gene regulation in species throughout the tree
30 of life. By delivering the Cas9 protein and appropriate guide RNAs into a cell, the organism's genome can be cut at any desired location.

In a further embodiment of the present invention the cell does or does not have α -1,6-fucosyltransferase activity.

35

The cell of the present invention may be a cell that does not comprise the gene of interest to be expressed or the cell may comprise the gene of interest to be expressed. The cell that does not comprise the gene of interest to be expressed is usually called "a naked cell".

5

The host cell of the present invention may be any host, so long as it can express an antibody molecule. Examples include a yeast cell, an animal cell, an insect cell, a plant cell and the like.

- 10 In one embodiment of the present invention is the cell selected from the group consisting of CHO, NS0, SP2/0, YB2/0, YB2/3HL.P2.G11.16Ag.20, NSO, SP2/0-Ag14, BHK cell derived from a syrian hamster kidney tissue, antibody-producing hybridoma cell, human leukemia cell line (Namalwa cell), an embryonic stem cell, and fertilized egg cell.

15

The cell can be an isolated cell or in cell culture or a cell line.

The protein of interest can be various types of protein, and in particular proteins that benefit from being expressed as glycoproteins

20

In a preferred embodiment the protein is erythropoietin (EPO).

In another embodiment is the protein α 1-antitrypsin.

- 25 In one aspect of the present invention is the protein a recombinant blood factor.

In one embodiment of the present invention is the recombinant blood factor selected from the group consisting of one or more of factor VIII, factor IX, factor XIII A-subunit, thrombin, and factor VIIa.

30

In one aspect of the present invention is the protein a recombinant thrombolytic, anticoagulant or another blood-related product.

- In one embodiment of the present invention is the recombinant thrombolytic,
35 anticoagulant or another blood-related product selected from the group consisting

of one or more of tissue plasminogen activator (tPA), hirudin, antithrombin, plasmin, plasma kallikrein inhibitor, and activated protein C.

In one aspect of the present invention is the protein a recombinant hormone.

5

In one embodiment of the present invention is the recombinant hormone selected from the group consisting of one or more of insulin, insulin degludec, human growth hormone, somatropin, pegvisomant, follicle-stimulating hormone, follitropin alfa, corifollitropin alfa, follitropin beta, metreleptin, liraglutide, parathyroid hormone, lutropin, teriparatide, nesiritide, and glucagon.

10

In one aspect of the present invention is the protein a recombinant growth hormone.

15 In one embodiment of the present invention is the recombinant growth hormone selected from the group consisting of one or more of EPO, filgrastim, sargramostim, mecaserim, and palifermin.

In one aspect of the present invention is the protein human growth hormone.

20

In one aspect of the present invention the protein is a glycosylated variant of human growth hormone.

In one aspect of the present invention is the protein a Recombinant interferon, interleukin or tumor necrosis factor.

25

In one embodiment of the present invention is the Recombinant interferon, interleukin or tumor necrosis factor selected from the group consisting of one or more of interferon alfa, PEGinterferon alfa, PEGinterferon alfa-2a, PEGinterferon alfa, interferon beta-1b, alglucosidase alfa, and laronidase.

30

In one aspect of the present invention is the protein an antigen or vaccine component.

In one aspect of the present invention is the protein of the present invention relevant for a disease or disorder selected from the group consisting of one or more of Hemophilia A, Hemophilia B, Acute myocardial infarction, heparinassociated thrombocytopenia, venous thrombosis, Symptomatic
5 vitreomacular adhesion/vitreomacular traction, Acute angioedema, Hereditary antithrombin deficiency, Hereditary angioedema, sepsis, diabetes, diabetes mellitus, Growth failure/growth hormone deficiency, infertility/subfertility, Type 2 diabetes, Osteoporosis, Hypoglycemia, Paget's disease, cancer, Anemia, Neutropenia, Hepatitis C, and Hyperuricemia, Rheumatoid arthritis, hepatitis A
10 and B, Arthritis, colitis, Crohn's, psoriasis, ankylosing spondylitis, Ulcerative colitis.

In one embodiment of the present invention the protein is an antibody.

15 In a preferred embodiment of the present invention is the antibody an IgG antibody.

In the present invention, the antibody molecule includes any molecule, so long as it comprises the Fc region of an antibody. Examples include an antibody, an
20 antibody fragment, a fusion protein comprising an Fc region, and the like.

The antibody is a protein which is produced in the living body by immune reaction as a result of exogenous antigen stimulation and has an activity to specifically bind to the antigen. Examples of the antibody include an antibody secreted by a
25 hybridoma cell prepared from a spleen cell of an animal immunized with an antigen; an antibody prepared by a genetic recombination technique, namely an antibody obtained by introducing an antibody gene-inserted antibody expression vector into a host cell; and the like. Specific examples include an antibody produced by a hybridoma, a humanized antibody, a human antibody and the like.

30

A hybridoma is a cell which is obtained by cell fusion between a B cell obtained by immunizing a mammal other than human with an antigen and a myeloma cell derived from mouse or the like and can produce a monoclonal antibody having the desired antigen specificity.

35

Examples of the humanized antibody include a human chimeric antibody, a human CDR-grafted antibody and the like.

A human chimeric antibody is an antibody which comprises an antibody heavy chain variable region (hereinafter referred to as "HV" or "VH", the heavy chain being "H chain") and an antibody light chain variable region (hereinafter referred to as "LV" or "VL", the light chain being "L chain"), both of an animal other than human, a human antibody heavy chain constant region (hereinafter also referred to as "CH") and a human antibody light chain constant region (hereinafter also referred to as "CL"). As the animal other than human, any animal such as mouse, rat, hamster, rabbit or the like can be used, so long as a hybridoma can be prepared therefrom.

The human chimeric antibody can be produced by obtaining cDNA's encoding VH and VL from a monoclonal antibody-producing hybridoma, inserting them into an expression vector for host cell having genes encoding human antibody CH and human antibody CL to thereby construct a human chimeric antibody expression vector, and then introducing the vector into a host cell to express the antibody.

As the CH of human chimeric antibody, any CH can be used, so long as it belongs to human immunoglobulin (hereinafter referred to as "hIg") can be used. But those belonging to the hIgG class are preferable and any one of the subclasses belonging to the hIgG class, such as hIgG1, hIgG2, hIgG3 and hIgG4, can be used. Also, as the CL of human chimeric antibody, any CL can be used, so long as it belongs to the hIg class, and those belonging to the κ class or λ class can also be used.

A human CDR-grafted antibody is an antibody in which amino acid sequences of CDR's of VH and VL of an antibody derived from an animal other than human are grafted into appropriate positions of VH and VL of a human antibody.

The human CDR-grafted antibody can be produced by constructing cDNA's encoding V regions in which CDR's of VH and VL of an antibody derived from an animal other than human are grafted into CDR's of VH and VL of a human antibody, inserting them into an expression vector for host cell having genes

encoding human antibody CH and human antibody CL to thereby construct a human CDR-grafted antibody expression vector, and then introducing the expression vector into a host cell to express the human CDR-grafted antibody.

- 5 As the CH of human CDR-grafted antibody, any CH can be used, so long as it belongs to the hIg, but those of the hIgG class are preferable and any one of the subclasses belonging to the hIgG class, such as hIgG1, hIgG2, hIgG3 and hIgG4, can be used. Also, as the CL of human CDR-grafted antibody, any CL can be used, so long as it belongs to the hIg class, and those belonging to the κ class or λ class
10 can also be used.

A human antibody is originally an antibody naturally existing in the human body, but it also includes antibodies obtained from a human antibody phage library, a human antibody-producing transgenic animal and a human antibody-producing
15 transgenic plant, which are prepared based on the recent advance in genetic engineering, cell engineering and developmental engineering techniques.

Regarding the antibody existing in the human body, a lymphocyte capable of producing the antibody can be cultured by isolating a human peripheral blood
20 lymphocyte, immortalizing it by its infection with EB virus or the like and then cloning it, and the antibody can be purified from the culture.

The human antibody phage library is a library in which antibody fragments such as Fab, single chain antibody and the like are expressed on the phage surface by
25 inserting a gene encoding an antibody prepared from a human B cell into a phage gene. A phage expressing an antibody fragment having the desired antigen binding activity can be recovered from the library, using its activity to bind to an antigen-immobilized substrate as the marker. The antibody fragment can be converted further into a human antibody molecule comprising two full B chains
30 and two full L chains by genetic engineering techniques.

A human antibody-producing transgenic non-human animal is an animal in which a human antibody gene is introduced into cells. Specifically, a human antibody-producing transgenic animal can be prepared by introducing a human antibody
35 gene into ES cell of a mouse, transplanting the ES cell into an early stage embryo

of other mouse and then developing it. By introducing a human chimeric antibody gene into a fertilized egg and developing it, the transgenic animal can be also prepared. Regarding the preparation method of a human antibody from the human antibody-producing transgenic animal, the human antibody can be
5 produced and accumulated in a culture by obtaining a human antibody-producing hybridoma by a hybridoma preparation method usually carried out in mammals other than human and then culturing it.

Examples of the transgenic non-human animal include cattle, sheep, goat, pig,
10 horse, mouse, rat, fowl, monkey, rabbit and the like.

Another aspect of the present invention relates to a method for producing an antibody composition, which comprises culturing the mammalian cell according to the present invention in a medium to produce and accumulate an antibody
15 composition in the culture; and recovering the antibody composition from the culture

Also, in the present invention, it is preferable that the antibody is an antibody which recognizes a tumor-related antigen, an antibody which recognizes an
20 allergy- or inflammation-related antigen, an antibody which recognizes circulatory organ disease-related antigen, an antibody which recognizes an autoimmune disease-related antigen or an antibody which recognizes a viral or bacterial infection-related antigen, and a human antibody which belongs to the IgG class is preferable.

25

An antibody fragment is a fragment which comprises the Fc region of an antibody. Examples of the antibody fragment include an H chain monomer, an H chain dimer and the like.

30 A fusion protein comprising an Fc region is a composition in which an antibody comprising the Fc region of an antibody or the antibody fragment is fused with a protein such as an enzyme, a cytokine or the like.

One embodiment of the present invention relates to a composition comprising the
35 antibody of the present invention, also referred to as antibody composition.

In one embodiment of the present invention shows the antibody or antibody composition high ADCC activity.

5 In the present invention, the ADCC activity is a cytotoxic activity in which an antibody bound to a cell surface antigen on a tumor cell in the living body activate an effector cell through an Fc receptor existing on the antibody Fc region and effector cell surface and thereby obstruct the tumor cell and the like.

The antibody composition of the present invention has potent antibody-dependent
10 cell-mediated cytotoxic activity (ADCC). An antibody having potent antibody-dependent cell-mediated cytotoxic activity is useful for preventing and treating various diseases including cancers, inflammatory diseases, immune diseases such as autoimmune diseases, allergies and the like, circulatory organ diseases and viral or bacterial infections.

15

In the case of cancers, namely malignant tumors, cancer cells grow. General anti-tumor agents inhibit the growth of cancer cells. In contrast, an antibody having potent antibody-dependent cell-mediated cytotoxic activity can treat cancers by injuring cancer cells through its cell killing effect, and therefore, it is more
20 effective as a therapeutic agent than the general anti-tumor agents.

In immune diseases such as inflammatory diseases, autoimmune diseases, allergies and the like, in vivo reactions of the diseases are induced by the release of a mediator molecule by immunocytes, so that the allergy reaction can be
25 inhibited by eliminating immunocytes using an antibody having potent antibody-dependent cell-mediated cytotoxic activity.

Examples of the circulatory organ diseases include arteriosclerosis and the like. The arteriosclerosis is treated using balloon catheter at present, but circulatory
30 organ diseases can be prevented and treated by inhibiting growth of arterial cells in restricture after treatment using an antibody having potent antibody-dependent cell-mediated cytotoxic activity.

Various diseases including viral and bacterial infections can be prevented and treated by inhibiting proliferation of cells infected with a virus or bacterium using an antibody having potent antibody-dependent cell-mediated cytotoxic activity.

- 5 The antibodies or antibody compositions of the present invention may there for be use to treat immune diseases, cancer viral or bacterial infections or other diseases or disorders mentioned above.

The medicament comprising the antibody composition of the present invention
10 can be administered as a therapeutic agent alone, but generally, it is preferable to provide it as a pharmaceutical formulation produced by an appropriate method well known in the technical field of manufacturing pharmacy, by mixing it with at least one pharmaceutically acceptable carrier.

- 15 It is desirable to select a route of administration which is most effective in treatment. Examples include oral administration and parenteral administration, such as buccal, tracheal, rectal, subcutaneous, intramuscular, intravenous or the like. In an antibody preparation, intravenous administration is preferable.

- 20 The dosage form includes sprays, capsules, tablets, granules, syrups, emulsions, suppositories, injections, ointments, tapes and the like.

Examples of the pharmaceutical preparation suitable for oral administration include emulsions, syrups, capsules, tablets, powders, granules and the like.

25

- Liquid preparations, such as emulsions and syrups, can be produced using, as additives, water; saccharides, such as sucrose, sorbitol, fructose, etc.; glycols, such as polyethylene glycol, propylene glycol, etc.; oils, such as sesame oil, olive oil, soybean oil, etc.; antiseptics, such as p-hydroxybenzoic acid esters, etc.;
30 flavors, such as strawberry flavor, peppermint, etc.; and the like.

- Capsules, tablets, powders, granules and the like can be produced using, as additive, fillers, such as lactose, glucose, sucrose, mannitol, etc.; disintegrating agents, such as starch, sodium alginate, etc.; lubricants, such as magnesium
35 stearate, talc, etc.; binders, such as polyvinyl alcohol, hydroxypropylcellulose,

gelatin, etc.; surfactants, such as fatty acid ester, etc.; plasticizers, such as glycerine, etc.; and the like.

Examples of the pharmaceutical preparation suitable for parenteral administration
5 include injections, suppositories, sprays and the like.

Injections may be prepared using a carrier, such as a salt solution, a glucose solution, a mixture of both thereof or the like. Also, powdered injections can be prepared by freeze-drying the antibody composition in the usual way and adding
10 sodium chloride thereto.

Suppositories may be prepared using a carrier such as cacao butter, hydrogenated fat, carboxylic acid or the like.

Also, sprays may be prepared using the antibody composition as such or using a
15 carrier which does not stimulate the buccal or airway mucous membrane of the patient and can facilitate absorption of the antibody composition by dispersing it as fine particles.

Examples of the carrier include lactose, glycerol and the like. Depending on the
20 properties of the antibody composition and the carrier, it is possible to produce pharmaceutical preparations such as aerosols, dry powders and the like. In addition, the components exemplified as additives for oral preparations can also be added to the parenteral preparations.

25 Although the clinical dose or the frequency of administration varies depending on the objective therapeutic effect, administration method, treating period, age, body weight and the like, it is usually 10 µg/kg to 20 mg/kg per day and per adult.

Definitions

30 General Glycobiology

Basic glycobiology principles and definitions are described in Varki et al. Essentials of Glycobiology, 2nd edition, 2009.

“N-glycosylation” refers to the attachment of the sugar molecule oligosaccharide
35 known as glycan to a nitrogen atom residue of a protein.

"O-glycosylation" refers to the attachment of a sugar molecule to an oxygen atom in an amino acid residue in a protein.

- 5 "Galactosylation" means enzymatic addition of a galactose residue to lipids, carbohydrates or proteins.

"Sialylation" is the enzymatic addition of a neuraminic acid residue.

- 10 "Neuraminic acid" is a 9-carbon monosaccharide, a derivative of a ketononose.

"Monoantennary" N-linked glycan is a engineered N-glycan consist of the N-glycan core (Man α 1-6(Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc β 1-Asn-X-Ser/Thr) that elongated with a single GlcNAc residue linked to C-2 and of the mannose α 1-3.

- 15 The single GlcNAc residue can be further elongated for example with Gal or Gal and NeuAc residues.

"Biantennary" N-linked glycan is the simplest of the complex N-linked glycans consist of the N-glycan core (Man α 1-6(Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc β 1-

- 20 Asn-X-Ser/Thr) elongated with two GlcNAc residues linked to C-2 and of the mannose α 1-3 and the mannose α 1-6. This core structure can then be elongated or modified by various glycan structures.

"Triantennary" N-linked glycans are formed when an additional GlcNAc residue is

- 25 added to either the C-4 of the core mannose α 1-3 or the C-6 of the core mannose α 1-6 of the bi-antennary core structure. This structure can then be elongated or modified by various glycan structures.

"Tetraantennary" N-linked glycans are formed when two additional GlcNAc

- 30 residues are added to either the C-4 of the core mannose α 1-3 or the C-6 of the core mannose α 1-6 of the bi-antennary core structure. This core structure can then be elongated or modified by various glycan structures.

"Poly-LacNAc" poly-N-acetyllactosamine ([Gal β 1-4GlcNAc] $_n$; $n \geq 2$).

"Glycoprofiling" means characterization of glycan structures resident on a biological molecule or cell.

"Glycosylation pathway" refers to assembly of the mono-saccharides into complex carbohydrates by the stepwise action of a group of enzymes, known as glycosyltransferases.

"Biosynthetic Step" means addition of a monosaccharide to glycan structure

10 "Glycosyltransferases" are enzymes that catalyze the formation of the glycosidic linkage to form a glycoside. These enzymes utilize 'activated' sugar phosphates as glycosyl donors, and catalyze glycosyl group transfer to a nucleophilic group, usually an alcohol. The product of glycosyl transfer may be an O-, N-, S-, or C-glycoside; the glycoside may be part of a monosaccharide, oligosaccharide, or
15 polysaccharide.

"Glycosylation capacity" means the ability to produce an amount of a specific glycan structure by a given cell or a given glycosylation process.

20 "Glycoconjugate" is a macromolecule that contains monosaccharides covalently linked to proteins or lipids.

"HEP" is an abbreviation for heparosan. Heparosan (HEP) is a natural sugar polymer comprising (-GlcUA-1,4-GlcNAc-1,4-) re-peats. It belongs to the
25 glycosaminoglycan polysaccharide family and is a negatively charged polymer at physiological pH. It can be found in the capsule of certain bacteria's but it is also found in higher vertebrate where it serves as precursor for the natural polymers heparin and heparan sulphate.

30 "PEG" is an abbreviation for polyethylene glycol. PEG polymers are generally used for increasing the half-life of therapeutic molecule. With their high hydrodynamic volume, PEG polymers increases the effective size of drugs and thus slows their clearance from the bloodstream via kidney filtration or by shielding the protein drug towards clearance receptors and proteolytical degradation.

"XTEN" is well defined peptide sequences based on a limited subset of amino acids, and assembled into longer repeating sequences. XTEN also increases the size of therapeutic molecules and prolongs their presence in the bloodstream.

"GlycoPEGylation" is the process of covalently attaching PEG to glycans of a
5 protein of interest.

"Enzymatic GlycoPEGylation" is the process of covalently attaching PEG to glycans of a protein of interest using a glycosyltransferase and suitable PEGylated glycosyl transfer groups, such as PEGylated NeuAc-CMP molecules.

10

"Simple(r) glycan structure" is a glycan structure containing fewer mono-saccharides and/or having lower mass and/or having fewer antennae.

"Human like glycosylation" means more sialic acids with α 2,6 linkage (more α 2,6 sialyltransferase enzyme) and/or less sialic acids with α 2,3 linkage and/or more
15 N-acetylneuraminic acid (Neu5Ac) and/or less N-glycolylneuraminic acid (Neu5Gc).

"Reconstruction" refers to Inserting exogeneous gene(s) into cells to obtain modified glycostructures. Typically target cells are producing simple glycan
20 structures as result of deconstruction.

"Deconstruction" means obtaining cells producing a simpler glycan structures by single or stacked knock out of glycosyltransferases

25 "Modified glycan profile" refers to change in number, type or position of oligosaccharides in glycans on a given glycoprotein.

More "homogeneous glycosylation" means that the proportion of identical glycan structures observed by glycoprofiling a given protein expressed in one cell is
30 larger than the proportion of identical glycan structures observed by glycoprofiling the same protein expressed in another cell.

General DNA, mol. biol. Any of various techniques used for separating and recombining segments of DNA or genes, commonly by use of a restriction enzyme
35 to cut a DNA fragment from donor DNA and inserting it into a plasmid or viral

DNA. Using these techniques, DNA coding for a protein of interest is recombined/cloned (using PCR and/or restriction enzymes and DNA ligases or ligation independent methods such as USER cloning) into a plasmid (known as an expression vector), which can subsequently be introduced into a cell by

5 transfection using a variety of transfection methods such as calcium phosphate transfection, electroporation, microinjection and liposome transfection. Overview and supplementary information and methods for constructing synthetic DNA sequences, insertion into plasmid vectors and subsequent transfection into cells can be found in Ausubel et al, 2003 and/or Sambrook & Russell, 2001.

10

"Gene" refers to a DNA region (including exons and introns) encoding a gene product, as well as all DNA regions which regulate the production of the gene product, whether or not such regulatory sequences are adjacent to coding and/or transcribed sequences or situated far away from the gene which function they

15 regulate. Accordingly, a gene includes, but is not necessarily limited to, promoter sequences, terminators, translational regulatory sequences such as ribosome binding sites and internal ribosome entry sites, enhancers, silencers, insulators, boundary elements, replication origins, matrix attachment sites, and locus control regions.

20

"Targeted gene modifications", "gene editing" or "genome editing"

Gene editing or genome editing refer to a process by which a specific chromosomal sequence is changed. The edited chromosomal sequence may comprise an insertion of at least one nucleotide, a deletion of at least one

25 nucleotide, and/or a substitution of at least one nucleotide. Generally, genome editing inserts, replaces or removes nucleic acids from a genome using artificially engineered nucleases such as Zinc finger nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), the CRISPR/Cas system, and engineered meganuclease re-engineered homing endonucleases. Genome editing

30 principles are described in Steentoft et al and gene editing methods are described in references therein and also broadly used and thus known to person skilled in the art.

"Endogenous" sequence/gene/protein refers to a chromosomal sequence or gene or protein that is native to the cell or originating from within the cell or organism analyzed.

- 5 "Exogenous" sequence or gene refers to a chromosomal sequence that is not native to the cell, or a chromosomal sequence whose native chromosomal location is in a different location in a chromosome or originating from outside the cell or organism analyzed.
- 10 "Cleavage" refers to the breakage of the covalent backbone of a DNA molecule. Cleavage can be initiated by a variety of methods including, but not limited to, enzymatic or chemical hydrolysis of a phosphodiester bond. Both single-stranded cleavage and double-stranded cleavage are possible, and double-stranded cleavage can occur as a result of two distinct single-stranded cleavage events.
- 15 DNA cleavage can result in the production of either blunt ends or staggered ends. In certain embodiments, fusion polypeptides are used for targeted double-stranded DNA cleavage.

- "Inactivated chromosomal sequence" refer to genome sequence that has been edited resulting in loss of function of a given gene product. The gene is said to be knocked out.
- 20

"Heterologous" refers to an entity that is not native to the cell or species of interest.

25

- The terms "nucleic acid" and "polynucleotide" refer to a deoxyribonucleotide or ribonucleotide polymer, in linear or circular conformation, and in either single- or double-stranded form. For the purposes of the present disclosure, these terms are not to be construed as limiting with respect to the length of a polymer. The terms can encompass known analogs of natural nucleotides, as well as nucleotides that are modified in the base, sugar and/or phosphate moieties (e.g., phosphorothioate backbones). In general, an analog of a particular nucleotide has the same base-pairing specificity; i.e., an analog of A will base-pair with T.
- 30

35

The term "nucleotide" refers to deoxyribonucleotides or ribonucleotides. The nucleotides may be standard nucleotides (i.e., adenosine, guanosine, cytidine, thymidine, and uridine) or nucleotide analogs. A nucleotide analog refers to a nucleotide having a modified purine or pyrimidine base or a modified ribose moiety. A nucleotide analog may be a naturally occurring nucleotide (e.g., inosine) or a non-naturally occurring nucleotide.

The terms "polypeptide" and "protein" are used interchangeably to refer to a polymer of amino acid residues. These terms may also refer to glycosylated variants of the "polypeptide" or "protein", also termed "glycoprotein". "polypeptide", "protein" and "glycoprotein" is used interchangeably throughout this disclosure.

The term "recombination" refers to a process of exchange of genetic information between two polynucleotides. For the purposes of this disclosure, "homologous recombination" refers to the specialized form of such exchange that takes place, for example, during repair of double-strand breaks in cells. This process requires sequence similarity between the two polynucleotides, uses a "donor" or "exchange" molecule to template repair of a "target" molecule (i.e., the one that experienced the double-strand break), and is variously known as "non-crossover gene conversion" or "short tract gene conversion," because it leads to the transfer of genetic information from the donor to the target. Without being bound by any particular theory, such transfer can involve mismatch correction of heteroduplex DNA that forms between the broken target and the donor, and/or "synthesis-dependent strand annealing," in which the donor is used to resynthesize genetic information that will become part of the target, and/or related processes. Such specialized homologous recombination often results in an alteration of the sequence of the target molecule such that part or all of the sequence of the donor polynucleotide is incorporated into the target polynucleotide.

30

As used herein, the terms "target site" or "target sequence" refer to a nucleic acid sequence that defines a portion of a chromosomal sequence to be edited and to which a targeting endonuclease is engineered to recognize, bind, and cleave.

"Targeted integration" is the method by which exogenous nucleic acid elements are specifically integrated into defined loci of the cellular genome. Target specific double stranded breaks are introduced in the genome by genome editing nucleases that allow for integration of exogenously delivered donor nucleic acid element into the double stranded break site. Thereby the exogenously delivered donor nucleic acid element is stably integrated into the defined locus of the cellular genome.

Sequence identity Techniques for determining nucleic acid and amino acid sequence identity are known in the art. Typically, such techniques include determining the nucleotide sequence of the mRNA for a gene and/or determining the amino acid sequence encoded thereby, and comparing these sequences to a second nucleotide or amino acid sequence. Genomic sequences can also be determined and compared in this fashion. In general, identity refers to an exact nucleotide-to-nucleotide or amino acid-to-amino acid correspondence of two polynucleotides or polypeptide sequences, respectively. Two or more sequences (polynucleotide or amino acid) can be compared by determining their percent identity. The percent identity of two sequences, whether nucleic acid or amino acid sequences, is the number of exact matches between two aligned sequences divided by the length of the shorter sequences and multiplied by 100. An approximate alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman, *Advances in Applied Mathematics* 2:482- 489 (1981). This algorithm can be applied to amino acid sequences by using the scoring matrix developed by Dayhoff, *Atlas of Protein Sequences and Structure*, M. O. Dayhoff ed., 5 suppl. 3:353-358, National Biomedical Research Foundation, Washington, D.C., USA, and normalized by Gribskov, *Nucl. Acids Res.* 14(6):6745-6763 (1986). An exemplary implementation of this algorithm to determine percent identity of a sequence is provided by the Genetics Computer Group (Madison, Wis.) in the "BestFit" utility application. Other suitable programs for calculating the percent identity or similarity between sequences are generally known in the art, for example, another alignment program is BLAST, used with default parameters. For example, BLASTN and BLASTP can be used using the following default parameters: genetic code=standard; filter=none; strand=both; cutoff=60; expect=10; Matrix=BLOSUM62; Descriptions=50 sequences; sort

by=HIGH SCORE; Databases=non-redundant,
GenBank+EMBL+DDBJ+PDB+GenBank CDS translations+Swiss
protein+Spupdate+PIR. Details of these programs can be found on the GenBank
website. With respect to sequences described herein, the range of desired degrees
5 of sequence identity is approximately 80% to 100% and any integer value
therebetween. Typically the percent identities between sequences are at least 70-
75%, preferably 80- 82%, more preferably 85-90%, even more preferably 92%,
still more preferably 95%, and most preferably 98% sequence identity.

10 It should be noted that embodiments and features described in the context of one
of the aspects of the present invention also apply to the other aspects of the
invention.

All patent and non-patent references cited in the present application, are hereby
15 incorporated by reference in their entirety.

The invention will now be described in further details in the following non-limiting
examples.

Examples

EXAMPLE 1

Determining the glycosyltransferase repertoire expressed in a mammalian CHO cell line.

- 5 CHO-K1 was obtained from ATCC and CHO-GS (CHOZN GS^{-/-} (Glutamine Synthase) clone produced by ZFN KO) was obtained from Sigma-Aldrich, St. Louis, MO. All CHO media, supplements and other reagents used were obtained from Sigma-Aldrich unless otherwise specified. CHO-GS cells were maintained as suspension cultures in EX-CELL CHO CD Fusion serum and animal component free
- 10 media, supplemented with 4mM L-glutamine. CHO cells were seeded at 0,25x10⁶cells/ml in 6 well plate and harvested at exponential phase 48h post inoculation for total RNA extraction with RNeasy mini kit (Qiagen). RNA integrity and quality were checked by 2100-Bioanalyser (Agilent Technologies). Library construction and next generation sequencing was performed using Illumina HiSeq
- 15 2000 System (Illumina, USA) under standard conditions as recommended by the RNASeq service provider. The aligned data was used to calculate the distribution of reads on CHO reference genes and coverage analysis was performed. Only alignment results that passed QC were used for downstream gene expression analysis.
- 20 RNAseq analysis was performed on two common CHO lines (CHO-K1, CHO-GS) and two independent CHO-GS triple *mgat4a/4b/5* KO clones (ZFN91-1C8, ZFN91-2A6) (TABLE 3). The reported RNAseq analysis of CHO-K1 was included for reference, and this was largely similar to our analyses except that the relative expression levels were higher. Importantly, a few genes including e.g. *galnt1* and
- 25 *mgat4b*, which was reported not to be expressed in CHO-K1 previously (Xu, Nagarajan et al. 2011), were found to be expressed. Moreover, in the case of *mgat4b* this gene was found to be essential for the glycoengineering experiments carried out here.

- An important observation of the analysis of CHO transcriptomes was that the
- 30 expression profiles of glycosyltransferases in the two triple KO clones analyses were identical to those of the parental CHO lines, demonstrating that the targeted ko gene editing performed in the CHO cell lines did not alter expression of other glycosyltransferase genes.

EXAMPLE 2**Gene inactivation of glycosyltransferase genes in the N-glycosylation pathway (Deconstruction).**

All the glycosyltransferase gene targeted inactivation were performed in CHO-GS
5 and/or in CHO-K1 and cells were grown as described in Example 1. Cells were
seeded at 0.5×10^6 cells/mL in T25 flask (NUNC, Denmark) one day prior to
transfection. 2×10^6 cells and 2 μ g endotoxin free plasmid DNA of each ZFN
(Sigma, USA) were used for transfection. Each ZFN was tagged with GFP and
Crimson by a 2A linker (Duda, Lonowski et al. 2014). Transfections were
10 conducted by electroporation using Amaxa kit V and program U24 with Amaxa
Nucleofector 2B (Lonza, Switzerland). Electroporated cells were subsequently
plated in 3 mL growth media in a 6-well plate. Cells were moved to 30°C for a 24h
cold shock. 72h post nucleofection the 10-15% highest labeled cell pool for both
GFP and Crimson were enriched by FACS. We utilized recent developed methods
15 for enriching KO clones by FACS (GFP/Crimson tagged ZFNs) (Duda, Lonowski et
al. 2014) and high throughput screening by an amplicon labeling strategy (IDAA)
(Zhang, Steentoft et al. submitted). Cells were single cell FACS sorted again one
week later to obtain single clones in round bottom 96 well plates. KO clones were
identified by IDAA, and when possible also by immunocytology with appropriate
20 lectins and monoclonal antibodies. Selected clones were further verified by TOPO
cloning of PCR products (Invitrogen, US) for in detail Sanger sequencing
characterization of mutations introduced in individual TOPO clones. The strategy
enabled fast screening and selection of KO clones with appropriate inactivation
mutations as outlined herein, and on average we selected 2-5 clones from each
25 targeting event.

The majority of KO clones exhibited out of frame causing insertions and/or
deletions (indels) in the range of ± 20 bps, and most targeted genes were present
with two alleles, while some (*mgat4B* and *mgat5*) were present with 1 or 3 alleles,
respectively (TABLE 4).

30 TABLE 4 lists all individual and stacked glycosyltransferase gene inactivation
events and the order (ancestry line) of stacking of glycosyltransferase gene
inactivation in cells to produce a deconstruction matrix of the N-glycosylation
pathway.

EXAMPLE 3**Gene insertion of glycosyltransferase genes in the N-glycosylation pathway (Reconstruction).**

Target specific integration (knockin/KI) was directed towards the CHO Safe Harbor
5 #1 locus (S.Bahr et al., BMC Proceedings 2013, 7(Suppl 6):P3.doi:10.1186/1753-6561-7-S6-P3). A modified ObLiGaRe strategy (33) was used where two inverted ZFN binding sites flank the donor plasmid gene of interest to be knocked in. Firstly a shuttle vector designated ObLiGaRe-2X-Ins was synthesized (TABLE 5).

ObLiGaRe-2X-Ins was designed in such a way, that any cDNA sequence encoding
10 protein of interest can be inserted into a multiple cloning site where transcription is driven by CMV IE promotor and a BgH terminator. In order to minimize epigenetic silencing, two insulator elements flanking the transcription unit were inserted. In addition an AAVS1 "landing pad" was included at the 3' end of ObLiGaRe-2X-Ins just upstream of the 3' inverted ZFN binding site. A full
15 ST6GAL1 open reading frame was inserted directionally into ObLiGaRe-2X-Ins generating ObLiGaRe-2X-Ins-ST6GAL1 (TABLE 5). Transfection and sorting of clones were performed as described in EXAMPLES 1 and 2. Clones were initially screened by positive SNA lectin staining and selected clones further analyzed by 5' and 3' junction PCR to confirm correct targeted integration event into
20 Safeharbor#1 site in the CHO *st3gal4/6* KO clones. The allelic copy number of integration was determined by WT allelic PCR. Subsequently, full human MGAT4A open reading frame was inserted directionally into 2nd allele of Safe Harbor#1 in ST6GAL1 KI clone, followed by inserting full human MGAT5 to AAVS1 "landing pad", which was included at the 3' end of ObLiGaRe-2X-Ins just upstream of the
25 3' inverted ZFN binding site (TABLE 5).

EXAMPLE 4**Recombinant production of human EPO and IgG in gene edited mammalian CHO cells.**

Expression constructs containing the entire coding sequence of human EPO and a
30 therapeutic IgG cloned into pcDNA3.1/myc-His (C-terminal tags) and pBUDCE4.1 (Invitrogen), respectively, were synthesized by Genewiz, USA. EPO is a 166 amino acid protein with N-glycans at Asn24, Asn38, and Asn83, and IgG has a single N-glycan at Asn297, respectively. Wt or KO CHO cells were transfected with EPO or IgG and maintained at 37 °C as suspension cultures in EX-CELL CHO CD Fusion

serum-free media, supplemented with 4mM L-glutamine and 400ug/ml Zeocin in 5% CO₂ incubator. Clones were scaled up and grown in T75 flasks at a seeding density of 0,5x10⁶ cells/ml, and harvested 72h or 96h later. Cell viability was higher than 90% in all reporter expressing clones and viable cell density are
5 between 2-3x10⁶ cells/ml upon harvest. Cells were removed by centrifugation at 300g for 5 min, and the supernatant were stored at -80°C. His-tagged recombinant human EPO was purified by nickel affinity purification (Invitrogen, US). Media was mixed 3:1 (v/v) in 4x binding buffer (200 mM Tris, pH 8.0, 1.2 M NaCl) and applied to 0.3ml packed NiNTA agarose (Invitrogen), pre-equilibrated in
10 binding buffer (50 mM Tris, pH 8.0, 300 mM NaCl). The column was washed with binding buffer and then bound protein was eluted with binding buffer with additional 250 mM imidazole. Fractions containing EPO were determined by SDS-PAGE and further purified on a reverse-phase HPLC purification with a Jupiter C4 column (5 µm, 300 Å, column 250 x 4.6 mm) (Phenomenex), using 0.1%
15 trifluoroacetic acid (TFA) and a gradient of 10–100% acetonitrile. IgG was purified by HiTrap™ Protein G HP (GE Healthcare, US) pre-equilibrated/washed in PBS and eluted with 0.1 M Glycine (pH 2.7). Purity of protein was evaluated by Coomassie staining of SDS-PAGE gels, and proteins were quantified by BCA Protein Assay Kit (Thermo Scientific, Rockford, US).

20 **EXAMPLE 4A**

Recombinant production of human GH and FVII in gene edited mammalian CHO cells

Four expression constructs containing the entire coding sequence for human GH (hGH) and human Factor VII (hFVII) are cloned into pCGS3 vector (Sigma-
25 Aldrich), that contains a glutamine synthetase (GS) selection cassette. Four hGH constructs carry different individual N-glycosylation sites at L93N, L101T or in combination L93N-L101T and the control without any (hGH WT). The constructs have been described before. Three hFVII constructs have removed different individual N-glycosylation sites at N145Q, N322Q or in combination
30 N145Q_N322Q and the control (hFVII WT). Suspension cells CHO-GS WT, CHO-GS with KO mgat2/4a/4b/5 (allowing production of N-glycans with monoantennary structure), CHO-GS with KO of B3gnt2/mgat4a/4b/5/ST3gal4/6 (allowing production of N-glycans with biantennary structure without poly-LacNAc and without a2,3sia capping), CHO-GS with KI of ST6gal1 in KO of

mgat4a/4b/5/ST3gal4/6 (allowing production of N-glycans with biantennary structure without poly-LacNAc and with α 2,6sia capping) and CHO-GS with KO of B3gnt2/mgat4a/4b/5 (allowing production of N-glycans with biantennary structure without poly-LacNAc and with α 2,3sia capping) are grown in E125 shaker flasks in
5 30ml EX-CELL CD CHO medium (Sigma-Aldrich) with 4mM L-Glutamine (Thermo Fisher Scientific) to a density of 2×10^6 cells/ml. The constructions of the strains are described in EXAMPLE 2 and 3. 2 μ g of each construct is transfected into each CHO strain individually with the Nucleofector II device (Kit V, program U-024, LONZA). After nucleofection, the cells are cultivated in 2ml EX-CELL CD CHO
10 medium 20% conditioned medium and 4mM L-Glutamine in 6well plates. 24 hours post transfection 10^5 cells of each transfection sample are transferred to an individual 24well and cultivated in 500 μ l EX-CELL CD CHO medium without L-Glutamine (selection start point). After 10-21 days the cells reach 80% confluence and are splitted 1:5 to a new 24well (1st split). After 10-14 days the cells reach
15 80% confluence and are splitted 1:5 to a new 24well (2nd split). After 7-9 days the cells reach 80% confluence and are splitted 1:10 to a new 24well (3rd split). Cells are expanded to 6well format (2ml culture volume) and T25 flasks (6ml culture volume) and are then transferred to E125 flasks (30ml culture volume) for three days cultivation. Each culture is splitted to 0.4×10^6 cells/ml in 30ml EX-CELL CD
20 CHO medium, with 20% CH CD Efficient Feed B Liquid Nutrient Supplement (Thermo Fisher Scientific) without L-Glutamine and with and without 25mM MSX in E125 shaker flasks. Vitamin K is added at 5 μ g/ml to all hFVII cultures. 7 days after cultivation cells reach densities between $11-1,5 \times 10^6$ cells/ml. Cultures are harvested into 50ml flacon tubes and spin down at 200g for 5min. The
25 supernatant is collected and stored at -20C until further analysis.

The purification methods of hGH and hFVII are described elsewhere (WO 2007026021 A1 and WO 1997010887 A1). N-glycan profiling of purified hGH and hFVII are done like for EPO and IgG in EXAMPLE 5.

30 **EXAMPLE 5**

N-glycan profiling of purified EPO and IgG.

Approximately 25 μ g purified EPO was digested with 2 U PNGase F (Roche Diagnostics, Mannheim, Germany) in 50 mM ammonium bicarbonate at 37°C for overnight. For IgG, 35 μ g samples were initially digested (overnight, 37°C) with

trypsin in 50 mM ammonium bicarbonate, and then heated at 95°C for 15 min and cooled to RT before PNGase F digestion as above. Released N-glycans were separated from intact EPO or IgG tryptic digest using in-house packed Stagetips (Empore disk-C18, 3M) and incubated in 100 mM ammonium acetate, pH 5.0 for 5 60 min at 22°C. The N-glycans were split in three equal aliquots. One-third was dried in glass vials and permethylated as described previously (Ciucanu and Costello 2003) and the remainder was saved for the native N-glycan analysis. Permethylated N-glycans were extracted with chloroform and desalted by washing the organic phase repeatedly with deionized water. The organic phase was 10 evaporated over a stream of nitrogen gas and the permethylated N-glycans were dissolved in 20 µL 50% (v/v) methanol. Finally, 1 µL sample was co-crystallized with 1 µL matrix composed of 2,5-dihydroxybenzoic acid (10 mg/ml) in 70% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid and 2.5 mM sodium acetate. Permethylated N-glycans were analyzed by positive mode MALDI-TOF (Autoflex 15 Speed, BrukerDaltronics, Bremen) operated in the reflector mode with data acquisition (2000 shots/spot) in the m/z 1000-7000 mass range. Glycan compositional analysis of all spectra was performed by the SysBioWare platform and is presented in Supplementary Table 4 (Vakhrushev, Dadimov et al. 2009). Released native N-glycans were reconstituted in MeOH/H₂O (1/1; v/v, containing 20 50mM Ammonium bicarbonate) at the concentration of 0.5 pmol/µl and analyzed on an Orbitrap Fusion MS (Thermo San Jose, USA). Samples were directly infused via EASY-Spray and analysed by negative ion mode. Flow rate was set to 500nl/min, spray voltage 1,900-2,100 V, and ion transfer tube temperature 275°C. Precursor ions were selected (isolation width, m/z 3) and subjected to 25 HCD fragmentation at 35% normalized collision energy (default charge state, $z=2$). All spectra were recorded at resolution 120,000.

EXAMPLE 6

Inactivation of glycosyltransferase genes by tandem single stranded oligo deoxy nucleotide (ssODN)/ZFN precise multi exon gene editing.

30 In this example, gene inactivation is performed by eliminating exons encoding the N-terminal cytoplasmic tail, transmembrane region, and parts of the stem region of the targeted glycosyltransferase gene. The target region deletion is mediated by use of the tandem single stranded oligo deoxy nucleotide (ssODN)/ZFN approach (Duda, Lonowski et al. 2014). The ZFN target site is selected in or as

close as possible around the sequence coding for the transmembrane region of the glycosyltransferase. As an example CHO *mgat4a* is targeted using CHO *mgat4a* ComposR ZFN's (Sigma) and an ssODN possessing 60bp homology arm sequences flanking the 5' UTR region including translational start site and 3' flanking *mgat4a* exon2 ZFN target site (*mgata* ssODN: gagagagattggtcttgcttgctcatcaccaacgtatgaaccagtgtgatggtgaaatgagtcGCCGTGCAGGA GCAGCAACTAACGGAAGTAACACTGCATTGACTACATTTTCAGGTAC) (SEQ ID NO:54) (5' UTR region shown in lower case, partial ZFN cut site in lower case italics and 3' flanking exons 2 ZFN binding site in upper case), thus removing the exon 1-2 encoding cytoplasmic tail, transmembrane domain and stem of *mgat4a*. Gene inactivation is performed in CHO-GS as described in Example 2 including *mgata* ssODN. Transfections were conducted by electroporation using Amaxa kit V and program U24 with Amaxa Nucleofector 2B (Lonza, Switzerland). Electroporated cells were subsequently placed in 3 mL growth media in a 6-well plate. Cells were moved to 30°C for a 24h cold shock. 72h post nucleofection the 10-15% highest labeled cell pool for both GFP and Crimson were enriched by FACS. Cells were single cell FACS sorted again one week later to obtain single clones in round bottom 96 well plates. Enrichment of KO clones was performed by FACS (GFP/Crimson tagged ZFNs). Clones are analyzed by PCR using primers Mgataex1F (5'- TATCCACTGTGTTGCTTGCTG-3') (SEQ ID NO:55)/Mgataex2R (5'- Actgctcttcagaggtcctg d-3') (SEQ ID NO:56), only detecting correctly target deleted clones. Mono/bi-allelic targeting was determined by PCR wt allele presence detection using primers Mgataex2F (5'-gaacgccttcgaatagctgaacatagg-3') (SEQ ID NO:57)/Mgataex2R. Genetic PCR results are validated by Southern blot analysis using an intron2 specific probe detecting correct removal of 37KBp intron1 target region carrying diagnostic KpnI sites.

Example 7

Inactivation of glycosyltransferase genes by multi-exon dual

30 CRISPR/Cas9 gene editing.

Gene inactivation is ensured by removal of exons encoding the cytoplasmic tail/transmembrane and parts of the stem encoded sequences of the desired gene needed to be inactivated. Target region deletion is mediated by use of a pairs of CRISPR/Cas9-gRNAs targeting the flanking regions encoding *Mgat4a* signal sequence, transmembrane anchoring and stem -regions. CHO *Mgat4a* is targeted

using CHO *Mgat4a* CRISPR/Cas9 gRNA's *Mgat4aEx1gRNA* (5'-GGTATACCACATGGCAAAATGGG-3') (SEQ ID NO:58) specific for exon1 and *Mgat4aEx2gRNA* (5'-GTCCAACAGTTTCGCCGTGCAGG-3') (SEQ ID NO:59) specific for exon2. Both gRNA's were cloned into the BbsI (NEB, USA) gRNA target site of px458 (Addgene, USA) encoding GFP tagged *S.pyogenes* Cas9 and U6 promoter driven gRNA cassette, generating px458-CRISPR-*Mgat4aex1gRNA* and px458-CRISPR- *Mgat4aex2gRNA*. Precise gene editing by nucleofection using 2ug of these CRISPR/Cas9-*Mgat4a* editing tools and target selection validation was performed in CHO-GS cells as described in Examples 2 and 6.

10

Example 8

Enzymatic GlycoPEGylation of glycoproteins monoantennary N-glycans.

KO of *mgat2* in combination with *mgat4a/4b/5* in CHO cells resulted in homogeneous monoantennary N-glycans when EPO was expressed (FIG 21).
 15 Reintroduction of human MGAT4A as well as MGAT4A and 5 in combination by knockin resulted in tri and tetraantennary N-glycans, respectively (FIG 21), demonstrating that the monoantennary N-glycan obtained is linked β 1-2 to the α 1-3Man branch controlled by *mgat1*. Enzymatic glycopegylation or transfer of other compounds by sialyltransferases and CMP-(PEG)NeuAc or relevant modified
 20 donor was tested using EPO as an example (FIGs 22-23).

Material and Methods – Glycovariants of EPO-Myc-His6 were produced in CHO with KO *mgat2/4a/4b/5* and purified on a nickel-NTA column and buffer exchanged into 100 mM Tris, 150 mM NaCl, 0.005 % NaN₃, pH 7.2 buffer as described in Example 4. Wild type EPO-Myc-His6 with normal glycosylation profile
 25 was produced in non modified CHO cells and in similar way purified on a nickel-NTA column before buffer exchanged into 100 mM Tris, 150 mM NaCl, 0.005 % NaN₃, pH 7.2 buffer. Protein concentrations were determined by NanoDrop Lite Spectrophotometer (Thermo Scientific™). Sialyltransferase (ST3Gal-III; 1.35 U/ml) in 20 mM HEPES, 120 mM NaCl, 50 % glycerol, pH 7.0 stock solution was
 30 obtained from FujiFilm Diosynth Biotechnologies, Japan. Sialidase (*Artherobacter urifaciens*) was produced at Novo Nordisk A/S and used as a 200 U/ml stock solution in 20 mM HEPES, 120 mM NaCl, 50 % glycerol, pH 7.0. Support bound neuraminidase (*Clostridium perfringens*) on agarose was obtained from Sigma (Cat. # N5254-10UN). The resin was washed extensively in MilliQ water before

use. 10k-PSC (cytidine-5'-monophosphoryl-[5-(N-10kDa-methoxypolyoxyethylene-oxycarboxamido)glycylamido-3,5-dideoxy-D-glycero-n-galacto-2-nonulopyranosurate], Lot: 3605-A-R0-01-61-1) was obtained from Albany Molecular Research, Inc, Albany, New York 12203, US. 10k-PSC was produced as described in WO03031464. The tris-buffer used in examples below contained 50 mM tris-HCl, 150 mM NaCl, pH 7.6.

SDS-PAGE gel electrophoresis was performed on NuPage precast gels (NuPage 7 % Tris-Acetate Gel, 15 wells, Invitrogen cat. No. EA03555BOX) using NuPage Tris-Acetate SDS Running buffer (Invitrogen cat. No. # LA0041). Samples (0.15 mg/ml) were added NuPage Sample Reducing Agent (Invitrogen cat. No. NP0004) and LDS sample buffer (Invitrogen cat. No. NP0008) and heated to 70°C for 10 min before gel loading. HiMark HMW (Invitrogen cat. No. LC6060) was used as standard. Electrophoresis was performed at 150 V, 120 mA & 25 W for 60 min. Gels were stained with SimplyBlue SafeStain (Invitrogen cat. No. LC5688) according to Invitrogen protocols. Coomassie stained gels were analysed by densitometry using GelQuant software and methods described previously (Rehbein and Schwalbe 2015) (FIG 23).

HPLC analysis of GlycoPEGylated EPO-Myc-His6 conjugates of the invention was performed as follows. Samples were analysed in non-reduced form. A Zorbax 300SB-C3 column (4.6x50 mm; 3.5 µm Agilent, Cat. No.: 865973-909) was used. Column was operated at 30°C. 2.5 µg sample was injected, and column eluted with a water (A) – acetonitrile (B) solvent system containing 0.1% trifluoroacetic acid. The gradient program was as follows: 0 min (25% B); 4 min (25% B); 14 min (46% B); 35 min (52% B); 40 min (90% B); 40.1 min (25% B).

Enzymatic glycoPEGylation of EPO-Myc-His6 variants was performed by either an one-pot method using sialidase in combination with ST3Gal-III, or by a sequential method where the EPO-Myc-His6 variant was first reacted with agarose-neuraminidase gel for 3h at 25°C, then after transferring the supernatant to a fresh vial reacted with the ST3Gal-III and PSC reagent. The one-pot method relies on the fact that *Artherobacter urifaciens* sialidase is unable to desialylate PEGylated sialosides. Use of recombinant glycoproteins with monoantennary N-

glycans without sialic acid capping as invented eliminates the need for prior desialylation.

GlycoPEGylation of recombinant human erythropoietin - Recombinant wildtype

- 5 EPO-Myc-His6 produced in CHO cell lines was dissolved in 100 mM Tris, 150 mM NaCl, 0.005 % NaN₃ (pH 7.2) to a concentration of 1.17 mg/ml. 10k-PSC reagent (2.0 mg) was dissolved in 100 ul 50 mM Tris-HCl, 150 mM NaCl (pH 7.6) to a concentration of 20 mg/ml. ST3Gal-III and AUS were both dissolved in 20 mM HEPES, 120 mM NaCl, 50 % glycerol, pH 7.0 to a volumetric concentration of 1.35
- 10 U/ml and 200 U/ml respectively. Reagents were mixed according to scheme below:

	Unit	Exp.1	Exp.2	Exp.3	Exp.4	Exp.5
wtEPO	ug	10	10	10	10	10
10k-PSC	eq	1	5	10	25	50
ST3Gal3	mU/ug wtEPO	0.196	0.196	0.196	0.196	0.196
Sialidase	U/ml	5	5	5	5	5
Tris buffer	ul	9.0	8.4	7.6	5.3	1.3
Final volume	ul	28.45	28.45	28.45	28.45	28.45
Final [wtEPO]	mg/ml	0.35	0.35	0.35	0.35	0.35

- Reactions were incubated for 18h at 32°C. Samples were then analysed by HPLC
- 15 and SDS-PAGE (FIG 22, lanes 5-9).

GlycoPEGylation of erythropoietin produced in CHO cells with KO of

- mgat2/4A/4B/5* - Recombinant wtEPO-Myc-His6 glycosylation variant produced in Mgat2/4a/4b/5 knock-out CHO cell line was dissolved in 100 mM Tris, 150 mM
- 20 NaCl, pH 7.2 to a concentration of 0.585 mg/ml. 10k-PSC reagent (2.0 mg) was dissolved in 100 ul 50 mM Tris-HCl, 150 mM NaCl, pH 7.6 to a concentration of 20 mg/ml. ST3GalIII and AUS were both dissolved in 20 mM HEPES, 120 mM NaCl, 50 % glycerol, pH 7.0 to a volumetric concentration of 1.35 U/ml and 200 U/ml respectively. Reagents were mixed according to scheme below:

	Unit	Exp.1	Exp.2	Exp.3	Exp.4	Exp.5
EPO-Myc-His6	ug	10	10	10	10	10
10k-PSC	eq	1	5	10	25	50
ST3Gal3	mU/ug EPO	0.196	0.196	0.196	0.196	0.196
Sialidase	U/ml	5	5	5	5	5
Tris buffer	ul	9.0	8.4	7.6	5.3	1.3
Final volume	ul	28.45	28.45	28.45	28.45	28.45
Final [EPO]	mg/ml	0.35	0.35	0.35	0.35	0.35

Reactions were incubated for 18h at 32°C. Samples were then analysed by HPLC and SDS-PAGE (FIG 22, lanes 11-15).

- 5 Two step glycoPEGylation reaction of recombinant human erythropoietin - EPO-Myc-His6 (272 ug) in 100 mM Tris, 150 mM NaCl, 0.005 % NaN₃, pH 7.2 (0.214 mg/ml) was treated with prewashed immobilized sialidase (150 ul suspension, 150 mU) for 3h at room temperature. Resin was then removed by filtration. The filtrate was analysed by LC-MS to confirm formation of asialo EPO-Myc-His6 in
- 10 monoantennary N-glycan form (calculated mass 25,464.97 Da, analysis 25,465.30 Da).

Calc. average mass	Found	Assignment
26921.23 Da	26921.53 Da	Penta sialylated EPO-myc-His6 containing 3 mono antennary N-glycanes and one O-glycan
26629.98 Da	26630.34 Da	Tetra sialylated EPO-myc-His6 containing 3 mono antennary N-glycanes and one O-glycan
26338.72 Da	26339.16 Da	Tri sialylated EPO-myc-His6 containing 3 mono antennary N-glycanes and one O-glycan
25464.97 Da	25465.30 Da	Desialylated (neuraminidase treated) EPO-myc-His6 containing 3 mono antennary N-glycanes and one O-glycan

The filtrate was then added 10k-PSC reagent dissolved in 100 ul 50 mM Tris-HCl, 150 mM NaCl, pH 7.6. ST3Gal-III and AUS were both dissolved in 20 mM HEPES, 120 mM NaCl, 50 % glycerol, pH 7.0 to a volumetric concentration of 1.35 U/ml 5 and 200 U/ml respectively. Reagents were mixed according to scheme below:

	Unit	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
asialo EPO-Myc-His6	ug	20	20	20	20	20
10k-PSC	eq	1	3	5	10	20
St3Gal3	mU/ug EPO	0.196	0.196	0.196	0.196	0.196
Tris buffer	ul	14.0	12.5	11.0	7.4	0
Final volume	ul	117.6	117.6	117.6	117.6	117.6
Final [EPO]	mg/ml	0.17	0.17	0.17	0.17	0.17

Reactions were incubated for 20h at 32°C. Samples were then analysed by HPLC and SDS-PAGE.

10

EXAMPLE 9

GlycoPEGylation of monoantennary N-glycans FVIIa

Glycovariants of hFVII N145Q, N322Q or in combination N145Q_N322Q and the control (hFVII WT) produced in CHO with KO mgat2/4a/4b/5 are glycoPEGylated 15 on the mono-antennary N-glycan by a 2 step enzymatic process. The FVIIa protein is initially buffer exchanged into a 25 mM HEPES buffer containing approximately 10 mM CaCl₂, 100 mM NaCl (pH 7.0). The buffer exchange is performed using a Slide-A-Lyzer cassette (Thermo Scientific) with a 10kD cut-off. To the FVIIa protein is then added sialidase (1.0 units per mg of FVIIa) and the 20 reaction mixture is incubated at room temperature for an hour. The asialo FVIIa is isolated using the procedure described by Thim, L et al. Biochemistry 1988, 27, 7785-779. FVIIa protein concentration is at this point determined by NanoDrop Lite Spectrophotometer (Thermo Scientific™) against a known FVIIa reference. 5 equivalents of 10k-PSC reagent produced as described in WO03031464 is then 25 added in 50 mM Tris-HCl, 150 mM NaCl (pH 7.6), followed by addition of ST3Gal-III enzyme (0.2 units per mg of asialo FVIIa protein). The reaction is incubated for 18h at 32°C. Excessive 10k-PSC reagent is removed by ion exchange chromatography: the reaction mixture is loaded on to a HiTrap Q ion-exchange

column (Amersham Bioscience) pre-equilibrated in 25 mM GlyGly, 50 mM NaCl, pH 8.0. The column is eluted with 25 mM GlyGly, 50 mM NaCl, pH 8.0 (buffer A) over 10 cv; followed by 25 mM GlyGly, 50 mM NaCl, 25 mM CaCl₂, pH 8.0 (buffer B) over 15 cv at constant flow and temperature (5°C). The eluent is monitored by absorbance at 280 nm. Fractions containing protein conjugate are pooled and pH adjusted to 6.0 using 1N HCl. Non pegylated FVIIa is then removed from glycopegylated FVIIa variant(s) by size exclusion chromatography on a Superdex 200 column (Amersham Bioscience) pre-equilibrated in 25 mM GlyGly, 50 mM NaCl, 25 mM CaCl₂, pH 6.0. The column is eluted with 25 mM GlyGly, 50 mM NaCl, 25 mM CaCl₂, pH 6.0 while monitoring absorbance at 280 nm. Fractions are analysed on SDS-PAGE (4-12% Bis-Tris NuPAGE gels) stained with Simple Blue as described by Invitrogen. Fractions containing PEGylated FVIIa are pooled and concentrated by filtering through an Amicon Ultra™-15 (10K MWCO) centrifuge filter at 4000 rpm, 10°C.

15

EXAMPLE 10**GlycoPEGylation of hGH glycovariant**

Glycovariants with individual N-glycosylation sites at L93N, L101T or in combination L93N-L101T of hGH and the control without any (hGH WT) produced in CHO with KO mgat2/4a/4b/5 are glycoPEGylated on the mono-antennary N-glycan by a 2 step enzymatic process similar to the previous example. The hGH protein is initially buffer exchanged into a 25 mM HEPES buffer (pH 7.2). Support bound neuraminidase (Clostridium perfringens) on agarose (Sigma Cat. # N5254-10UN) is added and the reaction mixture is incubated at room temperature for one hour. The agarose support is then filtered off, and the hGH protein is added 10k-PSC (WO03031464) reagent (5 equivalents relative to hGH protein) followed by ST3Gal-III enzyme (0.2 units per mg of hGH protein). The reaction is incubated for 18h at 32°C. The glycoPEGylated hGH protein is isolated by a combination of anionic exchange and size exclusion chromatography as described in the previous example. Fractions are analysed on SDS-PAGE stained with Simple Blue as described by Invitrogen. Fractions containing PEGylated hGH are pooled and concentrated by filtering through an Amicon Ultra™-15 (10K MWCO) centrifuge filter at 4000 rpm, 10°C. The glycoPEGylated hGH protein can alternatively be purified by RP-HPLC as described by da Silva Freitas et al. Bioconjugate Chem. 2013, 24, 456–463.

Table 4. ZFN design and sequence analysis of CHO glycoengineered clones.

Clone	Insert & Del	Alignment*
KO:mgat4A		
mgat4A	ZFN	TTCTGAGTTGAATGCCATTGTCCAACAgtt-----
-WT		tcGCCGTGCAGGAGCAGCAACTAACGGAAG (SEQ ID NO:9)
mgat4A	-5bp	TTCTGAGTTGAATGCCATTGTCCAACAgt-----
-allele1		CCGTGCAGGAGCAGCAACTAACGGAAG (SEQ ID NO:10)
mgat4A	+13bp	TTCTGAGTTGAATGCCATTGTCCAACAgttGAATTCTAGATGAtcGCCGTGCAGG
-allele2		AGCAGCAACTAACGGAAG (SEQ ID NO:11)
KO:mgat4A/4B		
mgat4A	ZFN	TTCTGAGTTGAATGCCATTGTCCAACAgtt-----
-WT		tcGCCGTGCAGGAGCAGCAACTAACGGAAG (SEQ ID NO:9)
mgat4A	-5bp	TTCTGAGTTGAATGCCATTGTCCAACAgt-----
-allele1		CCGTGCAGGAGCAGCAACTAACGGAAG (SEQ ID NO:10)
mgat4A	+13bp	TTCTGAGTTGAATGCCATTGTCCAACAgttGAATTCTAGATGAtcGCCGTGCAGG
-allele2		AGCAGCAACTAACGGAAG (SEQ ID NO:11)
mgat4B	ZFN	<u>GCCCTCCAGCAGCCCTCTgaggaCTGGATGATCCTGGAGTT</u> (SEQ ID
-WT		<u>NO:12)</u>
mgat4B	-7bp	GCCCTCCAGCAGCCCTCTg-----GATGATCCTGGAGTT (SEQ ID
-allele1		NO:13)
KO:mgat5		
mgat5-	ZFN	GGGGGATGATGCTTCTGCACTTCACCATCCAgt-----
WT		cagcgGACTCAGCCTGAGAGCAGCTCCATGTT (SEQ ID NO:17)
mgat5-	+14bp	GGGGGATGATGCTTCTGCACTTCACCATCCAgtcaTGAATTCTAGATGAgcgGACT
allele1		CAGCCTGAGAGCAGCTCCATGTT (SEQ ID NO:18)
mgat5-	-1bp	GGGGGATGATGCTTCTGCACTTCACCATCCAgt-----
allele2		agcgGACTCAGCCTGAGAGCAGCTCCATGTT (SEQ ID NO:19)
mgat5-	+61bp	GGGGGATGATGCTTCTGCACTTCACCATCCAgtcag (SEQ ID NO:20)-----
allele3		(+61bp)--cgGACTCAGCCTGAGAGCAGCTCCATGTT (SEQ ID NO:21)
KO:mgat4A/4B/5		
mgat4A	ZFN	TTCTGAGTTGAATGCCATTGTCCAACAgtt-----
-WT		tcGCCGTGCAGGAGCAGCAACTAACGGAAG (SEQ ID NO:9)

mgat4A -5bp TTCTGAGTTGAATGCCATTGTCCAACA~~g~~-----
 -*allele1* CCGTGCAGGAGCAGCAACTAACGGAAG (SEQ ID NO:10)
mgat4A +13bp TTCTGAGTTGAATGCCATTGTCCAACA~~g~~ttGAATTCTAGATGATcGCCGTGCAGG
 -*allele2* AGCAGCAACTAACGGAAG (SEQ ID NO:11)
mgat4B ZFN GCCCTCCAGCAGCCCTCTgaggaCTGGATGATCCTGGAGTT (SEQ ID
 -*WT* NO:12)
mgat4B -7bp GCCCTCCAGCAGCCCTCTg-----GATGATCCTGGAGTT (SEQ ID
 -*allele1* NO:13)
mgat5- ZFN GGGGGATGATGCTTCTGCACTTCACCATCCA~~g~~----
WT cagcgGACTCAGCCTGAGAGCAGCTCCATGTT (SEQ ID NO:17)
mgat5- +4bp GGGGGATGATGCTTCTGCACTTCACCATCCA~~g~~cagcCAGCgGACTCAGCCTGAGA
allele1 GCAGCTCCATGTT (SEQ ID NO:62)
mgat5- -4bp GGGGGATGATGCTTCTGCACTTCACCATCCA~~g~~-----
allele2 gGACTCAGCCTGAGAGCAGCTCCATGTT (SEQ ID NO:63)
mgat5- -16bp GGGGGATGATGCTTCTGCACTTC-----
allele3 CTCAGCCTGAGAGCAGCTCCATGTT (SEQ ID NO:64)

KO:B3gnt1
B3gnt1 ZFN TGCAGCTGCTCTACCTGTC-----gctgcTCTCCGGACTGCACG (SEQ
 -*WT* ID NO:25)
B3gnt1 +11bp TGCAGCTGCTCTACCTGTCgagcTGCTCTACCTGTCTCCGGACTGCACG (SEQ
 -*allele1* ID NO:26)
B3gnt1 -5bp TGCAGCTGCTCTACCTGTC-----TCTCCGGACTGCACG (SEQ
 -*allele2* ID NO:27)

KO:B3gnt2
B3gnt2 ZFN TTCAGCCCTTCCcgggcGTACTGGAACAGAGAGCA (SEQ ID NO:28)
 -*WT*
B3gnt2 -1bp TTCAGCCCTTCC-gggcGTACTGGAACAGAGAGCA (SEQ ID NO:29)
 -*allele1*
B3gnt2 -4bp TTCAGCCCTTCCcg----TACTGGAACAGAGAGCA (SEQ ID NO:30)
 -*allele2*

KO:B4galt1
B4galt ZFN TGCATCCGGTCCTACAGCgccagc----AACTGGACTATGGTA (SEQ ID
 1-*WT* NO:31)
B4galt +4bp TGCATCCGGTCCTACAGCgccagcCAGCAACTGGACTATGGTA (SEQ ID
 1-*allele1* NO:32)

KO:B4galt2

B4galt ZFN CAGCCCCGCCACTTTgcc-----atcGCCATGGACAAGTTTGGCT
 2-WT (SEQ ID NO:33)
B4galt
 2- +73bp CAGCCCCGCCACTTTgcc (SEQ ID NO:34) -- (+73bp) --
allele1 atcGCCATGGACAAGTTTGGCT (SEQ ID NO:35)

KO:B4galt3

B4galt ZFN CTAGCCCTCAAGTCAGGAtgt-----tgCGGAGGCTGCTGGAGAGG (SEQ ID
 3-WT NO:36)
B4galt
 3- +5bp CTAGCCCTCAAGTCAGGAtgtCGTGTtgCGGAGGCTGCTGGAGAGG (SEQ ID
allele1 NO:37)
B4galt
 3- +2bp CTAGCCCTCAAGTCAGGAtgt---tgCCCGGAGGCTGCTGGAGAGG (SEQ ID
allele2 NO:38)

KO:B4galt4

B4galt ZFN AACTGGGACTGCTTTat-----attcCACGATGTGGACCTGGTG (SEQ ID
 4-WT NO:39)
B4galt
 4- +1bp AACTGGGACTGCTTTat---TattcCACGATGTGGACCTGGTG (SEQ ID
allele1 NO:40)
B4galt
 4- +4bp AACTGGGACTGCTTTatattTATTcCACGATGTGGACCTGGTG (SEQ ID
allele2 NO:41)

KO:B4galt1/2

B4galt ZFN TGCATCCGGTCCTACAGCgccagc-----AACTGGACTATGGTA (SEQ ID
 1-WT NO:31)
B4galt
 1- +4bp TGCATCCGGTCCTACAGCgccagcCAGCAACTGGACTATGGTA (SEQ ID
allele1 NO:32)
B4galt ZFN CAGCCCCGCCACTTTgccatcGCCATGGACAAGTTTGGCT (SEQ ID NO:33)
 2-WT
B4galt
 2- -8bp CAGCCCCGCCAC-----cGCCATGGACAAGTTTGGCT (SEQ ID NO:65)
allele1

B4galt

2- -14bp CAGCCC-----cGCCATGGACAAGTTTGGCT (SEQ ID NO:66)
allele2

KO:st3Gal3

st3Gal ZFN CTCTCTCTTTGTCCTTGCTggcttCAAATGGCAGGACTTCAAG (SEQ ID
 3-WT NO:42)

st3Gal
 3- -5bp CTCTCTCTTTGTCCTTGCT-----CAAATGGCAGGACTTCAAG (SEQ ID
allele1 NO:43)

st3Gal
 3- -1bp CTCTCTCTTTGTCCTTGCTggct-CAAATGGCAGGACTTCAAG (SEQ ID
allele2 NO:44)

KO:st3Gal4

st3Gal ZFN GGCAGCCTCCAGTGTCGTCgttgt----gTTGTGGTGGGGAATGGGC (SEQ ID
 4-WT NO:45)

st3Gal
 4- +4bp GGCAGCCTCCAGTGTCGTCgttgtTTGTgTTGTGGTGGGGAATGGGC (SEQ ID
allele1 NO:46)

st3Gal
 4- -4bp GGCAGCCTCCAGTGTCGTCg-----gTTGTGGTGGGGAATGGGC (SEQ ID
allele2 NO:47)

KO:st3Gal6

st3Gal ZFN CGGTACCTCTGATTTTGCTttgccCTATGGGACAAGGCC (SEQ ID NO:48)
 6-WT

st3Gal
 6- -4bp CGGTACCTCTGATTTTGCTt----CTATGGGACAAGGCC (SEQ ID NO:49)
allele1

st3Gal
 6- -22bp CGGTACCTCTGA-----AGGCC (SEQ ID NO:50)
allele2

KO:st3Gal3/4

st3Gal ZFN CTCTCTCTTTGTCCTTGCTggcttCAAATGGCAGGACTTCAAG (SEQ ID
 3-WT NO:42)

st3Gal
 3- -5bp CTCTCTCTTTGTCCTTGct-----CAAATGGCAGGACTTCAAG (SEQ ID NO:43)
allele1

st3Gal
 3- -1bp CTCTCTCTTTGTCCTTGctggct-CAAATGGCAGGACTTCAAG (SEQ ID NO:44)
allele2

st3Gal
 4-WT ZFN GGCAGCCTCCAGTGTCGTCgttgt-----gTTGTGGTGGGGAATGGGC (SEQ ID NO:45)
st3Gal
 4- -4bp GGCAGCCTCCAGTGTCGTCg-----gTTGTGGTGGGGAATGGGC (SEQ ID NO:67)
allele1

st3Gal
 4- +5bp GGCAGCCTCCAGTGTCGTCgACACGttgtgTTGTGGTGGGGAATGGGC (SEQ ID NO:69)
allele2

KO:st3Gal4/6

st3Gal
 4-WT ZFN GGCAGCCTCCAGTGTCGTCgttgt----gTTGTGGTGGGGAATGGGC (SEQ ID NO:45)
st3Gal
 4- +4bp GGCAGCCTCCAGTGTCGTCgttgtTTGTgTTGTGGTGGGGAATGGGC (SEQ ID NO:46)
allele1

st3Gal
 4- -4bp GGCAGCCTCCAGTGTCGTCg-----gTTGTGGTGGGGAATGGGC (SEQ ID NO:47)
allele2

st3Gal
 6-WT ZFN CGGTACCTCTGATTTTGCT-ttgccCTATGGGACAAGGCC (SEQ ID NO:48)
st3Gal
 6- +1bp CGGTACCTCTGATTTTGCTTttgccCTATGGGACAAGGCC (SEQ ID NO:70)
allele1

st3Gal
 6- -7bp CGGTACCTCTGATTTTG-----CTATGGGACAAGGCC (SEQ ID NO:72)
allele2

KO:B3gnt2/mgat4A/4B/5

mgat4A
 -WT ZFN TTCTGAGTTGAATGCCATTGTCCAACAgtt-----
 tCGCCGTGCAGGAGCAGCAACTAACGGAAG (SEQ ID NO:9)
mgat4A
 -5bp TTCTGAGTTGAATGCCATTGTCCAACAg-----
 -*allele1* CCGTGCAGGAGCAGCAACTAACGGAAG (SEQ ID NO:10)

mgat4A +13bp TTCTGAGTTGAATGCCATTGTCCAACAgttGAATTCTAGATGATcGCCGTGCAGG
 -*allele2* AGCAGCAACTAACGGAAG (SEQ ID NO:11)
mgat4B ZFN GCCCTCCAGCAGCCCTCTgaggaCTGGATGATCCTGGAGTT (SEQ ID
 -*WT* NO:12)
mgat4B -7bp GCCCTCCAGCAGCCCTCTg-----GATGATCCTGGAGTT (SEQ ID
 -*allele1* NO:13)
mgat5- ZFN GGGGGATGATGCTTCTGCACTTCACCATCCAgt----
WT cagcgGACTCAGCCTGAGAGCAGCTCCATGTT (SEQ ID NO:17)
mgat5- +4bp GGGGGATGATGCTTCTGCACTTCACCATCCAgtcagcCAGCgGACTCAGCCTGAGA
allele1 GCAGCTCCATGTT (SEQ ID NO:62)
mgat5- -4bp GGGGGATGATGCTTCTGCACTTCACCATCCAgt-----
allele2 gGACTCAGCCTGAGAGCAGCTCCATGTT (SEQ ID NO:63)
mgat5- -16bp GGGGGATGATGCTTCTGCACTTC-----
allele3 CTCAGCCTGAGAGCAGCTCCATGTT (SEQ ID NO:64)
B3gnt2 ZFN TTCAGCCCTTCC--cgggcGTACTGGAACAGAGAGCA (SEQ ID NO:28)
 -*WT*
B3gnt2 +2bp TTCAGCCCTTCCCCcgggcGTACTGGAACAGAGAGCA (SEQ ID NO:73)
 -*allele1*
B3gnt2 +1bp TTCAGCCCTTCCC-cgggcGTACTGGAACAGAGAGCA (SEQ ID NO:74)
 -*allele2*

KO:st3Gal4/6/mgat4A/4B/5
mgat4A ZFN TTCTGAGTTGAATGCCATTGTCCAACAgtt-----
 -*WT* tcGCCGTGCAGGAGCAGCAACTAACGGAAG (SEQ ID NO:9)
mgat4A -5bp TTCTGAGTTGAATGCCATTGTCCAACAgt-----
 -*allele1* CCGTGCAGGAGCAGCAACTAACGGAAG (SEQ ID NO:10)
mgat4A +13bp TTCTGAGTTGAATGCCATTGTCCAACAgttGAATTCTAGATGATcGCCGTGCAGG
 -*allele2* AGCAGCAACTAACGGAAG (SEQ ID NO:11)
mgat4B ZFN GCCCTCCAGCAGCCCTCTgaggaCTGGATGATCCTGGAGTT (SEQ ID
 -*WT* NO:12)
mgat4B -7bp GCCCTCCAGCAGCCCTCTg-----GATGATCCTGGAGTT (SEQ ID
 -*allele1* NO:13)
mgat5- ZFN GGGGGATGATGCTTCTGCACTTCACCATCCAgt----
WT cagcgGACTCAGCCTGAGAGCAGCTCCATGTT (SEQ ID NO:17)
mgat5- +4bp GGGGGATGATGCTTCTGCACTTCACCATCCAgtcagcCAGCgGACTCAGCCTGAGA
allele1 GCAGCTCCATGTT (SEQ ID NO:62)
mgat5- -4bp GGGGGATGATGCTTCTGCACTTCACCATCCAgt-----
allele2 gGACTCAGCCTGAGAGCAGCTCCATGTT (SEQ ID NO:63)

mgat5- GGGGGATGATGCTTCTGCACTTC-----
alle3 -16bp CTCAGCCTGAGAGCAGCTCCATGTT (SEQ ID NO:64)
st3Gal ZFN GGCAGCCTCCAGTGTCGTCgttgt----gTTGTGGTGGGGAATGGGC (SEQ ID
4-WT NO:45)
st3Gal GGCAGCCTCCAGTGTCGTCgttgt-----gGTGGGGAATGGGC (SEQ ID
4- -5bp NO:68)
alle1
st3Gal GGCAGCCTCCAGTGTCGTCgTTGTttgtgTTGTGGTGGGGAATGGGC (SEQ ID
4- +4bp NO:46)
alle2
st3Gal ZFN CGGTACCTCTGATTTTGCTttgccCTATGGGACAAGGCC (SEQ ID NO:48)
6-WT
st3Gal CGGTACCTCTGATTTTGCTt----CTATGGGACAAGGCC (SEQ ID NO:49)
6- -4bp
alle1
st3Gal CGGTACCTCTGATTTTGCTttg--CTATGGGACAAGGCC (SEQ ID NO:71)
6- -2bp
alle2

KI:ST6GAL1/KO:st3gal4/6

st3Gal ZFN GGCAGCCTCCAGTGTCGTCgttgt----gTTGTGGTGGGGAATGGGC (SEQ ID
4-WT NO:45)
st3Gal GGCAGCCTCCAGTGTCGTCgttgtTTGTgTTGTGGTGGGGAATGGGC (SEQ ID
4- +4bp NO:46)
alle1
st3Gal GGCAGCCTCCAGTGTCGTCg-----gTTGTGGTGGGGAATGGGC (SEQ ID
4- -4bp NO:47)
alle2
st3Gal ZFN CGGTACCTCTGATTTTGCT-ttgccCTATGGGACAAGGCC (SEQ ID NO:48)
6-WT
st3Gal CGGTACCTCTGATTTTGCTTttgccCTATGGGACAAGGCC (SEQ ID NO:70)
6- +1bp
alle1
st3Gal CGGTACCTCTGATTTTG-----CTATGGGACAAGGCC (SEQ ID NO:72)
6- -7bp
alle2

KI:ST6GAL1/KO:st3gal4/6/mgat4A/4B/5

<i>mgat4A</i>		TTCTGAGTTGAATGCCATTGTCCAACAgtt-----
-WT	ZFN	tcGCCGTGCAGGAGCAGCAACTAACGGAAG (SEQ ID NO:9)
<i>mgat4A</i>		TTCTGAGTTGAATGCCATTGTCCAACAgtt-----
-allele1	-5bp	CCGTGCAGGAGCAGCAACTAACGGAAG (SEQ ID NO:10)
<i>mgat4A</i>		TTCTGAGTTGAATGCCATTGTCCAACAgttGAATTCTAGATGATcGCCGTGCAGG
-allele2	+13bp	AGCAGCAACTAACGGAAG (SEQ ID NO:11)
<i>mgat4B</i>		<u>GCCCTCCAGCAGCCCTCTgaggaCTGGATGATCCTGGAGTT</u> (SEQ ID
-WT	ZFN	<u>NO:12)</u>
<i>mgat4B</i>		GCCCTCCAGCAGCCCTCTg-----GATGATCCTGGAGTT (SEQ ID
-allele1	-7bp	NO:13)
<i>mgat5-</i>		GGGGGATGATGCTTCTGCACTTCACCATCCAg----
WT	ZFN	cagcg <u>GACTCAGCCTGAGAGCAGCTCCATGTT</u> (SEQ ID NO:17)
<i>mgat5-</i>		GGGGGATGATGCTTCTGCACTTCACCATCCAgcagcCAGCgGACTCAGCCTGAGA
allele1	+4bp	GCAGCTCCATGTT (SEQ ID NO:62)
<i>mgat5-</i>		GGGGGATGATGCTTCTGCACTTCACCATCCAg-----
allele2	-4bp	gGACTCAGCCTGAGAGCAGCTCCATGTT (SEQ ID NO:63)
<i>mgat5-</i>		GGGGGATGATGCTTCTGCACTTC-----
allele3	-16bp	CTCAGCCTGAGAGCAGCTCCATGTT (SEQ ID NO:64)
<i>st3Gal</i>		<u>GGCAGCCTCCAGTGTCGTCgttgttgt----gTTGTGGTGGGGAATGGGC</u> (SEQ ID
4-WT	ZFN	<u>NO:45)</u>
<i>st3Gal</i>		GGCAGCCTCCAGTGTCGTCgttgttgt-----gGTGGGGAATGGGC (SEQ ID
4-	-5bp	NO:68)
<i>st3Gal</i>		GGCAGCCTCCAGTGTCGTCgTTGTttgtgTTGTGGTGGGGAATGGGC (SEQ ID
4-	+4bp	NO:46)
<i>st3Gal</i>		
6-WT	ZFN	<u>CGGTACCTCTGATTTTGCTttgccCTATGGGACAAGGCC</u> (SEQ ID NO:48)
<i>st3Gal</i>		
6-	-4bp	CGGTACCTCTGATTTTGCTt----CTATGGGACAAGGCC (SEQ ID NO:49)
<i>st3Gal</i>		
6-	-2bp	CGGTACCTCTGATTTTGCTttg--CTATGGGACAAGGCC (SEQ ID NO:71)
<i>st3Gal</i>		
allele2		
<i>KO:mgat2</i>		
<i>mgat2-</i>		<u>TCCTTTGTCGCCCATTGCTgctccagaggacgaagCCGCAGGCGGCCACCACGA</u>
WT	TALEN	<u>(SEQ ID NO:3)</u>

mgat2- TCCTTTGTCGCCCCATTGCTgctcc----gacgaagccgcaggcgccaccacga
allele1 -4bp (SEQ ID NO:61)

KO:mgat2/stgal4/6

mgat2- TCCTTTGTCGCCCCATTGCTgctccagaggacgaagCCGCAGGCGGCCACCACGA
WT TALEN (SEQ ID NO:3)

mgat2- TCCTTTGTCGCCCCATTGCTgctccag----cgaagCCGCAGGCGGCCACCACGA
allele1 -4bp (SEQ ID NO:78)

st3Gal GGCAGCCTCCAGTGTCTGTCgtttgt----gTTGTGGTGGGGAATGGGC (SEQ ID
4-WT ZFN NO:45)

st3Gal GGCAGCCTCCAGTGTCTGTCgtttgtTTGTgTTGTGGTGGGGAATGGGC (SEQ ID
4- +4bp NO:46)

st3Gal GGCAGCCTCCAGTGTCTGTCg-----gTTGTGGTGGGGAATGGGC (SEQ ID
4- -4bp NO:47)

st3Gal CGGTACCTCTGATTTTGCT-ttgccCTATGGGACAAGGCC (SEQ ID NO:48)
6-WT ZFN

st3Gal CGGTACCTCTGATTTTGCTtttgccCTATGGGACAAGGCC (SEQ ID NO:70)
6- +1bp
allele1

st3Gal CGGTACCTCTGATTTTG-----CTATGGGACAAGGCC (SEQ ID NO:72)
6- -7bp
allele2

KO:mgat2/mgat4A/4B/5

mgat2- TCCTTTGTCGCCCCATTGCTgctccagaggacgaagCCGCAGGCGGCCACCACGA
WT TALEN (SEQ ID NO:3)

mgat2- TCCTTTGTCGCCCCATTGCTgctccag----cgaagCCGCAGGCGGCCACCACGA
allele1 -4bp (SEQ ID NO:78)

mgat4A TTCTGAGTTGAATGCCATTGTCCAACAgtt-----
-WT ZFN tcGCCGTGCAGGAGCAGCAACTAACGGAAG (SEQ ID NO:9)

mgat4A TTCTGAGTTGAATGCCATTGTCCAACAg-----
-allele1 -5bp CCGTGCAGGAGCAGCAACTAACGGAAG (SEQ ID NO:10)

mgat4A TTCTGAGTTGAATGCCATTGTCCAACAgttGAATTCTAGATGatcGCCGTGCAGG
-allele2 +13bp AGCAGCAACTAACGGAAG (SEQ ID NO:11)

mgat4B GCCCTCCAGCAGCCCTCTgaggaCTGGATGATCCTGGAGTT (SEQ ID
-WT ZFN NO:12)

mgat4B -7bp GCCCTCCAGCAGCCCTCTg-----GATGATCCTGGAGTT (SEQ ID NO:13)
-allele1
mgat5- ZFN GGGGGATGATGCTTCTGCACTTCACCATCCA~~g~~----
WT cagcgGACTCAGCCTGAGAGCAGCTCCATGTT (SEQ ID NO:17)
mgat5- +4bp GGGGGATGATGCTTCTGCACTTCACCATCCA~~g~~cagcCAGCgGACTCAGCCTGAGA
allele1 GCAGCTCCATGTT (SEQ ID NO:62)
mgat5- -4bp GGGGGATGATGCTTCTGCACTTCACCATCCA~~g~~-----
allele2 gGACTCAGCCTGAGAGCAGCTCCATGTT (SEQ ID NO:63)
mgat5- -16bp GGGGGATGATGCTTCTGCACTTC-----
allele3 CTCAGCCTGAGAGCAGCTCCATGTT (SEQ ID NO:64)

KO:mgat2/st3gal4/6/mgat4A/4B/5
mgat2- TALEN TCCTTTGTCGCCCATTGCTgctccagaggacgaagCCGCAGGCGGCCACCACGA
WT (SEQ ID NO:3)
mgat2- -5bp TCCTTTGTCGCCCATTGCTgctcca-----cgaaGCCGCAGGCGGCCACCACGA
allele1 (SEQ ID NO:4)
mgat4A ZFN TTCTGAGTTGAATGCCATTGTCCAACA~~g~~tt-----
-WT tcGCCGTGCAGGAGCAGCAACTAACGGAAG (SEQ ID NO:9)
mgat4A -5bp TTCTGAGTTGAATGCCATTGTCCAACA~~g~~-----
-allele1 CCGTGCAGGAGCAGCAACTAACGGAAG (SEQ ID NO:10)
mgat4A +13bp TTCTGAGTTGAATGCCATTGTCCAACA~~g~~ttGAATTCTAGATGAtcGCCGTGCAGG
-allele2 AGCAGCAACTAACGGAAG (SEQ ID NO:11)
mgat4B ZFN GCCCTCCAGCAGCCCTCTgaggaCTGGATGATCCTGGAGTT (SEQ ID NO:12)
-WT
mgat4B -7bp GCCCTCCAGCAGCCCTCTg-----GATGATCCTGGAGTT (SEQ ID NO:13)
-allele1
mgat5- ZFN GGGGGATGATGCTTCTGCACTTCACCATCCA~~g~~----
WT cagcgGACTCAGCCTGAGAGCAGCTCCATGTT (SEQ ID NO:17)
mgat5- +4bp GGGGGATGATGCTTCTGCACTTCACCATCCA~~g~~cagcCAGCgGACTCAGCCTGAGA
allele1 GCAGCTCCATGTT (SEQ ID NO:62)
mgat5- -4bp GGGGGATGATGCTTCTGCACTTCACCATCCA~~g~~-----
allele2 gGACTCAGCCTGAGAGCAGCTCCATGTT (SEQ ID NO:63)
mgat5- -16bp GGGGGATGATGCTTCTGCACTTC-----
allele3 CTCAGCCTGAGAGCAGCTCCATGTT (SEQ ID NO:64)
st3Gal ZFN GGCAGCCTCCAGTGTCGTCgtttgt----gTTGTGGTGGGGAATGGGC (SEQ ID NO:45)
4-WT
st3Gal -5bp GGCAGCCTCCAGTGTCGTCgtttgt-----gGTGGGGAATGGGC (SEQ ID NO:68)
4-
allele1

st3Gal
 4- +4bp GGCAGCCTCCAGTGTCTGTCgTTGTttgtgTTGTGGTGGGGAATGGGC (SEQ ID NO:46)
allele2
st3Gal
 6-WT ZFN CGGTACCTCTGATTTTGCTttgccCTATGGGACAAGGCC (SEQ ID NO:48)
st3Gal
 6- -4bp CGGTACCTCTGATTTTGCTt----CTATGGGACAAGGCC (SEQ ID NO:49)
allele1
st3Gal
 6- -2bp CGGTACCTCTGATTTTGCTttg--CTATGGGACAAGGCC (SEQ ID NO:71)
allele2

KO:fut8
fut8- CRISP CAAATACTTGATCCGTCCACAACCT-TGGCTGGAAAGGGAA (SEQ ID NO:51)
 WT R
fut8- CAAATACTTGATCCGTCCACAACC--TGGCTGGAAAGGGAA (SEQ ID NO:52)
allele1 -1bp
fut8- CAAATACTTGATCCGTCCACAACCTTTGGCTGGAAAGGGAA (SEQ ID NO:53)
allele2 +1bp

KO:fut8/B4galt1
fut8- CRISP CAAATACTTGATCCGTCCACAACCT-TGGCTGGAAAGGGAA (SEQ ID NO:51)
 WT R
fut8- CAAATACTTGATCCGTCCACAA-----GGCTGGAAAGGGAA (SEQ ID NO:77)
allele1 -4bp
fut8- CAAATACTTGATCCGTCCACAACCTTTGGCTGGAAAGGGAA (SEQ ID NO:53)
allele2 +1bp
B4galt ZFN TGCATCCGGTCCTACAGCgccagc-----AACTGGACTATGGTA (SEQ ID NO:31)
 1-WT
B4galt TGCATCCGGTCCTACAGCgccagcCAGCAACTGGACTATGGTA (SEQ ID NO:32)
 1- +4bp
allele1

KO:mgat3
mgat3- ZFN TTCTTGGAACCACTTCCCAaccggt-----GGCCGGCAGGATGGC (SEQ ID NO:5)
 WT
mgat3- TTCTTGGAACCACT-----ATGGC (SEQ ID NO:6)
allele1 -21bp

mgat3- +293b TTCCTGGACCACTGATACcc (SEQ ID NO:7) --+293bp--
allele2 p cggtGGCCGGCAGGATGGC (SEQ ID NO:8)

KO:mgat4C

mgat4C
 -WT ZFN ATACTTCAGACTATtatgtAATGCTCGAAGATGATGTT (SEQ ID NO:14)

mgat4C
 -*allele1* -13bp ATACTTCAGACT-----CGAAGATGATGTT (SEQ ID NO:15)

mgat4C
 -*allele2* -8bp ATACTTCAGACTAT-----GCTCGAAGATGATGTT (SEQ ID NO:16)

KO:mgat5B

mgat5B
 -WT ZFN CGTGGCGCCCTCCGCAAGatgagtGACCTGCTGGAGCTG (SEQ ID NO:22)

mgat5B
 -*allele1* -7bp CAGCTCCAGCAGGT-----CTTGCGGAGGGCGCCACG (SEQ ID NO:23)

mgat5B
 -*allele2* -5bp CAGCTCCAGCAGGT-----ATCTTGCGGAGGGCGCCACG (SEQ ID NO:24)

KO:B3gnt8

B3gnt8
 -WT TALEN TGGTCCAGAGATAGCTAATgaagcttctaggggtgGAGAAGCTGGGGCTGCTGA
 (SEQ ID NO:75)

B3gnt8
 -*allele1* -17bp TGGTCCAGAGG-----AGGGTGGAGAAGCTGGGGCTGCTGA
 (SEQ ID NO:76)

KO:mgat1

mgat1-
 WT ZFN AACAAGTTCAAGTTCccagcaGCTGTGGTAGTGGAGGAC (SEQ ID NO:1)

mgat1-
allele1 -2bp AACAAGTTCAAGTTCc--gcaGCTGTGGTAGTGGAGGAC (SEQ ID NO:2)

Gene targeting region underlined.

Table 5

tgcgcgcttttcggtgatgacggtgaaaacctctgacacatgcagctcccggagacggtcacagcttgtctgta
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5 gaaaataccgcatcaggcgccattcgccattcaggctgcgcaactgttggaagggcgatcgggtgcgggcctc
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cacacgcgtccatgaaagggcggttgcatgctgtccggagtctcagcgttataaccagaagtgcctgggtcg
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5 tatcaagtgagaaatcacctatgagtgcgactgaatccggtgagaatggcaaaagtttatgcatttctttcca
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10 agaggcataaattccgtcagccagtttagtctgacctctcatctgtaacatcattggcaacgctacctttgc
catgtttcagaaacaactctggcgcatcgggcttccatacaatcgatagattgtcgcacctgattgcccgcac
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gtttcccggttgaaatgtggctcatactcttctttttcaatattattgaagcatttatcagggttattgtctca
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15 gccacctgacgtctaagaaaccattattatcatgacattaacctataaaaaataggcggtatcacgaggcccttt
cgtc (SEQ ID NO:60)

Claims

1. A cell comprising one or more glycosyltransferase genes that have been inactivated, and that has new and/or more homogeneous stable glycosylation capacities, wherein mgat2 has been knocked out (KO), and with or without KO of
5 mgat4A and/or mgat4B and/or mgat5 allowing production of N-glycans with monoantennary structure.
2. A cell comprising one or more glycosyltransferase genes that have been inactivated, and that has new and/or more homogeneous stable glycosylation
10 capacities, wherein mgat2 has been knocked out (KO), and with or without KO of mgat4A and/or mgat4B and/or mgat5 in combination with KO of sialyltransferases allowing production of N-glycans with monoantennary structure and without sialic acid capping.
- 15 3. A cell comprising one or more glycosyltransferase genes that have been inactivated, and that has new and/or more homogeneous stable glycosylation capacities, wherein mgat2 has been knocked out (KO), and with or without KO of mgat4A and/or mgat4B and/or mgat5 in combination with KO of sialyltransferases and B3gnt2 allowing production N-glycans with monoantennary structure and
20 without sialic acid capping and without poly-LacNAc.
4. The cell according to any one of claims 1-3, wherein the cell is a mammalian cell or an insect cell.
- 25 5. The cell according to any one of claims 1-4, wherein the cell is derived from Chinese hamster ovary or from human kidney.
6. The cell according to any one of claims 1-5, wherein the cell is selected from the group consisting of CHO, NS0, SP2/0, YB2/0, CHO-K1, CHO-DXB11, CHO-
30 DG44, CHO-S, HEK293, HUVEC, HKB, PER-C6, NS0, or derivatives of any of these cells.
7. The cell according to any one of claims 1-6, wherein the cell is a CHO cell.

8. The cell according to any one of claims 1-7, furthermore encoding an exogenous protein of interest.
9. A method for the production of di- and triPEGylated EPO glycoproteins, the
5 method comprising the step of;
- enzymatic glycoPEGylation of glycoengineered EPO variants.
10. A method for the production of recombinant glycoproteins, comprising the step of:
10 - generating a cell with specific glycosylation properties, wherein specific glycosylation property is the capacity for monoantennary N-glycan synthesis.
11. The method according to any one of claims 9-10, wherein the cell is selected
15 from any one according to claims 1-8.
12. A glycoprotein obtainable from the methods of any one of claims 9-11.
13. A glycoprotein according to claim 12 having glycostructure outcome with one
20 or more of following changes selected from the group consisting of simpler glycan structure, more homogeneous product, more sialic acids per molecule, non-sialylated, non-galactosylated, more homogeneous bi-antennary, more homogeneous monoantennary, more homogeneous triantennary, more homogeneous without poly-LacNAc, higher productivity in cell culture, new stable
25 homogeneous glycosylation capacities, more human glycostructure, more homogeneous glycosylation and improved ADCC targeting of IgG, modified fucose level, no fucose, improved substrate for generating glycoconjugates.
14. A glycoprotein according to any one of claims 12-13, which is a homogeneous
30 glycoconjugate produced from a glycoprotein having a simplified glycan profile.
15. A glycoprotein according to any one of claims 12-14, which is a homogeneous glycoprotein conjugate comprising a polymer selected from, PEG, HEP, XTEN, PSA, HES.

15. A glycoprotein according to any one of claims 12-15, which is a homogeneous PEGylated protein conjugate produced from a protein variant according to the invention having a simplified glycan profile.
- 5 17. A glycoprotein according to any one of claims 12-16, which is a homogeneous PEGylated EPO conjugate produced from an EPO variant having a simplified glycan profile.
18. A glycoprotein according to any one of claims 12-17, which is a simplified enzymatic glycoPEGylation process using glycoengineered EPO variants that
10 provides high yield of di- and triPEGylated EPO forms.
19. A glycoprotein according to any one of claims 12-18, which is a protein conjugate according to the invention comprising FII, FV, FVIIa, FVIII, FIX, FX,
15 FXIII, hGH, a Fab fragment of an antibody, or a Fc domain of an antibody.
20. A glycoprotein according to any one of claims 12-19, which is a protein-protein conjugate produced from a glycoprotein with a simplified glycan profile.
- 20 21. A glycoprotein according to any one of claims 12-20, which is a protein-protein conjugate produced from a glycoprotein with a simplified glycan profile comprising a Fab fragment and one of either hGH, FII, FV, FVIIa, FVIII, FIX, FX or FXIII.
- 25 22. A glycoprotein according to any one of claims 12-21, which is a protein-protein conjugate produced from a glycoprotein with a simplified glycan profile comprising a Fc Domain and one of either hGH, FII, FV, FVIIa, FVIII, FIX, FX or FXIII.
- 30 23. A glycoprotein according to any one of claims 12-22, which is a homogeneous PEGylated EPO conjugate produced from an EPO variant having a simplified glycan profile.
24. Use of recombinant glycoproteins comprising monoantennary N-glycans for
35 enzymatic modification of polypeptides.

A. O-glycan

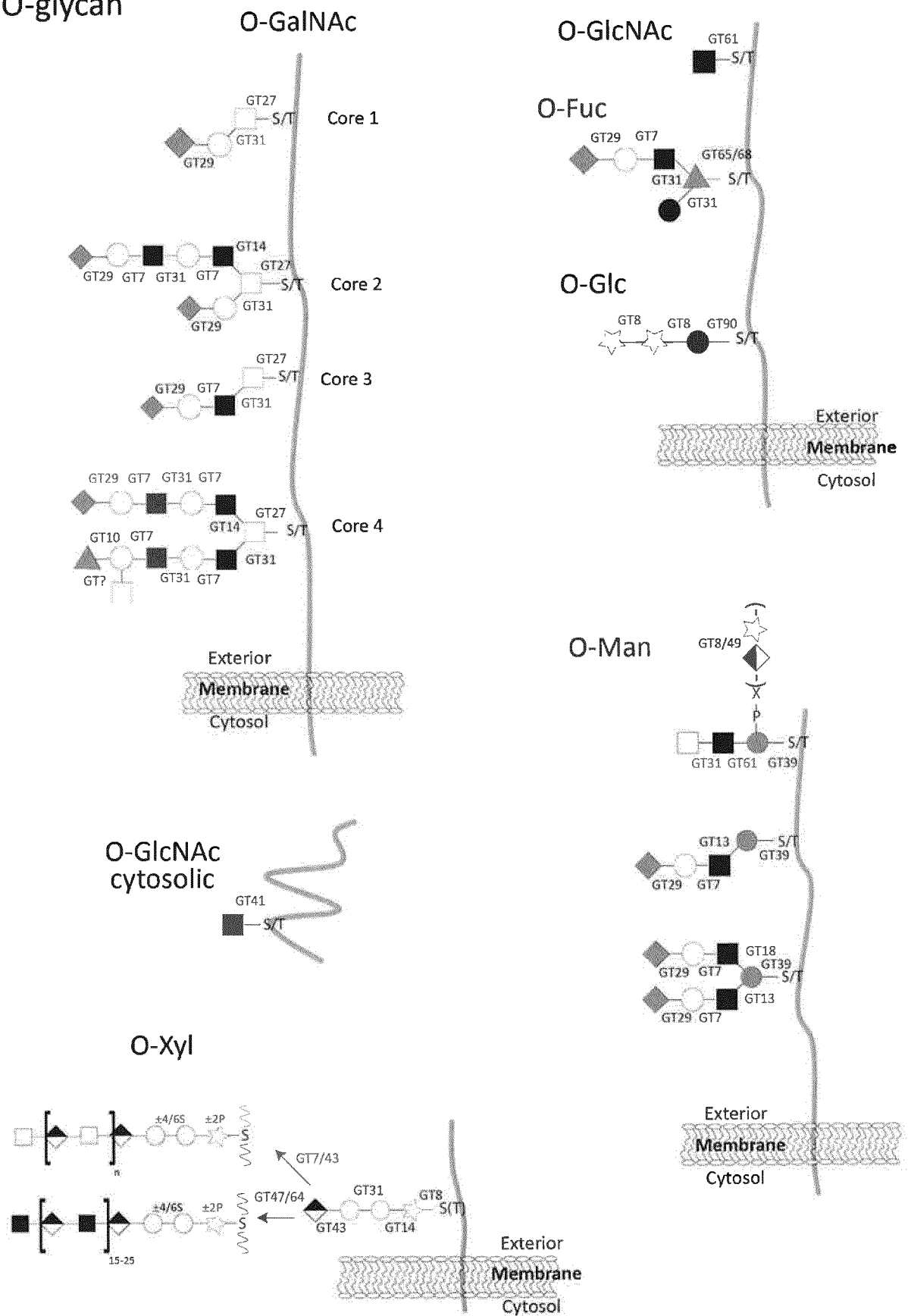
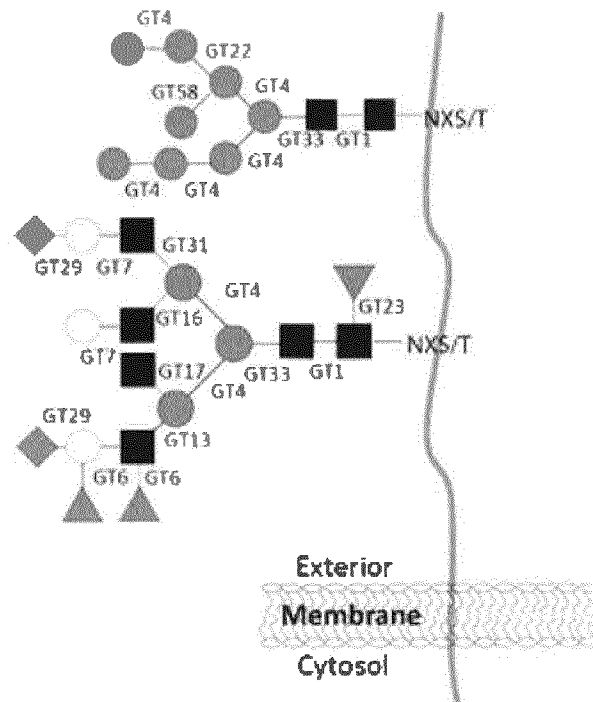
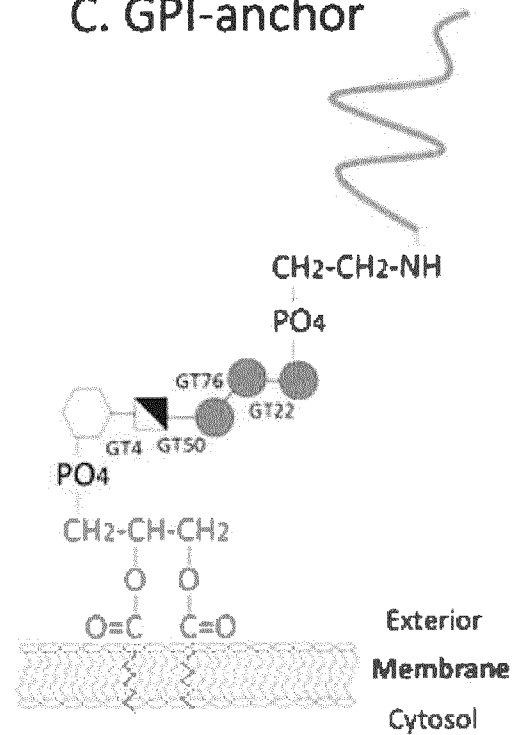


Fig. 1A

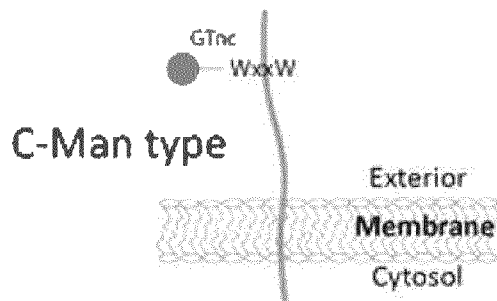
B. N-glycan



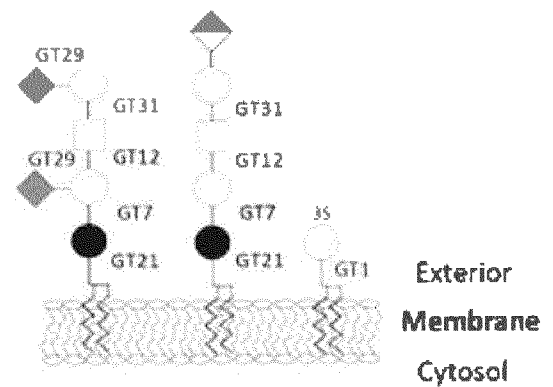
C. GPI-anchor



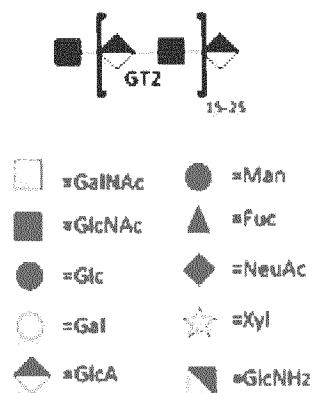
D. C-glycan



E. Glycosphingolipids



F. Hyaluronan



G. HO-Lysine glycan/O-Gal

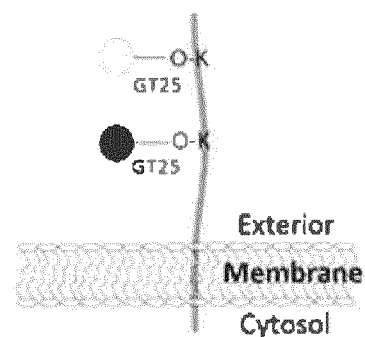
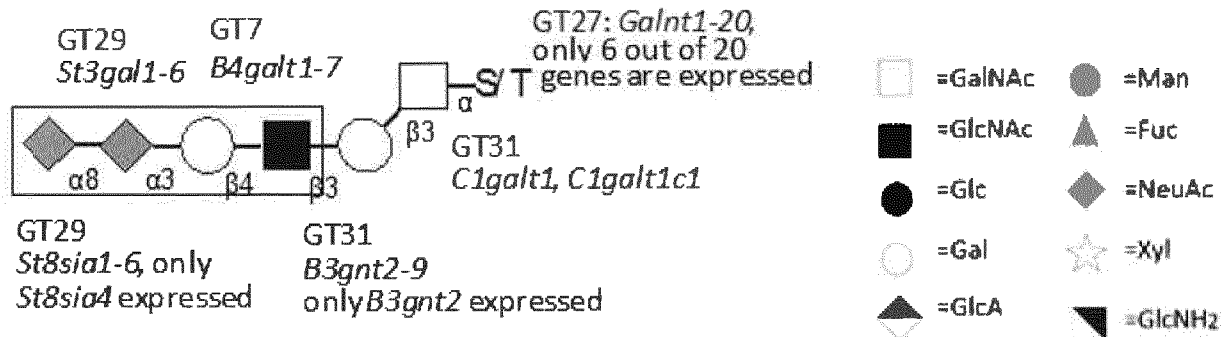


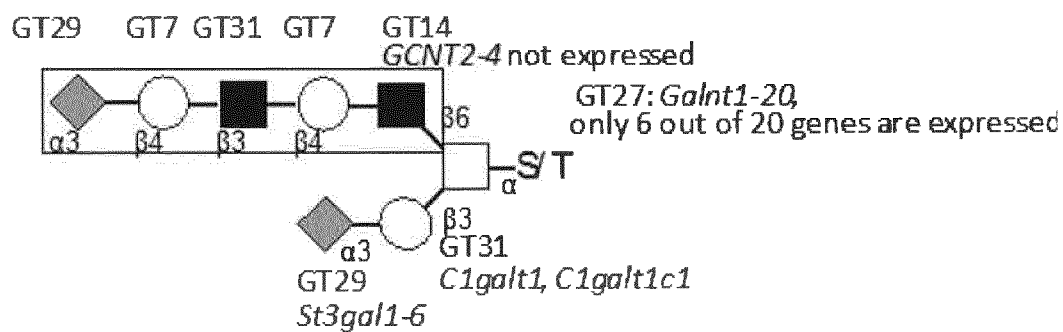
Fig. 1B-G

O-GalNAc

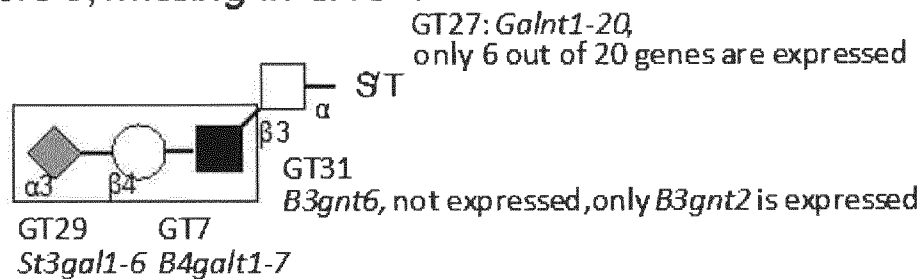
Core 1, only short structures are synthesized



Core 2, missing in CHO-GS/K1



Core 3, missing in CHO-GS/K1



Core 4, missing in CHO-GS/K1

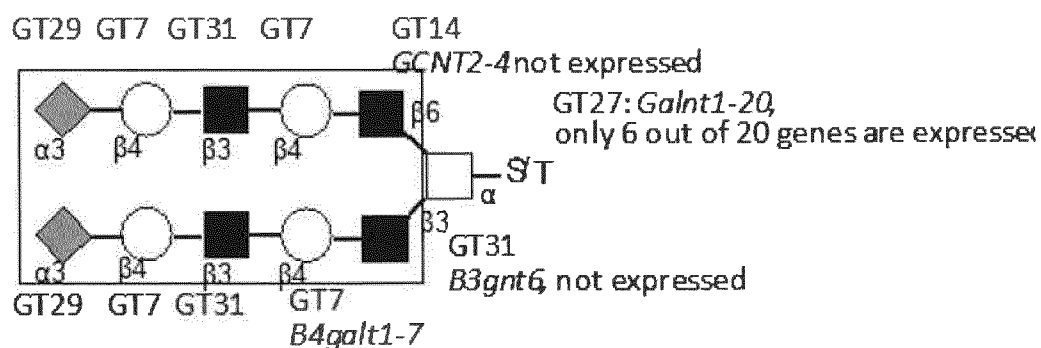


Fig. 2A1

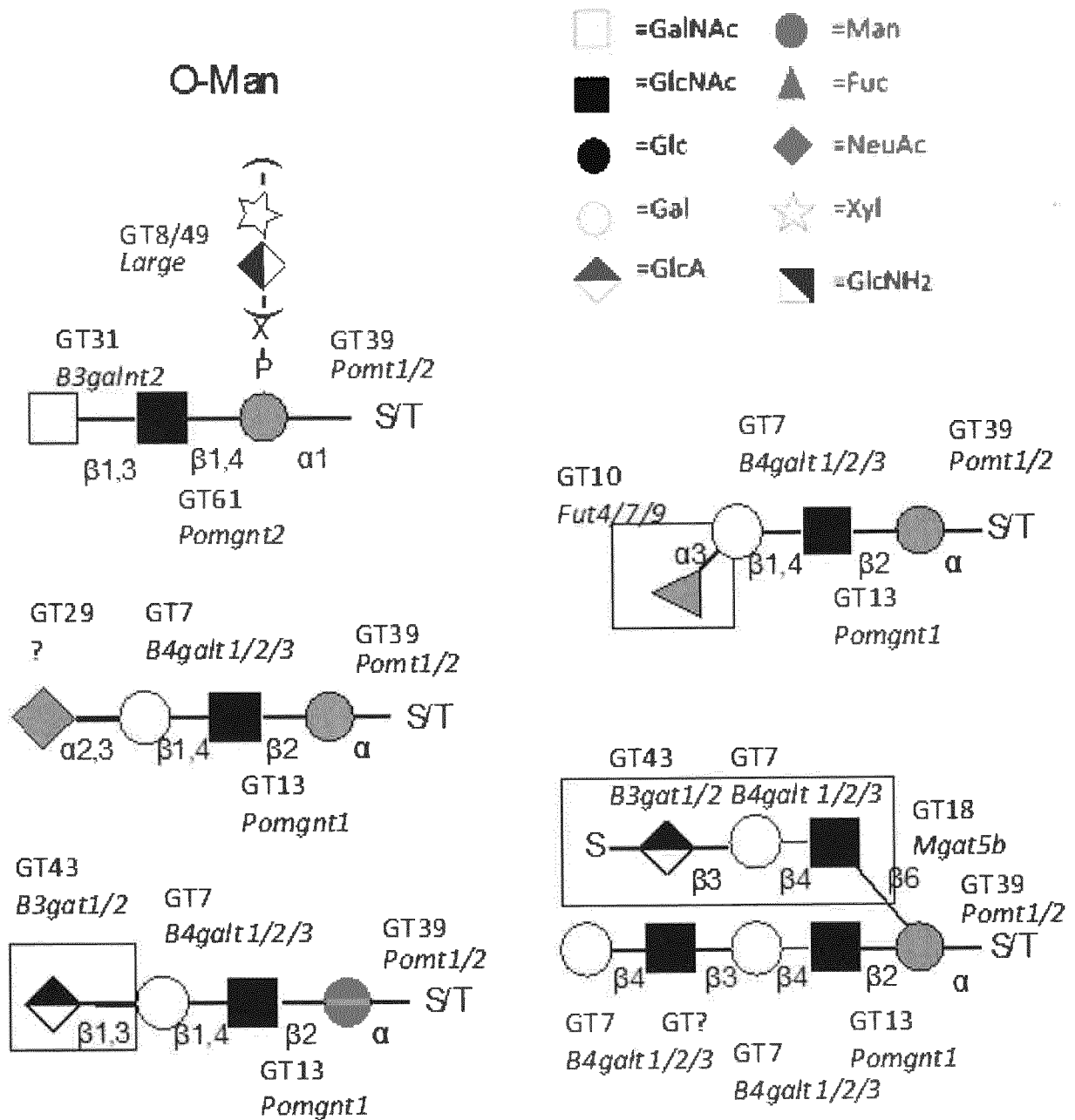


Fig. 2A2

5/35

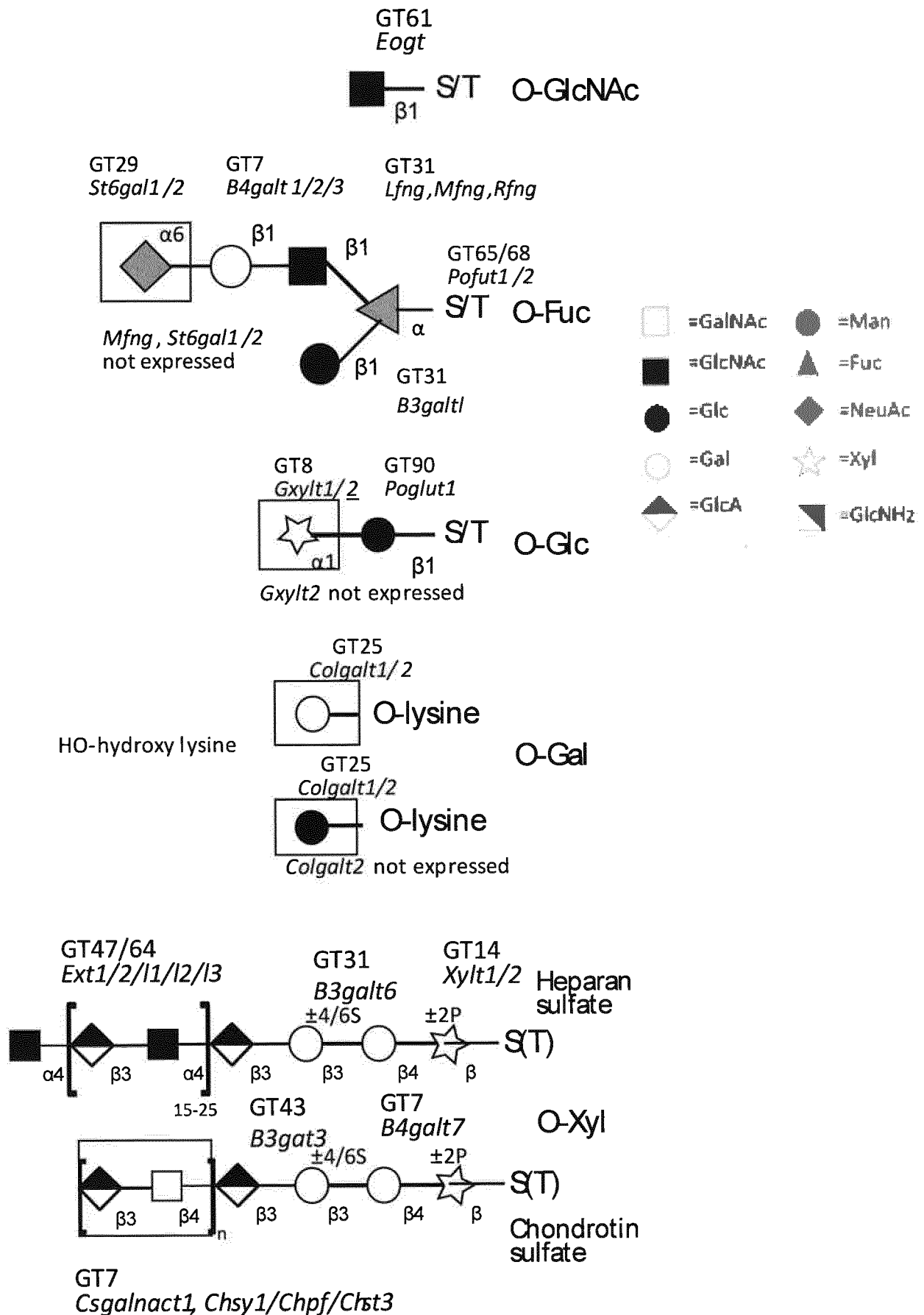
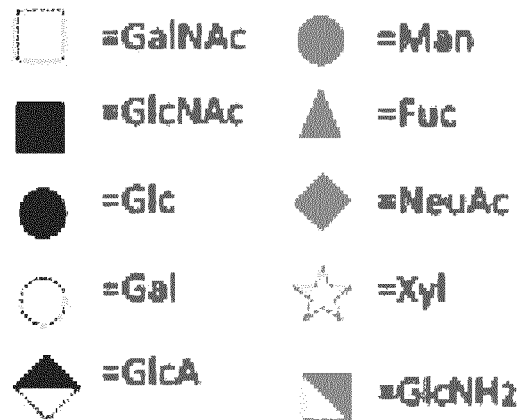
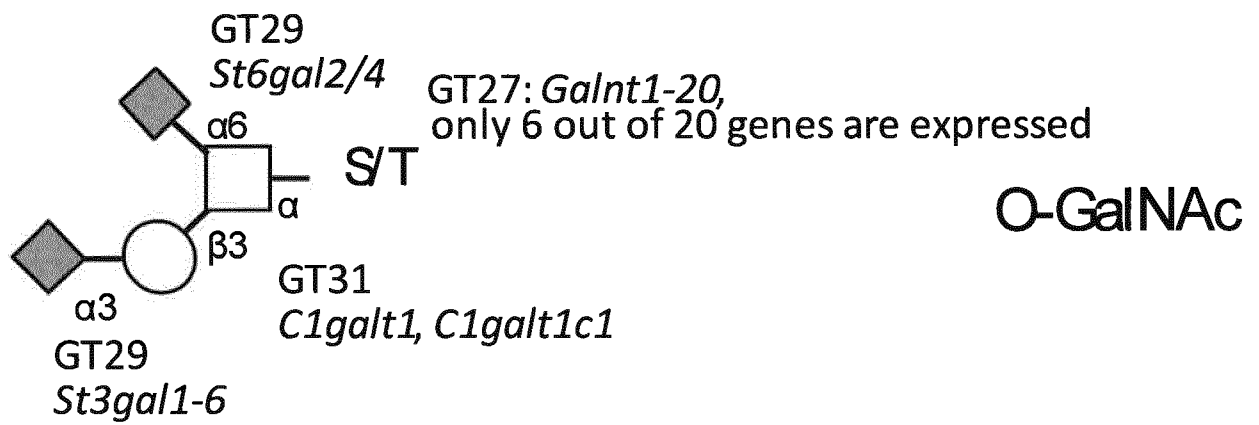


Fig. 2A3

CHO-GS/K1 O-GalNAc structures



B N-glycan

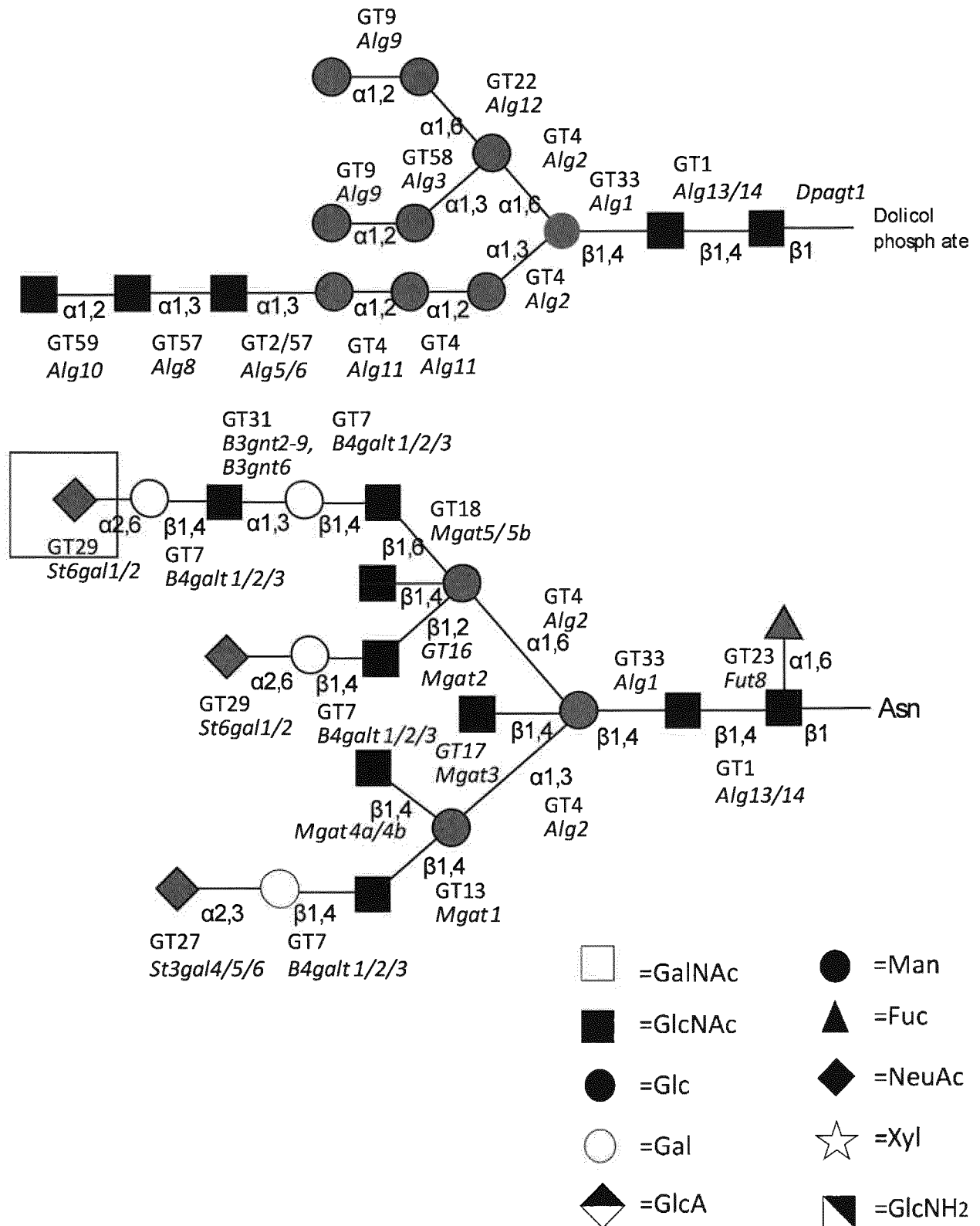


Fig. 2B

C Sphingolipids

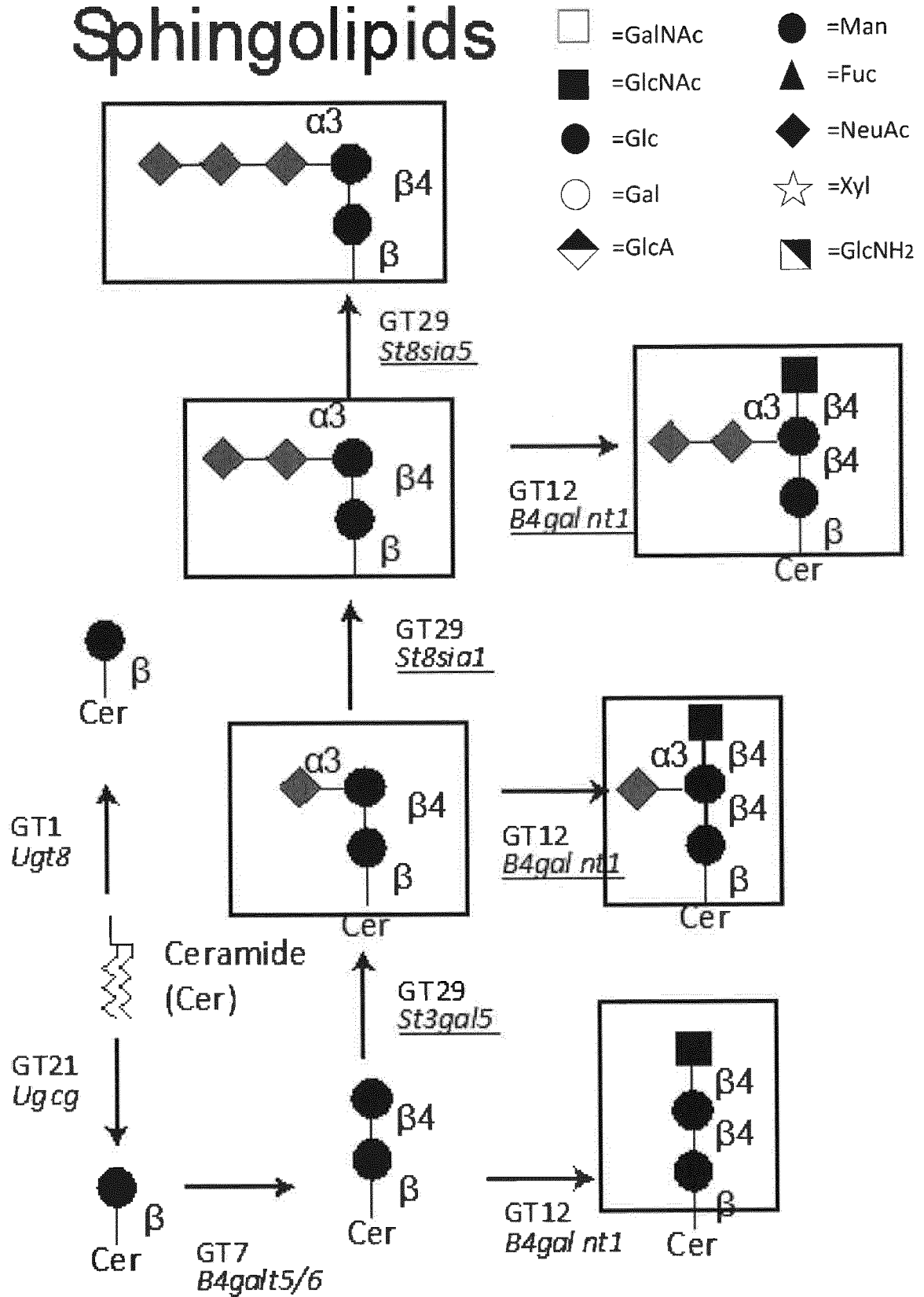
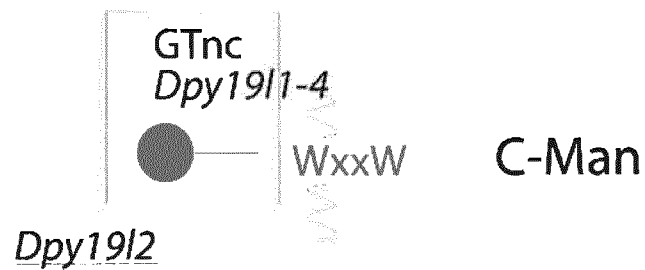


Fig. 2C

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D C-glycan



E GPI-anchor

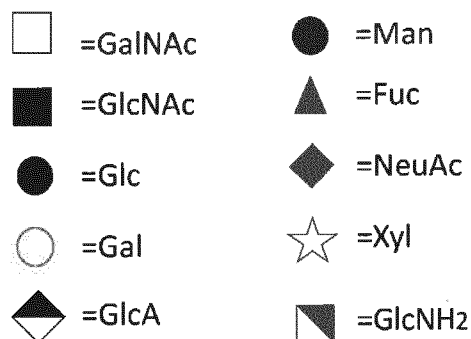
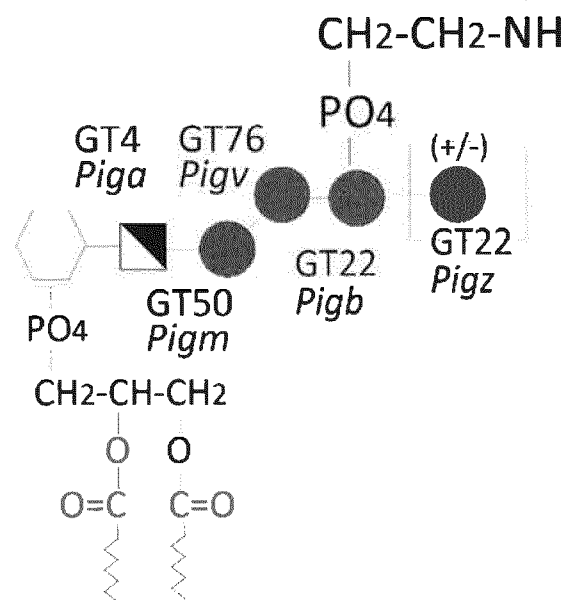


Fig. 2D-E

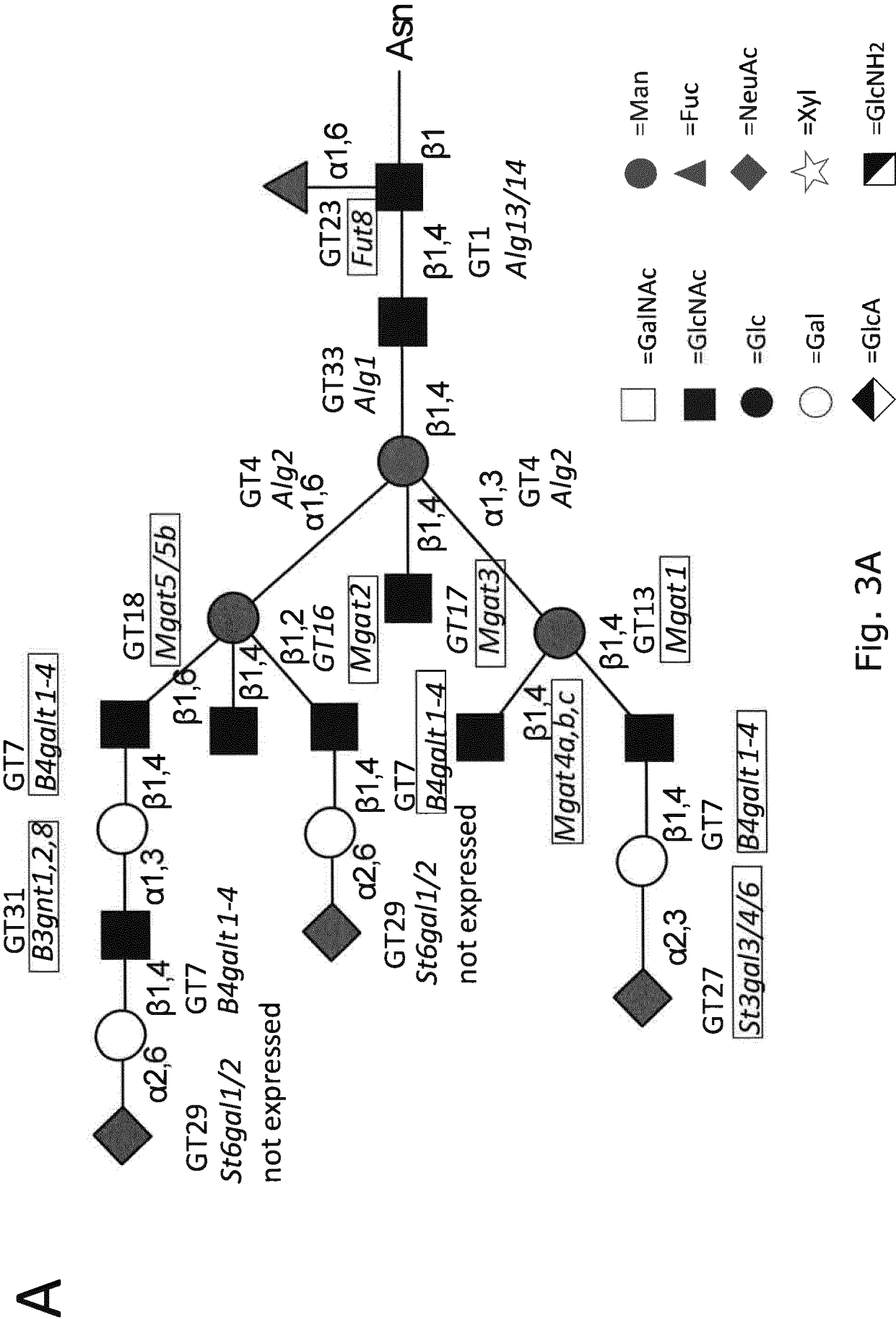


Fig. 3A

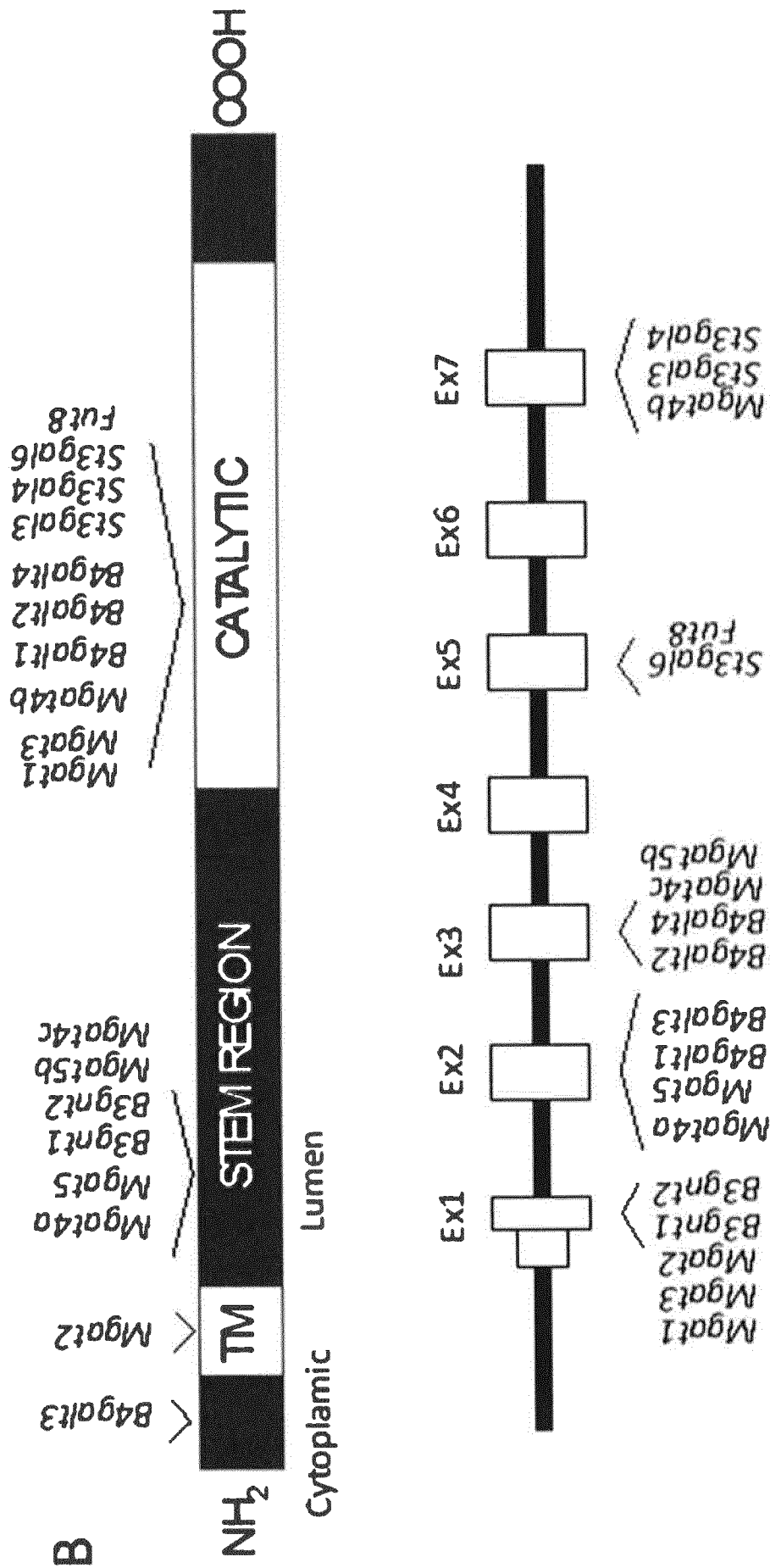


Fig. 3B

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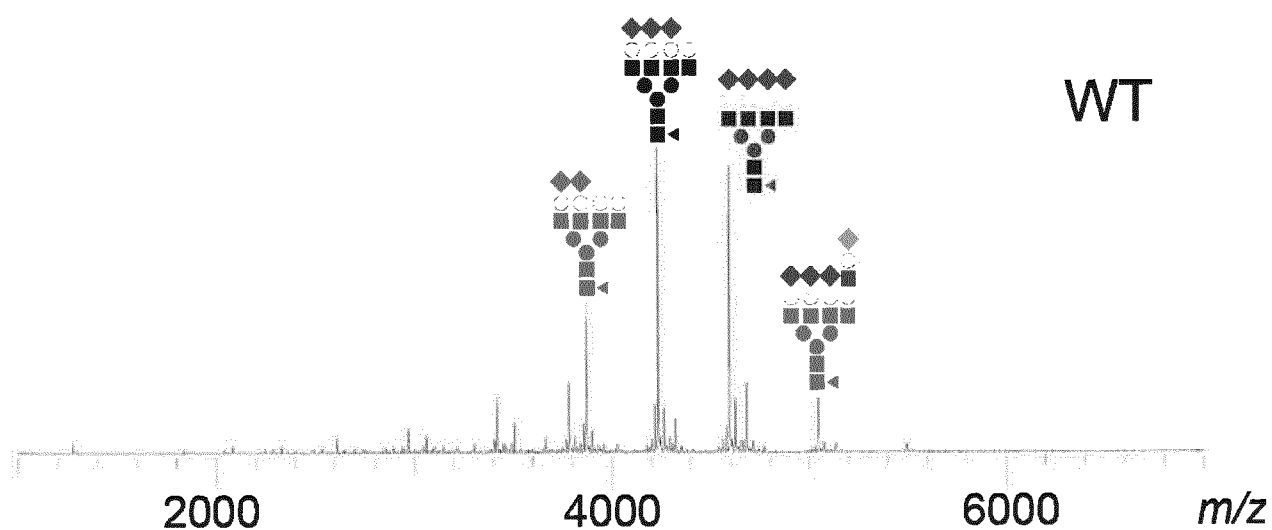


Fig. 4

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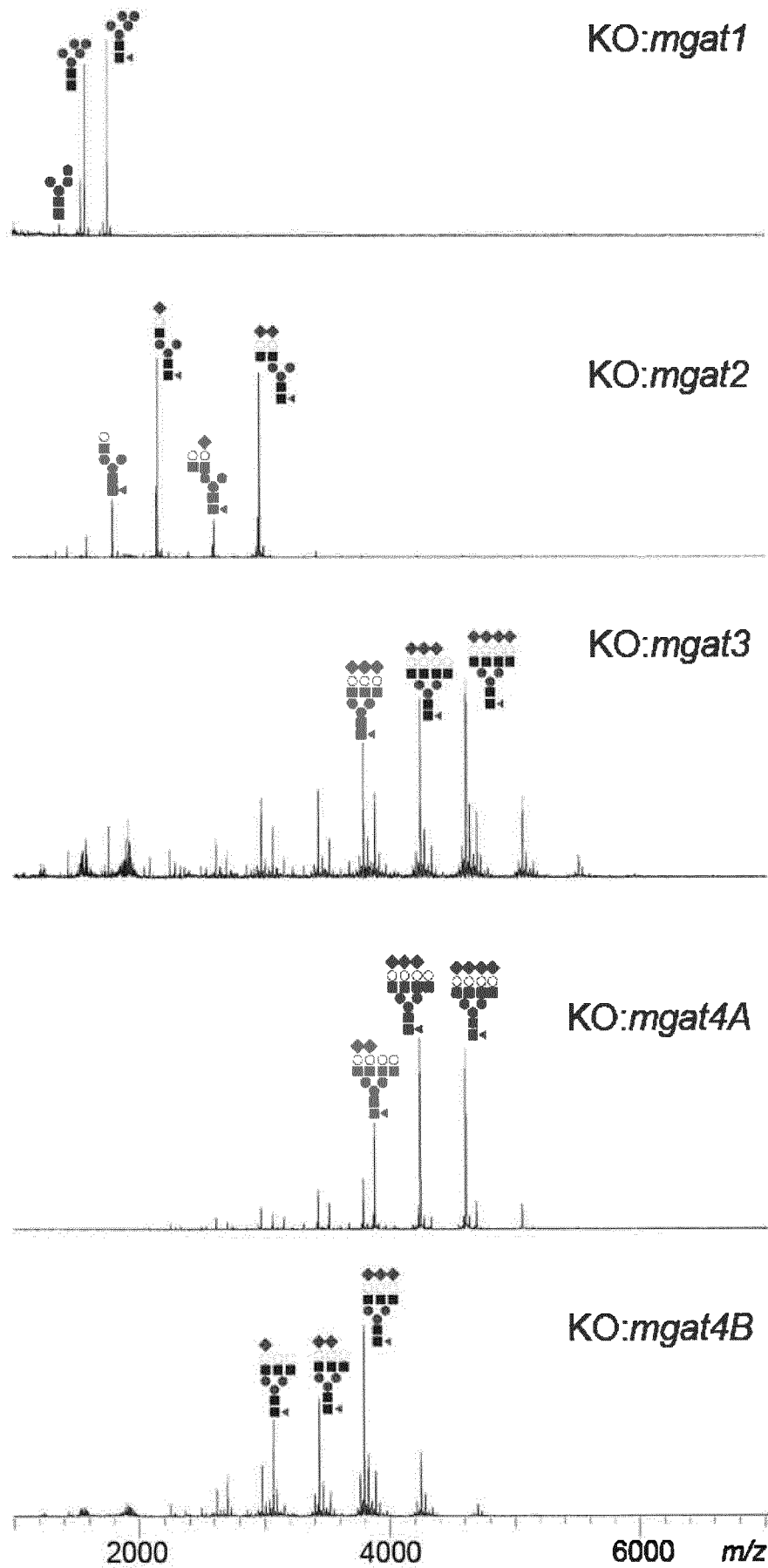


Fig. 5A

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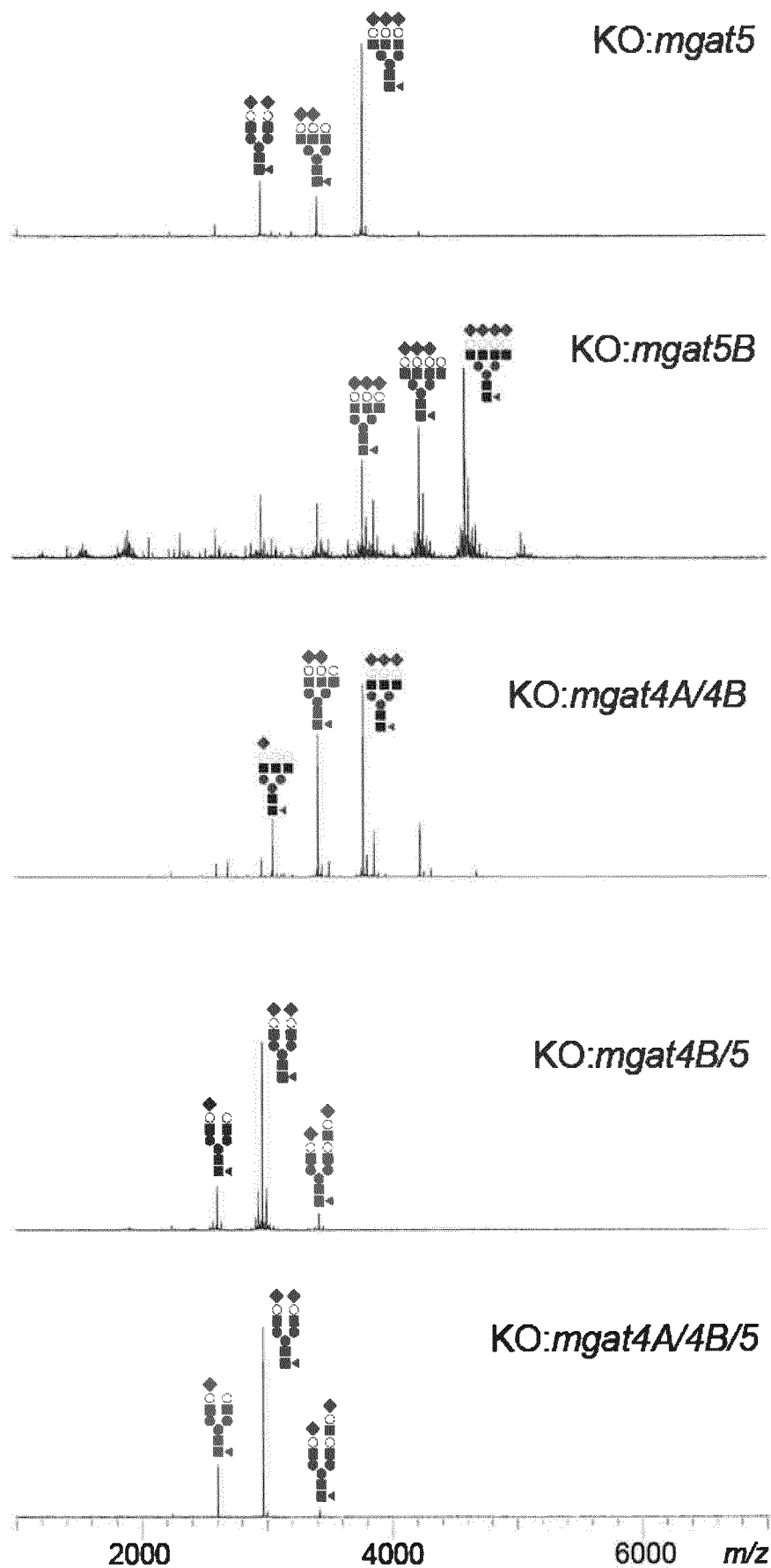


Fig. 5B

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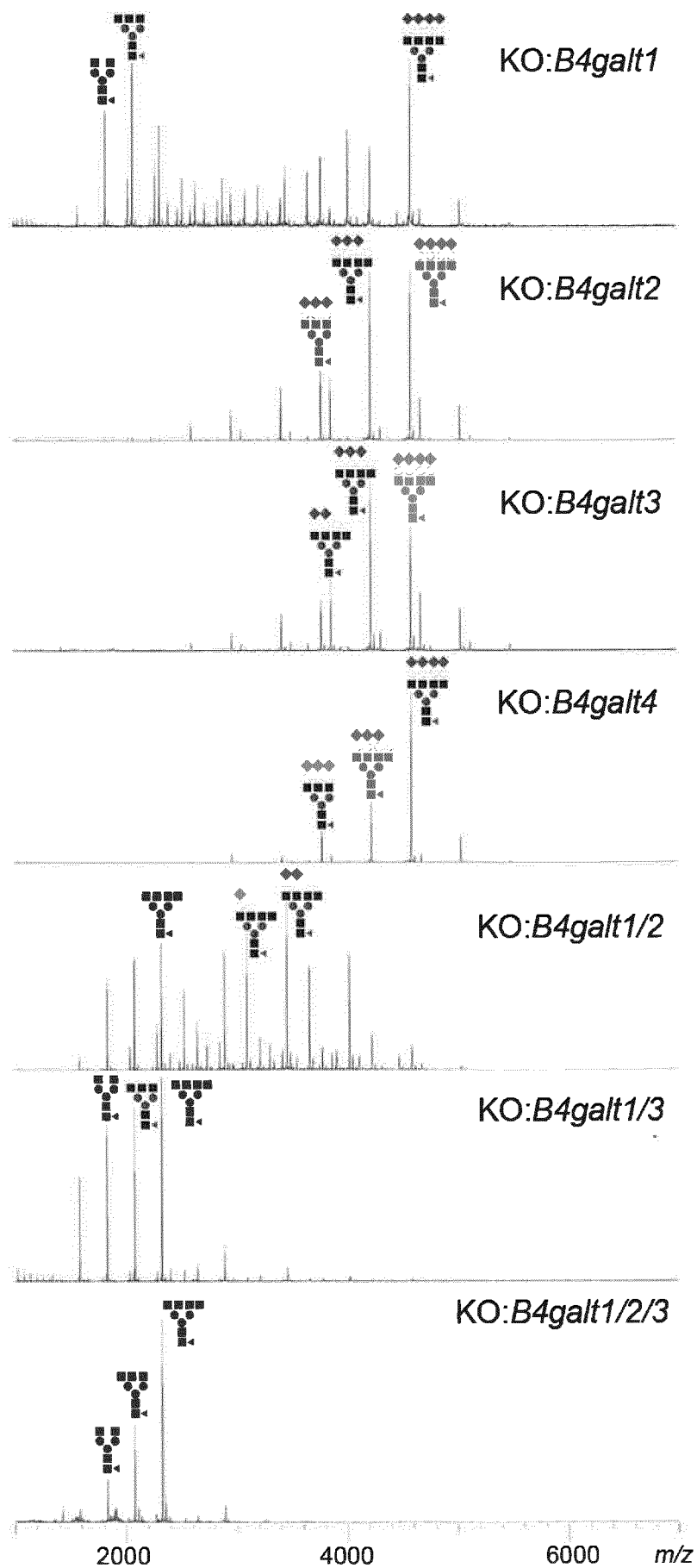


Fig. 6

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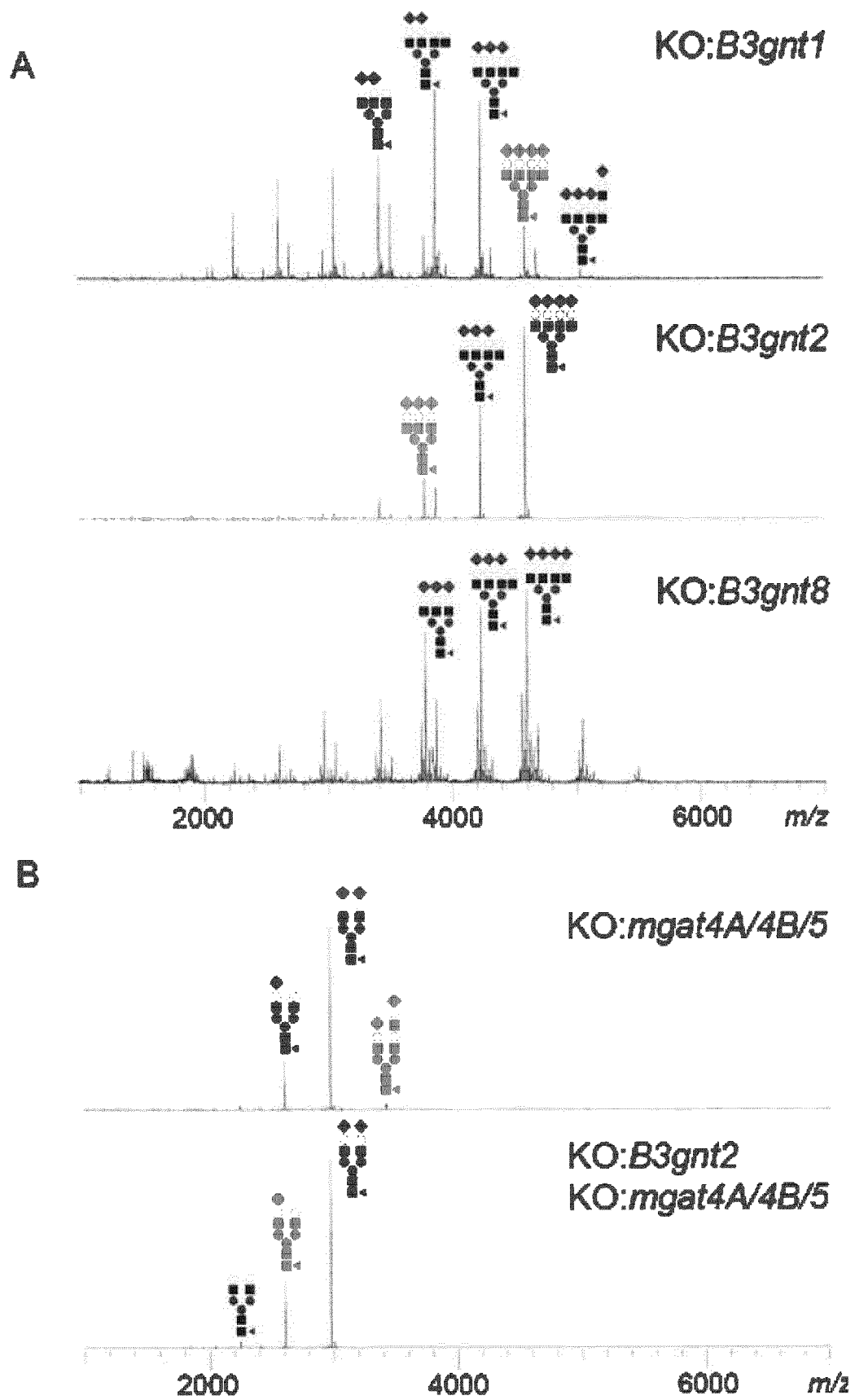


Fig. 7

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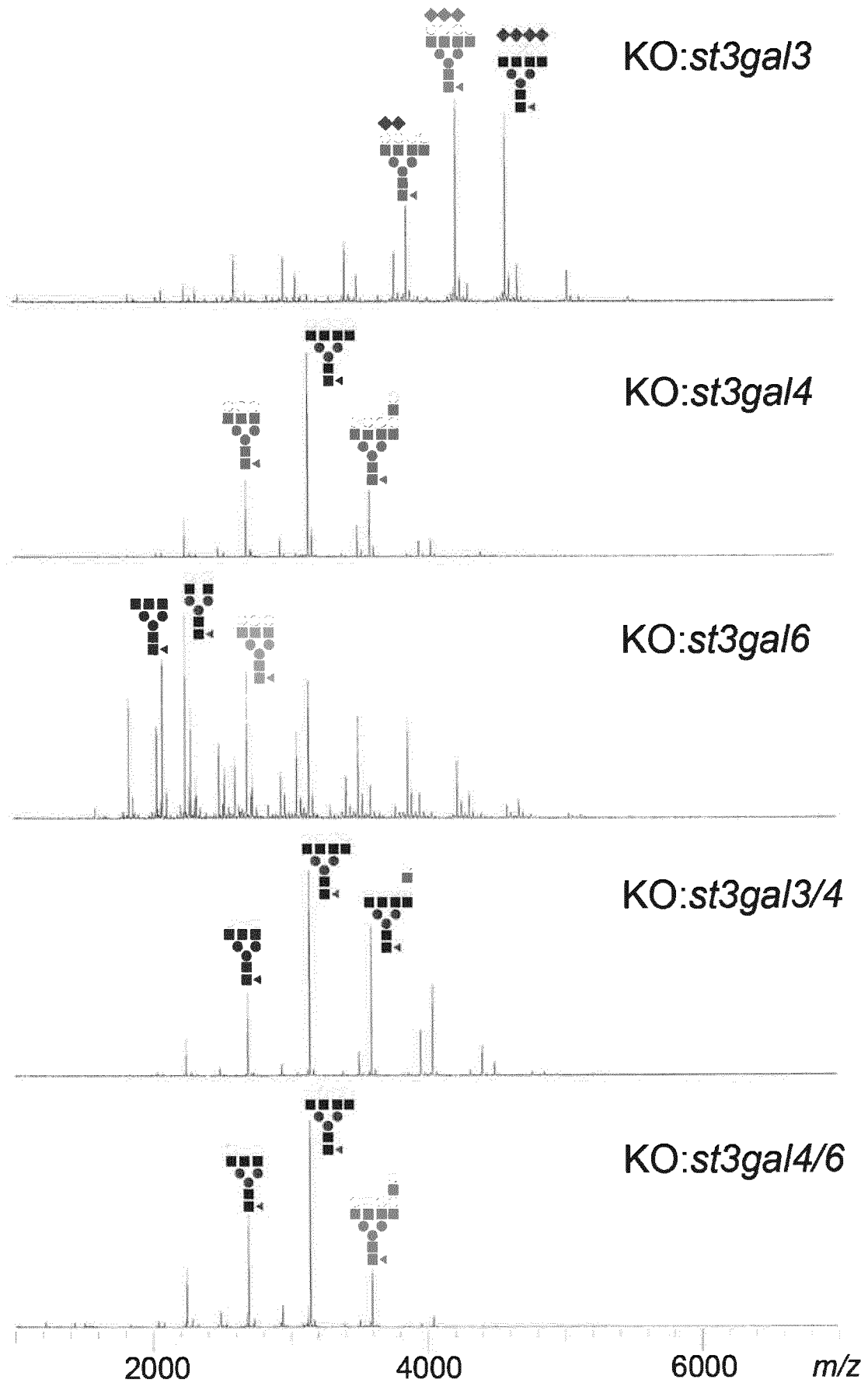


Fig. 8

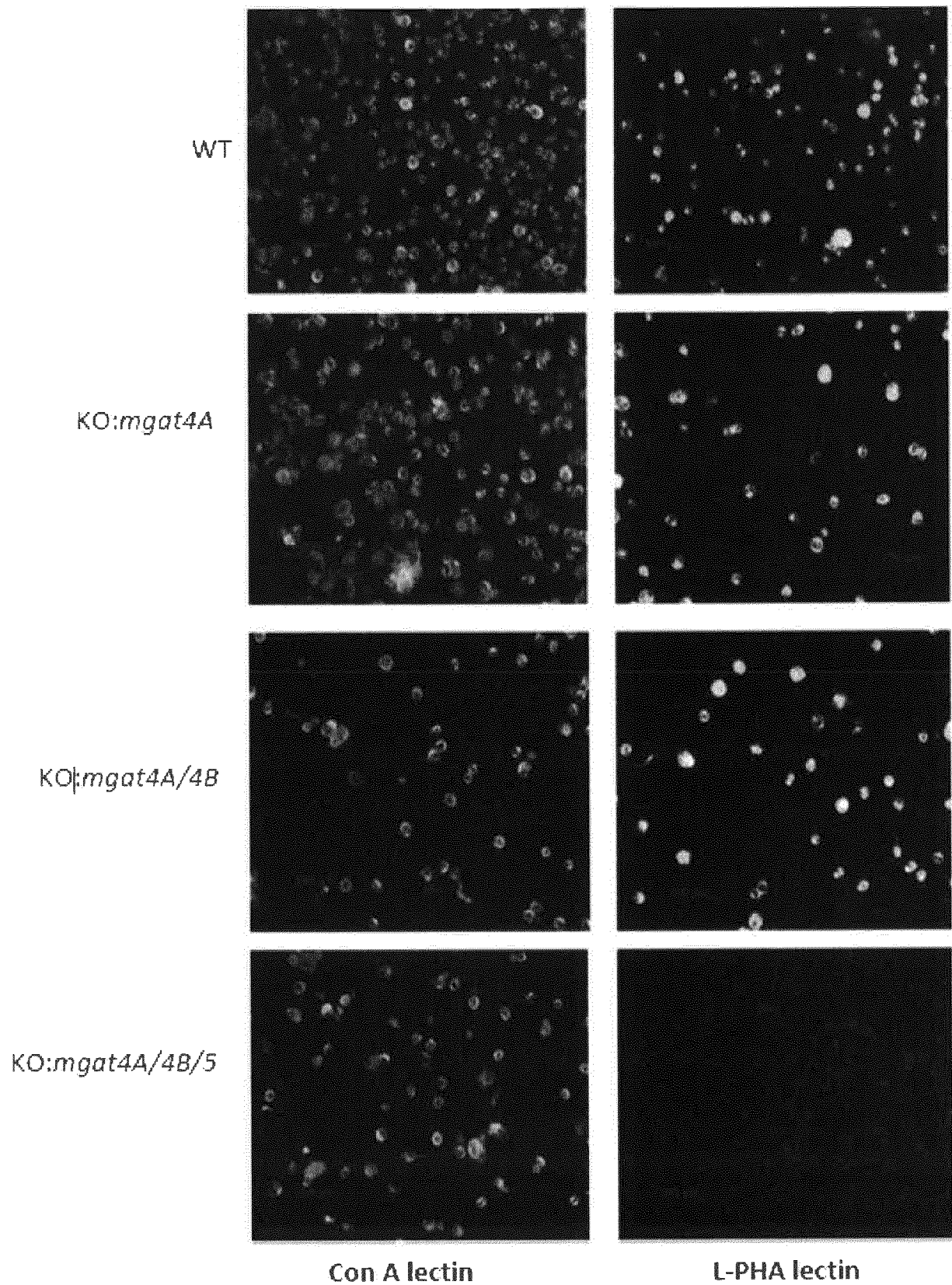


Fig. 9

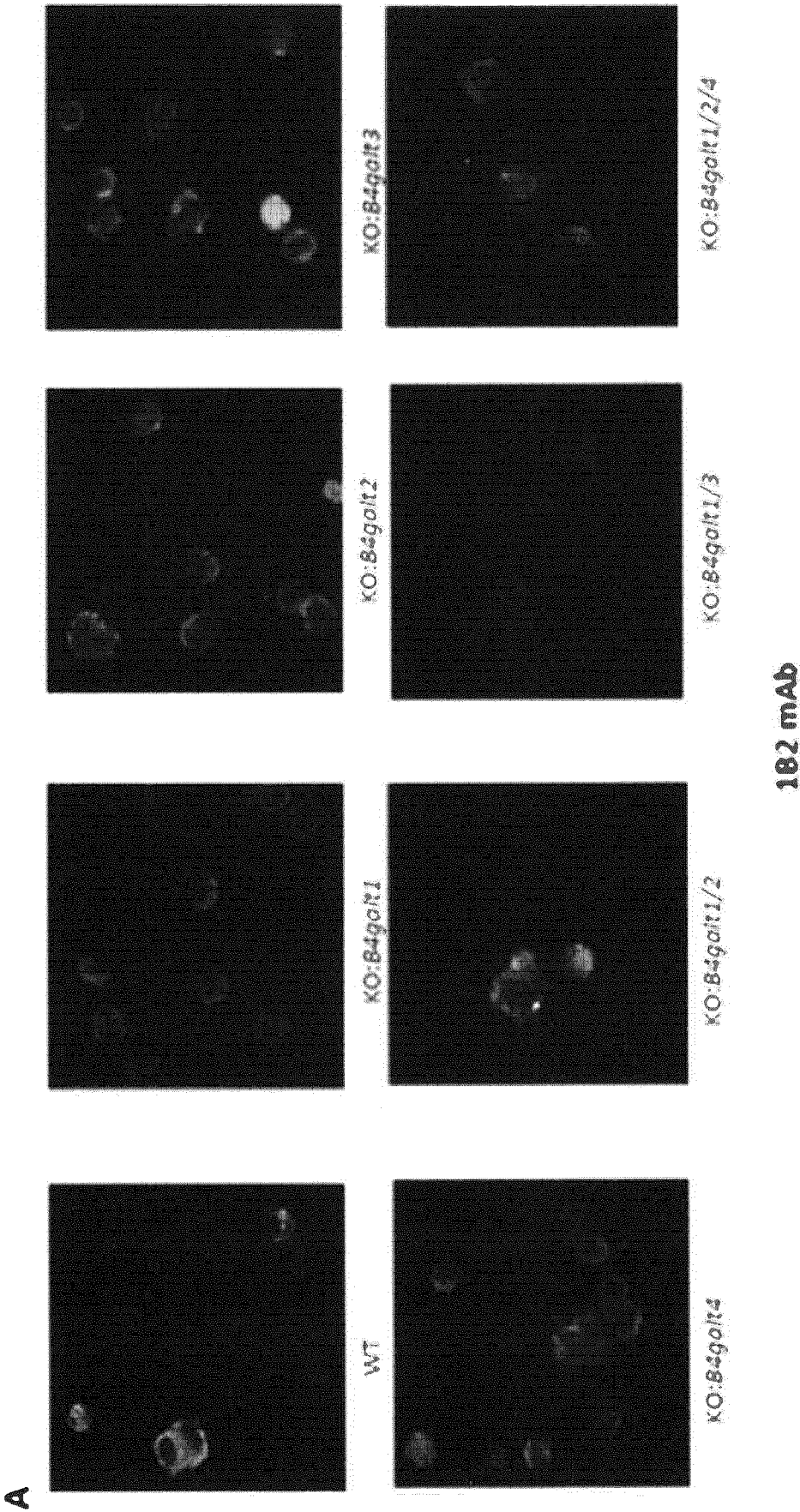


Fig. 10A

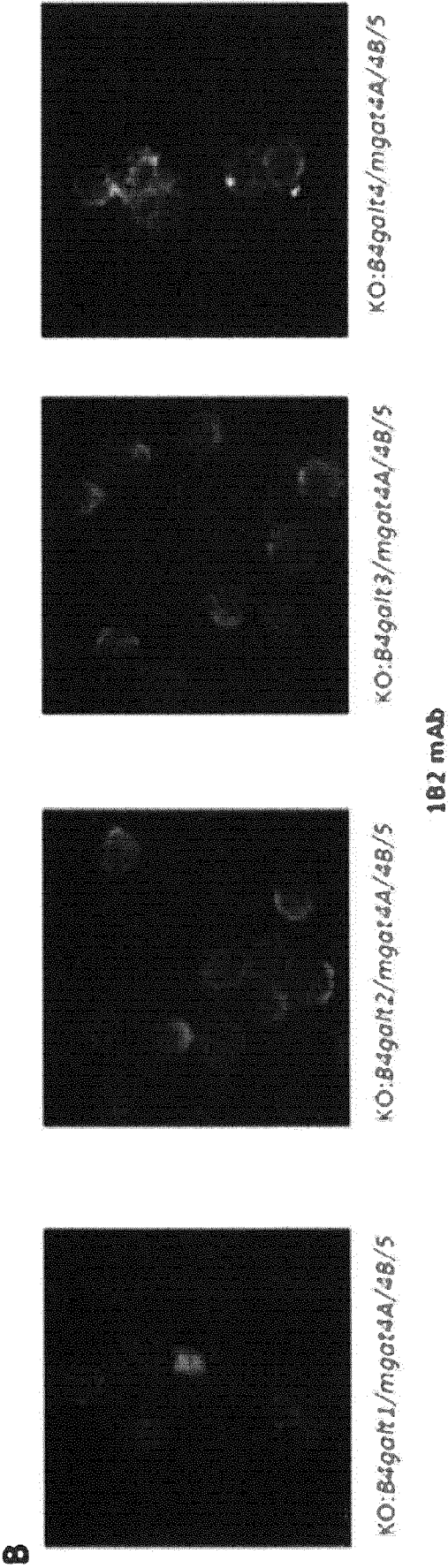


Fig. 10B

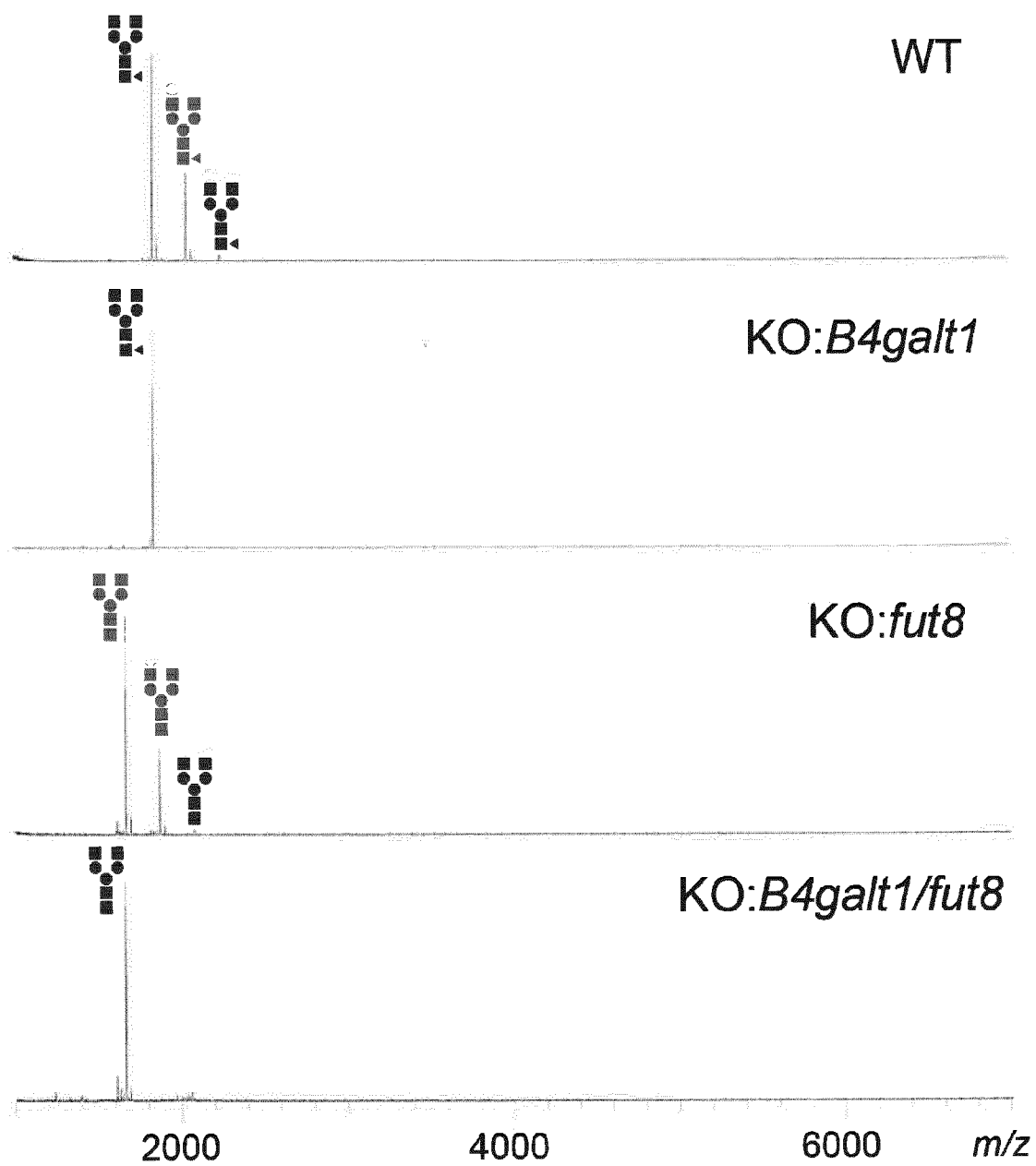


Fig. 11

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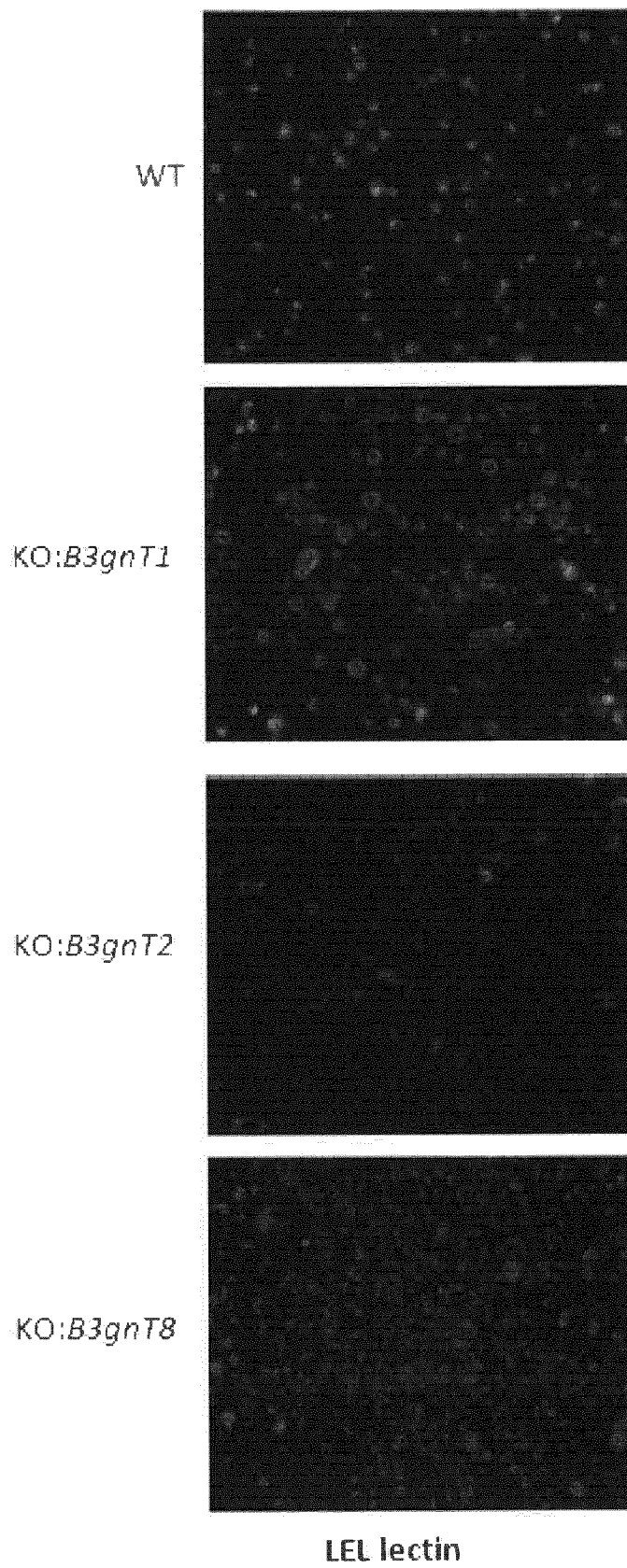


Fig. 12

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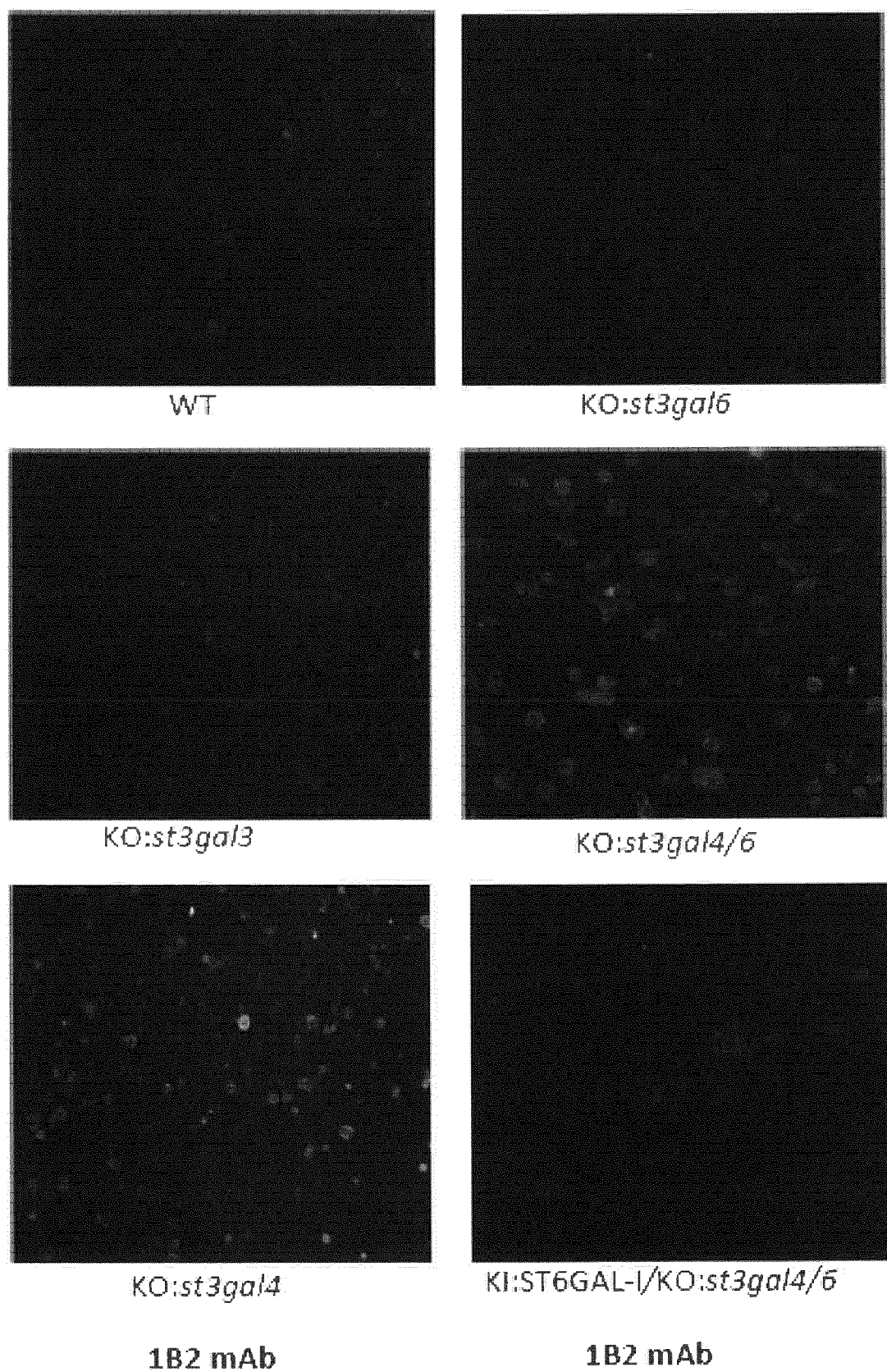


Fig. 13

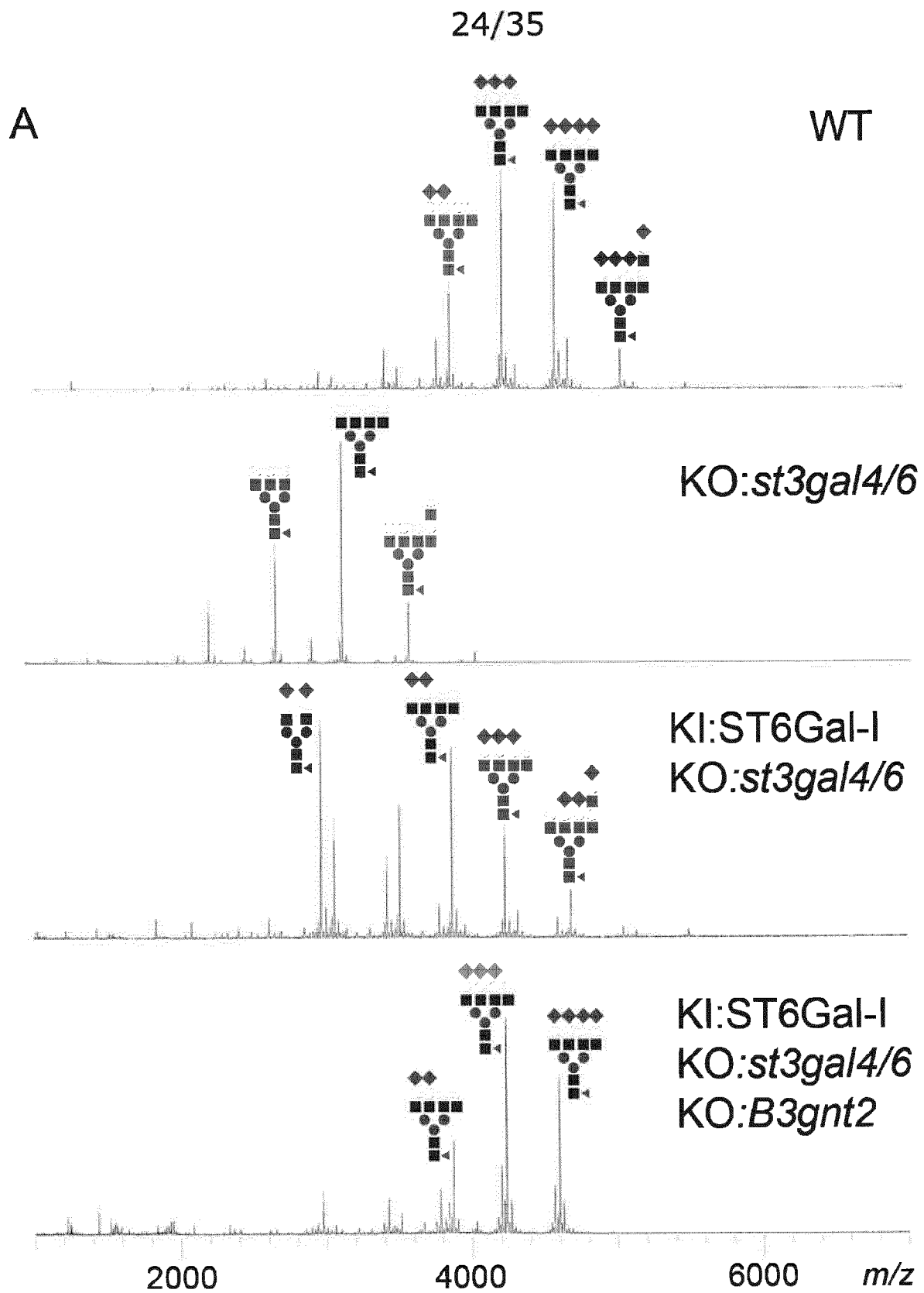


Fig. 14A

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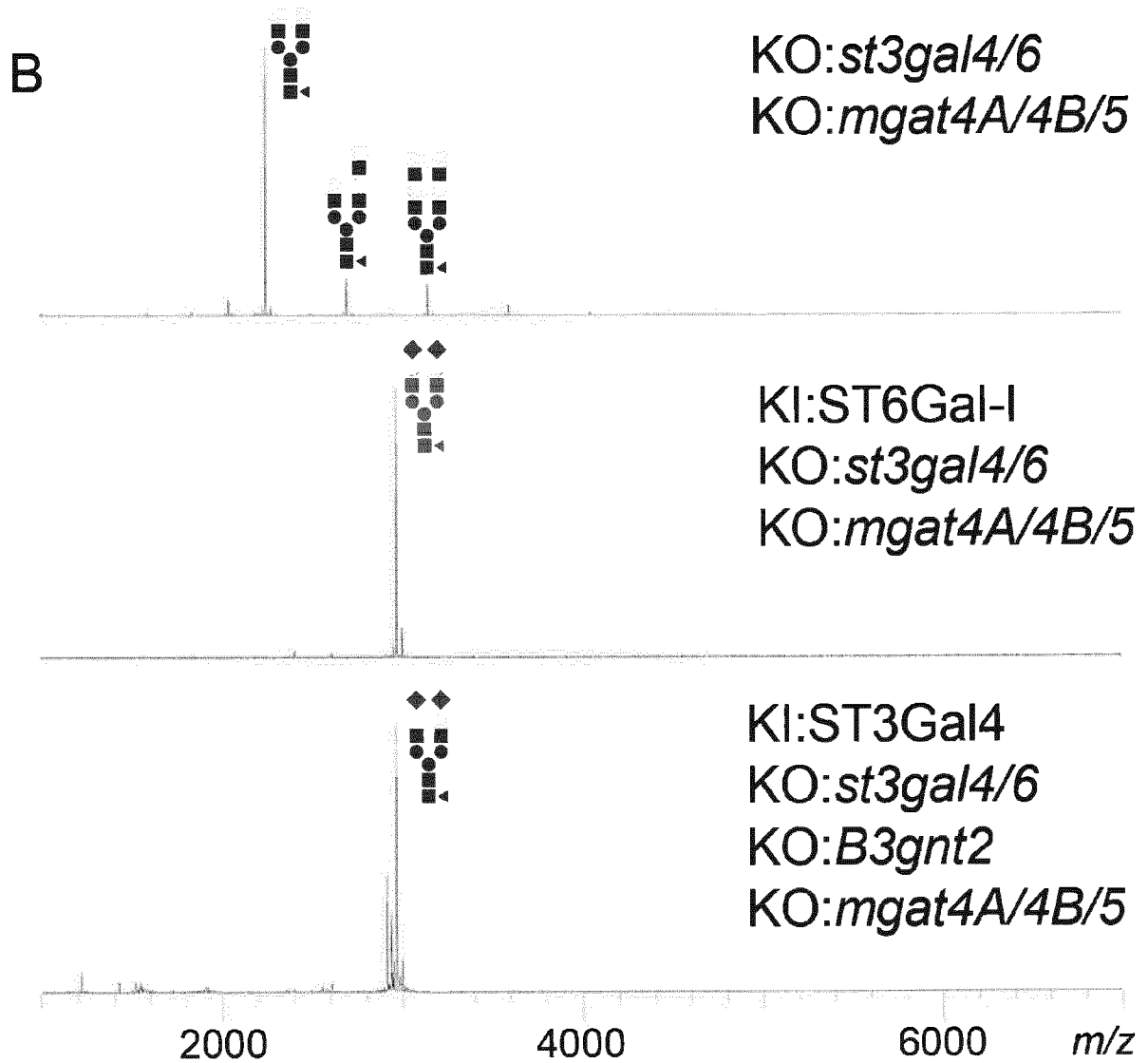


Fig. 14B

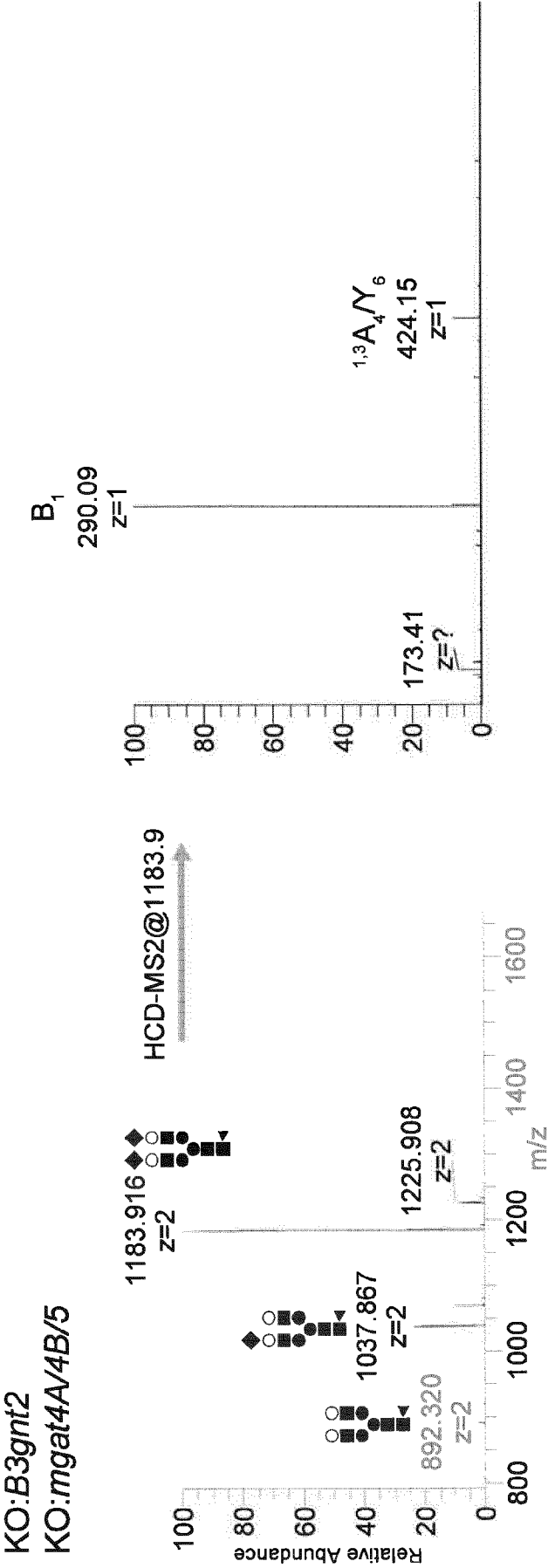


Fig. 15A

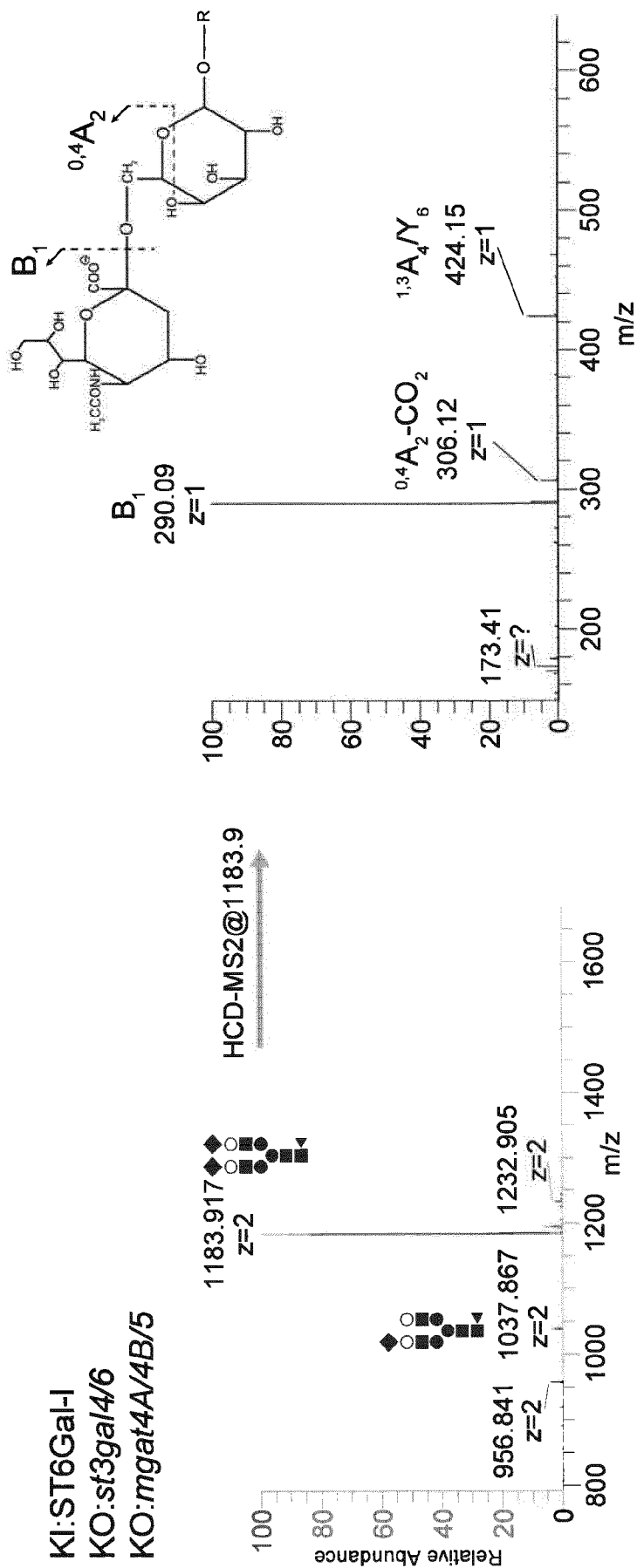


Fig. 15B

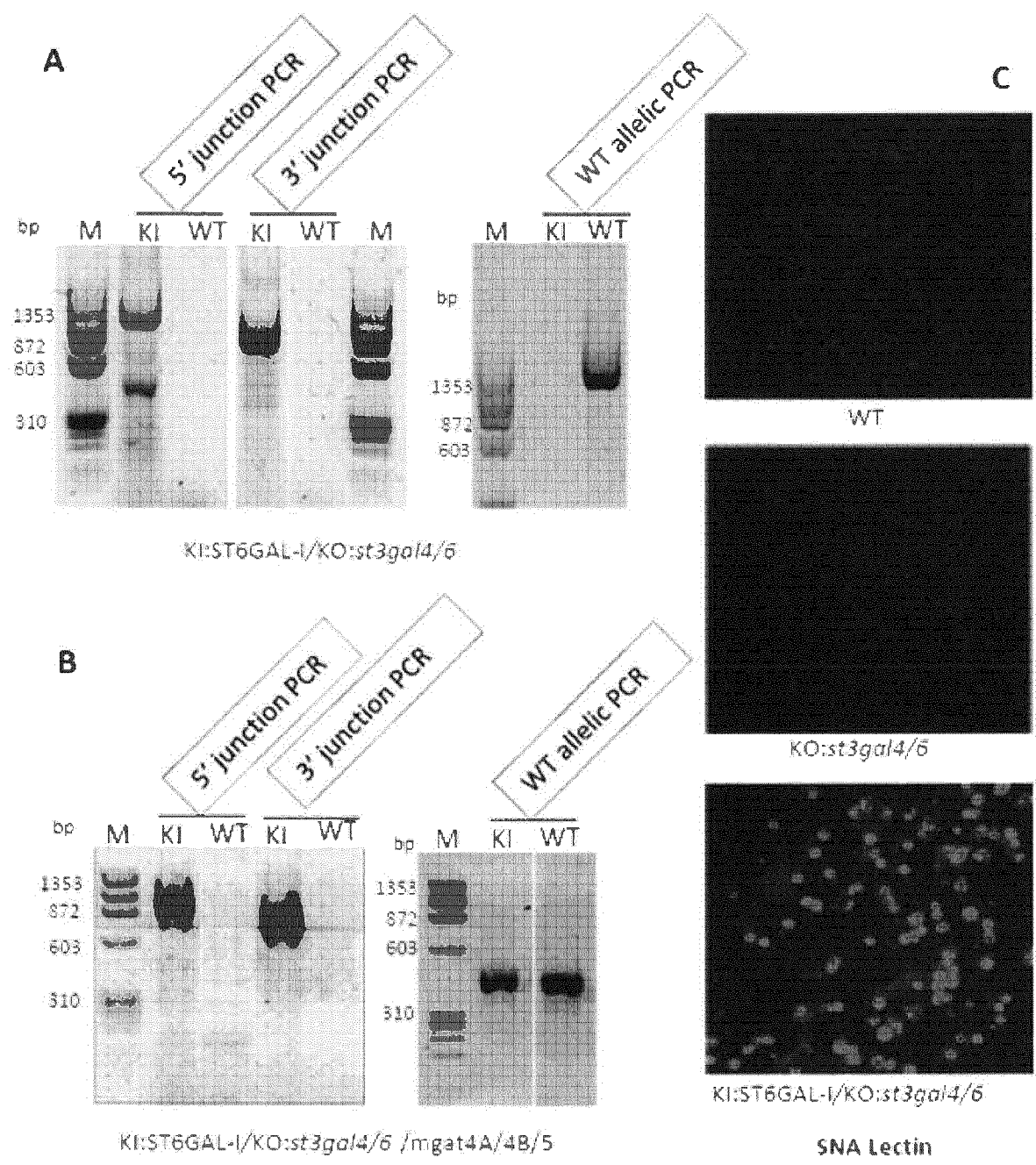


Fig. 16

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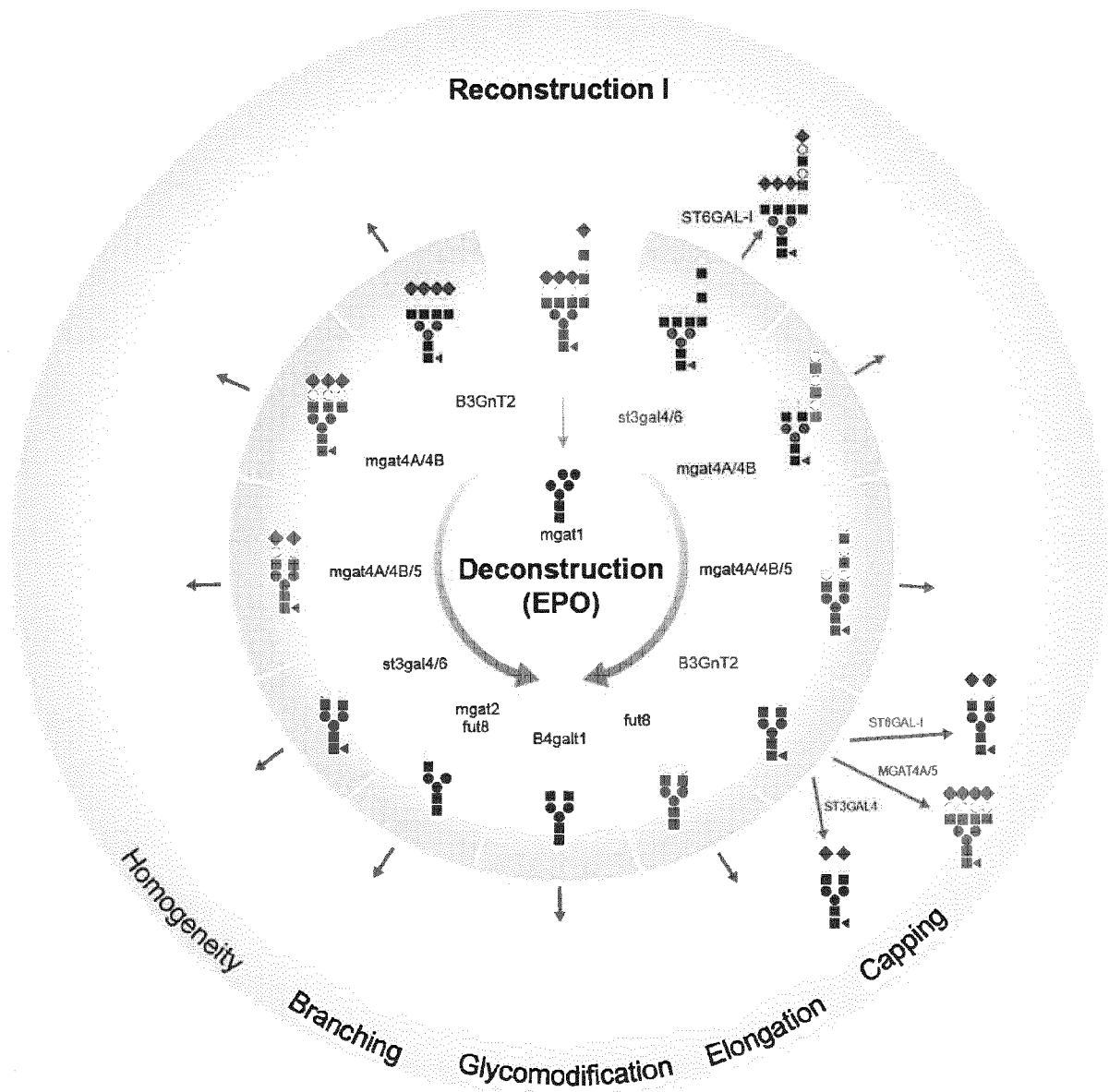


Fig. 17

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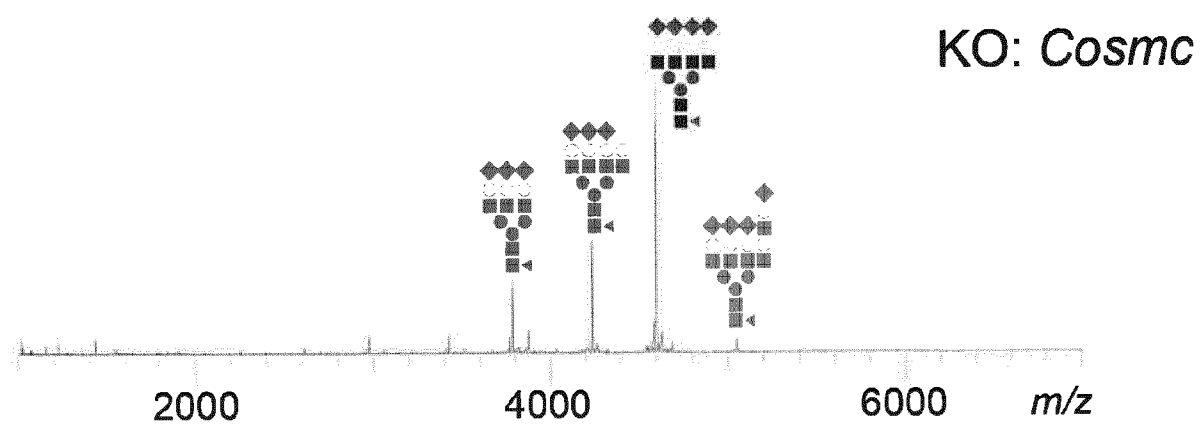


Fig. 18

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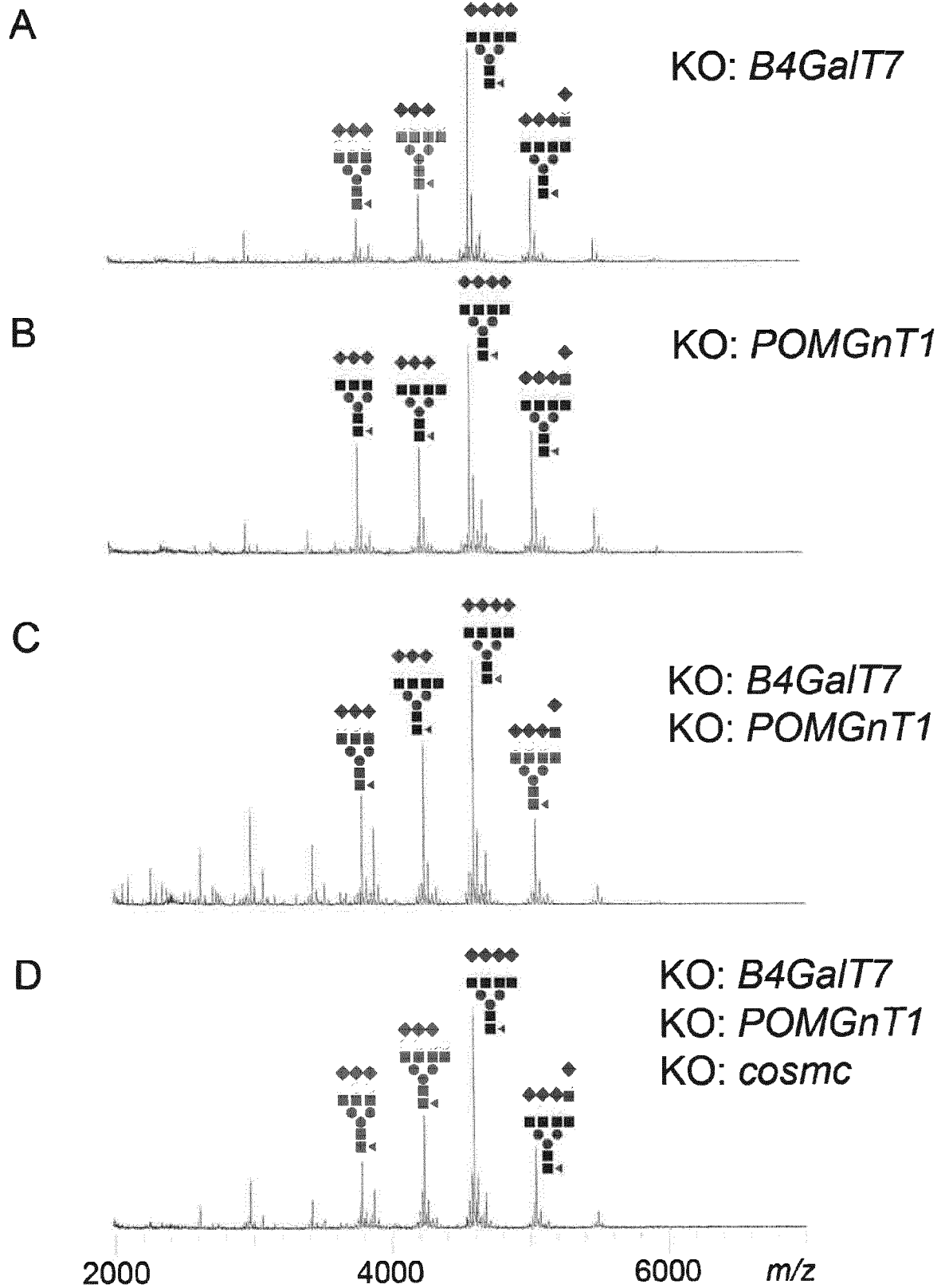


Fig. 19

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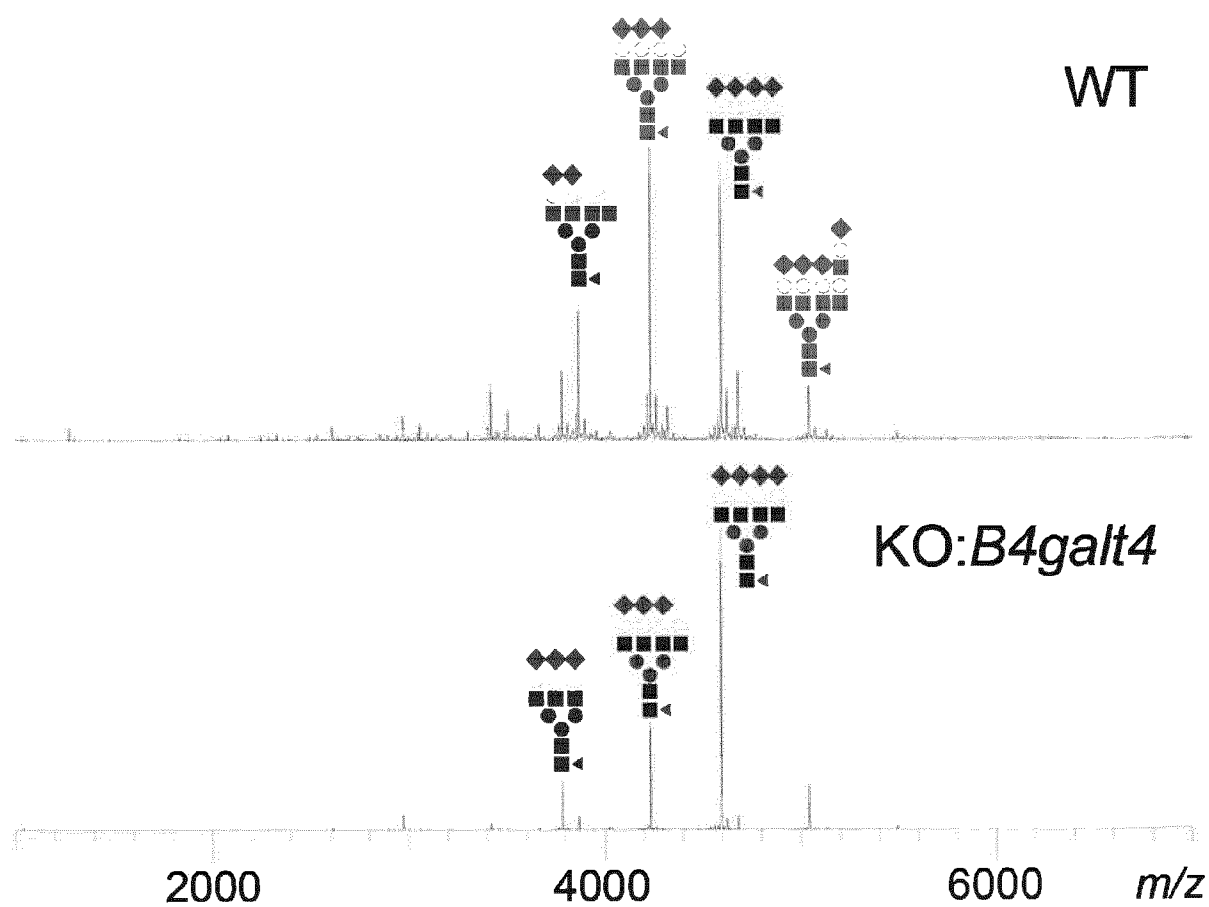


Fig. 20

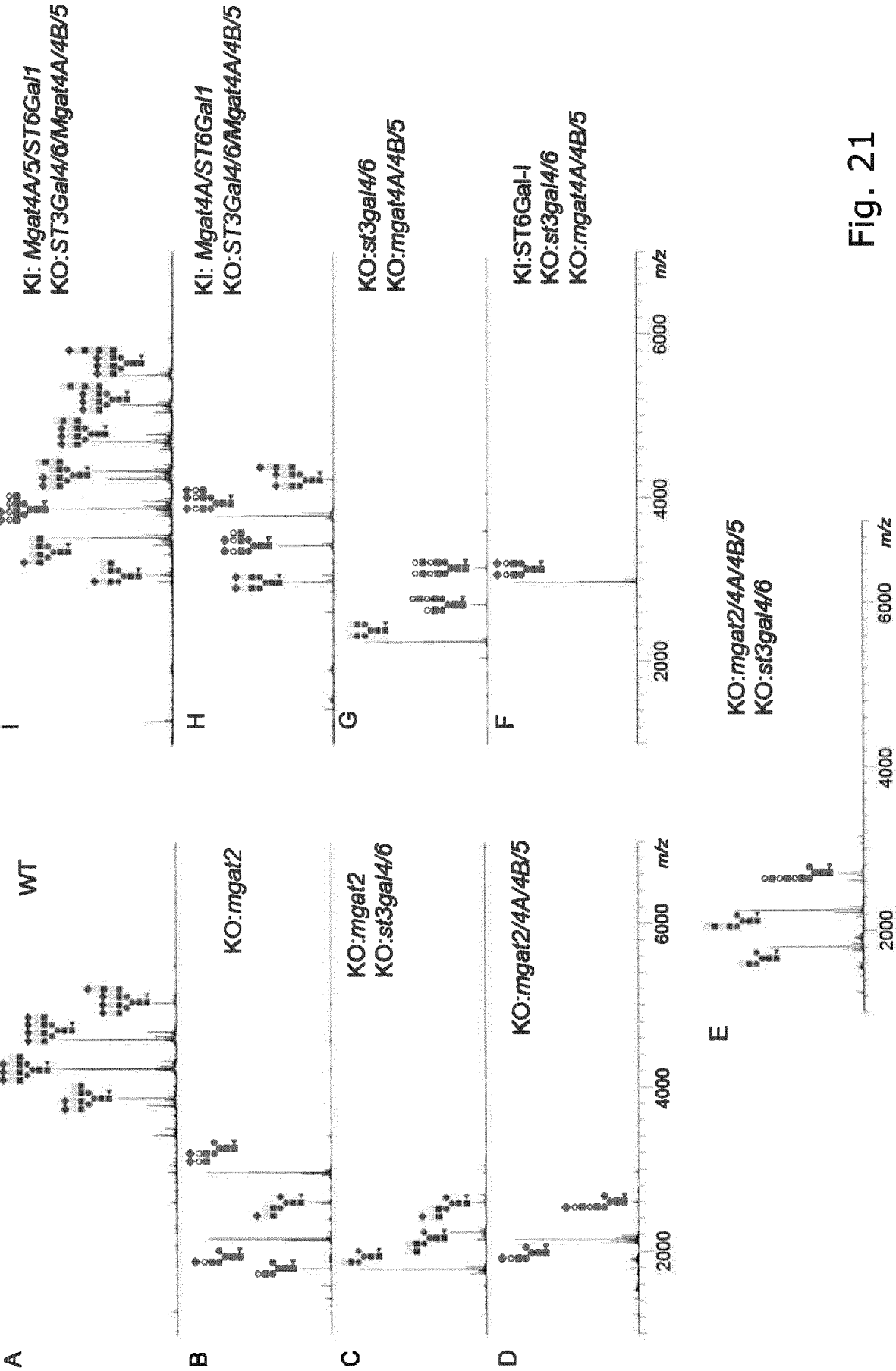


Fig. 21

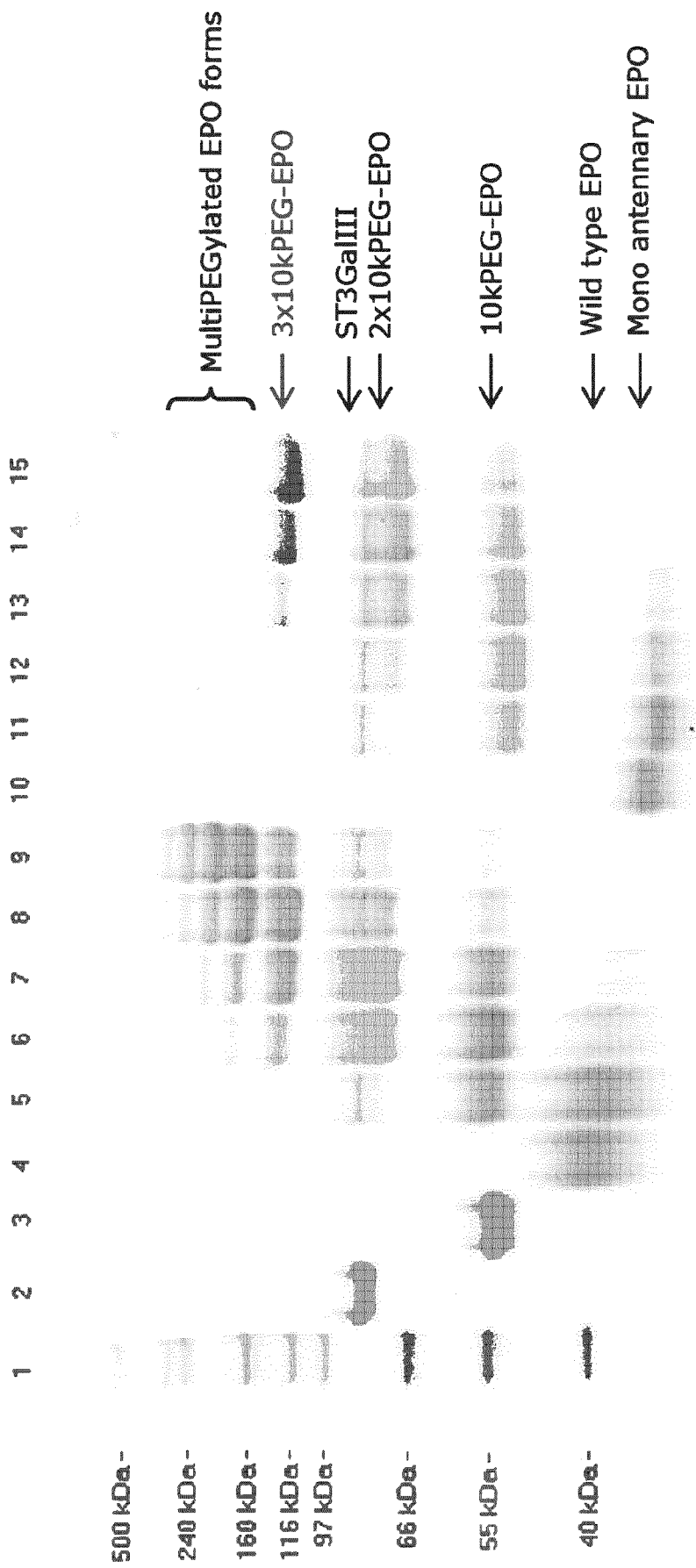
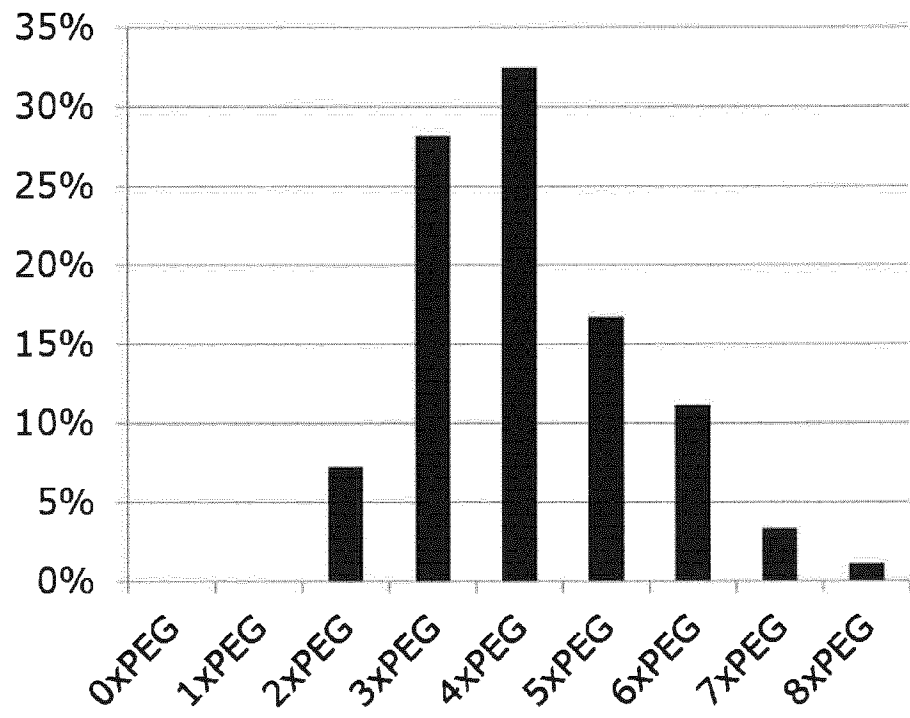


Fig. 22

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A



B

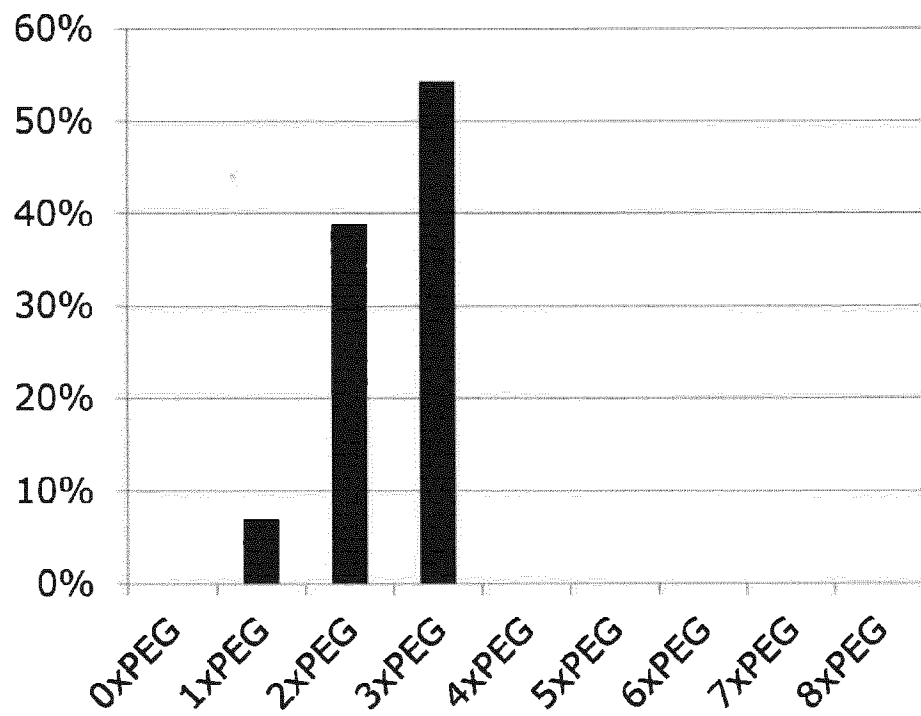


Fig. 23

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/063753

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P21/00 C12N15/90
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2010/074888 A1 (ARAGANE KATSUMI [JP] ET AL) 25 March 2010 (2010-03-25) abstract paragraphs [0001], [0029], [0040], [0041], [0055] example 1	1-8
X,P	WO 2015/134488 A1 (SIGMA ALDRICH CO LLC [US]) 11 September 2015 (2015-09-11) abstract paragraphs [0013], [0014], [0058] claim 4 ----- -/-	1-8



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

31 August 2016

Date of mailing of the international search report

11/11/2016

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Chavanne, Franz

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2016/063753

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	ZHANG YANG ET AL: "Engineered CHO cells for production of diverse, homogeneous glycoproteins", NATURE BIOTECHNOLOGY, vol. 33, no. 8, 1 August 2015 (2015-08-01) , pages 842-844, XP055298874, US ISSN: 1087-0156, DOI: 10.1038/nbt.3280 page 844 figure 2	1-8
E	----- WO 2016/091268 A2 (UNIV COPENHAGEN [DK]; NOVO NORDISK AS [DK]) 16 June 2016 (2016-06-16) abstract page 31, line 1 - line 11 page 41, line 6 - line 15 page 42, line 31 - line 35 page 80, line 19 - line 27 page 83, line 10 - line 22 page 94, line 13 - line 16 page 95, line 27 - line 29 page 97, line 10 - line 31 example 9 figure 35 claims 1,16,43,59-64 -----	1-8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2016/063753

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-8

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-8

A cell comprising one or more glycosyltransferase genes that have been inactivated, and that has new and/or more homogenous stable glycosylation capacities, wherein mgat2 has been knock out, and with or without KO of mgat4A and/or mgat4B and/or mgat5 allowing production of N-glycans with monoantennary structure.

2. claims: 9(completely); 12-23(partially)

A method for the production of di- and triPEGylated EPO glycoproteins; glycoproteins obtainable from said method

3. claims: 10, 11(completely); 12-23(partially)

A method for the production of recombinant glycoproteins; glycoprotein obtainable from said method

4. claim: 24

Use of recombinant glycoproteins comprising monoantennary N-glycans for enzymatic modification of polypeptides

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2016/063753

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2010074888 A1	25-03-2010	US 2010074888 A1	25-03-2010
		WO 2008093464 A1	07-08-2008
WO 2015134488 A1	11-09-2015	SG 11201607038T A	29-09-2016
		WO 2015134488 A1	11-09-2015
WO 2016091268 A2	16-06-2016	NONE	