

MODIFIED CHO CELLS AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority of Singapore provisional application no.
5 10202302799U, filed 29 September 2023, the contents of it being hereby incorporated by
reference in its entirety for all purposes.

FIELD OF THE INVENTION

[0002] The present invention relates generally to the field of biotechnology. In particular, the
10 present invention relates to engineered Chinese hamster ovary cells and the use of the same in
protein production.

BACKGROUND OF THE INVENTION

[0003] Protein glycosylation is the most abundant form of post-translational modification that
15 is known to significantly impact protein folding, stability, and activity. Many biotherapeutics,
including antibodies, cytokines, enzymes, hormones, and erythropoietin, are glycoproteins that
contain glycans as part of their structures. The glycan composition of these biotherapeutics had
been modified to enhance their safety and efficacy.

[0004] Biotherapeutics are generally produced recombinantly in mammalian cell culture
20 systems, with Chinese hamster ovary (CHO) cells commonly used due to their ability to generate
human-like glycosylation patterns. However, the complex nature of the glycosylation machinery
in mammalian cells often leads to undesirable heterogeneity in the carbohydrate determinants of
the recombinant therapeutics produced. Therefore, there is an unmet need for cell lines to
produce improved biotherapeutics.

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SUMMARY OF THE INVENTION

[0005] In one aspect, the present disclosure refers to a modified mammalian cell comprising
at least 3 gene mutations, wherein one of the gene mutations is MAN2A1, wherein the remaining
gene mutations are selected from the group consisting of SLC35C1, MGAT1, MAN2A2,
30 MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1,
or homologues thereof.

[0006] In another aspect, the present disclosure refers to the use of the modified mammalian
cells as disclosed herein in the manufacture of glycosylated proteins, wherein the glycosylated
proteins comprise high-mannose type *N*-glycans.

[0007] In yet another aspect, the present disclosure provides a kit for expressing glycosylated proteins comprising high-mannose type *N*-glycans, wherein the kit comprises the modified mammalian cells as disclosed herein and at least one buffer.

5 BRIEF DESCRIPTION OF THE DRAWINGS

[0008] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings, in which:

[0009] **Figure 1** shows chromatograms that show the composition of *N*-glycans attached to erythropoietin Fc (EPO-Fc) produced in the Chinese hamster ovary-K1 (CHO-K1) cell line (Figure 1a) and various Chinese hamster ovary glycosylation mutants (Figures 1b to 1z-a). Erythropoietin Fc was stably produced in these Chinese hamster ovary glycosylation mutants and purified using a protein A column. The *N*-glycans were released from erythropoietin Fc using PNGase F and labelled with *Rapi*Flour-MS (RFMS) for HILIC-UPLC-QTOF-MS analysis. Only major peaks are annotated in the chromatographs. Minor peaks in each sample, which represent less abundant glycans, are detailed in the ID table. The glycan structures presented in the figure are represented by monosaccharide symbols as follows: a circle in a darker shade represents mannose; a circle in a lighter shade represents galactose; a square represents *N*-acetylglucosamine; a triangle represents fucose; and a diamond represents *N*-acetylneuraminic acid.

[0010] **Figure 2** shows chromatograms that analyse the composition of *N*-glycans attached to trastuzumab produced in the Chinese hamster ovary-K1 (CHO-K1) cell line (Figure 2a) and various Chinese hamster ovary glycosylation mutants (Figures 2b to 2z-a). Recombinant trastuzumab was stably produced in these Chinese hamster ovary glycosylation mutants and purified using a protein A column. The *N*-glycans were released from trastuzumab using PNGase F and labelled with *Rapi*Flour-MS (RFMS) for HILIC-UPLC-QTOF-MS analysis. Only major peaks are annotated in the chromatographs. Minor peaks in each sample, which represent less abundant glycans, are detailed in the ID table. The glycan structures presented in the figure are represented by monosaccharide symbols as follows: a circle in a darker shade represents mannose; a circle in a lighter shade represents galactose; a square represents *N*-acetylglucosamine; a triangle represents fucose; and a diamond represents *N*-acetylneuraminic acid.

[0011] **Figure 3** shows an iso-electric focusing gel image from genetic complementary assay for identifying mutated glycogenes using erythropoietin as a model molecule. Constructs

expressing human erythropoietin were co-transfected with another construct expressing a glycogene to be tested. Two-days post transfection, the conditioned media were de-salted and analysed by an iso-electric focusing gel.

- [0012] **Figure 4** shows a roadmap for the generation of Chinese hamster ovary glycosylation mutants. The specific genome editing techniques used to generate these mutants are indicated in the roadmap. MAA: *Maackia amurensis* agglutinin; RCA-I: *Ricinus communis* agglutinin-I; ZFNs: Zinc Finger Nucleases; TALENs: Transcription Activator-Like Effector Nucleases; and CRISPR-Cas9: Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated Protein 9.
- 10 [0013] **Figure 5** shows the glycan profiles of α -mannosidase-treated erythropoietin Fc produced from CHO-gmt22 cells. Erythropoietin Fc was stably produced in CHO-gmt22 cells and purified using a protein A column. Figure 5a shows chromatogram showing the composition of *N*-glycans released by Peptide:N-glycosidase F (PNGase F) and labelled with *Rapi*Fluor-MS (RFMS) for HILIC-UPLC-QTOF-MS analysis. Figure 5b shows chromatogram of the sample
- 15 shown in Figure 5a, which was treated with α -mannosidase before HILIC-UPLC-QTOF-MS analysis. The glycan structures presented in the figure are represented by monosaccharide symbols as follows: a circle in a darker shade represents mannose; a circle in a lighter shade represents galactose; a square represents *N*-acetylglucosamine; and a diamond represents *N*-acetylneuraminic acid.
- 20 [0014] **Figure 6** shows the glycan profiles of erythropoietin Fc (EPO-Fc) protein and trastuzumab produced from Chinese hamster ovary-K1 (CHO-K1) cell line and various Chinese hamster ovary glycosylation mutants (CHO-gmts) generated for Man9 *N*-glycan production. Erythropoietin Fc protein and trastuzumab were stably expressed in various Chinese hamster ovary cell lines, purified, and their *N*-glycan composition was analysed using HILIC-UPLC-
- 25 QTOF-MS. Glycan composition results are shown for erythropoietin Fc (left) and trastuzumab (right). Figure 6a and 6b: CHO-K1; Figure 6c and 6d: CHO-gmt18; Figure 6e and 6f: CHO-gmt19; Figure 6g and 6h: CHO-gmt20; Figure 6i and 6j: CHO-gmt26; Figure 6k and 6l: CHO-gmt29; Figure 6m and 6n: CHO-gmt32; and Figure 6o and 6p: CHO-gmt35. The glycan structures presented in the figure are represented by monosaccharide symbols as follows: a circle
- 30 in a darker shade represents mannose; a circle in a lighter shade represents galactose; a square represents *N*-acetylglucosamine; a triangle represents fucose; and a diamond represents *N*-acetylneuraminic acid.

[0015] **Figure 7** shows graphs that demonstrate how different Fc glycans can affect the interaction between the Fc region of an antibody and Fc γ receptor (Fc γ R). Figure 7a shows a

graph demonstrating the K_D (dissociation constant) value of trastuzumab's interaction with Fc γ RIIIa, analysed with BiaCore. The detailed K_D data are summarised in Table 4. Figure 7b shows a graph illustrating the fold change in trastuzumab's interaction with Fc γ RIIIa, determined using a standard enzyme-linked immunosorbent assay (ELISA). Figure 7c shows a graph demonstrating the fold change in the interaction between trastuzumab and Fc γ RI, determined using a standard enzyme-linked immunosorbent assay (ELISA). Figure 7d shows a graph demonstrating the fold change in the interaction between trastuzumab and Fc γ RIIa, determined using a standard enzyme-linked immunosorbent assay (ELISA).

[0016] **Figure 8** shows the chromatograms of the composition of *N*-glycans on trastuzumab produced from the Chinese hamster ovary-K1 (CHO-K1) cell line (Figure 8a) and various Chinese hamster ovary glycosylation mutants with β -galactosyltransferase 1 (GalT1) and *N*-acetylglucosaminyltransferases III (GnT-III) overexpressed (Figures 8b to 8j). Trastuzumab was stably produced in these Chinese hamster ovary glycosylation mutants with the co-expression of either GalT1 or GnT-III. Recombinant trastuzumab was purified using a protein A column. The *N*-glycans were released by PNGase F and labelled with RapiFluor-MS (RFMS) for HILIC-UPLC-QTOF-MS analysis. The glycan structures presented in the figure are represented by monosaccharide symbols as follows: a circle in a darker shade represents mannose; a circle in a lighter shade represents galactose; a square represents *N*-acetylglucosamine; a triangle represents fucose; and a diamond represents *N*-acetylneuraminic acid.

[0017] **Figure 9** shows the *in vitro* antibody-dependent cellular cytotoxicity (ADCC) analysis of glycoengineered trastuzumab on breast cancer cell lines. Figure 9a shows chromatogram from fluorescence-activated cell sorting (FACS) analysis of Her2 expression levels using commercial Herceptin in MDA-MB-468, MDA-MB-231, MDA-MB-453, HCC1954, and SK-BR-3 breast cancer cell lines. Figures 9b to 9f show graphs comparing the fold of induction of cell-based antibody-dependent cellular cytotoxicity activity of trastuzumab produced from CHO-K1 and CHO-gmt3 cells with the commercial drug Herceptin. Figures 9g to 9h show graphs comparing the fold of induction of antibody-dependent cellular cytotoxicity activity of trastuzumab from CHO-K1, CHO-gmt2, and CHO-gmt9 on HCC1954 and MDA-MB-453 breast cancer cell lines. Figures 9i to 9m show graphs comparing the fold of induction of cell-based antibody-dependent cellular cytotoxicity activity of trastuzumab produced from CHO-K1 and various Chinese hamster ovary glycosylation mutants in SK-BR-3 breast cancer cell line.

[0018] **Figure 10** shows the effect of human immunodeficiency virus (HIV) gp140 glycans on interactions with anti-HIV broadly neutralising anti-HIV antibodies (bnAbs). Figure 10a

- shows the comparison of glycosylation patterns of BG505 SOSIP.664 trimers produced from CHO-K1, CHO-gmt4, and CHO-gmt35 cells. Figures 10b to 10f show the binding of these BG505 SOSIP.664 trimers to broadly neutralising anti-HIV antibodies, studied using enzyme-linked immunosorbent assay (ELISA). Figure 10b shows the interaction with the antibody 2G12;
- 5 Figure 10c shows the interaction with the antibody PG9; Figure 10d shows the interaction with the antibody PG16; Figure 10e shows the interaction with the antibody PGT121; and Figure 10f shows the interaction with the antibody PGT128. The glycan structures presented in the figure are represented by monosaccharide symbols as follows: a circle in a darker shade represents mannose; and a square represents *N*-acetylglucosamine.
- 10 [0019] **Figure 11** shows the chromatograms of the composition of *N*-glycans on trimeric Env gp140 of human immunodeficiency virus (HIV) produced from CHO-K1, CHO-gmt4, and CHO-gmt35 cells. Figure 11a to 11c show the composition of *N*-glycans on purified gp140 produced from three cell lines, which were released by PNGase F and labelled with *Rapi*Flour-MS (RFMS) for HILIC-UPLC-QTOF-MS analysis. Figure 11a shows the *N*-glycans on trimeric Env
- 15 gp140 of human immunodeficiency virus from CHO-K1. Figure 11b shows the *N*-glycans on trimeric Env gp140 of human immunodeficiency virus from CHO-gmt4. Figure 11c shows the *N*-glycans on trimeric Env gp140 of human immunodeficiency virus from CHO-gmt35. The glycan structures presented in the figure are represented by monosaccharide symbols as follows: a circle in a darker shade represents mannose; a circle in a lighter shade represents galactose; a
- 20 square represents *N*-acetylglucosamine; a triangle represents fucose; and a diamond represents *N*-acetylneuraminic acid.
- [0020] **Figure 12** shows β -glucocerebrosidase produced from CHO-gmt4, CHO-gmt17, and CHO-17x exhibits uniform Man5 glycans. Figure 12a shows the glycan profiles of recombinant β -glucocerebrosidase (GCase) expressed and purified from CHO-gmt4, CHO-gmt17, and CHO-
- 25 17x, as determined using HILIC-UPLC-QTOF-MS. Figure 12b shows a graph of the quantitative site-specific glycan analysis of recombinant β -glucocerebrosidase from CHO-gmt4, CHO-gmt17, and CHO-gmt17x cells. The glycosylation sites are indicated, with the Y-axis showing the percentage of glycan composition. The glycan structures presented in the figure are represented by monosaccharide symbols as follows: a circle in a darker shade represents
- 30 mannose; a square represents *N*-acetylglucosamine; and a triangle represents fucose. Man 3: *N*-Glycan with 3 mannose, Man 4: *N*-Glycan with 4 mannose, Man 4F: *N*-Glycan with 4 mannose and 1 Fucose, Man 5: *N*-Glycan with 5 mannose, Man 5F: *N*-Glycan with 5 mannose and 1 fucose, and Man 6: *N*-Glycan with 6 mannose.

[0021] **Figure 13** shows a chromatogram confirming the presence of bisecting structures in trastuzumab produced by CHO-gmt9 cells expressing GnT-III. MS/MS was performed on the glycan with the mass of 916.3809 m/z, charge 2 eluting at 13.58 min in CHO-gmt9+GnT-III. Presence of the bisecting structure was confirmed by the diagnostic fragment ion 1101.49 m/z (551.25 m/z for charge 2) which is specific to bisecting glycans detected through MS/MS. This ion represents a sequential linkage of a GlcNAc, a mannose, and two GlcNAcs

DEFINITIONS

[0022] As used herein, the term “homologues” refers to genes that share sequence homologies within or between different species. Homologous genes originate from a common origin through speciation, genetic duplication or horizontal gene transfer. Homologous genes can also include paralogs and orthologs.

[0023] As used herein, the term “glycosylation” refers to a post-translational modification (PTM) in which carbohydrate molecules, known as glycans, are covalently attached to proteins or lipids. There are two types of glycosylation, namely, *N*-linked glycosylation and *O*-linked glycosylation. *N*-linked glycosylation refers to the attachment of glycans to the nitrogen atom of an asparagine residue in a protein within the consensus sequence Asn-X-Ser/Thr, where X can be any amino acid except proline. On the other hand, *O*-linked glycosylation refers to the attachment of glycans to the oxygen atom of serine or threonine residues in a protein. In the present disclosure, the term “glycosylation” refers to *N*-linked glycosylation.

[0024] As used herein, the term “*N*-glycan” refers to carbohydrate structures covalently attached to a molecule via the asparagine residue by an *N*-glycosidic bond. This attachment occurs in specific locations on the molecules and is catalysed by enzymes called glycosyltransferases. *N*-glycans can be classified into several types: high mannose-type glycans are characterised by a high content of mannose residues and minimal branching; complex-type glycans have a more elaborate structure with multiple branches, including, for example but not limited to, *N*-acetylglucosamine, galactose, fucose, and sialic acid; and hybrid-type glycans combine features a mix of mannose-rich and branched structures. Additionally, specific high mannose glycans like, for example, Man5 *N*-glycan and Man9 *N*-glycan, refer to a *N*-linked glycan that consists of a core oligosaccharide with five and nine mannose sugar residues, respectively.

[0025] As used herein, the term “glycogene” refers to a gene that encodes an enzyme involved in the biosynthesis, modification, or processing of glycans. Examples of these enzymes include, but are not limited to, MAN2A1 (α -mannosidase II), SLC35C1 (GDP-fucose

transporter), MAN2A2 (α -mannosidase II X), MAN1A1 (α -mannosidase IA), MAN1A2 (α -mannosidase IB), MAN1C1 (α -mannosidase IC), MANEA (Endo α -mannosidase I), MAN1B1 (ER α -mannosidase I), EDEM1 (ER degradation-enhancing α -mannosidase-like protein 1), EDEM2 (ER degradation-enhancing α -mannosidase-like protein 2), EDEM3 (ER degradation-enhancing α -mannosidase-like protein 3), MGAT1 (GnT1 / α -1,3-mannosyl-glycoprotein 2- β -N-acetylglucosaminyltransferase), and MAN2B1 (Lysosomal α -mannosidase).

[0026] As used herein, the term “genetically modified cells” refers to glycosylation mutants with mutations in the genes responsible for glycosylation. Specifically, the term refers to a cell wherein one or more genes involved in one or more glycosylation pathway(s) have been genetically modified, altered, mutated, or engineered, which can lead to abnormal glycosylation patterns. These gene mutations can be, for example, but not limited to, an insertion (also called an insertion mutation) that involves the addition of one or more nucleotides into a gene; a deletion (also called a deletion mutation) that involves the deletion of one or more nucleotides from the gene or deletion of the entire gene from the genome; a point mutation that involves a change in a single nucleotide base in the gene by addition, deletion, or substitution; a frameshift mutation that alters the reading frame of the codons that occurs when nucleotides are inserted or deleted from the gene in numbers that are not multiples of three; a knock-out mutation where one or more specific genes are inactivated or “knocked-out” from an organism, and combinations thereof. These mutations cause disruption of a gene coding sequence, leading to the inactivation of the genes responsible for glycosylation. The term “gene inactivation” or “loss-of-function mutation”, in the context of the present disclosure means that the gene in question is rendered non-functional, thereby preventing it from being expressed or producing its corresponding (functional) protein. In some examples, the gene is so mutated that the resulting protein is non-functional. Several techniques can be used to achieve gene activation, such as including, but not limited to, Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated Protein 9 (CRISPR-Cas9), Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and RNA interference (RNAi). These genetic mutations can affect various aspects of glycosylation, including the addition, trimming, or modification of glycan structures. In another example, the glycosylation mutants disclosed herein can also comprise naturally occurring mutations in the genes responsible for glycosylation. In one example, the modified mammalian cell for use in expression systems to produce recombinant glycoproteins with specific glycan structures include, but are not limited to, Chinese hamster ovary (CHO) cells, Baby Hamster Kidney (BHK) cells, NS0 or Sp2/0 murine myeloma cells, and human embryonic kidney 293 (HEK293) cells.

[0027] As used herein, the term “*Maackia amurensis* agglutinin (MAA)” refers to a type of lectin that specifically targets and binds to a particular glycan structure, namely α 2,3-linked sialic acid, found on glycoproteins and glycolipids. *Maackia amurensis* agglutinin binds specifically to α 2,3-linked sialic acid, but not sialic acid in an α 2,6 linkage. In the case of Chinese hamster ovary (CHO) cells, which naturally have α 2,3-linked sialic acid on their surface, exposure of CHO cells to *Maackia amurensis* agglutinin had been shown to be toxic. This toxicity likely results from *Maackia amurensis* agglutinin binding to these sialic acids and triggering cell death through a process called apoptosis. In this disclosure, *Maackia amurensis* agglutinin was used to isolate Chinese hamster ovary (CHO) glycosylation mutants that are resistant to the toxic effects of *Maackia amurensis* agglutinin. Specifically, *Maackia amurensis* agglutinin was used to isolate Chinese hamster ovary (CHO) glycosylation mutants that do not have α 2,3-linked sialic acid on their surfaces, which made them resistant to the lectin’s toxic effects.

[0028] As used herein, the term “Chinese hamster ovary (CHO) cell mutants” or “modified Chinese hamster ovary (CHO) cells” refers to Chinese hamster ovary cells that have genetic mutations or modifications affecting their glycosylation processes. Specifically, the term refers to a cell wherein one or more genes involved in the glycosylation pathways have been altered, modified, mutated, or engineered, which can lead to abnormal glycosylation patterns.

[0029] As used herein, the term “biotherapeutics” refers to therapeutic products derived from biological sources or produced using biological processes. Examples of biotherapeutics include, but are not limited to, antibodies, recombinant proteins, immunomodulatory molecules, cytokines, growth factors, enzymes, hormones, erythropoietin, cell and gene therapy products, cell-based products, protein-based products, gene-silencing/editing therapies, tissue-engineered products, stem cell therapies, and vaccines.

[0030] As used herein, the monosaccharide abbreviations, including but not limited to glucose (Glc), galactose (Gal), mannose (Man), fucose (Fuc), *N*-acetylneuraminic acid (Neu5Ac), and *N*-acetylglucosamine (GlcNAc).

[0031] Further, the terms “CHO cell mutants”, “CHO glycosylation mutants (CHO gmts)”, and “modified Chinese hamster ovary (CHO) cells” are used interchangeably in the present disclosure. All these terms refer to Chinese hamster ovary (CHO) cells that have been genetically modified or mutated as disclosed above.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

- [0032] Protein glycosylation is the most common yet complicated post-translational modification that is critical for protein folding, stability, and activity. The inherent heterogeneity of biotherapeutics due to glycosylation can impact their safety and efficacy. Therefore, modifying glycan structures of biotherapeutics is important for ensuring drug safety and efficacy.
- 5 For instance, in the case of antibody therapeutics, the removal of core fucose from the *N*-glycan can enhance its affinity towards FcγRIIIa on natural killer (NK) cells, thereby improving its antibody-dependent cellular cytotoxicity (ADCC) activity. Additionally, Cerezyme, a recombinant human β-glucocerebrosidase (GCase), used to treat Gaucher's disease, requires enzymatic modification of its *N*-glycans to expose core mannose residues. These residues can be
- 10 recognised by mannose receptors and incorporated into macrophages, where its substrate, Glc-Cer, is accumulated.
- [0033] Furthermore, the human immunodeficiency virus (HIV) envelope glycoprotein, gp120, plays a key role in virus entry into CD4+ cells. The gp120 is heavily glycosylated, primarily with immature high mannose type glycans, while only a small fraction of the gp120 *N*-
- 15 glycans are processed to complex-type glycans. Studies have shown that a significant proportion of broadly neutralising anti-HIV antibodies (bnAbs) bind to glycan or glycan-peptide combinations on gp120. Therefore, producing gp120 with specific glycans attached is important for studying broadly neutralising anti-HIV antibodies and advancing understanding of HIV infection and immune response.
- 20 [0034] Mammalian cell expression systems are generally preferred for producing biotherapeutics because the glycans produced in these systems closely resemble those found in human cells, thus reducing the risk of potential adverse immunological reactions to the biotherapeutics.
- [0035] The present disclosure describes a modified mammalian cell for use in expression
- 25 systems to produce recombinant glycoproteins with specific glycan structures. Examples of such mammalian cells include, but are not limited to, Chinese hamster ovary (CHO) cells, Baby Hamster Kidney (BHK) cells, NS0 or Sp2/0 murine myeloma cells, and human embryonic kidney 293 (HEK293) cells. In one example, the modified mammalian cells for use in expression systems are Chinese hamster ovary (CHO) cells. In one example, the CHO cell is CHO-K1 as
- 30 designated according to the ATCC catalogue. In one example, there is described a modified Chinese hamster ovary (CHO) cell for use in expression systems to enhance the cell's ability to produce glycoproteins with specific glycan structures. In one example, the glycan structures are homogenous. In another example, the glycan structures are homogeneous Man5 *N*-glycans and/or homogeneous Man9 *N*-glycans.

[0036] In one example, the modified mammalian cell is a glycosylation mutant with mutations in the genes responsible for glycosylation. In one example, the genetic mutations can be, but not limited to, an insertion (also called an insertion mutation) that involves the addition of one or more nucleotides into a segment of DNA; a deletion (also called a deletion mutation) that involves the deletion of one or more nucleotides from the gene or deletion of the entire gene from the genome; a point mutation that involves a change in a single nucleotide base in the DNA sequence by addition, deletion, or substitution; a frameshift mutation that alters the reading frame of the codons that occurs when nucleotides are inserted or deleted from the DNA sequence in numbers that are not multiples of three; a knock-out mutation where one or more specific genes are inactivated or “knocked-out” from an organism, and combinations thereof. In one example, there is described a modified mammalian cell comprising at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, or at least 13 gene mutations. In another example, the target of the gene mutation, can be, but is not limited to, MAN2A1, SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof, as well as combinations thereof.

[0037] In one example, the modified mammalian cell comprises at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, or at least 13 gene mutations. In one example, the target of the gene mutation can be, but is not limited to, MAN2A1, SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof, and combinations thereof.

[0038] In one example, the modified mammalian cell comprises at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, or at least 13 gene mutations, wherein one of the gene mutations is MAN2A1 or a homologue thereof, wherein the remaining gene mutations are selected from the group consisting of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof. In one example, the modified mammalian cell comprises at least 3 gene mutations, wherein one of the gene mutations is MAN2A1 or a homologue thereof, wherein the remaining gene mutations are selected from the group consisting of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof. In one example, the modified mammalian cell comprises at least 4 gene mutations, wherein one of the gene mutations is MAN2A1 or a homologue thereof, wherein the remaining gene mutations are selected from the group consisting

of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof. In one example, the modified mammalian cell comprises at least 5 gene mutations, wherein one of the gene mutations is MAN2A1 or a homologue thereof, wherein the remaining gene mutations are selected from the group consisting of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof. In one example, the modified mammalian cell comprises at least 6 gene mutations, wherein one of the gene mutations is MAN2A1 or a homologue thereof, wherein the remaining gene mutations are selected from the group consisting of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof. In one example, the modified mammalian cell comprises at least 7 gene mutations, wherein one of the gene mutations is MAN2A1 or a homologue thereof, wherein the remaining gene mutations are selected from the group consisting of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof. In one example, the modified mammalian cell comprises at least 8 gene mutations, wherein one of the gene mutations is MAN2A1 or a homologue thereof, wherein the remaining gene mutations are selected from the group consisting of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof. In one example, the modified mammalian cell comprises at least 9 gene mutations, wherein one of the gene mutations is MAN2A1 or a homologue thereof, wherein the remaining gene mutations are selected from the group consisting of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof. In one example, the modified mammalian cell comprises at least 10 gene mutations, wherein one of the gene mutations is MAN2A1 or a homologue thereof, wherein the remaining gene mutations are selected from the group consisting of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof. In one example, the modified mammalian cell comprises at least 11 gene mutations, wherein one of the gene mutations is MAN2A1 or a homologue thereof, wherein the remaining gene mutations are selected from the group consisting of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof. In one example, the modified mammalian cell comprises at least 12 gene mutations, wherein one of the gene mutations is MAN2A1 or a homologue thereof, wherein the remaining gene mutations are selected from the group consisting of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof.

MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof. In one example, the modified mammalian cell comprises at least 13 gene mutations, wherein one of the gene mutations is MAN2A1 or a homologue thereof, wherein the remaining gene mutations are selected from the group consisting of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof.

[0039] In one example, the modified mammalian cell comprises 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13 gene mutations. In one example, the modified mammalian cell comprises 3 gene mutations. In one example, the modified mammalian cell comprises 4 gene mutations. In one example, the modified mammalian cell comprises 5 gene mutations. In one example, the modified mammalian cell comprises 6 gene mutations. In one example, the modified mammalian cell comprises 7 gene mutations. In one example, the modified mammalian cell comprises 8 gene mutations. In one example, the modified mammalian cell comprises 9 gene mutations. In one example, the modified mammalian cell comprises 10 gene mutations. In one example, the modified mammalian cell comprises 11 gene mutations. In one example, the modified mammalian cell comprises 12 gene mutations. In one example, the modified mammalian cell comprises 13 gene mutations. In one example, the target of the gene mutation can be, but is not limited to, MAN2A1, SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof, and combinations thereof.

[0040] In one example, the modified mammalian cell comprises 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13 gene mutations, wherein one of the gene mutations is MAN2A1 or a homologue thereof, wherein the remaining gene mutations are selected from the group consisting of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof. In one example, the modified mammalian cell comprises 3 gene mutations, wherein one of the gene mutations is MAN2A1 or a homologue thereof, wherein the remaining gene mutations are selected from the group consisting of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof. In one example, the modified mammalian cell comprises 4 gene mutations, wherein one of the gene mutations is MAN2A1 or a homologue thereof, wherein the remaining gene mutations are selected from the group consisting of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof. In one example, the modified mammalian cell comprises 5 gene mutations, wherein one of the gene mutations is MAN2A1 or a homologue thereof, wherein the remaining gene mutations are selected from the group consisting of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof.

thereof, wherein the remaining gene mutations are selected from the group consisting of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof. In one example, the modified mammalian cell comprises 6 gene mutations, wherein one of the gene mutations is MAN2A1 or a homologue thereof, wherein the remaining gene mutations are selected from the group consisting of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof. In one example, the modified mammalian cell comprises 7 gene mutations, wherein one of the gene mutations is MAN2A1 or a homologue thereof, wherein the remaining gene mutations are selected from the group consisting of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof. In one example, the modified mammalian cell comprises 8 gene mutations, wherein one of the gene mutations is MAN2A1 or a homologue thereof, wherein the remaining gene mutations are selected from the group consisting of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof. In one example, the modified mammalian cell comprises 9 gene mutations, wherein one of the gene mutations is MAN2A1 or a homologue thereof, wherein the remaining gene mutations are selected from the group consisting of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof. In one example, the modified mammalian cell comprises 10 gene mutations, wherein one of the gene mutations is MAN2A1 or a homologue thereof, wherein the remaining gene mutations are selected from the group consisting of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof. In one example, the modified mammalian cell comprises 11 gene mutations, wherein one of the gene mutations is MAN2A1 or a homologue thereof, wherein the remaining gene mutations are selected from the group consisting of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof. In one example, the modified mammalian cell comprises 12 gene mutations, wherein one of the gene mutations is MAN2A1 or a homologue thereof, wherein the remaining gene mutations are selected from the group consisting of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof. In one example, the modified mammalian cell comprises 13 gene mutations, wherein one of the gene mutations is MAN2A1 or a homologue thereof, wherein the remaining gene mutations are selected from the

- group consisting of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof.
- [0041] In one example, the modified mammalian cell comprises three (3) gene mutations, wherein the mutations are present in the following three genes: SLC35C1, MGAT1, MAN2A1, or homologues thereof. In another example, the modified mammalian cell comprises three (3) gene mutations, wherein the three gene that comprise mutations are SLC35C1, MGAT1, MAN2A1, or homologues thereof. In another example, the mutated genes comprise mutations as shown in SEQ ID NO: 11 and/or SEQ ID NO: 12; and SEQ ID NO: 41 and/or SEQ ID NO: 42. In another example, the MGAT1 gene is not expressed. In another example, the mutations disclosed herein can be mapped to corresponding positions or regions in a homologue sequence. In one example, the proteins expressed by the modified mammalian cell comprise of high-mannose type *N*-glycans. In another example, the high-mannose type *N*-glycans comprise of at least 90% Man5 *N*-glycans. In yet another example, the proteins expressed by the modified mammalian cell comprise of high-mannose type *N*-glycans, wherein the high-mannose type *N*-glycans comprise of at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 100% Man5 *N*-glycans.
- [0042] In one example, the modified mammalian cell comprises four (4) gene mutations, wherein the mutations are present in the following genes: SLC35C1, MGAT1, MAN2A1, MAN2A2, or homologues thereof. In another example, the modified mammalian cell comprises four (4) gene mutations, wherein the four genes that comprise mutations are SLC35C1, MGAT1, MAN2A1, MAN2A2, or homologues thereof. In another example, the mutated genes comprise mutation as shown in SEQ ID NO: 11 and/or SEQ ID NO: 12; SEQ ID NO: 41 and/or SEQ ID NO: 42; and SEQ ID NO: 47. In another example, the MGAT1 gene is not expressed. In another example, the mutations disclosed herein can be mapped to corresponding positions or regions in a homologue sequence. In one example, the proteins expressed by the modified mammalian cell comprise of high-mannose type *N*-glycans. In another example, the high-mannose type *N*-glycans comprise of at least 90% Man5 *N*-glycans. In another example, the proteins expressed by the modified mammalian cell comprise of high-mannose type *N*-glycans, wherein the high-mannose type *N*-glycans comprise of at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 100% Man5 *N*-glycans.
- [0043] In one example, the modified mammalian cell comprises ten (10) gene mutations, wherein the mutations are present in the following genes: MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2A1, MAN2A2, or homologues thereof.

In another example, the modified mammalian cell comprises ten (10) gene mutations, wherein the ten genes that comprise mutations are MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2A1, MAN2A2, or homologues thereof. In another example, the mutated genes comprise mutation as shown in SEQ ID NO: 58 and/or SEQ ID NO: 59; SEQ ID NO: 64; SEQ ID NO: 69 and/or SEQ ID NO: 70; SEQ ID NO: 73; SEQ ID NO: 78, SEQ ID NO: 79, and/or SEQ ID NO: 80; SEQ ID NO: 82; SEQ ID NO: 84; SEQ ID NO: 86; SEQ ID NO: 43; and SEQ ID NO: 48 and/or SEQ ID NO: 49. In another example, the mutations disclosed herein can be mapped to corresponding positions or regions in a homologue sequence. In one example, the proteins expressed by the modified mammalian cell comprise of high-mannose type *N*-glycans. In another example, the proteins expressed by the modified mammalian cell comprise of high-mannose type *N*-glycans, wherein the high-mannose type *N*-glycans comprise of at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 100% Man9 *N*-glycans. In another example, the high-mannose type *N*-glycans is at least 94% Man9 *N*-glycans.

[0044] In one example, the modified mammalian cell comprises eleven (11) gene mutations, wherein the mutations are present in the following eleven genes: MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2A1, MAN2A2, MAN2B1, or homologues thereof. In another example, the modified mammalian cell comprises eleven (11) gene mutations, wherein the eleven genes that comprise mutations are MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2A1, MAN2A2, MAN2B1, or homologues thereof. In another example, the mutated genes comprise mutations as shown in SEQ ID NO: 58 and/or SEQ ID NO: 59; SEQ ID NO: 64; SEQ ID NO: 69 and/or SEQ ID NO: 70; SEQ ID NO: 73; SEQ ID NO: 78, SEQ ID NO: 79, and/or SEQ ID NO: 80; SEQ ID NO: 82; SEQ ID NO: 84; SEQ ID NO: 86; SEQ ID NO: 39, SEQ ID NO: 44 and/or SEQ ID NO: 45; SEQ ID NO: 50 and/or SEQ ID NO: 51; and SEQ ID NO: 53 and/or SEQ ID NO: 54. In another example, the mutations disclosed herein can be mapped to corresponding positions or regions in a homologue sequence. In one example, the proteins expressed by the modified mammalian cell comprise of high-mannose type *N*-glycans. In another example, the high-mannose type *N*-glycans is at least 94% Man9 *N*-glycans. In another example, the proteins expressed by the modified mammalian cell comprise of high-mannose type *N*-glycans, wherein the high-mannose type *N*-glycans comprise of at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 100% Man9 *N*-glycans.

[0045] Protein glycosylation is a post-translation modification that involves various metabolic pathways and a variety of enzymes, leading to undesirable heterogeneity in the glycans attached to proteins. Efforts have been made to reduce the inherent difficulty of generating specific homogenous glycoproteins. In one example, disclosed herein is the use of cytotoxic lectin

5 *Maackia amurensis* agglutinin (MAA) to isolate glycosylation mutants. In one example, the glycosylation mutants that do not have α 2,3-linked sialic acid on their surfaces are isolated using the cytotoxic lectin *Maackia amurensis* agglutinin (MAA). Also disclosed herein is the use of *Ricinus communis* agglutinin-I (RCA-I) to isolate glycosylation mutants with inactive *N*-acetylglucosaminyltransferase I (GnT-I). In one example, disclosed herein is a Chinese hamster

10 ovary glycosylation mutant isolated using *Ricinus communis* agglutinin-I (RCA-I). In one example, the Chinese hamster ovary glycosylation mutant is isolated using *Ricinus communis* agglutinin-I (RCA-I) which has an inactive *N*-acetylglucosaminyltransferase I (GnT-I).

[0046] Advancements in genome editing technologies have improved the ability to generate glycosylation mutants capable of producing recombinant glycoproteins with specific glycan

15 structures. In one example, genetic modifications of the modified mammalian cells are performed through gene editing methods including, but not limited to, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas system, Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and RNA interference (RNAi). These gene modifications can affect various aspects of glycosylation, including the addition,

20 trimming, or modification of glycan structures. In one example, the present disclosure describes the method of Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated Protein 9 (CRISPR-Cas9) system to delete genes.

[0047] Also disclosed herein is a panel of Chinese hamster ovary glycosylation mutants generated by either cytotoxic lectin selection or genome editing. The gene mutations of the

25 various Chinese hamster ovary glycosylation mutants are listed in Table 1, while Table 2 provides a list of their corresponding gene mutations.

[0048] **Table 1. CHO glycosylation mutants and the corresponding inactivated genes.** Mutated genes in Chinese hamster ovary cell glycosylation mutants (CHO-gmt). Mutated genes in CHO cell glycosylation mutants are represented by gene symbols in the table. The

30 corresponding names of the gene symbols are as follows: SLC35A1 (CMP sialic acid transporter), SLC35A2 (UDP galactose transporter), SLC35C1 (GDP fucose transporter), MGAT1 (GnTI / α -1,3-mannosyl-glycoprotein 2- β -N-acetylglucosaminyltransferase), MGAT2 (GnTII / α -1,6-mannosyl-glycoprotein 2- β -N-acetylglucosaminyltransferase), MGAT4A (GnTIVa / α -1,3-mannosyl-glycoprotein 4- β -N-acetylglucosaminyltransferase A), MGAT4B

(GnTIVb / α -1,3-mannosyl-glycoprotein 4- β -N-acetylglucosaminyltransferase B), MGAT5 (GnTV / α -1,6-mannosylglycoprotein 6- β -N-acetylglucosaminyltransferase), MAN2A1 (α -mannosidase II), MAN2A2 (α -mannosidase II X), MAN1A1 (α -mannosidase IA), MAN1A2 (α -mannosidase IB), MAN1C1 (α -mannosidase IC), MANEA (endo α -mannosidase I), MAN1B1 (ER α -mannosidase I), EDEM1 (ER degradation-enhancing α -mannosidase-like protein 1), EDEM2 (ER degradation-enhancing α -mannosidase-like protein 2), EDEM3 (ER degradation-enhancing α -mannosidase-like protein 3), MAN2B1 (lysosomal α -mannosidase).

Cell line	Mutations
CHO-K1	
CHO-gmt1	SLC35A1
CHO-gmt2	SLC35A2
CHO-gmt3	SLC35C1
CHO-gmt4	MGAT1
CHO-gmt5	SLC35A1, SLC35C1
CHO-gmt6	SLC35A1, MGAT1
CHO-gmt7	SLC35A2, MGAT1
CHO-gmt8	SLC35A1, SLC35C1, MGAT1
CHO-gmt9	SLC35A2, SLC35C1
CHO-gmt10	SLC35A1, SLC35A2
CHO-gmt11	SLC35A1, SLC35A2, MGAT1
CHO-gmt12	SLC35C1, MGAT1
CHO-gmt13	SLC35A2, MGAT2
CHO-gmt14	SLC35A2, SLC35C1, MGAT2
CHO-gmt15	SLC35A2, MGAT4A, MGAT4B, MGAT5
CHO-gmt17	SLC35C1, MGAT1, MAN2A1
CHO-gmt17x	SLC35C1, MGAT1, MAN2A1, MAN2A2
CHO-gmt18	MAN1A1, MAN1A2, MAN1C1, MANEA
CHO-gmt19	MAN1A1, MAN1A2, MAN1C1, MAN1B1
CHO-gmt20	MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1
CHO-gmt21	MGAT2
CHO-gmt22	SLC35C1, MGAT2
CHO-gmt26	MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3
CHO-gmt29	MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1
CHO-gmt30	MAN2A1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, EDEM1, EDEM2, EDEM3
CHO-gmt32	MAN2A1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3
CHO-gmt35	MAN2A1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1

[0049] **Table 2. Mutations identified in target genes.** The wild-type gene sequences are provided for comparison. Insertion mutations are in bold font, while deletion mutations as dashed lines. The underlined sequences represent the target editing sites (for example, when using CRISPR or TALEN). Multiple mutants from the same original clone that share the same

gene mutation are grouped together. Allelic mutations (if applicable) for one allele are show in “unnamed” boxes beneath the mutations shown for the other allele.

Gene	Cell line	Partial sequence	Remarks
Slc35a1	CHO-K1	TGGTGTATGCTGTTT C AGAATAACATGGCTTTCC (SEQ ID NO: 1)	
	CHO-gmt1, gmt5, gmt8	TGGTGTATGCTGTTT A GAATAACATGGCTTTCC (SEQ ID NO: 2)	C>T
	CHO-K1	TTTATTATTGCTTCTAGGTTACCTATCAACTGAAGA (SEQ ID NO: 3)	
	CHO-gmt6	TTTATTATTGCTTCTACGTTACCTATCAACTGAAGA (SEQ ID NO: 4)	G>C (intron 3' splice site)
	CHO-K1	CATTCAAATGTATCTGT C AGGGATCGTGGTGACSTTAGTTGGTACCTACTTA TCAGATGGAGC (SEQ ID NO: 5)	
	CHO-gmt10	CATTCAAATGTATCTGT C AGGGATCG----- CTTATCAGATGGAGC (SEQ ID NO: 6; the remaining sequence after deletion corresponds to SEQ ID NO: 5)	-22
	CHO-gmt11	CATTCAAATGTA----- CCTACTTATCAGATGGAGC (SEQ ID NO: 7; the remaining sequence after deletion corresponds to SEQ ID NO: 5)	-33
Slc35a2	CHO-K1	CGCCTCTTTGGCTTTCACCTGGACCCATTA (SEQ ID NO: 8)	
	CHO-gmt2, gmt7, gmt9, gmt10, gmt11, gmt13, gmt14, gmt15	CGCCTCTTTGGCTT T CACCTGGACCCATTA (SEQ ID NO: 9)	+1
Slc35c1	CHO-K1	ATGATAAGTTTCAATAACCTCTGCCTCAAGTACGTAGGGGTGGCCTTCTACA ACGTGGGG (SEQ ID NO: 10)	
	CHO-gmt3, gmt12, gmt17	ATGATAAGTTTCAATAACCTCTGCCTCAAG- CGTAGGGGTGGCCTTCTACAACGTGGGG (SEQ ID NO: 11; the remaining sequence after deletion corresponds to SEQ ID NO: 10)	-2
		ATGATAAGTTTCAATAACCT----- ---TGGGG (SEQ ID NO: 12; the remaining sequence after deletion corresponds to SEQ ID NO: 10)	-35
	CHO-gmt5, gmt8	ATGATAAGTTTCAATAACCTCTGCCTCAA--- CGTAGGGGTGGCCTTCTACAACGTGGGG (SEQ ID NO: 13; the remaining sequence after deletion corresponds to SEQ ID NO: 10)	-3
		ATG----- CCTTCTACAACGTGGGG (SEQ ID NO: 14; the remaining sequence after deletion corresponds to SEQ ID NO: 10)	-40
	CHO-gmt9, gmt14	ATGATAAGTTTCAATAACCTCTGCCTCAAG T AGTACGTAGGGGTGGCCTTCT ACAACGTGGGG (SEQ ID NO: 15)	+3
Mgat1		ATGATAAGTTTCAATAACCTCTGCCTCAAG TAA STACGTAGGGGTGGCCTTCTACAACGTGGGG (SEQ ID NO: 16)	+4
	CHO-K1	AGTTCATCAAGCTGAACCGAGTTCGTGTCTTTACCCCA (SEQ ID NO: 17)	
	CHO-gmt4	AGTTCATCAAGCTGAACCGAG T AGTTCGTGTCTTTACCCCA (SEQ ID NO: 18)	C>T
	CHO-K1	CCCCGTGTGCCTGCGACCCCTCACCAGCCGTGATCCC (SEQ ID NO: 19)	
	CHO-gmt7, gmt11	CCCCGTGTGCCTGCGACCCCTCACCAGCCGTGATCCC (SEQ ID NO: 20)	+1
	CHO-K1	CATCATTGT C AGCCAGGACTGCGGGC (SEQ ID NO: 21; AAAGTGAGGACCAATGATCAGAAGGAGCTGGGGG (SEQ ID NO: 22)	
	CHO-gmt8	CATCATTGT C AG G CAGGACTGCGGGC (SEQ ID NO: 23); AAAGTGAGSACCGATGATCAGAAGGAGCTGGGGG (SEQ ID NO: 24)	C>G, A>G
	CHO-gmt6		not express

			ed
	CHO-gmt12, gmt17		not express ed
Mgat2	CHO-K1	CCAGGTGCATAACCGGCCGAATACCTCAGGCTGCTGCTAGACTCGCTTCGA AAA (SEQ ID NO: 25)	
	CHO-gmt13	CCAGGTGCATA----- CCTCAGGCTGCTGCTAGACTCGCTTCGAAAA (SEQ ID NO: 26; the remaining sequence after deletion corresponds to SEQ ID NO: 25)	-13
	CHO-gmt14	CCAGGTGCATAACCGGCCG- AATACCTCAGGCTGCTGCTAGACTCGCTTCGAAAA (SEQ ID NO: 27; the remaining sequence after deletion corresponds to SEQ ID NO: 25)	-1
		CCAGG----- CTGCTGCTAGACTCGCTTCGAAAA (SEQ ID NO: 28; the remaining sequence after deletion corresponds to SEQ ID NO: 25)	-26
Mgat4a	CHO-K1	GCTCTTCCAGCATGTGGGTCTACATTCGTCACCTGTCGGGGAAAAATCCAGAAA (SEQ ID NO: 29)	
	CHO-gmt15	GCTCTTCCAGCATGTGGGTCTACATTCGTCACCT-TCGGGGAAAAATCCAGAAA (SEQ ID NO: 30; the remaining sequence after deletion corresponds to SEQ ID NO: 29)	-1
		GCTCTTCCAGCATGTGGGTCTACATTCGTC----- ...GAAAGSTCAGTGACAATGGAATTC (SEQ ID NO: 31; the remaining sequence after deletion corresponds to SEQ ID NO: 29)	-304
Mgat4b	CHO-K1	CCAGCAAAATCTAACCTGTCCCTTCCCCAACCCAGAGGATCCGCGACTG AAGCCGTGGAA (SEQ ID NO: 32)	
	CHO-gmt15	CCAGCAAAATCTAACCTG----- AAGCCGTGGAA (SEQ ID NO: 33; the remaining sequence after deletion corresponds to SEQ ID NO: 32)	-34
Mgat5	CHO-K1	GCATGAGGAGTCCGGTGGATGAGACTTCGGATCCGGCGAATGGCTGATGCG TGGATCCAA (SEQ ID NO: 34)	
	CHO-gmt15	GCATGAGGAGTCCGGTGGATGAGACTTCGGATCCGGCGAATGGCTGATGCG TGGATCCAA (SEQ ID NO: 35)	+1
Man2a1	CHO-K1	TTCCAGCTCCTACGCTAAGACGTCCCGGTGGGATCTGCTGTTCTCA (SEQ ID NO: 36)	
	CHO-gmt29	TTCCAGCTCCTACGCTAAGACG AT CCCGGTGGGATCTGCTGTTCTCA (SEQ ID NO: 37)	+1
		TTCCAGCTCCTACGCTAAGACG TT CCCGGTGGGATCTGCTGTTCTCA (SEQ ID NO: 38)	+1
	CHO-gmt35	TTCCAGCTCCTACGCTAAGACG AT CCCGGTGGGATCTGCTGTTCTCA (SEQ ID NO: 39)	+1
		TTCCAGCTCCTACGCTAAGACG TT CCCGGTGGGATCTGCTGTTCTCA (SEQ ID NO: 40)	+1
	CHO-gmt17, gmt17X	AAACGGATGGATAGAATCATGGAATCGCGC CT TGAGGTACGAGGCCTTGAAA (SEQ ID NO: 41)	+1
		AAACGGATGGATAGAATCAT-----TGAGGTACGAGGCCTTGAAA (SEQ ID NO: 42; the remaining sequence after deletion corresponds to SEQ ID NO: 36)	-11
	CHO-gmt32	AAACGGATGGATAGAATCATGGAATCGCGC CT TGAGGTACGAGGCCTTGAAA (SEQ ID NO: 43)	+1
	CHO-gmt35	AAACGGATGGATAGAATCATGGAATCGCG-----AGGCCTTGAAA (SEQ ID NO: 44; the remaining sequence after deletion corresponds to SEQ ID NO: 36)	-11
		AAACGGATGGATAGAATCATGGAATCGCG CTT TGAGGTACGAGGCCTTGAAA (SEQ ID NO: 45)	+1
Man2a2	CHO-K1	TGTCCAAGCTGCAGGAAGACCCCTCGACGACGCTTTCTCTGGGCAGAAGTCTC CTTCTTTGCCAAGT (SEQ ID NO: 46)	
	CHO-gmt17X	TGTCCAAGCTGCAGGAAGACCCCTCGACCGACGCTTTCTCTGGGCAGAAGTCT CCTTCTTTGCCAAGT (SEQ ID NO: 47)	+1
	CHO-gmt32	TGTCCAAGCTGCAGGAAGACCCCTCGACCGACGCTTTCTCTGGGCAGAAGTCT CCTTCTTTGCCAAGT (SEQ ID NO: 48)	+1
		TGTCCAAGCTGCAGGAAGACCCCTCGAC CG CGACGCTTTCTCTGGGCAGAAGTCT TCCTTCTTTGCCAAGT (SEQ ID NO: 49)	+2

	CHO-gmt35	TGTCCAAGCTGCAGGAAGACCCCTCGACCGACGCTTCTCTGGGCAGAACTCTCCTTCTTTGCCAAGT (SEQ ID NO: 50)	+1
		TGTCCAAGCTGCAGGAAGACCCCTCGA- AAGCG -- TCTCTGGGCAGAACTCTCCTTCTTTGCCAAGT (SEQ ID NO: 51; the remaining sequence after deletion corresponds to SEQ ID NO: 46)	-8, +5
Man2b1	CHO-K1	TTCCAGCTCCTACGSTAAGACGTCCCGGTGGGATCTGCTGTTCTCA (SEQ ID NO: 52)	
	CHO-gmt35	TTCCAGCTCCTACGSTAAGACG AT CCCGGTGGGATCTGCTGTTCTCA (SEQ ID NO: 53)	+1
		TTCCAGCTCCTACGSTAAGACG TT CCCGGTGGGATCTGCTGTTCTCA (SEQ ID NO: 54)	+1
Man1a1	CHO-K1	TGTTTTTGAAGTAAATATACGCTTCGTTCGGTGGACTGCTGTCA (SEQ ID NO: 55)	
	CHO-gmt18	TGTTTTTGAAGTAAATATACGCTTCGT TC GGTGGACTGCTGTCA (SEQ ID NO: 56)	+1
	CHO-gmt19	-----TCGGTGGACTGCTGTCA (SEQ ID NO: 57; the remaining sequence after deletion corresponds to SEQ ID NO: 55)	-26
	CHO-gmt20, gmt26, gmt29, gmt32, gmt35	TGTTTTTGAAGTAAATATACGCTTCGT G CGGTGGACTGCTGTCA (SEQ ID NO: 58)	+1
		TGTTTTTGAAGTAAATATACGCTTCGT GGATCTTCTGCTGTACACAGGCC TTAAATTTTCATCTTTGAGTGACTGCCCAACACAGACACCCCGACATTT GACACTCAATGACATGGAGCTTAAGGAAGGGTATGGTCTGGACACCCCAAC ACTGGAGGTTAGGTACACCCATAGTAGTAGGCGAGACGGTGGACTGCTGTCA (SEQ ID NO: 59)	+165
Man1a2	CHO-K1	AAAAACCCCGGAGTCTTCCTGATCCATGGACCCGACGAACACAGACACAGGTTCGTTTGTTTTT (SEQ ID NO: 60)	
	CHO-gmt18	AAAAACCCCGGAGTCTTCCTGATCCATGGAA ACCCGACGAACACAGACACAGG TTCGTTTGTTTTT (SEQ ID NO: 61)	+1
		AAAAACCCCGGAGTCTTCCTGATCCATGGAA- TCGACGAACACAGACACAGGTTTCGTTTGTTTTT (SEQ ID NO: 62)	-2, +1
	CHO-gmt19	AAAAACCCCGGAGTCTTCCTGATCCATGGAG CAAGAGGGTGATTCTGGCCGA CGCGAACCTGGATAAGGTCTCAGCGCTACAAACAGCACCCGAGACAAACCC ATCAGGGAGCAGGCCGAGAATATCATAATACTAGTCAATAATCAATGTCAA CGCGTATATCTGGCCCGTACATCGCGAAACCCGACGAACACAGACACA GGTTCGTTTGTTTTT (SEQ ID NO: 63)	+155
	CHO-gmt20, gmt26, gmt29, gmt32, gmt35	AAAAACCCCGGAGTCTTCCTGATCCATGGAA ACCCGACGAACACAGACACAGG TTCGTTTGTTTTT (SEQ ID NO: 64)	+1
Man1c1	CHO-K1	TTTGCCTGGCAGAATTACCGCCGTTATGCGATGGGCAAGAATGAGCTCCGGCCACTCACAAA (SEQ ID NO: 65)	
	CHO-gmt18	TTTGCCTGGCAGAATTACCGCCGTTA---- ATGGGCAAGAATGAGCTCCGGCCACTCACAAA (SEQ ID NO: 66; the remaining sequence after deletion corresponds to SEQ ID NO: 65)	-4
	CHO-gmt19	TTTGCCTGGCAGAATTACCGCCGTTAT GCGCGATGGGCAAGAATGAGCTCCG GCCACTCACAAA (SEQ ID NO: 67)	+2
		TTTGCCTGGCAGAATTACCGCCGTTAT- CGATGGGCAAGAATGAGCTCCGGCCACTCACAAA (SEQ ID NO: 68; the remaining sequence after deletion corresponds to SEQ ID NO: 65)	-1
	CHO-gmt20, gmt26, gmt29, gmt32, gmt35	TTTGCCTGGCAGAATTACCGCCGTTA-- CGATGGGCAAGAATGAGCTCCGGCCACTCACAAA (SEQ ID NO: 69; the remaining sequence after deletion corresponds to SEQ ID NO: 65)	-2
		TTTGCCTGGCAGAATTACCGCCGTTATG----- TGAGCTCCGGCCACTCACAAA (SEQ ID NO: 70; the remaining sequence after deletion corresponds to SEQ ID NO: 65)	-13
Manea	CHO-K1	AAAGCCTAATGTGTGGCCAATCTGTTAACACCTTCAGGATCTCAGAGTGTT	

		CGCAAT (SEQ ID NO: 71)	
	CHO-gmt18	AAAGCCTAATGTGTGGGCCAATCTGTTAACA- CTTCAGGATCTCAGAGTGTTCGCAAT (SEQ ID NO: 72; the remaining sequence after deletion corresponds to SEQ ID NO: 71)	-1
	CHO-gmt20, gmt26, gmt29, gmt32, gmt35	AAAGCCTAATGTGTGGGCCAATCTGTTAACAC- TTCAGGATCTCAGAGTGTTCGCAAT (SEQ ID NO: 73; the remaining sequence after deletion corresponds to SEQ ID NO: 71)	-1
Man1b1	CHO-K1	GACAGGCTGAGCCCTGAGATTGCACACTTCAACCTGTACACTCGGGCGGAT CATAAGGATGTGGAGGTCAAGGTGAGCCTGGGCATAGGCAGCATTGGGTAA GGGTGATGCCAGCATGGGTGGATACTCCCTAAGCCTCCTGCTCTGCCCT (SEQ ID NO: 74)	
	CHO-gmt19	CACATGGATCTGGCTCAGGCACTCATGGAGACCTGCTATGAGATGAACCGAC AGATGGAGACAGGTCAAGGTGAGCCTGGGCATAGGCAGCATTGGGTAAAGGG TGATGCCAGCATGGGTGGATACTCCCTAAGCCTCCTGCTCTGCCCT (SEQ ID NO: 75)	various indels
		CACATGGATCTGGCTCAGGCACTCATGGAGACCTGCTATGAGATGAACCGGC AGATGGAGACAGGTCTGAGCCCTGAGATTGCACACTTCAACCTGTACACTCG GGCGGCCCT (SEQ ID NO: 76)	various indels
	CHO-K1	GAGATTGCACACTTCAACCTGTACACTCGGGCGGATCATAAGGATGTGGGAG GTCAAGG (SEQ ID NO: 77)	
	CHO-gmt20, gmt26, gmt29, gmt32, gmt35	GAGATTGCACACTTCAACCTGTACACTCGGGC----- AAGGATGTGGAGGTCAAGG (SEQ ID NO: 78; the remaining sequence after deletion corresponds to SEQ ID NO: 77)	-7
		GAGATTGCACACTTCAACCTGTACATTTCGGGCGG----- GATGTGGGAGGTCAAGG (SEQ ID NO: 79; the remaining sequence after deletion corresponds to SEQ ID NO: 77)	-8
		GAGATTGCACACTTCAACCTGTACATTTCGGGTGG----- GATGTGGGAGGTCAAGG (SEQ ID NO: 80; the remaining sequence after deletion corresponds to SEQ ID NO: 77)	-8
Edem1	CHO-K1	CGGTACGGGGCCCTCCCTGAGCGCTATAACTGGCAACTCCAGGCCCC (SEQ ID NO: 81)	
	CHO-gmt20, gmt26, gmt29, gmt32, gmt35	CGGTAC-----TGGCAACTCCAGGCCCC (SEQ ID NO: 82; the remaining sequence after deletion corresponds to SEQ ID NO: 81)	-24
Edem2	CHO-K1	TGCACCATGGTGCGCCAGGCCCGAGCGCACCGCGCCCGACCCGCCCCACTA CAGGTGAA (SEQ ID NO: 83)	
	CHO-gmt20, gmt26, gmt29, gmt32, gmt35	CGGTAC-----TGGCAACTCCAGGCCCC (SEQ ID NO: 84; the remaining sequence after deletion corresponds to SEQ ID NO: 83)	-24
Edem3	CHO-K1	TTTCATGCAGGAGATGATTGAACTGTCAAGTCAGCAACAAAAAGAGCAGCAGC TACCTCCACGAGCCGTACAAATTATTTCCACCCGTTTTTTGGCAGGGTAGT CTTAAC (SEQ ID NO: 85)	
	CHO-gmt20, gmt26, gmt29, gmt32, gmt35	TTTCATGCAGGAGATGATTGAACTGTCAAGTCAGCAACAAAAAGAGCAGCAGC TACCTCCACGAGGCCGTACAAATTATTTCCACCCGTTTTTTGGCAGGGTAG TCTTAAC (SEQ ID NO: 86)	+1

[0050] Glycosylation abilities of each of the mutant CHO cells disclosed herein were evaluated using two model proteins: erythropoietin-Fc fusion (EPO-Fc) and an IgG1 antibody, trastuzumab. In the present disclosure, Hydrophilic Interaction Liquid Chromatography-Ultra
5 Performance Liquid Chromatography-Quadrupole Time-of-Flight mass spectrometry (HILIC-

UPLC-QTOF-MS) was utilised to quantify the abundance of each glycan. As compared to the commonly used Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF), the quantitative analysis disclosed herein demonstrates an improvement over structural characterisation alone of glycans by providing detailed insights into both the composition and quantification of individual glycans within glycan mixtures.

[0051] Disclosed herein is a modified mammalian cell is engineered to express a glycosylated protein, wherein the glycosylated proteins comprise high-mannose type *N*-glycans. In one example, the modified mammalian cell disclosed herein can be used to express, but is not limited to, antibodies, recombinant proteins, immunomodulatory molecules, cytokines, growth factors, enzymes, hormones, erythropoietin, cell and gene therapy products, cell-based products, protein-based products, gene-silencing/editing therapies, tissue-engineered products, stem cell therapies, and vaccines.

[0052] In one example, the present disclosure disclosed the influence of specific Fc glycans on antibody interactions with various Fc γ receptors and their impact on antibody-dependent cellular cytotoxicity (ADCC) activation. In another example, the present disclosure describes the influence of gp120 *N*-glycans on the interaction with broadly neutralising anti-gp120 antibodies using trimeric gp120 produced in CHO cell mutants. In yet another example, the CHO cell glycosylation mutants generated in the present disclosure demonstrated the production of human β -glucocerebrosidase with homogenous Man5 *N*-glycans.

20 **Chinese hamster ovary (CHO) cells with mutated nucleotide sugar transporters**

[0053] Nucleotide sugar transporters transfer nucleotide sugars from the nucleus or cytosol to the endoplasmic reticulum or Golgi apparatus, where they serve as substrates for glycosylation. In this disclosure, the cytotoxic lectin *Maackia amurensis* agglutinin (MAA) was used to isolate two Chinese hamster ovary cell glycosylation mutants, CHO-gmt1 (CHO-glycosylation mutant 1) and CHO-gmt2 (CHO-glycosylation mutant 2). The CHO-gmt2 disclosed herein lacks a functional UDP-galactose transporter. Using genome editing techniques, for example, such as the genome editing techniques disclosed herein, two other mutants, CHO-gmt3 and CHO-gmt5 were generated by inactivating the GDP-fucose transporter gene in CHO-K1 and CHO-gmt1 cells.

30 [0054] In this disclosure, hydrophilic interaction liquid chromatography-ultra performance liquid chromatography-quadrupole time-of-flight mass spectrometry (HILIC-UPLC-QTOF-MS) was used to analyse *N*-glycans from two model molecules, erythropoietin Fc (EPO-Fc) and trastuzumab, produced in various CHO mutants. The use of HILIC-UPLC-QTOF-MS was preferred over the commonly used MALDI-TOF due to its ability to provide insights into both

the composition and quantification of individual glycans within glycan mixtures. In the present disclosure, two model molecules, erythropoietin Fc (EPO-Fc) and trastuzumab, were produced in various Chinese hamster ovary cell glycosylation mutants (CHO-gmts). Fig. 1 and Fig. 2 show the glycan profiles of erythropoietin Fc or trastuzumab from these Chinese hamster ovary cell glycosylation mutants, along with quantitative HILIC-UPLC-QTOF-MS analysis of the different glycans.

[0055] The present disclosure demonstrated that CHO-K1 cells efficiently produced highly branched, core-fucosylated, and highly sialylated *N*-glycans on erythropoietin Fc (Fig. 1a). The asparagine 297 (Asn297) in the Fc of erythropoietin Fc had been mutated to alanine Ala, resulting in *N*-glycans exclusively originating from the erythropoietin segment. In contrast, CHO-K1-produced trastuzumab predominantly features sialic acid-free, core-fucosylated bi-antennary complex glycans with varying galactose residues (0, 1, or 2) (Fig. 2a). The *N*-glycans from CHO-gmt1-produced erythropoietin Fc and trastuzumab completely lack sialic acid, as shown in Fig. 1b and Fig. 2b.

[0056] Using the erythropoietin (EPO) rescue assay, a MAA-resistant CHO cell line with an inactive UDP-galactose transporter gene (*Slc35a2*) (Fig. 3) was identified. Sequencing of the *Slc35a2* cDNA revealed a 1-nucleotide insertion mutation causing a frameshift in the gene (Table 2). CHO cells lacking a functional UDP-galactose transporter are designated as CHO-gmt2 cells. Erythropoietin Fc from CHO-gmt2 cells mostly lacked galactose (Fig. 1c). Similarly, trastuzumab *N*-glycan structures from CHO-gmt2 cells were primarily fucosylated and devoid galactose (Fig. 2c), indicating that the UDP-galactose transporter gene (*Slc35a2*) was inactive.

[0057] Also disclosed herein are CHO-gmt3 cells, wherein the *Slc35c1* gene encoding GDP-fucose transporter, was inactivated in CHO-K1 cells using Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) and/or Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated Protein 9 (CRISPR-Cas9). Erythropoietin Fc and trastuzumab *N*-glycans from CHO-gmt3 cells lacked core fucose (Fig. 1d, Fig. 2d).

[0058] Another CHO-glycosylation mutant disclosed herein is CHO-gmt5 cells, which are generated using ZFN-mediated *Slc35c1* gene inactivation in CHO-gmt1 cells, producing *N*-glycans devoid of sialic acid and fucose (Fig. 1f, Fig. 2f). Additionally, CHO-gmt9 cells, generated by Zinc Finger Nucleases-mediated inactivation of the *Slc35c1* gene in CHO-gmt2 cells, produce fucose-free and galactose-free *N*-glycans on erythropoietin Fc and trastuzumab (Fig. 1j, Fig. 2j).

[0059] Fig. 4 shows a detailed overview of the CHO glycosylation mutants disclosed herein, as well as the mutations involved.

CHO cells with dysfunctional GnT-I gene

[0060] In this disclosure, *Ricinus communis* agglutinin-I (RCA-I) was used to isolate CHO-gmt4 cells. Using *Ricinus communis* agglutinin-I, the present disclosure demonstrated that only CHO cells with inactive *N*-acetylglucosaminyltransferase I (GnT-I) could survive RCA-I treatment. The erythropoietin rescue assay further revealed that over 200 surviving clones lacked functional *MGAT1* gene, which encodes *N*-acetylglucosaminyltransferase I. cDNA sequencing confirmed the presence of *N*-acetylglucosaminyltransferase I mutations in all RCA-I-resistant clones. The *N*-glycans of erythropoietin Fc produced from CHO-gmt4 were mainly fucosylated and non-fucosylated Man5 glycans, along with paucimannose structures like Man4 and Man3 and their fucosylated forms (Fig. 1e). In contrast, trastuzumab *N*-glycan structures from CHO-gmt4 were primarily fucose-free Man5 glycans, with minor amounts of Man4 and Man3 glycans (Fig. 2c). Without being bound by theory, it is thought that these findings disclosed herein indicate that the *N*-glycans on erythropoietin Fc are more susceptible to additional processing, like fucosylation, than those on IgG antibodies.

CHO cells with dysfunctional nucleotide sugar transporters and GnT-I

[0061] As compared to CHO-K1 cells, the CHO glycosylation mutants described in this disclosure, including CHO-gmt1, CHO-gmt2 and CHO-gmt5 cells, showed greater RCA-I sensitivity. The RCA-I-resistant cells were further isolated from each of these mutant lines. The isolated RCA-I-resistant cells were named CHO-gmt6, CHO-gmt7, and CHO-gmt8. Erythropoietin rescue assay and cDNA sequencing confirmed all mutants carried a mutated *N*-acetylglucosaminyltransferase I gene (Table 2; Fig. 3). It was shown that CHO-gmt6 had dysfunctional SLC35A1 and MGAT1, CHO-gmt7 lacked SLC35A2 and MGAT1 activities, and CHO-gmt8 lacked functional SLC35A1, SLC35C1, and MGAT1 (Table 2). Due to MGAT1 impairment, erythropoietin-Fc *N*-glycans from CHO-gmt4, CHO-gmt6, CHO-gmt7, and CHO-gmt8 cells shared similar glycoprofiles among the erythropoietin-Fc generated in these glycosylation mutants, primarily comprising Man5 glycan (Fig. 1c, g, h, i). One advantage of the CHO glycosylation mutants disclosed herein is that they generate *N*-glycans with consistent structures, while their *O*-glycans and glycolipids can be different. Trastuzumab *N*-glycans from CHO-gmt4, CHO-gmt6, CHO-gmt7, and CHO-gmt8 cells exhibited uniform fucose-free Man5 *N*-glycans (Fig. 2e, g, h, i). The consistency in the *N*-glycan structures generated using the CHO glycosylation mutants disclosed herein is important in biotherapeutics production to ensure, for

example, batch-to-batch consistency of the overall glycosylation during the production of biotherapeutics.

- [0062] Using CRISPR-Cas9, CHO-gmt10 and CHO-gmt11 cells were generated by disrupting the *Slc35A1* gene in CHO-gmt2 and CHO-gmt7 cells. Table 3 provides details of the targeted sites by ZFNs and CRISPR-Cas9 in this study. In CHO-gmt10 cells, erythropoietin Fc *N*-glycans are mostly fucosylated galactose-free (Fig. 1k), while trastuzumab *N*-glycans are primarily a) glycans with one fucose but without terminal galactose (G0F) and b) glycans without additional fucose or galactose (G0) glycans (Fig. 2k). In CHO-gmt11 cells, the *N*-glycan profile resembles that of CHO-gmt4 cells in both erythropoietin Fc and trastuzumab, due to impaired MGAT1 activity (Fig. 1l; Fig. 2l).

[0063] **Table 3. CRISPR target sites for generating CHO glycosylation mutants.** Exon sequences are shown in black. Intron sequences are in bold font.

SEQ ID NO.	Gene	Target Sequence	Remarks
SEQ ID NO: 87	Slc35c1	tAACCTCTGCCTCAAGTACGTAGGGGTGGCCt	Exon 1
SEQ ID NO: 88	Slc35a1	tcaGGGATCGTGGTGACGTAGTTGGtac	Exon 5
SEQ ID NO: 89	Mgat2	taaCCGGCCGGAATACCTCAGGCTGctgc	Exon 1
SEQ ID NO: 90	Mgat4a	tggGTCTACATTCGTCACTGTCGGGGGaaa	Exon 10
SEQ ID NO: 91	Mgat4b	cca CCCA G AGGATCCGCGACTGAAG C cgt	Intron 1 - Exon 2; anti-sense strand
SEQ ID NO: 92	Mgat5	gatGAGACTTCGGATCCGGCGAATGGctg	Exon 6
SEQ ID NO: 93	Man2a1	ataGAATCATGGAATCGCGCTTGAG G tac	Exon 9 - Intron 9
SEQ ID NO: 94	Man2a2	gacCCTCGACGACGCTTTCTCTGGGCaga	Exon 4; anti-sense strand
SEQ ID NO: 95	Man1a1	gaaGTAAATATACGCTTCGTGCGGTGGact	Exon 4
SEQ ID NO: 96	Man1a2	gatCCATGGACCCGACGAACACAGACaca	Exon 1; anti-sense strand
SEQ ID NO: 97	Manic1	gcaGAATTACCGCCGTTATGCGATGGgca	Exon 2
SEQ ID NO: 98	Manea	tggGCCAATCTGTTAACACCTTCAGGatc	Exon 4
SEQ ID NO: 99	Man1b1	cctGTACACTCGGGCGGATCATAAGGatg	Exon 8
SEQ ID NO: 100	Man2b1	ccaGCTCCTACGGTAAGACGTCCCGGtgg	Exon 14
SEQ ID NO: 101	Edem1	cctCCCTGAGCGCTATAACTGGCAACtcc	Exon 7; anti-sense strand
SEQ ID NO: 102	Edem2	ggcCCCGACGGCACCGCGCCCGACCCcgc	Exon 1; anti-sense strand

SEQ ID NO: 103	Edem3	cctCCACGAGCCGTACAAATTATTTCCca	Exon 13; anti-sense strand
SEQ ID NO: 87	Sle35c1	tAACCTCTGCCTCAAGTACGTAGGGGTGGCCt	Exon 1
SEQ ID NO: 88	Sle35al	tcaGGGATCGTGGTGACGTTAGTTGGtac	Exon 5
SEQ ID NO: 89	Mgat2	taaCCGGCCGGAATACCTCAGGCTGCtgc	Exon 1
SEQ ID NO: 90	Mgat4a	tggGTCTACATTCGTCACTGTCGGGGGaaa	Exon 10
SEQ ID NO: 91	Mgat4b	cca CCCA G AGGATCCGCGACTGAAGC cgt	Intron 1- Exon 2; anti-sense strand
SEQ ID NO: 92	Mgat5	gatGAGACTTCGGATCCGGCGAATGGctg	Exon 6
SEQ ID NO: 93	Man2a1	ataGAATCATGGAATCGCGCTTGAG Gtac	Exon 9 - Intron 9
SEQ ID NO: 94	Man2a2	gacCCTCGACGACGCTTTCTCTGGGCaga	Exon 4; anti-sense strand
SEQ ID NO: 95	Man1a1	gaaGTAAATATACGCTTCGTGCGTGact	Exon 4
SEQ ID NO: 96	Man1a2	gatCCATGGACCCGACGAACACAGACaca	Exon 1; anti-sense strand
SEQ ID NO: 97	Man1e1	gcaGAATTACCGCCGTTATGCGATGGgca	Exon 2
SEQ ID NO: 98	Manea	tggGCCAATCTGTTAACACCTTCAGGatc	Exon 4
SEQ ID NO: 99	Man1b1	cctGTACACTCGGGCGGATCATAAGGatg	Exon 8
SEQ ID NO: 100	Man2b1	ccaGCTCCTACGGTAAGACGTCCCGGtgg	Exon 14
SEQ ID NO: 101	Edem1	cctCCCTGAGCGCTATAACTGGCAACtcc	Exon 7; anti-sense strand
SEQ ID NO: 102	Edem2	ggcCCCCGACGGCACCGCGCCCGACCCcgc	Exon 1; anti-sense strand
SEQ ID NO: 103	Edem3	cctCCACGAGCCGTACAAATTATTTCCca	Exon 13; anti-sense strand

CHO cell mutants that produce homogenous fucose-free Man5 N-glycans

[0064] The CHO-gmt4 cells produced fucosylated Man5 glycans on erythropoietin Fc. In order to produce fucose-free glycans, CHO-gmt3 cells were treated with RCA-I, resulting in CHO-gmt12 cells. Erythropoietin Fc from CHO-gmt12 featured fucose-free Man5 and Man4 glycans (Fig. 1m), while trastuzumab from CHO-gmt12 exhibited mainly fucose-free Man5 glycans (Fig. 2m).

[0065] The *Man2a1* gene, encoding α -mannosidase II, in CHO-gmt12 was disrupted with CRISPR-Cas9, resulting in CHO-gmt17 cells. Analysis of erythropoietin Fc produced in CHO-gmt17 showed predominant (90.68%) Man5 structures (Fig. 1q), with small amounts (2.8%) of Man4 and Man3 glycans. Trastuzumab from CHO-gmt17 contained nearly exclusive Man5

glycans (Fig. 2q). Subsequently, CHO-gmt17x was generated by using CRISPR-Cas9 to target *Man2a2* in CHO-gmt17 cells. MAN2A2, or α -mannosidase IIx, plays a role in removing mannose residues from Man5 glycans. Most glycans on erythropoietin Fc from CHO-gmt17x were primarily fucose-free Man5 glycans (95.38%) (Fig. 1r), whereas CHO-gmt17x-produced trastuzumab comprised 99% Man5 glycans (Fig. 2r).

CHO cell mutants that produce *N*-glycans with reduced branches

[0066] CHO-gmt15 disclosed herein was generated using CRISPR-Cas9 system to inactivate *Mgat4a*, *Mgat4b*, and *Mgat5* genes in CHO-gmt2 cells. These genes are known to add extra branches of GlcNAc to bi-antennary complex *N*-glycans, leading to tri- and tetra-antennary complex *N*-glycan formation. Analysis of *N*-glycans in CHO-gmt15 cells revealed predominantly fucosylated bi-antennary GlcNAc-terminated *N*-glycans in both erythropoietin Fc and trastuzumab. Furthermore, fucosylated mono-antennary GlcNAc-terminated *N*-glycans were found in erythropoietin Fc (Fig. 1p, Fig. 2p). Minimal quantities of mono-galactosylated *N*-glycans (along with sialylation) were identified, consistent with findings in other CHO-gmt2-derived cell lines. Without being bound by theory, these findings are thought to suggest that inactivation of the *Mgat4a*, *Mgat4b*, and *Mgat5* genes produced *N*-glycans with reduced branches.

[0067] CHO-gmt13 and CHO-gmt14 were generated by CRISPR-Cas9 targeting of the *Mgat2* gene in CHO-gmt2 and CHO-gmt9 cells, respectively. GnTII / α -1,6-mannosyl-glycoprotein 2- β -N-acetylglucosaminyltransferase (MGAT2) adds the second GlcNAc residue to the core *N*-glycan at the α 1,6-linked mannose, forming a β 1,2 linkage. *N*-glycans on the erythropoietin Fc from CHO-gmt13 cells are shown in Fig. 1n. Absence of MGAT2 leaves the α 1,6-linked mannose of the core *N*-glycan unmodified. In CHO-gmt13 cells, the two GlcNAc residues are linked to the α 1,3-linked mannose arm, deviating from the typical bi-antennary structure yet maintaining the same molecular mass. *N*-glycans on trastuzumab from CHO-gmt13 cells exhibited primarily fucosylated core structure with a single GlcNAc residue (Fig. 2n). The *N*-glycan profiles of erythropoietin Fc and trastuzumab from CHO-gmt14 cells resemble those from CHO-gmt13 cells, with the exception that they lack fucose (Fig. 1o, Fig. 2o). Among all mutants, trastuzumab from CHO-gmt14 cells carried the smallest *N*-glycan, consisting of six sugar residues.

[0068] Similarly, CHO-gmt21 and CHO-gmt22 resulted from CRISPR-Cas9 targeting *Mgat2* in CHO-K1 and CHO-gmt3 cells. Analysing *N*-glycan structures of CHO-gmt21-produced erythropoietin Fc revealed attachment of the branches to the α 1,3-linked mannose of the core

structure, with the α 1,6-linked mannose arm remains unchanged (Fig. 1v). Trastuzumab produced in CHO-gmt21 cells showed mainly mono-antennary glycans, with varied galactose and sialic acid levels (Fig. 2v). The *N*-glycan profiles of erythropoietin Fc and trastuzumab from CHO-gmt22 cells closely resemble those from CHO-gmt21 cells, except for lacking fucose (Fig. 1w, Fig. 2w). Complex type *N*-glycans on erythropoietin Fc produced in CHO-gmt13, CHO-gmt14, CHO-gmt21, and CHO-gmt22 are all linked to the α 1,3-linked mannose, leaving the α 1,6-linked mannose arm of the core structure unmodified. Mannosidase treatment confirmed these structures. Fig. 5 illustrates the mannosidase treatment of CHO-gmt22-produced *N*-glycans as an example, confirming the presence of unblocked mannose residue. Mannosidase treatments yield consistent results for erythropoietin Fc from CHO-gmt13, CHO-gmt14, and CHO-gmt21. Without being bound by theory, these findings are thought to suggest that GnT-II prevents *N*-acetylglucosaminyltransferase-V (GnT-V) from adding GlcNAc to the α 1,6-linked mannose arm in EPO-Fc. It was noted that *N*-glycan proportion in CHO-gmt21 and CHO-gmt22-produced trastuzumab exhibited higher sialylated single arm *N*-glycans than CHO-K1 cells and CHO-gmt3 cells. Without being bound by theory, it is thought that mono-antennary *N*-glycans on IgG antibodies enhanced sialyltransferase accessibility, leading to increased sialylation levels.

Generation of CHO cell mutants that produce homogenous Man9 *N*-glycans

[0069] MAN1B1 (ER α -mannosidase I) is known to generate Man8 isomer B, while MANEA (Golgi endo- α -mannosidase) is known to generate Man8 isomer A. Subsequently, MAN1A1, MAN1A2, and MAN1C1, namely Golgi α 1-2 mannosidase IA, IB and IC, trim Man8 to Man5 in the Golgi. Following the addition of a GlcNAc residue to the α 1-3Man arm of the Man5 glycan by GnT-I, the α 1-3 and α 1-6-linked mannose residues on the α 1-6Man arm are removed by α -mannosidase II and α -mannosidase IIx (MAN2A1, MAN2A2). The Man5 (Man₅GlcNAc₂) glycan plays a role as an intermediate in the *N*-glycosylation pathway. Mutants unable to reach this stage are incapable of producing complex or hybrid type *N*-glycans.

[0070] Using CRISPR-Cas9, three mutants were generated: CHO-gmt18, CHO-gmt19, and CHO-gmt20. In CHO-gmt18 cells, *Man1a1*, *Man1a2*, *Man1c1*, and *Manea* genes were disrupted, while *Man1b1* remained unchanged. In CHO-gmt19 cells, *Man1a1*, *Man1a2*, *Man1c1* and *Man1b1* genes were inactivated, with *Manea* gene functional. In CHO-gmt20 cells had all 5 genes (*Man1a1*, *Man1a2*, *Man1c1*, *Man1b1*, and *Manea*) knocked out. Fig. 6 illustrates the generation of CHO-gmt35 cells producing uniform Man9 *N*-glycans. The left and right columns in Fig. 6, display the *N*-glycan profiles of erythropoietin Fc and trastuzumab generated in various CHO glycosylation mutants, respectively. Without being bound by theory, although

CRISPR/Cas9 knocked out these α -mannosidases in the mutant lines, the presence of complex *N*-glycans in erythropoietin Fc suggests that trimming of mannose residues to Man5 glycans was not fully prevented.

[0071] Glycans linked to erythropoietin Fc and trastuzumab from wild-type CHO-K1 cells are shown as controls in Fig. 6a and b, respectively. Erythropoietin Fc glycans from CHO-gmt18, CHO-gmt19, and CHO-gmt20 are shown in Fig. 6c, e, and g, respectively. It was shown that the erythropoietin Fc produced from these CHO glycosylation mutants exhibit higher levels of high-mannose type glycans (CHO-gmt18, 51%; CHO-gmt19, 65.1%; CHO-gmt20, 75.2%) compared to those produced in CHO-K1 cells (0.73%). Erythropoietin Fc from all three mutants comprised Man6, Man7 and Man8 glycans, while Man9 glycans are only present in CHO-gmt19 and CHO-gmt20 cells. Fig. 1s, t, u illustrate detailed glycan profiles from these three mutants.

[0072] Conversely, CHO-gmt18, CHO-gmt19, and CHO-gmt20 produced Man6, Man7, and Man8 glycans on trastuzumab without complex glycans. Only trastuzumab produced in CHO-gmt19 and CHO-gmt20 cells had Man9 glycans attached (Fig. 6d, f, and h). Fig. 2s, t, and u show detailed glycans attached to trastuzumab in these three mutants. Without being bound by theory, these findings are thought to suggest that knocking out *Man1b1* is more effective than *Manea* in preventing mannose trimming. Notably, no complex *N*-glycans were seen on trastuzumab from all three mutants, suggesting trimming mannosidases are less effective on antibody glycans, likely due to structural constraints in the Fc region.

[0073] The present disclosure describes the roles of the five mannosidases in normal mannose trimming. Yet, upon their inactivation, it is thought that other α -mannosidases are likely to engage in this process. For example, ER degradation-enhancing α -mannosidase-like protein 1, 2, and 3 (EDEM1, EDEM2, EDEM3) are known as α -mannosidase that remove mannose residues from misfolded glycoproteins. To test this, CHO-gmt26 cells were generated using CRISPR-Cas9 targeting *Edem1*, *Edem2*, and *Edem3* genes in CHO-gmt20 cells. Comparing the erythropoietin Fc analyses between CHO-gmt20 and CHO-gmt26, the total high-mannose glycan population remained similar (75.2% vs. 74.1%). However, the Man9 content in erythropoietin Fc from CHO-gmt26 rose from 15.2% to 28.2% (Fig. 6g, Fig. 6i, Fig. 1u, and Fig. 1x). Man9 *N*-glycan quantity on trastuzumab from CHO-gmt26 exceeded those from CHO-gmt20 cells (Fig. 6h, Fig. 6j, Fig. 2u, and Fig. 2x). Without being bound by theory, it is thought that these findings disclosed herein suggest that EDEMs possess α 1,2-mannosidase activities for correctly folded glycoproteins and antibodies.

[0074] Without being bound by theory, it is thought that MAN2B1, a class II α -mannosidases, resides in lysosomes and participates in the catabolic pathway of *N*-glycans

released during glycoprotein turnover. This enzyme exhibits broad specificity, capable of cleaving all α -mannosidic linkages. MAN2A1 and MAN2A2, Golgi-resident class II α -mannosidases, are important for converting Man5GlcNAc2 to Man3GlcNAc2. This is achieved by removing α 1,3- and α 1,6-linked mannose residues from the α 1,6-Man arm of the core structure. The knockout of these genes during the creation of CHO-gmt17x from CHO-gmt4, as discussed earlier, suggest their proposed activities of removing mannose residues from N-glycans. Three more mutants were generated in CHO-gmt26 cells using CRISPR-Cas9. Three more mutants were generated in CHO-gmt26 cells using CRISPR-Cas9: CHO-gmt29 targeted Man2b1, CHO-gmt32 targeted Man2a1 and Man2a2, and CHO-gmt35 targeted Man2b1, Man2a1, and Man2a2.

[0075] N-glycans analysis of erythropoietin Fc and trastuzumab from CHO-gmt29 revealed no difference compared to CHO-gmt26 (Fig. 6k and Fig. 1y; Fig. 6l and Fig. 2y), suggesting that inactivating MAN2B1 alone in this genetic background had no clear impact on mannose trimming. This shows that, in one example, mutations claimed herein have a synergistic effect that is shown to be advantageous compared to individual mutations.

[0076] When both Man2a1 and Man2a2 were inactivated (CHO-gmt32), high-mannose glycan levels were observed in CHO-gmt32 cells, rising from 74.06% to 100%, with Man9 glycans accounting for 97.94% (Fig. 6m and Fig. 1z). Trastuzumab N-glycans from CHO-gmt32 cells showed a complete conversion of Man8 glycans to Man9 glycans, unlike those in CHO-gmt26 cells (Fig. 6n and Fig. 2z). Thus, MAN2A1 and MAN2A2 have been shown to contribute to mannose trimming in the absence of other α -mannosidases. In the comparison of erythropoietin Fc N-glycans from CHO-gmt35 and CHO-gmt32 cells, the amount of Man8 glycans decreased from 2.06% in CHO-gmt32 to 1.33% in CHO-gmt35 (Fig. 6o and Fig. 1z-a). The amount of Man9 from trastuzumab produced in CHO-gmt32 and CHO-gmt35 remained consistent at about 100% (Fig. 6p and Fig. 2z-a). Without being bound by theory, this finding is thought to suggest that all other mannosidases can contribute to mannose trimming, except for MAN2B1.

[0077] In one example, the present disclosure describes a method of generating Chinese hamster ovary glycosylation mutants that produce homogeneous Man9 N-glycans. Based on what is known in the art, trimming of Man9 to Man5 N-glycans in mammalian cells requires ER α -mannosidase I (MAN1B1), endo α -mannosidase I (MANEA), and Golgi α -mannosidase IA, IB, IC (MAN1A1, MAN1A2, MAN1C1). However, the present disclosure describes that when these genes were inactivated, the CHO cell mutants (CHO-gmt18, CHO-gmt19, and CHO-gmt20) still produced a large amount of complex N-glycans, suggesting that the conversion of

Man9 to Man5 *N*-glycans was not completely inhibited. In another example, the present disclosure describes that all other α -mannosidases in the genome (including EDEM1, EDEM2, EDEM3, MAN2B1, MAN2A1 and MAN2A2) had to be inactivated in order to produce homogeneous Man9 *N*-glycans (as shown, for example, in CHO-gmt35). In one example, the *N*-glycans attached to both erythropoietin Fc and human IgG antibodies produced in CHO-gmt35 cells are highly homogenous (>98%) Man9 *N*-glycans.

Impact of Fc glycans on Fc-FcγRIIIa interactions

[0078] The Fc region of IgG activates diverse cellular responses by interacting with Fc gamma receptors (FcγRs). Glycosylation on Asn297 of IgG1 is crucial for Fc conformation and FcγR binding. Relevant FcγRs include FcγRI (CD64), FcγRIIa-c (CD32a-c), and FcγRIIIa-b (CD16a-b). The present disclosure describes how different glycans impact Fc-FcγRI, IIa, and IIIa interactions using CHO cell glycosylation mutants-derived trastuzumab.

[0079] The Fc-FcγRIIIa (CD16a) interaction is crucial for antibody dependent cellular cytotoxicity (ADCC) activity. Removing core fucose from the Fc *N*-glycan enhances this interaction. To study trastuzumab's interaction with FcγRIIIa, human FcγRIIIa's extracellular domain with a His-tagged was produced in CHO cells. The glycoengineered trastuzumab were immobilized on the Surface Plasmon Resonance (SPR) sensor chip. The interaction between trastuzumab and FcγRIIIa was assessed using Biacore. The binding affinity (KD) determined by Biacore are summarised in Table 4 and illustrated graphically in Fig. 7a. Furthermore, ELISA was performed to compare antibody and FcγRIIIa binding. The His-tagged FcγRIIIa extracellular domain was incubated with nickel-coated plates, and antibody-FcγRIIIa binding was quantified via standard ELISA (Fig. 7b). Both methods exhibited consistent trends. The declining KD values from Biacore correlated with enhanced antibody binding seen in ELISA (Fig. 7a, b). These findings indicate ELISA's reliability in evaluating Fc-FcγRIIIa interactions under these conditions. Removing core fucose from the *N*-glycan increased Fc-FcγRIIIa affinity. Mutants lacking core fucose (CHO-gmt3, CHO-gmt5 and CHO-gmt9) showed a substantial affinity boost for FcγRIIIa. The obtained SPR data indicated that the enhanced affinity of the afucosylated antibody for FcγRIIIa is mainly attributed to a reduced off rate. This is evident from the reduced KD values: 69 nM, 46 nM and 80 nM for CHO-gmt3, CHO-gmt5, and CHO-gmt9-produced trastuzumab, respectively, in contrast to the 560 nM KD from CHO-K1-produced trastuzumab (Table 4).

[0080] **Table 4. On- and Off-rates and equilibrium constants from Biacore binding experiments.** The glycoengineered trastuzumab were immobilised on the Surface Plasmon

Resonance (SPR) sensor chip. His-tagged extra-cellular domain of FcγRIIIa was used to its interaction with trastuzumab. The On- and Off-rates, and the equilibrium constants are shown in the table.

Glycosylation mutant	k_{on} (mM ⁻¹ s ⁻¹)	k_{off} (s ⁻¹)	*K _D (nM)	K _D (nM)
CHO-K1	46.3	0.00492	106.2634989	560
CHO-gmt1	30.9	0.002942	95.21035599	644.6
CHO-gmt2	92.8	0.007838	84.4612069	365
CHO-gmt3	46	0.003129	68.02173913	68.98
CHO-gmt4	100	0.01308	130.8	128.5
CHO-gmt5	59.6	0.003063	51.39261745	45.71
CHO-gmt6	51.8	0.01275	246.1389961	194.8
CHO-gmt7	35.5	0.01271	358.028169	183.4
CHO-gmt8	355	0.0133	37.46478873	200.4
CHO-gmt9	151	0.012	79.47019868	80
CHO-gmt13	61.2	0.01245	203.4313725	779.4
CHO-gmt18	74.2	0.008503	114.5956873	136.2
CHO-gmt19	68.8	0.008143	118.3575581	136.9
CHO-gmt20	96.1	0.0107	111.3423517	151.6
CHO-gmt21	89.9	0.03167	352.2803115	799
CHO-gmt22	86.9	0.004546	52.31300345	109.8
CHO-gmt23	64	0.005086	79.46875	107.5
CHO-gmt35	79.6	0.007169	90.06281407	157.5
CHO-K1 + GalT	39.2	0.004312	110	257
CHO-gmt3 + GalT	46.9	0.00191	40.7249467	53.35
CHO-K1 + GnTIII	90.6	0.005575	61.53421634	513
CHO-gmt2 + GnTIII	41.9	0.006778	161.7661098	338.4
CHO-gmt9 + GnTIII	44.6	0.003109	69.70852018	57.23
CHO-gmt13 + GnTIII	35.8	0.005195	145.1117318	338.4
CHO-gmt21 + GnTIII	38.5	0.005614	145.8181818	273.3
CHO-gmt22 + GnTIII	40.8	0.003446	84.46078431	75.5
CHO-gmt23 + GnTIII	39	0.003609	92.53846154	82.4

- [0081] Trastuzumab from CHO-gmt4 and CHO-gmt8 cells, lacking GnT-I activity, displayed afucosylated Man3-5 glycans (Fig. 2e, i). This led to increased FcγRIIIa affinity, with KD values of 129 nM and 200 nM for trastuzumab from CHO-gmt4 and CHO-gmt8, respectively (Fig. 7a). Comparable outcomes were observed with other high mannose glycans, such as CHO-gmt18, 19, 35, as shown by Biacore and ELISA analyses (Fig. 7a, 7b). However, the enhancement was not as pronounced as seen in CHO-gmt3, which produces afucosylated complex type biantennary N-glycans. Trastuzumab from CHO-gmt4, 8, 18, 19, 35 all lack core-fucose, potentially enhancing binding. Without being bound by theory, it is thought that this suggests that the larger high mannose glycan might not fit as effectively into the interaction pocket as the afucosylated complex biantennary N-glycans.
- [0082] It is of note that, compared to trastuzumab from CHO-gmt2 and CHO-K1 cells, the presence of single-armed N-glycans on trastuzumab produced by CHO-gmt13 and CHO-gmt21 cells decreased FcγRIIIa affinity to 780 nM and 799 nM, respectively, regardless of galactose on the glycan (Fig. 7a). This decrease in FcγRIIIa affinity was confirmed by the ELISA (Fig. 7b). Without being bound by theory, it is thought that the smaller single-armed glycan structure may hinder effective interaction with FcγRIIIa. This is evident from afucosylated single-arms glycoforms from CHO-gmt22 and CHO-gmt23 cells, which did not reach the same affinity as CHO-gmt3 (Fig. 7a, b). GnT-III overexpression and the addition of bisecting GlcNAc improved the trastuzumab's affinity for FcγRIIIa from CHO-gmt13 and CHO-gmt21. Without being bound by theory, it is thought that this enhancement is likely due to the bisecting GlcNAc, which may increase the interaction with N-glycan on FcγRIIIa.

Impact of GalT1 and GnT-III overexpression on Fc glycan structures and FcγRIIIa interaction

- [0083] Co-expression of b-1,4-galactosyltransferase 1 (B4GalT1) in CHO-K1 and CHO-gmt3 cells led to an increase in galactosylation levels of the antibody, from 51% to 77% and from 27% to 80%, respectively (Fig. 8a, b). This increase in galactosylation was accompanied by a slight improvement in affinity, with KD values decreasing from 560 nM to 257 nM for CHO-K1 cells and from 69 nM to 53 nM for CHO-gmt3 cells, respectively (Table 4, Fig. 7a). These findings were further supported by ELISA results (Fig. 7b). These findings align with a previous study showing improved FcγRIIIa affinity through increased galactosylation.
- [0084] GnT-III overexpression in CHO-K1, CHO-gmt2 and CHO-gmt9 cells led to an increase in bisecting GlcNAc levels from 0% to 87% to 97% (Fig. 8c, d, e). HILIC-UPLC-QTOF-MS/MS analysis was conducted to distinguish between bisecting GlcNAc and tri-

antennary glycans with identical molecular mass. Bisecting GlcNAc presence was verified through HILIC-UPLC-QTOF-MS/MS analysis, as demonstrated in Figure 13. Bisecting GlcNAc presence appeared to have no impact on fucosylation in trastuzumab produced in CHO-K1 and CHO-gmt2 cells (Fig. 2a, Fig. 2c, Fig. 8c, and Fig. 8d). However, it significantly reduced fucosylation in CHO-gmt13 (from 93 to 74%) and CHO-gmt21 cells (from 92 to 70%) (Fig. 2n, Fig. 2v, Fig. 8g, and Fig. 8i). GnT-III overexpressing and introducing bisecting GlcNAc in CHO-K1, CHO-gmt2, CHO-gmt9, CHO-gmt13, and CHO-gmt21 cells all enhanced their FcγRIIIa binding affinity (Fig. 7a and Table 4).

Impact of Fc glycan structures on Fc-FcγRI and FcγIIa interactions

[0085] To explore Fc glycans on Fc-FcγRI and FcγIIa interactions, the interaction affinities between FcγRI and FcγRIIa extracellular domains and trastuzumab glycosylation variants were assessed using ELISA. As shown in Fig. 7c, d, glycans on trastuzumab clearly impacts its interaction with FcγRI and FcγRIIa, though somewhat less pronounced than with FcγRIIIa. In Fig. 7c, trastuzumab glycovariants are ordered by their FcγRI affinity, high to low. The same arrangement demonstrates a corresponding decrease in FcγRIIa affinity (Fig. 7d). This similarity implies a consistent glycan-related impact on the binding to both receptors. For example, GnT-III overexpression in CHO-gmt23 did not affect FcγRIIIa binding (Fig. 7a, b), but it did reduce Fc affinity for both FcγRI and FcγRIIa (Fig. 7c, d). Overexpressing GalT1 in CHO-K1 and CHO-gmt3, moderately raised binding affinity for both FcγRI and FcγRIIa (Fig. 7c, d). Thus, GalT1 overexpression enhances Fc binding affinity for all three receptors. However, trastuzumab variants from CHO-gmt2 and CHO-gmt9 cells, lacking galactose, displayed comparable affinity to CHO-K1 and CHO-gmt3-produced variants for both receptors, indicating that reducing galactosylation does not lower its affinity. Furthermore, the results indicated that core fucose presence does not affect Fc-FcγRI and Fc-FcγRIIa interactions. This suggests a distinction in the interaction between Fc glycan and FcγRIIIa compared to FcγRI and FcγRIIa. Previous studies highlighted the role of Fc glycan in antibody-FcγRI interaction. The present disclosure not only validated their findings but also indicated a similar interaction mode between Fc glycan and FcγRI/FcγRIIa.

Influence of Fc glycans on trastuzumab-induced antibody-dependent cellular cytotoxicity (ADCC) in breast cancer cell lines

[0086] Trastuzumab targets overexpressed HER2 antigen in breast cancer cells. The present disclosure describes the use of the antibody-dependent cellular cytotoxicity (ADCC) Reporter Bioassay kit to study trastuzumab glycovariant cytotoxicity in breast cancer cells, in order to

assess Fc glycan's impact on ADCC. In this disclosure, the HER2 levels of five human breast cancer cell lines (SK-BR-3, HCC1954, MDA-MB-231, MDA-MB-453, MDA-MB-468) were assessed via fluorescence-activated cell sorting (FACS) analysis using commercial Herceptin (Fig. 9a). Breast cancer cell lines exhibited diverse HER2 expression. MDA-MB-468, a triple-negative breast cancer cell line, served as a Her2 expression-negative control. Results indicate MDA-MB-231 had the lowest Her2 expression, while SK-BR-3 displayed the highest Her2 expression. Fig. 9b, c, d, e, and f show antibody-dependent cellular cytotoxicity (ADCC) Reporter Bioassay results for trastuzumab from CHO-K1 and CHO-gmt3 cells, and Herceptin, tested on five breast cancer cell lines. Among the cell lines MB-231, MDA-MB-453, HCC1954, and SK-BR-3, afucosylated trastuzumab from CHO-gmt3 showed stronger response than trastuzumab from CHO-K1 and Herceptin. Her2-negative MDA-MB-468 cells did not exhibit ADCC induction with any antibodies (Fig. 9b). One observation was the inverse correlation between Her2 levels and antibody-mediated cell killing response. Relative to Herceptin and trastuzumab from CHO-K1 cells, trastuzumab from CHO-gmt3 exhibited greater ADCC induction: around 7-fold in MDA-MB-231 cells, 2.5-fold in MDA-MB-453 cells, and 1.5-fold in HCC1954 and SK-BR-3 cells (Fig. 9c, b, d, e, and f). Previous studies showed that afucosylation of IgG1 antibodies enhances antibody-dependent cellular cytotoxicity activity for low antigen density, compared to normally glycosylated antibodies. Thus, investigating afucosylated trastuzumab or rituximab in patients with low HER2 or CD20 levels could be promising. HCC1954 and MDA-MB-453 cell lines exhibited similar Her2 expression, with HCC1954 showing slightly elevated levels. Consequently, the antibodies induced stronger ADCC response in HCC1954 (Fig. 9g) compared to MDA-MB-453 cells (Fig. 9h). Moreover, SK-BR-3 cells, selected for its highest Her2 expression, served as the target cells for testing a panel of trastuzumab glycovariants. Trastuzumab from CHO-gmt3 (afucosylated and galactosylated) and CHO-gmt9 (predominantly afucosylated and agalactosylated) triggered nearly identical ADCC levels, showing a 3.8-fold increase in maximum ADCC compared to CHO-K1-produced antibody (Fig. 9i). Therefore, CHO-gmt9, as disclosed herein, serves as a host for producing targeted anticancer antibodies by enhancing antibody-dependent cellular cytotoxicity through consistent *N*-glycan profiles and minimising batch variations (Fig. 2j).

[0087] The effect of high mannose, bisecting GlcNAc, and mono-antennary complex structures on antibody-dependent cellular cytotoxicity were demonstrated in the highly responsive SK-BR-3 target cells (Fig. 9j, k, l, m). Trastuzumab from CHO-gmt4 and CHO-gmt8 cells, dominated by fucose-free Man5 *N*-glycans (Fig. 2e, i), showed a 1.3-fold rise in maximum antibody-dependent cellular cytotoxicity response versus CHO-K1 cell-produced trastuzumab

(Fig. 9j). The introduction of 68% bisecting glycans into trastuzumab by expressing GnT-III in CHO-gmt3 cells (Fig. 8e) led to a slight increase in maximum antibody-dependent cellular cytotoxicity compared to trastuzumab produced in CHO-gmt3 cells (Fig. 9k). Trastuzumab from CHO-gmt14 cells, with galactose-free and fucose-free single-arm glycans, exhibited a 1.6-fold higher maximum ADCC than CHO-K1-produced trastuzumab (Fig. 9l). These findings align closely with the Biacore dinging data (Fig. 7A). Furthermore, in CHO-gmt14 cells, GnT-III overexpression led to a 1.8-fold increase in ADCC compared to CHO-K1-produced trastuzumab (Fig. 9l). Trastuzumab derived from CHO-gmt3 and CHO-gmt14 cells, co-expressing GnT-III, showed the highest ADCC activity among all tested glycovariants (Fig. 9m). Trastuzumab from CHO-gmt14 with overexpressed GnT-III elicited the highest level of ADCC signalling in SK-BR-3 cells.

Glycoengineered trimeric HIV-1 envelope glycoprotein (Env) for studying glycan specificity of bnAbs against HIV

[0088] Most broadly neutralizing antibodies (bnAbs) against HIV target the trimeric HIV envelope glycoprotein, consisting of gp120 and gp41. This HIV envelope glycoprotein trimer is shielded by dense glycans, often impacting bnAb-antigen interactions. BG505 SOSIP.664 gp140 is a recombinant soluble trimeric protein, closely mimicking the native membrane bound envelope glycoprotein spike of HIV BG505 strain. Like the native envelope glycoprotein, BG505 SOSIP.664 gp140 has occupied glycosylation sites with premature high mannose type *N*-glycans.

[0089] In this disclosure, the His-tagged BG505 SOSIP.664 gp140 (SOSIP Env) was produced in CHO-K1, CHO-gmt4 and CHO-gmt35 cells. Following purification with Ni⁺⁺-column, N-glycans were released with PNGase F and analysed using LC-MS. Fig. 10 shows the acquired glycan profiles. SOSIP Env protein from CHO-K1 cells (SOSIP Env-K1) primarily features high mannose-type glycans, with some small complex glycan peaks. Man9 stands out as the most abundant type of high mannose glycans (Fig. 10a). The SOSIP Env protein from CHO-gmt4 cells (SOSIP Env-Gmt4) lacks complex type N-glycans, due to the absence of GnT-I activity. The distribution of high mannose-type glycans differs from CHO-K1 cells, with Man5 being the predominant form. The SOSIP Env-Gmt35 protein produced in CHO-gmt35 cells (SOSIP Env-Gmt35) is a complex *N*-glycan-free SOSIP envelope glycoprotein that exclusively features Man9 glycans (Fig. 10a). Fig. 11a, b, c, d display the detailed *N*-glycan distribution of SOSIP envelope glycoprotein produced by these three cell lines.

[0090] The present disclosure produced five well-studied bnAbs targeting gp120 using CHO cells: 2G12, PGT121, PGT128, PG9, and PG16. Table 5 contains amino acid sequences of these

five antibodies. 2G12 binds the terminal dimannose structure of Man9 glycans via an unusual heavy chain domain exchange. PG9 penetrate the glycan shield through its long CDR3 region, fitting between two *N*-glycans to access the protein backbone on the V1/V2 region. PG16, a close relative of GP9, shares many binding characteristics with PG9, including binding to the same Asn160 glycan. However, PG16 distinguishes itself from PG9 by also binding to a sialylated complex type structure in the second *N*-glycan. The glycan binding specificity of PGT121 is more complex. It did not bind Man9 *N*-glycan on glycan arrays but did bind complex-type *N*-glycan on glycan arrays and in crystal structures. However, PGT121 recognised high-mannose-only HIV envelops, whether isolated or in virions. Like PG9, PGT128 interacts with two high-mannose type *N*-glycans (Man9 and Man5), and the V3 loop region upon binding to an engineered gp120 outer domain.

[0091] **Table 5. Amino acid sequences of five anti-HIV bnAbs.** The variable regions of the antibodies were obtained from publicly available database. The heavy chains were converted to IgG1 with the introduction of constant region and signal peptides. The heavy chains were converted to full-length light chains with the introduction of either kappa or lambda light chain constant regions.

2G12	kappa	MDMRVPAQLLGLLLWLSGARCDVMTQSPSTLSASVGDITITICRASQSIETWLAWYQQKPGKAPKLL IYKASTLKTGVPSRFSGSGSGTEFTLTISGLQFDDFATYHCQHYAGYSATFGQGRVEIKRTVAAPSVF IFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSSTYLSSTLTLSKAD YEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 104)
	IgG1	MEFGLSWVFLVALFRGVQCEVQLVESGGGLVKAGGSLILSCGVSNFRISAHTMNWVRRVPGGGLWVAS ISTSTYRDYADAVKGRFTVSRDDLEDFVYLQMHKMRVEDTAIYYCARKGSDRLSDNDPFDAGPGTVV TVSPASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTWNSGALTSGVHTFPAVLQSSGLYSL SSVTVTPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTL MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPEN NYKTTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSQSVMEALHNHYTQKSLSLSPGK (SEQ ID NO: 105)
PGT1 21	lambda	MAWTVLLGLLSHCTGSVTSYVLTQPSDISVAPGETARISCCEKSLGSRVQWYQHRAGQAPSLIITYNN QDRPSGIPERFSGSPDSPFGTTATLTITSVEAGDEADYYCHIWDSRVPTKWVFGGGTTTLTVLGQPKAAP SVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTPPSKQSNKYAASSYLSLTP EQWKSHKSYSCQVTHEGSTVEKTVAPTECS (SEQ ID NO: 106)

	lgG1	MEFGLSWVFLVALFRGVQCQMQLQESGPGLVKPFSETLSLTCSVSGASISDSYWSWIRRSPGKGLEWIGY VHKSGDTNYSPLKSRVNLSDLTSKNQVLSLVAATAADSGKYCARTLHGRRIYGIVAFNEWFTYFYM DVWNGTQVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV LQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFL FPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVVFSCSVMEALHNHYTQKSLSLSPGK (SEQ ID NO: 107)
PGT1 28	lambda	MAWALLLLTLLTQGTGSWAQSALTQPPSASGSPGQSITISCTGTSNNFVSWYQQHAGKAPKLVYDVK RPSGVPDRFSGSKSGNTASLTIVSGLQTDDEAVYYCGSLVGNWDVIFGGGTKLTVLGQPKAAPSVTLFPP SSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNKYAASSYLSLTPEQWKSHK SYSCQVTHEGSTVEKTVAPTECS (SEQ ID NO: 108)
	lgG1	MEFGLSWVFLVALFRGVQCEPQIQESGPTLVEASETSLTCAVSGDSTAACNSFWGVRQPPGKGLEWV GSLSHCASYWNRGWTYHNP SLKSRLLTALDTPKNLVFLKLSVTAADTATYYCARFGGEVLRVYDWPKP AWVDLWGRGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPS VFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTV LHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDI AVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVVFSCSVMEALHNHYTQKSLSLSPG K (SEQ ID NO: 109)
PG9	lambda	MAWALLLLTLLTQGTGSWAQSALTQPASVSGSPGQSITISCNSTNDVGGYESVSWYQQHPGKAPKVI YDVSKRPSGVSNRFGSKSGNTASLTISGLQAEDEGDYCKSLTSTRRRVFGTGKLTVLGQPKAAPSV TLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNKYAASSYLSLTPEQ WKSHKSYSCQVTHEGSTVEKTVAPTECS (SEQ ID NO: 110)
	lgG1	MEFGLSWVFLVALFRGVQCQRLVESGGGVVQPGSSRLSCLASGDFSRQGMHWVRQAPGQGLEWVAFI KYDGSEKYHADSVWGRSLISRDNSKDTLYLQMNSLRVEDTATYFCVREAGGPDYRNGYNYYDFYDGYN YHYMDVWGKGTITVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHT FPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGP SVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSD IAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVVFSCSVMEALHNHYTQKSLSLSP GK (SEQ ID NO: 111)
PG16	lambda	MAWALLLLTLLTQGTGSWAQSALTQPASVSGSPGQTITISCNSTSDVGGFDSVSWYQQSPGKAPKVMV FDVSHRPSGISNRFSGSKSGNTASLTISGLHIEDEGDYFCSSLTDRSHRIFGGGKVTVLGQPKAAPSV TLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNKYAASSYLSLTPEQ WKSHKSYSCQVTHEGSTVEKTVAPTECS (SEQ ID NO: 112)

lgG1	MEFGLSWVFLVALFRGVQCQEQLVESGGGVVQPGGSLRLSCLASGFTFHKYGMHWVRQAPGKGLEWVAL ISDDGMRKYHSDSMWGRVTISRDNKNTLYLQFSSLKVEDTAMFFCAREAGGPIWHDDVKYYDFNDGYY NYHYMDVWGKGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVH TFP AVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKFSNTKVDKKVEPKSCDKTHTCPPCPAPELLGG PSVFLFPPKPKDTLMISRTEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVL TVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSQSVMEALHNHYTQKSLSLS PGK (SEQ ID NO: 113)
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[0092] To assess how gp120 glycans affect binding affinities of five bnAbs, the present disclosure conducted ELISA on SOSIP envelope glycoprotein from CHO-K1, CHO-gmt4, and CHO-gmt35 cells. As expected, 2G12 consistently bound to all three proteins: SOSIP Env-K1, SOSIP Env-Gmt4, and SOSIP Env-Gmt35. This suggests that the Man9 glycans recognised by 2G12 remain unaffected in CHO-gmt4-produced SOSIP Env, despite a notable reduction in total Man9 content in CHO-gmt4-produced SOSIP Env (Fig. 10b). PG9 exhibited comparable affinity for both SOSIP Env-K1 and SOSIP Env-Gmt4, suggesting its binding is not dependent on complex type glycans. However, its binding to SOSIP Env-Gmt35 declined, suggesting a potential interference due to the presence of a large Man9 glycan (Fig. 10c). PG16 failed to bind SOSIP Env-Gmt35, likely due to the hindrance from a large Man9 glycan. Its affinity was lower than PG9 when binding to SOSIP Env-CHO-K1 and SOSIP Env-CHO-Gmt4. Interestingly, PG16 bound more strongly to SOSIP Env-CHO-Gmt4 than to SOSIP Env-CHO-K1 (Fig. 10d). Since PG16 is believed to interact with sialic acid on complex glycans, the removal of complex glycans in SOSIP Env-CHO-Gmt4 should reduce the interaction. The smaller high mannose glycans in SOSIP Env-CHO-Gmt4 may contribute to the enhanced binding, though this connection needs further investigation. PGT121 displayed comparable binding to SOSIP Env from three cell lines, suggesting that complex type *N*-glycans are not crucial for PGT121 to bind gp120 (Fig. 10e). Hence, high-mannose *N*-glycan is crucial for PGT121's binding to gp120, despite earlier suggestions of potential interaction with complex type glycans. PGT128, targeting the terminal dimannose of Man9 *N*-glycan, showed similar binding profiles to all three SOSIP Env proteins (Fig. 10f). Without being bound by theory, it is thought that these findings disclosed herein indicate that HIV envelope glycoproteins produced in these mutants can be valuable tools for probing the role of glycans in the recognition of broadly neutralizing antibodies.

Production of β -glucocerebrosidase (GCase) with uniform Man5 glycans using CHO-gmt17x cells

[0093] Recombinant β -glucocerebrosidase production in CHO-gmt4 cells eliminates the need for enzymatic treatment to obtain mannose-terminated *N*-glycans. Stable β -glucocerebrosidase-producing CHO-gmt4 transfectants were generated in the present disclosure and a high-producing clone was isolated. The purified β -glucocerebrosidase from CHO-gmt4 cells primarily consists of Man5 glycans, with fucosylated Man5, Man4 and fucosylated Man4 glycans detected. Similar to generating CHO-gmt17 cells, additional mutations were introduced into β -glucocerebrosidase-producing CHO-gmt4 cells by knocking out the GDP-fucose transporter and α -mannosidase II genes. Next, the α -mannosidase IIx gene in CHO-gmt17 cells was knocked out, resulting in β -glucocerebrosidase-producing CHO-gmt17x cells (Fig. 12a). β -glucocerebrosidase protein possesses five putative *N*-glycosylation sequons, with the first four sites confirmed to be glycosylated. The quantitative analysis reveals that glycans derived from CHO-gmt4 cells display the least homogeneity, comprising 76.78% of Man5, 13.51% of Man4, 6.23% of F(6)Man5, and 3.48% F(6)Man4. Glycans from CHO-17 cells comprise 87.31% Man5 and 12.69% Man4, whereas glycans from CHO-17x cells exhibit exceptional homogeneity with 95.34% Man5 and 4.66% Man. To elucidate the glycan distribution at each glycosylation sites, a site-specific glycosylation analysis was performed, and the results are shown in Fig. 12b. Analysis of the results confirmed the non-glycosylation at asparagine 501 (Asn501). The exclusive presence of Man5 *N*-glycans at asparagine 58 (Asn58) across all three cell lines indicates the absence of further *N*-glycan processing at this site. Without being bound by theory, it is thought that these findings disclosed herein indicates that Asn58 in β -glucocerebrosidase may have restricted access to modification enzymes, possibly due to structural constraints, despite its proximity to the N-terminus and absence of apparent obstruction by other protein regions. Conversely, glycans at the remaining three glycosylation sites underwent additional processing involving fucose addition and mannose residue removal. Glycan heterogeneity decreases with the inactivation of more genes in CHO-17 and CHO-gmt17x cells.

[0094] The present disclosure developed and characterised a panel of CHO glycosylation mutant cell lines capable of producing glycans with defined structures. Erythropoietin Fc and trastuzumab were produced in each CHO mutant, and the attached *N*-glycans were quantitatively analysed using LC-MS. The present disclosure provides a model molecule revealed that achieving homogenous Man9 *N*-glycan production requires the inactivation of nearly all the α -mannosidases in the genome except lysosomal MAN2B1. CHO-gmt4, CHO-gmt6, CHO-7, and CHO-gmt11 cells all lack GnT-I activity and produce predominantly Man5 *N*-glycans, their O-glycan and glycolipid profiles are different. This disclosure showed the lack of GnT-II activity

prevents GnT-V from adding GlcNAc to the core structure, causing branches to attach exclusively to the α 1,3-linked mannose, leaving the α 1,6-linked mannose free. The antibody's mono-antennary glycan has a greater sialic acid content than bi-antennary glycans produced in CHO-K1 cells. With trastuzumab as a model IgG1 antibody, the present disclosure analysed how Fc *N*-glycans affect interactions between the Fc region and three FcγRs. Adding bisecting GlcNAc with GnT-III enhanced trastuzumab's affinity to FcγRIIIa in all tested mutants. Using trastuzumab glycovariants, the present disclosure demonstrated how Fc glycans influence the interaction between Fc and FcγRI/FcγRIIa, revealing a shared interaction mechanism for both FcγRI and FcγRIIa. The trastuzumab glycovariants showed a clear correlation between their binding affinity to FcγRIIIa and their *in vitro* ADCC cell-killing efficacy. In this disclosure, a soluble trimeric HIV Env gp140 protein was produced based on the sequence of the HIV BG505 strain using CHO-K1, CHO-gmt4, and CHO-gmt35 cells. The glycan dependency of five bnAbs against HIV spike protein was confirmed using these trimeric gp140 proteins. These results strongly suggest that HIV Env produced in these mutants can be valuable tools for studying the role of glycans in bnAbs' recognition.

[0095] In most of these mutants, multiple genes have been inactivated. For instance, in CHO-gmt35 cells, up to eleven genes were inactivated using CRISPR-Cas9 technique. Traditional mouse gene knockout methods are inadequate for achieving this, as inactivating many of these glycodegenes would be lethal to the animal. However, at the cellular level, these mutations do not impact cell growth. All mutants have been adapted to serum-free suspension cultures and stably transfected for antibody and glycoprotein production.

[0096] The use of erythropoietin Fc and an IgG1 antibody as model molecules for assessing the effect of each mutation on *N*-glycans proved highly successful. The three *N*-glycans attached to erythropoietin are readily accessible for glycan modifications in the Golgi, resulting in a mixture of highly processed *N*-glycans. The Fc *N*-glycans, located between the two CH2 domains, encounter structural constraints that limit the activity of glycosidases and glycotransferases. Consequently, the Fc glycans exhibit less complexity compared to those of erythropoietin Fc. The two model molecules complement each other, creating a system for assessing the influence of various glycodegenes.

[0097] HILIC-UPLC-QTOF-MS was used for quantitative analysis of glycan structure produced by each mutant, highlighting its clear advantage over the commonly used MALDI-TOF method. This advantage was particularly notable in cells that overexpressed galactosyltransferase, as well as during the development of CHO-gmt17x and CHO-gmt35 cells.

- [0098] The production of a wide range of homogeneous Fc glycans, biologics incorporating both Man5 and Man9, glycoengineered gp140 specifically designed for studying bnAbs, all clearly highlights their vast potential for driving advancements in glycobiology and biotechnology.
- 5 [0099] In one example, erythropoietin Fc exhibited exclusively homogeneous Man9 glycans only when nearly all α -mannosidases in the genome were inactivated. In another example, trastuzumab produced by these mutants provided insights into how Fc *N*-glycans impact the antibody's interaction with Fc γ R1 and Fc γ R2a, Fc γ R3a, and their influence on ADCC. When studying Fc glycans in Fc-Fc γ R1 and Fc γ R2a interactions, a consistent glycan-related influence
- 10 had been observed on binding to both receptors. In one example, the CHO mutants produced trimeric gp120 demonstrated distinct reactivity with multiple broadly neutralizing anti-HIV antibodies, confirming the involvement of gp120 glycans in interactions with specific bnAbs. In a further example, one of the mutants produced human β -glucocerebrosidase with uniform Man5 *N*-glycans, showcasing its potential for glycoengineered therapeutic production.
- 15 [00100] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and
- 20 expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled
- 25 in the art, and that such modifications and variations are considered to be within the scope of this invention.
- [00101] As used in this application, the singular form "a," "an," and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a genetic marker" includes a plurality of genetic markers, including mixtures and combinations thereof.
- 30 [00102] As used herein, the term "about", in the context of concentrations of components of the formulations, typically means +/- 5% of the stated value, more typically +/- 4% of the stated value, more typically +/- 3% of the stated value, more typically, +/- 2% of the stated value, even more typically +/- 1% of the stated value, and even more typically +/- 0.5% of the stated value.

- [00103] Throughout this disclosure, certain embodiments may be disclosed in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the disclosed ranges. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.
- 10 [00104] Certain embodiments may also be described broadly and generically herein. Each of the narrower species and sub-generic groupings falling within the generic disclosure also form part of the disclosure. This includes the generic description of the embodiments with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.
- 15 [00105] The invention has been described broadly and generically herein. Each of the narrower species and sub-generic groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.
- 20 [00106] Other embodiments are within the following claims and non-limiting examples. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

25

EXPERIMENTAL SECTION

Methods

Cell cultures

- [00107] Adherent CHO-K1 cells and CHO glycosylation mutant cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen/Gibco) supplemented with 10% fetal bovine serum (FBS) (Invitrogen/Gibco, New Zealand), at 37°C with 5% CO₂. Suspension CHO-K1 cells and glycosylation mutant cells were cultured in a chemically defined, serum-free 50/50 medium, comprising a 1:1 ratio mixture of HyClone PF-CHO Multi-powder system (GE Healthcare) and CD CHO medium (Thermo Fisher Scientific). The cells were incubated in a
- 30

37°C, 8% CO₂ shaking incubator. The 50/50 medium was supplemented with 0.05% Pluronic F-68 acid (Thermo Fisher Scientific) and 6 mM L-glutamine (Thermo Fisher Scientific).

Cell lines

[00108] The Chinese hamster ovary-K1 (CHO-K1) cell lines used in this work were obtained from two different sources: CHO-K1 (ATCC) was purchased from the American Type Culture Collection (ATCC), while CHO-K1 (Mich) was obtained from Dr. Donald K. MacCallum at the University of Michigan in Ann Arbor.

[00109] Human breast cancer cell lines were obtained from ATCC (ATCC) and cultured according to their respective recommended protocols.

10 Generation of CHO glycosylation mutants (CHO-gmts)

[00110] The process for generating Chinese hamster ovary glycosylation mutants, including the genome editing techniques used to generate these Chinese hamster ovary glycosylation mutants and the mutated genes, is demonstrated in Figure 4. As indicated in Figure 4, CHO-gmt1 cells were isolated from CHO-K1 cells via *Maackia amurensis* agglutinin (MAA) treatment. CHO-gmt2 cells were similarly isolated through *Maackia amurensis* agglutinin selection, while CHO-gmt3 cells were generated from CHO-K1 cells using different genome editing techniques, including Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated Protein 9 (CRISPR-Cas9), Zinc Finger Nucleases (ZFNs), and Transcription Activator-Like Effector Nucleases (TALENs). CHO-gmt4 cells were isolated from CHO-K1 cells using *Ricinus communis* agglutinin-I (RCA-I) selection. CHO-gmt5 cells were generated by Zinc Finger Nucleases-mediated knockout of the Slc35c1 gene in CHO-gmt1 cells. CHO-gmt6, CHO-gmt7, and CHO-gmt8 cells were isolated from CHO-gmt1, CHO-gmt2, and CHO-gmt5 cells through *Ricinus communis* agglutinin-I selection.

[00111] To generate other CHO glycosylation mutants in this disclosure, CRISPR-Cas9 technology was used to knock out various glycosyltransferases, sugar nucleotide transporters, and mannosidases in different CHO cell lines. Potential CRISPR-Cas9 target sites in each gene were identified using the website <http://staff.biosustain.dtu.dk/laeb/crispy/>. Target sequences were selected near the translational start site and with minimal predicted off-target sites. CRISPR-Cas9 constructs were designed using the GeneArt™ CRISPR Nuclease Vector with OFP Reporter Kit (Thermo Fisher Scientific), following the manufacturer's protocol. The target sequences are provided in Table 2. The methods and steps of generating the CHO glycosylation mutants are summarised in Figure 4.

FACS analysis and cell sorting

[00112] Two days after transfection with CRISPR-Cas9 constructs, CHO cells expressing orange fluorescent protein (OFP) were individually sorted into 96-well plates using FACS Aria III (Becton Dickinson). Briefly, the adherent cells were detached from propagation flasks using Trypsin-EDTA (0.25%) (Thermo Fisher Scientific), centrifuged at $200 \times g$ for 5 min, and washed twice with cold DPBS. The cells were then resuspended in serum-supplemented growth medium and prepared for sorting.

[00113] FACS analyses were also performed using FITC-conjugated MAA and Concanavalin A (Con A) lectins to evaluate mannosidases knockout mutants during the development of CHO-gmt35 cells. MAA is a lectin with a specific affinity for α 2,3-linked sialic acid to galactose residues in complex *N*-glycans, and it binds to sialic acid-containing *N*-glycans on CHO cells. Con A specifically recognise α -mannose residues on cell surface. In wild-type CHO-K1 cells, FITC-MAA exhibits the strongest binding, while FITC-Con A displays the lowest binding. In mannosidases knockout cells, as complex glycans decrease and high mannose type glycans increase on CHO cells, the binding patterns of the two lectins reversed. These lectins served as indicators for identifying Man9-producing CHO mutants, eliminating the need for *N*-glycan structure analysis to evaluate the results. For instance, during the creation of CHO-gmt26 from CHO-gmt20 cells achieved by knocking out EDEM1, EDEM2, and EDEM3, FITC-Con A exhibited maximal staining and MAA-FITC displayed minimal staining only when all three genes were inactivated. The lectin staining procedure was conducted at 4 °C, unless otherwise stated. Briefly, 1×10^6 cells were centrifuged at $200 \times g$ for 5 min and subsequently washed twice with cold DPBS. Next, the cells were incubated in blocking solution (1% (w/v) BSA in DPBS) for 30 min. This was followed by incubation in 8 μ g/ml FITC-MAA, or FITC-Con A, in the blocking solution. The treated cells were washed twice with cold DPBS, and then resuspended in serum-supplemented growth medium and subjected to FACS sorting.

25 **Analyses of genomic DNA and cDNAs in CHO glycosylation mutants**

[00114] After treatment with various reagents like lectins or CRISPR-Cas9 constructs, single cells were isolated, cultured to confluence, and genomic DNA was extracted from the clones using QuickExtract™ DNA Extraction Solution (Epicentre). Next, we PCR-amplified and sequenced the targeted genomic regions to detect mutations, with the sequencing primers for various detailed in Table 3. PCR amplicons were cloned using the Zero Blunt™ TOPO™ PCR Cloning Kit for Sequencing (Thermo Fisher Scientific) and then sequenced to confirm mutation presence.

[00115] Mutant cell lines isolated with cytotoxic lectins like *Maackia amurensis* agglutinin and *Ricinus communis* agglutinin-I were first analysed using the erythropoietin (EPO) rescue

assay to identify the mutated gene. Briefly, mutant CHO cells are transfected with constructs expressing human erythropoietin and a glycogene. If the glycogene complements the mutated gene, erythropoietin will undergo full glycosylation, which is clearly visualized on an isoelectric focusing (IEF) gel. After identifying the mutated gene, mRNA was isolated and the open reading frame was sequenced to pinpoint the specific mutation. In brief, total RNA was extracted with the RNeasy Mini Kit (Qiagen). Then, cDNA was synthesised, and the target gene was amplified using gene-specific primers and the SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Thermo Fisher Scientific). The amplicons were then sequenced to identify mutations. Genome editing-generated mutations were determined using the same method, and a summary of all mutations is available in Table 2. Each gene mutation is compared to the corresponding sequence in wild-type CHO-K1 cells. For genes with multiple alleles identified, each allele's mutation is listed. Cell lines from the same parental cell line with shared mutation(s) are grouped together in Table 2.

Glutamine synthetase (GS) knockout from CHO cell

[00116] The glutamine synthetase (GS) gene was knocked out in CHO-K1 and CHO-gmt cells using Transcription Activator-Like Effector Nucleases (TALEN)-mediated gene editing as previously described.

T7E1 mismatch assay

[00117] Genomic DNA from CHO cells transfected with Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated Protein 9 (CRISPR-Cas9), Zinc Finger Nucleases (ZFNs), or Transcription Activator-Like Effector Nucleases (TALENs) was extracted using the DNeasy Blood & Tissue Kit (Qiagen) at 48 to 72-hours post-transfection for T7E1 mismatch assay.

Expression constructs

[00118] The human erythropoietin (EPO) open reading frame (ORF) was cloned into the pcDNA3.1 expression vector (Thermo Fisher Scientific). The DNA sequence encoding erythropoietin Fc fusion protein was constructed and an IRES sequence was used to link the glutamine synthetase (GS) selection marker downstream of the open reading frames (ORFs).

[00119] The heavy chain and the light chain of trastuzumab, with optimised signal peptides, were cloned into the pcDNA3.1 vector, in a tricistronic configuration as described previously. The light chain and heavy chain sequences of bnAbs 2G12, PG9, PG16, PGT121, and PGT128 (Table 5) were also cloned into pcDNA3.1 vector, using glutamine synthetase (GS) as the selection marker in the same tricistronic configuration.

- [00120] The ectodomains of FcγRI (NM_000566.3, residue 1-292), FcγRIIa (NM_001136219.1, residue 1-217), and FcγRIIIa (NM_000569, residue 1-208) were cloned into the pcDNA3.1 vector with a C-terminal 6x His tag. GS_{R324C} served as the selection marker, fused downstream of expressed proteins in a bicistronic configuration using an IRES sequence.
- 5 [00121] The coding sequence of 6x His-tagged BG505 SOSIP.664 gp140 Env (SOSIP Env) was cloned into the pcDNA 3.1 vector driven the CMV promoter. GS served as the selection marker, fused with an IRES sequence in a bicistronic arrangement. The Chinese hamster furin protease (NM_001243986.1) was cloned into the same vector under the SV40 promoter using the following primers: Forward primer:
- 10 CAACACCCGGGGCCACCATGGAGCTGAGGCCCTGGTTGCTATGGGTGG (SEQ ID NO: 114), Reverse primer: GGTGCCCCGGGTCAAAGGGCGCTCTGGTCTTTGATAAAGGC (SEQ ID NO: 115). The furin sequence was then linked to the Neomycin selection marker using an IRES sequence.

Transfection of adherent CHO cells

- 15 [00122] Adherent CHO cells were seeded into 6-well plates at a density of 3×10^5 cells/ml one day prior to transfection. On the day of transfection, cells were transfected using Lipofectamine LTX with Plus reagent (Thermo Fisher Scientific) following the manufacturer's protocol. Briefly, Lipofectamine LTX was diluted in Opti-MEM (Thermo Fisher Scientific), and DNA along with Plus reagent were diluted separately in Opti-MEM. The suspensions were then
- 20 combined, mixed, and added onto the cells.

Adaptation of adherent CHO cells to serum-free suspension culture

- [00123] Adherent CHO cells were gradually transitioned to serum-free suspension culture by serially diluting the serum content in the growth medium over several passages with the appropriate suspension medium ratios. At 2.5% serum, the cell lines were transferred into shake
- 25 flasks and cultured in incubator shaker until they were fully adapted to serum-free media.

Stable transfection of suspension cells

- [00124] Suspension CHO cells were electroporated using the SG Cell line 4D-Nucleofector® X Kit (LONZA) in the 4D-Nucleofector System (LONZA), following the manufacturer's protocol. Briefly, 1×10^7 cells were transfected with 5 μg of DNA in 100 μl Nucleofector
- 30 Solution SG. Two days after transfection, the medium was switched to glutamine-free 50/50 medium for selection. After the recovery period, the medium was supplemented with 25 μM L-methionine sulfoximine (MSX) (Merck) to selectively culture cells with the GS selection marker. Subsequently, cells were allowed to recover and grow until reaching confluence. Cells with the

attenuated GS selection marker (GS_{R324C}) underwent one round of glutamine-free selection without MSX.

- [00125] To produce HIV SOSIP Env, the construct was electroporated into CHO-K1, CHO-gmt4, and CHO-gmt35 cells at a ratio of 10 µg plasmid DNA per ten million cells, using the SG Cell line 4D-Nucleofector® X Kit from LONZA. Selection was performed using glutamine-free 50/50 media supplemented with 600 µg/ml Geneticin (Thermo Fisher Scientific).

Isoelectric focusing (IEF) analysis of EPO

[00126] Immobilised pH gradient (IPG) IEF was performed following a previously published protocol.

10 Large-scale production of recombinant proteins and antibodies

- [00127] Recombinant erythropoietin Fc, trastuzumab, 2G12, PGT121, PGT128, PG9, PG16, and BG505 SOSIP.664 gp140 were produced in large-scale in different CHO cell lines. After stable transfection with constructs encoding the recombinant proteins, large-scale cultures of 100 to 240 ml were completed. Cultures were grown for approximately 6-7 days. When cell viability was still above 90%, the supernatants were collected, filtered using Nalgene™ Rapid-Flow™ filter units with 0.2 µm PES membrane (Thermo Fisher Scientific), and stored at 4°C for purification.

Protein A purification of antibodies and erythropoietin Fc

- [00128] Erythropoietin Fc purification utilized HiTrap Protein A 5 ml column (GE Healthcare, Chicago, IL), while trastuzumab and bnAbs purification employed HiTrap MabSelect SuRe 5 ml column (GE Healthcare), with an ÄKTA purifier (GE Healthcare). On the purification day, supernatants from large-scale culture were equilibrated with 0.5 volume of 20 mM phosphate buffer (pH 7.2). The column was pre-equilibrated with the equilibration buffer, and then the supernatant was injected into the column. Subsequently, the column was washed with equilibration buffer to remove unbound proteins. Erythropoietin Fc purification used 100 mM glycine pH 3.0 for elution, while trastuzumab and bnAbs purification used 100 mM acetic acid pH 3.5 for elution. Fractions with the highest UV₂₈₀ readings were pooled, and the pH was adjusted to pH 6.6 with 1 M Trizma. Protein quantification was performed using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific).

30 Immobilized metal affinity chromatography for purification of recombinant His-tagged proteins

[00129] The supernatant containing His-tagged FcγRs and SOSIP Env were purified using His HiTrap HP column (GE Healthcare Life Sciences) with the ÄKTA purification system (GE Healthcare Life Sciences). Briefly, the column was equilibrated with 20 mM sodium phosphate

buffer pH 7.4 containing 500 mM NaCl and 20 mM imidazole. Then, the pre-filtered supernatant containing recombinant His-tagged protein was loaded onto the column. The bound column was washed with 20 mM sodium phosphate buffer pH 7.4 containing 500 mM NaCl and 40 mM imidazole. The proteins were eluted by increasing the imidazole concentration to 200 mM. The

5 FcγRs were buffer exchanged with 20 mM sodium phosphate pH 7 buffer, and the concentration was determined using NanoDrop 2000.

Purification of BG505 SOSIP.664 trimer

[00130] Approximately 200 ml of harvested supernatant containing BG505 SOSIP.664 trimer was diluted 1:1 with binding buffer (20mM sodium phosphate, 0.5M sodium chloride, 20mM

10 imidazole pH 7.4). Samples were loaded onto a pre-equilibrated HisTrap HP 1 ml column (Cytiva) at a flow rate of 0.8ml/min on an AKTA purifier UPC10. Columns were washed with wash buffer (20mM sodium phosphate, 0.5M sodium chloride, 40mM imidazole pH 7.4) for 20 column volume (CV) at 0.8ml/min. Gradient elution was performed from 40 mM to 200 mM imidazole in buffer (20mM sodium phosphate, 0.5M sodium chloride) over 30 CV at 1 ml/min,

15 with 2 ml fractions collected. Selected fractions were then pooled and buffer-exchanged to pH 8.2 using 20 mM Tris buffer with 75 mM sodium chloride.

Biacore analysis

[00131] The binding affinities and kinetics between the glycoengineered antibodies and FcγRIIIa ectodomain were measured using a Biacore T200 instrument. The antibody was

20 immobilized on a CM5 sensor chip (GE Life Sciences) using standard amine coupling techniques, and all SPR measurements were performed at 25 °C. Flow line 1 served as a blank for all sensor chips, while all experimental runs were conducted using a binding buffer containing 20 mM 3-morpholinopropane-1-sulfonic acid (MOPS), 100 mM sodium chloride, pH 7.4. Purified FcγRIIIa at different concentrations was diluted in the running buffer (20 mM

25 MOPS, 100 mM sodium chloride, pH 7.4) and injected over all 4 flow cells at a flow rate of 20 μl/min for 1a duration of 10 minutes. The dissociation phase continued for 40 minutes, and surface regeneration was not required as the response rate returned to baseline at the end of each cycle. The FcγRIIIa analyte concentration was serially diluted two-fold from 2000 nM to 15.625 nM. Sensorgrams were analysed using Biacore T200 Evaluation Software, and all sensorgrams

30 were generated by subtracting the blank injection response from the analyte injected flow cell responses. Equilibrium fitting was done to obtain the dissociation constant (K_D) using the maximum response unit (R_{max}) from each measured concentration range. Association rates (k_{on}) and dissociation rates (k_{off}) were calculated using a 1:1 binding model fitting. k_{on} was determined by measuring the slope of the line fitted to the observed association rates, while k_{off} was

determined by averaging the observed dissociation rates. The data shown represents the average of two experiments for each condition.

ADCC reporter bioassay

[00132] Trastuzumab's ADCC activity was assessed using the ADCC Reporter Bioassay (Promega, Fitchburg, WI) by following the manufacturer's protocol. In the assay, a panel of breast cancer cell lines (MDA-MB-468, MDA-MB-231, MDA-MB-453, HCC1954, and SK-BR-3) was used as target cells instead of the WIL-S cells provided in the kit. Target cells were detached from propagation flasks using Accutase[®] solution (Merck, Germany) and seeded in 96-well plates at a density of 1.25×10^4 cells / 100 μ l medium / well, approximately 20-24 hours before the assay. This ensured an effector cell to target cells ratio (E:T ratio) of 6:1. After incubating the ADCC Bioassay Effector Cells, luminescence was measured using the Tecan Infinite[®] 200 microplate reader (Tecan Trading AG, Switzerland). The results were analysed, and data graphed as Fold of Induction ((induced – background) / (control – background)) versus Log₁₀ [antibody] and fitted to a 4PL curve using GraphPad Prism[®] software (GraphPad Software). Each experiment was triplicated, yielding consistent results. results with similar trends were obtained. One was shown in the figure.

ELISA analysis to determine the binding affinity of trastuzumab to different FcγRs

[00133] A nickel coated plate (Pierce[™]) was incubated with either 0.5 μ g/ml of His-tagged FcγRI or His-tagged FcγRIIa, or 0.2 μ g/well of His-tagged FcγRIIIa for 2 hours at room temperature. The plate was then washed three times with PBST (PBS/0.05% Tween-20). Glycoengineered trastuzumab samples were added to each well and incubated for 2-hour at 37 °C. Unbound antibodies were then removed by washing the wells three times with PBST. The detection antibody (Goat POD-anti human IgG (H+L), Jackson ImmunoResearch) was added and incubated at room temperature for 2 hours, followed by washing. Substrate solution (1 step ultra TMB-ELISA, Thermo Fisher Scientific) was added to each well, and the reaction was stopped with 2 M sulfuric acid. Absorbance at 450 nm was then measured with SoftMax Pro software and a VersaMax ELISA Microplate reader. Each experiment was triplicated, and the mean reading was recorded.

ELISA to assess the binding affinities between the SOSIP Env and the bnAbs

[00134] Purified His-tagged SOSIP Env from CHO-K1, CHO-gmt4 and CHO-gmt35 were coated onto Nunc MaxiSorp[™] 96-well plate at 2 mg/ml (50 μ l/well) in 1x PBS for 2 hours at room temperature. Unbound SOSIP Env proteins were washed off with TBS-0.05% Tween-20 (TBST), followed by overnight blocking with 3% BSA/TBST at 4 °C or 1-hour room temperature block. After washing off unbound BSA with TBST, Protein A purified bnAbs from

CHO-K1 stable pools were added at 4-fold dilutions (starting at 10 µg/ml) and incubated for 1.5 hours at room temperature. Unbound bnAbs were removed with three TBST washes. Bound bnAbs were detected using Horse radish peroxidase-conjugated rabbit anti-human IgG (H+L) (Jackson ImmunoResearch) in 1% BSA/TBST at 1:5000 for 1.5 hours at room temperature, followed by five TBST washes before adding the substrate solution (1-step™ Ultra TMB-ELISA Substrate solution, Thermo Fisher Scientific). The colorimetric reaction was stopped with 0.2 M sulfuric acid, and the plates were read at 450 nm using Tecan Pro with Infinite 7.1 software. Each experiment was triplicated, and the mean reading was recorded.

[00135]

10 Glycan analysis by *HILIC-UPLC-QTOF*

[00136] *N*-glycans were released from 15 µg of intact purified protein by PNGase F treatment followed by fluorescent labelling and HILIC clean up using Glycoworks RapiFluor-MS (RFMS) *N*-glycan kit (Waters Corporation, Milford, MA, US) according to the manufacturer's protocol. The fluorescent labelled *N*-glycans were then dried down and reconstituted sequentially in 9 µL water, 10 µL dimethyl formamide (DMF), and 21 µL LC-MS grade acetonitrile (ACN) (> 99.9% purity, Fisher Chemical, Waltham, MA, US) (total of 40 µL).

[00137] Ten microliters (10 µL) of the reconstituted glycans were injected onto a Waters LC-MS/MS system which consists of a H-class Acquity UPLC system connected to a Xevo G2-S quadrupole-time of flight (QTOF) mass spectrometer, controlled by Waters UNIFI Biopharmaceutical software (version 1.8, Waters Corporation). Separation was performed across a Waters Acquity UPLC glycan BEH amide column (2.1 mm x 150 mm, 1.7 µm) using a gradient of buffer A (50 mM ammonium formate (pH 4.4)) and buffer B (100% ACN) with the following multi-step gradient; 75–51% B for 40 min at 0.4 mL/min, 51% B to 0% B in 1.5 min, 0% B for 3 min while flow rate is adjusted down from 0.4 to 0.2 mL/min, 0–75% B in 3.6 min at 0.2 mL/min, 75% B for 4.5 min at 0.2 mL/min, and 75% B for another 7.4 min while flow rate was increased from 0.2 back to 0.4 mL/min. Glycan signal was detected at excitation wavelength of 265 nm and emission wavelength of 425 nm. The retention time of each chromatographic peak was converted into a glucose unit (GU) by fitting into a calibration curve established by an external standard of RFMS-labelled dextran ladder (Waters Corporation, Milford, MA, US) using a cubic spline polynomial. The GU value of each chromatographic peak was search against the RFMS database (Waters Corporation) for assignment of possible *N*-glycan structures in the UNIFI software. Mass spectrum data was also used to identify the correct structure based on accurate mass confirmation (5 ppm error). Full MS scan was acquired in positive mode in the range of *m/z* 100–2000 using a capillary voltage of 2750 V, cone voltage at 15 V, source

temperature at 120 °C, desolvation gas flow at 800 L/H, desolvation temperature at 300 °C and scan time of 1 s. Glu-fibrinopeptide (m/z 785.8421) was introduced as a lock spray mass to calibrate the mass accuracy of the instrument during the run.

[00138] MSMS was performed from the mass range of m/z 150 to 2000 at a scan time of 0.5 s for charge states 1 to 3 and ion intensity of 2000. Ramp collision energy was set to 10 eV to 30 eV.

[00139] Most peaks contained 1 *N*-glycan and this case LC fluorescent peak area was used to quantitate using the UNIFI software.

Mannosidase digestion

10 [00140] RFMS labelled samples were dried down and reconstituted in a mixture of glycobuffer 4, zinc and α 1-2,3,6 mannosidase (New England Biolabs) and incubated for 72 h at 37 °C to ensure complete digestion. HILIC clean up using Glycoworks HILIC μ Elution plate (Waters Corporation) was performed following digestion.

Site-specific analysis of glucocerebrosidase *N*-glycopeptides by LC-MS/MS

15 [00141] Sample preparation for *N*-glycopeptide analysis was performed using the filter-aided sample preparation (FASP) method adapted from Wiśniewski et al. In brief, 30 μ g of the protein was first denatured and reduced using 4 M urea and dithiothreitol (DTT) at 56 °C for 30 min. The protein was then transferred to a 10 K MWCO filter (Pall Corporation) and centrifuged at 14,000 g for 10 min at room temperature to remove the urea and DTT followed by addition of
20 iodoacetamide for alkylation in the dark for 30 min. After 30 min, the iodoacetamide was removed by centrifugation and the protein which remained on filter was washed thrice with 50 mM ammonium bicarbonate. Trypsin (sequencing grade from Promega, Madison, WI, US) was then added in 1:20 ratio (enzyme/ protein) and incubated at 37 °C for 16 h. The digested products were collected subsequently as flow-through from the filter by centrifugation. The peptides were
25 dried down and reconstituted in 0.1% formic acid (Merck, Darmstadt, Germany) for analysis on LC-MS/MS.

[00142] The treated sample was injected and analysed by nanospray LC-MS/MS on an Orbitrap Fusion Tribrid (Thermo Fisher Scientific, Waltham, MA, US) with an Easy-Spray source coupled to a nano LC system (Ultimate 3000 RSLCnano, Thermo Fisher Scientific) with
30 a C18 trap column, (Acclaim PepMap100 C18, 5 μ m, 5 mm x 300 μ m, Thermo Fisher Scientific). Glycopeptides were enriched onto the trap column using a loading solvent of 0.1% trifluoroacetic acid (TFA) (Merck, Darmstadt, Germany) at a flow rate of 15 μ L/min for 5 min before being eluted and separated on a 50 μ m x 15 cm Easy-Spray C18, 2 μ m nano column (Thermo Fisher Scientific) at a flow rate of 300 nL/min using the following multi-step gradient;

4% B for 5.5 min, 4 to 95% B in 49.5 min, 95% B for 7 min, 95–4% B in 2 min, 4% B for 15 min. Solvent A is 0.1% formic acid and solvent B is 0.1% formic acid in ACN.

[00143] Full FT-MS scan was acquired in the range of m/z 400–3000, with the source voltage set at 2000 V, ion transfer tube temperature at 300 °C, resolution at 120 K, AGC target of 4×10^5 and maximum injection time of 50 ms. MS/MS experiment was carried out using HCD with the following parameters: top speed 5 s, charge state 2–7, dynamic exclusion at 15 s with mass tolerance of ± 10 ppm, intensity greater the 5×10^4 , isolation window of 1.6 m/z , collision energy at 20% and 40%, resolution of 30 K, AGC target of 1×10^5 and maximum injection time of 100 ms.

10 *N*-glycopeptide data analysis

[00144] Identification and quantification of glycopeptides and their glycosylation sites were performed using Byonic (Protein Metrics Inc., ver. 3.1.0) integrated into Proteome Discoverer (v2.2.0.388, Thermo Fisher Scientific) as well as manual inspection of the raw mass spectral data using the software Xcalibur (ver. 4.1.50, Thermo Fisher Scientific). The following parameters settings were used with the enzyme for sample digestion set to trypsin (full), two maximum missed cleavages, precursor mass tolerance at 15 ppm, fragment mass tolerance at 20 ppm, common modifications (3 per peptide) were dynamic methionine oxidation, asparagine and glutamine deamidation and static cysteine carbamidomethylation, rare modifications were set to one *N*-glycan per peptide and searched against an in-house created *N*-glycan library database comprising of 20 glycans with 1% false discovery rate.

[00145] Glycopeptides were identified by searching their MSMS fragmentation. In particular, the presence of oxonium ions (m/z 204.0867, 366.1396), peptide sequence (b- and y-ions fragmentation), elongation of the oligosaccharide as well as, the complete intact mass of the glycopeptides was checked manually for complete agreement.

CLAIMS

1. A modified mammalian cell comprising at least 3 gene mutations, wherein one of the gene mutations is MAN2A1, wherein the remaining gene mutations are selected from the group consisting of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2B1, or homologues thereof.
2. The modified mammalian cell of claim 1 comprising at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, or at least 12 mutations.
3. The modified mammalian cell of any one of the preceding claims, wherein the cell comprises 3, 4, 10, or 11 gene mutations.
4. The modified mammalian cell of any one of the preceding claims, wherein the mammalian cell is selected from the group consisting of Chinese hamster ovarian (CHO) cell, Baby Hamster Kidney (BHK) cells, NS0 or Sp2/0 murine myeloma cells, and human embryonic kidney 293 (HEK293) cells.
5. The modified mammalian cell of any one of the preceding claims, wherein the mammalian cell is a Chinese hamster ovarian (CHO) cell.
6. The modified mammalian cell of any one of claims 4 to 5, wherein the CHO cell is CHO-K1 as designated according to the ATCC catalogue.
7. The modified mammalian cell according to any one of the preceding claims, wherein the gene mutations are selected from the group consisting of SLC35C1, MGAT1, MAN2A2, MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, and MAN2B1.
8. The modified mammalian cell of any one of the preceding claims, wherein the gene mutations comprise or consist of SLC35C1, MGAT1, and MAN2A1.

9. The modified mammalian cell of any one of claims 1 to 7, wherein the gene mutations comprise or consist of SLC35C1, MGAT1, MAN2A1, and MAN2A2.
10. The modified mammalian cell of any one of claims 8 to 9, wherein proteins expressed by said cell comprise at least 90% Man5 N-glycans.
11. The modified mammalian cell of claim 10, wherein the proteins expressed by said cell comprise at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% Man5 N-glycans.
12. The modified mammalian cell of any one of claims 1 to 7, wherein the gene mutations comprise or consist of MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2A1, and MAN2A2.
13. The modified mammalian cell of any one of claims 1 to 7, wherein the gene mutations comprise or consist of MAN1A1, MAN1A2, MAN1C1, MANEA, MAN1B1, EDEM1, EDEM2, EDEM3, MAN2A1, MAN2A2, and MAN2B1.
14. The modified mammalian cell of any one of claims 12 to 13, wherein proteins expressed by said cell comprise at least 94% Man9 N-glycans.
15. The modified mammalian cell of claim 14, wherein the wherein proteins expressed by said cell comprise at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% Man9 N-glycans.
16. The modified mammalian cell of any one of the preceding claims, wherein the genes are deleted using a CRISPR-Cas9 system.
17. The modified mammalian cell of any one of the preceding claims, wherein the modified mammalian cell is engineered to express a glycosylated protein, wherein the glycosylated proteins comprise high-mannose type N-glycans.
18. The modified mammalian cell of claim 17, wherein the protein is erythropoietin.

19. The modified mammalian cell of claim 18, wherein the erythropoietin is conjugated to an Fc fragment.
- 5 20. The modified mammalian cell of claim 17, wherein the protein is an antibody.
21. The modified mammalian cell of claim 20, wherein the antibody is a human IgG1 antibody.
- 10 22. The modified mammalian cell of claim 21, wherein the human IgG1 antibody is trastuzumab.
23. Use of the modified mammalian cells according to claims 1 to 22 in the manufacture of glycosylated proteins, wherein the glycosylated proteins comprise high-mannose type N-glycans.
- 15 24. The use of claim 23, wherein the glycosylated proteins comprise a Man5 N-glycan or Man9 N-glycan.
- 20 25. The use of claim 24, wherein the glycosylated proteins comprise at least 90% Man5 N-glycans.
26. The use of claim 24, wherein the glycosylated proteins comprise at least 94% Man9 N-glycans.
- 25 27. The use according to any one of claims 23 to 26, wherein the glycosylated proteins are selected from the group consisting of cytokines, antibodies, broadly neutralizing antibodies (bnAbs), antibody-drug-conjugates (ADCs), and combinations thereof.
- 30 28. The use according to claim 27, wherein the cytokine is erythropoietin.
29. The use according to claim 28, wherein the erythropoietin is conjugated to an Fc fragment.

30. The use according to claim 27, wherein the antibody is a human IgG1 antibody.

31. The use according to claim 30, wherein the human IgG1 antibody is trastuzumab.

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32. A kit for expressing glycosylated proteins comprising high-mannose type N-glycans, wherein the kit comprises the modified mammalian cells according to claims 1 to 22 and at least one buffer.

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