

Methods for Producing Recombinant Glycoproteins with Modified Glycosylation

CROSS-REFERENCE TO RELATED APPLICATION

5 This application claims the benefit of U.S. Provisional Application No. 61/954,337, filed on March 17, 2014, the content of which is hereby incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

10 Glycosylation is important to the structures and functions of glycoproteins. For example, glycosylation is suggested to affect protein folding (and thus stability) and/or bioactivities of glycoproteins. The demand of therapeutic recombinant glycoproteins, especially monoclonal antibodies, robustly grows in the recent two decades. Previous studies reveal that minor differences in glycan structures of recombinant glycoproteins may impact
15 on the biological activities and pharmacokinetics of the glycoproteins. For example, Darbepoetin alfa is a hyper-glycosylated analog of recombinant human erythropoietin (EPO) with two extra N-linked glycosylation sites. The extra N-glycosylation increases the percentage of the molecular mass in carbohydrates and significantly extends the serum half-life of Darbepoetin alfa, as compared to endogenous and recombinant EPO. In addition, for a
20 therapeutic antibody whose efficacy mainly relies on antibody-dependent cell cytotoxicity (ADCC), both chemo-enzymatic and genetic approaches to remove the core fucose residue on the Fc portion have been developed to increase the potency of the ADCC effect induced by that antibody.

25 However, currently available methods for remodeling glycosylation often require multiple enzymes and/or multiple steps, resulting in high costs for manufacturing glyco-engineered recombinant proteins.

SUMMARY OF THE INVENTION

30 The present disclosure is based on the development of genetically engineered host animal cells capable of producing glycoproteins such as antibodies having modified glycosylation, including defucosylation and monoglycosylation. Such host animal cells were

engineered to overly express one or more of fucosidases, endoglycosidases, or both.

Unexpectedly, changes to the cellular glycosylation machinery in the host animal cells did not result in adverse effects in relation to glycoprotein synthesis and host cell growth.

Accordingly, the present disclosure provides a genetically engineered host animal cell (e.g., a mammalian cell), which overly expresses a fucosidase, an endoglycosidase, or both, wherein the host animal cell produces glycoproteins having modified glycosylation as compared with the wild-type counterpart. In some examples, the fucosidase can be a mammalian fucosidase or a bacterial fucosidase, for example, human FUCA1, human FUCA2, *Cricetulus griseus* fucosidase, alpha-L-1 *Chryseobacterium meningosepticum* α 1,6-fucosidase, or bacterial fucosidase BF3242. Alternatively or in addition, the endoglycosidase can be an Endo S enzyme, e.g., an enzyme comprising the amino acid sequence of SEQ ID NO:11. In some examples, the genetically engineered host animal cell expresses (i) human FUCA1, human FUCA2, *Cricetulus griseus* fucosidase, alpha-L-1 *Chryseobacterium meningosepticum* α 1,6-fucosidase, or bacterial fucosidase BF3242, and (ii) an Endo S (such as SEQ ID NO:11).

The genetically engineered host animal cell described herein may further express a glycoprotein, which can be exogenous (not expressed in the native animal cell of the same type). Examples include, but are not limited to, an antibody, an Fc-fusion protein, a cytokine, a hormone, a growth factor, or an enzyme.

In some examples, the genetically engineered host animal cell is a mammalian cell, e.g., a Chinese hamster ovary (CHO) cell, a rat myeloma cell, a baby hamster kidney (BHK) cell, a hybridoma cell, a Namalwa cell, an embryonic stem cell, or a fertilized egg.

Also described herein are methods for producing glycoproteins having modified glycosylation patterns (e.g., defucosylated or mono-glycosylated) using any of the genetically engineered host animal cells described herein. The method may comprise (i) providing a host animal cell expressing (a) a glycoprotein, and (b) a fucosidase, an endoglycosidase, or both; culturing the host animal cell under conditions allowing for producing the glycoprotein and the fucosidase, the endoglycosidase, or both; (ii) collecting the host animal cell or the culturing supernatant for isolating the glycoprotein, and optionally (iii) isolating the glycoprotein. The method may further comprise (iv) analyzing the glycosylation pattern of

the glycoprotein.

Further, the present disclosure features a method for preparing any of the genetically engineered host animal cells described herein. The method may comprise (i) introducing into an animal cell one or more expression vectors, which collectively encode a fucosidase, an endoglycosidase, or both, and optionally (ii) introducing into the animal cell an expression vector encoding a glycoprotein. The method may further comprise selecting transformed cells expressing the fucosidase, the endoglycosidase, and the glycoprotein.

The details of one or more embodiments of the invention are set forth in the description below. Other features or advantages of the present invention will be apparent from the following drawings and detailed description of several embodiments, and also from the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic illustration showing the structures of various N-glycans. A: typical N-linked glycans of glycoproteins. B: defucosylated N-glycans. C: N-glycans having a mono-N-acetylglucosamine reducing sugar (monoglycosylated).

Figure 2 is a schematic illustration of an exemplary expression vector for producing a fucosidase. A: map of an exemplary plasmid that carries a fucosidase gene. B: an exemplary expression cassette for expressing a fucosidase or an endoglycosidase S.

Figure 3 includes diagrams showing the production of homogenous afucosylated mono-sugar (GlcNAc) antibody h4B12 by transient expression of the antibody in host cells engineered to express a fucosidase and/or an endoglycosidase. A: a schematic illustration of producing homogenous afucosylated mono-sugar (GlcNAc) antibodies. B: a chart showing the glycosylation of antibody 4B12 produced in host cells engineered to express human FUCA1, human FUCA2, *C. griseus* fucosidase, alpha-L-1, or *C. meningosepticum* α 1,6-fucosidase, as determined by LC/MS/MS. C: a diagram showing the transient expression of a fucosidase or an endoglycosidase as indicated in CHO cells detected by Western blot. D: a chart showing antibody h4B12 produced in CHO cells expressing a fucosidase or an endoglycosidase as indicated, the antibody having homogenous fucose-free mono-sugar (GlcNAc) glycoform as determined by LC/MS/MS. A homogenous N-glycan refers to the

ratio of that N-glycan (*e.g.*, an afucosylated N-glycan) to the total N-glycans in a glycoprotein such as an antibody.

Figure 4 includes diagrams showing the production of homogenous afucosylated mono-sugar (GlcNAc) antibody rituximab by transient expression of the antibody in host cells engineered to express a fucosidase and/or an endoglycosidase. A: a chart showing the glycosylation of antibody rituximab produced in host cells engineered to express various fucosidases or endoglycosidases as indicated. B: a diagram showing the transient expression of a fucosidase (left lane) or an endoglycosidase (right lane) as indicated in CHO cells as detected by Westernblot.

DETAILED DESCRIPTION OF THE INVENTION

Disclosed herein are genetically engineered host animal cells such as mammalian cells capable of producing glycoproteins (*e.g.*, exogenous glycoproteins such as antibodies) having modified glycosylation patterns (*e.g.*, modified N-glycosylation patterns such as defucosylated N-glycans or mono-sugar glycans). Such host animal cells may be engineered to overly express a fucosidase, an endoglycosidase, or both. Optionally, the host animal cell is also engineered to express an exogenous glycoprotein such as an antibody.

The structure of a typical complex N-glycan of glycoproteins produced in wild-type mammalian cells is shown in Figure 1, panel A. Such a complex N-glycan contains a N-acetylglucosamine (GlcNAc) residue attached to a glycosylation site, an asparagine residue (Asn) of a glycoprotein, and a fucose residue is attached to that Asn residue in an alpha1,6-linkage. The genetically engineered host animal cells are capable of producing glycoproteins (*e.g.*, endogenous or exogenous) having modified N-glycans, such as defucosylated N-glycans, an example of which is provided in Figure 1, panel B, and mono-sugar N-glycans, in which only a GlcNAc residue is attached to the Asn glycosylation site (Figure 1, panel C). A glycoprotein having modified glycosylation refers to a glycoprotein carrying at least one glycan such as an N-glycan that is structurally different from glycans of the glycoprotein produced in the wild-type counterpart of the genetically engineered host animal cell. A defucosylated glycan refers to any glycan that does not contain an alpha1,6-fucose residue or any fucose residue.

A. Fucosidase

A fucosidase is an enzyme that breaks down fucose. This enzyme cleaves fucose residues from a glycan containing such. The fucosidase for use in making the genetically engineered host animal cells can be a mammalian fucosidase or a bacterial fucosidase. In some embodiments, the fucosidase is a wild-type enzyme, *e.g.*, a wild-type bacterial enzyme or a wild-type mammalian enzyme such as a human enzyme. The amino acid sequences and encoding nucleotide sequences of a number of exemplary fucosidases are provided below (including a His-tag at the C-terminus):

Human Fucosidase FUCA1:

Amino Acid Sequence (SEQ ID NO:1)

MRVPAQLLGLLLLWLPGARCQPPRRYTPDWPSLDSRPLPAWFDEAKFGVFIHWGVFSVPA
WGSEWFWWHWQGEGRPQYQRFMRDNYPPGFSYADFGPQFTARFFHPEEWADLFQAAGAKY
VVLTTKHHEGETNWPSFVSWNWSKDVGPHRDLVGELGTALRKRNIRYGLYHSLEWFHP
LYLLDKKNGFKTQHFVSAKTMPELYDLVNSYKPDLIWSDGEWECPDITYWNSTNFLSWLYN
DSPVKDEVVVNDRWGQNCCHGGYYNCEDEKFKPQSLPDHKWEMCTSIDKFSWGYRRDMA
LSDVTEESEIISELVQTVSLGGNYLLNIGPTKDGLIVPIFQERLLAVGKWLSEINGEAIYA
SKPWRVQWEKNTTSVWYTSKGSAYVAIFLHWPENGLNLESPITTTSTTKITMLGIQGDLE
WSTDPDKGLFISLPQLPPSAVPAEFAWTIKLTGVKHHHHHH

Nucleotide Sequence (SEQ ID NO:2; codon optimized)

atgagagtgcctgctcagctgctgggactgctgctgctgtggctgcctgggtgctagatgc
cagccccctcgagatacacccctgactggccttcctggactccagacctctgcccgtc
tggtttgacgaggccaagttcggcgtgttcacactggggcgtgttctccgtgcctgcc
tggggctctgagtgggttctggtggcattggcagggcgagggcagacctcagtaccagcgg
ttcatgcgggacaactacccccctggcttctcctacgccgacttcggccctcagttcacc
gcccgttcttccaccctgaggaatgggcccgatctgttccaggccgctggcgccaaatac
gtggtgctgaccaccaagcaccacgagggttcaccaactggccctcccccggtgtcctgg
aactggaactctaaggacgtgggccccaccgggatctcgtgggagaactgggaaccgcc
ctgcggaagcgggaacatcagatacggcctgtaccactccctgctggaatgggtccacccc
ctgtacctgctggacaagaagaacggcttcaagaccagcacttcgtgtccgccaagacc
atgcccagagctgtacgacctcgtgaactcctacaagcccagacctgatttgagcgacggc
gagtgggagtgccccgacacctattggaactccaccaactttctgtcctgggtgtacaac
gactccctgtgaaggacgaggtggtcgtgaacgacagatggggccagaactgctcctgt
caccacggcggctactacaactgcgaggacaagttcaagccccagtcctgcccgaccac
aagtgggagatgtgcacctctatcgacaagttctcctggggctaccggcgggacatggcc
ctgtctgatgtgaccgaggaatccgagatcatctccgagctgggtgcagaccgtgtccctg
ggcggcaactacctgctgaacatcgccctaccaaggacggcctgatcgtgcccatcttc
caggaacggctgctggcgtgggcaagtggctgtctatcaacggcgaggccatctacgcc
tccaagccttggcgagtgcagtgaggagaagaacaccacctccgtgtggtacacctccaag

ggctctgccgtgtacgccatcttcctgcactggcccgagaacggcgtgctgaacctggaa
tcccccatcaccacctctaccaccaagatcaccatgctgggcacccagggcgacctgaag
tgggtccaccgacctgacaagggcctgttcctcctcctgccccagctgcctccttccgct
gtgcctgctgagttcgctggaccatcaagctgaccggcgtgaagcaccaccaccatcac
cattga

Human Fucosidase FUCA2:

Amino Acid Sequence (SEQ ID NO:3)

10 MRVPAQLLGLLLLWLPGARCHSATRFDPTWESLDARQLPAWFDQAKFGIFIHGVSFVPS
FGSEWFWWYQKEKIPKYVEFMKDNYPSPFKYEDFGPLFTAKFFNANQWADIFQASGAKY
IVLTSKHHEGFTLWGSEYSWNWNAIDEGPKRDIVKELEVAIRNRDRLRGLYYSLEFWFH
PLFLEDESSSFHKRQFPVSKTLPELYELVNNYQPEVLWSDGDGGAPDQYWNSTGFLAWLY
15 NESPVVRGTVVTNDRWGAGSICKHGGFYTCSDRYNPGHLLPHKWENCMTIDKLSWGYRREA
GISDYLTIEELVKQLVETVSCGNLLMNIGPTLDGTISVVFEERLRQMGSWLKVNGEAIY
ETHTWRSQNDTVTPDVWYTSKPKEKLVYAIFLKWPTSGQLFLGHPKAILGATEVKLLGHG
QPLNWISLEQNGIMVELPQLTIHQMPCKWGWALALTNVIHHHHHH

Nucleotide Sequence (SEQ ID NO:4; codon optimized)

20 atgagagtgcctgctcagctgctgggactgctgctgctgtggctgcctggcgctagatgc
cactccgccaccagattcgacccccacctgggagctctctggacgccagacagctgcccgt
tggtttgaccaggccaagttcggcacatcttcactccactggggcgtgttctccgtgcccagc
ttcggctctgagtgggtctggtggtactggcagaaagagaagatccccaatacgtggag
ttcatgaaggacaactacccccccagctttaagtacgaggacttcggccccctgttcacc
25 gccaagttcttcaacgccaaccagtgggcccagacatcttcaggcctctggcgccaagtac
atcgtgctgacctccaagcaccacgagggcttcaccctgtggggctccgagtactcctgg
aactggaacgccatcgacgagggccccaagcgggacatcgtgaaagaactggaagtggcc
atccggaaccggaccgacctgagattcggcctgtactactcctgttcgagtggttccac
cccctgtttctggaagatgagtcctccagcttccacaagcggcagttccccgtgtccaag
30 accctgcccagagctgtacgagctcgtgaacaactaccagcccaggtgctgtggagtgc
ggggatgggtggtgccccgatcagtagtgaactctaccggttctctggcctggctgtac
aacgagtctcctgtgcggggcaccgtcgtgaccaacgatagatggggcgctggctccatc
tgcaagcacggcggcttctacacctgttccgaccggtacaaccccgcccatctgctgcct
cacaagtgggagaactgcatgaccatcgacaagctgtcctggggctacagaagagaggcc
35 ggcattctccgactacctgacaatcgaggaaactcgtgaagcagctgggtgaaaccgtgtcc
tgcggcggcaacctgctgatgaacatcggccctaccctggacggcaccatctccgtgggtg
ttcgaggaacggctgcggcagatgggctcctgggtgaaagtgaacggcgaggccatctac
gagacacacacctggcgggtcccagaacgacaccgtgacccctgacgtgtggtacaccagc
aagcccaaagaaaagctggtgtatgccatcttctgaagtggcctacctccggccagctg
40 ttctggggccacctaaaggctatcctggggcgccaccgaagtgaactgctgggccatgga
cagccccctgaactggatctccctggaacagaacggcatcatgggtggaactgcccagctg
accatccatcagatgccctgcaaattggggctggggcctggccctgaccaacgtgatccac
catcaccaccaccactga

Cricetulus griseus (Chinese Hamster) Fucosidase FUCA2

Amino Acid Sequence (SEQ ID NO:5)

5 MRVPAQLLGLLLLWLPGARCKSSRRYDPTWESLDRRPLPSWFDQAKFGIFIHWGVFSVPS
FGSEFWWWYWQKEKRPKFVDFMNNNYPFGFKYEDFGVLFTAKFFNASQWADILQASGAKY
LVLTSKHHEGFTLWGSEYSWNWNAVDEGPKRDIVKELKVAITKNTDLRFGLYYSLFEWFH
PLFLEDKLSSFQKRQFPI SKMLPELYELVNKYQPDILWTDGDGGAPDRYWNSTGFLAWLY
NESPVRNTTVTNDRWGAGSICKHGGYYTCSDRYNPGHLLPHKWENCMTIDQFSWGYRREA
10 VISDYLTIEELVKQLVETVACGGNLLMNIGPTLDGIIPVIFEERLRQMGMWLKVNGEAIY
ETQPWRSQNDTATPDVWYTYKPEEKIVYAI FLKWPVSRELFL EQPIGSLGETEVALLGEG
KPLTWTSLKPNGI IVELPQLTLHQMPCKWGWTLALTNVTHHHHHH

Nucleotide Sequence (SEQ ID NO:6; codon optimized)

15 atgagagtgcctgctcagctgctgggactgctgctgctgtggctgcctggcgctagatgc
aagtcctctcggagatacgacccccacctgggagtccttgacagaaggcctctgcccagt
tggttcgaccaggccaagttcggcatcttcacccactggggcggtgttctccgtgcccagc
ttcggctctgagtgggtctggtggtactggcagaaagagaagcggcccaagttcgtggac
20 ttcatgaacaacaactacccccctggctttaagtacgaggacttcggcgctgctgttcacc
gccaagttcttcaacgcctcccagtgggccgacatcctgcaggcttcggcgctaaagtac
ctggtgctgacctccaagcaccacgagggtttaccctgtggggctccgagtactcctgg
aactggaacgcctggacgagggccctaagcgggacatcgtgaaagaactgaaggtggcc
atcaccaagaacaccgacctgagattcggcctgtactactcctgttcgagtggttccac
25 cccctgtttctggaagataagctgtccagcttccagaagcggcagttcccatctccaag
atgctgcccagagctgtacgagctcgtgaacaagtaccagcctgacatcctgtggaccgac
ggggatggtggcgcccctgacagatactggaactctaccggcttcttgccctggctgtac
aacgagtcctcctgtgcggaacaccgtcgtgaccaacgacagatggggcgctggctccatc
tgcaagcacggcggtactacacctgttccgaccggtacaaccccgcccatctgctgcct
30 cacaagtgggagaactgcatgacaatcgaccagttctcctggggctaccggcgcgaggcc
gtgatctctgactacctgaccatcgaggaaactcgtgaagcagctggtgaaaccgtggcc
tgtggcggaacctgctgatgaacatcggccctaccctggacggcatcatccccgtgatc
ttcgaggaacggctgcggcagatgggcatgtggctgaaagtgaacggcgaggccatctac
gagacacagccttggcggtcccagaacgacaccgccacacctgacgtgtggtacacctac
35 aagcccgaagagaagatcgtgtacgccatcttccctgaagtggcccgctgtccagagagctg
tttctggaacagcccatcggtccctggggcgagacagaagtggctctgctggggcgagggc
aagcctctgacctggacctccctgaagcccaatggcatcatcgtggaactgccccagctg
accctgcaccagatgccctgtaaattggggctggaccctggccctgaccaacgtgaccac
caccacctcaccactga
40

Chryseobacterium meningosepticum α 1,6-Fucosidase

Amino Acid Sequence (SEQ ID NO:7)

45

MRVPAQLLGLLLLWLPGARCHNVSEGYEKPADPLVVQNLEQWQDLKFLFMHWGTYSQWG
IVESWSLCPEDESWTQRKPEHGKSYNEYVKNYENLQTTFNVPVQFNPQKWADATKKAGMKY
VVFTTKHHDFAMFDTKQSDYKITSSKTPFSKNPKADVAKEIFNTFRDNGFRIGAYFSKP
DWHSDDYWWSYFPPKDRNVNYDPQKYPARWENFKKFTFNQLNEITSNYGKIDILWLDGGW
5 VRPFHTIDPNIEWQRTIKVEQDIDMDKIGTMARKNQPGIIIVDRTVPGKWENYVTPEQAV
PEHALSIPWESCITMGDSFSYVPNDNYKSSQKIIETLIRIISRGGNYLMNIAPGPNGDYD
AVVYERLKEISGWMDKNQSAVFTTRALAPYHESDFYTTQSKDGKIVNVFHISEKSNIYQAP
SELSFSIPENINPKTVKVLGISSQIKWKKKGKNKIHVQLPEERTKLNYSTVIQITQHSHHHH
H

10 Nucleotide Sequence (SEQ ID NO:8; codon optimized)

atgagagtgcctgctcagctgctgggactgctgctgctgtggctgcctggcgctagatgc
cacaatgtgtccgaggggtacgagaagcccgccgaccctctgggtgggtgcagaacctggaa
cagtggcaggacctgaagttcggcctgttcactgcactggggcacctactcccagtggggc
15 atcgtggaatcctggtcctgtgccctgaggacgagtccttggaaccagcggaagcctgag
cacggcaagtcctacaacgagtacgtgaagaactacgagaacctgcagaccaccttcaac
cccgtgcagttcaacccccagaagtgggcccgcacgccaccaagaaagccggcatgaaatac
gtgggtgttcaccaccaagcaccacgacggcttcgccatgttcgacaccaagcagtcgcgac
tacaagatcacctcctccaagaccccccttcagcaagaaccccaaggccgacgtggccaaa
20 gagattttcaacaccttcgggacaacggcttcgggatcggcgcctacttctccaagcct
gactggcactccgacgactactggtggtcctacttcccaccaaggaccggaacgtgaac
tacgaccctcagaaataccccgccagatgggagaacttcaagaagttcaccttcaatcag
ctgaacgagatcaccagcaactacggcaagatcgacatcctgtggctggacggcggtatgg
gtgcgacccttccacaccatcgaccccaacatcgagtggcagcgaccatcaaggtggaa
25 caggacatcgacatggacaagatcggcaccatggcccgaagaaccagcccggcatcatc
atcgtggaccggaccgtgcctggcaagtgggagaattacgtgacccccgagcaggccgtg
cctgagcatgccctgtctatcccttgggagtcctgtatcacaatgggcgacagcttctcc
tacgtgccccaacgacaactacaagtcctcccagaagatcatcgagacactgatcaggatc
atctccagaggcggcaactacctgatgaatatcgcccctggccccaacggcgactacgac
30 gctgtggtgtacgagcggctgaaagaaatctccggctggatggataagaaccagtcgcgc
gtgtttaccacccgggctctggcccttaccacgagtcgcgacttctactacaccagtc
aaggacggaaagatcgtgaacgtgttccacatctccgagaagtccaactaccaggccccc
tccgagctgtccttcagcatccccgagaacatcaaccccaagaccgtgaaggtgctgggc
atctccagccagatcaagtggagaagaagggaacaagatccacgtgcagctgcccgag
35 gaacggaccaagctgaactactccaccgtgatccagatcaccagcaccaccaccatcac
cactga

40 *Bacterial Fucosidase BF3242*

Amino Acid Sequence (SEQ ID NO:9)

MRVPAQLLGLLLLWLPGARCQQKYQPTEANLKARSEFQDNKFGIFLHWGLYAMLATGEWT

MTNNNLNYKEYAKLAGGFYPSKFDADKWVA AIKASGAKYICFTTRHHEGF SMFDTKYSDY
 NIVKATPFKRDVVKELADACAKHGIKLHFFYYSHIDWYREDAPQGRTGRRTGRPNPKGDWK
 SYYQFMNNQLTELLTNYGPIGAIWFDGWWDQDINPDFDWELPEQYALIHRLQPACLVGNN
 HHQTPFAGEDIQIFERDLP GENTAGLSGQSVSHLPLETCETMNGMWGYKITDQNYKSTKT
 5 LIHYLVKAAGKDANLLMNIGPQPDGELPEVAVQRLKEVGEWMSKYGETIYGTRGGLVAPH
 DWGVTTQKGNKLYVHILNLQDKALFLPIVDKKVKAVVFADKTPVRFTKNKEGIVLELAK
 VPTDVDYVVELTIDHHHHHH

Nucleotide Sequence (SEQ ID NO:10; codon optimized)

10 atgagagtgcctgctcagctgctgggactgctgctgctgtggctgcctggtgctagatgc
 cagcagaagtaccagcccaccgaggccaacctgaaggccagatccgagttccaggacaac
 aagttcggcatcttcctgcactggggcctgtacgccatgctggctactggcgagtggacc
 atgaccaacaacaacctgaactacaaagagtacgctaagctggctggcggttctacccc
 tccaagttcgacgcccgaacaatgggtggccgccatcaaggcctctggcgccaagtacatc
 15 tgcttcaccacccggcaccacgagggttctccatgttcgacaccaagtactccgactac
 aacatcgtgaaggccaccccccttcaagcgggacgtcgtgaaagagctggccgacgctgc
 gctaagcacggcatcaagctgcacttctactactcccacatcgactggtacagagaggac
 gccccccagggcagaaccggcagaagaacaggcagacccaaccccaaggcgactggaag
 tcctactaccagtttatgaacaaccagctgaccgagctgctgaccaactacggccccatc
 20 ggcgccattttggttcgacgggtggtgggaccaggacatcaaccccgacttcgactgggag
 ctgcccagagcagtacgccctgatccacagactgcagcccgcctgtctcgtgggcaacaac
 caccaccagaccccccttgccggcgaggacatccagattttcgagcgggatctgcccggc
 gagaacaccgctggactgtctggccagtcctgttccatctgcccctggaaacctgcgag
 acaatgaacggcatgtggggctacaagatcacccagaccagaactacaagtcaccaagaca
 25 ctgatccactacctcgtgaaagccgctggcaaggacgccaacctgctgatgaacatcggc
 cccagcctgacggcgagctgcctgaagtggctgtgcagcggctgaaagaagtgggagag
 tggatgtctaagtacggcgagactatctacggcaccagaggcgccctggtggccccctcat
 gattggggcgtgaccaccacagaagggcaacaagctgtacgtgcacatcctgaacctgcag
 gacaaggccctgttcctgcccacgtgggacaagaaagtgaagaaagccgtgggtgttcgcc
 30 gacaagacccccgtgcggttcaccaagaacaaagagggcatcgtgctggaactggccaag
 gtgccaccgacgtggactacgtggtggaactgaccatcgaccaccatcatcaccaccac
 tga

In some embodiments, the fucosidase can be an enzyme (*e.g.*, a wild-type enzyme)
 35 that share at least 85% (*e.g.*, 90%, 93%, 95%, 96%, 97%, 98%, or 99%) sequence identity as
 compared with any of the exemplary fucosidases provided above (*e.g.*, SEQ ID NO:1, SEQ
 ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, as well as other fucosidases
 described herein).

The “percent identity” of two amino acid sequences is determined using the algorithm
 40 of Karlin and Altschul Proc. Natl. Acad. Sci. USA 87:2264-68, 1990, modified as in Karlin
 and Altschul Proc. Natl. Acad. Sci. USA 90:5873-77, 1993. Such an algorithm is

incorporated into the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. J. Mol. Biol. 215:403-10, 1990. BLAST protein searches can be performed with the XBLAST program, score=50, wordlength=3 to obtain amino acid sequences homologous to the protein molecules of the invention. Where gaps exist between two sequences, Gapped BLAST can be utilized as described in Altschul et al., Nucleic Acids Res. 25(17):3389-3402, 1997. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used.

Mammalian fucosidases that can be used in constructing the genetically engineered host animal cells include, but are not limited to, those disclosed under GenBank Accession Nos. NP_114409.2, XP_003811598.1, AAH03060.1, EHH53333.1, XP_001127152.1, XP_010360962.1, XP_006084558.1, XP_004263802.1, XP_007171384.1, XP_006075254.1, XP_010982011.1, NP_001004218.1, and XP_010964137.1.

Bacterial fucosidases that can be used in constructing the genetically engineered host animal cells include, but are not limited to, those disclosed under GenBank Accession Nos. WP_008769537.1, WP_032568292.1, EYA08300.1, WP_005780841.1, EXY26528.1, WP_044654435.1, WP_029425671.1, WP_022470316.1, CDA84816.1, WP_004307183.1, and WP_008025871.1.

B. Endoglycosidase

An Endoglycosidase is an enzyme that breaks the glycosidic bonds between two sugar monomers in a glycan, thereby releasing oligosaccharides from glycoproteins or glycolipids. Endoglycosidase for use in the present disclosure (e.g., a wild-type enzyme) includes endoglycosidase D, endoglycosidase F, endoglycosidase F1, endoglycosidase F2, endoglycosidase H, and endoglycosidase S. Exemplary endoglycosidase enzymes of each subgenus are provided in the table below:

Entry	Entry name	Protein names	Gene names	Organism
P36911	EBA1_ELIME	Endo-beta-N-acetylglucosaminidase F1 (EC 3.2.1.96) (Di-N-acetylchitobiosyl beta-N-acetylglucosaminidase F1) (Endoglycosidase F1) (Mannosyl-	endOF1	Elizabethkingia meningoseptica (Chryseobacterium meningosepticum)

		glycoprotein endo-beta-N-acetylglucosaminidase F1)		
P36913	EBA3_ELIME	Endo-beta-N-acetylglucosaminidase F3 (EC 3.2.1.96) (Di-N-acetylchitobiosyl beta-N-acetylglucosaminidase F3) (Endoglycosidase F3) (Mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase F3)	endOF3	Elizabethkingia meningoseptica (Chryseobacterium meningosepticum)
P36912	EBA2_ELIME	Endo-beta-N-acetylglucosaminidase F2 (EC 3.2.1.96) (Di-N-acetylchitobiosyl beta-N-acetylglucosaminidase F2) (Endoglycosidase F2) (Mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase F2)	endOF2	Elizabethkingia meningoseptica (Chryseobacterium meningosepticum)
P04067	EBAG_STRPL	Endo-beta-N-acetylglucosaminidase H (EC 3.2.1.96) (DI-N-acetylchitobiosyl beta-N-acetylglucosaminidase H) (Endoglycosidase H) (Endo H) (Mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase H)		Streptomyces plicatus
TOJJ04	TOJJ04_STRSZ	Endoglycosidase (EndoS) (EC 3.2.1.96)	M837_00287	Streptococcus equi subsp. zooepidemicus SzS31A1

Other suitable endoglycosidase enzymes include those that share at least 85% (*e.g.*, 90%, 95%, 98%, or 99%) sequence identity to any of the enzymes described herein. Enzymes having a high sequence homology (*e.g.*, at least 85% sequence identity) with any of the above-listed endoglycosidase are expected to possess the same biological activity. Such enzymes (*e.g.*, wild-type enzymes) may be retrieved from a gene database such as GenBank using one of the above listed enzymes as a query.

In some embodiments, the endoglycosidase described herein is an Endo S enzyme. Endo S is an endoglycosidase that specifically cleaves N-glycans at the first GlcNAc residues attached to the Asn glycosylation sites of Fc domains in native IgG molecules, resulting in monoglycosylated IgG molecules, *i.e.*, an IgG molecule having a single GlcNAc attached to an Asn glycosylation site. The amino acid sequence and the encoding nucleotide sequence are provided below:

Endo S Amino Acid Sequence (SEQ ID NO:11):

MRVPAQLLGLLLLWLPGARCAQHDSLRVKAEDKVVQTSPPSVSAIDDLHYLSENSKKEFK
EGLSKAGEVPEKLDILSKAQQADKQAKVLAEMKVPEKIAMKPLKGPLYGGYFRTWHDKT
5 SDPAEKDKVNSMGELPKEVDLAFVFHDWTKDYSLFWQELATKHVPTLNKQGTRVIRTIPW
RFLAGGDHSGIAEDTQKYPNTPEGNKALAKAIVDEYVYKYNLDGLDVDIERDSIPKVNGK
ESNENIQRSIAVFEEIGKLIGPKGADKSRLFIMDSTYMADKNPLIERGAPYIDLLLQVY
GIQGEKGDWDPVARKPEKTMEERWESYSKYIRPEQYMGVFSFYENAGSGNLWYDINERK
DDHNPLNSEIAGTRAERYAKWQPKTGGVKGKGFISYRIDRDGVAHQPKKVSDDEKRTNKAI
10 KDITDGIVKSDYKVSALKKVMENDKSYELIDQKDFDPKALREAVIAQVGSRRGDLERFN
GTLRLDNPDIKSLEGLNKLKLLAKLELIGLSQITKLDSSVLPENIKPTKDTLVSVLETYK
NDDRKEEAKAIPQVALTISGLTGLKELNLAGFDRDSLALGIDAASLTSLKVDLSKNKLDL
AAGTENRQIFDVMLSTVSNRVGSNEQTVTFDHQKPTGHYPNTYGTTSRLRPVGEKIDLQ
SOLLFGTVTNQGTLINSEADYKAYQEQLIAGRRFVDPGYAYKNFAVTYDAYKVRVTDSTL
15 GVTDEKKLSTSKEETYKVEFFSPTNGTKPVHEAKVVVGAEKTMMVNLAAGATVIKSDSHE
NAKKVFDGAIEYNPLSFSSKTSITFEFKEPGLVKYWRFFNDITRKDDYIKEAKLEAFVGH
LEDDSKVKDSLEKSTEWVTVSDYSGEAQEFSQPLDNISAKYWRVTVDTKGGRYSSPSLPE
LQILGYRLPLTHDYKDDDDK

20 Endo S Nucleotide Sequence (SEQ ID NO:12; codon optimized)

atgagagtgccctgctcagctgctgggcctgctgctgctgctggctgacctggtgctagatgc
gcccagcacgactccctgatcagagtgaggccgaggacaaggtggtgcagacctccct
tccgtgtccgccatcgacgacctgcactacctgtccgagaactccaagaaagagttcaaa
gagggcctgtccaaggccggcgaggtgcccgaagagctgaaggacatcctgagcaaggct
25 cagcaggccgacaagcaggccaaggtgctggccgagatgaagggtgccagagaagatcgcc
atgaagcccctgaagggccctctgtacggcggtacttcagaacctggcacgacaagacc
tccgacccccgccgagaaggacaaagtgaactccatgggagctgcccgaagaggtggac
ctggccttctgtgttccacgactggaccaaggactactccctgttctggcaggaactggcc
accaagcacgtgcccaccctgaacaagcagggcaccagagtgatccggacaatcccctgg
30 cggtttctggctggcgggcaccactctggaatcgccgaggatacccagaagtaccccaac
acccccgagggcaacaaggccctggctaaggccatcggtggacgagtagctgtacaagtac
aacctggacggcctggacgtggacatcgagcgggactccatccctaaagtgaacggcaaa
gagtcacaacgagaacatccagcgggtctatcgccgtgttcgaggaaatcggcaagctgatc
ggccccaagggcgccgacaagtcccggtgttcatcatggactccacctacatggccgat
35 aagaacccccctgatcgagagaggcgcccccttacatcgatctgctgctggtgcaggtgtac
ggcatccaggggcgagaagggcgattgggaccctgtggcccggaagcctgaaaagaccatg
gaagagagatgggagtcctactccaagtacatccggcccagcagtagtatggtgggattc
agcttctacgaggaaaacgccgggtccggcaacctgtggtacgacatcaacgagcgggaag
gacgaccacaaccctctgaactccgagatcgccggcaccgggctgagagatacgctaag
40 tggcagcccaagaccggcgagtgaggggcgcatcttctcctacgccatcgatagggat
ggcgtggcccaccagcctaagaaggtgtccgacgacgagaagcggaccaacaaggctatc
aaggacatcaccgacggcctcgtagagtccgactacaaggtgtccaaagccctgaagaaa
gtgatggaaaacgacaagagctacgagctgatcgaccagaaggacttccccgataaggcc
ctgcgcgagggcgtgattgctcaagtgggctccagacggggcgacctggaaagattcaac

ggcaccctgcggtggacaacccccgacatcaagtccttgggaaggcctgaacaaactgaag
 aagctggccaagctggaactgatcggactgtcccagatcacaaagctggactcctccgtg
 ctgcctgagaacatcaagcccaccaaggacaccctgggtgtccgtgctggaaacctacaag
 aacgacgaccggaagaggaagccaaggccatccctcaggtggccctgaccatctctggc
 5 ctgaccggcctgaaagagctgaatctggccggccttcgaccgggattccctggctggaatc
 gatgccgcctctctgacctccctggaaaaagtggacctgtctaagaacaagctggatctg
 gctgccggcaccgagaaccggcagatcttcgacgtgatgctgtccaccgtgtccaacaga
 gtgggcagcaacgagcagaccgtgaccttcgaccaccagaagcccaccggccactaccct
 aacacctacggcaccacctccctgagactgcctgtgggcgagggcaagatcgacctgcag
 10 tcccagctgctgttcggcaccgtgaccaaccagggcacactgatcaactccgaggccgat
 tacaaggcctaccaggaacagctgatcgctgggcgagattcgtggaccctggctacgct
 tacaagaacttcgccgtgacctacgatgcctacaaagtgcgctgaccgactccaccctg
 ggcgtgacagacgaaaagaagctgagcacctccaaagaagagacatacaaggtggaattc
 ttctccccccaccaatggcaccaagcctgtgcatgaggctaagggtggtcgtgggcgcccag
 15 aaaaccatgatgggtcaacctggccgctgggcgccaccgtgatcaagtctgactctcacgag
 aatgccaaaaaggtgttcgacggcgccatcgagtacaatcctctgagcttctccagcaag
 accagcatcaccttcgagtttaagaacccggcctcgtgaaatactggcgggttcttcaac
 gatataccccgcaaggacgactacatcaaagaggctaagctggaagccttcgtgggcat
 ctggaagatgactccaaagtgaaggactctctggaaaagtccaccgagtgggtcaccgtg
 20 tctgactactctggcgaggccaggaattctcccagcccctggacaacatctccgccaaag
 tattggagagtgaccgtggacaccaagggcgacgggtacagctctcctagcctgcccag
 ctgcagatcctgggctacagactgcctctgaccacgactataaggacgacgacgacaaa
 tga

In some embodiments, an Endo S enzyme described herein can be an enzyme (*e.g.*, a
 25 wild-type enzyme) that share at least 85% (*e.g.*, 90%, 93%, 95%, 96%, 97%, 98%, or 99%)
 sequence identity as compared with SEQ ID NO:11. Examples include, but are not limited
 to, those described under GenBank Accession Nos. EQB24254.1, WP_037584019.1,
 WP_012679043.1, and ADC53484.1.

30 C. Genetically Engineered Host Animal Cells

The host animal cells described herein are genetically engineered to overly express
 one or more enzymes having specific glycan-modifying activities (*e.g.*, glycosidase or glycol-
 transferase). A genetically engineered host animal cell is an animal cell that carry exogenous
 (non-native) genetic materials, such as exogenous genes encoding one or more of the
 35 fucosidase and endoglycosidase described herein. A host cell that overly expresses an
 enzyme refers to a genetically engineered host cell that expresses the enzyme in a level
 greater (*e.g.*, 20%, 50%, 80%, 100%, 2-fold, 5-fold, 10-fold, 50-fold, 100-fold, 1,000-fold,
 10⁴-fold, or 10⁵-fold higher) than that of the enzyme in the wild-type counterpart of the host

cell, *i.e.*, the same type of cell that does not contain the same genetic modification as the genetically engineered host cell. In some embodiments, a gene encoding an exogenous enzyme as described herein can be introduced into a suitable parent animal cell to produce the genetically engineered host animal cell described herein. An exogenous enzyme refers to
5 an enzyme that does not exist in the parent cell used for making the engineered host animal cell.

Genetically engineered host animal cells as described herein, which are capable of producing glycoproteins having modified glycosylation as compared with the wild-type counterpart, can be prepared by the routine recombinant technology. In some instances, a
10 strong promoter can be inserted upstream to an endogenous fucosidase and/or endoglycosidase gene to enhance its expression. In other instances, exogenous genetic materials encoding one or more of fucosidases and/or endoglycosidases can be introduced into a parent host cell to produce the genetically engineered host animal cells as described herein.

15 A gene encoding a fucosidase or endoglycosidase as described herein can be inserted into a suitable expression vector (*e.g.*, a viral vector or a non-viral vector) using methods well known in the art. Sambrook et al., *Molecular Cloning, A Laboratory Manual*, 3rd Ed., Cold Spring Harbor Laboratory Press. For example, the gene and the vector can be contacted, under suitable conditions, with a restriction enzyme to create complementary ends on each
20 molecule that can pair with each other and be joined together with a ligase. Alternatively, synthetic nucleic acid linkers can be ligated to the termini of a gene. These synthetic linkers contain nucleic acid sequences that correspond to a particular restriction site in the vector. In some embodiments, the gene of the fucosidase or endoglycosidase is contained in an expression cassette comprising one or more of the following elements: a Kozak sequence and
25 a signal peptide sequence, which are located at the N-terminus of the enzyme, and a protein tag (*e.g.*, FLAG, His-tag, include chitin binding protein (CBP), maltose binding protein (MBP), and glutathione-S-transferase (GST)). The protein tag can be located at either the N-terminus or C-terminus of the enzyme. See, *e.g.*, Figure 2, panel B.

Additionally, the expression vector can contain, for example, some or all of the
30 following: a selectable marker gene, such as the neomycin gene for selection of stable or

transient transfectants in mammalian cells; enhancer/promoter sequences from the immediate early gene of human CMV for high levels of transcription; transcription termination and RNA processing signals from SV40 for mRNA stability; SV40 polyoma origins of replication and ColE1 for proper episomal replication; versatile multiple cloning sites; and T7 and SP6 RNA promoters for in vitro transcription of sense and antisense RNA. Suitable vectors and methods for producing vectors containing transgenes are well known and available in the art. Sambrook et al., Molecular Cloning, A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory Press.

If two or more enzymes are to be used in constructing the host animal cells described herein, for example, two or more fucosidases, two or more endoglycosidases, or a combination of fucosidase and endoglycosidase, genes encoding the two or more enzymes can be inserted into separate express vectors or inserted into a common express vector designed for producing multiple proteins.

Expression vectors for producing the fucosidase and/or endoglycosidase may be introduced into suitable parent host cells, including, but are not limited to, murine myeloma cells (e.g., NSO cells), Chinese Hamster Ovary (CHO) cells, human embryonic kidney cells (e.g., HEK293), and human retinoblastoma cells (e.g., PER.C6). Selection of a suitable host cell line, which is within the knowledge of those skilled in the art, would depend on the balance between the need for high productivity and the need for producing the product having desired properties.

In some instances, the expression vectors can be designed such that they can incorporate into the genome of cells by homologous or non-homologous recombination by methods known in the art. Methods for transferring expression vectors into the parent host cells include, but are not limited to, viral mediated gene transfer, liposome mediated transfer, transformation, transfection and transduction, e.g., viral mediated gene transfer such as the use of vectors based on DNA viruses such as adenovirus, adeno-associated virus and herpes virus, as well as retroviral based vectors. Examples of modes of gene transfer include e.g., naked DNA, CaPO₄ precipitation, DEAE dextran, electroporation, protoplast fusion, lipofection, cell microinjection, and viral vectors, adjuvant-assisted DNA, gene gun, catheters. In one example, a viral vector is used. To enhance delivery of non-viral vectors to

a cell, the nucleic acid or protein can be conjugated to antibodies or binding fragments thereof which bind cell surface antigens. Liposomes that also include a targeting antibody or fragment thereof can be used in the methods described herein.

A “viral vector” as described herein refers to a recombinantly produced virus or viral particle that comprises a polynucleotide to be delivered into a host cell, either *in vivo*, *ex vivo* or *in vitro*. Examples of viral vectors include retroviral vectors such as lentiviral vectors, adenovirus vectors, adeno-associated virus vectors and the like. In aspects where gene transfer is mediated by a retroviral vector, a vector construct refers to the polynucleotide comprising the retroviral genome or part thereof, and a therapeutic gene.

The genetically engineered animal host cells can comprise the use of an expression cassette created for either constitutive or inducible expression of the introduced gene(s). Such an expression cassette can include regulatory elements such as a promoter, an initiation codon, a stop codon, and a polyadenylation signal. The elements can be operably linked to the gene encoding the surface protein of interest such that the gene is operational (*e.g.*, is expressed) in the host cells.

A variety of promoters can be used for expression of the fucosidase and/or endoglycosidase (as well as any exogenous glycoproteins as described herein). Promoters that can be used to express the protein are well known in the art, including, but not limited to, cytomegalovirus (CMV) intermediate early promoter, a viral LTR such as the Rous sarcoma virus LTR, HIV-LTR, HTLV-1 LTR, the simian virus 40 (SV40) early promoter, *E. coli* lac UV5 promoter and the herpes simplex tk virus promoter.

Regulatable promoters can also be used. Such regulatable promoters include those using the tetracycline repressor (tetR) [Gossen, M., and Bujard, H., *Proc. Natl. Acad. Sci. USA* 89:5547-5551 (1992); Yao, F. et al., *Human Gene Therapy*, 9:1939-1950 (1998); Shockelt, P., et al., *Proc. Natl. Acad. Sci. USA*, 92:6522-6526 (1995)]. Other systems include FK506 dimer, VP16 or p65 using estradiol, RU486, diphenol murislerone or rapamycin. Inducible systems are available from Invitrogen, Clontech and Ariad.

The effectiveness of some inducible promoters can be increased over time. In such cases one can enhance the effectiveness of such systems by inserting multiple repressors in tandem, *e.g.*, TetR linked to a TetR by an internal ribosome entry site (IRES). Alternatively,

one can wait at least 3 days before screening for the desired function. While some silencing may occur, it can be minimized by using a suitable number of cells, preferably at least 1×10^4 , more preferably at least 1×10^5 , still more preferably at least 1×10^6 , and even more preferably at least 1×10^7 . One can enhance expression of desired proteins by known means to enhance the effectiveness of this system. For example, using the Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE). See Loeb, V. E., et al., Human Gene Therapy 10:2295-2305 (1999); Zufferey, R., et al., J. of Virol. 73:2886-2892 (1999); Donello, J. E., et al., J. of Virol. 72:5085-5092 (1998).

Examples of polyadenylation signals useful to practice the methods described herein include, but are not limited to, human collagen I polyadenylation signal, human collagen II polyadenylation signal, and SV40 polyadenylation signal.

The exogenous genetic material that includes fucosidase gene and/or endoglycosidase gene (as well as a glycoprotein gene as described herein) operably linked to the regulatory elements may remain present in the cell as a functioning cytoplasmic molecule, a functioning episomal molecule or it may integrate into the cell's chromosomal DNA. Exogenous genetic material may be introduced into cells where it remains as separate genetic material in the form of a plasmid. Alternatively, linear DNA, which can integrate into the chromosome, may be introduced into the cell. When introducing DNA into the cell, reagents, which promote DNA integration into chromosomes, may be added. DNA sequences, which are useful to promote integration, may also be included in the DNA molecule. Alternatively, RNA may be introduced into the cell.

Selectable markers can be used to monitor uptake of the desired transgene into the host animal cells described herein. These marker genes can be under the control of any promoter or an inducible promoter. These are known in the art and include genes that change the sensitivity of a cell to a stimulus such as a nutrient, an antibiotic, *etc.* Genes include those for neo, puro, tk, multiple drug resistance (MDR), *etc.* Other genes express proteins that can readily be screened for such as green fluorescent protein (GFP), blue fluorescent protein (BFP), luciferase, and LacZ.

D. Producing Glycoproteins Having Modified Glycosylation

The genetically engineered host animal cells can be used for producing glycoproteins (*e.g.*, endogenous or exogenous) having modified glycosylation patterns. In some embodiments, the parent host cell for use to producing the engineered host animal cells described above already carries a gene(s) encoding an exogenous glycoprotein. In other
5 embodiments, a gene or multiple genes encoding a glycoprotein of interest can be introduced into the genetically engineered host animal cells that express one or more fucosidase and/or endoglycosidase by methods known in the art or described herein.

Genetically engineered host animal cells capable of producing both a glycoprotein of interest and one or more of fucosidases and/or endoglycosidases can be cultured under
10 suitable conditions allowing for expression of these proteins. The cells and/or the culture medium can be collected and the glycoprotein of interest can be isolated and purified from the cells and/or the culture medium by routine technology. The glycosylation pattern of the glycoprotein thus produced can be determined by routine technology, *e.g.*, LC/MS/MS, to confirm modification of glycosylation.

15 In some examples, the glycoprotein of interest is an antibody. Exemplary antibodies include, but are not limited to, abciximab (glycoprotein IIb/IIIa; cardiovascular disease), adalimumab (TNF- α , various auto-immune disorders, *e.g.*, rheumatoid arthritis), alemtuzumab (CD52; chronic lymphocytic leukemia), basiliximab (IL-2R α receptor (CD25); transplant rejection), bevacizumab (vascular endothelial growth factor A; various cancers,
20 *e.g.*, colorectal cancer, non-small cell lung cancer, glioblastoma, kidney cancer; wet age-related macular degeneration), catumaxomab, cetuximab (EGF receptor, various cancers, *e.g.*, colorectal cancer, head and neck cancer), certolizumab (*e.g.*, certolizumab pegol) (TNF alpha; Crohn's disease, rheumatoid arthritis), Daclizumab (IL-2R α receptor (CD25); transplant rejection), eculizumab (complement protein C5; paroxysmal nocturnal
25 hemoglobinuria), efalizumab (CD11a; psoriasis), gemtuzumab (CD33; acute myelogenous leukemia (*e.g.*, with calicheamicin)), ibritumomab tiuxetan (CD20; Non-Hodgkin lymphoma (*e.g.*, with yttrium-90 or indium-111)), infliximab (TNF alpha; various autoimmune disorders, *e.g.*, rheumatoid arthritis) Muromonab-CD3 (T Cell CD3 receptor; transplant rejection), natalizumab (alpha-4 (α 4) integrin; multiple sclerosis, Crohn's disease),
30 omalizumab (IgE; allergy-related asthma), palivizumab (epitope of RSV F protein;

Respiratory Syncytial Virus infection), panitumumab (EGF receptor; cancer, e.g., colorectal cancer), ranibizumab (vascular endothelial growth factor A; wet age-related macular degeneration), rituximab (CD20; Non-Hodgkin lymphoma), tositumomab (CD20; Non-Hodgkin lymphoma), trastuzumab (ErbB2; breast cancer).

5 In some examples, the glycoprotein of interest is a cytokine. Examples include, but are not limited to, interferons (e.g., IFN- α , IFN- β , or IFN- γ), interleukins (e.g., IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-12), and colony stimulating factors (e.g., G-CSF, GM-CSF, M-CSF). The IFN can be, e.g., interferon alpha 2a or interferon alpha 2b. See, e.g., Mott HR and Campbell ID. “*Four-helix bundle growth factors and their receptors: protein-protein*”
10 *interactions.*” Curr Opin Struct Biol. 1995 Feb;5(1):114-21; Chaiken IM, Williams WV. “*Identifying structure-function relationships in four-helix bundle cytokines: towards de novo mimetics design.*” Trends Biotechnol. 1996 Oct;14(10):369-75; Klaus W, et al., “*The three-dimensional high resolution structure of human interferon alpha-2a determined by heteronuclear NMR spectroscopy in solution*”. J. Mol Biol., 274(4):661-75, 1997, for further
15 discussion of certain of these cytokines.

The protein of interest may also be a cytokine protein that has a similar structure to one or more of the afore-mentioned cytokines. For example, the cytokine can be an IL-6 class cytokine such as leukemia inhibitory factor (LIF) or oncostatin M. In some embodiments, the cytokine is one that in nature binds to a receptor that comprises a GP130
20 signal transducing subunit. Other four-helix bundle proteins of interest include growth hormone (GH), prolactin (PRL), and placental lactogen. In some embodiments, the target protein is an erythropoiesis stimulating agent, e.g., (EPO), which is also a four-helix bundle cytokine. In some embodiments, an erythropoiesis stimulating agent is an EPO variant, e.g., darbepoetin alfa, also termed novel erythropoiesis stimulating protein (NESP), which is
25 engineered to contain five N-linked carbohydrate chains (two more than recombinant HuEPO). In some embodiments, the protein comprises five helices. For example, the protein can be an interferon beta, e.g., interferon beta-1a or interferon beta-1b, which (as will be appreciated) is often classified as a four-helix bundle cytokine. In some embodiments, a target protein is IL-9, IL-10, IL-11, IL-13, or IL-15. See, e.g., Hunter, CA, Nature Reviews

Immunology 5, 521-531, 2005, for discussion of certain cytokines. See also Paul, WE (ed.), Fundamental Immunology, Lippincott Williams & Wilkins; 6th ed., 2008.

In addition, the protein of interest may be a protein that is approved by the US Food & Drug Administration (or an equivalent regulatory authority such as the European Medicines
5 Evaluation Agency) for use in treating a disease or disorder in humans. Such proteins may or may not be one for which a PEGylated version has been tested in clinical trials and/or has been approved for marketing. In some instances, the protein of interest is an Fc-fusion protein, including, but not limited to, abatacept, etanercept, IL-2-Fc fusion protein, CD80-Fc fusion protein, and PDL1-Fc fusion protein.

10 Further, the protein of interest may be a neurotrophic factor, *i.e.*, a factor that promotes survival, development and/or function of neural lineage cells (which term as used herein includes neural progenitor cells, neurons, and glial cells, *e.g.*, astrocytes, oligodendrocytes, microglia). For example, in some embodiments, the target protein is a factor that promotes neurite outgrowth. In some embodiments, the protein is ciliary
15 neurotrophic factor (CNTF; a four-helix bundle protein) or an analog thereof such as Axokine, which is a modified version of human Ciliary neurotrophic factor with a 15 amino acid truncation of the C terminus and two amino acid substitutions, which is three to five times more potent than CNTF in in vitro and in vivo assays and has improved stability properties.

20 Alternatively, the protein of interest can be an enzyme, *e.g.*, an enzyme that is important in metabolism or other physiological processes. As is known in the art, deficiencies of enzymes or other proteins can lead to a variety of disease. Such diseases include diseases associated with defects in carbohydrate metabolism, amino acid metabolism, organic acid metabolism, porphyrin metabolism, purine or pyrimidine metabolism, lysosomal
25 storage disorders, blood clotting, etc. Examples include Fabry disease, Gaucher disease, Pompe disease, adenosine deaminase deficiency, asparaginase deficiency, porphyria, hemophilia, and hereditary angioedema. In some embodiments, a protein is a clotting or coagulation factor, (*e.g.*, factor VII, VIIa, VIII or IX). In other embodiments a protein is an enzyme that plays a role in carbohydrate metabolism, amino acid metabolism, organic acid
30 metabolism, porphyrin metabolism, purine or pyrimidine metabolism, and/or lysosomal

storage, wherein exogenous administration of the enzyme at least in part alleviates the disease.

Further, the protein of interest can be a hormone, such as insulin, growth hormone, Luteinizing hormone, follicle-stimulating hormone, and thyroid-stimulating hormone. The protein of interest can also be a growth factor, including, but not limited to, adrenomedullin (AM), angiopoietin (Ang), autocrine motility factor, bone morphogenetic proteins (BMPs), brain-derived neurotrophic factor (BDNF), epidermal growth factor (EGF), erythropoietin (EPO) fibroblast growth factor (FGF), glial cell line-derived neurotrophic factor (GDNF), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), growth differentiation factor-9 (GDF9), healing factor, hepatocyte growth factor (HGF) hepatoma-derived growth factor (HDGF), insulin-like growth factor (IGF), keratinocyte growth factor (KGF), migration-stimulating factor (MSF), myostatin (GDF-8), nerve growth factor (NGF) and other neurotrophins, platelet-derived growth factor (PDGF), thrombopoietin (TPO), transforming growth factor alpha(TGF- α), transforming growth factor beta(TGF- β), tumor necrosis factor-alpha(TNF- α), vascular endothelial growth factor (VEGF), and placental growth factor (PGF).

Without further elaboration, it is believed that one skilled in the art can, based on the above description, utilize the present invention to its fullest extent. The following specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever. All publications cited herein are incorporated by reference for the purposes or subject matter referenced herein.

EXAMPLES

METHODS

i. Construction of expression vectors for producing a fucosidase or an endoglycosidase.

In order to construct expression vectors for a fucosidase or an endoglycosidase, fucosidase or endoglycosidase gene was isolated by routine technology and subjected to codon optimization based on codon usage of hamster cells. The synthetic genes were prepared by GeneArt Corp. and cloned into pcDNA3.1 B(-) Myc-His vector (Invitrogen, US) at restriction sites Bgl II/EcoR I. (Figure 2, panel A). The expression cassette of an alpha-fucosidase comprises, from 5' to 3', a Kozak sequence, an Igk leader sequence, the coding

sequence of the fucosidase, and a sequence encoding a His-tag. Figure 2, panel B. The expression cassette of an endoglycosidase comprises, from 5' to 3', a Kozak sequence, an Igk leader sequence, the coding sequence of the endoglycosidase, and a sequence encoding a Flag tag. Figure 2, panel B.

5 *ii. Preparation of defucosylated antibody*

An antibody producing cell line was maintained at $0.3\sim 3.0 \times 10^6$ viable cells/mL in a complete medium, CD FortiCHO™ medium supplemented with 8 mM L-glutamine and anti-Clumping Agent at 1:100 dilution (Life Technologies, USA). Cells were maintained on a
10 shaking platform setting at 130-150rpm in an 8% CO₂ incubator.

To produce defucosylated antibodies, the antibody-producing cells noted above were transfected with the expression vector encoding an alpha-fucosidase described above by FreeStyleMAX reagent (Life Technologies, USA) according to manufacturer's protocol. Transfected cells were cultured in a medium comprising 4g/L of glucose and the medium was
15 changed every other day. The cells were harvested when the cell viability was dropped below 70% . Clarified culture supernatant was collected and purified by Protein A Chromatography.

iii. Analysis of glycosylation of antibodies

20 Recombinant antibodies prepared according to the methods described herein were reduced, alkylated, and digested overnight with trypsin in the presence of 25 mM ammonium bicarbonate buffer (pH~8) at 37 °C. PNGase F solution (3 µL, Roche) was added to 200 µL of the digested sample and the mixture was incubated for another 16 hours at 37 °C. The released glycans were separated from the peptides using a Sep-Pak® C18 cartridge (Waters).
25 The Sep-Pak C18 was washed with acetonitrile, followed by water. The PNGaseF digested sample was loaded onto the cartridge and the released glycans were eluted with 1% ethanol while the peptides remained bound to the Sep-Pak C18. The released protein oligosaccharides were first purified using a porous graphite carbon column (PhyNexus) and then permethylated. All mass spectrometry experiments were performed using an Orbitrap
30 Fusion Tribrid mass spectrometer via direct infusion into the nano-electrospray source.

RESULTS

1. Production of antibodies h4B12, rituximab, and omalizumab having mono-sugar (GlcNAc) glycoform

5 A monosaccharide glycovariant could be made from the aforementioned di-sugar variant by a fucosidase cleavage reaction. Search from a number of available enzymes and glycol-peptide analysis by LC/MS/MS indicated that, with optimized cleavage reaction conditions, an efficient de-fucosidation could be achieved using an α -1,6-fucosidase, and that a higher cleavage efficiency is associated with a lower NF/N ratio. Alternatively, a mono-sugar glycovariant could be obtained with two reaction enzymes combined in sequence, including 10 an endoglycosidase (Endo S) and an α -1,6-fucosidase. The resultant mono-GlcNAc glycovariant was shown in Figure 3, panel A.

The results show that Endo-S removed >90% N-linked glycans of the heavy chain of h4B12, rituximab, and omalizumab produced in the engineered CHO cells described herein.

15 The defucosylation ability of five different types of fucosidases: FUCA1, FUCA2, *Cricetulus griseus* fucosidase, α -L-1, *Chryseobacterium meningosepticum* α 1,6-Fucosidase, and BF3242 are 5.8%, 9.1%, 17.7%, 11.5 and 68%, respectively, in relation to h4B12 antibodies produced in the CHO cells expressing each of the fucosidase. Figure 3, panel B. The expression of the enzymes was detected by Western blot as shown in Figure 3, panel C.

20 2-deoxy-2-fluoro L-Fucose is a fluorinated fucose analog. It can be metabolized inside host cells to generate a substrate-based inhibitor of fucosyltransferases. When culturing antibody-producing CHO cells transiently expressing fucosidase BF3242 and Endo-S, 99.89% of the N-glycans linked to the antibody produced in the CHO cells are monoglycosylated (GlcNAc-Ig-Fc). Figure 3, panel D.

25 CHO-35D6 cells, which produce rituximab, were stably transfected with an expression vector for producing BF3242 and Endo-S. The cells were cultured in the presence of 100~400 μ g/ml G418. The antibody thus produced contains 17-19% of GlcNAc-Ig-Fc. Figure 4, panel A.

Enzyme expression was detected by Western blot as shown in Figure 4, panel B.

30 Similar results were observed in omalizumab produced in CHO cells engineered to express both a fucosidase and Endo S.

Such efficiency represents an important step for transglycosylation in the preparation of antibodies with homogeneous glycan form.

OTHER EMBODIMENTS

5 All of the features disclosed in this specification may be combined in any combination. Each feature disclosed in this specification may be replaced by an alternative feature serving the same, equivalent, or similar purpose. Thus, unless expressly stated otherwise, each feature disclosed is only an example of a generic series of equivalent or similar features.

10 From the above description, one skilled in the art can easily ascertain the essential characteristics of the present invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, other embodiments are also within the claims.

What is claimed is:

1. A genetically engineered host animal cell, which expresses a fucosidase, an endoglycosidase, or both, wherein the engineered host animal cell produces glycoproteins having modified glycosylation.

2. The genetically engineered host animal cell of claim 1, wherein the fucosidase is a mammalian fucosidase or a bacterial fucosidase.

3. The genetically engineered host animal cell of claim 1 or claim 2, wherein the endoglycosidase is an Endo S enzyme.

4. The genetically engineered host animal cell of any of claims 1-3, which further expresses a glycoprotein.

5. The genetically engineered host animal cell of claim 4, wherein the glycoprotein is exogenous.

6. The genetically engineered host animal cell of claim 4 or claim 5, wherein the glycoprotein is an antibody, an Fc-fusion protein, a cytokine, a hormone, a growth factor, or an enzyme.

7. The genetically engineered host animal cell of claim 6, wherein the glycoprotein is an antibody.

8. The genetically engineered host animal cell of any of claims 1-7, wherein the animal cell is a mammalian cell.

9. The genetically engineered host animal cell of claim 8, wherein the mammalian cell is a Chinese hamster ovary (CHO) cell, a rat myeloma cell, a baby hamster kidney (BHK) cell, a hybridoma cell, a Namalwa cell, an embryonic stem cell, or a fertilized

egg.

10. The genetically engineered host animal cell of any of claims 1-9, which expresses both a fucosidase and an endoglycosidase.

5

11. The genetically engineered host animal cell of any of claims 1-10, which expresses (i) human FUCA1, human FUCA2, *Cricetulus griseus* fucosidase, alpha-L-1 *Chryseobacterium meningosepticum* α 1,6-fucosidase, or bacterial fucosidase BF3242, and (ii) an Endo S.

10

12. A method for producing a defucosylated glycoprotein, comprising:
providing a genetically engineered host animal cell expressing (a) a glycoprotein, and (b) a fucosidase, an endoglycosidase, or both;
culturing the host animal cell under conditions allowing for producing the glycoprotein and the fucosidase, the endoglycosidase, or both; and
collecting the host animal cell or the culture supernatant for isolating the glycoprotein.

15

13. The method of claim 12, wherein the fucosidase is a mammalian fucosidase or a bacterial fucosidase.

20

14. The method of claim 12 or claim 13, wherein the endoglycosidase is an Endo S enzyme.

15. The method of any of claims 11-14, wherein the glycoprotein is exogenous.

25

16. The method of claim 15, wherein the glycoprotein is an antibody, an Fc-fusion protein, a cytokine, a hormone, a growth factor, or an enzyme.

17. The method of claim 16, wherein the glycoprotein is an antibody.

30

18. The method of any of claims 12-17, wherein the genetically engineered host animal cell is a mammalian cell.

19. The method of claim 18, wherein the mammalian cell is a Chinese hamster
5 ovary (CHO) cell, a rat myeloma cell, a baby hamster kidney (BHK) cell, a hybridoma cell, a Namalwa cell, an embryonic stem cell, or a fertilized egg.

20. The method of any of claims 12-19, wherein the genetically engineered host animal cell expresses both a fucosidase and an endoglycosidase.

21. The method of any of claims 12-20, further comprising isolating the
10 glycoprotein.

22. The method of any of claims 12-21, further comprising analyzing the
15 glycosylation pattern of the glycoprotein.

23. A method for preparing the genetically engineered host animal cell of claim 1, comprising introducing into an animal cell one or more expression vectors, which collectively encode a fucosidase, an endoglycosidase, or both.

24. The method of claim 23, further comprising introducing into the animal cell an
20 expression vector encoding a glycoprotein.