demannosylation (either by separate enzymes or a single enzyme) are required to achieve mammalian cellular uptake of glycoproteins.

Disclosed is~~In one aspect, this document features~~ a method for uncapping a mannose-1-phospho-6-mannose linkage or moiety and demannosylating a phosphorylated N-glycan on a glycoprotein. The method includes providing the glycoprotein having a phosphorylated N-glycan containing the mannose-1-phospho-6-mannose linkage or moiety; and contacting the glycoprotein with a mannosidase capable of (i) hydrolyzing a mannose-1-phospho-6-mannose linkage or moiety to mannose-6-phosphate and (ii) hydrolyzing a terminal alpha-1,2 mannose, alpha-1,3 mannose and/or alpha-1,6 mannose linkage or moiety. In one aspect, there is provided a method for uncapping mannose-1-phospho-6-mannose moieties and demannosylating phosphorylated N-glycans on a glycoprotein, said method comprising

a) providing said glycoprotein having phosphorylated N-glycans containing said mannose-1-phospho-6-mannose moiety; and

b) contacting said glycoprotein with a mannosidase that (i) hydrolyzes a mannose-1-phospho-6-mannose moiety to mannose-6-phosphate and (ii) hydrolyzes terminal alpha-1,2 mannose, alpha-1,3 mannose and/or alpha-1,6 mannose linkages.

The mannosidase can be a family 38 glycosyl hydrolase. The mannosidase can be from *Canavalia ensiformis* or *Yarrowia lipolytica*.

This document also features a method of demannosylating phosphorylated N-glycans. The method includes providing a glyoprotein comprising a phosphorylated N-glycan; and contacting the glycoprotein with a mannosidase capable of (i) hydrolyzing a mannose-1-phospho-6-mannose linkage or moiety to mannose-6-phosphate and (ii) hydrolyzing a terminal alpha-1,2 mannose, alpha-1,3 mannose and/or alpha-1,6 mannose linkage or moiety. In one aspect, there is provided a method of demannosylating phosphorylated N-glycans, said method comprising

a) providing a glycoprotein comprising phosphorylated N-glycans; and

b) contacting said glycoprotein with a mannosidase that (i) hydrolyzes a mannose-1-phospho-6-mannose moeity to mannose-6-phosphate and (ii) hydrolyzes terminal alpha-1,2 mannose, alpha-1,3 mannose and/or alpha-1,6 mannose linkages.

The mannosidase can be a family 38 glycosyl hydrolase. The mannosidase can be from *Canavalia ensiformis* or *Yarrowia lipolytica*.

The methods described herein can further include after the providing and contacting steps, contacting a mammalian cell with the glycoprotein that includes the demannosylated phosphorylated N-glycan, wherein, after the contacting, the glycoprotein is transported to the interior of the mammalian cell (e.g., a human cell).

The methods described herein further can include isolating the glycoprotein produced in the methods. The protein can be a human protein expressed in a fungal organism. For example, the fungal organism can be *Yarrowia lipolytica* or *Arxula adeninivorans*. The fungal organism also can be a methylotrophic yeast (e.g., *Pichia pastoris*, *Pichia methanolica*, *Oogataea minuta*, or *Hansenula polymorpha*) or a filamentous fungus (e.g., *Aspergillus caesiellus, Aspergillus candidus, Aspergillus carneus, Aspergillus clavatus, Aspergillus deflectus, Aspergillus flavus, Aspergillus fumigatus, Aspergillus glaucus, Aspergillus nidulans, Aspergillus niger, Aspergillus*

ochraceus, Aspergillus oryzae, Aspergillus parasiticus, Aspergillus penicilloides, Aspergillus restrictus, Aspergillus sojae, Aspergillus sydowi, Aspergillus tamari, Aspergillus terreus, Aspergillus ustus, or *Aspergillus versicolor*). The protein can be a pathogen protein, a lysosomal protein, a growth factor, a cytokine, a chemokine, an antibody or antigen-binding fragment thereof, or a fusion protein. For example, the lysosomal protein can be a lysosomal enzyme such as a lysosomal enzyme associated with a lysosomal storage disorder (LSD). A LSD can be Fabry's disease, mucopolysaccharidosis I, Farber disease, Gaucher disease, GM1-gangliosidosis, Tay-Sachs disease, Sandhoff disease, GM2 activator disease, Krabbe disease, metachromatic leukodystrophy, Niemann-Pick disease, Scheie disease, Hunter disease, Sanfilippo disease, Morquio disease, Maroteaux-Lamy disease, hyaluronidase deficiency, aspartylglucosaminuria, fucosidosis, mannosidosis, Schindler disease, sialidosis type 1, Pompe disease, Pycnodysostosis, ceroid lipofuscinosis, cholesterol ester storage disease, Wolman disease, Multiple sulfatase deficiency, galactosialidosis, mucolipidosis, cystinosis, sialic acid storage disorder, chylomicron retention disease with Marinesco-Sjögren syndrome, Hermansky-Pudlak syndrome, Chediak-Higashi syndrome, Danon disease, or Geleophysic dysplasia.

This document also features a method of producing a target protein having an uncapped mannose-6-phosphate linkage or moiety and demannosylated phosphorylated N-glycans in a fungal organism. The method includes providing a fungal cell genetically engineered to include a nucleic acid encoding a mannosidase that can hydrolyze a mannose-1-phospho-6-mannose linkage or moiety to a phospho-6-mannose moiety and hydrolyze a terminal alpha-1,2 mannose, alpha-1,3 mannose and/or alpha-1,6 mannose linkage or moiety of such a phosphate containing glycan; and introducing into the cell a nucleic acid encoding a target protein.

In one aspect, there is provided an isolated fungal cell genetically engineered to produce glycoproteins comprising

demannosylated phosphorylated N-glycans, said fungal cell comprising a nucleic acid encoding a mannosidase, said mannosidase being capable of (i) hydrolyzing a mannose-1-phospho-6-mannose moiety to mannose-6-phosphate and (ii) hydrolyzing a terminal alpha-1,2 mannose, alpha-1,3 mannose and/or alpha-1,6 mannose linkage.

This document also features an isolated fungal cell genetically engineered to produce glycoproteins that include an uncapped mannose-6-phosphate and a demannosylated phosphorylated N-glycan. The fungal cell can be *Yarrowia lipolytica* or *Arxula adeninivorans*. The fungal cell also can be a methylotrophic yeast (e.g., *Pichia pastoris*, *Pichia methanolica*, *Oogataea minuta*, or *Hansenula polymorpha*) or a

chylomicron retention disease with Marinesco-Sjögren syndrome, Hermansky-Pudlak syndrome, Chediak-Higashi syndrome, Danon disease, or Geleophysic dysplasia.

A polypeptide capable of promoting mannosyl phosphorylation can be a MNN4 polypeptide (e.g., a *Yarrowia liplytica*, *S. cerevisiae*, *Ogataea minuta*, *Pichia pastoris*, or *C. albicans* polypeptide). The polypeptide capable of promoting mannosyl phosphorylation can be a *P. pastoris* PNO1 polypeptide.

Disclosed is~~In yet another aspect, this document features~~ a substantially pure culture of *Yarrowia lipolytica*, *Pichia pastoris*, *Hansenula polymorpha*, *Ogataea minuta*, *Pichia methanolica*, *Arxula adeninivorans*, or *Aspergillus niger* cells, a substantial number of which are genetically engineered to produce glycoproteins that contain uncapped mannose-6-phosphate linkages or moieties and demannosylated phosphorylated N-glycans. Substantial number indicates that more than about 40% of the total number of viable cells in the culture are genetically engineered. The cells can include a nucleic acid encoding a mannosidase, the mannosidase capable of (i) hydrolyzing a mannose-1-phospho-6-mannose linkage or moiety to mannose-6-phosphate and (ii) hydrolyzing a terminal alpha-1,2 mannose, alpha-1,3 mannose and/or alpha-1,6 mannose linkage or moiety. The cells further can include a nucleic acid encoding a polypeptide capable of promoting mannosyl phosphorylation. The cells can be genetically engineered to be deficient in OCH1 activity. The cells further can include a nucleic acid encoding a polypeptide capable of promoting mannosyl phosphorylation, and can be genetically engineered to be deficient in OCH1 activity. The mannosidase can include a secretion signal and/or a targeting signal to target the mannosidase to an intracellular compartment.

This document also features a method of directing a glycoprotein to the interior of a mammalian cell. The method includes providing a glycoprotein wherein its mannose-6-phosphate linkages have been demannosylated, and contacting the cell with the demannosylated glycoprotein. The glycoprotein can be demannosylated with a family 47 or family 92 glycosyl hydrolase. The glycoprotein can be demannosylated with a mannosidase from *Aspergillus satoi* or *Cellulosimicrobium cellulans*. The glycoprotein can be demannosylated with a family 38 glycosyl hydrolase such as a mannosidase from *Canavalia ensiformis* or *Yarrowia lipolytica*.

~~In another aspect, t~~This document also features a method of directing a glycoprotein to the interior of a mammalian cell. The method includes providing a glycoprotein having a phosphorylated N-glycan, wherein the glycoprotein does not substantially bind to a mannose-6-phosphate receptor on the cell; contacting the glycoprotein with a mannosidase capable of hydrolyzing a terminal alpha-1,2 mannose linkage or moiety when the underlying mannose is phosphorylated to produce a demannosylated glycoprotein, wherein the glycoprotein after the demannosylation, substantially binds to the mannose-6-phosphate receptor on the cell; and contacting the cell with the demannosylated glycoprotein. The glycoprotein can be demannosylated with a family 47 or family 92 glycosyl hydrolase. The mannosidase can be from *Aspergillus satoi* or *Cellulosimicrobium cellulans*. The glycoprotein can be demannosylated with a family 38 glycosyl hydrolase such as a mannosidase from *Canavalia ensiformis* or *Yarrowia lipolytica*.

In one aspect, there is provided a method of directing a glycoprotein to the interior of a mammalian cell, said method comprising

a) providing a glycoprotein having a phosphorylated N-glycan comprising a terminal mannose residue bound by an alpha 1,2 linkage to an underlying mannose residue, wherein the underlying mannose is phosphorylated at the 6 position, the phosphate residue bound to the underlying mannose is uncapped, and said glycoprotein does not substantially bind to a mannose-6-phosphate receptor on said cell;

b) contacting said glycoprotein with a mannosidase that hydrolyzes a terminal alpha-1,2 mannose linkage when the underlying mannose is phosphorylated, to produce a demannosylated glycoprotein, wherein said glycoprotein, after said demannosylation, substantially binds to said mannose-6-phosphate receptor on said cell; and

c) contacting said cell with said demannosylated glycoprotein,

wherein the mannosidase does not have the ability to uncap a phosphate residue bound to the 6 position of a mannose residue.

In another aspect, there is provided a method of directing a glycoprotein to the interior of a mammalian cell, said method comprising

a) providing a glycoprotein having a phosphorylated N-glycan comprising a terminal mannose residue bound by an alpha 1,2 linkage to an underlying mannose residue, wherein the underlying mannose is phosphorylated at the 6 position, the phosphate residue bound to the underlying mannose is uncapped, and said glycoprotein does not substantially bind to a mannose-6-phosphate receptor on said cell;

b) contacting said glycoprotein with a mannosidase that hydrolyzes a terminal alpha-1,2 mannose linkage when the underlying mannose is phosphorylated, to produce a demannosylated glycoprotein, wherein said glycoprotein, after said demannosylation, substantially binds to said mannose-6-phosphate receptor on said cell; and

c) contacting said cell with said demannosylated glycoprotein,

wherein the mannosidase uncaps a phosphate residue bound to the 6 position of a mannose residue.

~~In yet another aspect, t~~This document further features a method of converting a glycoprotein from a first form that does not substantially bind to a mannose-6-phosphate receptor on a mammalian cell to a second form that does substantially bind to a mannose-6-phosphate receptor on a mammalian cell, wherein in the first form, the glycoprotein includes one or more N-glycans containing one or more terminal mannose residues that are linked at the 1 position to a mannose residue that contains a phosphate residue at the 6 position. The method includes contacting the first form of the glycoprotein with a mannosidase that demannosylates terminal mannose residues.

In one aspect, there is provided a method of converting a glycoprotein from a first form that does not substantially bind to a mannose-6-phosphate receptor on a mammalian cell to a second form that does substantially bind to a mannose-6-phosphate receptor on a mammalian cell, wherein in the first form, the glycoprotein comprises one or more N-glycans, each containing one or more terminal mannose residues that are linked at the 1 position to an underlying mannose residue that contains a phosphate residue at the 6 position and the phosphate residue is uncapped, the method comprising contacting the first form of the glycoprotein with a mannosidase that removes said one or more terminal mannose residues, wherein the mannosidase does not have the ability to uncap a phosphate residue bound to the 6 position of a mannose residue.

In another aspect, there is provided a method of converting a glycoprotein from a first form that does not substantially bind to a mannose-6-phosphate receptor on a mammalian cell to a second form that does substantially bind to a mannose-6-phosphate receptor on a mammalian cell, wherein in the first form, the glycoprotein comprises one or more N-glycans, each containing one or more terminal mannose residues that are linked at the 1 position to an underlying mannose residue that contains a phosphate residue at the 6 position and the phosphate residue is uncapped, the method comprising contacting the first form of the glycoprotein with a mannosidase that removes said one or more terminal mannose residues, wherein the mannosidase also uncaps a phosphate residue bound to the 6 position of a mannose residue. The mannosidase can have uncapping and demannosylating activities. For example, the mannosidase can be from *Canavalia ensiformis* or *Yarrowia lipolytica*. In some embodiments, the mannosidase does not have uncapping activity (e.g., a mannosidase from *Aspergillus satoi* or *Cellulosimicrobium cellulans*).

This document also features a method of directing a glycoprotein to the interior of a mammalian cell, the glycoprotein includes one or more mannose-1-phospho-6-mannose linkages or moieties. The method includes contacting the cell with the glycoprotein after (a) uncapping the one or more mannose-1-phospho-6-mannose linkages or moieties to mannose-6-phosphate on the glycoprotein, wherein, after uncapping, the glycoprotein

does not substantially bind to a mannose-6-phosphate receptor on the cell and, after step (a), (b) demannosylating phosphorylated N-glycans on the glycoprotein, wherein after both the uncapping and the demannosylation, the glycoprotein does substantially bind to a mannose-6-phosphate receptor on the cell. Steps (a) and (b) can be catalyzed by two different enzymes (e.g., a *Cellulosimicrobium cellulans* mannosidase such as CcMan5 and a *Canavalia ensiformis* mannosidase) or by a single enzyme.

In one aspect, there is provided a method of directing a glycoprotein to the interior of a mammalian cell, the glycoprotein comprising a mannose-1-phospho-6-mannose moiety, wherein the mannose residue that has a phosphate residue bound at the 6 position is linked to a terminal mannose residue at the 1 position of the terminal mannose residue, the method comprising contacting the cell with the glycoprotein after it has undergone:

(a) uncapping of the mannose-1-phospho-6-mannose moiety to mannose-6-phosphate on the glycoprotein; and

(b) removal of the terminal mannose residue,

wherein the glycoprotein that has undergone (a) and not (b) or (b) but not (a) does not substantially bind to a mannose-6-phosphate receptor on the cell,

wherein the glycoprotein that undergone (a) and (b) does substantially bind to a mannose-6-phosphate receptor on the cell, and

wherein steps (a) and (b) are catalyzed by different enzymes.

In another aspect, there is provided a method of directing a glycoprotein to the interior of a mammalian cell, the glycoprotein comprising a mannose-1-phospho-6-mannose moiety, wherein the mannose residue that has a phosphate residue bound at the 6 position is linked to a terminal mannose residue at the 1 position of the terminal mannose residue, the method comprising contacting the cell with the glycoprotein after it has undergone:

(a) uncapping of the mannose-1-phospho-6-mannose moiety to mannose-6-phosphate on the glycoprotein; and

(b) removal of the terminal mannose residue,

wherein the glycoprotein that has undergone (a) and not (b) or (b) but not (a) does not substantially bind to a mannose-6-phosphate receptor on the cell,

wherein the glycoprotein that undergone (a) and (b) does substantially bind to a mannose-6-phosphate receptor on the cell, and

wherein steps (a) and (b) are catalyzed by a single enzyme.

~~In another aspect, t~~This document also features a method of directing a glycoprotein to the interior of a mammalian cell. The method includes providing a glycoprotein having uncapped and demannosylated phosphorylated N-glycans, and contacting the mammalian cell with the glycoprotein.

In the methods described herein, the glycoprotein can be a human protein.

In the methods described herein, the glycoprotein can be a pathogen protein, a lysosomal protein, a growth factor, a cytokine, a chemokine, an antibody or antigen-binding fragment thereof, or a fusion protein. The lysosomal protein can be a lysosomal enzyme (e.g., acid alpha glucosidase or alpha galactosidase). The glycoprotein can be associated with a LSD (e.g., Fabry's disease, mucopolysaccharidosis I, Farber disease, Gaucher disease, GM1-gangliosidosis, Tay-Sachs disease, Sandhoff disease, GM2 activator disease, Krabbe disease, metachromatic leukodystrophy, Niemann-Pick disease, Scheie disease, Hunter disease, Sanfilippo disease, Morquio disease, Maroteaux-Lamy disease, hyaluronidase deficiency, aspartylglucosaminuria, fucosidosis, mannosidosis, Schindler disease, sialidosis type 1, Pompe disease, Pycnodysostosis, ceroid lipofuscinosis, cholesterol ester storage disease, Wolman disease, Multiple sulfatase deficiency, galactosialidosis, mucolipidosis, cystinosis, sialic acid storage disorder, chylomicron retention disease with Marinesco-Sjögren syndrome, Hermansky-Pudlak syndrome, Chediak-Higashi syndrome, Danon disease, or Geleophysic dysplasia).

The document also features a glycoprotein capable of being transported to the interior of a mammalian cell, wherein the glycoprotein has been treated with any of the methods described herein, as well as a mammalian cell (e.g., human cell) that includes such a glycoprotein.

In one aspect, there is provided a glycoprotein capable of being transported to the interior of a mammalian cell, wherein said glycoprotein has been treated with the method as defined herein, and

wherein said glycoprotein contains a N-glycan in which a phosphate residue bound to an underlying mannose residue is uncapped and in which the underlying mannose residue is not bound to an additional mannose residue.

In one aspect, there is provided a mammalian cell comprising said glycoprotein as defined herein.

In one aspect, there is provided the glycoprotein as defined herein, for use in a method comprising administering the glycoprotein to a subject in need thereof.

In another aspect, there is provided~~this document features~~ a method of treatment ~~that includes~~, said method comprising administering the~~such a~~ glycoprotein as described herein to a subject in need thereof.