Piirainen, Mari A.; Frey, Alexander D.

**The Impact of Glycoengineering on the Endoplasmic Reticulum Quality Control System in Yeasts**

*DOI:* 10.3389/fmolb.2022.910709

Published: 02/06/2022

*Document Version*
Publisher's PDF, also known as Version of record

*Published under the following license:*
CC BY

*Please cite the original version:*
Piirainen, M. A., & Frey, A. D. (2022). The Impact of Glycoengineering on the Endoplasmic Reticulum Quality Control System in Yeasts. *Frontiers in Molecular Biosciences*, 9, 1-8. [910709].
https://doi.org/10.3389/fmolb.2022.910709

This material is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of the repository collections is not permitted, except that material may be duplicated by you for your research use or educational purposes in electronic or print form. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone who is not an authorised user.
The Impact of Glycoengineering on the Endoplasmic Reticulum Quality Control System in Yeasts

Mari A. Piirainen1 and Alexander D. Frey1,2*

1Department of Bioproducts and Biosystems, Aalto University, Espoo, Finland, 2Kemistintie 1, Aalto University, Otakaari, Finland

Yeasts are widely used and established production hosts for biopharmaceuticals. Despite of tremendous advances on creating human-type N-glycosylation, N-glycosylated biopharmaceuticals manufactured with yeasts are missing on the market. The N-linked glycans fulfill several purposes. They are essential for the properties of the final protein product for example modulating half-lives or interactions with cellular components. Still, while the protein is being formed in the endoplasmic reticulum, specific glycan intermediates play crucial roles in the folding of or disposal of proteins which failed to fold. Despite of this intricate interplay between glycan intermediates and the cellular machinery, many of the glycoengineering approaches are based on modifications of the N-glycan processing steps in the endoplasmic reticulum (ER). These N-glycans deviate from the canonical structures required for interactions with the lectins of the ER quality control system. In this review we provide a concise overview on the N-glycan biosynthesis, glycan-dependent protein folding and quality control systems and the wide array glycoengineering approaches. Furthermore, we discuss how the current glycoengineering approaches partially or fully by-pass glycan-dependent protein folding mechanisms or create structures that mimic the glycan epitope required for ER associated protein degradation.

Keywords: protein N-glycosylation, endoplasmic reticulum associated protein degradation (ERAD), endoplasmic reticulum quality control (ERQC), yeast, glycoengineering

INTRODUCTION

Yeasts are widely used and established production hosts for biopharmaceuticals. Saccharomyces cerevisiae is by far the most utilized yeast species for therapeutic proteins and has been approved as a production host for 35 different products. Following by a large margin, Pichia pastoris is used for the production of three, while Hansenula polymorpha is utilized for producing two therapeutic proteins. The yeast-based biopharmaceuticals currently in market include various insulin products and vaccines, but also other peptide hormones, a blood clotting factor subunit and a recombinant urate oxidase (Walsh, 2018). So far, N-glycosylated biopharmaceuticals manufactured by yeasts are currently missing on the market.

The intricacy of the N-glycan biosynthesis pathways and the wide range of end products of this modification renders the engineering of glycosylation process very demanding. Despite the heterogeneity of N-glycan structures, the formation of a lipid-linked oligosaccharide (LLO), the transfer of the glycan to a protein, and the trimming of the N-glycan during protein folding are highly conserved processes and thus N-glycan maturation in the Golgi apparatus starts from the same structure.
The N-glycans fulfill several purposes, they modulate the properties of the protein product, support protein folding through binding to calnexin and export of folded glycoproteins and provide the necessary cues to the endoplasmic reticulum associated protein degradation (ERAD) machinery to initiate protein turnover. The currently applied glycoengineering approaches affect the N-glycan structure comprising terminal α-1-3 linked and α-1-6 linked mannoses (Huffaker and Robbins, 1982; Couto et al., 1984; O’Reilly et al., 2006). Then, Alg1p adds two α-1-2 linked mannose residues to the α-1-3 arm of the LLO initiating the A branch (Cipullo et al., 2001; O’Reilly et al., 2006). The resulting Manα1GlcNAc2-P-P-Dol is translocated to the luminal side of the ER. Efforts to identify the flipase and elucidate the mechanism of the translocation reaction through in vivo and in vitro experiments produced conflicting results and the identity of the flipase and the specific role of Rfl1p remains unresolved (Helenius et al., 2002; Frank et al., 2008; Sanyal et al., 2008; Rush et al., 2009; Verchère et al., 2021).

On the luminal side of the ER, the LLO precursor is further branched and elongated. The α-1-6 linked arm of the LLO receives an α-1-3 linked mannose by Alg3p (Huffaker and Robbins, 1983; Aebi et al., 1996b; Sharma et al., 2001) forming the B branch which is capped with an α-1-2 linked mannose by Alg9p (Burd and Aebi, 2005). A third branch, C branch, is formed as Alg12p transfers an α-1-6 linked mannose to the α-1-6 arm of the core glycan (Burd and Aebi, 1999). Again Alg9p catalyzes the addition of a terminal mannose (Frank and Aebi, 2005). The final steps of the LLO biosynthesis include the addition of three glucose residues to the A branch. The first two glucose residues are added via α-1-3 linkages by Alg6p and Alg8p and the third via an α-1-2 linkage by Alg10p, respectively (Runge and Robbins, 1986; Stagljar et al., 1994; Reiss et al., 1996; Burda and Aebi, 1998).

The complete Glc3Man9GlcNAc2 oligosaccharide is transferred en bloc via an N-glycosidic bond to an asparagine residue in the acceptor sequon Asn-X-Ser/Thr by the oligosaccharyltransferase (OST) complex. To ensure that only completely assembled LLOs are transferred, the OST in most eukaryotes has a strong preference for the Glc3Man9GlcNAc2 LLO, and the outermost glucose together with the chitobiose stem (GlcNAc2) of the LLO are important determinants for the substrate recognition (Burd et al., 1999).

**N-Glycan Trimming Reactions Involved in Protein Folding and Degradation**

The following glycan trimming reactions play important roles in protein folding and marking of misfolded proteins for ERAD. First, two glucose residues are very rapidly removed from the Glc3Man9GlcNAc2 glycan (Jakob et al., 1998). The resulting Glc3Man9GlcNAc2 glycan is specifically recognized by the lectin calnexin (Cne1p) (Parlati et al., 1995; Xu et al., 2004). Removal of the last glucose residue of the N-glycan by the glucosidase heterodimer Glp2p/Glp1p releases the protein from calnexin (Trombetta et al., 1996; Wilkinson et al., 2006).
some yeasts, an α1-3 linked glucose residue can be re-transferred to the Manα3GlcNAc2 glycans by a UDP-glucose:glycoprotein glucosyltransferase (UGGT) enabling re-binding to calnexin and extending the time to correctly fold. However for example, S. cerevisiae lacks UGGT (Fernández et al., 1994), making the removal of the last glucose residue an irreversible process. After the removal of the glucose residues, one mannose residue is removed from the B branch of the N-glycan by the α1-2 mannosidase Mns1p (Camirand et al., 1991). Mns1p is a relatively slowly acting enzyme (Jakob et al., 1998), and this trimming step is thought to act as a timer mechanism for glycoprotein folding, giving the protein a sufficient time window for obtaining its native conformation (Jakob et al., 1998; Helenius and Aebi, 2004). Once the protein has reached its native conformation, the Manα3GlcNAc2 glycans acts as a determinant for cargo recruitment into COPII vesicles by the mannose lectins Emp47p and Emp46p (Sato and Nakano, 2002) (Figure 1B). Based on structural similarities to related human proteins and extensive testing of their glycan specificities, the α1-2 linked mannose of the A-branch was suggested to serve as binding motif for interaction with these mannose lectins (Sato et al., 2006; Kamiya et al., 2008).

However, after trimming by Mns1p, a second mannose residue can be cleaved from the C branch by the α1-2 mannosidase activity of the Htm1p-Pdi1p complex (Pfeiffer et al., 2016). As a result of this reaction, ManαGlcnAc2 glycans containing an exposed terminal α1-6 linked mannose residue are formed (Clerc et al., 2009; Gauss et al., 2011; Pfeiffer et al., 2016). The Htm1p-Pdi1p complex preferentially demannosylates ManαGlcnAc2 glycans of partially unstructured polypeptides (Liu et al., 2016; Pfeiffer et al., 2016).

Recognition of Endoplasmic Reticulum Associated Protein Degradation Substrates and Retrotranslocation

From three different ERAD systems, ERAD-L is responsible for eliminating ER luminal proteins which comprise also any secreted recombinant protein (Carvalho et al., 2006). The presence of a terminal α1-6 linked mannose residue of the Manα3GlcNAc2 glycan is an important determinant for ERAD targeting of misfolded proteins (Quan et al., 2008; Kniss et al., 2018). However, also other glycans displaying a terminal α1-6 linked mannose, for example such forms as generated through ALG3 deletion are recognized by Yos9p (Szathmary et al., 2005; Clerc et al., 2009) (Figure 1B). When this residue is displayed, it can be recognized by the lectin domain of Yos9p (Friedmann et al., 2002; Buschhorn et al., 2004). In addition, Yos9p has affinity for unstructured or hydrophobic regions of misfolded proteins (Smith et al., 2014). Thus, a bipartite signal requiring both aberrant protein structures and the α1-6 linked mannose signal is utilized for targeting of a glycoprotein to ERAD (Xie et al., 2009).

The HRD1 complex consists of the transmembrane proteins Hrd1p, Hrd3p, Uaa1p, Ubx2p and Der1p and the cytosolic components Cdc48p, Npl4p and Ufd1p and mediates retrotranslocation into the cytosol and ubiquitylation (Carvalho et al., 2006). Recognition by Yos9p leads to targeting of the misfolded protein towards the HRD1 complex (Martinez Benitez et al., 2011). It was suggested that Hrd3p recognizes sequences downstream of the glycosylation site that are not in their native state and Yos9p makes this more specific by only targeting misfolded glycoproteins to it (Gauss et al., 2006; Mehnert et al., 2015; Wu et al., 2020). The misfolded protein then associates with the close by Der1p, and it was proposed that this association leads to the unfolding and insertion of the substrate into substrate channel of the translocon complex that is formed by Hrd1p and Der1 (Carvalho et al., 2010; Mehnert et al., 2013; Vasic et al., 2020; Wu et al., 2020).

When the substrate emerges on the cytosolic side of the HRD1 complex, the ubiquitination step is performed by Hrd1p and this polyubiquitination prevents the slipping back of the protein into the ER lumen (Hiller et al., 1996; Friedlander et al., 2000; Bays et al., 2001; Metzger et al., 2013). When ubiquitinated, the substrate polypeptide is pulled away from the ER membrane by a complex of Cdc48p, Ufd1p and Npl4p (Wolf and Stolz, 2012) and it is directed to degradation by the 26S proteasome after removal of the N-glycans by Png1p (Suzuki et al., 2000; Hirsch et al., 2003).

Glycoengineering Strategies in Yeasts

The humanization of the yeast N-glycans consists of two main objectives, first, to create suitable N-glycan structures that can act as substrates for the subsequent glycan maturation steps and, second, to introduce the required mammalian mannosidase and glycosyltransferase activities. Here, we focus on the steps and approaches leading to the formation of the simplest complex-type N-glycan GlcNα2Manα3GlcNAc2. Undesired and not well documented side effects of the glycoengineering are often growth defects that can stem from the approach chosen for generation of the glycan acceptor and from the introduction of the required glycan processing enzymes into the Golgi apparatus (Jacobs et al., 2009; Song et al., 2010; Parsaie Nasab et al., 2013).

In mammals, the first glycan processing steps taking place in the Golgi apparatus are the trimming of the remaining α1-2 linked mannosides from Manα3GlcNAc2 by α-1,2-mannosidases IA, IB and IC (Bause et al., 1993; Tremblay et al., 1998; Tremblay and Herscovics, 2000). After mannosidase I trimming, the Manα3GlcNAc2 glycan receives a β1-2 linked GlcNAc residue from GlcNAc transferase I (GnTI), forming a hybrid-type GlcNAcManα3GlcNAc2 glycan (Oppenheimer and Hill, 1981). The pathway towards complex-type glycans proceeds by trimming of the two remaining mannoside residues from the B and C branches by α-mannosidases II and IIX (Tabas and Kornfeld, 1978; Misago et al., 1995; Shah et al., 2008). The α1-6 linked mannose of the GlcNα3Manα3GlcNAc2 glycan receives a β1-2 linked GlcNAc residue from GlcNAc transferase II (GnTII), forming the complex-type GlcNα3Manα3GlcNAc2 glycan (Bendiak and Schachter, 1987). Depending on the sequence of these processing steps, Manα3GlcNAc2, and Manα3GlcNAc2, glycans can be selected as starting point for humanization of N-glycosylation pathway in the Golgi apparatus. Furthermore, from in vitro experiments it is known that GnTII can also act on Manα3GlcNAc2 glycan substrates (Vella et al., 1984) and thus
Piirainen and Frey

Tailored Glycans Impact ERQC

ALG3

in the Golgi apparatus by GnTI and route A and B converge. By deletion of α glucosylation of the A-branch. Arrows without names: multiple steps; arrows with names: specific enzymes are marked. Man$_	ext{$_{1}$}$GlcNA$_	ext{$_{2}$}$ glycans can serve as third road towards the formation of complex-type N-glycans.

Utilizing the Man$_	ext{$_{1,2}$}$GlcNA$_	ext{$_{2}$}$ as the starting structure, the mammalian N-glycosylation pathway must be reproduced introducing mannosidase activities (ManI and ManII) and glycosyltransferases (GnTI and GnTII) into the Golgi apparatus (Figure 2, route A). By applying an extensive screening approach appropriate genetic constructs for optimal enzymatic activities were identified that led to a strain producing proteins with complex-type GlcNA$_	ext{$_{2}$}$Man$_	ext{$_{1,2}$}$GlcNA$_	ext{$_{2}$}$ of very high homogeneity in $P$. pastoris (Hamilton et al., 2003).

Man$_	ext{$_{1,2}$}$GlcNA$_	ext{$_{2}$}$ glycans formed in the ER can serve as the substrate for GnTI. This approach starts from native ER glycan forms but includes a heterologous α-1,2 mannosidase activity for the trimming of all α-1,2 linked mannosides (Figure 2, route B). This strategy was first implemented in $S$. cerevisiae, where a HDEL-tagged α-1,2 mannosidase from Aspergillus saitoi was expressed leading to formation of Man$_	ext{$_{1,2}$}$GlcNA$_	ext{$_{2}$}$ structures and later adapted in $P$. pastoris, where the expression of an HDEL-tagged α-1,2 mannosidase from Trichoderma reesei eliminated over 85% of the α-1,2 linked mannosides (Chiba et al., 1998; Callewaert et al., 2001). Using this strategy ManII, GnTI and GnTII activities are required in the Golgi apparatus and an increasing N-glycan heterogeneity was observed with each additional glycan processing step introduced (Jacobs et al., 2009).

In $S$. cerevisiae and $P$. pastoris, using the Man$_	ext{$_{1,2}$}$GlcNA$_	ext{$_{2}$}$ and Man$_	ext{$_{1,2}$}$GlcNA$_	ext{$_{2}$}$ structures as starting point required deletions of genes encoding interfering mannosyltransferases including Och1p, and in $S$. cerevisiae additionally the mannosyltransferase Mnn1p and phosphomannosyltransferase Mnn4p in order to increase homogeneity of the glycans structure (Chiba et al., 1998; Hamilton et al., 2003; Vervecken et al., 2004).

Alternatively, a Man$_	ext{$_{1,2}$}$GlcNA$_	ext{$_{2}$}$ glycan can be generated in the ER. This can be achieved through deletion of ALG3 gene and expression of a heterologous α-1,2 mannosidase activity in the ER (Figure 2, route C). In this approach the α-1,6 linked mannose which serves as acceptor for GnTI is formed during LLO biosynthesis. In order to remove the A-branch, expression of a heterologous mannosidase from $A$. saitoi was used and yielded glycoproteins predominantly containing the Man$_	ext{$_{1,2}$}$GlcNA$_	ext{$_{2}$}$ glycan. This approach was implemented in $P$. pastoris, $H$. polymorpha, $Y$. lipolytica, $A$. niger and $A$. nidulans, respectively, however, was not effective in $K$. marxiani (Bobrowicz et al., 2004; Kainz et al., 2008; Oh et al., 2008; De Pourcq et al., 2012b; Lee et al., 2020; Anyaogu et al., 2021). Using this strategy, GnTI and GnTII activities are required in the Golgi apparatus.

Incomplete glycosylation of the A-branch, and thus hypoglycosylation, and inefficient glucosidase I and II trimming are associated with ALG3 deletion in various yeasts (Verostek et al., 1993; Aebi et al., 1996a; Bobrowicz et al., 2004; De Pourcq et al., 2012a; Anyaogu et al., 2021). The low glycosylation site occupancy was tackled by the overexpression of glycosyltransferase ALG6, which was earlier shown to partially restore the glycosylation deficiency in $S$. cerevisiae (Burdas et al., 2019).
while the interfering glucose residues were eliminated by the overexpression of α-glucosidase II activities (De Pourcq et al., 2012a; Anyaogu et al., 2021). Alternatively, in P. pastoris, the low glycosylation site occupancy was compensated by overexpression of single-subunit OST from Leishmania major (Choi et al., 2012).

Direct biosynthetic generation of Man₃GlcNAc₂ glycan can be achieved by the deletion of ALG3 and ALG11 genes terminating the LLO biosynthesis at a very early stage. The Man₃GlcNAc₂ glycan contains a terminal α-1,3 linked and α-1,6 linked mannose residue, respectively, that serve as acceptor for GnTI and GnTII (Figure 2, route D). This strategy has been utilized in H. polymorpha and S. cerevisiae (Song et al., 2010; Parsaie Nasab et al., 2013; Piirainen et al., 2021).

Due to the strong modification of the LLO biosynthesis, measures to compensate for the resulting hypoglycosylation phenotypes are required. In both organisms this was achieved by improving the flipping of the LLO into the ER lumen and in S. cerevisiae by additionally overexpressing a single-subunit OST (Song et al., 2010; Parsaie Nasab et al., 2013). Furthermore, a number of mannosyltransferases compete with GnTI and GnTII for the Man₃GlcNAc₂ glycan in the Golgi apparatus leading to glycan structures with additional mannose residues that precluded the complete processing into complex type N-glycans and reduce glycan homogeneity (Song et al., 2010; Parsaie Nasab et al., 2013; Piirainen et al., 2016, 2021).

**DISCUSSION**

Most of the glycoengineering approaches developed for yeasts proceed via the formation of non-native, truncated glycan structures in the ER. To date it is unclear if and to which extent these deviating structures might impact the quality of the produced glycoproteins or affect their turnover by ERAD. Most of the studies on ER protein quality control systems were done with artificial folding-deficient glycoproteins. However, how about folding-proficient glycoproteins that would be ultimately produced in glycoengineered yeasts? Among these relevant proteins are antibodies, and the comparably smaller glycoengineered P. pastoris or S. cerevisiae (Hamilton et al., 2006; Li et al., 2006; Jacobs et al., 2009; Parsaie Nasab et al., 2013; Piirainen et al., 2021). Importantly, these glycoproteins do not depend on glycans for proper folding and can be manufactured either as aglycones, in glycosylation incompetent host cells such as Escherichia coli or in vitro systems (Higgins, 2010; Yin et al., 2012). Thus, it is plausible that the altered glycan structures do not directly lead to misfolding of these proteins. However, they might affect the folding kinetics and lead to undesired interaction with the ERAD system.

IgG molecules have been shown to interact with molecular chaperones, protein disulfide isomerases and peptidylprolyl isomerase activities. Moreover, UGGT, and small amounts of calnexin were detected in samples of immunoprecipitated IgG indicating the involvement of the calnexin cycle in the folding of IgG (Meunier et al., 2002). In vitro studies with yeast Cne1p and chemically unfolded egg yolk IgY carrying Glc₃Man₃GlcNAc₂ structures indicated that Cne1p reduces aggregation (Xu et al., 2004a). Whether and to which extent the antibody folding process is dependent on Cne1p in vivo is unknown. Glycan structures formed in both glycoengineering approaches that are based on altering the LLO biosynthesis partially or completely bypass the calnexin-dependent protein folding (Figure 2, route C and D).

The exposed α-1,6 linked mannose on the Man₃GlcNAc₂ glycan is the canonical glycan motif recognized by Yos9p (Szathmary et al., 2005; Clerc et al., 2009). The identical exposed α-1,6 linked mannose is also generated by expression of α,1,2 mannosidase activity in ER that trims glycans to a Man₃GlcNAc₂ structure but lacks the complete A-branch (Figure 2, route B). Whether this glycan is recognized by Yos9p is unknown. Alternatively, exposed α-1,6 linked mannose residues, such as the one generated by ALG3 deletion are recognized by Yos9p (Szathmary et al., 2005; Clerc et al., 2009). The identical α-1,6 linked mannose is present in ALG3 deletion expressing α,1,2 mannosidase activity and ALG11 ALG3 deletion strains, though the interaction of this glycan with Yos9p remains unknown (Figure 2, route C,D). Overall, the glycoengineering approaches have the potential to generate glycan structures that are recognized by Yos9p without the need for prior processing by Htm1p-Pdi1p complex.

What is the impact of ERAD system on the clearance of an IgG? In wild-type S. cerevisiae, deletion of ERAD components HRD1, HRD3, YOS9, HMT1 and UBC7 delayed IgG cellular clearance. Moreover, antibody clearance in an ALG3 deletion strain with functional ERAD was comparable to the clearance in wild-type strain indicating that an exposed α-1,6 linked mannose is sufficient for ER clearance (de Ruijter and Frey, 2015). Furthermore, deletion of HRD1, but not of HTM1 or YOS9 reduced clearance rates in an ALG3 ALG11 deletion strain (Piirainen and Frey, 2020). This indicates that glycan-independent mechanisms additionally can contribute to IgG clearance in yeast.

In summary, the modifications introduced by glycoengineering into the N-glycan processing pathway have the potential to impair glycan-dependent protein folding, i.e., by by-passing calnexin and to generate glycan structure that are recognized by ERAD. However, our current understanding is incomplete and further studies will be required.

**AUTHOR CONTRIBUTIONS**

MP collected the majority of the background information. AF and MP contributed equally to the writing of the manuscript.
REFERENCES

Aebi, M., Gassenhuber, J., Domdey, H., and Heesen, S. t. (1996a). Cloning and Characterization of the ALG3 Gene of Saccharomyces cerevisiae. Glycobiology 6, 439–444. doi:10.1093/glycob/6.4.439

Aebi, M., Gassenhuber, J., Domdey, H., and Heesen, S. t. (1996b). Cloning and Characterization of the ALG4 Gene of Saccharomyces cerevisiae. Glycobiology 6, 439–444. doi:10.1093/glycob/6.4.439

Anyaogu, D. C., Hansen, A. H., Hoof, J. B., Majewska, N. I., Contesini, F. J., Paul, J. T., et al. (2021). Glycoengineering of Asparaginyl Galtid proteins to Produce Precursor for Humanized N-Glycan Structures. Metab. Eng. 67, 153–163. doi:10.1016/j.ymben.2021.06.001

Barnes, G., Hansen, W. J., Holcomb, C. L., and Rine, J. (1984). Asparagine-linked Cytosolic Side of the Endoplasmic Reticulum. J. Biol. Chem. 261, 21828–21840. doi:10.1074/jbc.M101896200

Clerc, S., Hirsch, C., Oggeri, D. M., Deprez, P., Jakob, C., Sommer, T., et al. (2009). Htm1 Protein Generates the N-Glycan Signal for Glycoprotein Degradation in the Endoplasmic Reticulum. J. Cell. Biol. 184, 159–172. doi:10.1083/mbc. 200809198

Couto, J. R., Hufnaker, T. C., and Robbins, P. W. (1984). Cloning and Expression of Escherichia coli of a Yeast Mannosyltransferase from the Asparagine-Linked Glycosylation Pathway. J. Biol. Chem. 259, 378–382. doi:10.1002/1661-9258(19940602)269:1163–1163

de Pourcq, K., Tiels, P., Van Hecke, A., Geyssens, S., Verweecken, W., and Callewaert, N. (2012a). Engineering Yeast Glycolipid. Production of Complex Human Glycoproteins Homogeneously Modified with the Universal Man3GlcNAc2 N-Glycan Core. Proc. Natl. Acad. Sci. U.S.A. 109, 3979–3984. doi:10.1172/journal.pone.0039976

de Pourcq, K., Verweecken, W., Dewerte, I., Valseva, A., Van Hecke, A., and Callewaert, N. (2012b). Engineering the Yeast Yarriwia Lipidityca for the Production of Therapeutic Proteins Homogeneously Glycosylated with Man5GlcNAc2 and Man6GlcNAc2. Microb. Cell. Fact. 11, 53. doi:10.1186/1475-2859-11-53

Fernández, F. S., Trombetta, S. E., Hellman, U., and Parodi, A. J. (1994). Purification to Homogeneity of UDP-Glucose:glycoprotein Glycosyltransferase from Schizosaccharomyces pombe and Apparent Absence of the Enzyme From Saccharomyces cerevisiae. J. Biol. Chem. 269, 30701

Frank, C. G., and Aebi, M. (2005). ALG9 Mannosyltransferase Is Involved in Two Different Steps of Lipid-Linked Oligosaccharide Biosynthesis. Glycobiology 15, 1156–1163. doi:10.1074/jbc.g500000200

Frank, C. G., Sanay, S., Rush, J. S., Waechter, C. J., and Menon, A. K. (2008). Does Rft1 Flip an N-Glycan Lipid Precursor? Nature 454, E3–E4. doi:10.1038/ nature07165

Friedlander, R., Jarosch, E., Urban, J., Volkwein, C., and Sommer, T. (2000). A Regulatory Link between ER-Associated Protein Degradation and the Unfolded-Protein Response. Nat. Cell. Biol. 2, 379–384. doi:10.1038/35017001

Friedlander, E., Salzberg, Y., Weinberger, A., Shaltiel, S., and Gerst, J. E. (2002). YOS9, the Putative Yeast Homolog of a Gene Amplified in Osteosarcomas, Is Involved in the Endoplasmic Reticulum (ER)-Golgi Transport of GPI-Anchor Proteins. J. Biol. Chem. 277, 35274–35281. doi:10.1074/jbc.M201044200

Gauss, R., Jarosch, E., Sommer, T., and Hirsch, C. (2006). A Complex of Yos9p and the HRD Ligase Integrates Endoplasmic Reticulum Quality Control into the Degradation Machinery. Nat. Cell. Biol. 8, 849–858. doi:10.1038/ nb1144

Gauss, R., Kanehara, K., Carvalho, P., Ng, D. T. W., and Aebi, M. (2011). A Complex of Pdi1p and the Mannosidase Htm1p Initiates Clearance of Unfolded Glycoproteins from the Endoplasmic Reticulum. Mol. Cell. 42, 782–793. doi:10.1016/j.molcel.2011.04.027

Hamilton, S. R., Bobrowicz, P., Bobrowicz, B., Davidson, R. C., Li, H., Mitchell, T., et al. (2003). Production of Complex Human Glycoproteins in Yeast. Science 301, 1244–1246. doi:10.1126/science.1088166

Hamilton, S. R., Davidson, R. C., Sethuraman, N., Nett, J. H., Jiang, Y., Rios, S., et al. (2006). Humanization of Yeast to Produce Complex Terminally Sialylated Glycoproteins. Science 313, 1441–1443. doi:10.1126/science.1130256
Helenius, A., and Aebi, M. (2004). Roles of N-Linked Glycans in the Endoplasmic Reticulum. Annu. Rev. Biochem. 73, 1019–1049. doi:10.1146/annurev.biochem.73.011303.073752

Helenius, J., Ng, D. T. W., Marolda, C. L., Walter, P., Valvano, M. A., and Aebi, M. (2002). Translocation of Lipid-Linked Oligosaccharides across the ER Membrane Requires RIP1 Protein. Nature 415, 447–450. doi:10.1038/415447a

Higgins, E. (2010). Carbohydrate Analysis throughout the Development of a Protein Therapeutic. Glycoconj J. 27, 211–225. doi:10.1007/s10719-009-9261-x

Hiller, M. M., Finger, A., Schweiger, M., and Wolf, D. H. (1996). ER Degradation of a Misfolded Luminal Protein by the Cytosolic Ubiquitin-Proteasome Pathway. Science 273, 1725–1728. doi:10.1126/science.273.5282.1725

Hirsch, C., Blom, D., and Ploegh, H. L. (2003). A Role for N-Glycanase in the Cytosolic Turnover of Glycoproteins. EMBO J. 22, 1036–1046. doi:10.1093/emboj/cdg107

Hufnaker, T. C., and Robbins, P. W. (1982). Temperature-sensitive Yeast Mutants Deficient in Asparaginyl-Linked Glycosylation. J. Biol. Chem. 257, 3203–3210. doi:10.1016/S0021-9258(19)81096-7

Hufnaker, T. C., and Robbins, P. W. (1983). Yeast Mutants Deficient in Protein Glycosylation. Proc. Natl. Acad. Sci. U.S.A. 80, 7466–7470. doi:10.1073/pnas.80.24.7466

Jacobson, P. P., Geysens, S., Vervecken, W., Contreras, R., and Callewaert, N. (2009). Engineering Complex-type N-Glycosylation in Pichia pastoris Using Glycomethodology Technology. Nat. Protoc. 4, 58–70. doi:10.1038/nprot.2008.213

Jakob, C. A., Burda, P., Roth, J., and Aebi, M. (1998). Degradation of Misfolded Endoplasmic Reticulum Glycoproteins in Saccharomyces Cerevisiae Is Determined by a Specific Oligosaccharide Structure. J. Cell Biol. 142, 1223–1233. doi:10.1083/jcb.142.5.1223

Kainz, E., Gallmeister, A., Hatzi, C., Nett, J. H., Li, H., Schinko, T., et al. (2008). N-glycan Modification in Aspergillus Species. Appl. Environ. Microbiol. 74, 1076–1086. doi:10.1128/AEM.01038-07

Kamiya, Y., Kamiya, D., Yamamoto, K., Nyfeler, B., Hauri, H.-P., and Kato, K. (2008). Molecular Basis of Sugar Recognition by the Human L-type Lectins Lectin Yos9. Structural Investigation of Glycan Recognition by the ERAD Quality Control System Htm1 in Saccharomyces Cerevisiae. EMBO J. 27, 244–253. doi:10.1038/sj.emboj.7601744

Kniss, A., Kazemi, A., Lohr, F., Berger, M., Rogov, V. V., Günert, P., et al. (2018). Structural Investigation of Glycan Recognition by the ERAD Quality Control Lecin Yos9. J. Biol. Chem. 72, 1–10. doi:10.1016/s1088-1802-0201-6

Kuruczinska, M. A., and Robbins, P. W. (1987). Protein Glycosylation in Yeast: Transcript Heterogeneity of the ALG7 Gene. Proc. Natl. Acad. Sci. U.S.A. 84, 2145–2149. doi:10.1073/pnas.84.8.2145

Lee, M.-H., Hsu, T.-L., Lin, J.-J., Lin, Y.-J., Kuo, Y.-Y., Eto, S., et al. (2015). Molecular Cloning and Expression of cDNAs Encoding Human Alpha-Mannosidase II and a Previously Unrecognized Alpha-Mannosidase IX Isozyme. Proc. Natl. Acad. Sci. U.S.A. 92, 11766–11770. doi:10.1073/pnas.92.25.11766

O’Reilly, M. K., Zhang, G., and Imperiali, B. (2006). In Vitro evidence for the Dual Function of Alg2 and Alg11: Essential Mannosyltransferases in N-Linked Glycoprotein Biosynthesis. Biochemistry 45, 9593–9603. doi:10.1021/bi0608780

Oh, D.-B., Park, J.-S., Kim, M. W., Cheon, S. A., Kim, E. J., Moon, H. Y., et al. (2008). Glycoengineering of the Methylotrophic yeast Hansenula polymorpha for the Production of Glycoproteins with Trimmannosyl coreN-Glycan by Blocking Core Oligosaccharide Assembly. Biotechnol. J. 3, 659–668. doi:10.1002/biot.200700252

Oppenheimer, C. L., and Hill, R. L. (1981). Purification and Characterization of a Rabbit Liver Alpha 1 Goes to 3 Mannoside Beta 1 Goes to 2 N-Acetylgalcosaminyltransferase. J. Biol. Chem. 256, 799–804. doi:10.1016/S0021-9258(19)70047-7

Parlati, F., Dominguez, M., Bergeron, J. J. M., and Thomas, D. Y. (1995). Saccharomyces cerevisiae CNE1 Encodes an Endoplasmic Reticulum (ER) Membrane Protein with Sequence Similarity to Calnexin and Calreticulin and Functions as a Constituent of the ER Quality Control Apparatus. J. Biol. Chem. 270, 244–253. doi:10.1074/jbc.270.1.244

Poirin, A. M., Boer, H., De Ruiter, J. C., and Frey, A. D. (2016). A Dual Approach for Improving Homogeneity of a Human-type N-Glycan Structure in Saccharomyces cerevisiae. Glycoconjug. J. 33, 189–199. doi:10.1007/s10719-016-9656-4

Poirain, M. A., and Frey, A. D. (2020). Investigating the Role of ERAD on Antibody Processing in Glycoengineered Saccharomyces cerevisiae. FEMS Yeast Res. 20, 1–11. doi:10.1093/femsyr/foaa002

Poirain, M. A., Salminen, H., and Frey, A. D. (2021). Production of Galactosylated Complex-type N-Glycans in Glycoengineered Saccharomyces cerevisiae. Appl. Microbiol. Biotechnol. 106, 301–315. doi:10.1007/s00253-021-11727-9

Quan, E. M., Kamiya, Y., Kamiya, D., Denic, V., Weibezahn, J., Kato, K., et al. (2008). Defining the Glycan Destruction Signal for Endoplasmic Reticulum-Associated Degradation. Mol. Cell. 32, 870–877. doi:10.1016/j.molcel.2008.11.017

Reiss, G., Heesen, S. t., Zimmerman, J., Robbins, P., and Aebi, M. (1996). Isolation of the ALG6 Locus of Saccharomyces cerevisiae Required for Glucosylation in the N-Linked Glycosylation Pathway. Glycobiology 6, 493–498. doi:10.1093/glyco/6.5.493

Rungo, K. W., and Robbins, P. W. (1986). A New Yeast Mutation in the Glucosylation Steps of the Asparagine-Linked Glycosylation Pathway. Formation of a Novel Asparagine-Linked Oligosaccharide Containing Two Glucose Residues. J. Biol. Chem. 261, 15582–15590. doi:10.1002/1096-9028.19866754-7

Rush, J. S., Gao, N., Lehrman, M. A., Matveev, S., and Waechter, C. J. (2005). Suppression of RFI Expression Does Not Impair the Transbiliary Movement of ManSigLeAc2-P-P-Dolichol in Sealed Microsomes from Yeast. J. Biol. Chem. 280, 19833–19842. doi:10.1074/jbc.M109.008933

Sanyal, S., Frank, C. G., and Menon, A. K. (2008). Distinct Flippases Translocate Glycerophospholipids and Oligosaccharide Diphosphate Dolichols across the Endoplasmic Reticulum. Biochemistry 47, 7937–7946. doi:10.1021/bi800723n

Sato, K., and Nakano, A. (2002). Emp47p and its Close Homolog Emp46p Have a Tyrosine-Containing Endoplasmic Reticulum Exit Signal and Function in Glycoprotein Secretion in Saccharomyces cerevisiae. MBoC 13, 2518–2532. doi:10.1091/mboct-02-001-027
Satoh, T., Sato, K., Kanoh, A., Yamashita, K., Yamada, Y., Igarashi, N., et al. (2006). Structures of the Carbohydrate Recognition Domain of C2β+ independent Cargo Receptors Emp6p and Emp1p. J. Biol. Chem. 281, 10410–10419. doi:10.1074/jbc.M512358200

Shah, N., Kuntz, D. A., and Rose, D. R. (2008). Golgi α-mannosidasase II Cleaves Two Sugars Sequentially in the Same Catalytic Site. Proc. Natl. Acad. Sci. U.S.A. 105, 9570–9575. doi:10.1073/pnas.0802206105

Sharma, C. B., Knauer, R., and Lehle, L. (2001). Biosynthesis of Lipid-Linked N-Glycans. J. Biol. Chem. 286, 30212–30218. doi:10.1074/jbc.M106106200

Stagljar, I., Te Heesen, S., and Aebi, M. (1994). New Phenotype of Mutations Deletion of the Yeast Golgi Man6-10GlcNAc2 Processing Intermediates on Secreted Invertase. J. Biol. Chem. 269, 12104–12115. doi:10.1016/0021-9258(94)90314-3

Verrecken, W., Kaigorodov, V., Callewaert, N., Geyssens, S., De Vusser, K., and Contreras, R. (2004). In Vivo synthesis of Mammalian-like, Hybrid-type N-Glycans in Pichia pastoris. Appl. Environ. Microbiol. 70, 2639–2646. doi:10.1128/AEM.70.5.2639

Walsh, G. (2018). Biopharmaceutical Benchmarks 2018. Nat. Biotechnol. 36, 1136–1145. doi:10.1038/nbt.4305

Wilkinson, B. M., Purwanji, J., and Stirling, C. J. (2006). Yeast GTB1 Encodes a Subunit of Glucosidase II Required for Glycoprotein Processing in the Endoplasmic Reticulum. J. Biol. Chem. 281, 6325–6333. doi:10.1074/jbc.M510455200

Wolf, D. H., and Zolst, A. (2012). The Cdc48 Machine in Endoplasmic Reticulum Associated Protein Degradation. Biochimica Biophysica Acta (BBA) - Mol. Cell. Res. 1823, 117–124. doi:10.1016/j.jbmarc.2011.09.002

Wu, X., Siggel, M., Ovchinnikov, S., Mi, W., Svetlov, V., Nudler, E., et al. (2020). Structural Basis of ER-Associated Protein Degradation Mediated by the Hrd1 Ubiquitin Ligase Complex. Science 368. doi:10.1126/science.aaaz449

Xie, W., Kanehara, K., Sayeed, A., and Ng, D. T. W. (2009). Intrinsinc Conformational Determinants of Signal Peptide Misfolding to the Hrd1/Htm1 Endoplasmic Reticulum-Associated Degradation System. MBoC 20, 3317–3329. doi:10.1093/mboce/o9.03-0231

Xu, X., Azakami, H., and Kato, A. (2004a). P-domain and Lectin Site Are Involved in the Chaperone Function ofSaccharomyces CerevisiaeCalnexin Homologue. FEBS Lett. 570, 155–160. doi:10.1016/j.febslet.2004.06.039

Xu, X., Kanbara, K., Azakami, H., and Kato, A. (2004b). Expression and Characterization of Saccharomyces cerevisiae Cne1p, a Calnexin Homologue. J. Biochem. 135, 615–618. doi:10.1093/jb/mvh074

Yin, G., Garces, E. D., Yang, J., Zhang, J., Tran, C., Steiner, A. R., et al. (2012). Aglycosylated Antibodies and Antibody Fragments Produced in a Scalable In Vitro Transcription-Translation System. MAbs 4, 217–225. doi:10.4161/mabs.4.2.19202

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher’s Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Piirainen and Frey. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.