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APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY



Production of galactosylated complex-type N-glycans in glycoengineered *Saccharomyces cerevisiae*

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Abstract

N-glycosylation is an important posttranslational modification affecting the properties and quality of therapeutic proteins. Glycoengineering in yeast aims to produce proteins carrying human-compatible glycosylation, enabling the production of therapeutic proteins in yeasts. In this work, we demonstrate further development and characterization of a glycoengineering strategy in a *Saccharomyces cerevisiae* $\Delta alg3 \Delta alg11$ strain where a truncated Man₃GlcNAc₂ glycan precursor is formed due to a disrupted lipid-linked oligosaccharide synthesis pathway. We produced galactosylated complex-type and hybrid-like N-glycans by expressing a human galactosyltransferase fusion protein both with and without a UDP-glucose 4-epimerase domain from *Schizosaccharomyces pombe*. Our results showed that the presence of the UDP-glucose 4-epimerase domain was beneficial for the production of digalactosylated complex-type glycans also when extracellular galactose was supplied, suggesting that the positive impact of the UDP-glucose 4-epimerase domain on the galactosylation process can be linked to other processes than its catalytic activity. Moreover, optimization of the expression of human GlcNAc transferases I and II and supplementation of glucosamine in the growth medium increased the formation of galactosylated complex-type glycans. Additionally, we provide further characterization of the interfering mannosylation taking place in the glycoengineered yeast strain.

Key points

- Glycoengineered Saccharomyces cerevisiae can form galactosylated N-glycans.
- Genetic constructs impact the activities of the expressed glycosyltransferases.
- Growth medium supplementation increases formation of target N-glycan structure.

Keywords Galactosyltransferase · Glucosamine · Glycoengineering · UDP-glucose 4-epimerase · Yeast

Introduction

Therapeutic proteins are a fast-growing product segment in the pharmaceutical industry, consisting of products such as antibodies, hormones, and vaccines. The increasing production of therapeutic proteins can provide opportunities to develop alternative production platforms for the predominantly mammalian cell-based production processes. Yeasts have many advantages over mammalian cells in biotechnological processes, including

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their fast growth in inexpensive growth media, ease of genetic manipulation, and lower risk of contamination. Various therapeutic proteins are currently produced by yeast, mainly by *Saccharomyces cerevisiae* (Walsh 2018).

A significant proportion of therapeutic proteins contain N-glycans. N-glycosylation is a heterogeneous and speciesspecific posttranslational modification, and the presence of N-glycans as well as their structures can have a significant impact on the properties of a protein. For example, the glycan pattern in antibodies can affect their therapeutic efficacy (Kurogochi et al. 2015; Reusch and Tejada 2015). In addition, darbepoetin alpha, a hyperglycosylated variant of recombinant human erythropoietin, has an increased in vivo activity and serum half-life (Egrie and Browne 2001). The differences in the native N-glycosylation between yeast and mammalian cells currently prevent the use of yeast for the production of therapeutic glycoproteins, as nonhuman

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N-glycosylation may compromise the therapeutic efficacy and safety of the product.

The N-glycan biosynthesis begins in the endoplasmic reticulum (ER) where in a highly conserved process, a Glc₃Man₉GlcNAc₂ lipid-linked oligosaccharide (LLO) is formed, transferred to a nascent protein by an oligosaccharyltransferase (OST) complex, and trimmed to Man₈GlcNAc₂ structure containing three branches during protein folding. Glycans are matured in a species-specific manner as the protein proceeds in the secretory pathway to the Golgi apparatus. The N-glycans in yeast proteins are large and hypermannosylated. In contrast, mammalian glycoproteins mostly contain hybrid and complex-type N-glycans. The maturation of hybrid and complex-type N-glycans begins with the trimming of three α 1-2 linked mannose residues, forming Man₅GlcNAc₂. Next, a GlcNAc residue is attached via a β 1-2 linkage to the α 1-3 linked mannose of the A branch by N-acetylglucosaminyltransferase I (GnTI), forming a hybrid-type GlcNAc₁Man₅GlcNAc₂ glycan. Complex-type glycans are formed by further mannosidase trimming followed by the addition of a second GlcNAc residue to the exposed α 1-6 linked mannose of GlcNAcMan₃GlcNAc₂ by N-acetylglucosaminyltransferase II (GnTII) (Figure S1). The branches of the resulting GlcNAc₂Man₃GlcNAc₂ (G0) or hybrid-type GlcNAc1Man5GlcNAc2 glycans can be further elongated by β1-4-galactosyltransferase (GalT), transferring galactose to the terminal GlcNAc residues. Further on, the galactosylated branches can be capped with sialic acids. In addition to these modifications, additional branching of the glycan core as well as core fucosylation can occur.

During the past two decades, the N-glycosylation pathways of various yeasts have been engineered aiming to enable the production of therapeutic proteins with humancompatible N-glycosylation. An essential step in yeast glycoengineering is to create suitable intermediate N-glycan structures that can act as substrates for the subsequent mammalian-type glycan maturation steps. Some glycoengineering approaches have relied on replicating the glycan trimming reactions of the mammalian glycosylation pathway by expressing mannosidases and blocking the formation of the yeast-specific outer chain by OCH1 deletion (Choi et al. 2003; Hamilton et al. 2003; Vervecken et al. 2004). An alternative approach is to either partially or completely bypass the glycan trimming steps. This can be achieved by deleting mannosyltransferases in the LLO biosynthesis pathway, preventing the elongation of the LLO branches (Bobrowicz et al. 2004; Cheon et al. 2012; De Pourcq et al. 2012; Wang et al. 2013). In S. cerevisiae, deletion of ALG3 and ALG11 genes (Parsaie Nasab et al. 2013) resulted in a strain that forms a truncated trimannosyl core structure (Man₃GlcNAc₂) in the ER. This glycan can be directly converted to complex-type glycans without any trimming reactions.

After a suitable precursor glycan is formed, hybrid and complex-type glycans are generated in yeasts by expressing mammalian N-acetylglucosaminyltransferases, galactosyltransferases, and sialyltransferases. Glycosyltransferases in the Golgi apparatus are type II transmembrane proteins, and their N-terminal cytoplasmic, transmembrane, and stem domains direct the transferases into certain Golgi cisternae (Tu and Banfield 2010). When expressing glycosyltransferases of mammalian origin in yeast, their correct localization is ensured by fusing their catalytic domains to the localization sequences of yeast endogenous glycosyltransferases. Mammalian glycosyltransferases also utilize nucleotide sugars including UDP-GlcNAc and UDP-galactose not present in the Golgi apparatus of most yeasts and improving their availability has often been required to obtain efficient conversions by the mammalian glycosyltransferases. Expression of a UDP-GlcNAc transporter from Kluyveromyces lactis (Yea4, alternatively called Mnn2-2) has increased the amount of hybrid and complex-type glycans in S. cerevisiae and other yeasts (Choi et al. 2003; Bobrowicz et al. 2004; Wang et al. 2013; Piirainen et al. 2016). In addition, incorporation of a UDP-glucose 4-epimerase from Schizosaccharomyces pombe into a GalT fusion protein has provided efficient N-glycan galactosylation (Bobrowicz et al. 2004; Jacobs et al. 2009; Wang et al. 2013). With these and additional modifications, yeast strains forming hybrid and complex-type glycans with terminal GlcNAc (Callewaert et al. 2001; Choi et al. 2003; Hamilton et al. 2003; Cheon et al. 2012; Parsaie Nasab et al. 2013), galactose (Vervecken et al. 2004; Bobrowicz et al. 2004; Wang et al. 2013), and even sialic acid residues (Hamilton et al. 2006) have been developed.

We have previously reported that complex-type glycans bearing terminal GlcNAc residues can be generated in *S. cerevisiae* and that their amount can be increased by enhancing the transport of UDP-GlcNAc to the Golgi apparatus (Parsaie Nasab et al. 2013; Piirainen et al. 2016). In this work, we build on this glycoengineering approach and demonstrate galactosylation of hybrid-like and complextype N-glycans by the expression of a galactosyltransferase fusion protein. In addition, we show that the formation of these glycans can be enhanced by optimization of expression vectors and cultivation medium.

Materials and methods

Strains

S. cerevisiae host strain and plasmids used in this study are presented in Table 1. Strain YMP14 (Piirainen et al. 2016) is derived from the glycoengineered strain YAF39 that contains deletions of *ALG3* and *ALG11* genes, the artificial flippase

Name	Genotype/description	Reference
SS328	MATα ade2-101 his3Δ200 lys2-801 ura3-52	ATCC® MYA193 TM
YMP17	SS328 Δalg3::HIS3 Δalg11::HIS3 Δleu2::KanMX4::LbSTT3_3::Flc2* Δmnn1::loxP, UV-mut	This work
pEK7	Empty low copy plasmid, LEU2 selection marker	de Ruijter et al. (2016)
pAK3	ORF for Kre2p-GnTI fusion under control of GAL1 promoter, URA3 selection marker, low copy plasmid	This work
pAX428	ORFs for Kre2p-GnTI and Mnn2p-GnTII fusions under control of <i>GAL1-10</i> promoter, <i>URA3</i> selection marker, high copy plasmid	Parsaie Nasab et al. (2013)
pSKH01	ORFs for Kre2p-GnTI and Mnn2p-GnTII fusions under control of <i>GAL1</i> promoters, <i>URA3</i> selection marker, low copy plasmid	This work
pAF21	ORFs for Kre2p-GnTI and Mnn2p-GnTII fusions under control of <i>GAL1</i> promoters, KlYea4p under control of <i>GPD</i> promoter, <i>URA3</i> selection marker, low copy plasmid	This work
pAF22	ORFs for Kre2p-GnTI fusion under control of <i>GAL1</i> promoter, KlYea4p under control of <i>GPD</i> promoter, <i>URA3</i> selection marker, low copy plasmid	This work
pSR01	ORF for Mnn2p-Uge1p-GalT fusion under control of <i>GAL1</i> promoter, <i>LEU2</i> selection marker, low copy plasmid	This work
pSR02	ORF for Mnn2p-GalT fusion under control of GAL1 promoter, LEU2 selection marker, low copy plasmid	This work

Flc2*, and an oligosaccharyltransferase from *Leishmania brasiliensis*, and has been UV-mutagenized for improved growth (Parsaie Nasab et al. 2013). YMP17 was generated from strain YMP14 by removing the nourseothricin selection marker from *MNN1* locus with Cre recombinase (Hegemann and Heick 2011).

Cloning of overexpression constructs

All recombinant DNA work was done using *Escherichia coli* TOP10 (Invitrogen, Waltham, MA, USA) as the cloning host. Empty plasmid pEK7 containing a *LEU2* selection marker was used to complement for auxotrophies in strains where no gene expression with a *LEU2* plasmid took place (de Ruijter et al. 2016). Expression vectors are based on pRS plasmid series (Mumberg et al. 1995), and oligonucleotides used for generating the plasmids are listed in Table S1.

Human GlcNAc transferase I and II fusion proteins with yeast endogenous localization sequences have been constructed by Parsaie Nasab et al. (2013). GlcNAc transferase I fusion protein (GnTI) consists of S. cerevisiae Kre2p targeting sequence, encompassing the N-terminal cytoplasmic, transmembrane and stem domains, the catalytic domain of human GlcNAc transferase I, and a C-terminal FLAG tag, and GlcNAc transferase II fusion protein (GnTII) consists of S. cerevisiae Mnn2p targeting sequence, the catalytic domain of human GlcNAc transferase II, and a C-terminal FLAG tag. A low copy number expression vector for GnTI was constructed by inserting the ORF encoding Kre2p-GnTI-FLAG into SpeI and XhoI sites of pRS416-GAL by standard cloning procedures, creating plasmid pAK3. A low copy number vector for coexpression of GnTI and GnTII under the control of GAL1 promoter (GnTI/II vector) was constructed in two steps. First, ORF encoding Mnn2p-GnTII-FLAG was amplified from pAX428 (Parsaie Nasab et al. 2013) by PCR with oligonucleotides OMP97 and OMP98 and inserted into *SpeI* and *XhoI* site of pRS416-GAL with ELIC cloning (Koskela and Frey 2014), creating plasmid pMP27. Next, a DNA fragment containing *GAL1* promoter, ORF encoding Kre2p-GnTI-FLAG, and *CYC1* terminator was amplified from pAK3 by PCR with oligonucleotides OMP108 and OMP109 and inserted into *Eco5*3kI site of pMP27 with ELIC cloning, creating plasmid pSKH01.

The expression cassette for UDP-GlcNAc transporter from K. lactis (Yea4) was combined with GnTI and GnTI/ II expression vectors as follows. First, a fragment containing GPD1 promoter and KlYEA4 ORF was excised from pMP002 (Piirainen et al. 2016) with SacI and XhoI and inserted into SacI and XhoI site of pMP27, creating plasmid pAF19 where the KlYEA4 expression construct is in an expression vector containing URA3 selection marker. Next, a DNA fragment containing GAL1 promoter, ORF encoding Mnn2p-GnTII-FLAG, and CYC1 terminator was excised from pMP27 with Eco53kI and SbfI and inserted into NaeI and SbfI site of pAF19, creating plasmid pAF20. A DNA fragment containing GAL1 promoter, ORF encoding Kre2p-GnTI-FLAG, and CYC1 terminator was then amplified from pAK3 with oligonucleotides OMP104 and OMP105 and inserted into NaeI site of pAF19 and pAF20, creating plasmids pAF22 and pAF21, respectively.

For expression of human β 1-4 galactosyltransferase 1 (GalT, Uniprot ID P15291) and UDP-glucose 4-epimerase from *S. pombe* (Uge1, Uniprot ID Q9Y7X5), a codon-optimized ORF encoding a fusion protein (Uge1-GalT) containing Uge1, GSGG linker peptide, and amino acids 44–398 of GalT was synthesized (Eurofins Genomics) and the sequence is deposited (GenBank accession number OK129337). To generate expression constructs for GalT both with and

without Uge1, ORFs encoding Uge1-GalT or GalT were amplified by PCR with oligonucleotide pairs OMP88 and OMP90, or OMP89 and OMP90, respectively. The PCR fragments were inserted by ELIC cloning into *XhoI* and *Bmg*BI-digested pAK2, consisting of pRS415-GAL plasmid backbone and ORF encoding amino acids 1–36 of *S. cerevisiae* Mnn2p localization sequence. The resulting plasmids pSR01 and pSR02 contain the ORFs encoding Mnn2p-Uge1-GalT and Mnn2p-GalT fusion proteins, respectively, under the control of *GAL1* promoter and *CYC1* terminator.

Cultivation of yeast strains

S. cerevisiae strains were cultivated at 28 °C in chemically defined medium (0.67% yeast nitrogen base without amino acids and Hopkins dropout mixture lacking leucine and uracil) supplemented with 0.2 M sorbitol and with 2% raffinose as a carbon source. For N-glycan analysis of cell wall and secreted proteins, 3 ml precultures were grown at 230 rpm for 48–72 h. Cells were diluted to an OD_{600} of 0.2 in 20 ml culture volume and grown until OD_{600} of at least 1 was reached. Cells were harvested, resuspended to an OD_{600} of 1 in 20 ml of fresh medium supplemented with 2% galactose for induction of GnTI, GnTII, and GalT expression, and grown for 24 h. Induction medium for growth medium supplementation tests and for N-glycan analysis of secreted proteins was supplemented with 15 mM glucosamine.

N-glycan sample preparation

N-glycans were isolated from the cell wall proteins and secreted proteins. Cell wall proteins were isolated and prepared for N-glycan analysis as previously described (Piirainen et al. 2019). Briefly, 50 OD_{600} units of the cells were lysed using 0.5 mm glass beads in 10 mM Tris-HCl buffer pH 7.4 containing protease inhibitor cocktail (Complete EDTA-free, Roche, Basel, Switzerland) and 1 mM phenylmethanesulfonyl fluoride. Covalently linked cell wall material was collected by centrifugation (16,000 g, 1 min), and the reduction and alkylation of cysteines were performed at 37 °C in 10 mM dithiotreitol in denaturing buffer (2 M thiourea, 7 M urea, 2% sodium dodecyl sulphate, 50 mM Tris-HCl pH 8) followed by addition of iodoacetamide to a final concentration of 24 mM. The pellet was washed five times with the denaturing buffer and five times with water. For the analysis of the N-glycans on secreted proteins, 15 ml of culture medium was cleared by centrifugation, and the supernatant was concentrated and washed with water to a volume of 200-300 µl using centrifugal concentrators with a molecular weight cut-off of 10,000 (Sartorius, Göttingen, Germany).

N-glycans of both cell wall and secreted proteins were released in 200 μl of reaction mixture containing 1 μl

peptide-N-glycosidase F (500 U/µl PNGase F, glycerol free, New England Biolabs, Ipswich, MA, USA), glycoprotein denaturing buffer, GlycoBuffer 2, and 1% NP-40 at 37 °C with shaking at 230 rpm for 16 h. Secreted protein samples were denatured in glycoprotein denaturing buffer (New England Biolabs, Ipswich, MA, USA) for 10 min at 95 °C prior to the PNGase F reaction. N-glycans were purified with C18 and graphitized carbon columns (Supelclean ENVI-18 and ENVI-carb, Sigma Aldrich, St. Louis, MO, USA) as described in Piirainen et al. (2019). For MS/MS glycan analysis, N-glycan samples were permethylated as described earlier (Piirainen et al. 2019), except that chloroform was replaced by dichloromethane.

Glycosidase reactions

 α 1-2-mannosidase (Agilent, Santa Clara, CA, USA) and β1-4-galactosidase (New England Biolabs, Ipswich, MA, USA) reactions were performed according to manufacturer's instructions, and N-glycans were purified from the reaction mixture with HyperSepTM Hypercarb microscale solid phase extraction tips (Sigma Aldrich, St. Louis, MO, USA). The tips were washed 5 times with 20 µl of 95% acetonitrile and equilibrated 5 times with 20 µl of 2% acetonitrile. Samples were loaded to the tips by pipetting 20–50 times, the tips were washed 10 times with 20 µl of 2% acetonitrile, and glycans were eluted by pipetting 10 times with 10 µl of 70% acetonitrile, repeating elution by pipetting 3 times, and combining the eluate with the first eluate. The samples were dried with air flow at 60 °C.

MALDI-TOF

N-glycan samples were dissolved in 10 µl of water prior to MS analysis. Equal amounts of sample and matrix solution (20 mg/ml of super-DHB [9:1 mixture of 2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid] in 30% acetonitrile, 0.1% trifluoroacetic acid, and 1 mM NaCl) were mixed and spotted onto a target plate. N-glycans and LLOs were analyzed by UltrafleXtreme MALDI-TOF MS operated in positive ion and reflector mode (Bruker Daltonics, Billerica, MA, USA). Permethylated N-glycan samples were dissolved in 5% acetonitrile containing 0.1% trifluoroacetic acid and spotted onto a target plate with the super-DHB matrix. MS/MS spectra were acquired in positive LIFT mode with high-energy CID using argon as a collision gas. For MS/MS measurements in negative ion mode, samples were applied as described earlier (Domann et al. 2012) with minor modifications. In short, 0.5 µl of N-glycan sample was spotted onto a target plate followed by 0.3 µl of THAP matrix (150 mg/ ml 2',4',6'-trihydroxyacetophenone monohydrate in acetone) and 0.5 µl of 1 M ammonium nitrate. MS/MS spectra were acquired in negative LIFT mode with CID using argon as a collision gas. Annotation of glycans in figures follows the symbol nomenclature for glycans (Varki et al. 2015), depicting hexoses as filled circles (glucose: blue; mannose: green; galactose: yellow) and N-acetylhexoses as filled squares (N-acetylglucosamine: blue).

Data analysis

Table 2Efficiencies of GnTI,GnTII, and GalT in cell wall

N-glycan samples

The efficiencies of the GnTI, GnTII, and GalT reactions were calculated based on the relative glycan abundances obtained from MALDI-TOF MS measurements of nontreated N-glycan samples and N-glycan samples sequentially digested with α 1-2 mannosidase and β 1-4 galactosidase. The enzymatic efficiencies were defined as the total relative abundance of glycan structures processed by GnTI, GnTII, or GalT per the total relative abundance of potential substrates available for the respective enzymes. All glycans containing an α 1-3 linked mannose residue (Man₃₋₆GlcNAc₂, Gal₀₋₁GlcNAc₁Man₃₋₄GlcNAc₂, Gal₀₋₂GlcNAc₂Man₃GlcNAc₂) were considered as substrates for GnTI, all glycans processed by GnTI were considered as substrates for GnTII, and all *β*1-2 linked GlcNAc residues were considered as substrates for GalT. Statistical significance of the differences in the relative glycan abundances between strains was evaluated by Student's t test.

Results

Optimization of the GlcNAc₂Man₃GlcNAc₂ synthesis pathway

We have previously shown that complex-type GlcNAc₂Man₃GlcNAc₂ (G0) glycans can be formed in a glycoengineered $\Delta alg3 \Delta alg11 S$. cerevisiae strain and that its amount can be increased by the expression of a UDP-GlcNAc transporter from K. lactis (Yea4) (Parsaie Nasab et al. 2013; Piirainen et al. 2016). When aiming to develop the strain further to obtain galactosylated N-glycans, the maximal achievable level of galactosylation depends on the amount of substrate glycans available for GalT. It is therefore important to optimize the glycan modification steps preceding galactosylation. We systematically tested a number of existing and new plasmids for the expression of GnTI, GnTII, and Yea4 in strain YMP17, which is a derivative of a glycoengineered strain YMP14 producing less interfering mannosylation due to MNN1 deletion (Piirainen et al. 2016). The N-glycan pattern of cell wall proteins in these strains was analyzed by MALDI-TOF MS and relative abundances of the N-glycan structures were calculated (Table S2). To evaluate the different genetic constructs, we also estimated the efficiencies of each glycan-modifying reaction based on the MALDI-TOF MS data by calculating the amount of glycans processed by each glycosyltransferase relative to the amount of their theoretically available substrate sites (Table 2).

When GnTI was expressed in strain YMP17, approximately half of total cell wall N-glycans received a

Expressed enzymes	GnTI efficiency ^a (%)	GnTII efficiency ^a (%)	GalT efficiency ^b (%)
GnTI	53.1 ± 0.8	n/a	n/a
GnTI + Yea4	50.3 ± 3.8	n/a	n/a
GnTI + Yea4 + GalT	51.3 ± 2.2	n/a	77.5
GnTI + Yea4 + Uge1-GalT	57.0 ± 1.1	n/a	83.2
GnTI+II (high copy plasmid)	32.6 ± 3.2	70.4 ± 6.4	n/a
GnTI+II	46.5 ± 3.9	77.9 ± 4.0	n/a
GnTI+II+Yea4	50.7 ± 2.7	92.2 ± 0.4	n/a
GnTI+II+Yea4 with glucosamine	50.5	92.2	n/a
GnTI + II + Yea4 + GalT	37.7 ± 2.5	50.6 ± 2.1	58.6
GnTI + II + Yea4 + Uge1-GalT	41.8 ± 2.7	62.9 ± 8.4	60.3
GnTI + II + Yea4 + Uge1-GalT with glucosamine	53.3 ± 0.2	87.6 ± 0.8	64.1

^aThe data represent the mean \pm SEM of 3–5 biological replicates, except for the strain expressing GnTI+II+Yea4 with glucosamine the mean of 2 biological replicates is shown. ^bGalT efficiency was calculated from galactosidase digestions performed to a representative sample

n/a, not applicable

GlcNAc residue, and 39% of these glycans additionally contained a fourth mannose residue (Table S2). These GlcNAcMan₃₋₄GlcNAc₂ structures resemble mammalian hybrid-type glycans except that due to the ALG3 deletion and endogenous mannosyltransferase activity of S. cerevisiae, the α 1-6 linked arm of the trimannosyl core contains no mannose extensions or a single additional mannose whereas the α 1-6 linked arm of hybrid-type glycans contains one α 1-3 linked and one α 1-6 linked mannose residue. The glycans not processed by GnTI predominantly consisted of Man₄GlcNAc₂ structures that had received one additional mannose residue to the trimannosyl core (Fig. 1). The fourth mannose residue in both Man₄GlcNAc₂ and GlcNAcMan₄GlcNAc₂ structures was confirmed to be α 1-2 linked (Fig. 2), which is in accordance with earlier findings of the interfering mannose (Parsaie Nasab et al. 2013).

Next, we coexpressed Yea4 with GnTI to test whether the positive effects of Yea4 on glycan pattern reported earlier are related to the activity of GnTI. When Yea4 was coexpressed with GnTI, the relative abundance of glycans processed by GnTI did not change significantly (Table 2). However, the relative abundance of GlcNAcMan₄GlcNAc₂ decreased (Fig. 1, Table S2), only composing 26% of all glycans processed by GnTI. Thus, expression of a UDP-GlcNAc transporter did not increase the GnTI activity but decreased mannosylation of the GlcNAcMan₃GlcNAc₂ structure. The decreased mannosylation was only observed in glycans processed by GnTI, as the relative amounts of Man₃GlcNAc₂ and Man₄GlcNAc₂ remained unchanged.

In our earlier study, GnTI and GnTII were expressed in a high copy number vector using a bidirectional *GAL1-10* promoter, placing GnTI and GnTII under *GAL1* and *GAL10* promoters, respectively (Parsaie Nasab et al. 2013). When this vector was expressed in YMP17, the relative abundance of the G0 glycan was 21% (Fig. 1, Table S3). The efficiency



Fig. 2 MALDI-TOF MS spectra of N-glycans isolated from cell wall proteins of YMP17 expressing GnTI in plasmid pAK3 before (A) and after (B) α 1-2 mannosidase treatment

of the GnTI reaction in this strain was only 33% (Table 2), significantly lower than obtained with the low copy number GnTI expression vector. We therefore wondered if changing the expression construct for GnTI and GnTII to a low copy number version could improve the yield of complex-type glycans, and created a low copy number vector containing both GnTI and GnTII under *GAL1* promoter. With the new GnTI/II expression vector, the relative abundance of the G0 glycan increased to 32% (Fig. 1, Table S3). This improvement was partially caused by an increased GnTI efficiency, which was nearly as high as when expressed without GnTII. The relative abundance of Man₄GlcNAc₂ decreased correspondingly, although biological variation was high. In addition, the amount of glycans processed by GnTII increased.



Fig. 1 Relative abundances of N-glycans isolated from the cell walls of YMP17 expressing GnTI (pAK3), GnTI and *KIYEA4* (pAF22), GnTI and GnTII under *GAL1-10* promoter in a high copy number plasmid (pAX428), GnTI and GnTII in a low copy number plasmid

(pSKH01), and GnTI, GnTII, and *KIYEA4* in a low copy number plasmid (pAF21) measured by MALDI-TOF MS. The data represent the mean \pm SEM of 3–5 biological replicates. *p* <0.05 (*), *p* <0.01 (**), *p* <0.001 (***)

Taking into account that GnTII only accepts glycans processed by GnTI as its substrate (Vella et al. 1984), the efficiency of the GnTII reaction was 70% with the high copy number version and 78% with the new low copy GnTI/II expression vector (Table 2).

After demonstrating that the new version of the GnTI/II expression vector provided increased relative abundances of G0 glycans, we incorporated the expression of the UDP-GlcNAc transporter to this vector to further increase its amounts. With Yea4 coexpression, the relative abundance of G0 increased to 43% (Fig. 1, Table S3), exceeding the levels of the previously predominant Man₄GlcNAc₂ structure. Yea4 expression did not significantly increase the efficiency of GnTI, similarly to our observations without GnTII expression. However, GnTII efficiency of over 90% was obtained when Yea4 was expressed (Table 2). In addition, very low amounts of incompletely processed or interfering structures other than Man₄GlcNAc₂ were seen. Thus, the coexpression of Yea4 with GnTI and GnTII led to a very high GnTII activity in our glycoengineered system, but the activity of GnTI was modest and mostly unaffected by the expression of Yea4 or GnTII.

Galactosylation of hybrid-like and complex glycans in *S. cerevisiae*

Next, we aimed to test whether the complex and hybrid-like glycans formed in S. cerevisiae could be galactosylated. We constructed a tripartite fusion protein (Uge1-GalT) consisting of Mnn2p targeting sequence, UDP-glucose 4-epimerase from S. pombe, a GSGG linker peptide, and GalT that was used successfully for galactosylation of N-glycans in Pichia pastoris and Hansenula polymorpha (Bobrowicz et al. 2004; Wang et al. 2013). To determine the importance of the epimerase domain in our expression system, we also created a corresponding fusion protein lacking the UDP-glucose 4-epimerase domain (GalT). We first confirmed by Western blot that both Uge1-GalT and GalT fusion proteins were expressed intracellularly and appeared intact (Figure S2). Next, we coexpressed the GalT and Uge1-GalT constructs with GnTI and Yea4 and analyzed the cell wall N-glycans with MALDI-TOF MS. When GalT or Uge1-GalT was expressed, the relative abundances of the signals at m/z 1298 and 1460 increased, corresponding to the sodium adducts of GlcNAcMan₄GlcNAc₂ or GalGlcNAcMan₃GlcNAc₂, and GlcNAcMan₅GlcNAc₂ or GalGlcNAcMan₄GlcNAc₂, respectively. Correspondingly, the relative abundance of GlcNAcMan₃GlcNAc₂ structure dropped from 35 to under 10% upon expression of Uge1-GalT and GalT (Fig. 3 and Fig. 5, Table S2). Enzymatic digestions of the N-glycans with α 1-2 mannosidase and β 1-4 galactosidase revealed that nearly 90% of the signal intensity at m/z 1298 and 1460 arose from galactosylated structures, and approximately 80%



Fig. 3 Relative abundances of N-glycans isolated from the cell walls of YMP17 expressing GnTI and *KlYEA4* (pAF22), GnTI, *KlYEA4* and GalT (pAF22+pSR02), and GnTI, *KlYEA4*, and Uge1-GalT (pAF22+pSR01) measured by MALDI-TOF MS. The data represent the mean \pm SEM of three biological replicates

of all potential GalT substrate sites, i.e., β 1-2 linked Glc-NAc residues, had received a β 1-4 linked galactose residue (Table 2). The presence of the UDP-glucose 4-epimerase domain had at most a minor positive effect on the extent of galactosylation. In addition, the efficiency of the GnTI reaction was not hampered by the expression of GalT or Uge1-GalT. In fact, the GnTI efficiency was even slightly increased when coexpressed with Uge1-GalT. Thus, the expression of GalT or Uge1-GalT does not interfere with GnTI activity and glycans processed with GnTI can be efficiently galactosylated in glycoengineered *S. cerevisiae* strain YMP17 both with and without the UDP-glucose 4-epimerase.

After demonstrating that the GalT and Uge1-GalT constructs had satisfactory in vivo activity towards hybrid-like glycans in S. cerevisiae, we next coexpressed them with GnTI, GnTII, and Yea4. As a result, mono- and digalactosylated complex-type glycans (Gal₁₋₂GlcNAc₂Man₃GlcNAc₂ or G1 and G2, respectively) were formed (Figs. 4 and 5). A higher relative abundance of G2 glycans was obtained with the Uge1-GalT construct compared to the construct without the epimerase (11.5% and 5.1%, respectively, Fig. 4, Table S3). However, the relative abundance of G2 was rather low with both constructs, and several incompletely processed as well as mannosylated structures were present. Sequential α 1-2 mannosidase and β 1-4 galactosidase digestion revealed that approximately 60% of all β 1-2 linked GlcNAc residues were processed by GalT or Uge1-GalT, which was less than when expressing GalT or Uge1-GalT with GnTI and Yea4 only (Table 2). A vast majority of the signals at m/z 1298 and 1461 arose from galactosylated **Fig. 4** Relative abundances of N-glycans isolated from the cell walls of YMP17 expressing GnTI, GnTII, and *K1YEA4* (pAF21), GnTI, GnTII, *K1YEA4*, and GalT (pAF21+pSR02), and GnTI, GnTII, *K1YEA4*, and Uge1-GalT (pAF21+pSR01) measured by MALDI-TOF MS. The data represent the mean \pm SEM of three biological replicates. *p* < 0.05 (*), *p* < 0.01 (**)



60

50

40

30

20

10

0

609

933

Relative abundance (%)

Fig. 5 Representative MALDI-TOF MS spectra of N-glycans isolated from cell wall proteins in strain YMP17 expressing pAF22 (**A**), pAF21 (**B**), pAF22 and pSR02 (**C**), and pAF21 and pSR02 (**D**).

hybrid-like structures, as observed earlier. However, only 27% of the available G0 structures were converted to G2 glycans by the GalT construct (Table 2). When the Uge1-GalT construct was used instead, the conversion from G0 to G2 was higher (46%, Table 2). Thus, the UDP-glucose 4-epimerase domain did not seem to impact the overall efficiency of galactosylation but its presence increased the yield of G2 structures (Table S3).

Analysis of the efficiencies of the GnTI and GnTII reactions preceding the galactosylation step revealed that the GnTI efficiency decreased significantly when



MALDI-TOF MS spectra of **C** and **D** after β 1-4 galactosidase digestion (**E** and **F**, respectively)

coexpressed with GnTII and GalT (Table 2), resulting in increased relative abundances of $Man_3GlcNAc_2$ and $Man_4GlcNAc_2$ structures (Fig. 4, Table S3). In addition, the GnTII efficiency decreased even more, as shown by the appearance of the hybrid-like GlcNAcMan_4GlcNAc_2 and GalGlcNAcMan_4GlcNAc_2 glycans. When the UDPglucose 4-epimerase domain was included, both GnTI and GnTII processing efficiencies were somewhat increased (42% and 63%, respectively) but remained significantly lower than without GalT expression (Table 2). Therefore, the coexpression of GnTI, GnTII, GalT, and Yea4 seemed to have a negative impact on the activities of all three glycosyltransferases, and the presence of the Uge1p domain partially mitigated this effect. To confirm that GnTI and GnTII were expressed normally in the presence of the GalT or Uge1-GalT expression vector, we analyzed intracellular GnTI and GnTII levels by Western blot (Figure S2). Both GnTI and GnTII were detected albeit the apparent molecular weight of GnTII was slightly lower than expected, and their levels were not notably changed by GalT or Uge1-GalT expression. In order to better understand the physiological consequences of the overexpression of glycosyltranferases, we also measured the growth of the strains expressing GnTI only, GnTI and GnTII, and GnTI, GnTII, and UGE-GalT, respectively, to analyze whether the expression of multiple glycosyltransferases has a negative impact on viability. The expression of UGE-GalT did not hamper growth compared to the expression of GnTI or coexpression of GnTI and GnTII only; in fact, the growth rate was higher when UGE-GalT was expressed (Figure S3).

Optimizing Gal₂GlcNAc₂Man₃GlcNAc₂ synthesis by growth medium supplementation

After defining the optimal genetic constructs, we tested whether the formation of G0 and G2 glycans could be further improved by growth medium optimization. Among several growth medium adjustments tested, we found that supplementation of 15 mM glucosamine doubled the relative abundance of formed G2 glycans in cell wall N-glycan samples (Fig. 6, Table S3). In these samples, the efficiencies of GnTI and GnTII reactions (53% and 88%, respectively) were significantly improved compared to samples grown without glucosamine and comparable to the efficiencies obtained without Uge1-GalT expression (Table 2). Thus, glucosamine supplementation seemed to compensate for the loss of GnTI and GnTII activities caused by GalT expression. However, supplementation of glucosamine had no impact on the glycan pattern when only GnTI, GnTII, and Yea4 were coexpressed.

We then utilized the optimized genetic constructs and growth medium to analyze the N-glycan pattern of the total secreted proteins, which is likely to provide a better representation of the glycan pattern in recombinantly produced proteins. As a result, we obtained nearly 50% relative abundance of the G0 structure when GnTI, GnTII, and Yea4 were expressed (Table S4). When coexpression of Uge1-GalT was included, G2 was the most prevalent glycan structure with up to 30% relative abundance (Fig. 7, Table S4). Compared to cell wall samples, N-glycans from secreted proteins generally contained higher amounts of the initial Man₃GlcNAc₂



Fig. 7 Representative MALDI-TOF MS spectra of N-glycans isolated from secreted proteins in strain YMP17 expressing GnTI, GnTII, and *KlYEA4* (**A**) and GnTI, GnTII, *KlYEA4*, and Uge1-GalT (**B**) grown in the presence of 15 mM glucosamine



Fig. 6 Relative abundances of N-glycans isolated from the cell walls of YMP17 expressing GnTI, GnTII, and *KIYEA4* (pAF21) or GnTI, GnTII, *KIYEA4*, and Uge1-GalT (pAF21+pSR01) with and without glucosamine (GlcN) measured by MALDI-TOF MS. The data

represent the mean \pm SEM of three biological replicates, except for YMP17 expressing pAF21 with glucosamine the mean of two biological replicates is shown. p < 0.05 (*), p < 0.001 (***)

glycans that were neither processed by GnTI nor mannosylated (Parsaie Nasab et al. 2013 and Tables S3 and S4). However, glucosamine supplementation restored the relative abundance of $Man_3GlcNAc_2$ in secreted proteins close to the levels found in cell wall samples.

Finally, to demonstrate the expression of a therapeutically relevant recombinant protein carrying galactosylated complex-type N-glycans, an IgG molecule was expressed in the strain with the optimized genetic constructs for GnTI, GnTII, and GalT activities and its N-glycans were analyzed. The N-glycan pattern of the expressed IgG contained galactosylated G1 and G2 structures along with G0 and other incompletely processed structures, although their relative abundances differed somewhat from the glycan patterns of cell wall and total secreted proteins (Figure S4).

The interfering mannose is linked to the α1-6 branch of the trimannosyl core

Throughout our efforts to create GlcNAc-containing and galactosylated glycans, a significant amount of interfering mannosylation took place despite the optimization of G0-G2 biosynthesis pathway, as seen by the persistent presence of the Man₄GlcNAc₂ structure. The additional mannose can interfere with the GlcNAc transferase activities, and individual deletions of several known or putative α 1-2 mannosylation (Parsaie Nasab et al. 2013; Piirainen et al. 2016). Therefore, we aimed to gather more detailed information on the structure and formation of these interfering structures.

To confirm that no Man₄GlcNAc₂ is formed already during the LLO biosynthesis, we isolated LLOs from strain YMP17 and analyzed them with MALDI-TOF MS. LLOs of an $\Delta alg 3 \Delta alg 11$ yeast strain have been analyzed earlier by HPLC with [³H]mannose labeling (Parsaie Nasab et al. 2013), and the strain was reported to produce exclusively Man₃GlcNAc₂ LLOs. However, strain YMP17 contains several additional modifications compared to the strain analyzed earlier. Most importantly, the strain has been UV-mutagenized for improved growth. Additionally, as Man₃GlcNAc₂-PP-Dol is a poor substrate for the flippase and OST complex in the LLO biosynthesis pathway, the reduced glycosylation efficiency has been compensated by expression of an artificial flippase and the OST from Leishmania brasiliensis (Parsaie Nasab et al. 2013). Further on, the MNN1 gene was also deleted to eliminate the formation of Man₅GlcNAc₂ and larger glycans (Piirainen et al. 2016). A MALDI-TOF MS spectrum of LLOs in YMP17 (Figure S5) shows the presence of Man₃GlcNAc₂ and a minor amount of Man₂GlcNAc₂, corresponding to the earlier results from the original $\Delta alg3 \Delta alg11$ strain (Parsaie Nasab et al. 2013) and confirming that Man₄GlcNAc₂ is only formed after the transfer of the glycan to a protein. N-glycan analysis of cell wall proteins in YMP17 in turn shows that Man₃GlcNAc₂ was almost entirely converted to Man₄GlcNAc₂ (Figure S5).

Two different isomeric structures can form Man₄GlcNAc₂ glycans, as the fourth mannose can be linked either to the Man α 1-3 branch or to the Man α 1-6 branch of the trimannosyl core. The formation of GlcNAcMan₄GlcNAc₂ upon expression of GnTI indicates that at least a part of the mannosyltransferase activity links the fourth mannose to the α 1-6 linked branch, as the α 1-3 linked branch has been processed by GnTI. However, it cannot be excluded that Man₄GlcNAc₂ is a mixture of both isomers. Especially mannosylation taking place in the α 1-3 linked branch would result in interference with the GnTI reaction. Furthermore, it is not known whether a single or multiple mannosyltransferases contribute to the mannosylation. We performed highenergy CID MS/MS analysis of permethylated and underivatized Man₄GlcNAc₂ in positive and negative ion mode, respectively, to find out the position of the fourth mannose (Stephens et al. 2004; Domann et al. 2012). In-depth MS/MS analysis of Man₄GlcNAc₂ glycans from various strains by negative and positive CID revealed several diagnostic fragment ions indicating the presence of the additional mannose residue in the α 1-6 arm of the trimannosyl core (Figure S6). Only weak or inconclusive fragmentation corresponding to the presence of the fourth mannose in the α 1-3 arm was found. Thus, even though the presence of a second isomer could not be conclusively ruled out, the data suggest that at least a majority of Man₄GlcNAc₂ glycans contain the additional mannose in the α 1-6 arm.

Discussion

In this study, we conducted a comprehensive analysis on the factors affecting N-glycan processing and introduced a further step to the biosynthesis of human-compatible N-glycans in glycoengineered S. cerevisiae. Galactose residues serve important functions in N-glycans, such as enhancing the biological activity of antibodies (Reusch and Tejada 2015). Thus, we aimed to include the galactosylation step into an S. cerevisiae strain that can form complex-type N-glycans with terminal GlcNAc residues (Parsaie Nasab et al. 2013). We tested two alternative galactosyltransferase constructs: Golgi-targeted human GalT and a corresponding fusion protein developed by Bobrowicz et al. (2004) that additionally contains Uge1p from S. pombe, a yeast that natively produces galactose-containing glycans. Uge1p catalyzes the epimerization of UDP-glucose into UDP-galactose, which is used as a sugar donor for galactosyltransferases (Suzuki et al. 2010). In glycoengineered P. pastoris, expression of human GalT resulted in inefficient galactosylation, possibly due to a lack of UDP-galactose in the Golgi apparatus (Vervecken et al. 2004; Bobrowicz et al. 2004). Bobrowicz et al. reasoned that a Golgi-localized Uge1p could generate a local UDP-galactose supply for the GalT, and the Uge1-GalT fusion construct enabled efficient formation of Gal₂GlcNAc₂Man₃GlcNAc₂ glycans. This construct has also been used in other glycoengineered yeast strains (Jacobs et al. 2009; Wang et al. 2013).

In our experiments, both galactosyltransferase constructs provided efficient galactosylation of hybrid-like glycans. We used galactose as an inducer for the expression of the glycosyltransferases, which also provides an extracellular source of UDP-galactose precursor that can be imported into the cytosol and converted into UDP-galactose by Gal1p, Gal10p, and Gal7 (Lohr et al. 1995). It seems that in the presence of extracellular galactose, UDP-glucose 4-epimerase activity is not required for maintaining sufficient UDP-galactose levels in the Golgi apparatus. This is in agreement with the finding that UDP-galactose can be transported into the Golgi apparatus in S. cerevisiae although no UDP-galactose transporters have yet been identified (Roy et al. 1998). Also in S. pombe, UGE1 was only required for cell surface galactosylation when grown in the absence of galactose (Suzuki et al. 2010). In contrast, P. pastoris cannot assimilate galactose due to the loss of galactose assimilating enzymes and therefore UDP-glucose 4-epimerase is critical for efficient galactosylation in this yeast.

Interestingly, the presence of the Uge1p domain increased the formation of complex-type G2 glycans even though the availability of UDP-galactose did not seem to be limited and the overall galactosylation efficiency was not strongly affected by Uge1p. This finding raises an interesting question about the role of the UDP-glucose 4-epimerase domain. The role of Golgi-localized Uge1p for the galactosylation reaction has also been questioned earlier, as NAD⁺ required for the catalytic activity of Uge1p is not known to be imported into the secretory pathway (De Pourcq et al. 2010). Also, our results suggest that rather than the epimerization reaction per se, the role of Uge1 in N-glycan galactosylation might be related to other aspects of the glycan maturation pathway, such as the activity or substrate specificity of GalT or the activity of GnTII.

The presence of the Uge1p domain increased the conversion from G0 to G2, which was relatively low compared to the extent of galactosylation in hybrid-like glycans. GalT does not process the acceptor sites at the α 1-3 and α 1-6 linked branches of the N-glycan with equal efficiencies (Pâquet et al. 1984; Blanken et al. 1984), and the presence of multiple substrate sites makes galactosylation of G0 kinetically a complex process (McDonald et al. 2018). Our data suggest that the inclusion of the Uge1p domain in the GalT construct possibly improved the efficiency of this process. The Uge1p domain can induce various structural changes to the GalT fusion protein potentially affecting the catalytic activity or branch specificity of GalT.

These structural factors include the location of the catalytic domain relative to the membrane and the flexibility of the protein. GalT is also shown to exist in a dynamic equilibrium between monomeric and homodimeric states (Harrus et al. 2018), and this equilibrium could be affected by the Uge1p domain.

The Uge1p domain also mitigated the decrease in the GnTII activity that occurred upon GalT expression. The decreased activity of GnTII in the presence of GalT probably results from the competition of GalT and GnTII for the glycan substrates. GnTI, GnTII, and GalT need to process Man₃GlcNAc₂ in a sequential order to form G2 because GnTII requires the GlcNAcMan₃GlcNAc₂ structure created by GnTI as its substrate (Vella et al. 1984) and is unable to process galactosylated GalGlcNAcMan₃GlcNAc₂ structures (Bendiak and Schachter 1987; Kadirvelraj et al. 2018). However, GalT can also galactosylate hybrid-type glycans that have not been processed by GnTII. Localization of GalT too early in the secretory pathway relative to GnTII can cause premature galactosylation, preventing further glycan processing by GnTII. In our strains, GalT and GnTII were both expressed as fusions with Mnn2p targeting sequences. Despite some uncertainty regarding the localization of GnTII due to its unexpectedly low apparent molecular weight, the localization of GnTII and GalT activities overlapped as both complex-type Gal₁₋₂GlcNAc₂Man₃GlcNAc₂ and hybrid-like GalGlcNAcMan₃₋₄GlcNAc₂ glycans were formed, indicating that galactosylation took place both before and after the GnTII step. Mnn2p localization sequence was used for GnTII and Uge1-GalT also in *H. polymorpha*, where both hybrid and complex galactosylated glycans were formed similarly to our results (Wang et al. 2013). However, the level of premature galactosylation was very low in our strains when the optimal genetic constructs and growth medium were used. As the negative effect of GalT on GnTII efficiency was partially mitigated by the Uge1p domain, we could speculate that perhaps the inclusion of Uge1p domain in the GalT fusion protein might have an impact on either the activity of GnTII or the relative localization of GnTII and GalT, enabling a higher amount of GnTII processing prior to galactosylation.

Coexpression of GnTI, GnTII, Yea4, and GalT or Uge1-GalT decreased the efficiencies of all three glycosyltransferases compared to strains expressing only two of the glycosyltransferases. In addition to the factors mentioned above, the lowered efficiency of all three glycosyltransferases might also be caused by a limited capacity of a yeast cell to express heterologous enzymes. We expressed GnTI, GnTII, and GalT under the control of *GAL1* promoter that requires a transcriptional activator Gal4p for expression (Traven et al. 2006). When this promoter is used in multiple copies for heterologous gene expression, the amount of regulatory proteins can become limiting (Schultz et al. 1987), and as a consequence lower the expression levels of all three glycosyltransferases.

We also improved the efficiencies of GnTI and GnTII reactions, which create the substrate glycans for GalT, by changing their expression construct to a low copy number plasmid. In the cloning process, the GAL10 promoter for GnTII was replaced by GAL1 whereas the promoter for GnTI remained as GAL1. The lower copy number improved the GnTI activity, but it is not clear whether also the promoter change affected GnTII expression. Data regarding the relative strength of GAL1 and GAL10 promoters is not consistent, as GAL10 promoter has resulted in lower (West et al. 1984; Yocum et al. 1984; Park et al. 2000), higher (Partow et al. 2010), or approximately equal expression levels compared to GAL1 promoter (Cartwright et al. 1994; Elison et al. 2018). Expressing high levels of heterologous proteins and maintaining a high number of plasmid DNA can cause metabolic burden (Karim et al. 2013), affecting the general fitness of the cell and various cellular processes including glycan maturation. Lowering the copy number seemed to maintain sufficient intracellular glycosyltransferase levels while minimizing the cellular stress caused by their expression.

Expression of the K. lactis UDP-GlcNAc transporter improved the formation of G0 glycans as also reported earlier, suggesting that the supply of UDP-GlcNAc in the Golgi apparatus might be limited in yeast. Yeast cells can import extracellular glucosamine and convert it to UDP-GlcNAc, resulting in an elevated intracellular UDP-GlcNAc concentration (Bulik et al. 2003). We hypothesized that an additional increase in the intracellular UDP-GlcNAc concentration could further increase the formation of complex-type glycans. Interestingly, extracellular glucosamine had no impact in strains expressing GnTI, GnTII, and Yea4 but a positive impact was seen when the expression of Uge1-GalT was included. Without extracellular glucosamine, the GnTI and GnTII efficiencies were relatively low in this strain due to the negative effects of GalT on these glycosyltransferases, but glucosamine supplementation seemed to compensate for this activity loss. Taken together with the observation that the coexpression of Yea4 only increased the efficiency of GnTII, our results suggest that high GnTII activities can be obtained by improving the expression vector and the UDP-GlcNAc supply but an unidentified factor seems to limit the efficiency of GnTI to approximately 50%.

The limited GnTI activity could be connected to the $Man_4GlcNAc_2$ glycans that were formed in significant amounts in addition to the hybrid-like and complex glycans. Elimination of the additional α 1-2 linked mannose residue has been attempted by deletion of various known or putative α 1-2 mannosyltransferase genes including *MNN2*, *MNN5*, *KRE2*, *KTR1*, and *KTR3* (Parsaie Nasab et al. 2013) without success. We also tested deletions of *MNN9* and *VAN1*, members of the mannan polymerase complex I that elongates

the α 1-6 linked N-glycan outer chain and has been reported to also have α 1-2 mannosyltransferase activity (Stolz and Munro 2002). However, no effects on glycan pattern were seen (unpublished data). It is possible that the mannosylation is either caused by an uncharacterized enzyme or multiple mannosyltransferases with overlapping activities. However, interfering α 1-2 linked mannosylation has also been observed in other glycoengineered yeasts and eliminated or largely reduced by expression of GnTI and GnTII (Hamilton et al. 2003; Bobrowicz et al. 2004; Wang et al. 2013), suggesting that the mannosylation could also be prevented if sufficiently active and optimally localized GlcNAc transferases are used.

The position of the additional mannose in the trimannosyl core and the localization of the mannosyltransferase activity relative to GnTI and GnTII determines if screening of potential mannosyltransferase deletions or optimized expression and localization of GnTI or GnTII would more likely eliminate the interfering mannosylation. Our MS/MS data showed that the additional mannose residue was at least mostly linked to the α 1-6 arm of the trimannosyl core. Thus, this mannose residue does not directly block the acceptor site for GnTI. Although the activity of GnTI towards Man₄GlcNAc₂ is not known, experimental data on various mammalian GnTIs suggests that GnTI can process glycans with varying α 1-6 branch structures, including structures with glycosidic linkages at the C2 of the α 1-6 mannose, albeit at somewhat lower activity than the native substrate glycan Man₅GlcNAc₂ (Oppenheimer et al. 1981; Oppenheimer and Hill 1981; Vella et al. 1984). Thus, if Man₄GlcNAc₂ glycans were formed in the glycan maturation process before the GnTI step, we assume that they would likely be processed by GnTI at least to some extent. However, very little GlcNAcMan₄GlcNAc₂ was formed when GnTI, GnTII, and Yea4 were coexpressed, suggesting that only Man₃GlcNAc₂ glycans are encountered by GnTI and the glycans not processed by GnTI would be converted to Man₄GlcNAc₂ later in the Golgi apparatus. Also our preliminary N-glycan data from whole cell extracts supports this presumption. Whole cell extracts that contain intracellular N-glycan intermediates in addition to readily processed cell wall N-glycans had a lower relative abundance of Man₄GlcNAc₂ compared to the cell wall samples, supporting the assumption that the α 1-2-mannosyltransferase activity is not likely to occur very early in the secretory pathway (unpublished results). Thus, optimizing the catalytic activity of GnTI would be a primary target for further engineering. Based on the relative abundances of various glycans found in our samples, we constructed a route from Man₃GlcNAc₂ to G2 and interfering structures (Fig. 8).

The data in this work mostly represent the average glycan patterns found in cell wall and secreted proteins. While the cell wall protein fraction contains mostly GPI-anchored



proteins, the secreted proteins represent a pool of soluble proteins. We expect that the observed glycan patterns provide realistic views on the glycan pattern to be expected for a recombinant protein. Indeed, our data on the glycan pattern of a purified recombinant antibody expressed in the yeast cells supports the notion that cell wall and secreted proteins are good indicators for the glycan pattern although a larger fraction of Man₃GlcNAc₂ remained unmodified. However, as glycoforms between different proteins and even between glycosylation sites within a protein can vary, the glycosylation pattern needs to be evaluated individually for each protein of interest when considering the potential applications of glycoengineered yeast in therapeutic protein production. Most therapeutic glycoproteins are a mixture of different glycoforms, and the range of required or acceptable glycoforms can vary depending on the product and the host cell line (Goh and Ng 2018). For example, the N-glycans of therapeutic antibodies are typically incompletely galactosylated and predominantly composed of fucosylated and nonfucosylated G0, G1, and G2 structures (Reusch and Tejada 2015). Thus, instead of homogeneous production of single glycoforms, the ability to produce glycan patterns comparable to current production platforms using yeast could be more advantageous. In addition to optimizing the glycosylation pattern, increasing the production levels of foreign proteins in yeast is essential in the context of therapeutic protein production, and our research group has also accomplished improvements in this area (Koskela et al. 2020).

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Author contribution MP designed the genetic constructs, conducted the experiments with HS, supervised preliminary experiments, and interpreted the results together with HS. HS conducted preliminary experiments, MS/MS measurements, and analyzed the MS/MS data. AF conducted all experiments related to the antibody expression. MP had primary responsibility for writing the manuscript. HS and AF critically reviewed the manuscript. All authors approved the manuscript.

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Data availability All materials are shared with academic institutions upon request and execution of a material transfer agreement.

Code availability Not applicable.

Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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References

- Bendiak B, Schachter H (1987) Control of glycoprotein synthesis. Kinetic mechanism, substrate specificity, and inhibition characteristics of UDP-N-acetylglucosamine:alpha-D-mannoside beta 1–2 N-acetylglucosaminyltransferase II from rat liver. J Biol Chem 262:5784–5790
- Blanken WM, van Vliet A, van den Eijnden DH (1984) Branch specificity of bovine colostrum and calf thymus UDP-Gal: N-acetylglucosaminide beta-1,4-galactosyltransferase. J Biol Chem 259:15131–15135
- Bobrowicz P, Davidson RC, Li H, Potgieter TI, Nett JH, Hamilton SR, Stadheim TA, Miele RG, Bobrowicz B, Mitchell T, Rausch S, Renfer E, Wildt S (2004) Engineering of an artificial glycosylation pathway blocked in core oligosaccharide assembly in the yeast *Pichia pastoris*: production of complex humanized glycoproteins with terminal galactose. Glycobiology 14:757– 766. https://doi.org/10.1093/glycob/cwh104
- Bulik DA, Olczak M, Lucero HA, Osmond BC, Robbins PW, Specht CA (2003) Chitin synthesis in Saccharomyces cerevisiae in response to supplementation of growth medium with glucosamine and cell wall stress. Eukaryot Cell 2:886–900. https://doi. org/10.1128/EC.2.5.886-900.2003
- Callewaert N, Laroy W, Cadirgi H, Geysens S, Saelens X, Min Jou W, Contreras R (2001) Use of HDEL-tagged *Trichoderma reesei* mannosyl oligosaccharide 1,2-alpha-D-mannosidase for N-glycan engineering in *Pichia pastoris*. FEBS Lett 503:173–178. https://doi.org/10.1016/s0014-5793(01)02676-x
- Cartwright CP, Li Y, Zhu Y-S, Kang Y-S, Tipper DJ (1994) Use of β-lactamase as a secreted reporter of promoter function in yeast. Yeast 10:497–508. https://doi.org/10.1002/yea.320100409
- Cheon SA, Kim H, Oh DB, Kwon O, Kang HA (2012) Remodeling of the glycosylation pathway in the methylotrophic yeast *Hansenula polymorpha* to produce human hybrid-type N-glycans. J Microbiol 50:341–348. https://doi.org/10.1007/ s12275-012-2097-2
- Choi B-K, Bobrowicz P, Davidson RC, Hamilton SR, Kung DH, Li H, Miele RG, Nett JH, Wildt S, Gerngross TU (2003) Use of combinatorial genetic libraries to humanize N-linked glycosylation in the yeast *Pichia pastoris*. Proc Natl Acad Sci U S A 100:5022–5027. https://doi.org/10.1073/pnas.0931263100
- De Pourcq K, De Schutter K, Callewaert N (2010) Engineering of glycosylation in yeast and other fungi: current state and perspectives. Appl Microbiol Biotechnol 87:1617–1631. https://doi.org/ 10.1007/s00253-010-2721-1
- De Pourcq K, Tiels P, Van Hecke A, Geysens S, Vervecken W, Callewaert N (2012) Engineering *Yarrowia lipolytica* to produce glycoproteins homogeneously modified with the universal Man3GlcNAc2 N-glycan core. PLoS One 7:e39976. https://doi. org/10.1371/journal.pone.0039976

- de Ruijter JC, Koskela EV, Frey AD (2016) Enhancing antibody folding and secretion by tailoring the Saccharomyces cerevisiae endoplasmic reticulum. Microb Cell Fact 15:87. https://doi.org/ 10.1186/s12934-016-0488-5
- Domann P, Spencer DIR, Harvey DJ (2012) Production and fragmentation of negative ions from neutral N-linked carbohydrates ionized by matrix-assisted laser desorption/ionization. Rapid Commun Mass Spectrom 26:469–479. https://doi.org/10.1002/rcm.5322
- Egrie JC, Browne JK (2001) Development and characterization of novel erythropoiesis stimulating protein (NESP). Br J Cancer 84:3–10. https://doi.org/10.1054/bjoc.2001.1746
- Elison GL, Xue Y, Song R, Acar M (2018) Insights into bidirectional gene expression control using the canonical GAL1/GAL10 promoter. Cell Rep 25:737–748. https://doi.org/10.1016/j.celrep. 2018.09.050
- Goh JB, Ng SK (2018) Impact of host cell line choice on glycan profile. Crit Rev Biotechnol 38:851–867. https://doi.org/10.1080/ 07388551.2017.1416577
- Hamilton SR, Bobrowicz P, Bobrowicz B, Davidson RC, Li H, Mitchell T, Nett JH, Rausch S, Stadheim TA, Wischnewski H, Wildt S, Gerngross TU (2003) Production of complex human glycoproteins in yeast. Science 301:1244–1246. https://doi.org/ 10.1126/science.1088166
- Hamilton SR, Davidson RC, Sethuraman N, Nett JH, Jiang Y, Rios S, Bobrowicz P, Stadheim TA, Li H, Choi B-K, Hopkins D, Wischnewski H, Roser J, Mitchell T, Strawbridge RR, Hoopes J, Wildt S, Gerngross TU (2006) Humanization of yeast to produce complex terminally sialylated glycoproteins. Science 313:1441–1443. https://doi.org/10.1126/science.1130256
- Harrus D, Khoder-Agha F, Peltoniemi M, Hassinen A, Ruddock L, Kellokumpu S, Glumoff T (2018) The dimeric structure of wildtype human glycosyltransferase B4GalT1. PLoS ONE 13:1–17. https://doi.org/10.1371/journal.pone.0205571
- Hegemann JH, Heick SB (2011) Delete and repeat: a comprehensive toolkit for sequential gene knockout in the budding yeast Saccharomyces cerevisiae. Methods Mol Biol 765:189–206. https:// doi.org/10.1007/978-1-61779-197-0
- Jacobs PP, Geysens S, Vervecken W, Contreras R, Callewaert N (2009) Engineering complex-type N-glycosylation in *Pichia pastoris* using GlycoSwitch technology. Nat Protoc 4:58–70. https://doi.org/10.1038/nprot.2008.213
- Kadirvelraj R, Yang JY, Sanders JH, Liu L, Ramiah A, Prabhakar PK, Boons GJ, Wood ZA, Moremen KW (2018) Human N-acetylglucosaminyltransferase II substrate recognition uses a modular architecture that includes a convergent exosite. Proc Natl Acad Sci U S A 115:4637–4642. https://doi.org/10.1073/ pnas.1716988115
- Karim AS, Curran KA, Alper HS (2013) Characterization of plasmid burden and copy number in *Saccharomyces cerevisiae* for optimization of metabolic engineering applications. FEMS Yeast Res 13:107–116. https://doi.org/10.1111/1567-1364.12016
- Koskela EV, Frey AD (2014) Homologous recombinatorial cloning without the creation of single-stranded ends: exonuclease and ligation-independent cloning (ELIC). Mol Biotechnol 57:233–240. https://doi.org/10.1007/s12033-014-9817-2
- Koskela EV, Gonzalez Salcedo A, Piirainen MA, Iivonen HA, Salminen H, Frey AD (2020) Mining data from plasma cell differentiation identified novel genes for engineering of a yeast antibody factory. Front Bioeng Biotechnol 8:1–16. https://doi.org/10.3389/ fbioe.2020.00255
- Kurogochi M, Mori M, Osumi K, Tojino M, Sugawara SI, Takashima S, Hirose Y, Tsukimura W, Mizuno M, Amano J, Matsuda A, Tomita M, Takayanagi A, Shoda SI, Shirai T (2015) Glycoengineered monoclonal antibodies with homogeneous glycan (M3, G0, G2, and A2) using a chemoenzymatic approach have different affinities for FcγRIIIa and variable antibody-dependent cellular

cytotoxicity activities. PLoS ONE 10:1–24. https://doi.org/10. 1371/journal.pone.0132848

- Lohr D, Venkov P, Zlatanova J (1995) Transcriptional regulation in the yeast GAL gene family: a complex genetic network. FASEB J 9:777–787. https://doi.org/10.1096/fasebj.9.9.7601342
- McDonald AG, Tipton KF, Davey GP (2018) A mechanism for bistability in glycosylation. PLoS Comput Biol 14:1–15. https://doi.org/ 10.1371/journal.pcbi.1006348
- Mumberg D, Müller R, Funk M (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene 156:119–122. https://doi.org/10.1016/0378-1119(95)00037-7
- Oppenheimer CL, Eckhardt AE, Hill RL (1981) The nonidentity of porcine N-acetylglucosaminyltransferases I and II. J Biol Chem 256:11477–11482
- Oppenheimer CL, Hill RL (1981) Purification and characterization of a rabbit liver alpha $1 \rightarrow 3$ mannoside beta $1 \rightarrow 2$ N-acetylglucosaminyltransferase. J Biol Chem 256:799–804
- Pâquet MR, Narasimhan S, Schachter H, Moscarello MA (1984) Branch specificity of purified rat liver Golgi UDP-ga1actose: N-acetylglucosamine. J Biol Chem 6:4716–4721
- Park EH, Shin YM, Lim YY, Kwon TH, Kim DH, Yang MS (2000) Expression of glucose oxidase by using recombinant yeast. J Biotechnol 81:35–44. https://doi.org/10.1016/S0168-1656(00) 00266-2
- Parsaie Nasab F, Aebi M, Bernhard G, Frey AD (2013) A combined system for engineering glycosylation efficiency and glycan structure in *Saccharomyces cerevisiae*. Appl Environ Microbiol 79:997–1007. https://doi.org/10.1128/AEM.02817-12
- Partow S, Siewers V, Bjørn S, Nielsen J, Maury J (2010) Characterization of different promoters for designing a new expression vector in Saccharomyces cerevisiae. Yeast 27:955–964. https://doi.org/ 10.1002/yea.1806
- Piirainen MA, Boer H, de Ruijter JC, Frey AD (2016) A dual approach for improving homogeneity of a human-type N-glycan structure in *Saccharomyces cerevisiae*. Glycoconj J 33:189–199. https://doi. org/10.1007/s10719-016-9656-4
- Piirainen MA, Salminen H, Frey AD (2019) Tailoring N-glycan biosynthesis for production of therapeutic proteins in *Saccharomyces cerevisiae*. In: Gasser B, Mattanovich D (eds) Recombinant protein production in yeast. Springer, New York, pp 227–241
- Reusch D, Tejada ML (2015) Fc glycans of therapeutic antibodies as critical quality attributes. Glycobiology 25:1325–1334. https:// doi.org/10.1093/glycob/cwv065
- Roy SK, Yoko-o T, Ikenaga H, Jigami Y (1998) Functional evidence for UDP-galactose transporter in *Saccharomyces cerevisiae* through the in vivo galactosylation and in vitro transport assay. J Biol Chem 273:2583–2590. https://doi.org/10.1074/jbc.273.5.2583
- Schultz LD, Hofmann KJ, Mylin LM, Montgomery DL, Ellis RW, Hopper JE (1987) Regulated overproduction of the GAL4 gene product greatly increases expression from galactose-inducible promoters on multi-copy expression vectors in yeast. Gene 61:123–133. https://doi.org/10.1016/0378-1119(87)90107-7

- Stephens E, Maslen SL, Green LG, Williams DH (2004) Fragmentation characteristics of neutral N-linked glycans using a MALDI-TOF/TOF tandem mass spectrometer. Anal Chem 76:2343–2354. https://doi.org/10.1021/ac030333p
- Stolz J, Munro S (2002) The components of the Saccharomyces cerevisiae mannosyltransferase complex M-Pol I have distinct functions in mannan synthesis. J Biol Chem 277:44801–44808
- Suzuki S, Matsuzawa T, Nukigi Y, Takegawa K, Tanaka N (2010) Characterization of two different types of UDP-glucose/-galactose 4-epimerase involved in galactosylation in fission yeast. Microbiology 156:708–718. https://doi.org/10.1099/mic.0.035279-0
- Traven A, Jelicic B, Sopta M (2006) Yeast Gal4: a transcriptional paradigm revisited. EMBO Rep 7:496–499. https://doi.org/10.1038/ sj.embor.7400679
- Tu L, Banfield DK (2010) Localization of Golgi-resident glycosyltransferases. Cell Mol Life Sci 67:29–41. https://doi.org/10.1007/ s00018-009-0126-z
- Varki A, Cummings RD, Aebi M, Packer NH, Seeberger PH, Esko JD, Stanley P, Hart G, Darvill A, Kinoshita T, Prestegard JJ, Schnaar RL, Freeze HH, Marth JD, Bertozzi CR, Etzler ME, Frank M, Vliegenthart JFG, Lütteke T, Perez S, Bolton E, Rudd P, Paulson J, Kanehisa M, Toukach P, Aoki-Kinoshita KF, Dell A, Narimatsu H, York W, Taniguchi N, Kornfeld S (2015) Symbol nomenclature for graphical representations of glycans. Glycobiology 25:1323– 1324. https://doi.org/10.1093/glycob/cwv091
- Vella GJ, Paulsen H, Schachter H (1984) Control of glycoprotein synthesis. IX. A terminal Manα1-3Manβ1-sequence in the substrate is the minimum requirement for UDP-N-acetyl-Dglucosamine:α-D-mannoside (GlcNAc to Manα1-3) β2-Nacetylglucosaminyltransferase I. Can J Biochem Cell Biol 62:409– 417. https://doi.org/10.1139/084-056
- Vervecken W, Kaigorodov V, Callewaert N, Geysens S, De Vusser K, Contreras R (2004) In vivo synthesis of mammalian-like, hybrid-type N-glycans in *Pichia pastoris*. Appl Environ Microbiol 70:2639–2646. https://doi.org/10.1128/AEM.70.5.2639
- Walsh G (2018) Biopharmaceutical benchmarks 2018. Nat Biotechnol 36:1136–1145. https://doi.org/10.1038/nbt.4305
- Wang H, Song HL, Wang Q, Qiu BS (2013) Expression of glycoproteins bearing complex human-like glycans with galactose terminal in *Hansenula polymorpha*. World J Microbiol Biotechnol 29:447– 458. https://doi.org/10.1007/s11274-012-1197-9
- West RW, Yocum RR, Ptashne M (1984) Saccharomyces cerevisiae GAL1-GAL10 divergent promoter region: location and function of the upstream activating sequence UASG. Mol Cell Biol 4:2467–2478. https://doi.org/10.1128/mcb.4.11.2467
- Yocum RR, Hanley S, West R, Ptashne M (1984) Use of lacZ fusions to delimit regulatory elements of the inducible divergent GAL1-GAL10 promoter in *Saccharomyces cerevisiae*. Mol Cell Biol 4:1985–1998. https://doi.org/10.1128/mcb.4.10.1985

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