Engineering *Yarrowia lipolytica* to Produce Glycoproteins Homogeneously Modified with the Universal Man$_3$GlcNAc$_2$ N-Glycan Core

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**Abstract**

*Yarrowia lipolytica* is a dimorphic yeast that efficiently secretes various heterologous proteins and is classified as “generally recognized as safe.” Therefore, it is an attractive protein production host. However, yeasts modify glycoproteins with non-human high mannose-type N-glycans. These structures reduce the protein half-life in vivo and can be immunogenic in man. Here, we describe how we genetically engineered N-glycan biosynthesis in *Yarrowia lipolytica* so that it produces Man$_3$GlcNAc$_2$ structures on its glycoproteins. We obtained unprecedented levels of homogeneity of this glycan structure. This is the ideal starting point for building human-like sugars. Disruption of the ALG3 gene resulted in modification of proteins mainly with Man$_5$GlcNAc$_2$ and GlcMan$_5$GlcNAc$_2$ glycans, and to a lesser extent with Glc$_2$Man$_5$GlcNAc$_2$ glycans. To avoid underoccupancy of glycosylation sites, we concomitantly overexpressed ALG6. We also explored several approaches to remove the terminal glucose residues, which hamper further humanization of N-glycosylation; overexpression of the heterodimeric *Apergillus niger* glucosidase II proved to be the most effective approach. Finally, we overexpressed an α-1,2-mannosidase to obtain Man$_3$GlcNAc$_2$ structures, which are substrates for the synthesis of complex-type glycans. The final *Yarrowia lipolytica* strain produces proteins glycosylated with the trimannosyl core N-glycan (Man$_3$GlcNAc$_2$), which is the common core of all complex-type N-glycans. All these glycans can be constructed on the obtained trimannosyl N-glycan using either in vivo or in vitro modification with the appropriate glycosyltransferases. The results demonstrate the high potential of *Yarrowia lipolytica* to be developed as an efficient expression system for the production of glycoproteins with humanized glycans.

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**Introduction**

There is increasing demand for efficient expression systems for the economical production of biopharmaceuticals. The properties of recombinant biopharmaceutical proteins can be fine-tuned by manipulating the glycan structures attached to them. However, versatile production methods for producing specific glycoforms are few and involve mostly laborious in vitro pathway engineering.

To rapidly generate different glycoforms of a particular biopharmaceutical for functional studies and subsequent production, it would be valuable to have a microbial expression system that produces N-glycoproteins homogenously modified with the Man$_3$GlcNAc$_2$ N-glycan core. This core is common to all mammalian N-glycan structures, and any complex type N-glycan can be built in vitro on this core using the appropriate glycosyltransferases and sugar-nucleotide donors. However, no convenient expression system producing this Man$_3$GlcNAc$_2$ core is currently available. Our objective was to engineer the yeast *Yarrowia lipolytica* for this purpose.

Yeasts combine the ease of genetic manipulation and up-scaling of microbial cultures with the ability to secrete and modify proteins with the major eukaryotic post-translational modifications. *Saccharomyces cerevisiae* and the methylotrophic yeasts *Pichia pastoris* and *Hansenula polymorpha* are the most frequently used yeast hosts for recombinant protein production, but there is growing interest in the dimorphic yeast *Yarrowia lipolytica*. This yeast can grow to high cell density on long-chain fatty acids. The promoters of acyl-CoA oxidase (POX) genes are strongly induced on this carbon source and are therefore used to drive heterologous gene expression. Moreover, *Y. lipolytica* has long been used for the production of lipases for the agro-food industry and is therefore classified as GRAS (generally regarded as safe).

To generate a *Y. lipolytica* strain producing Man$_3$GlcNAc$_2$ on its glycoproteins, we engineered the ER-localized components of the N-glycosylation pathway. At the cytoplasmic side of the ER membrane, N-glycosylation starts with the synthesis of a dolichol linked glycan precursor (Figure 1A). The intermediate Man$_5$GlcNAc$_2$-PP-Dol structure flips to the luminal side of the ER,
where it is further elongated, first by the α-1,3-mannosyltransferase Alg3p, and then by other mannosyltransferases until ManαGlcNAc2 is formed. This dolichol linked sugar is then glucosylated by the α-1,3-glucosyltransferase Alg6p, after which two more glucose residues are added. The resultant glycans (Glc3ManαGlcNAc2) is transferred to the nascent polypeptide chain (Figure 1A) [1].

In a process of quality control for protein folding [2], all glucose residues are trimmed sequentially. The first two glucose molecules are removed rapidly by the consecutive action of glucosidase I and II, whereas the last α-1,3-linked glucose residue is removed more slowly by glucosidase II (GII). Monoglucosylated proteins are recognized by calnexin and/or calreticulin. These ER chaperones aid the folding of the glycoprotein and do not reassociate with the glycoprotein once the last glucose residue is removed by GII. If the glycoprotein does not fold properly, it is glucosylated again by the UDP-glucose:glycoprotein glucosyltransferase, after which it again binds calnexin and/or calreticulin and reenters the folding cycle.

When the glycoprotein is correctly folded and the sugars are trimmed to ManαGlcNAc2 by ER mannosidase I, the protein proceeds along the secretory pathway. In the Golgi apparatus of yeasts, the ManαGlcNAc2 N-glycans are further extended by the addition of mannose and phospho-mannose residues. This elongation is initiated by the α-1,6-mannosyltransferase Och1p [3,4]. In contrast, higher eukaryotes first trim the glycans to Glc3ManαGlcNAc2 by Golgi mannosidases I and then further modify them to complex type glycans [5–7].

Several methods can be envisioned to engineer yeast for the production of homogeneous, universal glycan ‘scaffolds’ on which different types of eukarotic N-glycans can be built [8]. One approach is to engineer only Golgi-localized processes so that the more essential ER-localized steps of the N-glycosylation pathway are not affected. This has been successfully implemented in P. pastoris [9,10]. Another approach is to interfere with the ER steps of the pathway. This is particularly attractive at the ALG3 step: disruption of ALG3 is expected to lead to the glucosylation of proteins with ManαGlcNAc2 N-glycans, which should be easy to trim to ManαGlcNAc2 with an α-1,2-mannosidase (Figure 1B). ManαGlcNAc2 is the common core of all types of eukarotic N-glycans and provides an ideal scaffold for in vitro or in vivo synthesis of different glycoforms.

However, at least in S. cerevisiae [11,12], the situation is complicated because ManαGlcNAc2-PP-Dol is glucosylated by Alg6p less efficiently than ManGlcNAc2-PP-Dol. Glucosylation of the N-glycan precursor is important for its efficient transfer to nascent proteins by oligosaccharyltransferase, and reduced glucosylation diminishes this transfer. Previous studies have not addressed this shortcoming of this otherwise attractive engineering approach. Here, we report that the glucose residues on glycoproteins produced in alg3 strains are not removed efficiently by Yarrowia GII, and we describe the engineering strategy we used to solve this problem (Figure 1B). Through this integrated ‘systems engineering’ approach, we succeeded in creating a glyco-engineered Y. lipolytica strain that produces glycoproteins homogeneously modified with the trimannosyl core N-glycan (Manα-

Results

ALG3 Gene Knock-out

In order to alter Y. lipolytica to produce heterologous proteins glycosylated with ManαGlcNAc2, we interfered with biosynthesis of the core N-glycan (Figure 1B, step1). Elimination of Alg3p α-1,3-mannosyltransferase prevents the addition of an α-1,3-

Compensation for Underoccupancy of the N-glycan Sites by Overexpressing ALG6

The alg3 mutation in S. cerevisiae causes underoccupancy of N-glycosylation sites [12,13,15–17]. Efficient transfer of the dolichol linked N-glycan precursor to a protein by the oligosaccharyltransferase complex (OST) requires the triglucosyl glycopeptide on the dolichol-linked precursor [1,18]. The first glucosyltransferase, Alg6p, can glucosylate the ManGlcNAc2-PP-Dol structure in alg3 S. cerevisiae [12], but with low efficiency. This results in underglucosylation of the dolichol linked precursor, poor transfer by OST, and reduced occupancy of N-glycosylation sites. Anticipating this problem, we incorporated an Alg6p constitutive overexpression cassette in the alg3 knock-out vector (Figure 1B, step2). The resultant vector (pYLalg3PUT-ALG6) was transformed into WT Y. lipolytica MTLY60, yielding strain YLA3–A6 (Table 1). Upon DSA-FACE analysis of the N-glycans derived from this mutant strain YLA3 (Table 1). This indicates that Alg6p activity was indeed augmented and clearly shows that the
A Glycan processing in ER

B Glycan engineering in ER

C Golgi Glycosyl-T's
endogenous \textit{Y. lipolytica} GII activity was insufficient to deglucosylate its suboptimal Glc$_1$-Man$_2$GlcNAc$_2$ substrates.

To evaluate the underoccupancy of N-glycosylation sites in our different strains, we examined the N-glycosylation of overexpressed \textit{Y. lipolytica} lipase 2 (LIP2), which has two glycosylation sites \[19,20\]. We analyzed the pattern of secreted proteins before and after N-deglycosylation with PNGaseF. For the wild type strain, a single LIP2 band with a smear of hyper-N-glycosylation is observed (Figure 4, lane 3). In the \textit{alg3} knock-out strain, LIP2 is found in two bands (Figure 4, lane 7), the top one at the same MW as the non-hyperglycosylated wild type-produced protein, and the bottom one at an intermediate position between the wild type-produced protein and the fully de-N-glycosylated protein. The bottom band is much less abundant in the preparation from the \textit{alg3} mutant strain overexpressing Alg6p (Figure 4, lane 5). The bands are separated by 1–2 kDa and they collapse into one band when the N-glycans are removed by PNGaseF digestion (Figure 4, lane 4, 6 and 8). These results indicate that the N-glycosylation sites are underoccupied in the \textit{alg3} mutant. As intended, overexpression of Alg6p largely compensates for this underoccupancy, because only one band is visible on the protein gel (Figure 4, lane 5). It should be noted that this phenotype was observed in cells in mid-log phase of growth, and that it was much less pronounced in stationary-phase cells (data not shown). The difference is probably due to the considerably slower flux of proteins through the N-glycosylation pathway in stationary phase.

Interestingly, no hyperglycosylation of LIP2 was seen in the \textit{alg3} and \textit{alg3}ALG6 strains, which means that our strategy need not involve knocking out any Golgi mannosyltransferases to obtain homogeneous glycosylation, contrary to previous approaches \[9,10\]. Consequently, we solved the underglycosylation problem of the \textit{alg3} mutant by overexpressing Alg6p, but this was at the expense of further augmenting the fraction of undesired glycosylated Man$_2$GlcNAc$_2$ derivatives.

### Removal of Capping Glucoses

In strains in which \textit{alg3} is disrupted, the N-glycans are capped by GII-hydrolyzable glucose residues. This type of capping is more pronounced when the \textit{ALG6} gene is overexpressed. Since the presence of these glucose residues prevents conversion of Man$_2$GlcNAc$_2$ to Man$_2$GlcNAc$_2$ by an introduced \(\alpha\)-1,2-mannosidase (Figure 1B, step 4), our next objective was to eliminate those glucose residues by further in vivo engineering.

### Removal of Capping Glucose Residues: Mutanase and \textit{T. brucei} GII

We examined the possibility of using the mutanase of \textit{Trichoderma harzianum} to remove the capping glucose residues on the Man$_2$GlcNAc$_2$ glycans. Both unwanted glucose residues are \(\alpha\)-1,3-linked to the rest of the sugar, and this mutanase has \(\alpha\)-1,3-glucosidase activity. A dilution series of the Novozyme 234 mutanase preparation was added to the oligosaccharides derived from the YLA3–A6 strain (Man$_2$GlcNAc$_2$, GlcMan$_2$GlcNAc$_2$ and Glc$_2$Man$_2$GlcNAc$_2$). The DSA-FACE profile (Figure 5B, panel G) shows that Glc$_2$Man$_2$GlcNAc$_2$ was effectively hydrolyzed to GlcMan$_2$GlcNAc$_2$. However, Glc$_2$Man$_2$GlcNAc$_2$ was not deglucosylated further. It should be noted that Man$_2$GlcNAc$_2$ was also trimmed, most probably by a contaminating mannosidase in the crude enzyme mixture. Since complete deglucosylation could not be obtained with this mutanase, we abandoned this approach.

### Table 1. \textit{Y. lipolytica} strains used in this study.

| \textit{Y.l.} strains | Genotype | Reference |
|----------------------|----------|-----------|
| MTLY60               | MatA ura3-302 leu2-270 xpr2-322_lip2_lip7_lip8 | Pickers et al, 2005 |
| YLA3                 | MTLY60 with \textit{alg3}::URA3 | This work |
| YLA3–A6              | MTLY60 with \textit{alg3}::ALG6-URA3 | This work |
| YLTBGIIA             | As YLA3–A6 overexpr of Tb GII \(\alpha\) | This work |
| YLTBGIIAHDDEL        | As YLA3–A6 overexpr of Tb GII \(\alpha\) HDEL | This work |
| YLTBpreGIIAHDDEL     | As YLA3–A6 overexpr of LIP2pre Tb GII \(\alpha\) HDEL | This work |
| YLYGLIA              | As YLA3–A6 overexpr of YI GII \(\alpha\) | This work |
| YLYGLIAHDDEL         | As YLA3–A6 overexpr of YI GII \(\alpha\) HDEL | This work |
| YLYGLIIAB            | As YLA3–A6 overexpr of YI GII \(\alpha\), \(\beta\) | This work |
| YLANGIIA             | As YLA3–A6 overexpr of An GII \(\alpha\) | This work |
| YLANGIIAB            | As YLA3–A6 overexpr of An GII \(\alpha\), \(\beta\) | This work |
| YLMAN                | As YLANGIIA overexpr of \(\alpha\)-1,2-mannosidase | This work |

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As an alternative strategy, we overexpressed the *T. brucei* GII α-subunit. *T. brucei* uses a dual N-glycosylation system that can transfer both Man9GlcNAc2 and Man5GlcNAc2 to proteins (Figure 5A) [21]. Furthermore, unlike organisms that exclusively transfer Glc3Man9GlcNAc2, the GII enzyme in *T. brucei* uses GlcMan5GlcNAc2 as a preferred substrate [22]. Therefore, we tested whether the *T. brucei* enzyme can deglucosylate these structures in our engineered strains. We transformed the YLA3–A6 strain with pYLHmAXTbGIIa, which resulted in a YLTBGIIA strain (Table 1) and analyzed its cell wall mannoprotein glycans. No deglucosylation was observed (Figure 5B, panel D). As GII is heterodimeric [23], we considered the possibility that the α-subunit of *T. brucei* GII cannot dimerize with the β-subunit of *Y. lipolytica* GII and would thus not be retained in the endoplasmic reticulum. So we introduced an HDEL ER-retrieval tag at the C-terminus of the α-subunit of *T. brucei* GII. Moreover, we expressed the *T. brucei* enzyme once with its own signal peptide and once with the *Y. lipolytica* LIP2 signal peptide in the YLA3–A6 strain (yielding strains YLTBGIIAHDEL and YLTBpreGIIAHDEL, respectively) (Table 1). N-glycan analysis of the clones overexpressing the HDEL-tagged α-subunit showed reduced abundance of the mono-glucosylated Man5GlcNAc2 peak (Figure 5B, panel E and F), whereas the di-glucosylated Man9GlcNAc2 structure was not hydrolyzed. Evidently, this latter structure is not a substrate for the *T. brucei* GII. Consequently, this engineering approach also did not solve our problem, so we abandoned it.

**Figure 2. Identification of N-glycans by exoglycosidase digestion and DSA-FACE analysis.** A: Oligomaltose reference. B, N-glycans from RNaseB reference. C–G, N-glycans from different strains: C, MTLY60 wild type strain; D, alg3 knock-out strain; E, The same as D but treated with α-1,2-mannosidase; F, The same as D but treated with JB α-mannosidase; G, The same as D but treated with glucosidase II. The N-glycan structures in the alg3 knock-out strain are consistent with Man9GlcNAc2, GlcMan5GlcNAc2 and Glc2Man5GlcNAc2.

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Removal of capping glucoses by overexpression of the endogenous GII. To eliminate mono- and di-glucosylated Man5GlcNAc2 structures in vivo, the YLA3–A6 strain was genetically engineered to overexpress the *Y. lipolytica* GII. This enzyme is a heterodimer consisting of two subunits, of which the α-subunit is catalytically active [23] and contains a GH31 family domain [24]. We started by overexpressing the α-subunit in our YLA3–A6 strain. Glucosylation of the various glycans in the resultant strain, YLYLGIIA, was not reduced (Figure 3, panel F versus panel E).

It is believed that the β-subunit, which contains an HDEL tag, serves primarily to retain the α-subunit in the ER [23, 25–27]. Therefore, we first tried mimicking the β-subunit’s function by adding an HDEL tag to the C-terminus of the α-subunit of the *Y. lipolytica* GII. This way, the tag would serve to retrieve the enzyme from the Golgi apparatus to the ER via COPI vesicles and thereby help to maintain the enzyme at its site of action. Again, α-glucose removal was not improved in any of the transformation clones of the resultant YLYLGIIAHDG strain (Figure 3, panel G).

Several studies have indicated the necessity of the β-subunit of the GII complex for maturation, solubility, stability and enzymatic activity on natural substrates [25–29]. Overexpression of the α subunit of *Y. lipolytica* GII alone was not sufficient to reduce the unwanted glucosylation on the Man9GlcNAc2 glycan. Therefore we simultaneously overexpressed the β-subunit in two strains that
overexpress the \textit{Y. lipolytica} GI\textsubscript{I} \(\alpha\)-subunit with or without HDEL tag and we tested both the hp4d and the TEF promoter. We retained the clone with the best glycan profile, \textit{i.e.} the one that removed \(\alpha\)-glucose most efficiently. The best result was obtained in a strain that overexpressed the \textit{Y. lipolytica} GI\textsubscript{II} \(\alpha\)-subunit with the HDEL tag, with a slightly improved effect when the \textit{Y. lipolytica} GI\textsubscript{II} \(\beta\)-subunit was expressed from the TEF promoter compared to the hp4d promoter. Therefore, we created a strain that overexpressed both the \textit{Y. lipolytica} GI\textsubscript{I} \(\alpha\) and \(\beta\)-subunit driven by the TEF promoter. The strain was named YLYLGIIAB (Figure 3, panel H). However, though overexpression of both \(\alpha\) and \(\beta\)-subunits of \textit{Y. lipolytica} GI\textsubscript{II} significantly reduced the proportion of glucosylated Man\textsubscript{5}GlcNAc\textsubscript{2}, it was still insufficiently effective for homogeneous glycoprotein production.

![Figure 3. DSA-FACE analysis of engineered \textit{Y. lipolytica} strains.](image)

A, oligomaltose reference. B–K, N-glycans derived from different sources: B, bovine RNaseB reference; C, MTLY60 wild type strain; D, alg3 knock-out strain; E, alg3 mutant strain overexpressing Alg6p. F–I, the alg3 mutant strain overexpressing Alg6p engineered with: F, \textit{Y. lipolytica} GI\textsubscript{I}A; G, \textit{Y. lipolytica} GI\textsubscript{II}A HDEL-tagged; H, both \(\alpha\) and \(\beta\) subunits of \textit{Y. lipolytica} GI\textsubscript{II}; I, the HDEL-tagged \textit{A. niger} GI\textsubscript{II}; J, both \(\alpha\) and \(\beta\) subunits of \textit{A. niger} GI\textsubscript{I}. K, The latter strain engineered with an HDEL-tagged \textit{T. reesei} \(\alpha\)-1,2-mannosidase. This fully engineered strain produces glycoproteins with more than 85% trimannosyl core N-glycans.

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Discussion

\textit{Y. lipolytica} has emerged as a suitable system for heterologous protein expression [33]. With the increasing importance of yeasts

![Figure 4. SDS-PAGE evaluation of underoccupancy of N-glycan sites in lipase 2 after inactivation of \textit{alg3}](image)

1. Wild-type strain (WT, MTLY60). 3, The same as lane 1 but overexpressing lipase2. 5, The alg3 knock-out strain overexpressing lipase2 and Alg6p. 7, The alg3 knock-out strain overexpressing lipase2. Lanes 2, 4, 6 and 8, the same as 1, 3, 5, and 7, respectively, but treated with PNGaseF. A hyperglycosylation smear is observed when lipase2 is overexpressed in the WT strain. For the alg3 mutant strain expressing lipase2, two distinct bands are visible, which is consistent with site underoccupancy largely compensated for by Alg6p overexpression. Lane 9: PNGaseF preparation used for the digestions shown in Lanes 2, 4, 6 and 8.

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Removal of capping glucoses by overexpression of the \textit{A. niger} GI\textsubscript{II}. Kainz and colleagues [30] recently reported that knockout of \textit{ALGC}, the \textit{ALG3} homologue in the filamentous fungus \textit{A. niger}, leads to the synthesis of Man\textsubscript{5}GlcNAc\textsubscript{2} glycans. In \textit{in vitro} digestion of these glycans with \(\alpha\)-1,2-mannosidase gave almost exclusively Man\textsubscript{3}GlcNAc\textsubscript{2} [30]. Hence, no glucosylated glycan structures were detected when the \textit{ALG3} gene was disrupted in \textit{A. niger}. Therefore, we assumed that the GI\textsubscript{II} of \textit{A. niger} can cope better with the alterations in N-glycan substrate structures caused by inactivation of the ER-mannosyltransferase Alg3p. Indeed, overexpression of the HDEL-tagged \(\alpha\)-subunit of \textit{A. niger} GI\textsubscript{II} alone in our \textit{Y. lipolytica} GI\textsubscript{II} strain overexpressing Alg6p, \textit{i.e.} YLA3–A6, resulted in trimming of the glucosylated Man\textsubscript{5}GlcNAc\textsubscript{2} forms in the newly made YLANGIIIA strain (Figure 3, panel I). No differences were seen between the strains that overexpressed the \(\alpha\)-subunit of \textit{A. niger} GI\textsubscript{II} under control of the TEF or under control of the hp4d promoter (data not shown). We subsequently overexpressed the \(\beta\)-subunit of \textit{A. niger} GI\textsubscript{II} in the YLANGIIIA strain that overexpressed the HDEL-tagged \(\alpha\)-subunit of \textit{A. niger} GI\textsubscript{II}, also under control of the TEF promoter. The resultant strain was named YLANGIIAB. Analysis of the glycan structures on glycoproteins produced by this strain showed very efficient conversion of glucosylated to non-glucosylated Man\textsubscript{5}GlcNAc\textsubscript{2} glycan structures (Figure 3, panel J), which represented about 80% of the total cell wall mannoprotein N-glycan pool.

Overexpression of ER-targeted \(\alpha\)-1,2-mannosidase Leads to Production of Man\textsubscript{5}GlcNAc\textsubscript{2}

As a final step in our N-glycan engineering (Figure 1B, step 4), we aimed at converting Man\textsubscript{5}GlcNAc\textsubscript{2} to core Man\textsubscript{3}GlcNAc\textsubscript{2} glycan structures. Therefore, we overexpressed a \textit{Y. lipolytica}-optimized ER-targeted \textit{T. reesei} \(\alpha\)-1,2-mannosidase [31,32] in the alg3 knock-out strain overexpressing Alg6p and the \textit{A. niger} GI\textsubscript{II}/\textit{\beta}, \textit{i.e.} YLANGIIAB. The resulting strain, YLMAN, produces homogeneous Man\textsubscript{5}GlcNAc\textsubscript{2} (>85%) (Figure 3, panel K).
Engineering *Y. lipolytica* for Man$_3$GlcNAc$_2$-Proteins

A  **N-glycosylation in *T. brucei***

B  **Sugars *T. brucei* and mutanase**

Glucose Units

| Glucose Units | Oligomaltose | RNaseB | YLA3-A6 | YLTBGIIA | YLTBGIIAHDEL | YLTBpreGIIAHDEL | Mutanase |
|---------------|-------------|--------|---------|----------|---------------|-----------------|----------|
| 5             | ![Oligomaltose](#) | ![RNaseB](#) | ![YLA3-A6](#) | ![YLTBGIIA](#) | ![YLTBGIIAHDEL](#) | ![YLTBpreGIIAHDEL](#) | ![Mutanase](#) |
| 10            | ![Oligomaltose](#) | ![RNaseB](#) | ![YLA3-A6](#) | ![YLTBGIIA](#) | ![YLTBGIIAHDEL](#) | ![YLTBpreGIIAHDEL](#) | ![Mutanase](#) |
Man9GlcNAc2 and Man5GlcNAc2 can be transferred to proteins. Next, these proteins are reglucosylated and deglucosylated in the folding cycle by ALG3 glycosylation site occupancy in the lipase secreted by the overexpression of ALG6 constitutively overexpressing the [16,34]. We anticipated this problem and avoided it by oligosaccharide is a major structural determinant in the specificity of proteins.

NAc2 was shown to contain glucose, which is consistent with additional glycan structures: GlcMan 5GlcNAc2 and the expected Man5GlcNAc2 (dolichol-linked type) as well as two converted to any desired mammalian N-glycan using Golgi glycosyltransferases and GII, respectively. (Figure 5. T. brucei GII and mutanase tested as engineering approach. (A) The dual N-glycosylation system in T. brucei. Both Man9GlcNAc2 and Man5GlcNAc2 can be transferred to proteins. Next, these proteins are reglucosylated and deglucosylated in the folding cycle by glucosyltransferase and GII, respectively. (B) DSA-FACE analysis of reference N-glycans and N-glycans derived from strains engineered with T. brucei GII or treated with mutanase. A. Oligomaltose reference. B. N-glycans from RNaseB reference. C. N-glycans from the alg3 mutant strain overexpressing Alg6p. D-F. N-glycan from the alg3 mutant strain overexpressing Alg6p and engineered in different ways: D. engineered with T. brucei GII, E. engineered with T. brucei GII with HDEL tag; F. engineered with T. brucei GII with HDEL tag and pre-lip2 signal. G. N-glycans derived from the alg3 mutant strain overexpressing Alg6p treated with mutanase. doi:10.1371/journal.pone.0039976.g005)

as an alternative host for recombinant protein production, it has become important to glyco-engineer yeasts for production of humanized glycans for therapeutic purposes. We aimed to engineer the Yarrowia ER glycosylation pathway for the production of the Man3GlcNAc2 core N-glycan structure, which can be converted to any desired mammalian N-glycan using Golgi glycosyltransferases (Figure 1C).

Upon disruption of the ALG3 gene in Y. lipolytica, we observed the expected Man4GlcNAc2 (dolichol-linked type) as well as two additional glycan structures: GlcMan9GlcNAc2 and Glc2Man5GlcNAc2. Both glucose residues could be removed in vitro by purified rat liver GIH. It has also been reported that N-glycosylation sites of secretory proteins are underoccupied in alg3 mutants [12,13,15–17]. Various studies have shown that the glucose residues on the lipid-linked oligosaccharide facilitate the transfer of the oligosaccharide to protein [1,18]. Nonglucosylated or partially glucosylated oligosaccharides can be transferred to protein, but with a reduced efficiency. In alg3 mutants of baker’s yeast, the resulting Man4GlcNAc2 lipid-linked glycan is not glucosylated efficiently [12]. Apparently, the 6’ branch of the oligosaccharide is a major structural determinant in the specificity and activity of the Alg6p, dolichol-P-Glc-Man9GlcNAc2-2-DDol glycosyl transferase, which is the first glucosyltransferase in the ER [16,34]. We anticipated this problem and avoided it by constitutively overexpressing the Y. lipolytica ALG6 gene. Indeed, overexpression of ALG6 largely remedied the defect in N-glycosylation site occupancy in the lipase secreted by the alg3 mutant. However, this complemented strain secreted proteins with more Man4GlcNAc2 glucosylation, most likely because of the transfer of a larger fraction of nonglucosylated Man3GlcNAc2 to proteins.

Remarkably and beneficially, Y. lipolytica Golgi glucosyltransferases does not seem to further modify the glycans upon disruption of the ALG3 gene. This was also reflected in the increased homogeneity of secreted LIP2 lipase on SDS-PAGE gels. Most likely, YOCh1p does not recognize the ER-type Man5GlcNAc2 or its glucosylated derivatives.

In contrast, N-glycans released from an alg3och1 mutant strain of P. pastoris contain the expected HexGlcNAc2 structure, as well as large quantities of glycans of higher molecular weight ranging from HexaGlcNAc2 to Hexa12GlcNAc2 [35]. Upon treatment with α-1,2-mannosidase, the Man5GlcNAc2 was converted to Man7GlcNAc2, which is consistent with the alg3 Man5 structure. The other glycans, however, were mostly resistant to treatment with broad-specificity α-mannosidase. Amongst these, only Hexa12GlcNAc2 was shown to contain glucose, which is consistent with a GlcMan5GlcNAc2 structure [35]. The presence of larger structures implies the existence of P. pastoris Golgi glucosyltransferases capable of acting on these substantially truncated substrates. This is clearly different from the situation in Yarrowia. In a S. cerevisiae alg3sec18 mutant, a substantial proportion of the glycan chains on the model protein invertase were the mono-, di- and triglucosylated Man9GlcNAc2 structures [12,16,36].

In contrast, in the plant Arabidopsis thaliana, an alg3gl mutant yielded Man5GlcNAc2 glycans, which led to the hypothesis that an aberrant Man5GlcNAc2 structure, once it is transferred to a protein, is trimmed by the Golgi α-1,2-mannosidase [37]. Similarly, analysis of whole cell extracts from the filamentous fungus A. niger algC knock-out (the ALG3 homologue) revealed the presence of Man5GlcNAc2 N-glycans [30]. Moreover, proteins secreted by an alg3 mutant of the yeast Hansenula polymorpha contain almost no glucosylated glycans [38]: model glycoproteins contain predominantly Man5GlcNAc2. The less abundant Hexa8GlcNAc2 structures can be almost completely converted to Man3GlcNAc2 by in vitro digests with α-1,2- and α-1,6-mannosidases. Deletion of the endogenous OCH1 gene encoding the initiating α-1,6-mannosyltransferase decreases the overall abundance of Hexa8GlcNAc2 structures and only a minor fraction of Hexa8GlcNAc2 remains. This Hexa8GlcNAc2 glycan quite likely contains a capping glucose residue [38].

The presence of glucose residues on the alg3 Man5GlcNAc2 glycans implies either the existence of an endogenous glucosyltransferase or, more likely, insufficient activity of ER-resident GIH, which normally cleaves both α1,3-linked glucose residues successively from Glc1Man5GlcNAc2. GIH’s substrate specificity includes the 6’ pentamannosyl branch of its glucose-containing oligosaccharide substrates. Its activity seems to decrease with reduction of the number of mannoses on the 6’ branch of the N-glycan substrate. Mammalian GIH activity was several times higher with Glc1Man5GlcNAc2 as substrate than with Glc1Man9GlcNAc2. Moreover, oligosaccharides lacking the fourth or fifth mannose residues on the 6’ branch were very poor substrates [39]. Similar results were obtained by other investigators [40–43]. More recently, it was found that the rate of GIH-mediated trimming is specifically dependent on the presence of the α-1,2-linked mannose on the C-arm [44]. The β-subunit of GIH contains a mannose-6-phosphate-homology (MRH) domain that recognizes carbohydrates and contributes to substrate recognition [45]. Sequence alignments indicated that all residues involved in mannose binding in the MRH domain are conserved in GIH β, except for those that interact with the phosphate group. Indeed, there is evidence that the GIH β-subunit plays a key role in enhancing the specific activity of the heterodimeric GIH enzyme towards natural N-glycan substrates [28,29,46–49].

From all the above observations, it can be concluded that, GIH of Y. lipolytica is much more specific for its natural substrate than, for example, the GIH of A. thaliana or A. niger. Here, we used this broader substrate specificity of A. niger GIH to reduce the glucosylation of our YLA3–A6 strain.

The feasibility of our integrated system’s engineering approach illustrates the current level of understanding of the N-glycosylation pathway’s intricacies. We anticipate that this strain will find use in the structure-function analysis of N-glycan modifications in many settings, such as in the fine-tuning of biopharmaceutical protein N-glycans to particular therapeutic goals.

Materials and Methods

Strains, Reagents and Culture Conditions

Escherichia coli strains MC1061, TOP10, and DH5α were used for the amplification of recombinant plasmid DNA.
Yarrowia lipolytica MTLY60 (Table 1) [50] was used as parent strain. All yeast strains were cultured at 28 °C. They were grown on YPD (20 g/L dextrose, 20 g/L bacto-peptone and 10 g/L yeast extract) or MM (1.7 g/L yeast nitrogen base (YNB) without amino acids and ammonium sulfate, 10 g/L glucose, 5 g/L NH4Cl, 50 mM K2/Na3 phosphate buffer pH 6.0, and 7.7 g/L Complex Serum-free Medium (CSM); for selection of Ura+ and Leu+ transformants, 7.1 g/L CSM –ura or CSM –leu was added instead of CSM.

Standard Genetic Techniques

For transformation of Y. lipolytica, competent cells were prepared as described [51]. Briefly, cells were pretreated with lithium acetate and incubated with the DNA to be transformed together with salmon sperm carrier DNA. PEG 4000 was added, and after a heat shock at 42 °C, cells were plated on selective plates.

Genomic DNA was isolated using the MasterPure™ Yeast DNA Purification Kit according to the instructions of the manufacturer (Epicenter Biotechnologies). PCR amplification was performed in a volume of 50 μL containing 20 mM Tris-HCl pH 8.4, 50 mM KCl, different concentrations of MgCl2, 0.4 mM of dNTPs, 50 ng of template DNA, 50 pmol of primers, and 2.5 units of either Taq or Pfu DNA polymerase. Cycling conditions were as follows: denaturation at 94 °C for 15 min followed by hot start at 80 °C and 30 cycles of 94 °C for 45 s, suitable annealing temperature for 45 s, and extension at 72 °C for 1 min per kb, followed by 10 min of final extension at 72 °C.

DNA fragments in PCR reactions and those recovered from gels were purified using Nucleospin extract II (Macherey-Nagel).

Vector Construction

**Knocking out the ALG3 gene.** We used a knock-out strategy that makes use of the Cre-lox recombination system, which facilitates efficient marker rescue [52]. The genomic region upstream of the ALG3 ORF (GenBank Accession No: XM_500467; Genolevures: YALI0B03652g) was amplified from genomic DNA of Y. lipolytica MTLY60 by PCR with primers ALG3Pfw and ALG3Prv (Table 2) using Taq polymerase (Invitrogen, Carlsbad, CA, USA). The overhanging A was removed with T4 DNA polymerase (Fermentas, Burlington, Ontario, Canada). The genomic region downstream of the ALG3 ORF was amplified from genomic DNA of Y. lipolytica MTLY60 by PCR with primers ALG3Tfw and ALG3Trv (Table 2) using Pfu DNA polymerase (Fermentas). The presence of overlapping primer sequences containing I-SceI restriction sites allowed the linking of the fragments by PCR with primers ALG3Pfw and ALG3Prv using Taq polymerase. This co-amplification was then subcloned in pCR-2.1-TOPO-TA (Invitrogen, Carlsbad, CA, USA) and sequenced. It was then cloned between the NotI and Pdi sites in a derivative of pbGluScriptISK (Stratagene, Cedar Creek, Texas, USA) to yield pBLUYlalg3P3PT. Next, the URA3 selection marker flanked by lox sites originating from pKS-LPR-URA3 [52] (a gift from J.M. Nicaud, INRA) was inserted in the introduced I-SceI site between upstream and downstream regions, yielding pYlalg3P3PUT. Similarly, pYlalg3P3LT was constructed by exchanging the URA3 cassette in pYlalg3P3UT with the LEU2 selection marker from pKS-LPR-LEU2 be means of I-SceI digestion.

**Cloning the ALG6 gene.** The ORF (1725 bp) of ALG6 together with the 413-bp downstream region (GenBank Accession No: XM_500467; Genolevures: YALI0B03652g) was cloned from genomic DNA of Y. lipolytica MTLY60 by PCR with primers ALG6Pfw and ALG6Prv (Table 2) using Pfu DNA polymerase. The amplified fragment was cloned in pCR-Blunt-II-TOPO (Invitrogen) by PCR with primers TbGlucII fw and TbGlucII rv (Table 2) using Pfu DNA polymerase. The PCR fragment was cloned in pCR-Blunt-II-TOPO (Invitrogen, Carlsbad, CA, USA) and confirmed by Sanger sequencing. Next, it was cloned into pDONR201. The amplified fragment was cloned as a cassette in pYlalg3PUT with the LEU2 selection marker yielding pYLHmXXYYGIIa. To add the HDEL coding sequence to the ORF of the GII subunit of Y. lipolytica, a PCR was performed on the obtained plasmid pYLHmXXYYGIIa with primers YlGlicII fw and YlGlicII rv (Table 2) and the amplified fragment was cloned as described above for the version without HDEL tag.

**Cloning the GII alpha-subunit of Trypanosoma brucei with and without HDEL tag.** The ORF (2421 bp) of the GII α-subunit gene was amplified from genomic DNA of T. brucei (GenBank Accession No: AJ865333; a gift from Stijn Roge, Institute of Tropical Medicine, Antwerp) by PCR with primers TbGlucII fw and TbGlucII rv (Table 2) using Pfu DNA polymerase. The amplified fragment was cloned in pCR-Blunt-II-TOPO (Invitrogen, Carlsbad, CA, USA) and confirmed by sequencing. Next, it was subcloned into pDONR201. The amplified fragment was cloned in pCR-Blunt-II-TOPO (Invitrogen, Carlsbad, CA, USA) and confirmed by sequencing. Next, it was subcloned into pCR-Blunt-II-TOPO (Invitrogen, Carlsbad, CA, USA) and confirmed by sequencing. Next, it was subcloned into pDONR201. The amplified fragment was cloned as a cassette in pYlalg3PUT with the LEU2 selection marker for protein expression controlled by the hp4d promoter and the URA3 marker, yielding pYLHmXXYHΔGIIa. To add an HDEL tag to the T. brucei GII α-subunit, PCR was performed on the obtained plasmid with primers TbGlicII fw and TbGlicII rv (Table 2) and the amplified fragment was cloned in the same way as without HDEL tag.

**Cloning the GII beta-subunit of Y. lipolytica.** The ORF (1288 bp) of the GII β-subunit gene was cloned from genomic DNA of Y. lipolytica MTLY60 (GenBank Accession No: XM_500467; Genolevures: YALI0B03652g) by PCR with primers YlGlicII fw and YlGlicII rv (Table 2) and Pfu DNA polymerase. Two other vectors [pYLHII and pYLTL] carrying the LEU2 selection marker were constructed for protein expression controlled by the hp4d or TEF promoter, respectively. Next, the ORF of Y. lipolytica GII β-subunit was cloned in pYLHmXXYHΔGIIa in these vectors, yielding pYLHmLYGIIIB and pYLTLYGIIIB.

**Cloning the GII alpha-subunit of Aspergillus niger.** cDNA for a fusion of the ORF of the α-subunit of A. niger GII and an HDEL tag, flanked by SmalI and AvrII, was synthesized by Geneart AG (Regensburg, Germany). The sequence was codon-optimized for expression in Y. lipolytica. First, two intermediate vectors were constructed, pYLTXUL2pre and pYLUXUL2pre, by introducing the pre sequence of LIP2 in pYLHmXXY and pYLHmXXY. The latter was derived from pYLHmXXY by replacing the hp4d promoter by the TEF promoter. The introduction of the pre sequence of LIP2 was performed by annealing two primers (Table 2) and cloning them BamHI–AvrII in pYLHmXXY and pYLHmXXXY. The above-
Table 2. Primers used in this study.

| Primer name | Sequence (5’→3’) | Restriction site |
|-------------|------------------|------------------|
| ALG3fw | CAGTCGGCGCGCCTGCTCTTTCCTCATCAGAATGACCT | NotI |
| ALG3rv | CATTACCACTGTTAGACTCTGTTAGCTCGCC | XbaI |
| ALG3fw | GTAGGGATACAAGGGTTAATGCTCTCAAGGACGAGGACCAGGAAGACGAGACCTTAT | NotI |
| ALG3rv | GACCTAATTAACCATCTGAGCTGACATCGTACCCACATCTGTCGTC | XbaI |
| ALG6fw | CAGTGGATCGTACAGCTCTCATTAATGCTCC | BamHI |
| ALG6rv | GACCTAGAAGGCCCTAGATGTAAGGTGCTACT | AvrII |
| YlGlucHfw | GTCCGATTCTATGAAAGACGTTGAATGCGGGC | BamHI |
| YlGlucHrv | CTAGCCTAGTTAAGGAGACATGAGTGGCAAG | AvrII |
| TbGlucHfw | GTCCGATCTGATCAGTCTGTGCTAATGCGT | BamHI |
| TbGlucHrv | CTAGCCTAGTTAAGGAGACATGAGTGGCAAG | AvrII |
| YlGlucHDw | CTAGCCTAGTTAAGGAGACATGAGTGGCAAG | AvrII |
| YlGlucDfw | GTCCGATCTGATCAGTCTGTGCTAATGCGT | BamHI |
| YlGlucDrv | CTAGCCTAGTTAAGGAGACATGAGTGGCAAG | AvrII |
| LIP2prefw | GATGCTGAGGGGAAAGATGGGATGACGCTGTCCTCAGGTT | BamHI |
| LIP2prerv | CTAGCCTAGTTAAGGAGACATGAGTGGCAAG | AvrII |

Restriction sites in “ refer to overhanging parts of them.

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mentioned cDNA of the glucosidase α-subunit of A. niger flanked by SacII and AvrII was cloned in the corresponding restriction sites of pYLTUXL2pre and pYLHUXL2pre after SacII digestion + T4 DNA polymerase blunting and AvrII digestion. The resultant plasmids (pYLTUXL2preAnGlucIIa and pYLHUXL2-preAnGlucIIa, respectively) were confirmed by sequencing.

Cloning the GII beta-subunit of A. niger. The coding sequence for the β-subunit of A. niger GII flanked by Eco47III and AvrII restriction sites was synthesized by GeneArt AG (Regensburg, Germany) as cDNA codon-optimized for expression in Y. lipolytica. Two intermediate vectors (pYLTUXL2pre and pYLHUXL2pre) were constructed by introducing the pre sequence of LIP2 in pYLTL and pYLHLL, respectively, as described above. The resultant plasmids (pYLTUXL2preAnGlucIIa and pYLHUXL2-preAnGlucIIa, respectively) were confirmed by sequencing.

Cloning the Trichoderma reesei α,1,2-mannosidase with HDEL tag. We used an expression plasmid derived from pYLTUXL2preManHDEL [32] by digestion with I-SceI followed by replacement of the URA3 selection marker with the hygromycin selection marker (obtained from pKS-LPR-HYG, a gift from J.M. Nicaud, INRA) [52]. The resultant plasmid, pYLTTHygL2pre-ManHDEL, contains the T. reesei α,1,2-mannosidase coding sequence codon-optimized for Y. lipolytica, under control of a TEF promoter, preceded by the T. lipolytica LIP2 pre signal sequence, and C-terminally tagged with an HDEL retrieval sequence.

Selection marker rescue. In all plasmids, the selection marker cassette is flanked by loxP and loxR sites to facilitate marker rescue by transient overexpression of the Cre recombinase. For overexpression of Cre recombinase, we used pRRQ2 (a gift from J.M. Nicaud, INRA) [52], which expresses the enzyme under control of the hp4d promoter and carries the LEU2 resistance gene.

Preparation of Mannoproteins, N-glycan Analysis and Exoglycosidase Digests

Yeast strains were inoculated and grown overnight in 10 mL of standard YPD medium in 50 mL Falcon tubes rotating at 250 rpm in a 28°C incubator. The cells were then pelleted at 4000 rpm in a cooled Eppendorf 5010R centrifuge. The supernatants were removed, and the cells were first washed with 2 mL of 0.9% NaCl solution followed by two washes with 2 mL of water and subsequently resuspended in 1.5 mL of 0.02 M sodium citrate pH 7 in an Eppendorf tube. After autoclaving for 90 min at 121°C, they were vortexed and the cellular debris was spun down. Then the supernatants were collected and the mannoproteins were precipitated overnight with four volumes of methanol at 4°C on a rotating wheel. After centrifugation, the pellets were allowed to dry and then dissolved in 50 μL of water.

The whole 50 μL of the cell wall protein solution was used to prepare N-glycans labeled with 8-amino-1,3,6-trisulphonic acid (APTS) according to a published method [55]. Then, fluorophore-assisted carbohydrate electrophoresis (FACE) was performed with an ABI 3130 DNA sequencer.

For the exoglycosidase digests, one tenth of the prepared APTS-labeled N-glycans was used. Exoglycosidase treatment of APTS-labeled glycan was performed with a purified rat liver mixture of α and β (5 mU/mL, a gift from Dr. Terry Butters, Glycobiology Institute, Department of Biochemistry, Oxford, UK) [56]. Equal volumes of enzyme (in 80 mM triethylamine buffer, pH 7, containing 0.15 M NaCl and 10% glycerol) and sample were incubated together at 37°C overnight. The samples were then vacuum dried, resuspended in 10 μL of water, and analyzed on the ABI 3130 DNA sequencer.
PNGaseF Treatment of Glycoproteins

Proteins in the T. harzianum culture medium were precipitated with two volumes of ice-cold acetone. After incubation on ice for 20 min and centrifugation at 14,000 rpm for 5 min, the supernatant was removed and the protein pellet was resuspended in 100 μL of 50 mM Tris-HCl, pH 8. SDS and β-mercaptoethanol were added to a final concentration of 0.5% and 1%, respectively. Samples were incubated for 5 min at 100°C, after which G7 buffer (10× buffer, New England Biolabs), NP-40 (final concentration of 1%), complete protease inhibitor (Roche) and in-house produced PNGaseF (15 IU/BMB milliliters) were added. After overnight incubation at 37°C, proteins were precipitated by the deoxycholate/trichloroacetic acid (DOC/TCA) procedure, resuspended in 2× Laemmli buffer, and analyzed by SDS-PAGE.

In vitro Digestion with Trichoderma Harzianum Mutanase

T. harzianum mutanase Novozym 234, L1412 was obtained from Sigma-Aldrich Corporation, Spruce St., St. Louis, MO, USA. A stock solution of the enzyme (10 g/L) was prepared by dissolving 40 mg in 4 mL of 5 mM NH4Ac pH 5 buffer. Five serial five-fold dilutions were made, and the final dilution (0.2 μL) was used to treat 0.3 μL of APTS-labeled N-glycans in a total volume of 10 μL buffered to a final concentration of 50 mM NH4Ac pH 5. This reaction mixture was incubated overnight at 37°C and analyzed on an ABI 3130 DNA sequencer after desalting on a Sephadex G10 column [35].

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Author Contributions

Conceived and designed the experiments: KDP PT SG WV NC. Performed the experiments: KDP AVH. Analyzed the data: KDP PT NC. Wrote the paper: KDP.

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