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Investigating the role of ERAD on antibody processing in glycoengineered 
*Saccharomyces cerevisiae*

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Abstract

N-glycosylation plays an important role in the endoplasmic reticulum quality control (ERQC). N-glycan biosynthesis pathways have been engineered in yeasts and fungi to enable the production of therapeutic glycoproteins with human-compatible N-glycosylation, and some glycoengineering approaches alter the synthesis of the lipid-linked oligosaccharide (LLO). Because the effects of LLO engineering on ERQC are currently unknown, we characterized intracellular processing of IgG in glycoengineered Δalg3 Δalg11 Saccharomyces cerevisiae strain and analyzed how altered LLO structures affect endoplasmic reticulum-associated degradation (ERAD). Intracellular IgG light and heavy chain molecules expressed in Δalg3 Δalg11 strain are ERAD substrates and targeted to ERAD independently of Yos9p and Htm1p, whereas in the presence of ALG3 ERAD targeting is dependent of Yos9p but does not require Htm1p. Blocking of ERAD accumulated ER and post-Golgi forms of IgG and increased glycosylation of mα secretion signal but did not improve IgG secretion. Our results show ERA targeting of a heterologous glycoprotein in yeast, and suggest that proteins in the ER can be targeted to ERAD via other mechanisms than the Htm1p-Yos9p-dependent route when the LLO biosynthesis is altered.

Introduction

The endoplasmic reticulum (ER) is the starting point of the secretory pathway where protein folding takes place. Many chaperones and their cofactors assist the protein folding process in the ER by binding to hydrophobic regions of nascent proteins (Thibault and Ng 2012). However, some polypeptide molecules fail to reach their correctly folded state. Misfolding can be caused by mutations or environmental stress but it is also a common event in cells in normal conditions (Helenius and Aebi 2001). Accumulation of misfolded proteins can have detrimental effects on the viability and homeostasis of the cell (Powers et al. 2009; Geiler-Samerotte et al. 2011), and therefore cells need to be able to dispose of terminally misfolded proteins. To serve this purpose, ER contains a quality control system (ERQC) that assists protein folding, monitors the folding state of proteins, retains immature proteins in the ER, and targets terminally misfolded proteins to ER-associated protein degradation (ERAD).

A majority of secreted proteins receives an N-glycan at one or more N-glycosylation sites (Asn-X-Ser/Thr, X≠Pro) upon entering the ER lumen, and N-glycans play an important role in the ERQC system. After glycan transfer, two glucose residues from the Glc3Man9GlcNAc2 glycan
are trimmed by glucosidases I and II, forming Glc-Man\(_2\)GlcNAc\(_2\). In yeast, this glycan is recognized and bound by calnexin, a molecular chaperone that promotes folding and prevents aggregation and ER export of incompletely folded glycoproteins (Helenius and Aebi 2004). Further removal of the third glucose residue by glucosidase II causes dissociation of the protein from calnexin. Next, mannosidase MnsIp removes a mannose residue, creating Man\(_8\)GlcNAc\(_2\). The glycan trimming steps occur relatively slowly and they are thought to provide the protein a sufficient time window to obtain its native conformation. Further trimming of the glycan by mannosidase Htm1p (also called Mnl1p) reveals a terminal \(\alpha1-6\) linked mannose, which is a critical signal for targeting the glycoprotein to ERAD. This mannose signal is recognized by Yos9p, a lectin-like receptor that interacts with Hrd3p (Gauss et al. 2006). After a dual recognition of the glycan signal and misfolded regions, the protein is directed to translocation and ubiquitination by a protein complex consisting of Hrd1p, Hrd3p, Usa1p, Der1p and Yos9p (Carvalho, Goder and Rapoport 2006; Gauss et al. 2006; Quan et al. 2008). Finally, the protein is degraded in the cytosol by the proteasome.

In addition to its role in ERQC, N-glycosylation is an important posttranslational modification from the perspective of therapeutic protein production. N-glycosylation is very common in therapeutic proteins, and both the presence and structures of N-glycans can affect the activity, stability and immunogenicity of the product. The early steps of N-glycan biosynthesis and trimming that also play a role in ERQC are conserved among eukaryotes but the branches of the N-glycans are matured in a species-specific manner in the Golgi apparatus. As a result, all N-glycans share a common Man\(_8\)GlcNAc\(_2\) core that can contain a wide variety of modifications: N-glycans present in yeast proteins are often hypermannosylated, whereas mammalian N-glycans are of complex type and their antennae typically consist of N-acetylglucosamine, galactose and sialic acids.

The aim to produce antibodies and other therapeutic glycoproteins in non-mammalian hosts such as yeast has led to the engineering of their N-glycosylation pathways. An essential step in obtaining human-compatible N-glycosylation is to create suitable intermediate N-glycan structures that can act as substrates for mammalian glycosyltransferases. Formation of these intermediate structures in yeasts and fungi requires the elimination of certain mannose residues and can be obtained by several different approaches. Some approaches rely merely on modifying
the N-glycan maturation steps in the Golgi apparatus by the expression of mannosidases and
deletion of Golgi-localized mannosyltransferases, most importantly \textit{OCH1} (Hamilton \textit{et al.} 2003; Vervecken \textit{et al.} 2004; Tomimoto \textit{et al.} 2013). However, other glycoengineering approaches already target the biosynthesis of the lipid-linked oligosaccharide (LLO) in the ER. These approaches include the deletion of the \textit{ALG3} gene either alone or in combination with \textit{OCH1}, and expression of mannosidases in the ER or Golgi apparatus (Bobrowicz \textit{et al.} 2004; Kainz \textit{et al.} 2008; Cheon \textit{et al.} 2012; De Pourcq \textit{et al.} 2012). Another approach in \textit{Saccharomyces cerevisiae} significantly shortens the LLO biosynthesis by deletion of two ER mannosyltransferases, \textit{ALG3} and \textit{ALG11} (Parsaie Nasab \textit{et al.} 2013). This approach does not require glycan trimming in the Golgi apparatus since the truncated Man$_3$GlcNAc$_2$ structure can directly serve as an acceptor for human glycosyltransferases.

The possible effects of altered LLO biosynthesis from the perspective of recombinant protein production have not yet been studied (Piirainen \textit{et al.} 2014). The deletion of \textit{ALG3} and \textit{ALG11} genes prevents the formation of the native glycan intermediates that participate in ERQC. Deletion of \textit{ALG3} prevents the formation of the B and C branches that contain the substrate for Htm1p (Fig. 1). Instead, an alternative α1-6 linked mannose is present, which may directly act as a binding substrate for Yos9p without prior trimming by Ms1p and Htm1p (Quan \textit{et al.} 2008). In the absence of Alg11p, the A branch that interacts with calnexin is not formed (Absmanner \textit{et al.} 2010).

In this work, we investigated the possible effects of the Δ\textit{alg3} Δ\textit{alg11} glycoengineering approach on ER quality control and the role of ERAD in antibody processing and secretion in yeast \textit{S. cerevisiae}. ERAD has mainly been investigated using misfolding prone model proteins such as carboxypeptidase Y mutant CPY*(Finger, Knop and Wolf 1993), but we aimed to characterize the role of ERAD in a normally folding protein that is also relevant in terms of the applications of glycoengineered yeast. We therefore expressed full-length IgG in a Δ\textit{alg3} Δ\textit{alg11} \textit{S. cerevisiae} strain developed earlier and tested how disruptions in the ERAD pathway and compensation of \textit{ALG3} and \textit{ALG11} deletions affect intracellular protein processing and secretion by using a cellular clearance assay and by monitoring the processing of matα secretion signal.
Materials and methods

Generating yeast strains and plasmids

All yeast strains are derived from the wild-type strain SS328 and are listed in table 1. The strains generated in this study are based on YAF39 that lacks ALG3 and ALG11 genes (Parsaie Nasab et al. 2013). PEP4 and either of the ERAD genes YOS9, HTM1 and HRD1 were deleted by homologous recombination with a PCR product containing NatNT2 selection marker, loxP recombinase sites and flanking regions for the target genes (Hegemann and Heick 2011). PCR primers used to generate the deletion cassettes are listed in Table S1. Transformation was done by the lithium acetate method, and 100 µg/ml nourseothricin (Jena Bioscience, Jena, Germany) was used for selection. Prior to YOS9, HTM1 and HRD1 deletions, the NatNT2 selection marker was removed from PEP4 locus with Cre recombinase as described earlier (Hegemann and Heick 2011).

The ALG11 ORF was amplified with PCR from genomic DNA of S. cerevisiae strain BY4742 (ATCC® 201389™) with primers OMP68 and OMP69, digested with XbaI and XhoI and ligated into the XbaI/XhoI site of pRS415-TEF (Mumberg, Müller and Funk 1995), creating plasmid pMP24. The ALG3 ORF and 1 kb upstream region were amplified with PCR from genomic DNA of S. cerevisiae strain BY4742 with primers OMP86 and OMP67 and inserted into the SacI/XhoI site of pRS415-TEF (Mumberg, Müller and Funk 1995) with ELIC cloning (Koskela and Frey 2014), replacing the TEF1 promoter and creating plasmid pMP25. For the coexpression of ALG3 and ALG11 in a single plasmid, ALG11 expression cassette containing TEF1 promoter and CYC1 terminator was amplified by PCR from plasmid pMP24 with primers OMP95 and OMP96 and inserted into the EheI site of pMP25 with ELIC cloning, creating plasmid pMP28. Plasmid pAX538 that contains the heavy and light chain sequences of anti-HyHEL IgG fused with wild-type MATα pre- and propeptide sequences (pre-pro-HC and pre-pro-LC) has been described elsewhere (de Ruijter et al. 2018). Empty vectors pEK7 and pEK17 were used to complement for auxotrophies in strains where ALG3, ALG11 or IgG were not expressed, and these vectors have been described elsewhere (de Ruijter, Koskela and Frey 2016).

Growth of yeast strains

All yeast strains were grown at 28 °C. Empty yeast strains were grown in YPD medium supplemented with 0.2 M sorbitol. Yeast strains containing plasmids were grown in selective SD
medium lacking uracil and leucine and supplemented with 0.2 M sorbitol. For IgG expression, strains were grown in SD -Ura -Leu medium supplemented with 0.2 M sorbitol, 0.2 M sodium phosphate buffer, pH 6.5 and 50 mg l⁻¹ bovine serum albumin (BSA), using 2 % raffinose as a carbon source. IgG expression was induced with 2 % galactose.

**Growth assays**

Growth phenotypes were tested with a spotting assay and by measuring growth curves in liquid medium. For both assays, 3 ml precultures of strains containing the IgG expression vector were grown in selective SD medium in 24 well plates for 32-48 h. For spotting assay, cultures were diluted to OD₆₀₀ 0.1, and 4 µl of 1:5 serially diluted cultures were spotted on selective SD agar plates with and without 0.2 M sorbitol. Plates were incubated at 28 °C or 37 °C for 4 days. Growth curves were measured in a 96 well plate format. Precultures were diluted to OD₆₀₀ of 0.1 in IgG expression medium without BSA with 2 % galactose, and 100 µl of cultures were incubated in a 96 well round bottom plate at 28 °C with shaking in linear mode in Cytation 3 multi-mode reader (Bio-Tek, Winooski, VT, USA). OD₆₀₀ was measured every 15 minutes for 92 hours.

**ER clearance assay**

Cellular clearance assay was based on an earlier study (de Ruijter and Frey 2015). IgG expression was induced in exponentially growing yeast cultures with 2 % galactose when OD₆₀₀ 0.7-1.0 was reached. After three hours of expression, IgG expression was quenched by adding 2 % glucose. Two OD₆₀₀ units of cells were collected by centrifugation (5 min 9000 g) at 0, 1, 2 and 3 hours after the glucose addition.

Cell extracts were prepared as described earlier (de Ruijter and Frey 2015) with minor modifications. Two OD₆₀₀ units of cells was used per sample, cell lysis and washing of the glass beads were performed with 200 µl of lysis buffer, and samples were resuspended in 50 µl of SDS-PAGE sample buffer (62.5 mM Tris-HCl pH 6.8, 2 % SDS, 10 % glycerol, 50 mM DTT and 0.005 % bromophenol blue). For deglycosylation, the lysed cell pellet was denatured and treated with PNGase F or Endo H (New England Biolabs, Ipswich, MA, USA) according to manufacturer’s instructions.
Western blotting was performed for 10 µl of samples, corresponding to 0.4 OD$_{600}$ units of cells. Samples were run on a 12.5 % SDS-PAGE gel and transferred onto a nitrocellulose membrane. Membranes were stained with Ponceau S (0.1 % Ponceau S, 1 % acetic acid) after transfer. IgG heavy chain was detected with 1:4000 dilution of peroxidase conjugated anti-human IgG (Fc specific) antibody produced in goat (Sigma-Aldrich, St. Louis, MO, USA), and IgG light chain was detected with 1:10 000 dilution of peroxidase conjugated anti-human kappa light chains (bound and free) antibody produced in goat (Sigma-Aldrich). Signals were detected with Supersignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA).

Signals were quantified with Image Studio Lite (Li-Cor, Lincoln, NE, USA). To account for variation in sample loading and transfer efficiency, IgG heavy and light chain signals were corrected to Ponceau S stained total protein in the molecular weight range of 25-75 kDa. For each time point, the corrected IgG heavy and light chain signals were normalized to the corresponding IgG signal at 0h.

Because biomass growth occurred during the clearance assay, the increase in biomass after quenching IgG expression caused dilution of the IgG signals also without any protein degradation or secretion. In order to correct for this IgG signal reduction caused by growth, we calculated the proportion of “pre-glucose” biomass in the samples by dividing the OD$_{600}$ values at each time point with the OD$_{600}$ values at the time point of glucose addition. IgG signal decrease caused by degradation and secretion was then calculated by dividing the normalized IgG signals with the amount of “pre-glucose” biomass in the sample.

**IgG production**

A small-scale IgG production cultivation was performed in a 24 well plate format in 3 ml culture volume. 3 ml precultures were grown in selective SD medium in 24 well plates for 32-48 h. Cultures were diluted to OD$_{600}$ of 1.0, and IgG expression was induced with 2 % galactose for 18h. Culture supernatants were adjusted to 1x PBT buffer, and the IgG concentrations were analyzed by ELISA as described earlier (de Ruijter and Frey 2015).
Results

Reconstitution of the LLO biosynthesis in Δalg3 Δalg11 yeast strain
In order to characterize the role of altered N-glycan structures for ERAD in IgG-expressing glycoengineered yeast, we created a set of strains differing in the LLO biosynthesis and combined them with deletions of ERAD components. S. cerevisiae strain YMP28 was used as a basis for the strain set. Strain YMP28 contains the deletions of ALG3, ALG11 and PEP4 genes and its progenitor has been UV-mutagenized and selected for improved growth. In addition, the strain contains an oligosaccharyltransferase (OST) from Leishmania braziliensis and the artificial flippase Flc2*p that have more flexible glycan acceptor requirements than the endogenous OST complex and flippase of yeast and therefore compensate for the lowered glycosylation efficiency caused by ALG3 and ALG11 deletions (Parsaie Nasab et al. 2013). YMP28 generates a truncated Man₃GlcNAc₂ LLO lacking the canonical ERAD glycan signal that sequentially interacts with Htm1p and Yos9p, and contains a terminal α1-6 linked mannose instead of the B and C branches. N-glycans containing this α1-6 mannose can be bound by Yos9p (Szathmary et al. 2005; Quan et al. 2008) and do not require prior mannosidase trimming for ERAD targeting (Clerc et al. 2009; Xie et al. 2009; Gauss et al. 2011). We restored the formation of the native B and C branches and thus the glycan timer mechanism to this strain by plasmid-based expression of ALG3. In addition, a strain with native LLO biosynthesis was reconstructed by plasmid-based expression of both ALG3 and ALG11. In order to study the effect of disruptions in the ERAD pathway in these three LLO variant strains, we introduced deletions of HTM1, YOS9 and HRD1 genes. This strain set allowed us to compare the effects of ERAD deletions on antibody production with and without the canonical ERAD glycan signals.

In order to verify that the plasmid-based expression system for ALG3 and ALG11 corresponds sufficiently to the expression of ALG3 and ALG11 in wild-type yeast, we isolated LLOs from the three LLO variant strains and analyzed them with MALDI-TOF (Fig. S1). YMP28 (Δalg3 Δalg11) contained mainly Man₃GlcNAc₂ LLOs and no larger LLOs were detected. When ALG3 was expressed, the predominant LLO signal remained as Man₃GlcNAc₂, corresponding to the structure formed on the cytoplasmic side of the ER. In addition, small amounts of Man₆GlcNAc₂ and Man₇GlcNAc₂ LLOs were detected, indicating the formation of B and C branches. Same LLOs were also detected in a Δalg11 control strain as well as in earlier results obtained from [³H]-
mannose labeled samples (Helenius et al. 2002). To identify which LLOs were transferred to
proteins in the strain expressing ALG3, we also analyzed N-linked glycans isolated from whole
cell protein extracts with MALDI-TOF and tested the sensitivity of intracellular glycoproteins to
endoglycosidase H, which only cleaves high-mannose or hybrid-type glycans (Trimble and
Tarentino 1991). Man₆GlcNAc₂ and Man₇GlcNAc₂ were the predominant N-linked glycans found
in this strain (Fig. S1B). In addition, N-glycosylated IgG molecules expressed in this strain were
sensitive to Endo H cleavage, whereas without the expression of ALG3 the glycoproteins were
Endo H resistant (Fig. S2). These findings support the conclusion that episomal expression of
ALG3 restored the formation of the B and C branches in N-glycans. As a next step towards
reconstituting the LLO biosynthesis, ALG11 was expressed in YMP28. As a result, Man₅GlcNAc₂
LLO was predominantly formed, corresponding to the synthesis of the A branch consistently with
a ∆alg3 control strain and LLO structures reported earlier (Sharma, Knauer and Lehle 2001).
Next, we combined the expression of ALG3 and ALG11 to fully regenerate the native LLO
biosynthesis. When ALG3 and ALG11 were coexpressed, LLO structures up to Hex₁₂GlcNAc₂
were detected but at different relative abundances than in wild-type yeast. An α-mannosidase
digestion of the LLOs revealed that mono- and diglucosylated LLOs were the most abundant
structures in the strain with reconstituted LLO biosynthesis. Even though protozoan OSTs are able
to transfer also truncated LLOs to proteins, based on the relative abundances of the incomplete
LLO structures it is fair to assume that a majority of N-glycans in the reconstructed strain with
ALG3 and ALG11 coexpression contains the sugar residues required for interaction with calnexin
and Yos9p, enabling normal ERQC. Thus, plasmid-based expression of ALG3 and ALG11 genes
sufficiently restored the LLO biosynthesis.

Modifications in LLO biosynthesis and deletion of HRD1 affect growth
Sensitivity of our strain set to temperature and osmolarity was tested with a spotting assay. Strains
were grown in 28 °C and 37 °C in SD medium with and without the supplementation of sorbitol
as an osmotic stabilizer (Fig. 2). Growth was also monitored in liquid cultures in inducing
conditions (Fig. 3). Overall, modifications on the LLO biosynthesis had a stronger effect on
growth than ERAD deletions. Expression of ALG11 had an impact on temperature tolerance, as
has been reported earlier (Cipollo et al. 2001). At an elevated temperature of 37 °C, strains that
did not contain a functional ALG11 gene failed to grow without the supplementation of sorbitol,
and addition of an osmotic stabilizer partially restored growth. In contrast, the expression of ALG3
affected growth negatively in both solid and liquid cultures, and coexpression of \textit{ALG11} with 
\textit{ALG3} compensated for this growth defect. \textit{HRD1} deletion was the only ERAD modification that 
had an impact on growth phenotypes: \textit{Δhrd1} strains displayed weaker growth than other ERAD 
versions, as seen in both elevated temperature and IgG-expressing conditions. Strains lacking both 
\textit{ALG11} and \textit{HRD1} failed to grow completely at elevated temperature even in the presence of 
sorbitol.

\textbf{Processing of IgG polypeptides is affected by modifications in LLO biosynthesis and ERAD}

In order to follow both the clearance and processing of IgG in the secretory pathway of our strain 
set, we expressed IgG heavy and light chains with \textit{mata} secretion signal, which drives 
posttranslational translocation (Rothblatt \textit{et al.} 1987; Rapoport \textit{et al.} 1998). The secretion signal 
contains a 19-amino acid pre region which is cleaved as the polypeptide is translocated into the 
ER, and a 64-amino acid pro region that contains three N-glycosylation sites and is cleaved in the 
late Golgi apparatus by Kex2p before secreting the target protein (Caplan \textit{et al.} 1991). Due to 
these processing steps of the pre-pro region, we could gain information on the subcellular location 
of the emerging IgG molecules in the secretory pathway and discriminate between IgG 
polypeptides residing in the ER or early secretory pathway and post-Golgi IgG molecules. Figure 
4 shows representative Western blot data of the intracellular IgG heavy and light chain signals of 
our strain set after three hours of IgG expression. Signals corresponding to the mature cleaved 
form of heavy and light chains as well as both nonglycosylated and glycosylated pro-LC and pro-
HC forms were detected, as confirmed by a PNGase F digestion of the samples (Fig. 4C and 4D). 
In addition, a varying extent of N-glycosylation in the pro region of the light chains could be 
distinguished (Fig. 4B). Even though the pro region contains three N-glycosylation sites (Caplan 
\textit{et al.} 1991), we only detected two different glycosylated forms of pro-LC in addition to the 
nonglycosylated form. Although the number of N-glycans in these forms could not be confirmed, 
we refer to the two N-glycosylated signals with higher and lower molecular weight as fully and 
partially glycosylated forms, respectively.

The total amounts and forms of intracellularly accumulated IgG were affected by the disruption of 
ERAD: the deletion of \textit{HRD1} increased the accumulation of pro-HC in \textit{Δalg3 Δalg11} strain and in 
the \textit{ALG3}-expressing LLO variant strain but not when \textit{ALG3} and \textit{ALG11} were coexpressed. 
Additionally, the deletion of \textit{YOS9} increased pro-HC accumulation in the \textit{ALG3}-expressing LLO
variant strain. Furthermore, YOS9 and HRD1 deletions increased the intracellular amount of the mature heavy chain form in all three LLO variant strains. A similar but weaker trend also recurred for light chain signals, where the proportion of the mature form was higher than in heavy chain.

The presence of Hrd1p also had an impact on the N-glycosylation site occupancy, as the proportion of fully glycosylated pro-LC increased upon HRD1 deletion (Fig. 4B and Fig. S3). In strains that did not express ALG11 the relative amount of partially glycosylated pro-LC decreased correspondingly, while in the LLO variant strain coexpressing ALG3 and ALG11 a corresponding decrease of nonglycosylated pro-LC took place. In the strain coexpressing ALG3 and ALG11, increased N-glycosylation site occupancy caused by HRD1 deletion was also seen in pro-HC. This LLO variant strain generates larger N-glycans than the other two LLO variant strains, providing sufficient resolution between nonglycosylated and glycosylated pro-HC forms.

In addition to the HRD1 deletion, the expression of ALG3 and ALG11 affected the glycosylation site occupancy of the pro region, as each LLO variant strain showed a distinct glycosylation pattern of pro-LC (Fig. 4B and Fig. S3). In Δalg3 Δalg11 genetic background the partially glycosylated form was predominant, contributing approximately to half of the total pro-LC signal, while nonglycosylated and fully glycosylated forms were equally abundant. When ALG3 was expressed, the amounts of fully and partially glycosylated forms were equally high and only a minor fraction of pro-LC was nonglycosylated. In contrast, the coexpression of ALG3 and ALG11 caused significant underglycosylation as the majority of pro-LC was nonglycosylated, and underglycosylation of the heavy chain was also detected. In addition, the mature HC form in the LLO variant strain containing ALG3 and ALG11 consisted of a mixture of glycosylated and nonglycosylated forms (Fig. 4C).

**Role of ERAD in IgG production depends on presence and structure of N-glycans**

In order to investigate the role of ERAD in antibody production in our glycoengineered yeast strain in more detail, we followed the rate of IgG heavy and light chain clearance from the cell using a cellular clearance assay developed earlier (de Ruijter and Frey 2015). The assay utilizes the ability of glucose to repress gene expression driven by GAL1 promoter. In contrast to classical clearance assays based on cycloheximide treatment, bulk protein flow is not affected in our assay.
IgG production was induced with galactose for three hours and then quenched by the addition of glucose.

We first aimed to confirm that glucose addition efficiently stops the translation of new IgG polypeptides. We measured the mRNA levels of IgG heavy and light chains prior to and during their expression and after the glucose addition in all three LLO variant strains with intact ERAD and in Δalg3 Δalg11 strain with HRD1 deletion using RT-qPCR (Fig. S4). After the addition of glucose, IgG mRNA was rapidly degraded: heavy chain mRNA reached pre-induction levels already in 30 minutes after the glucose addition, while light chain mRNA was degraded somewhat slower but reached very low levels (less than 5% of the levels during induction) within one hour after the glucose addition. We could therefore establish that essentially all IgG polypeptides detected in our clearance assay originate from IgG gene expression during galactose induction and no residual gene expression takes place after the addition of glucose. Additionally, IgG expression levels did not vary significantly between strains during induction (data not shown). Consequently, we can presume that the observed changes in IgG polypeptide amounts in our clearance assay are a result of protein secretion, degradation and dilution from cellular growth.

To characterize the clearance rates of intracellular IgG heavy and light chains with a focus on the early secretory pathway, we quantified the pro-HC and pro-LC signals obtained from a clearance assay with a monitoring time of 3 hours. Representative Western blots from a clearance assay of Δalg3 Δalg11 LLO variant strains are shown in Figure 5, where the pro-HC and pro-LC signal decrease can be seen. Clearance curves shown in Figure 6 represent pro-HC and pro-LC signals normalized to total protein and the corresponding signal before the repression of IgG expression and corrected for the signal decrease caused by cellular growth. Except for strains where the signal decrease was negligible, clearance curves mostly followed first order decay kinetics ($R^2 > 0.9$, $p < 0.05$). The clearance constants and clearance half-lives of all strains are listed in Table S2.

First, we tested if the IgG expressed in yeast is an ERAD substrate and to which extent. From the selected ERAD genes, YOS9 and HTM1 encode proteins that more specifically interact with N-glycans of misfolded proteins, whereas HRD1 encodes a central component of the complex that is responsible for ubiquitination and retrotranslocation of aberrant ER luminal and transmembrane proteins and is also involved in the degradation of certain nonglycosylated proteins (Kanehara,
Xie and Ng 2010). Delayed IgG clearance caused by HRD1 deletion therefore indicates that targeting to proteasomal degradation is a substantial route for intracellular IgG processing.

HRD1 deletion had a very clear impact on IgG clearance in the Δalg3 Δalg11 genetic background, essentially stopping all clearance of heavy chain and somewhat lowering the clearance rate of light chain (Fig. 6A and 6D). HRD1 deletion also delayed the clearance of heavy and light chains in the ALG3-expressing LLO variant strain (Fig. 6B and 6E). Surprisingly, the deletion of HRD1 or other ERAD components had no impact on IgG clearance when ALG3 and ALG11 were coexpressed (Fig. 6C and 6F). In addition, IgG clearance in this LLO variant strain was delayed in comparison with strains lacking the ALG11 gene (Table S2). Therefore, intracellular IgG polypeptides are cleared by ERAD in Δalg3 Δalg11 and ALG3-expressing LLO variant strains, but not in the presence of both ALG3 and ALG11.

Next, we investigated more closely the glycan-specific parts of ERAD in the strains that were affected by HRD1 deletion. As expected, IgG clearance remained unchanged upon HTM1 deletion in Δalg3 Δalg11 strain that does not form the substrate for Htm1p. However, the deletion of HTM1 also had no impact on IgG clearance in the ALG3-expressing LLO variant strain that can form N-glycans with intact B and C branches. Thus, Htm1p activity is not required for ERAD of IgG in our yeast strains even if the substrate glycan for Htm1p would be available.

Interestingly, YOS9 deletion had no effect on the IgG clearance rate in Δalg3 Δalg11 strain even though N-glycans formed in this strain contain a “non-canonical” terminal α1-6 mannose that can be recognized by Yos9p in vitro (Quan et al. 2008). However, when the B and C branches were reintroduced by the expression of ALG3, the deletion of YOS9 decelerated IgG clearance in a similar extent to HRD1 deletion. Therefore, Hrd1p-dependent degradation of IgG in Δalg3 Δalg11 strain is independent of both Htm1p and Yos9p, whereas when the canonical glycan timer signal is reconstituted, ERAD of IgG is dependent on Yos9p but does not require Htm1p activity.

**Modifications in LLO biosynthesis affect IgG secretion**

Intracellular proteins can be cleared from the secretory pathway by two routes: degradation and secretion. To estimate the secretory capacity of our strain set and to test whether the deletion of ERAD components has an impact on IgG secretion, we analyzed the antibody concentrations in culture supernatants in a small-scale IgG production experiment. In Figure 7, IgG concentrations
in culture supernatant normalized to final OD_{600} after 18 hours of expression are shown. Expression of *ALG3* led to slightly increased IgG secretion, whereas IgG levels were significantly lowered upon the coexpression of *ALG3* and *ALG11*. ERAD deletions caused no significant effects on secretion although increased IgG levels upon *HRD1* deletion were observed in the LLO variant strain coexpressing *ALG3* and *ALG11*.

**Discussion**

In order to enable the efficient production of human-compatible glycoproteins in glycoengineered yeast, it is necessary to understand the processing of the target protein in the secretory pathway. ERAD is an integral part of a protein quality control system that relies on N-glycan signals. However, the role and function of ERAD in glycoengineered yeast that skips native N-glycan processing and instead forms a truncated Man$_3$GlcNAc$_2$ structure are unknown. In addition, ERAD has mostly been studied using folding defective model proteins such as a CPY* (Finger, Knop and Wolf 1993). In the context of recombinant protein production, a target protein that can fold correctly but might challenge the processing capacity of the ER could give a better representation of the cellular events and the role of ERAD. Because production of full-length antibodies is both a challenging task for a yeast cell and a potential application for glycoengineered yeast, we selected IgG as a target protein.

In order to gain a better understanding on the intracellular processing of IgG produced in a glycoengineered yeast strain with *ALG3* and *ALG11* deletions, we measured how deletions of ERAD components affect the intracellular clearance, processing and secretion of IgG. We found that IgG heavy and light chains are ERAD substrates in Δalg3 Δalg11 strain but ERAD targeting is independent of Yos9p and Htm1p. Since the Man$_3$GlcNAc$_2$ glycan does not contain a substrate for Htm1p, the dispensability of Htm1p for IgG processing was anticipated. However, the finding that neither Yos9p was required for the ERAD targeting of IgG was unexpected. ERAD targeting in Δalg3 Δalg11 yeast strain has not been studied earlier in vivo, but based on studies in Δalg3 mutant strains, the lack of B and C branches should not inhibit the binding of Yos9p to a glycan and a terminal α1-6 mannose residue can be recognized by Yos9p regardless of its location in the N-glycan (Szathmary et al. 2005; Clerc et al. 2009; Xie et al. 2009; Zhang et al. 2017). In addition, an in vitro study revealed that Yos9p binds to Man$_3$GlcNAc$_2$ with a similar affinity as to
native ERAD signal structures (Quan et al. 2008). We therefore expected that the Δalg3 Δalg11 strain merely lacks the glycan timer mechanism, potentially causing a premature ERAD targeting of proteins. However, the degradation of intracellular IgG in this glycoengineered yeast strain does not follow the typical Yos9p-mediated and N-glycan-dependent ERAD-L pathway.

If Yos9p-mediated ERAD targeting does not take place in Δalg3 Δalg11 strain, how are intracellular IgG molecules targeted to ERAD? Yos9p-dependent ERAD is not the only possible mechanism for the degradation of ER luminal proteins. A fraction of the HRD complex does not contain Yos9p, and Hrd1p-dependent ubiquitination can also take place without Yos9p (Gauss et al. 2006). Furthermore, a nonglycosylated deletion variant of a folding-defective proteinase A mutant can be targeted to ERAD independently of Yos9p (Kanehara, Xie and Ng 2010). In addition, findings on cytosolic free oligosaccharides suggest that also many glycoproteins might be degraded independently of the Htm1p-Yos9p directed ERAD: structures containing a terminal α1-6 mannose only compose a minor fraction of the oligosaccharide pool originating mostly from ERAD-targeted glycoproteins (Hirayama et al. 2010). However, no ERAD targeting took place in the ALG3 and ALG11-coexpressing LLO variant strain where a majority of IgG molecules was nonglycosylated, suggesting that the presence of an N-glycan is required for ERAD targeting of IgG even though Man3GlcNAc2 does not directly act as an ERAD signal for Yos9p. Therefore, a completely glycan-independent ERAD targeting mechanism does not seem likely. Elucidating the precise mechanism of ERAD targeting in Δalg3 Δalg11 genetic background requires further research.

Interestingly, when the B and C branches were restored by the expression of ALG3, IgG remained as an ERAD substrate but this time Yos9p was required for ERAD targeting. Thus, it seems that IgG can be cleared via either a Yos9p-dependent or a Yos9p-independent route, depending on the presence of the B and C branches. However, since both Δalg3 Δalg11 strain and the ALG3-expressing LLO variant strain can present a terminal α1-6 mannose signal it is rather odd that only one of these strains displays Yos9p-dependent ERAD. The Man5GlcNAc2 glycan lacking two mannose residues from the A branch has an increased affinity to Yos9p in vitro compared with Man3GlcNAc2 or N-glycans found in wild-type yeast (Quan et al. 2008). The reason for the enhanced affinity of Yos9p to the Man5GlcNAc2 structure is unclear, as this glycan has not been found in wild-type yeast. However, Man5GlcNAc2 can form in the ALG3-expressing LLO variant
strain after α1-2 mannosidase processing of the B and C branches. Perhaps the increased affinity of Yos9p to this Man$_5$GlcNAc$_2$ structure can explain why Yos9p participates in ERAD targeting in the LLO variant strain that contains ALG3 but not in $\Delta$alg3 $\Delta$alg11 strain. Another factor that could potentially affect the function of ERAD components is their own N-glycosylation, as both Yos9p and Htm1p are glycoproteins (Friedmann et al. 2002; Sakoh-Nakatogawa, Nishikawa and Endo 2009). Thus far, the importance of glycosylation for these proteins has not been studied, but it may be possible that altered N-glycosylation of Yos9p and Htm1p in our glycoengineered strains would affect the folding or stability of these ERAD components, thereby affecting the function of the ERAD system.

Another surprising finding in the LLO variant strain with restored B and C branches was that Yos9p-dependent ERAD targeting was independent of Htm1p. According to the current model for glycoprotein ERAD, the cleavage of an α1-2 mannose from the C branch by Htm1p is required for recognition by Yos9p and for subsequent ERAD targeting of a misfolded glycoprotein. Our results are contradicting to this model and various earlier reports, where the deletion of HTM1 caused a delayed clearance of N-glycosylated ERAD substrates CPY* and Pdr5* (Quan et al. 2008; Clerc et al. 2009; Martinez Benitez et al. 2011; Liu, Fujimori and Weissman 2016; Pfeiffer et al. 2016). However, the overexpression of HTM1 has no effect on the clearance of normally folding glycoproteins whereas it increases the clearance rate of ERAD substrates CPY* and Erg3-Myc (Pfeiffer et al. 2016), suggesting that the importance of Htm1p for ERAD targeting can differ for misfolding prone model proteins and native proteins. Furthermore, earlier experiments conducted in our group showed that HTM1 deletion does not affect IgG clearance rate significantly in wild-type yeast (de Ruijter and Frey 2015).

As confirmed by MALDI-TOF analysis, the B and C branches of N-glycans are formed normally upon episomal expression of ALG3. Thus, no binding substrate for Yos9p should be available without prior α1-2 mannosidase activity. Since the binding specificity of Yos9p to a terminal α1-6 linked mannose is quite well established, it is unlikely that another sugar residue could act as a binding substrate for Yos9p in the absence of a terminal α1-6 mannose. Yos9p can also interact with ERAD client proteins independently of its lectin function (Bhamidipati et al. 2005; Denic, Quan and Weissman 2006; Izawa et al. 2012). However, as Yos9p was dispensable for ERAD targeting in $\Delta$alg3 $\Delta$alg11 strain, the role of Yos9p in ERAD targeting of IgG in the presence of
ALG3 seems to be linked to the B and C branches of N-glycans. How Yos9p can participate in ERAD targeting without Htm1p is therefore an interesting question. One possible explanation could be an overlapping activity of another ER mannosidase with Htm1p, and evidence suggesting the presence of additional mannosidase activities in the ER exists. Apart from Htm1p, Mns1p is the only ER protein with confirmed α1-2 mannosidase activity. In addition to processing the B branch, Mns1p is also able to cleave α1-2 mannose from the C branch in vitro in a prolonged incubation (Herscovics, Romero and Tremblay 2002), making it a potential candidate to compensate for the loss of Htm1p activity although this activity has not been proven in vivo. In addition, a putative α1-2 mannosidase Mnl2p has been found in the ER (Martinez Benitez et al. 2011). Mnl2p partially compensates the role of Htm1p in its absence and it has been proposed to play a role together with Htm1p in generating the ER degradation signal (Martinez Benitez et al. 2011). Given that IgG is not an innately misfolding protein and presumably targeted for ERAD in a much smaller extent than mutant ERAD model substrates, even a relatively low mannosidase activity could be sufficient to compensate for the loss of Htm1p in our experimental setup. A low overlapping activity of Mnl2p with Htm1p could offer an alternative explanation to its partial compensating effect on the clearance of CPY* in the absence of Htm1p (Martinez Benitez et al. 2011).

When the complete LLO biosynthesis was reconstructed by the coexpression of ALG3 and ALG11, no ERAD targeting of IgG was observed during the clearance assay. The N-glycosylation site occupancy of IgG molecules was also low, and the cause for underglycosylation in this strain is unclear. Transfer of the reconstituted LLOs to proteins should not be hindered because strain YMP28 contains the yeast native OST in addition to the OST from L. braziliensis and Flc2*p that are more flexible in their substrate specificities. Nevertheless, due to the low N-glycosylation efficiency of the IgG molecule glycan-dependent ERAD cannot take place in a significant extent this strain. As no ERAD was observed, N-glycosylation seems to be essential for the ERAD targeting of IgG molecules. N-glycosylation site occupancy of the heavy chain itself is not strictly required for the correct folding and secretion of IgG, as aglycosylated IgG molecules can be successfully produced (Rakestraw et al. 2009). However, N-glycosylation of the pro region can be more critical to secretory processing, as lack of N-glycosylation in the pro region delays clearance and decreases the secretion of mata (Caplan et al. 1991). When HRD1 was deleted in this LLO variant strain, we observed an increase both in IgG secretion and in N-glycosylation site
occupancy of pro-HC and pro-LC. We therefore suspect that the low N-glycosylation site occupancy of IgG in the LLO variant strain coexpressing ALG3 and ALG11 not only causes lack of ERAD targeting but also reduced secretion.

HRD1 deletion had a negative impact on growth in all LLO variant strains even though HRD1 deletion did not affect IgG clearance when ALG3 and ALG11 were coexpressed. Even though the effects of this gene were not seen in IgG clearance due to underglycosylation of the pro region, HRD1 may still have an important function for the cell, possibly via non-ERAD related indirect effects. Another interesting effect of HRD1 deletion was an increase in the amount of fully glycosylated pro-LC and pro-HC. This effect is probably not a direct consequence of the blocked retrotranslocation and ubiquitination but might rather reflect an unknown effect of HRD1 on the N-glycan transfer process. Human HRD1 has been shown to affect N-glycosylation efficiency in mammalian cells where its expression inhibits N-glycosylation of posttranslationally glycosylated ABCG5/8 transporters in a mechanism that is independent of the E3 ubiquitin ligase activity of HRD1 (Suzuki et al. 2014). We speculate that perhaps yeast Hrd1p could similarly to its human homologue have an inhibitory effect on N-glycosylation for certain proteins.

From the perspective of recombinant protein production, ER presents a possible bottleneck in the secretory pathway. Although ERAD has an important role for the quality control of produced proteins, nonoptimal ERAD function might also have a negative impact on the secretion of potentially slowly folding proteins. Pfeffer et al. found that more than half of intracellularly produced recombinant protein in Pichia pastoris is degraded, raising a possibility that excessive protein degradation might limit protein secretion (Pfeffer et al. 2012). Effect of ERAD deletions on the production of antibodies or antibody fragments has been tested in S. cerevisiae and P. pastoris (de Ruijter and Frey 2015; Zahr, Mattanovich and Gasser 2018). In both organisms, the deletion of ERAD components has mainly negative effects on target protein secretion. Also in our experiments, ERAD disruptions caused no clear changes on secretion with the exception of the somewhat increased secretion caused by HRD1 deletion in the LLO variant strain containing ALG3 and ALG11. However, this effect is more likely linked to N-glycosylation site occupancy than to changes in ERAD, as discussed above. The ERAD mutations that decreased IgG clearance also increased the accumulation of intracellular pro-HC. Thus, other processes in the ER or early Golgi apparatus seem to limit IgG secretion rather than ERAD.
In conclusion, our results show that antibody molecules can be targeted to ERAD by at least two different mechanisms in yeast strains where the LLO biosynthesis has been modified and that the participation of Yos9p to ERAD targeting depends on the presence of intact N-glycan B and C branches. These findings reflect the notion that ERAD seems to be a more complex process than the current model suggests and perhaps different mechanisms of ERAD targeting can dominate depending on the target protein, N-glycan structure and glycosylation efficiency.

**Funding**

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**Acknowledgements**

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**References**


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Figure 1. Schematic representation of the LLO structures that are transferred to proteins in the ER by different LLO variant strains. LLO formed in wild-type yeast, showing the nomenclature of A, B and C branches, in Δalg3 Δalg11 genetic background, and in Δalg11 genetic background. Blue squares, N-acetylglucosamine; green circles, mannose; blue circles, glucose.

Figure 2. Growth phenotypes of the ERAD mutant and LLO variant strain set in solid growth medium. The strain set consists of glycoengineered Δalg3 Δalg11 strain YMP28 (control) with Δyos9, Δhtm1 or Δhrd1 deletions, an antibody expression vector and an empty vector (Δalg3 Δalg11), expression vector for ALG3 (+ALG3) or coexpression vector for ALG3 and ALG11 (+ALG3 +ALG11). Cultures were serially diluted on selective SD medium with and without sorbitol supplementation and grown at 28 or 37 °C for four days.
Figure 3. Growth of ERAD mutant and LLO variant strain set during antibody expression.

OD$_{600}$ was measured during growth at 28 °C in buffered selective SD medium containing 2% galactose in a 96 well plate format. Growth of ERAD mutants in Δalg3 Δalg11 genetic background (A), with the expression of ALG3 (B) and with the coexpression of ALG3 and ALG11 (C). Growth of LLO variant strains without ERAD deletions (D), with Δyos9 deletion (E), with Δhtm1 deletion (F) and with Δhrd1 deletion (G). Data represent mean of three biological replicates.
Figure 4. Intracellular IgG signals of the ERAD mutant and LLO variant strain set. Cell extracts were prepared after three hours of IgG expression, run on 12.5 % (A, B, D) or 7.5 % (C) SDS-PAGE gels, blotted onto nitrocellulose membranes, and detected with IgG heavy and light chain antibodies. A representative Western blot of IgG heavy chain (A) and light chain (B) signals of the ERAD mutant and LLO variant strain set. IgG heavy chain signals in the LLO variant coexpressing ALG3 and ALG11 with Δhrd1 deletion with and without PNGase F treatment (C). IgG light chain signals in Δalg3 Δalg11 strain with Δhrd1 deletion with and without PNGase F treatment (D). Glycosylated forms are indicated with triangles.

Figure 5. Western blot analysis of intracellular IgG clearance. IgG was expressed in exponentially growing cells for three hours, after which IgG expression was repressed by the addition of 2% glucose. Intracellular IgG levels were measured by Western blot from cell extract
samples collected until three hours after the glucose addition. Representative Western blots of IgG clearance in Δalg3 Δalg11 strain YMP28 (control) with Δyos9, Δhtm1 or Δhrd1 deletions with heavy chain (A) and light chain (B) detection. Ponceau S staining was used for the normalization of IgG signals, and representative samples of strain YMP28 stained with Ponceau S are shown (C). Glycosylated forms are indicated with triangles.

Figure 6. Quantitative analysis of intracellular IgG clearance. IgG was expressed in exponentially growing cells for three hours, after which IgG expression was repressed by the addition of 2% glucose and intracellular IgG levels were monitored by Western blot for three hours. IgG heavy and light chain signals were normalized to total protein and signal intensity at zero hours and corrected for IgG dilution due to the biomass increase. Relative heavy chain and light chain amounts of ERAD mutants in Δalg3 Δalg11 strain (A and D), Δalg3 Δalg11 strain expressing ALG3 (B and E) and Δalg3 Δalg11 strain coexpressing ALG3 and ALG11 (C and F), respectively. Data represent the mean ± SEM of 3-5 biological replicates. p < 0.05 (*), p<0.001
(***) compared with the control strain two hours after the glucose addition analyzed by Student’s t test.

**Figure 7. Analysis of IgG production in the ERAD mutant and LLO variant strain set.** IgG was expressed for 18 hours in cells grown in a 24 well plate format. IgG concentrations were measured using ELISA and normalized to OD600. Data represent the mean ± SEM of three biological replicates. p < 0.01 (**) compared to Δalg3 Δalg11 strain analyzed by Student’s t test.

**Tables**

Table 1. List of yeast strains used in this work.

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Supporting information for:

Investigating the role of ERAD on antibody processing in glycoengineered *Saccharomyces cerevisiae*

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Supplementary methods

Analysis of lipid-linked and N-linked oligosaccharides

Lipid-linked oligosaccharides (LLOs) were isolated as described by Zufferey et al. (1995) with some modifications. In short, 50 OD\textsubscript{600} units of cells grown to a late exponential phase were lysed by vortexing with glass beads for 5 minutes, and vortexing was repeated after adding 50 µl of DM (dichloromethane:methanol, 3:2). The cell suspension was washed four times with 4 ml of DM by vortexing for 5 min and centrifuging (5 min 4500 g). Washing was repeated four times with 4 ml of UP (dichloromethane:methanol:water, 3:48:47) containing 4 mM MgCl\textsubscript{2}, and two times with 4 ml of UP. LLOs were repeatedly extracted with 4 ml and two times 3 ml of DMW (dichloromethane:methanol:water, 10:10:3) by vortexing for 10 min and centrifuging (5 min 4500 g), and dried under nitrogen gas at 37°C. For the release of oligosaccharides from lipids, LLOs were dissolved in 35 µl isopropanol and hydrolysed by incubating for 45 minutes at 95 °C with 1 ml of 20 mM HCl. Released oligosaccharides were extracted with 5 ml of DM, and extraction was repeated by the addition of 3 ml of DM and 400 µl of water. Samples were dried under nitrogen gas at 60 °C and dissolved in 80 µl of water. Jack bean mannosidase (Sigma-Aldrich, St. Louis, MO, USA) digestion of LLO samples was performed in 20 µl reaction volume in 100 mM sodium acetate buffer with 20 mM ZnSO\textsubscript{4}, pH5 with 50 mU/µl enzyme concentration. The reaction mixture was incubated at 37 °C for 18 hours. Prior to MS analysis, LLO samples were purified with HyperSep Hypercarb SPE tips (Thermo Scientific) and concentrated by evaporation.

N-linked glycans were isolated from whole cell extracts prepared with trichloroacetic acid (TCA) precipitation. 50 OD\textsubscript{600} units of cells in 250 µl of 20 % TCA were lysed by vortexing with glass beads. The resulting suspension was collected, and the glass beads were sequentially washed with 300 µl and 700 µl of 5% TCA. Precipitated proteins were collected by centrifugation (10 min 14 000 g), and the pellet was washed with 750 µl of cold ethanol and centrifuged as previously. For deglycosylation, the sample was denatured and treated with PNGase F overnight (New England Biolabs, Ipswich, MA, USA) according to manufacturer’s instructions. The released glycans were purified using C18 and graphitized carbon columns as described earlier (Piirainen et al. 2016).

Purified N-glycans and LLOs were measured with UltraflxeXtreme MALDI-TOF MS (Bruker Daltonics, Billerica, MA, USA) in positive ion and reflector mode using 2,5-dihydroxybenzoic acid (DHB) or 9:1 mixture of DHB and 2-hydroxy-5-methoxybenzoic acid as a matrix.
mRNA analysis

IgG expression was induced in exponentially growing yeast cultures with 2% galactose. After three hours of expression, IgG expression was quenched by the addition of 2% glucose, and 2 ml samples were collected by centrifugation (3 min 9000 g) at 0, 15, 30, 45 and 60 minutes after the glucose addition.

RNA was isolated from cell pellets with RNeasy® Mini Kit (Qiagen, Hilden, Germany), and approximately 5 µg of RNA was treated with TURBO DNA-free™ DNAse (Thermo Fisher Scientific, Waltham, MA, USA). 500 ng of RNA was converted to cDNA with Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific). qPCR was performed in CFXConnect thermocycler (Bio-Rad, Hercules, CA, USA) with Maxima SYBR green/ROX qPCR Master Mix (Thermo Fisher Scientific) using 10 ng of cDNA as a template. IgG heavy chain, IgG light chain and actin were amplified with primer pairs OMP91 and OMP92, OMP93 and OMP94, and OAF1 and OAF2, respectively. Expression levels of IgG heavy and light chains were normalized to actin levels.
Supplementary figures

Figure S1. Lipid-linked oligosaccharide (LLO) and N-glycan structures of glycoengineered Δalg3 Δalg11 strain with episomal expression of ALG3 and ALG11. LLOs were isolated from strain YMP28 (Δalg3 Δalg11) containing expression vectors for ALG3 and ALG11 and analyzed with MALDI-TOF MS. LLOs of YMP28 (A). LLOs of YMP28 with ALG3 expression vector (upper panel), a corresponding control strain YG1365 (SS328 Δalg11, Helenius et al. 2002) (middle panel) and N-linked glycans of YMP28 with ALG3 expression vector (lower panel) (B). LLOs of YMP28 with ALG11 expression vector (upper panel) and a corresponding control strain YJR12 (SS328 Δalg3, de Ruijter and Frey 2015) (C). LLOs of YMP28 with the ALG3 and ALG11 coexpression vector (upper panel) and wild-type control strain SS328 (lower panel) (D). LLOs of
YMP28 with the ALG3 and ALG11 coexpression vector digested with jack bean mannosidase (D, upper panel inlay) reveal the extent of glucosylation in the LLO pool. M and Hex indicate the amount of mannoses or hexoses in the LLOs and N-glycans, respectively.

**Figure S2. Sensitivity of N-glycans to endoglycosidase treatment in glycoengineered ∆alg3 ∆alg11 strain with episomal expression of ALG3.** Cell extracts were prepared after three hours of IgG expression, incubated overnight in the absence (-) or presence (+) of Endo H or PNGase F, blotted onto a nitrocellulose membrane from 12.5 % SDS-PAGE gel, and detected with an IgG light chain antibody. IgG light chain signals in strain YMP28 (∆alg3 ∆alg11) and YMP28 with ALG3 expression vector (+ALG3) with and without Endo H treatment (A). IgG light chain signals in ∆alg3 ∆alg11 strain with ∆hrd1 deletion with and without PNGase F treatment (B, identical with Figure 4D). Glycosylated pro-LC forms are indicated with triangles.

**Figure S3. Glycosylation efficiencies of intracellular pro-LC in the ERAD mutant and LLO variant strain set.** Cell extracts were prepared after 3 hours of IgG expression, run on 12.5 %
SDS-PAGE gel, blotted onto nitrocellulose membranes, and detected with an IgG light chain antibody. Data represent the mean relative signal intensities of nonglycosylated, partially glycosylated, and fully glycosylated pro-LC forms in Δalg3 Δalg11 strain YMP28 (control) with Δyos9, Δhtm1 or Δhrd1 deletions, with ALG3 expression vector (+ALG3) and with ALG3 and ALG11 coexpression vector (+ALG3 +ALG11) from 3-5 biological replicates.

Figure S4. IgG heavy and light chain mRNA levels after the quenching of IgG expression.
IgG was expressed in exponentially growing yeast cells for three hours, and mRNA levels were measured by RT-qPCR after the glucose repression of the GAL1 promoter based IgG expression. Actin-normalized mRNA levels of IgG heavy chain (A) and light chain (B) measured from strains YMP28 (Δalg3 Δalg11), YMP32 (+Δhrd1), YMP28 with ALG3 expression vector (+ALG3), and YMP28 with ALG3 and ALG11 coexpression vector (+ALG3 +ALG11) relative to the mRNA levels at the time point of glucose addition. Data represent mean of two biological replicates.
**Supplementary tables**

**Table S1. List of oligonucleotides used in this work.**

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Table S2. Clearance constants ($k_c$) and half-lives ($t_{1/2}$) of intracellular IgG heavy and light chain polypeptides. All strains are derived from strain $\Delta$alg3 $\Delta$alg11 strain YMP28, and additional ERAD deletions and plasmid-based expression of $ALG3$ and $ALG11$ are indicated. The clearance constants were calculated by linear regression (least square sum method) of the ln-transformed relative growth-corrected signals. The clearance half-life was calculated as $\ln(2)$ divided by the clearance constant.

<table>
<thead>
<tr>
<th>LLO compensation</th>
<th>ERAD deletion</th>
<th>HC $k_c$</th>
<th>LC $k_c$</th>
<th>HC $t_{1/2}$ (h)</th>
<th>LC $t_{1/2}$ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>0.339</td>
<td>0.241</td>
<td>2.046</td>
<td>2.881</td>
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<td>-</td>
<td>$\Delta$yos9</td>
<td>0.394</td>
<td>0.151</td>
<td>1.759</td>
<td>4.605</td>
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<tr>
<td>-</td>
<td>$\Delta$htm1</td>
<td>0.584</td>
<td>0.257</td>
<td>1.186</td>
<td>2.697</td>
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<tr>
<td>-</td>
<td>$\Delta$hrd1</td>
<td>n/a</td>
<td>0.085</td>
<td>n/a</td>
<td>8.166</td>
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<tr>
<td>$ALG3$</td>
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<td>0.431</td>
<td>0.862</td>
<td>1.609</td>
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<tr>
<td>$ALG3$</td>
<td>$\Delta$yos9</td>
<td>0.477</td>
<td>0.165</td>
<td>1.453</td>
<td>4.195</td>
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<tr>
<td>$ALG3$</td>
<td>$\Delta$htm1</td>
<td>0.826</td>
<td>0.298</td>
<td>0.839</td>
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<tr>
<td>$ALG3$</td>
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<td>0.108*</td>
<td>2.100</td>
<td>6.418</td>
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<tr>
<td>$ALG3$+ALG11</td>
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<td>0.273</td>
<td>0.100</td>
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<td>6.945</td>
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<td>0.065</td>
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<tr>
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<td>2.282</td>
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<tr>
<td>$ALG3$+ALG11</td>
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<td>0.207</td>
<td>0.103</td>
<td>3.355</td>
<td>6.723</td>
</tr>
</tbody>
</table>

* $p$ value of linear fit between 0.05 and 0.1
n/a = no degradation or degradation did not follow first order kinetics

References


Zufferey R, Knauer R, Burda P et al. STT3, a highly conserved protein required for yeast