30. James, P., Halladay, J. & Craig, E. A. Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics* **144,** 1425–1436 (1996).

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The authors declare that they have no competing financial interests.

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Translocation of lipid-linked oligosaccharides across the ER membrane requires Rft1 protein

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***N*-linked glycosylation of proteins in eukaryotic cells follows a highly conserved pathway. The tetradecasaccharide substrate ($Glc_3Man_9GlcNAc_2$) is first assembled at the membrane of the endoplasmic reticulum (ER) as a dolichylpyrophosphate (Dol-PP)-linked intermediate, and then transferred to nascent polypeptide chains in the lumen of the ER[1]. The assembly of the oligosaccharide starts on the cytoplasmic side of the ER membrane with the synthesis of a $Man_5GlcNAc_2$-PP-Dol intermediate. This lipid-linked intermediate is then translocated across the membrane so that the oligosaccharides face the lumen of the ER, where the biosynthesis of $Glc_3Man_9GlcNAc_2$-PP-Dol continues to completion. The fully assembled oligosaccharide is transferred to selected asparagine residues of target proteins. The transmembrane movement of lipid-linked $Man_5GlcNAc_2$ oligosaccharide is of fundamental importance in this biosynthetic pathway, and similar processes involving phospholipids and glycolipids are essential in all types of cells[2–4]. The process is predicted to be catalysed by proteins, termed flippases, which to date have remained elusive[2–4]. Here we provide evidence that yeast *RFT1* encodes an evolutionarily conserved protein required for the translocation of $Man_5GlcNAc_2$-PP-Dol from the cytoplasmic to the lumenal leaflet of the ER membrane.**

Asymmetric lipid distribution is a fundamental characteristic of biological lipid bilayers. Lipids are the building blocks of membranes, and are involved in many processes including signalling, membrane polarity, biosynthesis and intracellular traffic, where membrane sidedness is critical. Several P-type ATPase and ABC transporters in addition to a fatty-acid transporter have been implicated in lipid translocation at the plasma membrane; however, translocators involved in membrane biogenesis have yet to be identified[5,6]. One of the most striking examples of lipid flipping is the translocation of the $Man_5GlcNAc_2$-PP-Dol intermediate from the cytosolic side of the ER membrane to the lumen before the completion of the biosynthesis of $Glc_3Man_9GlcNAc_2$-PP-Dol[7,8] (Fig. 1). In this case, a very large and polar oligosaccharide moiety must be translocated across the hydrophobic interior of the bilayer.

There are two lines of evidence suggesting that $Man_5GlcNAc_2$-PP-Dol is the intermediate that is translocated across the ER membrane[7]. The first is based on monosaccharide donors. Biosynthesis of $Man_5GlcNAc_2$-PP-Dol requires membrane-associated glycosyltransferases and cytosolic nucleotide-activated sugar substrates, UDP-GlcNAc and GDP-Man. In contrast, the subsequent transferases, starting with the *ALG3* mannosyltransferase, use Dol-P-Man and Dol-P-Glc precursors that are available in the lumen of the ER[8]. The absence of GDP-mannose within the ER lumen implies that the synthesis of $Man_5GlcNAc_2$-PP-Dol must occur on the cytosolic side of the ER membrane[9]. The second line of evidence is based on a binding study. When the mannose-specific lectin, concanavalin A, was added to intact 'right-side-out' ER vesicles, only $Man_5GlcNAc_2$-PP-Dol and smaller intermediates bound to it, indicating that $Man_6GlcNAc_2$ and larger intermediates were sequestered in the lumen of the ER[10].

The translocation of $Man_5GlcNAc_2$-PP-Dol has long been thought to be protein-mediated. The unassisted transmembrane movement of polyisoprenol-linked sugars in artificial liposomes is extremely slow[11,12]. The putative flippase catalysing this reaction is expected to be encoded by an essential gene, as the absence of PP-Dol-linked oligosaccharides in the ER lumen would halt all *N*-linked protein glycosylation—an essential process. PP-Dol-linked oligosaccharides that are incompletely assembled on the cytoplasmic side of the ER membrane (for example, $Man_3GlcNAc_2$-PP-Dol and $Man_4GlcNAc_2$-PP-Dol) can still be transferred to the ER lumen and from there to protein, suggesting that the flippase is not strictly specific for $Man_5GlcNAc_2$-PP-Dol but can operate, albeit inefficiently, with relaxed substrate specificity[13,14].

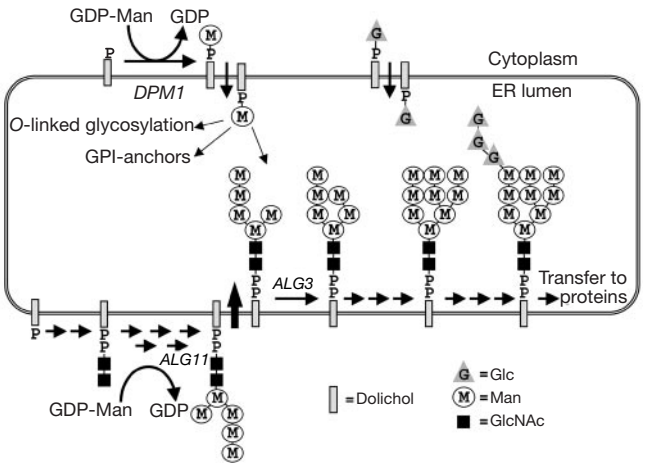

Figure 1 Biosynthesis of the *N*-linked oligosaccharides requires enzymatic reactions on both sides of the ER membrane. The lipid dolichylpyrophosphate serves as the membrane-bound carrier of the oligosaccharides. *ALG* gene products, including Alg3 and Alg11, catalyse the stepwise transfer of single monosaccharides. On the cytoplasmic side, activated sugars are used to generate the branched oligosaccharide, $Man_5GlcNAc_2$, which is translocated into the ER. Man-P-Dol and Glc-P-Dol are synthesized on the cytoplasmic side and are also translocated into the ER where they are used in the synthesis of full-length oligosaccharide. Man-P-Dol, the product of the *DPM1* enzyme in yeast, is also required for the synthesis of GPI-anchors and *O*-mannosylation of proteins.

In a previous study, we identified a mutant in *Saccharomyces cerevisiae* with a screen designed to detect cellular defects that require an intact unfolded protein response[15]. We showed that the mutant strain was deficient in *N*-linked glycosylation[15]. The mutation mapped to *RFT1*, which encodes an essential protein with multiple transmembrane domains that is localized in the ER (data not shown and M. J. Cai, personal communication). Homologues of *RFT1* are found in all eukaryotic genomes sequenced to date, with the notable exception of *Plasmodium falciparum*, a eukaryotic parasite that lacks *N*-glycoproteins[16].

To analyse the function of Rft1 in more detail, we constructed a haploid strain where the *GAL1-10* promoter replaced the endogenous *RFT1* promoter. Transfer of this strain to a glucose-containing medium repressed Rft1 expression and resulted in a severe reduction of growth. To assess the glycosylation defect in the mutant strain, we analysed carboxypeptidase Y (CPY), a well-characterized vacuolar protease carrying four *N*-linked oligosaccharides[17]. At different time points after repression of *RFT1*, CPY became increasingly hypoglycosylated, lacking up to three or even all four *N*-linked oligosaccharides (Fig. 2a). We next analysed the oligosaccharide PP-Dol precursors present in this strain at different time points after *RFT1* repression. To this end, aliquots of the same cultures were labelled with [^{3}H]-mannose, lipid-linked oligosaccharides were isolated, and the oligosaccharide moieties were cleaved off and separated by high-performance liquid chromatography (HPLC). As shown in Fig. 2b, depletion of Rft1 resulted in a significant accumulation of the $Man_5GlcNAc_2$ precursor, at the expense of the more completely assembled $Man_8GlcNAc_2$ and $Glc_3Man_9GlcNAc_2$ forms.

In principle, there are several possible defects that could lead to hypoglycosylation of *N*-glycoproteins with the concomitant accumulation of lipid-linked $Man_5GlcNAc_2$ intermediates (see Fig. 1). The effect could be explained by: (1) a deficiency in the mannosyltransferase Alg3; (2) a decreased availability of the substrate of Alg3, Dol-P-Man; or (3) a block in translocation of the lipid-linked $Man_5GlcNAc_2$ intermediate across the membrane.

The first two of these possibilities are ruled out by the following observations. First, and consistent with our previous work[18], *Δalg3* strains accumulate lipid-linked $Man_5GlcNAc_2$. $Man_5GlcNAc_2$ in *Δalg3* cells is transferred to proteins in the ER, resulting in CPY glycoforms that are completely resistant to endo-β-*N*-acetylglucosaminidase H (EndoH) digestion[18] (Fig. 3, top panel). In contrast, Rft1 depletion resulted in hypoglycosylated CPY that remained sensitive to EndoH digestion (Fig. 3, top panel), indicating that any residual glycosylation involved the transfer of fully assembled core oligosaccharide units. Thus, *RFT1*-depleted cells did not lack *ALG3* activity.

The second observation is that the ER-lumenal pool of Dol-P-Man, the substrate from which mannoses are transferred to lipid-linked oligosaccharides in the ER lumen, is not diminished. Specific leaky mutations in Dol-P-Man synthase, encoded by a single essential locus, *DPM1*, display a temperature-sensitive growth phenotype and have a very low pool of Dol-P-Man. This low level of Dol-P-Man not only affects *N*-linked but also *O*-linked protein glycosylation, as Dol-P-Man is the primary substrate in the *O*-glycosylation process in the ER of fungal cells[19]. As expected, *dpm1-6* mutant cells displayed a heterogeneous population of hypoglycosylated CPY molecules, indicative of a deficiency in *N*-linked protein glycosylation (Fig. 3, top panel, lanes 7 and 8). Furthermore, depletion of Dol-P-Man in the *dpm1* mutant also resulted in altered *O*-linked mannosylation, as visualized by the presence of unglycosylated or partially glycosylated chitinase in these cells (Fig. 3, bottom panel, lanes 7 and 8)[20]. Chitinase is an extensively *O*-mannosylated protein whose mobility, as measured in an SDS–polyacrylamide gel electrophoresis (PAGE) gel, shifts from a relative molecular mass of 130,000 to 60,000 (M_r 130K and 60K, respectively) in the absence of *O*-mannosylation[20]. Importantly, depletion of Rft1 had no effect on chitinase glycosylation (Fig. 3, bottom panel, lanes 3 and 4), demonstrating that there is a normal pool of Dol-P-Man in these cells. As expected, the same result was observed in the Alg3-deficient strain (Fig. 3, bottom panel, lanes 5 and 6). Depletion of Rft1 did not affect the level of the glycosylphosphatidylinositol (GPI)-anchored Gas1 protein (data not shown). Biosynthesis of the GPI-anchor is sensitive to deficient Dol-P-Man synthesis activity, because the core mannosyl residues of the GPI-

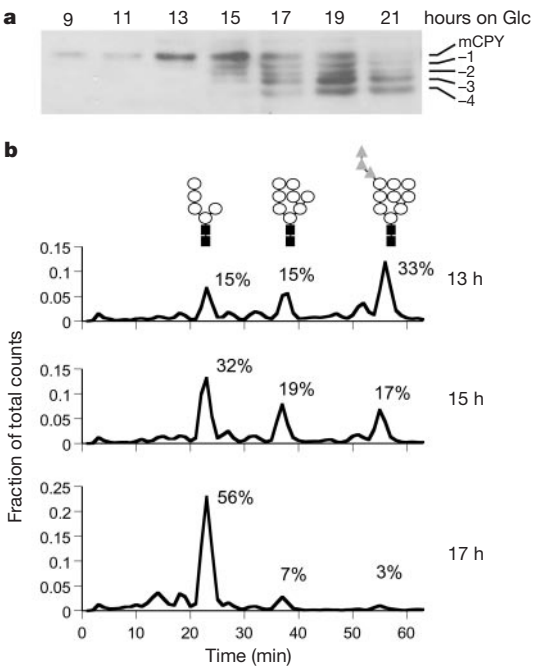

Figure 2 *RFT1* repression reduces *N*-linked glycosylation by altering the synthesis of lipid-linked oligosaccharides. The yeast strain used is YG1137, where the glucose-repressible *GAL1-10* promoter has replaced the *RFT1* promoter. **a**, Western blot analysis of CPY in YG1137 cultures grown in glucose-containing medium for the indicated times. Bands representing fully glycosylated (mCPY) and hypoglycosylated forms (lacking up to four oligosaccharides) of CPY are indicated. **b**, HPLC trace of metabolically labelled lipid-linked oligosaccharide intermediates from the same *RFT1*-repressed cells at 13, 15 and 17 h after shift to glucose-containing medium. Major HPLC peaks corresponding to $Man_5GlcNAc_2$, $Man_8GlcNAc_2$ and $Glc_3Man_9GlcNAc_2$ are marked. The percentage of total signal (average of two measurements) in marked peaks is indicated. Man, GlcNAc and Glc are indicated by circles, squares and triangles, respectively.

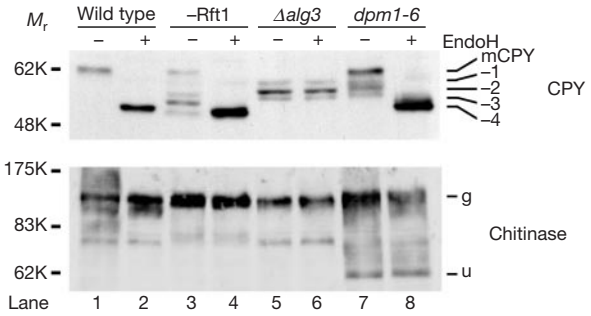

Figure 3 *RFT1* repression results in a distinct protein glycosylation defect. Western blot analysis of protein extracts from wild-type (SS328), –Rft1 (YG1137 grown for 24 h in glucose), *Δalg3* (YG248) and *dpm1-6* mutant (grown for 4 h at 37 °C) strains are shown. Protein extracts were incubated overnight in the presence (+) or absence (–) of EndoH. Blots were probed with antibodies against CPY (top panel) and chitinase (bottom panel). Mobility of fully glycosylated (mCPY) and hypoglycosylated forms of CPY are marked in the top panel, whereas fully glycosylated (g) and unglycosylated (u) forms of chitinase are specified in the bottom panel.

anchors are derived from Dol-P-Man[19].

Taken together, these results point to the possibility that the phenotypes observed on Rft1 depletion result strictly from impaired $Man_5GlcNAc_2$-PP-Dol translocation across the ER membrane. This hypothesis is supported further by the following findings. *ALG11* encodes a GDP-Man-dependent mannosyltransferase, which is required for cytoplasmic synthesis of $Man_5GlcNAc_2$-PP-Dol intermediate[14]. *Δalg11* cells grow very slowly, are temperature sensitive, and contain severely hypoglycosylated proteins. These mutant cells accumulate $Man_3GlcNAc_2$-PP-Dol, which is then translocated, albeit inefficiently, across the ER membrane. Once inside the ER, lumenal mannosyltransferases add four additional Dol-P-Man-derived mannose residues, resulting in $Man_7GlcNAc_2$ oligosaccharides. Therefore, analysis of lipid-linked oligosaccharides in *Δalg11* cells reveals two major peaks, representing $Man_3GlcNAc_2$ and $Man_7GlcNAc_2$ (Fig. 4a). Notably, $Man_3GlcNAc_2$ was shown to face the cytosol[10], whereas $Man_7GlcNAc_2$ faces the ER lumen, suggesting that translocation into the ER lumen of the incomplete $Man_3GlcNAc_2$ is the rate-limiting step in the biosynthesis of $Man_7GlcNAc_2$ (ref. 14). We proposed that an increased amount of the postulated flippase might therefore compensate for the *ALG11* deletion-induced defect, resulting in an increased formation of $Man_7GlcNAc_2$-PP-Dol and protein glycosylation.

To test this hypothesis, we introduced *RFT1* on a multicopy plasmid into *Δalg11* cells. As shown in Fig. 4a, overexpression of Rft1 resulted in a reduction of the relative levels of $Man_3GlcNAc_2$ with a concomitant increase in $Man_7GlcNAc_2$. This shift of the steady-state concentration of a cytoplasmically exposed oligosaccharide species to ER-lumenally exposed forms shows directly that overexpression of *RFT1* affects the topological distribution of the lipid-linked oligosaccharide.

We next examined asparagine-linked oligosaccharides in *Δalg11* cells and *Δalg11* cells that overexpress *RFT1*, after digesting protein fractions with peptide-*N*-glycosidase F (PNGaseF). Analysis of the released oligosaccharides revealed predominantly $Man_6GlcNAc_2$ in both strains (Fig. 4b), which probably results from the trimming of

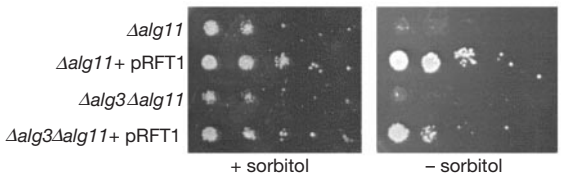

Figure 5 Overexpression of *RFT1* improves growth of *Δalg11* (YG1365) and *Δalg3Δalg11* (YG1363) strains. *Δalg11* and *Δalg3Δalg11* strains bearing either YEp352 or pJH01 (+pRFT1) were spotted onto SD-ura plates (with or without sorbitol) in rows. Each row consists of a serial dilution of indicated strain. The plates were incubated for 3 days at 30 °C.

protein-bound $Man_7GlcNAc_2$ by ER mannosidase[14,21]. We conclude from these results that, as in wild-type cells, ER-lumenally oriented $Man_7GlcNAc_2$—but not cytoplasmically oriented $Man_3GlcNAc_2$—served as substrate for protein glycosylation. Moreover, overexpression of Rft1 in *Δalg11* cells resulted in a shift towards CPY glycoforms containing more oligosaccharide units (Fig. 4b, compare lanes 3 and 5), demonstrating an improved glycosylation efficiency that is consistent with an increased pool of available substrate in the ER lumen. Notably, the oligosaccharides present on CPY molecules were sensitive to EndoH (Fig. 4b, lanes 3–6), supporting the conclusion that $Man_7GlcNAc_2$ and not $Man_3GlcNAc_2$ was transferred to protein. The increased glycosylation associated with *RFT1* overexpression also coincided with a partial suppression of the *Δalg11* growth defect (Fig. 5). The overexpression of *RFT1* only altered the relative level of $Man_7GlcNAc_2$ to $Man_3GlcNAc_2$ oligosaccharide, but did not result in the appearance of a novel lipid-linked oligosaccharide.

To rule out the possibility that overexpression of *RFT1* has an effect on lumenal mannose transferases, we constructed a *Δalg3Δalg11* double mutant strain. In these cells, irrespective of *RFT1* overexpression, the lipid-linked $Man_3GlcNAc_2$ oligosaccharide was the largest oligonucleotide observed (data not shown).

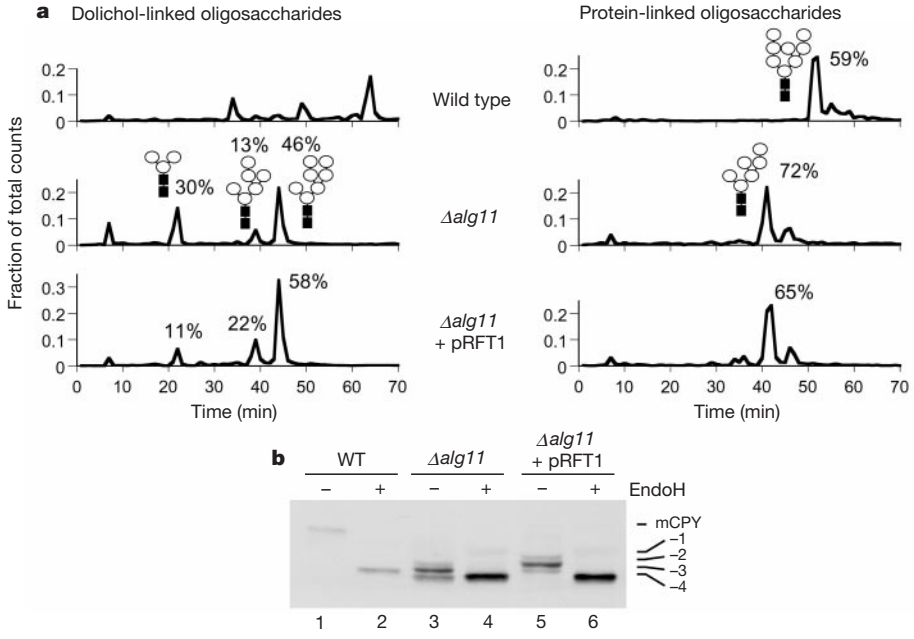

Figure 4 Overexpression of *RFT1* improves *N*-linked glycosylation in a *Δalg11* strain. **a**, HPLC traces of [^{3}H]-mannose-labelled lipid-linked oligosaccharides and N-linked oligosaccharides from wild-type (WT) cells and *Δalg11* cells (YG1365) bearing either an empty plasmid (*Δalg11*) or *RFT1* on a multicopy plasmid (*Δalg11* + pRFT1). Major HPLC peaks corresponding to $Man_3GlcNAc_2$, $Man_6GlcNAc_2$ and $Man_7GlcNAc_2$ of the lipid-linked oligosaccharide fraction, and $Man_8GlcNAc_2$ and $Man_6GlcNAc_2$ of N-linked oligosaccharide fraction are marked. See Fig. 2b legend for definition of the symbols. In wild-type cells, lipid-linked $Glc_3Man_9GlcNAc_2$, $Man_8GlcNAc_2$ and $Man_5GlcNAc_2$ represent the predominant oligosaccharides. **b**, Proteins from the same cultures as **a** were extracted, incubated overnight in the presence (+) or absence (–) of EndoH, blotted, and the membranes probed with CPY antibody. Bands representing fully glycosylated (mCPY) and hypoglycosylated forms of CPY are indicated.

Again, we observed partial restoration of growth (see Fig. 5) and increased glycosylation of CPY (data not shown) on *RFT1* overexpression. These results further support the conclusion that Rft1 does not affect the structures of the lipid-linked oligosaccharides but rather their topological disposition in the ER membrane, thereby affecting the availability of the oligosaccharide substrate for *N*-glycosylation.

The experiments in this study support the hypothesis that Rft1 is required directly for membrane translocation of the $Man_5GlcNAc_2$ oligosaccharide across the ER membrane. The overexpression experiments in the *alg11* mutant (Fig. 4) suggest that Rft1 is the limiting component for the flipping reaction *in vivo* and that no additional factors may be required for its function. Rtf1 is therefore an excellent candidate protein for the $Man_5GlcNAc_2$-PP-Dol flippase in the ER membrane. Of note, the results indicate that the translocation of Dol-P-Man[7,22] across the ER membrane is independent of Rft1. Similar to the plasma membrane phospholipid scramblase[23], the fatty acid transporter FATP[24], and the bacterial Wzx protein[25], the amino-acid sequence of Rft1 does not reveal ATP-binding domains. Further genetic and biochemical experiments should allow us to address the mechanisms that make the translocation of polar and bulky substrates—such as the $Man_5GlcNAc_2$-PP-Dol intermediate—across the membrane possible, and whether membrane asymmetry is critical for the process. □

Methods

Yeast medium and methods

We used standard yeast medium and genetic techniques[26]. With the exception of YG1137, all strains were grown on YPD medium unless otherwise stated. Strain YG1137 was maintained on YPGal. YG1363 and YG1365 were grown in medium supplemented with 1 M sorbitol unless otherwise stated.

Lipid- and protein-linked oligosaccharide analysis

Lipid-linked oligosaccharides were labelled, extracted and analysed as described[27]. In brief, yeast cells (50 ml culture with an absorbance at 546 nm of 1) were grown in YPD and incubated in medium containing [^{3}H]-mannose before lyses with organic solvents. Lipid-linked oligosaccharide was extracted using organic solvents and oligosaccharides were released by mild acid hydrolysis. The released oligosaccharides were analysed by HPLC using an NH_2 column with flow-through counting. We plotted the number of counts per minute divided by total counts in the run. The percentage of total signal in a sample is the average using two measurements. *N*-linked oligosaccharide was purified from cell debris after lipid-linked oligosaccharide extraction. Protein of the debris pellet was solubilized (10 min at 100 °C) in 0.2 ml 1% SDS, 50 mM Tris-HCl, 1% β-mercaptoethanol. After centrifugation (2 min at 15,000*g*) supernatant was supplemented to 1% (v/v) NP40 in 0.25 ml and protein-linked oligosaccharides were digested off using PNGaseF (2 units, overnight at 37 °C). Proteins were precipitated with 0.75 ml ethanol and samples were spun for 20 min at 15,000*g*. The supernatant was dried and resuspended in 0.2 ml 70:30 acetonitrile:water, 0.1 ml of which was analysed by HPLC as above.

Strain construction

The endogenous *RFT1* promoter was replaced with the *GAL1-10* promoter region using the polymerase chain reaction (PCR)-based method[28] in the background yeast strain SS328XSS330, yielding strain YG1133 (*MAT**a**/α ade2-201/ade2-201 ura3-52/ura3-52 his3Δ200/his3Δ200 tyr1/+ lys2-801/+, Pgal-rft1::KanMX/+*). YG1133 was sporulated and tetrads were dissected on YPGal to obtain a haploid strain with *RFT1* under the *GAL1-10* promoter control, YG1137 (*MATα ade2-201 ura3-52 his3Δ200 lys2-801 Pgal-rft1::KanMX*). Similarly, the entire *ALG11* open reading frame was replaced in SS328XSS330 by integration of a PCR product containing the *S. cerevisiae HIS3* locus. Transformed yeast strain YG1141 (*MAT**a**/α ade2-201/ade2-201 ura3-52/ura3-52 his3Δ200/his3Δ200 tyr1/+ lys2-801/+ Δalg11::HIS3/+*) was sporulated and tetrads were dissected to obtain a *Δalg11* haploid, YG1361 (*MATα ade2-201 ura3-52 his3Δ200 Δalg11::HIS3*), which was mated with YG248 (ref. 18). The resulting diploid YG1362 (*MAT**a**/α ade2-201/ade2-201 ura3-52/ura3-52 his3Δ200/his3Δ200 lys2-801/+ Δalg3::HIS3 Δalg11::HIS3/+*) was sporulated on YPD plates containing 1 M sorbitol to obtain the haploid strains YG1365 (*MATα ade2-101 ura3-52 his3Δ200 Δalg11::HIS3*) and YG1363 (*MATα ade2-101 ura3-52 his3Δ200 lys2-801 Δalg3::HIS3 Δalg11::HIS3*). YG248 (*MATα Δalg3::HIS3 ade2-101 his3Δ200 lys2-801 ura3-52*)[18] and the temperature-sensitive dpm1-6 (*MATα dpm1::LEU2 leu2-3 lys2 ura3 trp1 pDM8-6*)[19] strains were used.

Plasmid construction

To construct the Rft1 overexpression plasmid pJH01, the 2.8-kilobase (kb) *Eco*RI–*Sph*I fragment containing the Rft1 locus of pDN387 (ref. 15) was ligated into the same sites of Yep352, a high-copy yeast plasmid.

Protein analysis

We performed protein extraction, EndoH treatment and western analysis as described[18]. Antibodies against CPY and chitinase were diluted 3,000- and 20,000-fold, respectively.

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1. Kornfeld, R. & Kornfeld, S. Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* **54,** 631–664 (1985).
2. Bugg, T. D. & Brandish, P. E. From peptidoglycan to glycoproteins: common features of lipid-linked oligosaccharide biosynthesis. *FEMS Microbiol. Lett.* **119,** 255–262 (1994).
3. Higgins, C. F. Flip-flop: the transmembrane translocation of lipids. *Cell* **79,** 393–395 (1994).
4. Sprong, H., van der Sluijs, P. & van Meer, G. How proteins move lipids and lipids move proteins. *Nature Rev. Mol. Cell Biol.* **2,** 504–513 (2001).
5. Hettema, E. H. & Tabak, H. F. Transport of fatty acids and metabolites across the peroxisomal membrane. *Biochim. Biophys. Acta* **1486,** 18–27 (2000).
6. Borst, P., Zelcer, N. & van Helvoort, A. ABC transporters in lipid transport. *Biochim. Biophys. Acta* **1486,** 128–144 (2000).
7. Abeijon, C. & Hirschberg, C. B. Topography of glycosylation reactions in the endoplasmic reticulum. *Trends. Biochem. Sci.* **17,** 32–36 (1992).
8. Burda, P. & Aebi, M. The dolichol pathway of N-glycosylation. *Biochim. Biophys. Acta* **1426,** 239–257 (1999).
9. Perez, M. & Hirschberg, C. B. Topography of glycosylation reactions in the rough endoplasmic reticulum membrane. *J. Biol. Chem.* **261,** 6822–6830 (1986).
10. Snider, M. D. & Rogers, O. C. Transmembrane movement of oligosaccharide-lipids during glycoprotein synthesis. *Cell* **36,** 753–761 (1984).
11. McCloskey, M. A. & Troy, F. A. Paramagnetic isoprenoid carrier lipids. 1. Chemical synthesis and incorporation into model membranes. *Biochemistry* **19,** 2056–2060 (1980).
12. Hanover, J. A. & Lennarz, W. J. The topological orientation of N,N′- diacetylchitobiosylpyrophosphoryldolichol in artificial and natural membranes. *J. Biol. Chem.* **254,** 9237–9246 (1979).
13. Huffaker, T. C. & Robbins, P. W. Yeast mutants deficient in protein glycosylation. *Proc. Natl. Acad. Sci. USA* **80,** 7466–7470 (1983).
14. Cipollo, J. F., Trimble, R. B., Chi, J. H., Yan, Q. & Dean, N. The Yeast ALG11 gene specifies addition of the terminal alpha 1,2-Man to the Man5GlcNAc2-PP-dolichol N-glycosylation intermediate formed on the cytosolic side of the endoplasmic reticulum. *J. Biol. Chem.* **276,** 21828–21840 (2001).
15. Ng, D. T., Spear, E. D. & Walter, P. The unfolded protein response regulates multiple aspects of secretory and membrane protein biogenesis and endoplasmic reticulum quality control. *J. Cell Biol.* **150,** 77–88 (2000).
16. Davidson, E. A. & Gowda, D. C. Glycobiology of *Plasmodium falciparum*. *Biochimie* **83,** 601–604 (2001).
17. Hasilik, A. & Tanner, W. Carbohydrate moiety of carboxypeptidase Y and perturbation of its biosynthesis. *Eur. J. Biochem.* **91,** 567–575 (1978).
18. Aebi, M., Gassenhuber, J., Domdey, H. & te Heesen, S. Cloning and characterization of the *ALG3* gene of *Saccharomyces cerevisiae*. *Glycobiology* **6,** 439–444 (1996).
19. Orlean, P. Dolichol phosphate mannose synthase is required *in vivo* for glycosyl phosphatidylinositol membrane anchoring, O-mannosylation, and N-glycosylation of protein in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* **10,** 5796–5805 (1990).
20. Orlean, P., Kuranda, M. J. & Albright, C. F. Analysis of glycoproteins from *Saccharomyces cerevisiae*. *Methods Enzymol.* **194,** 682–697 (1991).
21. Jelinek-Kelly, S. & Herscovics, A. Glycoprotein biosynthesis in *Saccharomyces cerevisiae*. Purification of the alpha-mannosidase which removes one specific mannose residue from $Man_9GlcNAc$. *J. Biol. Chem.* **263,** 14757–14763 (1988).
22. Rush, J. S. & Wächter, C. J. Transmembrane movement of a water-soluble analogue of mannosylphosphoryldolichol is mediated by an endoplasmic reticulum protein. *J. Cell Biol.* **130,** 529–536 (1995).
23. Zhou, Q. *et al.* Molecular cloning of human plasma membrane phospholipid scramblase. A protein mediating transbilayer movement of plasma membrane phospholipids. *J. Biol. Chem.* **272,** 18240–18244 (1997).
24. Schaffer, J. E. & Lodish, H. F. Expression cloning and characterization of a novel adipocyte long chain fatty acid transport protein. *Cell* **79,** 427–436 (1994).
25. Feldman, M. F. *et al.* The activity of a putative polyisoprenol-linked sugar translocase (Wzx) involved in *Escherichia coli* O antigen assembly is independent of the chemical structure of the O repeat. *J. Biol. Chem.* **274,** 35129–35138 (1999).
26. Guthrie, C. & Fink, G. R. Guide to yeast genetics and molecular biology. *Methods Enzymol.* **194,** 3–37 (1991).
27. Zufferey, R. *et al. STT3*, a highly conserved protein required for yeast oligosaccharyltransferase activity *in vitro*. *EMBO J.* **14,** 4949–4960 (1995).
28. Wach, A. *et al.* in *Methods in Microbiology: Yeast Gene Analysis* (eds Tuite, M. & Brown, A. J.) 67–81 (Academic, San Diego, 1998).

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Competing interests statement

The authors declare that they have no competing financial interests.

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