Mammalian STT3A/B oligosaccharyltransferases segregate N-glycosylation at the translocon from lipid-linked oligosaccharide hydrolysis

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Oligosaccharyltransferases (OSTs) N-glycosylate proteins by transferring oligosaccharides from lipid-linked oligosaccharides (LLOs) to asparaginyl residues of Asn-Xaa-Ser/Thr acceptor sequons. Mammals have OST isoforms with STT3A or STT3B catalytic subunits for cotranslational or posttranslational N-glycosylation, respectively. OSTs also hydrolyze LLOs, forming free oligosaccharides (fOSs). It has been unclear whether hydrolysis is due to one or both OSTs, segregated from N-glycosylation, and/or regulated. Transfer and hydrolysis were assayed in permeabilized HEK293 kidney and Huh7.5.1 liver cells lacking STT3A or STT3B. Transfer by both STT3A-OST and STT3B-OST with synthetic acceptors was robust. LLO hydrolysis by STT3B-OST was readily detected and surprisingly modulated: Without acceptors, STT3B-OST hydrolyzed Glc₃Man₉GlcNAc₂-LLO but not Man₉GlcNAc₂-LLO, yet it hydrolyzed both LLOs with acceptors present. In contrast, LLO hydrolysis by STT3A-OST was negligible. STT3A-OST however may be regulatory, because it suppressed STT3B-OST-dependent fOSs. TREX1, a negative innate immunity factor that diminishes immunogenic fOSs derived from LLOs, acted through STT3B-OST as well. In summary, only STT3B-OST hydrolyzes LLOs, depending upon LLO quality and acceptor site occupancy. TREX1 and STT3A suppress STT3B-OST-dependent fOSs. Without strict kinetic limitations during posttranslational N-glycosylation, STT3B-OST can thus moonlight for LLO hydrolysis. In contrast, the STT3A-OST/translocon complex preserves LLOs for temporally fastidious cotranslational N-glycosylation.

dolichol | glycosylation | oligosaccharyltransferase | STT3A | STT3B

Asparagine (N)-linked glycosylation occurs in all three domains of life—eukaryotes, bacteria, and archaea—with conservation of mechanism and topology. N-glycans serve multiple purposes ranging from the folding and function of individual glycoproteins, to cell–cell interactions and signaling (1). N-glycosylation begins with synthesis of an oligosaccharide on a polyisoprenyl phosphate carrier, i.e., a lipid-linked oligosaccharide (LLO), with the polyisoprene chain embedded in the membrane and the oligosaccharide unit displayed in an aqueous environment. In metazoans and many other eukaryotes (2) the LLO is glucose₃mannose₉N-acetylglucosamine₂-P-P-dolichol_{C95} (G₃M₉-LLO) with the oligosaccharide facing the endoplasmic reticulum (ER) lumen. G₃M₉-LLO is the donor for N-glycosylation of asparaginyl residues in select Asn-Xaa-Ser/Thr/Cys sequons of polypeptides, also luminal, by membranous oligosaccharyltransferases (OSTs). These are single subunit enzymes in bacteria, archaea, and some single-cell eukaryotes, and multisubunit complexes in other single-cell eukaryotes such as *Saccharomyces cerevisiae* and all metazoans (3, 4).

Most sequons are N-glycosylated cotranslationally as they emerge from the translocon into the ER lumen, but a minority are N-glycosylated posttranslationally after synthesis of the polypeptide is complete. The latter include sequons within ~50- to 55-aa residues of the carboxyl terminus (5), which are inside the translocon at the time of ribosomal release, and sequons unavailable for cotranslational

modification due to nearby disulfide bonds (6). Mammalian OSTs evolved several features to manage the co/posttranslational workload (7): (i) Mammalian OST complexes have eight to nine subunits and exist as one of two major isoforms with either STT3A or STT3B catalytic subunits. (ii) STT3A-OST glycosylates cotranslationally, with DC2 and KCP2 subunits that direct STT3A-OST to the translocon (8–10). (iii) STT3B-OST glycosylates posttranslationally, is unassociated with the translocon, and has an oxidoreductase-type subunit—MagT1 or TUSC3 (6, 9, 10). (iv) These distinct roles are evident in two human *Congenital Disorders of Glycosylation*, STT3A-CDG and STT3B-CDG (11). (v) Both isoforms recognize G₃M₉-LLO, but STT3A-OST is less tolerant of the intermediate M₉-LLO as a donor than is STT3B-OST (12). STT3A-OST and STT3B-OST are thus highly related, but function with important catalytic and temporal differences.

Unphosphorylated N-linked-type free oligosaccharides (fOSs) are released by hydrolysis of LLO pyrophosphate bonds under conditions that permit N-glycosylation by OSTs (13). In vitro LLO hydrolysis mirrors N-glycosylation regarding LLO structure and concentration dependence, and requirement for divalent metal ion. Thus, OST was suggested to cause the hydrolysis, but a separate LLO hydrolase was also possible (14). Our group reported “OST-like” LLO hydrolysis in the context of the phosphomannomutase 2 deficiency *PMM2-CDG* (15, 16), Herpes simplex virus-1 infection (17), and Three-Prime Repair Exonuclease 1 (TREX1) dysfunction

Significance

Specialized sugar polymers (oligosaccharides) are necessary for life at the protein, organelle, cell, and organism levels. Processes for degrading oligosaccharides enhance their repertoire of functions, but a potential problem is short circuiting between degradation and synthesis. With the endoplasmic reticulum of mammalian cells, we show how lipid-linked oligosaccharides (LLOs) meant for attachment to proteins are segregated from an immunogenic process that involves their degradation to free oligosaccharides. Specifically, the STT3A isoform of oligosaccharyltransferase only transfers oligosaccharides, while the STT3B isoform can hydrolyze LLOs when not preoccupied with attachment of oligosaccharides to protein. This allows cells to perform both important processes in concert, without problems due to competition.

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Fig. 2. STT3B-OST selectively hydrolyzes G₃M₉-LLO when acceptor is absent. OST products and LLOs were analyzed with permeabilized STT3A-KO cells after 30-min incubations. UDP-Glc was included for G₃M₉-LLO or omitted for M₉-LLO. Some G₃M₉-LLO was detected even with UDP-Glc omitted. (A) FACE gels of samples from duplicate STT3A-KO dishes. Single arrowhead indicates G₃M₉Gn₂; double arrowhead indicates M₉Gn₂ (two exposures are shown). *SI Appendix, Fig. S1* displays an expansion of this experiment including WT and STT3B-KO cells. (B and C) Graphs ($n = 4-6$) of hydrolysis of G₃M₉-LLO (+UDP-Glc, B) or M₉-LLO (-UDP-Glc, C) by STT3A-KO cells, with results from hydrolysis-incompetent STT3B-KO cells plotted for comparison. ns, not significant.

AP showed that the two STT3B-OST activities competed for the same LLO pool, and both were attenuated by NGI-1. Some inactive sequons can interact with OST (40), but this was inconsequential for hydrolysis because free G₃M₉Gn₂ was formed equally with CP or no peptide.

LLO hydrolysis is thus a distinguishing feature of STT3B-OST, not a general OST side reaction. STT3A-OST, docked at the translocon, must cotranslationally N-glycosylate sequons during the brief moment they emerge from the translocation tunnel. LLO hydrolysis would sabotage this function. On the other hand, STT3B-OST acts posttranslationally without a fastidious kinetic requirement, and should tolerate “toggling” between transfer and hydrolysis.

Hydrolysis by STT3B-OST Is LLO Specific and Acceptor Dependent. We anticipated that the LLO glycan specificity of STT3B-OST’s hydrolase activity would emulate its transferase activity, which used both G₃M₉-LLO and M₉-LLO efficiently (12). This was tested by omitting UDP-glucose (UDP-Glc), the precursor of glucose-P-dolichol needed to convert M₉-LLO to G₃M₉-LLO (Fig. 2 and *SI Appendix, Fig. S1 A and B*). LLOs synthesized with UDP-Glc were mostly G₃M₉-LLO, with small amounts of M₉-LLO and other LLO intermediates. Without UDP-Glc, LLOs were primarily M₉-LLO, but traces of G₃M₉-LLO were detected, perhaps from residual G₃M₉-LLO/glucose-P-dolichol present at the beginnings of reactions, or UDP-Glc impurities in the GDP-mannose or UDP-GlcNAc. STT3A-OST’s transferase activity (STT3B-KO cells) favored G₃M₉-LLO over M₉-LLO at two donor ratios (depending on UDP-Glc addition). STT3B-OST, unlike STT3A-OST, used G₃M₉-LLO and M₉-LLO as transferase donors equally. Thus, the STT3A-OST and STT3B-OST LLO specificities in HEK293 knockouts mirrored measurements with these OST complexes isolated biochemically (12).

With M₉-LLO, like G₃M₉-LLO, hydrolysis by STT3A-OST was insignificant (*SI Appendix, Fig. S1 A and B*), but results for STT3B-OST were unanticipated. With CP or no peptide, STT3B-OST strongly preferred G₃M₉-LLO for hydrolysis over M₉-LLO. Even when UDP-Glc was omitted, STT3B-OST generated mostly free G₃M₉Gn₂, attributable to residual G₃M₉-LLO in the assay, and free M₉Gn₂ was essentially undetectable. In the presence of acceptor

peptide, however, STT3B-OST became unselective for M₉-LLO vs. G₃M₉-LLO as hydrolysis substrates, paralleling its transferase specificity. As expected addition of acceptor allowed transfer to out-compete hydrolysis. STT3B-OST’s unexpected acceptor-dependent LLO hydrolysis specificity implicates a role for EL5 (*Discussion*).

Hepatocyte STT3B-OST, Not STT3A-OST, Is an LLO Hydrolase. We next tested the role of STT3A and STT3B in hepatocytes, which are major secretory cells producing most serum N-glycoproteins and are physiologically sensitive to OST dysfunction (11). OST subunit knockouts and rescue lines with hepatocyte-derived Huh7.5.1 cells (41) were thus used to examine LLO hydrolysis. Consistent with HEK293 cells, transferase activity was evident in all Huh7.5.1 STT3A/B knockouts and rescue lines, and LLO hydrolysis was insignificant in STT3B knockouts (Fig. 3). Importantly, LLO hydrolysis was restored by rescue of the STT3B knockout with normal (catalytically active) STT3B, but not with transferase-dead STT3B bearing point mutations for three evolutionarily conserved catalytic residues (41). STT3B-OST is therefore the sole LLO hydrolase in hepatocytes and kidney cells, with hydrolysis most likely at the catalytic site rather than a separate allosteric site (12, 42).

STT3A Diminishes STT3B-OST-Dependent fOSs. Since STT3B-OST hydrolyzes LLOs, we expected fOSs to be depleted in STT3B-KO cells. Indeed, several fOSs were more abundant in STT3A-KO than in STT3B-KO cells (Fig. 4A and *SI Appendix, Fig. S3A*). However, control cell fOSs were similar to those in STT3B-KO cells, not STT3A-KO cells. Cellular fOSs therefore unexpectedly increased with the absence of STT3A, not the presence of STT3B. Acting cotranslationally, STT3A-OST should aid N-glycan-dependent folding. Glycoproteins normally bearing multiple N-glycans, but missing one if posttranslational glycosylation by STT3B-OST failed to fully compensate in STT3A-KO cells, could become candidates for ERAD. Subsequent cytosolic digestion by PNGase, ENGase, and mannosidases (27, 36) of additional ERAD substrates might elevate fOSs in STT3A-KO cells, supplementing the normally small contribution of ERAD to mammalian fOSs (22).

Fig. 3. LLO hydrolysis in hepatocytes requires transferase-competent STT3B-OST. OST activities were determined (Fig. 1) with permeabilized control, STT3A-KO, or STT3B-KO Huh7 cells, including STT3B-KO with transfected normal or transferase-incompetent (“catalytically dead,” STT3Bcat) forms of STT3B. (A) FACE gels with duplicate samples. (B) Quantitation ($n = 4$). ns, not significant.

Fig. 4. STT3A suppresses fOSs generated by STT3B-OST. fOSs from intact cells were analyzed by FACE. fOSs of interest are numbered and lettered according to migration relative to glucose oligomers G_{4-7} . (A) fOSs no. 4 and no. 5 in knockouts normalized to total protein, then the average value for WT HEK cells. (B) fOSs no. 4 and no. 5 in STT3A-KO cells were diminished by NGI-1 (5 μ M) and TN (5 μ g/mL). Companion FACE gels for A and B are in [SI Appendix, Fig. S3](#). (C) WT HEK and STT3A-KO cells were grown in the absence (*Upper*) or presence (*Lower*) of KF + CSN. Total fOSs were harvested from intact cells, or separate luminal and cytosolic pools were harvested after permeabilization.

Elevation of STT3A-KO fOS levels was sensitive to NGI-1 and the LLO synthesis inhibitor tunicamycin (TN) (Fig. 4B and [SI Appendix, Fig. S3B](#)), demonstrating origination from LLOs. They varied in size corresponding to Glc₃₋₈ standards. The migrations of the two most prominent fOSs (Fig. 4C and [SI Appendix, Fig. S3](#); near Glc₄ and Glc₅) were consistent with M₃Gn₁₋₂-M₅Gn₁₋₂ structures which would form by partial trimming inside the ER lumen and further trimming by cytosolic/lysosomal glycosidases (13). Since STT3A-KO cells treated with ALO lost a subset of the Glc₃₋₈ fOSs, they were a luminal/cytosolic mixture (Fig. 4C). Thus, the fOSs increased in STT3A-KO cells could be due to either LLO hydrolysis or PNGase/ENGase digestion of ERAD substrates.

PNase is inhibited by z-VAD-FMK, a caspase inhibitor (43, 44), and in preliminary experiments it suppressed STT3A-KO fOSs. However, a control caspase inhibitor (Q-VD-Oph) which does not affect PNase (43, 45) gave similar inhibition. This prompted an alternative approach for differentiating between LLOs and ERAD as the fOS source. Glycoprotein ERAD requires processing of N-linked oligosaccharides by kifunensine (KF)-sensitive ER mannosidase(s) (46). This forms structures with exposed α 1,6-linked mannosyl residues in the ER lumen for OS9/XTP3-B binding (26). Retrotranslocation of such ERAD substrates and PNase digestion releases ERAD-derived fOSs into the cytosol. KF should therefore attenuate cytosolic ERAD-associated fOSs (22). By comparison, cytosolic fOSs from LLO hydrolysis require removal of

glucosyl residues by CSN-sensitive glucosidases I and II to exit the ER (13). As indicated previously (22) a mixture of KF and CSN should thus diminish LLO-derived cytosolic fOSs originating by either hydrolysis or N-glycosylation/ERAD. However, new and larger fOSs are expected only for LLO hydrolysis, with some being luminal. Inhibition of ERAD should simply eliminate PNGase-dependent fOSs with no new species (*SI Appendix, Fig. S3C*).

Fig. 4C displays fOSs after mixed KF/CSN treatment of both control and STT3A-KO cells, allowing STT3A-KO specific fOSs to be deduced by comparison. KF/CSN increased total fOSs, diminished two cytosolic fOSs (nos. 4 and 5), and caused the appearance of one cytosolic (no. 8) and one luminal (no. 6b) fOS. We conclude that the fOSs increased in STT3A-KO cells result from STT3B-OST-dependent LLO hydrolysis, not ERAD. STT3A thus unexpectedly suppresses this fOS pool (*Discussion*).

TREX1 Suppresses fOSs Generated by STT3B-OST. TREX1 (DNase III) suppresses inappropriate activation of cGAS-STING innate immunity signaling (47). We reported a second TREX1 function requiring its carboxyl-terminal tail anchor, which localizes TREX1 to the cytosolic face of the ER and may explain TREX1 diseases with mutations that retain DNase activity (18). TREX1 knockout in mouse embryonic fibroblasts (MEFs) increases LLO hydrolase activity and LLO-derived fOSs, which interestingly activate expression of IFN-stimulated genes (18).

To test potential connections between TREX1 and STT3B-OST-specific LLO hydrolysis, we took advantage of fortuitously insignificant TREX1 levels in HEK293 cells (18). After stably expressing human TREX1 in control HEK293, STT3A-KO, and STT3B-KO cells, fOSs were analyzed. High cell density attenuated TREX1 effects on fOSs (*SI Appendix, Fig. S44*), but at low cell density, control and STT3A-KO cells revealed suppression of STT3B-OST-dependent fOSs by TREX1 (Fig. 5). STT3B-KO fOSs were unaffected. The two STT3A-KO fOSs diminished most by TREX1 (Fig. 5*A*, *arrows*) may be part of the STT3A-suppressible fOS pool (Fig. 4*C*

Fig. 5. TREX1 suppresses fO5s only in cells expressing STT3B. Human TREX1 was expressed in HEK lines as indicated, and after growth at optimal cell density (SI Appendix, Fig. S4A) fO5s were harvested from intact cells. (A) FACE gel showing fO5s suppressed by TREX1, arrows highlighting fO5s no. 4 and no. 5. (B) Quantitation of suppression of fO5s no. 4 + no. 5 by TREX1 ($n = 6$). ns, not significant.

and *SI Appendix, Fig. S3A*), and the HEK293 cell density effect is possibly related to mitosis-dependent phosphorylation of TREX1's C terminus (48). The fOS results with HEK293 cells were next expanded with STT3A and STT3B knockdowns in TREX1-KO MEFs. Target mRNAs were lowered by >90%, but only knockdown of STT3B suppressed fOSs in TREX1-KO MEFs, reaching fOS levels in WT MEFs (*SI Appendix, Fig. S4B*). In both HEK293 and MEF cells TREX1, a negative regulator of both innate immunity and LLO-derived fOSs, was thus linked to STT3B-OST.

Discussion

Spiro and Anumula suggested that mammalian OST was also a LLO hydrolase (14). Szymanski and coworkers, working with *C. jejuni* which has a single subunit OST (19), and then Suzuki's group, working with *S. cerevisiae* which has a single OST complex (20), both demonstrated directly that those OSTs catalyzed LLO hydrolysis. This left little doubt that mammalian OST also hydrolyzed LLO, but important questions remained: whether the STT3A and/or STT3B-OST isoforms hydrolyzed LLOs; whether N-glycosylation was shielded from competing LLO hydrolysis; and whether LLO hydrolysis was biologically useful or regulatable.

Multiple Factors Shield N-Linked Glycosylation from LLO Hydrolysis.

In both kidney and liver cells LLOs were significantly hydrolyzed by STT3B-OST, not STT3A-OST, and thus hydrolysis is not a simple catalytic error generally affecting OSTs. Possibly STT3B-OST allows water into the catalytic site while STT3A-OST excludes it. Although the catalytic mechanism proposed for transfer of glycan by OST (30, 49) may not be easily reconciled with hydrolysis, the high concentration of water in the aqueous ER lumen could drive LLO hydrolysis by STT3B-OST. Alternatively, a latent LLO hydrolysis activity by STT3A-OST might be disabled upon docking to the translocon (9) perhaps via allosteric interaction or creation of a water-tight seal. Our data do not distinguish these models. Therefore, it will be interesting to see whether LLO hydrolysis is altered by losses of the STT3A-OST-specific subunits DC2 and KPC2, which are involved in translocon docking (8, 9), as well as by the STT3B-OST-specific oxidoreductase subunits MagT1 and TUSC3 needed for cellular STT3B-OST function with nascent polypeptides but not catalysis with simple acceptors by the isolated complex (6, 12).

STT3A-OST must cotranslationally glycosylate acceptor sequons as they emerge from the translocation tunnel near STT3A-OST's catalytic site, and therefore STT3A-OST appears to reserve LLOs for N-glycosylation rather than hydrolyze them. In contrast, STT3B-OST glycosylates sequons posttranslationally, and should accommodate modest delays due to lack of bound, intact LLO. STT3B-OST thus appears amenable to LLO hydrolysis "moonlighting." Transferase competition in the presence of acceptor also weakened hydrolysis by STT3B-OST. These properties of mammalian OSTs should shield both cotranslational and posttranslational N-glycosylation from LLO hydrolysis.

Regulatory Potential of STT3A and TREX1. STT3A-OST does not hydrolyze LLOs, and in HEK293 cells STT3A knockout surprisingly elevated the TN- and NGI-1-sensitive fOSs linked to STT3B-OST and independent of ERAD. Such indirect modulation of STT3B-OST function by STT3A suggests that STT3A-OST may N-glycosylate a factor needed for fOS metabolism or transport. STT3A's impact on HEK293 fOSs was also emulated by TREX1: like STT3A knockout, loss of TREX1 increased STT3B-OST-dependent fOSs. In MEFs, TREX1 knockout increases fOS levels and LLO hydrolysis (18). We reconfirmed here (*SI Appendix, Fig. S5*) the increased LLO hydrolysis activity in TREX1-KO MEFs (18), but we did not detect altered LLO hydrolysis in control or STT3A-KO HEK293 cells transfected with TREX1, even though fOSs were diminished (Fig. 5). Consequently, TREX1 may have two modes for modulating fOSs, only one regulating LLO hydrolysis, but both impacting TREX1's suppression of IFN-stimulated genes (18).

External Loop 5 and LLO Selectivity. LLO hydrolysis specificity for STT3B-OST was surprisingly acceptor dependent (Fig. 2). *Campylobacter lari* PglB protein (a single subunit OST) has a disordered loop, designated EL5, involved in binding of both LLO donors and peptide acceptors (29, 30, 50). EL5 is near a pocket proposed to accommodate the glycan of LLO and becomes ordered in its amino or carboxyl portions when LLO or acceptor peptides bind, respectively. The two conformational changes are modular and interactive. EL5 is most compact with both substrates bound, and suited to restrict access/egress of LLO to/from the pocket. In a proof-of-principle experiment with PglB, a lipid-linked monosaccharide maneuvered past/under EL5 more readily than a larger oligosaccharide (30). High-resolution structures of the *S. cerevisiae* and mammalian OST complexes have not probed EL5 function further (8, 49, 51). It thus remains unclear how the respective EL5s might explain STT3A-OST's transferase preference for G₃M₉-LLO over M₉-LLO, and/or the absence of a transferase preference by STT3B-OST (12).

In acceptor-free conditions, we found that STT3B-OST was surprisingly selective during LLO hydrolysis, resembling the transferase activity of STT3A-OST rather than STT3B-OST; G₃M₉-LLO was hydrolyzed but not M₉-LLO. However, with acceptor peptide present, LLO hydrolysis by STT3B-OST mirrored its own transferase activity to utilize both G₃M₉-LLO and M₉-LLO. In this way, STT3B-OST emulated STT3B-like PglB of *C. jejuni*, which hydrolyzes LLO in response to reduced osmotic stress, and seems more selective for full-length LLO as a hydrolase than as a transferase (20).

Extrapolating from the properties of *C. lari* PglB (29, 30, 50), we envision both the polyisoprenoid and oligosaccharide portions of LLO interacting with STT3B-OST while EL5 is still fully disordered. This would permit the oligosaccharide (independent of size) full access to its pocket. LLO-induced amino-terminal modular compaction of EL5 might then selectively hinder egress of oligosaccharide from the pocket. The larger dolichol-linked G₃M₉Gn₂ would be trapped and subject to hydrolysis, while the smaller dolichol-linked M₉Gn₂ might pass through/under the partially compacted EL5 to dissociate from STT3B-OST without hydrolysis—a "catch-and-release" mechanism. Upon binding of peptide in addition to LLO, full ordering of EL5 in both its amino and carboxyl portions would form a more restrictive compact loop. M₉-LLO as well as G₃M₉-LLO would then be trapped and available for both hydrolysis and transfer by STT3B-OST, as we observed (Fig. 2).

Summary. STT3B-OST, but not STT3A-OST, hydrolyzes LLO to generate fOSs which can be suppressed by STT3A and TREX1. Additional features of STT3B-OST allow toggling between transferase and hydrolysis modes. N-glycosylation, especially at the translocon, is thus protected from competing LLO hydrolysis which generates regulatable fOSs (*SI Appendix, Fig. S6*).

Materials and Methods

Cell Culture. Human HEK293-WT, STT3A-KO, and STT3B-KO kidney lines (37) and WT TREX1-KO mouse fibroblasts (18) were grown in DMEM (low glucose; Thermo Fisher no. 11885092) supplemented with 10% FBS (Atlanta Biological). Human Huh7.5.1 WT, STT3A-KO, STT3B-KO, and rescue hepatocyte lines (41) were grown in DMEM (high glucose; Thermo Fisher no. 11995065) with 10% heat-inactivated FBS, 1× L-glutamine and 1× nonessential amino acids. Cells were cultured in a humidified 5% CO₂ atmosphere, then harvested directly with methanol for FACE analysis of saccharides (39) or permeabilized (32).

FACE. Saccharide classes including LLOs, N-glycans, fOSs, and monosaccharides were isolated from methanolic sonicates of live or permeabilized cells and analyzed by FACE (38, 39). LLO glycans were released from dolichol with mild acid, and N-glycans were released with endo H (New England Biolabs no. P0703L) for glycopeptides or PNGase F (Prozyme no. GKE-5003) for glycoproteins. Glycans were labeled with 7-amino-1,3-naphthalenedisulfonic acid (ANDS) (AnaSpec no. 81529), monosaccharides were labeled with 2-aminoacridone (AMAC) (Sigma no. 06627), and samples were normalized to total cellular protein for FACE

analysis. Gel images were acquired with a UVP Chemidoc-It II scanner, and saccharides were quantified with VisionWorks software.

Permeabilization of Plasma Membranes with Pore-Forming Toxin. Cells in 10-cm dishes were refed within 24 h before ALO treatment to achieve 70–90% confluence, and permeabilized with ALO as described for streptolysin O (32). Dishes on ice were rinsed twice with ice-cold PBS, 20 nM ALO (4 mL, ice-cold in PBS) was added for 4 min with periodic swirling on ice, and dishes were rinsed again with ice-cold PBS. Dishes were then incubated for 4 min at 37 °C with prewarmed transport buffer (TB) (78 mM KCl, 4 mM MgCl₂, and 50 mM K-Hepes, pH 7.2), then placed directly on ice for 10 min. If desired the conditioned TB was collected to obtain diffusible cytosolic contents, while the cell bodies were harvested for luminal contents or used for OST reactions.

OST Reactions. ALO-permeabilized cells were incubated for 30–60 min in TB containing 0 or 400 μM UDP-Glc, 200 μM UDP-GlcNAc, 50 μM GDP-Man,

0.2 mM AMP, 10 mg/mL CSN, and 10 mg/mL DMN, with 50 μM CP/AP and 5 μM NGI-1 as indicated. After discarding the reaction buffer, cell bodies were scraped into methanol with sonication, followed by isolation of glycopeptides, fOSs, and LLOs all from the same dishes for direct comparison.

Statistics. Graphpad Prism 7 was used to calculate mean ± SD and perform statistical analyses. Where two groups were compared, a two-tailed *t* test was used. For multiple groups, ordinary one-way ANOVA (no matching or pairing) was performed using Tukey's multiple comparisons test. For both the ANOVA and the *t* test, *P* < 0.05 was considered significant.

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- Varki A (2017) Biological roles of glycans. *Glycobiology* 27:3–49.
- Samuelson J, et al. (2005) The diversity of dolichol-linked precursors to Asn-linked glycans likely results from secondary loss of sets of glycosyltransferases. *Proc Natl Acad Sci USA* 102:1548–1553.
- Helenius A, Aebi M (2004) Roles of N-linked glycans in the endoplasmic reticulum. *Annu Rev Biochem* 73:1019–1049.
- Kelleher DJ, Gilmore R (2006) An evolving view of the eukaryotic oligosaccharyltransferase. *Glycobiology* 16:47R–62R.
- Shrimal S, Trueman SF, Gilmore R (2013) Extreme C-terminal sites are posttranslocationally glycosylated by the STT3B isoform of the OST. *J Cell Biol* 201:81–95.
- Cherepanova NA, Shrimal S, Gilmore R (2014) Oxidoreductase activity is necessary for N-glycosylation of cysteine-proximal acceptor sites in glycoproteins. *J Cell Biol* 206:525–539.
- Cherepanova N, Shrimal S, Gilmore R (2016) N-linked glycosylation and homeostasis of the endoplasmic reticulum. *Curr Opin Cell Biol* 41:57–65.
- Braunger K, et al. (2018) Structural basis for coupling protein transport and N-glycosylation at the mammalian endoplasmic reticulum. *Science* 360:215–219.
- Shrimal S, Cherepanova NA, Gilmore R (2017) DC2 and KCP2 mediate the interaction between the oligosaccharyltransferase and the ER translocon. *J Cell Biol* 216:3625–3638.
- Ruiz-Canada C, Kelleher DJ, Gilmore R (2009) Cotranslational and posttranslational N-glycosylation of polypeptides by distinct mammalian OST isoforms. *Cell* 136:272–283.
- Shrimal S, Ng BG, Losfeld M-E, Gilmore R, Freeze HH (2013) Mutations in STT3A and STT3B cause two congenital disorders of glycosylation. *Hum Mol Genet* 22:4638–4645.
- Kelleher DJ, Karaoglu D, Mandon EC, Gilmore R (2003) Oligosaccharyltransferase isoforms that contain different catalytic STT3 subunits have distinct enzymatic properties. *Mol Cell* 12:101–111.
- Chantret I, Moore SEH (2008) Free oligosaccharide regulation during mammalian protein N-glycosylation. *Glycobiology* 18:210–224.
- Anumula KR, Spiro RG (1983) Release of glucose-containing polymannose oligosaccharides during glycoprotein biosynthesis. Studies with thyroid microsomal enzymes and slices. *J Biol Chem* 258:15274–15282.
- Gao N, Shang J, Lehrman MA (2005) Analysis of glycosylation in CDG-Ia fibroblasts by fluorophore-assisted carbohydrate electrophoresis: Implications for extracellular glucose and intracellular mannose 6-phosphate. *J Biol Chem* 280:17901–17909.
- Cline A, et al. (2012) A zebrafish model of PMM2-CDG reveals altered neurogenesis and a substrate-accumulation mechanism for N-linked glycosylation deficiency. *Mol Biol Cell* 23:4175–4187.
- Gao N, et al. (2011) Mannose-6-phosphate regulates destruction of lipid-linked oligosaccharides. *Mol Biol Cell* 22:2994–3009.
- Hasan M, et al. (2015) Cytosolic nuclease TREX1 regulates oligosaccharyltransferase activity independent of nuclease activity to suppress immune activation. *Immunity* 43:463–474.
- Harada Y, et al. (2013) Eukaryotic oligosaccharyltransferase generates free oligosaccharides during N-glycosylation. *J Biol Chem* 288:32673–32684.
- Nothaft H, Liu X, McNally DJ, Li J, Szymanski CM (2009) Study of free oligosaccharides derived from the bacterial N-glycosylation pathway. *Proc Natl Acad Sci USA* 106:15019–15024.
- Barre Y, et al. (2017) A conserved DGGK motif is essential for the function of the PglB oligosaccharyltransferase from *Campylobacter jejuni*. *Glycobiology* 27:978–989.
- Harada Y, Masahara-Negishi Y, Suzuki T (2015) Cytosolic-free oligosaccharides are predominantly generated by the degradation of dolichol-linked oligosaccharides in mammalian cells. *Glycobiology* 25:1196–1205.
- Suzuki T, et al. (2002) Endo-β-N-acetylglucosaminidase, an enzyme involved in processing of free oligosaccharides in the cytosol. *Proc Natl Acad Sci USA* 99:9691–9696.
- Huang C, et al. (2015) Endo-β-N-acetylglucosaminidase forms N-GlcNAc protein aggregates during ER-associated degradation in Ngly1-defective cells. *Proc Natl Acad Sci USA* 112:1398–1403.
- Suzuki T, Funakoshi Y (2006) Free N-linked oligosaccharide chains: Formation and degradation. *Glycoconj J* 23:291–302.
- Quan EM, et al. (2008) Defining the glycan destruction signal for endoplasmic reticulum-associated degradation. *Mol Cell* 32:870–877.
- Hebert DN, Molinari M (2012) Flagging and docking: Dual roles for N-glycans in protein quality control and cellular proteostasis. *Trends Biochem Sci* 37:404–410.
- Suzuki T, Kitajima K, Emori Y, Inoue Y, Inoue S (1997) Site-specific de-N-glycosylation of diglycosylated ovalbumin in hen oviduct by endogenous peptide: N-glycanase as a quality control system for newly synthesized proteins. *Proc Natl Acad Sci USA* 94:6244–6249.
- Lizak C, Gerber S, Numao S, Aebi M, Locher KP (2011) X-ray structure of a bacterial oligosaccharyltransferase. *Nature* 474:350–355.
- Napiórkowska M, et al. (2017) Molecular basis of lipid-linked oligosaccharide recognition and processing by bacterial oligosaccharyltransferase. *Nat Struct Mol Biol* 24:1100–1106.
- Martys JL, Shevell T, McGraw TE (1995) Studies of transferrin recycling reconstituted in streptolysin O permeabilized Chinese hamster ovary cells. *J Biol Chem* 270:25976–25984.
- Anand M, et al. (2001) Requirement of the Lec35 gene for all known classes of monosaccharide-P-dolichol-dependent glycosyltransferase reactions in mammals. *Mol Biol Cell* 12:487–501.
- Gao N, Lehrman MA (2002) Coupling of the dolichol-P-P-oligosaccharide pathway to translation by perturbation-sensitive regulation of the initiating enzyme, GlcNAc-1-P transferase. *J Biol Chem* 277:39425–39435.
- Gao N, Shang J, Lehrman MA (2008) Unexpected basis for impaired Glc3Man9GlcNAc2-P-P-dolichol biosynthesis by elevated expression of GlcNAc-1-P transferase. *Glycobiology* 18:125–134.
- Lopez-Sambrooks C, et al. (2016) Oligosaccharyltransferase inhibition induces senescence in RTK-driven tumor cells. *Nat Chem Biol* 12:1023–1030.
- Harada Y, Hirayama H, Suzuki T (2015) Generation and degradation of free asparagine-linked glycans. *Cell Mol Life Sci* 72:2509–2533.
- Cherepanova NA, Gilmore R (2016) Mammalian cells lacking either the cotranslational or posttranslational oligosaccharyltransferase complex display substrate-dependent defects in asparagine linked glycosylation. *Sci Rep* 6:20946.
- Gao N, Lehrman MA (2006) Non-radioactive analysis of lipid-linked oligosaccharide compositions by fluorophore-assisted carbohydrate electrophoresis. *Methods Enzymol* 415:3–20.
- Gao N, Holmes J, Lehrman MA (2013) Letter to the glycoforum: Improved protocols for preparing lipid-linked and related saccharides for fluorophore-assisted carbohydrate electrophoresis (FACE). *Glycobiology* 23:1111.
- Nilsson I, et al. (2003) Photocross-linking of nascent chains to the STT3 subunit of the oligosaccharyltransferase complex. *J Cell Biol* 161:715–725.
- Marceau CD, et al. (2016) Genetic dissection of Flaviviridae host factors through genome-scale CRISPR screens. *Nature* 535:159–163.
- Karaoglu D, Kelleher DJ, Gilmore R (2001) Allosteric regulation provides a molecular mechanism for preferential utilization of the fully assembled dolichol-linked oligosaccharide by the yeast oligosaccharyltransferase. *Biochemistry* 40:12193–12206.
- Misaghi S, Pacold ME, Blom D, Ploegh HL, Korbel GA (2004) Using a small molecule inhibitor of peptide: N-glycanase to probe its role in glycoprotein turnover. *Chem Biol* 11:1677–1687.
- Misaghi S, Korbel GA, Kessler B, Spooner E, Ploegh HL (2006) z-VAD-fmk inhibits peptide:N-glycanase and may result in ER stress. *Cell Death Differ* 13:163–165.
- Caserta TM, Smith AN, Gultice AD, Reedy MA, Brown TL (2003) Q-VD-OPh, a broad spectrum caspase inhibitor with potent antiapoptotic properties. *Apoptosis* 8:345–352.
- Liu Y, Choudhury P, Cabral CM, Sifers RN (1999) Oligosaccharide modification in the early secretory pathway directs the selection of a misfolded glycoprotein for degradation by the proteasome. *J Biol Chem* 274:5861–5867.
- Hasan M, et al. (2013) Trex1 regulates lysosomal biogenesis and interferon-independent activation of antiviral genes. *Nat Immunol* 14:61–71.
- Kucej M, Fermainnt CS, Yang K, Irizarry-Caro RA, Yan N (2017) Mitotic phosphorylation of TREX1 C terminus disrupts TREX1 regulation of the oligosaccharyltransferase complex. *Cell Rep* 18:2600–2607.
- Wild R, et al. (2018) Structure of the yeast oligosaccharyltransferase complex gives insight into eukaryotic N-glycosylation. *Science* 359:545–550.
- Shrimal S, Cherepanova NA, Gilmore R (2017) One flexible loop in OST lassos both substrates. *Nat Struct Mol Biol* 24:1009–1010.
- Bai L, Wang T, Zhao G, Kovach A, Li H (2018) The atomic structure of a eukaryotic oligosaccharyltransferase complex. *Nature* 555:328–333.