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SPOTLIGHT

A pyrophosphatase that regulates lipid precursors of *N*-glycosylation

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The oligosaccharide used for protein *N*-glycosylation in the ER is built as a glycolipid. A recent study by Li, Suzuki, and colleagues (https://doi.org/10.1083/jcb.202501239) identifies a long-sought enzyme that hydrolyzes this lipid as part of a possible homeostatic/quality control mechanism.

Protein *N*-glycosylation—the attachment of an oligosaccharide chain to an asparagine residue—is essential for life as it affects the structure, quality control, trafficking, and function of the majority of proteins that enter the eukaryotic secretory pathway, including antibodies, signaling receptors, and cell adhesion molecules. It occurs in the lumen of the ER by a highly conserved multistep process in which an oligosaccharide chain (glycan) is first assembled on a dolichol lipid carrier, the glycan being attached via a pyrophosphate (-PP-) bond to the lipid. This glycolipid is termed oligosaccharide-PP-dolichol (alternatively dolichol-linked oligosaccharide, or simply DLO). Next, the glycan is transferred from DLO to specific asparagine residues—within the sequence motif NXS/T (X = any amino acid except proline)—in nascent proteins by the enzyme oligosaccharyltransferase (OST) (Fig. 1, reaction 1) (1).

Free oligosaccharides are inevitably released during *N*-glycosylation and subsequent glycoprotein quality control and can be viewed as metabolic waste, the cost of doing business. Thus, neutral oligosaccharides are produced from DLOs in the ER lumen by the hydrolytic activity of OST (Fig. 1, reaction 2), as well as in the cytoplasm, where they are cleaved off retrotranslocated misfolded *N*-glycoproteins by the glyco-amidase Ngly1. The former are transported to the cytoplasm, and in both cases, the oligosaccharides are degraded. Although the glycan is lost in this process, the dolichol-PP generated by OST action is converted to dolichol-P by the enzyme Cax4 (Fig. 1) and recycled for use in DLO biosynthesis. It is worth noting that dolichol is metabolically expensive—synthesis of a typical 100-carbon dolichol chain from acetate is coupled to the hydrolysis of >60 ATPs—and recycling is key to having sufficient quantities of this molecule available.

Surprisingly, oligosaccharide monophosphates (alternatively termed phosphorylated oligosaccharides, or POSs) are also produced. These are derived from DLOs by the action of a pyrophosphatase (DLO-PPase, EC 3.6.1.44) (Fig. 1, reaction 4), whose activity was originally recognized in the 1970s–1980s (2, 3, 4). What is the biological relevance of this enzyme? Its existence is startling, as its unregulated activity would seem to run counter to DLO biosynthesis, compromising the source of oligosaccharide for *N*-glycosylation. However, POS levels are normally low and become elevated only when DLO biosynthesis is impaired, such as in cultured mammalian cells grown in low glucose (5) or in the cells of patients with certain congenital disorders of glycosylation where immature DLOs accumulate (6, 7). This suggests that DLO-PPase activity may serve a protective or homeostatic function. Progress on this question has been slow because the molecular identity of DLO-PPase was not known despite significant advances almost a decade ago (4, 8).

In this issue of *JCB*, Shengtao Li, Tadashi Suzuki and coworkers now report the identification of DLO-PPase from budding yeast, naming the enzyme Llp1 (lipid-linked oligosaccharide pyrophosphatase) (9). They show that Llp1 is a Golgi-localized membrane protein, with its active site facing the lumen (Fig. 1). The subcellular segregation of Llp1 in the Golgi from the DLO biosynthetic pathway in the ER provides a clear means to prevent uncontrolled destruction of DLOs in the ER, consistent with a regulatory role for this enigmatic enzyme.

The discovery of Llp1 by Li et al. (9) includes several elements, and it is worth highlighting these before turning to the details of their paper and the bigger picture. The authors make use of yeast, as DLO biosynthesis in this system is essentially identical to that found in mammalian cells (1). DLO assembly starts with the phosphorylation of dolichol to dolichol-P, followed by the transfer of a sugar phosphate (*N*-acetylglucosamine-phosphate, GlcNAc-P) to generate GlcNAc-PP-dolichol. Subsequent glycosyltransfer reactions, catalyzed by a series of Alg proteins, add mannose (Man) and glucose (Glc) to generate mature DLO ($Glc_3Man_9GlcNAc_2$-PP-dolichol) for use in *N*-glycosylation. Of particular note, the pathway in both yeast and mammals is topologically split across the ER membrane. The first seven steps produce the lipid intermediate $Man_5GlcNAc_2$-PP-dolichol (M5-DLO) on the cytoplasmic face of the ER, whereas the remaining seven reactions that extend M5-DLO to mature DLO ($Glc_3Man_9GlcNAc_2$-PP-dolichol) occur on the luminal side. For this to

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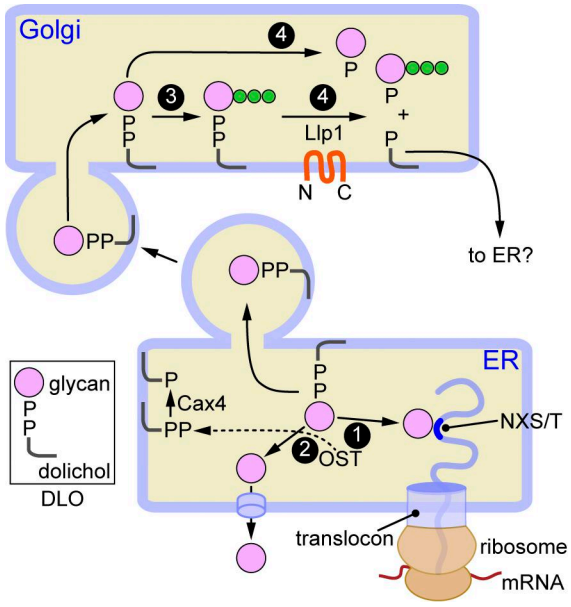

Figure 1. **DLO catabolism in yeast.** OST transfers the glycan from DLO to *N*-glycosylate nascent proteins at an NXS/T motif (reaction 1). OST also hydrolyzes DLO to produce neutral glycans (reaction 2), which are transported into the cytoplasm and degraded. Reactions 1 and 2 generate dolichol-PP (dotted line), which is processed by Cax4 to dolichol-P and recycled for DLO biosynthesis. DLO also moves to the Golgi apparatus, likely by vesicular transport, where a polymannose chain (green beads) is added (reaction 3). The DLO-PPase Llp1, a small membrane protein (in red), cleaves both unmodified and mannosylated DLOs to release POSs and dolichol-P (reaction 4). The latter may be recycled to the ER.

happen, M5-DLO must flip across the ER membrane, a process catalyzed by a specific lipid channel or scramblase that prefers M5-DLO over DLOs with smaller, larger, or aberrant glycans (10). Thus, DLOs at different stages of maturation are available on both sides of the ER membrane.

We now turn to the paper. Li et al. (9) begin by showing that POSs in yeast cells are oligosaccharide monophosphates, as expected for the products of a pyrophosphatase enzyme acting on DLOs. The POSs are found in membrane fractions, suggesting that they are produced by a luminal pyrophosphatase reaction. Consistent with this, Li et al. show that POSs are elevated in yeast strains that lack luminally oriented glycosyltransferases responsible for elongating M5-DLO to mature DLO. These strains accumulate incomplete DLOs and a proportional high amount of the corresponding POSs. For example, cells that lack the Alg3 mannosyltransferase, which converts M5-DLO to M6-DLO on the luminal side of the ER, accumulate M5-DLO as well as the corresponding POS ($Man_5GlcNAc_2$-P). Importantly, they also accumulate unusually large POSs carrying polymannose chains that are typically installed by Golgi-localized mannosyltransferases. The takeaway from these data is that even though DLOs are synthesized in the ER, the structures of the resulting POSs reflect exposure of DLOs and/or POSs to Golgi enzymes. Thus, DLOs must traffic to the Golgi apparatus to encounter DLO-PPase.

Li et al. (9) next set out to purify DLO-PPase from yeast microsomes, using an assay in which they monitored conversion of exogenously supplied M5-DLO to $Man_5GlcNAc_2$-P. Strangely, they found a significant portion (~20%) of the activity in the supernatant fraction after salt-washing the membranes and capitalized on this observation to arrive at a sufficiently enriched fraction that they subjected to shotgun proteomics, eventually identifying 33 protein candidates. On testing each of these by determining POS levels in the corresponding yeast deletion strain, they identified Llp1 as a DLO-PPase. Deletion of the *LLP1* gene eliminated POS production as well as DLO-PPase activity in microsomes, both being restored by overexpression of the protein in the deletion strain. A high-confidence model of Llp1 generated using AlphaFold predicts a protein with four transmembrane spans. Protease protection assays show that both the N and C terminus of the protein are exposed to the cytoplasm. Subcellular fractionation experiments indicate that Llp1 co-fractionates with a Golgi marker and is clearly separated from the ER.

Interestingly, sequence analyses show that Llp1 is related to a family of proteins resembling VanZ, a protein of previously unknown function encoded within the vancomycin resistance gene cluster in bacteria. Vancomycin binds lipid II, a DLO-like molecule that provides the building block for bacterial peptidoglycan (cell wall). Li et al. (9) purified a bacterial VanZ homolog and showed that it too has DLO-PPase activity, although its natural substrate would be expected to be lipid II. It is possible that VanZ may act to normalize lipid II levels should they become dysregulated, or if abnormal lipid II variants accumulate, similar to the proposal that Llp1 regulates DLO levels. Llp1, VanZ, and other family members share several conserved motifs—in Llp1, these are all located on the luminal side of the protein, consistent with the site of POS production noted above.

The discovery of Llp1 is only the end of the beginning of this story of DLO catabolism. Many questions remain. Surprisingly, mammalian cells do not have an Llp1 homolog, and despite extensive conservation of the core glycosylation machinery between yeast and mammals, the latter catabolize DLOs somewhat differently. Mammalian POSs are found in the cytoplasm, not within the endomembrane system (4, 5, 6). These POSs may be generated luminally, as in yeast, and then exported to the cytoplasm, as seen for neutral oligosaccharides generated by OST-mediated hydrolysis of DLOs (Fig. 1), but thus far there is no direct evidence for this possibility (6). It seems more likely that mammalian POSs are generated by a cytoplasmically oriented DLO-PPase, distinct from the activity discovered by Li et al. (9) in yeast. However, similar to the situation in yeast, the mammalian DLO-PPase activity fractionates with Golgi markers (4), preventing it from a potentially destructive direct encounter with the DLO biosynthetic pathway in the ER. The DLOs consumed by this enzyme are likely delivered to the Golgi by vesicular transport (as shown for yeast in Fig. 1). To add to the complexity of the situation in mammalian cells, brefeldin A treatment, which collapses some of the Golgi compartment into the ER, produces luminal POSs in addition to the expected cytoplasmic pool. These POSs are modified by Golgi glycosyltransferases, analogous to the polymannose modifications seen in

yeast POSs. What is the origin of luminal POSs in BFA-treated cells? Perhaps there are two DLO-PPases, each one oriented differently in the membrane, with the luminally oriented enzyme being revealed/activated only during BFA-induced compartment mixing. These points will only be resolved through identification of the mammalian enzymes and resolution of their subcellular location and membrane topology, following the yeast example.

Finally, there are outstanding questions about the biological relevance of this DLO catabolic process. It may be that the pyrophosphatase activity is simply a cellular mechanism to conserve and recycle metabolically expensive dolichol-P, extracting it from immature or abnormal DLOs that cannot be used for *N*-glycosylation or from DLOs that escape the ER. An intriguing further possibility is that POSs themselves may have bioactivity, either within the cell as suggested by Li et al. (9) and others, or upon secretion into the extracellular medium.

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