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A tale of short tails, through thick and thin: investigating the sorting mechanisms of Golgi enzymes

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The Golgi apparatus is an important site for the modification of most secreted and membrane proteins. Glycan processing is the major class of modification and is mediated by a large number of Golgi-resident glycosyltransferases and glycosidases. These Golgi enzymes are largely type II transmembrane domain (TMD) proteins consisting of a short N-terminal cytosolic tail, a relatively short TMD and a lumenal 'stem/stalk' region which acts as a spacer between the catalytic domain and the lipid bilayer. The cytosolic tail, TMD, and stem together make what is termed the CTS domain which is responsible for the specific localisation of these enzymes within sub-Golgi compartments via multiple mechanisms. In addition, the catalytic domains of some Golgi enzymes are secreted as a consequence of proteolytic cleavage within their TMDs or stem regions. Finally, there is evidence to suggest that when the retention of Golgi enzymes is perturbed they are targeted for lysosomal degradation.

Keywords: cisternal maturation; COPI; glycosylation; glycosyltransferase; golgi; golgi enzyme; membrane thickness; sorting motifs

Secreted and membrane proteins are synthesised at the endoplasmic reticulum (ER) where they are integrated into the ER membrane or released into the ER lumen [1]. In the ER, newly synthesised proteins undergo protein folding and the addition of the N-linked glycan core structure [2]. Glycosylation involves the modification of specific protein residues with a wide range of different carbohydrate moieties, often acting in pathways of sequential modification [2]. Glycosylation modifications can exhibit a vast degree of heterogeneity, and can have significant implications for a protein's stability or function [3]. From the ER, these proteins are targeted to the different corners of the secretory pathway, for example some proteins are destined for retention in the ER while others are targeted to the plasma membrane or endolysosomal system via the Golgi apparatus. In addition to the ER, the Golgi apparatus functions as a major site of glycan addition and processing. As proteins progress through the Golgi apparatus, they are subjected to processing of N-linked glycans and the addition of O-linked glycans [2]. This is mediated by a large number of different glycosylation enzymes which display variable distributions across the Golgi cisternae.

Golgi-resident glycosylation enzymes belong to two subcategories: glycosyltransferases and glycosidases [4]. These enzymes exhibit substantial diversity, not only across eukaryota but within eukaryotic genomes [5]. For example, humans have over 300 genes encoding glycosylation enzymes while a plant genome can contain well in excess of 400 such genes [4,6]. A near universal feature of Golgi enzymes is that they are type II transmembrane proteins, characterised by having a short cytoplasmic tail at their N terminus (generally 5–20 amino acids), a single transmembrane domain (TMD) and a disordered stem/stalk region which acts

Abbreviations
EM, electron microscopy; ER, endoplasmic reticulum; MHD, μ-homology domain; SPPL3, signal peptide protease like-3; TGN, trans-Golgi network; TMD, transmembrane domain.

as a spacer between the bilayer and the luminally facing catalytic domain. Many Golgi enzymes are able to form homo or heterodimers through interactions via their TMDs and lumenal domains [7,8]. The catalytic domains have diverse sequences and structures and are responsible for enzymatic and substrate specificity [9]. The region comprising the cytoplasmic tail, TMD and stem/stalk has been termed the CTS domain and it is this domain which is responsible for the Golgi-targeting of these enzymes (Fig. 1) [10]. Over decades of research, each component of the CTS domain has been implicated in distinct Golgi-targeting mechanisms. These different CTS domains are also responsible for the specific sub-Golgi distributions of different enzymes. It is worth noting that particular enzymes are generally not restricted to single cisternae but are spread over 2–3 cisternae with their positions broadly corresponding to their order in their pathway of action. While the CTS domain is clearly sufficient for Golgi-localisation, what is uncertain is the relative contribution of the different parts of the CTS domain.

Any model for how Golgi enzymes are retained in the Golgi has to be integrated into what is known about membrane traffic within the Golgi. Transport vesicles leave the trans-Golgi network (TGN) for various destinations and so Golgi enzymes will need to avoid entering these along with cargo. In addition, there are transport vesicles that move between cisternae, and it is now widely believed that these carry Golgi enzymes back to earlier cisternae as cargo is moved forward in cisternae that progress toward the TGN – the cisternal maturation model (Fig. 2). This model posits that the Golgi cisternae are constantly maturing or progressing from a cis-Golgi cisterna to a trans-Golgi cisterna [11]. As cisternae mature, they develop a more 'trans-like' profile in terms of their protein and lipid content [11]. In order to maintain the polarity in cisternal content across the Golgi, Golgi-residents are selectively recycled back to earlier cisternae in vesicles generated by COPI, a vesicle coat made up of coatomer subunits. Cisternal maturation in combination with a vesicular counter-flow serves to provide stasis and ensure the correct localisation of Golgi enzymes [11]. Thus, the Golgi enzyme sorting machinery must not just ensure that Golgi enzymes are retained within the organelle, but also that the different enzymes are distributed to a differing subset of cisternae. This review will focus on the role that the cytoplasmic tails and TMDs of Golgi enzymes play in their sorting in the context of the cisternal maturation model.

Transmembrane domain-dependent sorting

Membrane thickness model

Initially the TMDs of a handful of Golgi enzymes were shown to be important for their Golgi-localisation, despite there being no discernible consensus in their primary sequences [12–16]. An initial comparison, based on

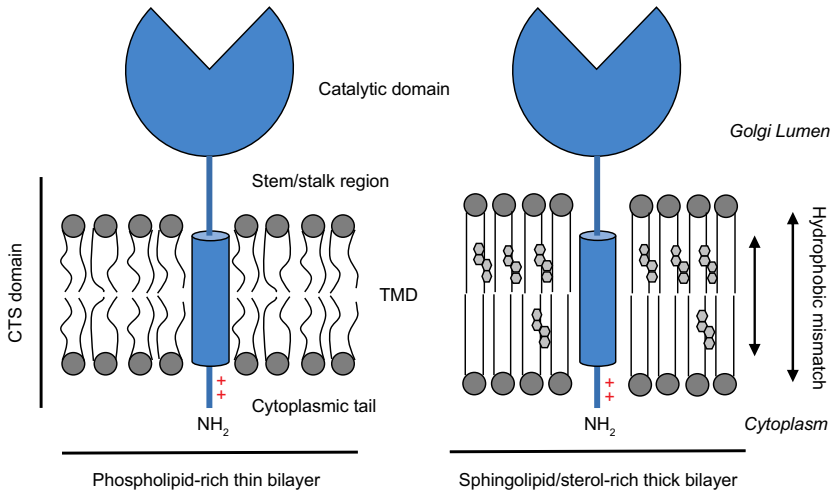

Fig. 1. Topology of Golgi enzymes and their residence in membranes of varying lipid content and thickness. The 'CTS' domain corresponds to the cytoplasmic tail, TMD and stem/stalk region. The residues of the N-terminal cytoplasmic tail are characteristically basic and can interact with coatomer directly or indirectly. The TMDs of Golgi enzymes are relatively short and consequently favour residence in membranes that are phospholipid-rich, disordered and hence thin. In contrast, the acyl chains of sphingolipid/sterol-rich membranes are saturated resulting in greater order, tighter chain packing and therefore greater bilayer thickness. Residence in thicker, sphingolipid/sterol-rich, membranes is energetically unfavourable due to an incompatibility between the width of the bilayer and the length of the hydrophobic TMD.

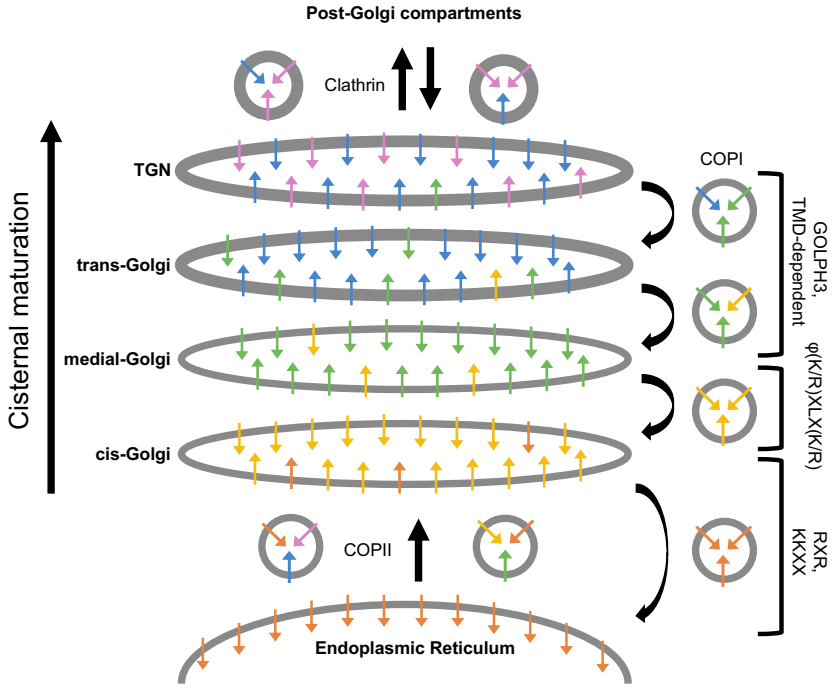

Fig. 2. Cisternal maturation model. Golgi cisternae progressively mature from cis to trans as they gradually transition from phospholipid-rich membranes containing cis-Golgi resident proteins to sphingolipid/sterol-rich membranes containing trans-Golgi residents. In order to maintain this polarity, lipids and Golgi-residents are subject to selective retrograde trafficking in COPI vesicles to their correct, earlier, cisternae of residence. This selective vesicular transport is likely to be mediated by several, nonmutually exclusive, mechanisms. It is likely that these different mechanisms are complementary and are of different prominence at different places within the Golgi stack. ER-resident cargo proteins are targeted for COPI-dependent retrograde transport from the Golgi to the ER by cytoplasmic KKXX or RXR ER-retrieval sequences. For intra-Golgi vesicular traffic, several cis-Golgi enzymes harbour a φ(K/R)XLX(K/R) consensus motif through which they interact directly with the δ/ζ1-COP subunits of coatomer. It is likely that the φ(K/R)XLX(K/R) motif is important for recycling within the early Golgi. At the late Golgi, GOLPH3 is recruited to the membrane by PtdIns4P where it can simultaneously interact with coatomer and the cytoplasmic tails of a different subset of Golgi enzymes to promote their segregation into COPI vesicles. Also at the late-Golgi, an increase in sphingolipid/sterol content results in a concomitant increase in membrane thickness. This favours the segregation of Golgi enzymes with short TMDs into phospholipid-rich budding COPI vesicles. In contrast, at the TGN clathrin-coated vesicles destined for post-Golgi compartments are enriched in sphingolipids/sterols which serves to exclude Golgi enzymes with short TMDs from these carriers.

a limited collection of available sequences, showed that Golgi enzymes exhibited shorter TMDs when compared to plasma membrane-resident type II transmembrane proteins [15]. Subsequent analysis, conducted on an expansive dataset of fungal and vertebrate sequences, showed that across eukaryotes, TMD length of bitopic membrane proteins generally increased in correlation with later residence in the secretory pathway [17]. The most striking difference was observed when comparing the length of the TMDs of ER/Golgi-residents versus post-Golgi-residents. Furthermore, it was shown that there is a preference for bulkier residues in the exoplasmic half of the TMDs of Golgi-residents when compared to that of their plasma membrane counterparts [17]. It is not yet fully resolved whether the plasma membrane really is thicker than the membranes of the ER and Golgi, but such an increase has at least been seen by electron microscopy (EM) including a study based on EM of frozen unfixed cells [18–20]. It was thus hypothesised that the short TMDs of the Golgi enzymes were retained at the Golgi as a result of their favoured residence in thinner membranes of the early secretory pathway [15]. In other words, it is more energetically favourable to have a short hydrophobic α-helix residing in a compatibly thin hydrophobic bilayer such as that of the ER and Golgi than in a thicker post-Golgi bilayer (see Fig. 1). It is worth noting that this hydrophobic mismatch of a long TMD in a short bilayer is thought to be more tolerable and energetically favourable than that of a short TMD in a thicker bilayer [21,22]. This could account for the traffic of biosynthetic cargo with long TMDs which are synthesised and inserted into the membrane at the ER and must traverse the secretory pathway to post-Golgi destinations.

The short TMDs of Golgi enzymes may favour residence in a thinner membrane of the early secretory

pathway, but what would cause a difference in membrane thickness? The lipid compositions of different membranes are not uniform throughout the secretory pathway [23]. Membranes of the ER are characteristically phospholipid-rich with low levels of sphingolipids and sterols [24]. However, while cis-Golgi cisternae are broadly comparable to ER membranes in their lipid composition, there is an increase in sphingolipid and sterol content in the trans-Golgi and on to post-Golgi compartments [25]. This variation in membrane composition is driven in part by a number of factors. While cholesterol is synthesised at the ER, or delivered to the ER from lysosomes, most is then transported to later secretory compartments via non-vesicular transport [26–28]. Furthermore, the localisation of sphingolipid synthesis enzymes at the trans-Golgi and plasma membrane can account for a raised sphingolipid content at these sites (Fig. 1) [29]. Moreover, the gradient in phospholipids, sphingolipids and sterols could be driven by the selective vesicular transport of lipids between Golgi cisternae in the context of cisternal maturation (Fig. 2) [25]. In comparison to phospholipid-rich membranes, sphingolipid and sterol-rich membranes exhibit greater acyl chain order and bilayer thickness [29]. Therefore, with the change in lipid content throughout the secretory pathway there should be a concomitant increase in bilayer thickness and order, and it also been proposed that longer TMDs could themselves contribute to thickening the bilayer [17,19,21]. It is this variation in bilayer thickness throughout the Golgi which lends itself to the retention of its resident enzymes through their relatively short TMDs [15].

The cisternal maturation model states that as cisternae progress from cis to trans, Golgi-residents are cycled back via COPI-dependent vesicular transport [11]. How would the membrane thickness model work in the context of cisternal maturation? Experiments with liposomes show that at the appropriate concentrations, sphingolipids and sterols segregate away from phospholipid-rich regions by phase separation [30,31]. If this occurred *in vivo,* it could result in the formation of a lipid domain which favours residence of Golgi enzymes and another which favours cargo proteins destined for post-Golgi compartments [32]. It is worth noting that this lipid segregation is unlikely to occur at the ER and cis-Golgi owing to the low levels of sphingolipids and sterols [25]. However, it is also possible that lipid segregation is driven by high curvature such as that found at Golgi rims and during the biogenesis of COPI vesicles [31,33]. Ordered lipid domains consisting of sphingolipids and sterols favour flat cisternal membranes and are less tolerant of curvature than the relatively disordered phospholipid-rich lipid domains [34]. This phenomenon has been demonstrated in giant unilamellar vesicles composed of sphingolipids, sterols and phospholipids through the application of high curvature via the extrusion of nanotubules [31]. It has been reported that COPI vesicles formed *in vitro* from isolated mammalian Golgi stacks have lower levels of sterols and sphingolipids, and more phospholipids, than the Golgi from which they budded [33]. This would be consistent with the vesicles preferentially selecting ER-like lipids, although a caveat is that the lipid composition of the Golgi was determined for the whole stack whereas the vesicles may have been budding from only a subset of cisternae. Such selection would certainly favour the Golgi enzymes, with their short TMDs, segregating into the budding COPI vesicle [25]. It is worth noting that unlike COPI, clathrin-coated vesicles at the trans-Golgi network (TGN) actually enrich for sphingolipids and sterols which therefore may serve to exclude Golgi enzymes from post-Golgi carriers [35]. Further support for the importance of lipids in the retention of Golgi-residents comes from the findings that genetic or chemical disruption of sphingolipid synthesis causes the mislocalisation of Golgi enzymes [36,37].

A difference between the membrane thickness of COPI vesicles and that of their cisternal membrane of origin has long been observed in fixed samples [38]. Furthermore, new evidence has arisen from applying EM tomography to cryopreserved *Chlamydomonas reinhardtii* [19]. The tomograms reveal a clear difference between the membrane thickness of budding COPI vesicles and cisternal membranes at the medial/trans-Golgi [19]. Interestingly, while there appears to be no difference in membrane thickness between the cis and medial-Golgi, there is a notable increase in thickness between the medial and trans-Golgi [19]. This would suggest that TMD-dependent sorting at the Golgi primarily occurs at the medial/trans interface with their being insufficient levels of sphingolipids at the cis-Golgi to affect the bilayer or promote lipid segregation (Fig. 2) [25]. In contrast, at the trans-Golgi the sphingolipid content is sufficient for segregation to occur due to the local presence of sphingolipid synthases (Fig. 3A) [29]. This would suggest that the recycling of enzymes from early Golgi cisternae occurs through mechanisms independent of the physiochemical properties of the TMD.

Sequence-specific transmembrane domain sorting

Studies on the role of the TMDs in Golgi retention have examined only a limited range of proteins but

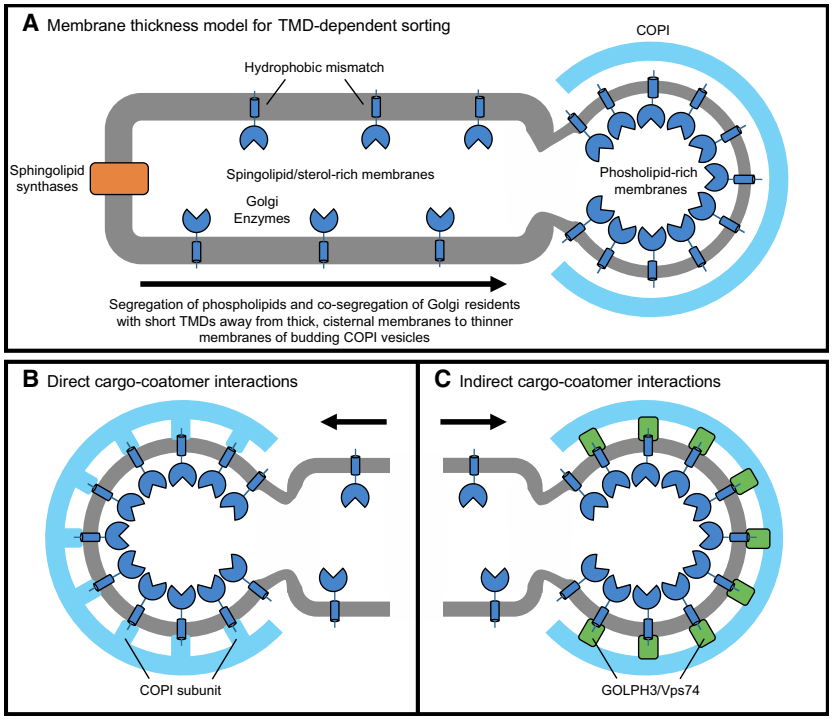

Fig. 3. Possible mechanisms of Golgi enzyme retention. (A) Sphingolipid synthases and selective vesicular transport of lipids drive a local increase in sphingolipid levels at the late-Golgi. The high curvature, or enrichment of short TMD proteins, associated with budding COPI vesicles may promote the segregation of phospholipids away from the sphingolipids and sterols of the cisterna and into the vesicular bud. It is subsequently energetically favourable for Golgi enzymes, with their relatively short TMDs, to segregate into the thinner phospholipid-rich membrane of the vesicular bud. Segregation of cargo can also be driven by the direct interaction of the cytoplasmic tails of the enzymes with various subunits of the COPI coat (B), or occur indirectly via COPI adaptors such as GOLPH3/Vps74 (C).

most have suggested that the key aspect for retention is the physiochemical properties of the TMD. However, recent studies conducted in plants highlighted a glutamine residue in the TMD of N-acetylglucosaminyltransferase I (GnTI) which was important for its targeting to the cis/medial-Golgi [39]. Mutagenesis of this residue caused GnTI to localise later in the Golgi and be directed to the vacuole for degradation [39]. It remains to be seen whether this residue regulates the localisation of the enzyme through an interaction with the membrane-spanning domain(s) of a proteinaceous COPI adaptor. On that note, a recent flurry of CRISPR screens in mammalian cells with readouts linked to glycosylation status has implicated the integral membrane protein TM9SF2 in the retention of a subset of Golgi enzymes [40–43]. TM9SF2 is a Golgi-localised nine transmembrane-spanning protein of unknown function which belongs in a family with three other proteins, all of which have a variety of orthologues across eukaryota [44]. Notably, all four TM9SF proteins have been detected in COPI vesicles generated from semi-intact cells [45]. Knockout of *TM9SF2* causes a global defect in glycosylation and the mislocalisation of various Golgi enzymes [40–43]. Furthermore, TM9SF2 was reported to interact with the enzyme Gb3 synthase (*A4GALT*) *in vitro* [41]. Interestingly, it was shown that TM9SF2 has a conserved KxD/E COPI-binding motif at its C terminus and disruption with point mutations or a C-terminal tag was sufficient to cause the protein to accumulate in post-Golgi compartments [44]. Could TM9SF2 and its relatives act as novel COPI adaptors by simultaneously interacting with the COPI coat and sequence motifs in the TMDs of Golgi enzymes? This would be analogous to the COPI adaptor Rer1 which detects ER-retrieval signals in the TMDs of a number of ER-resident proteins [46].

Cytoplasmic tail sorting motifs

VPS74/GOLPH3

Some of the early studies on Golgi enzyme retention that found a role for the TMD also found that the cytoplasmic tail could contribute to retention [12,13,47,48]. In some cases, the cytoplasmic tail on its own could impart a Golgi-sorting signal but the mechanism by which this signal was recognised was unclear [47,49]. This changed with the discovery that the yeast protein Vps74, a peripheral Golgi protein, could simultaneously interact with the cytoplasmic tails of a number of yeast mannosyltransferases and with coatomer (Fig. 3C) [50,51]. In the absence of Vps74, some Golgi-resident enzymes were mislocalised to the vacuole and glycosylation was perturbed [50,51]. Vps74 is conserved between fungi and metazoans and there are two mammalian orthologues: GOLPH3 and GOLPH3L. The mammalian orthologues can partially

rescue the phenotypes of yeast lacking Vps74 [50]. GOLPH3/Vps74 proteins interact with the δ-COP and β-COP components of the COPI coat through a conserved cluster of arginine residues within a short (≈ 50 amino acids) unstructured region near their N-termini [50,52]. Furthermore, the mammalian and yeast proteins share the ability to interact with the phosphoinositide lipid PtdIns4P which appears to be essential for their Golgi-localisation [36,53,54]. Levels of PtdIns4P increase from the cis- to the trans-Golgi [55]. It was demonstrated that GOLPH3/Vps74 proteins are recruited to the late-Golgi by PtdIns4P where they can interact with the cytoplasmic tails of Golgi enzymes. It is here that GOLPH3/Vps74 facilitates the retrograde transport of enzymes from late cisterna to the earlier Golgi compartments in which they normally reside [36]. In other words, GOLPH3 can be likened to a gatekeeper at the entrance to the post-Golgi compartments where it selectively sends Golgi enzymes backwards against the flow of the maturing queue of cisternae.

The consensus motif recognised by Vps74 was found to be (F/L)(L/I/V)XX(R/K) [50]; however, the recognition motif is less well-defined for GOLPH3 and GOLPH3L. To date, only a handful of enzymes have been shown to interact with GOLPH3 or have been shown to require GOLPH3 for their proper Golgi localisation (see Table 1) [56–59]. The work of several studies came to the consensus that their cytoplasmic tails share a motif loosely characterised as a membrane-proximal di-basic stretch preceded by a hydrophobic residue [56–59]. For example, C2GnT (*GCNT1*) was shown to interact with GOLPH3 via an LLLRR motif in its cytoplasmic tail, as was SialT (*ST6GAL1*) which has a similar LKK motif [56]. GOLPH3 is required for the incorporation of C2GnT and SialT into COPI vesicles *in vitro,* a requirement which was not shared with other COPI cargo [58]. In *Drosophila melanogaster*, the GOLPH3 ortholog was found to interact with, and be important for the trafficking of, the exostosin enzymes EXT1, EXT2 and EXTL3 [60]. It was also shown that the interaction of GOLPH3 with EXT1 and EXT2 was conserved in mammalian cells [60]. It is noteworthy that EXT1 and EXT2 form heterodimers [7]. In fact, several Golgi enzymes have been shown to heterodimerise with each other and possibly even form higher order oligomers [61]. Therefore, caution is needed when trying to identify sorting motifs in cytoplasmic tails given the possibility of enzymes interacting indirectly with GOLPH3 as part of a heterodimer. When considering the increase in complexity in mammalian Golgi enzyme biology relative to that of yeast it seems likely that only a small proportion of GOLPH3 clients are currently known. Interestingly, a bioinformatic analysis of Golgi-resident type II transmembrane proteins searching for a membrane-proximal di-hydrophobic, di-basic motif suggested the motif was present in 15% of proteins tested (33 enzymes) [62]. Further experimental interrogation is required to inform and compliment future bioinformatic analyses in order to elucidate the GOLPH3 recognition motif and complete client list.

Direct interactions between enzymes and coatomer

Several direct interactions have been described between the COPI coat and its cargo, many of which are associated with retrieval of ER residents from the cis-Golgi for return to the ER, as opposed to intra-Golgi trafficking of Golgi enzymes (Fig. 2). One of the best characterised ER-retrieval motifs is the dilysine motif: a membrane proximal KKXX or KXKXX sequence [66,67]. The motif is found at the cytoplasmic C terminus of many ER-resident type I transmembrane proteins and interacts directly with the α- and β'-COP subunits [68]. Another ER-retrieval motif is the RXR motif which is found in the cytoplasmic tails of integral membrane proteins of varying topology including multi-pass proteins that are components of multimeric complexes [69]. Often complete assembly of the multimeric complex masks the RXR motif and so suppresses ER-retrieval [69]. This serves to limit the premature progression of improperly assembled complexes through the secretory pathway. RXR motifs can also be masked by post-translational modification or the through the binding of regulatory proteins [69]. The position of the RXR motif in relation to the membrane appears critical for its function [70]. This can be illustrated for type II transmembrane proteins by comparing two different glycosylation enzymes. The poly-arginine stretch ^{3}RGERRRR^{9} in the cytoplasmic tail of human glucosidase-1 (*MOGS*) is positioned 29 residues distal to the membrane and is sufficient to target the protein to the ER [71]. In contrast, a similar ^{1}MRRRSR^{6} motif positioned immediately proximal to the membrane in the tail of GalNAc-T2 ((*GALNT2*) is sufficient for Golgi-targeting [63]. The molecular identity of the receptors and/or coat components involved in either arginine-based interaction is unclear but it would be surprising if it was not COPI-dependent. This intriguing bifurcation of the sorting signal, apparently based on membrane proximity alone, suggests the positioning of the cytoplasmic tails in the context of the structure of coatomer is functionally critical.

Table 1. Reported direct and indirect interactions between the cytoplasmic tails of Golgi enzymes and coatomer. All cytoplasmic tail sequences represent the extreme N terminus preceding the TMD as assigned according to UniProt with the exception of *GALNT8* [64]. Note that annotations of TMDs are not always precise and so the positions of the membrane proximal end of the cytoplasmic tails should be viewed with caution. Underlined residues highlight a proposed GOLPH3 recognition motif characterised by a membrane-proximal consensus sequence consisting of a hydrophobic residue upstream of a di-basic motif [62]. Residues in bold highlight a φ(K/R)XLX(K/R) motif corresponding to a δ-COP/ζ1-COP consensus binding motif [63]. Highlighted residues correspond to a WX(n1-6)(W/F) motif shown to bind to δ-COP [65]

Golgi enzyme gene	Cytoplasmic tail sequence	Reported interactor(s)
EXT1[a]	MQAKKRY	GOLPH3 [60]
EXT2[a]	MCASVKYNIRGPALIPRMKTKHRIY	GOLPH3 [60]
GALNT3	MAH**LKRLVK**LHIKRHYHKK	δ-COP [63], ζ1-COP [63]
GALNT4	MAVRWTWAGKSC	δ-COP [63]
GALNT6	**MRLLRR**RH	δ-COP [63]
GALNT8	MMF**WRKLPK**	δ-COP [63], ζ1-COP? [63]
GALNT12	MWGRTARRRCPRELRRGRE	GOLPH3 [57]
GCNT1	M**LRTLL**RRR	GOLPH3 [56,58], δ-COP [63], ζ1-COP [63]
GNPTAB	M**LFKLLQR**QTYTCLSHRYGLY	δ-COP [63], ζ1-COP [63]
POMGNT1	MDDWKPSPLIKPFGARKKRSWYLTWKYKLTNQRALRR	GOLPH3 [57]
ST6GAL1	MIHTNLKKK	GOLPH3 [58,59]
ST3GAL4	MVSKSRWK	GOLPH3 [59]

[a]Note that EXT1 and EXT2 form heterodimers so the ability of either tail to interact directly with GOLPH3 is not clear.

Recently a direct interaction between the cytoplasmic tails of Golgi enzymes and coatomer has been reported (Fig. 3B) [63]. Based on a previous observation that disease mutations in the cytoplasmic tail of GlcNAc-1-phosphotransferase (Ptase/*GNPTAB*) caused its mislocalisation from the Golgi to the lysosome, Liu and colleagues used a proximity-dependent labelling approach to find cytoplasmic tail-interacting proteins [63,72]. They leveraged the topology of Ptase, a double-pass enzyme with a cytoplasmic N- and C terminus, by placing the promiscuous biotin ligase BioID2 on the C terminus so as to biotinylate proteins that interacted with the N terminus [63]. After isolating and sequencing the interacting proteins, they found that, amongst others, the δ-COP and ζ1-COP subunits of coatomer were top hits when comparing the wild-type protein to the disease mutants [63]. Notably GOLPH3 was absent from the list of hits suggesting that the interaction with coatomer was GOLPH3-independent [63]. The cytoplasmic tail of Ptase and various cis-Golgi resident enzymes were found to bind directly to the μ-homology domain (MHD) of δ-COP while some also bound to ζ1-COP (see Table 1) [63]. Interestingly, the tails interacted with the binding pocket on δ-COP MHD that is structurally analogous to the binding pocket of the μ subunit of the AP-2 clathrin adaptor which recognises the endocytic sorting motif Yxxφ in cytoplasmic tails [63,73]. The δ-COP/ζ1-COP consensus binding motif was identified as φ(K/R)XLX(K/R) and it was shown that mutagenesis of this motif was, in most instances, sufficient to both ablate coatomer binding *in vitro* and disrupt Golgi-localisation *in vivo* [63]. Curiously, C2GnT, which was previously reported to interact with coatomer indirectly via GOLPH3, was also found to interact directly with coatomer [56,58,63]. The cytoplasmic tail of C2GnT, appears to have a putative GOLPH3 binding site that overlaps the δ-COP/ζ1-COP binding motifs, suggesting redundancy in the sorting signals in the cytoplasmic tail of at least one Golgi enzyme (Table 1), and disruption of both is required to ablate an interaction with coatomer [63]. However, it should be noted that in this study deletion of GOLPH3 did not affect the Golgi localisation of C2GnT, in contrast to what had been reported previously [54,61]. However, neither study examined the GOLPH3's closely related paralogue GOLPH3L. The binding properties of this protein have not been reported, but if it does bind C2GnT1 it may be that its expression level varies between cell lines.

Liu and colleagues also demonstrated *in vitro* binding between a peptide corresponding to the cytoplasmic tail of GalNAc-T4 (*GALNT4*) and δ-COP MHD via a Wx(n1-6)W/F motif to a binding pocket distinct from that which recognises φ(K/R)XLX(K/R) in other Golgi enzymes [63,65]. The Wx(n1-6)W/F motif is an evolutionarily conserved δ-COP MHD-interacting motif which has been described in a number of proteins involved in COPI biology including ARFGAP1, Scyl1 and Dsl1 [65,74–76]. It remains to be seen whether this interaction occurs *in vivo* with the full coat complex and the tail in the context of a membrane. Various

structures of coatomer suggest there may be sufficient flexibility in δ-COP and the COPI coat as a whole to accommodate for a direct interaction with the cytoplasmic tails [19,77,78]. Another curiosity is that Wx(n1-6)W/F motifs often feature amongst highly acidic stretches [65]; however, this does not appear to be the case for the motif in the tail of GalNAc-T4. Regardless, this potentially presents a novel COPI cargo-sorting motif in the context of Golgi enzymes and it will be interesting to explore its prevalence. For example, the motif appears to be present in the tails of POMGnT1 (Table 1) and a number of human fucosyltransferases, while a disease-causing missense mutation in *B3GALT6* appears to generate a synthetic Wx(n1-6)W/F motif in its cytoplasmic tail [79].

All the enzymes that have been shown to bind directly to coatomer are cis-Golgi residents while several of the GOLPH3 clients are localised later in the mammalian Golgi stack. Are these different sorting signals important for segregating different enzymes into vesicles that bud from different cisternae (Fig. 2)? In other words, are enzymes with a δ-COP/ζ1-COP-binding motif sorted from earlier cisterna, such as the medial-Golgi, while those with a GOLPH3-binding motif are sorted from the late-Golgi? This may result in distinct subclasses of COPI vesicles, defined by their cisterna of origin and their variable cargo loads, which serve to fine-tune the localisation of enzymes across the Golgi cisternae. Enzymes like C2GnT will be retained by both sorting motifs and benefit from redundant mechanisms to keep them from the late-Golgi. What is not clear is how the distinction is achieved between COPI vesicles associated with intra-Golgi traffic and those associated with ER-retrieval. These different subtypes of vesicle must differ in cargo content yet both have been proposed to select cargo by direct interactions between cargo and the COPI coat subunits. It is conceivable that there are differences in the conformation of the COPI coat in different sub-Golgi sites to facilitate distinct modes of direct cargo interaction and selection.

The membrane thickness model and tail-dependent sorting

So how do cytoplasmic tail-dependent mechanisms of Golgi-enzyme sorting fit alongside mechanisms of TMD-based retention based on the membrane thickness model? It seems quite possible that Golgi enzymes have multiple distinct, complimentary and additive mechanisms for Golgi-targeting (Fig. 3). The short TMDs of the Golgi enzymes could promote their segregation into the thinner and less ordered membranes of budding COPI vesicles. This would occur at the late-Golgi where the sphingolipid and cholesterol content is sufficiently high that most of the bilayer is thicker and ordered. The direct and/or indirect interaction of the cytoplasmic tails with coatomer then serves to contribute to this segregation, either by promoting segregation in its own right or by stabilising those which have segregated as a result of lipid composition. Once budding is complete, the vesicle loaded with Golgi enzymes and phospholipids can fuse with an earlier cisterna in order to maintain the correct lipid and protein distribution between the Golgi cisternae. We suggest that TMD-dependent and GOLPH3-dependent sorting occurs predominately at the late-Golgi while direct tail interactions with the δ/ζ1-COP subunits are important for sorting within the earlier part of the Golgi. At the cis-Golgi; dilysine, diarginine and other ER-retrieval mechanisms are important for retrograde trafficking from the Golgi to the ER. Evidence for a complimentary relationship between tail-dependent and TMD-dependent sorting is that genetic disruption of GOLPH3/Vps74 and genetic or chemical disruption of sphingolipid biosynthesis result in similar defects in Golgi enzyme sorting [36,37]. Evidence for an additive relationship between the two mechanisms is that Vps74 has negative genetic interactions with several genes involved in sphingolipid and phospholipid synthesis [36].

Turnover of mislocalised Golgi enzymes

A common feature across eukaryotic systems is that the loss of retention of Golgi enzymes, whether through perturbations in cytoplasmic tail- or TMD-dependent targeting mechanisms, often results in their targeting to the lysosome for degradation [39,50,51,60]. Many questions remain as to the details of this degradation mechanism. It is unclear whether enzymes leaking out of the Golgi take a direct path to endosomes and hence lysosomes or whether they first travel to the plasma membrane where they are rapidly endocytosed. In either case there must be cues for the detection of Golgi enzyme escapees. One possibility is that the lumenal portion of the enzymes is recognised in the context of the late-Golgi or post-Golgi compartments. The apparent general nature of lysosomal-targeting makes this mode of recognition seem unlikely owing to the great sequence and structural diversity of the lumenal domains of Golgi enzymes [2,9]. In contrast, the short cytoplasmic tails and short TMDs are features conserved across Golgi enzymes and across eukaryota. Is the hydrophobic mismatch of a Golgi enzyme's short TMD in a thicker late-Golgi or

post-Golgi membrane bilayer somehow sensed by a receptor? In yeast, a complex containing the Golgi-resident multi-pass transmembrane protein Tul1 has been implicated in vacuolar targeting of membrane proteins [80,81]. Tul1 is an E3 ubiquitin ligase that recognises membrane proteins with exposed polar residues within the bilayer and ubiquitinates their cytosolic lysines for targeting to the vacuole for degradation [81]. It is conceivable that enzymes of the Golgi which have leaked out to the TGN are recognised by Tul1 as a consequence of hydrophobic mismatch. However, despite the apparent conservation and general nature of the lysosomal targeting of mislocalised Golgi enzymes, a Tul1 relative in metazoans is missing [81]. Furthermore, many Golgi enzymes do not have any lysine residues in their cytosolic tails to serve as a substrate for ubiquitination. While it has been shown for other substrates that the N-terminal amine group can be ubiquitinated to initiate degradation [82], none of the known Tul1 substrates are ubiquitinated in this way (only one of which is a Golgi enzyme – Ktr1) [80]. Could another unknown factor be recognising hydrophobic mismatch at the late-Golgi? It would be informative to determine whether lysosomal-targeted Golgi enzymes are ubiquitinated. It is also worth noting that Golgi enzymes, as with all membrane proteins, generally have an enrichment of basic residues in their membrane-proximal cytoplasmic segments in accordance with the 'positive-inside rule' [83,84]. Could these cytosolic basic residues also contribute to the mechanism of detection and processing at the trans-Golgi or TGN (other than lysine residues serving as ubiquitin ligase substrates)? Data from existing genetic screens for Vps74 interactors or crosslinking experiments of Golgi enzyme reporters in GOLPH3/Vps74 knockout cells may be a good place to start the search for unknown factors.

Secretion of Golgi enzymes

Although Golgi enzymes normally reside in the Golgi in their full length form, they can undergo proteolytic cleavage by proteases residing in the secretory pathway [85]. Proteolysis occurs either in the disordered stem region or within the TMD allowing the liberated catalytic domain to traverse the secretory pathway to the extracellular environment [86,87]. In the absence of the appropriate donor nucleoside diphosphate sugars normally found in the Golgi lumen, the catalytic domains of the Golgi enzymes are unlikely to have significant enzymatic activity in the extracellular space, although they may retain the ability to bind their substrate glycans. Thus, the proteolytic cleavage of Golgi enzymes may serve to downregulate their function in the Golgi, or possibly even prevent escapees from binding glycans when they reach the surface [87]. The catalytic domains of various Golgi enzymes can be found in plasma [88,89] or in the media of cultured cells [90,91].

Recently, signal peptide protease like-3 (SPPL3), a Golgi-resident member of the SPP/SPPL family of proteins, has been implicated in the cleavage of Golgi enzymes [87]. SPP/SPPL proteases are highly conserved intramembrane proteases with a preference for substrates with a type II topology [92]. Initially, it was demonstrated that genetic manipulation of SPPL3 levels in tissue culture cells and knockout mice resulted in changes in the cleavage of a handful of N-glycosylation enzymes which led to a resultant change in global glycosylation [87]. Subsequent analysis of the secretome of SPPL3-deficient or -overexpressing cell lines revealed a plethora of new substrates, mostly Golgi enzymes associated with both N- and O-glycosylation [93]. Peptide analysis of the secreted enzymes revealed that the cleavage site was within the TMD towards the lumenal face with a preference for M or Y residues at position one [93]. While it appears that SPPL3 is the primary protease responsible for the cleavage of Golgi enzymes, similar analyses of the secretome of BACE1-inhibited [94] and SPPL2C-overexpressing cells [95] revealed a handful of Golgi enzyme substrates for these proteases, some of which were shared with SPPL3 [93]. This suggests that some enzymes are subject to cleavage by multiple different proteases. Interestingly, a number of the apparent substrates for SPPL2C are SNARE proteins and it has been suggested that the cleavage of SNAREs could indirectly alter the localisation of Golgi enzymes through the regulation of their trafficking [95].

Pathological and physiological implications of Golgi enzyme sorting

Glycosylation enzymes often act in a pathway of sequential sugar modifications and the efficiency of these pathways is thought to be dependent on the sequential compartmentalisation of these enzymes and their relative abundance. For example, the mislocalisation of a late-acting enzyme to an earlier part of the Golgi or the ER can result in the premature termination or excessive activation of glycan branching and such changes can have physiological consequences [96]. Similarly, a change in the levels of a particular enzyme, whether it be due to changes in extracellular secretion or lysosomal-degradation, can perturb glycan patterns. For example, knockdown of GalNAc-T3

(*GALNT3*) can trigger epithelial-mesenchymal transition through aberrant glycosylation of E-cadherin [97]. Furthermore, the relocation of GalNAc-T1 (*GALNT1*) from the Golgi to the ER drives an increase in O-glycosylation which leads to activation of matrix metalloproteinase MMP14 thus promoting tumour metastasis [98]. Over-expression of GOLPH3 is observed in a range of cancers and its oncogenic activity has been linked to the activation of AKT signalling, although the exact mechanism remains unclear [99]. Several studies have shown that a variation in GOLPH3 levels can modulate the trafficking and therefore activity of its Golgi enzyme clients leading to large-scale modification of the glycosylation status of the cell which influences various oncogenic pathways [56,59]. For example, knockdown of GOLPH3 in has been reported to cause a reduction in sialylation of various proteins including receptor tyrosine kinases and integrins resulting in a reduction in AKT signalling and cell motility [59]. These defects were linked to the interaction of GOLPH3 with its sialyltransferase clients and could be rescued with the overexpression of SialT. Moreover, overexpression of the GOLPH3 ortholog in *Drosophila melanogaster* was shown to impair heparan sulphate proteoglycan synthesis which lead to aberrant hedgehog glycosylation and subsequent morphogenic defects [60].

Conclusions and perspectives

The hunt for new coatomer adaptors

Despite the vast array of different Golgi enzymes with their variety of cytoplasmic tails and sub-Golgi localisation patterns, there are still only a few known direct or indirect interactions between the tails and the COPI coat. This is in contrast with the known diverse cargo interactions found for the distantly related clathrin/adaptor protein (AP) coats. This diversity reflects the ability of clathrin/AP coats to interact with an array of cargo-specific adaptors [100,101]. In contrast, recent proteomic profiling of COPI coats generated using different coatomer subunit isoforms suggested variation in core coat components was insufficient to alter cargo specificity [45]. While the core components of the COPI coat may not influence cargo selection directly, it is possible there are unknown cargo-specific COPI adaptors that fulfil this function. GOLPH3/Vps74 proteins have already been discovered and appear to function primarily in recycling cis/medial-residents from the late Golgi [50,51], but what other factors exist? It is of note in this context that unlike metazoa and fungi, plants lack an orthologue of Vps74 [50,51]. Since the cytoplasmic tails of these enzymes are also small, it is conceivable that any new adaptors will simultaneously interact with the lipid bilayer, as is the case for GOLPH3/Vps74. While coincidence detection by novel adaptors may pose a technical challenge in future affinity chromatography screens, it may be a key mechanism for coupling cargo selection with sub-Golgi localisation.

Concluding remarks

While considerable progress has been made in understanding the trafficking of Golgi enzymes over the past 30 years, it is clear there are still large gaps in our knowledge. Recent exciting structural insights into the COPI coat will help answer this question [19,77,78]. It remains to be seen if there are additional auxiliary coat adaptors to be discovered which provide a means for cargo selectivity and therefore enhance COPI vesicle diversity. The study of intra-Golgi trafficking has traditionally been technically challenging and at times fraught with controversy, in part, due to difficulties in resolving the short distances between cisternae by light microscopy. With recent advances in genome-wide CRISPR/Cas9 screens, super-resolution microscopy and other innovations such as expansion microscopy [102,103], hopefully these challenging questions will finally be answered definitively.

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