Conserved juxtamembrane domains in the yeast golgin Coy1 drive assembly of a megadalton-sized complex and mediate binding to tethering and SNARE proteins

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The architecture and organization of the Golgi complex depend on a family of coiled-coil proteins called golgins. Golgins are thought to form extended homodimers that are C-terminally anchored to Golgi membranes, whereas their N termini extend into the cytoplasm to initiate vesicle capture. Previously, we reported that the Saccharomyces cerevisiae golgin Coy1 contributes to intra-Golgi retrograde transport and binds to the conserved oligomeric Golgi (COG) complex and multiple retrograde Golgi Q-SNAREs (where SNARE is soluble NSF-attachment protein receptor). Here, using various engineered yeast strains, membrane protein extraction and fractionation methods, and in vitro binding assays, we mapped the Coy1 regions responsible for these activities. We also report that Coy1 assembles into a megadalton-size complex and that assembly of this complex depends on the most C-terminal coiled-coil and a conserved region between this coiled-coil and the transmembrane domain of Coy1. We found that this conserved region is necessary and sufficient for binding the SNARE protein Sed5 and the COG complex. Mutagenesis of conserved arginine residues within the C-terminal coiled-coil disrupted oligomerization, binding, and function of Coy1. Our findings indicate that the stable incorporation of Coy1 into a higher-order oligomer is required for its interactions and role in maintaining Golgi homeostasis. We propose that Coy1 assembles into a docking platform that directs COG-bound vesicles toward cognate SNAREs on the Golgi membrane.

The Golgi complex must accurately sort, process, and export secretory cargo while simultaneously maintaining its own composition to ensure continuous functionality. Transport specificity is crucial for the Golgi complex to properly sort cargo while maintaining its own homeostasis, and this feat is achieved through a combination of dedicated trafficking factors, including small Rab GTPase networks, multisubunit tethering complexes (MTCs), soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), and a family of coiled-coil proteins called golgins. Each class of proteins is associated with executing distinct steps in vesicle transport at the Golgi: Rabs and golgins initiate vesicle capture, specific SNARE combinations drive vesicle fusion, and MTCs at the Golgi function somewhere in-between these stages. However, our understanding of how these components collaborate with one another to execute the capture and fusion of specific vesicles at the Golgi remains incomplete.

Golgins confer an initial layer of specificity to vesicle transport at the Golgi complex. These proteins are C-terminally anchored to Golgi membranes and are primarily composed of coiled-coils, suggesting the N termini of a golgin could extend hundreds of nanometers away from the Golgi membrane. Based on this topology, golgins have long been suspected of functioning as vesicle tethers. These predictions have largely been borne out: most mammalian golgins are sufficient to initiate vesicle capture in vivo, and the extreme N termini of these golgins are essential for these tethering events. Although ongoing work still seeks to define the vesicular signatures recognized by each golgin, our understanding of how vesicles are captured at the Golgi has advanced significantly.

Less is known about how a tethered vesicle traverses the distance from the N terminus of a golgin to the Golgi membrane. One model proposes that vesicles selectively diffuse toward the Golgi membrane through interactions with distinct Rab-binding sites distributed across the length of each golgin. Golgin flexibility has also been implicated in delivering vesicles to Golgi membranes; for example, unstructured regions between the coiled-coils of GCC185 allow this protein’s N terminus to come within 40 nm of its C terminus. Beyond how a vesicle approaches the Golgi membrane, it also remains unclear how vesicle capture is coordinated with recruitment of the requisite fusion factors.

Previously, we identified a role for the golgin Coy1 in intra-Golgi retrograde transport in Saccharomyces cerevisiae. Munro and co-workers first characterized Coy1 and proposed a role for this golgin in Golgi transport based on genetic interactions with the SNARES GOS1 and SEC22. Subsequently, Warren and co-workers reported that the mammalian homolog...
of Coy1, CASP, specifically captured vesicles laden with recycled Golgi enzymes in vitro. However, Wong and Munro (7) later reported that CASP did not nucleate capture of any vesicle populations in their in vivo mitochondrial localization assay, calling into question this protein’s capacity to function as a vesicle tether and its role in Golgi transport. We reported that cells lacking COY1 display a defect in the retention of the cis-Golgi mannosyltransferase Och1 and that combining coy1/H9004 with other golgin mutations results in growth and glycosylation defects, consistent with a role for Coy1 in maintaining Golgi homeostasis. Coy1 physically interacts with all three Golgi retropgrade Q-SNAREs and with the COG complex, an MTC required for retrograde transport on the Golgi (2, 6, 13, 24), suggesting a role for this golgin in vesicle docking and/or SNAREpin assembly.

To better understand Coy1’s contribution to retrograde Golgi transport, we mapped the functionally essential regions of the protein. Here, we report that a conserved C-terminal region adjacent to Coy1’s integral membrane domain is necessary and sufficient to bind both the SNARE Sed5 and the COG complex. Immediately N-terminal to this binding domain is a coiled-coil domain that is required for stable integration of Coy1 into a megadalton-sized complex. Mutation of conserved motifs within the minimal binding domain disrupts Coy1 interactions and in vivo function. These consequences are also observed when conserved arginine residues within the C-terminal coiled-coil domain are mutated. These arginine mutations also reduce the assembly of Coy1 into a larger complex, suggesting that the interactions and function of Coy1 depend on stable oligomerization. Based on these findings, we propose that Coy1 assembles into a docking platform that links COG-bound vesicles to a cognate set of fusogens on Golgi membranes.

Results

Previously, we reported that the golgin Coy1 elutes in the void volume of a Superose 6 gel-filtration column, which has an exclusion limit of 4 MDa (24). Two possibilities could explain this elution pattern: either Coy1 is incorporated into a large complex or it forms a smaller but highly extended assembly, as has been reported for other coiled-coil proteins of the Golgi complex (25). To distinguish between these possibilities, we sedimented detergent-solubilized membrane proteins from semi-intact cells through a 5–45% sucrose gradient. We monitored the distribution of Coy1 alongside other protein complexes of known size. The COPII coat subunit, Sec13, and the α-subunit of coatomer, Cop1, were detected in the middle and earlier sections of the gradient, consistent with their reported sedimentation coefficients of 13 S and 7.8 S, respectively (26–28). The integral membrane protein Erv46, which assembles into a complex of ~300 kDa (29), was also found in the middle of the gradient, whereas the syntaxin Vam3 was detected in the earliest fractions, consistent with a reported sedimentation coefficient of 4 S (30). Strikingly, Coy1 was most enriched at the bottom of the gradient. The Coy1-enriched fractions were well-resolved from the COG and COPII complexes, both of which interact transiently with Coy1 (Fig. 1A) (24, 31). The integral membrane domain of Coy1 did not contribute to this sedimentation pattern, as Coy1∆TM was similarly enriched at the bottom of the gradient (Fig. 1B). In combination with our gel-filtration data (24), we conclude that the cytosolic domain of Coy1 assembles into a megadalton-sized complex.

To better understand how oligomerization of Coy1 affects its role in retrograde Golgi transport and interactions with the COG complex and SNAREs, we developed a mapping approach to identify the functionally essential regions of this protein.
First, we examined the contribution of Coy1’s predicted coiled-coil domains \((32, 33)\) by individually deleting them from a 2-Mb plasmid that expresses \(COY1\) under the strong constitutive \(PHO5\) promoter (Fig. 2A). After verifying expression of these constructs (Fig. 2B), we tested their ability to induce a growth defect in the \(gos1/\Delta\) strain. As reported previously (22), overexpression of \(COY1\) induced a strong growth defect in \(gos1/\Delta\) cells. This growth defect was still apparent in cells overexpressing Coy1\(CC1\), Coy1\(CC2\), or Coy1\(CC3\), but it was ameliorated in cells bearing the Coy1\(CC4\), Coy1\(CC5\), and Coy1\(CC6\) truncations (Fig. 2C). At elevated temperatures, a graded phenotype was apparent, with truncations closer to the C terminus of Coy1 eliciting a less severe phenotype than those near the N terminus. These results reveal that the middle and C-terminal coiled-coils are required for Coy1 toxicity in \(gos1/\Delta\) cells.

In a reciprocal approach, we examined whether these constructs could rescue the \(coy1/\Delta\ rud3/\Delta sgm1/\Delta\) strain, which cannot grow at high temperatures without plasmid-borne \(COY1\).
(24). Each transformant grew at a similar rate at lower temperatures, whereas cells bearing an empty vector were inviable at the restrictive temperature. Growth was rescued by the WT, ΔCC2, and ΔCC3 constructs. Some growth was apparent in cells carrying the Coy1ΔCC1 plasmid, but not to the same level as the former constructs, suggesting Coy1 function had been partially compromised. Cells bearing the ΔCC4, ΔCC5, and ΔCC6 plasmids were completely inviable (Fig. 2D). Thus, both the toxicity and rescue assays pinpointed these coiled-coils as essential for Coy1 function.

To analyze the contribution of these coiled-coils to oligomerization of Coy1, we resolved solubilized yeast membranes expressing these constructs as their sole copy of Coy1 on sucrose gradients. Notably, although these constructs were all highly overexpressed, the sedimentation pattern of plasmid-borne Coy1 remained comparable with that of the endogenously expressed protein (Fig. 2E). Smaller assemblies of Coy1 do not accumulate upon overexpression, which suggests that Coy1 assembles into a megadalton-sized complex on its own. Five of the six Coy1ΔCC constructs exhibited a similar sedimentation pattern, with a majority of each protein peaking after Cop1 (Fig. 2F). In contrast, Coy1ΔCC6 exhibited a broad distribution with one peak at the top of the gradient and a second minor peak at the same position as WT Coy1 (Fig. 2, E and F), suggesting that formation of this complex was strongly reduced but not abolished. This result indicates that the C-terminal coiled-coil of Coy1 is required for the stable incorporation of this protein into a higher-order oligomer.

We next analyzed whether these coiled-coil truncations affected binding of Coy1 to either Sed5 or the COG complex in bead-based pulldown assays. However, we were unable to identify a single coiled-coil domain that, when deleted, abolished these interactions.3 We instead tested whether truncation of the three functionally nonessential N-terminal coiled-coils (amino acids 1–210) or the entire coiled-coil region (1–535) affected these interactions (Fig. 3A). WT Coy1 was specifically recovered with GST–Sed5 and lobe A of the COG complex, which consists of Cog1, Cog2, Cog3, and Cog4 (24, 34). However, these interactions were not as robust as observed previously (24). This difference is likely attributable to the inclusion of the transmembrane domain in these N-terminal truncations, suggesting that Coy1’s integral membrane domain may limit these interactions or that some binding activity is lost following detergent solubilization. The first truncation, spanning residues 211–679, bound to Sed5 and COG to a similar extent as the full-length protein. Surprisingly, specific interactions with Sed5 and COG were not only preserved in the 536–679 truncation, these interactions appeared far more robust than in the other constructs (Fig. 3, B and D). Relative to WT Coy1, recovery of this C-terminal fragment was on average nearly three times higher with Sed5 and 15-fold higher with COG (Fig. 3, C and E). These interactions were abolished when Coy1 was C-terminally truncated at residue 535 (Fig. 3, F–I), indicating that Coy1(536–679) is both necessary and sufficient to interact with Sed5 and the COG complex.

Figure 3. C terminus of Coy1 is necessary and sufficient to bind the COG complex and Sed5 in vitro. A, diagram of constructs used in this study. B, C terminus of Coy1 is sufficient to selectively bind GST–Sed5. Recombinant Sed5 and Bet1 were immobilized on GSH-agarose resin and incubated with solubilized membrane extracts from pGAL-COY1(1–679)-3xHA (CBY3484), pGAL-COY1(211–679)-3xHA (CBYS544), and pGAL-COY1(536–679)-3xHA (CBYS545) as described under “Experimental procedures.” After washing the resin, bound proteins were eluted with sample buffer. 1% of the reaction inputs and 20% of the eluates were indicated Coy1 constructs with either Bet1 or Sed5 was calculated from six independent experiments described in "Experimental procedures." After washing the resin, bound proteins were eluted with sample buffer. 1% of the reaction inputs and 20% of the eluates were resolved on 11.5% SDS-polyacrylamide gels and immunoblotted for HA. C, truncation of coiled-coil domains increases binding to Sed5. Mean recovery of the indicated Coy1 constructs with either Bet1 or Sed5 was calculated from six independent experiments described in "Experimental procedures." D, C terminus of Coy1 is sufficient to selectively bind the COG complex. Binding assays with recombinant COG complex on Ni-NTA resin resolved on 11.5% SDS-polyacrylamide gels and immunoblotted for HA. C, truncation of coiled-coil domains increases binding to Sed5. Mean recovery of Coy1 with Ni-NTA resin without (−COG) and with (+COG) recombinant COG complex was quantified from six independent experiments described in D. E, truncation of coiled-coil domains improves binding of Coy1 to the COG complex. Mean recovery of Coy1 with Ni-NTA resin without (−) or with (+) recombinant COG complex was quantified from six independent experiments described in D. F, C terminus is essential for Coy1 to bind Sed5. SNARE-binding assays using cytosols from coy1Δ semi-intact cells (CBY2660) expressing the indicated plasmid-borne Coy1 constructs were performed as in B. G, mean recovery of Coy1 constructs from three independent experiments described in F. H, C terminus of Coy1 is essential for COG complex binding. Binding to COG was performed as described in D using cytosol as in F. I, mean recovery of Coy1 with or without COG, quantified from three independent experiments described in H. In all panels of this figure, error bars indicate standard deviation, and statistical analyses were performed with two-way ANOVA followed by Tukey’s test, with n.s., not significant; **, p < 0.005; ****, p < 0.0001.

3 N. S. Anderson and C. Barlowe, unpublished observations.
To complement these in vitro binding assays, we tested whether the cytosolic portion of Coy1’s C-terminal binding domain (CBD) localized to Golgi membranes. We constructed plasmids encoding this domain sandwiched between an N-terminal superfolder GFP variant (35) and a C-terminal 3xHA tag with the intent of visualizing its localization in vivo (Fig. 4A). However, we were unable to detect expression of this fusion protein when encoded on a centromeric plasmid. Overexpression of this construct from a 2-μm vector yielded a detectable signal, although the abundance of this fusion protein was reduced relative to the other Coy1 truncations (Fig. 4B). We elected to examine the distribution of this fusion protein through bulk fractionation assays instead of live-cell imaging.

Figure 4. C-terminal domain of Coy1 confers Golgi membrane targeting information. A, diagram of constructs used in the experiments described in this figure and Fig. 5. B, comparison of expression levels. Samples of semi-intact coy1Δ cells expressing the indicated constructs under the constitutive TPI promoter were resolved on 11.5% gels and immunoblotted with anti-HA and anti-Sec19 (loading control). C, C-terminal domain of Coy1 confers membrane targeting information. Semi-intact cells expressing the indicated constructs were resuspended in buffer and centrifuged at 54,000 rpm as described under “Experimental procedures.” Equivalent amounts of the soluble (S) and membrane-pellet (P) fractions were resolved on 11.5% SDS-polyacrylamide gels and immunoblotted for HA, Sec19, and Yet3. D, quantification of average membrane association of the HA-tagged constructs and, as a control, Sec19 from five independent experiments as described in C. Error bars indicate standard deviation, and statistical analyses were performed with two-way ANOVA followed by Dunnett’s test with ****, p < 0.0001. E, C-terminal domain of Coy1 is necessary and sufficient for Golgi membrane targeting. Membranes from coy1Δ cells expressing the indicated constructs were separated over 18–60% sucrose gradients. Fractions were collected from the top of gradients and resolved on 11.5% gels for immunoblotting against the HA epitope and the integral Golgi (Och1) and ER (Yet3) membrane proteins. The blots depicted represent one of three independent experiments. F, densitometric quantification of blots displayed in E, showing distribution of Coy1-HA, Och1, and Yet3. The majority of Coy1(1–613) cofractionates with the Golgi marker Och1. Coy1(1–535) stays at the top of the gradient, and GFP-Coy1(536–613) is enriched in the Golgi fractions.
assay (36, 37). As expected, GFP was largely detected in the cytosolic fraction, although ∼18% could be detected in the pellet, reflecting some carryover with membrane proteins. Full-length Coy1 was also exclusively detected in the pellet, whereas 91% of Coy1ΔTM was also found in the membrane fraction, as reported previously (24). In contrast, the Coy1(1–535) construct that lacks the CBD exhibited a primarily cytosolic distribution, with only 26% remaining in the membrane pellet (Fig. 4, C and D). Despite low levels of expression (Fig. 4B), over 60% of the GFP–CBD construct was detected in the membrane pellet (Fig. 4, C and D). This construct was not as strongly associated with membranes as Coy1ΔTM, suggesting that other cytosolic regions of Coy1 may stabilize interactions with membranes. Regardless, these data support a role for Coy1’s C-terminal domain in engaging with other membrane-associated factors.

Next, we examined whether the CBD specifically associated with Golgi membranes by resolving organelles from lysed semi-intact coy1Δ cells expressing the cytosolic Coy1 constructs on sucrose gradients. In each experiment, Golgi membranes marked by Och1 were enriched in the middle of the gradient, whereas denser ER membranes marked by Yet3 pelleted at the bottom of the tube. Although Coy1ΔTM exhibited a broad sedimentation profile, most of this protein co-fractionated with Golgi membranes, as reported previously (24). In contrast, Coy1(1–535) fractionated at the top of the gradient, reflecting its cytosolic localization. Strikingly, GFP–CBD was consistently enriched in the Golgi membrane fractions (Fig. 4, E and F). The strong association of this construct with Golgi membrane fractions provides further support for the CBD in interacting with other Golgi-associated proteins, including but not limited to Sed5 and the COG complex.

To analyze the contribution of the CBD to Coy1 oligomerization, coy1Δ cells expressing these same plasmids were detergent-solubilized, and protein complexes were resolved on sucrose gradients. As observed previously, full-length Coy1 was enriched in the bottom of the gradient (Fig. 5). Coy1ΔTM also sediments as a large oligomer, although it exhibits a split distribution between the top and bottom of the gradient (Fig. 5B). This result contrasts with the sedimentation pattern observed when Coy1ΔTM is expressed from its endogenous locus (Fig. 1B), suggesting that the integral membrane domain stabilizes oligomerization when Coy1 levels are very high. Both the Coy1(1–535) and GFP–CBD constructs were solely detected in the earliest fractions of the gradient, indicating that the CBD is necessary but not sufficient for oligomerization of Coy1 (Fig. 5).

To analyze the functional relevance of the CBD, we first tested whether this region could induce a growth defect in gos1Δ. We constructed a series of Coy1 constructs in which Coy1 was N-terminally truncated either after the dispensable N-terminal coiled-coils (encompassing residues 233–679), or the first (379–679), second (494–679), or third (536–679) essential coiled-coils. Expression of full-length Coy1 and the first truncated construct (233–679) caused a strong growth defect in gos1Δ, consistent with this N-terminal region being dispensable for Coy1 toxicity. Cells expressing the 379–679 construct grew slightly less robustly than those carrying an empty vector. Notably, expression of the 494–679 construct resulted in a more severe growth defect than the preceding truncation, suggesting that residues 379–493 limit the toxicity of Coy1. Cells expressing the membrane-anchored CBD(536–679) phenocopied the empty vector control (Fig. S1). These results indicate that full Coy1 activity requires its three most C-terminal coiled-coils, but partial activity is still apparent until the most C-terminal coiled-coil of Coy1 is truncated.

The C terminus of Coy1 belongs to a conserved protein family termed the CASP C-terminal region (Pfam entry: PF08172) (38). Alignment of this family using the program Jalview revealed a series of highly conserved residues within the last
coiled-coil domain and the CBD of Coy1 (39). We asked whether these conserved residues were important for Coy1 functionality. We built a series of COY1 constructs on a pRS416 pGAL1 backbone in which either the 496QRDRFRX503, 536YERIRY541, or 604KXXXR613 motifs were mutagenized to alanines, with X denoting residues that were not mutagenized (Fig. 6A). Each of these constructs was highly overexpressed relative to endogenous Coy1 (Fig. S2). Overexpression of WT
Coy1 induced a strong growth defect in gos1Δ as expected, but cells expressing the mutant Coy1 variants all grew comparatively well. The Coy1 K5K:KXXR construct induced an intermediate phenotype, yielding cells that grew more robustly than those expressing WT Coy1, but not as well as those carrying either an empty vector or the other two Coy1 motif mutations (Fig. 6B). In a reciprocal experiment, we examined whether these constructs could complement the glycosylation defects observed in the coy1Δ sgm1Δ strain rather than the temperature sensitivity of the coy1Δ sgm1Δ rud3Δ strain. The apparent molecular weights of two secretory glycoproteins, Gas1 and CPY, are reduced in coy1Δ sgm1Δ, and this defect can be corrected by supplying COY1 on a plasmid (24). However, a plasmid encoding the QRDRFRXR Coy1 construct failed to rescue Gas1 and CPY glycosylation, whereas expression of the YERIRY and K5K:KXXR constructs resulted in an intermediate phenotype (Fig. 6C). We conclude that the conserved motifs in Coy1’s C-terminal coiled-coil and binding domain are required for optimal Coy1 function in Golgi transport.

We next analyzed whether these motif mutations affected Coy1’s ability to bind Sed5 or the COG complex. Each Coy1 variant was recovered with GST–Sed5 and not with GST–Bet1, but recovery of the mutant proteins was reduced relative to WT Coy1ΔTM. Notably, recovery of Coy1 with Sed5 was more severely impaired in the presence of the QRDRFRXR and K5K:KXXR mutations than with the YERIRY mutation (Fig. 6, D and E). A similar pattern was observed when binding to COG was monitored: the QRDRFRXR and K5K:KXXR mutations reduced recovery of Coy1ΔTM, whereas the YERIRY mutation did not significantly affect binding (Fig. 6, F and G). Thus, the conserved QRDRFRXR and K5K:KXXR motifs are essential for Coy1’s ability to efficiently bind Sed5 and the COG complex.

Membrane association of Coy1ΔTM constructs was also monitored as an alternative means of analyzing the impact of the indicated mutations on Coy1’s protein–protein interactions. As expected, Coy1ΔTM remains enriched in the membrane fraction, whereas the QRDRFRXR and YERIRY mutations result in a shift to the cytosolic fraction (Fig. 6, H and I). Although these mutations disrupt membrane association of Coy1ΔTM, they do not do so as drastically as truncation of the entire C-terminal binding domain (Fig. 4, C and D). This result suggests that some of Coy1’s interactions with membrane-associated proteins are preserved when these mutations are present. The K5K:KXXR mutation did not appreciably disrupt membrane association of Coy1ΔTM (Fig. 6, H and I). However, protein levels of the Coy1ΔTM K5K:KXXR construct were reduced by nearly 85% relative to the other constructs, suggesting that this mutation may destabilize the protein and result in its degradation. This instability appeared to arise from the combination of the K5K:KXXR and ΔTM mutations, as the full-length Coy1 K5K:KXXR protein is expressed at levels comparable with the WT protein and the other mutants (Fig. S2).

We next tested whether these mutations affected Coy1’s capacity to oligomerize by monitoring sedimentation of solubilized membranes from coy1Δ cells that expressed full-length Coy1 on a centromeric plasmid under the TPI promoter. As a control, we also monitored sedimentation of Cop1, which peaked in the same fractions in each experiment. WT Coy1 was enriched in the bottom of the sucrose gradient, as were the YERIRY and K5K:KXXR mutants, indicating that these residues are not involved in Coy1 oligomerization. The QRDRFRXR mutation resulted in a shift of Coy1 to the first fractions of the gradients, along with a minor secondary peak at the bottom of the gradient (Fig. S3), similar to the sedimentation pattern observed when the entire C-terminal coiled-coil of Coy1 (ΔCC6) is truncated (Fig. 2, E and F). We conclude that the conserved QRDRFRXR motif is essential for stable oligomerization of Coy1.

Finally, we examined whether single point mutations within the QRDRFRXR motif could disrupt Coy1’s function, oligomerization, and interactions. We focused on residues predicted to lie within the hydrophobic core of this coiled-coil domain, as these residues often determine a coiled-coil’s oligomeric state (40, 41). Within this motif, Phe-500 and either Arg-497 or Arg-503, depending on the register, were mapped by the coiled-coil prediction software Waggawagga to the hydrophobic core of the C-terminal coiled-coil domain (Fig. 7A) (42). As bulky and charged residues are infrequently found in this hydrophobic position, we asked whether converting these residues to the more commonly observed leucine affected Coy1’s function (43). Growth of the gos1Δ strain was still inhibited by overexpression of COY1 F500L, whereas cells expressing the R497L or R503L mutation grew as well as those carrying the full QRDRFRXR mutated construct (Fig. 7B). Moreover, the R497L...
and R503L Coy1 constructs failed to completely restore glycosylation of Gas1 and CPY in coy1Δ sgm1Δ, indicating that these point mutations partially disrupt Coy1's function (Fig. 7C). Like the QRDRFRXR mutation (Fig. 6, D–G), the R497L and R503L mutations reduced the binding of Coy1ΔTM to both Sed5 (Fig. 7, D and E) and the COG complex (Fig. 7, F and G). Finally, the R497L and R503L mutations shifted Coy1’s sedimentation pattern toward the top of the gradient (Fig. 8) in a similar manner as the motif mutation and C-terminal coiled-coil deletion (Fig. 2, E and F, and Fig. S3). These results indicate that Arg-497 and Arg-503 are required for stable oligomerization of Coy1 and suggest that the assembly of Coy1 into a higher-order structure is essential for its interactions and in vivo function.

Discussion

Transport specificity underlies compartmentation of the Golgi complex and depends on the activity of golgins, SNAREs, and MTCs, among other factors (4, 10, 15). A coherent understanding of how these components interface together to execute vesicle capture and fusion at the Golgi remains lacking. The work described here supports a model in which the golgin Coy1 assembles into a docking platform that directs tethering factors toward the requisite fusion machinery to maintain Golgi homeostasis.

Golgins are often depicted as rod-shaped homodimers (16). Our gel-filtration and sucrose-gradient sedimentation assays of

**Figure 7. Interactions and oligomerization of Coy1 depend on conserved arginine residues within the C-terminal coiled-coil.** A, alignment of the coiled-coil sequences surrounding the QRDRFRXR motif from various eukaryotes. The predicted heptad repeat registers with the hydrophobic positions highlighted are depicted above. Conserved arginine residues at positions 497 or 503 are predicted to map to the hydrophobic core. B, R497L and R503L mutations disrupt Coy1 toxicity. Growth rate assays of gos1Δ cells carrying either an empty vector (e.v.) or plasmids encoding Coy1 constructs under the GAL1 promoter were set up at 30 °C on minimal media with either glucose or galactose as the sole carbon source. Expression of WT Coy1 induces a growth defect that is ameliorated by the QRDRFRXR motif mutation. This growth defect is also attenuated by the R497L and R503L point mutations, and it persists in the presence of the F500L mutation. C, R497L and R503L point mutations disrupt the function of Coy1 in Golgi transport. Lysates from coy1Δ sgm1Δ cells expressing either an empty vector or plasmids indicated the Coy1 constructs were resolved over 8% gels and blotted against Gas1, Coy1, and CPY. Och1 and Cop1 were monitored as loading controls.

D, R497L and R503L mutations weaken the interaction between Coy1 and Sed5. SNARE-binding assays using cytosol from coy1Δ cells expressing the indicated Coy1ΔTM constructs were conducted as described in Fig. 3F. E, quantification of Coy1 recovery from D (n = 5). Error bars indicate standard deviation. Statistical analyses were performed with two-way ANOVA followed by Tukey’s test with n.s., not significant; ***, p < 0.001 and **, p < 0.005.

F, binding of COG by Coy1 depends on Arg-497 and Arg-503. In vitro COG-binding assays using cytosol from coy1Δ cells expressing the indicated Coy1ΔTM constructs were conducted as described in Fig. 3H. G, quantification of Coy1 recovery from F (n = 7). Statistical tests were performed as described in E.
yeast membrane extracts indicate that Coy1 is instead incorporated into a much larger complex (24). This sedimentation pattern cannot be attributed to aggregation induced by the integral membrane domain of Coy1, as endogenously-expressed Coy1ΔTM sediments at a similar position as full-length Coy1. Furthermore, the observation that Coy1 sediments at one or two discrete positions suggests that this protein is not indiscriminately forming oligomers but is instead forming a complex of a defined size (Fig. 1, A and B). Determining the exact oligomeric state of Coy1 will require subjecting the purified, active complex to more precise techniques such as analytical ultracentrifugation or native MS (44–46).

Notably, overexpression of full-length Coy1 from either a multicopy plasmid (Fig. 2A) or by the strong GAL1 promoter does not result in an accumulation of unassembled Coy1 in either sucrose-gradient or gel-filtration assays, respectively (24). If Coy1 was present in a heteromeric complex, its overexpression would be expected to saturate the availability of other binding partners and lead to an accumulation of unassembled Coy1. This result has not been observed, suggesting that Coy1 assembles into the core of this complex on its own. However, this does not exclude the possibility that other subunits decorate this complex.

Golgins are thought to act in vesicle transport by N-terminally capturing incoming vesicles. In contrast, our genetic toxicity and complementation assays have highlighted the importance of the C-terminal half of Coy1. Truncation of any of the putative coiled-coil domains between residues 233 and 535 diminished Coy1’s capacity to induce a growth defect in the gos1Δ strain, whereas the same constructs also failed to rescue viability of the coy1Δ rud3Δ sgm1Δ strain at a restrictive temperature (Fig. 2, C and D). Notably, gos1Δ cells overexpressing a Coy1 truncation that lacks its N-terminal 232 residues exhibit a growth defect that is as robust as cells expressing full-length Coy1 (Fig. S1). The dispensability of Coy1’s N terminus in these assays does not explicitly exclude a function for this region in membrane trafficking or other processes. However, the essentiality of the middle and C-terminal regions suggests a more membrane-proximal role for Coy1 than other golgin proteins. We have identified two discrete regions of Coy1 that mediate different protein–protein interactions. One region spanning 78 residues between Coy1’s C-terminal coiled-coil and its transmembrane domain is both necessary and sufficient for Coy1’s interactions with the SNARE Sed5 and the COG complex, a region we have termed the C-terminal binding domain (Fig. 3). Complementing these in vitro binding experiments, membrane fractionation assays revealed a tight association between the CBD and Golgi membranes (Fig. 4). The strength of this association raises the possibility that the CBD interacts with other unidentified Golgi-associated factors. This inference is lent further support by the observation that the motif mutations that disrupted binding to Sed5 and COG (discussed below) only reduced membrane association of Coy1ΔTM by 15% at most, whereas the full CBD truncation diminished membrane association to nearly background levels (Figs. 4, C and D, and 6, H and I). Uncovering the interaction landscape of the CBD should improve our understanding of the contribution of Coy1 to Golgi homeostasis.

The CBD is functionally essential, as COY1 constructs in which the highly-conserved \textsuperscript{536}YERIRY\textsuperscript{541} or \textsuperscript{606}K\textsubscript{5}X\textsubscript{3}KXXR\textsuperscript{613} sequences were mutagenized to a series of alanine residues both failed to induce a growth defect in gos1Δ and only partially rescued the CPY and Gas1 glycosylation defects in coy1Δ sgm1Δ (Fig. 6, A–C). The K\textsubscript{5}X\textsubscript{3}KXXR mutation also weakened the interactions between Coy1ΔTM and both Sed5 and the COG complex, indicating the first correlation between Coy1’s in vivo function and its engagement of these interaction partners. The YERIRY mutation only modestly affected binding to Sed5 and had no effect on binding of the COG complex (Fig. 6, D–G), but it also modestly destabilized Coy1ΔTM’s membrane association (Fig. 6, H and I), suggesting that this mutation disrupts interactions between Coy1 and factors that have yet to be identified. Neither of these mutations affected oligomerization of Coy1 (Fig. S3), suggesting that any interactions disrupted by these mutations are transient rather than stable, a conclusion supported by the observation that Coy1 and Cog3 are well-resolved from one another on both sucrose gradients (Fig. 1A) and by gel filtration (24). Understanding how these motifs affect
Coy1’s engagement of Sed5 and the COG complex will require structural approaches. The capability of the CBD to bind both Sed5 and the COG complex alongside its proximity to Coy1’s transmembrane domain suggests a role for Coy1 in directing tethered vesicles to cognate fusion factors. Vesicle tethering is likely initiated by other golgins that act in retrograde transport, such as Rud3 or Sgm1 \((7, 47–51)\). Once tethered, Coy1’s CBD could provide a landmark that directs vesicles toward the Golgi membrane. The COG complex has been proposed to reel in tethered vesicles toward the Golgi membranes, based on the presence of N- and C-terminal COG-binding sites on the mammalian homolog of Sgm1 \((52)\). The CBD of Coy1 not only provides a more membrane-proximal site for COG-bound vesicles, but it could also recruit Sed5 and potentially other cognate SNAREs into the vicinity of the COG-bound vesicle. It is unclear whether a single CBD can bind to COG and Sed5 simultaneously, but oligomerization of Coy1 could provide a mechanism to cluster these tethering and fusion factors together.

Higher-order assembly of Coy1 also depends on the CBD, as truncation of this region completely shifts Coy1 to the earliest fractions of the gradient. However, the GFP–CBD construct migrates at the top of the gradient, indicating that although this region is necessary for oligomerization of Coy1, it cannot form a higher-order structure on its own \((5)\). Notably, mutations within the CBD that disrupt the binding of Coy1 to Sed5 and COG had no detectable effect on the size of the Coy1 complex, suggesting that binding of the CBD to these proteins does not contribute substantial bulk to the size of the Coy1 complex \((Fig. 6, A–G, and Fig. 5)\). The fact that this domain does not form a larger complex on its own suggests that oligomerization of Coy1 depends on coordination between the CBD and Coy1’s coiled-coil domains.

Indeed, the most C-terminal coiled-coil domain is also required for Coy1 to stably assemble into a higher-order structure \((Fig. 2)\). Mutation of conserved residues within this coiled-coil reduce oligomerization of Coy1 and disrupt its \textit{in vivo} function, suggesting that oligomerization is essential for Coy1 to contribute to Golgi retrograde transport \((Figs. 6, A–C, 7, A–C, and 8 and Fig. S3)\). It is unclear whether the CBD, the C-terminal coiled-coil, or both domains form the oligomerization interface. However, the finding that truncation of the CBD, but not the C-terminal coiled-coil, completely shifts Coy1 to the top of the gradient suggests that the CBD forms the primary oligomerization interface. The milder shift observed when the C-terminal coiled-coil is deleted or mutated is consistent with a role for this domain in either stabilizing or promoting assembly of this complex. Although coiled-coils typically assemble into dimers, trimers, or tetramers, larger assemblies braced by coiled-coils have recently been discovered in native and engineered proteins \((53–56)\). These structures offer a framework for considering how the C-terminal coiled-coil may enable oligomerization of Coy1. Further studying how the C-terminal coiled-coil domain and CBD collaborate to define the oligomeric state of Coy1 will be essential to understanding its function in retrograde transport at the Golgi complex.

Our data suggest that the interactions between the CBD, COG complex, and Sed5 are influenced by Coy1’s coiled-coils. A Coy1 construct lacking the coiled-coil domains binds these interaction partners more robustly than full-length Coy1, suggesting that these interactions are autoinhibited by the coiled-coil region. As this effect is only observed after truncation of the entire coiled-coil region and is not apparent when only the most N-terminal coiled-coil domains are truncated \((Fig. 3, A–D)\), this restriction is likely attributable to the middle coiled-coil domains.

Unexpectedly, although Coy1 binds the COG complex and Sed5 when its N-terminal coiled-coils are truncated \((Figs. 6, A–E)\), the QDRDFRFX, R497L, and R503L mutations all attenuate these interactions when the N terminus of Coy1 is intact \((Figs. 6, D–G, and 7, D–G)\). As these mutations also destabilize the Coy1 complex \((Fig. 8 and Fig. S3)\), these results suggest that oligomerization is required for Coy1 to engage with interaction partners. Oligomerization is not essential for binding \textit{per se}, as the CBD alone does not sediment as a large particle, yet it robustly interacts with Sed5 and the COG complex. Moreover, the observation that full-length Coy1 persists as an oligomer yet only modestly interacts with Sed5 and the COG complex suggests that oligomerization alone does not enable these interactions \((Figs. 1 and 3, B–E)\). Rather, we speculate that oligomerization is a prerequisite for relief of Coy1 autoinhibition. Restricting the activity of Coy1 until it has been incorporated into a larger complex could serve to prevent premature interactions between Coy1 and SNAREs like Sed5 that could interfere with trafficking between the ER and Golgi complex. This model is consistent with the observation that the Coy1(494–679) construct, which includes only the most C-terminal coiled-coil, elicits a more pronounced growth defect in \textit{gos1Δ} cells than the 379–679 construct, which additionally includes the middle coiled-coil domains \((Fig. S1)\).

Once assembled into a large complex, we speculate that Coy1 persists in a closed conformation until a signal, perhaps conveyed after the capture of a vesicle by another tethering factor, triggers rearrangement of the coiled-coil domains in a manner that relieves autoinhibition of Coy1. This conformational change would expose binding sites for the COG complex and Golgi retrograde SNAREs and provide a means for a captured vesicle to dock and initiate SNAREpin assembly. Mapping intra- and inter-molecular interactions between Coy1’s coiled-coil domains and the CBD will be essential to better understand how these regions define the activation and binding mechanisms of Coy1.

Why would oligomerization of Coy1 into a megadalton-sized complex be essential for its interactions and \textit{in vivo} function? Studies on exocytotic and vacuolar fusion have established a role for oligomeric SNARE-binding proteins in scaffolding the assembly of multiple SNAREpins to promote stable opening of a fusion pore \((30, 57–61)\). Additionally, a recent report on the homotypic fusion and vacuole protein-sorting complex (HOPS) revealed that after SNAREpin assembly, HOPS drives the transition from hemifusion to fusion pore opening. This latter function can also be fulfilled by simply affixing large ligands, such as antibodies or the 850-kDa enzyme phosphofructokinase, to the SNAREpin. Molecular dynamics simulations suggested that during hemifusion, bulky SNARE-bound proteins like HOPS introduce a curvature strain between...
apposing membranes that is relieved by the opening of the fusion pore (62). It remains untested whether this finding is applicable to other organelles. Still, the precedents established by these studies offer some guidance on how to further explore whether oligomerization of Coy1 contributes to membrane fusion or SNAREpin assembly in a reconstituted system.

**Experimental procedures**

**Growth media**

Yeast cultures were grown inYP (2% bacto-peptone, 1% bacto-yeast extract (BD Biosciences)). Cultures carrying plasmids were maintained in auxotrophic selective media (0.7% yeast nitrogen base without amino acids (BD Biosciences)) with Complete Supplement Media (MP Biomedicals, Solon, OH). Unless otherwise indicated, cultures were grown in 2% glucose. To induce expression of constructs under the GAL1 promoter, cultures were first grown overnight in 3% raffinose and then back-diluted in media with 2% galactose (Millipore Sigma).

**Yeast strain construction**

Transformations into yeast were performed using the lithium acetate method (63, 64). Yeast strains used in this study are listed in Table 1. The pGAL-COY1 (211–679)-3xHA (CBY5544) and pGAL-COY1 (536–679)-3xHA (CBY2674) strains were constructed through gene targeting (65). The pFA6a-kamnxx6-pGAL1 cassette was amplified with the primers NA046 and NA047 or NA205 and NA206, respectively, which were designed to N-terminally truncate Coy1 at the indicated residues. The fragments were transformed into COY1–3xHA (CBY2674). Transformants were subjected to selection on YPD with 0.5 μg/ml G418 and screened by immunoblotting (Thermo Fisher Scientific, Waltham, MA).

**Plasmid construction**

Plasmids used in this study are listed in Table 2, and primers are listed in Table S1 of the supporting information. The sequence of the constructs described below were verified by Sanger cycle sequencing by the Dartmouth Molecular Biology Shared Resource facility.

To construct the Coy1ΔCC plasmids, predicted coiled-coil domains were identified using Marcol (32, 33) and deleted from the COY1 sequence on pRS426 COY1 (22) using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the modifications described by Liu and Naismith (66). Coy1ΔCC1 was constructed with primers NA046 and NA047; ΔCC2 with NA065 and NA066; ΔCC3 with NA036 and NA037; ΔCC4 with p038 and p039; ΔCC5 with NA050 and NA051; and ΔCC6 with NA052 and NA053.

The pRS425 pTiPI plasmid was built by amplifying the TPI promoter from YIPlac204-TC-Sec7-6xDsRed-M1 (67) (Addgene 25448) using SWP37 and SWP38. This sequence was inserted into pRS425 through SacII and Xmal sites. The sequence encoding the 3xHA epitope and ADH1 terminator were subsequently amplified from pFA6a-3xHA-His3MX6 with NA178 and NA179 (65). This fragment was ligated into pRS425 pTiPI through Xhol/Apal sites, generating pRS425 pTiPI-3xHA.

The pRS425 pGAL COY1 (1–613)-3xHA and pRS425 pGAL COY1 (1–535)-3xHA plasmids were constructed by amplifying

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**Table 1**

Yeast strains used in this study

| Strains       | Genotype                          | Ref.     |
|---------------|-----------------------------------|----------|
| CBY740 (BY4742) | MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 | 83       |
| CBY2660       | CBY740 with coy1Δ downX6          | Research Genetics |
| CBY2674       | CBY740 with COY1–3xHA-His3MX6     | 24       |
| CBY2679       | CBY740 with gos1Δ upX6            | Research Genetics |
| CBY3484       | CBY740 with COY1 (1–613)-3xHA-His3MX6 | 24       |
| CBY4102       | CBY740 with coy1Δ upX6 sgp1Δ hphMX6 | 24       |
| CBY4103       | CBY740 with coy1Δ downX6 rad3Δ XanMX6 sgp1Δ hphMX6 | 24       |
| CBY5544       | CBY2674 with pGAL-COY1 (211–679)  | This study |
| CBY5545       | CBY 2674 with kanMX6-pGAL-COY1 (536–679) | This study |

**Table 2**

Plasmids used in this study

| Strain no.r | Plasmid name               | Ref.     |
|-------------|----------------------------|----------|
| CBB307      | pRS425                     | 84       |
| CBB308      | pRS426                     | 84       |
| CBB3547     | YIPlac204-TC-Sec7-6xDsRed-M1 | 67       |
| CBB3733     | pRS426 pPHO5 Coy1           | 22       |
| CBB4067     | pRS316 pTPI GFP P4M         | S. Weiss |
| CBB4241     | pSH7                       | 68       |
| CBB4262     | pRS426 pPHO5 Coy1ΔCC3       | This study |
| CBB4264     | pRS426 pPHO5 Coy1ΔCC4       | This study |
| CBB4296     | pRS426 pPHO5 Coy1ΔCC1       | This study |
| CBB4297     | pRS426 pPHO5 Coy1ΔCC2       | This study |
| CBB4298     | pRS426 pPHO5 Coy1ΔCC5       | This study |
| CBB4299     | pRS426 pPHO5 Coy1ΔCC6       | This study |
| CBB4930     | pRS425 pTPI                | S. Weiss |
| CBB5075     | pRS425 pTPI 3xHA           | This study |
| CBB5427     | pRS416 pGAL Coy1 KX,KX,R    | This study |
| CBB5461     | pRS425 pGAL Coy1 (1–613) 3xHA | This study |
| CBB5462     | pRS425 pGAL Coy1 (1–535) 3xHA | This study |
| CBB5473     | pRS416 pGAL qDRDRFRX         | This study |
| CBB5474     | pRS416 pGAL Coy1 YERIRY     | This study |
| CBB5475     | pFA6a-link-GFPEnvy-SpiH1sG  | 35       |
| CBB5522     | pRS425 pTPI GFPEnvy 3xHA     | This study |
| CBB5524     | pRS425 pTPI GFPEnvy Coy1 (536–613) 3xHA | This study |
| CBB5569     | pRS316 pTPI Coy1 3xHA       | This study |
| CBB5570     | pRS316 pTPI Coy1 3xHA QDRFRX | This study |
| CBB5571     | pRS316 pTPI Coy1 3xHA YERIRY | This study |
| CBB5572     | pRS316 pTPI Coy1 3xHA KX,KX,R | This study |
| CBB5573     | pRS316 pTPI Coy1 (1–613) 3xHA | This study |
| CBB5575     | pRS316 pTPI Coy1 (1–613) 3xHA QDRFRX | This study |
| CBB5576     | pRS316 pTPI Coy1 (1–613) 3xHA KX,KX,R | This study |
| CBB5577     | pRS316 pTPI Coy1 (1–535) 3xHA | This study |
| CBB5581     | pRS416 pGAL Coy1 3xHA       | This study |
| CBB5582     | pRS416 pGAL Coy1 (233–679) 3xHA | This study |
| CBB5583     | pRS416 pGAL Coy1 (379–679) 3xHA | This study |
| CBB5584     | pRS416 pGAL Coy1 (494–679) 3xHA | This study |
| CBB5585     | pRS416 pGAL Coy1 (536–679) 3xHA | This study |
| CBB5624     | pRS416 pGAL Coy1 R497L      | This study |
| CBB5625     | pRS416 pGAL Coy1 R503L      | This study |
| CBB5626     | pRS416 pGAL Coy1 F500L       | This study |
| CBB5629     | pRS316 pTPI Coy1–3xHA R497L  | This study |
| CBB5643     | pRS316 pTPI Coy1–3xHA R503L  | This study |
| CBB5626     | pRS316 pTPI Coy1 (1–613) 3xHA R497L | This study |
| CBB5627     | pRS316 pTPI Coy1 (1–613) 3xHA R503L | This study |

* S. Weiss, unpublished observations.

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EDITORS’ PICK: Dissection of Coy1 functional domains
the promoter and indicated regions of COY1 from pRS416 pGAL COY1 using NA231 and either NA233 or NA234, respectively. Fragments were then inserted into pRS425 pTPI 3xHA through XbaI and Xhol sites.

To construct pRS416 pGAL COY1, COY1 was amplified from pRS426 COY1 with NA215 and NA218 (22). The fragment was then inserted into the pSH47 backbone through XbaI/Xhol overhangs (68).

The pRS425 pTPI-COY1–3xHA constructs encoding residues 1–679 or 1–535 were built by amplifying the indicated regions from pRS416 pGAL COY1 with NA245 and either NA232 or NA234, whereas the region encoding residues 1–613 was amplified from pRS426 pPHOS COY1 with NA164 and NA177. The fragments were subsequently ligated into pRS425 pTPI-3xHA through Xmal and Xhol sites. The pRS425 pTPI-GFP-3xHA construct was built by amplifying the GFPeny ORF from pFA6a-link-GFPeny-SpHis5 (35) (Addgene 60782) with NA244 and NA256 and then inserting this fragment into pRS425 pTPI-xHA through Xmal and SalI sites. The pRS425 pTPI-COY1(1–613)-3xHA from pRS425 pGAL COY1(1–613)-3xHA with NA179 and NA248 and then ligation this fragment into pRS425 pTPI-GFP-3xHA.

COY1 plasmids with point mutations were constructed using the megaprimer method with modifications (69, 70). Megaprimers with the QRDFRFRX, YERIRY, KXkXXRR, R497L, F500L, and R503L mutations were amplified with NA218 and the mutagenic primers NA240, NA241, NA239, NA269, NA268, and NA270. Megaprimers were gel-purified and used with NA215 to amplify the full COY1 ORF. The fragments were then inserted into the pSH47 backbone through Xbal/Xhol overhangs, generating pRS416 pGAL COY1 plasmids bearing the indicated point mutations. The COY1 sequence was then amplified from these plasmids with NA245 and NA232 and ligated into pRS316 pTPI-COY1(1–613)-3xHA, generating the pRS316 pTPI-COY1–3xHA mutant plasmids. The pRS416 plasmids were also used as templates to amplify pGAL-COY1(1–613) using NA242 and NA245 for the QRDFRFRX and YERIRY constructs and NA250 and NA251 for the KXkXXRR construct. These fragments were then ligated into pRS425 pTPI-3xHA through SalC and Xhol sites, yielding the pRS425 pGAL COY1(1–613) 3xHA plasmids with the indicated point mutations. This COY1(1–613)-3xHA sequence was subsequently amplified from this pRS425 series using p242 and p245 and ligated into pRS316 pTPI through Xmal and Kpnl sites, yielding pRS316 pTPI COY1(1–613)-3xHA point mutant constructs.

Plasmids expressing N-terminal truncations of Coy1 were constructed by amplifying the COY1–3xHA sequence from pRS425 pGAL-COY1–3xHA. NA242 and either NA215(1–679), NA258(233–679), NA259(379–679), NA261(494–679), or NA224(536–679) were used as primers. The constructs were ligated into pSH47 through XbaI and Kpnl sites.

Membrane preparation and yeast cell lysis

Yeast semi-intact cells were prepared as described previously (71). To prepare yeast whole-cell lysates from small scale cultures, 1 OD600 unit of mid-log phase yeast cells were pelleted at 4,000 rpm in an Eppendorf 5424 centrifuge for 5 min. Pellets were resuspended in 200 µl of JR lysis buffer (25 mM HEPES, pH 7.0, 50 mM KOAc, 2 mM EDTA, 1.8 mM DTT, 1.8 mM PMSF) and diluted with an equivalent volume of 5% SDS-PAGE sample buffer (125 mM Tris, pH 6.8, 30% glycerol, 4% SDS, 0.01% bromphenol blue). 100 µl of acid-washed beads were added to the samples, which were then lysed in a Mini-Beadbeater-16 (Biospec, Bartlesville, OK). Lysates were centrifuged at 14,000 × rpm in an Eppendorf 5424 centrifuge for 10 min, and the supernatant was recovered, boiled at 95 °C for 5 min, and resolved on SDS-polyacrylamide gels. Lysates from 1-liter cultures were prepared by liquid nitrogen lysis as described previously (24).

Antibodies and immunoblotting

Polyclonal antibodies against Cog2 and Cog3 (72), the COPI complex (73), Coy1 (24), GST–Yet3 (37), Erv46 (74), Sec13 (75), Sec19 (76), and Vam3 (77) have all been previously reported. Monoclonal anti-HA (HA.11 antibodies were purchased from BioLegend (San Diego, CA). All primary antibodies were diluted 1:1000 except for GST–Yet3 and Cog2 antibodies, which were used at 1:5000. Secondary donkey anti-rabbit and sheep anti-mouse antibodies were purchased from GE Healthcare and used at a 1:10,000 concentration. Immunoblot signals were developed with Supersignal West Pico chemiluminescent substrate (Thermo Fisher Scientific), detected with a GelBox Chemi XR5, and quantified with Syngene GeneTools densitometric analysis software (Syngene, Frederick, MD). Sucrose gradient distributions were plotted with Microsoft Excel (Microsoft, Redmond, WA). All other graphs and all statistical analyses were conducted with Graphpad Prism (Graphpad Software, San Diego, CA).

Membrane and protein fractionation

Membrane association assays were conducted as described previously with modifications (24). In brief, coy1Δ cells expressing the indicated constructs under the constitutive TPI promoter on 2-µm or centromeric plasmids (in Figs. 4 and 5, respectively) were grown in selective media overnight and diluted to 0.1 OD600/ml in 50 ml of YPD. Cells were harvested in mid-log phase and converted to semi-intact cells. 0.1-ml aliquots were prepared from the semi-intact cells and frozen in liquid nitrogen. Cells were subsequently resuspended in 1 ml of B88 (150 mM KOAc, 20 mM HEPES, pH 7.0, 5 mM MgOAc, 250 mM sorbitol) + 1 mM PMSF and centrifuged at 54,000 rpm in a TLA 100.3 rotor for 20 min at 4 °C. Samples of the supernatant were diluted 1:1 in 5% SDS-PAGE sample buffer, and the remaining supernatant was aspirated off. After resuspending the membrane pellet in 1.1 ml of B88, samples of the pellet were diluted in sample buffer. Supernatant and pellet fractions were then resolved on 11% SDS-polyacrylamide gels and analyzed by immunoblotting with antiserum directed against either Coy1, the HA epitope, Sec19, or Erv46.

Fractionation of proteins on sucrose gradients was conducted as described previously with modifications (73, 77–79). 5–45% continuous sucrose gradients with 25 mM HEPES, pH 8.0, 150 mM KOAc, and 1% Triton X-100 were prepared in 14 × 89-mm Ultra-Clear tubes (Beckman Coulter). 25 OD600 units of
the indicated yeast strains were converted to semi-intact cells and resuspended in 0.75 ml of buffer (25 mM HEPES, pH 8.0, 150 mM KOAc, 5 mM EDTA, 1 mM PMSF, 1 mM DTT) and then diluted with an equivalent volume of the same buffer supplemented with 2% Triton X-100. Samples were vortexed and incubated on ice for 15 min and then centrifuged at 54,000 rpm in a TLA 100.3 rotor for 20 min at 4 °C. 0.5 ml of the supernatant was layered on top of the gradient, and samples were centrifuged at 220,000 × g for 12 h at 4 °C in an SW41 rotor. 0.8-ml fractions were collected from the top to the bottom of the gradient, and fractions were diluted 1:1 in SDS-PAGE sample buffer and boiled. Samples were then resolved on 11% SDS-polyacrylamide gels and analyzed by immunoblotting with antisera against Cog1, Cog3, and Vam3. Fractionation of organelles over sucrose gradients was performed as described previously (24, 80).

Protein purification and in vitro binding assays

Recombinant proteins were purified from *Escherichia coli* as described previously (5, 24, 34, 81, 82). Binding assays were set up as described with modifications (24). 15 μl of Ni-NTA or GSH-agarose resin per reaction was washed with 1 ml of binding buffer (25 mM HEPES, pH 7.4, 150 mM KOAc, 10 mM β-mercaptoethanol, 250 mM sorbitol, 0.1% Triton X-100, and, for the Ni-NTA reactions, 30 mM imidazole) three times and incubated with 50 μg of purified protein per reaction for 1 h at room temperature with nutation and then washed three times with 1 ml of binding buffer again. The immobilized proteins were set aside while cell extract or cytosol was prepared.

For binding assays using Coy1 with an intact transmembrane domain (Fig. 3, B–E), 50-ml cultures of pGAL-COY1–3xHA (CY2674), pGAL-COY1 (211–679) (CBY5544), and pGAL-COY1 (536–679) (CBY5545) were grown to mid-log in YPD at 30 °C and then diluted to 0.1 OD₆₀₀/ml in 1 liter of YP + 2% galactose. Growth was continued for 16 h at 30 °C, and cells were subsequently harvested by centrifugation, lysed in a blender with liquid nitrogen, and divided into aliquots corresponding to 50 mg wet weight. Cells were later resuspended in buffer (0.5 ml of 25 mM HEPES, pH 7.4, 150 mM KOAc, 10 mM β-mercaptoethanol, 250 mM sorbitol, 1 mM PMSF and, for the COG binding assays, 30 mM imidazole) and then diluted with an equivalent volume of buffer supplemented with 2% Triton X-100. Cells were briefly vortexed, incubated on ice for 15 min, and centrifuged at 54,000 rpm in a TLA 100.3 rotor for 20 min at 4 °C. 0.5 ml of the solubilized extract was then incubated with the immobilized proteins for 3 h at 4 °C, washed with 1 ml of binding buffer, and eluted by boiling in 5 × sample buffer. The eluate was subsequently resolved on 11.5% gels and analyzed by immunoblotting with monoclonal antisera against HA to monitor recovery of Coy1 and polyclonal antibodies against GST and Cog2.

For all other binding assays, coy1Δ cells carrying 2-μm plasmids encoding the COY1ΔTM constructs expressed under the GAL1 promoter were grown overnight in selective media with 3% raffinose as the sole carbon source. Cells were then back-diluted to 0.1 OD₆₀₀/ml in 50 ml of YP + 2% galactose, grown to mid-log, and converted to semi-intact cells. Semi-intact cells were resuspended in 0.5 ml of binding buffer and snap-frozen in liquid nitrogen in 25-μl aliquots. Aliquots were resuspended in 1.1 ml of binding buffer + 1 mM PMSF and centrifuged at 54,000 rpm in a TLA 100.3 rotor for 20 min at 4 °C. 0.5 ml of the supernatant was incubated with the immobilized proteins, washed, eluted, and analyzed as described above.

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References

1. Glick, B. S., and Luini, A. (2011) Models for Golgi traffic: a critical assessment. *Cold Spring Harb. Perspect. Biol.* 3, a005215 CrossRef Medline

2. Fisher, P., and Ungar, D. (2016) Bridging the gap between glycosylation and vesicle traffic. *Front. Cell Dev. Biol.* 4, 15 CrossRef Medline

3. Mizuno-Yamasaki, E., Rivera-Molina, F., and Novick, P. (2012) GTPase network in membrane traffic. *Annu. Rev. Biochem.* 81, 637–659 CrossRef Medline

4. Yu, I. M., andHughson, F. M. (2010) Tethering factors as organizers of intracellular vesicular traffic. *Annu. Rev. Cell Dev. Biol.* 26, 137–156 CrossRef Medline

5. Parlati, F., McNew, J. A., Fukuda, R., Miller, R., Söllner, T. H., and Rothman, J. E. (2000) Topological restriction of SNARE-dependent membrane fusion. *Nature* 407, 194–198 CrossRef Medline

6. Parlati, F., Varlamov, O., Paz, K., McNew, J. A., Hurtado, D., Söllner, T. H., and Rothman, J. E. (2002) Distinct SNARE complexes mediating membrane fusion in Golgi transport based on combinatorial specificity. *Proc. Natl. Acad. Sci. U.S.A.* 99, 5424–5429 CrossRef Medline

7. Wong, M., and Munro, S. (2014) Membrane trafficking. The specificity of vesicle traffic to the Golgi is encoded in the golgin coiled-coil proteins. *Science* 346, 1256898 CrossRef Medline

8. Magdeléine, M., Gautier, R., Gounon, P., Barelli, H., Vanni, S., and Antonny, B. (2016) A filter at the entrance of the Golgi that selects vesicles according to size and bulk lipid composition. *Elife* 5, e16988 CrossRef Medline

9. Yuan, H., Davis, S., Ferro-Novick, S., and Novick, P. (2017) Rewiring a Rab regulatory network reveals a possible inhibitory role for the vesicle tether, Uso1. *Proc. Natl. Acad. Sci. U.S.A.* 114, E8637–E8645 CrossRef Medline

10. Gillingham, A. K. (2018) At the ends of their tethers! How coiled-coil proteins capture vesicles at the Golgi. *Biochem. Soc. Trans.* 46, 43–50 CrossRef Medline

11. Malsam, J., and Söllner, T. H. (2011) Organization of SNAREs within the Golgi stack. *Cold Spring Harb. Perspect. Biol.* 3, a005249 CrossRef Medline

12. Siniossoglou, S., and Pelham, H. R. (2001) An effector of Ypt6p binds the COG complex, a Ypt1p effector required for retrograde intra-Golgi trafficking, and interacts with Golgi SNAREs and COPI vesicle coat proteins. *Annu. Rev. Biochem.* 70, 591–599 CrossRef Medline

13. Suvorova, E. S., Duden, R., and Lupashin, V. V. (2002) The Sec34/Sec35p complex, a Ypt6p effector required for retrograde intra-Golgi trafficking, interacts with Golgi SNAREs and COPI vesicle coat proteins. *J. Cell Biol.* 157, 631–643 CrossRef Medline

14. Willett, R., Kudtyk, T., Pokrovskaya, I., Schönherr, R., Ungar, D., Duden, R., and Lupashin, V. (2013) COG complexes form spatial landmarks for distinct SNARE complexes. *Nat. Commun.* 4, 1553 CrossRef Medline

15. Dubke, M. L., and Munson, M. (2016) The secret life of tethers: the role of tethering factors in SNARE complex regulation. *Front. Cell Dev. Biol.* 4, 42 CrossRef Medline

16. Gillingham, A. K., and Munro, S. (2016) Finding the Golgi: golgin coiled-coil proteins show the way. *Trends Cell Biol.* 26, 399–408 CrossRef Medline
17. Wong, M., Gillingham, A. K., and Munro, S. (2017) The golgin coiled-coil proteins capture different types of transport carriers via distinct N-terminal motifs. BMC Biol. 15, 3 CrossRef Medline

18. Shin, J. J. H., Gillingham, A. K., Begum, F., Chadwick, J., and Munro, S. (2017) TBC1D23 is a bridging factor for endosomal vesicle capture by golgin at the trans-Golgi. Nat. Cell Biol. 19, 1424–1432 CrossRef Medline

19. Sinka, R., Gillingham, A. K., Kondylis, V., and Munro, S. (2008) Golgi coiled-coil proteins contain multiple binding sites for Rab family G proteins. J. Cell Biol. 183, 607–615 CrossRef Medline

20. Munro, S. (2011) The golgin coiled-coil proteins of the Golgi apparatus. Cold Spring Harb. Perspect. Biol. 3, a005256 CrossRef Medline

21. Cheung, P. Y., Limouze, C., Mabuchi, H., and Pfeffer, S. R. (2015) Protein flexibility is required for vesicle tethering at the Golgi. Elife 4, e12790 CrossRef Medline

22. Gillingham, A. K., Pfeifer, A. C., and Munro, S. (2002) CASP, the alternatively spliced product of the gene encoding the CCAAT-displacement protein transcription factor, is a Golgi membrane protein related to gian-...
icosahedral bacteriophage 4X174 forms a tail for DNA transport during infection. Nature 505, 432–435 CrossRef Medline
57. Shi, L., Shen, Q. T., Kiel, A., Wang, H. W., Melia, T. J., Rothman, J. E., and Pincet, F. (2012) SNARE proteins: one to fuse and three to keep the nascent fusion pore open. Science 335, 1355–1359 CrossRef Medline
58. Wang, J., Bello, O., Auclair, S. M., Wang, J., Coleman, J., Pincet, F., Krishnakumar, S. S., Sindelar, C. V., and Rothman, J. E. (2014) Calcium sensitive ring-like oligomers formed by synaptotagmin. Proc. Natl. Acad. Sci. U.S.A. 111, 13966–13971 CrossRef Medline
59. Wang, J., Li, F., Bello, O. D., Sindelar, C. V., Pincet, F., Krishnakumar, S. S., and Rothman, J. E. (2017) Circular oligomerization is an intrinsic property of synaptotagmin. Elife 6, e27441 CrossRef Medline
60. Orr, A., Song, H., Rusin, S. F., Kettenbach, A. N., and Wickner, W. (2017) HOPS catalyzes the interassembly of each vacuolar SNARE into a SNARE complex. Mol. Biol. Cell 28, 975–983 CrossRef Medline
61. Bello, O. D., Jouannot, O., Chaudhuri, A., Stroeva, E., Coleman, J., Volynski, K. E., Rothman, J. E., and Krishnakumar, S. S. (2018) Synaptotagmin oligomerization is essential for calcium control of regulated exocytosis. Proc. Natl. Acad. Sci. U.S.A. 115, E7624–E7631 CrossRef Medline
62. D’Agostino, M., Risselada, H. J., Lürick, A., Ungermann, C., and Mayer, A. (2017) A tethering complex drives the terminal stage of SNARE-dependent membrane fusion. Nature 551, 634–638 CrossRef Medline
63. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) Transformation of intact yeast cells with alkali cations. J. Bacteriol. 153, 163–168 Medline
64. Gietz, R. D., and Schiestl, R. H. (2007) Quick and easy yeast transformation protocol. Yeast 24, 2519–2524 CrossRef Medline
65. Longtine, M. S., McKenzie, A. M., Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J. R. (1998) Additional modules for use with the LiAc/SS carrier DNA/PEG method. Yeast 15, 119–122 CrossRef Medline
66. Loset, E., Reinke, C. A., Jellen, J., Strongin, D. E., Bevis, B. J., and Glick, B. S. (2006) Golgi maturation visualized in living yeast. Nature 441, 1002–1006 CrossRef Medline
67. Güldener, U., Heck, S., Fielder, T., Beinheimer, I., and Hegemann, J. H. (1996) A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res. 24, 2519–2524 CrossRef Medline
68. Sarkar, G., and Sommer, S. S. (1998) The “megaprimer” method of site-directed mutagenesis. BioTechniques 60, 404–407 Medline
69. Xu, Z., Colosimo, A., and Gruenert, D. C. (2003) Site-directed mutagenesis using the megaprimer method. Methods Mol. Biol. 235, 203–207 CrossRef Medline
70. Christianson, T. W., Sikorski, R. S., Darte, M., Shero, J. H., and Hieter, P. (1992) Multifunctional yeast high-copy-number shuttle vectors. Gene 2, 119–122 Medline
71. Baker, D., Hicke, L., Rexach, M., Schleyer, M., and Schekman, R. (1988) Reconstitution of SEC gene product-dependent intercompartamental protein transport. Cell 54, 335–344 CrossRef Medline
72. Ballew, N., Liu, Y., and Barlowe, C. (2005) A Rab requirement is not bypassed in SLY1–20 suppression. Mol. Biol. Cell 16, 1839–1849 CrossRef Medline
73. Duden, R., Hosobuchi, M., Hamamoto, S., Winey, M., Byers, B., and Schekman, R. (1994) Yeast β- and β′-coat proteins (COP). Two coatomer subunits essential for endoplasmic reticulum-to-Golgi protein traffic. J. Biol. Chem. 269, 24486–24495 Medline
74. Otte, S., Belden, W. J., Heidtman, M., Liu, J., Jensen, O. N., and Barlowe, C. (2001) Erv41p and Erv46p: new components of COPII vesicles involved in transport between the ER and Golgi complex. J. Cell Biol. 152, 503–518 CrossRef Medline
75. Salama, N. R., Yeung, T., and Schekman, R. W. (1993) The Sec13p complex and reconstitution of vesicle budding from the ER with purified cytosolic proteins. EMBO J. 12, 4073–4082 CrossRef Medline
76. Barlowe, C. (1997) Coupled ER to Golgi transport reconstituted with purified cytosolic proteins. J. Cell Biol. 139, 1097–1108 CrossRef Medline
77. Ungermann, C., Sato, K., and Wickner, W. (1998) Defining the functions of trans-SNARE pairs. Nature 396, 543–548 CrossRef Medline
78. Duden, R., Griffiths, G., Frank, R., Argos, P., and Kreis, T. E. (1991) β-COP, a 110 kD protein associated with non-clathrin-coated vesicles and the Golgi complex, shows homology to β-adaptin. Cell 64, 649–665 CrossRef Medline
79. Conibear, E., and Stevens, T. H. (2000) Vps55p, Vps53p, and Vps54p form a novel multisubunit complex required for protein sorting at the yeast late Golgi. Mol. Biol. Cell 11, 305–323 CrossRef Medline
80. Powers, J., and Barlowe, C. (1998) Transport of axl2p depends on erv14p, an ER-vesicle protein related to the Drosophila cornichon gene product. J. Cell Biol. 142, 1209–1222 CrossRef Medline
81. Tsui, M. M., and Banfield, D. K. (2000) Yeast Golgi SNARE interactions are promiscuous. J. Cell Sci. 113, 145–152 Medline
82. Peng, R., and Gallwitz, D. (2002) Sly1 protein bound to Golgi syntaxin Sec5p allows assembly and contributes to specificity of SNARE fusion complexes. J. Cell Biol. 157, 645–655 CrossRef Medline
83. Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., and Boeke, J. D. (1998) Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast 15, 115–132 CrossRef Medline
84. Christianson, T. W., Sikorski, R. S., Darte, M., Shero, J. H., and Hieter, P. (1992) Multifunctional yeast high-copy-number shuttle vectors. Gene 2, 119–122 Medline

EDITORS’ PICK: Dissection of Coy1 functional domains