**RESEARCH ARTICLE SUMMARY**

**MEMBRANE TRAFFICKING**

The specificity of vesicle traffic to the Golgi is encoded in the golgin coiled-coil proteins

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**INTRODUCTION:** The eukaryotic cell contains membrane-bound organelles with distinct functionality and composition. Preservation of organelle identity depends on the highly selective transfer of proteins and lipids between compartments. Central to this are transport carriers called vesicles. Mechanisms are required not only for the selective incorporation of specific cargos into vesicles as they bud off a donor organelle but also for the correct delivery to an acceptor organelle. SNARE proteins on the vesicle and destination organelle drive membrane fusion after arrival and have been implicated in contributing to specificity in choice of organelle. However, upstream of the fusion step, a process called tethering is thought to initially attach the vesicle to the destination organelle and then bring it close to allow the SNARE proteins on opposite membranes to interact. The importance of tethering in conferring specificity to membrane traffic is currently unclear.

**RATIONALE:** To study the contribution of tethering to specificity in membrane trafficking, we focused on the Golgi apparatus. The Golgi complex is a multicompartment organelle at the intersection of secretory and endocytic trafficking pathways and so receives vesicles from a range of destinations. A family of well-conserved large coiled-coil proteins on the Golgi, the golgins, have been suggested to function as vesicle tethers at the Golgi. However, mild phenotypes of golgin mutants have presented a challenge for elucidating their in vivo roles. We thus used a relocation strategy to test for their sufficiency rather than necessity in vesicle tethering. Ten mammalian golgins that are conserved outside of vertebrates and found on different regions of the Golgi were ectopically expressed at the mitochondria through attachment to a mitochondrial transmembrane domain in place of their C-terminal Golgi targeting domain. We then used the distribution of cargo-laden vesicles originating from different locations as a readout for the golgins’ tethering activity.

**RESULTS:** We demonstrate that subsets of golgins are capable of redirecting particular endogenous or exogenous cargo destined for the Golgi to an ectopic site, the mitochondria. Specifically, golgin-97, golgin-245, and GCC88 were able to capture endosome-to-Golgi cargos; GM130 and GMAP210 were able to capture endoplasmic reticulum (ER)–to-Golgi cargos; and golgin-84, TMF, and GMAP210 were able to capture Golgi resident proteins. Furthermore, electron microscopy yielded ultrastructural evidence for the accumulation of vesicular membranes around mitochondria decorated with specific golgins. These data suggest that not only do the golgins capture vesicles, they also exhibit specificity toward vesicles of different origins—from the endosomes, from the ER, or from within the Golgi itself.

**CONCLUSION:** We have been able to demonstrate that relocation of specific golgins is sufficient to reroute specific classes of transport vesicles to an ectopic site. Thus, most golgins are sufficient to nucleate a specific tethering process, and hence they are likely to make a major contribution to the specificity of vesicle traffic arriving at the Golgi. In addition, this relocation system may be a useful tool for isolating specific transport vesicles that are normally short-lived, hence providing a route to further understanding of specificity in membrane traffic.

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Golgins are sufficient to capture specific classes of vesicle. We hypothesized that if the golgins tether vesicles destined for the Golgi, then their relocation to mitochondria should result in ectopic capture of specific classes of vesicle. Immunofluorescence demonstrates that the presence of a single golgin on mitochondria results in the ectopic capture of a specific cargo. Electron microscopy reveals that vesicles accumulate around these mitochondria.

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The specificity of vesicle traffic to the Golgi is encoded in the golgin coiled-coil proteins

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The Golgi apparatus is a multicompartment central sorting station at the intersection of secretory and endocytic vesicular traffic. The mechanisms that permit cargo-loaded transport vesicles from different origins to selectively access different Golgi compartments are incompletely understood. We developed a rerouting and capture assay to investigate systematically the vesicle-tethering activities of 10 widely conserved golgin coiled-coil proteins. We find that subsets of golgins with distinct localizations on the Golgi surface have capture activities toward vesicles of different origins. These findings demonstrate that golgins act as tethers in vivo, and hence the specificity we find to be encoded in this tethering is likely to make a major contribution to the organization of membrane traffic at the Golgi apparatus.

The functionality of the membrane-bound organelles of eukaryotic cells is determined by their constituent proteins and lipids. A major determinant of organelle composition in the endomembrane system is the highly selective transfer of cargo-laden vesicles between compartments. Not only must the correct cargo be collected at the origin, the transport vesicle must be selectively delivered to the correct destination. SNARE proteins on the vesicle and destination organelle assembly to drive membrane fusion (1). The diversity of cellular SNAREs, their differential localization, and their selective pairwise interactions implicate them in contributing specificity to membrane traffic (2–4). However, the relatively small size of SNAREs means that they can only interact after a vesicle is closely apposed to its potential destination. A process that they can only interact after a vesicle is closely apposed to its potential destination. A process called tethering is thought to attach the vesicle to the organelle before SNARE complex assembly (5–7). The degree to which organelle tethers confer specificity rather than efficiency to membrane traffic is currently unclear.

To investigate this problem, we focused on the Golgi apparatus, the central sorting station in the endomembrane system (8). The Golgi is a stack of distinct cisternae arranged from cis to trans, and particular vesicles appear to preferentially fuse with different cisternae within the stack. For example, vesicles derived from the endoplasmic reticulum (ER) deliver cargo to the cis face of the Golgi, whereas those from the endocytic pathway deliver cargo to the trans face of the Golgi. Moreover, vesicles mediate selective transfer of contents between Golgi cisternae. Thus, providing spatial cues to vesicles arriving at the Golgi is critical for endomembrane traffic and represents an ideal system for investigating the basis of vesicle targeting specificity.

The golgins, large well-conserved coiled-coil proteins anchored to the membrane via their C termini (9–11), have been suggested to act as vesicle tethers, but in vivo evidence for this role is lacking (12, 13). In addition, other roles have been proposed for some golgins, such as recruiting kinases or cytoskeletal regulators (14, 15). Golgin mutants typically have mild or tissue-specific phenotypes, leaving their function uncertain (16, 17). We hypothesized that the golgins have overlapping functions in tethering, which may obscure their function when any one is missing. We therefore developed methods to test their sufficiency, rather than necessity, in selective vesicle tethering.

**Experimental strategy**

Our strategy was to attach golgins to a different structure in the cell and then test whether this was sufficient to redirect Golgi-bound carriers to the ectopic destination (Fig. 1A). We chose mitochondria because they display a distinctive localization, are distributed throughout the cell, and do not normally capture transport vesicles. We tested 10 mammalian golgins chosen for their conservation outside of vertebrates and spatial distribution in different Golgi regions (Fig. 1B). Ectopic golgin localization was accomplished by either direct or induced attachment to a mitochondrial transmembrane domain in place of the C-terminal Golgi targeting domain (Fig. 1C). The direct fusion approach was more straightforward (Fig. 1D and fig. S1), whereas the induced targeting strategy provided tight temporal control (Fig. 1, C and E). The golgins GM130 and GCC185 are known to bind soluble cytosolic proteins [p15 and CLASP, respectively (14, 18)], and in both cases, relocation of the golgin resulted in the binding partner being found on the mitochondria as well as the Golgi; this suggested that relocation had not grossly perturbed the structure of the golgin (fig. S2). For two of the golgins (GM130 and GMAP-210), a high expression level of the mitochondrial form caused the Golgi to become fragmented, which could be due to their titrating away factors that contribute to Golgi integrity (such as p15) or due to the effect of their relocation on ER-to-Golgi traffic (see below).

Vesicles originating from different locations were analyzed for localization upon ectopic expression of individual golgins. The identity of originating vesicles was marked by either endogenous or exogenous cargo selective to that site. Analysis of all combinations of originating vesicles with each of the 10 golgins was anticipated to provide a comprehensive view of their capacity to provide landmarks for vesicular traffic. We present these findings by successively analyzing transport vesicles of endosomal, ER, and intra-Golgi origin.

**Capture of endosome-to-Golgi carriers**

One of the best-characterized cargos that traffic from endosomes back to the Golgi is the cation-dependent mannose 6-phosphate receptor (CD-MPR) that delivers newly made lysosomal hydrolases to endosomes and then returns to the Golgi (19). At steady state, most of this receptor is in the Golgi apparatus, but mitochondrial forms of three of the golgins caused endogenous CD-MPR to accumulate on mitochondria (Fig. 2A). These were golgin-97, golgin-245, and GCC88, three of the four golgins that share a C-terminal GRIP domain that mediates binding to Arl1, a G protein of the trans side of the Golgi (9, 11). In each case, CD-MPR distribution was the sum of the Golgi and mitochondrial distribution, and quantitation confirmed that the effect was seen in most of the transfected cells (Fig. 2B). The remaining seven golgins had no detectable effect on the distribution of the CD-MPR (Fig. 1A). The same three golgins could also induce a redistribution of the SNARE protein Vti1a, another cargo of these retrograde carriers (20) (fig. S3). Other endogenous proteins that travel along this retrograde route were also affected, including the cation-independent MPR and TGN46, a protein of unknown function that has both Golgi retention and endosome retrieval signals (21) (fig. S4). These effects did not simply reflect capture of endosomes on the mitochondria, because both early and late endosomal markers did not relocate (fig. S5). Finally, to confirm that the carriers were moving from endosomes to Golgi, we expressed the golgins in a cell line expressing a CD8-MPR chimera that recycles efficiently from the surface to the Golgi via endosomes (22). When CD8 antibodies were bound to the surface and followed after endocytosis, the carriers containing the antibodies could be captured by the mitochondrial golgins early after initiation of uptake; this finding indicates that the carriers were moving from endosomes to the Golgi (fig. S6).

The above experiments used golgins that were constitutively targeted to mitochondria...
Fig. 1. Strategy for relocation of the golgins to mitochondria. (A) An in vivo assay for golgin tethering activity. Normally vesicles from various origins move to find the Golgi. If a particular class of vesicles can be captured by a specific golgin, then presenting that golgin on mitochondria should allow ectopic capture of that class of vesicle. (B) The 10 human golgin coiled-coil proteins that have orthologs outside of vertebrates. (C) Scheme for relocating golgins to mitochondria in a constitutive or inducible manner, illustrated for the GRIP domain protein golgin-97. The C-terminal transmembrane domain of monoamine oxidase (MOA) is sufficient for targeting to the outer mitochondrial membrane (40). Inducible relocation was performed using the rapamycin-controlled heterodimerization domains from FKBP and FRB (41). (D) COS cells expressing representative golgin-mito fusion proteins stained for a hemagglutinin (HA) epitope located between the golgin and the organelle targeting motif. Costaining for a mitochondrial marker, MTC02, and a Golgi marker, ZFPL1 (cis), indicates correct relocation of each of the golgins to the outer membrane of the mitochondria. Representative regions of mitochondria are magnified (Golgi marker omitted, therefore showing green and red channels only). Scale bars, 10 μm. (E) COS cells coexpressing reroutable golgin-97 with FKBP-GFP-MAO with or without 200 nM rapamycin treatment for 15 min. Reroutable golgin-97 was detected by staining for a HA epitope tag between the golgin and the FRB domain. Scale bars, 10 μm.
in transiently transfected cells, and so the golgins required 24 to 36 hours to accumulate to readily detectable levels. When the golgins were instead acutely located to mitochondria by means of the rapamycin-dependent inducible system, we observed a rapid relocation of the retrograde cargo such as CD-MPR and Vti1a within 15 min of addition of rapamycin (Fig. 2C and fig. S3B). This relocation was seen

**Fig. 2. Golgin-97, golgin-245, and GCC88 relocate endosome-to-TGN cargos to the mitochondria.** (A) COS cells expressing the indicated golgin-mito (mitochondria-targeted golgins) were costained for an HA epitope to detect the recombinant golgins, endogenous CD-MPR, and ZFPL1 (cis) to mark the Golgi. Representative regions are shown magnified. Intensity plots of signal intensity (y axis) against distance in μm (x axis) show occurrence of overlap between channels. Respective signals from the mitochondria and the Golgi are indicated above each plot by letters M (for mitochondria) and G (for Golgi). (B) Plot for mean CD-MPR labeling intensity within the mitochondria segment (the golgins) or elsewhere in the cell (background labeling); N ≥ 30. (C) COS cells coexpressing reroutable golgin-97 with FKBP-GFP-MAO were treated with 200 nM rapamycin for 0 or 15 min and costained for endogenous CD-MPR. Intensity plot as in (A). Scale bars, 10 μm.
with the same three GRIP domain golgins, which suggests that the effect is a direct consequence of the ectopic location of the golgins (fig. S7).

Capture of ER-to-Golgi carriers

A second major class of carriers that arrive at the Golgi are those that deliver newly made secreted and membrane proteins from the ER. Proteins leave the ER from exit sites found both adjacent to the Golgi and scattered throughout the cytoplasm, and from the latter sites carriers move along microtubules to reach the Golgi (23). To follow these carriers, we made use of a green fluorescent protein (GFP)–tagged secreted protein that accumulates in the ER until released by a small molecule (24). Upon release, the reporter relocates to the Golgi within 10 to 15 min and then leaves for the surface; this behavior was not perturbed by the mitochondrial forms of 8 of the 10 golgins tested (Fig. 3A). However, for the golgins GM130 and GMAP-210, we observed association of GFP-labeled structures with mitochondria (Fig. 3, B and C). This association appeared to gradually reduce after an initial peak; to quantify this, we used live cell imaging to compare the GFP-labeled cargo to mCherry-labeled mitochondrial golgins in individual cells over time (figs. S8 to S11). Quantification revealed that GM130 and GMAP-210 caused an association of ER-derived carriers with mitochondria that was specific but also transient, presumably reflecting the carriers being captured and then eventually released, perhaps by being pulled away by the motors that move them toward the Golgi (Fig. 4A).

The two golgins able to capture pre-Golgi carriers are both found on the cis side of the Golgi, consistent with a role in ER-to-Golgi traffic (25, 26). GM130 interacts via its N-terminal 74 residues with the tethering factor p115 (18). A mitochondrial form of GM130 lacking residues 1 to 74 no longer relocated p115 to mitochondria and showed reduced Golgi fragmentation (Fig. 3C and fig. S2). Nonetheless, this construct could still transiently capture ER-derived carriers, which suggests that GM130 has some vesicle tethering activity independent of p115 and also indicates that the ectopic capture of ER-derived cargo is not simply a consequence of Golgi fragmentation (Figs. 3C and 4A). To strengthen the latter conclusion, we also followed ER-derived cargo in cells in which the Golgi had been fragmented by depolymerizing microtubules. Despite the Golgi being fragmented, capture was not observed with a control golgin but could be clearly detected for GM130 (Fig. 4, B to D, and movies S1 and S2).

Capture of carriers containing Golgi residents

The Golgi stack is surrounded by vesicles that are thought to recycle Golgi resident proteins back to earlier parts of the stack as the cisternae mature, and it has also been proposed that some may carry secretory cargo forward through the stack (27). We thus looked for relocation of Golgi resident proteins by the relocated golgins. With
Fig. 4. Live cell imaging of ectopic capture of ER-derived carriers by GMAP210 and GM130. (A) Graphs of GFP fluorescence (y axis) against time (x axis) demonstrate time courses for secretion of the GFP-FM4-hGH reporter by C1 cells that were transiently expressing mitochondria-targeted golgins. Average GFP fluorescence intensity over the entire cell (whole cell) or the mitochondrial region (mitochondria) was determined for several cells over time, with the results presented as percentages of the fluorescence intensity at t = 0. Data are means ± SEM; golgin-84 (N = 5), GMAP-210 (N = 7), GM130 (N = 6), and GM130delN75 (N = 7). Stills from a representative data set from each are shown in figs. S8 to S11. (B and C) C1-HeLa cells transiently expressing mitochondria-targeted mCherry-golgin-97 (B) or mCherry-GM130 (C) were pretreated with 0.5 μM nocodazole for 3 hours to depolymerize microtubules prior to being incubated at 37°C for the indicated times with a mix of nocodazole and D/D solubilizer to induce secretion of the GFP-FM4-hGH reporter. Images were acquired every 2 min for 140 min (movies S1 and S2). Transient association of GFP-FM4-hGH with mitochondria can be seen for GM130 (illustrated by arrowheads) but not for golgin-97. Scale bars, 5 μm. (D) Quantitation of the relative association of the GFP-FM4-hGH reporter with mitochondria over time in nocodazole-treated cells as in (B) and (C). Data are means ± SEM (N = 4 for both).
Fig. 5. Mitochondrial TMF and golgin-84 relocate Golgi membrane proteins. (A) Confocal micrographs of COS cells expressing TMF-mito (HA) and stained for a Golgi enzyme, GalNAc-T2, and a Golgi marker, GCC88 (trans), without, or following, 6 hours of nocodazole treatment as indicated. (B and C) COS cells expressing the indicated golgin-mito were treated with nocodazole for 6 hours and costained for GalNAc-T2 and a Golgi marker, GCC88 (trans). (D) COS cells expressing golgin-84-mito were treated with 0.5 μM nocodazole for 6 hours and labeled for two of the endogenous Golgi proteins GalNAcT2, TGN46, or COPI as indicated. In all cases, solid arrows indicate Golgi ministacks positive for both Golgi markers, with these being distinct from mitochondria; open arrows indicate structures on mitochondria positive for GalNAcT2 but not the other marker. (E) COS cells coexpressing golgin-84Acterm-FRB and FKBP-MAO were treated with 0.5 μM nocodazole for 3 hours followed by 200 nM rapamycin (and nocodazole) to direct dimerization and then fixed as indicated and stained for endogenous GalNAcT2 and TGN46. Solid arrows indicate Golgi ministacks positive for both markers; open arrows indicate GalNAcT2 captured onto mitochondria after 15 min. Scale bars, 10 μm.
ectopic TMF, the endogenous Golgi enzyme GalNAc-T2 could be detected on mitochondria as well as on the Golgi, but unlike the carriers described above, this accumulation was only seen on those mitochondria near the Golgi stack (Fig. 5A). This may reflect the fact that intra-Golgi carriers do not need to move far from the Golgi, and so most only encounter the few mitochondria that are close to the stack, whereas carriers coming to the Golgi from the ER and endosomes can travel long distances along microtubules and hence pass many mitochondria. To increase the probability of intra-Golgi carriers encountering mitochondria, we used nocodazole to remove microtubules and thus scatter the Golgi into many ministacks located throughout the cytoplasm and thus in the proximity of many more mitochondria. To increase the probability of intra-Golgi carriers encountering mitochondria, we used nocodazole to remove microtubules and thus scatter the Golgi into many ministacks located throughout the cytoplasm and thus in the proximity of many more mitochondria. In such cells, we could see robust relocation of GalNac-T2 to mitochondria by TMF (Fig. 5A) as well as by golgin-84 and GMAP-210, but not by any other golgin (Fig. 5, B and C, and fig. S12). Analysis of further Golgi markers confirmed that the ministacks are distinct from the GalNac-T2 captured on mitochondria, because the markers from different cisternae are much closer to each other than to the GalNac-T2 on the mitochondria (Fig. 5D). Finally, after acute relocation of golgin-84 to mitochondria by means of the rapamycin-based dimerization, GalNAc-T2 could be clearly detected on mitochondria after 15 min, indicating that this capture in nocodazole-treated cells is unlikely to be due to an indirect effect arising over many hours (Fig. 5E).

We next examined a range of different Golgi resident membrane proteins in nocodazole-treated cells and again found relocation by specific golgins, with strikingly different patterns seen for different residents. The cis-Golgi membrane protein ZFPL1 was efficiently relocated by golgin-84 and GMAP-210, and to a lesser extent by TMF, but was unaffected by the other seven golgins (Fig. 6A and fig. S13). In contrast, the TGN resident protein TGN46 was relocated by TMF but not by golgin-84 or GMAP-210 (Fig. 6A). Quantitation of colocalization with mitochondria confirmed these differential relocalizations (Fig. 6B). TGN46 has two targeting signals: one in its cytoplasmic tail that mediates retrieval from endosomes (hence TGN46 is in the endosome-to-Golgi carriers captured by the GRIP golgins), and a second transmembrane signal that confers retention in the trans-Golgi, probably by directing recycling within the stack (21).

The golgins giantin, CASP, and golgin-84 are themselves Golgi resident membrane proteins; if Golgi cisternae were to undergo maturation, then these three golgins would have to recycle to earlier cisternae in transport vesicles. Indeed, endogenous giantin was relocated to mitochondria by golgin-84 and GMAP-210, and although golgin-84 could not be tested with itself, it was also relocated by GMAP-210 (Fig. 6, C and D, and fig. S14). It is possible that these golgins interact directly to mediate tethering, but this remains to be determined. Taken together, these results show that three of the golgins are able to relocate resident membrane proteins of the Golgi to mitochondria. This presumably reflects the capture of TGN46 signal within the HA-positive mitochondria segment (the golgins) or whole cell (background). N ≥ 30. (C and D) COS cells expressing the indicated golgin-mito were treated with nocodazole for 6 hours and costained for either transmembrane golgins, giantin or golgin-84, and a Golgi marker, GCC88. Intensity plots of signal intensity (y axis) against distance in μm (x axis) show occurrence of overlap between the markers. Scale bars, 10 μm (magnified boxes, 5 μm).
of carrier vesicles containing these proteins, with the pattern of proteins captured varying between individual golgins.

**Electron microscopy reveals carrier capture by golgin-coated mitochondria**

In the experiments described above, endogenous membrane proteins were found to be relocated to mitochondria by specific golgins. If this is the case, then membrane-bound carriers accumulating around mitochondria should be detectable by electron microscopy (EM). To identify transfected cells, we expressed the mitochondrial golgins from a bicistronic plasmid that also expressed a form of APEX peroxidase that is targeted to the mitochondrial matrix (Fig. 7A). This peroxidase directs the precipitation of diaminobenzidine to form a dark deposit that is visible in EM sections (Fig. 7B). We initially examined the effect of coexpressing golgin-97, which we had found to efficiently capture endosome-derived proteins. In these cells, the mitochondria were surrounded by circular and oval membrane profiles that were absent from control cells expressing APEX alone (Fig. 7, C and D). The structures were typically 60 to 80 nm in diameter and often accumulated between adjacent mitochondria, but could also be seen around isolated parts of the mitochondrial surface.
Such membrane capture was also observed with other golgins that can relocate membrane proteins, including GCC88, TMF, GMAP-210, golgin-245, and golgin-84 (Fig. 7, F and G, and fig. S15). In contrast, we did not observe membranes accumulating around mitochondria coated with giantin or GCC35, consistent with their lack of effect on any of the markers tested (fig. S16). The tendency for the captured tubulovesicular structures to accumulate between mitochondria is presumably a result of their simultaneous engagement with golgins attached to adjacent mitochondria. This “zippering” effect resulted in clustering of mitochondria and explains the striped appearance of golgins on mitochondrial clusters that we had observed by immunofluorescence (Fig. 1D). In some cells, the mitochondrial clustering was so effective that all were collected together, separated by lines of captured vesicles that were often at a regular spacing between the mitochondria (Fig. 7E).

Discussion

Multiple classes of intracellular transport vesicles deliver cargo specifically to the Golgi apparatus. Our results show that relocation of specific golgin-coated proteins is sufficient to redirect specific classes of vesicles to an ectopic location (Fig. 8A). This provides clear evidence for at least seven of the golgins being bona fide vesicle tethers. However, the golgins not only capture vesicles but also can clearly distinguish among vesicles of different origins. This implies that the location of golgins specifies the point of vesicle tethering, and indicates that the machinery that recruits the golgins to specific cisternae of the Golgi helps to define their identity as the correct target organelle for incoming vesicles (Fig. 8B). This does not preclude other factors such as SNARE complex formation also making a contribution to the specificity of membrane traffic, but at least in this case, SNARE complex formation does not appear to be necessary for recognition of the destination organelle.

Our studies also reveal a degree of redundancy within the golgins; subsets of golgins apparently share tethering activity. For instance, golgin-97, golgin-245, and GCC88 all capture endosome-to-Golgi cargo, and GM130 and GMAP-210 both capture ER-to-Golgi carriers. This partial redundancy would be consistent with the proposal that they act collectively to surround different regions of the Golgi with docking sites for particular vesicle types (30). Such a model would be loosely analogous to the capture of cytoplasmic-to-nuclear cargo by the multiple FG repeat proteins of the nuclear pore (31). Three of the 10 golgins (CASP, GCC85, and giantin) did not show tethering activity in our assay; it is possible that they have other roles such as microtubule organization, as has been suggested for GCC85 (14). However, it is also possible that they can act as tethers, but only in conjunction with other golgins or after Golgi-specific modification (32, 33).

The fact that golgins can distinguish different classes of carrier vesicle implies that each class displays one or more unique features that allow it to be recognized. There are a range of plausible candidates for these vesicle features, such as the Rab guanosine triphosphatases (30, 34, 35). Indeed, further proteins may bind directly to the golgins to help capture the vesicle even if the golgins are themselves sufficient to nucleate a functional tethering entity. However, irrespective of what else is involved in completing the entire tether, our findings should make it easier to identify and isolate specific classes of carrier vesicles. These key mediators of traffic are small and normally short-lived, but the ectopic golgins cause these carriers to accumulate in a docked state; this allows their contents to be readily probed by immunolabeling and also suggests routes to isolation for proteomic analysis. In the case of the Golgi, our studies are already informative: Trans-Golgi residents preferred TMF, whereas cis- and medial Golgi residents preferred golgin-84 and GMAP-210. Because the golgins were presented in the same way (i.e., on mitochondria), this finding indicates that vesicles from different parts of the stack must display different golgin-binding features, and hence their destination within the stack is directed at least in part by molecular interactions and not simply by spatial proximity. This may be important for allowing the structure of the Golgi to vary between tissues and yet still maintain cisternal identity. In addition, the finding that GMAP-210 can bind both ER-derived vesicles and those containing Golgi residents raises the intriguing possibility that it could act as a scaffold for the assembly of new cisternae. As such, our findings not only reveal that golgins are sufficient to confer specificity to the tethering of Golgi destined vesicles but also provide new tools to investigate the organization of membrane traffic in this complex and much-debated organelle (36).

Materials and methods

Plasmids

C-terminally truncated golgins were provided by A. Gillingham [human GMAP210, golgin-84, and CASP (33, 37)] or PCR-amplified from full-length cDNAs kindly provided by others [human GCC88, GCC185, and golgin-245 from P. Gleeson (38); human golgin-97 from F. Barr; mouse TMF and rat GM130 from M. Lowe; human giantin from A. Linstedt (39)]. Human MAO-A C-terminal TMD (481-528) was PCR-amplified from human cDNA.

Truncated golgins were as follows: GCC88ΔC-term (1-Ala77); GCC185ΔC-term (1-Ser1535); golgin-97ΔC-term (1-Val84); golgin-245ΔC-term (1-Gly263);
TMFAC-term (1-Thr<sup>74</sup>); giantinαC-term (1-Cys<sup>220</sup>); golgin-84αC-term (1-Ala<sup>175</sup>); CASPAC-term (1-Arg<sup>38</sup>); GM30αC-term (1-Leu<sup>52</sup>); GM30δN75αC-term (Met<sup>52</sup>-Leu<sup>58</sup>); GMAP210αC-term (1-Leu<sup>52</sup>).

GolginαC-term-GAGA linker-HA-MAO, golginαC-term-GAGA (or GAGAMS) linker-HA-FRB, mCherry-GAGA (or GAGGS) linker-golginαC-term-GAGA linker-HA-MAO were generated in pcDNA3.1+ (Clontech) from the respective monoclonic plasmids.

**Antibodies**

Antibodies used in this study included mouse CD-9 (22d4), Developmental Studies Hybridoma Bank (DSHB); mouse CD8 (UCHT1, Sigma); rabbit anti-CLASP1 (gift from A. Akhmanova); rat β-COP [23C (TCP-1)]; mouse EEA1 (610457, BD Biosciences); mouse ERGIC-53 ([ALX-804-602-C100, Enzo Life Sciences]; mouse GαLαC (UH-4, gift from U. Mandel and H. Clausen), rabbit GCC88 (HPA001232, Sigma); rabbit GCC185 (HPA035849, Sigma); rabbit giantin (HPA011008, Sigma); rabbit GM130 (1837-1, Epitomics); mouse GM130 (610823, BD Biosciences); mouse golgin-245 (611281, BD Biosciences); rabbit golgin-84 (HPA000992, Sigma); rabbit HA (Y-11, Santa Cruz Biotechnology); rat HA (3F10, Roche); mouse LAMP1 (H4A3, DSHB); mouse MTCO2 (ab3298, Abcam); mouse p115 (612260, BD Biosciences); sheep TGN46 (AHP500, AbD serotec); rabbit TMF (HPA008729, Sigma); mouse Vtiba (611220, BD Biosciences); and rabbit ZFPL1 (HPA014099, Sigma).

**Cell culture, transient transfections, and treatments**

COS-7 cells, CD8-CIMPR HeLa cells (22), CT-HeLa cells expressing GFP-FM4-4HGH (24), and mouse embryonic fibroblasts (MEFs) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin at 37°C and 5% CO<sub>2</sub>. COS-7 cells and MEFs were transiently transfected with FKHBP and FRB domains with each of these domains at 37°C for various incubation times with rapamycin (Sigma; 100 nM from 2.74 mM stock in DMSO). To depolymerize microtubules, we treated cells at 37°C for 2 to 6 hours with nocodazole (Sigma, 1 μM from a 1 mM stock in DMSO). To induce secretion in CT-HeLa cells, we treated cells with D/D solubilizer (Clontech, 0.5 μM) at 37°C for various incubation times (24).

HeLa cells stably expressing the CD8-CIMPR reporter construct (22) were transfected with golgin-mito constructs 24 to 48 hours before the internalization assay and plated onto multispecies microscope slides 12 to 36 hours after transfection. Slides were incubated at 4°C for 20 minutes to stop all trafficking and washed with phosphate-buffered saline (PBS). Mouse antibody to CD8 (Sigma) was applied in DMEM for 30 minutes at 4°C to bind onto the cell surface. After two washes with PBS, antibody uptake was induced by replacing PBS with prewarmed DMEM followed by incubation at 37°C prior to fixation with 4% formaldehyde in PBS.

**Immunofluorescence and live cell imaging**

COS-7 cells, HeLa cells, and MEFs were fixed with 4% formaldehyde in PBS for 30 minutes, permeabilized in 0.5% Triton X-100 for 10 minutes, and blocked in blocking buffer (20% FCS, 0.5% Tween-20 in PBS) for 30 minutes. Primary and secondary antibodies were applied in blocking buffer for 1 hour; cells were washed twice with PBS and mounted under a cover slip in Vectashield mounting medium (Vector Labs). Images were acquired with a Zeiss LSM780 confocal microscope using a 63× APOCHROMAT oil-immersion objective.

Quantitation of mitochondrial capture in fixed cells was performed with Imaris 7.4.0 (Bitplane). First, the mitochondria and Golgi regions of each transfected cell were segmented by the HA staining pattern and ZFPL1 staining, respectively. Next, the latter segment was subtracted from the former segment to create a new mitochondria segment that had excluded any overlapping Golgi regions. Mean intensity of the molecule of interest on this segment was determined as an arbitrary, but relative, value. Background staining levels were obtained by quantifying mean signal intensity of the former segment to create a new mitochondrial segment and the other segment from the whole cell.

**APEX and electron microscopy**

Cells grown on glass-bottom petri dishes (MatTek) were fixed at room temperature with 2% glutaraldehyde (EM grade, Agar Scientific) in 0.1 M cacodylate buffer, pH 7.4 (CB). Cells were then incubated on ice for 30 to 60 minutes, rinsed twice with chilled CB, and blocked with 50 mM glycine in CB on ice for 10 minutes. To start the APEX-catalyzed oxidation of 3,3-diaminobenzidine (DAB), we added a freshly prepared solution of DAB free base (0.5 mg/ml, Sigma) in HCl and 0.03% (10 mM) H<sub>2</sub>O<sub>2</sub> (Sigma) to the cells. Formation of reaction product (a brownish precipitate) was monitored by light microscopy. After 15 minutes, the reaction was halted by washing the cells twice with chilled CB. Samples were postfixed with 1% osmium tetroxide in CB on ice for 1 hour, washed five times with distilled water, and dehydrated in ascending series of acetones (30%, 50%, 70%, 90%, 100%) at room temperature. The dehydrated samples were infiltrated and embedded in CY212 resin. Areas containing brown precipitates were excised and mounted on dummy blocks and sectioned parallel to the substratum. Pale gold 70-nm sections were contrasted with saturated aqueous uranyl acetate and Reynolds lead citrate. Electron micrographs were recorded at 80 kV or 120 kV on a FEI Tecnai Spirit TEM.
The specificity of vesicle traffic to the Golgi is encoded in the golgin coiled-coil proteins
Mie Wong and Sean Munro
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