Membrane adhesion dictates Golgi stacking and cisternal morphology

Intaek Lee1,2, Neeraj Tiwari1, Myyun Hwa Dunlop1, Morven Graham, Xinran Liu, and James E. Rothman2

Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06520

Contributed by James E. Rothman, December 27, 2013 (sent for review November 26, 2013)

Two classes of proteins that bind to each other and to Golgi membranes have been implicated in the adhesion of Golgi cisternae to each other to form their characteristic stacks: Golgi reassembly and stacking proteins 55 and 65 (GRASP55 and GRASP65) and Golgin of 45 kDa and Golgi matrix protein of 130 kDa. We report here that efficient stacking occurs in the absence of GRASP65/55 when either Golgin is overexpressed, as judged by quantitative electron microscopy. The Golgi stacks in these GRASP-deficient HeLa cells were normal both in morphology and in anterograde cargo transport. This suggests the simple hypothesis that the total amount of adhesive energy gluing cisternae dictates Golgi cisternal stacking, irrespective of which molecules mediate the adhesive process. In support of this hypothesis, we show that adding artificial adhesive energy between cisternae and mitochondria by dimerizing rapamycin-binding domain and FK506-binding protein domains that are attached to cisternal adhesive proteins allows mitochondria to invade the stack and even replace Golgi cisternae within a few hours. These results indicate that although Golgi stacking is a highly complicated process involving a large number of adhesive and regulatory proteins, the overriding principle of a Golgi stack assembly is likely to be quite simple. From this simplified perspective, we propose a model, based on cisternal adhesion and cisternal maturation as the two core principles, illustrating how the most ancient form of Golgi stacking might have occurred using only weak cisternal adhesive processes because of the differential between the rate of influx and outflux of membrane transport through the Golgi.

Significance

Stacking of cisternae in the mammalian Golgi apparatus is known to be a highly complicated process that involves a large number of adhesive proteins, including Golgi reassembly and stacking proteins (GRASPs) and Golgin tethers, as well as coordinated disassembly/reassembly of Golgi stacks during mitotic cell division. Using extensive quantitative analysis of electron microscopic data, we show here that Golgi stacking with flattened cisternae is maintained in HeLa cells depleted of GRASP65/55 proteins when the GRASP-deficient cells are complemented by exogenous expression of either Golgi matrix protein of 130 kDa or Golgin45. These results strongly suggest that the Golgi stack assembly and cisternal morphology are governed by simple membrane adhesion at the core, explaining how Golgi stacking occurs in organisms, which do not express (or use) GRASP-type adhesion proteins such as plants and yeast.

Author contributions: I.L. and J.E.R. designed research; I.L., N.T., M.H.D., M.G., and X.L. performed research; I.L. contributed new reagents/analytic tools; I.L., N.T., and M.H.D. analyzed data; and I.L. and J.E.R. wrote the paper.

The authors declare no conflict of interest.

1L.L., N.T., and M.H.D. contributed equally to this work.

2To whom correspondence may be addressed. E-mail: james.rothman@yale.edu or intaek.lee@yale.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1323895111/-/DCSupplemental.
for which these four adhesive proteins either are not found or are not important for stacking.

**Results**

**Simultaneous Depletion of GRASP65/55 or GM130/Golgin45 Disrupts Cisternal Flatness of Golgi Stacks but Does Not Result in Golgi Unstacking.** A recent study reported that double knockdown of GRASP65/55 results in complete unstacking of Golgi in HeLa cells (15). To better understand the relative contribution of Golgins and GRASPs in Golgi stack assembly and to find out whether GRASPs are both necessary and sufficient for Golgi stacking in vivo, we decided to simultaneously deplete both GRASPs or both Golgins and study the effects on Golgi stacking, using electron microscopy. In contrast to the earlier report, we observed that simultaneous depletion of GRASP65/55 or their Golgin binding partners, GM130/Golgin45, results in disruption of Golgi cisternal flatness (cf. EM photos in Fig. 1 A–C) without significant Golgi unstacking. As shown in Fig. S1, double depletion

Fig. 1. Double knockdown of GRASP65/55 or GM130/Golgin45 leads to significant disruption in Golgi cisternal flatness, but not Golgi disassembly. (A–C) Representative EM micrographs of HeLa cells treated with siRNAs against indicated adhesive proteins for 96 h. (Scale bar, 0.5 μm.) Control siRNA (A); GRASP65/55 siRNA (B); GM130/Golgin45 siRNA (C). Double knockdown of the two GRASPs or two Golgins resulted in significant disruption of Golgi cisternal flatness, but we did not observe significant Golgi unstacking under any double-knockdown conditions. For morphological criteria of Golgi unstacking, we looked for significant separation of cisternae from main body of the Golgi or significant vesiculation of the Golgi at the EM level. (D and E) K-S plots showing distribution of maximum cisternal luminal width in double-knockdown cells (D) or in single-knockdown cells (E), based on luminal width measurement using ImageJ software. (F) Table summarizing maximum luminal width measurement for both single- and double-knockdown cells. Results are expressed as the mean ± SEM. Numbers in parenthesis indicate the number of Golgi cisternae for which luminal width was measured. Note that all analysis passed the Student t test (P < 0.05), except for Golgin45-depleted cells. G, Golgi; n, nucleus; M, mitochondria; tER, transitional endoplasmic reticulum.

Fig. 2. Disrupted Golgi cisternal flatness in the double-depletion cells can be reversed by rescue transfection. (A–D) K-S plots showing rescue of Golgi cisternal flatness by exogenous expression of the RNAi-resistant form of GRASP65 (A) or GRASP55 (B) in cells depleted of both GRASPs and by exogenous expression of the RNAi-resistant form of GM130 (C) or Golgin45 (D) in cells depleted of both Golgins. Representative electron micrographs of Golgi stacks in cells rescued with indicated cDNAs are shown in the right panel of each graph. (Scale bar, 0.5 μm.) (E) Table summarizing the results from rescue experiments. Results are expressed as the mean ± SEM. Numbers in parenthesis indicate the number of Golgi cisternae that luminal width was measured.

![Image of EM micrographs](https://www.pnas.org/doi/10.1073/pnas.1323895111)
of GRASPs resulted in a significant reduction of Golgins, and vice versa. These phenomena suggest that Golgins and GRASPs are likely to affect the stability of each other to a certain extent (GM130–GRASP65 and Golgin45–GRASP55), mainly because of the role they play in the membrane association of their binding partners. Importantly, however, even with this additional reduction in the level of other adhesion proteins, we did not observe any significant Golgi unstacking in either GRASP or Golgin double-depleted HeLa cells. To exclude the possibility that this difference is a result of siRNA oligos used for GRASP65/55 depletion, we designed an additional set of siRNA oligos against GRASP65 and GRASP55, respectively. The efficiency of GRASP65/55 double knockdown routinely reached above 98% for both sets of siRNAs against GRASP65/55 (Figs. S1 and S2). Neither siRNA combinations led to significant unstacking of the Golgi when examined under EM. We have no explanation for this discrepancy, although we also observed a significant unstacking of the Golgi in a small percentage (<1%) of the knockdown cells we analyzed.

Morphologically, we noticed that Golgi membranes appeared to have lost the characteristic flattened cisternae. As a way to quantify the change in Golgi cisternal morphology, we measured the maximum luminal width of the Golgi cisternae (see Fig. S1 for an example). In control siRNA-transfected cells, the average maximum luminal width of Golgi cisternae was around 59 ± 3 nm (Fig. 1F). In single-knockdown cells, we observed a small but consistent increase (~10–15 nm) in the average luminal width, whereas GRASP65/55 or GM130/Golgin45 double depletion led to a two- to threefold increase in the luminal width (Fig. 1D and E) for Kolmogorov–Smirnov (K-S) plots; see Fig. S1 for frequency distribution graphs.

We reasoned that increased cisternal width in these knockdown cells might be a result of a block in anterograde cargo transport through the Golgi. To test this, we used CD8 fused to conditional aggregation domain (24) to assess the efficiency of bulk cargo transport under these knockdown conditions. Surprisingly, we found that GRASP65/55 double knockdown consistently led to a ~30–40% increase in CD8 transport to the plasma membrane compared with control cells and GM130/Golgin45 depleted cells (Fig. S2; see Fig. S3 for raw data), suggesting that the increased cisternal width in these cells is not likely to be caused by accumulation of anterograde cargo within the lumen of the Golgi cisterna. If this is true, is it possible that Golgins may be able to substitute for GRASPs either entirely or partially in maintaining Golgi stacking and cisternal morphology? To test this, we exogenously expressed Golgins in GRASP65/55-depleted HeLa cells to see whether Golgins can restore cisternal flatness in GRASP-depleted HeLa cells. Remarkably, crossing the functional barrier exogenously expressing GRASP65 or GRASP55 in GRASPs double-depleted cells (Fig. 2 A and B) or by expressing either Golgin GM130 or Golgin45 in Golgin double-depleted cells (Fig. 2 C and D; see Fig. 2E for summary). The average maximum luminal width for rescued Golgi cisternae was very close to the values we saw in single-knockdown cells. The knockdown and rescue expressions were confirmed by Western blots, as shown in Fig. S5. Altogether, this is consistent with the idea that GRASP55 and GRASP65 play a complementary role in Golgi stacking and cisternal morphology, as can Golgin45 and GM130, despite their differing physiological cis-trans distributions.

Are GRASP Proteins Dispensable for Golgi Stacking? As shown in the triple-knockdown experiments (Fig. S4), there was a strong indication that GRASPs and Golgins may be playing functionally complementary roles in Golgi stacking and cisternal morphology. Results in Golgi Disassembly. These findings naturally led us to the question of whether the changes in cisternal flatness in the GRASP- or Golgin-depleted cells are associated with Golgi stacking. In other words, the disrupted cisternal flatness may or may not be indicative of the progression toward Golgi disassembly in these cells. To find an answer to this seemingly elusive question, we tested whether additional depletion of Golgin45 or GM130 leads to Golgi disassembly in GRASP65/55-depleted HeLa cells. If so, the result would suggest that both cisternal flatness and cisternal stacking are determined by the collective cisternal adhesive process from these GRASPs and Golgins. In support of this idea, we found that triple knockdown of GRASP65/55 and Golgin45 (and, to a lesser extent, GM130) in HeLa cells results in significant Golgi unstacking (Fig. S4 A and B). Qualitatively, depletion of Golgin45/GRASP65/55 led to almost complete Golgi disassembly, but only partial Golgi unstacking was observed in GM130/GRASP65/55-depleted cells (compare EM photos in Fig. S4 A and B).

Additional Knockdown of Golgin45 in GRASP65/55-Depleted Cells Results in Golgi Disassembly. These findings naturally led us to the question of whether the changes in cisternal flatness in the GRASP- or Golgin-depleted cells are associated with Golgi stacking. In other words, the disrupted cisternal flatness may or may not be indicative of the progression toward Golgi disassembly in these cells. To find an answer to this seemingly elusive question, we tested whether additional depletion of Golgin45 or GM130 leads to Golgi disassembly in GRASP65/55-depleted HeLa cells. If so, the result would suggest that both cisternal flatness and cisternal stacking are determined by the collective cisternal adhesive process from these GRASPs and Golgins. In support of this idea, we found that triple knockdown of GRASP65/55 and Golgin45 (and, to a lesser extent, GM130) in HeLa cells results in significant Golgi unstacking (Fig. S4 A and B). Qualitatively, depletion of Golgin45/GRASP65/55 led to almost complete Golgi disassembly, but only partial Golgi unstacking was observed in GM130/GRASP65/55-depleted cells (compare EM photos in Fig. S4 A and B).

Is the Disruption in Golgi Cisternal Flatness Reversible? We then asked whether the disruption in cisternal flatness can be restored by rescue transfection. To do this, we expressed an RNAi-resistant form of each adhesion protein in double-knockdown cells that eliminate both GRASP proteins or both Golgins. As expected, Golgi stack morphology was restored by either
between GRASPs and Golgins was equally effective. Expressing either Golgin protein in GRASPs double-depleted cells restored the Golgi stack morphology, as judged qualitatively by EM (Fig. 3 A and B, Right). Golgi cisternal flatness was not restored by expressing trans-Golgi network localized Golgin97 (Fig. 3C). As shown in Fig. S6, we also performed reverse experiments, in which we depleted GM130/Golgin45, followed by exogenous expression of GRASP55-GFP. Although the degree of restoration is smaller compared with the effect of Golgin overexpression in GRASP-depleted cells, we still found a significant restoration in cisternal flatness in these cells ($P < 0.05$).

We measured maximum cisternal luminal width to quantify the degree of restoration (see K-S plots in Fig. 3 A–D). Exogenous expression of Golgin45 in GRASP65/55-depleted cells resulted in Golgi with a 61 ± 2 nm average maximum cisternal luminal width (from 155 ± 7 nm in GRASP65/55-depleted cells). Compared with control siRNA-transfected cells (59 ± 3 nm), there was essentially no significant difference. Thus, our results strongly indicate that the GRASPs and Golgin45 (and, to a lesser extent, GM130) are likely to play a functionally redundant and complementary role for Golgi stacking under physiological conditions. We measured the anterograde cargo transport in these cells (Fig. S6D) and found that the amount of CD8 transported to the plasma membrane in these cells is slightly lower compared with GRASP65/55-depleted cells.

### Golgin45-Dependent Restoration of Golgi Cisternal Flatness and Morphology Is Not Cell Type-Specific.

To see whether these observations are restricted to HeLa cells, we tested human fibrosarcoma HT1080 cells depleted of either GRASP65/55 or GM130/Golgin45 for 96 h and processed these knockdown cells for EM. We found that the morphological changes in HT1080 cells depleted of GRASPs or Golgins were essentially identical (Fig. S7A). No significant unstacking of the Golgi was observed in either knockdown cell. Exogenous expression of Golgin45 in GRASP65/55-depleted HT-1080 for 18 h resulted in similar restoration of Golgi cisternal flatness as in HeLa cells (Fig. S7 A–C). Because HT-1080 cells are known to secrete matrix metalloproteinase MMP-2 abundantly, the knockdown cells were tested for the secretion of endogenous MMP-2. Strikingly, we found that both GRASP and Golgin double-depleted HT1080 cells secreted significantly increased amounts of endogenous MMP-2 ($\sim$1.5-fold increase; Fig. S7 E and F) during the 4-h secretion assays compared with the control transfected cells. These results suggest that flattening of Golgi cisternae might have a role in determining the rate of anterograde cargo transport through the Golgi.

### Can an Ectopic Adhesion Process Introduce Another Organelle into the Golgi Stack?

Our results so far have suggested that cisternal adhesion, irrespective of its molecular source, is likely to be the overriding principle of Golgi stack assembly. If so, then adding adhesive bonds between Golgi cisternae and another organelle (mitochondria) should lead to the formation of hybrid stacks within a few hours, in which mitochondria can be incorporated into preexisting interphase Golgi stacks as if they were Golgi cisternae, provided the ectopic binding energy exceeds that normally stabilizing the Golgi stack.

To engineer ectopic adhesion between Golgi cisternae and mitochondria that can be controlled pharmacologically, we used a “knock-sideways” approach (13, 25) involving the ligand-induced formation of heterodimers between the FK506-binding protein (FKBP) and a rapamycin-binding domain (FRB). These two proteins bind to each other when a rapamycin analog (AP21967, which simultaneously binds to the FRB and FKBP domains) is added to cells. The Golgi adhesion protein GRASP55 was expressed as a hybrid protein with FKBP (and a fluorescent protein [tag blue fluorescent protein (tagBFP)]) to enable visualization (Fig. S8A). FRB was expressed as a hybrid with a protein targeted to the mitochondrial outer membrane (OMP25) also tagged with the myc epitope. In this way, the expressed GRASP55 hybrid protein should relocalize from the Golgi (and cytosol if present in excess) to the mitochondria when the ligand AP21967 is added to the cells (Fig. S8B). More specifically, endogenous GRASPs were replaced by the GRASP55 hybrid protein by using GRASP double-depleted cells that also express an RNAi-resistant form of the GRASP55-FKBP-tagBFP hybrid. To simplify the analysis and focus on the effects of ectopic adhesion on the stack (compared with the ribbon), these cells were

![Fig. 4. Golgi–mitochondria hybrid stack formation driven by engineered ectopic adhesive interaction.](image) Representative electron micrographs from knock-sideways experiments. (A) HeLa cells treated with siRNAs against GRASP65/55 for 72 h were rescued with GRASP55-FKBP-tagBFP for 24 h, followed by 3 h of treatment with Nocodazole (A); GRASP55-FKBP-tagBFP-rescued cells treated with AP21967 for indicated time to rapidly target the exogenous GRASP55 fusion protein onto mitochondria, which leads to Golgi–mitochondria hybrid stacks (B–D). G, Golgi; n, nucleus; M, mitochondria.
treated with nocodazole to produce ministacks before initiating knock-sideways with AP21967.

Electron microscopy was then used to follow the fate of pre-existing Golgi stacks and mitochondria (Fig. 4 A–D). The result was a dramatic reorganization of the Golgi stacks within 30–60 min after addition of ligand (2 μM), in which mitochondria cluster around ministacks at earlier times (Fig. 4B) and then invade them at later times, often forming hybrid stacks (Fig. 4 C and D). In some cases, Golgi cisternae appeared to wrap around mitochondria similar to a hot dog bun wrapping around a sausage. The diversity of the resulting hybrid organelles makes it impossible to quantify the results. However, it is nonetheless clear that these results support the idea that established Golgi stacks can be dissected by ectopic adhesion to mitochondria within a few hours. This implies that the stacks had been stabilized by reversible adhesion that could then be overcome at equilibrium by an engineered alternative.

**Discussion**

In this study, we report that cisternal adhesion is likely to be a key governing principle that dictates the two characteristic features of the Golgi apparatus (flattened cisternae and cisternal stacking) as illustrated in Fig. 5A along with EM photos, showing examples of Golgi stacks at different stages of cisternal adhesion, and although GRASPs seem to play an important role in Golgi stacking under physiological conditions, they are not absolutely essential even in mammalian cells in the sense that they could be replaced with Golgins, as shown in our experiments. This implies

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**Fig. 5.** Illustrations describing the concept of the adhesion model and the simplest form of cisternal stacking that could occur via cisternal adhesion and Rab conversion as two fundamental principles of stacking mechanism. (A) Illustration explaining diverse morphologies of Golgi stacks based on the adhesion model: Golgi cisternae are analogous to a water-filled balloon that is densely covered with Velcro-like molecules (tethering proteins, GRASPs, or other adhesive proteins). At the low adhesive condition, two of these water-filled balloons (cisternae) would be barely adhered together. As the adhesive energy is progressively increased broadly across the surface of cisternae, the cisternae gets flattened as the cisternal adhesive strength overcomes the osmotic pressure within the cisternae. Examples of Golgi EM photos for progressively adhesive conditions are shown here. (Scale bar, 500 nm.) (B) Illustration depicting the simplest form of cisternal stacking, based on the adhesion model and Rab conversion. According to this model, cisternal stacking occurs because of significantly faster ER-to-Golgi anterograde cargo transport compared with cargo export from the trans-Golgi to the plasma membrane. Only weak tethering or adhesive processes may suffice if the rate of the ER-Golgi transport far exceeds that of Golgi–plasma membrane transport. Cisternal maturation initiates the stacking of newly forming cis-cisternae to now more distal cisternae.
that Golgi stacking may be possible even in organisms lacking GRASP-type adhesive proteins.

In the case of yeast (S. pombe) and plants, in which none of the homologs for these four proteins have been either identified or found to be necessary for Golgi stacking, similar adhesive proteins or new adhesive interactions of existing Golgi proteins could potentially mediate cisternal adhesion. It is possible that even weak tethering processes between neighboring cisternae could promote cisternal stacking (theoretically (i.e., multiple tether–Rab binding, tether–SNARE binding, etc.). In Fig. 5B, we propose a model illustrating how the most primitive form of cisternal stacking might have occurred by this kind of weak tethering (adhesion) processes and Rab conversion, a compartmental maturation process, in which one Rab-GTP recruits the Rab–GTP exchange factor for the subsequent Rab protein and is known to be well-conserved through the evolution from yeast to mammals (26–29). This model requires that the cargo export from the trans-Golgi be slower than cargo import into the cis-Golgi. In this case, the slower the older (distal) cisternae dis-sipates from the trans side of the Golgi via cargo export to the plasma membrane, the better the chance of cisternal stacking because newly formed cis-cisternae would accumulate at the cis face of now more distal cisternae. Naturally, the number of cisternae per stack would be determined by dynamic ratio of cargo influx/outflux through the Golgi in a given cell type.

One of the reasons that newly formed nascent cis-cisternae may stack with older cis-cisternae (rather than fuse with older cis-cisternae) could be cisternal maturation (i.e., Rab conversion) of older cis-cisternae. Thus, newly forming cis-cisternae continues to grow in volume as a result of rapid membrane influx from the endoplasmic reticulum (ER), until Rab conversion (i.e., RabA → RabB) is fully completed, at which point the matured cisternae begins to stack with more nascent cis-cisternae. In this way, the Golgi stack could be built from the trans side of the Golgi toward the cis side in terms of a purely mechanistic point of view.

If one assumes that Golgi stacking could occur as described here, why do these Golgins and GRASPs play crucial roles in mammalian and fly Golgi stacking under physiological conditions? The answer to this question may be found from the fact that yeast and plant Golgi stacks do not undergo mitotic disassembly/reassembly, whereas mammalian and fly Golgi stacks do. It is possible that the necessity for rapid mitotic disassembly/reassembly of Golgi stacks might have brought about the evolutionary adaptation of this particular group of adhesive proteins for Golgi stacking in mammals and fly. Thus, yeast and plants may be using the more ancient strategy (i.e., multiple weak adhesive processes) for building stable Golgi stacks, whereas mammals and flies have adapted a small group of proteins for Golgi stacks that can be dynamically disassembled and reassembled in sync with mitotic cell division.

Materials and Methods

Reagents and Antibodies. All common reagents were purchased from Sigma-Aldrich unless otherwise mentioned. The following antibodies were used: mouse monoclonal GM130 (BD Transduction Laboratories), goat polyclonal GRASP56 (Santa Cruz Biotechnology), rabbit polyclonal GRASP55 (Proteintech Inc.), rabbit polyclonal p115 (Santa Cruz Biotechnology), HRP-conjugated mouse β-actin antibody (GenScript). Rabbit polyclonal Golgin97 (AbCam). Rabbit polyclonal Golgin45 antibody was made by injecting synthetic Golgin45 peptide (AA40–53) conjugated to KLH (GenScript). pcDNA-hGRASP55-GFP was kindly provided by Adam Linstedt (Carnegie Mellon University). Rat GM130 cDNA was obtained from Nobuhiro Nakamura (Kyoto Sangyo University). Human Golgin45 cDNA was purchased from Addgene. Human GRASP65 cDNA was from Yanzhuang Wang (University of Michigan, Ann Arbor, MI). Human Golgin97 cDNA was obtained from Sean Munro (MRC Laboratories, Cambridge, United Kingdom).

Acknowledgments. We thank Adam Linstedt, Gregory Lavieu, Nobuhiro Nakamura, Yanzhuang Wang, and Sean Munro for kindly sharing reagents and Stuart Kornfeld for critical reading of the manuscript. We are especially grateful to Vivek Malhotra for insightful inputs and discussion during the preparation of this manuscript.

References

Supporting Information

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SI Materials and Methods

Cell Culture and Treatments. HeLa cells were grown in DMEM supplemented with 10% (wt/vol) FBS (Invitrogen) at 37 °C. For transfection with siRNA, cells were plated onto 6-well plates 24 h before transfection. We performed the transfection using DharmaFect-1 (HeLa) or DharmaFect-4 (HT1080) (Dharmacon), according to manufacturer’s instruction. cDNA transfection was done using standard protocol, using Lipofectamine2000 (Invitrogen) or FugeneHD (Promega). Confocal images were obtained using a LSM510 confocal microscope (Carl Zeiss).

Sequences of siRNA Oligos Used in the Knockdown Experiments. All siRNA oligos were purchased from Integrated DNA Technology, and the target sequences were as follows: human Golgin of 45 kDa (Golgin45) (GCATCATAGTCTTCCAGTCCATGG), human Golgin45 (5′UTR for rescue experiments) (CGGAGAAUAG-GAAUCUAGAGGGU), human Golgi matrix protein of 130 kDa (GM130) (GGACAATGCTGCTACTCTACAACCA), Golgi reassembly and stacking protein 55 (GRASP55) oligo 1 (CTGC-GAGAGACCTCAGTCACACCAA), GRASP55 oligo 2 (CCACC-GAGGAACATCAGGAATTGAAC), GRASP65 oligo 1 (CCTGAGACCCCTCAGTCCACACA), and GRASP65 oligo 2 (CTGAGTGTCGGCACCATTGGT).

Sample Preparation and Image Acquisition for Electron Microscopy/ Tomography. The transfected cells were fixed in 2.5% (wt/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4 for 1 h. They were then rinsed in 0.1 M sodium cacodylate buffer and scraped and pelleted in 2% (wt/vol) agar. Samples were trimmed and postfixed in 1% osmium tetroxide for 1 h, en bloc stained in 2% (wt/vol) uranyl acetate in maleate buffer at pH 5.2 for a further hour, rinsed and then dehydrated in an ethanol series, infiltrated with resin (Embed812 Electron Microscopy Science), and baked overnight at 60 °C. Hardened blocks were cut using a Leica UltraCut UC7, and 60-nm sections were collected onto formvar/carbon-coated nickel grids and stained using 2% (wt/vol) uranyl acetate and lead citrate. These were viewed using a FEI Tecnai Biotwin transmission electron microscope (TEM) at 80 kV. Images were taken using Morada CCD and iTEM (Olympus) software, typically at 26,000 × magnification. For electron tomography, 250-nm sections were collected on formvar/carbon copper grids labeled on both sides with 10-nm gold particles. A tomography tilt series was acquired using FEI Express 3D software on an FEI Tecnai TF20 FEG TEM at 200 kV. Images were reconstructed using IMOD software (University of Colorado).

Knock-Sideways Experiments. Knock-sideways experiments were carried out as described previously by others (1, 2) with few modifications. Cells were transfected with siRNAs against GRASP65 and GRASP55 and incubated for 72 h. We then performed the rescue transfection with a siRNA-resistant form of GRASP55-FKBP6-binding protein (FKBP)-tag blue fluorescent protein (tagBFP) and rapamycin-binding domain (FRB)-myc mitochondrial outer membrane (OMP25) for 18 h. Cells were treated with 1 μg/mL Nocodazole for 3 h, followed by 2 μM AP21998 for the indicated time to induce the targeting of GRASP55-FKBP-tagBFP onto mitochondria outer membrane (OMP25) for 18 h. Cells were then split into another 6-well plate the following day before transfection. We performed knock-down experiments were carried out as described previously by others (1, 2) with few modifications. Cells were transfected with siRNAs against GRASP65 and GRASP55 and incubated for 72 h. We then performed the rescue transfection with a siRNA-resistant form of GRASP55-FKBP6-binding protein (FKBP)-tag blue fluorescent protein (tagBFP) and rapamycin-binding domain (FRB)-myc mitochondrial outer membrane (OMP25) for 18 h. Cells were treated with 1 μg/mL Nocodazole for 3 h, followed by 2 μM AP21998 for the indicated time to induce the targeting of GRASP55-FKBP-tagBFP onto mitochondria outer membranes, followed by fixing and processing for confocal microscopy or EM analysis.

Flow Cytometry Procedure. Flow cytometry was performed using a Becton Dickinson FACSCalibur. For surface immunofluorescence stainings, 1–2 × 10⁶ cells were washed once in ice-cold PBS/0.5% BSA with 2 mM EDTA (FACS buffer), followed by incubation with a saturating amount of the allophycocyanin (APC)-conjugated anti-human CD8a (clone OKT8; eBioscience) for 30 min at 4 °C. After incubation, cells were washed twice with ice-cold FACS buffer and cells were filtered using polystyrene round-bottom 12 × 75 mm BD Falcon tubes. Data were acquired through a live gate with accumulation of at least 200,000 events. The data were analyzed Using FlowJo software (Tree Star). During analysis, GFP-positive cells were gated and only GFP-positive cells were further quantified for CD8-APC positive staining.

MMP-2 Secretion Assays. HT1080 cells were grown in 6-well plates overnight and transfected with the indicated siRNAs the next day. Cells were then split into another 6-well plate the following day and cultured for 72 h. Culture medium was replaced with 500 μL Opti-MEM (per well) and incubated to collect endogenous matrix metalloproteinase MMP-2 secreted into the medium for indicated times. We then collected the conditioned media and concentrated the secreted MMP-2 by methanol-chloroform precipitation using 100 μg BSA as carrier. The collected MMP-2-containing samples were then mixed with SDS sample buffer for analysis by SDS/PAGE and Western blots. Cells were also collected and analyzed by Western blots for data normalization. MMP-2 antibody was obtained from cell signaling.

Anterograde Cargo Transport Assays. We used conditional aggregation domain (CAD; FM4) (3) fused to CD8 and GFP to determine the relative efficiency of anterograde cargo transport. Cells were treated with indicated siRNAs for 72 h. We then transfected cargo protein construct plus any indicated cDNAs for 18 h, followed by induction of AP21998 (solubilizer drug) to release the cargo molecules from the endoplasmic reticulum (ER) to Golgi to the plasma membrane. Cells were harvested by EDTA in PBS, followed by staining with anti-CD8 antibody conjugated to APC to detect cell surface CD8. Stained cells were analyzed by flow cytometry, using a FACSCalibur flow cytometer (BD Biosciences).

Image Analysis on Electron Micrographs and Data Presentation. Unless otherwise stated, we used digital electron micrographs in tiff format and ImageJ software (National Institutes of Health) to perform Golgi area analysis and cisternal luminal width measurement. See Fig. S1 for detailed illustrations on how we determined various standard parameters to calculate metric values from arbitrary unit values in ImageJ. To comprehensively assess the functional relationship of the GRASP55 and the Golgins, a large number of cisternae and stacks in numerous sections were studied extensively. We performed two independent experiments for each data set in the figures. This allowed us to establish the statistical distribution of data obtained, which can be represented overall by its mean and SEM, or in greater detail by its frequency distribution (e.g., Fig. S1). For convenient comparison of numerous frequency distributions representing different conditions, we represent each frequency distribution with a normalized saturation plot [Kolmogorov–Smirnov plot (K-S plot); ref. 4] of the cumulative area under the frequency distribution curve as a function of the maximum cisternal luminal width.


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obtained the unit value of maximum vertical luminal width of each cisterna for conversion into micromillimeter scale. Frequency distribution histograms for maximum cisternal luminal width analysis for both single and double knockdown (KD) samples (Right). Western blots on mid- and lower left panel show the knockdown efficiency in the single-depletion experiments for all four proteins and the GRASPs double-knockdown experiments. Knockdown efficiency was typically above 98% for all proteins.

Fig. S2. (A) Western blots showing the knockdown (KD) efficiency of GRASP65/55 double-depletion experiments using the second set of siRNA oligos. (B) Bar graph summarizing anterograde cargo transport assays for double-depletion experiments. Cells were treated with the indicated siRNAs for 72 h, followed by transfection with CD8-GFP-FM4 for 18 h. We used this construct to determine the cargo transport efficiency in these cells, using flow cytometry (FCM). These experiments were repeated twice for confirmation. GRASP65/55-depleted cells showed approximately ∼40% increase in cargo transport (**P < 0.05) compared with control and GM130/Golgin45-depleted cells. See Fig. S3 for raw FCM data.
Fig. S3. Histograms showing the representative raw data from the CD8 anterograde transport assays. The figures show the CD8 cell surface expression profile in the absence of drug (Left) and in presence of drug AP21998 (Right). HeLa cells were transiently transfected with CD8-GFP-CAD into the respective knockdown cells for 18 h, followed by cell surface expression analysis using APC-conjugated CD8 antibody by FCM. GFP-positive cells were gated using FlowJo software and GFP-positive cells were further analyzed for the CD8-positive population, as shown by histogram. Transfected cells were treated with either ethanol or disaggregating drug AP21998 for 3 h at 37 °C, which releases the cargo CD8-GFP-CAD from ER to the cell surface. Experiments were repeated twice and are highly reproducible.
Fig. S4. Simultaneous depletion of Golgin45 and GRASP65/55 results in significant Golgi unsticking. (A) Representative EM photo showing significant Golgi disassembly in HeLa cells with Golgin45/GRASP65/55 triple knockdown. (B) Triple knockdown of GM130 and GRASP65/55 leads to partial Golgi disassembly. HeLa cells were treated with indicated siRNAs for 96 h, followed by processing for EM study. (Scale bar, 1 μm.) Arrow heads indicate the area of Golgi EM photos where Golgi unstacking is evident.

Fig. S5. Immunoblots showing knockdown and rescue of the indicated adhesion proteins. Cells were transfected with the indicated siRNAs for 72 h, followed by rescue transfection with RNAi-resistant adhesion protein cDNAs for 18 h. Cells were then either fixed for EM analysis or lysed for SDS/PAGE and immunoblots. *Nonspecific bands.
Fig. S6. (A) A representative electron micrograph of Golgi stacks showing the rescue of Golgi cisternal flatness by exogenous expression of GRASP55 in cells depleted of GM130/Golgin45. (Scale bar, 1 μm.) G, Golgi apparatus. (B) K-S plots showing rescue of Golgi cisternal flatness by exogenous expression of GRASP55 in the cells depleted of both GM130 and Golgin45. (C) Table summarizing the results from substitution experiments. Results are expressed as the mean ± SEM (P < 0.05). Numbers in parentheses indicate the number of Golgi cisternae that the luminal width was measured. (D) Bar graph summarizing anterograde cargo secretion assays for substitution experiments. Cells were treated with the indicated siRNAs for 72 h, followed by transfection with the indicated Golgin cDNAs plus CD8-GFP-CAD for 18 h. We used the same procedure as in Fig. 1 to determine the cargo transport efficiency in these cells using FCM. These experiments were repeated twice for confirmation (**P < 0.05).
Fig. S7. Golgin45-dependent restoration of Golgi cisternal flatness in GRASP65/55-depleted cells is not cell type-specific. (A) Representative EM photos showing the disruption of Golgi cisternal flatness in GRASPs or Golgin-depleted HT1080 cells. HT1080 cells were transfected with the indicated siRNA combinations and grown for 96 h before fixation for EM study. Exogenous expression of Golgin45 for 12 h restores Golgi stacking in GRASP65/55-depleted HT1080 cells, as in HeLa cells. (Lower right) EM photo. (B) Table showing the summary of maximum luminal width measurements in HT1080 cells under various knockdown (KD) conditions (the number in parenthesis indicates the number of Golgi cisternae measured). Results are expressed as the mean ± SEM. (C) Histogram representation of maximum cisternal luminal width measurement in human fibrosarcoma HT1080, depleted of GRASP65/55 or GM130/Golgin45 by siRNA. (D) Western blots showing the knockdown efficiency of GRASPs and Golgins in HT1080 used for the experiments. (E) Representative MMP-2 secretion assays. (Upper) MMP-2 secreted into the medium during the 4-h experiment. Cells were lysed and probed for intracellular MMP-2 (Middle) and GAPDH (Lower), used as a loading control. (F) Line graph showing the summary of two independent experiments. MMP-2 secretion increased ~1.5-fold in both GRASPs and Golgin-double-depleted HT1080 cells. The densitometric values from secreted MMP-2 were normalized to the values from GAPDH blot.
Fig. S8. (A) Schematics of knock-sideways of GRASP55 to mitochondria for the Golgi-mitochondria hybrid stacking; design of GRASP55-FKBP-tagBFP and FRB-myc-OMP25 constructs. (B) GRASP55 is rapidly relocated from Golgi to mitochondria outer membranes, using drug-induced dimerization of the FKBP-FRB domain. The diagram shows three different outcomes: Golgi stack-mitochondria interaction, single unstacked cisternae recruited onto mitochondria, and mitochondria aggregation via GRASP55 oligomerization on mitochondria outer membranes.