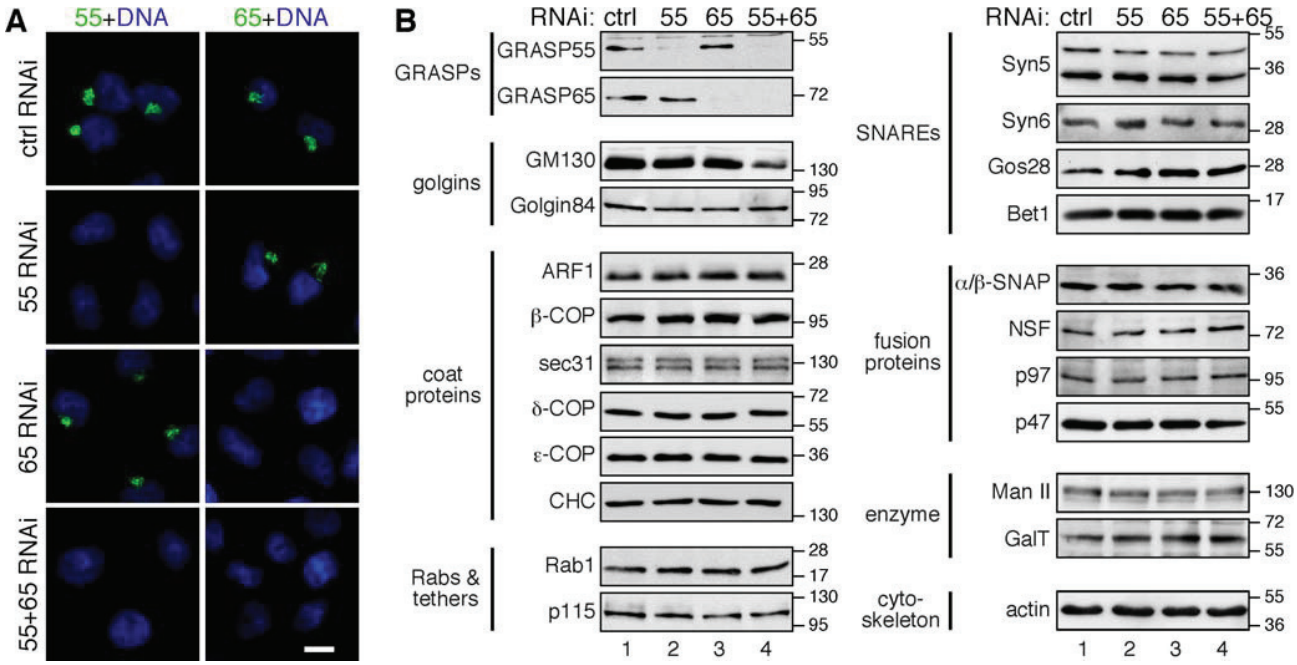
Supplementary Figures

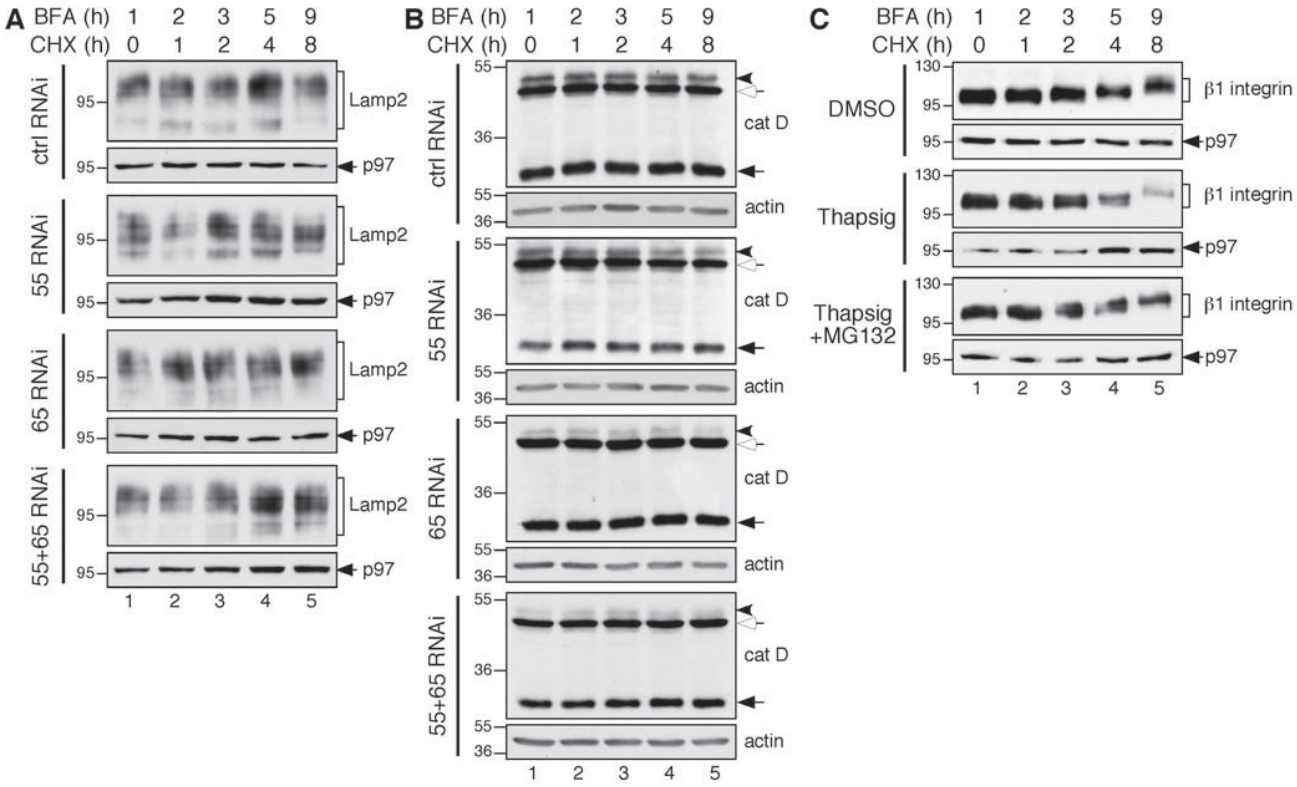
Supplementary Figure S1



Supplementary Figure S1. GRASP depletion does not affect the level of membrane trafficking-related proteins in the cell.

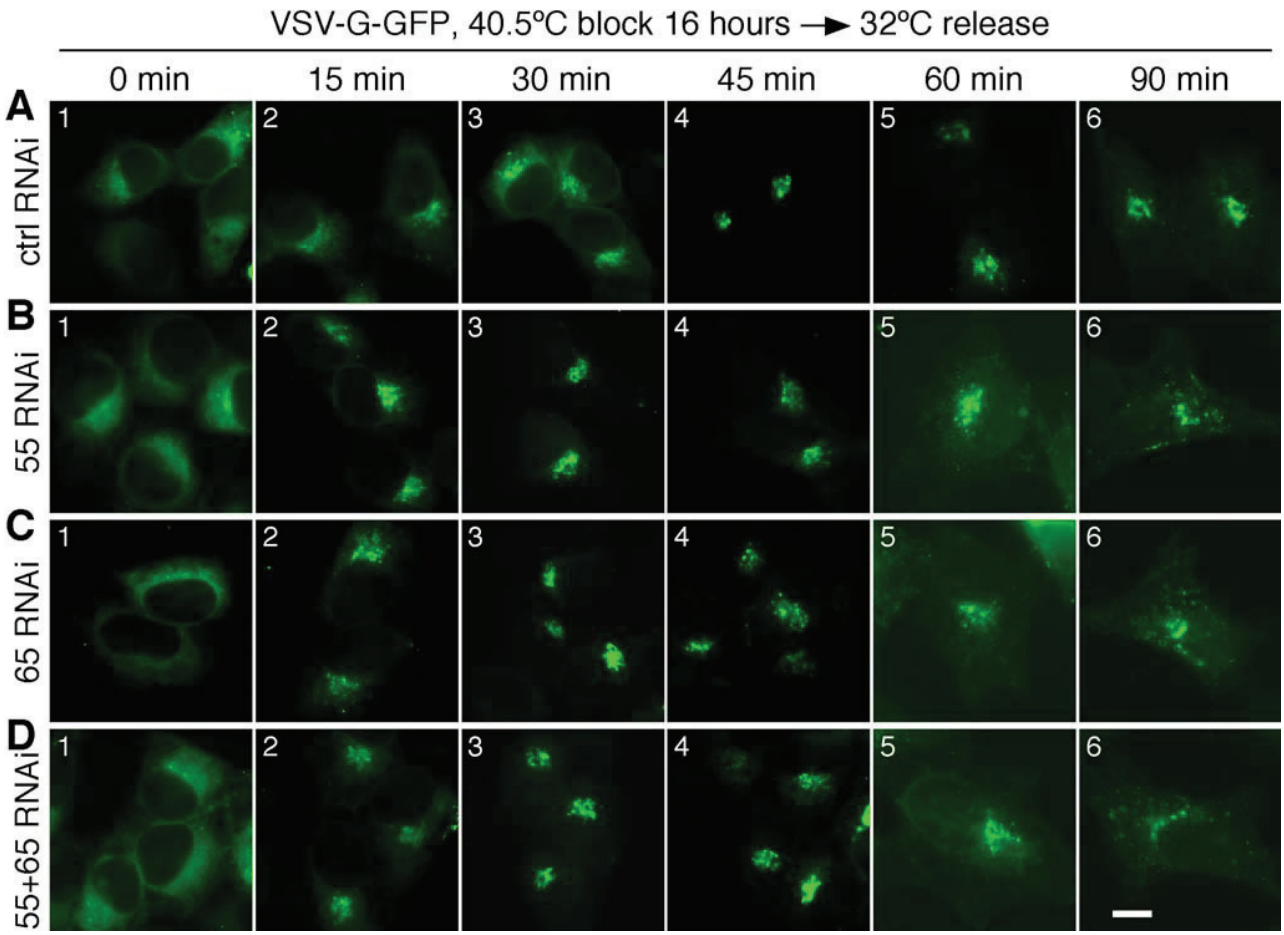
(A) Fluorescence images of GRASP depleted cells. GRASPs are shown in green and DNA in blue. Scale bar, 10 μm. (B) The effect of GRASP65 and GRASP55 depletion on cellular proteins. HeLa cells transfected with the indicated RNAi were lysed and analyzed for indicated proteins by Western blots. Molecular weight standards (kDa) are shown on the right.

Supplementary Figure S2



Supplementary Figure S2. GRASP depletion does not result in ERAD of lamp2 and cathepsin D. HeLa cells transfected with indicated RNAi were first treated with BFA for 1 hour followed by the addition of cycloheximide (CHX) into the medium and incubated for the indicated time period. Proteins in the cell lysate were analyzed by Western blot of the same gels for Lamp2 and p97 (A), or for cathepsin D (cat D) and actin (B). Arrowheads, open arrows and closed arrows in (B) indicate immature, intermediate and mature forms of cathepsin D, respectively. Note that GRASP depletion does not affect the stability of these proteins. (C) ER stress induced by thapsigargin treatment causes ERAD of β1 integrin. HeLa cells were first treated with DMSO (as control), 2 μM thapsigargin (thapsig), or with 2 μM thapsigargin and 10 μM MG132 for 3 hours, then treated with 5 μg/ml BFA for 1 h followed by the addition of 100 μM cycloheximide (CHX) into the medium and incubated for the indicated time periods. Shown are Western blots of cell lysate for β1 integrin and p97 on the same blots. Molecular weight standards (kDa) are shown on the left.

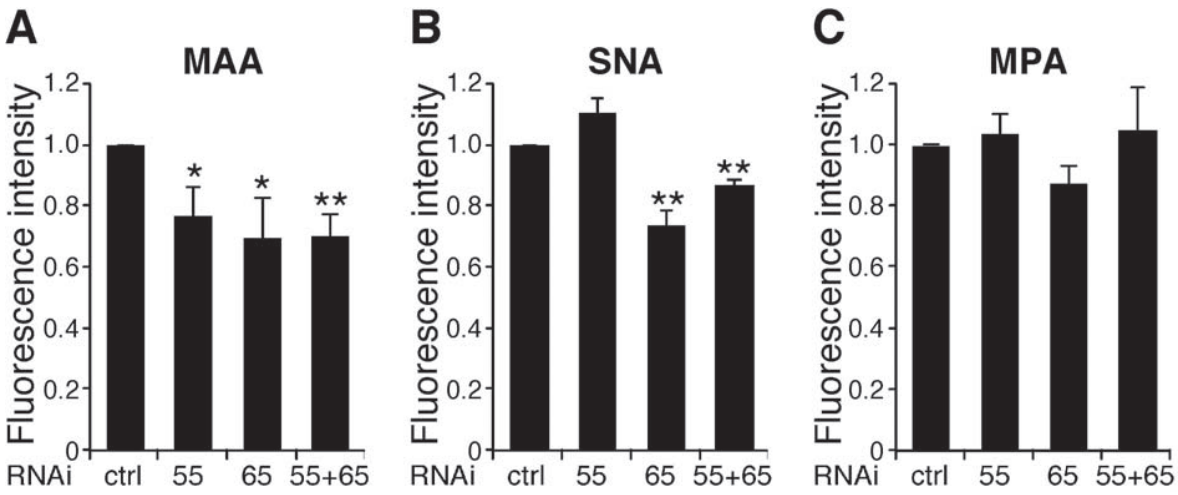
Supplementary Figure S3



Supplementary Figure S3. GRASP depletion accelerates VSV-G trafficking through the Golgi membranes.

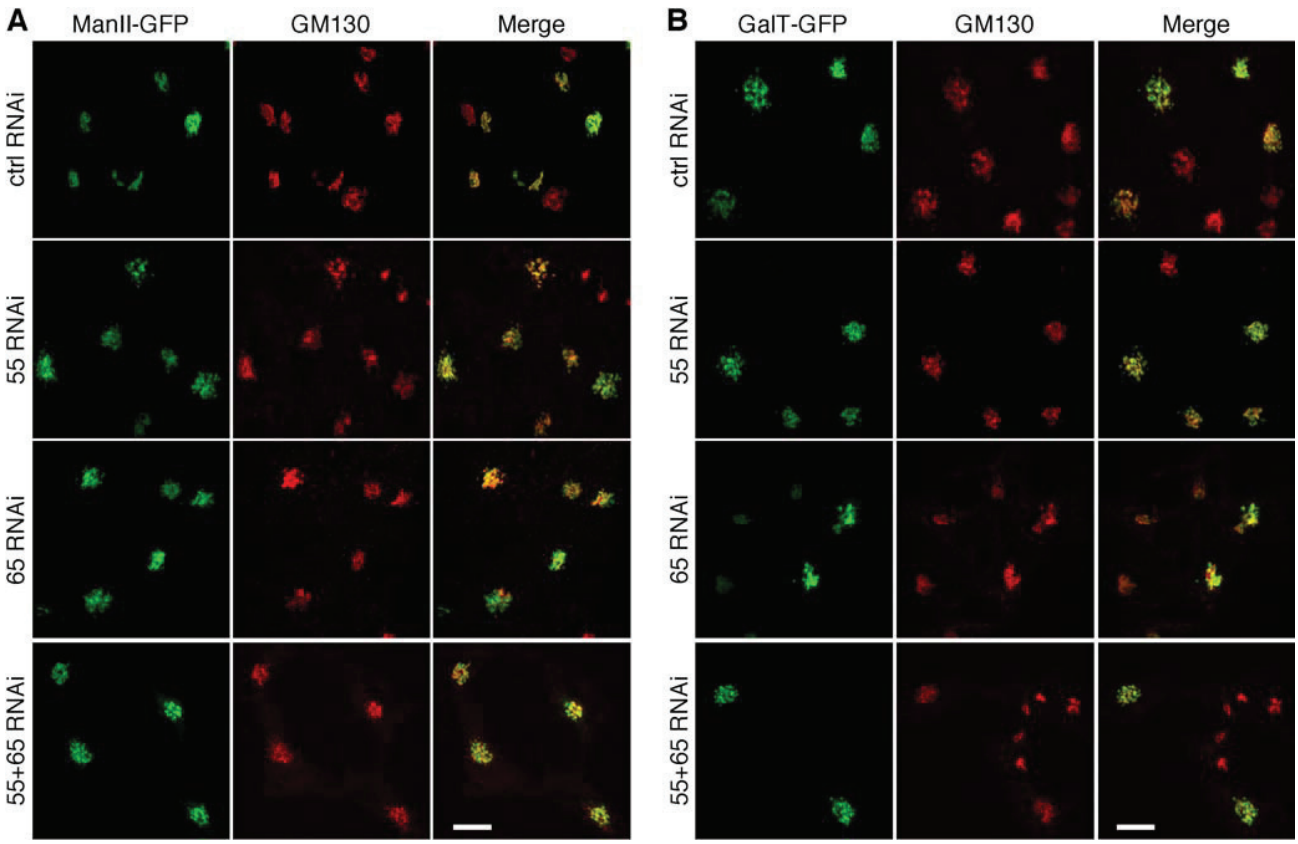
HeLa cells transfected with indicated RNAi were infected with VSV-G ts045-GFP adenovirus and incubated at 40.5°C for 16 h. Cells were shifted to 32ºC, incubated for indicated time period (chase), and analyzed by fluorescence microscopy for VSV-G-GFP. Scale bar, 10 μm.

Supplementary Figure S4



Supplementary Figure S4. GRASP depletion affects glycosylation of cell surface proteins.
(A–C) Lectin staining on the plasma membrane of cells treated with the indicated RNAi was determined using flow cytometry. The data are presented as the mean±SEM normalized against control RNAi-treated cells from three independent experiments. Note that MAA and SNA staining decreased by the depletion of the GRASPs.

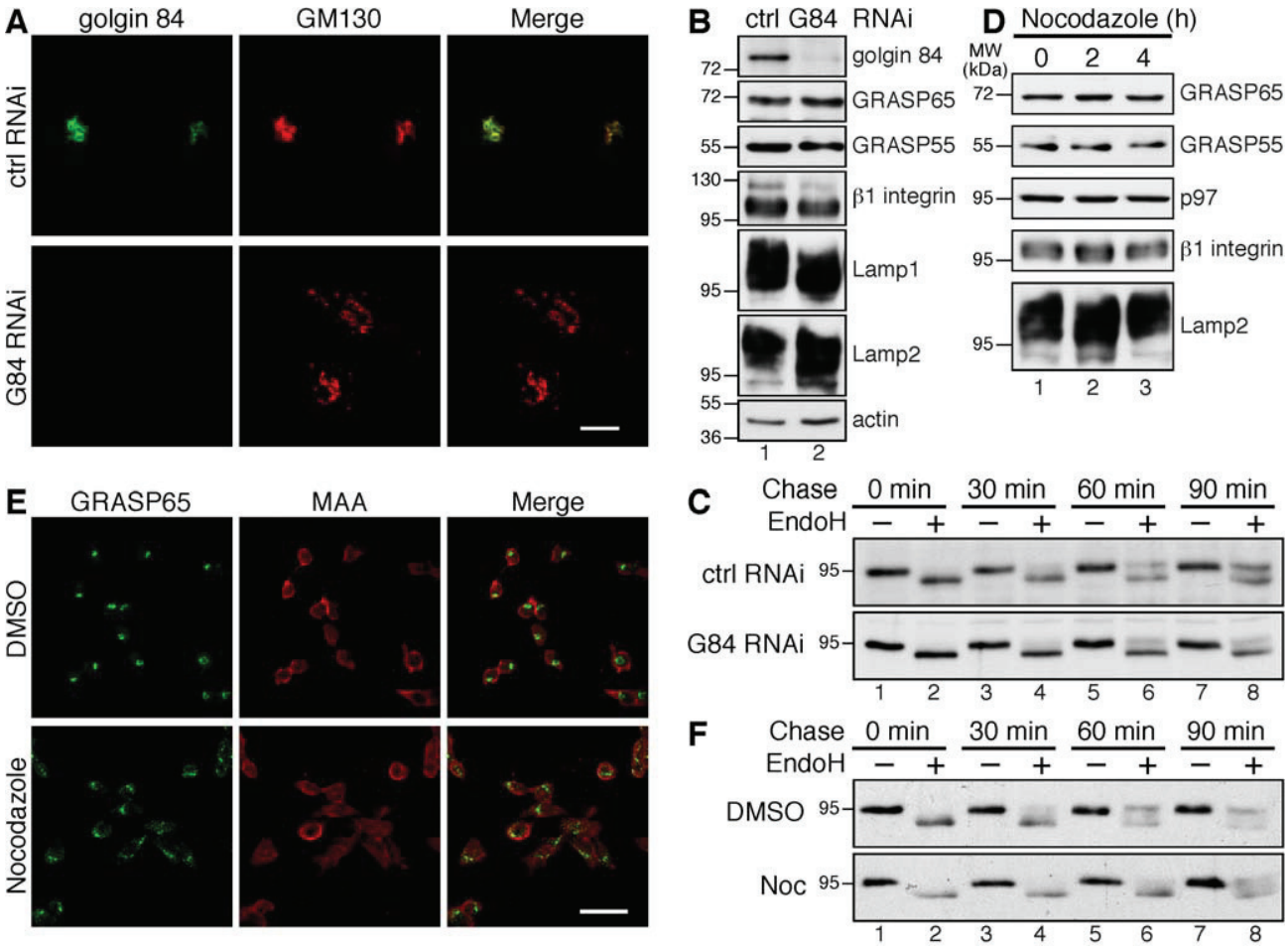
Supplementary Figure S5



Supplementary Figure S5. Exogenously expressed Golgi enzymes are localized to the Golgi membranes in GRASP depleted cells.

(A) Mannosidase II (ManII)-GFP localizes to the Golgi in GRASP-depleted cells. HeLa cells treated with the indicated RNAi were transfected with a ManII-GFP plasmid and analyzed by immunofluorescence microscopy for GM130. Note that ManII-GFP colocalized with GM130 in all cells. (B) Galactosyltransferase (GalT)-GFP localizes to the Golgi in GRASP-depleted cells. Scale bars in (A) and (B), 10 μm.

Supplementary Figure S6

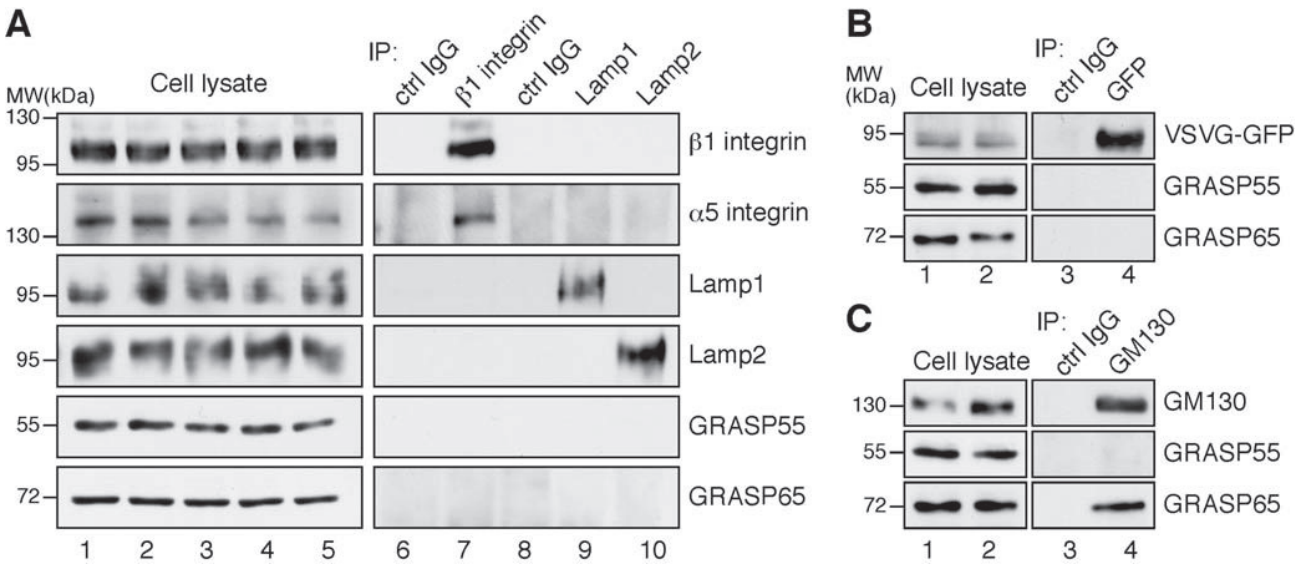


Supplementary Figure S6. Golgi ribbon unlinking by golgin 84 depletion and by nocodazole treatment decreases protein trafficking.

(A) Golgin 84 depletion results in Golgi ribbon unlinking. Shown are fluorescence images of golgin 84-depleted cells. Scale bar, 10 μm. (B) The effect of golgin 84 depletion on cellular proteins. HeLa cells transfected with the indicated RNAi were lysed and analyzed for indicated proteins by Western blots. (C) Golgin 84 depletion decreases VSV-G trafficking. HeLa cells transfected with indicated RNAi were infected with VSV-G ts045-GFP adenovirus and incubated at 40.5°C for 16 h. Cells were shifted to 32ºC for indicated time period, cell lysates were treated with EndoH and analyzed by Western blot for VSV-G-GFP. (D) The effect of nocodazole treatment on cellular proteins. Cells were treated with 500 ng/ml nocodazole for indicated time periods followed by Western blots. (E) Nocodazole treatment results in Golgi fragmentation but has no impact on cell surface glycosylation. Non-permeabilized HeLa cells treated with DMSO or with 500 ng/ml nocodazole for 2 h were exposed

to TRITC-conjugated MAA. The cells were then fixed, stained with GRASP65 antibodies, and visualized using a confocal microscope. Scale bar, 50 μm. (F) Nocodazole treatment slows down VSV-G trafficking analyzed as in (C), nocodazole was added 1 h before the temperature shift to 32ºC. Molecular weight standards (kDa) are shown on the left.

Supplementary Figure S7



Supplementary Figure S7. Marker proteins used in this study do not interact with GRASP55/65. (A-C) HeLa cells were solubilized and immunoprecipitated with indicated antibodies followed by Western blot analysis for indicated proteins. Cells used in (B) were infected by VSV-G ts045-GFP adenovirus. Molecular weight standards (kDa) are shown on the left.

Supplementary Methods

Cell culture, transfections and treatment

rtTA HeLa m2 cells were grown in DMEM (Invitrogen) containing 10% fetal bovine serum and L-glutamine at 37°C in a 5% CO_2 incubator. Knockdown transfections were performed in HeLa cells plated at 40% confluency, following the manufacturer's instructions. For cells in six-well plates, 3 µl of a 50 µM RNAi stock was added to 250 µl of Opti-MEM. In a separate tube, 5 µl of transfectamine RNAiMAX (Invitrogen) was mixed with 250 µl of Opti-MEM and incubated for 5 min at room temperature. The two mixtures were combined and incubated at room temperature for 20 min, then added to the cells in 2 ml DMEM containing 10% fetal bovine serum. RNAi for human GRASP55 (AACTGTCGAGAAGTGATTATT)[17] and GRASP65 (CCTGAAGGCACTACTGAAAGCCAAT)[13] were purchased from Ambion and Invitrogen, respectively. Control non-specific RNAi were purchased from Ambion. RNAi for human Golgin-84 (AAGTAGGATCTCGGACACCAG)[36] were purchased from Invitrogen. For BFA, cycloheximide (CHX) or nocodazole treatment, 5 µg/ml BFA, 100 µM (28.1 µg/ml) CHX or 500 ng/ml nocodazole was directly added into the tissue culture medium and incubated for indicated time. Cells were lysed and analyzed by Western blotting.

Subcellular fractionation

HeLa cells were transfected with indicated RNAi for 72 hours, washed with PBS, collected in homogenization buffer (0.25 M sucrose, 10 mM HEPES, pH7.2, 1 mM $Mg(OAc)_2$, 1 mM EDTA, 0.5 mM PMSF, and protease inhibitor cocktail) with a cell scraper, and homogenized using a ball-bearing homogenizer to ~80% breakage examined by trypan blue exclusion. Homogenate was centrifuged for 10 min at 1000 *g* and 4ºC. Postnuclear supernatant (PNS) was subjected to ultracentrifugation for 1 h in a TLA55 rotor at 55,000 rpm (135,000 *g*) and 4ºC to separate membranes from cytosol. The cytosol was methanol/chloroform precipitated and both cytosol and membrane pellets were dissolved in equal volumes of SDS buffer. Equal proportions of the membranes and cytosol were loaded onto SDS-PAGE and analyzed by Western blot using indicated antibodies. Protein bands were quantified using the NIH ImageJ software.

Microscopy

Fluorescence images were captured with a Leica SP5 confocal laser-scanning microscope using a 100× oil lens or a Zeiss Observer Z1 epifluorescence microscope using a 63× oil lens. Phase contrast images were taken with a Zeiss Observer Z1 microscope using a 40× lens. For images captured using the

confocal microscope, each image was a maximum projection from a z-stack. Pictures were assembled in Adobe Photoshop.

VSV-G transport

A population of HeLa cells that stably expressed VSV-G-GFP (*ts*O45) in an inducible manner was established using a retroviral system, as described previously[14], and enriched by fluorescence-activated cell sorting. Cells were transfected with control, GRASP55, GRASP65, or both GRASP55 and GRASP65 RNAi and incubated at 37°C. Eighty hours after transfection, 1 μg/ml doxycycline was added, and the cells were incubated at 40.5°C. Fifteen hours later, 100 μM cycloheximide was added to inhibit protein synthesis. One hour after the addition of cycloheximide, the cells were shifted to 32°C to release the VSV-G proteins from the ER. After incubation for an indicated time period at 32°C, the cells were harvested in PBS containing 20 mM EDTA and fixed with 3.7% paraformaldehyde. The cells were blocked in PGA (PBS containing 0.2% gelatin and 0.04% azide) buffer, and surface VSV-G-GFP was labeled with antibodies specifically directed against its extracellular domain[56] at 4°C overnight. After washing with PGA buffer, the cells were incubated with a Cy5-conjugated goat-anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories) for 30 min at room temperature. The fluorescence intensities of GFP and Cy5 were analyzed on a FACScalibur flow cytometer (BD Biosciences). The amount of VSV-G on the cell surface after subtracting the background was normalized based on the GFP intensity.

To monitor VSV-G trafficking in the cell, we infected HeLa cells using VSV-G ts045-GFP adenovirus (kindly provided by David Sheff and Heike Fölsch). Briefly, HeLa cells were transfected with indicated RNAi for 72 h, and VSV-G ts045-GFP adenovirus was directly added into the medium with a MOI of 10 and cells were incubated for 16 h at 40.5˚C. Cells were further treated with 100 μM cycloheximide for 1 hour and incubated at 32˚C for indicated time points. Cells were dissolved in 1x glycoprotein denaturing buffer and the collected samples were treated with EndoH (NEB, 20 U for each reaction) at 37˚C for 1 h as indicated. Reactions were stopped by mixing with SDS buffer and boiling. Proteins were separated on 7.5% SDS-PAGE and analyzed by Western blot for GFP antibody (Sigma). Bands on the gel were quantified with NIH ImageJ software and the percentage of EndoH resistant form of VSV-G with respect to total VSV-G was calculated. For fluorescence microscopy, cells at different time points of 32ºC incubation were fixed in 3.7% paraformaldehyde and mounted on moviol. Fluorescence images were captured with Zeiss Observer Z1 epifluorescence microscope. To specifically determine intra-Golgi trafficking, GRASP-depleted cells were infected by VSV-G ts045-

GFP adenovirus and incubated at 40.5ºC for 16 h. Cells were shifted to 15ºC for 2 h to allow accumulation of VSV-G in the ERGIC compartment. Cells were then incubated at 20ºC to allow intra-Golgi trafficking of VSV-G. Cells were fixed at different time points and stained for GalT1. Colocalization of VSV-G with GalT was quantified using the "colocalization analysis" plugin of the NIH ImageJ software.

Supplementary References

56. Henry, L. & Sheff, D.R. Rab8 regulates basolateral secretory, but not recycling, traffic at the recycling endosome. *Mol Biol Cell* **19**, 2059-2068 (2008).