## METHODS

### Yeast in vitro transport and vesicle binding assays

For the vesicle binding assays, the permeabilized cells were pelleted after the in vitro transport reaction as described before[6]. The conditions used to remove the cells did not pellet slowly sedimenting membranes that contain radiolabeled cargo. The supernatant was transferred to a new tube that contained 40 µl of a 50% slurry of TRAPPI-containing beads[6]. The final volume of the reaction was adjusted to 500 µl with TBPS (25 mM HEPES (pH 7.2), 115 mM potassium acetate, 2.5 mM magnesium acetate, 250 mM sorbitol plus protease inhibitors) and the reaction was incubated for 2 h at 4°C. The beads were washed three times with 750 µl of TBPS and counted. The counts from a no vesicle control reaction (reaction with apyrase +/- Golgi) were subtracted as background. The binding of vesicles formed with cytosol was considered to be 100%, and the amount of binding (+/- Golgi) was adjusted to equal Concanavalin A (ConA) counts (equal vesicles). Two stage transport assays were also performed. Vesicles were formed in the absence of Golgi, the cells were pelleted and an equal number of vesicles were incubated with or without Golgi before the binding assay was performed.

For reactions with IC261, the ATP concentration of the 10X ATP stock was lowered to 1.7 mM ATP. For the vesicle tethering assay, free vesicles were separated from vesicles that bound to the Golgi on a sucrose velocity gradient as described before[6].

### In vitro kinase assay

Purified GST, GST-Sec23p and GST-Sec24p (5µg), immobilized on beads, were incubated with 250ng of $His_6$-Hrr25p or catalytically inactive $His_6$-Hrr25p K38A or no

kinase in kinase assay buffer (50 mM Hepes pH 7.4, 2 mM EDTA, 10 mM $MgCl_2$, 1 mM DTT, 5 mM cold ATP, 2.5 μCi $\gamma P^{32}$-ATP, 100 μM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate and protease inhibitors) for 1 h at 30°C. The beads were washed twice with 1X PBS and eluted in 25 μl of sample buffer by heating to 100°C for 5 min. The samples were analyzed by autoradiography.

**Nucleotide loading of $His_6$-Δ23-Sar1p**

$His_6$-Δ23-Sar1p was loaded with the desired nucleotide (GDP or GTPγS) overnight at 4°C in the following buffer: 20 mM Hepes pH 7.2, 150 mM NaCl, 1 mM $MgCl_2$ with 0.1 mM of nucleotide.

**In vitro binding assays with recombinant proteins**

For the binding experiments with the phosphomimetic mutations in GST-Sec23p, equimolar amounts (0.1 μM) of GST, GST-Sec23p beads +/- phosphomimetic mutations were incubated with either 5-10 nM of $His_6$-Δ23Sar1p- GTPγS (nucleotide was loaded as described above) or 10 nM of purified TRAPPI[6] in binding buffer I (25 mM Hepes pH 7.2, 150 mM NaCl, 2% Triton X-100, 1mM DTT, 2 mM EDTA, 0.5 mM $MgCl_2$ and protease inhibitors) for 3-4 h at 4°C. The beads were washed 3X with binding buffer I and eluted in 25 μl of sample buffer by heating to 100°C for 5 min.

For TRAPPI displacement assay, 0.5 μM of TRAPPI was incubated overnight at 4°C in binding buffer I with 0.1 μM of GST-Sec23p or GST. The next day, the beads were washed three times in binding buffer I and resuspended in binding buffer II (25 mM Hepes pH 7.2, 150 mM NaCl, 0.1% Triton X-100, 1mM DTT, 2 mM EDTA, 0.5 mM

$MgCl_2$ and protease inhibitors). Increasing amounts (0-0.25 μM) of purified $His_6$-Hrr25p were added to the reactions and incubated for 4 h at 4°C. Subsequently, the supernatants were aliquoted into fresh tubes and heated in sample buffer to 100°C for 5 min. The beads were washed 3X with binding buffer I and eluted as described above.

For the competition assay between Hrr25p and TRAPPI, 0.3 μM of GST-Sec23p or GST beads were incubated with 0.25 μM of purified TRAPPI and/or 0.15 μM of $His_6$-Hrr25p in binding buffer I for 3-4 h at 4°C. For the competition assay between Sar1p and TRAPPI, 0.1 μM of GST-Sec23p or GST beads were incubated with 10 nM of purified $His_6$-Δ23Sar1p-GTPγS with increasing concentrations (0-50 nM) of TRAPPI as above. For the reciprocal experiment, GST fusion proteins were incubated with 10 nM of TRAPPI and increasing concentrations (0-50 nM) of $His_6$-Δ23Sar1p-GTPγS. The beads were then washed 3X with binding buffer I and eluted as above. The molarity of TRAPPI was calculated based on the amount of Trs33p in the complex.

**Yeast immunofluorescence microscopy**

Cells expressing Vrg4p-GFP or Sec7p-GFP and Hrr25p-RFP were grown to an $OD_{599}$ of 0.5-1.5 in YPD medium. One to two $OD_{599}$ units were pelleted and resuspended in 25 μl of YPD medium. Cells were examined with a Carl Zeiss Observer Z.1 spinning-disk confocal fluorescence microscope using DIC, GFP, or RFP filters with a 100X oil-immersion objective. Images were captured with a Zeiss AxioCam MRm and analyzed using AxioVision Rel. 4.7 software. At least 300 puncta (and 100 cells) were examined in 3 separate experiments that were used to calculate the S.D. shown in Fig. 2**a**.

**Growth conditions for the *hrr25* mutant**

SFNY1941 (*MATa ura3-52 lys2-801 ade2-101 trpΔ63 his3-Δ200 leu2-Δ1hrr25Δ::loxP-kanMX-loxP pKK204(2μ pGAL-3HA-HRR25$^{degron}$*) was grown in YP-Raf-Gal (2% Raffinose, 0.5% Galactose) medium to $OD_{599}$=1-2. A total of 500 $OD_{599}$ units of cells were pelleted under sterile conditions and shifted to either YP-Raf-Gal or YPD (YP +2% glucose) medium for 10 h.

**Differential centrifugation experiment**

A total of 100 $OD_{599}$ units of cells were pelleted, resuspended in 2 ml of spheroplast buffer (1.4 M sorbitol, 100 mM sodium phosphate pH 7.5, 0.35% 2-mercaptoethanol and 0.5 mg/ml zymolyase) and incubated for 30 min at 37°C. The spheroplasted cells were then divided into four 0.5 ml aliquots and centrifuged over a 1 ml sorbitol cushion (1.7 M sorbitol, 100 mM HEPES pH 7.2) for 5 min at 6400 rpm at 4°C in a microfuge. The supernatant was removed and the four pellets were resuspended in 1 ml of lysis buffer (100 mM HEPES pH 7.2, 1 mM EGTA, 0.2 mM DTT, 1 mM PMSF and protease inhibitors) and lysed using a Dounce homogenizer. The lysate was centrifuged for 2 min at 500 g at 4°C in a microfuge and the supernatant was transferred to a new tube. An aliquot of this fraction (100 µl) was mixed with 50 µl of 3X sample buffer (Total fraction-T) and heated at 100$^{o}$C for 5 min, while the remaining portion (600 µl) was centrifuged for 90 min at 40,000 rpm at 4°C in a Beckman SW55 Ti rotor. The lipid layer was removed and 100 µl of the supernatant (S) was mixed with 50 µl of 3X sample buffer and heated at 100$^{o}$C for 5 min. The pellet (P) was resuspended in 500 µl of lysis

buffer and 100 µl was mixed with 50 µl of 3X sample buffer and heated at 100$^{o}$C for 5 min.

**Immunoprecipitation assay to detect phosphorylation**

SFNY1941 was grown as described above and whole cell lysates (10 mg) were immunoprecipitated with anti-Sec24 antibody. The immunoprecipitates were then immunoblotted with anti-phosphoSer/Thr (BD Biosciences, 1:500 dilution), anti-Sec24 (1:1000 dilution) and anti-Sec23 antibodies (1:1000 dilution).

**Mass spectrometric analysis:** *In vitro* phosphorylated GST-Sec23p was trypsin-digested and subjected to LC-MS/MS analysis as described previously[23].

**Generation of Sec23p phosphomimetic mutations:** The phosphomimetic mutations (S742D, T747E and S742D/T747E) in GST-Sec23p were generated by the two-step PCR method for site-directed mutagenesis using **pPE124**[24] as the template. Mutations in **pRS414**-*SEC23* (S742D/T747E) were generated on **pCF364**[52] using the QuikChange™ Site-directed mutagenesis kit (Agilent Technologies). All constructs were verified by sequencing.

**Analysis of mBet3 and mSec23 on mammalian COPII vesicles**

A detailed description of the generation, immuno-isolation, and immunoblotting of COPII transport intermediates derived from semi-intact normal rat kidney (NRK) cells is described elsewhere[17]. Briefly, a VSV-G-myc construct was introduced by electroporation and its expression in the ER was amplified using vaccinia virus VTF-7 at

41°C. After permeabilization, the VSV-G-myc-expressing cells or control untransfected NRK cells were suspended in a vesicle budding cocktail and incubated at 32°C for 30 min. Subsequently, the donor cells were removed by sedimentation. For the p115 experiment, the supernatant (which contains released COPII vesicles) was then incubated with purified full-length $His_6$-p115[26] for 60 min at 32°C. The p115 preparation was functional in tests of interactions with ER/Golgi SNAREs (not shown). The suspension of transport intermediates was then subjected to immunoisolation using anti-myc antibody. Proteins were eluted from the beads using 0.1M glycine pH 2.5, neutralized, concentrated, and analyzed on a 4-20% gradient SDS polyacrylamide gel followed by western blot analysis. To quantitate the abundance of mBet3 and mSec23 on the isolated vesicles and to normalize to vesicle yield, we divided the band intensity by the signal for the cargo marker VSV-G-myc and syntaxin 5 for each lane. The cargo-normalized mBet3 and mSec23 signals were then expressed as a percentage relative to the GTP condition. GMP-PNP was used at 100μM concentration.

**Analyzing ER-Golgi traffic in vivo in the presence of IC261**

NRK cells were electroporated with a plasmid encoding VSV-G ts045-GFP, plated on glass coverslips in 6-well plates and incubated overnight at 40°C. Ten min before the temperature shift, 100 μM IC261 (solubilized in DMSO) or DMSO was added to the medium at 40°C. After 10 min, the coverslips were either fixed in 4% paraformaldehyde for 30 min, or shifted to 15°C medium containing IC261or DMSO. At 15°C, VSV-G-GFP can leave the ER, but accumulates in swollen peripheral ER/Golgi interface structures that only slowly move toward the Golgi area[27]. The 15°C treatment makes ER

exit, pre-Golgi assembly and transport to the Golgi more resolvable. Coverslips were fixed after 30 or 60 min at 15°C.

After fixation, coverslips were treated twice for 10 min with 0.1 M glycine and then the samples were permeabilized in BSA/goat serum blocking solution containing 0.35 % saponin. All subsequent antibody incubations and washes were carried out in blocking solution containing saponin. Primary antibody incubations included the anti-rbet1 mouse monoclonal antibody 16G6[28] and a rabbit polyclonal antisera against gpp130 (Covance Research Products, Princeton, NJ). 16G6 is known to label rbet1 more intensely when the antigen accumulates at peripheral sites[29]. Secondary antibodies were goat anti-mouse-cy3 and goat anti-rabbit-cy5. DAPI was also included during the secondary antibody incubation. Following extensive washing, the coverslips were mounted in Slow-fade Gold mounting medium (Invitrogen) and imaged using the widefield microscope and instrumentation methods described before[29]. Briefly, each field of cells were captured in four colors (GFP, cy3, cy5, and DAPI) at 21 focal planes through the sample. Image stacks were then deconvolved using the Huygens algorithm (Scientific Volume Imaging, The Netherlands). Maximum intensity projections from five consecutive image planes were used for quantitation and display.

To quantitate the transfer from the ER to pre-Golgi structures, each set of images was opened as an image stack in the Openlab program (Improvision, UK). The extracellular background was subtracted from each image, and then a roughly square region of interest (ROI) that abutted the nucleus and extended approximately three-quarters of the distance to the edge of the cell was hand-drawn. This ROI was chosen such that it constituted a region of cytoplasm completely free of any Golgi labeling. The

rbet1 image was used to define a pre-Golgi mask within this larger ROI using an intensity threshold of 4-6x the rbet1 labeling background. The pre-Golgi mask was then subtracted from the original ROI to create an ER mask. The pre-Golgi and ER mask were sequentially applied to the VSV-G-GFP image to determine the average maximum intensity of cargo spots in the pre-Golgi compartment, and the average intensity of cargo in the ER, respectively. The ratio of these two parameters, average maximum intensity in the pre-Golgi compartment divided by average intensity in the ER, was determined for each of the twenty randomly sampled images from each condition.

To quantitate the concentration of pre-Golgi structures near the Golgi, a Golgi mask was derived from the gpp130 image using an intensity threshold of 10% of maximum intensity for the Golgi area of interest. A total punctate cargo mask was derived from the VSV-G-GFP image by choosing an intensity threshold for each cell sample such that a faint punctate spot would be captured in the mask but residual diffuse ER labeling would not. This cargo mask was then superimposed on the Golgi mask and all cargo-containing objects that did not partially or completely overlap with a Golgi object were deleted from the cargo mask. This peri-Golgi cargo mask, as well as the original total cargo mask were sequentially applied to the VSV-G-GFP image to determine the total intensity of cargo spots in the peri-Golgi region and in the whole cell, respectively. The ratio of these two parameters, total intensity of cargo spots in the peri-Golgi region divided by total intensity of cargo spots in the cell, was determined for each of the 20 randomly sampled images analyzed for each condition.

For both quantitations, the average raw ratio for DMSO-treated cells from a given experiment was defined as 100% and each individual ratio value from that experiment

was expressed relative to 100%. This normalization step allowed combination of quantitations from independent experiments to produce the values for Fig. 5c and 5e.

Note, all experiments in the manuscript were performed at least 3 times or more on separate days.

**References**


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