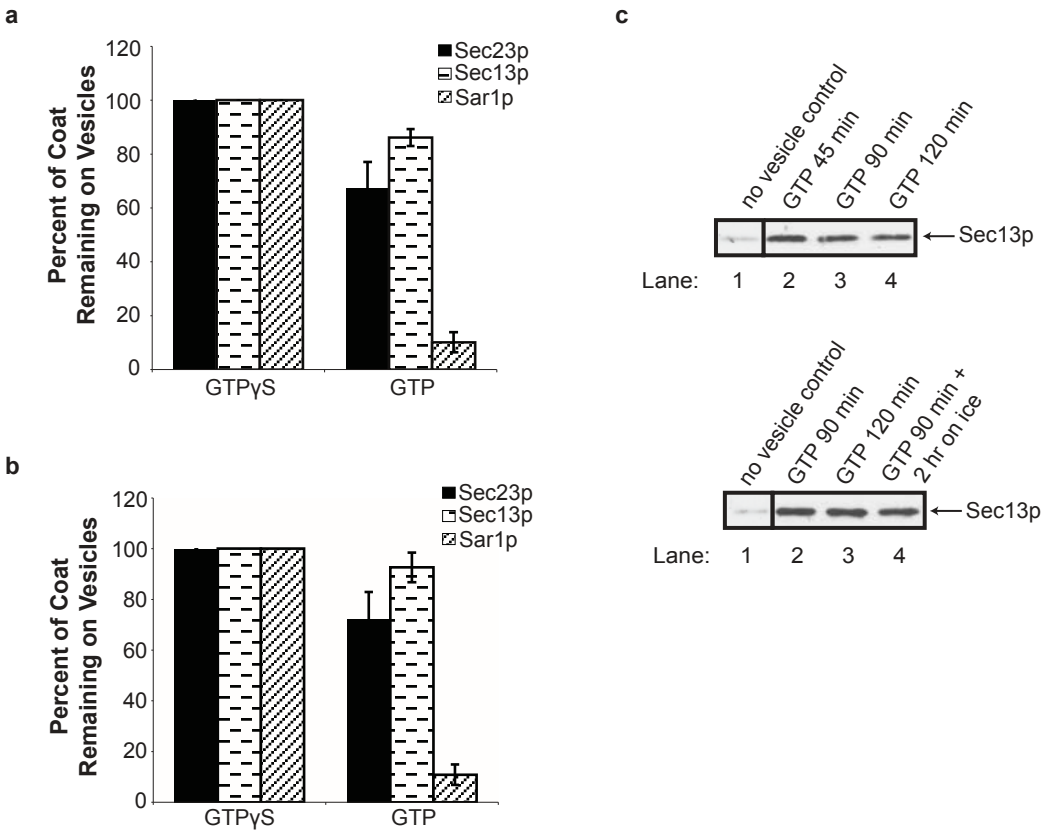


**Fig. S1. Sec13p and Sec23p, but not Sar1p, are retained on COPII vesicles after they bud.** To ascertain if the outer shell of the COPII coat is retained on vesicles, COPII vesicles, containing radiolabeled cargo (pro-α-factor), were generated in vitro with cytosol and permeabilized yeast donor cells (PYCs). At the end of the reaction, the PYCs were pelleted and the vesicles remaining in the supernatant were gel filtered and pelleted prior to western blot analysis. The amounts of Sec23p (outer shell), Sec13p (inner shell) and Sar1p on vesicles, formed with GTP were compared to GTPγS (100% coat), a non-hydrolyzable GTP analogue that locks the coat on vesicles. The amount of coat on the vesicles was normalized to Bos1p in **a** or Con A counts in **b**. **c**, The outer shell of the COPII coat is stably associated with vesicles after they bud. Top, Vesicles were formed with PYCs and cytosol for the indicated times. The PYCs were pelleted and the vesicles remaining in the supernatant were blotted for Sec13p after gel filtration. Bottom, Vesicles were formed for 90 (lane 2) or 120 (lane 3) min, the PYCs were pelleted and the vesicles in the supernatant were incubated for 2 h on ice (lane 4). Lane 1 is a no vesicle control, reaction with apyrase. Error bars represent S.D., N=3.

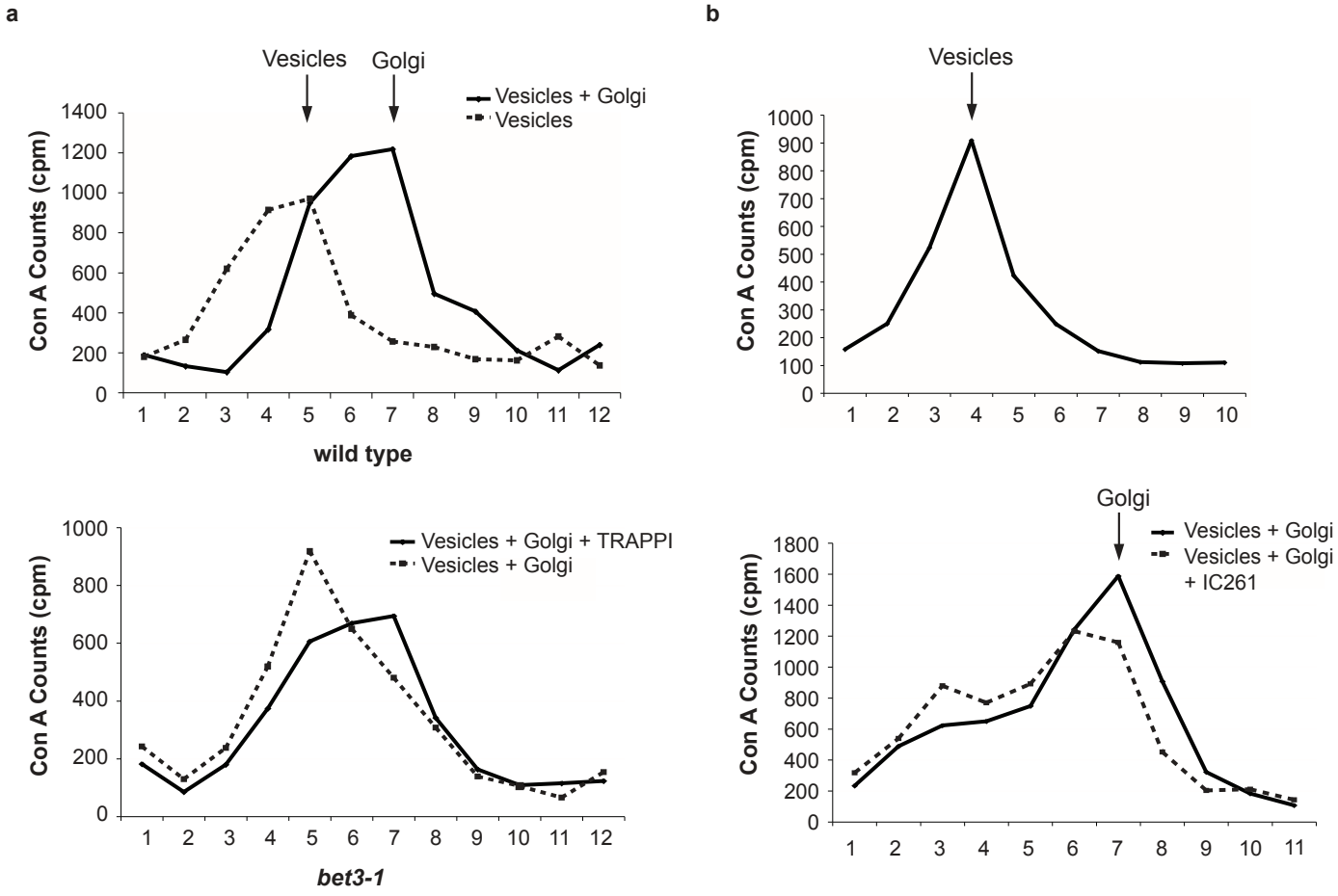


**Fig. S2. IC261 blocks fusion and not vesicle tethering. a**, TRAPPI rescues the *bet3-1* tethering defect in vitro. Top, Vesicles formed from wild type donor cells and cytosol were incubated without (dotted line) or with (black line) Golgi membranes. Bottom, Vesicles formed from *bet3-1* (SFNY596, *MATa bet3-1 ura3-52 leu2-3,112*) donor cells, cytosol and Golgi without (dotted line) or with (black line) recombinant TRAPPI (10 μg). TRAPPI was incubated with fractions for 20 min on ice before the assay was initiated. In the presence of TRAPPI, vesicle tethering was restored (see vesicles shift to the Golgi peak). **b**, Top, To determine where wild type vesicles migrate, vesicles formed from wild type donor cells and cytosol were incubated without Golgi and fractionated on a sucrose velocity gradient. Bottom, to address if IC261 blocks vesicles tethering or membrane fusion, in vitro transport assays were performed in the presence of Golgi +/- 400 μM IC261 and fractionated on a sucrose velocity gradient to separate free vesicles from vesicles bound to the Golgi.

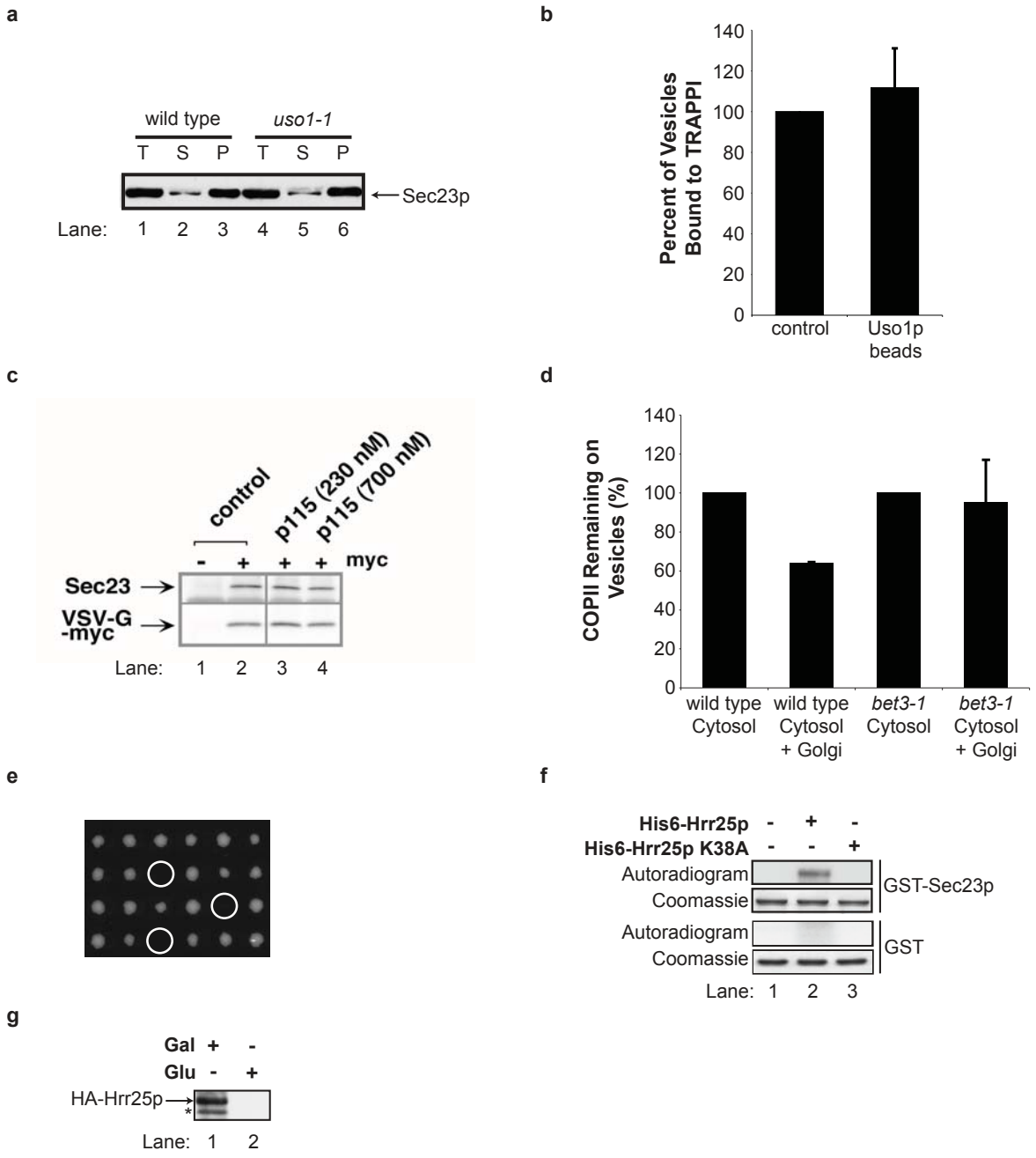


**Fig. S3. Uso1p/p115 does not appear to participate in COPII vesicle uncoating. a**, To determine if Uso1p participates in COPII vesicle uncoating in vivo, wild type (lanes 1-3) and the temperature-sensitive *uso1-1* mutant (lanes 4-6) were grown overnight to early log phase and shifted to 37 $^{o}$C for 2 h. Total (T) lysates were prepared and centrifuged at 150,000 g as described in the **METHODS**. The supernatant (S), pellet (P) and total (T) lysates were then analyzed by western blot analysis. **b**, To determine if Uso1p participates in COPII vesicle uncoating in vitro, vesicles formed in vitro were incubated with control or Uso1p-containing beads. TAP-tagged Uso1p (SFNY1686, *USO1-TAP* from Open Biosystems) and control

beads (SFNY1357, *MATα leu2-3,112 ura3-52 trp1::TRP1 YPR105C-TAP)* were prepared as described before[6]. Subsequently, the beads were pelleted and the vesicles remaining in the supernatant were incubated with TRAPPI-containing beads. Membranes, containing radiolabeled cargo, that bound to the TRAPPI-containing beads were counted in a scintillation counter. Error bars represent S.D., N=4. **c**, Purified recombinant p115 does not release Sec23 from mammalian COPII vesicles. Vesicles containing VSV-G-myc were immunoisolated with anti-myc antibody subsequent to an incubation with 230 nM (lane 3), 700 nM (lane 4), or no p115 (lane 2); lane 1, untagged control. Vesicles incubated with 230 nM p115 retained 79 $\pm$ 7 % (N=2) of the Sec23 relative to lane 2 (no p115), while vesicles incubated with 700 nM p115 retained 81 $\pm$ 7 % (N=2). Concentrations in excess of 1 μM p115 also did not lead to the release of Sec23 from vesicles (not shown). **d,** Measuring vesicle uncoating in vitro. COPII vesicles were generated in vitro from PYCs prepared from cells containing Sec13p-GFP (DBY1034-S13G)[30]. At the end of the reaction, the PYCs were pelleted and the vesicles remaining in the supernatant were gel filtered and pelleted prior to western blot analysis. The amounts of Sec13p-GFP on vesicles formed with cytosol (100% coat) was compared to the amount formed with cytosol and Golgi. The cytosol and Golgi used to form vesicles were prepared from either wild type (N=2; bars show the range) or *bet3-1*(S.D., N=3) cells. **e**, *HRR25* is essential for growth. A diploid strain in which one copy of *HRR25* was disrupted was transformed with a balancing plasmid (*CEN URA3 HRR25*), sporulated and dissected. Spores that contained the disrupted gene, but not the balancing plasmid, stopped growing at the two cell stage (see white circles on plate). **f**, GST-Sec23p and GST were incubated without (lane 1), or with $His_6$-Hrr25p (lane 2) or $His_6$-Hrr25p-K38A (lane 3) and $\gamma P^{32}$-ATP. The top panel is a lighter exposure of the top panel in Fig. 2**b**. **g**, Lysates prepared from *hrr25* cells grown in galactose (lane 1) or 10 h in glucose (lane 2) containing medium were immunoblotted with anti-HA antibody.

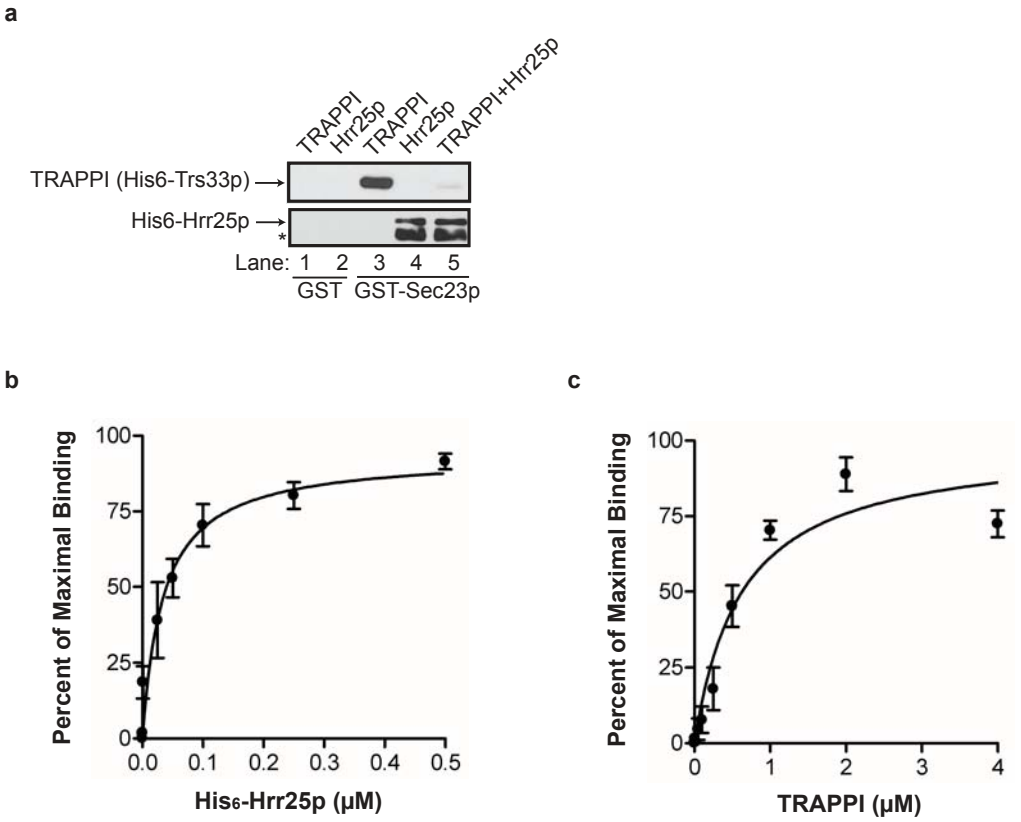


**Fig. S4. Hrr25p binds to Sec23p with greater affinity than TRAPPI. a**, $His_6$-Hrr25p competes with TRAPPI for binding to GST-Sec23p (compare lane 5 with lanes 3 and 4). No binding was observed to GST (lanes 1 and 2). The starred band is a degradation product of Hrr25p. Additional information is provided in the **METHODS**. To determine the dissociation constants of $His_6$-Hrr25p:GST-Sec23p and TRAPPI:GST-Sec23p, increasing amounts of $His_6$-Hrr25p (**b**) and TRAPPI (**c**) were incubated with 100 nM GST-Sec23p. The amount of protein bound to GST-Sec23p for each concentration was calculated as the percent of maximal binding. The graphs were generated and the dissociation constants ($K_d$) were estimated by fitting the results into a non-linear regression equation using the Graph Pad Prism software. The error bars represent S.E.M., N=3-4. The dissociation constants of $His_6$-Hrr25p:GST-Sec23p and TRAPPI:GST-Sec23p were estimated to be 0.043 ± 0.009 μM and 0.63 ± 0.15 μM, respectively.

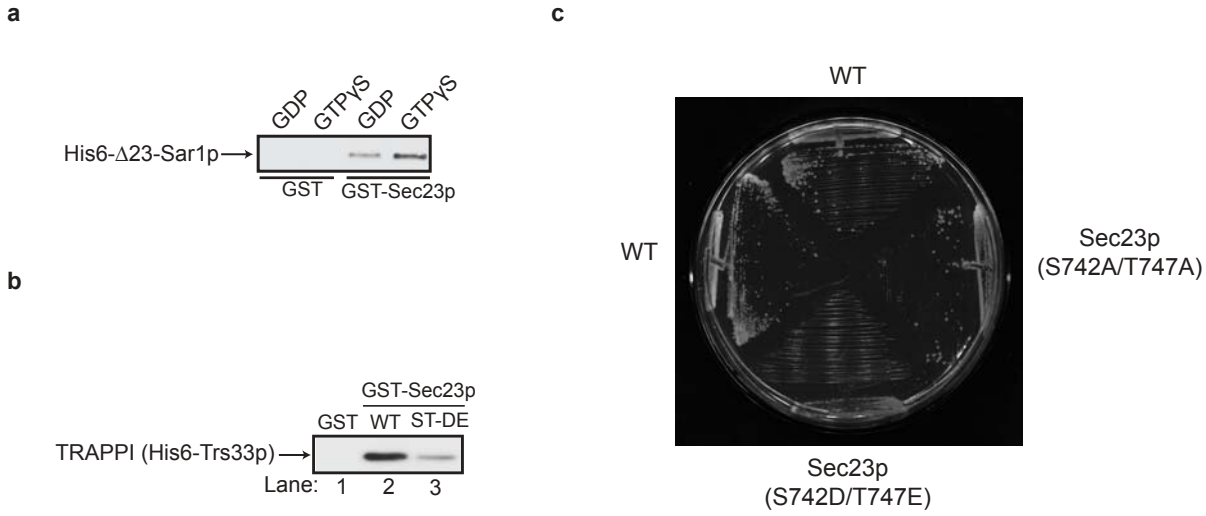


**Fig S5. The *sec23* phosphomimetic mutations impair growth. a,** GST or GST-Sec23p beads were incubated with 10 nM of $His_6$-Δ23Sar1p-GDP or $His_6$-Δ23Sar1p-GTPγS. **b**, Binding conditions were the same as in Fig. 3**b**. The concentration of TRAPPI was 10 nM in the binding experiment shown. **c**, Site-directed mutagenesis was used to introduce the S742D/T747E or the non-phosphorylated S742A/T747A double mutations into plasmid **pCF364** (**pRS414**, *SEC23*). LMY570 (*Mata ade2-101oc his3-Δ200 leu2-Δ1 lys2-801am trp1-Δ63 ura3-52 SEC23::HIS3 SEC23-URA3*) was then transformed with **pCF364** or **pCF364** harboring the double mutations, and the strains were cured of the *SEC23-URA3* plasmid on 5-FOA plates. The growth of the resulting strains on a YPD plate at 33 $^{o}$C is shown.

**Supplementary References**

30. Rossanese, O.W. et al. Golgi structure correlates with transitional endoplasmic reticulum organization in Pichia pastoris and Saccharomyces cerevisiae. *J. Cell Biol* **145**, 69-81 (1999).