SUPPLEMENTAL MATERIALS

Requirement for Golgi-localized PI(4)P in fusion of COPII vesicles with Golgi compartments

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SUPPLEMENTAL MATERIALS AND METHODS

Determination of Sac1^{WT} phosphatase activity on semi-intact cell membranes under *in vitro* pre-treatment conditions

Washed semi-intact cell membranes were treated with 50 μM Sac1^{WT} or 50 μM Sac1^{C392S} as in Figure 4. Membrane lipids were extracted as described (Gray *et al.*, 2003) with minor modifications: 1.5 A_{280} units (~1.03 OD_{600} units) of treated membranes were independently precipitated in quadruplicate with 5% TCA, 1 mM EDTA in 1.5 ml on ice for 15 min followed by centrifugation. The pellet was washed twice with 1.5 ml ice-cold 5% TCA, 1 mM EDTA, and acidic lipids were extracted and dried. Lipids were reconstituted with 10 μl 2:1:0.8 CH_3OH:$CHCl_3$:H_2O by vortexing and sonication for 5 min at 30ºC on a B2200R-1 Branson ultrasonic bath (Branson Ultrasonic Corporation, Danbury, CT). Lipids (4 μl) were spotted on a PI(4)P Mass Strip Kit™ membrane as indicated by the manufacturer (Echelon Biosciences, Salt Lake City, UT), and developed by chemiluminescence and densitometric analysis as described for immunoblots. A Mann-Whitney-Wilcoxon test to compare the medians was performed on the observed results.

NEM inactivation of Sac1^{WT} phosphatase activity

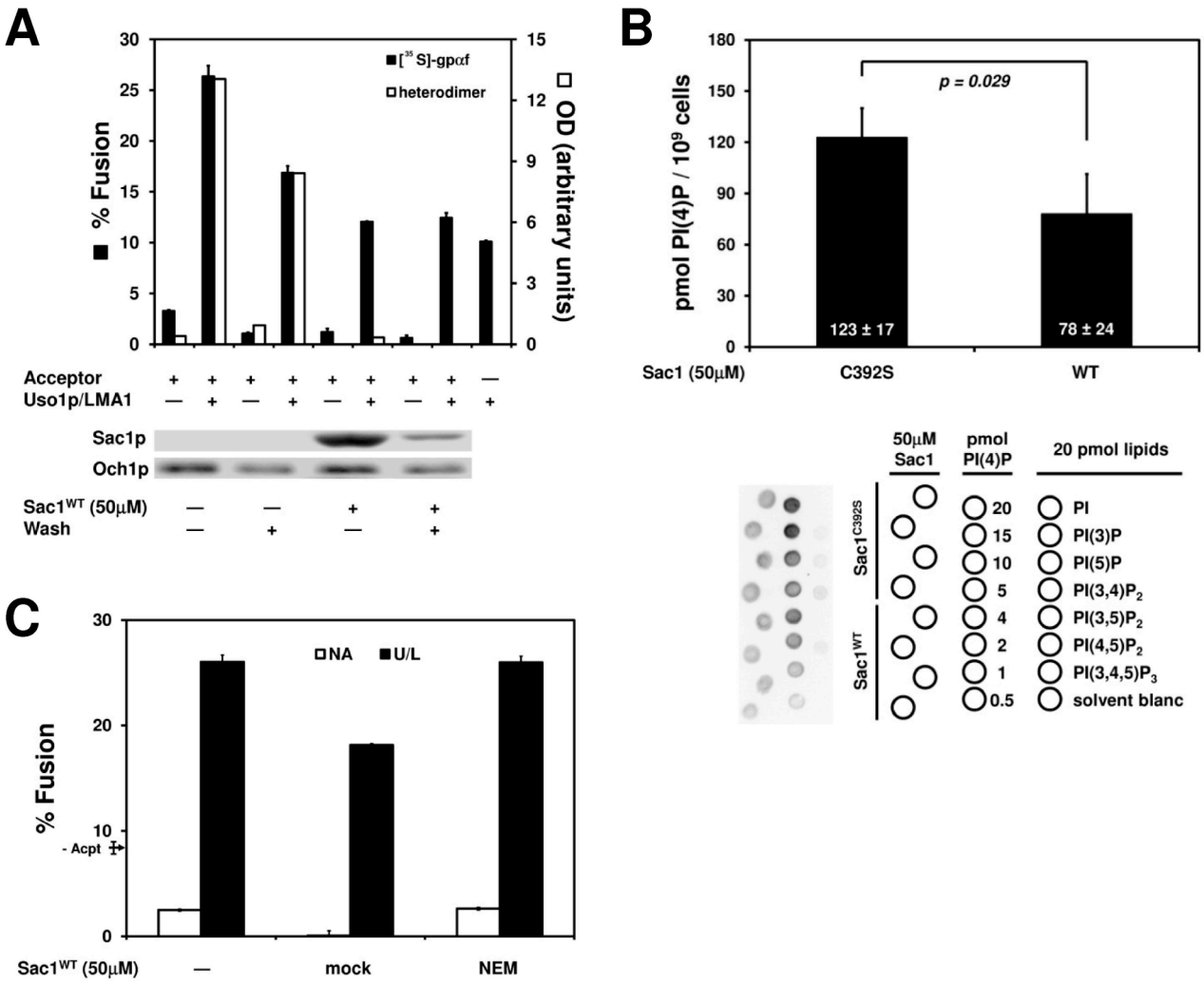
To inactivate Sac1^{WT}, protein (600 μg) was incubated at 8ºC for 10 min with 50 mM NEM (Maehama *et al.*, 2000). Excess NEM was quenched with 100 mM DTT at 8ºC for 10 min, and then dialyzed overnight against buffer 88 without sorbitol containing 0.5 mM DTT. A mock inactivation was performed in parallel using ethanol carrier in place of NEM.

SUPPLEMENTAL TABLE S1. CANDIDATE STRAINS TESTED FOR FUSION DEFECTS

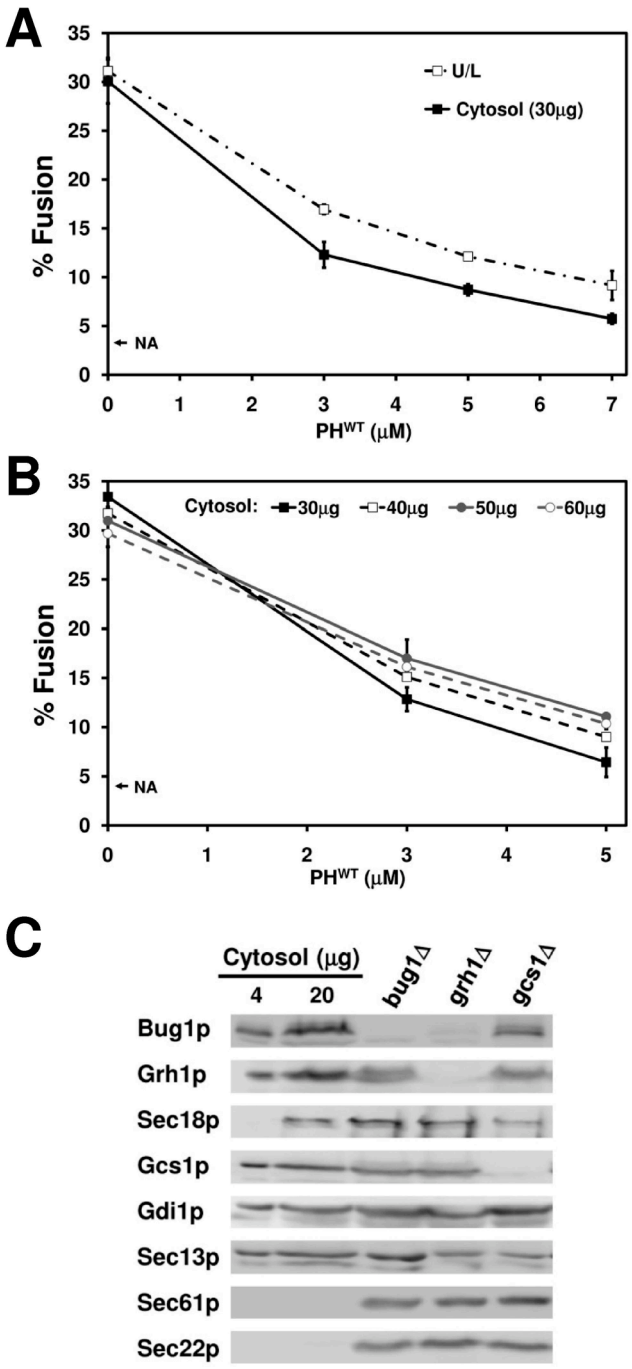
Strain	Genotype	Study reporting PI(4)P binding[1]
atg26Δ	BY4742; *atg26Δ::KAN^R*	(Yu *et al.*, 2004)
bem3Δ	BY4742; *bem3Δ::KAN^R*	(Zhu *et al.*, 2001)
caf120Δ	BY4742; *caf120Δ::KAN^R*	(Yu *et al.*, 2004)
cla4Δ	BY4742; *cla4Δ::KAN^R*	(Yu *et al.*, 2004)
cog5Δ	BY4742; *cog5Δ::KAN^R*	(Zhu *et al.*, 2001)
gcs1Δ	BY4742; *gcs1Δ::KAN^R*	(Zhu *et al.*, 2001)
osh1Δ	BY4742; *osh1Δ::KAN^R*	(Yu *et al.*, 2004)
osh2Δ	BY4742; *osh2Δ::KAN^R*	(Yu *et al.*, 2004)
osh3Δ	BY4742; *osh3Δ::KAN^R*	(Yu *et al.*, 2004)
osh1Δ2Δ3Δ	SEY6210; *osh1Δ::URA3 osh2Δ::URA3 osh3Δ::LYS2*	
osh4Δ	BY4742; *osh4Δ::KAN^R*	(Zhu *et al.*, 2001)
osh6Δ	BY4742; *osh6Δ::KAN^R*	(Zhu *et al.*, 2001)
pdr16Δ	BY4742; *pdr16Δ::KAN^R*	(Zhu *et al.*, 2001)
pdr17Δ	BY4742; *pdr17Δ::KAN^R*	(Zhu *et al.*, 2001)
rgc1Δ	BY4742; *rgc1Δ::KAN^R*	(Yu *et al.*, 2004)
scs2Δ	BY4742; *scs2Δ::KAN^R*	(Zhu *et al.*, 2001)
skg3Δ	BY4742; *skg3Δ::KAN^R*	(Yu *et al.*, 2004)
spo14Δ	BY4742; *spo14Δ::KAN^R*	(Yu *et al.*, 2004)
syt1Δ	BY4742; *syt1Δ::KAN^R*	(Yu *et al.*, 2004)
tus1Δ	BY4742; *tus1Δ::KAN^R*	(Yu *et al.*, 2004)
vtc4Δ	BY4742; *vtc4Δ::KAN^R*	(Zhu *et al.*, 2001)
yel1Δ	BY4742; *yel1Δ::KAN^R*	(Yu *et al.*, 2004)
yhr131cΔ	BY4742; *yhr131cΔ::KAN^R*	(Yu *et al.*, 2004)
ypr091cΔ	BY4742; *ypr091cΔ::KAN^R*	(Yu *et al.*, 2004)

[1]Refers to the original study that reported binding of the indicated protein to PI(4)P.

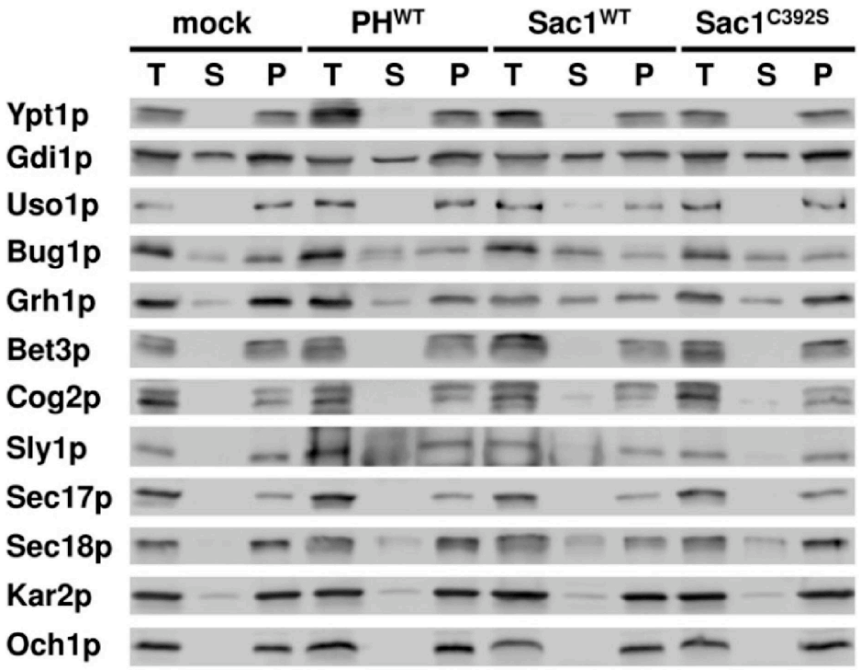
All of the above strains are from the Research Genetics Collection (Brachmann *et al.*, 1998), except for the *osh1Δ2Δ3Δ* strain (Beh *et al.*, 2001). Because our results showed that PI(4)P was required on Golgi membranes for fusion with COPII vesicles, we performed two-stage fusion assays chasing wild type vesicles into acceptor semi-intact cell membranes prepared from the indicated deletion mutants compared to their isogenic wild type. All deletion strains tested above displayed normal fusion levels except for the *gcs1Δ* mutant. Semi-intact cell membranes from the *gcs1Δ* strain exhibited a 47% decrease in fusion efficiency but displayed normal levels of COPII vesicle budding and tethering *in vitro*.



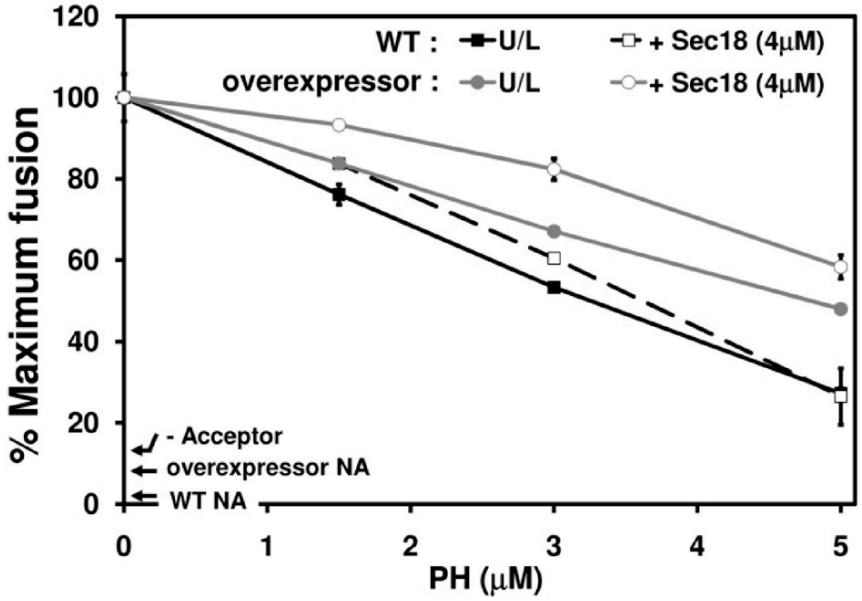
Supplemental Figure S1. The Sac1^{WT} phosphatase activity is required for inhibition of fusion. (A) Washing excess Sac1^{WT} does not rescue fusion inhibition or formation of cross-linked tran-SNARE complexes. Experiment as in Figure 4D monitored formation of cross-linked Bet1p^{I83C}-Sec22p^{D153C} heterodimers (open bars) and Golgi-modified [^{35}S]gpαf fusion (black bars). Ice controls were subtracted as in Figure 4D. Aliquots of treated membranes were immunoblotted for Sac1p using an anti-penta-His antibody and for the Golgi membrane protein Och1p (loading control). Note that the wash removed ~78% Sac1^{WT}. (B) Sac1^{WT} is catalytically active on semi-intact cell membranes *in vitro* under pre-treatment conditions. Washed semi-intact cell membranes were treated with 50 μM Sac1^{WT} or 50 μM Sac1^{C392S} as in Figure 4, lipids extracted and levels of PI(4)P/cell quantified using a lipid overlay assay (shown below). Note that PI(4)P levels are reduced by ~37% (*p=0.029*). (C) NEM inactivation of the catalytic phosphatase activity in Sac1^{WT} reverses inhibition. Semi-intact cell acceptor membranes pretreated with buffer, 50 μM mock-inactivated Sac1^{WT} or 50 μM NEM-inactivated Sac1^{WT} as in Figure 4 were used in two-stage fusion reactions as described in Figure 4A. Note that the mock treated Sac1^{WT} inhibited fusion while the NEM-inactivated Sac1^{WT} did not. The arrow indicates background fusion in the absence of added acceptor membranes.



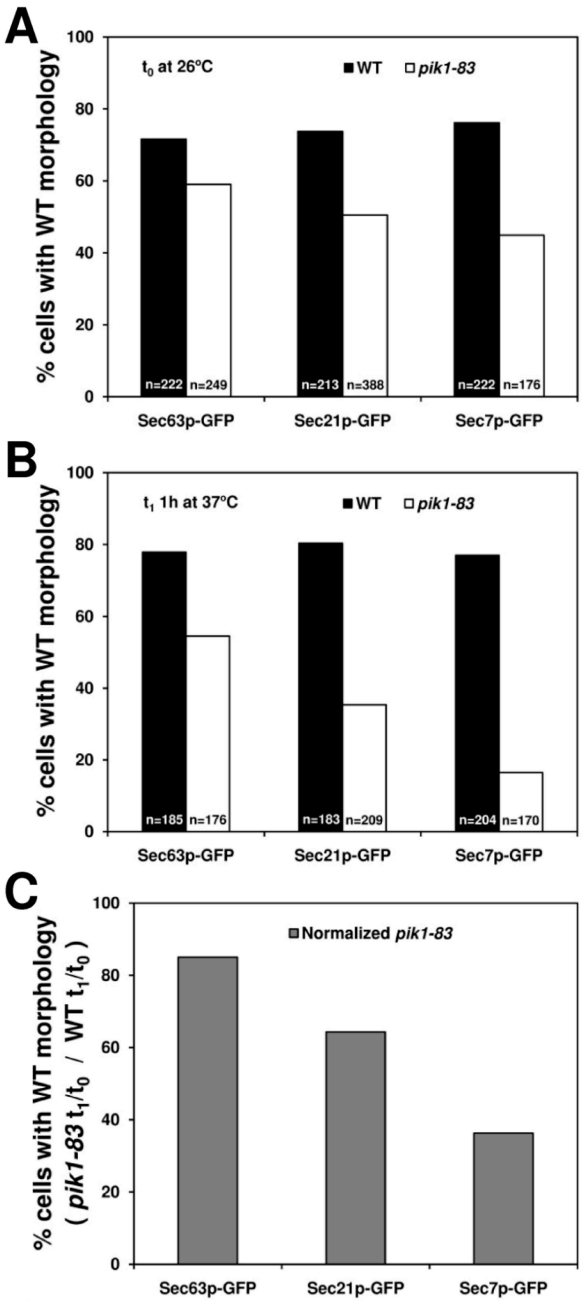
Supplemental Figure S2. Cytosol does not reverse PH^{WT} inhibition of transport. (A) Two-stage fusion reactions reconstituted with cytosol are sensitive to inhibition with PH^{WT} domain. Semi-intact cell acceptor membranes pre-treated with indicated amounts of PH^{WT} for 20 min at 4ºC were used in two-stage fusion reactions as in Figure 1B. (B) Increasing concentrations of cytosol do not reverse inhibition of fusion by PH^{WT} domain. Two-stage fusion reactions using increasing concentrations of cytosol were performed as in panel A. (C) Immunoblot analysis of cytosol. Two different amounts of cytosol were compared to aliquots of semi-intact cell membranes prepared from the indicated deletion strains (Brachmann *et al.*, 1998). Note that Grh1p, Bug1p, Sec18p and Gcs1p are enriched in this cytosol. Antibodies against Gcs1p have been described (Lewis *et al.*, 2004). Cytosol was prepared as described (Barlowe, 1997).



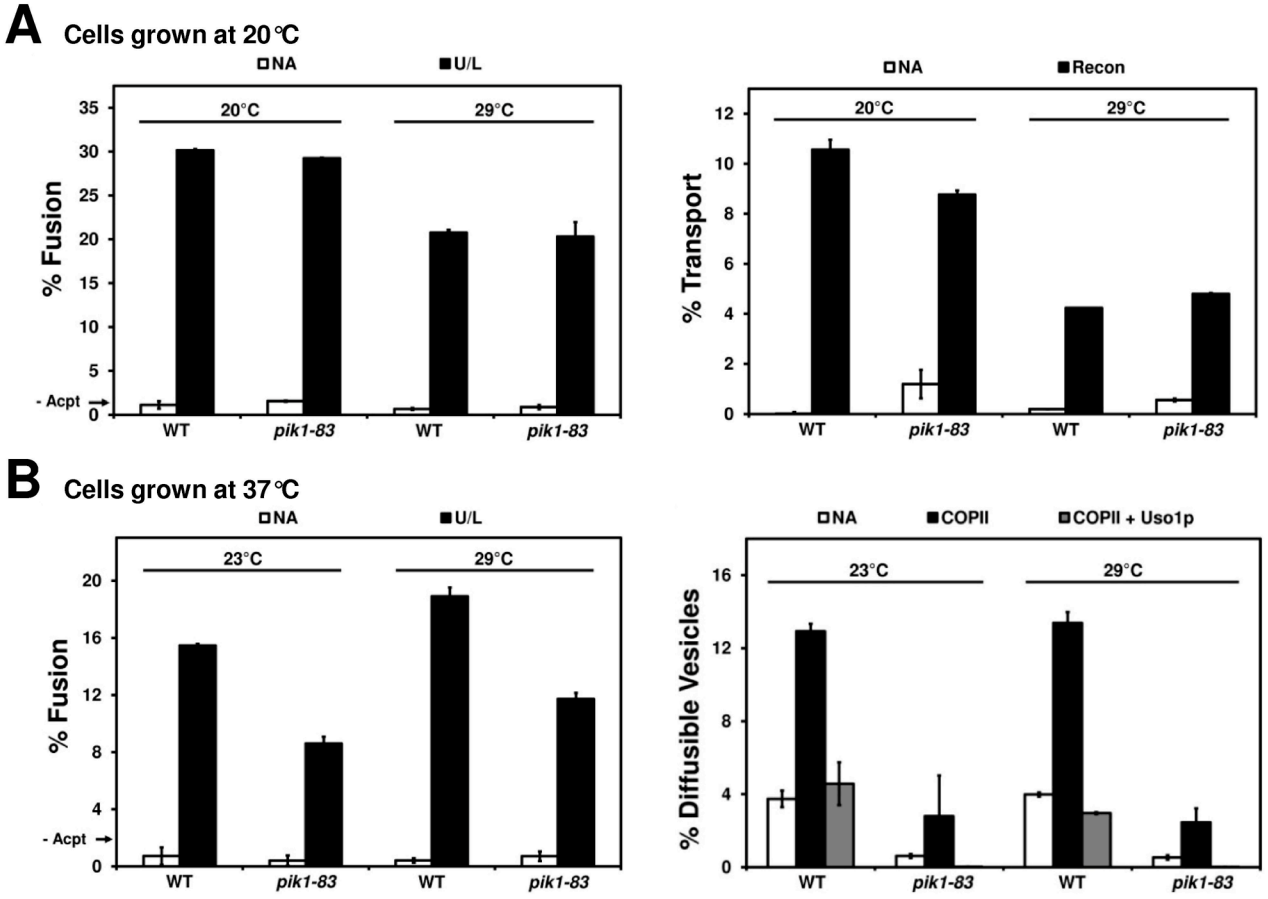
Supplemental Figure S3. Membrane association of transport factors after treatment of semi-intact cells with PH^{WT}, $Sac1^{WT}$ or $Sac1^{C392S}$. Semi-intact cells (65 A_{280} units/ml) were pretreated with 50 μM $Sac1^{WT}$ for 30 min at 12ºC and then diluted in buffer 88 to a final concentration of 12.5 A_{280} units/ml. In parallel, semi-intact cells (12.5 A_{280} units/ml) were incubated on ice for 20 min with buffer, 7 μM PH^{WT} or 5 μM $Sac1^{C392S}$. All reactions were then incubated for 30 min at 23ºC and separated into soluble (S100) and pellet (P100) fractions for immunoblot analysis of the Rab GTPase Ypt1p and Rab GDP dissociation inhibitor Gdi1p; the tethering factors Uso1p, Bug1p, Grh1p, Bet3p (TRAPP complex), and Cog2p (COG complex); the fusion factors Sly1p (SM protein), Sec17 (α-SNAP) and Sec18 (NSF); and the control proteins Kar2p (soluble luminal ER chaperone) and Och1p (Golgi membrane marker). Note that these treatments shifted a low level of Sec18p, Grh1p and Bug1p into the soluble fraction whereas other factors were not affected. In the Sly1p immunoblot PH^{WT} was detected as a cross-reactive species.



Supplemental Figure S4. Overexpression of anterograde ER-Golgi fusion factors partially rescues PH^{WT} inhibition. Data in Figure 7C plotted relative to the control transport reaction in the absence of inhibitor. NA is background fusion in the absence of fusion factors. The - Acceptor condition indicates the background fusion in the absence of added semi-intact cell acceptor membranes.



Supplemental Figure S5. Quantification of ER and Golgi morphologies in wild type and *pik1-83* cells from Figure 8. (A) ER and Golgi morphologies in GFP-tagged strains after growth at 26ºC and (B) after shift to 37ºC for 1 h. To quantify ER morphology, cells expressing Sec63p-GFP were scored for the appearance of elaborated and gapped ER structures compared to wild type cells, which exhibited a typical perinuclear and peripheral ER morphology (Prinz *et al.*, 2000). Cis-Golgi structures were assessed in cells expressing Sec21p-GFP where wild type morphology corresponded to 3-8 medium-sized puncta per cell; and trans-Golgi structures in Sec7p-GFP expressing wild type cells corresponded to 2-6 larger puncta per cell (Rossanese *et al.*, 2001). Abnormal Golgi morphologies included smaller and more numerous puncta, as well as ring-like structures that may correspond to Berkeley bodies. (C) Data from panel B was normalized to panel A (t_1/t_0), and the *pik1-83* t_1/t_0 data was then normalized to WT t_1/t_0 to account for the defective morphologies at 26ºC. The morphological defects in *pik1-83* cells shifted to the restrictive temperature of 37ºC are severe at trans-Golgi compartments, moderate at cis-Golgi compartments and minor at ER compartments.



Supplemental Figure S6. Analysis of *pik1-83* membranes in cell free transport assays. (A) Semi-intact cells were prepared from wild-type (WT) or *pik1-83* cells grown at 20ºC. LEFT: Two-stage fusion reactions performed as in Figure 1B using wild-type COPII vesicles and the indicated semi-intact cell acceptor membranes. The reactions were incubated at 20ºC or 29ºC (highest temperature at which the *in vitro* assay remains active). RIGHT: One-stage transport reactions as in Figure 1A were incubated at 20ºC or 29ºC. Note that there are no significant fusion or transport defects in *pik1-83* membranes compared to WT. NA is the background level of transport in the absence of transport factors. The "-Acpt" condition represents the background level of fusion in the absence of added acceptor membranes. (B) Semi-intact cells were prepared from WT or *pik1-83* cells grown at 26ºC and then shifted to 37ºC for 1 h. Two-stage fusion reactions (LEFT) and diffusible vesicle experiments (RIGHT) were conducted as in panel A and Figure 1C, respectively, and incubated at 23ºC or 29ºC. The *pik1-83* semi-intact cell membranes displayed defects in vesicle fusion (~44% inhibition) and budding (~80% inhibition) after cells were grown at 37ºC for 1 h. (A-B) Note that the *pik1-83* membranes did not display thermosensitive defects *in vitro* and suggests that the *pik1-83* encoded enzyme remains active at 29ºC in vitro or that there is a sufficient pool of PI(4)P on acceptor membranes such that continual synthesis is not needed under conditions of the cell free transport assay. However, after shifting cells to 37ºC for 1 hr, the semi-intact cell membranes displayed defects in vesicle budding and fusion suggesting that depletion of PI(4)P *in vivo* compromises ER and Golgi membrane function in these cell free assays.

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