Lysophospholipids Facilitate COPII Vesicle Formation

Graphical Abstract

Highlights

- COPII mutant sec12-4 is rescued by the overexpression of an ER resident phospholipase
- Lipidomic analysis of COPII vesicles shows enrichment in lysophospholipids
- Recruitment of COPII proteins to liposomes increases in presence of lysophospholipids
- Lysophosphatidylinositol lowers the rigidity of membranes in vitro

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In Brief

Melero et al. show that lysophospholipids are enriched in COPII vesicles and facilitate their formation. These highly conical lipids can lower the energy required to deform membranes and increase the recruitment of COPII coats to giant liposomes in vitro. Their results show that lysophosphatidylinositol strongly facilitates COPII vesicle formation.

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Lysophospholipids Facilitate COPII Vesicle Formation

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SUMMARY

Coat protein complex II (COPII) proteins form vesicles from the endoplasmic reticulum to export cargo molecules to the Golgi apparatus. Among the many proteins involved in this process, Sec12 is a key regulator, functioning as the guanosine diphosphate (GDP) exchange factor for Sar1p, the small guanosine triphosphatase (GTPase) Sar1 in coordination with cargo proteins at ER exit sites (ERES). GTP-bound Sar1 binds membranes through an N-terminal amphipathic-helix, whose insertion can induce tubulation in vitro [2, 3]. Helix insertion creates spontaneous curvature [4], which could result in the lowering of the membrane bending rigidity upon Sar1 binding [5]. Thus, membrane bending by Sar1p will depend on its density, and regulators of Sar1p membrane binding strongly affect COPII budding. For example, Sec12, the GDP exchange factor (GEF) for Sar1, stimulates the loading of GTP onto Sar1, increasing Sar1 association with the membrane and initiating coat formation. Temperature-sensitive sec12 mutants have disorganized ERES, which cause accumulation of COPII machinery and cargo dispersal throughout the ER [6, 7].

The primary function of Sar1-GTP is to recruit the heterodimer Sec23/24. Sec24 acts as a cargo adaptor [8] whereas Sec23 is a GTPase-activating protein (GAP) for Sar1 [9]. The subcomplex Sar1-GTP/Sec23/Sec24 serves as a platform for recruitment of the heterotetramer Sec13/31 [10]. Sec31 is the essential structural component forming the polyhedral structure [11], whereas Sec13 is proposed to help Sec31 to bend the membrane by rigidifying the coat [12]. Furthermore, Sec31 stimulates the GAP activity of Sec23 [13, 14], promoting COPII uncoating by Sar1 deactivation. GTP hydrolysis by Sar1 may also promote fission, but this mechanism remains unclear [2, 3]. Thus, bending of the membrane by the COPII machinery seems to combine induction of spontaneous curvature by the membrane insertion of Sar1p amphipathic helix and scabbling by the rigid coat of Sec13/31–Sec23/24. This mechanism is similar to clathrin in vesicle formation, whose action can be promoted by lowering the bending rigidity or membrane tension [15].

Notably, bending rigidity strongly depends on the lipid composition [16], and thus lipid modifications or sorting during COPII assembly could be expected to affect budding. Mammalian Sar1 was found to enhance the activity of both phospholipase D and phospholipase A1 p125 [17, 18]. These lipases generate phosphatidic acid (PA) and lysophospholipids, respectively. Interestingly, in vitro, Sar1 membrane binding increases when PA and lysophospholipids are present [19]. Thus, conical lipids may promote COPII budding by facilitating Sar1 binding and initiation. Moreover, blocking de novo fatty acid synthesis had a similar effect to the sec12-4 mutant phenotype, altering the distribution of ERES in wild-type yeast [7].

Surprisingly, Funato and collaborators found that the sec12-4 conditional mutation could be rescued by deletions of the lipid transferases osh2, osh3, and osh4 [20]. Here, we explore the mechanistic basis for this suppression, finding that osh2, osh3, and osh4 cells have a lipid composition enriched in lysophospholipids, which may rescue the sec12-4 mutant by enhancing COPII

INTRODUCTION

Vesicular transport from the endoplasmic reticulum (ER) to the Golgi apparatus is mediated by the coat protein complex II (COPII) [1]. COPII assembly is triggered by the small guanosine triphosphatase (GTPase) Sar1 in coordination with cargo proteins at ER exit sites (ERES). GTP-bound Sar1 binds membranes through an N-terminal amphipathic-helix, whose insertion can induce tubulation in vitro [2, 3]. Helix insertion creates spontaneous curvature [4], which could result in the lowering of the membrane bending rigidity upon Sar1 binding [5]. Thus, membrane bending by Sar1p will depend on its density, and regulators of Sar1p membrane binding strongly affect COPII budding. For example, Sec12, the GDP exchange factor (GEF) for Sar1, stimulates the loading of GTP onto Sar1, increasing Sar1 association with the membrane and initiating coat formation. Temperature-sensitive sec12 mutants have disorganized ERES, which cause accumulation of COPII machinery and cargo dispersal throughout the ER [6, 7].

The primary function of Sar1-GTP is to recruit the heterodimer Sec23/24. Sec24 acts as a cargo adaptor [8] whereas Sec23 is a GTPase-activating protein (GAP) for Sar1 [9]. The subcomplex Sar1-GTP/Sec23/Sec24 serves as a platform for recruitment of the heterotetramer Sec13/31 [10]. Sec31 is the essential structural component forming the polyhedral structure [11], whereas Sec13 is proposed to help Sec31 to bend the membrane by rigidifying the coat [12]. Furthermore, Sec31 stimulates the GAP activity of Sec23 [13, 14], promoting COPII uncoating by Sar1 deactivation. GTP hydrolysis by Sar1 may also promote fission, but this mechanism remains unclear [2, 3]. Thus, bending of the membrane by the COPII machinery seems to combine induction of spontaneous curvature by the membrane insertion of Sar1p amphipathic helix and scabbling by the rigid coat of Sec13/31–Sec23/24. This mechanism is similar to clathrin in vesicle formation, whose action can be promoted by lowering the bending rigidity or membrane tension [15].

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Surprisingly, Funato and collaborators found that the sec12-4 conditional mutation could be rescued by deletions of the lipid transferases osh2, osh3, and osh4 [20]. Here, we explore the mechanistic basis for this suppression, finding that osh2, osh3, and osh4 cells have a lipid composition enriched in lysophospholipids, which may rescue the sec12-4 mutant by enhancing COPII
vesicle formation. Indeed, overexpression of a specific isoform of phospholipase B, which increased the cellular levels of lysophospholipids, also rescued sec12-4. Moreover, we found that lysophospholipids are enriched in COP II vesicles formed in vitro from microsomes. By reconstituting COP II assembly on giant liposomes with purified proteins, we found that lysophosphatidylinositol (lysoPI) can dramatically increase the binding of Sar1 and Sec13/31 and lower membrane bending rigidity, which supports a role for conical lipids in the recruitment of the COP II machinery in vivo.

RESULTS

Defects in COP II Vesicle Formation Are Rescued by Lysophospholipid Accumulation

Δosh2 Δosh3 Δosh4 s12-4 mutants survive high temperature better than sec12-4 mutants alone (Figure S1A) [20]. Because Osh proteins are proposed to participate in lipid homeostasis, we analyzed the glycerophospholipid composition of this strain and related strains (same genetic background) grown at permissive temperature (24°C) by mass spectrometry to determine whether the amounts of specific lipids correlated with the rescue phenotype. We detected an increase in lysoPI in Δosh2 Δosh3 Δosh4 sec12-4 strain of 60% compared to wild-type and sec12-4 strain (Figure S1B). Other major phospholipid species were altered, but the changes were not of the same magnitude (Figure S1A; Table S1). In our measurements, lysoPI16:0 was the primary isoform of lysophospholipids with 1.6 mol % in Δosh2 Δosh3 Δosh4 s12-4 strain compared to 0.7 mol % in sec12-4 strain (Figure 1B). Interestingly, lysoPI18:0 abundance is increased as well. Phospholipases A generate lysophospholipids from glycerophospholipids. There are three phospholipases A in yeast with relevant activities: Plb1p, Plb2p and Plb3p. However, Plb1p and Plb2p also degrade lysophospholipids to fatty acids and a polar head group [21, 22]. To test whether lysophospholipids could rescue the sec12-4 mutant, we overexpressed PLB1, PLB2, and PLB3 in wild-type and the temperature-sensitive sec12-4 strains. At restrictive temperatures, sec12-4 overexpressing PLB3 was able to grow at 33°C and had limited survival at 35°C (Figure 1A). Overexpression of PLB1 rescued sec12-4 (Figure S1C) but with low reproducibility, whereas overexpression of PLB2 was lethal at any temperature (data not shown). Furthermore, testing the maturation of carboxypeptidase Y (CPY) through the secretory pathway by western blot, we observed that the pre-CPY form that accumulated in the sec12-4 mutant was partially reduced at both restrictive and non-permissive temperatures when PLB3 was overexpressed (Figure S1D), indicating a partial suppression of the transport defect. Interestingly, PLB3 overexpression did not rescue other sec mutants tested (Figure S2), in particular mutants of the COP II coat components, SNARE proteins, or COPI components. To confirm whether lysophospholipid levels were modified by the overexpression of PLB3, we analyzed the glycerophospholipid composition at permissive temperature, as the sec12-4 strain could not be grown at non-permissive temperature. Interestingly, overexpression of PLB3 caused a decrease in phosphatidylinositol (PI) and an increase in lysoPI16:0 in sec12-4 strains (Figures S1B and 1B), consistent with the fact that Plb3p uses PI and phosphatidylserine (PS) as its main substrates [21]. We detected as well an increase in the major species

**Figure 1. Overexpression of PLB3 Rescues sec12-4 Mutant Phenotypes**

(A) Overexpression of PLB3 rescues temperature-sensitive growth defect of sec12-4 mutant. Five-fold serial dilution of 1 optical density 600 (OD600)/mL of yeast culture was spotted onto YPD plates and were incubated at specified temperatures for 3 days. (B) Relative lysoPI species amount over the total of lysophospholipids in several strains. Usual lipid yield for cell extracts was 6 mmol. Error bars represent SD. *p < 0.05 and ***p < 0.001. (C) Overexpression of PLB3 prevents ERES relocalization caused by sec12-4 at 30°C. Strains expressing SEC13-GFP were used to visualize the ERES at 24°C and 30°C. The scale bars represent 5 μm. (D) ERES per cell were counted and averaged from randomly chosen cells for each strain (wild-type [WT], n = 10; sec12-4, n = 13; WT + pPLB3, n = 15; sec12-4 + pPLB3, n = 13). Error bars represent SD. See also Figures S1 and S2 and Table S1.
of phosphatidylcholine (PC) and decrease of PE (Figure S1B; Table S1). The findings that osn deletions and PLB3 overexpression rescued sec12-4 strains and had a lipid composition enriched in lysoPI suggest that lysoPI levels may overcome the sec12-4 temperature-sensitive growth phenotype.

In further support of this idea, the defects in ERES formation displayed by sec12-4 strains are rescued by PLB3 overexpression. To visualize the ERES, we used strains expressing SEC13-GFP, which increases the temperature sensitivity of sec12-4 mutant from 33°C to 30°C (data not shown). In wild-type strains, ERES form small dots distributed on the cortical ER and nuclear envelope [23]. This distribution is similar at 24°C and 30°C (Figure 1C). Sec12-4 ERES have a wild-type distribution at 24°C. However, at 30°C, sec12-4 ERES collapsed into one or two foci per cell (Figures 1C and 1D). We found that sec12-4 cells overexpressing PLB3 had dispersed ERES at 30°C resembling the wild-type (Figure 1C). Only few cells had intermediate phenotypes, with partially dispersed ERES increasing the average number of ERES per cell in sec12-4 PLB3 (Figure 1D).

These data support a role of lysophospholipids in facilitating COP II budding. We next wondered whether we could observe a relative enrichment of lysophospholipids within COP II vesicles, which could further support a role for specific lipids in COP II budding.

**Lipidomics of COP II Vesicles Reveals an Enrichment of Lysophospholipids**

To investigate whether lysophospholipid levels in the membrane changed upon COP II budding, we first measured the abundance of lysophospholipids in microsomes. Our microsome fraction was highly enriched in ER membranes, with low mitochondrial contamination (Figure S3A). We measured the microsome lipid composition with electrospray ionization coupled to mass spectrometry (ESI-MS) (see STAR Methods). In accordance with previously published measurements [24], we found that our microsomes were enriched in PE and had lower PS compared to the total cell (Figure 2A). With our extraction method, we measured a cellular lysophospholipid content of less than 0.5 mol % of glycerophospholipids. Interestingly, microsomal membranes contained 1 mol % of lysophospholipids. Furthermore, the lysophospholipid profile was different between microsomal membranes and whole-cell membranes: lysophosphatidylethanolamine (lysoPE) was the major lysophospholipid in the microsomal membranes, whereas lysophosphatidylcholine (lysoPC) and lysoPE were of equal abundance in the whole-cell lipidome. We concluded from these measurements that specific lysophospholipids are enriched in microsomal membranes.

Because lysophospholipids are components of the ER membranes and may participate in the rescue of sec12 mutants, we speculated that they may be enriched in the membrane of budded vesicles. We thus measured the lipid composition of COP II vesicles generated through in vitro budding assays using microsomes and purified COP II components (see STAR Methods and [1]). Budding assay efficiency was assessed by western blot (Figure S3B). We extracted lipids as described previously from the COP II vesicle fraction isolated by centrifugation [25]. Levels of extracted lipids from the vesicular fraction of budding assays in which purified COP II proteins were omitted allowed us to compute the background amounts of lipids (Figure S3C), which was then subtracted from lipid values obtained for other conditions. Furthermore, we extracted lipids from the budded microsomes (Figure 2B).

Interestingly, the major glycerophospholipids did not change substantially in budded vesicles except for PS, whose abundance is significantly reduced in microsomes post-budding and COP II vesicles (Figure 2B). Strikingly, lysophospholipids are substantially enriched in microsomes post-budding and in COP II vesicles, with a more abundant level in the latter (Figure 2B). Our in vitro generated COP II vesicles have 4 mol % of...
lysophospholipids on average, which is the largest value of all membrane sources measured. LysoPE is the major lysophospholipid, followed by lysoPC and lysoPI in almost equal abundance (Figure 2B). LysoPI and lysoPC were specifically enriched in COPII vesicles, having 2 times more lysoPC and 3 times more lysoPI than microsomes. Remarkably, vesicles had 1.6 times more lysoPC and 2 times more lysoPI than microsomes post-budding (Figure 2C). In contrast, lysoPE was much less enriched in COPII vesicles and thus behaved like other glycerophospholipids. We conclude that lysophospholipids are enriched in COPII vesicles, in particular lysoPI and lysoPC. To understand the potential effect of this lysophospholipid enrichment on the recruitment of COPII and on the deformability of the lipid membrane, we studied the recruitment of COPII components and membrane elastic properties using giant unilamellar vesicles (GUVs).

**LysoPI Increases the Binding of COPII Proteins to Membranes and Lowers Membrane Bending Rigidity**

The bending rigidity of lipid membranes is determined by their composition [16, 26, 27], and lysophospholipids have been shown to lower membrane bending rigidity [28]. Therefore, local accumulation of lysophospholipids at the ERES may facilitate COPII-mediated membrane deformation. Furthermore, the presence of lysophospholipids could generate curvature stress [29], which could help the insertion of Sar1 and hence the recruitment of COPII coats.

To test this hypothesis, we reconstituted COPII coat binding to membranes using GUVs, which allowed us to study effects of membrane composition on COPII binding. We used a modified Major Mix lipid composition [19], which contained 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) 33%, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) 23%, soy PI 33%, 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) 8%, and 1,2-dioleoyl-sn-glycero-3-phosphate (DOPA) 5%, and a Major Mix + lysoPI, which contains 10% of soy lysoPI. We chose lysoPI because of its high spontaneous curvature and of its enrichment in COPII vesicles (see above).

First, we studied the binding of COPII proteins to GUVs [5, 30]. Sar1p was fluorescently labeled with Alexa-647 and Sec13/31 with Alexa 488. Sar1 membrane binding was promoted by exchanging GDP with guanylyl imidodiphosphate (GMP-PNP) using EDTA [5]. Within minutes after incubation of the protein mix with GUVs, both Sar1p and Sec13/31 complex bound to the surface of the GUVs. Interestingly, Sar1p covered the membrane surface rather homogeneously, with some higher intensity punctae (Figure 3A; Videos S1 and S2), whereas Sec13/31 complex binding formed discrete punctae that colocalized with the brightest Sar1p punctae (Figure 3A). To quantify the signal of COPII binding, we acquired time-lapse images from 5 GUVs per condition. The frames were temporally averaged and GUVs linearized. The average intensity of Sar1p and Sec13/31 complex were high with GMP-PNP and strongly reduced without it (Figures 3A and 3C).

With the same approach, we studied the binding of COPII proteins on GUVs containing lysoPI (Figure 3A). Both Sar1 and Sec13/31 binding was stronger in the presence of lysoPI than in the conditions tested previously. Sar1 distribution was rather similar whereas the distribution of Sec13/31 complex was different. We found that, in the presence of lysoPI, Sec13/31 complex covers the complete surface of the GUV. Nevertheless, brighter puncta were found colocalized with Sar1 puncta. Sar1p and Sec13/31 complex signals increased dramatically with lysoPI (Figure 3C), showing that lysophospholipids can increase COPII protein binding. The increase in binding of Sec13/31 due to lysoPI was greater than the increase for Sar1 (Figure 3C), suggesting that lysophospholipids could also facilitate coat assembly. Remarkably, we did not observe large membrane deformations in any of the conditions tested [30, 31], although some of the puncta could contain small highly curved membrane structures (Figure 3A, lower panels; Videos S3 and S4).

With our experimental setup, we did not directly observe Sec23/24; however, we tested whether the absence of Sec23/24 affects the binding of the other COPII proteins. In conditions without lysoPI, omission of Sec23/24 allowed binding of Sar1 but no substantial Sec13/31 binding (Figures 4A and 4B). We tested as well the effect of omitting Sec23/24 under conditions with lysoPI. Interestingly, Sar1 binding was not improved by the presence of lysophospholipids in the absence of Sec23/24 (Figure 4A). To reach similar membrane binding as in Figure 3C, the full set of COPII proteins was required. Finally, Sec13/31 was not recruited to GUVs with lysoPI in the absence of sec23/24 (Figure 4B). To test whether lysophospholipids decrease membrane bending rigidity, we measured bending rigidity of GUVs using an aspiration pipette and an optical tweezer (Figure 3D; see STAR Methods and [32, 33]). The force (f) required to pull tubes out of GUVs is proportional to the square root of the bending rigidity (κ) and of the membrane tension (σ), with $f = 2\pi \sqrt{2\kappa \sigma}$ [34]. We fixed membrane tension with an aspiration pipette and measured the force required to pull a tube using the optical tweezer (Figure 3D). The slope of the plot of the squared force versus membrane tension gives membrane bending rigidity of a GUV (Figure 3D). We first measured bending rigidities of single palmitoyl-oleyl phosphatidylcholine (POPC) GUVs (Figures 3D and 3E). Then, lysoPI was injected in the proximity of the GUV, and force measurements were repeated (Figure 3D). During injection of lysoPI, we observed a large extension of the membrane tongue aspirated in the pipette, revealing the insertion of lysoPI in the membrane (Figure 3G). After stabilization of the tongue increase, which suggested that the incorporation of lysoPI had reached equilibrium, we measured the bending rigidities of the same GUVs: we observed a consistent drop of bending rigidity of 26% on average (Figure 3F). We conclude that the presence of lysoPI lowers the bending rigidity of the membrane and thus the energy cost to deform it.

**DISCUSSION**

In this study, we provide evidence for a direct cooperation between highly conical lysophospholipids and COPII machinery (Figure 5). We show that the sec12-4 mutant is rescued by over-expression of a phospholipase, PLB3. This caused a modest increase in the cellular levels of lysoPI, similar to those found in osh2ΔΔΔΔ sec12-4 cells, and the lysophospholipids were enriched in ER membranes. Furthermore, the relief of sec12-4 growth defects seems to be directly linked to the abundance of lysophospholipids, as osh2ΔΔΔΔ sec12-4 cells accumulate...
more lysoPI and suppresses growth defects better than Plb3. Phospholipases have been directly implicated in membrane budding in cells by producing conical lipids [17, 18, 35], suggesting that lysophospholipids could help budding by COPII in conditions of defective coat machinery. Encouraged by these observations, we measured the lipid composition of in vitro generated COPII vesicles, which were also enriched in lysophospholipids, especially lysoPC and lysoPI. These two lysophospholipids are the most conical phospholipids in the cell when compared to other lysophospholipids [36]. Our method may have underestimated the true proportion of lysophospholipids in COPII vesicles, because we used saturating concentrations of COPII proteins, which could reduce the lipid selectivity and the lipid enrichment that occurs under physiological conditions. A fundamental question that arises from our data concerns the localization of lysophospholipids.

The enrichment of lysophospholipids in COPII vesicles could be explained by different mechanisms. The membrane curvature during vesicle formation could trigger curvature-driven lipid sorting of lysophospholipids toward the nascent vesicle. In addition, lipids with preference for disordered membranes could play a role in the enrichment of lysophospholipids in a highly curved membrane structure, such as a COPII vesicle [32, 37], preventing their diffusion toward the rest of the ER.

Another possibility is that lysoPI production is specifically located or activated in COPII buds: curvature may activate membrane-remodeling enzymes that would produce lysophospholipids in the nascent vesicle. This latter mechanism is consistent with the observation that lysoPI increases the binding of COPII to membrane surface and lowers bending rigidity.

Figure 3. LysoPI Increases the Binding of COPII to Membrane Surface and Lowers Bending Rigidity

(A) Confocal images of COPII proteins binding to giant liposomes. GUVs were made of 36.5% DOPC, 20% DOPE, 30% soy PI, 8% DOPS, 5% DOPA, and 0.05% fluorescent rhodamine-PE. To test the effect of lysophospholipids, a similar mix, including 10% lysoPI, was added over the final weight of lipids and used in similar conditions. COPII proteins were mixed in a separate tube (Sar1 2.38 μM, Sec23/24 320 nM, and Sec13/31 562 nM) and incubated with GMP-PNP and EDTA to facilitate nucleotide exchange. After this short incubation, the protein mix was pipetted into the chamber with GUVs. Time-lapse images were taken for each GUV, 5 frames per second, 100 frames per channel. In total, four conditions were tested: with or without lysoPI combined with or without GMP-PNP. Scale bar, 10 μm.

(B) After time averaging, the resulting GUV image was linearized and the intensity profile across the membrane was plotted. Background was subtracted and normalized the signal from inside the GUV to 0.

(C) Intensity profiles obtained from the procedure shown in (B) for different conditions tested. Each profile is the average of five GUV profiles. Error bars represent SEM.

(D) Scheme of experimental setup. An aspiration micropipette (left down) is used to set tension by aspirating a tongue from the GUV. A microbead trapped by an optical tweezer (right) is used to pull a nanotube from the GUV. A second micropipette (left up) injects lysoPI locally.

(E) Linear variation of the force squared \( f^2 \) required to pull a tube from a vesicle as a function of membrane tension \( \sigma \) (N.m\(^{-1}\)) applied by the aspiration micropipette. Line slopes are proportional to the bending rigidity \( k \). Measurements were done on the same GUV before lysoPI was injected and afterward.

(F) Average bending rigidity before and after injection of lysoPI (n = 5). Error bars represent SD.

(G) Time-lapse of lysoPI injection (120 μM) in the vicinity of a GUV aspirated within a micropipette. Upon injection of lysoPI, a force drop is detected (not shown) and the aspiration tongue elongates until it stabilizes. The scale bar represents 10 μm.

See also Videos S1, S2, S3, and S4.
with our finding that microsomes post-budding accumulated lysophospholipids only when incubated with an ATP regeneration system (Figures 2B and S3C), which is required for membrane deformation, but not for lipid hydrolysis: phospholipases have no direct requirement for ATP or GTP to hydrolyze lipids [21]. It has been shown that curvature and lipid packing defects stimulate the enzymatic activity of phospholipases A2 (PLA2), a homolog of yeast PLB proteins [38, 39], and similar behaviors have been found for sphingomyelinases [40]. As PLBs are glycosylphosphatidylinositol (GPI)-anchored proteins, which must enter into COPII vesicles for their transport, they may be activated upon arrival in negative curvature domains, such as the rim of ERES [23, 41], suggesting the possibility of generation of lysophospholipids at COPII vesicle formation sites.

Our findings on the lipid composition of vesicles led us to characterize the lipid-dependent interactions of COPII proteins with artificial liposomes. Using GUVs lacking lysophospholipids, both Sar1p and Sec13/31 complex were only able to bind when a non-hydrolyzable form of GTP was added to the assay. Whereas Sar1p was distributed over the entire surface of the GUV, Sec13/31 complex was restricted to areas where Sar1p was more abundant. Strikingly, the presence of lysophospholipids allowed the recruitment of Sec13/31 complex over the entire surface of the membrane in a Sar1- and Sec23/24-dependent manner. Our reconstitutions omitting Sec23/24 (Figure 4A) show that Sar1 binding is enhanced only in the presence of the full coat and lysoPI. Multiple binding sites for Sar1p to the full coat may participate in increasing Sar1p density in the presence of the full coat and lysoPI. Alternatively, the use of GMP-PNP in our reconstitution experiments may artificially increase Sar1p concentration by blocking the GAP activity of the full coat onto Sar1p. Furthermore, lysophospholipids cannot bypass the need for Sar1 (Figure 4B). Thus, the efficient binding of Sar1p to the membrane as well as generation of curvature and Sec13/31 assembly strongly depend on the presence of lysophospholipids. It is possible that the COPII coat is adapted to highly disordered membranes (Figure 5B), as seen for clathrin coats [15].

Another interesting feature from our reconstitution assays is that lysoPI increased binding of COPII proteins to the membrane surface in the absence of GMP-PNP. This binding is especially significant for Sar1 and was previously described [19]. This has important implications when explaining the suppression of the

Figure 4. Full COPII Coat Is Required for Optimal Binding to Membrane Surface
(A) Averaged intensity profiles of Sar1 and Sec13/31 membrane binding with different lipid composition and conditions omitting Sec23/24 or with full COPII coats. Intensity profiles from several GUVs (n ≥ 13) were obtained following the procedure shown in Figure 3B. Error bars represent SEM.
(B) Averaged intensity profile of Sec13/31 membrane binding with different lipid composition and conditions omitting Sar1 or with full COPII coats. Intensity profiles from several GUVs (n ≥ 10) were obtained following the procedure shown in Figure 3B. Error bars represent SEM.

Figure 5. Lysophospholipids Decrease Membrane Bending Rigidity and Facilitate COPII Recruitment to Exit Sites
(A) Diagram of surface area of phosphatidylinositol 32:1 and surface area of lysophosphatidylinositol 16:0.
(B) Deformable ER membrane with lysophospholipids facilitates the recruitment of COPII proteins. Insertion of Sar1 on the outer leaflet of the ER and subsequent recruitment of Sec23/24 could trigger the sorting of lysoPI toward disordered membrane (1). Enrichment in lysoPI lowers membrane rigidity and facilitates high curvature deformation by COPII proteins (2). Scission of vesicles is enriched in lysoPI (3).
sec12-4 mutant. Strains carrying this mutation suffer from low Sar1p recruitment efficiency. If lysophospholipids can increase the binding of Sar1p to the ER surface, this could overcome a deficiency in nucleotide exchange by Sec12. Indeed, recent reports have suggested that the Sar1 amphipathic helix might be permanently exposed, allowing limited binding to the membrane even in the GDP state of the protein, whereas the Sar1-GTP state would stabilize the interaction with the membrane [43]. In this context, lysophospholipids would help to stabilize Sar1p interaction with the ER surface under poor GTP exchange conditions.

Membrane bending is a challenging task for the COPII machinery. Cargo crowding in the vesicular lumen opposes membrane bending by coat proteins [12, 44, 45]. Lipids are key to membrane bending, as the elasticity of membranes is directly linked to their composition [26]. Thus, membrane remodeling with non-bilayer lipids, such as lysophospholipids, could introduce curvature stress, which lowers the bending rigidity and softens the membrane (Figure 5). This has been tested for LysoPC and LysoPE [28, 29], but no measurements have been done for LysoPI. With our tube-pulling assay, we found that adding lysoPI to pure POPC vesicles lowers bending rigidity by 26%. With our technical approach, we cannot control the mol % of lysoPI adsorbed by individual GUVs; however, the consistency of the difference in bending rigidity suggests that saturation was reached on each GUV. Lipidomics of in vitro generated vesicles showed that LysoPC and LysoPI are enriched in COPII, in contrast to LysoPE. Interestingly, LysoPE has been found to have little effect on the spontaneous curvature and the elasticity of membranes in vitro [29]. Altogether, these data suggest a role for conical lysophospholipids in COPII vesicle formation, most likely by increasing the elasticity of ER membrane at the ERES and facilitating the interaction of COPII proteins with the outer leaflet.

In summary, we propose that COPII coat subunits cooperate with lipids and lipid remodeling enzymes to achieve highly curved membrane geometries. We have found evidence of lysophospholipids in COPII vesicles at higher ratios than in microsomes, and we speculate that such lipids will be found enriched at the ERES. As COPII coats have limited membrane-bending capacity [12, 36, 46], efficient Sar1 GTPase cycles and a highly deformable membrane composition are probably needed to efficiently generate a vesicle intermediate and to achieve membrane fission. Most likely, specific lipids and membrane-remodeling enzymes participate to optimize the process.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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  - Bending rigidity measurements
  - Proteins and microsomes purification
- **QUANTIFICATION AND STATISTICAL ANALYSIS**

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes three figures, one table, and four videos and can be found with this article online at https://doi.org/10.1016/j.cub.2018.04.076.

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**AUTHOR CONTRIBUTIONS**

Conceptualization, H.R. and A.R.; Investigation, A.M., N.C., and T.K.; Resources, I.R., K.F., and C.B.; Writing – Original Draft, A.M.; Writing – Review & Editing, H.R., A.R., C.B., and K.F.; Visualization, A.M.; Supervision, H.R., A.R., C.B., and K.F.; Project Administration, H.R. and A.R.; Funding Acquisition, H.R. and A.R.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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**STAR METHODS**

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit polyclonal Anti-Sac1 | Barlowe Lab | N/A |
| Rabbit polyclonal Anti-Erv46 | Barlowe Lab | N/A |
| Rabbit polyclonal Anti-Emp24 | Riezman Lab | N/A |
| Rabbit polyclonal Anti-Sec22 | Barlowe Lab | N/A |
| Rabbit polyclonal Anti-Bos1 | Riezman Lab | N/A |
| Rabbit polyclonal Anti-Cor1 | Pfanner Lab | N/A |
| **Bacterial and Virus Strains** |        |            |
| RSB805              | [47]   | N/A        |
| CBB205              | [1]    | N/A        |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| GTP                 | Sigma-Aldrich | 10106399001 |
| ATP                 | Sigma-Aldrich | 10519979001 |
| GDP-Mannose         | Sigma-Aldrich | G5131 |
| Creatine Phosphate  | Sigma-Aldrich | 27920 |
| Creatine Phosphokinase | Sigma-Aldrich | C3755-17.5KU |
| 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) | Avanti Polar Lipids (Alabaster, AL) | 850725C |
| 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) | Avanti Polar Lipids (Alabaster, AL) | 850375C |
| Soy L-α-phosphatidylcholine (PC) | Avanti Polar Lipids (Alabaster, AL) | 840044C |
| 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) | Avanti Polar Lipids (Alabaster, AL) | 840035C |
| 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) | Avanti Polar Lipids (Alabaster, AL) | 810150C |
| DSPE-PEG(2000) Biotin | Avanti Polar Lipids (Alabaster, AL) | 880129C |
| Soy L-α-lysophosphatidylcholine (lysoPC) | Avanti Polar Lipids (Alabaster, AL) | 850090P |
| Alexa® 647 maleimide | Molecular Probes | A20347 |
| TFP-Alexa® 488 | Life technology | A37563 |
| Avidin               | Sigma-Aldrich | A9275 |
| Casein               | Sigma-Aldrich | C6905-1G |
| **Experimental Models: Organisms/Strains** |        |            |
| YPH500 (MATα ade2 trp1 ural3 leu2 his3 lys2) | [23] | N/A |
| YPH499 (MATa ade2 trp1 ural3 leu2 his3 lys2) | Riezman Lab | N/A |
| SMY80 (MATa sec12-4 ade2 trp1 ural3 leu2 his3 lys2) | [23] | N/A |
| KMY111 (SMY80 SEC13-GFP::TRP1) | [23] | N/A |
| FKY387 (MATα osh2Δ::HIS3 osh3Δ::URA3 osh4Δ::TRP1 his3 leu2 lys2 trp1 ural3 bar1) | [20] | N/A |
| FKY387 (MATα sec12-4 osh2Δ::TRP1 osh3Δ::URA3 osh4Δ::HIS3 his3 leu2 lys2 trp1 ural3 bar1) | [20] | N/A |
| BY4742               | [48]   | N/A        |
| RSY1069              | [49]   | N/A        |
| CBY120               | Barlowe Lab | N/A |
| **Recombinant DNA**  |        |            |
| pRS425-TDH3          | Riezman Lab | N/A |
| pRS425-TDH3-PLB1     | This study | N/A |
| pRS425-TDH3-PLB2     | This study | N/A |
| pRS425-TDH3-PLB3     | This study | N/A |

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Aurélien Roux (aurelien.roux@unige.ch).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Saccharomyces cerevisiae strains were cultured on YPD media made of 1% yeast extract, 2% bactopeptone, 2% glucose and 0.2% MES hydrate or synthetic media containing yeast nitrogen base (without amino acids and with ammonium sulfate) 0.67%, glucose 0.2% and -LEU drop out amino acid mix. Growth temperatures ranged from 24°C to 37°C depending on the experiment.

METHOD DETAILS

Permissive temperature spot assay

S. cerevisiae strains were viability tested at several temperatures (25-30-32-35-37°C) with or without PLB3 overexpression. Fivefold serial dilution of 1 OD600/mL of yeast culture were spotted onto SD -URA plates and incubated at indicate temperatures for 3 to 6 days.

ERES visualization

YPH499 was transformed with YPCLAC-SEC13mch to visualize ERES in wild-type yeast. KMY111 [23] was transformed with pRS425-PLB3 and used to visualize ERES in the sec12-4 mutant. KMY111 alone was used as a negative control. Strains were grown at 24°C in minimal media until 0.5 OD600/mL and then each culture was divided in two. One half was placed at 30°C while the rest remained at 24°C. The cultures were incubated 30 min at the respective temperatures and afterward imaged with a Zeiss Axio Z1 epifluorescence microscope. Random cells were chosen and well defined endoplasmic reticulum (ER) exit sites (ERES) were counted manually. The samples from the culture incubated at 30°C were visualized using 30°C glass slides pre-warmed at 30°C. The 4 strains were observed and analyzed in the same way as described before.

Lipid extraction and analysis from yeast cultures

Strains YPH499, SMY80 and SMY80 transformed with pRS425-TDH3-PLB3 plasmid were grown on selective media overnight at 24°C. At 0.5 OD600/mL cells were collected and resuspended in YPD (For 1L: glucose 20 g, Bacto peptone 10 g, Bacto yeast extract 20 g, MES hydrate 1.95 g and 40 mg each of Tryptophan, Uracil and Adenine) and incubated at 24°C. Cells were collected when cultures reached 1 OD600/mL and processed for lipid extraction. The cultures were transferred to 50 mL FALCON tubes and cellular metabolism was stopped with trichloroacetic acid (5% final), incubated for at least 10 min on ice and after centrifugation for 5 min at 800 xg, cells were washed with 10 mL 5% TCA and centrifuged for 5 min at 800 xg. The pellet was resuspended in 5 mL water and the appropriate amount to have 25 OD (1 OD = 10⁸ cells) was transferred into a 10 mL glass tubes and centrifuged for 5 min at 800 xg. The supernatant was removed and the pellets were frozen in the tubes at −80°C.

The lipids were extracted as previously described [50] the pellets were thawed and 500 μL glass beads were added together with 25 μL of internal standard mix (7.5 nmol of 17:0/14:1 PC, 7.5 nmol of 17:0/14:1 PE, 6.0 nmol of 17:0/14:1 PI, 4.0 nmol of 17:0/14:1 PS purchased from Avanti Polar Lipids [Alabaster, AL]). Then, 1.5 mL extraction solvent (ethanol, water, diethyl ether, pyridine, and 4.2 N ammonium hydroxide [15:15:5:1:0.018, vol/vol]) was added. The sample was vortexed for 6 min on a multi vortexer (Labtek International, Christchurch, New Zealand). Samples were incubated for 20 min at 60°C, then centrifuged for 5 min at 800 g. The supernatant was transferred to a clean 13 mm glass tube (Corning) with a Teflon-lined cap. The extraction was repeated once by adding extraction solvent to the beads. The samples were dried under a flow of N₂ and stored at −80°C.

The analysis was done as described [50] with minor modifications. Glycropospholipids were analyzed by using a Triple Stage Quadrupole (TSQ) Vantage Mass Spectrometer (Thermo Scientific) equipped with a robotic nanoflow ion source, Nanomate HD (Advion Biosciences, Ithaca, NY). Lipid extracts were dissolved in 500 μL of chloroform:methanol:water (2:7:1, vol/vol/vol) for both positive- and negative-ion-mode mass analysis. Samples were analyzed by direct infusion. Glycroospholipids were detected by multiple reaction monitoring as described.
In vitro vesicle formation assay

Preparation of microsomes

All steps are done at 4°C except incubation for vesicle generation. Two aliquots of microsomes per budding reaction, including two per negative control, were thawed on ice. Each microsome aliquot was in the range of 3.2 mg/mL of protein. Once the microsomes were thawed, all aliquots were combined in one single siliconized Eppendorf tube. Microsomes were incubated with ATP (1 mM) at 6°C for 10 min and after this placed on ice for 5 min. The, microsomes were carefully washed with 2 volumes of B88 and centrifuged at 19000 x g for 3 min. Washes were repeated 3 times. After the last centrifugation, the supernatant was aspirated and the pellet of microsomes was resuspended in 200 μL per budding reaction and aliquoted in siliconized Eppendorf tubes.

In vitro vesicle formation

The vesicle formation assay was adapted from [1, 49]. The standard budding reaction was composed of 200 μL of microsomes from the same batch (3.2 μg/μL of protein as absorbance by OD280), 50 μL of B88 buffer without potassium acetate, 10 μL Sec23/24 (stock at 1.5 mg/mL), 15 μL of Sec13/31 (stock at 1.5 mg/mL), 12 μL of Sar1 (stock at 3.3 mg/mL) and GTP 1mM (Sigma) previously combined with an ATP regeneration system composed of ATP 1mM (Sigma), GDP-mannose 50 μM (Sigma), creatine phosphate 40 mM (Sigma), and creatine phosphokinase 2 mg/mL (Sigma C3755-17.5KU). Finally, B88 buffer was added to reach 500 μL volume. The mix was gently vortexed and vesicle formation was started by placing the tubes in a water-bath at 23°C for 25 min. To finish the reaction, all tubes were placed on ice for 5 min. After this, 5% from each reaction was taken and combined together and labeled as Total fraction. All samples (excluding totals) were centrifuged at 19,000 xg for 3 min at 4°C. Afterward, 450 μL of supernatant were taken carefully, avoiding the pellet of microsomes. The pellets were kept at 4°C or immediately frozen in liquid N2. The supernatant was divided into two aliquots placed in different ultracentrifuge tubes. All samples were centrifuged at 55,000 rpm for 12 min at 4°C in a Beckman TLA 100.3 rotor. Finally, all supernatants were discarded and vesicular fraction was immediately frozen in liquid N2 and stored at −80°C.

As a negative control, we incubated the microsomes in similar conditions as the other budding reactions but without adding COPII proteins and addition of apyrase to eliminate nucleoside triphosphates and any residual budding coming from the microsomes. This allowed us to obtain the background of proteins and lipids generated during the assay. All budding reactions were scaled up two-fold to generate enough material for western blot and lipidomic analysis. We extracted the lipids following the same procedure. The lipid content obtained in the negative control reactions was subtracted from the lipid content found in COPII vesicle fractions, both generated with the same microsome batch.

The vesicular pellet was not visible to the eye, but was tightly bound to the bottom of the tube after ultracentrifugation. Therefore, the pellet of the fraction called “totals” was used as a reference to assess the solubilization of vesicular pellets. Each sample was resuspended in 35 μL of 5x Laemml sample buffer [51], then boiled for 3 min at 95°C and finally vigorously vortexed. The whole process was repeated 3 times or until “totals” sample was fully dissolved. Then, 5 μL of each sample were used to analyze the vesicular markers by SDS-PAGE at 10 or 12.5% acrylamide concentration. We used rabbit antibodies against COPII markers and ER resident proteins. The analysis of several of these proteins in the same western blot allowed us to judge the efficiency of the vesicle formation assay by comparison between negative and positive control reactions and in comparison with the fraction called “totals,” equivalent to 5% of the original protein content in the microsomal batch.

Lipid extraction and analysis of cells, microsomes and COPII vesicles

Strain BY4742 was used for all experiments related to in vitro vesicle formation assay. Yeast were grown in YPD (For 1L: glucose 20 g, Bacto peptone 10 g, Bacto yeast extract 20 g, MES hydrate 1.95 g) to ~1.5 OD600/mL and were processed to spheroplasts. Before adding lyticase to the sample, an aliquot of 25 OD (1 OD = 10⁸ cells) was taken and frozen with liquid N2 when lyticase reaction was finished. Microsome pellets (~350 μg in proteins mass) were used for glycerophospholipid analysis. As for vesicles and budding reaction backgrounds, at the end of budding assay the sample was divided in two and one aliquot was used for glycerophospholipid analysis.

MTBE lipid extraction [25, 52] with some modifications was done for all samples. Samples were thawed on ice and resuspended in 100 μL of water. 360 μL of methanol and a mix of internal standards was added (7.5 nmol of 17:0/14:1 PC, 7.5 nmol of 17:0/14:1 PE, 6.0 nmol of 17:0/14:1 PI, 4.0 nmol of 17:0/14:1 PS purchased from Avanti Polar Lipids [Alabaster, AL]). 200 μL of glass beads were added to cell samples. Samples were vortexed and 1 mL of MTBE was added. Samples were placed for 10 min on a disruptor genie at 4°C (Scientific industries, Bohemia, NY). After vortexing, samples were transferred to 2 mL Eppendorf tubes. This was followed by an incubation for 1 hr at room temperature (RT) on a shaker. Phase separation was induced by addition of 200 μL MS-grade water. After 10 min of incubation at RT samples were centrifuged at 1,000 g for 10 min. The upper (organic) phase was transferred into a 13mm glass tube (Corning) with a Teflon-lined cap and the lower phase was re-extracted with 400 μL of a MTBE/MeOH/H₂O mixture (10:3:1.5). Samples were vortexed, incubated for 10 min at RT, and centrifuged for 10 min at 1000 g. The upper phase was collected and the combined organic phases were dried in a CentriVap Vacuum Concentrator (Labconco, MO). The analysis of glycerophospholipids was done as described for the previous extraction (see Yeast sample collection and extraction from yeast cultures).
**COPII reconstitution assays**

**GUV electroformation**

Major-minor mix was done as described in [19, 30] with few modifications. Mixes of 2 mg/mL of lipids were combined by mol percent: 36.5% 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 20% 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 30% soy L-α-phosphatidylinositol (PI), 8% 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), 5% 1,2-dioleoyl-sn-glycero-3-phosphate (DOPA), 0.5% 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) and 0.015% of DSPE-PEG(2000) Biotin were purchased from Avanti Polar Lipids (Alabaster, AL). When GUVs were prepared with lysophospholipids, 10% soy L-α-lysophosphatidylinositol (LysOPI). The mix was dried under nitrogen flow and resuspended in chloroform.

Lipid mixes were deposited (20 µg) on two indium-tin oxide coated (ITO) slides (10 µm each) and dried for 30min at 30 °C in a vacuum chamber. The ITO slides were washed 5 times with 40 µL B88 (469 mOsm). After this, 10 µL of GUVs were pipetted, letting them sit 2 min on the chamber floor. Immediately after, 10 µL of 4 mg/mL casein (Sigma-Aldrich C6905-1G) was added to block GUVs binding. No less than 5 washes with B88 469 mOsm were done. The COPII proteins mix was Sar1-Alexa647 at 0.05 µg/µL, Sec13/31-Alexa488 at 0.09 µg/µL and Sar1 nucleotide exchange. The protein mix was incubated 5 min at room temperature < 23 °C before pipetting it into the microfluidic chamber (ibidi µ-Slide) with GUVs.

Microscopy was performed using a Nikon Eclipse Ti confocal spinning disc microscope with 100x magnification. Images were analyzed with Fiji.

**Bending rigidity measurements**

To measure the effect of LysOPI on membrane bending rigidity, the tube pulling method was employed on giant unilamellar vesicles [53]. The vesicle is held with a micropipette connected to a micromanipulator (MP-285, Sutter Instrument, Novato, CA, USA) and a pressure control system (water reservoir attached to a Zaber linear actuator). The membrane tether is extracted through a streptavidin coated bead (0.05 µm diameter, Spherotech, Lake Forest, IL, USA) held with a custom made optical trap using a 5W laser beam (ML5-CW-P-TKS-OTS, Manlight, Lannion, France) focused through a 100x 1.3NA oil immersion objective. The aspiration pressure DP can be varied and is directly related to the vesicle membrane tension σ.

The aspiration pressure DP can be varied and is directly related to the vesicle membrane tension σ. The force F can be computed through Hooke’s law F = k*Dx where Dx is the displacement of the from its equilibrium position. The aspiration pressure DP can be varied and is directly related to the vesicle membrane tension sigma through the Laplace law, yielding Sigma = ΔP/2*(1/(1/RadiusPipette-1/RadiusVesicle)). The force required to maintain the nanotube is classically linked with the membrane tension sigma (σ) and the membrane bending rigidity κ (κ) through the relation F = 2πκ/(2κπ). The slope of the curves F^2 vs membrane (κ) is given by a way to measure κ.

Kappa measurements have several sources of variation, (salt concentration, pipetting reproducibility, batch vesicles, size, error of radius measurements (pipette/vesicle)), leading to usually wide distributions of κ measurements. To best measure the effect of LysOPI, we performed a relative measurement, by measuring κ on the same vesicle, first without LysOPI, then by incorporated LysOPI using a second pipette that flows a solution with solubilized LysOPI. This reduces many sources of variation and yields the most significant results (identical buffer, bead, pipette, batch of vesicle). Practically, POPC vesicles supplemented with 0.1% of rhodamine PE and 0.015% of DSPE-PEG(2000) Biotin were grown by electroformation for 1H at 1V 10Hz in a sucrose solution adjusted to 240 mOsm. Then an experiment chamber was filled with 0.4 mg/mL casein a solution of NaCl 100mM HEPES 20 mM pH 7.4, beads, and vesicles to an appropriate density for the experiment, and the micropipette holding vesicle is placed on the microscope field of view. The injection pipette was filled with a solution containing NaCl 100mM HEPES 20 mM pH 7.4 and LysOPI (Avanti 850090) solubilized at 120 µM. The flow of the injection pipette was adjusted to perturb minimally the bead (Force of the flow < 0.5 pN). During an experiment the injection pipette was held well above the focal plane, which prevents injection of LysOPI. A first set of pressure steps is applied to the vesicle with a membrane nanotube, yielding a value for κ without lysis injected (green curves Figure 3E). Then the injection pipette is slowly approached toward the observation chamber until a force drop is detected, which is caused by LysOPI insertion.

Proteins and microsomes purification

Lyticase was produced from the bacterial strain RSB805. An overnight culture was diluted to 1/100 in 6 l and incubated at 37 °C. At 0.5 OD600 expression was induced by adding IPTG to 0.4mM. The culture was incubated for 5 hr. Cells were harvested and washed.
with 25mM Tris pH 7.4. Then centrifuged and resuspended with 1/50 of the original volume in 25mM Tris pH 7.4 with EDTA 2mM final concentration. An equal volume of 40% sucrose, 25mM Tris pH 7.4 was added. The mix was mixed gently for no more than 20 min. Cells were harvested in a GSA rotor at 7,500 rpm for 10 min. Afterward the supernatant was aspirated making sure there was no remaining liquid. Cells were resuspended in 1/50 of the original volume in ice cold 0.5 mM MgSO4, mixed gently and incubated 20 min on ice. The mix was centrifuged down in a SS34 rotor at 10,000 rpm for 10 min. The supernatant containing the lyticase was aliquoted in 10 mL aliquots and frozen in liquid nitrogen.

Sar1p was purified from CBB205 transformed with PTY40, a GST-Sar1p fusion expression plasmid. The strain was plated on LB plus ampicillin and grow at 37°C overnight. 200 mL of LB media plus ampicillin were inoculated. At 0.7 OD600/mL, expression was induced by addition of IPTG (0.5mM final). The culture was incubated 3 hr at 37°C. After this, cells were collected by centrifugation and the pellet was frozen at ~80°C. The pellet was resuspended in TBS solution (Tris 50mM, NaCl 150mM, MgCl2 5mM plus 1% Triton x100, at pH 7.4). Afterward, the sample was sonicated 3 times for 3 min and centrifuged at 12,000 xg for 30 min and the supernatant was collected. A bed of 7 mL of glutathione Sepharose beads was added and incubated with rotatory agitation for 1 hr at 4°C. TBS (no detergent) and glutathione 30mM were added at pH 7.4. Fractions of 1 mL were manually collected and analyzed by SDS-PAGE and stained with Coomassie blue. Peak fractions were pooled and 150 μL of PreScission at 1.5 mg/mL were added. The supernatant was dialyzed 2 hr against B88 buffer (HEPES 20mM, sorbitol 250 mM, potassium acetate 150 mM, magnesium acetate 5 mM, pH 6.8) plus GDP 5 μM. A bed of 7 mL of glutathione Sepharose beads was added. The mix was incubated 1 hr at 4°C with rotatory agitation. After this, the supernatant was injected in a BioRad GST-kit column, collecting the flow through. Protein concentration was estimated by BCA and aliquots of 10 μL were frozen in liquid N2 and stored at ~80°C.

Sec23/24 was purified using the yeast strain RSY1069. A yeast pre-culture was grown overnight in SD+Ade+His at 30°C. Yeast were inoculated at 0.005 OD600/mL in 2 × 1 l SD+Ade+His cultures and grown in a fermentor at 30°C with air supply. Cells were collected at 1.65 OD600/mL and collected by centrifugation at 5 krpm, 5 min at 4°C. Pellets were frozen at ~80°C. Approximately 40 gr of yeast were obtained. Cells were pulverized using a Retsch MixerMill 400, cooled with liquid nitrogen. The powder was re-suspended in 100 mL lysis buffer (HEPES KOH 50mM, potassium acetate 750mM, EGTA 0.1 mM, PMSF 0.5 mM, β-mercaptoethanol 1.4mM, pH 7) with protease inhibitor cocktail. Then it was centrifuged at 40,000 xg for 1 hr. After centrifugation, the lipid top layer was removed by aspiration. The supernatant was loaded onto a Hitrap Iacm 1 mL column, then washed with MES 50mM, Potassium Acetate 750mM, EGTA 0.1 mM, imidazole 40mM, glycerol 10% and β-mercaptoethanol 1.4 mM (pH 7), then washed with HEPES 50mM, potassium acetate 500mM, EGTA 0.1 mM, imidazole 40mM, glycerol 10% and β-mercaptoethanol 1.4 mM (pH 7). After a wash with HEPES 50mM, potassium acetate 500mM, EGTA 0.1 mM, imidazole 100mM, glycerol 10% and β-mercaptoethanol 1.4 mM (pH 7) proteins were eluted with HEPES 50mM, potassium acetate 500mM, EGTA 0.1 mM, imidazole 400mM, glycerol 10% and β-mercaptoethanol 1.4 mM (pH 7). Fractions of 1 mL were collected and analyzed by SDS-PAGE to determine the fractions containing proteins. The fractions were combined and dialyzed overnight in HEPES 50 mM, potassium acetate 500mM, EGTA 0.1 mM, imidazole 100mM, glycerol 10% and β-mercaptoethanol 1.4mM (pH 7). The product of dialysis was loaded onto an ion-exchange (HiTrap DEAE) 1 mL column. The loading buffer was HEPES 50mM, potassium acetate 500mM, EGTA 0.1 mM, imidazole 50mM and glycerol 10% (pH 7). The elution buffer was HEPES 50mM, potassium acetate 1M, EGTA 0.1 mM, imidazole 50mM and glycerol 10% (pH 7). The elution was achieved with 80% elution buffer 20% injection buffer. Fractions of 1 mL were collected. SDS-PAGE analysis was used to determine the fractions containing Sec23/24 complex. Peak fractions were pooled and dialyzed against HEPES 50mM, Potassium acetate 800mM and magnesium acetate 5mM (pH 7.4).

Sec13/31 was purified from CBY120. The strain was cultured in 10 mL SD-Ura at 30°C. About 24 hr later, cells were inoculated into 2L SD-Ura and incubated at 30°C. The following day, the culture was inoculated into 19 L of YPD in a fermentor with air supply to a final OD600 mL of 0.12. The cells were collected the next morning at 2.3 OD600/mL and after centrifugation the pellet (approx. 90 g) was frozen at ~80°C. Cells were pulverized using a Retsch MixerMill 400, cooled with liquid nitrogen after resuspension in cold B88 buffer and centrifuged at 10,000 xg for 10min. After this, the supernatant was further centrifuged at 100,000 xg for 1 hr. The top layer of lipids was removed by aspiration and the rest of the supernatant was loaded onto a histidine trap Iacm column of 5 mL. Two buffers were used, Buffer A (HEPES 20mM, potassium acetate 150mM, pH 7), and Buffer B (HEPES 20mM, potassium acetate 150mM, imidazole 200mM, pH 7). After injection of the column, a wash was done with 92.5% Buffer A and 7.5% Buffer B (15 mM imidazole final). Elution was done with 100% Buffer B. Fractions of 5 mL were collected. After analysis by SDS-PAGE, peak fractions 10 to 12 were combined. The samples were loaded onto a MonoQ column of 5 mL. Buffers used were Buffer A (HEPES 20mM, magnesium acetate 1mM, pH 7) and Buffer B (HEPES 20mM, potassium acetate 1M, magnesium acetate 1mM, pH 7). The column was equilibrated with 70% Buffer A and 30% Buffer B. The elution was achieved by increasing the concentration of Buffer B, 1% per minute during the first 14 min and then 5% per minute during the last 5 min. Final elution was with 100% Fractions of 1 mL were taken. A Coomassie staining of SDS-PAGE was done to find the peak fractions. The preparation turned out to give two different pools, and only the second pool was found to be active for budding assay.

Microsomes, enriched in ER membranes, were obtained from homogenized cells and were used as donor membranes for in vitro reactions or analysis of ER protein and lipid composition [49]. In our studies, the wild-type yeast strain BY4742 was grown in 1L YPD cultures to densities of 1.5 to 1.8 OD600/mL. The cells were collected by centrifugation and treated with lyticase to remove the cell wall. After no more than 30 min of treatment, 85% of spheroplasting efficiency was achieved as measured by light absorbance in a 1:100 dilution into water at 600 nm. The resulting spheroplasts were collected by centrifugation and frozen at ~80°C overnight. Afterward, the pellet of spheroplasts was thawed on ice and resuspended in lysis buffer (HEPES 20mM pH 7.4, EDTA 2mM, sorbitol 0.1M, potassium acetate 50mM, DTT 1mM, PMSF 1mM). The spheroplasts were homogenized using a motorized dounce
homogenizer (9 strokes) and the homogenate was first subjected to a low speed centrifugation (5000 xg, 5 min, 4°C) to remove most of the nuclei, plasma membrane and mitochondria, which were discarded. The supernatant was then centrifuged (27,000 xg, 10 min, 4°C) to collect the microsomes. The pellet was resuspended and microsomes were further purified on a two-step sucrose gradient (bottom layer 1.5M, top layer 1.2M) using ultracentrifugation in a swinging bucket rotor (100,000x g, 1 hr, 4°C). Microsomes were collected at the interphase between the two sucrose phases, discarding other membranes by aspiration. This process usually generated 3.2 mg/mL of proteins as measured by UV light spectrophotometer, which is considered a good working condition for in vitro vesicle formation assay. Microsomes were finally aliquoted and frozen at −80°C.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Several replica experiments were carried out for each experiment. For each experiment the number of replicas is indicated in each correspondent figure legend.

GraphPad Prism 7 was used to determine median, standard deviation, standard error and statistical significance. Statistical significances were determined applying a multiple t test using the Holm-Sidak method, with alpha = 0.05. Computations assume that all rows are samples from populations with the same scatter (SD). * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001.

No randomization/stratification, blinding, sample-size estimation or inclusion/exclusion criteria were done for any experiment.