

Minimal membrane docking requirements revealed by reconstitution of Rab GTPase-dependent membrane fusion from purified components

Christopher Stroupe¹, Christopher M. Hickey¹, Joji Mima, Amy S. Burfeind², and William Wickner³

Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755

This Feature Article is part of a series identified by the Editorial Board as reporting findings of exceptional significance.

Edited by Thomas C. Südhof, Stanford University School of Medicine, Palo Alto, CA, and approved September 16, 2009 (received for review April 7, 2009)

Rab GTPases and their effectors mediate docking, the initial contact of intracellular membranes preceding bilayer fusion. However, it has been unclear whether Rab proteins and effectors are sufficient for intermembrane interactions. We have recently reported reconstituted membrane fusion that requires yeast vacuolar SNAREs, lipids, and the homotypic fusion and vacuole protein sorting (HOPS)/class C Vps complex, an effector and guanine nucleotide exchange factor for the yeast vacuolar Rab GTPase Ypt7p. We now report reconstitution of lysis-free membrane fusion that requires purified GTP-bound Ypt7p, HOPS complex, vacuolar SNAREs, ATP hydrolysis, and the SNARE disassembly catalysts Sec17p and Sec18p. We use this reconstituted system to show that SNAREs and Sec17p/Sec18p, and Ypt7p and the HOPS complex, are required for stable intermembrane interactions and that the three vacuolar Q-SNAREs are sufficient for these interactions.

biochemical reconstitution | Rab effector

Rab GTPases are central regulators of intracellular membrane trafficking (1), mediating vesicle formation (2, 3) and transport (4), and membrane docking (5). Like other small GTPases, Rab proteins cycle between GTP-bound and GDP-bound forms (6); this cycling is regulated by guanine nucleotide exchange factors (GEFs) (7) and GTPase-activating proteins (GAPs) (8). In their GTP-bound forms, Rab GTPases interact with a diverse set of proteins and protein complexes termed effectors (9). Despite the wealth of data regarding the physical interactions in which Rab GTPases participate, Rab function is poorly understood, for want of systems for studying Rab proteins and their effectors in the context of chemically defined membrane fusion.

Membrane fusion reactions have been reconstituted from purified components *in vitro*, including Ca²⁺- and polyethylene glycol-stimulated fusion (10, 11), viral fusion (12), and SNARE-mediated fusion (13). Regulation of SNARE-dependent membrane fusion by the SNARE-interacting protein Sec1/Munc18 (SM) (14, 15) and the SNARE-binding proteins synaptotagmin and complexin (16–25) has also been reconstituted. We have recently reported reconstituted membrane fusion that requires yeast vacuolar SNAREs, regulatory lipids, and the homotypic vacuole fusion and protein sorting (HOPS)/class C vacuole protein sorting (class C Vps) complex, a six-subunit effector and GEF for the yeast vacuolar Rab GTPase Ypt7p (26–28). The Vps33p subunit of the HOPS complex is a SM protein (27), whereas the Vps39p subunit contains the Ypt7p GEF activity (26).

We now report reconstitution of membrane fusion that requires purified Ypt7p, HOPS complex, vacuolar SNAREs (Vam3p, Vti1p, Vam7p, and Nyv1p), ATP hydrolysis, and the SNARE chaperones Sec17p and Sec18p [the yeast homologs of soluble *N*-ethylmaleimide sensitive factor attachment proteins (SNAPs) and *N*-ethylmaleimide sensitive factor (NSF), respectively]. Membrane fusion is not accompanied by lysis, and only Ypt7p that is in its GTP-bound state can support fusion.

Previous studies of vacuole fusion have used assays of content mixing (29, 30). In this study, we use an assay of proteoliposome lipid mixing under conditions in which the proteoliposome membranes remain intact, shielding lumenally oriented lipids from external aqueous probes. This fusion also preserves the curvature of the initial liposome population, an independent measure of lysis-free fusion. The lipid mixing that we measure therefore represents authentic membrane fusion. Biochemical reconstitution of Rab5-dependent membrane fusion using purified components has also recently been reported (31).

We have used our *in vitro* system to investigate the molecular mechanisms of membrane docking, a key intermediate in membrane fusion. Docking is the close association of two membranes before fusion and has been proposed to consist of two steps: tethering, a reversible, Rab GTPase-dependent and SNARE association-independent association, followed by assembly of membrane-bridging “transSNARE” complexes (32–34). Both Ypt7p and the HOPS complex are required for vacuole docking *in vitro* (5, 35), and in general Rab GTPases and their effectors play a key role in bringing membranes in proximity before fusion (9). The HOPS complex might link apposed membranes, because it has a myriad of binding partners in addition to Ypt7p: SNARE complexes (36), the SNAREs Vam3p (37) and Vam7p (35) in their monomeric states, and phosphoinositides (35). However, it remains unclear whether Ypt7p–HOPS complex interactions or, more broadly, Rab–effector interactions mediate tethering by forming a direct physical link between membranes. We show in this study that Ypt7p and the HOPS complex are required for clustering of reconstituted proteoliposomes, but we also find that they are insufficient for this clustering; Sec17p, Sec18p, and vacuolar SNAREs are required as well, and the three vacuolar Q-SNAREs (38) are sufficient.

Results

We have recently reported proteoliposome fusion that requires SNAREs and the HOPS complex, but not Ypt7p (28). These proteoliposomes were made using the “standard” method, in which dried lipids are dissolved in a detergent solution of proteins, followed by removal of detergent (39). We reasoned that a different method of proteoliposome preparation might result in proteoliposomes that exhibit the physiological require-

Author contributions: C.S., C.M.H., J.M., A.S.B., and W.W. designed research; C.S., C.M.H., J.M., and A.S.B. performed research; C.S., C.M.H., J.M., and A.S.B. contributed new reagents/analytic tools; C.S. and W.W. analyzed data; and C.S. and W.W. wrote the paper.

The authors declare no conflict of interest.

¹C.S. and C.M.H. contributed equally to this work.

²Present address: Institute for Protein Research, Osaka University, Suita, Osaka, Japan.

³To whom correspondence should be addressed. E-mail: william.wickner@dartmouth.edu.

This article is a PNAS Direct Submission.

This article contains supporting information online at www.pnas.org/cgi/content/full/0903801106/DCSupplemental.

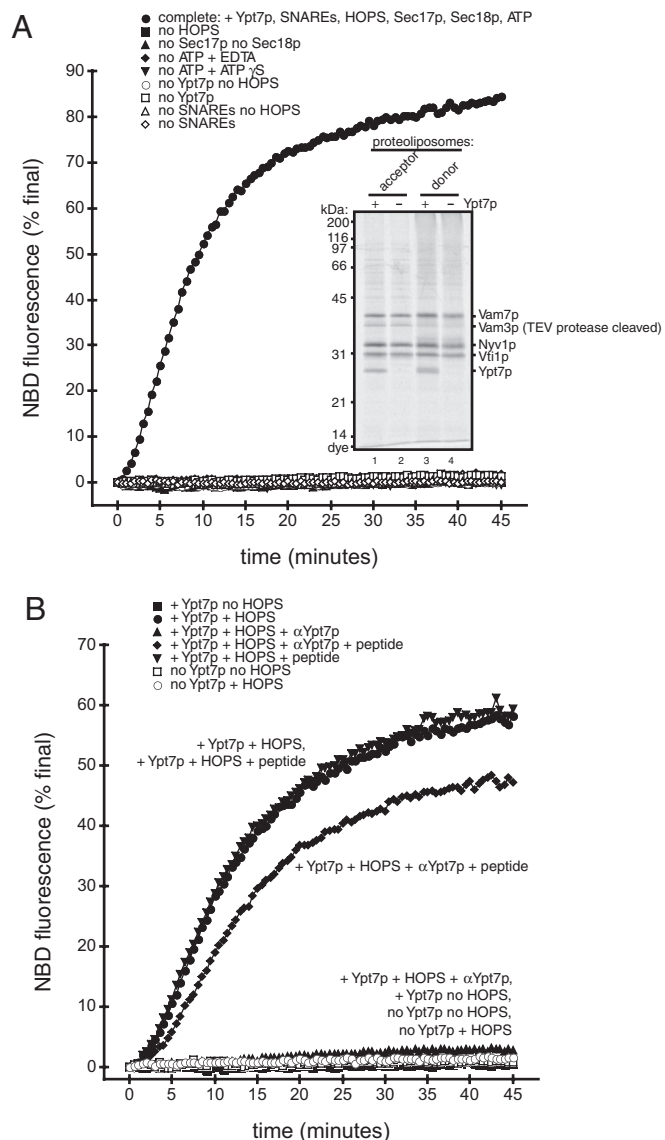


Fig. 1. Ypt7p-dependent lipid mixing. (A) Lipid mixing of direct-method proteoliposomes requires Ypt7p, HOPS, Sec17p/Sec18p, ATP hydrolysis, and SNAREs. Fusion reactions (see *Methods*) used proteoliposomes of indicated composition and lacked the indicated soluble factors; any omitted components were replaced by their buffers. (*Inset*) Acceptor and donor proteoliposomes with SNAREs, with or without Ypt7p, were analyzed by SDS/PAGE and Sypro Ruby staining (5 nmol of total lipids per lane). (B) Anti-Ypt7p antibodies block lipid mixing. Anti-Ypt7p (1.2 M final; \blacktriangle), a mixture of anti-Ypt7p peptide (12 μ M final; \blacklozenge), Ypt7p peptide alone (12 M final; \blacktriangledown), or RB150 (all others) were preincubated with the indicated proteoliposomes for 10 min at 27 °C. MgCl₂, ATP, Sec17p, Sec18p, and HOPS complex or HOPS buffer, as indicated, were then added and reactions were carried out as described in *Methods*.

ment for Ypt7p for membrane fusion. We therefore made proteoliposomes by using “direct” addition, incubation of protein/detergent solutions with preformed protein-free liposomes at a low detergent concentration, followed by detergent removal (39), to incorporate Ypt7p (40) and the vacuolar SNARE proteins Vam3p, Vti1p, Vam7p, and Nyv1p into liposomes that had been extruded from lipid mixtures similar in composition to vacuole membranes (see *Methods*). We also made proteoliposomes bearing SNAREs but lacking Ypt7p. The presence or absence of Ypt7p has no effect on the efficiency of incorporation of SNAREs (Fig. 1*A Inset*). The resulting proteoliposomes are

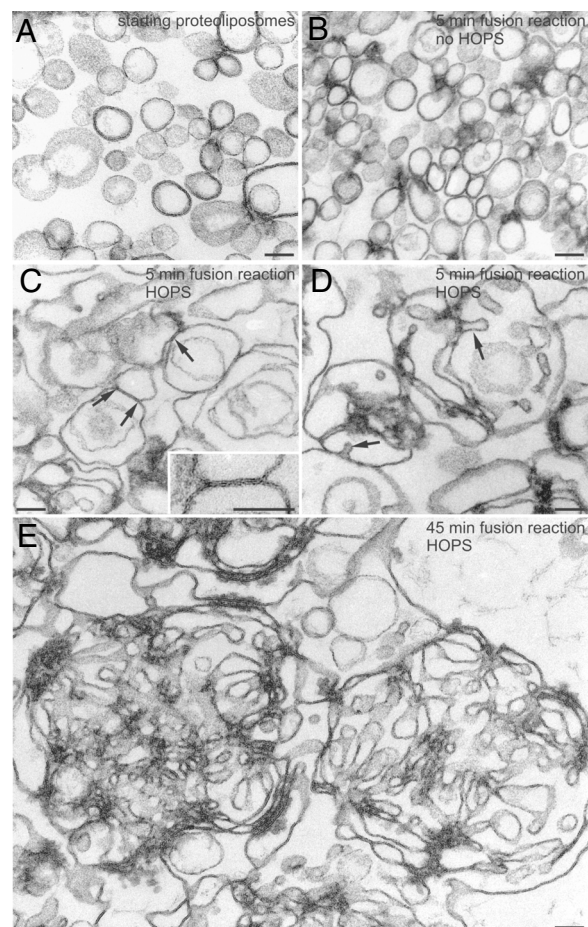


Fig. 2. Electron microscopy analysis of proteoliposomes and fusion reactions. Donor proteoliposomes or donor-only fusion reactions were prepared as described. After 5 or 45 min at 27 °C for the reactions, and without incubation for the proteoliposomes alone, glutaraldehyde was added to a final concentration of 0.1% from a 2% stock in 0.1 M sodium phosphate, pH 7.3. Reactions were incubated at room temperature for 30 min, then centrifuged for 15 min at 14,000 rpm in an Eppendorf (Hamburg, Germany) 5415C microcentrifuge at 4 °C. Pellets were covered with 450 μ L of 1% low melting point agarose, then processed for transmission electron microscopy as described (79). (A) Starting proteoliposomes not incubated under fusion conditions (mean size = 114 \pm 3 nm). (B) Proteoliposomes incubated for 5 min in a fusion reaction without HOPS complex. (C and D) Proteoliposomes incubated for 5 min in a fusion reaction with HOPS. Liposomes are larger and pleomorphic, possibly as a result of incipient fusion. In C, the arrows point to regions where juxtaposed liposomes have established a close contact. The area delimited by two arrows is shown at higher magnification in the *Inset*. Note that the external leaflets of the proteoliposome membranes in this region have apparently merged into a single osmiophilic line resulting in the formation of a pentalaminar structure suggestive of a fusion event. (D) Large liposomes are frequently endowed with membrane infoldings, resulting in the formation of tubular structures (arrows). (E) Proteoliposomes incubated for 45 min in the fusion reaction with HOPS. Fused liposomes have generated tangled skeins of tubular membranes. (Scale bars, 100 nm.)

114 \pm 3 nm in diameter, as estimated by thin-section electron microscopy, and most are unilamellar (Fig. 2*A*).

To assay membrane fusion, each combination of proteins was incorporated into two types of liposomes: “donor” liposomes, which contain quenching concentrations (1.5 mole percent each) of rhodamine- and nitrobenzoxadiazole (NBD)-conjugated phosphatidylethanolamines, and “acceptor” liposomes lacking these fluorophores (41). In donor liposomes, NBD fluorescence is quenched by Förster resonance energy transfer (FRET) to rhodamine. Upon fusion of donor and acceptor liposomes, the

surface concentration of NBD- and rhodamine-conjugated lipids is reduced, FRET is abrogated, and NBD fluorescence increases (41).

Fusion of the membranes of these proteoliposomes requires Ypt7p and SNAREs, purified HOPS complex, and the SNARE chaperones Sec17p and Sec18p (Fig. 1A and Fig. S1). ATP hydrolysis is also required, because EDTA or the poorly hydrolyzable ATP analog ATP γ S blocks fusion. Neither ATP γ S nor EDTA inhibits HOPS function. HOPS-stimulated fusion of standard-method proteoliposomes bearing only Nyv1p with proteoliposomes bearing Vam3p, Vam7p, and Vti1p, which does not require ATP or Sec17p/Sec18p (28), is not inhibited by ATP γ S or EDTA (Fig. S2). As an additional test of the requirement for Ypt7p for fusion, an anti-Ypt7p antibody (42) reduces fusion nearly to background levels (Fig. 1B). Preincubation of this antibody with the peptide against which it had been raised (42) relieves inhibition, whereas the peptide has no stimulatory activity (Fig. 1B); thus, antibody inhibition is specific for Ypt7p.

Lysis of vacuolar membranes has been observed when vacuolar SNAREs are overexpressed (43). Neuronal SNAREs can also cause proteoliposome lysis at high concentrations (44). We therefore used electron microscopy to examine whether lipid mixing (Fig. 1A) is accompanied by an increase in proteoliposome size. We observed little change in size when Ypt7p- and SNARE-bearing proteoliposomes were incubated under fusion conditions but without HOPS complex (Fig. 2B). However, HOPS induces a marked increase in proteoliposome size within only 5 min (Fig. 2C), accompanied by invaginations of the larger proteoliposomes (Fig. 2D). We also observed pentalaminar structures that could be fusion intermediates (Fig. 2C *Inset*). After 45 min of incubation with HOPS, the large proteoliposomes are extensively folded (Fig. 2E and Fig. S3). These invaginations and folds are consistent with a fusion reaction that preserves the high curvature of the small starting proteoliposomes (Fig. 2A and B), that is, fusion without lysis.

We also used sodium dithionite to test whether Ypt7p-dependent proteoliposome lipid mixing is caused by lysis followed by coalescence of membrane fragments (28). This reducing agent destroys only accessible NBD fluorescence because it crosses intact lipid bilayers very slowly (45). In aqueous solution, the reducing activity of sodium dithionite decays fully within 30 min (28). Sodium dithionite decreases the fluorescence of a mixture of donor and acceptor proteoliposomes (Fig. 3). If this mixture is incubated at 27 °C until the sodium dithionite has decayed, and then HOPS complex, Sec17p, Sec18p, and ATP are added to trigger lipid mixing (Fig. 3), NBD fluorescence increases at the same rate as in a reaction containing fresh sodium dithionite (Fig. 3). Thus, lipid mixing does not cause proteoliposome lysis and access of sodium dithionite to the NBD on the interior leaflet of the proteoliposome membranes. This assay therefore demonstrates authentic fusion of the inner membrane monolayer without interruption of the membrane permeability barrier.

We next investigated whether the nucleotide-binding state of Ypt7p regulates membrane fusion. Added GTP is not necessary for proteoliposome fusion (Fig. 1A), nor for fusion of purified vacuoles (46). Is purified Ypt7p already in its GTP-bound form, and is this GTP-bound Ypt7p required for proteoliposome fusion? To test these questions, we used Gyp1–46 (47), a catalytic fragment of a GAP that stimulates GTP hydrolysis by Ypt7p (8). Ypt7p- and HOPS complex-dependent fusion (Fig. 4) is inhibited by Gyp1–46, suggesting that functional Ypt7p is GTP-bound. To show that this inhibition is caused by modulation of Ypt7p-bound nucleotide we used GTP γ S, a slowly hydrolyzable GTP analog that prevents inhibition of vacuole fusion by Gyp1–46 (46) but does not block fusion (30). GTP γ S relieves inhibition of proteoliposome fusion by Gyp1–46 but has little effect on fusion in the absence of Gyp1–46 (Fig. 4). As a

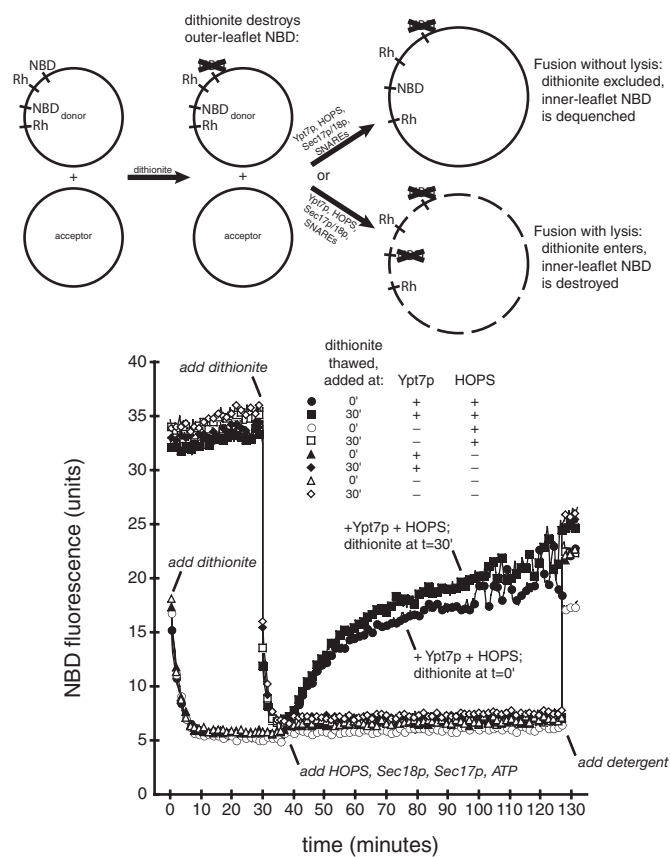


Fig. 3. Lipid mixing is not accompanied by lysis. Fusion reactions (see *Methods*) used proteoliposomes with SNAREs, with Ypt7p (filled symbols) or without Ypt7p (open symbols). Sodium dithionite (40 mM; Sigma) was prepared by addition of solid sodium dithionite to ice-cold RB150+, frozen immediately in aliquots, stored at -80°C , and thawed just before use. At $t = 0$, one set of proteoliposomes in RB150+ (13.2 μL ; circles and triangles) received freshly thawed sodium dithionite (2 μL) and was incubated at 27 °C. A second set of proteoliposomes (13.2 μL ; squares and diamonds) was incubated at 27 °C without sodium dithionite. At $t = 30$ min, freshly thawed sodium dithionite (2 μL) was added, at room temperature, to this second set of proteoliposomes, and reactions were returned to 27 °C. At $t = 37$ min, MgCl_2 , ATP, Sec17p, Sec18p, and HOPS complex (circles and squares) or HOPS buffer (triangles and diamonds) were added, in a total volume of 4.8 μL , at room temperature. Reactions were incubated at 27 °C for 60 min, followed by addition of 2 μL of 1% Thesit and 5-min incubation at 27 °C. Raw fluorescence units are shown.

control for nonspecific effects of GTP γ S we added UTP γ S, which does not relieve inhibition by Gyp1–46 (Fig. 4). Thus, GTP-bound Ypt7p is required for proteoliposome fusion.

Requirements for Intermembrane Interactions. Ypt7p and the HOPS complex are required for vacuole docking (5, 35), but it is unclear whether they suffice. We therefore used microscopy to find the minimal set of factors required for clustering of proteoliposomes. Fusion reactions using proteoliposomes with or without Ypt7p, and with or without SNAREs, each in the presence or absence of HOPS complex, were imaged and the area occupied by each cluster of proteoliposomes in a field was measured with ImageJ (National Institutes of Health, Bethesda). Cumulative distribution plots for each treatment are shown in Fig. 5A, and representative images are shown in Fig. S4A; histogram analysis of selected distributions is presented in Fig. S4B. Individual proteoliposomes are too small to allow measurement of their size by this method. It is likely that some fusion occurs in the clustering assay using proteoliposomes bearing

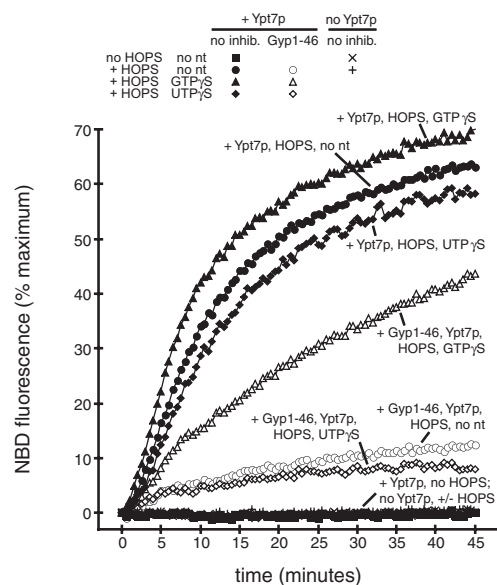


Fig. 4. Lipid mixing requires GTP-bound Ypt7p. Fusion reactions (see *Methods*) used proteoliposomes with SNAREs, with or without Ypt7p as indicated. During the preincubation, reactions received RB150 (squares and circles), GTP γ S (100 μ M final; triangles), or UTP γ S (100 μ M final; diamonds), and RB150 (filled symbols) or Gyp1-46 (2 μ M final; open symbols). The symbols for reactions without Ypt7p are behind the filled squares and show no detectable increase in NBD fluorescence.

SNAREs. However, fusion cannot occur without clustering (see below). Thus, if an increase in cluster size is detected, then clustering must have taken place, regardless of whether the cluster consists of small unfused proteoliposomes, larger fused proteoliposomes, or a combination of the two.

In the presence of ATP, Sec17p, and Sec18p, proteoliposomes containing SNAREs form large clusters in a Ypt7p- and HOPS complex-dependent manner (Fig. 5A). Clustering of proteoliposomes without SNAREs, however, is not stimulated by Ypt7p and HOPS complex (Fig. 5A). HOPS, along with Ypt7p and the SNAREs, also induces a significant increase in the distribution of mean fluorescence intensity in proteoliposome clusters (Fig. S4C), indicative of an increase in the number of proteoliposomes in these clusters. Thus, Ypt7p and the HOPS complex are insufficient for stable membrane-membrane interactions under the conditions of our assay; SNAREs are also required. In several cases, distributions of cluster areas for reactions using SNARE-free proteoliposomes are significantly different from distributions for reactions using SNARE-containing proteoliposomes without Ypt7p and/or HOPS complex (Fig. S4D). However, the median cluster sizes in these cases differ only slightly (14–33%), in contrast with the large differences (825–1,380%) in median size between reactions using SNARE- and Ypt7p-bearing proteoliposomes with HOPS complex and all other reactions (Fig. S4D).

Do these large clusters represent an on-pathway intermediate of membrane fusion? The lipid-mixing assay (Fig. 1A) shows that most of the proteoliposomes in a reaction undergo fusion. At the same time, the clustering assay (Fig. 5A) shows that most of the proteoliposomes in a reaction enter into larger clusters: the size distribution for the “complete” reaction diverges from the distributions for the reactions lacking Ypt7p and/or HOPS at roughly the 10th percentile. The small clusters that make up only 10% of the clusters in the complete reaction cannot account for the extent of lipid mixing shown in Fig. 1A. Thus, the proteoliposomes in the larger clusters must fuse to generate such a large extent of lipid mixing. The physiological nature of the clustering

reaction is also strongly supported by the fact that it requires SNAREs, a Rab GTPase, and the HOPS complex, which are required for fusion *in vivo* and on isolated vacuoles (27, 48, 49).

How do the SNARE proteins mediate proteoliposome clustering? SNAREs can form “*trans*” complexes that bridge the space between membranes (34). Because SNARE-dependent proteoliposome fusion requires Sec17p and ATP hydrolysis by Sec18p (Fig. 1A), the SNAREs are likely to be in “*cis*” complexes, residing in the same membrane, at the beginning of a fusion reaction. Disassembly of these complexes by Sec17p and Sec18p (50) would be required for the formation of *cis* complexes containing only the three vacuolar Q-SNAREs (38) or of transSNARE complexes containing three Q-SNAREs and one R-SNARE (38, 51). We therefore performed clustering assays in the absence of Sec17p and Sec18p. Without these factors, Ypt7p-containing proteoliposomes do not exhibit HOPS complex-dependent clustering (Fig. S4E). Replacement of ATP with ATP γ S, to block ATP hydrolysis by Sec18p, has the same effect (Fig. S4F).

To test whether “3Q” *cis* complexes or “3Q:1R” *trans* complexes mediate clustering, we made proteoliposomes bearing only the three vacuolar Q-SNAREs, Vam3p, Vti1p, and Vam7p (38). When Ypt7p is also present in these proteoliposomes, the HOPS complex induces a large increase in cluster size (Fig. 5B). No increase in size is induced by the HOPS complex when Ypt7p is not present (Fig. 5B). Therefore, the three vacuolar Q-SNAREs are sufficient for Ypt7p- and HOPS-dependent intermembrane interactions in the absence of membrane fusion (Fig. 5B *Inset*).

We next asked whether SNAREs promote proteoliposome clustering via recruitment of the HOPS complex to membranes by measuring HOPS binding to the direct-method proteoliposomes used in the clustering analysis. These proteoliposomes exhibit Ypt7p-dependent HOPS complex binding (Fig. 6, bars 1 and 2) that is stimulated by SNAREs (Fig. 6, bars 1–4). Without Sec17p and Sec18p, however, HOPS still binds efficiently to proteoliposomes (Fig. 6, bars 1 and 5), although clustering is abrogated in the absence of Sec17p and Sec18p (Fig. S4E). Thus, HOPS-proteoliposome binding is insufficient for proteoliposome clustering. We therefore conclude that the Ypt7p and the HOPS complex act together with a 3Q *cis*-SNARE complex of Vam3p, Vti1p, and Vam7p to mediate stable intermembrane interactions under the conditions of our assay.

Basis of Ypt7p Requirement for Fusion. We next turned to the question of why the direct-method proteoliposomes presented here show the physiological requirement for Ypt7p for fusion whereas standard-method proteoliposomes do not (28). We found that differences in SNARE levels, or lipidic contaminants, have no measurable effect on Ypt7p dependence, whereas differences in cardiolipin levels and in the ability of proteoliposomes to bind HOPS complex are major factors in their dependence on Ypt7p for fusion.

SNARE levels or lipidic contaminants do not impact the extent of Ypt7p dependence for proteoliposome fusion. Direct-method proteoliposomes have lower levels of Vam3p than the other SNAREs (Fig. 1A *Inset*). However, standard-method proteoliposomes made with 25% of the usual level of Vam3p, which do not fuse efficiently, do not show enhanced dependence on Ypt7p for fusion (Fig. S5A). We therefore compared the lipid composition of standard-method and direct-method proteoliposomes by using mass spectrometry (Fig. S5B and C). The only major lipid present in the standard-method proteoliposome analysis but not in the direct-method analysis (Fig. S5B) was identified as erucylamide, a fatty acid amide used in the manufacture of plastic films (52). However, erucylamide does not induce fusion of direct-method proteoliposomes lacking Ypt7p (Fig. S5D).

By contrast, both cardiolipin levels and HOPS complex-binding activity contribute to the extent of Ypt7p dependence for fusion. Cardiolipin levels are lower in direct-method proteoli-

dependent manner (Fig. 6, bars 1 and 2); this binding is stimulated by SNAREs (Fig. 6, bars 1–4) but is unaffected by Sec17p and Sec18p (Fig. 6, bar 5). In contrast, standard-method proteoliposomes bind HOPS complex even in the absence of Ypt7p (Fig. 6, bars 7, 8, and 10), although HOPS binding to standard-method proteoliposomes is stimulated by Ypt7p (Fig. 6, bars 6 and 9). Standard-method liposomes without Ypt7p or SNAREs also bind HOPS complex (Fig. 6, bar 8), demonstrating a direct interaction between HOPS and liposome membranes. Standard-method proteoliposomes made with lowered cardiolipin levels bind HOPS to nearly the same extent as standard-method proteoliposomes made with normal amounts of cardiolipin, both with and without Ypt7p (Fig. 6, bars 6, 7, 9, and 10); thus, cardiolipin exerts its effect by modulating the propensity of membranes to fuse, not by altering HOPS complex-proteoliposome binding.

We conclude that the difference in requirement for Ypt7p for fusion between standard-method and direct-method proteoliposomes has two bases: the difference in requirement for Ypt7p for HOPS complex recruitment to membranes and the difference in cardiolipin content between standard-method and direct-method proteoliposomes.

Discussion

Reconstituted proteoliposomes can be prepared with more rigorous control of lipid and protein composition than purified organelles, which rely on intracellular transport for delivery of their constituents. Deletion or mutation of genes encoding trafficking proteins can affect delivery of other factors, complicating the interpretation of experiments using purified organelles derived from mutant sources. Reconstitution of Ypt7p-dependent membrane fusion and clustering therefore provides a chemically defined system for functionally dissecting the molecular interactions underlying docking.

Docking has been proposed to take place in two stages: tethering, a Rab GTPase-dependent, SNARE-independent, and reversible association, followed by trans-SNARE interactions (32, 34). Tethering may be mediated by “tethering factors” that interact simultaneously with binding partners, including Rab proteins, in apposed membranes (9, 54–57). However, no proposed Rab-dependent tethering factor has ever, to our knowledge, been shown to have direct membrane-bridging activity in a chemically defined membrane tethering reaction. In this study, proteoliposomes bearing Ypt7p alone cannot cluster in the presence of HOPS complex; SNAREs and Sec17p/Sec18p are also required, and the three vacuolar Q-SNAREs Vam3p, Vti1p, and Vam7p are sufficient), for intermembrane interactions (Fig. 5). We conclude that Ypt7p and the HOPS complex are insufficient for stable membrane-membrane associations in our assay, and that a Q-SNARE complex acts together with Ypt7p and the HOPS complex to bring membranes into proximity before fusion.

Many studies have suggested a role for SNARE proteins in pre-fusion membrane association. Antibodies against Sec18p and removal of ATP prevent vacuole docking; both of these treatments inhibit *cis*-SNARE complex disassembly (5, 50) and would be expected to block formation of 3Q *cis*-SNARE or 3Q:1R trans-SNARE complexes. In an *in vitro* assay for formation of the vesicular tubular cluster, an intermediate in transport from the endoplasmic reticulum (ER) to the Golgi apparatus, antibodies against Syntaxin 5, a homolog of Vam3p, inhibit vesicle “coisolation,” as does addition of a dominant negative mutant of α -SNAP that blocks NSF SNARE complex disassembly activity (58, 59). Docking of synaptic vesicles to the plasma membrane in *Caenorhabditis elegans* neuromuscular junctions also requires syntaxin (60). SNARE-dependent (21, 61) and syntaxin-, synaptotagmin I-, phosphatidylserine-, and Ca^{2+} -dependent (19) proteoliposome clustering have also been reported, although in both cases this clustering was independent of Rab GTPases and effectors.

Other studies, however, have suggested that SNAREs are not required for intermembrane associations. Vacuoles lacking Vam3p are able to dock, and this docking is not inhibited by antibodies against Nyv1p (33). Also, vacuoles lacking Nyv1p can dock (62). However, these results do not preclude the action of other SNAREs in docking: Pep12p, a homolog of Vam3p, and Ykt6p, a homolog of Nyv1p, both can enter vacuolar SNARE complexes (36, 63). Other studies of the involvement of SNAREs in docking have used intact organelles (32, 64), which are likely to contain multiple sets of SNAREs that could also form “noncanonical” SNARE complexes (65) that mediate docking but not membrane fusion. Moreover, temperature-sensitive SNARE mutants that permit docking of ER-derived vesicles after incubation at a restrictive temperature that blocks membrane fusion (32) may be defective for fusion, but not for docking, at restrictive temperatures. Finally, a golgin protein acting in consort with an Arf GTPase (66), and several synaptotagmin isoforms (67, 68), can induce SNARE-independent, but also Rab- and Rab effector-independent, liposome clustering.

We have proposed that Ypt7p and the HOPS complex do not form a direct, stable bridge between membranes, but rather mediate docking by acting in consort with a 3Q complex consisting of Vam3p, Vti1p, and Vam7p. We cannot, however, eliminate the possibility that HOPS and Ypt7p mediate a transient intermembrane interaction that is too labile to be detected but that is an obligate intermediate before stable docking mediated by HOPS, Ypt7p, and a 3Q SNARE complex. Nor can we rule out the possibility that other vacuolar proteins and lipids, not included in this reconstitution, contribute to SNARE-independent membrane associations, although no other vacuolar tethering factors have yet been reported. Finally, care should be taken when interpreting results obtained with proteoliposomes that are smaller than vacuoles. However, the region of contact between docked vacuoles, the vertex ring (69), is a region of high curvature, and thus the proteoliposomes used in this study are likely to be an appropriate model for this site of intermembrane contact.

We have found that differences in cardiolipin content and HOPS complex binding activity underlie the difference in Ypt7p dependence for fusion of standard-method and direct-method proteoliposomes. Our standard-method proteoliposomes have higher cardiolipin content than our direct-method proteoliposomes (Fig. S5C). Cardiolipin stimulates Ypt7p-independent fusion of standard-method proteoliposomes (Fig. S5E), consistent with the finding that Mg^{2+} -cardiolipin can mediate liposome fusion without leakage (70). Does cardiolipin play a role in vacuole fusion *in vivo*? Cardiolipin is synthesized exclusively in the inner membrane of mitochondria (71); its reported presence in vacuoles in lipid analysis of purified organelles (72) is therefore likely caused by mitochondrial contamination. Furthermore, vacuoles from yeast lacking cardiolipin synthase have normal morphology at 30 °C (73). At 37 °C, cardiolipin-deficient yeast have abnormal vacuole morphology, but this defect is suppressed by deletion of the gene encoding the sodium/proton exchanger Nhx1p or the gene encoding the mitochondrial signaling protein Rtg2p (73). Thus, cardiolipin is unlikely to be involved in vacuole fusion.

Differences in HOPS complex binding and fusion requirements between direct-method and standard-method proteoliposomes provide an opportunity to define Rab GTPase function. Ypt7p is required for HOPS binding to direct-method proteoliposomes, whereas standard-method proteoliposomes bind HOPS robustly even in the absence of Ypt7p (Fig. 6). Furthermore, direct-method proteoliposomes require Ypt7p for fusion (Fig. 1A) whereas standard-method proteoliposomes do not (28). SNAREs are also not required for HOPS binding to standard-method liposomes (Fig. 6). Thus, the HOPS complex binds standard-method proteoliposomes via direct interactions with the membrane. These interactions may be mediated by phosphoinositides, which bind the HOPS complex (35), or the interaction of highly curved membranes with the ArfGAP1 lipid

packing sensor motif in residues 356–379 of the Vps41p subunit of the HOPS complex (74). Although more work will be required to learn the molecular basis for the difference in requirements for HOPS complex binding to direct-method and standard-method proteoliposomes, GTP-bound Ypt7p is required for HOPS complex association with the vacuole (46); thus, this requirement for direct-method proteoliposomes reflects a central physiological function of Ypt7p. We have recently found that that phosphorylation of the Vps41p subunit of the HOPS complex by the casein kinase I homolog Yck3p (75) abrogates HOPS-membrane interactions and causes Ypt7p dependence for fusion of standard-method proteoliposomes (40). This result is consistent with the finding, both by Mima et al. (28) and shown here, that Ypt7p-independent HOPS-proteoliposome interactions (Fig. 6) can support Ypt7p-independent membrane fusion (Fig. S5E). These results demonstrate that the primary function of Ypt7p is recruitment of the HOPS complex to membranes.

The studies presented here suggest a working model for vacuole tethering. *Cis* 3Q:1R SNARE complexes are disassembled by Sec17p/18p, allowing the assembly of *cis* 3Q SNARE complexes. The HOPS complex associates with membranes via its direct affinities for SNAREs, Ypt7p:GTP, and vacuolar lipids, but is optimally activated for tethering by associations with Ypt7p:GTP and the 3Q *cis*-SNARE complex. The vacuolar proteins and lipids that directly interact in *trans* during tethering are not known, but it is likely that tethering is needed for rapid formation of trans-SNARE complexes and subsequent fusion.

Methods

Reagents. His₆-Sec18p (28), his₆-Sec17p (28), and anti-Ypt7p and Ypt7p peptide (42) have been described. Gyp1–46 (47) was the gift of Vincent Starai (University of Georgia, Athens). ATP γ S and GTP γ S were from Roche, and UTP γ S was from Jena Bioscience. Nucleotides (as Mg²⁺ salts), Gyp1–46, GDI (guanosine nucleotide dissociation inhibitor), and anti-Ypt7p were in RB150 [20 mM NaHepes (pH 7.4), 150 mM NaCl, 10% (vol/vol) glycerol], Sec17p and Sec18p were in buffers as described (76, 77), and Ypt7p peptide was in 20 mM Pipes-KOH (pH 6.8) 200 mM sorbitol. Phosphoinositides were from Echelon Research, ergosterol was from Sigma, fluorescent lipids were from Invitrogen, and all other lipids were from Avanti Polar Lipids. Overexpression and purification of HOPS complex are described in *SI Text*. Primers used for plasmid and strain construction are in [Table S1](#).

Direct Incorporation of Proteins into Liposomes. All lipids were dissolved in chloroform except for phosphoinositides, which were dissolved in 1:2:0.8 chloroform/methanol/water. Lipids were mixed in glass tubes at the following mole percentages (28, 72, 78): 43% 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), 18% 1-palmitoyl-2-oleoyl phosphatidylethanolamine (POPE), 18% soy PI, 4.4% 1-palmitoyl-2-oleoyl phosphatidylserine (POPS), 2% 1-palmitoyl-2-oleoyl phosphatidic acid (POPA), 1.6% heart cardiolipin, 8% ergosterol, 1% each PI (3)P, PI(4)P, PI(4,5)P₂, dansyl-phosphatidylethanolamine (PE); for donor lipids, 41% POPC, 18% POPE, 18% soy PI, 4.4% POPS, 2% POPA, 1.6% heart cardiolipin, 8% ergosterol, 1% each PI (3)P, PI(4)P, PI(4,5)P₂, 1.5% each NBD-PE, Rhodamine-PE. Lipids were dried under a stream of N₂ gas followed by vacuum, then suspended to a final concentration of 10 mM in RB150+ (RB150 with 1 mM MgCl₂) by incubation on ice for 1 h with occasional vortexing, followed by 10 freeze–thaw cycles. (Lipids were often stored at –80 °C under N₂ gas after the last freeze.) Lipids were then passed 11 times through a 25-mm diameter, 1- μ m pore filter (Nucleopore Track-Etch Membrane; Whatman) in an ER-1 extruder (Eastern Scientific) at room temperature. Dansyl-PE or NBD-PE and rhodamine-PE fluorescence were used to measure the concentration of extruded lipids ($\lambda_{ex}/\lambda_{em}$ 350/540 and 540/586 nm, respectively).

The molar ratios of proteins to lipids in incorporation reactions were 1:667 (SNAREs) and 1:1,333 (Ypt7p) for acceptor liposomes and 1:1,000 (SNAREs) and 1:2,000 (Ypt7p) for donor liposomes. Before protein incorporation, GST-Vam3p, Nyv1p, and Vti1p (28) were mixed and dialyzed for 4–6 h, using Fisherbrand dialysis tubing with a molecular mass cutoff of 6–8 kDa and a volume/cm of 1.67 mL (Thermo Fisher Scientific), into mock Ypt7p buffer. (Mock Ypt7p buffer and Vam7 buffer were used in place of SNAREs for

SNARE-free liposomes.) This mixture was then supplemented with Vam7p (28), tobacco etch virus (TEV) protease (at a 1:1 molar ratio to GST-Vam3p) and either Ypt7p or mock Ypt7p buffer. Proteins were mixed with extruded liposomes that were diluted with RB150+ such that the incorporation reaction had an R_{eff} , the ratio of the difference between the n-octyl- β -D-glucopyranoside concentration and its critical micelle concentration (CMC) to the lipid concentration (39), of 0.2 for donor liposomes and 0.3 for acceptor liposomes. (The CMC of n-octyl- β -D-glucopyranoside was considered to be 18.5 mM.) The volume of the liposome solution was calculated by using the following formula:

$$V_{liposomes} = \frac{V_{protein} ([\beta OG]_{protein} - CMC) - R_{eff} M_{lipids}}{CMC}$$

where $V_{protein}$ is the volume of the protein solution, $[\beta OG]_{protein}$ is the n-octyl- β -D-glucopyranoside concentration in the protein solution, and M_{lipids} is the number of moles of lipids in the liposome solution. In a typical preparation, 795 μ L of proteins (1.5 nmol of each GST-Vam3p, Vti1p, Nyv1p, Vam7p, and TEV protease; 0.75 nmol of Ypt7p or an equivalent volume of mock Ypt7p buffer; 32.4 mM n-octyl- β -D-glucopyranoside) were mixed with 581.4 μ L of acceptor lipids (1 μ mol). Protein/lipid/detergent mixtures were incubated on ice for 1 h then dialyzed into RB150+ at 4 °C overnight. For small-scale incorporations, Slide-A-Lyzer Mini Dialysis units with a 10-kDa cutoff (Pierce) were used, whereas for large-scale incorporations Fisher dialysis tubing with a cutoff of 6–8 kDa and a volume/cm of 1.67 mL was used.

Dialyzed proteoliposomes were mixed with 80% Histodenz in RB150+ to a final concentration of 35% or 40% Histodenz and covered with 30% Histodenz in RB150+, then by RB150+; volumes and centrifuge tubes depended on the scale of the incorporation reaction. In the example above, the lipid/protein/detergent mixture (1,376.4 μ L) was mixed with 1.1 mL of 80% Histodenz (35.5% Histodenz final) in an 11 \times 60-mm ultracentrifuge tube (Beckman no. 328874); 0.6 mL of 30% Histodenz then 0.6 mL of RB150+ were layered over this mixture. Gradients were centrifuged in a Beckman (Palo Alto, CA) SW-60 rotor at 55,000 rpm at 4 °C for 3 h or in a TLS-55 rotor at 50,000 rpm at 4 °C for 2 h. Proteoliposomes were harvested from the top interface of each gradient and dialyzed against RB150+ overnight at 4 °C. Lipid concentrations were measured as described above. Efficiency of protein incorporation was assessed by SDS/PAGE and Sypro Ruby (Invitrogen) staining; in all cases, the presence or absence of Ypt7p in incorporation reactions made no detectable difference in the efficiency of SNARE incorporation (Fig. 1A *Inset*).

Standard method proteoliposomes were prepared as in Mima et al. (28) except that Ypt7p was added to the initial lipid/detergent/protein solution at a 1:2,000 Ypt7p/lipid molar ratio or the same volume of mock Ypt7p buffer was added to the initial lipid/detergent/protein solution.

Proteoliposome Fusion Reactions. Fusion was performed in 384-well plates (Corning no. 3676) at 27 °C. Complete reactions were in RB150 with: acceptor proteoliposomes, 0.36 mM total lipids; donor proteoliposomes, 0.03 mM total lipids; 1 mM ATP-Mg²⁺; 5 mM free MgCl₂; Sec18p, 150 nM hexamer; 50 nM Sec17p; 34 nM HOPS complex. Proteoliposomes, inhibitors, and inhibitor-reversal agents were mixed in a total volume of 15.2 μ L on ice, then placed at 27 °C for 10 min. Reactions were moved to room temperature and MgCl₂, ATP, Sec18p, Sec17p, and HOPS (or HOPS buffer) were added, premixed in a volume of 4.8 μ L. Reactions were returned to 27 °C and fluorescence ($\lambda_{ex}/\lambda_{em}$ 460/538 nm) was measured for 60 min. Thesit (2 μ L of a 1% solution) was added and fluorescence was measured after 5 min at 27 °C. Dequenching was calculated as described (28). Each graph represents data from one experiment representative of three or more experiments. For representation of fusion data as the normalized sum of dequenching values, each dequenching curve was first adjusted by subtracting the minimum dequenching value for that particular condition from every point in the curve. Adjusted dequenching values for 0–45 min were then added. Each sum was then normalized by dividing it by the average sum of all of the complete dequenching reactions by using proteoliposomes from the same preparation and multiplying by 100. Normalized values were averaged; means and standard deviations are presented in [Fig. S2](#).

ACKNOWLEDGMENTS. We thank the Pole Facultaire de Microscopie Electronique (PFMU) at the University of Geneva Medical School for access to electron microscopy equipment, Vincent Starai for Gyp1–46, and Reza Kordestani and Christian Raetz (Duke University, Durham, NC) for lipid analysis. This work was supported by National Institutes of Health Grant GM23377.

1. Pfeffer SR (2001) Rab GTPases: Specifying and deciphering organelle identity and function. *Trends Cell Biol* 11:487–491.

2. Carroll KS, et al. (2001) Role of Rab9 GTPase in facilitating receptor recruitment by TIP47. *Science* 292:1373–1376.

3. Pagano A, Crottet P, Prescianotto-Baschong C, Spiess M (2004) In vitro formation of recycling vesicles from endosomes requires adaptor protein-1/clathrin and is regulated by rab4 and the connector rabaptin-5. *Mol Biol Cell* 15:4990–5000.
4. Bahadoran P, et al. (2001) Rab27a: A key to melanosome transport in human melanocytes. *J Cell Biol* 152:843–850.
5. Mayer A, Wickner W (1997) Docking of yeast vacuoles is catalyzed by the Ras-like GTPase Ypt7p after symmetric priming by Sec18p (NSF). *J Cell Biol* 136:307–317.
6. Kabcnell AK, Goud B, Northup JK, Novick PJ (1990) Binding and hydrolysis of guanine nucleotides by Sec4p, a yeast protein involved in the regulation of vesicular traffic. *J Biol Chem* 265:9366–9372.
7. Soldati T, Shapiro AD, Svejstrup AB, Pfeffer SR (1994) Membrane targeting of the small GTPase Rab9 is accompanied by nucleotide exchange. *Nature* 369:76–78.
8. Du LL, Collins RN, Novick PJ (1998) Identification of a Sec4p GTPase-activating protein (GAP) as a novel member of a Rab GAP family. *J Biol Chem* 273:3253–3256.
9. Grosshans BL, Ortiz D, Novick P (2006) Rabs and their effectors: Achieving specificity in membrane traffic. *Proc Natl Acad Sci USA* 103:11821–11827.
10. Parente RA, Lentz BR (1986) Rate and extent of poly(ethylene glycol)-induced large vesicle fusion monitored by bilayer and internal contents mixing. *Biochemistry* 25:6678–6688.
11. Papahadjopoulos D, Vail WJ, Pangborn WA, Poste G (1976) Studies on membrane fusion. II. Induction of fusion in pure phospholipid membranes by calcium ions and other divalent metals. *Biochim Biophys Acta* 448:265–283.
12. Harmsen MC, et al. (1985) Reconstitution and fusogenic properties of Sendai virus envelopes. *Eur J Biochem* 149:591–599.
13. Weber T, et al. (1998) SNAREpins: Minimal machinery for membrane fusion. *Cell* 92:759–772.
14. Scott BL, et al. (2004) Sec1p directly stimulates SNARE-mediated membrane fusion in vitro. *J Cell Biol* 167:75–85.
15. Shen J, et al. (2007) Selective activation of cognate SNAREpins by Sec1/Munc18 proteins. *Cell* 128:183–195.
16. Giraud CG, Eng WS, Melia TJ, Rothman JE (2006) A clamping mechanism involved in SNARE-dependent exocytosis. *Science* 313:676–680.
17. Schaub JR, et al. (2006) Hemifusion arrest by complexin is relieved by Ca²⁺-synaptotagmin I. *Nat Struct Mol Biol* 13:748–750.
18. Tang J, et al. (2006) A complexin/synaptotagmin 1 switch controls fast synaptic vesicle exocytosis. *Cell* 126:1175–1187.
19. Bhalla A, Chicka MC, Tucker WC, Chapman ER (2006) Ca²⁺-synaptotagmin directly regulates t-SNARE function during reconstituted membrane fusion. *Nat Struct Mol Biol* 13:323–330.
20. Chicka MC, Hui E, Liu H, Chapman ER (2008) Synaptotagmin arrests the SNARE complex before triggering fast, efficient membrane fusion in response to Ca²⁺. *Nat Struct Mol Biol* 15:827–835.
21. Yoon TY, et al. (2008) Complexin and Ca²⁺ stimulate SNARE-mediated membrane fusion. *Nat Struct Mol Biol* 15:707–713.
22. Malsam J, et al. (2009) The carboxyl-terminal domain of complexin I stimulates liposome fusion. *Proc Natl Acad Sci USA* 106:2001–2006.
23. Maximov A, et al. (2009) Complexin controls the force transfer from SNARE complexes to membranes in fusion. *Science* 323:516–521.
24. Chicka MC, Chapman ER (2009) Concurrent binding of complexin and synaptotagmin to liposome-embedded SNARE complexes. *Biochemistry* 48:657–659.
25. Dai H, Shen N, Arac D, Rizo J (2007) A quaternary SNARE-synaptotagmin-Ca²⁺-phospholipid complex in neurotransmitter release. *J Mol Biol* 367:848–863.
26. Wurmser AE, Sato TK, Emr SD (2000) New component of the vacuolar class C-Vps complex couples nucleotide exchange on the Ypt7 GTPase to SNARE-dependent docking and fusion. *J Cell Biol* 151:551–562.
27. Seals DF, et al. (2000) A Ypt/Rab effector complex containing the Sec1 homolog Vps33p is required for homotypic vacuole fusion. *Proc Natl Acad Sci USA* 97:9402–9407.
28. Mima J, et al. (2008) Reconstituted membrane fusion requires regulatory lipids, SNAREs, and synergistic SNARE chaperones. *EMBO J* 27:2031–2042.
29. Haas A (1995) A quantitative assay to measure homotypic vacuole fusion in vitro. *Methods Cell Sci* 17:283–294.
30. Jun Y, Wickner W (2007) Assays of vacuole fusion resolve the stages of docking, lipid mixing, and content mixing. *Proc Natl Acad Sci USA* 104:13010–13015.
31. Ohya T, et al. (2009) Reconstitution of Rab- and SNARE-dependent membrane fusion by synthetic endosomes. *Nature* 459:1091–1097.
32. Cao X, Ballew N, Barlowe C (1998) Initial docking of ER-derived vesicles requires Uso1p and Ypt1p but is independent of SNARE proteins. *EMBO J* 17:2156–2165.
33. Ungermann C, Sato K, Wickner W (1998) Defining the functions of trans-SNARE pairs. *Nature* 396:543–548.
34. Collins KM, Wickner WT (2007) Trans-SNARE complex assembly and yeast vacuole membrane fusion. *Proc Natl Acad Sci USA* 104:8755–8760.
35. Stroupe C, Collins KM, Fratti RA, Wickner W (2006) Purification of active HOPS complex reveals its affinities for phosphoinositides and the SNARE Vam7p. *EMBO J* 25:1579–1589.
36. Collins KM, Thorngren NL, Fratti RA, Wickner WT (2005) Sec17p and HOPS, in distinct SNARE complexes, mediate SNARE complex disruption or assembly for fusion. *EMBO J* 24:1775–1786.
37. Dulubova I, et al. (1999) A conformational switch in syntaxin during exocytosis: Role of munc18. *EMBO J* 18:4372–4382.
38. Fasshauer D, Sutton RB, Brunger AT, Jahn R (1998) Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. *Proc Natl Acad Sci USA* 95:15781–15786.
39. Rigaud JL, Levy D (2003) Reconstitution of membrane proteins into liposomes. *Methods Enzymol* 372:65–86.
40. Hickey CM, Stroupe C, Wickner W (2009) The major role of the Rab Ypt7p in vacuole fusion is supporting HOPS membrane association. *J Biol Chem* 284:16118–16125.
41. Struck DK, Hoekstra D, Pagano RE (1981) Use of resonance energy transfer to monitor membrane fusion. *Biochemistry* 20:4093–4099.
42. Eitzen G, Thorngren N, Wickner W (2001) Rho1p and Cdc42p act after Ypt7p to regulate vacuole docking. *EMBO J* 20:5650–5656.
43. Starai VJ, Jun Y, Wickner W (2007) Excess vacuolar SNAREs drive lysis and Rab bypass fusion. *Proc Natl Acad Sci USA* 104:13551–13558.
44. Dennison SM, Bowen ME, Brunger AT, Lentz BR (2006) Neuronal SNAREs do not trigger fusion between synthetic membranes but do promote PEG-mediated membrane fusion. *Biophys J* 90:1661–1675.
45. McIntyre JC, Sleight RG (1991) Fluorescence assay for phospholipid membrane asymmetry. *Biochemistry* 30:11819–11827.
46. Eitzen G, et al. (2000) Sequential action of two GTPases to promote vacuole docking and fusion. *EMBO J* 19:6713–6720.
47. Albert S, Will E, Gallwitz D (1999) Identification of the catalytic domains and their functionally critical arginine residues of two yeast GTPase-activating proteins specific for Ypt/Rab transport GTPases. *EMBO J* 18:5216–5225.
48. Wickner W, Haas A (2000) Yeast homotypic vacuole fusion: A window on organelle trafficking mechanisms. *Annu Rev Biochem* 69:247–275.
49. Seelye ES, et al. (2002) Genomic analysis of homotypic vacuole fusion. *Mol Biol Cell* 13:782–794.
50. Ungermann C, Nichols BJ, Pelham HR, Wickner W (1998) A vacuolar v-t-SNARE complex, the predominant form in vivo and on isolated vacuoles, is disassembled and activated for docking and fusion. *J Cell Biol* 140:61–69.
51. Parlati F, et al. (2000) Topological restriction of SNARE-dependent membrane fusion. *Nature* 407:194–198.
52. Peloso CW, O'Connor MJ, Bigger SW, Scheirs J (1998) Characterizing the degradation of the polymer slip additive erucamide in the presence of inorganic antiblock agents. *Polym Degrad Stab* 62:285–290.
53. Rand RP, Sengupta S (1972) Cardiolipin forms hexagonal structures with divalent cations. *Biochim Biophys Acta* 255:484–492.
54. Kümmel D, Heinemann U (2008) Diversity in structure and function of tethering complexes: Evidence for different mechanisms in vesicular transport regulation. *Curr Protein Pept Sci* 9:197–209.
55. Whyte JR, Munro S (2002) Vesicle tethering complexes in membrane traffic. *J Cell Sci* 115:2627–2637.
56. Pfeffer S (2001) Vesicle tethering factors united. *Mol Cell* 8:729–730.
57. Sztul E, Lupashin V (2006) Role of tethering factors in secretory membrane traffic. *Am J Physiol* 290:C11–C26.
58. Bentley M, et al. (2006) SNARE status regulates tether recruitment and function in homotypic COPII vesicle fusion. *J Biol Chem* 281:38825–38833.
59. Barnard RJ, Morgan A, Burgoyne RD (1997) Stimulation of NSF ATPase activity by α -SNAP is required for SNARE complex disassembly and exocytosis. *J Cell Biol* 139:875–883.
60. Hammarlund M, et al. (2007) Open syntaxin docks synaptic vesicles. *PLoS Biol* 5:e198.
61. Tareste D, Shen J, Melia TJ, Rothman JE (2008) SNAREpin/Munc18 promotes adhesion and fusion of large vesicles to giant membranes. *Proc Natl Acad Sci USA* 105:2380–2385.
62. Wang L, Merz AJ, Collins KM, Wickner W (2003) Hierarchy of protein assembly at the vertex ring domain for yeast vacuole docking and fusion. *J Cell Biol* 160:365–374.
63. Gerrard SR, Mecklem AB, Stevens TH (2000) The yeast endosomal t-SNARE, Pep12p, functions in the absence of its transmembrane domain. *Traffic* 1:45–55.
64. Geumann U, et al. (2008) SNARE function is not involved in early endosome docking. *Mol Biol Cell* 19:5327–5337.
65. Bethani I, et al. (2007) The specificity of SNARE pairing in biological membranes is mediated by both proofreading and spatial segregation. *EMBO J* 26:3981–3992.
66. Drin G, et al. (2008) Asymmetric tethering of flat and curved lipid membranes by a golgin. *Science* 320:670–673.
67. Araç D, et al. (2006) Close membrane-membrane proximity induced by Ca²⁺-dependent multivalent binding of synaptotagmin-1 to phospholipids. *Nat Struct Mol Biol* 13:209–217.
68. Connell E, et al. (2008) Cross-linking of phospholipid membranes is a conserved property of calcium-sensitive synaptotagmins. *J Mol Biol* 380:42–50.
69. Wang L, Seeley ES, Wickner W, Merz AJ (2002) Vacuole fusion at a ring of vertex docking sites leaves membrane fragments within the organelle. *Cell* 108:357–369.
70. Ortiz A, Killian JA, Verkleij AJ, Wilschut J (1999) Membrane fusion and the lamellar-to-inverted-hexagonal phase transition in cardiolipin vesicle systems induced by divalent cations. *Biophys J* 77:2003–2014.
71. Schlame M, Haldar D (1993) Cardiolipin is synthesized on the matrix side of the inner membrane in rat liver mitochondria. *J Biol Chem* 268:74–79.
72. Zinser E, et al. (1991) Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote *Saccharomyces cerevisiae*. *J Bacteriol* 173:2026–2034.
73. Chen S, Tarsio M, Kane PM, Greenberg ML (2008) Cardiolipin mediates cross-talk between mitochondria and the vacuole. *Mol Biol Cell* 19:5047–5058.
74. Drin G, et al. (2007) A general amphipathic α -helical motif for sensing membrane curvature. *Nat Struct Mol Biol* 14:138–146.
75. LaGrassa TJ, Ungermann C (2005) The vacuolar kinase Yck3 maintains organelle fragmentation by regulating the HOPS tethering complex. *J Cell Biol* 168:401–414.
76. Thorngren N, et al. (2004) A soluble SNARE drives rapid docking, bypassing ATP and Sec17/18p for vacuole fusion. *EMBO J* 23:2765–2776.
77. Haas A, Wickner W (1996) Homotypic vacuole fusion requires Sec17p (yeast α -SNAP) and Sec18p (yeast NSF). *EMBO J* 15:3296–3305.
78. Schneider R, et al. (1999) Electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis of the lipid molecular species composition of yeast subcellular membranes reveals acyl chain-based sorting/remodeling of distinct molecular species en route to the plasma membrane. *J Cell Biol* 146:741–754.
79. Orci L, et al. (1993) Budding from Golgi membranes requires the coatamer complex of nonclathrin coat proteins. *Nature* 362:648–652.