Improved reconstitution of yeast vacuole fusion with physiological SNARE concentrations reveals an asymmetric Rab(GTP) requirement

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ABSTRACT In vitro reconstitution of homotypic yeast vacuole fusion from purified components enables detailed study of membrane fusion mechanisms. Current reconstitutions have yet to faithfully replicate the fusion process in at least three respects: 1) The density of SNARE proteins required for fusion in vitro is substantially higher than on the organelle. 2) Substantial lysis accompanies reconstituted fusion. 3) The Rab GTPase Ypt7 is essential in vivo but often dispensable in vitro. Here we report that changes in fatty acyl chain composition dramatically lower the density of SNAREs that are required for fusion. By providing more physiological lipids with a lower phase transition temperature, we achieved efficient fusion with SNARE concentrations as low as on the native organelle. Although fused proteoliposomes became unstable at elevated SNARE concentrations, releasing their content after fusion had occurred, reconstructed proteoliposomes with substantially reduced SNARE concentrations fused without concomitant lysis. The Rab GTPase Ypt7 is essential on both membranes for proteoliposome fusion to occur at these SNARE concentrations. Strikingly, it was only critical for Ypt7 to be GTP loaded on membranes bearing the R-SNARE Nyv1, whereas the bound nucleotide of Ypt7 was irrelevant on membranes bearing the Q-SNAREs Vam3 and Vti1.

INTRODUCTION
Intracellular membrane fusion is essential for endocytic and exocytic vesicular traffic. Fusion is regulated and catalyzed by families of proteins that are conserved among all eukaryotic cells. Organellar-specific Rab/Ypt-family GTPases bind effector proteins to tether membranes, allowing associations in-trans among soluble N–ethylmaleimide–sensitive factor attachment protein receptor (SNARE) proteins anchored to apposed membranes (Gray et al., 2006). SNAREs are defined by their heptad-repeat SNARE domains bearing either a central arginyl or glutaminyl residue and are categorized accordingly as R- or Qa/Qb/Qc-SNAREs (Fasshauer et al., 1998; Kloepper et al., 2007). R- and Qa-SNAREs bind to conserved regions of Sec1/Munc18-family proteins, which likely catalyze assembly of a complex of all four SNAREs (Baker et al., 2015). Completion of such trans-SNARE associations, termed docking, brings the bilayers into close apposition. Membrane fusion converts trans-SNARE complexes into cis-SNARE complexes, which are then disassembled by the ATP-driven chaperone NSF/Sec18 and its cochaperone, α-SNAP/Sec17 (Mayer et al., 1996). The regulated interactions among these fusion catalysts have been studied in systems ranging from baker’s yeast to the neuronal synapse.

Homotypic membrane fusion among the vacuoles (lysosomes) of Saccharomyces cerevisiae requires each of these conserved proteins. Vacuoles are tethered for fusion by the Rab Ypt7 and by a heterohexameric protein complex termed the homotypic fusion and vacuole protein sorting (HOPS) complex (Hickey et al., 2009). Two of the HOPS subunits (Vps39 and Vps41) have direct affinity for Ypt7 (Brett et al., 2008; Plemel et al., 2011), and the subunit Vps33 is a member of the Sec1/Munc18 family (Baker et al., 2015). Vacuolar fusion uses four SNAREs: the R-SNARE Nyv1, the Qa-SNARE Vam3, the Qb-SNARE Vti1, and the Qc-SNARE Vam7. Three of these SNAREs are integrally membrane anchored at their C-terminus, whereas Vam7 is a peripheral membrane protein that is bound to membranes by its affinities for acidic lipids,
1,2-dipalmitoyl-sn-glycero-3-phospho-(1-myoinositol-3-phosphate) (PI(3)P), HOPS, and the other SNAREs (Lee et al., 2006; Strope et al., 2006; Karunakaran and Wickner, 2013). HOPS- and Ypt7-dependent tethering draws apposed membranes together, and a ring-shaped microdomain enriched in the SNAREs, Rab, HOPS, and certain lipids (phosphoinositides, ergosterol, and diacylglycerol [DAG]) forms around the docked, apposed membranes (Wang et al., 2002, 2003; Fratti et al., 2004). Vacuole fusion depends on Sec17/Sec18 to liberate SNAREs from cis-complexes (Mayer et al., 1996) and on HOPS to promote the formation of SNARE complexes in-trans (Zick and Wickner, 2013; Baker et al., 2015). Finally, the lipid rearrangements of fusion itself are strongly dependent on small–head group lipids, which can be accommodated in the presumed nonbilayer fusion intermediate structures (Zick et al., 2014) while affecting membrane fluidity (Dawaliby et al., 2016).

Vacuole fusion has been studied extensively in vivo (Wada et al., 1992), in vitro with the purified organelle (reviewed in Wickner, 2010), and more recently with reconstituted proteoliposomes of defined lipids and purified proteins. Although it requires most of the same components, the fusion of proteoliposomes has differed from that of the native membrane in that it has required far higher molar ratios of SNAREs to lipid (Zick et al., 2014, 2015b), has not shown as strict a dependence on Ypt7 (Orr et al., 2015), and has exhibited far more lysis (Zucchi and Zick, 2011) than is seen with wild-type vacuoles (Starai et al., 2007). We now report that each of these characteristics is dramatically affected by preparing the proteoliposomes with lipids of different fatty acyl chain compositions, creating a more fluid model membrane that better resembles the native organelle (Schneiter et al., 1999). Fusion is now seen at physiological levels of SNAREs, lysis is reduced and occurs only well after fusion, and Ypt7 is essential for fusion as Ypt7(GTP) in-trans to the R-SNARE and either Ypt7(GTP) or Ypt7(GDP) in-trans to the Q-SNAREs. This asymmetry may orient HOPS for productive association with SNAREs.

RESULTS

The vacuolar membrane is composed of a complex mixture of lipids (Zinser and Daum, 1995). Lipid diversity is not limited to the polar head groups. There is also a striking variability among the membrane phospholipids regarding length and saturation of the fatty acyl chains (Schneiter et al., 1999), with only a minor fraction of the fatty acyl chains being saturated (Figure 1A). Lipids within

FIGURE 1: Increased membrane fluidity lowers the SNARE density that is required for RPL fusion. (A) Summary of fatty acyl chain compositions found in yeast vacuoles, as described in Schneiter et al. (1999). Fatty acid compositions of phospholipids are grouped by chain length and number of double bonds (e.g., 34:2, composed of palmitoleic acid [C16:1] and oleic acid [C18:1]). (B) Phase transition temperatures of multiple PC species (Marsh, 2013), which can be used as a surrogate measure of membrane fluidity. (C–H) Fusion reactions of RPLs composed of vacuolar mimic lipids (PC, PE, PI, PS, PA, ergosterol, DAG, PI(3)P) bearing Nyv1 (R) or Vam3/Vti1 (2Q) at various SNARE:lipid ratios (1:1000–1:32,000) and Ypt7(GTP) at a protein:lipid ratio of 1:2000 were incubated with 50 nM HOPS, 50 nM Sec18, 600 nM Sec17, and 100 nM Vam7 for 30 min at 27ºC. The species of PC (47.6 mol%) was chosen as indicated, with all other lipids unaltered. (I, J) Fusion reactions of RPLs as in E and H with a SNARE:lipid ratio of 1:1000 (concentration of each membrane-embedded SNARE in reaction, 250 nM) were performed in the presence of indicated levels of Vam7 (1–4096 nM). Kinetic curves of content mixing assays in this and subsequent figures are representative of at least three experiments.
FIGURE 2: The lipid side-chain choice did not substantially affect the amount of trans-SNARE complex formation. Fusion reactions of R- and 2Q-RPLs with SNARE:lipid ratios of 1:2000, 1:8000, or 1:32,000 and PC lipid side-chain choice of 16:0 18:1 (palmitoyl-oleoyl [PO]) or 18:2 18:2 (18:2; compare with Figure 1, C and F) were incubated for 20 min at 27°C with 50 nM HOPS and 100 nM Vam7Δ3 (complete reaction), only 50 nM HOPS (–V7), or only 100 nM Vam7Δ3 (–H). Reactions (20 μl for SNARE:lipid ratios of 1:2000, 80 μl for SNARE:lipid ratios of 1:8000, 320 μl for SNARE:lipid ratios of 1:32,000) were diluted in β-octylglucoside buffer (1% wt/vol final) to a final volume of 0.5 ml and incubated with 20 μl of protein A magnetic beads and 20 μg of affinity-purified αVam3 antibody for 2 h at room temperature. The beads were washed three times with 1 ml of β-octylglucoside buffer, and the samples were eluted in 100 μl of SDS sample buffer at 95°C for 5 min. Samples were analyzed by SDS–PAGE and Western blot with αNv1 antibody.

the membrane bilayer engage through van der Waals interactions among neighboring hydrophobic chains. Both chain length and the number of unsaturated double bonds, which kink the otherwise straight hydrocarbon chains, determine the strength of those intermolecular interactions and influence the biophysical properties of a membrane. The relative mobility of individual molecules within a lipid bilayer determines its fluidity and viscoelastic behavior in response to bilayer deformations (Wu et al., 2015). The phase transition temperature (the temperature at which the phase behavior of the membrane transitions from a solid to a liquid state) is a surrogate measure of the degree of membrane fluidity and is intimately linked to acyl chain length and saturation (Figure 1B).

Membrane fluidity modulates fusion competence

To test what effect the fatty acyl chain composition of a bilayer has on the ability of SNAREs to drive membrane fusion, we prepared reconstituted proteoliposomes (RPLs) of vacuolar mimic lipid (VML) composition with SNAREs at a variety of protein:lipid ratios and with phosphatidylcholine (PC), the most abundant phospholipid, bearing different fatty acyl chains (Figure 1, C–H). Membranes containing the PC with the highest phase transition temperature that we tested (14:0-PC) showed almost no fusion activity (Figure 1C), even at a molar SNARE:lipid ratio of 1:1000. RPLs with a PC species of intermediate fluidity (Figure 1, D and E) showed considerable fusion activity as long as SNAREs were present at high concentrations. Reducing the SNARE concentration under these conditions, however, resulted in a gradual loss of fusion activity. At a molar SNARE:lipid ratio of 1:32,000, which is similar to the density of SNARE proteins that is found on the organelle (Zick et al., 2014, 2015b), no detectable fusion activity remained. Membranes with PC species that contained a double bond in both fatty acyl chains retained at least some fusion activity even at the lowest SNARE densities (Figure 1, F–H). Membranes containing 16:1 PC, the most abundant form of PC found on native vacuolar membranes (Figure 1A), demonstrated good fusion activity even at low SNARE:lipid ratios (Figure 1G). Because RPLs that were made with 16:1 PC were unstable during storage at −80°C (the content marker proteins were released within days), we used another PC species with similar characteristics for this practical reason, even though it is not found in yeast, which does not have the ability to synthesize polyunsaturated fatty acids. RPLs containing 18:2 PC, which also has a low phase transition temperature and did not exhibit leakage upon storage, had considerable fusion activity at reduced SNARE levels (Figure 1H). The influence of membrane fluidity on fusion activity can also be seen when the soluble Qc-SNARE Vam7 is added at limiting concentrations to RPLs bearing the other three SNAREs at molar protein:lipid ratios of 1:1000 (Figure 1, I and J). Membranes containing 16:0 18:1 PC require considerably higher concentrations of Vam7 for efficient fusion than membranes containing 18:2 PC (e.g., compare Figure 1, I, 256 nM Vam7, vs. J, 4 nM Vam7).

To determine whether membrane fluidity affects fusion capacity indirectly by modulating the propensity of SNAREs to engage in trans complexes or directly by allowing fusion to be catalyzed by fewer trans-SNARE complexes, we determined the amount of trans-SNARE complexes that formed when using RPLs with either 16:0 18:1 PC or 18:2 PC at various SNARE concentrations. For this, we performed fusion reactions with a form of Vam7 that had a C-terminal truncation (Vam7Δ3), which allows stable trans-SNARE complex formation but prevents fusion (Schwartz and Merz, 2009), and analyzed the association of Vam3 and Nv1 by coimmunoprecipitation with antibody to the Qa SNARE Vam3 (Figure 2). To maintain comparable conditions during the immunoprecipitations regardless of the SNARE density on the membranes, we increased the scale of the initial reactions for RPLs with reduced SNARE density to compensate for the imbalance (i.e., SNAREs at 1:2000, 1x scale; SNAREs at 1:8000, 4x scale; SNAREs at 1:32,000, 16x scale). Proteoliposomes were incubated under fusion conditions, solubilized with detergent, and assayed for the Nv1 bound to Vam3 by coimmunoprecipitation with antibody to Vam3. The amount of trans complex that formed during a 20-min incubation was not significantly altered by the species of PC that was present in the RPLs (compare lane 11 vs. 12; 13 vs. 14; and 15 vs. 16). When Vam7 or HOPS was omitted, no trans-SNARE complex was detectable (lanes 7–10). This indicates that membrane fluidity directly affects the capacity of lipid bilayers to fuse rather than modulating the efficiency of trans-SNARE complex formation.

Membranes with elevated SNARE levels are unstable after fusion

Having achieved fusion of reconstituted membranes with significantly reduced SNARE densities, we reexamined some of the earlier inconsistencies between our in vitro reconstitution model and fusion of the isolated organelle. It was shown previously (Starai et al., 2007) that little lysis occurs during the fusion of vacuoles from wild-type
yeast, but markedly enhanced lysis accompanies the fusion of vacuoles from strains that overexpressed the vacuolar SNAREs. This fusion had lost Ypt7(GTP) dependence as well. Lysis and loss of Ypt7(GTP) dependence also accompanied the fusion of proteoliposomes reconstituted with high SNARE levels (Zucchi and Zick, 2011; Orr et al., 2015). To reexamine the lysis component of the reconstituted fusion reaction, we performed fusion reactions with SNAREs at high levels, as had been used in prior studies (R- and 2Q-RPLs with 1:2000 M SNARE:lipid ratios, 100 nM Vam7). Fusion reactions, but the content did not remain intralumenal over time. (A, B) Fusion reactions of R and 2Q RPLs (molar SNARE:lipid ratio 1:2000, Ypt7(GTP):lipid ratio 1:8000) were performed in the presence of 0, 1, 2, 4, 8, 16, 32, or 64 μM unlabeled streptavidin. Reactions contained 50 nM HOPS, 50 nM Sec18, 600 nM Sec17, and 100 nM Vam7. (C, D) Fusion reactions as in A with RPLs that contained luminal neuraminidase (20 μg/ml) were incubated for various times and then diluted and centrifuged to pellet the RPLs. Pre-spin and postspin supernatant samples were mixed with MuNANA substrate solution (containing 4-methylumbelliferyl-α-d-neuraminic acid [50 μM final] and Thesit [0.2% wt/vol final]) in a 384-well plate, and the fluorescence signal that resulted from substrate hydrolysis by neuraminidase was recorded for 20 min. Neuraminidase activity that was recovered in the supernatant fraction is displayed as averages and SDs from three separate experiments. The pre-spin samples were defined as 100% activity.

To test whether the lysis of fused proteoliposomes was invariant at each level of SNARE pairing and fusion, we introduced modified assays able to measure lysis directly (Figure 4). RPLs bearing the R-SNARE Nyv1, Marina-Blue-PE, and luminal biotinylated PhycoE were incubated with RPLs bearing the Q-SNAREs Vam3 and Vti1 and nitrobenzoxadiazole (NBD)-PE and had one of three luminal compositions: 1) Cy-labeled streptavidin (Sa-Cy5), 2) no content marker (empty), or 3) unlabeled streptavidin. The level of the Qc SNARE Vam7 was systematically varied from 0.25 to 16 nM, regulating the capacity for trans-SNARE pairing. All incubations were in the presence of external Sa-Cy5. To avoid the substantial background signal caused by the large quantity of external Sa-Cy5, we measured the signal (PhycoE quenching) only if the fusion event was preceded by fusion. Conducting such experiments in the presence of increasing amounts of Vam7 revealed that the Qc SNARE Vam7 detected very little lysis occurred after fusion, as there was little detectable signal when unlabeled streptavidin was entrapped in 2Q-RPLs (Figure 4C). Of note, there was almost no lysis at 1 nM or less Vam7, which, however, displayed a considerable fusion signal (e.g., compare purple curves in Figure 4, A and B). At higher Vam7 levels, which induced more robust fusion, a higher level of lysis was detected. The lipid mixing signals (Figure 4, bottom) showed that the different content markers did not adversely affect the overall reaction. Lysis was also exclusively seen at high SNARE levels when the molar ratio of Nyv1 to lipids was varied and the concentration of Vam7 was invariant (Supplemental Figure S1).

Ypt7(GTP) is essential for fusion at physiological SNARE concentrations

Because SNARE overexpression also enabled Δypt7 vacuoles to fuse (Starai et al., 2007), we reevaluated the Ypt7 requirement in the reconstituted system and how it relates to the level of SNAREs and Ypt7 that are present on the RPLs (Figure 5). At high SNARE concentrations, fusion occurred regardless of the nucleotide state of Ypt7. This may reflect the direct affinity of HOPS for the vacuolar SNAREs (Stoupe et al., 2006; Baker et al., 2015), bypassing the physiological role of Ypt7 as a HOPS receptor. When the concentrations of SNAREs were reduced, fusion occurred only when Ypt7 was loaded with GTP (blue curves) and was lost when Ypt7 was present in its GDP-loaded state (red curves). The Rab GTPase Ypt7 was present in these reconstitutions at concentrations approximately fivefold higher than found on the organelle. When SNAREs are present at the physiological levels of 1:32,000 molar ratio to lipids, the capacity...
Thus molecular understanding of the fusion reaction requires that it components that are integral cannot be removed and added at will.

Strikingly, Ypt7, while strictly required on both fusion partners, only needed to be in its GTP-loaded form on membranes that bore the trans-SNARE complexes. It is noteworthy that fusion can occur without lysis when the ability to form trans-SNARE complex is restricted, for example, by limiting the concentration of the soluble SNAP Vam7. This reduction in trans-SNARE complexes may eliminate the existence of simultaneous, competing fusion sites at multiple membrane interfaces. Whether such competition could occur in vivo is unclear, but the regular occurrence of even minor amounts of lysis and the uncontrolled release of vacuolar enzymes into the cytosol would have devastating consequences. The formation of trans-SNARE complexes thus has to happen in a highly regulated manner, catalyzed by specific machinery such as HOPS, rather than being driven by mass effect among highly concentrated SNAPs. Reconstitutions of fusion need to consider the physiological levels of each protein, the lipid composition, and the possibility of lysis.

At the newly established standard conditions (molar ratios of SNAPs: lipid 1:32,000 and Ypt7:lipid 1:8000), fusion exhibits a strict dependence on Ypt7, as seen in vivo. Curiously, we discovered an asymmetry of required guanine nucleotide bound to the Ypt7 with respect to it being adjacent to R- versus Q-SNARE interactions. It is noteworthy that fusion can occur without lysis when the ability to form trans-SNARE complex is restricted, for example, by limiting the concentration of the soluble SNAP Vam7. This reduction in trans-SNARE complexes may eliminate the existence of simultaneous, competing fusion sites at multiple membrane interfaces. Whether such competition could occur in vivo is unclear, but the regular occurrence of even minor amounts of lysis and the uncontrolled release of vacuolar enzymes into the cytosol would have devastating consequences. The formation of trans-SNARE complexes thus has to happen in a highly regulated manner, catalyzed by specific machinery such as HOPS, rather than being driven by mass effect among highly concentrated SNAPs. Reconstitutions of fusion need to consider the physiological levels of each protein, the lipid composition, and the possibility of lysis.

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FIGURE 4: The current reconstitution of membrane fusion with RPLs leads to an unstable fusion product. (A–C) A modification of the PhycoE- and Sa-Cy5–based content mixing assay allows the discrimination of when lysis occurs. RPLs containing biotinylated PhycoE were subjected to fusion reactions with RPLs containing (A) Cy5-labeled streptavidin (luminal concentration, 8 μM), (B) no content marker, or (C) unlabeled streptavidin (luminal concentration, 32 μM), all in the presence of extralumenal Cy5-labeled streptavidin (4 μM). Fusion reactions of R and Q RPLs (molar SNARE:lipid ratio 1:2000, Ypt7(GTP):lipid ratio 1:8000) were performed in the presence of 0–16 nM Vam7, 50 nM HOPS, 50 nM Sec18, and 600 nM Sec17. Top, quenching data of PhycoE as a result of its binding interaction with Sa-Cy5. Bottom, corresponding lipid mixing data (quenching of Marina-Blue by NBD) from the same reactions.
of Vps39 and Vps41 with two Ypt7 molecules on two separate membranes might be of central importance for tethering but less so for HOPS catalysis of trans-SNARE complex assembly. Whether HOPS remains bound to two Ypt7 molecules throughout the fusion cycle is unclear. Specifically orienting the HOPS complex in a preferred conformation with respect to the R- and Q-SNAREs through selective interaction of Vps41, the HOPS subunit that is adjacent to the SM subunit Vps33 (Brocker et al., 2012), with Ypt7(GTP) might aid its catalysis of trans-SNARE complex formation.

Several important lessons have emerged from reconstituted vacuolar fusion. The spontaneous assembly of the four-helical coiled-coil trans-SNARE complex is very inefficient, so that SNAREs alone give very slow fusion and only at unphysiologically high concentrations that induce substantial lysis. SNARE complex assembly is likely catalyzed by HOPS, with a central role of its Vps33 SM subunit binding the SNARE domains of Vam3 and Nyv1 (Baker et al., 2015). Independent tethering is required (Zick and Wickner, 2014) and performed by HOPS based on the affinity of two of its subunits for Ypt7 (Brett et al., 2008), with contributions of undetermined magnitude from the affinity of HOPS for phosphoinositides, other acidic lipids, and SNAREs (Stroupe et al., 2006; Orr et al., 2015). Small–head group lipids that are nonbilayer prone and fluid fatty acyl chains are also crucial to lowering the energy barrier for fusion (Figure 1; Zick et al., 2014). We recently reported that Sec17 (α-SNAP) can associate with trans-SNARE complexes and trigger the fusion event via an N-terminal apolar loop (Zick et al., 2015a).

Further iterations and refinements of the reconstituted fusion system are necessary to determine any roles of sphingolipids, lipid asymmetry, and membrane curvature in fusion. Additional components that stabilize membranes against postfusion lysis might still be missing. If lysis is due to an inability to adjust the ratio of inner leaflet to outer leaflet lipids, then it may be cured by the addition of a lipid flipase or by the budding of small vesicles, as occurs at the organelle to recycle components to the endosome. Finally, additional layers of regulation and their interplay should be reconstituted, such as GAP and GEF cycling of the guanine nucleotide on Ypt7 and regulation of HOPS by the vacuolar Yck3 kinase (LaGrassa and Ungermann, 2005; Brett et al., 2008; Nordmann et al., 2010; Lawrence et al., 2014) and unidentified phosphatase(s).

Two other intracellular membrane fusion events have been reconstituted with purified components. Fusion has been reconstituted with neuronal SNAREs, NSF and SNAP, Munc18-1, Munc13, and synaptotagmin-1, the sensor for calcium (Ma et al., 2013). Other essential components, such as complexin and Rab3α, are being tested in such reconstitutions. Elements of neuronal fusion, such as complexin, synaptotagmin, and calcium, are required only at the synapse, making this a special, albeit particularly important, case of intracellular fusion. The other example is the Rab- and SNARE-dependent reconstitution of endosomal fusion (Ohya et al., 2009). Further studies are needed in this system to establish the role of each factor and which functions are shared, or distinct, from vacuolar or neuronal fusion. Embracing both the complexity of biological systems and the rigor of chemically defined reconstitution will be a fruitful guiding principle in studies of membrane fusion.

**MATERIALS AND METHODS**

**Proteins and reagents**

The purification of HOPS, prenylated Ypt7 (Zick and Wickner, 2013), Vam7, Sec17 (Schwartz and Merz, 2009), Sec18 (Haas and Wickner, 1996), and neuraminidase (Zucchi and Zick, 2011) were as described. Membrane-anchored vacuolar SNAREs (Vam3, Vti1, Nyv1) were isolated (Mima et al., 2008) and exchanged into octylglucoside buffer (Zucchi and Zick, 2011). Proteins were frozen in aliquots in liquid nitrogen and stored at ~80°C. Most lipids were obtained from Avanti Polar Lipids (Alabaster, AL), with the exception of ergosterol, which was from Sigma-Aldrich (St. Louis, MO), PI(3)P, which was from Avanti Polar Lipids (Alabaster, AL), with the exception of ergosterol, which was from Sigma-Aldrich (St. Louis, MO). 

**FIGURE 5:** The nucleotide state of the Rab GTPase Ypt7 is critical for fusion at reduced SNARE densities. Fusion reactions of RPLs bearing all four SNAREs (molar SNARE:lipid ratio 1:1000–1:128,000, Ypt7:lipid ratio 1:2000) were performed in the presence of 50 nM HOPS, 50 nM Sec18, and 600 nM Sec17. Ypt7 was nucleotide exchanged into its GTP or GDP form on both RPLs before addition of Sec17, Sec18, and HOPS. RPLs with a molar SNARE:lipid ratio of 1:32,000 and an average RPL diameter of ~200 nm (Zick and Wickner, 2014) carry ~10 copies of each SNARE. The horizontal axis represents 0–30 min, and the vertical axis represents 0–100% content mixing.
FIGURE 6: A titration of Ypt7 shows its critical importance for fusion at moderate SNARE concentrations. Fusion reactions of R and 2Q RPLs (molar SNARE:lipid ratio 1:32,000, Ypt7:lipid ratio 1:2000–1:32,000) were performed in the presence of 0–100 nM Vam7, 50 nM HOPS, 50 nM Sec18, and 600 nM Sec17. Ypt7, where present, was nucleotide exchanged into its GTP or GDP form on both RPLs before addition of Sec17, Sec18, and HOPS. The horizontal axis represents 0–30 min, and the vertical axis represents 0–60% content mixing.

Echelon Biosciences (Salt Lake City, UT), and the fluorescent lipids (Marina Blue–1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine [DHPE] and NBD-DHPE), which were from Thermo Fisher Scientific (Waltham, MA). Cy5-derivatized streptavidin was purchased from KPL (now SeraCare Life Sciences, Milford, MA) and biotinylated PhycoE and unlabeled streptavidin from Thermo Fisher Scientific. Protein concentrations were determined by Bio-Rad Hercules (Hercules, CA) protein assay, which is based on the Bradford dye-binding method (Bradford, 1976).

Proteoliposome preparation
Proteoliposomes were prepared (Zick et al., 2014) from mixed micellar solutions (containing 50 mM β-octyl-glucoside) by detergent dialysis (20-kDa cutoff membrane) in RB150/Mg2+ (20 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid NaOH, pH 7.4, 150 mM NaCl, 1 mM MgCl2, 10% glycerol [vol/vol]) with individual SNAREs and prenylated Ypt7 at various molar protein:lipid ratios. Lipids dissolved in chloroform were mixed in vials containing β-octyl-glucoside at the following proportions. For vacuum mixed lipids (16:0 18:1), 44.8–47.6 mol% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, 18 mol% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine, 18 mol% soy l-α-phosphatidylinoisitol (Pl), 4.4 mol% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine, 2 mol% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate, 1 mol% 1,2-dipalmitoyl-sn-glycerol, 8 mol% ergosterol, and 1 mol% Pl(3P). For VML (18:2) compositions, as for RPLs used in Figures 3–7, lipids were mixed in the same proportions as described but with the dilinoleoyl forms of PC, phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidic acid (PA). All proteoliposomes throughout this study contained 0.2 mol% Marina-Blue-PE or 3 mol% NBD-PE.

Fusion assay
Fusion reactions were assembled in 20 μl. Fusion assay pairs of RPLs (each 250 μM lipid) in RB150, 5 μM streptavidin, 1 mM EDTA, and 10 μM GDP or GTP were preincubated for 10 min at 27°C before addition of 1.25 mM MgCl2 to exchange Ypt7 to its GTP- or GDP-bound form. For reactions with asymmetric Ypt7 disposition, R- and 2Q-RPLs were nucleotide exchanged separately and mixed only immediately before reactions were started. Of the preincubated RPLs, 14 μl (or 2 x 7 μl) was transferred to wells of 384-well plates, and soluble components (6 μl; e.g., HOPS, Sec17, Sec18, ATP, Vam7) or their respective buffers were added to initiate reactions. All reactions contained 0.5% (wt/vol) defatted bovine serum albumin, 5 mM reduced glutathione, and 1 mM dithiothreitol. Plates were incubated at 27°C in a fluorescence plate reader, and content mixing signals (PhycoE–Cy5-FRET: excitation [ex], 565 nm; emission [em], 670 nm; cutoff, 590 nm), or lipid mixing (Marina-Blue quenching) signals (ex, 370 nm; em, 465 nm; cutoff, 420 nm) were recorded at intervals of 10–30 s in a SpectraMax Gemini XPS (Molecular Devices, Sunnyvale, CA) fluorescence plate reader. Maximal content mixing values were determined after addition of 0.2% (wt/vol) Thesit to samples that had not received streptavidin.

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REFERENCES
Baker RW, Jeffrey PD, Zick M, Phillips BP, Wickner WT, Hughson FM (2015).
A direct role for the Sec1/Munc18-family protein Vps33 as a template for SNARE assembly. Science 349, 1111–1114.
Bradford MM (1976). Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72, 248–254.
Brett CL, Pielme RL, Lobinger BT, Vignali M, Fields S, Merz AJ (2008).
Efficient termination of vacuolar Rab GTPase signaling requires coordinated action by a GAP and a protein kinase. J Cell Biol 182, 1141–1151.
Brocker C, Kuhlee A, Gatsogiannis C, Balderhaar HJ, Honscher C, Engelbrecht-Vandere S, Ungermann C, Rauner S (2012). Molecular architecture of the multisubunit homotypic fusion and vacuole protein sorting (HOPS) tethering complex. Proc Natl Acad Sci USA 109, 1991–1996.
Dawaliby R, Trubbia C, Delporte C, Noyon C, Ruysschaert JM, Van Antwerp P, Govaerts C (2016). Phosphatidylethanolamine is a key regulator of membrane fluidity in eukaryotic cells. J Biol Chem 291, 3658–3667.

Fahsahler 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.

Fratti RA, Jun Y, Merz AJ, Margolis N, Wickner W (2004). Interdependent assembly of specific regulatory lipids and membrane fusion proteins into the vertex ring domain of docked vacuoles. J Cell Biol 167, 1087–1098.

Fukuda R, McNew JA, Weber T, Parlati F, Engel T, Nickel W, Rothman JE, Sollner TH (2000). Functional architecture of an intramembrane t-SNARE. Nature 407, 198–202.

Grosshans BL, Ortiz D, Novick P (2006). Rab and their effectors: achieving specificity in membrane traffic. Proc Natl Acad Sci USA 103, 11821–11827.

Haas A, Wickner W (1996). Homotypic vacuole fusion requires Sec17p (yeast alpha-SNAP) and Sec18p (yeast NSF). EMBO J 15, 3296–3305.

Hickey CM, Strope 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.

Karunakaran V, Wickner W (2013). Fusion proteins and select lipids cooperate as membrane receptors for the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) Vam7p. J Biol Chem 288, 28557–28566.

Kleoppe TH, Kenle CN, Fasshauer D (2007). An elaborate classification of SNARE proteins sheds light on the conservation of the eukaryotic endomembrane system. Mol Biol Cell 18, 3463–3471.

LaGrassa TJ, Ungermann C (2005). The vacuolar kinase Yck3 maintains organelle fragmentation by regulating the HOPS tethering complex. J Cell Biol 168, 401–414.

Lawrence G, Brown CC, Flood BA, Karunakaran S, Cabrera M, Nordmann M, Ungermann C, Fratti RA (2014). Dynamic association of the PI3P-interacting Mon1-Ccz1 GEF with vacuoles is controlled through its phosphorylation by the type 1 casein kinase Yck3. Mol Biol Cell 25, 1608–1619.

Lee SA, Kovacs J, Stahelin RV, Cheever ML, Overduin M, Setty TG, Burd WC, Liao ST, Poulter SC, Ploegh HL, Overduin M, Setty TG, Burd WC, Liao ST, Poulter SC, Ploegh HL (2005). The vacuolar kinase Yck3 maintains organelle fragmentation by regulating the HOPS tethering complex. J Cell Biol 168, 401–414.

Mayer A, Wickner W, Haas A (1996). Sec17p (yeast alpha-SNAP) and Sec18p (yeast NSF) drive release of Sec17p (yeast NSF). EMBO J 15, 3296–3305.

Zick M, Stroupe C, Orr A, Douville D, Wickner WT (2015b). Correction: Rab-GTP for membrane binding and to catalyze tethering and fusion. Mol Biol Cell 26, 305–315.

Plemele RL, Lobinger BT, Brett CL, Angers CG, Nickerson DP, Pausell A, Sprague D, Merz AJ (2011). Subunit organization and Rab interactions of Vps-C protein complexes that control endolysosomal membrane traffic. Mol Biol Cell 22, 1353–1363.

Schneiter R, Brugger B, Sandhoff R, Zellnig G, Leber A, Lampl M, Athenstaedt K, Hrastnik C, Eder S, Daum G, 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.

Schwarz ML, Merz AJ (2009). Capture and release of partially zipped trans-SNARE complexes on intact organelles. J Cell Biol 185, 535–549.

Stari VJ, Jun Y, Wickner W (2007). Excess vacuolar SNAREs drive lysis and Rab bypass fusion. Proc Natl Acad Sci USA 104, 13551–13558.

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.

Wada Y, Ohsumi Y, Anraku Y (1992). Genes for directing vacuolar morphology in Saccharomyces cerevisiae. I. Isolation and characterization of two classes of vam mutants. J Biol Chem 267, 18665–18670.

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.

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.

Wickner W (2010). Membrane fusion: five lipids, four SNAREs, three chaperones, two nucleotides, and a Rab, all dancing in a ring on yeast vacuoles. Annu Rev Cell Dev Biol 26, 115–136.

Wu SH, Shankagowt S, Bivas R, Wu S, Povinelli ML, Malmstadt N (2015). Viscoelastic deformation of lipid bilayer vesicles. Soft Matter 11, 7385–7391.

Zick M, Orr A, Schwartz ML, Merz AJ, Wickner WT (2015a). Sec17 can trigger fusion of trans-SNARE paired membranes without Sec18. Proc Natl Acad Sci USA 112, E2290–E2297.

Zick M, Stroupe C, Orr A, Douville D, Wickner WT (2014). Membranes linked by trans-SNARE complexes require lipids prone to non-bilayer structure for progression to fusion. eLife 3, e01879.

Zick M, Stroupe C, Orr A, Douville D, Wickner WT (2015b). Correction: membranes linked by trans-SNARE complexes require lipids prone to non-bilayer structure for progression to fusion. eLife 4, doi: 10.7554/eLife.08843.

Zick M, Wickner W (2013). The tethering complex HOPS catalyzes assembly of the soluble SNARE Vam7 into fusogenic trans-SNARE complexes. Mol Biol Cell 24, 3746–3753.

Zick M, Wickner WT (2014). A distinct tethering step is vital for vacuole fusion. eLife 3, e03251.

Zinser E, Daum G (1995). Isolation and biochemical characterization of organelles from the yeast, Saccharomyces cerevisiae. Yeast 11, 493–536.

Zucchi PC, Zick M (2011). Membrane fusion catalyzed by a Rab, SNAREs, and SNARE chaperones is accompanied by enhanced permeability to small molecules and by lysis. Mol Biol Cell 22, 4635–4646.