Asymmetric Rab activation of vacuolar HOPS to catalyze SNARE complex assembly

Thomas Torng, Hongki Song, and William Wickner*
Department of Biochemistry and Cell Biology, Geisel School of Medicine at Dartmouth, Hanover, NH 03755

ABSTRACT Intracellular membrane fusion requires Rab-family GTPases, their effector tethers, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, and SNARE chaperones of the Sec1/Munc18 (SM), Sec17/α-SNAP, and Sec18/NSF families. We have developed an assay using fluorescence resonance energy transfer to measure SNARE complex formation in real time. We now show that yeast vacuolar SNAREs assemble spontaneously into RQaQbQc complexes when the R- and Qa-SNAREs are concentrated in the same micelles or in *cis* on the same membrane. When SNAREs are free in solution or are tethered to distinct membranes, assembly requires catalysis by HOPS, the vacuolar SM and tethering complex. The Rab Ypt7 and vacuole lipids together allosterically activate the bound HOPS for catalyzing SNARE assembly, even if none of the SNAREs are membrane bound. HOPS-dependent fusion between proteoliposomes bearing R- or Qa-SNAREs shows a strict requirement for Ypt7 on the R-SNARE proteoliposomes but not on the Qa-SNARE proteoliposomes. This asymmetry is reflected in the strikingly different capacity of Ypt7 in *cis* to either the R- or Qa-SNARE to stimulate SNARE complex assembly. Membrane-bound Ypt7 activates HOPS to catalyze 4-SNARE complex assembly when it is on the same membrane as the R-SNARE but not the Qa-SNARE, thus explaining the asymmetric need for Ypt7 for fusion.

INTRODUCTION

Membrane fusion is essential for intracellular vesicular traffic, hormone secretion, and neurotransmission. Fusion follows conserved mechanisms, catalyzed by conserved protein families in all eukaryotes. SNARE (*N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins associate via their heptad-repeat SNARE domains to form a four-helical coiled-coil *trans*-SNARE complex anchored to apposed membranes. SNAREs are named R, Qa, Qb, or Qc according to the central arginyl or glutaminyl residue in the SNARE domain (Fasshauer *et al.*, 1998). The spontaneous assembly of functional *trans*-SNARE complexes for fusion is rare at physiological concentrations (Zick and Wickner, 2014; Song and Wickner, 2019).

Rab-family GTPases accelerate fusion by recruiting effector proteins. One function of effectors is to tether two membranes into proximity (Grosshans *et al.*, 2006) to promote functional *trans*-SNARE complex assembly. It has been unclear whether there are additional links between Rab function and SNARE complex assembly.

The homotypic fusion of vacuoles (lysosomes) in *Saccharomyces cerevisiae* offers several advantages for studying membrane fusion. A complete reconstituted system of vacuole fusion reveals a minimal set of required proteins for successive rounds of membrane fusion (Mima *et al.*, 2008; Zick and Wickner, 2016). The SNAREs Nyv1 (R), Vam3 (Qa), and Vti1 (Qb)—hereafter simply termed R, Qa, and Qb—are integral membrane proteins, while Vam7 (Qc) is soluble. The Rab Ypt7 recruits HOPS, the heterohexameric homotypic fusion and vacuole protein sorting complex, to the membrane (Hickey *et al.*, 2009). HOPS subunits Vps39 and Vps41 have direct affinity for Ypt7 (Ostrowicz *et al.*, 2010). HOPS also has direct affinity for each of the four vacuolar SNAREs (Song *et al.*, 2020), including affinity for the R- and Qa-SNAREs via the HOPS Sec1/Munc18 family subunit Vps33 (Baker *et al.*, 2015). Acidic lipids stimulate fusion by recruiting HOPS and the Qc-SNARE (Cheever *et al.*, 2001; Stroupe *et al.*, 2006), while nonbilayer lipids such as ergosterol are thought to lower the energy barrier for the bilayer rearrangements required for fusion (Zick *et al.*, 2014). After fusion is complete,

SNARE complexes are disassembled by the NSF/Sec18 and α-SNAP/Sec17 chaperones (Söllner *et al.*, 1993; Mayer *et al.*, 1996).

Ypt7 may serve purposes beyond tethering. Fusion shows a stricter requirement for GTP-bound Ypt7 on the membrane bearing the R-SNARE than on the membrane bearing the Qa-SNARE (Zick and Wickner, 2016). This observation has proven difficult to explain with only assays of fusion per se. We have therefore adapted a fluorescence resonance energy transfer (FRET) assay (Weninger *et al.*, 2003; Shin *et al.*, 2014) to directly measure vacuolar SNARE complex assembly. We now report that Ypt7 and vacuolar lipids act together to allosterically activate HOPS to catalyze 4-SNARE complex assembly. This activation is asymmetric with respect to which SNARE is bound to the same proteoliposome as Ypt7. SNAREs without their membrane anchors do not assemble spontaneously at an appreciable rate, nor does HOPS increase this rate significantly. However, HOPS bound to Ypt7 and vacuolar lipids can assemble 4-SNARE complexes from SNAREs that are soluble or membrane bound. While Ypt7 promotes the HOPS-dependent formation of 4-SNARE complexes when the R-SNARE is present on the same membrane, Ypt7 prevents complex formation if the R-SNARE is bound to a separate membrane. In contrast, Ypt7 has little effect on HOPS-catalyzed 4-SNARE assembly when the Qa-SNARE is membrane bound.

RESULTS

Proteoliposomes bearing transmembrane R-SNARE and lumenally entrapped biotin-*R*-phycoerythrin were mixed with proteoliposomes carrying transmembrane Qa-, Qb-, and Qc-SNAREs and lumenally entrapped Cy5-streptavidin, with molar ratios of SNARE to lipid of 1:2000. Ypt7 was present on one, both, or neither proteoliposome at a molar ratio to lipid of 1:8000. Fusion, indicated by an increase in FRET between the lumenal fluorophores, was assayed in the presence of HOPS. Fusion was stimulated by the presence of Ypt7 on the same membrane as the R-SNARE. In contrast, Ypt7 bound to the same membrane as the Q-SNAREs had no effect (Figure 1; the mean FRET is plotted with shaded regions showing the SD of three replicates, in this and subsequent figures). Thus, Ypt7 supports HOPS-mediated fusion in an asymmetric manner, dependent on its location relative to the R- and Qa-SNAREs (Zick and Wickner, 2016).

Vacuolar SNARE domains alone do not suffice for rapid assembly of a four-SNARE complex

We sought to explain this asymmetry by measuring SNARE complex assembly in real time with a FRET-based assay. Because the vacuolar Qb N-domain and transmembrane anchors are not essential for fusion (Song and Wickner, 2017), we isolated the SNARE domain of the Qb-SNARE without its N-terminal or transmembrane domains, with an MBP tag for purification, and with a unique cysteine directly upstream of the SNARE domain. This cysteine was derivatized with Alexa Fluor 568, yielding a construct hereafter termed *Qb (Figure 2A). The Qc-SNARE, labeled with Oregon Green 488 at its single native cysteine, is termed *Qc. Proximity of these fluorescent SNAREs was measured by their FRET signal, obtained by exciting near the excitation peak of Oregon Green 488 (490 nm) and measuring emission near the emission peak of Alexa 568 (615 nm). For each reaction, the contributions of the individual fluorophores to the FRET channel were measured, then subtracted from the raw FRET signal to reveal the true FRET signal as a direct measure of SNARE complex assembly.

We first tested whether a FRET signal between *Qb and *Qc was indicative of a four-SNARE complex. When *Qb and *Qc were
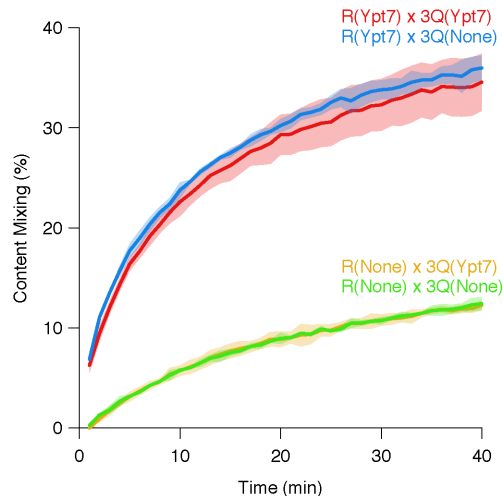

FIGURE 1: The Ypt7 requirement for fusion is asymmetric with respect to the R- and Qa-SNAREs. Proteoliposomes (1 mM lipid) bearing R- or QaQbQc-SNAREs and Ypt7 where indicated (at 1:2000 and 1:8000 molar ratios to lipids for the SNAREs and for Ypt7, respectively) were separately nucleotide exchanged by incubating for 10 min at 27°C with 10 μM streptavidin, 2 mM EDTA, and 20 μM GTP, followed by adding 4 mM MgCl$_2$. Proteoliposomes were incubated separately for 10 min at 27°C in a plate reader, then 5 μl of each were mixed with HOPS for a total volume of 20 μl. Final concentrations were 250 μM total lipid of each proteoliposome, 50 nM HOPS, 5 μM streptavidin, 1 mM EDTA, 10 μM GTP, and 3 mM MgCl$_2$. Content mixing signals were measured every minute with a SpectraMax fluorescence plate reader. Maximal content mixing was assayed after addition of 0.2% (wt/vol) Thesit to samples without streptavidin. For this figure and subsequent figures, shaded regions behind each curve mark the standard deviations from three replicates.

combined in detergent with full-length R and Qa, a FRET signal rapidly developed, suggesting spontaneous assembly of a SNARE complex (Figure 2B, curve a). The FRET signal did not develop if Sec17 and Sec18 were added at the beginning of the reaction (curve c), as Sec17 and Sec18 disassemble SNARE complexes (Söllner *et al.*, 1993). Furthermore, the FRET signal was rapidly reduced by late addition of Sec17 and Sec18, but not by Sec18 alone (curves g and h), indicating that this complex is not simply an aggregate but is recognized by Sec17 and Sec18. The development of the FRET signal was prevented by an excess of nonfluorescent Qb-SNARE (curve b), but once established the FRET signal did not decrease when excess Qb-SNARE was added late (curve i), indicating that Qb-SNAREs in solution cannot exchange with Qb-SNAREs in a SNARE complex. The development of the FRET signal was dependent on the presence of both the R- and Qa-SNAREs (curves d–f). Though full-length Qa-, Qb-, and Qc-SNAREs can assemble into a 3Q complex (Fukuda *et al.*, 2000), the SNARE domain alone of Qb, as used in this assay, lacks its N-terminal domain and is unable to form a 3Q complex (Supplemental Figure 1). As the R-, Qb-, and Qc-SNAREs do not form a stable complex (Song *et al.*, 2020), the FRET signal in this assay is an unequivocal indicator of a four-SNARE complex.

The ability of SNARE domains to spontaneously assemble into a four-SNARE complex is strongly influenced by their coenrichment in common micelles through their C-terminal membrane anchors. In these reactions, R and Qa are spatially limited to micelles by their apolar transmembrane domains, concentrating two of the reactants for four-SNARE assembly. Indeed, the rate of the reaction dropped dramatically when one or both of the full-length R- and Qa-SNAREs
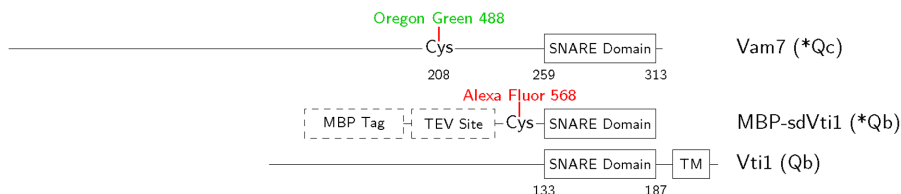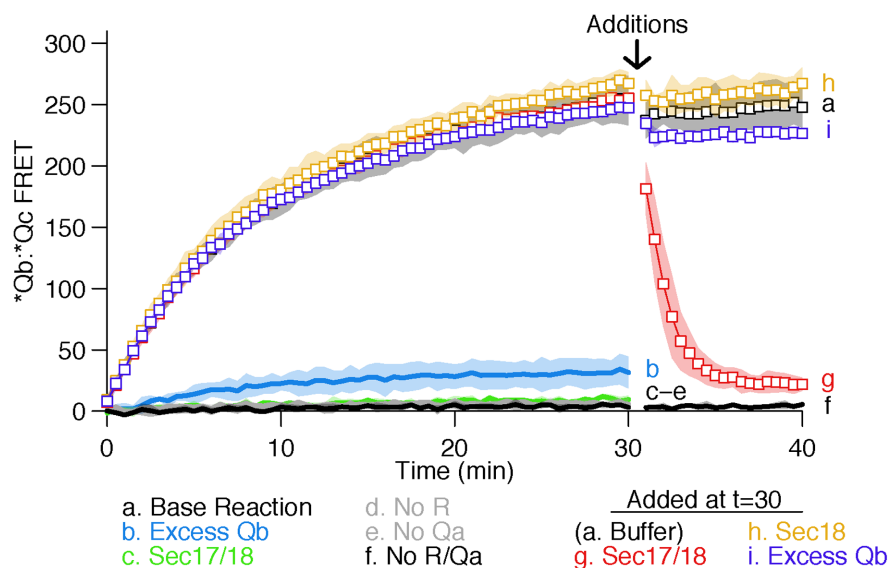
FIGURE 2: Fluorescently tagged Qb- and Qc-SNAREs report four-SNARE assembly in real time. (A) Schematic of the fluorescent Qb- and Qc-SNAREs used in this study. Full-length Qb is shown for comparison. (B) SNARE complex assembly reactions were performed in RB150/0.5% β-octylglucoside. Base reaction: *Qb and *Qc (1 μM each) were mixed with 1 μM full-length R and Qa in 20 μl, followed by an additional 4 μl of RB150/0.5% β-octylglucoside at 30 min. Reactions g–i received 4 μl of RB150 containing either 10.7 μM Sec17, 3.5 μM Sec18, 5 mM ATP, and 5 mM MgCl$_2$, or Sec18, ATP, and MgCl$_2$, or 45 μM underivatized MBP-Qb at 30 min. Reactions b and c also contained underivatized MBP-Qb or Sec17, Sec18, ATP, and MgCl$_2$ from the beginning of the reaction. Reactions d–f were missing R, Qa, or both.
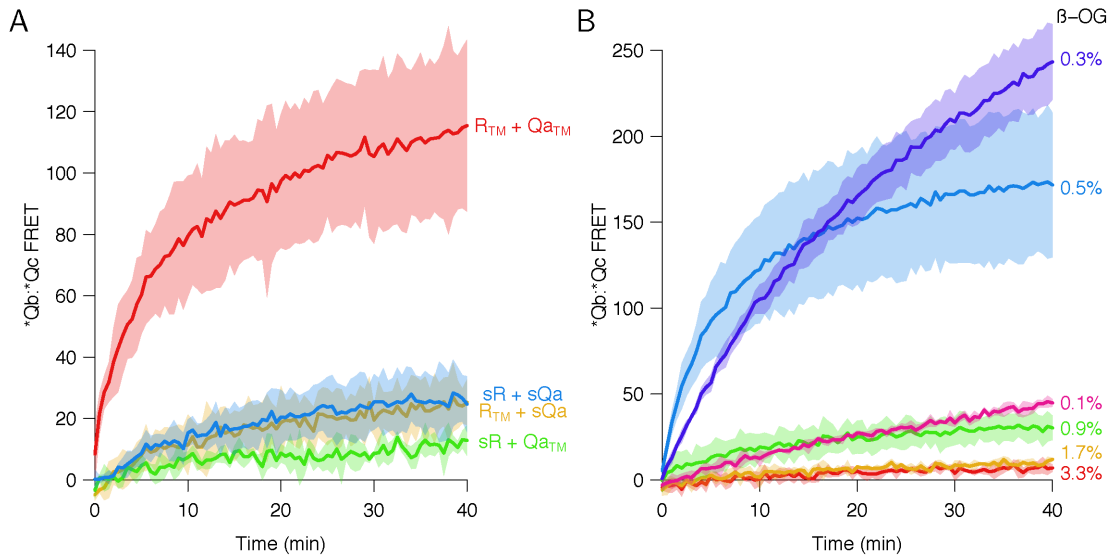
Ypt7 activates HOPS for four-SNARE assembly

We next examined the function of Ypt7 in the assembly of four SNAREs deprived of their membrane anchors and in aqueous solution. The spontaneous assembly of these four soluble SNAREs is very slow, regardless of membrane composition (Figure 4, blue curve, and Figure 5A, curve d). However, the assembly rate was enhanced when HOPS and membranes of vacuole lipids bearing Ypt7 were present (Figure 5A, curve a). As seen in detergent micellar solution with full-length R and Qa bearing their apolar domains (Figure 2A), SNARE assembly with all four soluble SNAREs required both R and Qa (Figure 5A, curves b and c), but here it also required HOPS (Figure 5A, curve a vs. curve d). HOPS only supported SNARE complex assembly when Ypt7 was bound to proteoliposomes bearing a vacuolar mixture of lipids (Ypt7/VML); HOPS with Ypt7 integrally bound to PC liposomes (Ypt7/PC), protein-free liposomes of vacuole lipids only (Naked/VML), or a combination of the two failed to evoke a FRET signal (Figure 5A, curve a vs. curves g, h, and i). Ypt7/PC proteoliposomes and Naked/VML liposomes did not prevent HOPS from being activated by Ypt7/VML proteoliposomes for the assembly of four soluble SNAREs (Figure 5A, curves e and f), likely due to the multiplicative affinities of HOPS for Ypt7 and for vacuole lipids.

Does Ypt7 promote four-SNARE assembly simply by recruiting HOPS to the membrane, or is it a ligand that allosterically activates HOPS to assemble the four-SNARE complex? We compared four-SNARE assembly at different concentrations of HOPS and with different proteoliposome species. On proteoliposomes of VML composition that bore Ypt7, HOPS stimulated four-SNARE assembly at high or low HOPS concentrations (Figure 5B, red curves). Proteoliposomes of PC composition and/or lacking Ypt7 did not support HOPS-dependent four-SNARE assembly (Figure 5, A and B). We also compared HOPS binding to these proteoliposomes. Proteoliposomes were mixed with HOPS, incubated, and then floated through a density gradient (Orr *et al.*, 2017). The HOPS bound to proteoliposomes was quantified by immunoblot. The amount of HOPS bound to VML proteoliposomes bearing Ypt7 at 150 nM HOPS was less than the amount bound to VML protein-free liposomes or Ypt7/PC proteoliposomes at 300 nM HOPS (Figure 5C), yet the former had greater potency to support SNARE complex assembly (Figure 5B). Thus, Ypt7 and vacuolar lipids do not function merely to recruit HOPS to membranes for four-SNARE assembly but also activate HOPS to work with these soluble SNARE substrates.

HOPS and Ypt7 asymmetrically activate SNARE assembly on membrane-bound R- and Qa-SNAREs

To investigate the asymmetric fusion activity of Ypt7 with respect to the SNAREs (Figure 1), we prepared proteoliposomes of VML composition bearing Ypt7 and either the R- or Qa-SNARE and studied

with transmembrane (TM) anchors were replaced by their soluble counterparts sR or sQa (Figure 3A). The rate of four-SNARE complex assembly was also inhibited by high detergent concentration with classic surface dilution kinetics (Carman *et al.*, 1995), as the R- and Qa-SNAREs are less frequently localized to a common detergent micelle at high detergent concentrations (Figure 3B). Low detergent concentration also inhibited four-SNARE assembly (Figure 3B), as the SNAREs were no longer soluble. Mutual affinities among SNARE domains, though necessary, are not sufficient for meaningful rates of spontaneous SNARE complex assembly without concentrating the SNAREs in common micelles.

The dependence of spontaneous SNARE assembly on their concentration through limiting detergent micelles has a parallel for assembly on proteoliposomes as well. When full-length R and Qa were both present on the same proteoliposome, a four-SNARE complex formed spontaneously (Figure 4, red curve). When one or neither of these SNAREs was membrane bound, a four-SNARE complex formed far more slowly (Figure 4, blue, orange, and green curves). Similarly, if the R and Qa were membrane bound to different proteoliposomes, no four-SNARE complex was formed (Figure 4, black curve).
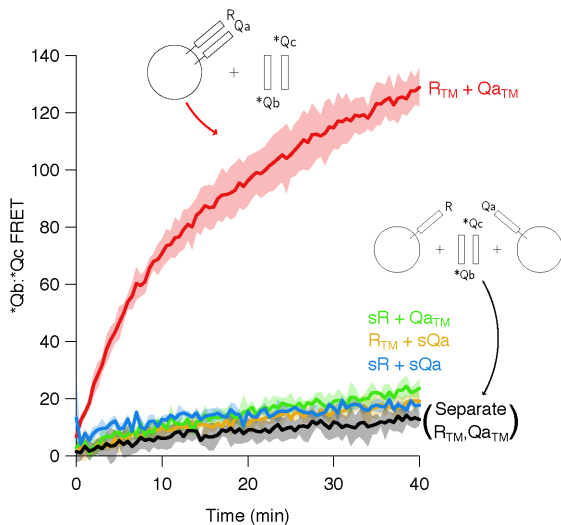
FIGURE 3: Coenrichment of full-length SNAREs in common detergent micelles is needed to promote SNARE complex assembly. (A) *Qb and *Qc (1 µM each) were mixed with 1 µM soluble (sR, sQa) or full-length transmembrane-anchored (TM) R and Qa in RB150/0.5% β-octylglucoside. (B) *Qb, *Qc, full-length R and Qa (1 µM each), and the final indicated concentrations of β-octylglucoside were mixed in RB150.

their capacity for SNARE assembly with our FRET assay. HOPS stimulated four-SNARE assembly without Ypt7 (Figure 6, A and B, filled vs. open red symbols). However, Ypt7 markedly stimulated HOPS-dependent four-SNARE assembly when both Ypt7 and R were bound to the same membrane (Figure 6A, blue vs. red filled symbols). In contrast, Ypt7 had almost no effect when Qa was membrane bound

FIGURE 4: SNARE complexes spontaneously form when R and Qa are on a common membrane. (A) *Qb and *Qc (1 µM each) were mixed with proteoliposomes (1 mM lipid each) bearing either R, Qa, both, or neither. Soluble versions of R and Qa (sR, sQa; 0.5 µM) were added to proteoliposomes not bearing that SNARE. Membrane-bound R and Qa bear the TM suffix here to emphasize the presence of their transmembrane hydrophobic anchor. The black curve contains two species of proteoliposomes, bearing either R or Qa (2 mM total lipid). For this figure and all subsequent figures involving proteoliposomes, the molar ratio to lipid for each protein is 1:2000 and the proteoliposomes are incubated with EDTA to nucleotide-exchange their Ypt7 to bear GTP before the start of the reaction (see *Materials and Methods*).

(Figure 6B, blue vs. red filled symbols). Thus, HOPS is asymmetrically activated for SNARE complex assembly by Ypt7, as it is highly active when R is in *cis* to Ypt7 but not when Qa is in *cis* to Ypt7.

Fusion requires the formation of complexes in *trans*. Would Ypt7 in *trans* to SNAREs exhibit the same behavior as in *cis*? As HOPS has affinities for Ypt7 (Seals *et al.*, 2000), vacuole lipids (Figure 5C), and SNAREs (Song *et al.*, 2020), we conducted reactions involving sequential component additions. Reactions began with HOPS and proteoliposomes bearing either R or Qa (Figure 7, A and B, respectively). Either soluble SNAREs (Figure 7, open symbols) or competing liposomes (closed symbols), which were either protein-free liposomes (Naked, red symbols) or Ypt7-proteoliposomes (blue symbols), were added after 10 minutes. The reaction was allowed to proceed for an additional 10 minutes before the remaining components, the competing Ypt7-proteoliposomes, naked liposomes, or soluble SNAREs, were added. HOPS supported SNARE complex assembly on R- or Qa-proteoliposomes, and the addition of naked liposomes had no effect (Figure 7, red symbols). Naked liposomes acted as negative controls for Ypt7-proteoliposomes as they have no effect on four-SNARE assembly alone (Figure 5). When the R-SNARE was membrane bound, proteoliposomes bearing Ypt7 blocked four-SNARE assembly, whether added before the SNAREs (Figure 7A, filled blue symbols) or after (open blue symbols). In contrast, when the Qa-SNARE was membrane bound, the addition of Ypt7 in *trans* had little effect (Figure 7B, blue vs. red symbols). Thus Ypt7 stimulates HOPS activity for four-SNARE assembly when the R-SNARE is present in *cis* but inhibits activity if the R-SNARE is in *trans*, whereas Ypt7 in *cis* or in *trans* has little effect on the capacity of HOPS to promote assembly of a four-SNARE complex with membrane-bound Qa. These data suggest an inherent hierarchy of functional associations of HOPS in the presence of vacuolar lipids, first with Ypt7 and R in *cis*, then with Qa in *trans*.
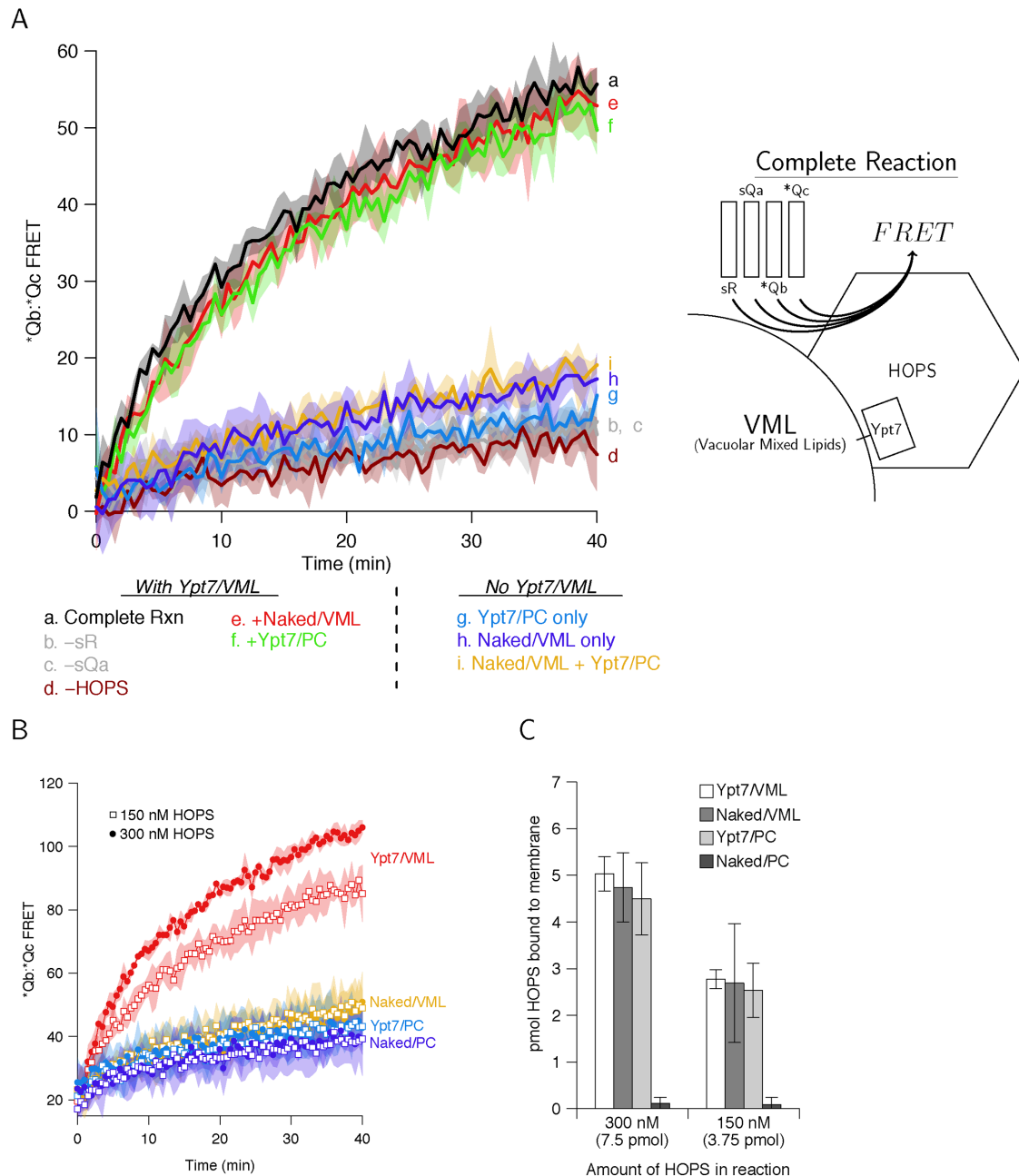
DISCUSSION

Biochemical studies are revealing a web of physical and functional interactions among membrane fusion proteins. For example, the neuronal 20S complex of fusion proteins includes SNAREs, NSF, and α-SNAP (Zhao *et al.*, 2015), and vacuolar HOPS has direct affinity for
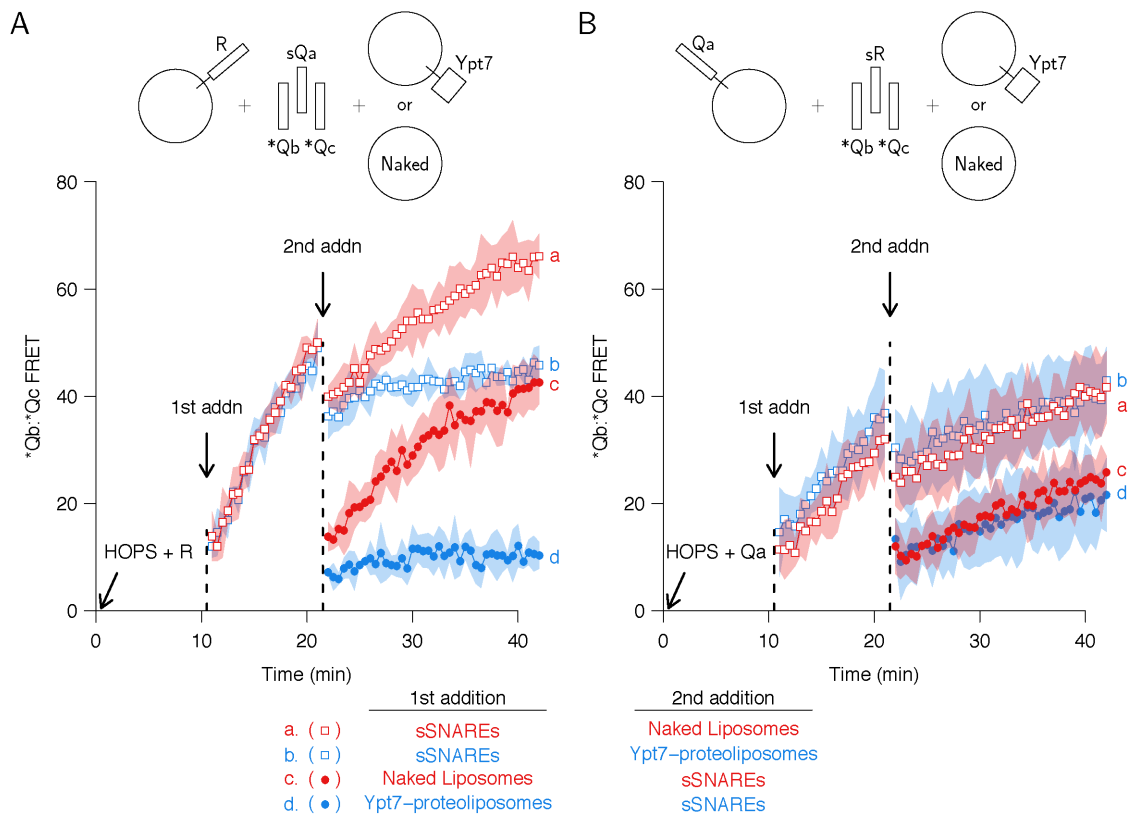
FIGURE 5: Ypt7 on liposomes of VML lipids activates HOPS for four-SNARE assembly. (A) Complete reaction: *Qb and *Qc (1 μM each) were mixed with 1 μM sR and sQa, 160 nM HOPS, and proteoliposomes (0.6 mM lipid) of VML composition bearing Ypt7. A schematic of the complete reaction is drawn on the right. Reactions b–d were missing sR, sQa, or HOPS as indicated. Reactions e and f contained an additional 0.6 mM of the indicated proteoliposomes. Reactions g–i contained each proteoliposome (0.6 mM lipid each) of the indicated composition in place of Ypt7/VML proteoliposomes. (B) *Qb and *Qc (1 μM) were mixed with 1.5 μM sR and sQa, proteoliposomes (1 mM lipid) of VML or PC composition and bearing Ypt7 or not, and HOPS diluted in HOPS buffer to 300 or 150 nM. (C) HOPS bound to liposomes was quantified as described (Orr *et al.*, 2017) with modifications. Briefly, liposomes (1 mM lipid) were nucleotide-exchanged in 20 μl, and then 5 μl of a solution of HOPS in HOPS buffer (Hickey and Wickner, 2010) was added. Samples were nutated for 40 min at room temperature, then a portion was floated through a Histodenz gradient (Orr *et al.*, 2017) by centrifugation (55,000 rpm, 4°C, 45 min, TLS55 rotor [Beckman Coulter]). Samples before and after flotation were solubilized in Thesit and analyzed for lipid content by rhodamine fluorescence. After normalizing for lipid concentration, samples were immunoblotted for Vps16 alongside a standard curve, and band intensities were quantified by UN-SCAN-IT v6.3 software.

phosphoinositides (Stroupe *et al.*, 2006), for each of the four SNAREs (Stroupe *et al.*, 2006; Baker *et al.*, 2015; Song *et al.*, 2020), and for two Ypt7s (Hickey and Wickner, 2010; Lürick *et al.*, 2017). Some Rab proteins have multiple effectors (Zerial and McBride, 2001), but

HOPS is the sole effector for the Rab Ypt7 for vacuolar fusion. The association between HOPS and Ypt7 on distinct membranes tethers the membranes (Hickey and Wickner, 2010), but is tethering the sole function of Ypt7? In such a model, tethering would bring HOPS into
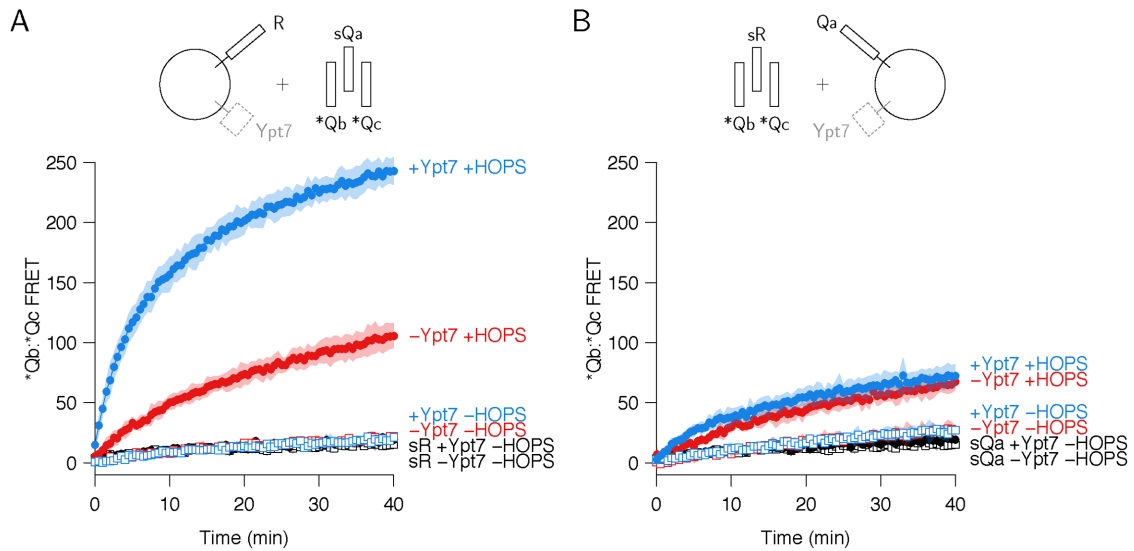
FIGURE 6: Ypt7 activates HOPS for four-SNARE assembly with membrane-bound R in *cis* but not with membrane-bound Qa. *Qb and *Qc (1 µM each) were mixed with proteoliposomes (1 mM lipid) bearing R (A), Qa (B), Ypt7 (blue), or no SNAREs (black) as indicated. Soluble versions of R and/or Qa (0.5 µM) were added when they were missing from the proteoliposomes. Reactions were given either 160 nM HOPS (filled symbols) or RB150 (open symbols). The black curves are with liposomes without bound SNAREs; therefore, the same curves are presented in A and B.

	1st addition
a. (□)	sSNAREs
b. (□)	sSNAREs
c. (●)	Naked Liposomes
d. (●)	Ypt7–proteoliposomes

	2nd addition
	Naked Liposomes
	Ypt7–proteoliposomes
	sSNAREs
	sSNAREs

FIGURE 7: Ypt7 prevents HOPS from working with R-SNARE in *trans* but not Qa-SNARE. Volume changes at each addition account for jumps in the FRET signal. Data is not shown for regions of the curves where fluorescent SNAREs were not present. HOPS (160 nM) was mixed with proteoliposomes (0.75 mM lipid) bearing (A) R-SNARE or (B) Qa-SNARE only. After 10 min, either *Qb, *Qc, and the missing sQa or sR, respectively (1 µM each), were added (open symbols), or proteoliposomes (0.75 mM lipid; filled symbols) bearing either Ypt7 (blue) or no protein (Naked, red) were added. After an additional 10 min, the proteoliposomes (open symbols) or soluble SNAREs (filled symbols) were added.

proximity with all four SNAREs, enhancing the capacity of HOPS to catalyze SNARE assembly, but the action of Ypt7 would be completed once tethering is done. If Ypt7 were only needed for tethering, its role would be symmetric with respect to the R- or Q-SNAREs on each membrane. Rather, we now show that Ypt7 and vacuolar lipids allosterically activate HOPS to assemble SNARE complexes (Figure 5), and that Ypt7 is needed asymmetrically with respect to the SNAREs, both for the activation of HOPS for four-SNARE assembly (Figures 6 and 7) and for fusion per se (Figure 1).

To study Ypt7-mediated activation of HOPS as a catalyst of SNARE assembly, we have adapted FRET assays of SNARE complex assembly (Weninger et al., 2003; Shin et al., 2014; Prinslow et al., 2019). With this assay, we find that SNARE domains are necessary, but not sufficient, for rapid SNARE complex assembly (Figures 3 and 5). SNAREs rely on their membrane anchor, whether localizing them to common micelles or to membranes, for spontaneous complex assembly. In experiments where the SNAREs are lacking their membrane anchor, HOPS can still catalyze SNARE complex assembly when it is allosterically activated by simultaneous associations with vacuolar lipids and Ypt7 (Figure 5). This activation is not explicable simply as concentrating the HOPS and SNAREs together through membrane tethering. Because the SNAREs are soluble in these experiments, tethering would not result in a greater concentration of SNAREs near HOPS. Tethering would also not explain the strikingly asymmetric activation of HOPS by Ypt7 and vacuolar lipids when a SNARE is membrane bound; Ypt7 strongly stimulates HOPS-dependent four-SNARE assembly if it is in cis to the R-SNARE (Figure 6A) but not if it is in cis to the Qa-SNARE (Figure 6B).

These findings are unexpected, as Rab proteins have been thought to merely recruit effector complexes such as HOPS to their target membranes and to provide membrane specificity. As HOPS is an asymmetric complex, it is plausible that Ypt7 activates HOPS by orienting it to bind to R-SNARE on the same membrane, but that this same action with Qa-SNARE on the membrane renders HOPS inactive. There is precedent for peripheral membrane proteins being catalytically activated by association with their integral membrane receptors. For example, the SecA subunit of bacterial preprotein translocase hydrolyzes ATP very slowly by itself, but association with specific membrane lipids, its integral SecYEG domain, and substrate strongly activates its ATP hydrolysis (Lill et al., 1990). Similarly, HOPS alone does not catalyze 4-SNARE assembly (Figure 5A; Hickey and Wickner, 2010) until it is activated by vacuolar lipids and Ypt7. There is also a precedent for tethering complexes to be activated by non-Rab GTPases. The Rho3/Cdc42 GTPases induce a conformational change in the exocyst complex, increasing its affinity many-fold for its cognate R-SNARE Snc2 (Rossi et al., 2020). Though unrelated to fusion, it has recently been shown that TORC2 (target of rapamycin complex-2) is directly activated by Rab proteins Vps21, Ypt52, and Ypt53 (Locke and Thorner, 2019).

In homotypic yeast vacuole fusion, both fusion partner membranes bear integral R and Qa. What can we infer about Ypt7-dependent HOPS-catalyzed trans-SNARE complex assembly on vacuoles? HOPS cannot be replaced for fusion by a synthetic tether unless the Q-SNAREs are preassembled, indicating that HOPS promotes functional four-SNARE complex assembly (Song and Wickner, 2019). Ypt7 on the same membrane as the R-SNARE must be GTP-bound for fusion to occur at physiological SNARE concentrations (Zick and Wickner, 2016). Thus, Ypt7(GTP)/R activation of HOPS may not only enhance four-SNARE assembly (Figure 6A), but also predispose HOPS to form functional complexes, presumably as parallel helical bundles (Weninger et al., 2003). However, four-

SNARE assembly is not favorable when Ypt7 and the R-SNARE are in trans (Figure 7A), likely due to a greater affinity of HOPS for Ypt7 than for the R-SNARE, and thus greatly reducing the HOPS interaction with the R-SNARE. Additionally, Ypt7 has little effect on the ability of HOPS to assemble SNARE complexes on membrane-bound Qa-SNARE, in cis (Figure 6B) or in trans (Figure 7B). Therefore, the asymmetric fusion behavior with respect to Ypt7 (Figure 1) appears to be purely due to a cis Ypt7/R activation of HOPS. In our working model, HOPS initially engages Ypt7, the lipids, and the R-SNARE on one membrane, and then engages the Q-SNAREs and Ypt7 in trans on the fusion partner membrane.

With this FRET assay, we have now gained deeper insight into the fundamental concepts of SNARE complex assembly, which can be summarized in three major points: 1) SNARE domains alone do not suffice for SNARE complex assembly, and either a) coenrichment in common detergent micelles (Figures 2 and 3), b) coenrichment in cis on common proteoliposomes (Figure 4), or c) coengagement by activated HOPS (Figure 5) is necessary. 2) HOPS is allosterically activated as a SNARE assembly catalyst by engagement with two ligands, the Rab Ypt7 and vacuolar lipids (Figure 5). 3) HOPS activation is asymmetric with respect to the SNARE that is in cis to the Ypt7 (Figure 6). We have purposely avoided studying trans-SNARE complexes and fusion due to several difficulties. Deeper illumination of the fusion pathway will require a more sensitive real-time assay of trans-SNARE complex and is thus not the focus of this article. Detecting these trans-SNARE complexes by FRET may be limited by the fact that trans-complexes only form between <5% of the vacuolar SNAREs in a reaction (Collins and Wickner, 2007). Additionally, fusion reconstitutions are optimally dependent on all the fusion factors when reconstitution is done with physiological ratios of SNARE:lipid, approximately 1:32,000 (Zick et al., 2014), which is too low for detection by current FRET methods. Other studies have succeeded in studying trans-SNARE complexes via FRET, but only by using high concentrations of SNAREs (Shin et al., 2014; Bae et al., 2013), or by employing single-molecule FRET microscopy (Weninger et al., 2003). In vacuole fusion, the function of Ypt7 is bypassed at high SNARE concentrations (Figure 6 of Zick and Wickner, 2016) and thus cannot be studied in this manner.

Is the activation of HOPS by its cognate Rab a model for all the Rabs of vesicular traffic and their effectors? The CORVET complex is homologous to HOPS and also has a Vps33 subunit (Paplowska et al., 2007); it will likely have the same features when interacting with its Rab Vps21. Sec1, the plasma membrane SM protein, is not an integral part of the exocyst, but binds to it (Morgera et al., 2012). It therefore would not be surprising if Sec1, to mediate SNARE assembly at the plasma membrane, is activated through association with the exocyst bound to its Rab Sec4. Rab5-dependent fusion of endosomes depends on multiple Rab5-effectors and has been reconstituted with purified components (Ohya et al., 2009), but it is unclear whether this Rab allosterically activates those effectors or solely acts through binding and localizing them. Nevertheless, it has been shown that these Rab5 effectors do activate the TORC2 complex directly (Locke and Thorner, 2019).

MATERIALS AND METHODS
Protein purification
Full-length Nyv1 (R) was purified and prepared in RB150 (20 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 10% [vol/vol] glycerol) with 1% β-octylglucoside as described (Mima et al., 2008; Schwartz and Merz, 2009; Zucchi and Zick, 2011). Full-length Vam3 (Qa) with a his$_6$ tag and a 3C site was purified as described (Izawa et al., 2012). Soluble Nyv1 (Thorngren et al., 2004) and soluble Vam3

(Song and Wickner, 2017) were purified with a GST tag and a TEV site as described, then cleaved with TEV protease and buffer exchanged into RB150 before use. Vam7 (Qc), Sec17 (Schwartz and Merz, 2009), Sec18 (Haas and Wickner, 1996), and HOPS (Hickey and Wickner, 2010) were purified as described. The SNARE domain of Vti1 (Qb) with an MBP tag, TEV site, and inserted cysteine was purified as described (Song and Wickner, 2017). The SNARE domain of Vti1 with a unique N-terminal cysteine was purchased from New England Peptide (Gardner, MA). GST-tagged Vam7 (Fratti *et al.*, 2007) and Ypt7 with a transmembrane anchor (Song and Wickner, 2019) were purified as described.

The single cysteine of Vam7 was derivatized with Oregon Green 488 maleimide, and the single cysteine of the MBP-Vti1 construct was derivatized with Alexa Fluor 568 maleimide (each fluorescent maleimide from Thermo Fisher Scientific, Waltham, MA) in a similar manner. Vam7 was first gel filtered in RB500 (RB150 but with 500 mM NaCl) with Sephadex G-50 Fine from GE Healthcare Life Sciences (Pittsburgh, PA) to remove dithiothreitol, then concentrated to 50–100 μM with Amicon Ultra 0.5 ml, 30K filters (Millipore, Tullagreen, Ireland). Approximately 10-fold molar excess of tris(2-carboxyethyl)phosphine and of the corresponding fluorescent maleimide were added to Vam7 and Vti1 and incubated overnight (at least 18 h) at room temperature in the dark. Maleimide concentrations were at least 0.9 mM for all preparations. Derivatization reactions were stopped with a 3-fold molar excess of cysteine over maleimide, and then the samples were gel filtered through Sephadex G-50 Fine (equilibrated with RB500 for Vam7, RB150 for Vti1) to separate the SNAREs from cysteine. Derivatized SNAREs were analyzed alongside underivatized SNAREs via SDS–PAGE/Coomassie-staining. Derivatized SNARE preparations showed a single band in a location distinct from the underivatized SNAREs, confirming that the SNAREs were completely derivatized.

Proteoliposome preparation

Most lipids were purchased from Avanti Polar Lipids (Alabaster, AL), with the exceptions of ergosterol from Sigma-Aldrich (St. Louis, MO), PI(3)P from Echelon Biosciences (Salt Lake City, UT), and Rhodamine-DHPE from Life Technologies (Carlsbad, CA). Biotin-*R*-phycoerythrin and streptavidin were purchased from Thermo Fisher Scientific, and Cy5-streptavidin was purchased from SeraCare Life Sciences (Milford, MA).

Proteoliposomes were prepared with four different lipid compositions. PC liposomes contained only 18:2 PC (1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine). Liposomes of VML composition (Mima *et al.*, 2008; Zick and Wickner, 2016) contained lipids in the following proportions: 47.6% 18:2 PC, 18% 18:2 PE (1,2-dilinoleoyl-*sn*-glycero-3-phosphoethanolamine), 18% Soy PI (L-α-phosphatidylinositol [Soy]), 8% ergosterol, 4.4% 18:2 PS (1,2-dilinoleoyl-*sn*-glycero-3-phospho-L-serine), 2% 18:2 PA (1,2-dilinoleoyl-*sn*-glycero-3-phosphate), 1% diacylglycerol (1,2-dipalmitoyl-*sn*-glycerol), and 1% PI(3)P (1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1′-myo-inositol-3′-phosphate)). Rh-DHPE containing versions of these two liposomes contained 1% Rh-DHPE and 1% less 18:2 PC.

Proteoliposomes were reconstituted in RB150 with 1 mM MgCl$_2$ by detergent dialysis as described (Zick and Wickner, 2013) with modifications. Proteoliposomes for fusion assays were prepared as described (Zick *et al.*, 2014) with SNARE:lipid ratios of 1:2000 and Ypt7:lipid ratios of 1:8000. To prepare proteoliposomes for SNARE assembly assays, lipids dissolved in chloroform were mixed with β-octylglucoside in methanol at a molar ratio of 1:12.5. This mixture was dried by a stream of nitrogen and then by speed-vac. Pellets were dissolved in 4-fold concentrated RB150/Mg to yield mixed

micellar solutions with 7.5 mM lipid. Aliquots (160 μl) of mixed micelles were mixed with SNAREs and Ypt7 at protein:lipid ratios of 1:2000 as indicated in figure legends to yield a total volume of 640 μl. The protein–lipid mixture was nutated for approximately 10 min, then added to 25 kDa cutoff, 12 mm flat width dialysis tubing (Spectrum Labs, Rancho Dominguez, CA). The tubing was knotted and dialyzed overnight (at least 18 h) at 4°C against 100 ml RB150/Mg and 0.6 g Biobeads SM-2 (Biorad, Hercules, CA) per mixture with continuous stirring. Proteoliposomes were then harvested after floatation through a Histodenz gradient (Zick and Wickner, 2013). Lipid phosphate was assayed, and samples were brought to a final concentration of 3–5 mM lipid in RB150/Mg before aliquots were frozen in liquid nitrogen.

SNARE assembly assays

Reactions (20 μl) were prepared as described in figure legends and performed in 384-well Corning black plates at 27°C in a SpectraMax Gemini XPS (Molecular Devices, Sunnyvale, CA) fluorescence plate reader with fluorescence recorded at intervals of 30 s. Automix was set to 1 s between reads, and read priority was set to column priority. Three channels were read simultaneously: Oregon Green 488 fluorescence (excitation [ex]: 497 nm; emission [em]: 527 nm; cutoff [c/o]: 515 nm), Alexa Fluor 568 fluorescence (ex: 568 nm; em: 605 nm; c/o: 590 nm), and FRET (ex: 490 nm; em: 615 nm; c/o: 590 nm). Bleedthrough ratios of Oregon Green 488 and Alexa Fluor 568 into the FRET channel were obtained by measuring *Qb or *Qc alone in the appropriate buffer, then dividing the raw FRET value by the raw value of the corresponding single fluorescence channel. For example, the ratio for *Qc was typically 0.0385, meaning that every fluorescence unit detected in the Oregon Green 488 channel contributed 0.0385 units of signal to the FRET channel. FRET values as reported were obtained by multiplying the bleedthrough ratio by the signal from the fluorescence channels for each fluorophore per reaction, and then subtracting those values from the raw FRET value. Small differences (<0.0010) in the bleedthrough ratio shifted the background value by as much as 20 units between experiments but otherwise had little effect on the data. All experiments contained 1 μM each of *Qb and *Qc in 20 μl. At this concentration, the fluorophores contributed about 350 units of background to the FRET channel, though the noise itself remains small (as evidenced by the standard deviations in each figure).

All proteoliposomes were first incubated in a mixture of GTP and EDTA in RB150 for 10 min at 27°C, then MgCl$_2$ was added to complete GTP exchange. This incubation was performed whether or not Ypt7 was present. Final concentrations were always 20 μM GTP, 1 mM EDTA, and enough MgCl$_2$ to achieve 0.5 mM unchelated Mg, regardless of proteoliposome concentration.

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