Sec18 binds the tethering/SM complex HOPS to engage the Qc-SNARE for membrane fusion

Amy Orr and William Wickner*
Department of Biochemistry and Cell Biology, Geisel School of Medicine at Dartmouth, Hanover, NH 03755-3844

ABSTRACT **Membrane fusion is regulated by Rab GTPases, their tethering effectors such as HOPS, SNARE proteins on each fusion partner, SM proteins to catalyze SNARE assembly, Sec17 (SNAP), and Sec18 (NSF). Though concentrated HOPS can support fusion without Sec18, we now report that fusion falls off sharply at lower HOPS levels, where direct Sec18 binding to HOPS restores fusion. This Sec18-dependent fusion needs adenine nucleotide but neither ATP hydrolysis nor Sec17. Sec18 enhances HOPS recognition of the Qc-SNARE. With high levels of HOPS, Qc has a Km for fusion of a few nM. Either lower HOPS levels, or substitution of a synthetic tether for HOPS, strikingly increases the Km for Qc to several hundred nM. With dilute HOPS, Sec18 returns the Km for Qc to low nM. In contrast, HOPS concentration and Sec18 have no effect on Qb-SNARE recognition. Just as Qc is required for fusion but not for the initial assembly of SNAREs in *trans*, impaired Qc recognition by limiting HOPS without Sec18 still allows substantial *trans*-SNARE assembly. Thus, in addition to the known Sec18 functions of disassembling SNARE complexes, oligomerizing Sec17 for membrane association, and allowing Sec17 to drive fusion without complete SNARE zippering, we report a fourth Sec18 function, the Sec17-independent binding of Sec18 to HOPS to enhance functional Qc-SNARE engagement.**

SIGNIFICANCE STATEMENT

- Sec18 (NSF) has been thought to only disassemble SNAREs when powered by ATP hydrolysis, and to only do this via its association with Sec17 (SNAP).
- The authors found that Sec18 binds the tethering/SM complex HOPS without Sec17 and enhances the membrane fusion system's affinity for the Qc SNARE without need for ATP hydrolysis.
- Discovery of this activity for Sec18 will lead to an examination of the functions of Sec18 in each membrane fusion event in organisms from yeast to humans.

Monitoring Editor
Elizabeth Miller
MRC Laboratory of Molecular Biology

Received: Feb 6, 2024
Revised: Mar 18, 2024
Accepted: Mar 22, 2024

New Hypothesis

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E24-02-0060) on March 27, 2024.

*Address correspondence to: William Wickner (Bill.Wickner@Dartmouth.edu).

Abbreviations used: HOPS, homotypic fusion and vacuole protein sorting; NSF, N-ethylmaleimide-sensitive fusion protein; Qa, the Qa-SNARE Vam3; Qb, the Qb-SNARE Vti1; Qc, the Qc-SNARE Vam7; R, the R-SNARE Nyv1; SNAP, soluble NSF-attachment protein; SNARE, soluble NSF-attachment protein receptor; VML, vacuolar mixed lipids.

INTRODUCTION

Membrane fusion on the endocytic and exocytic pathways is essential for cell compartmentation and growth, hormone secretion, and neurotransmission. The catalysts and mechanisms of fusion are conserved from yeast to humans. Rab family GTPases mark organelles and trafficking vesicles (Hutagalung and Novick, 2011). Rabs bind tethers which link membranes (Baker and Hughson, 2015). Sec1/Munc18 (SM family) proteins catalyze the assembly of SNARE proteins anchored to each fusion partner into four-helical coiled coils structures, the *trans*-SNARE complex (Parisotto *et al.*, 2014; Baker *et al.*, 2015; Orr *et al.*, 2017; Jiao *et al.*, 2018;

Stepien *et al.*, 2022). SNAREs are integral or peripheral membrane proteins with N-domains, SNARE domains, short but crucial juxtamembrane domains, and often C-terminal membrane anchor domains (Jahn and Scheller, 2006). SNAREs are in four conserved families: R, Qa, Qb, and Qc, with heptad-repeat apolar residues throughout their canonical SNARE domains (Fasshauer *et al.*, 1998). Though individual SNARE domains are random coil, they assemble into four-SNARE RQaQbQc complexes, zippering together in the N to C direction (Gao *et al.*, 2012) and converting each SNARE domain into α-helices as zippering proceeds (Sorensen *et al.*, 2006). SNARE zippering is driven by burying the heptad-repeat apolar residues of each SNARE domain into the interior of the four-helical bundle and away from water, allowing greater aqueous hydrogen bonding. SNARE complexes are in *cis* when each SNARE is anchored to the same membrane and in *trans* when the R-SNARE is anchored to one membrane and Q-SNAREs are bound to its tethered fusion partner. The initiation of *trans*-SNARE zippering is catalyzed by Sec1/Munc18 (SM) family proteins exploiting conserved binding sites for the R and Qa SNARE domains (Baker *et al.*, 2015; Stepien *et al.*, 2022). *trans*-SNARE complexes become *cis* upon membrane fusion, and these are disassembled by the combined action of SNARE-associated Sec17/SNAP with its bound Sec18/NSF ATPase (Söllner *et al.*, 1993; Cipriano *et al.*, 2013).

SEC17 and SEC18 were among the earliest genes identified in the classical screen for yeast temperature-sensitive mutants in the secretory pathway (Novick *et al.*, 1980). Their mammalian homologues SNAP and NSF (Griff *et al.*, 1992) were the earliest organelle fusion proteins purified with the assay of protected lumenal mixing of organelle contents. NSF was purified as a soluble protein needed for fusion (Block *et al.*, 1988) and SNAP supports its membrane association (Weidman *et al.*, 1989). Membrane-bound NSF and SNAP can be solubilized in detergent and isolated as a 20s particle (Söllner *et al.*, 1993). Isolation of the particle through an affinity tag on NSF allowed ATP-driven 20s particle disassembly, freeing the integral membrane **SNA**P **re**ceptor**s**, termed SNAREs (Söllner *et al.*, 1993). NSF and SNAP were thought to constitute an ATP-driven machine for membrane fusion, but studies with the fusion of isolated yeast vacuoles showed that fusion occurred after an early step of ATP-dependent release of Sec17/SNAP (Mayer *et al.*, 1996). Reconstitution studies (Weber *et al.*, 1998) showed that SNAREs alone could mediate fusion by zippering membranes together and perturbing them through bilayer-disrupting juxtamembrane domains (Rathore *et al.*, 2019). Sec17/SNAP and Sec18/NSF are required to disassemble the postfusion cis-SNARE complexes for subsequent fusion rounds. The roles of SNAREs to drive fusion and of Sec18/NSF and Sec17/SNAP for disassembly seemed clear, apparently acting in different parts of the membrane fusion pathway. However, as reported earlier for Sec17 (Schwartz and Merz, 2009; Zick *et al.*, 2015; Schwartz *et al.*, 2017; Song *et al.*, 2017; Song *et al.*, 2021) and described here for Sec18, both Sec17 and Sec18 have distinct and dramatic effects earlier in the fusion pathway.

Membrane fusion has received extensive study with vacuoles from *Saccharomyces cerevisiae*. Vacuoles undergo constant fission into smaller vacuoles and fusion to form large, often singular vacuoles. Vacuole fusion has been studied in vivo (Wada *et al.*, 1992), in vitro with the purified organelle (Haas *et al.*, 1994; Ungermann *et al.*, 1998a), and as reconstituted with defined lipids and pure recombinant proteins (Sato and Wickner, 1998; Mima *et al.*, 2008; Zick and Wickner, 2016). Fusion relies on a Rab (Ypt7), four SNAREs (R (Nyv1), Qa (Vam3), Qb (Vti1), and Qc (Vam7), referred to hereafter as simply: R, Qa, Qb, and Qc), the hexametric 660 kDa protein HOPS, Sec17, and Sec18. HOPS is comprised of single copies of Vps11, Vps16, Vps18, Vps33, Vps39, and Vps41 (Seals *et al.*, 2000; Wurmser *et al.*, 2000). Vps33 is the SM protein of the vacuole, while both Vps39 and Vps41 have direct affinity for Ypt7 (Brett *et al.*, 2008; Hickey and Wickner, 2010; Bröcker *et al.*, 2012). HOPS has direct affinity for each of the four SNAREs (Stroupe *et al.*, 2006; Baker *et al.*, 2015; Song *et al.*, 2020), for phosphoinositides (Stroupe *et al.*, 2006), for Ypt7 (Seals *et al.*, 2000), and for Sec17 (Song *et al.*, 2021). HOPS tethers two membranes by its affinity for their Rab Ypt7 (Stroupe *et al.*, 2006; Hickey and Wickner, 2010). When HOPS interacts with Ypt7, with R-SNARE on the same membrane, and with the lipids PI and PI3P, it is activated to catalyze the assembly of the Q-SNAREs in *trans* with this R (Torng *et al.*, 2020; Torng and Wickner, 2021). HOPS renders fusion resistant to Sec17/Sec18 (Mima *et al.*, 2008; Xu *et al.*, 2010; Song and Wickner, 2019). Mutations within the HOPS Vps33 subunit still allow it to tether membranes but increase the fusion Km for the Qc SNARE and remove the capacity of HOPS to render fusion resistant to Sec17/Sec18 (Baker *et al.*, 2015).

The proteins catalyzing fusion have multiple overlapping affinities. Sec17 has affinity for SNAREs, Sec18, HOPS, membrane lipid, and for other Sec17 molecules in the same complex with SNAREs. SNAREs have affinities for each other as well as for Sec17 and HOPS. HOPS can directly bind to two Ypt7 molecules, to each SNARE, to Sec17, and to phoshatidylinositol-3-phosphate. Each is enriched in an interdependent manner in ring-shaped structures surrounding docked vacuole membranes (Wang *et al.*, 2002; Fratti *et al.*, 2004). The catalysts of vacuole homotypic fusion have been purified and reconstituted in functional form (Mima *et al.*, 2008; Stroupe *et al.*, 2009). The structure of each fusion protein has been determined, including most recently HOPS (Shvarev *et al.*, 2022). Current efforts are focused on the sequence of fusion protein interactions and how these proteins work to give efficient and regulated fusion.

Schwartz and Merz (2009) showed with purified vacuoles that partial C-terminal truncation of the yeast vacuolar Qc SNARE caused an arrest of SNARE zippering, and made the pioneering discovery that the blocked fusion was restored by adding Sec17. Subsequent studies with reconstituted proteoliposomal fusion confirmed and extended these findings (Zick *et al.*, 2015; Schwartz *et al.*, 2017; Song *et al.*, 2017, 2021). SNAREs can be altered in several ways near their C-terminii to block fusion: shortening one or several Q-SNAREs in their SNARE domains by C-terminal deletion of several heptad repeats (Schwartz and Merz, 2009; Song *et al.*, 2020), conversion of the Qa SNARE domain apolar amino acyl residues to polar (Song *et al.*, 2021), swap of the juxtamembrane region between R and Qa SNAREs (Orr *et al.*, 2022), or having Qb as the sole anchored Q-SNARE (Wickner *et al.*, 2023). In each case, the block is bypassed by Sec17, and Sec18 addition only lowers the Sec17 Km (Orr and Wickner, 2022). This novel activity of Sec18 requires ATP, but nonhydrolyzable ATP analogues function just as well. This is in striking contrast to the SNARE disassembly activity of Sec18, which strictly requires ATP hydrolysis. Several Sec17 molecules, bound to Sec18, will stably bind to membrane bilayers, enriching the local Sec17 concentration to enhance SNARE association (Orr and Wickner, 2022). However, when a hydrophobic membrane-anchor sequence was added to the N-terminus of Sec17 and this recombinant form was assembled into proteoliposomes along with the Rab and SNAREs, the Sec17 stimulation of fusion still required Sec18 and ATP or ATPγS (Song *et al.*, 2017). Thus, Sec18 function extends beyond delivery of Sec17 to the membrane.

We now report that rapid fusion at low HOPS concentrations needs Sec18. This novel Sec18 role is specific for HOPS, because

Sec18 does not enhance fusion with limiting levels of a nonspecific tether. Sec18 binds directly to HOPS, and its support of HOPS-dependent fusion does not need Sec17 or ATP hydrolysis. Sec18 has dramatic effects on the functional recognition of the Qc-SNARE. With abundant HOPS, the fusion Km for Qc is a few nanomolar, while the Qc Km is several hundred nanomolar with either limiting HOPS or with a nonspecific tether. Sec18 restores fusion with limiting HOPS to a Qc Km in the nanomolar range. In contrast to these effects on Qc-SNARE recognition, the Km for Qb-SNARE is unaffected by the HOPS concentration or by Sec18. Just as *trans*-SNARE complex assembles in the absence of Qc SNARE without supporting fusion (Song *et al.*, 2020), impaired Qc-SNARE recognition due to limiting HOPS without Sec18 still allows substantial *trans*-SNARE complex formation without proportional fusion.

Limiting the concentrations of proteins which catalyze fusion has revealed successive layers of catalysis and regulation. Extremely high SNARE concentrations alone will mediate fusion (Weber *et al.*, 1998; Fukuda *et al.*, 2000), but at lower SNARE levels HOPS becomes essential for fusion (Mima *et al.*, 2008), and the Rab Ypt7 becomes necessary with physiologically low SNARE levels (Stroupe *et al.*, 2009; Zick and Wickner, 2016). Limiting SNARE zippering or SNARE anchoring reveals Sec17-driven fusion, and lowering HOPS levels now reveals a novel function for Sec18 which is independent of Sec17.

RESULTS

To study the effects of Sec17 and Sec18 on fusion with HOPS or with a synthetic tether, we prepared proteoliposomes from mixed micellar solutions of vacuole-mimic lipids, detergent, the vacuolar Rab, the R-SNARE or the three mixed Q-SNAREs, and either of two fluorescent proteins, biotinylated phycoerythrin or Cy5-labeled streptavidin. The proteoliposomes which formed after dialysis were isolated by floatation and bear the Rab, the R or Q SNAREs, and entrapped lumenal fluorescent protein. In mixtures of these proteoliposomes, the fluorescent proteins are initially separated by at least the thickness of two lipid bilayers, too far for FRET (fluorescent resonance energy transfer). Fusion and the attendant lumenal compartment mixing allows the biotin to bind streptavidin, bringing the attached Cy5 and phycoerythrin fluorophores into intimate proximity and generating a strong FRET signal as a measure of fusion (Zucchi and Zick, 2011). To prevent spurious signal from background lysis, fusion assays have a large excess of extralumenal nonfluorescent streptavidin. When the three Q-SNAREs are preassembled during proteoliposome preparation, bypassing the need for SNARE assembly catalysis by an SM protein or SM protein complex such as HOPS (Song and Wickner, 2019), fusion only needs a tether and the R and Q SNAREs on complementary proteoliposomes (Song and Wickner, 2019; Orr and Wickner, 2022). HOPS fulfills this function. Fusion between Ypt7/R and Ypt7/QaQbQc proteoliposomes is supported by 50 nM HOPS (Figure 1, open circles). It is modestly stimulated by Sec18 (filled circles), and Sec17 can further stimulate.

Spontaneous SNARE assembly yields are unaffected by Sec17/Sec18

Because Sec17 and Sec18 can suppress or activate fusion (Mima *et al.*, 2008), we asked whether they simply alter the capacity of SNAREs for uncatalyzed self-assembly. R, GST-Qa, Qb, and Qc, the four purified recombinant vacuolar SNAREs, were co-incubated for 1 hour in mixed micellar solution with β-octylglucoside. The GST-tagged Qa SNARE was then isolated with glutathione beads, and after washing the beads the other three SNAREs were seen to have assembled with GST-Qa into a stable complex (Figure 2, lane 2; lane 1 lacked GST-Qa). Sec17 associated with the four-SNARE bundle (lanes 3–7), but its addition had no effect on the yield of assembled complex (lanes 3–5), even when each of the four SNAREs had been separately preincubated with Sec17 for 30 min before they were mixed with each other (lanes 6 and 7). More Sec18 associated stably with SNAREs when Sec17 was also present (lanes 9–12), but Sec18 had little or no effect on the spontaneous, uncatalyzed yield of stable SNARE complexes in detergent mixed micellar solution. These findings contrast with those seen when SNAREs are membrane-bound. Sec17 and Sec18 strongly suppress the *trans*-association of membrane-anchored R and Q SNAREs when their liposomes are incubated with the synthetic tether GST-C1b (Wickner *et al.*, 2023), suggesting that there are distinct, important constraints on the assembly in *trans* of membrane-anchored SNAREs.

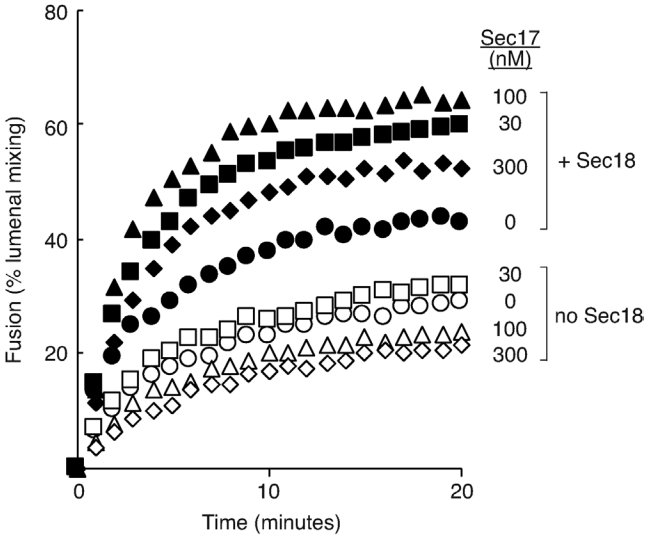

FIGURE 1: Sec18 stimulates proteoliposome fusion. Proteoliposomes were prepared as described in *Materials and Methods* with vacuolar lipids, Ypt7-tm, and either R or QaQbQc SNAREs. Fusion assays were performed as described in *Materials and Methods* with 50 nM HOPS, 1 mM Mg:ATPγS, the indicated concentrations of Sec17, and either without Sec18 or with 50 nM Sec18. The assay was performed three times. Statistical analysis of initial rates of fusion across the three repeats is in Supplemental Figure S1.

Sec18-dependent fusion without Sec17

Because we consistently observe a small stimulation of fusion by Sec18 alone under our standard fusion conditions (Figure 1A; Song *et al.*, 2017), we systematically varied the assay conditions to optimize the dependence of fusion on Sec18. With Ypt7/R and Ypt7/QaQbQc proteoliposomes, we saw little fusion at 2.5 to 10 nM HOPS unless Sec18 was present (Figure 3A; red bars with no Sec18, hatched or filled bars with Sec18; concentrations of Sec18 throughout refer to the hexamer). HOPS has been measured at 2000 copies per cell (Ho *et al.*, 2018), and cell volume estimates are from 67 to 309 fl, varying with ploidy, growth media, temperature, and cell cycle (Uchida *et al.*, 2011; Zakhartsev and Reuss, 2018). HOPS is thus present in cells at 10–50 nM, the range studied here. A similarly significant fusion stimulation by Sec18 in the presence of 5–20 nM HOPS was seen for Ypt7/QaQb proteoliposomes with added Qc (Figure 3B). By 50 nM HOPS, Sec18 gave a more modest stimulation of fusion (Figure 3B), as seen when all three Q-SNAREs had been preassembled during proteoliposome preparation (Figures 1 and 3A).

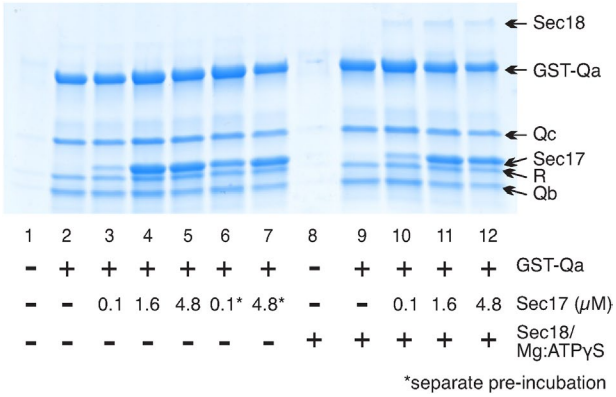

FIGURE 2: Neither Sec17 nor Sec18 affect the yield of 4-SNARE complexes in detergent mixed micellar solution. SNARE assembly incubations (50 µl) were performed (1 h, on ice) in buffer B (20 mM HEPES/NaOH, pH 7.4, 0.15 M NaCl, 10% (vol/vol) glycerol, 0.1 M β-octylglucoside, 20 mM imidazoleCl, 1 mM $MgCl_2$) with 4 µM each of GST-Qa, and wild-type untagged R, Qb, and Qc. Where indicated, Sec17 (0.1, 1.6, or 4.8 µM), Sec18 (50 nM), and Mg:ATPγS (1 mM) were added. For lanes 6 and 7, each of the four SNAREs were first mixed with the indicated concentration of Sec17, incubated for 30 min on ice, then mixed with each other. For all 12 samples, a 45 µl portion was mixed with glutathione resin prewashed with buffer B, nutated for 1 h at 4°C, then pulse-centrifuged to pellet the resin. Resin pellets were suspended four times in 0.5 ml buffer B, then collected by pulse centrifugation. Finally, liquid was withdrawn with a fine-tip pipette and the resin was dispersed in 25 µl of SDS–PAGE sample buffer, heated to 95°C for 4 min, and centrifuged to pellet the resin. Samples were analyzed by colloidal Coomassie SDS–PAGE. The assay was performed in triplicate and analysis of protein band quantification can be found in Supplemental Figure S2.

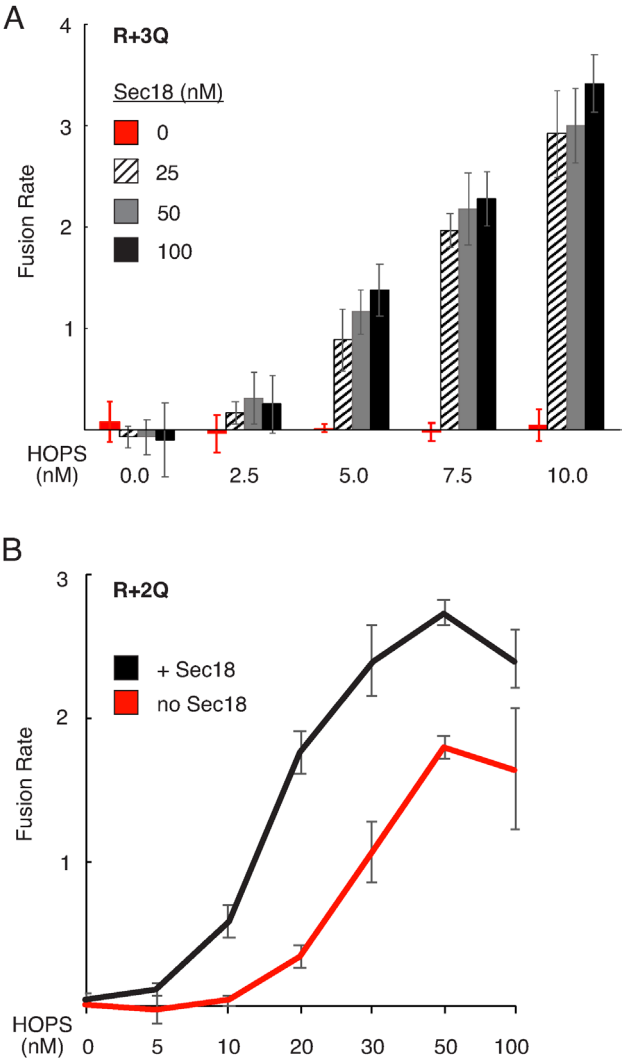

FIGURE 3: Sec18 strongly stimulates fusion at limiting HOPS concentrations. (A) Fusion of Ypt7/R and Ypt7/QaQbQc proteoliposomes was assayed with the indicated concentrations of HOPS and Sec18. All incubations had 1 mM Mg:ATPγS. The assay was done in triplicate, and initial rates of fusion were obtained by subtracting the per cent lumenal mixing at min 1 from that at min 6 and dividing by five. A typical full kinetics of fusion is shown in Supplemental Figure S3A. (B) As in part A, but with Ypt7/QaQb proteoliposomes, 100 nM Qc, and 25 nM Sec18 where indicated. The initial rates of fusion are shown, between min 1 and min 6, and were averaged across three replicate experiments. Typical fusion kinetics are shown in Supplemental Figure S3B.

Direct affinity of HOPS and Sec18

To test whether the synergy between Sec18 and HOPS for fusion might reflect their direct interaction, Sec18 and/or HOPS were incubated with either liposomes or with proteoliposomes, followed by (proteo)liposome floatation through a density gradient and assay of the bound Sec18 and HOPS (Figure 4). As reported (Song *et al.*, 2020), HOPS does not bind to liposomes of phosphatidylcholine, and neither does Sec18 (Figure 4A, lanes 1 and 5). Sec18 has direct affinities for 3Q-SNARE or 4-SNARE complexes (Figure 4A, lanes 2 and 3) even though Sec17 is absent, as expected from the modest capacity of Sec18 alone to disassemble SNARE complexes (Zick *et al.* (2015); Supplemental Figure S12). Sec18 also has direct affinity for Ypt7 (Figure 4A, lane 4). HOPS, bound to membranes by its direct affinity for Ypt7, supports substantial Sec18 binding (Figure 4A, lane 8).

In addition to the affinity of HOPS for Ypt7 (Figure 4A, lane 8; Seals *et al.*, 2000), HOPS binds to membranes through its affinity for acidic lipids, most notably phosphatidylinositol-3-phosphate (PI3P) which is present in vacuolar mixed lipids (VML; Figure 4B, lane 1; Stroupe *et al.*, 2006). Sec18 does not bind to VML liposomes (Figure 4B, lane 2), but binds to these liposomes in the presence of HOPS (Figure 4B, lane 3), establishing a direct HOPS:Sec18 association. As seen with liposomes of only PC (Figure 4A, lanes 1 vs. 4), Sec18 also has direct affinity for Ypt7 in the presence of VML lipids (Figure 4B, lanes 2 vs. 5).

Sec18 fusion stimulation is specific for HOPS

Because either HOPS or the synthetic tether GST-C1b support comparable fusion of proteoliposomes when the three Q-SNAREs had been preassembled (Wickner *et al.*, 2023), and Sec18 stimulates HOPS-mediated fusion at low HOPS concentrations (Figure 3A), we asked whether Sec18 would also stimulate fusion when the synthetic tether GST-C1b was at limiting concentrations. At each level of GST-C1b, Sec18 does not stimulate fusion (Figure 5), indicating that Sec18 fusion stimulation is specific to HOPS rather than simply to a limited level of tethering. Sec18 inhibition of fusion with GST-C1b tethering might reflect Sec18 direct binding to the SNAREs (Figure 4A, lanes 1–3).

Sec18 restoration of fusion is independent of Sec17

Sec17 enhances over 100-fold the capacity of Sec18 to disassemble *cis*-SNARE complexes for subsequent fusion (Vivona *et al.*, 2013;

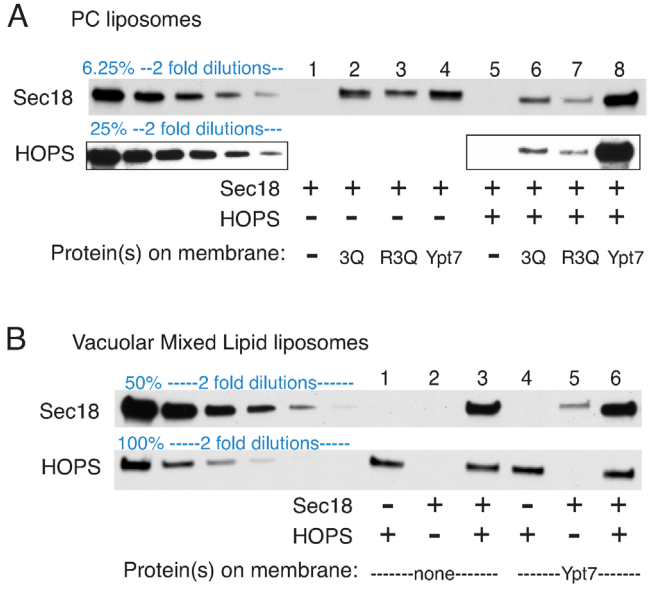

FIGURE 4: Sec18 binds directly to HOPS, as well as to the SNAREs and Ypt7. (A) Proteoliposomes of PC + 1% Rhodamine-DHPE were prepared as described in *Materials and Methods*, bearing either no membrane proteins, SNARE proteins at 1:2000 protein to lipid ratios, or Ypt7-tm at 1:4000 protein to lipid ratio. Assays of HOPS and Sec18 binding to [proteo]liposomes were as described in *Materials and Methods*, with 30 µl reactions receiving 1 mM ATP and 83 nM Sec18 followed by 500 nM HOPS, where added. (B) Proteoliposomes of vacuolar mimic lipid were prepared as described, bearing 1% Rhodamine-DHPE and either no membrane proteins, or Ypt7-tm at 1:2000 protein to lipid ratio. Binding assays were in 20 µl, with 1 mM Mg:ATP, and with final additions of 400 nM HOPS and/or 67 nM Sec18. HOPS was added before Sec18 when both were present. With the smaller volume reaction, the amount of 54% histodenz added was reduced to 60 µl, and the amount of sample placed in the tube and harvested from the tube was also adjusted to 60 µl. The middle gradient layers of 35% and 30% histodenz were increased to 90 µl each. These assays were done in triplicate. Western blots were quantified, as seen in Supplemental Figure S4.

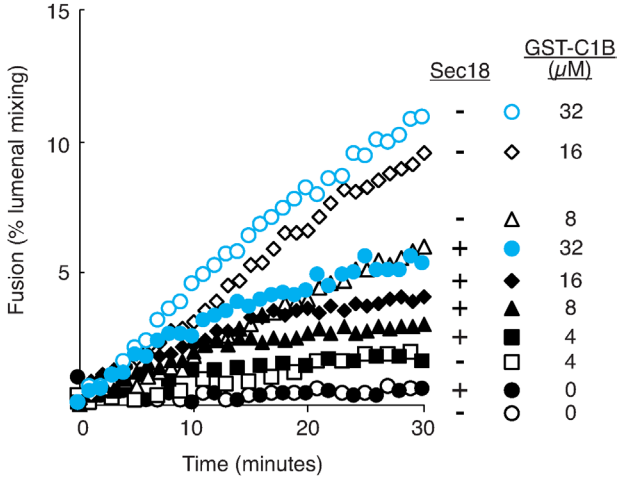

FIGURE 5: With limiting levels of a synthetic tether instead of HOPS, Sec18 does not stimulate fusion. The fusion of Ypt7/R and Ypt7/QaQbQc proteoliposomes was assayed as described in *Materials and Methods*, with 1 mM Mg:ATPγS, the indicated concentrations of GST-C1b, and 60 nM Sec18 where indicated (filled symbols). A representative kinetic assay is shown from triplicate assays. Analysis of the rates of fusion, with error bars, is in Supplemental Figure S5.

Zick *et al.*, 2015; Huang *et al.*, 2019), and promotes fusion in the absence of complete SNARE zippering (Song *et al.*, 2021). We, therefore, examined the capacity of Sec17 to help restore fusion at low HOPS concentrations. Without Sec18, there was no restoration of fusion by up to 1 µM Sec17 (Figure 6A). Even with limiting levels of Sec18 as well as HOPS, Sec17 gave only very modest fusion stimulation (Figure 6B). These small stimulations by Sec17 may reflect enhanced Sec18 delivery to membranes, but show that Sec17 is not central to the mechanism of Sec18 restoration of fusion at limiting HOPS levels.

Sec18 needs adenine nucleotide, but not ATP hydrolysis, to promote fusion with 10 nM HOPS

Sec18 needs ATP hydrolysis to disassemble SNARE complexes (Söllner *et al.*, 1993) and ATPγS will not suffice (Song *et al.*, 2017), but ATP hydrolysis is not needed for Sec18 to help Sec17 bypass the need for complete SNARE zippering for fusion (Song *et al.*, 2017). Because Sec18 is purified in the presence of ATP, an aliquot of Sec18 was desalted through a spin column in fusion reaction buffer Rb150 (20 mM HEPES/NaOH, pH 7.4, 150 mM NaCl, 10% [vol/vol] glycerol) at 4°C to remove unbound ATP, and aliquots were rapidly frozen in liquid nitrogen and stored at –80°C. With 10 nM HOPS, fusion supported by this filtered Sec18 (Figure 7, filled symbols) required the addition of either ATP, ATPγS, or ADP (diamonds, triangles, or circles; no adenine nucleotide is squares). Even without the spin column, dilution of the 2 µM purified Sec18 to the 10 nM concentration in the fusion reaction reduced the ATP to a level where added adenine nucleotide was needed (red open squares vs. triangles).

HOPS, alone or with proteoliposomes, is stable during fusion incubations

To test whether Sec18 supports fusion with limiting (10 nM) HOPS by stabilizing the HOPS, we asked whether HOPS is labile in our fusion incubations. HOPS was incubated separately from other fusion incubation components (Sec18, Mg:ATPγS, Qc, and proteoliposomes), then mixed with them after each had been separately incubated for 0, 1, 2, 4, 8, or 30 min at 27°C. Fusion began at the same rate immediately after mixing and independent of the time of separate incubation (Figure 8A). HOPS was equally stable during incubation with proteoliposomes before mixing with Sec18, Mg:ATPγS, and Qc (Figure 8B). Because Sec18-stimulated fusion with low HOPS levels begins without lag (Supplemental Figure S3), and HOPS is stable for up to 30 min, fusion activation by Sec18 is not simply due to HOPS stabilization.

Sec18 regulation of the Km for the Qc-SNARE

HOPS has direct affinity for the Qc-SNARE (Stroupe *et al.*, 2006) and catalyzes the entry of Qc into functional fusion complex (Zick and Wickner, 2013). Individual SNAREs are consumed during their assembly into SNARE complexes and are not recycled until after fusion, and then only if Sec17 and Sec18 are present. They are required for fusion, and thus could be considered as substrates for the reaction leading to fusion. The concentration of Qc needed for half maximal fusion, termed here the Km for Qc, is a measure of the functional avidity of the fusion apparatus for Qc. With ample HOPS (50 nM), the Km for Qc is ~3 nM (Figure 9A, in red), as reported (Schwartz and Merz, 2009; Schwartz *et al.*, 2017; Lee *et al.*, 2020). With a simple tether such as GST-C1b which lacks

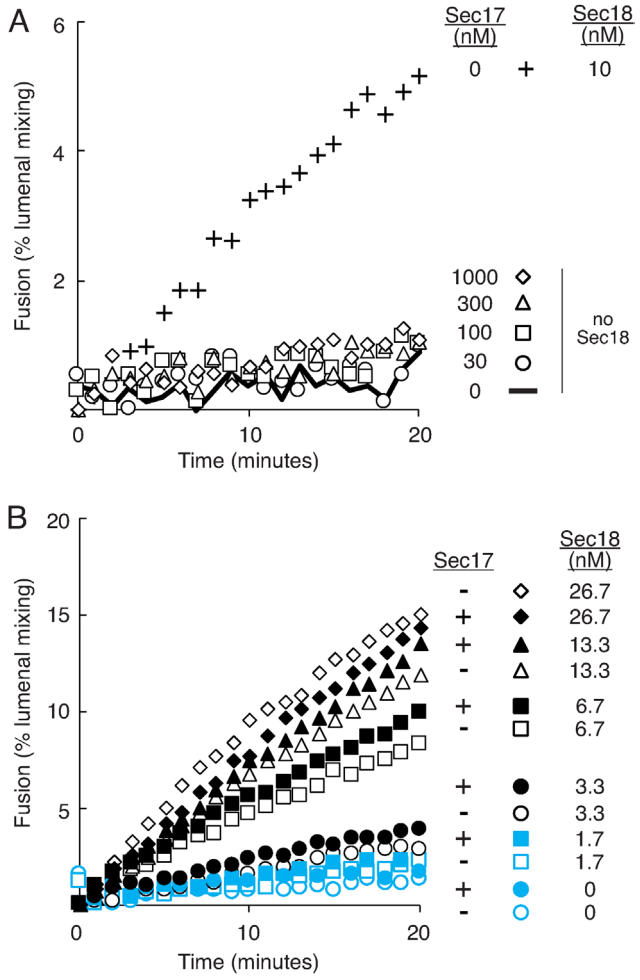

FIGURE 6: Sec17 does not substitute for Sec18 and is not needed for fusion when both HOPS and Sec18 are limiting for fusion. (A) Up to 1 μM Sec17 does not support fusion with 10 nM HOPS. The fusion of Ypt7/R and Ypt7/QaQb proteoliposomes was assayed as described in *Materials and Methods* with 10 nM HOPS, 100 nM Qc, 1 mM Mg:ATPγS, and either 10 nM Sec18 or the indicated concentrations of Sec17. (B) There is little stimulation by Sec17. Fusion was as in A, with 10 nM HOPS, 1 mM Mg:ATPγS, the indicated concentrations of Sec18, and 200 nM Sec17 where indicated. Assays in parts A and B were done in triplicate and average rates of fusion with error bars can be seen in Supplemental Figure S6.

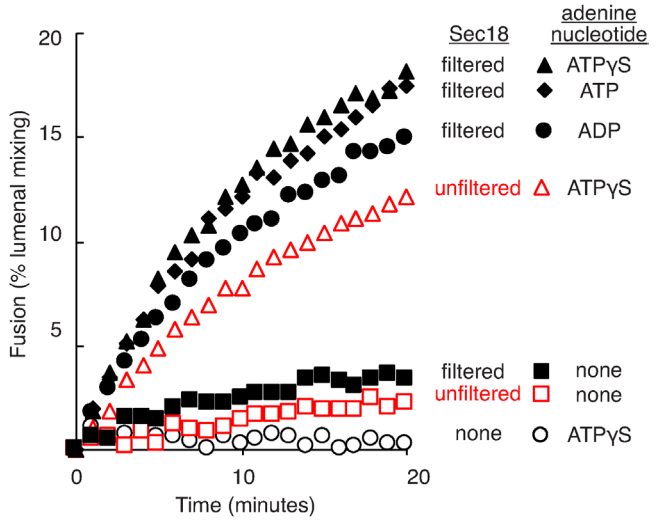

FIGURE 7: Sec18 needs adenine nucleotide, but not ATP hydrolysis, to support fusion with 10 nM HOPS. Fusion of Ypt7/R and Ypt7/QaQb proteoliposomes with 100 nM Qc and 10 nM HOPS was assayed with 10 nM Sec18 and 1 mM of the indicated adenine nucleotide. A portion of the Sec18 had been sedimented (filtered) through a spin gel filtration column in Rb150 to remove unbound ATP. Triplicate assays were performed and average representative fusion rates are shown with error bars in Supplemental Figure S7.

SNARE recognition, there is no catalysis of Qc entry into SNARE complex and the Km is far greater (Figure 9B). With 10 nM HOPS and no Sec18, there is some fusion with high levels of Qc, with a Km of ~400 nM (Figure 9C), a dramatic loss of functional Qc-SNARE affinity. The addition of Sec18 to incubations with 10 nM HOPS restores the affinity of the system for Qc to a Km of 3 nM (Figure 9D). Ypt7, which is the major membrane receptor for HOPS, and which activates the membrane-bound HOPS (Torng *et al.*, 2020) is also required for a low Km for Qc for fusion (Lee *et al.*, 2020).

Q-SNARE specificity

To explore whether Sec18 stimulation with limiting (10 nM) HOPS is specific for the Qc-SNARE or might extend to the Qb-SNARE as well, proteoliposomes were prepared with the Rab Ypt7 and with only one anchored SNARE, R or Qa. Fusion was assayed with 10 nM HOPS, soluble Qb (lacking its membrane anchor; Zick and Wickner, 2013), Qc, and either with or without added Sec18 and Mg:ATPγS (Figure 10). Half the incubations had 1 μM sQb and varied concentrations of Qc (Figure 10, A and B), and half had 1 μM Qc and varied concentrations of sQb (Figure 10, C and D). With 1 μM sQb, there was greater fusion at each Qc level with added Sec18 (Figure 10B) than without (Figure 10A). The addition of Sec18 not only stimulated fusion, but lowered the apparent Km for Qc from several hundred nanomolar (Figure 10A, no Sec18) to several nanomolar (Figure 10B, with Sec18), as seen for fusion with wild-type, membrane-anchored Qb (Figure 9). The other incubations (Figure 10, C and D) had 1 μM Qc and varied concentrations of sQb. At each sQb level, there was again greater fusion with added Sec18 (Figure 10D) than without (Figure 10C), but the apparent Km for sQb was ~ 10 nM in either case. Sec18 enhances the recognition of Qc far more than the recognition of Qb for fusion.

Stage of Sec18 action

HOPS binds to the Rab Ypt7 on each membrane to tether the bilayers (Hickey and Wickner, 2010), binds each of the four SNAREs (Stroupe *et al.*, 2006; Baker *et al.*, 2015; Song *et al.*, 2020), and catalyzes their assembly into fusion-competent *trans*-complexes (Baker *et al.*, 2015; Orr *et al.*, 2017; Jiao *et al.*, 2018), in parallel and in register. What is the contribution of Sec18 to these steps? To assay tethering, the Qc SNARE was omitted from incubations with Ypt7/R and Ypt7/QaQb proteoliposomes with the indicated concentrations of HOPS in the presence or absence of Sec18. Sec18 has little effect on tethering at each HOPS concentration (Figure 11A). Proteoliposomes were also prepared with phosphatidylcholine, either without incorporated proteins or bearing Ypt7, R-SNARE, or the three Q-SNAREs. HOPS does not bind to protein-free phosphatidylcholine liposomes, but has direct affinity for the Rab and SNAREs. Sec18 does not affect the binding of HOPS to Ypt7 or to the R-SNARE or three Q-SNAREs (Figure 11B).

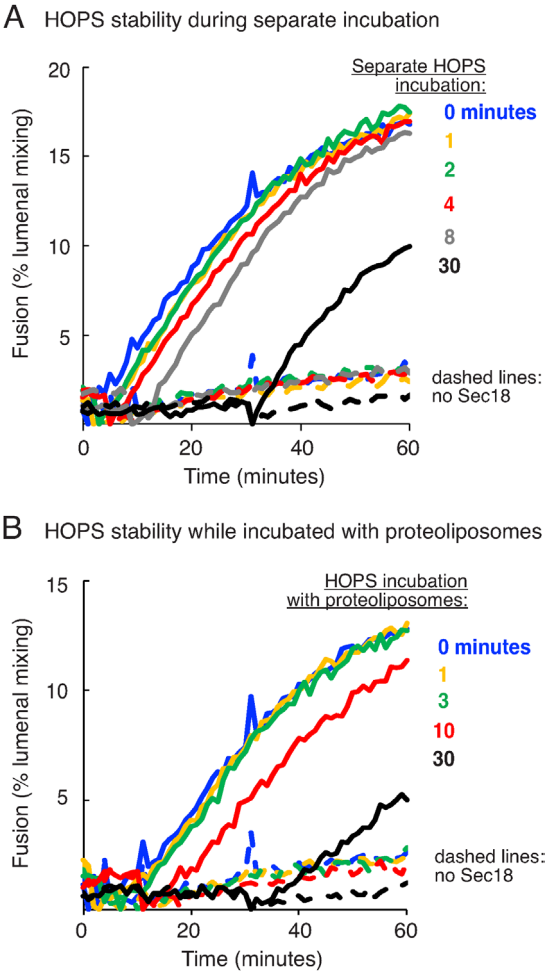

FIGURE 8: Sec18 is not needed to stabilize HOPS in solution or when mixed with proteoliposomes. (A) HOPS in Rb150 was incubated in wells of a 384 well plate, separate from wells containing 16 µl of the other fusion reaction constituents (nucleotide exchanged Ypt7/R and Ypt7/QaQb proteoliposomes, 1 mM Mg:ATPγS, Qc, and either Sec18 or Rb150 reaction buffer for 10 min at 27°C). After 0, 1, 2, 4, 8, or 30 min of continued incubation at 27°C, 4 µl of HOPS was transferred into the wells with the other components. (B) As in A, but HOPS and nucleotide exchanged proteoliposomes were incubated together in 16 µl for the indicated times separately from a mixture of the other fusion reaction constituents, whereupon 4 µl of the latter was added to the wells with the proteoliposomes and the HOPS. In Panels A and B, HOPS was 10 nM, Qc was 100 nM, and Sec18, where added, was 25 nM. These assays were performed in triplicate and representative kinetics are shown from one replicate. Fusion rates upon addition of timed components from the three replicates are shown with error bars in Supplemental Figure S8.

Sec18 and 10 nM HOPS cooperate for fusion (Figures 3 and 11C, top panel). Earlier studies show that approximately half the fusion-ready SNARE complexes form in the complete absence of Qc SNARE as in its presence, but with a severe loss of fusion (Song *et al.*, 2020). With 10 nM HOPS alone, is there still substantial *trans*-SNARE assembly with little fusion despite the increase in Km for Qc? The assembly of *trans*-SNARE complex between proteoliposomes which had been incubated without additional proteins, with 10 nM HOPS, with 25 nM Sec18, or with both, was assayed by solubilizing the membranes in detergent, immunoprecipitating the Qa SNARE, and measuring by immunoblot the Qa-bound R-SNARE (Figure 11C). HOPS, but not Sec18, is required for formation of SNARE complex (Figure 11C, bottom panel, lanes 3 and 4 vs. 1 and 2). To test whether these SNARE complexes were in *trans*, we employed MARCKS effector domain peptide, a lipid-ligand which allows trans-SNARE assembly but blocks fusion (Orr and Wickner, 2023). The same level of SNARE complex formed when fusion was blocked by the presence of MARCKS effector domain peptide as in its absence (lanes 4 vs. 8), showing that we were measuring R:Qa SNARE association in *trans*. Sec18 promoted *trans*-SNARE assembly but was not required (lanes 3 and 4), but there was a disproportionate decrease in the rate of fusion (Figure 11C, top panel) in the absence of Sec18, in accord with earlier studies when Qc had been simply withheld (Song *et al.*, 2020).

Multiple Sec18 functions

Sec18 acts throughout the catalytic cycle of membrane fusion. Several Sec17 molecules can bind to the hexameric Sec18, delivering a Sec17:Sec18 complex to membranes by the affinity of each Sec17 N-terminal apolar loop for lipids and thereby increasing the membrane surface concentrations of both proteins (Orr and Wickner, 2022). Sec18 strongly enhances the capacity of Sec17 to promote fusion in a manner independent of SNARE zippering (Song *et al.*, 2017). This is not solely due to enhanced Sec17 delivery to membranes, as it is even seen when Sec17 is artificially membrane-anchored (Song *et al.*, 2017). Our current study shows that Sec18 binds HOPS, enhancing the recognition of the Qc SNARE for fusion. After fusion, Sec18 binds Sec17 to strongly enhance the Sec18 capacity for SNARE complex disassembly (Söllner *et al.*, 1993; Zick *et al.*, 2015; Huang *et al.*, 2019), recycling the SNAREs for additional rounds of fusion. While Sec18 can directly interact with Sec17, SNAREs, and HOPS, the order of these interactions and their regulation require further study.

DISCUSSION

Our iterative progress in understanding membrane fusion parallels earlier understanding in fields such as transcription, where core RNA polymerase was seen to copy DNA but without proper regulation or accurate mRNA 5′ and 3′ ends. Other transcription factors require core polymerase but confer transcriptional regulation and faithful initiation and termination (Stillman, 1998). In a similar vein, the highest SNARE levels masked the need for HOPS (Fukuda *et al.*, 2000), lower SNARE levels revealed a need for HOPS but not for Ypt7 (Mima *et al.*, 2008), physiologically low SNARE levels reveal the Ypt7 requirement (Zick and Wickner, 2016), and we now report that higher levels of HOPS mask stimulation by Sec18. The earliest reconstitution of vacuole fusion (Fukuda *et al.*, 2000) used high SNARE:lipid ratios and gave fusion with substantial lysis (Zucchi and Zick, 2011; Zick and Wickner, 2014). At lower SNARE levels, fusion requires HOPS (Mima *et al.*, 2008), and at yet lower SNARE levels fusion needs Ypt7 as well and is not accompanied by lysis (Stroupe *et al.*, 2009; Zick and Wickner, 2016). Fusion is stimulated by Sec17 when there are suboptimal lipids (Zick *et al.*, 2015) or when the C-terminal region of Q-SNAREs is perturbed, either by deletion of several heptads near the C-terminus of the SNARE domain (Schwartz and Merz, 2009; Schwartz *et al.*, 2017; Song *et al.*, 2021), by swap of the juxtamembrane regions of the R and Qa SNAREs (Orr *et al.*, 2022), by substitution of polar amino acyl residues for apolar residues in the Qa SNARE domain (Song *et al.*, 2021), or by the sole Q-SNARE transmembrane anchor being on Qb rather than Qa (Wickner *et al.*, 2023). We now report that Sec18 binds HOPS to lower the HOPS concentration needed for membrane fusion to 10 nM. Sec18 does not enhance HOPS

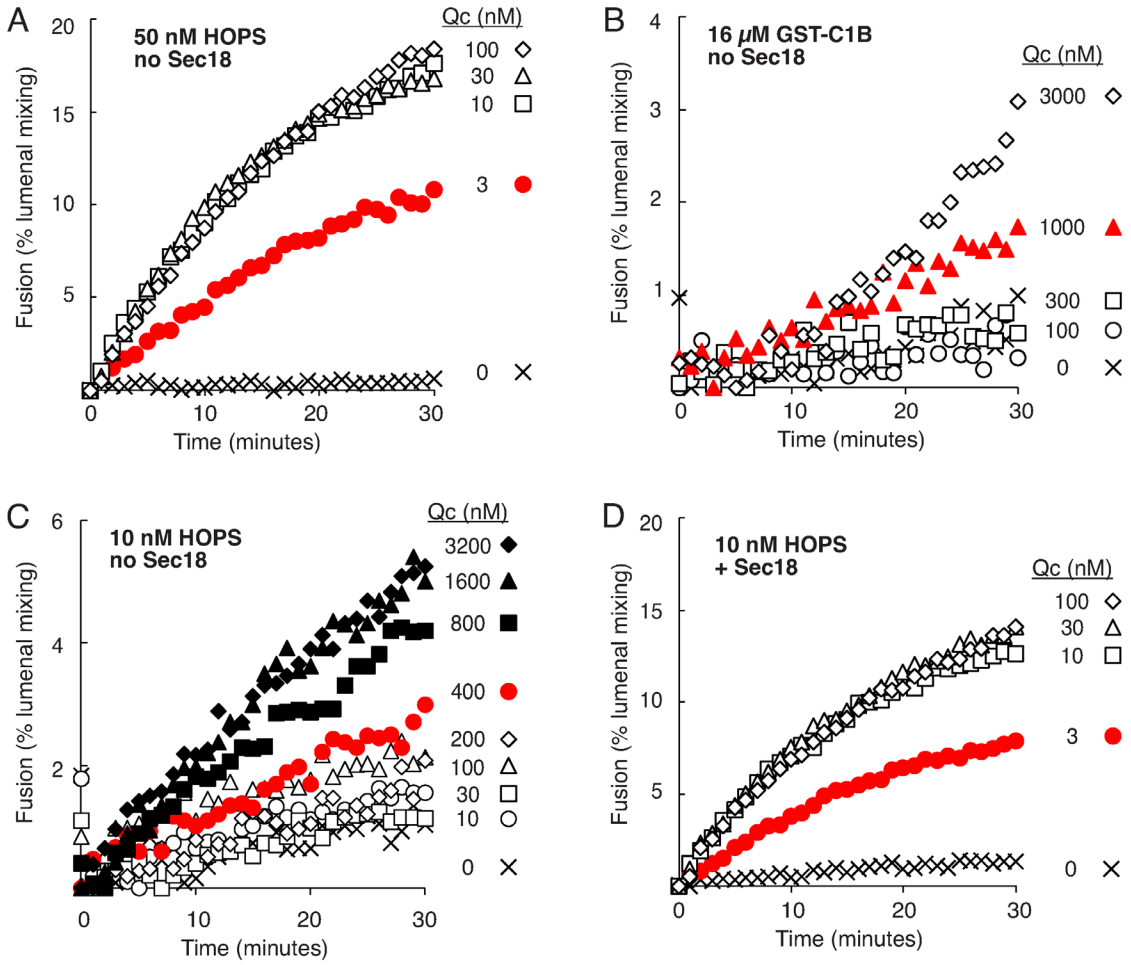

FIGURE 9: At limiting HOPS, Sec18 restores the low Km for Qc for fusion. (A) Fusion incubations of Ypt7/R and Ypt7/QaQb proteoliposomes with 50 nM HOPS had the indicated concentrations of Qc. The fusion kinetics with 3 nM Qc, its approximate Km, is shown in red. (B) Fusion incubations had Ypt7/R and Ypt7/QaQb proteoliposomes, 16 μM GST-C1b, and the indicated concentrations of Qc. The apparent Km for Qc is at least 1000 nM, shown in red. (C) Fusion incubations with the same proteoliposomes had 1 mM Mg:ATPγS, 10 nM HOPS, no Sec18 and the indicated concentrations of Qc; the approximate Km concentration is shown in red. (D) As in C, but with 25 nM Sec18, and with the indicated concentrations of Qc. The approximate Km concentration of Qc is shown in red. All assays were performed in triplicate, and rates of fusion can be seen with error bars in Supplemental Figure S9.

binding to the Rab or SNAREs (Figure 11B). Instead, Sec18 promotes functional Qc-SNARE recognition (Figures 9 and 10) and allows *trans*-SNARE complexes which HOPS has assembled to proceed to fusion. The mechanism of this Sec18 action is unknown but Sec18, which is hexameric, might contribute to fusion by binding several HOPS and thereby colocalizing several *trans*-SNARE complexes, might enhance fusion by localizing additional bulk at the fusion site (D'Agostino *et al.*, 2017), or may contribute to functional HOPS binding of Qc. As the highest SNARE levels mask the need for HOPS, and lower SNARE levels mask the need for Ypt7, so high HOPS concentrations mask the need for Sec18. These findings with Sec18 and the tethering complex HOPS have parallels to studies in mammalian cells of the assembly of a high molecular weight complex bearing the EEA1 tether and the Sec18 homologue NSF (McBride *et al.*, 1999), though formation of that complex required its Qa-SNARE and ATP hydrolysis. Another intriguing parallel is that the N-domain of SNAP-25, encompassing the neuronal Qb SNARE domain, can associate with the center of the oligomeric NSF ring (White *et al.*, 2018), though it is unclear whether the center of Sec18 binds the yeast vacuolar Qc-SNARE to enhance fusion.

Our current findings contrast in several regards with other aspects of Sec18 function: 1.) Sec18 was thought to only interact with other fusion proteins through Sec17 (Weidman *et al.*, 1989) or SNAREs (Zick *et al.*, 2015), but now is seen to also interact directly with HOPS (Figure 4). 2.) Sec17 has little or no effect on this activity of Sec18 (Figure 6). 3.) HOPS directly recognizes Qc (Stroupe *et al.*, 2006) and is the only tether to lower the Km for Qc (Zick and Wickner, 2013; Figure 5), but Sec18 is even required for fusion at limiting HOPS when Qc had been preassembled into complex with the other Q-SNAREs during proteoliposome preparation (Figure 3A). Thus, the effect of Sec18 isn't solely on activating HOPS to bring free, soluble Qc to the membrane or into complex with the other Q-SNAREs. 4.) This Sec18 effect is specific for recognition of Qc, not of Qb, as revealed in fusion reactions with Ypt7/R plus Ypt7/Qa proteoliposomes with limiting HOPS (Figure 10).

These initial observations leave many questions unanswered, such as why physiologically low concentrations of HOPS which

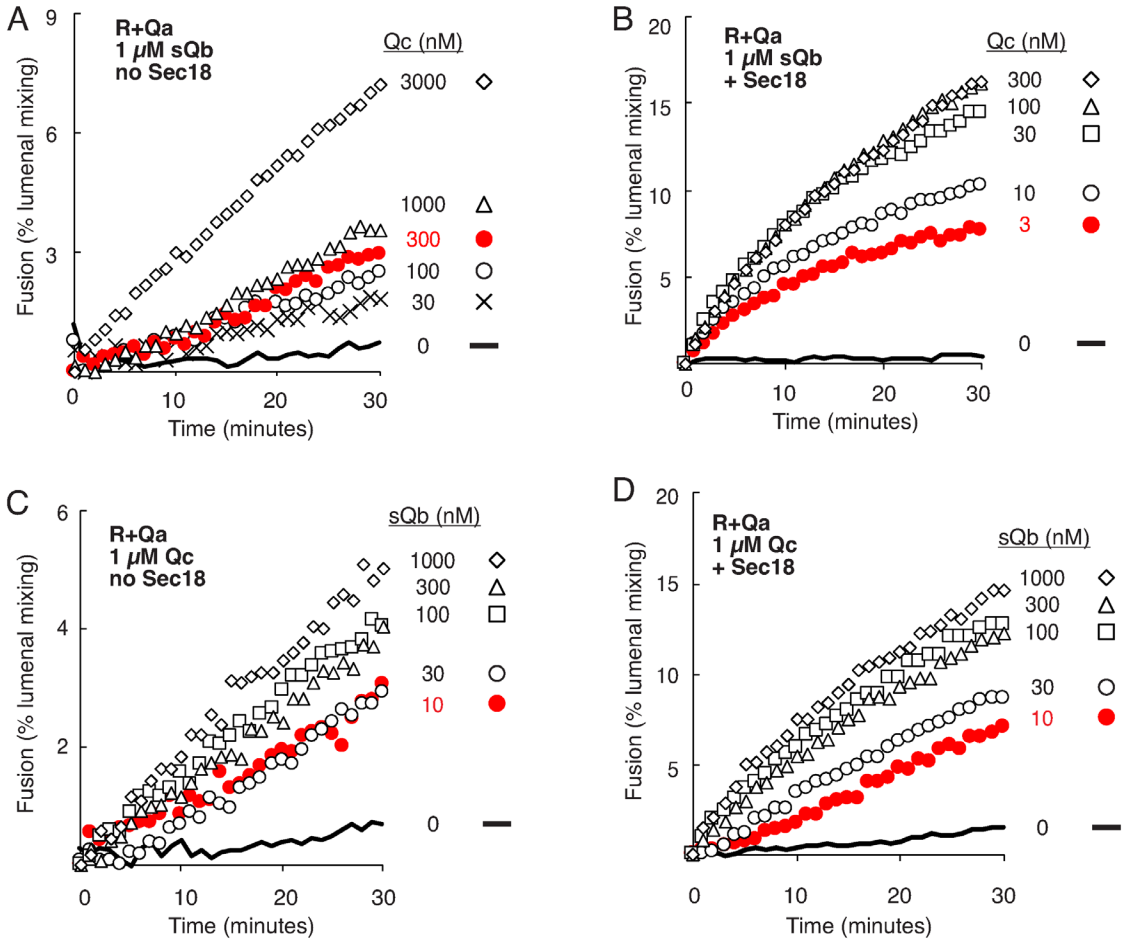

FIGURE 10: Sec18 governs the HOPS affinity for Qc but not for Qb. Fusion assays contained Ypt7/R and Ypt7/Qa proteoliposomes, 10 nM HOPS, and the indicated concentrations of sQb and Qc. (A) Fusion with 1 μM sQb, the indicated concentrations of Qc, and no Sec18; fusion with 300 nM Qc is shown in red. (B) As in A, but with 1 mM Mg:ATPγS and 25 nM Sec18. (C) Fusion with 1 μM Qc and the indicated concentrations of sQb, without Sec18. Fusion with the approximate Km concentration of sQb, 10 nM, is in red. (D) As in C, but with 1 mM Mg:ATPγS and 25 nM Sec18. Red symbols are for 10 nM sQb. All assays were done in triplicate and the average fusion rates with error bars are shown in Supplemental Figure S10.

support *trans*-SNARE assembly lose their potency for engaging Qc for fusion and how Sec18 functionally cooperates with HOPS. HOPS binds membranes through its combined affinities for PI3P, other acidic lipids, each SNARE, and Ypt7 on two membranes, but whether these associations are ordered and interdependent is largely unexplored. As HOPS concentration is reduced from 50 to 10 nM, it may pass through the affinity constant for some of these ligands, altering its mode of binding. The concentration of HOPS will determine its frequency of binding the right ligands on each membrane in the right order and in the right conformation. Because at 10 nM HOPS its overall binding to Ypt7, R, or 3Q is not affected by Sec18, HOPS alone may bind them incorrectly, in the wrong conformation or the wrong order. Our working model is that Sec18, as part of a Sec18:HOPS complex, binds to SNAREs including Qc and thereby guides 10 nM HOPS to productive associations. It is unclear how 50 nM HOPS performs the same function.

MATERIALS AND METHODS

Reagents

Lipids were from Avanti Polar Lipids (Alabaster, AL), Sigma-Aldrich (St. Louis, MO) and Echelon Biosciences (Salt Lake City, UT). Dialysis tubing was from Repligen Corporation (Waltham, MA). GTP, ATP, ADP, ATPγS and Histodenz were from Sigma-Aldrich. BioBeads-SM2 were purchased from BioRad (Hercules, CA), and β-octylglucoside was from Anatrace (Maumee, OH). Cy5-Streptavidin was from SeraCare (Milford, MA). Biotin-conjugated R-phycoerythrin was from Life Technologies Corporation (Eugene, OR) and unlabeled streptavidin was from Pierce Thermo Scientific (Rockford, IL). MARCKS Effector Domain peptide was from Vivitide (Gardner, MA).

Proteins

R (Nyv1), Qa (Vam3), Qb (Vti1), and Qc (Vam7; Mima *et al.*, 2008; Schwartz and Merz, 2009), sQb (Zick and Wickner, 2013), Sec17 (Schwartz and Merz, 2009), Sec18 (Haas and Wickner, 1996), Ypt7-tm and GST-Nyv1 (Song *et al.*, 2020), GST-C1B (Johnson *et al.*, 2000; Fratti *et al.*, 2004), and HOPS (Zick and Wickner, 2013) were purified as described, frozen in small aliquots in liquid nitrogen, and stored at –80°C.

Proteoliposomes

Proteoliposome preparation was as described (Orr and Wickner, 2022). In brief, β-octylglucoside in methanol in a 2 ml glass vial was mixed with chloroform solutions of each lipid, delivered with

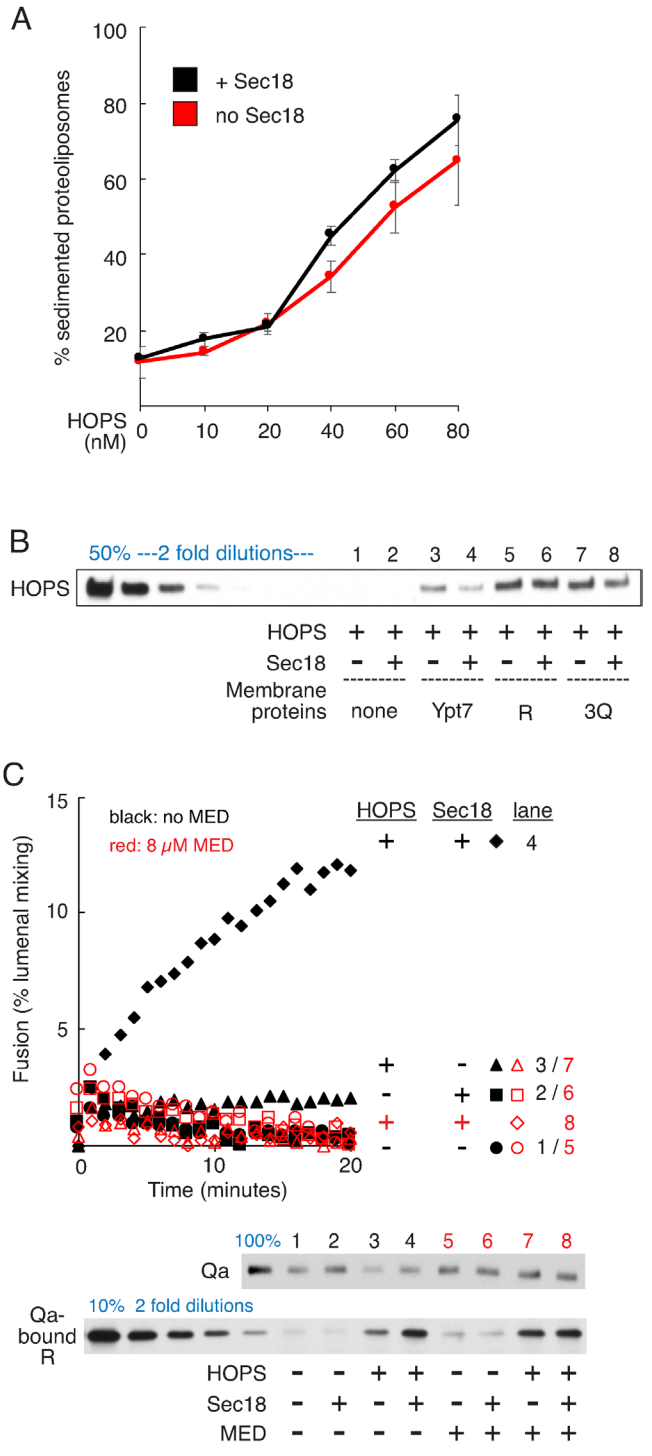

FIGURE 11: Stages of Sec18 action. (A) Tethering is not promoted by Sec18. Ypt7/R and Ypt7/QaQb proteoliposomes, of vacuolar mimic lipid composition but with 1% rhodamine-DHPE, were mixed with 1 mM Mg:ATPγS, the indicated concentration of HOPS, and with (or without) 25 nM Sec18, then incubated 20 min at 27°C. After addition of 80 µl of Rb150 with 1 mM $MgCl_2$ and five pipettings with a P200 Pipetteman set to 90 µl, 20 µl were withdrawn and the rest was centrifuged (5 min, 4°C, 6,000 × g) in a Microfuge. A 20 µl sample was carefully withdrawn from the top, and the rhodamine fluorescence of samples before and after centrifugation was measured. Error bars are shown for triplicate assays. (B) Sec18 does not affect the binding of HOPS to Ypt7, R, or 3Q. Proteoliposomes of PC + 1% rhodamine-DHPE were prepared without any membrane proteins, with Ypt7-tm at 1:8000 protein to lipid ratio, or with R or QaQbQc (3Q) at 1:2000 protein to lipid ratios. Assays (20 µl) of HOPS binding were performed as described in Methods and Figure 4B. Assays contained 1 mM Mg:ATPγS, 25 nM Sec18 (where added), and 10 nM HOPS (added last). (C) Sec18 acts after *trans*-SNARE complex assembly. Fusion reactions were conducted as described in *Materials and Methods*, with Ypt7/R and Ypt7/QaQb proteoliposomes and 10 nM HOPS, 100 nM Qc, 25 nM Sec18 where added, and 8 µM MARCKS effector domain peptide where indicated. In addition to the 20 µl reactions in the plate reader, larger-scale reactions (48 µl) were simultaneously incubated in a PCR strip in a 27°C water bath. After 20 min, the reactions in the plate reader were added to the corresponding reactions in the PCR strip and immunoprecipitations were conducted as described (Orr and Wickner, 2023). A typical kinetic assay is shown along with representative Western blots. All assays in Figure 11 were conducted in triplicate and western blots were analyzed with UN-SCAN-IT software. Quantifications of blot analysis in Panels B and C as well as average rates of fusion for Panel C can be found in Supplemental Figure S11 Panels B and C.

Hamilton syringes, and the chloroform/methanol solvent was removed under a stream of nitrogen for 30 min at room temperature. Vials were then centrifuged in vacuo for 3 h in a speedvac to complete solvent removal. The lipid pellets were overlaid with 400 µl of 50 mM HEPES/NaOH, pH 7.4, 375 mM NaCl, 25% glycerol, 2.5 mM $MgCl_2$, nutated at room temperature for 3 h to dissolve the pellet, and stored at –80°C. Aliquots of frozen lipid: detergent micellar solutions were thawed by nutation at room temperature for 30 min, then placed on ice and supplemented with 350 µl of purified Ypt7-tm and SNAREs in Rb150 (20 mM HEPES/NaOH, pH 7.4, 150 mM NaCl, 10% glycerol) with 1% β-octylglucoside and 250 µl of either Cy5-Streptavidin (for Ypt7/R proteoliposomes) or biotinylated phycoerythrin (for Ypt7/Q proteoliposomes). Unless otherwise noted, Ypt7-tm was added in a 1:8000 M ratio to lipid, Qc, where present, was added in a 1:2700 M ratio to lipid, and R, Qa, and Qb, where present, were added in a 1:16,000 M ratio to lipid. The higher level of Qc was used to drive more complete assembly of 3Q-SNARE complex. Each 1 ml mixed micellar solution was dialyzed in 12-mm flat width, 25 KDa MWCO dialysis tubing against 250 ml of Rb150 + 1 mM $MgCl_2$ with 1 gm of Bio-beads with stirring overnight at 4°C in the dark.

Fusion

Fusion assays (20 µl) were initiated by mixing nucleotide exchanged proteolipsomes with an equal volume of mixed soluble components. Ypt7/R and Ypt7/Q proteolipsomes (0.4 mM each, bearing either R, QaQbQc, QaQb, or only Qa) were mixed with 9 µM streptavidin, 1 mM EDTA, and 0.05 mM GTP, and incubated for 10 min in a 27°C water bath to strip the Ypt7-bound guanine nucleotide before receiving 3 mM $MgCl_2$ to allow binding of GTP. Aliquots of 10 µl of these proteolipsomes were placed in wells of a 384 well plate, separate from wells containing 13 µl of the mixed remaining soluble components, for example, HOPS or GST-C1b, soluble SNAREs, and where indicated, Sec18, Mg:ATPγS, and Sec17. The plate with these separate mixtures was incubated for 10 min at 27°C in a Gemini XPS plate reader (Molecular Devices, Sunnyvale, CA) and the reactions were started by transferring 10 µl of the soluble components to the nucleotide exchanged proteoliposomes with a multichannel pipette.

Protein: Liposome Binding Assays

Assays of protein binding to liposomes were conducted in Rb150 in 30 µl unless otherwise noted. Proteoliposomes (0.5 mM final lipid

phosphorus) were incubated for 10 min at 27°C with EDTA (1 mM) and GTP (0.1 mM), followed by the addition of $MgCl_2$ and defatted BSA (2 mM and 0.2% final concentrations, respectively). After 1 min, reactions received 1 mM final ATP (or ATPγS) and other soluble components indicated in the figure legends. Reactions were incubated at 27°C for 1 h, after which 90 µl of 54% iso-osmolar Histodenz in Rb150+Mg (Rb150 with 1 mM $MgCl_2$) was added and the tubes were vortexed gently. A portion (80 µl) of each was transferred to a Beckman 7 × 20 mm ultracentrifuge tube and overlaid with 80 µl of 35% Histodenz in Rb150+Mg, then 80 µl of 30% Histodenz in Rb150+Mg, and finally 50 µl of Rb150+Mg. Tubes were centrifuged in a TLS-55 swinging bucket rotor for 30 min at 55,000 rpm, and 80 µl was harvested at the 0–30% Histodenz interface. The starting samples and harvested samples received 5% Thesit to yield a 0.125% final concentration and the samples were nutated for 30 min. The percentage of lipid recovered was determined by comparing the Rhodamine fluorescence of 10 µl of the solubilized starting samples and 10 µl of the solubilized harvested samples. Samples were boiled for 5 min with a quarter volume of 5x SDS–PAGE sample buffer with ß-mercaptoethanol and analyzed by Western blot.

Other assays, such as membrane tethering and the association between R and Qa SNAREs, are described in figure legends.

ACKNOWLEDGMENTS

We thank Jeremy Thorner for helpful information, and Gus Lienhard, Christian Ungermann, Alex Merz, and Fred Hughson for fruitful discussions. This work was supported by National Institutes of Health grant 5R35GM118037.

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.

Baker RW, Hughson FM (2015). Chaperoning SNARE assembly and disassembly. Nat Rev Mol Cell Biol 17, 465–479.

Block MR, Glick BS, Wilcox CA, Wieland FT, Rothman JE (1988). Purification of an N-ethylmaleimide-sensitive protein catalyzing vesicular transport. Proc Natl Acad Sci USA 85, 7852–7856.

Brett CL, Plemel 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.

Bröcker C, Kuhlee A, Gatsogiannis C, Balderhaar HJK, Hönscher C, Engelbrecht-Vadre S, Ungermann C, Raunser S (2012). Molecular architecture of the multisubunit homotypic fusion and vacuole protein sorting (HOPS) tethering complex. Proc Natl Acad Sci USA 109, 1991–1996.

Cipriano DJ, Jung J, Vivona S, Fenn TD, Brunger AT, Bryant Z (2013). Processive ATP-driven substrate disassembly by the N-ethylmaleimide-sensitive factor (NSF) molecular machine. J Biol Chem 288, 23436–23445.

D'Agostino M, Risselada HJ, Lürick A, Ungermann C, Mayer A (2017). A tethering complex drives the terminal stage of SNARE-dependent membrane fusion. Nature 551, 634–638.

Fasshauer D, Sutton RBB, 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 R, 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, Söllner TH (2000). Functional architecture of an intracellular membrane t-SNARE. Nature 407, 198–202.

Gao Y, Zorman S, Gundersen G, Xi ZQ, Sirinakis G, Rothman JE, Zhang YL (2012). Single reconstituted neuronal SNARE complexes zipper in three distinct stages. Science 337, 1340–1343.

Griff IC, Schekman R, Rothman JE, Kaiser CA (1992). The yeast SEC17 gene product is functionally equivalent to mammalian α-SNAP protein. J Biol Chem 267, 12106–12115.

Haas A, Conradt B, Wickner W (1994). G-Protein ligands inhibit in vitro reactions of vacuole inheritance. J Cell Biol 126, 87–97.

Haas A, Scheglmann D, Lazar T, Gallwitz D, Wickner W (1995). The GTPase Ypt7p of *Saccharomyces cerevisiae* is required on both partner vacuoles for the homotypic fusion step of vacuole inheritance. EMBO J 14, 5258–5270.

Haas A, Wickner W (1996). Homotypic vacuole fusion requires Sec17 (yeast α-SNAP) and Sec18 (yeast NSF). EMBO J 15, 3296–3305.

Hickey CM, Wickner W (2010). HOPS initiates vacuole docking by tethering membranes before trans-SNARE complex assembly. Mol Biol Cell 21, 2297–2305.

Ho B, Baryshnikopva A, Brown GW (2018). Unification of protein abundance datasets yields a quantitative Saccharomyces cerevisiae dataset. Cell Sys 6, 192–205.

Huang X, Sun S, Wang X, Fan F, Zhou Q, Lu S, Cao Y, Wang Q-W, Dong M-Q, Yao J, Sui S-F (2019). Mechanistic insights into the SNARE complex disassembly. Sci Adv 5, aau8164.

Hutagalung AH, Novick PJ (2011). Rab GTPases in membrane traffic and cell physiology. Physiol Rev 91, 119–149.

Jahn R, Scheller RH (2006). SNAREs- engines for membrane fusion. Nat Rev Mol Cell Biol 7, 631–643.

Jiao J, He M, Port SA, Baker RW, Xu Y, Qu H, Xiong Y, Wang Y, Jin H, Eisemann TJ, *et al.* (2018). Munc18-1 catalyzes neuronal SNARE assembly by templating SNARE association. eLife 7, e41771.

Johnson JE, Giorgione J, Newton AC (2000). The C1 and C2 domains of protein kinase C are independent membrane targeting modules, with specificity for phosphatidylserine conferred by the C1 domain. Biochemistry 39, 11360–11369.

Lee M, Wickner W, Song H (2020). A Rab prenyl membrane-anchor allows effector recognition to be regulated by guanine nucleotide. Proc Natl Acad Sci USA 117, 7739–7744.

Mayer A, Wickner W, Haas A (1996). Sec18p (NSF)-driven release of Sec17p (a-SNAP) can precede docking and fusion of yeast vacuoles. Cell 85, 83–94.

McBride HM, Rybin V, Murphy C, Giner A, Teasdale R, Zerial M (1999). Oligomeric complexes link Rab5 effectors with NSF and drive membrane fusion via interactions between EEA1 and Syntaxin 13. Cell 98, 377–386.

Mima J, Hickey CM, Xu H, Jun Y, Wickner W (2008). Reconstituted membrane fusion requires regulatory lipids, SNAREs and synergistic SNARE chaperones. EMBO J 27, 2031–2042.

Novick P, Field C, Schekman R (1980). Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell 21, 205–215.

Orr A, Song H, Rusin SF, Kettenbach AN, Wickner W (2017). HOPS catalyzes the interdependent assembly of each vacuolar SNARE into a SNARE complex. Mol Biol Cell 28, 975–983.

Orr A, Wickner W (2022). Sec18 supports membrane fusion by promoting Sec17 membrane association. Mol Biol Cell 33, ar127.

Orr A, Song H, Wickner W (2022). Fusion with wild-type SNARE domains is controlled by juxtamembane domains, transmembrane anchors, and Sec17. Mol Biol Cell 33, ar38.

Orr A, Wickner W (2023). MARCKS effector domain, a reversible lipid ligand, illuminates late stages of membrane fusion. Mol Biol Cell 34, ar123.

Parisotto D, Pfau M, Scheutzow A, Wild K, Mayer MP, Malsam J, Sinning I, Söllner TH (2014). An extended helical conformation in domain 3a of Munc18-1 provides a template for SNARE (Soluble N-Ethylmaleimide-sensitive NSF attachment protein receptor) complex assembly. J Biol Chem 289, 9639–9650.

Rathore SS, Liu Y, Yu H, Wan C, Lee MS, Yin Q, Stowell MHB, Shen J (2019). Intracellular vesicle fusion requires a membrane-destabilizing peptide located at the juxtamembrane region of the v-SNARE. Cell Reports 29, 4583–4592.

Sato K, Wickner W (1998). Functional reconstitution of Ypt7p GTPase and a purified vacuole SNARE complex. Science 281, 700–702.

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

Schwartz ML, Nickerson DP, Lobingier BT, Plemel RL, Duan M, Angers CG, Zick M, Merz AJ (2017). Sec17 (α-SNAP) and an SM-tethering complex regulate the outcome of SNARE zippering in vitro and in vivo. eLife 6, e27396.

Seals DF, Eitzen G, Margolis N, Wickner WT, Price A (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.

Shvarev D, Schoppe J, König C, Perz A, Fülbrunn A, Kiontke S, Langemeyer L, Januliene D, Schnelle K, Kümmel D, *et al.* (2022). Structure of the

HOPS tethering complex, a lysosomal membrane fusion machinery. eLife 11, e80901.
Söllner T, Bennett MK, Whiteheart SW, Scheller RH, Rothman JE (1993). A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. Cell 75, 409–418.
Song H, Orr A, Duan M, Merz AJ, Wickner W (2017). Sec17/Sec18 act twice, enhancing membrane fusion and then disassembling cis-SNARE complexes. eLife 6, e26646.
Song H, Wickner W (2019). Tethering guides fusion-competent *trans*-SNARE assembly. Proc Natl Acad Sci USA 116, 13952–13957.
Song H, Orr AS, Lee M, Harner ME, Wickner WT (2020). HOPS recognizes each SNARE, assembling ternary *trans*-complexes for rapid fusion upon engagement with the 4th SNARE. eLife 9, e53559.
Song H, Torng TL, Orr AS, Brunger AT, Wickner WT (2021). Sec17/Sec18 can support membrane fusion without help from complete SNARE zippering. eLife 10, e67578.
Sorensen JB, Wiederhold K, Müller EM, Milosevic I, Nagy G, de Groot B, Grubmüller H, Fasshauer D (2006). Sequential N to C-terminal SNARE complex assembly drives priming and fusion of secretory vesicles. EMBO J 25, 955–966.
Stepien KP, Xu J, Zhang X, Bai X-C, Rio J (2022). SNARE assembly enlightened by cryo-EM structures of a synaptobrevin-Munc18-1-syntaxin-1 complex. Sci Adv 8, eabo5272.
Stillman B, (ed.) (1998). Mechanisms of transcription. Cold Spring Harbor Symposium Quantitative Biology Vol LXIII.
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.
Stroupe C, Hickey CM, Mima J, Burfeind AS, Wickner W (2009). Minimal membrane docking requirements revealed by reconstitution of Rab GTPase-dependent membrane fusion from purified components. Proc Natl Acad Sci USA 106, 17626–17633.
Torng T, Song H, Wickner W (2020). Asymmetric Rab activation of vacuolar HOPS to catalyze SNARE complex assembly. Mol Biol Cell 31, 1060–1068.
Torng T, Wickner W (2021). Phosphatidylinositol and phosphatidylinsolitol-3-phosphate activate HOPS to catalyze SNARE assembly, allowing small headgroup lipids to support the terminal steps of membrane fusion. Mol Biol Cell 32, ar19.
Uchida M, Sun Y, McDermott G, Knoechel C, Le Gros MA, Parkinson D, Drubin DG, Larabell CA (2011). Quantitative analysis of yeast internal architecture using soft X-ray tomography. Yeast 28, 227–236.
Ungermann C, Sato K, Wickner W (1998a). Defining the functions of *trans*-SNARE pairs. Nature 396, 543–548.
Ungermann C, Nichols BJ, Pelham HRB, Wickner W (1998b). 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.
Vivona S, Cipriano DJ, O'Leary S, Li YH, Fenn TD, Brunger AT (2013). Disassembly of all SNARE complexes by N-Ethylmaleimide-sensitive factor (NSF) is initiated by a conserved 1:1 interaction between α-soluble NSF binding protein (SNAP) and SNARE complex. J Biol Chem 34, 24984–24991.
Wada Y, Ohsumi Y, Anraku Y (1992). Genes for directing vacuole morphogenesis in Saccharomyces cerevisiae. I. Isolation and characterization of two classes of *vam* mutants. J Biol Chem 267, 18665–18670.
Wang L, Seeley ES, Wickner W, Merz AJ (2002). Vacuole fusion at a ring of docking sites leaves membrane fragments within the organelle. Cell 108, 357–369.
Weber T, Zemelman BV, McNew JA, Westermann B, Gmachi M, Parlati F, Söllner TH, Rothman JE (1998). SNARE pins: Minimal machinery for membrane fusion. Cell 92, 749–772.
Weidman PJ, Melancon P, Block MR, Rothman JE (1989). Binding of an N-ethylmaleimide-sensitive fusion protein to Golgi membranes requires both a soluble protein(s) and an integral membrane receptor. J Cell Biol 108, 1589–1596.
White KI, Zhao M, Choi UB, Pfuetzner RA, Brunger AT (2018). Structural principles of SNARE complex recognition by the AAA+ protein NSF. eLife 7, e38888.
Wickner W, Lopes K, Song H, Rizo J, Orr A (2023). Efficient fusion requires a membrane anchor on the vacuolar Qa-SNARE. Mol Biol Cell 34, ar88.
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.
Xu H, Jun Y, Thompson J, Yates J, Wickner W (2010). HOPS prevents the disassembly of trans-SNARE complexes by Sec17p/Sec18p during membrane fusion. EMBO J 29, 1948–1960.
Zakhartsev M, Reuss M (2018). Cell size and morphological properties of yeast Saccharomyces cerevisiae in relation to growth temperature. FEMS Yeast Research 18, 18, foy052.
Zick M, Orr A, Schwartz ML, Merz AJ, Wickner WT (2015). Sec17 can trigger fusion of trans-SNARE paired membranes without Sec18. Proc Natl Acad Sci USA 112, E2290–E2297.
Zick M, Wickner W (2013). The tethering complex HOPS catalyzes the 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 membrane fusion. eLife 3, e03251.
Zick M, Wickner W (2016). Improved reconstitution of yeast vacuole fusion with physiological SNARE concentrations reveals an asymmetric Rab(GTP) requirement. Mol Biol Cell 27, 2590–2597
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.