Sec17/Sec18 can support membrane fusion without help from completion of SNARE zippering

Hongki Song¹*, Thomas Torng¹*, Amy Orr¹, Axel T. Brunger², and William Wickner¹

¹Department of Biochemistry and Cell Biology
Geisel School of Medicine at Dartmouth
Hanover, NH 03755-3844

²Howard Hughes Medical Institute and
Department of Molecular and Cellular Physiology
Stanford University
Stanford, CA 94305

*Co-first authors

for Correspondence: William.Wickner@Dartmouth.edu

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Impact statement: Sec18(NSF) and Sec17(αSNAP) provide a parallel pathway to fusion that is independent of energy from SNARE zippering.
Abstract

Membrane fusion requires R-, Qa-, Qb-, and Qc-family SNAREs that zipper into RQaQbQc coiled coils, driven by the sequestration of apolar amino acids. Zippering has been thought to provide all the force driving fusion. Sec17/αSNAP can form an oligomeric assembly with SNAREs with the Sec17 C-terminus bound to Sec18/NSF, the central region bound to SNAREs, and a crucial apolar loop near the N-terminus poised to insert into membranes. We now report that Sec17 and Sec18 will drive robust fusion without requiring zippering completion. Zippering-driven fusion is blocked by deleting the C-terminal quarter of any Q-SNARE domain or by replacing the apolar amino acids of the Qa-SNARE which face the center of the 4-SNARE coiled coils with polar residues. These blocks, singly or combined, are bypassed by Sec17 and Sec18, and SNARE-dependent fusion is restored without help from completing zippering.
Membrane fusion requires Rab-family GTPases and SNARE proteins. SNAREs constitute four families, termed R, Qa, Qb, and Qc (Fasshauer et al., 1998). Each has an N-domain, an \( \alpha \)-helical SNARE domain of 50-60 aminoacyl residues with heptad-repeat apolar residues, and often a C-terminal membrane anchor. Each SNARE \( \alpha \)-helical turn is termed a "layer." The central "0-layer" of each fully-assembled SNARE complex has inwardly-oriented arginyl (for R-SNAREs) or glutaminyl (for Qa, Qb, and Qc SNAREs) residues, forming a polar center to the otherwise hydrophobic core of the 4-helical SNARE bundle (Sutton et al., 1998). The SNARE domain layers are numbered from the 0-layer, in the positive direction toward the SNARE C-termini and in the negative direction toward the N-domains. Prior to 4-SNARE assembly, individual SNARE domains are random-coil (Fasshauer et al., 1997; Hazzard et al., 1999). Sec1/Munc18 (SM) family proteins catalyze the N to C directional assembly of SNAREs anchored to each tethered membrane (Fiebig et al., 1999; Sørensen et al., 2006; Baker et al., 2015; Orr et al., 2017; Jiao et al., 2018). Each SNARE domain transitions from random coil to \( \alpha \)-helix as the heptad-repeat apolar aminoacyl residues become sequestered into the interior of the coiled coils (Fasshauer et al., 1997; Sutton et al., 1998). This hydrophobic collapse relies on the exclusion of water and is the driving force for SNARE assembly (Sørensen et al., 2006). Completion of SNARE zippering can release up to 40kBT per SNARE complex (Gao et al., 2012; Min et al, 2013; Zhang, 2017) to overcome the 40-90 kBT hydration barrier for membrane stalk formation, the dominant energy barrier for fusion (Aeffner et al., 2012). Upon fusion, the \textit{trans}-SNARE complex becomes a \textit{cis}-complex, anchored to the fused membrane bilayer. Sec17 (\( \alpha \)SNAP) and SNAREs are receptors for the Sec18 (NSF) AAA ATPase (Clary et al., 1990; Winter et al., 2009; Zick et al., 2015). Sec18 uses the energy from ATP binding and hydrolysis to disassemble SNAREs for further fusion cycles (Söllner et al., 1993; Ungermann et al., 1998; Zhao et al., 2015) and to disassemble dead-end SNARE complexes (Xu et al., 2010; Lai et al, 2017; Choi et al., 2018; Song and Wickner, 2019; Jun and Wickner, 2019).

The molecular interactions between Sec18/NSF, Sec17/\( \alpha \)SNAP, and neuronal SNAREs were illuminated by determination of their structures when assembled without membrane anchors into the NSF/\( \alpha \)SNAP/SNARE complex, also referred to as the 20s particle (Zhao et al., 2015; White et al., 2018). The heart of these structures is the 4-helical bundle of the R, Qa, Qb, and Qc SNARE domains. Between two and four Sec17/\( \alpha \)SNAP form a right-handed assembly surrounding the left-handed superhelical coiled coils of the SNARE complex. In this structure, the N-terminal apolar loop of each \( \alpha \)SNAP is poised to enter a lipid bilayer adjacent to the SNARE transmembrane domains.

Yeast vacuole fusion, a model of non-neuronal fusion, has been studied \textit{in vivo} (Wada et al., 1992), \textit{in vitro} with the isolated organelle (Wickner, 2010), and in a reconstituted proteoliposome-based reaction with purified components (Mima et al., 2008; Stroupe et al., 2009; Zick and Wickner, 2016). Each protein implicated by the \textit{in vivo} genetics is required for the reconstitution: the Rab Ypt7, the R-SNARE Nyv1 and Q-SNAREs Vam3, Vti1, and Vam7 (hereafter referred to as R, Qa, Qb, and Qc), and a large hexameric protein termed HOPS (homotypic fusion and vacuole protein sorting) with multiple direct affinities. Two HOPS subunits bind Ypt7, anchored on each membrane (Brett et al., 2008), to mediate tethering (Hickey and Wickner, 2010). A third HOPS subunit is Vps33, the vacuolar Sec1/Munc18 (SM) protein, with direct capacity to bind R and Qa.
SNARE domains, in parallel and in register (Baker et al., 2015). HOPS also has direct affinity for the Qb and Qc SNAREs (Stroupe et al., 2006; Song et al., 2020) and for vacuolar lipids (Orr et al., 2014). Some functions of HOPS correspond to fusion factors in other systems; neuronal Munc13 cooperates with Munc18 in SNARE assembly (Richmond et al., 2001; Ma et al., 2011; Lai et al., 2017). Munc18 corresponds to the HOPS subunit Vps33, but the protein which mediates the Munc13 function for vacuole fusion is unclear. The association of HOPS with Ypt7 and vacuolar lipids allosterically activates HOPS to catalyze SNARE assembly (Torng and Wickner, 2020). When the SNAREs are initially in 4-SNARE complexes on two apposed membranes, fusion requires Sec17, Sec18, and ATP to disassemble these cis-SNARE complexes and liberate the SNAREs for assembly into trans-complexes (Mayer et al., 1996; Nichols et al., 1997; Zick et al., 2015).

Though early studies suggested that disassembly of cis-SNARE complexes might be the sole function of Sec17 and Sec18 (Mayer et al., 1996), recent findings have broadened our understanding of their roles. While SNAREs are a core fusion machine (Weber et al., 1998), a complete fusion machine (Mima et al., 2008; Zick et al., 2016) also requires the Rab, its tethering effector, an SM-family protein, and the NSF/Sec18 and αSNAP/Sec17 SNARE chaperones. In this more complete context, Sec17 and Sec18 also contribute to fusion per se: 1. trans-SNARE complexes which form in a Ypt7-dependent manner between vacuoles bear Sec17 in comparable abundance to the SNAREs (Xu et al., 2010). 2. Fusion between proteoliposomes has been reconstituted with purified components. When tethering is by nonspecific agents, fusion is inhibited by Sec17, Sec18, and ATP (Mima et al., 2008; Song et al., 2017). However, Sec17, Sec18, and ATP stimulate fusion with HOPS (Mima et al., 2008; Zick et al., 2015; Song et al., 2017). 3. A pioneering study by Schwartz and Merz (2009) showed that the Qc3Δ deletion of several heptads at the C-terminus of the Qc SNARE blocks the fusion of isolated yeast vacuoles, but this block is overcome by the addition of Sec17. Qc3Δ, ending at the SNARE domain layer +3 and thus lacking layers +4 to +8, blocks vacuole fusion in vivo as well, and overexpression of Sec17 partially restores cellular vacuole morphology (Schwartz et al., 2017). Reconstituted in vitro fusion with limiting Sec17 concentrations, where Sec17 will not restore fusion, also requires Sec18 (Schwartz et al., 2017). It has been unclear whether the Sec17 bypass of this deletion of the C-terminal Qc region is particular to just this one SNARE or is general for any Q-SNARE, and whether Sec17 simply contributes its SNARE-binding energy to the energy of 3-SNARE zippering or whether it drives fusion by other means. 4. Fusion reactions with initially separate SNAREs can require Sec17, and this fusion is stimulated by Sec18 without ATP hydrolysis (Zick et al., 2015, Song et al., 2017). Sec17 alone stimulates the fusion of reconstituted proteoliposomes with wild-type SNAREs, and the degree of stimulation is a function of the lipid headgroup and fatty acyl composition (Zick et al., 2015). An intermediate in fusion accumulates during HOPS-dependent fusion without Sec17, allowing a sudden burst of fusion upon Sec17 addition (Zick et al., 2015). Single molecule pulling studies have also revealed that αSNAP stabilizes SNARE complexes (Ma et al., 2016, but see Ryu et al., 2015). However, the mechanism whereby HOPS-dependent fusion is stimulated by Sec17/Sec18 has been unclear.

Complete SNARE zippering is considered essential for SNARE-dependent fusion (Sørensen et al., 2006). We now report that Sec17 has a second mode of promoting fusion which can compensate for incomplete zippering. Fusion with SNAREs and HOPS is completely arrested when several heptads repeats in the C-terminal region of the SNARE core complex are removed from any one...
of the three Q-SNAREs. With any such C-terminally truncated Q-SNARE domain, or even with C-terminal truncations to both Qb and Qc, blocked fusion is restored by Sec17 and Sec18 without ATP hydrolysis. Association between the C-terminal heptads of the R and the single remaining full-length Qa-SNARE, each anchored to one of the docked membranes, would not yield the same assembly energy as with wild-type SNAREs (Sutton et al., 1998) and would contribute far less force towards the bilayer rearrangements of fusion. The N-terminal apolar loop of Sec17 is particularly important for this function of Sec17, and it may stabilize SNARE bundles or trigger fusion by insertion into lipid bilayers. Zippering-driven fusion is also arrested with full-length SNARE domains when the apolar, inward-facing residues of the Qa SNARE layers +4 to +8 are replaced by Ala, by Ser, or by Gly, but in each case fusion is restored by Sec17, Sec18, and nonhydrolyzable ATPγS. Strikingly, even fusion which is blocked by the concurrent replacement of apolar residues from the +4 to +8 layers of Qa and the deletion of the +4 to +8 layers of both Qb and Qc, removing all capacity for hydrophobic collapse between the +4 and +8 layers of the R and Q SNAREs, is fully restored by Sec17 and Sec18. We propose that Sec17 either creates a favorable folding environment for the assembly of the remaining full-length SNARE domains or directly promotes bilayer remodeling through insertion of the apolar loops of several SNARE-bound Sec17s or acts by a combination of these two mechanisms.
Results

Vacuole SNAREs (Figure 1A) have an N-domain and a SNARE domain. Several have a transmembrane (TM) anchor, but Qc lacks a hydrophobic membrane anchor, and instead associates with membranes through its affinities for the other SNAREs, for HOPS (Stroupe et al., 2006), and for phosphatidylinositol 3-phosphate through its N-terminal PX domain (Cheever et al., 2001). SNARE domain layers are numbered from the central 0-layer, as shown. Fusion requires that R- and at least one Q-SNARE be anchored to docked membranes (Song and Wickner, 2017). When they are, soluble forms of the other Q-SNAREs without membrane anchors, termed sQ (Figure 1A), will support Ypt7/HOPS-dependent fusion. Vacuolar SNAREs with C-terminal truncations, corresponding to partial zippering, form stable complexes (Figure 1-figure supplement 1), supporting their use in fusion studies. Based on the single particle cryoEM structure of the homologous human neuronal NSF/αSNAP/SNARE complex (Zhao et al., 2015), we modeled the associations of Sec17 (αSNAP) (Figure 1B) and Sec18 (NSF) with vacuolar SNAREs, viewed in profile or in an end-on view from the membrane (Figure 1C). In this model, we assume that four Sec17 monomers (Figure 1B) assemble together, surrounding the 4-SNARE coiled coil (Figure 1C). In contrast, only two αSNAP molecules have been observed in EM structures of the NSF/αSNAP/V7-SNARE complex (Zhao et al., 2015) and the NSF/αSNAP/SNARE complex that included the linker between the two SNAP-25 SNARE domains (White et al., 2018). The presence of the SNAP-25 linker in these two complexes may interfere with binding of the other two αSNAP molecules. Since the vacuolar SNARE complex does not contain a linker between SNARE domains, it is reasonable to postulate that four Sec17 molecules bind to the vacuolar SNARE complex, but the precise number of Sec17 molecules is yet to be determined for the Sec18/Sec17/vacuolar SNARE complex. Rapid fusion needs Sec17 and Sec18 in addition to HOPS and SNAREs (Song et al., 2017). To understand how they work together, we exploited direct assays of SNARE associations to show that incompletely zippered SNAREs can associate stably and that HOPS allows Sec17 to promote completion of zippering and SNARE complex stability. We then examined their functional relationships to show that SNARE-bound Sec17 and Sec18 can promote rapid fusion when energy from zippering is greatly reduced or lost.

Sec17 alters SNARE complex conformation. The capacity of vacuolar SNAREs to form stable partially-zippered structures raised the question of whether these SNAREs can zipper efficiently, especially when anchored to membranes or associated with other fusion proteins such as HOPS. To study the kinetics of SNARE interactions, we employed an ensemble fluorescence resonance energy transfer (FRET) assay of vacuolar SNARE associations (Torng and Wickner, 2020). The Qc-SNARE was prepared with a unique cysteinyl residue in any of 3 positions (Figure 2A), either the native Cys208 which is upstream (U) of the SNARE domain or, after substitution of serine for this cysteine, with Met250Cys at the N-terminal end of the SNARE domain or with Ala316Cys at the C-terminal end of the SNARE domain. Each was derivatized with Oregon Green 488. Fusion proteins were also prepared with a unique cysteine either immediately N-terminal, or C-terminal, to the Qb SNARE domain, and each was derivatized with Alexa Fluor 568. As a model for exploring the effects of HOPS and Sec17 on SNARE dynamics, these fluorescent proteins were co-incubated with proteoliposomes bearing Ypt7, R and Qa. cis-SNARE complex assembly can occur spontaneously on these proteoliposomes, but assembly is stimulated by HOPS, allowing direct comparison of HOPS-dependent and HOPS-independent SNARE assembly (Torng and Wickner, 2020). The average FRET efficiency in these studies is modest because they include in
bulk reactions all the fluorescent Qb and Qc, many of which do not enter SNARE complexes. SNARE complex assembly with HOPS gave a high average FRET efficiency when fluorophores were at the N-terminus of the Qb SNARE domain and at or near the N-terminal end of the Qc SNARE domain (Figure 2B, red and yellow curves). There was lower FRET efficiency when the fluorophores were at opposite ends of the Qc and Qb SNARE domains (Figure 2B, blue, green, and indigo). When both fluorophores were at the C-terminal ends of the Qb and Qc SNARE domains, a low signal was seen (Figure 2B, purple), similar to the average FRET efficiency when fluorophores were at opposite ends of the SNARE domains and thus suggesting incomplete zippering. After 1 hour, Sec17 was added to each reaction. Strikingly, Sec17 only enhanced the average FRET efficiency between C-terminal fluorophores, rapidly rising to the level seen when the fluorophores were together at the N-terminii of the SNARE domains (Figure 2B, purple; see also Figure 2-figure supplement 1), indicating a Sec17-induced change at the C-terminal end of the SNARE complex. Sec17/αSNAP may promote the zipperping of isolated SNARE domains (Ma et al., 2016), but is now seen to act in the context of membranes and HOPS.

Sec17-induced conformational change requires HOPS. Proteoliposomes with R, Qa, and Ypt7 (where indicated) were incubated with Qb-SNARE domain and Qc. Both SNARE domains were either fluorescently labeled at their N-termini (Figure 2C, bars 1-5) or at their C-termini (Figure 2C, bars 6-10). Incubations were in the presence or absence of HOPS. Sec17 addition after 1 hour did not enhance the average FRET efficiency between N-terminally disposed fluorophores in the presence or absence of HOPS (Figure 2C, bars 1-5), but stimulated the average FRET efficiency between SNARE domain C-terminal fluorophores in a HOPS-dependent manner (Figure 2C, bars 6-10), since the enhanced FRET between the SNAREs in the presence of HOPS and Sec17 (bar 9) is not seen without HOPS (bar 8) or without Sec17 (bar 10). This indicates a HOPS-dependent and Sec17-induced SNARE conformational change. This was diminished when zippering was inhibited by the absence of the +4 to +8 layers of sQa (Figure 2D, bar 1 vs. 3) or by the conversion of each inward-facing apolar residue of the full-length Qa SNARE domain layers +4 to +8 to Gly (Figure 2E, bar 1 vs. 3). The F22SM23S mutation of Sec17 (FSMS hereafter), diminishing the hydrophobicity of its N-domain loop (Song et al., 2017), reduced the Sec17 capacity for inducing HOPS-dependent conformational change (Figure 2F). We also examined the effects of other mutants of Sec17. The K159E,K163E mutation (KEKE hereafter) diminishes Sec17:SNARE association (Marz et al., 2003); one of these residues (Sec17 K159) is in a pair (αSNAP K122, K163) that abolishes disassembly of the neuronal SNARE complex by NSF/αSNAP (Zhao et al., 2015). The C-terminal L291A,L292A mutation of Sec17 (LALA hereafter) interferes with its cooperation with Sec18 for SNARE complex disassembly (Barnard et al., 1997; Schwartz and Merz, 2009; Zick et al., 2015), and 6AtoN is the conversion of 6 inward-facing acidic residues of Sec17 which face basic SNARE residues in the 20s structure (Figure 1C) to neutral residues. Neither KEKE, LALA, nor 6AtoN had a large effect on the capacity of Sec17 to promote HOPS-dependent conformational change (Figure 2F).

Since this Sec17-induced SNARE conformational change is seen with C-terminal fluors but not N-terminal fluors, requires HOPS as the SNARE assembly catalyst, and requires SNAREs that can zipper, a major part of the conformational change may be zippering itself. This might be spontaneous after Sec17-induced release of HOPS from SNAREs (Collins et al., 2015; Schwartz et al., 2017) or be promoted by Sec17 binding along the SNAREs (Figure 1C), reflecting its affinity for the C-terminal region of SNARE bundles (Ma et al., 2016). HOPS bound to SNAREs may
inhibit C-terminal zipperning, even though it helps to assemble the N-terminus of the four-helix bundle. Sec17 could then increase FRET because it displaces HOPS. Such C-terminal zipperning would be favored by Sec17 binding, but could also be spontaneous in the absence of these factors.

Sec17 also interacts with partially-zippered SNARE complex to promote SNARE complex stability. SNARE complex was assembled by HOPS on Ypt7/R proteoliposomes with soluble Qa, with the Qb SNARE domain labeled with a fluorophore at a cysteiny1 residue upstream of the SNARE domain, and with Qc of full length (w.t.) or with the 3Δ C-terminal truncation, each bearing a fluorophore at its native cysteine residue upstream of the SNARE domain. When the complex of proteoliposomes with these fluorescent Qb and Qc had full-length SNARE domains, it was stable whether or not it included Sec17, as there was no loss of average FRET efficiency after addition of excess nonfluorescent Qc (Figure 2G, bars 1 and 2; Figure 2-figure supplement 1G). In contrast, fluorescent Qc3Δ was "chased" by exchange with nonfluorescent Qc (bar 3), but Sec17 stabilized this SNARE complex, blocking the chase (bar 4). Thus, in the absence of Sec17, the assembly of Qc3Δ into partially-zippered SNARE complex is reversible. Each domain of Sec17 helps to stabilize Qc3Δ against exchange (bars 5-8), but especially the Sec17 N-terminal apolar loop (bar 5). The HOPS-dependent functions of Sec17, such as promotion of zipperning, may be aided by the direct affinity between these proteins (Figure 2-figure supplement 2).

**Generality of Sec17 and Sec18 bypass of arrested zipperning.** Sec17 can restore fusion when Qc has truncations at the C-terminal end of its SNARE domain (Schwartz and Merz, 2009), stimulated by Sec18 (Schwartz et al., 2017). We asked whether Sec17 can restore fusion with similar deletions of residues after the +3 layer in the other Q-SNAREs. Proteoliposomes bearing Ypt7 and R-SNARE were assayed for fusion with proteoliposomes bearing this Rab and any two anchored Q-SNAREs. Proteoliposome mixtures were incubated with HOPS and the soluble form of the remaining soluble Q-SNARE deprived of its membrane anchor and a C-terminal portion of its SNARE domain (Figure 3: A, Qc3Δ; B, sQb3Δ; C, sQa3Δ). As reported (Schwartz et al., 2017), there was no fusion with Qc3Δ unless 600 nM Sec17, 300 nM Sec18, and ATP or ATPγS were present (Figure 3A); these concentrations are in the physiological concentration ranges of Sec17 (150 to 1100 nM) and Sec18 (250-760 nM) (Ho et al., 2018). While ATP and its nonhydrolyzable analog ATPγS support comparable fusion with wild-type SNAREs (Song et al., 2017), hydrolyzable ATP inhibits fusion through Sec17/Sec18-mediated disassembly of SNARE complexes when defective SNAREs such as Qc3Δ are present, a proofreading function. The same pattern was seen for fusion with sQb3Δ (Figure 3B) and sQa3Δ (Figure 3C). The unique spatial disposition of the Sec17/αSNAP molecules with respect to each SNARE (Figure 1C, and Zhao et al., 2015) makes it unlikely that Sec17 could somehow fill each of the gaps left by each of these deletions to shield apolar residues within the SNARE bundle and thereby continue to drive zipperning, or that Sec17 binding could induce the remaining R and two Q +4 to +8 layers to somehow rotate to form a hydrophobic 2- or 3-layered core.

Fusion assays were also performed with Ypt7/R proteoliposomes and each of the three Ypt7/single anchored Q-SNARE proteoliposomes in the presence of HOPS and the other two soluble Q-SNAREs (Figure 4). With membrane-anchored Qa and with sQb and Qc having complete SNARE domains, there was HOPS-dependent fusion without further addition (Figure 4A, black line), though Sec17 and Sec18 with AMP-PNP, ATPγS, or ATP did stimulate (compare black curves, A-D). Deletion of the +4 to +8 layers from either soluble Qb or Qc abolished fusion (Figure 4A),
which was restored by Sec17 and Sec18 with either AMP-PNP, ATPγS, or ATP (B-D, red and blue curves). There was no fusion when both soluble Q-SNAREs had truncated SNARE domains (Figure 4A, orange), but strikingly the fusion was partially restored by Sec17 and Sec18 with ATP (Figure 4D, orange) and more fully restored when the adenine nucleotide was resistant to hydrolysis (Figure 4, B and C, orange). With 2 Q-SNAREs lacking the C-terminal portion of their SNARE domains, the apolar amino acyl residues of the remaining 2 SNAREs would not be as effectively shielded from water if they continued zippering together. Fusion could not be restored by Sec17 and Sec18 if either soluble SNARE was omitted instead of truncated (green and purple).

This fusion with sQb3Δ and Qc3Δ occurs in stages, initially sensitive to antibody to either the HOPS SM subunit Vps33 or to Sec18, then acquiring resistance to Vps33 antibody while remaining sensitive to the Sec18 ligand (Figure 4-figure supplement 2). When only the Qb-SNARE was membrane anchored, there was little fusion without Sec17 and Sec18 (Figure 4E, black curve). When the SNARE domain of sQa or Qc had been truncated, fusion was strictly dependent on non-hydrolyzable ATP analogs, and little fusion was seen with dual SNARE domain truncation. Similar patterns were seen with anchored Qc (Figure 4, I-L). We term the fusion induced by Sec17 and Sec18 in the presence of 3Δ SNARE domain truncations "zippering bypass fusion."

To explore the capacity of Sec17 and Sec18 to compensate for partial SNARE zippering, we assayed the fusion of Ypt7/R and Ypt7/Qa proteoliposomes with sQb3Δ, Qc3Δ, and HOPS, using various concentrations of wild-type or mutant Sec17, and with or without Sec18 and ATPγS (Figure 5). Without Sec17, fusion is not supported by Sec18 (A and B, blue curves). Limited fusion is possible with 1 or 2 μM wild-type Sec17 alone (A, black and red), but Sec18 allows faster fusion and with less Sec17 (A and B; tan). Fusion requires the apolar loop near the N-terminus of Sec17, as the F21S,M22S mutation (FSMS) blocks fusion entirely (C and D). The K159E,K163E mutation (KEKE) diminishes Sec17:SNARE association (Marz et al., 2003). The KEKE mutation prevents fusion without Sec18 (E), but a slow and limited fusion with KEKE-Sec17 is restored by Sec18 (F). The C-terminal L291A,L292A mutation of Sec17 (LALA), which interferes with its cooperation with Sec18 for SNARE complex disassembly (Barnard et al., 1997; Schwartz and Merz, 2009; Zick et al., 2015), diminishes zippering-bypass fusion (A vs. G, red curves), and a limited fusion is restored through the addition of Sec18 (H). These data suggest that Sec17 action directly requires its apolar loop domain, since the loss of this apolar region is not bypassed by Sec18. Sec18 may stimulate fusion by modulating the conformation of Sec17 associations with trans-SNARE complexes, but Sec18 is not simply promoting Sec17 binding, since it is still needed for fusion when Sec17 is joined to an integral N-terminal membrane anchor (Figure 5-figure supplement 2). Basic residues in the +3 to +8 layers near the C-terminus of the R and Qa SNARE domains are near acidic residues on the interior of the Sec17 assembly (Figure 5I). To determine whether Sec17 might rely on these ionic interactions to support fusion, we converted these acidic residues of Sec17 to alanine or serine, creating the mutant Sec17-E34S,E35S,D38S,E73A,D43A,E75A (termed Sec17 6 Acidic to Neutral, or Sec17-6AtN), but this mutant Sec17 still supports fusion (Figure 5, J and K). Interestingly, mutation of acidic residues of αSNAP near the C-terminal end of the neuronal SNARE complex also only had a modest effect on disassembly activity of NSF/αSNAP (Zhao et al., 2015).

**Fusion despite triply-crippled SNARE zippering.** Since SNARE zippering is driven by the sequestration of apolar residues into the interior of the 4-SNARE bundle, we examined the effect
of converting the apolar residues of the Qa SNARE domain +4 to +8 layers to Ala, to Ser, or to Gly. Fusion between Ypt7/R- and Ypt7/Qa-proteoliposomes in the presence of HOPS, sQb, and Qc but without Sec17 or Sec18 (Figure 6A, black curve), was diminished by replacing each of the +4 to +8 layer apolar residues of Qa with Ala (Figure 6A, green curve) and was abolished when they were replaced by Ser (blue curve) or by Gly (red curve). The persistence of some fusion with the Ala substitutions may reflect that two of the residues were already Ala, that Ala has the greatest propensity among the amino acids to form α-helices, Gly the least, and Ser is in between (Pace and Scholtz, 1998), and that alanine itself is the least hydrophilic of these three amino acids. When these same incubations were performed with Sec17, Sec18, and ATPγS, rapid and comparable fusion was seen in each case (Figure 6B). When hydrolyzable ATP was used instead of ATPγS, there was little effect on the fusion kinetics with wild-type SNARE domain sequences (Figure 6, B vs. C, black curves). In contrast, hydrolyzable ATP led to fusion inhibition when SNARE packing stability was reduced by substitution of apolar residues by Ala, Ser, or Gly (Figure 6C). Though the apolar residues are not required for fusion aided by Sec17 and Sec18 (Figure 6B), they apparently stabilize the SNAREs against ATP-driven proofreading disassembly (Figure 6C). To triply weaken the completion of zippering, the same proteoliposomes with wild-type Qa or the Qa +4 to +8 layer having small side-chain residues instead of apolar residues were incubated with HOPS, sQb3Δ and Qc3Δ, either without Sec17 or Sec18 (Figure 6D) or with Sec17, Sec18, and ATPγS (Figure 6E) or ATP (Figure 6F). Fusion was optimally supported by Sec17, Sec18, and ATPγS (Figure 6E). The independence of this fusion from energy derived by zippering is underscored by the similar fusion rates in all incubations with Sec17, Sec18, and ATPγS, whether with apolar or polar Qa +4 to +8 residues or with full-length or +4 to +8-truncated sQb and Qc (Figures 6, B vs. E). With the Qb and Qc SNARE domains truncated, and the Qa lacking apolar inward-facing amino acyl side-chains, little or no energy could be gained from the completion of R and mutant-Qa zippering. Thus Sec17 acts in three ways: triggering a zippering-dependent SNARE conformational change in the presence of HOPS and full-length SNARE domains (Figure 2), acting with Sec18 to promote fusion independent of energy from zippering (Figure 6), and supporting the disassembly of post-fusion cis-SNARE complexes by Sec18.
Discussion

The catalytic roles of fusion proteins have been gleaned from functional reconstitution studies. These studies initially showed that the zippering of concentrated SNAREs can drive slow fusion (Weber et al., 1998; Fukuda et al., 2000). As proteoliposome SNARE levels were lowered towards physiological levels, reconstituted vacuolar and neuronal fusion reactions required additional factors (Zick and Wickner, 2016; Stepien and Rizo, 2021). In addition to SNAREs, reconstituted neuronal fusion requires Munc18, Munc13, calcium, NSF, and αSNAP (Ma et al., 2013; Lai et al., 2017) while reconstituted vacuole fusion needs HOPS (Stroupe et al., 2006), the Rab Ypt7 (Stroupe et al., 2009; Ohya et al., 2009), and specific lipid head-group composition and fatty acyl fluidity (Zick and Wickner, 2016). HOPS and other SM proteins catalyze SNARE assembly (Baker et al., 2015; Orr et al., 2017; Jiao et al., 2018), regulated by an activated Rab (Zick and Wickner, 2016; Torng and Wickner, 2020), and may confer resistance to Sec17/αSNAP and Sec18/NSF-mediated trans-SNARE disassembly (Xu et al., 2010; Jun and Wickner, 2019). Sec17/αSNAP and Sec18/NSF stimulate fusion with HOPS and wild-type SNAREs (Mima et al., 2008; Song et al., 2017).

Fusion can be supported by either complete 4-SNARE zippering without Sec17 or Sec18, or by Sec17 and Sec18 association with only partially zippered SNAREs, but the most rapid fusion requires both (Song et al., 2017). While Sec17 and Sec18 are known to bypass the fusion blockade by Qc C-terminal truncation alone (Schwartz and Merz, 2009; Schwartz et al., 2017), the generality of this bypass with respect to any one Q-SNARE or even two Q-SNAREs (Figures 3, 4) shows that it is not specific to Qc alone. Moreover, when zippering with full-length SNARE domains is weakened by the substitution of small amino acyl residues for large apolar residues in the +4 to +8 layers of Qa, Sec17 and Sec18 will also restore fusion (Figure 6A, B). Strikingly, Sec17 and Sec18 drive fusion despite a triple blockade to complete zippering, namely the absence of two C-terminal Q-SNARE domains and the lack of apolarity in the third (Figure 6E). In the NSF/αSNAP/SNARE complex, the 4-SNAREs wrap around each other in a left-handed super-helix, and the Sec17’s wrap around them in a right-handed fashion, yet they form a specific structure (Zhao et al., 2015; White et al., 2018). It seems unlikely that residues from one or more Sec17 could substitute for the missing residues when 2 heptads are removed from the C-termini of one or even two Q-SNAREs and the bulky apolar residues are removed from the third.

From the earliest reconstitutions of HOPS-dependent fusion (Mima et al., 2008) and in subsequent studies (e.g. Zick et al., 2015), Sec17 and Sec18 gave strong stimulation. In contrast, Sec17 and Sec18 inhibit SNARE-only fusion, or fusion with nonphysiological tethers (Mima et al., 2008; Zick and Wickner, 2014; Schwartz et al., 2017; Song and Wickner, 2019). HOPS not only binds each SNARE, but has direct affinity for Sec17 (Figure 2-figure supplement 2). In model studies with cis-SNARE complexes, HOPS is necessary for Sec17 to enhance zippering per se (Figure 2C). HOPS:Sec17 binding may underlie their synergy for fusion, but further studies are needed to test this idea.

Earlier studies and our current work suggest a working model of vacuole membrane fusion, encompassing findings here and elsewhere (Figure 7). HOPS exploits the affinity of its Vps39 and Vps41 subunits for the Rab Ypt7 (Brett et al., 2008) on each fusion partner membrane (Figure 7A) to mediate [step1] tethering (Hickey and Wickner, 2010). Tethered membranes (Figure 7B) are a
prerequisite for SNARE assembly in an active conformation, likely a common N to C SNARE
domain orientation (Song and Wickner, 2019). HOPS has direct affinity for each of the 4 vacuolar
SNAREs (Song et al., 2020) and is allosterically activated by vacuolar lipids and Ypt7:GTP (Torng
and Wickner, 2020) as a catalyst of SNARE assembly [step2]. SNAREs begin to zipper (Figure
7C) from the N towards the C-terminal end of their SNARE domain. When any one Q-SNARE is
omitted, fusion intermediates assemble which undergo very rapid fusion when the missing Q-
SNARE is supplied (Song et al., 2020), suggesting that fusion without Sec17/Sec18 is rate-limited
by the completion of SNARE zipperping and/or the spontaneous release of bulky HOPS. Sec17 has
direct affinity for SNAREs (Söllner et al., 1993), HOPS (Figure 2-figure supplement 2), lipid
(Clary et al., 1990; Zick et al., 2015), and Sec18 (Söllner et al., 1993). Sec17 displaces HOPS from
the SNAREs [Figure 7, step 3], as shown by earlier studies: 1. Vacuolar SNAREs are found in
complex with Sec17 or with HOPS, but not with both, and Sec17 can displace HOPS from
SNAREs (Collins et al, 2005). 2. When vacuolar HOPS-dependent reconstituted fusion is arrested
by Qc3Δ, HOPS is bound to the incompletely-zippered SNARE complex until it is displaced by
Sec17 (Schwartz et al., 2017). 3. trans-SNARE complexes which assemble between isolated
vacuoles are largely associated with Sec17 (Xu et al., 2010). The Sec17 association with partially-
zippered trans-SNARE complex (Figure 7D) promotes conformational change (Figure 2) leading
to complete zippering. The Sec17: 4-SNARE complex will bind Sec18 [Step 4] through its
affinities for both SNAREs (Zick et al., 2015) and for Sec17. Sec18 regulates in some unknown
fashion the Sec17/αSNAP assembly into an oligomeric structure surrounding the SNARE complex
intermediate (Zhao et al., 2015), a trans-anchored Sec18/Sec17/SNARE pre-fusion complex
(Figure 7E). Some ATP hydrolysis-dependent disassembly can occur, which may represent
proofreading of incorrect and unstable trans-SNARE complexes (Choi et al., 2018). Sec17
oligomerization may also be stabilized or guided by the insertion of its N-terminal loop into the
membranes. While SNAREs can slowly complete zipperping and support fusion without Sec17 or
Sec18 (Mima et al., 2008; Song et al., 2015), and slow fusion can occur with Sec17 and Sec18
when sQb and Qc lack the C-terminal portion of their zipper domain (Figure 4D), optimal
fusion requires 4 complete SNARE domains, Sec17/αSNAP, and Sec18/NSF. The energy for
fusion [step 5] derives from multiple sources: the completion of SNARE zipperping, the binding
energies which create the Sec17 structure surrounding the SNAREs, and from the energy of bilayer
distortion through Sec17 apolar loop insertion. Cis-SNARE complexes (Figure 7F) formed by
fusion (Söllner et al., 1993; Mayer et al., 1996) are disassembled by Sec17, Sec18, and ATP [step
6], freeing SNAREs from each other (Figure 7A) for later assembly in trans. Each of these
components- the four SNAREs, the Rab Ypt7, HOPS, Sec17, and Sec18- are part of this ordered
pathway of associations and disassembly, constituting a holoenzyme for vacuole membrane fusion.

Our current studies reveal a general capacity of Sec17/αSNAP and Sec18/NSF to support fusion,
even when little or no energy would be derived from completion of zipperping. Sec17 provides a
cage-like environment (Chakraborty et al., 2010), albeit with side fenestrations (Schwartz et al.,
2017), which may favor SNARE zipperping or, where zipperping is blocked, allow the remaining
SNARE domains to attain positions and conformations which approximate zipperping. The apolar
N-domains of the four Sec17s are clearly essential for fusion (Figures 5 and Figure 5-figure
supplement 2), whether through positioning the Sec17s, facilitating their assembly, or directly
inserting as a "membrane wedge" and thereby contributing to bilayer disruption at the fusion site.
The continued need for this apolar loop when Sec17 is integrally membrane-anchored (Figure 5-
figure supplement 2) shows that it does not simply contribute to Sec17 membrane association.
Elements of "Sec17 cage" and "membrane wedge" action are not mutually exclusive. Further work will be needed to determine the relative energies of Sec17 and Sec18 binding to the assembling 4-SNARE trans-complex, each of four Sec17s inserting its apolar loops into the membrane, Sec17 forming lateral associations with other Sec17 molecules in the cage around the SNAREs, and complete SNARE zippering, as each of these helps to achieve the bilayer distortions of fusion.

Our current studies further our understanding of how Sec17(αSNAP), Sec18 (NSF), and SNAREs cooperate to promote membrane fusion. Sec17 has multiple functions: a. It supports Sec18 association with SNARE complexes for their ATP-driven disassembly for subsequent rounds of fusion (Ungermann et al., 1998; Choi et al., 2018). Sec17/αSNAP and Sec18/NSF are also part of a quality control system that for vacuoles includes HOPS (Starai et al., 2008) and in the neuronal system includes Munc18 and Munc13 (Ma et al., 2013; Lai et al., 2017). These disassembly reactions require ATP hydrolysis, and can be interrupted by the Sec17 LALA mutation. b. Sec17 promotes a conformational change which leads to complete SNARE zippering. While neuronal SNAREs which are properly assembled (i.e., involving the Munc18/Munc13 pathway) will fully zipper with high energy yield, this may not be true for all non-neuronal SNAREs. c. Sec17 promotes fusion even when SNARE zippering is incomplete. Sec17 uses the partially-zippered SNAREs as a platform to bind to the fusion site, and its apolar N-domain loop to trigger fusion. Sec18 also has multiple functions: a. Sec18/NSF drives ATP-driven SNARE disassembly (Zhao et al., 2015), blocked by the Sec17 LALA mutation. b. Sec18 supports Sec17 for direct promotion of fusion, built on a platform of partially-zippered SNAREs. This does not need ATP hydrolysis, and is insensitive to the LALA mutation. Thirdly, SNAREs also have multiple functions: a. They can completely zipper, thereby stressing the bilayer and triggering fusion (Weber et al., 1998; Sutton et al., 1998). As previously reported (Mima et al., 2008; Song et al., 2017), HOPS alone, without Sec17 or Sec18, will support slow SNARE-dependent fusion. b. SNAREs support the assembly of a microdomain where multiple fusion proteins and lipids become highly enriched (Fratti et al., 2004). c. As shown here, SNAREs form an essential platform for Sec17 and Sec18 to contribute to fusion independently of completion of zippering. With physiological levels of wild-type vacuolar SNAREs, rapid fusion requires Rab-activated HOPS, Sec17, Sec18, and ATP.

While intracellular fusion reactions share many requirements, such as for SNAREs and SM protein, there are major differences as well. Synaptic vesicle fusion and other calcium dependent secretion systems require calcium, synaptotagmin, Munc13, and complexin, none of which have their obvious counterparts in calcium independent systems. Vacuole and endosome fusion have their SM protein as an integral subunit of the tethering complex, which is not seen at other organelles. The similarities and differences in fusion pathways at each organelle will be clarified as each is more thoroughly studied.
### Materials and Methods

#### Key Resources Table

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PI3P was from Echelon (Salt Lake City, Utah), ergosterol from Sigma (St. Louis, MO), fluorescent lipids from Thermo Fisher (Waltham, MA), and other lipids were from Avanti (Alabaster, AL). Biobeads SM2 were from BioRad, Cy5-Streptavidin from SeraCare (Milford, MA), biotinylated phycoerythrin from Invitrogen (Eugene, OR) and underivatized streptavidin from Thermo Fisher. Spectrapor 6 dialysis tubing (7.5mm dia, 25 kDa cutoff) was from Spectrum Labs (Las Vegas, NV). Octyl-b-D-glucopyranoside was purchased from Anatrace (Maumee, OH).

### Mutant constructions.

Sec17 with 6 acidic amino acids mutated to neutral residues, GST-Sec17 (E34S, E35S, D38S, E73A, D43A, E75A), was generated by PCR with Phusion high-fidelity DNA polymerase (NEB). The DNA fragment was cloned into BamHI and SalI digested pGST parallel1 vector (Sheffield et al.,1999) with an In-Fusion kit (Takara Bio USA, Mountain View, CA). Using inverse PCR, pParallel1-GST-Sec17 mutant (E34S, E35S and D38S) was amplified with Phusion high-fidelity DNA polymerase (NEB) from a GST-Sec17 construct. The amplified linear DNA was re-circularized with an In-fusion kit (Takara Bio USA, Mountain View, CA). To generate Sec17 acidic to neutral mutants, pParallel1-GST-Sec17 mutant (E34S, E35S, and D38S) was amplified with a Sec17 E73A, D43A, and E75A mutant primer set using Phusion high-fidelity DNA polymerase (NEB) and re-circularized with an In-fusion kit (Takara Bio USA, Mountain View, CA).

For Sec17-E34S,E35S, D38S:

- For Sec17-E34S,E35S, D38S:
  - F TCGTCGGCTGCTTCTCTTTGTGTCCAAGCAGCCAC
  - R AGAAGCAGCCGACGAAAACCTTGATGATAGCAGGA

  For Sec17 E73A, D43A, E75A:

  - F GGTAATGCAGCCGCAGCAGGAAATACCTACGTAGA
  - R TGCGGCTGCATTACCAGCCTTTCTGATAGTCAG

  sQa3Δ, sQb3Δ. GST-sQa3Δ with amino acyl residues 1–235 and GST-sQb3Δ with amino acyl residues 1–160 were generated by PCR with Phusion high-fidelity DNA polymerase (NEB). DNA fragments were cloned into BamHI and SalI digested pGST parallel1 vector (Sheffield et al.,1999) with an In-Fusion kit (Takara Bio USA, Mountain View, CA).

For GST-sQa3Δ.
For GST-sQb3Δ,

To generate Qα with L238, M242, A245, L249, and A252 changed to Ala, Ser or Gly, the pParallel1 GST vector with Qα lacking residues 228-257 was generated by inverse PCR with pParallel1 GST-Qα (Mima et al, 2008) using Phusion high-fidelity DNA polymerase (NEB). The DNA duplex with mutations (Gly, Ser or Ala) of the +4 to +8 heptad repeats was cloned into the amplified vector bearing Qα 1-227 with an In-fusion kit (Takara Bio USA, Mountain View, CA).

For inverse PCR of pParallel1 GST and Qα 1-227

F: GACCAGCATCAGAGGGACCG
R: GTCTATGGGTGTACTTTGTT

For the Gly mutant of Qα

Sequence1:
GTAACCCACCATAGACGAGAAATATCTCGCATGGCCATGATAACGCGCAATGGGCA
CAAACCAAGGACCAGGCGACCAGCAGCATCAGAGG

Sequence2:
CCTCTGATGCTGGTGCTCTGGTGCTTTGTTTGCTATTCTGCGTTATCATGG
CCATGCGAGATATTCTCGTCTATGGGTGTTAC

For the Ser mutant of Qα

Sequence1:
GTAACCCACCATAGACGAGAAATATCTCGCATAGCCATGATAACGCGCAATGGGCA
CAAACAAAGGACCAGGCGACCAGCAGCATCAGAGG

Sequence2:
CCTCTGATGCTGGTGCTCTGGTGCTTTGTTTGCTATTCTGCGTTATCATGGGC
TATCGAGATATTCTCGTCTATGGGTGTTAC

For the Ala mutant of Qα

Sequence1:
GTAACCCACCATAGACGAGAAATATCTCGCATGCCATGATAACGCGCAATGGGCA
CAAACAAAGGACCAGGCGACCAGCAGCATCAGAGG

Sequence2:
CCTCTGATGCTGGTGCTCTGGTGCTTTGTTTGCTATTCTGCGTTATCATGGGC
GCATGCGAGATATTCTCGTCTATGGGTGTTAC

Vam7 mutants with cysteines inserted at the N- and C-termini of the SNARE domain were generated by inverse PCR with the Vam7 intein vector (Schwartz and Merz, 2009) and Phusion high-fidelity DNA polymerase (NEB). First, the native cysteine was removed by mutating it to serine (C208S) using the mutant primer set below. Vam7 mutants with cysteines inserted near the N- and C- termini of the SNARE domain, M250C and A316C, respectively, were generated from the cysteine-lacking plasmid in the same fashion.

For Vam7-C208S,

F: GAAAGCGATGACATTGGTACAGCAAACATAGCTCA
Protein isolation. HOPS and prenyl-Ypt7 (Zick and Wickner, 2013), Ypt7-TM (Song et al., 2020), Sec17 (Schwartz and Merz, 2009), TM-anchored Sec17 and TM-anchored Sec17-F21SM22S (Song et al., 2017), Sec18 (Mayer et al., 1996), wild-type vacuolar SNAREs and his6-R (Mima et al., 2008; Schwartz and Merz, 2009; Izawa et al., 2012), sQb (Zick and Wickner, 2013) Qc-C208SM250C, Qc-C208SA316C, and Qc3Δ (Schwartz and Merz, 2009) were purified as described.

Proteoliposome fusion. Proteoliposomes were prepared by detergent dialysis from β-octylglucoside mixed micelles for fusion assays (Song et al., 2017; Song et al., 2019) and SNARE assembly assays (Torng et al., 2020) as described. Briefly, for fusion assay, proteoliposomes (1 mM lipid) were prepared with membrane-bound Ypt7 and R at 1:8000 and 1:16,000 molar ratios to lipid, respectively, and with luminal biotin-phycoerythrin. Proteoliposomes were also prepared with membrane-bound Ypt7 and the indicated Q-SNAREs at 1:8000 and 1:16,000 molar ratios to lipid, respectively, plus luminal Cy5-streptavidin. These were incubated separately for 10 min at 27°C with 1μM GTP and 1 mM EDTA, then MgCl2 was added to 2 mM. After prewarming (27°C 10 min) the separately GTP-exchanged proteoliposomes in a 384 well plate, fusion reactions were initiated by mixing 5 μl of each proteoliposome preparation and supplementing with other fusion factors in volumes summing to 10 μl, continuing incubation at 27°C in a Spectramax fluorescent...
plate reader. Fusion incubations (20 µl) in RB150 (20 mM HEPES/NaOH, pH 7.4, 150 mM NaCl, 10% glycerol) had proteoliposomes (0.5 mM total lipid concentration), 50 nM HOPS, the indicated concentrations of sSNAREs, 400 or 600 nM Sec17, 300 nM Sec18, 1 mM ATP or its analogs, and 3 mM MgCl₂, as modified in each Figure legend.

**SNARE assembly assay.** Assays were performed as described previously (Torng et al., 2020) with one addendum. In brief, reactions (20 µL) were at 27 °C in a SpectraMax Gemini XPS (Molecular Devices) plate reader. Standard reactions include HOPS (160 nM), proteoliposomes [0.5 mM lipid, with SNARE and Ypt7 at a molar ratios of either 1:2000 or 1:4000 for Ypt7/R-proteoliposomes and Ypt7/RQa-proteoliposomes, respectively], and fluorescently-labeled Qb and Qc (1 µM), and sQa (1 µM) as necessary. These were incubated for 60 min, then Sec17 was added to 500 nM. Three fluorescence channels were read simultaneously at intervals of 30 s: the donor channel Oregon Green 488 from Qc (excitation [ex]: 497 nm; emission [em]: 527 nm; cutoff [c/o]: 515 nm), the acceptor channel Alexa Fluor 568 from Qb (ex: 568 nm; em: 605 nm; c/o: 590 nm), and the FRET channel (ex: 490 nm; em: 615 nm; c/o: 590 nm). For each timepoint, the bleed through-corrected FRET signal was obtained by subtracting the background signals coming from the donor and acceptor channels from the signal in the FRET channel as detailed in Torng et al. (2020). This was further corrected by dividing by the geometric mean of the donor and acceptor signals. The final corrected signal, reported as “Average FRET efficiency,” is a combined measure of the proportion of fluorescent SNAREs undergoing FRET and their average FRET efficiency.

**Molecular models:** MODELLER (Webb & Sali, 2016) was used to create individual homology models of the vacuolar SNARE complex (Nyv1, Vam3, Vti1, Vam7), and of Sec18 starting from the coordinates of synaptobrevin-2, SNAP-25, syntaxin-1A, and NSF in the cryo-EM structure of the neuronal NSF/αSNAP/SNARE complex (PDB ID 3J96) (Zhao et al., 2015). For Sec18, the linker between the N and D1 domains was deleted from the generated homology model since there was no information about these linkers in this structure (PDB ID 3J96) of the neuronal 20S complex.

The MODELLER protocol consisted of an alignment step (python script file align.py) and a modelling step (python script file modeler-input.py). The script files are shown here for synaptobrevin (nyv1):

```python
align.py

from modeller import *

env = environ()
aln = alignment(env)
mdl = model(env, file='sb.pdb', model_segment=('FIRST:K','LAST:K'))
aln.append_model(mdl, align_codes='sbK', atom_files='sb.pdb')
aln.append(file='target_sequence.pir', align_codes='nyv1')
aln.salign(local_alignment=True, rr_file='${LIB}/blosum62.sim.mat',
          gap_penalties_1d=(-600, -600),
          output='",
          align_block=15, # no. of seqs. in first MSA
          align_what='PROFILE',
```
aln.edit(edit_align_codes='nyv1', base_align_codes='rest', min_base_entries=1, overhang=0)
aln.write(file='nyv1.ali', alignment_format='PIR')
aln.write(file='nyv1.pap', alignment_format='PAP')

The homology models of Nyv1, Vam3, Vti1, Vam7 and Sec18, together with the crystal structure of Sec17 (PDB ID 1QQE) (Rice & Brunger, 1999) were fit into the Cryo-EM structure of the neuronal NSF/αSNAP/SNARE complex (PDB ID 3J96) (Zhao et al., 2015). The fit was performed by using the “align” feature of PyMol to individually superimpose the coordinates of the vacuolar proteins with the corresponding coordinates of the neuronal proteins in the structure of the neuronal NSF/αSNAP/SNARE complex.
Acknowledgements. We thank Gustav Lienhard, Charles Barlowe, Frederick Hughson, Michael Zick, Christian Ungermann, Jose Rizo, and Randy Schekman for insightful suggestions. This work was supported by grants R35GM118037 (to W.W.) and R37MH63105 (to A.T.B.) from the NIH.


Figure 1. Model of the Sec18/Sec17/vacuolar SNARE complex and Sec17 mutations. A. Schematic of the 4 yeast vacuolar SNAREs, the soluble Q-SNAREs (sQx), and their deletion derivatives sQxΔ lacking regions C-terminal to the +3 layer of the SNARE domain. B. Structure of Sec17 (Rice and Brunger, 1999; Schwartz et al., 2017). Residues mutated in certain experiments are highlighted. C. Modelling of the vacuolar Sec18/Sec17/SNARE complex. MODELLER (Webb & Sali, 2016) was used to create individual homology models of the vacuolar SNARE complex (Nyv1, Vam3, Vti1, Vam7), and of Sec18 starting from the coordinates of synaptobrevin-2, SNAP-25, syntaxin-1A and NSF in the Cryo-EM structure of the neuronal 20S complex (PDB ID 3J96) (Zhao et al., 2015). These homology models, together with the crystal structure of Sec17 (PDB ID 1QQE) (Rice & Brunger, 1999) were fit into the Cryo-EM structure of the neuronal 20S complex (PDB ID 3J96) (Zhao et al., 2015). We used PDB ID 3J96 because this Cryo-EM structure did not include the SNAP-25 linker and the Habc domain of syntaxin-1A. The vacuolar SNARE complex (Nyv1, Vam3, Vti1, Vam7) also does not include a linker between any of the SNARE motifs; in all structures of NSF/αSNAP/ternary SNARE complexes determined thus far, four αSNAP molecules are observed for SNARE complexes that do not contain a linker connecting two of the SNARE domains (White, Zhao, Choi, Pfuetzner, & Brunger, 2018), and we therefore included four Sec17 molecules in our model of the vacuolar 20S complex. In the PDB coordinate file supplied as Source Data File of the homology model of the Sec18/Sec17/vacuolar SNARE complex, Sec18 molecules, chains A-F; Sec17, chains G-J; Nyv1, chain K; Vam3, chain L; Vti1, chain M; Vam7, chain N. Colors: cyan: Nyv1; magenta: Vam3; yellow: Vti1; salmon: Vam7; gray, orange, green, slate: Sec17; yellow, magenta, gray, blue, salmon, green: Sec18. Cartoon representations are shown. Two views related by a 90 degree rotation are shown (left: sideview; right: membrane-end view). Figure 1B reproduced from Figure 5A Schwartz, et al. 2017, eLife, published under the Creative Commons Attribution 4.0 International Public License (CC BY 4.0; https://creativecommons.org/licenses/by/4.0/).

Figure 2. Sec17 modifies SNARE conformation in a HOPS- and zippering-dependent manner and stabilizes complexes with truncated SNARE domains. A. Schematic of fluorescently labeled SNARE constructs used in this study. SNAREs were derivateized as described previously (Torng et al., 2020). Wild-type Qc contains a single Cys residue at 208 at the upstream (U) position. (N)- and (C)-labeled constructs replace residues 250 and 316 with Cys, respectively, while also replacing Cys208 with Ser. Each Qc construct was derivatized from Oregon Green 488 as described (Torng et al., 2020). A fusion of maltose binding protein (MBP), a TEV site, and the Qb SNARE domain (residues 133-187) was expressed with an added cysteinyl residue immediately upstream or downstream of the SNARE domain. Each Qb construct was derivatized with Alexa Fluor 568. Qc and Qb labeled with a fluorescent probe at any position are written as *Qc and *Qb. B-G. Bar graphs are reported as the mean of the relative FRET change (%) per trial with propagated standard deviation for n=3 trials. The relative change was calculated from averaging 10 data points each from just before Sec17 addition and from the end of the measurement period 20 min later, except where indicated. See Supplementary Data for specific time points used as well as the statistics for the propagation of uncertainty. Also see Supplementary Figure for a bar graph representation for (B) and for the kinetic curves for (C-G).
complex. Ypt7/RQa proteoliposomes were incubated with pairs of *Qb and *Qc labeled at the N-, C-, or upstream (U)- locations as indicated in A. Curves are averages of n=3 trials, and the shaded regions behind each curve show the standard deviation per time point. C. Sec17-promoted zippering requires HOPS. RQa proteoliposomes were incubated with either the N-labeled *Qb/*Qc pair (left) or the C-labeled *Qb/*Qc pair (right), with Ypt7 and HOPS as indicated. A reaction with buffer (RB150) added instead of Sec17 serves as a negative control. D. Sec17 does not promote C-terminal zippering if the SNARE domain of Qa is truncated. Ypt7/R proteoliposomes were incubated with C-terminally labeled *Qb and *Qc and either soluble Qa or Qa3Δ, and the relative FRET change was calculated over 10 min after Sec17 or buffer addition. E. Sec17-induced zippering requires the apolar heptad repeat amino acyl residues in Qa SNARE domain layers +4 to +8. Proteoliposomes bearing Ypt7, R-SNARE, and either wild-type Qa or Qa with the +4 - +8 layers inwardly-oriented apolar residues mutated to Gly were incubated with C-terminally labeled *Qb and *Qc, and then Sec17 or its mutants were added at t=60 min. F. Sec17-driven SNARE conformational change is stunted by the FSMS mutation. Ypt/R proteoliposomes were incubated with sQa and C-terminally labeled *Qb and *Qc, then Sec17 or mutants as indicated were added at t = 60 min. G. Sec17 stabilizes incompletely-zipped SNARE complexes. Ypt7/R proteoliposomes were incubated with sQa and C-terminally-labeled *Qb and *Qc. Sec17 or the indicated mutants were added at t = 60 min. Non-fluorescent Qc (8.5 µM) was added at t = 80 min, and the loss of FRET over 35 min was measured starting immediately after addition of non-fluorescent Qc.

**Figure 3.** Fusion is blocked by deletion of the last 5 C-terminal SNARE domain layers from any single Q-SNARE and is restored by Sec17, Sec18, and ATP or ATPγS. A. Fusion incubations, as described in Methods, had Ypt7/R- and Ypt7/QaQb proteoliposomes (1:8,000 Ypt7:lipid molar ratio, 1:16,000 SNARE:l lipid molar ratios), 2µM Qc3Δ, and where indicated 600 nM Sec17, 300 nM Sec18, 1mM ATP (red) or ATPγS (blue). B. Fusion with 2µM sQb3Δ and with Ypt7/QaQc-tm proteoliposomes, but otherwise as in (A). C. Fusion with 2µM sQa3Δ and with Ypt7/QbQc-tm proteoliposomes, but otherwise as in (A). Mean and standard deviations from three independent experiments are shown in Figure 3-figure supplement 1.

**Figure 4.** Fusion with single membrane-anchored Q-SNAREs. A-D. Fusion incubations, as described in Methods, had Ypt7/R- and Ypt7/Qa- proteoliposomes (1:8,000 Ypt7-TM:l lipid molar ratio, 1:16,000 SNARE:l lipid molar ratios), 50 nM HOPS, 2 µM sQb or sQb3Δ, 2µM Qc or Qc3Δ, and where indicated 600 nM Sec17, 300 nM Sec18, and 1mM ATP, AMP-PNP, or ATPγS. E-H. Fusion incubations as for (A), but with Ypt7/Qb-proteoliposomes and 2µM sQa or sQa3Δ, 2µM Qc or Qc3Δ and Sec17, Sec18, and adenine nucleotide as indicated. I-L. Fusion incubations as for (A), but with Ypt7/Qc-tm-proteoliposomes and 2µM sQa or sQa3Δ, 2µM Qb or sQb3Δ and Sec17, Sec18, and adenine nucleotide as indicated. Mean and standard deviations from more than 3 independent experiments are shown in Figure 4-figure supplement 1.

**Figure 5.** Role of each domain of Sec17 in zippering-bypass fusion. A-H, J, K: Fusion between Ypt7/R and Ypt7/Qa proteoliposomes (1:8,000 Ypt7-TM:l lipid and 1:16,000 SNARE:l lipid molar ratios) was assayed with 50nM HOPS, 2µM sQb3Δ, 2µM Qc3Δ, the indicated concentration of wild-type or mutant Sec17, and with or without 250nM Sec18 and 1mM ATPγS. I. Ionic interactions between Sec17s and vacuolar SNAREs in the +4 to +8 layers in...
the model of the Sec18/Sec17/vacuolar SNARE complex (Figure 1). Colors: cyan: R; magenta: Qa; yellow: Qb; salmon: Qc; gray, orange, green, slate: Sec17; red: oxygen atoms; blue: nitrogen atoms. Cartoon representations are shown along with sidechains shown as thin lines. Thick lines (sticks) are interacting glutamate and aspartate (acidic) residues on the surface of Sec17 (aminoacyl residues 34, 35, 38, 73, 74, 75) that interact with the vacuolar SNARE complex lysine and arginine basic residues in each of the 4 SNAREs. Mean and standard deviations from four independent experiments are shown in Figure 5-figure supplement 1.

**Figure 6.** Sec17, Sec18, and ATPγS restore fusion to Ypt7/R and Ypt7/Qa proteoliposomes which were triply-crippled from completion of SNARE domain zippering by deletion of the +4 to +8 layers of the sQb and Qc SNAREs and by substitution of the apolar residues of the +4 to +8 layers of Qa, substituting Ala, Ser, or Gly for L238, M242, A245, L249, and A252. Fusion incubations (see Methods) had A-C. Ypt7/R- and Ypt7/Qa (w.t. (black), Gly mutant (blue), Ser mutant (red) or Ala mutant (green)) proteoliposomes (1:8,000 Ypt7-TM:lipid molar ratio, 1:16,000 SNARE:lipid molar ratios), 50nM HOPS, 2μM sQb (w.t.) and 2μM Qc (w.t.) (A-C) or D-F 2μM Qc3Δ and 2μM sQb3Δ. Sec17 and Sec18 buffers (A and D) or 600nM Sec17, 300nM Sec18, and 1mM ATPγS (B and E) or ATP (C and F) were also present. Kinetics shown are representative of 4 experiments. Mean and standard deviations from four independent experiments are shown in Figure 6-figure supplement 1.

**Figure 7.** Current working model. Catalyzed tethering and SNARE assembly (A-C) is followed by HOPS displacement by Sec17 (C and D) and further assembly of Sec17 and Sec18 on the partially-zippered SNARE complex, promoting completion of zippering and apolar Sec17 loop insertion (D-F), both of which promote fusion. See text for discussion.
Figure Supplement Legends

Figure 1-figure supplement 1. SNARE associations during partial zippering. Purified full-length vacuolar SNAREs will spontaneously, though slowly, assemble into a parallel 4-helical coiled coils structure which extends from the N-terminal (-7) layer of the SNARE domain to the C-terminal (+8) end of the SNARE domain. While the association of neuronal SNAREs has been studied extensively, the association of full-length or truncated vacuole SNAREs has received less study. Full-length R-SNARE, with a 6his-tag on its N-terminus, was mixed in octylglucoside with full-length Q-SNAREs, with soluble Q-SNAREs (sQ), or with sQ3Δs. Stable spontaneous complexes were isolated with nickel-NTA beads and examined by immunoblot. Complex formation with full-length SNAREs (lane 1) was not blocked by the absence of trans-membrane domain from Qa, Qb, or both (lanes 2-4) or by the 3Δ deletion in the C-terminal end of the SNARE domain in any one or two Q-SNAREs (lanes 5-10). A. Reactions (50µl) containing 2.5µM of each SNARE in Buffer 151 (20mM HEPES-NaOH pH 7.4, 150mM NaCl, 10% glycerol, 1% β-OG, 20mM imidazole) were nutated at 4˚C for 2 hours. A 40µl portion of each was transferred to a 0.5ml Eppendorf tube containing 20µl of a 50% slurry of Qiagen Nickel-NTA Agarose in Buffer 151. Suspensions were nutated at 4˚C for 1 hour. Loosely bound proteins were removed by four successive suspensions in 0.5ml Buffer 151, each followed by centrifugation (500xg, 6 min, 4˚C) and removal of the supernatant. Bound proteins were eluted by boiling the agarose in 50µl of SDS sample buffer (50mM TrisCl, pH 6.8, 5% glycerol, 2% sodium dodecyl sulfate, 0.2% bromophenol blue, 1% β-mercaptoethanol) for 5 minutes. Samples were analyzed by SDS-PAGE followed by immunoblotting. A typical result, and the mean and standard deviations from three independent experiments, are shown. B. Western blots of three identical experiments were scanned and analyzed with UN-SCAN-IT Software (Silk Scientific, Orem UT). Average pixel values were set at 100 for the all-wild-type positive control (lane 1). Standard deviations are shown.

Figure 2-figure supplement 1. Statistics and representative kinetic data for Figure 2. A. Schematic of fluorescently labeled SNARE constructs used in this study. B-G. Supplementary data for panels B-G in Figure 2. Subfigures are presented with the same subfigure labels and matching colors. All kinetic data are averages of 3 trials, and the shaded regions behind each curve show the standard deviation per time point. B. The effect of Sec17 on SNARE average FRET efficiency as a function of fluorophore position. The mean and standard deviation of three trials is shown for each reaction. C. Representative kinetic data showing the effect of HOPS and Ypt7 on Sec17-induced zippering. Data for the N-terminally labeled *Qb/*Qc pair is shown on the left, and data for the C-terminally labeled pair is shown on the right. Reactions with HOPS are in purple, and reactions without HOPS are in orange. As a negative control, buffer (RB150) was added instead of Sec17 for the reaction in gray. D. Representative kinetic data showing that Sec17 has only minor effect on the average FRET efficiency of SNARE complexes bearing truncated Qa SNARE. E. Representative kinetic data showing that Sec17 does not affect the average FRET efficiency of SNARE complexes bearing Qa-SNARE with a mutated C-terminal half. F. Representative kinetic data for the ability of Sec17 or mutants to promote C-terminal zippering of the SNARE complex. G. Representative kinetic data showing that Sec17 stabilizes incompletely-zippered SNARE complexes. Sec17, mutants, or buffer was added at t = 60 min (1), and non-fluorescent Qc was added at t = 80 min (2). Reactions containing wildtype Qc are shown with filled circles, and reactions with Qc3Δ are shown with open squares. The effect of wildtype Sec17 (red) is contrasted
with no Sec17 (gray) in the left-side graph, and the four Sec17 mutants used in this experiment are compared in the right-side graph.

**Figure 2—figure supplement 2.** Direct binding of Sec17 to HOPS. Since proteoliposome fusion promoted by synthetic tethers is inhibited by all concentrations of Sec17 whereas HOPS-dependent fusion is only inhibited by very high Sec17 levels (Song and Wickner, 2019) and Sec17-triggered zippering requires HOPS (Figure 2C), we tested whether HOPS might directly associate with Sec17. Phosphatidylcholine liposomes were prepared without protein (“naked”) or with TM-Sec17, a recombinant form of Sec17 with an N-terminal apolar transmembrane anchor (Song et al., 2017). These liposomes were incubated with HOPS, then floated through a density gradient. HOPS only bound in the presence of anchored Sec17, showing a direct and nearly stoichiometric binding between these proteins. A. Liposomes composed of PC/PS/NBD (83.5/15/1.5%) were prepared from mixed micellar solutions by detergent dialysis as described in Materials and Methods. Liposomes bore either no proteins or TM-Sec17 (Song et al 2017) at a 1:4000 protein to lipid molar ratio. Floatation assays were conducted as described in Song et al, 2020, Figure 2. In brief, 30μl reactions in RB150 contained 0.5mM liposomal lipid, 125nM integral TM-Sec17 where present, 0.2% defatted BSA, 1mM MgCl₂, and 500nM HOPS. Reactions were incubated (1hr, 30˚C) and liposomes floated through Histodenz (Sigma, St. Louis, MO) density medium. Collected samples were solubilized in 0.125% Thesit, adjusted for lipid recovered and analyzed for bound proteins by immunoblot for Vps16, a HOPS subunit. Because the reaction contained four times more HOPS than integrally bound TM-Sec17, we express the standard curve as the per cent of molar equivalence (to total liposomal TM-Sec17) of HOPS. B. Western blots from three repetitions of this experiment were analyzed by UN-SCAN-IT Software with standard curves. Mean values and standard deviations are shown.

**Figure 3—figure supplement 1.** Statistics for Figure 3. Fusion blocked by deletion of the C-terminal 5 layers of any Q-SNARE domain is restored by Sec17 and Sec18. A. Ypt7/R- and Ypt7/QaQb, B. Ypt7/R- and Ypt7/QaQc-tm, and C. Ypt7/R- and Ypt7/QbQc-tm. The experiment in Figure 3 was repeated in triplicate; mean values and standard deviations for fusion after 30 minutes are shown.

**Figure 4—figure supplement 1.** Statistics data for Figure 4. Fusion between Ypt7/R- and A-D. Ypt/Qa, E-H. Ypt7/Qb, or I-L. Ypt7/Qc-tm proteoliposomes. Fusion assays were described in Figure 4, repeated more than 3 times. Mean values and standard deviations for fusion after 20 minutes are shown.

**Figure 4—figure supplement 2.** Selective inhibitors reveal successive fusion intermediates. Ypt7/R- and Ypt7/Qa-proteoliposomes were co-incubated in the presence of HOPS and other fusion proteins or inhibitors, allowing resolution of an early, HOPS-dependent reaction stage from late, Sec18-dependent fusion. Incubations were in sets of 3, with either of 3 antibody preparations. When HOPS, Ypt7/R- and Ypt7/Qa-proteoliposomes were mixed with sQb3Δ, Qc3Δ, Sec17, Sec18, and ATPyS plus control IgG or specific antibody from the start of the incubation, fusion was blocked by affinity-purified antibody to either Sec18 (Haas and Wickner, 1996) or to Vps33 (Seals et al., 2000), the SM subunit of HOPS (A, Set 1). In each set, any of these components not present in the initial incubation was added after 30 min. When the proteoliposomes were incubated for 29 minutes with HOPS, sQb3Δ, and Qc3Δ prior to antibody addition, followed by Sec17,
Sec18, and ATPγS one minute later, the restored fusion remained sensitive to each affinity-purified antibody (Set 2). If however the initial 29 minute incubation also bore Qc3Δ, alone (Set 4) or in combination with other proteins (Sets 5-8), the restored fusion had acquired resistance to antibody to Vps33 prior to addition of the missing fusion proteins. Incubation with sQb3Δ did not confer resistance to anti-Vps33 (Set 3). Sec17 and Sec18 are not needed to form a reaction intermediate which is resistant to antibody to Vps33 (Set 4). In contrast, when fusion reactions were blocked by the single omission of either sQb3Δ (Set 5) or Qc3Δ (Set 3), any intermediates which formed were not resistant to antibody to Sec18. A very partial resistance was seen when all 4 SNAREs were present during the initial 29 minute incubation (Sets 7 and 8). The specificity of inhibition by antibody to Sec18 was confirmed by its failure to inhibit Sec18-independent fusion with full-length sQb and Qc (B and C) but there was complete inhibition of Sec18-dependent fusion reactions with sQb3Δ and Qc3Δ (D and E, red). Sec18 thus acts late in the fusion pathway, as incubations of the proteoliposomes with HOPS and Qc3Δ, which confers fusion-resistance to antibody to Vps33 (Set 4), remain sensitive to antibody to Sec18. A. Proteoliposomes with Ypt7/R and proteoliposomes with Ypt7/Qa (1:8,000 Ypt7-TM:lipid molar ratio, 1:16,000 SNARE:lipid molar ratios) were mixed with 50 nM HOPS at t=0. At either t = 0 or at t = 30, indicated subsets of 2μM sQb3Δ, 2μM Qc3Δ, 400nM Sec17, 300nM Sec18, and 1mM ATPγS were added to the incubations. To block Sec18 or Vps33, affinity-purified antibody (1μg) to each was added at t=0 (sample 1) or at t=29 (samples 2-8). As a control, 1μg IgG was added to separate samples as indicated. The average and standard deviations of fusion 10 min after addition of any missing components is shown from three independent experiments. B-E. Sec18 antibody or control IgG inhibition experiments were performed with Ypt7/R and Ypt7/Qa proteoliposomes with 50 nM HOPS, 2μM sQb and 2μM Qc (black curves) or 2μM sQb3Δ and 2μM Qc3Δ (red curves). 1μg IgG (filled circles) or αSec18 (open circles), and without (B and C) or with (D and E) 400nM Sec17, 300nM Sec18 and 1mM ATPγS as indicated. Kinetics shown are representative of 4 experiments. Mean and standard deviations from four independent experiments are shown. F-M. Representative experiments from A, Set 1 to 8, are shown.

**Figure 5-figure supplement 1.** Mean and standard deviations of fusion after 60 minutes from 4 independent assays as in Figure 5 are shown.

**Figure 5-figure supplement 2.** To test whether Sec18 is simply promoting fusion by contributing to the affinity of Sec17 for membranes which bear SNARE complexes, we prepared proteoliposomes with Ypt7, with R or Qa, and with either no Sec17, with an equimolar (to SNAREs) TM-Sec17 which bears an N-terminal hydrophobic transmembrane anchor domain derived from Qb, or with TM-Sec17-F21S,M22S (TM-FSMS hereafter). In the presence of HOPS, sQb3Δ, and Qc3Δ, membrane-anchored Sec17 supported zippering-bypass fusion which was strictly Sec18-dependent (A-C, no Sec18; D-F, with Sec18 and ATPγS). TM-Sec17 on both sets of proteoliposomes supported fusion (D and G, black) but fusion was not seen in the absence of TM-Sec17 (D and G, red), and only limited fusion was seen with TM-Sec17-FSMS (D and G, blue). Fusion was greatly diminished if TM-Sec17 or TM-Sec17-FSMS was only present on one of the fusion partner proteoliposomes (E and H), but there was substantial fusion if one of the proteoliposomes had TM-Sec17 and one had TM-Sec17-FSMS (F and I). The simplest model is that several Sec17 molecules are required to form the Sec17 assembly around the SNAREs, but that the hydrophobic Sec17 N-domain loop is only necessary on one membrane for fusion.
PNP functioned as well as ATPγS (compare G-I to D-F), but hydrolyzable ATP completely blocked fusion with membrane-anchored TM-Sec17 (J-L), similar to the lower activity of hydrolysable ATP with wildtype Sec17 (Figure 4), reflecting the proofreading activity of Sec17/Sec18. **M.** Statistics data for Figure 5-figure supplement 2, A-L. Fusion reactions had the indicated proteoliposomes, 50nM HOPS, 2μM each of sQb3Δ and Qc3Δ, and either Sec18 buffer (black), 1mM ATPγS and Sec18 (red), 1mM AMP-PNP and Sec18 (blue), or 1mM ATP and Sec18 (orange). Experiments were repeated in quadruplicate; mean values and standard deviations for fusion after 60 minutes are shown.

**Figure 6-figure supplement 1.** Fusion assays between Ypt7/R- and Ypt7/Qa with either Qa w.t. (black), Qa 5Ala mutant (green), Qa 5Gly mutant (blue), or Qa 5Ser mutant (red) were as described in Figure 6. **A-C.** Fusion with 2μM sQb and 2μM Qc. **D-F.** Fusion with 2μM sQbΔ and 2μM Qc3Δ. Experiments were repeated in quadruplicate; mean values and standard deviations for fusion after 30 minutes are shown.
Source Data

**Figure 1-Source Data 1.** Source data file (PDB) for Figure 1C.

**Figure 2-Source Data 1.** Source data file (Excel) for Figure 2B.

**Figure 3-Source Data 1.** Source data file (Excel) for Figure 3 A, B, and C.

**Figure 4-Source Data 1.** Source data file (Excel) for Figure 4 A-L.

**Figure 5-Source Data 1.** Source data file (Excel) for Figure 5 A-H, J, and K.

**Figure 6-Source Data 1.** Source data file (Excel) for Figure 6 A-F.
A. SNARE Terminology

R: N

<table>
<thead>
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<th>+3</th>
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<td>Qa: N</td>
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<td>TM</td>
<td>C</td>
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<tr>
<td>Qb: N</td>
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<tr>
<td>Qc: N</td>
<td>SNARE Domain</td>
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</table>

Soluble Q-SNARE (sQx)

sQx3Δ

Membrane Anchor

B. Sec17 structure

C. Modeled “Sec18/Sec17/Vacuolar SNARE” complex structure for yeast vacuoles

Membrane-proximal

Membrane-distal

Side View

Membrane End View
A. Undocked membranes, unassociated proteins

6. ATP hydrolysis & Complex disassembly

B. Tethered membranes

2. HOPS partially zippers SNAREs

C. HOPS partially-zippered trans-SNAREs

D. Sec17:trans-SNARE complex

E. Assembled pre-fusion complex

4. Assembly of Sec17, Sec18 on SNAREs

F. Sec17/Sec18/cis-SNARE complex

5. Sec17 loops insert + full zippering → Fusion!

Proofreading Disassembly

Slow Complete Zippering and Fusion
A Staged fusion reactions with selective inhibitors

<table>
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<tr>
<th>t=0'</th>
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<th>t=30'</th>
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<tr>
<td>Ypt7/R + Ypt7/Qa</td>
<td>sQb3Δ</td>
<td>Qc3Δ</td>
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<tr>
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Fusion 10min after addition of any missing components

- IgG 40%
- aVps33 2%
- aSec18 36%
- aVps33 2%
- aSec18 52%
- aVps33 6%
- aSec18 1%
- aVps33 55%
- aSec18 5%
- aVps33 39%
- aSec18 58%
- aVps33 1%
- aSec18 59%
- aVps33 5%
- aSec18 59%
- aVps33 20%
- aSec18 60%
- aVps33 47%
- aSec18 22%

B Fusion without Sec17 or Sec18

C Fusion with Sec17, Sec18, and ATPyS

D Set 1

E Set 2

F Set 3

G Set 4

H Set 5

I Set 6

J Set 7

K Set 8