Sec18 supports membrane fusion by promoting Sec17 membrane association

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ABSTRACT Membrane fusion is driven by Sec17, Sec18, and SNARE zippering. Sec17 bound to SNAREs promotes fusion through its membrane-proximal N-terminal apolar loop domain. At its membrane-distal end, Sec17 serves as a high-affinity receptor for Sec18. At that distance from the fusion site, it has been unclear how Sec18 can aid Sec17 to promote fusion. We now report that Sec18, with ATPγS, lowers the Km of Sec17 for fusion. A C-terminal and membrane-distal Sec17 mutation, L291A,L292A, diminishes Sec17 affinity for Sec18. High levels of wild-type Sec17 or Sec17-L291AL292A show equivalent fusion without Sec18, but Sec18 causes far less fusion enhancement with low levels of Sec17-L291AL292A than with wild-type Sec17. Another mutant, Sec17-F21SM22S, has reduced N-loop apolarity. Only very high levels of this mutant protein support fusion, but Sec18 still lowers the apparent fusion Km for Sec17-F21SM22S. Thus Sec18 stimulates fusion through Sec17 and acts at the well-described interface between Sec18 and Sec17. ATP acts as a ligand to activate Sec18 for Sec17-dependent fusion, but ATP hydrolysis is not required. Even without SNAREs, Sec18 and Sec17 exhibit interdependent stable association with lipids, with several Sec17 bound for each Sec18 hexamer, explaining how Sec18 stabilization of surface-concentrated clusters of Sec17 lowers the Sec17 Km for assembly with SNAREs. Each of the associations, between SNARE complex, Sec18, Sec17, and lipid, helps assemble the fusion machinery.

INTRODUCTION

Exocytic and endocytic vesicular trafficking requires conserved catalysts of membrane fusion. These include Rab GTPases, Rab-binding "effector" proteins which tether membranes, SNARE proteins which assemble in *cis* (when on the same membrane) or in *trans* between tethered membranes, and SNARE chaperones. Each SNARE consists of an N-domain, a characteristic heptad-repeat SNARE domain which assembles with others into stable 4-helical coiled coils with internally oriented apolar residues (Sutton *et al.*, 1998), a juxtamembrane (Jx) domain, and often a C-terminal apolar transmembrane anchor (Figure 1A). Four-SNARE complex assembly proceeds from the N- to C-termini of the SNARE domains, a process termed "zippering" (Sorensen *et al.*, 2006). Each of the four SNARE domains has an arginyl or glutaminyl residue, internally oriented and thus facing each other at the central, 0-layer of the 4-SNARE bundle. SNAREs are in R, Qa, Qb, and Qc conserved families (Fasshauer *et al.*, 1998), and 4-SNARE bundles are of RQaQbQc composition. Conserved SNARE chaperones include Sec17/SNAP, the hexameric Sec18/NSF ATPase, and proteins in the SM (Sec1/Munc18) family. SNAREs, SNAP/Sec17, and NSF/Sec18 assemble to form a 20s particle of known structure (Zhao *et al.*, 2015a). Several Sec17 molecules assemble around 4-SNARE bundles (Zhao *et al.*, 2015a,b), each with a crucial apolar N-domain loop that may insert into the membrane to promote fusion (Zick *et al.*, 2015; Song *et al.*, 2021). At its C-terminus, Sec17 serves as high-affinity Sec18 receptor (Weidman *et al.*, 1989). Sec18 is an ATPase which enhances the Sec17 stimulation of fusion (Zick *et al.*, 2015; Song *et al.*, 2017) and then disassembles SNARE complexes after fusion (Söllner *et al.*, 1993; Mayer *et al.*, 1996). SM proteins, either alone or in complex, catalyze SNARE assembly (Fiebig *et al.*, 1999; Sorensen *et al.*, 2006; Baker *et al.*, 2015; Orr *et al.*, 2017; Jiao *et al.*, 2018).

We study fusion mechanisms with vacuoles from *Saccharomyces cerevisiae*. A genetic screen for defective vacuole fusion
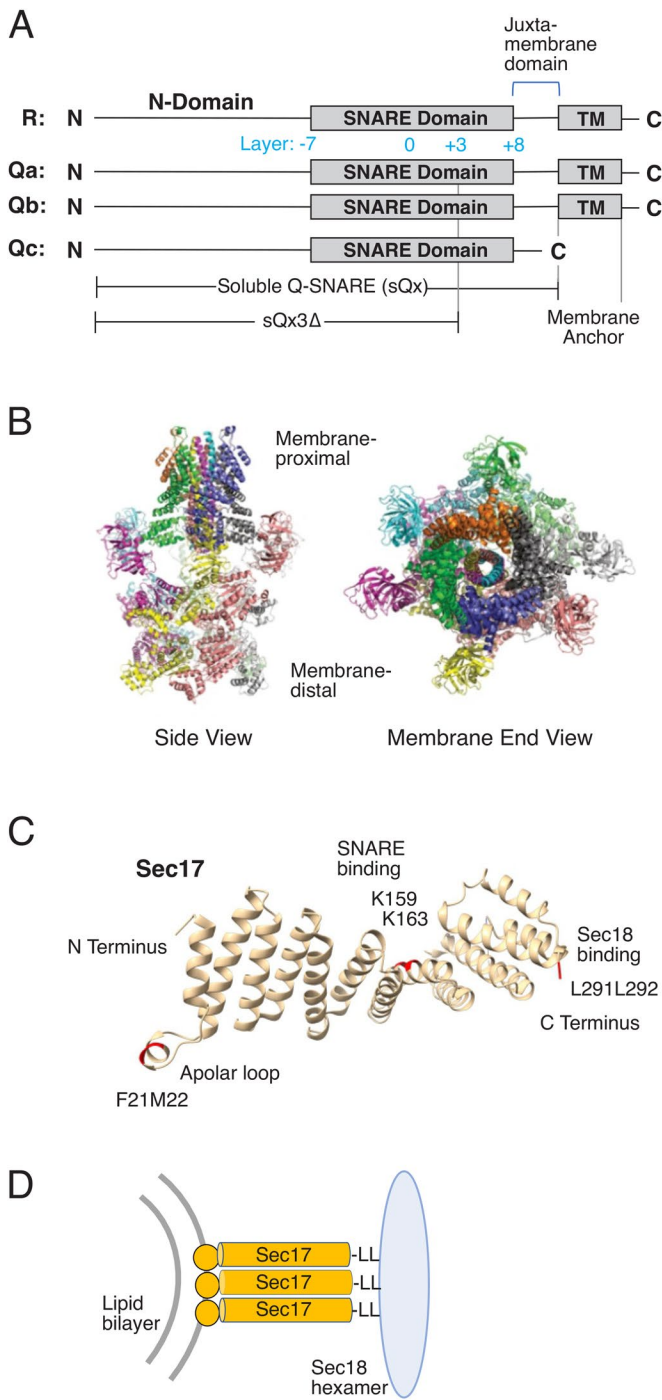
FIGURE 1: Structures of fusion catalysts. (A) Domains of vacuolar SNAREs; TM denotes trans-membrane anchor. (B) The yeast vacuolar 20s complex (Song et al., 2021) modeled on the neuronal 20s complex (Zhao et al., 2015a). The illustrations in A and B are from Song et al. (2021). (C) The structure of Sec17 (Rice and Brunger, 1999) here modeled in ChimeraX. (D) Working model of three Sec17 molecules bound by their N-terminal apolar loops to lipid and by their C-terminal dileucine motif to Sec18.

(Wada et al., 1992) identified nine vacuole fusion (VAC) genes, encoding two of the four vacuolar SNAREs, the Rab, and all six subunits of a protein termed HOPS (homotypic fusion and vacuole protein sorting). HOPS tethers membranes (Stroupe et al, 2006; Hickey and Wickner, 2010), binds each of the four vacuolar SNAREs

(Stroupe et al., 2006; Baker et al., 2015; Song et al., 2020), catalyzes SNARE assembly (Zick and Wickner, 2013; Baker et al., 2015; Orr et al., 2017; Jiao et al., 2018), and confers resistance to Sec17/Sec18-mediated disassembly of trans-SNARE complex (Xu et al., 2010). Extensive study of vacuole fusion in vivo and with the isolated organelle (Wickner, 2010) defined the proteins, lipids, and topology of fusion. Fusion was reconstituted with eight defined vacuolar lipids and physiological levels of purified recombinant proteins: the four SNAREs (Figure 1A), HOPS, Sec17, Sec18, and Ypt7 (Mima et al., 2008; Stroupe et al., 2009; Zick and Wickner, 2016). The Vps39 and Vps41 subunits of HOPS bind to the Rab Ypt7 on separate membranes to effect tethering (Brett et al., 2008; Brocker et al., 2012). The Vps33 subunit of HOPS is a member of the SM family which binds the R and Qa SNARE domains (Baker et al., 2015). HOPS also has direct affinity for the Qb and Qc vacuolar SNAREs (Stroupe et al, 2006; Song et al., 2020). HOPS is activated to catalyze the assembly of the trans RQaQbQc 4-SNARE complex by its associations in cis with Ypt7:GTP, phosphatidylinositol-3-phosphate, phosphatidylinositol (the major acidic vacuolar lipid), and the R-SNARE (Torng et al., 2020; Torng and Wickner, 2021). As shown (Zhao et al., 2015a) in the structure of the 20s complex (Figure 1B), several Sec17 molecules wrap around the 4-SNARE complex, with the apolar N-domain loops of Sec17 poised to insert into membranes at or near the site of fusion. Sec17 promotes fusion both by enhancing SNARE zippering (Ma et al., 2016; Song et al., 2021) and through direct action of the Sec17 apolar N-terminal loop on the membrane (Zick et al., 2015; Song et al., 2017, 2021). Sec17 also has a C-terminal binding site for Sec18 (Barnard et al., 1996; Barnard et al., 1997; Schwartz and Merz, 2009; see Figure 1B). When zippering is blocked (Schwartz and Merz, 2009; Zick et al., 2015; Song et al., 2021) or the Jx regions of the R and Qa SNAREs are swapped (Orr et al., 2022), the SNARE-bound Sec17 and Sec18 will still drive membrane fusion. Either complete SNARE zippering or Sec17 and Sec18 assembly on the platform of partially zippered SNAREs will suffice for slow fusion, but rapid fusion requires both full SNARE zippering and the Sec17/Sec18 chaperones (Song et al., 2017, 2021).

Sec17 binding sites have been well-defined through mutation of specific amino acyl residues which underlie its affinities for lipids, SNAREs, or Sec18 (Figure 1C). The L291AL292A (LALA) mutant near the C-terminus is at the site for Sec18 binding (Barnard et al., 1997; Schwartz and Merz, 2009), the F21SM22S (FSMS) mutant in the Sec17 apolar N-domain loop reduces its affinity for membrane lipids (Winter et al., 2009; Zick et al., 2015), and the K159EK163E (KEKE) mutant reduces its affinity for SNAREs (Marz et al., 2003; Song et al., 2017). Sec17 binding to the nascent fusion complex is favored by the ionic, hydrophobic, and Van der Waals forces of each of these multiple binding sites and opposed by the loss of spatial freedom, i.e., entropy. With multiple binding sites, there's only one entropy factor and hence tighter binding than the simple product of individual binding affinities. Studies of reconstituted fusion with wild-type Sec17 and these mutant proteins have advanced our understanding of how Sec17 contributes to fusion. Since Sec18 binds at a substantial distance from the two apposed bilayers, at the C-terminus of Sec17 and near the N-terminal end of the four SNARE domains, it has been unclear how it promotes fusion from this membrane-distal perch far from the site of lipid rearrangement during fusion.

Sec17 and Sec18 stimulate the rate of fusion with wild-type SNAREs but are not essential for fusion (Song et al., 2017). Energy from SNARE zippering is also not essential but stimulates the rate of fusion in the presence of Sec17 and Sec18 (Song

et al., 2021). Fusion requires Sec17/Sec18 when the completion of zippering is blocked either by C-terminal truncation of a Q-SNARE domain (Schwartz and Merz, 2009; Zick *et al.*, 2015; Song *et al.*, 2021) or by swapping the Jx regions between the R- and Qa-SNAREs (Orr *et al.*, 2022). We now report that Sec18, with ATPγS, lowers the Km for Sec17 to promote fusion. This action of Sec18 relies on the well-characterized site of Sec17:Sec18 association. Promotion of fusion by Sec18 requires ATP but not ATP hydrolysis. The cooperative assembly of SNAREs with Sec17 was previously studied in a model subreaction in which the four soluble SNAREs (deprived of their membrane anchors; Figure 1A) assemble with Sec17 molecules. The combined assembly of soluble SNAREs and Sec17 binds tightly to liposomes by the product of the modest affinities of the apolar loops of each of the several SNARE-bound Sec17s for lipids (Zick *et al.*, 2015; Song *et al.*, 2017), assayed after liposome floatation. With this liposome floatation assay for Sec17 oligomerization, we now find that Sec18 and Sec17 are interdependent for their association with lipids in the absence of SNAREs. Sec17 oligomerization can thus be promoted by either Sec18 or SNAREs or both. These physical and functional assays provide initial insights about how Sec18 promotes fusion.

RESULTS

Vacuolar SNAREs (Figure 1A) have N-domains, SNARE domains, Jx domains, and (except for the Qc-SNARE) apolar transmembrane anchors. To assay fusion, proteoliposomes are prepared with either of two lumenal markers, Cy5-labled streptavidin or the biotinylated fluorescent protein phycoerythrin. When these proteoliposomes are mixed, the two fluorophores are initially separated by at least the thickness of two membrane bilayers, precluding FRET (fluorescence resonance energy transfer). Upon fusion, the tight binding of biotin to streptavidin brings the bound Cy5 and phycoerythrin into intimate proximity, giving a strong FRET signal (Zucchi and Zick, 2011). The physiological ATP-driven disassembly of cis-SNARE complexes by Sec18 and Sec17 keeps SNAREs largely disassembled. Fusion can be studied from this initially fully disassembled state by combining proteoliposomes with Ypt7 and the R-SNARE (YR) with others bearing Ypt7 and the Qa-SNARE (YQa) as well as HOPS, soluble Qb-SNARE (sQb [Figure 1A], lacking its membrane anchor), and Qc, which inherently lacks an apolar membrane anchor. For simplicity, the proteoliposomes in this study are referred to only by their SNAREs, although they all bear Ypt7.

Sec18 shifts the Km for Sec17

Proteoliposomes with Jx regions which are swapped between the R and Qa SNAREs, termed R(JxQa) and Qa(JxR), require Sec17 for fusion (Orr *et al.*, 2022). With these proteoliposomes, fusion is restored by equivalent levels of either wild-type Sec17 (Figure 2, blue) or Sec17-LALA (red; See Supplement to Figure 2 for a typical set of kinetic traces). Sec18 and ATPγS alone does not stimulate fusion (Figure 2, green or black at 0 Sec17) but shifts the Km for Sec17 stimulation from 120 to 10 nM with little effect on the maximal rates of fusion. There is less shift by Sec18 of the Km for Sec17-LALA (red vs. black). Fusion is inhibited by the highest levels of Sec17, as reported (Wang *et al.*, 2000), blocking early stages of SNARE complex assembly (Zick *et al.*, 2015).

Fusion can also be blocked by C-terminal truncation of the SNARE domains of one or several Q-SNAREs (Song *et al.*, 2021). With Qc3Δ, a well-characterized C-terminal SNARE truncation (Schwartz and Merz, 2009), fusion is restored by high levels of
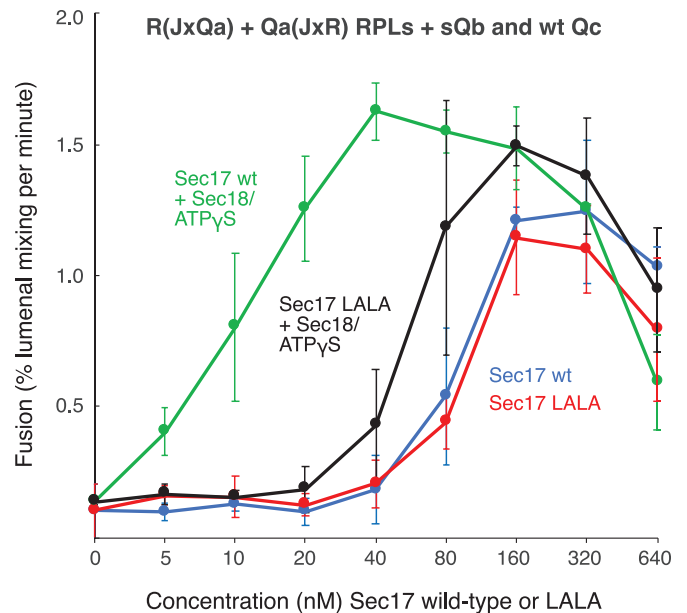

FIGURE 2: When fusion is inhibited by swap of the R and Qa Jx domains, Sec18 promotes fusion by lowering the Km for Sec17. Using proteoliposomes with Ypt7 and SNAREs bearing swapped Jx domains, termed R(JxQa) and Qa(JxR) as described in Orr *et al.* (2022), fusion reactions were conducted as described in *Materials and Methods*, with 1 mM Mg:ATPγS throughout, 100 nM each sQb and Qc, 50 nM HOPS, and with the indicated concentrations of wild-type Sec17 or Sec17-LALA and 300 nM Sec18 where added. Fusion rates were calculated as described in *Materials and Methods*. Means with error bars from three replicates are shown. An example of the kinetics of lumenal mixing is shown in Supplemental Figure S1.

wild-type Sec17 (Figure 3, blue) or Sec17-LALA (red). Sec18 reduces the Km for Sec17 ~ 10-fold (blue vs. green) but has less effect on fusion with Sec17-LALA (red vs. black). Thus Sec18, with ATPγS, promotes Sec17-dependent fusion through its known binding interface with Sec17.

Without Sec17, fusion is blocked by the exchange of the Jx regions of the R- and Qa-SNAREs (Orr *et al.*, 2022). Sec17-F21SM22S has reduced hydrophobicity of its N-loop (Barnard *et al.*, 1996; Schwartz and Merz, 2009) and only restores fusion to R(JxQa) and Qa(JxR) proteoliposomes with a Km of at least 1000 nM (Figure 4A, red). As seen with wild-type Sec17 (Figure 2), the addition of Sec18 and ATPγS also strongly reduces the Km for Sec17-FSMS (black). In contrast, Sec17-FSMS at any concentration is unable to restore fusion when zippering is blocked within the SNARE domain by the Qc3Δ mutant (Figure 4B, red and black) as noted (Song *et al.*, 2021).

Adenine nucleotide is an activating ligand to Sec18

ATP is present during Sec18 purification for Sec18 stability (Block *et al.*, 1988). To analyze the role of adenine nucleotide in Sec18 action during fusion, an aliquot of purified Sec18 was rapidly centrifuged through a short column of gel filtration resin into ATP-free buffer. Aliquots were frozen in liquid nitrogen and stored at –80°C, then thawed on ice immediately before use. The fusion of R(JxQa) and Qa(JxR) proteoliposomes was supported by HOPS, sQb, Qc, Sec17, ATP-free Sec18, and ATPγS (Figure 5, blue). Fusion was strongly suppressed by the omission of either Sec17, ATP-free Sec18, or Mg:ATPγS (red, black, or green, respectively). The restoration of each missing reactant after 20 min fully restored fusion, showing that Sec18 did not need ATPγS for stability during this time
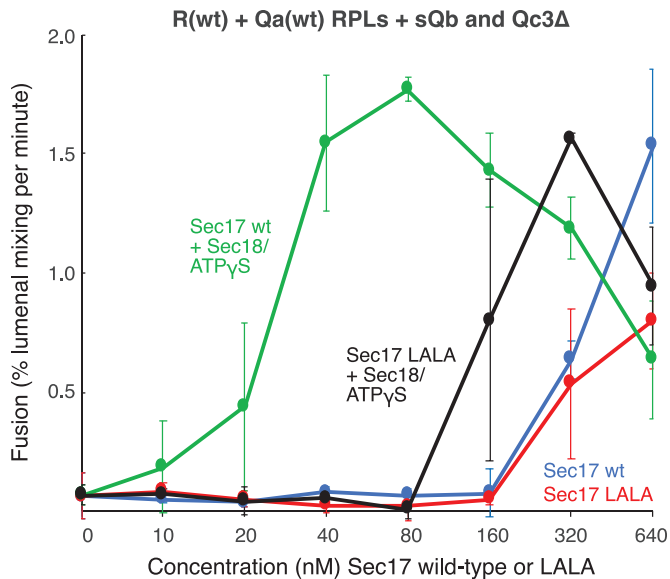
R(wt) + Qa(wt) RPLs + sQb and Qc3Δ

FIGURE 3: Sec18/ATPγS lowers the Km for Sec17 to restore fusion which is blocked by SNARE zippering arrest. The experiment is as described in Figure 2, except that fusion partner proteoliposomes bore wild-type R and Qa, and incubations had Qc3Δ instead of wild-type Qc. Means and standard deviations of fusion rates at each concentration of Sec17 and Sec17-LALA, with and without Sec18, are shown. A representative example of fusion kinetics, (the percentage of lumenal mixing at each minute) is presented in Supplemental Figure S2.

and incubation condition but does need it to support Sec17-dependent fusion. In addition to the well-established roles of adenine nucleotide in stabilizing Sec18 (Block *et al.*, 1988) and providing energy through hydrolysis for SNARE disassembly (Söllner *et al.*, 1993), it is also a ligand which activates Sec18 to promote Sec17 and SNARE-dependent fusion.

Sec18/ATPγS enhances the membrane binding of Sec17

We have previously reported an assay for the assembly of a subset of the fusion components: lipids, SNAREs, and Sec17 (Zick *et al.*, 2015). In this assay, protein-free liposomes were incubated with five recombinant proteins: the four soluble SNAREs (sSNAREs) and Sec17. After floatation, the liposome-bound proteins were assayed by SDS–PAGE and immunoblot. Sec17 and the sSNAREs are interdependent for their association with liposomes, and this multiprotein association with lipid relies on the apolar N-domain loop of Sec17 (Zick *et al.*, 2015; Song *et al.*, 2017). Since multiple SNAP, and presumably Sec17, will associate with a 4-SNARE complex (Zhao *et al.*, 2015a), this floatation assay likely reflects Sec17 oligomerization to align several Sec17 apolar loops to bind to membranes by the combined membrane affinities of each loop. We find that Sec18, with ATPγS, can replace the soluble SNAREs in this liposome-association assay, promoting Sec17 binding to membrane lipids with concomitant Sec18 membrane association (Figure 6, lanes 1 vs. 2). The enhancement of Sec17 binding to liposomes by Sec18/ATPγS is presumably due to the capacity of hexameric Sec18 to bind multiple Sec17 molecules (Zhao *et al.*, 2015a).

Distinct binding domains of Sec17

We have previously reported (Figure 3, Figure supplement 2 in Song *et al.*, 2017) that the assembly of wild-type Sec17 with the
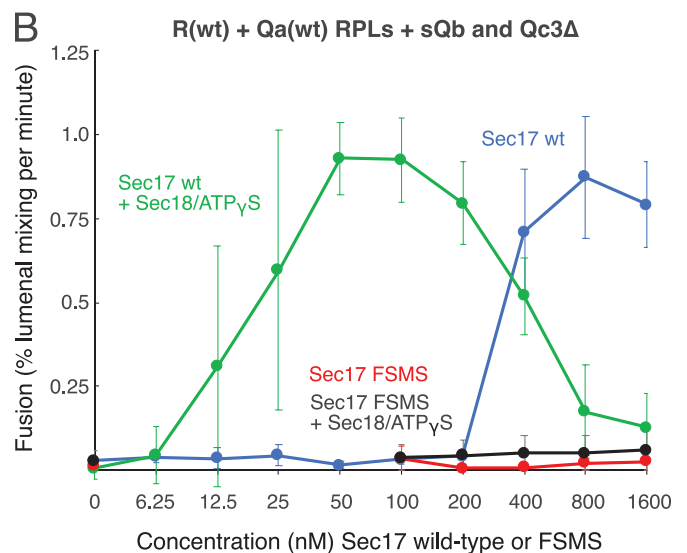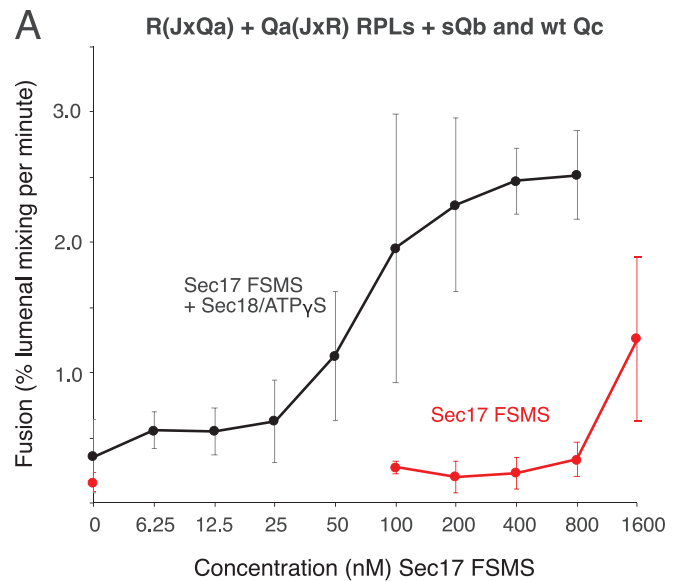

A R(JxQa) + Qa(JxR) RPLs + sQb and wt Qc

B R(wt) + Qa(wt) RPLs + sQb and Qc3Δ

FIGURE 4: Fusion with Sec17-FSMS. (A) With juxta-swapped R and Qa proteoliposomes, soluble Qb and wild-type Qc, Sec18/ATPγS dramatically lowers the Km for Sec17-FSMS. (B) When fusion is arrested by adding Qc3Δ to wild-type R and Qa proteoliposomes, Sec18 and Sec17-FSMS fail to rescue fusion. Fusion incubations were performed as described in *Materials and Methods* with either (A) juxta-swapped proteoliposomes and wild-type Qc or (B) wild-type proteoliposomes and Qc3Δ to prevent zippering and with the indicated concentrations of wild-type Sec17 or Sec17-FSMS, 100 nM each sQb and Qc, and with or without 300 nM Sec18. In this experiment, only treatments that contained Sec18 received 1 mM Mg:ATPγS. Means and standard deviations of fusion rates as described in Figure 2 are shown. A representative example of fusion as % of lumenal mixing for each panel is presented in Supplemental Figure S3.

soluble SNAREs to bind liposomes is unaffected by the LALA mutation in the absence of Sec18, as expected. The concomitant floatation of Sec17 and SNAREs was abolished by the Sec17 KEKE mutation that impairs Sec17:SNARE association or by the FSMS mutation that impairs Sec17:lipid association. In contrast, the Sec17 and Sec18 association with liposomes in the absence of SNAREs is
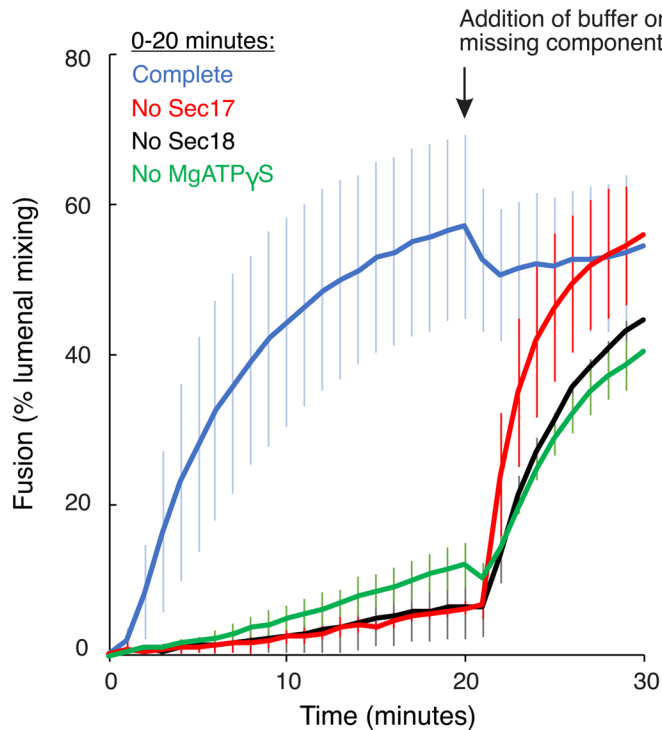
FIGURE 5: The stimulation of fusion at low (25 nM) Sec17 needs Sec18 and Mg:ATPγS. Fusion reactions were conducted as described in *Materials and Methods*, with modifications. The Ypt7 on juxtaswapped R and Qa proteoliposomes was nucleotide exchanged with GTP and 10 µl per reaction were distributed into wells of a 384 well plate. Four mixtures of the remaining soluble components were prepared, one with a complete mix of components (blue) and the others missing either the Sec17 (red), the Sec18 (black), or the Mg:ATPγS (green). These mixtures (13 µl) were distributed into empty wells of the 384 well plate, and the plate was preincubated for 10 min at 27°C, after which 10 µl of the soluble components were transferred to the wells of proteoliposomes at time 0. The complete fusion reactions (20 µl) had 0.21 mM R(JxQa) proteolipsomes, 0.21 mM Qa(JxR) proteoliposomes, 50 nM HOPS, 100 nM sQb, 100 nM Qc, 300 nM Sec18, 2 mM free MgCl$_2$, and 1 mM Mg:ATPγS. After 20 min of reading FRET, the drawer to the plate reader was opened and each well received 4 µl of Rb150 or the missing soluble component in Rb150 and FRET was recorded for another 10 min. Fusion with error bars from triplicate experiments is presented here.
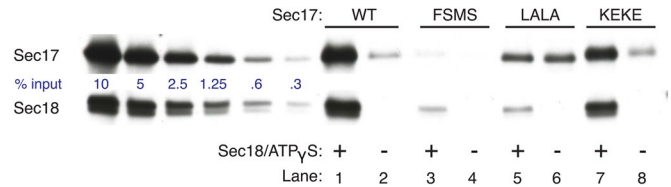

FIGURE 6: The affinity of Sec17 and Sec18/ATPγS for liposomes is dependent on their affinities for each other and on the hydrophobic loop of Sec17. Binding assays were conducted as described in *Materials and Methods* in the presence or absence of 2 µM Sec18, 1 mM Mg:ATPγS, and either wild-type Sec17 or the FSMS, the LALA, or the KEKE Sec17 mutants. Wild-type Sec17 lipid binding was greatly enhanced in the presence of Sec18. The KEKE mutant, which lowers Sec17:SNARE affinities, had little effect on these interdependent lipid bindings, whereas the FSMS mutant Sec17 all but abolished binding of either itself or Sec18. The lipid binding of the LALA mutant protein, with diminished binding to Sec18, was also greatly reduced. A representative Western blot is shown with standard curves of input. Quantified data with error bars from triplicate experiments can be found in Supplemental Figure S4.

insensitive to the KEKE mutation (Figure 6, lanes 1 vs. 7) but is strongly inhibited by the LALA or FSMS mutations (lanes 3-6). For unknown reasons, the LALA mutation allows a low level of spontaneous association between Sec17-LALA and liposomes (lane 6) without enhancement by Sec18 (lane 5). Sec17 exploits its independent and direct affinities for lipids, SNAREs, and Sec18/ATPγS to mediate their associations, reflected in the 20s complex (Figure 1B).

Stoichiometry of interdependent complex of Sec17, Sec18, and lipids

To compare the levels of Sec17 and Sec18 which are lipid bound, each protein was prepared with an N-terminal tag of six histidinyl residues. After protein-free liposomes were incubated with his$_6$-Sec17, his$_6$-Sec18, and ATPγS, the liposomes were recovered by floatation and assayed for bound proteins. We exploited the

common N-terminal his$_6$ tag as a shared epitope to assay the ratio of the two lipid-bound proteins and found that neither protein bound without the other, that is, they were interdependent for membrane association (Figure 7A, lanes 1 and 2 vs. lane 3). Serial twofold dilutions of the lane 3 sample of lipid-bound proteins showed that there was twice as much liposome-bound Sec18 polypeptide as Sec17 polypeptide (arrows indicate comparable band intensities, quantified in Figure 7B from replicate experiments by densitometry). Since Sec18 is hexameric, this corresponds to the interdependent stable lipid binding of ~ 3 mol of Sec17 for each mol of Sec18 hexamer. To confirm that both proteins had efficiently transferred from the SDS gel to the nitrocellulose filter, a control mixture of purified his$_6$-Sec17 and his$_6$-Sec18 was prepared and serial twofold dilutions were analyzed by SDS–PAGE. In contrast to a control gel which underwent the immunoblot procedure without electrophoretic transfer, both proteins were efficiently electrophoresed out of the SDS gel (Supplemental Figure S5A). A secondary nitrocellulose paper, placed behind the normal primary paper, showed that only 12% of Sec17 or Sec18 passed through the first paper (Supplemental Figure S5B).

DISCUSSION

Vacuole fusion is mediated by a cascade of protein associations. A Rab Ypt7 on each fusion partner, activated by GTP loading by the guanine nucleotide exchange protein Ccz1/Mon1 (Wang *et al.*, 2003; Nordmann *et al.*, 2010), binds to the Vps39 and Vps41 subunits of the HOPS complex (Brett *et al.*, 2008; Brocker *et al.*, 2012) to tether the two membranes (Hickey and Wickner, 2010). HOPS is allosterically activated by associations with Ypt7 and the R-SNARE on the same membrane and by the lipids PI and PI3P, enabling HOPS to catalyze the assembly in *trans* of the Q-SNAREs from the tethered membranes (Torng *et al.*, 2020; Torng and Wickner, 2021). Once the Q-SNAREs are assembled, they require no further catalysis for productive association with R-SNARE (Song and Wickner, 2019). Sec17 can displace HOPS from SNARE complexes (Collins *et al.*, 2005; Schwartz *et al.*, 2017) and is a major protein bound to *trans*-SNARE complexes (Xu *et al.*, 2010). HOPS prevents premature complex disassembly by Sec18, Sec17, and ATP (Xu *et al.*, 2010), but the precise timing of HOPS displacement from the SNAREs by Sec17 is unclear. Partially zippered *trans*-SNARE complexes can
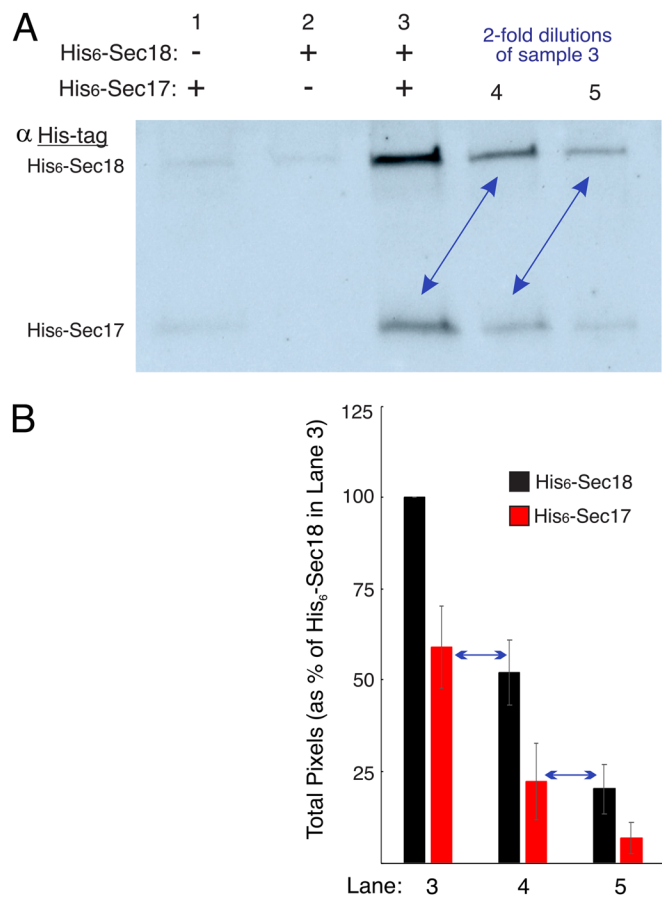
FIGURE 7: Sec17 and Sec18/ATPγS are interdependent for stable lipid binding, with an apparent stoichiometry of 3 Sec17 bound with each Sec18 hexamer. (A) Lipid binding assays were conducted as described in *Materials and Methods* with 1 mM Mg:ATPγS and 1 μM each of his$_6$-Sec17 and his$_6$-Sec18 where present. Floated samples were analyzed by Western blot with antibody to the His$_6$-tag on the N-terminus of each protein using THE™-His antibody (GenScript USA, Inc., Piscataway, NJ). Lanes 4 and 5 are serial twofold dilutions of the sample shown in lane 3. (B) Repeat experiments were quantified by scans of immunoblots normalized to the pixels in the undiluted sample's Sec18 band (black, lane 3).

complete zippering for slow fusion, though zippering is opposed by the energy of forcing the two bilayers together. Partially zippered SNAREs can also serve as a platform for the association of Sec17 and Sec18 to drive fusion (Zick *et al.*, 2015; Song *et al.*, 2021). Even without energy derived from zippering, fusion can be driven by SNARE-bound Sec17, and this can be enhanced by association with Sec18 (Song *et al.*, 2021). Since the binding site for Sec18 is at the membrane-distal C-terminus of Sec17, as shown with the LALA mutation (Winter *et al.*, 2009) and in the structure of the 20s complex of NSF/SNAP/SNAREs (Zhao *et al.*, 2015a), it has been unclear how Sec18 can contribute to Sec17-dependent, zippering-independent fusion.

The Sec17 N-domain apolar loop mediates its labile enrichment at membranes. We now report (Figures 6 and 7) that this is stabilized and enhanced by multimeric Sec17 association with Sec18/ATPγS. This may not activate Sec18 as an ATPase, since the association with SNAP does not support NSF ATP hydrolysis in the absence of SNAREs (Cipriano *et al.*, 2013). Several SNAP molecules are bound to each NSF in the 20s structure (Zhao *et al.*, 2015a), and we

find that several Sec17 molecules will assemble with Sec18 and lipids (Figure 7). This assembly of Sec18, several Sec17 molecules, and a lipid bilayer (Figure 1D) may be far more stable than any binding of monomeric Sec17 to another Sec17, to Sec18 or to lipids alone. The binding affinity of each individual Sec17 to remain in this complex may simply approximate the mathematical product of the several distinct Sec17 affinities: at the Sec17 N-domain apolar loop for lipids, at its C-terminal dileucine motif for Sec18 and by ionic interactions with its neighboring Sec17 molecules in the complex (Zhao *et al.*, 2015a,b). It is also possible that Sec17 binding to individual sites in the prefusion complex may alter its affinity for other sites. HOPS has direct affinity for Sec17 (Figure supplement 2 of Figure 2 of Song *et al.*, 2021), and Sec17 can in turn displace HOPS (Collins *et al.*, 2005; Schwartz *et al.*, 2017) and drive SNARE zippering (Ma *et al.*, 2016; Song *et al.*, 2021) as well as contribute directly to fusion through its apolar N-domain loop (Song *et al.*, 2017; ibid, 2021). This is a departure from a model in which the obligate order of assembly is that SNAREs must zipper first, then bind Sec17, and finally Sec18 binds to several SNARE-bound Sec17 molecules to form a structure like the 20s complex but anchored in *trans*. Our current studies show that Sec18 can assemble with several Sec17 molecules at their LALA-mutation sensitive C-terminal end and stably associate with lipid. The assembly of lipids, several molecules of Sec17, and hexameric Sec18 will be stabilized by multiple binding affinities as above. While each Sec17 may have readily reversible and weak binding to Sec18 and weak binding to lipid, it will have high affinity for the assembly. By this means, the enhanced concentration of lipid-bound Sec17 lowers the required overall concentration (Km) of Sec17 needed for interaction with SNAREs to promote fusion.

Each component in the prefusion complex has multiple direct interactions. The SNAREs assemble a coiled coil together; the Qc N-terminal PX domain binds PI3P in *trans*, contributing to the tethering function (Zick and Wickner, 2014); and the R and Q apolar anchors insert into the apposed membranes. Acidic residues on the surface of the SNARE coiled coils bind to basic residues of each Sec17 in the surrounding layer (Zhao *et al.*, 2015b; Marz *et al.*, 2003). Sec17 molecules also bind to the SNAREs through a conserved region which intercalates into the 4-SNARE bundle. The SNARE-bound Sec17 molecules may also bind to each other by ionic interactions between acidic residues on one edge of the curved Sec17 structure and basic residues on the edge of the adjacent Sec17 (Zhao *et al.*, 2015b). There are currently no data on the inherent stability of the assembly of these Sec17:Sec17 ionic interactions. Each Sec17 N-domain apolar loop binds to the lipid membrane with at least two functions: aiding the recruitment of Sec17 to the membrane (Zick *et al.*, 2015) and contributing to productive bilayer rearrangements in fusion (Song *et al.*, 2017). Sec17 is also the high-affinity receptor for Sec18 (Weidman *et al.*, 1989). Each Sec17 has five important interactions: with the bilayer, with the SNARE coiled coils, with each of its two Sec17 neighbors in the 20s structure, and with Sec18. The Sec18 hexamer is bound to each Sec17 and has been thought to only interact with SNAREs indirectly through Sec17. However, the finding (Zick *et al.*, 2015) that Sec18 alone has the capacity to unwind SNARE complexes implies that Sec18 also has direct affinity for SNAREs.

We propose that Sec18 enhances the capacity of low concentrations of Sec17 to drive fusion through Sec18 binding multiple Sec17s, strongly enhancing their membrane association. It is unclear whether oligomeric Sec17 would have higher affinity for partially zippered SNAREs or would dissociate at the membrane to give concentrated membrane-bound Sec17 molecules prior to SNARE

association. In either case, this mechanism is consistent with the finding that Sec18 alone (without HOPS or SNAREs) promotes stable Sec17 association with liposomes. This association relies on the Sec17 apolar loop, as it is diminished by the F21SM22S mutation in that loop (Figure 6). Oligomerization of Sec17 would multiplicatively enhance Sec17 membrane association just as Sec17 membrane association can be driven by SNARE associations (Zick *et al.*, 2015; Song *et al.*, 2021). It is unclear whether the assembly of multiple Sec17s with either sSNAREs or Sec18 is stabilized by the energy of Sec17:Sec17 binding.

We do not yet know whether Sec18 or NSF will have the same function for membrane fusion at other organelles. The only other fusion system analyzed with almost full reconstitution where this question might be addressed is the neuronal synapse. Reconstitution studies suggest that α-SNAP and NSF have important roles at the synapse (Ma *et al.*, 2013; Stepien and Rizo, 2021) which are distinct from those seen in yeast vacuoles. Synapses and vacuoles have shared SNARE functions, but synapses have distinct Ca-triggered chaperones, including Munc13, Complexin, and Synaptotagmin, and the synaptic SM protein Munc18-1 is not part of a stable complex like HOPS.

While incorrect *trans*-complex may be proofread by 17/18/ATP (Starai *et al.*, 2008; Xu *et al.*, 2010), we speculate that Sec17/Sec18 hydrolysis of ATP may otherwise be coupled to the completion of fusion and to the SNAREs assuming a postfusion *cis*-complex configuration. Since only a few percent of SNAREs assume a *trans*-configuration (Collins and Wickner, 2007), the technical challenges to test this idea have not yet been met.

MATERIALS AND METHODS

Reagents

ATPγS was from Sigma-Aldrich (St. Louis, MO); lipids and lumenal probes were purchased from previously cited sources (Zick and Wickner, 2016), and β-octylglucoside was from Anatrace (Maumee, OH). Dialysis was in Spectra/Por 6 tubing, 25kDa cutoff, 12 mm diameter, from Repligen Corporation (Waltham, MA). Biobeads SM-2 were from Bio-Rad Laboratories (Hercules, CA). Underivatized streptavidin was from Thermo Scientific (Waltham, MA).

Recombinant proteins

The purification of HOPS (Zick and Wickner, 2013), Ypt7 anchored by the recombinant transmembrane domain from Vti1, termed Ypt7-tm and expressed in *Escherichia coli* (Song *et al.*, 2020), the wild-type Qc SNARE (Schwartz and Merz, 2009) and the other 3 vacuolar SNAREs (Mima *et al.*, 2008), soluble Qb SNARE (Zick and Wickner, 2013), Sec17 (wild-type and mutants) and QcΔ3 (Schwartz and Merz, 2009), and His_6-tagged Sec18 and His_6-tagged Sec17 (Haas and Wickner, 1996) were performed as described. Aliquots of each protein were frozen in liquid nitrogen and stored at –80°C. The soluble Qb SNARE, purified by an MBP-tag, was cleaved from its tag by TEV protease prior to use in fusion assays.

Proteoliposome preparation

Using the dilinoleoyl (18:2) forms of PC, PE, PS, and PA, proteoliposomes were prepared as described (Zick and Wickner, 2016) except that fluorescent lipids were omitted and equivalent mol of 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine were added instead. In brief, vacuolar lipids were mixed in chloroform and methanol with β-octylglucoside and dried under a stream of nitrogen and then in a Speedvac. Mixed lipid/detergent pellets were rehydrated in an aqueous buffer, termed Rb150 (20 mM HEPES:NaOH, pH 7.4, 150 mM NaCl, 10% glycerol) with 1 mM $MgCl_2$, then mixed with Ypt7-tm and R- or Qa- SNAREs at a Ypt7:lipid molar ratio of 1:8000 and a SNARE:lipid molar ratio of 1:16,000. Portions (1 ml) were dialyzed at 4°C overnight in the dark with stirring against a 250-fold volume excess of Rb150+1 mM $MgCl_2$ with 1 g Biobeads per ml of sample dialyzed. Each was assayed for lipid phosphorus and diluted with Rb150 + 1 mM $MgCl_2$ to 2 mM lipid phosphorus, and 30-μl aliquots were frozen in liquid nitrogen and stored at –80°C.

Fusion assays

Unless otherwise noted, assays of fusion were in 16 μl prepared in two 8 μl additions: one containing GTP-exchanged proteoliposomes and the other containing the chaperones and various soluble components. First, YR and YQa proteoliposomes (0.44 mM each) were incubated together in Rb150 at 27°C after mixing with 10 μM streptavidin, 50 μM GTP, and 1 mM EDTA, followed, after 10 min, by the addition of 3 mM $MgCl_2$. The additional soluble components were also prepared in Rb150, containing (unless otherwise noted) 1 mM Mg:ATPγS, 100 nM sQb and Qc, 50 nM HOPS, 300 nM Sec18, and various levels of Sec17 as indicated. The two mixes (8 μl of the proteoliposome mix and 12 μl of the soluble component mix) were transferred into separate wells of a black 384-well plate (Corning, Incorporated, Kennebunk, ME) and incubated at 27°C for 10 min in a Molecular Devices SpectraMax GeminiXPS Plate Reader (Sunnyvale, CA). The reaction was then initiated by transferring 8 μl of each soluble component mix to the wells containing the proteoliposomes and reading FRET between the cy5-streptavidin and the biotin-Phycoerythrin (Ex: 565, Em: 670, CO: 630). Fusion rates are the percentage of lumenal mixing per minute at each concentration of Sec17 calculated by subtracting the percentage of maximal fusion at 5 min from that at 15 min and dividing by 10.

Lipid binding assays

Lipid binding assays were performed with protein-free liposomes of PC/PS/Rhodamine-PE (84, 15, and 1%, respectively). Reactions (30 μl) containing 0.5 mM of protein-free liposomes, 0.2% defatted bovine serum albumin (Sigma-Aldrich, St. Louis MO), 1 mM Mg:ATPγS, 1 μM Sec17, and either 0, 1, or 2 μM Sec18 were incubated for 1 h at 30°C. Reactions were moved to ice where they received 90 μl of 54% (weight/vol) Histodenz (Sigma-Aldrich, St. Louis, MO) in modified Rb150+Mg (20 mM HEPES-NaOH pH 7.4, 150 mM NaCl, 2% glycerol, 1 mM $MgCl_2$) and were vortexed gently. An 80-μl portion of each reaction was transferred to 7 × 20-mm polycarbonate tubes (Beckman Coulter, Brea, CA), then overlaid with 35, 30, then 0% Histodenz in modified Rb150+Mg (80, 80, and 50 μl, respectively). Reactions were centrifuged in a TLS-55 rotor for 30 min, 4°C, 55,000 rpm. Floated liposomes (80 μl) were harvested into 0.5 ml Eppendorf tubes where they received 2 μl of 5% Thesit. The remaining unspun starting samples (40 μl) received 1 μl of 5% Thesit and all samples were bath sonicated for 5 min and then nutated for 30 min at room temperature. The percentage of lipid recovered was determined by comparing the fluorescence of the solubilized starting samples to the solubilized harvested liposomes. Samples were analyzed by Western blot, with loading volumes adjusted to correct for the percentage recovered, and bands were quantified using UN-SCAN-IT Software (Silk Scientific, Orem, UT).

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