HOPS prevents the disassembly of *trans*-SNARE complexes by Sec17p/Sec18p during membrane fusion

Hao Xu¹, Youngsoo Jun²,
James Thompson³, John Yates³
and William Wickner^{1,*}

¹Department of Biochemistry, Dartmouth Medical School, Hanover, NH, USA, ²Department of Life Science, Gwangju Institute of Science and Technology, Buk-gu, Gwangju, Korea and ³Department of Chemical Physiology, Scripps Research Institute, La Jolla, CA, USA

SNARE-dependent membrane fusion requires the disassembly of *cis*-SNARE complexes (formed by SNAREs anchored to one membrane) followed by the assembly of *trans*-SNARE complexes (SNAREs anchored to two apposed membranes). Although SNARE complex disassembly and assembly might be thought to be opposing reactions, the proteins promoting disassembly (Sec17p/Sec18p) and assembly (the HOPS complex) work synergistically to support fusion. We now report that *trans*-SNARE complexes formed during vacuole fusion are largely associated with Sec17p. Using a reconstituted proteoliposome fusion system, we show that *trans*-SNARE complex, like *cis*-SNARE complex, is sensitive to Sec17p/Sec18p mediated disassembly. Strikingly, HOPS inhibits the disassembly of SNARE complexes in the *trans*-, but not in the *cis*-, configuration. This selective HOPS preservation of *trans*-SNARE complexes requires HOPS:SNARE recognition and is lost when the apposed bilayers are dissolved in Triton X-100; it is also observed during fusion of isolated vacuoles. HOPS thus directs the Sec17p/Sec18p chaperone system to maximize functional *trans*-SNARE complex for membrane fusion, a new role of tethering factors during membrane traffic.

The EMBO Journal (2010) 29, 1948–1960. doi:10.1038/emboj.2010.97; Published online 14 May 2010

Subject Categories: membranes & transport

Keywords: HOPS; membrane fusion; tethering factor; *trans*-SNARE complex; yeast vacuole

Introduction

Membrane fusion, the merger of two biological membranes without content leakage, is essential for protein transport along the exocytic and endocytic pathways in eukaryotic cells. Fusion requires conserved membrane-anchored proteins named SNAREs (α SNAP receptors) (Wickner and Schekman, 2008; Sudhof and Rothman, 2009), which reside

on both the donor and acceptor membranes. SNAREs form four helical coiled-coil bundles through their heptad-repeat SNARE domains. *Cis*-SNARE complexes are disassembled by Sec17p/ α SNAP and Sec18p/NSF in an ATP-dependent step called priming. Liberated SNAREs from apposed membranes form *trans*-SNARE complexes, an essential step for fusion. Other proteins cooperate with the SNAREs to achieve fusion. Rab GTPases and their effectors promote the tethering of donor and acceptor membranes (Grosshans *et al.*, 2006; Markgraf *et al.*, 2007; Hickey and Wickner, 2010) and thereby indirectly promote *trans*-SNARE complex formation. Sec1p/Munc18 (SM) proteins bind individual SNAREs as well as SNARE complexes and can influence fusion subreactions in either a negative or positive manner (Shen *et al.*, 2007; Malsam *et al.*, 2008; McNew, 2008; Deak *et al.*, 2009).

We have studied membrane fusion using yeast vacuoles (Ungermann *et al.*, 1998b; Collins and Wickner, 2007; Jun *et al.*, 2007). Isolated vacuoles bear all the components required for robust fusion, including SNAREs (Vam3p, Vti1p, Vam7p, and Nyv1p), SNARE disassembly chaperones (Sec17p and Sec18p), a Rab GTPase (Ypt7p), and HOPS (Wickner, 2002). HOPS, which is composed of six subunits, fulfills multiple roles in fusion. One subunit (Vps39p) is a guanine nucleotide exchange factor for Ypt7p and another (Vps33p) belongs to the SM protein family (Seals *et al.*, 2000; Wurmser *et al.*, 2000). HOPS has direct affinities for Ypt7p:GTP (Seals *et al.*, 2000), for vacuolar lipids (Stroupe *et al.*, 2006), and for SNAREs (*ibid.*). Each HOPS subunit is required for fusion, as mutation of its gene yields a highly fragmented vacuole morphology, indicating a fusion block (Wada *et al.*, 1992). Antibodies to any HOPS subunit inhibit fusion (Price *et al.*, 2000; Seals *et al.*, 2000) and, where tested, prevent *in vitro* formation of *trans*-SNARE complexes (Price *et al.*, 2000; Collins and Wickner, 2007).

Studies of HOPS are of general interest for understanding endomembrane traffic, because HOPS is conserved from yeast to plants to humans, and is a member of the family of large tethering factors that act at each fusion event along the secretory and endocytic pathways. In *Arabidopsis thaliana*, VCL1 (Vps16), AtVps11, and AtVps33 form a complex on the vacuole (Rojo *et al.*, 2003). Mutations in *Drosophila melanogaster* VPS18, VPS33, or VPS41 genes alter eye colour (Pulipparacharuvil *et al.*, 2005). In mice, the Vps33a gene regulates coat pigmentation (Suzuki *et al.*, 2003), and human mutation in VPS33B causes arthrogryposis-renal dysfunction-cholestasis syndrome (Gissen *et al.*, 2004). Like HOPS, which tethers endosomes and lysosomes/vacuoles, large multisubunit tethering factors regulate fusion at other organelles. CORVET acts at the endosome (Peplowska *et al.*, 2007), the exocyst regulates cell surface growth (Guo *et al.*, 1999), whereas COG, VFT/GARP, and TRAPP are essential for traffic to the Golgi and within that organelle (Sacher *et al.*, 1998; Siniosoglou and Pelham, 2002; Suvorova *et al.*, 2002).

*Corresponding author. Department of Biochemistry, Dartmouth Medical School, 7200 Vail Building, Room 425 Remsen, Hanover, NH 03755-3844, USA. Tel.: +1 603 650 1701; Fax: +1 603 650 1353; E-mail: bill.wickner@dartmouth.edu

Received: 15 March 2010; accepted: 20 April 2010; published online: 14 May 2010

Insights about HOPS function are thus of general interest for understanding intracellular trafficking at all organelles and in all organisms.

In vacuole fusion, HOPS, along with other accessory factors such as Sec17p and Sec18p, regulate *trans*-SNARE complexes. In one study, HOPS suppressed the fusion of vacuoles with SNAREs bearing 0-layer alterations but not vacuoles with wild-type SNAREs (Starai *et al*, 2008), distinguishing cognate and noncognate SNARE complexes. Another study used vacuoles that bore lipid-anchored Nyv1p (Jun *et al*, 2007). These vacuoles failed to fuse, but accumulated *trans*-SNARE complexes that were sensitive to added Sec18p, suggesting that *trans*-SNARE complexes formed under these conditions are not stable. This agrees with the observations from a vacuole-mimic reconstituted proteoliposome fusion system, where vacuolar SNAREs do not suffice for *trans*-SNARE complex formation or fusion in the presence of Sec17p/Sec18p unless HOPS is also added (Mima *et al*, 2008). Using a C-terminally truncated Vam7p (Vam7p-3Δ), Schwartz and Merz (2009) captured a nonfusogenic but partially zipped *trans*-SNARE complex on vacuoles. Strikingly, fusion was partially restored to these vacuoles by the addition of Sec17p in a Sec18p-independent manner. A large excess of Sec18p can even inhibit vacuole fusion (Ungermann *et al*, 1998b). Taken together, these studies show that Sec17p, Sec18p, and HOPS have access to *trans*-SNARE complexes.

We now report that the *trans*-SNARE complex formed during vacuole fusion is largely associated with Sec17p. Using reconstituted proteoliposomes, we demonstrate that *trans*-SNARE complex is subject to disassembly by Sec17p/Sec18p. HOPS inhibits the disassembly of the *trans*-SNARE complex, but not the *cis*-SNARE complex, and the integrity of the apposed membranes is required for this aspect of HOPS function. Our findings explain the synergy between Sec17p/Sec18p and HOPS for membrane fusion (Mima *et al*, 2008); Sec17p/Sec18p disassemble *cis*-SNARE complexes, but productive *trans*-SNARE complexes are protected by HOPS. As all membrane fusion events involve Sec17p/ α SNAP, Sec18p/NSF, SNAREs, large tethering complexes, and SM proteins, a similar mechanism may regulate *trans*-SNARE complexes on other organelles.

Results

Sec17p is associated with Vam3p during vacuole fusion

To determine the partners of the *trans*-SNARE complex, we searched for proteins that bind Vam3p in a fusion pathway dependent manner. Vacuoles from BJ3505 *nyv1Δ* (which has full-length Vam3p) and DKY6281 *vam3(ΔN)* (bearing N-terminally deleted Vam3p) were isolated and used in fusion reactions (Figure 1A). Neither initial vacuole population has Nyv1p associated with full-length Vam3p. After co-incubation, we prepared detergent extracts and immunoprecipitated the full-length Vam3p by using antibodies that are specific for the N-terminal domain of Vam3p. Sypro Ruby staining revealed several proteins that were specifically associated with Vam3p under fusion conditions (Figure 1B and C, lane 1). Mass spectrometry analysis (<http://yeastrc.org/pdr/>) indicated that these proteins include Nyv1p, Vti1p, Vam7p, and Sec17p.

Sec17p on vacuoles interacts with individual SNAREs as well as with the SNARE complex (Collins *et al*, 2005). However, Sec17p is released from vacuoles in the presence of Sec18p and ATP, an early event in the vacuole fusion reaction (Mayer *et al*, 1996), and Sec17p is not recovered with Vam3p when fusion (but not priming) is blocked (Figure 1D, lanes 6, 7, and 9). A portion of the Sec17p appeared to be recruited to Vam3p under conditions that permit fusion and the formation of new SNARE complexes (Figure 1D, lanes 5 and 8).

Trans-SNARE complex is largely associated with Sec17p

To determine whether Sec17p was associating with free Vam3p or with Vam3p in a SNARE complex, we again used vacuoles from BJ3505 *nyv1Δ* and DKY6281 *vam3(ΔN)* strains. At the end of the fusion reaction, we immunodepleted Sec17p from the vacuolar detergent extracts and asked whether SNARE complexes, as assayed by Nyv1p that was bound to full-length Vam3p, were removed through their association with Sec17p (Figure 2A). When fusion is robust (Figure 2B, lane I), virtually all the newly formed SNARE complexes are removed by Sec17p immunodepletion (Figure 2C, left panel, compare lane 10 with lane 9 or 11), showing that most of the newly formed SNARE complexes interact with Sec17p.

At least three types of SNARE complex form during these incubations: *trans*-SNARE complex, *cis*-SNARE complex that was *trans*- before fusion, and *cis*-SNARE complex that formed *de novo* after fusion and had never been *trans*-. To determine whether Sec17p is associated with the *trans*-SNARE complex, we exploited vacuoles from DKY6281 *vam3(ΔN) nyv1-CCIIM*. The transmembrane domain of Nyv1p on these vacuoles had been replaced by the Ykt6p prenylation motif (Jun *et al*, 2007). Vacuoles with lipid-anchored Nyv1p fuse poorly (Figure 2B, lane II) and thus a larger percentage of the new SNARE complex will be *trans*-SNARE complex as compared with reactions involving vacuoles with the wild-type Nyv1p. Earlier studies using Nyv1p-CCIIM vacuoles (Figure 6E of Jun *et al*, 2007) established that >50% of the new SNARE complex formed is *trans*-SNARE complex. Remarkably, immunoadsorption of Sec17p completely depleted the new SNARE complex from detergent extracts of docked NYV1-CCIIM vacuoles (Figure 2B, compare lane 21 with lane 20 or 22), showing that the new SNARE complex, including the *trans*-SNARE complex, is largely associated with Sec17p. What is the significance of the interaction between Sec17p and the *trans*-SNARE complex?

Trans-SNARE complex is sensitive to Sec17p/Sec18p

Earlier studies have shown that a partially lipid-anchored *trans*-SNARE complex formed during vacuole fusion is subject to Sec18p and Vam7p-dependent remodelling, implicating Sec17p (along with Sec18p) in disassembling the *trans*-SNARE complex (Jun *et al*, 2007). To study SNAREs with wild-type membrane anchors, we have exploited a reconstituted proteoliposome fusion system made from recombinant proteins and a set of pure lipids that closely match the composition of vacuole membranes (Mima *et al*, 2008). Fusion is measured by lipid mixing between proteoliposomes (Figure 3A and B). By placing Nyv1p on one set of liposomes and Vam3p and Vti1p on the other, we are able to bypass Sec17p/Sec18p-dependent priming and directly study new

Figure 1 Sec17p associates with Vam3p during fusion. **(A)** Assay scheme. Fusion reactions containing BJ3505 *nyv1Δ* vacuoles, DKY6281 *vam3(ΔN)* vacuoles, and 40 nM Vam7p were incubated at 27°C for 45 min with either no inhibitors, Gyp1-46 (117 μg/ml)/Gdi1p (30 μg/ml), or MED (10 μM). At the end of the reaction, Pho8p activity was assayed **(B)**. As control, BJ and DKY vacuoles were incubated separately, and the Pho8p activity from each was measured and added (lane 4). The rest of the vacuoles were solubilized in detergent and proteins co-immunoprecipitated with α Vam3 were subject to SDS-PAGE and Sypro Ruby staining **(C)** and to mass spectrometry analysis. Asterisks in **(C)** indicate unique bands. The arrow points to the Sec17p band. **(D)** The Sec17p association with Vam3p (without additional Vam7p) was assayed. BJ3505 *nyv1Δ* vacuoles (33 μg) and DKY6281 *vam3(ΔN)* vacuoles (33 μg) were incubated for 45 min with indicated reagents (117 μg/ml Gyp1-46, 30 μg/ml Gdi1p, 64 μg/ml α Vps33, 75 μg/ml α Ypt7, 67 μg/ml Ypt7 peptide) and 6 μg of vacuoles were analysed for Pho8p activity. The remainder was solubilized in detergent (see Materials and methods). Nyv1p and Sec17p that co-immunoprecipitated with Vam3p were analysed by immunoblot. Two regions of the same blot from one gel are shown; there is an empty space between lanes 5 and 6. See Supplementary Figure S1 for the original scan.

SNARE complex formation. In this assay, fusion (Figure 3A) and new SNARE complex formation (Figure 3C) require the addition of the soluble SNARE Vam7p. Both fusion and SNARE complex formation are stimulated by HOPS. It is noteworthy that similar rates of fusion can be achieved despite drastically different amounts of SNARE complex (compare Figure 3C, lanes 6 and 10, and the corresponding fusion in Figure 3A, filled circles and filled inverted triangles, respectively), suggesting that not all new SNARE complexes are functionally equal.

To resolve *trans*-SNARE complex formation from fusion and possible formation of post-fusion *cis*-SNARE complexes, we used a C-terminally truncated Vam7p (Vam7p-3Δ) that efficiently drives *trans*-SNARE complex formation on isolated vacuoles in the absence of fusion (Schwartz and Merz, 2009). In the reconstituted fusion system, Vam7p-3Δ supports *trans*-SNARE complex formation (Figure 3C, lanes 12–17) without fusion (Figure 3B). The yield of *trans*-SNARE complex

increases with increasing amounts of Vam7p-3Δ in the reaction. In addition, HOPS promotes the formation of *trans*-SNARE complexes in the presence of Vam7p-3Δ (Figure 3C). Vam7p-3Δ therefore permits study of *trans*-SNARE complex without the interference of post-fusion *cis*-SNARE complexes.

To determine whether *trans*-SNARE complexes can be disassembled by Sec17p and Sec18p, reactions were divided into two stages, assembly of *trans*-SNARE complexes followed by their disassembly by Sec17p/Sec18p. During the first stage incubation, 6% of the Nyv1p entered into *trans*-SNARE complex with Vam3p in the presence of Vam7p-3Δ (Figure 4, compare lanes 5 and 13). If the reaction was allowed to continue for another 30 min (second stage), more *trans*-SNARE complex was detected (Figure 4, compare lane 7 and 13). Sec17p and Sec18p added at the beginning of the second stage reduced the amount of Nyv1p associated with Vam3p to about 2% (Figure 4, lane 9), significantly

Figure 2 *Trans*-SNARE complex is largely associated with Sec17p. (A) Assay scheme. Vacuoles from BJ3505 *nyv1* Δ (93 μ g) were incubated with vacuoles (93 μ g) from DKY6281 *vam3*(Δ N) or DKY6281 *vam3*(Δ N) *nyv1*-CCIIM under standard fusion conditions for 45 min before an aliquot was assayed for Pho8p activity (B). The rest were solubilized in detergent and divided equally into three portions, each to be incubated with agarose resin conjugated with either α Porin I (a control IgG), α Sec17p, or no IgG. After 1 h incubation, supernatant was collected by centrifugation and incubated with fresh resin for the second time to ensure complete depletion of Sec17p (lanes 3, 7, 14, and 18). A small aliquot of the supernatant collected after the second incubation was set aside for western blotting, whereas the rest were incubated with immobilized α Vam3-N. Proteins co-precipitated with Vam3p were subject to SDS-PAGE and immunoblot analysis (C). Note that there are more Nyv1 molecules on DKY6281 *vam3*(Δ N) vacuoles than on DKY6281 *vam3*(Δ N) *nyv1*-CCIIM vacuoles (compare lanes 1 and 12), as reported earlier (Jun *et al*, 2007), but the amount of new SNARE complex formed in reactions I and II are comparable (compare lanes 11 and 22).

below the level reached in the first stage (6%), showing that Sec17p and Sec18p disassemble the complex already formed in the first stage. Please note that under our immunoprecipitation conditions, the Sec17p/Sec18p is inactivated by EDTA/RIPA buffer (Supplementary Figure S2), so that any SNARE complex disassembly we measure has occurred on the membrane rather than in detergent.

To control for SNARE complex assembly during the second stage of the reaction, we also performed this stage of the reaction with purified recombinant FYVE domain. FYVE domain inhibits vacuole fusion by binding to PI(3)P (Fratti *et al*, 2004), a regulatory lipid that is essential for *trans*-SNARE complex formation (Collins and Wickner, 2007). As FYVE domain inhibits the *trans*-SNARE complex formation on proteoliposomes (Figure 4, lane 6 versus 7), adding FYVE domain at the start of the second stage of the reaction allows direct evaluation of the effect of Sec17p and Sec18p on existing complexes. As shown in Figure 4 (lanes 10–12), Sec17p/Sec18p disassembles *trans*-SNARE complex in a concentration-dependent manner. This explains why Sec17p and Sec18p block the fusion of proteoliposomes in the absence of HOPS (Mima *et al*, 2008).

HOPS inhibits the Sec17/18-dependent disassembly of the *trans*-SNARE complex

To allow SNARE-dependent membrane fusion in the presence of Sec17p and Sec18p, the disassembly of *trans*-SNARE

complexes must be inhibited. HOPS and Sec17p/Sec18p synergistically promote proteoliposome fusion (Mima *et al*, 2008), suggesting that HOPS might regulate the Sec17p/Sec18p-mediated disassembly of *trans*-SNARE complex. As mentioned above, we allowed *trans*-SNARE complex to form in an initial incubation in the presence or absence of HOPS. To arrest further *trans*-SNARE complex assembly while examining HOPS function, we could not use FYVE domain as in Figure 4, as FYVE binds to phosphoinositides that are part of the HOPS membrane receptor (Burd and Emr, 1998; Kutateladze *et al*, 1999). We therefore added either CaCl₂ (10 mM) or KCl (250 mM), which effectively prevents fusion (data not shown) and *trans*-SNARE complex formation (Supplementary Figures S3 and S4). Without HOPS, *trans*-SNARE complexes are disassembled by Sec17p and Sec18p in the second stage of the reaction (lanes 9–12 in Figure 5A and B). The disassembly rate is slower in the presence of 250 mM KCl, presumably due to salt inhibition of Sec17p/Sec18p. Strikingly, *trans*-SNARE complexes are quite resistant to Sec17p/Sec18p-mediated disassembly in the presence of HOPS (lanes 5–8 in Figure 5A and B). There is a slight decrease of *trans*-SNARE complex at high concentrations of Sec17p/Sec18p (compare lane 8 with lane 5), suggesting that the HOPS inhibition of *trans*-SNARE complex disassembly is not absolute.

Sec17p and Sec18p can disassemble a complex of four vacuolar SNARE soluble domains and this disassembly is not

Figure 3 A truncated Vam7p (Vam7p-3Δ) arrests the fusion of proteoliposomes while allowing *trans*-SNARE complex formation. Proteoliposomes bearing Nyv1p were mixed with proteoliposomes carrying Vam3p and Vti1p. Wild-type Vam7p, Vam7p-3Δ, and/or HOPS (61 nM) was added as indicated (**A**, **B**). Fusion between donor and acceptor liposomes was measured by the dequenching of NBD-PE fluorescence (see Materials and methods). (**C**) At the end of the reaction, proteoliposomes were solubilized in detergent, and the proteins that adsorbed to immobilized αVam3 were analysed by SDS-PAGE and immunoblot.

affected by HOPS (Mima *et al*, 2008), suggesting that membrane integrity might be required for the HOPS-dependent preservation of the *trans*-SNARE complex. To directly address this, we modified the *trans*-SNARE complex disassembly assay by also preparing a parallel set of samples, which received Triton X-100 during the second stage of the reaction (Figure 6A, lanes 7–12). Triton X-100 solubilization of donor and acceptor membranes allowed Vam3p (from acceptor proteoliposome) and Nyv1p (from donor proteoliposomes) that had not been in *trans*-SNARE complex to form a complex in detergent despite the addition of excess soluble GST-Nyv1p to compete with Nyv1p. This led to the detection of a modest increase of Vam3p–Nyv1p association (compare Figure 6A, lanes 1 and 7, lanes 4 and 10). Strikingly, almost all these complexes were effectively disassembled by Sec17p/Sec18p regardless of the presence of HOPS (compare Figure 6A, lanes 7 and 9, lanes 10 and 12). *Trans*-SNARE complexes formed on apposed liposomes that were not subsequently exposed to detergent maintained their resistance to Sec17p/Sec18p in the presence of HOPS (Figure 6A, lanes 1–6). Thus, the HOPS-dependent preservation of *trans*-SNARE complexes appears to rely on the integrity of the two apposed membranes.

To address whether SNARE recognition is required for this new function of HOPS, we examined the effect of saturating HOPS for its capacity to bind SNAREs. We have previously

shown that HOPS interacts preferentially with Vam7p among the vacuolar SNAREs, and that this interaction is specific for the N-terminal PX domain of Vam7p (Stroupe *et al*, 2006). We therefore tested whether the addition of an excess of this PX domain would block HOPS preservation of *trans*-SNARE complexes. To avoid complications arising from the affinity of this PX domain for vacuolar PI(3)P, we used the Y42A mutant PX domain, which specifically lacks affinity for phosphoinositides (Cheever *et al*, 2001). Proteoliposomes bearing Vam3p and Vti1p were incubated with those bearing Nyv1p in the presence of Vam7p-3Δ to allow *trans*-SNARE complex assembly, then mixed with CaCl₂ to prevent further *trans*-SNARE complex assembly. After adding combinations of HOPS and/or Y42A-PX, a second incubation was performed that included Sec17p and Sec18p where indicated. The *trans*-SNARE complexes formed during the initial incubation were readily disassembled by added Sec17p/Sec18p (Figure 6B, lanes 4–6), and HOPS inhibited this disassembly (Figure 6B, lanes 1–3). Adding Y42A-PX alone had no effect on the Sec17p/Sec18p-mediated disassembly (Figure 6B, lanes 10–12), but it prevented HOPS from preserving the *trans*-SNARE complex (Figure 6B, lanes 7–9). Thus, HOPS appears to rely on both membrane bilayer integrity (Figure 6A) and on its interaction with SNAREs (Figure 6B) to preserve *trans*-SNARE complexes.

Figure 4 *Trans*-SNARE complex is disassembled by Sec17p/Sec18p. Donor proteoliposomes (50 μ M) bearing Nvy1p were mixed with acceptor proteoliposomes (400 μ M) bearing Vam3p and Vti1p (with 1 mM $MgCl_2$, 1 mM ATP and an ATP regenerating system). Samples received either 1.2 μ M Vam7p-3 Δ , 1.2 μ M Vam7p-3 Δ with 12 μ M FYVE, or control buffer. After 30 min at 27°C (Stage I), samples with Vam7p-3 Δ were either transferred to ice or incubated at 27°C for another 30 min without Sec17p/Sec18p (lanes 7 and 10) or with the addition of Sec17p/Sec18p (0.34 μ M/0.24 μ M or 0.68 μ M/0.24 μ M; lanes 8, 9, 11, and 12). Samples from lanes 10 to 12 also received FYVE at the end of Stage I to prevent the formation of new SNARE complex. At the end of Stage II, Vam7p-3 Δ (1.2 μ M) was added to the sample as shown in lane 1, which had not initially received Vam7p-3 Δ . All samples were assayed for *trans*-SNARE complex (see Materials and methods). The Nvy1p signal, normalized for the recovery of Vam3p, was averaged from three independent experiments. Error bars represent standard error of the mean (s.e.m.) calculated using Kaleidagraph. Note that lane 13 measures *trans*-SNARE complex formation in the first stage only. If the second stage reaction continues at 27°C without inhibitors, more *trans*-SNARE complex will form (compare lanes 13 and 7).

HOPS does not inhibit cis-SNARE complex disassembly

Is this effect of HOPS specific for *trans*-SNARE complexes or does HOPS also inhibit *cis*-SNARE complex disassembly by Sec17p/Sec18p? Proteoliposomes bearing Vam3p, Vam7p, Vti1p, and Nyv1p were incubated at low concentration to eliminate tethering and *trans*-SNARE complex formation (see Materials and methods). The disassembly of their *cis*-SNARE complexes was measured by the loss of association between Nyv1p and Vam3p. Approximately 60% of the Nyv1p on these proteoliposomes was initially associated with Vam3p (Figure 7A). Incubation with Sec17p and Sec18p reduced the level of Nyv1p associated with Vam3p to 25%. The complexes that were insensitive to Sec17p and Sec18p were likely oriented toward the liposome lumen and thus inaccessible to the SNARE chaperones. HOPS did not inhibit *cis*-SNARE complex disassembly at any Sec17p/Sec18p concentration tested (Figure 7A).

To more closely mimic the conditions used in the *trans*-SNARE complex disassembly assays (Figure 5) and, in particular, to account for the fact that the proportion of the SNAREs engaged in *trans* complexes (4–8% of total; Figures 5 and 6) is smaller than that of the *cis*-SNARE complex (60%; Figure 7A) in our reactions, we lowered the amount of 4-SNARE proteoliposomes in the *cis*-SNARE disassembly assay so that the concentrations of the SNARE complexes in the two were equivalent (see Material and methods). 4-SNARE proteoliposomes that bear either Vam7p-3Δ or Vam7p were pre-incubated with HOPS (or control buffer) for 30 min before the addition of Sec17p and Sec18p at the same concentrations used in the *trans*-SNARE complex disassembly assay. Sec17p and Sec18p effectively

disassembled *cis*-SNARE complexes in the presence or absence of HOPS (Figure 7B and C). Thus, the HOPS-mediated preservation of *trans*-SNARE complexes, which requires the integrity of the two apposed membranes, is specific to the *trans* conformation of the SNARE complex.

HOPS relieves Sec18-dependent inhibition of vacuole fusion

Is the HOPS-dependent preservation of *trans*-SNARE complexes on proteoliposomes bearing only pure fusion proteins also seen on the intact and chemically complex organelle? The fusion of vacuoles can be modulated by exogenous Sec18p (Figure 8A), as reported (Ungermann *et al*, 1998b). At low concentrations, Sec18p stimulates fusion, suggesting that vacuolar *cis*-SNARE complexes are not completely disassembled by the endogenous Sec18p. However, high Sec18p concentrations are inhibitory. As most vacuolar *trans*-SNARE complex is associated with Sec17p (Figure 2), the excess Sec18p may rapidly disassemble *trans*-SNARE complexes before they can promote membrane fusion. In accord with this idea, the addition of HOPS relieved the inhibitory effect of excessive Sec18p (Figure 8B). Vacuoles normally bear sufficient HOPS for fusion, and thus standard vacuole fusion reactions were not affected by the further addition of 10–40 nM HOPS. However, at even 10 nM, added HOPS prevented the inhibition by Sec18p. The fusion signal from reactions containing both Sec18p and HOPS is slightly higher, probably because Sec18p disassembled *cis*-SNARE complexes to promote fusion while not interfering with *trans*-SNARE complex function. Thus, HOPS preserves *trans*-SNARE

Figure 5 HOPS inhibits the disassembly of *trans*-SNARE complex by Sec17p/Sec18p. Donor proteoliposomes (50 μ M) bearing Nyv1p, acceptor proteoliposomes (400 μ M) bearing Vam3p and Vti1p, and 1.2 μ M Vam7p-3 Δ were mixed with 61 nM HOPS or its buffer alone. *Trans*-SNARE complex was allowed to form at 27°C for 30 min. Samples then received either 10 mM CaCl₂ (**A**) or 250 mM KCl (**B**) to stop the formation of new SNARE complex. Samples were then divided into four portions and received either Sec17p/Sec18p (0.68 μ M/0.24 μ M or 1.36 μ M/0.48 μ M) (lanes 7, 8, 11, and 12) or control buffer (lanes 5, 6, 9, and 10). After 30 min of further incubation at 27°C (except samples in lanes 6 and 10, which were placed on ice), *trans*-SNARE complex was assayed. Data from three experiments were analysed as in Figure 4. In both (**A**, **B**), the *P*-values between lanes 5 and 8 or 5 and 7 are larger than 0.25.

complexes on the intact organelle as well as with reconstituted proteoliposomes.

Discussion

Although HOPS is required for vacuole fusion, its mechanism of synergy with Sec17p/Sec18p has been unclear. Sec17p/Sec18p can disassemble either *cis*-SNARE complexes (Figure 7; Ungermann *et al*, 1998a) or *trans*-SNARE com-

plexes (Figures 4–6; Jun *et al*, 2007), and HOPS will associate with either *cis*-SNARE complexes (Collins *et al*, 2005) or *trans*-SNARE complexes (Figure 6B). HOPS promotes the tethering of vacuoles (Stroupe *et al*, 2006) or reconstituted proteoliposomes (Stroupe *et al*, 2009). Tethering is a prerequisite for *trans*-SNARE complex formation (Hickey and Wickner, 2010). It is not known whether HOPS directly catalyses *trans*-SNARE complex formation or makes the *trans*-SNARE complex more potent for fusion. We now report

Figure 6 HOPS preservation of *trans*-SNARE complex requires membrane integrity (A) and HOPS:Vam7p interaction (B). (A) *trans*-SNARE complex was formed at 27°C for 30 min as described in Figure 5. After receiving 10 mM CaCl₂, aliquots received various amounts of Sec17p/Sec18p (0/0, 0.68 μM/0.24 μM, or 1.36 μM/0.48 μM) with (lanes 7–12) or without (lanes 1–6) 1% Triton X-100. After 30 min of further incubation at 27°C, *trans*-SNARE complex was assayed. Data from five independent experiments were analysed. Error bars represent s.e.m. For samples in lanes 7–12, GST-Nyv1p was added before the addition of Triton X-100 to reduce new SNARE complex formation in detergent. (B) Proteoliposomes bearing Nyv1p (50 μM) and proteoliposomes (400 μM) bearing Vam3p and Vti1p were mixed with 1.2 μM Vam7p-3Δ at 27°C for 30 min to allow *trans*-SNARE complex formation. After receiving 10 mM CaCl₂, samples were aliquoted into tubes containing HOPS, HOPS with Y42A-PX, or control buffers. The final concentration of Y42A-PX is 10.8 μM, and thus the molar ratio of Y42A-PX:Vam7p-3Δ = 9:1. Each aliquot was further divided into three portions to receive Sec17p/Sec18p (0/0, 0.68 μM/0.24 μM, or 1.36 μM/0.48 μM). After 30 min of additional incubation at 27°C, *trans*-SNARE complex was assayed. Data from four independent experiments were analysed. Error bars represent s.e.m.

that HOPS inhibits the disassembly of *trans*-SNARE complexes by Sec17p and Sec18p, placing HOPS near the SNAREs at a step immediately before fusion. *Trans*-SNARE complexes may undergo multiple cycles of assembly and disassembly to allow the accumulation of cognate, functional *trans*-SNARE complexes. HOPS could create a physical barrier to stabilize functional *trans*-SNARE complexes against attack by Sec17p/Sec18p, allowing fusion to occur (Figure 9). HOPS association with *trans*-SNARE complex may be important for this protection. As both *trans*- and *cis*-SNARE complexes presumably have the same cytoplasmic helical bundle, it is reasonable that the selective protection of *trans*-SNARE complex by HOPS depends on the intact, apposed membranes of tethered vesicles (Figure 6). We hypothesize that HOPS protects the *trans*-SNARE complex only when it binds to both SNAREs and to the apposed membranes (Figure 9). Such a topology does not exist for *cis*-SNARE complexes,

which are formed by SNAREs on the same membrane, suggesting why *cis*-SNARE complexes are not protected by HOPS. As HOPS can proofread several structural features of *trans*-SNARE complexes, it may only protect functional *trans*-SNARE complexes.

A second, alternative model to explain HOPS action is that its association with *trans*-SNARE complexes causes a conformational change that renders the SNAREs resistant to Sec17p/Sec18p mediated disassembly, and this conformational change would reverse if the HOPS:*trans*-SNARE complex were solubilized in Triton X-100. We note, however, that Triton X-100 preserves the structure and activity of many membrane complexes, and specifically allows HOPS to remain with SNARE complexes (Collins *et al*, 2005). Further studies will be required to test these two models.

The dynamic nature of the vacuolar *trans*-SNARE complex is not limited to proteoliposomes. *Trans*-SNARE complexes

Figure 7 HOPS does not inhibit *cis*-SNARE complex disassembly. **(A)** 4-SNARE proteoliposomes (100 μ M lipids), 61 nM HOPS or its buffer, and various amounts of Sec17p/Sec18p (0/0, 0.17 μ M/0.06 μ M, 0.34 μ M/0.12 μ M, or 0.68 μ M/0.24 μ M) were incubated at 27°C for 30 min. The Nyv1p bound to Vam3p was assayed as described (see Materials and methods). Data from three independent experiments were analysed as in Figure 4. **(B, C)** To control for the effect of HOPS pre-incubation with proteoliposomes and to use the same amount SNARE complex as in the *trans*-SNARE complex disassembly assay, reduced amounts of 4-SNARE proteoliposomes (3.3 μ M lipids) bearing either Vam7p **(B)** or Vam7p-3Δ **(C)** were incubated with 61 nM HOPS or its buffer alone at 27°C for 30 min. Aliquots received control buffer or various amounts of Sec17p/Sec18p as in Figure 7A. After 30 min of further incubation at 27°C, *cis*-SNARE complex was assayed. The amount of *cis*-SNARE complex detected in the absence of Sec17p/Sec18p was set at 100% and was used to normalize the *cis*-SNARE complexes detected in the presence of Sec17p/Sec18p. Data from four independent experiments were analysed. Error bars represent s.e.m. At each Sec17p/Sec18p concentration, the differences between samples with or without HOPS were not statistically significant.

on vacuoles can undergo Sec17p and Sec18p-dependent remodelling during fusion (Jun *et al*, 2007). The finding that *trans*-SNARE complexes formed during vacuole fusion are largely associated with Sec17p (Figure 1) and that excessive Sec18p inhibits vacuole fusion (Figure 8; Ungermann *et al*, 1998b) suggests that most of these complexes are subject to remodelling. However, *trans*-SNARE complex remodelling is not essential for fusion under all circumstances. Fusion persists after Sec17p/Sec18p are inactivated or removed (Mayer *et al*, 1996), and both vacuoles and proteoliposomes are capable of fusion without Sec17p and Sec18p at all (Figure 3A; Thorngren *et al*, 2004; Mima *et al*, 2008). Thus, *trans*-SNARE complex remodelling is not absolutely required for merger of two biological membranes. However, wild-type vacuoles, which bear noncognate SNAREs such as Snc2p (Robinson *et al*, 2006; Collins and Wickner, 2007), are always subject to Sec17p and Sec18p action *in vivo*. Rapid *trans*-SNARE complex turnover coupled with selective stabilization by HOPS may optimize vacuole fusion in the cell.

Trans-SNARE complex remodelling may not be unique to vacuole fusion. Exocytosis in neuroendocrine PC-12 cells is

blocked by additional α SNAP after priming and docking, and extra NSF can relieve the blockade (Barszczewski *et al*, 2008), suggesting potential roles for these SNARE chaperones at a stage well beyond *cis*-SNARE complex disassembly. Studies of sea urchin eggs indicated that SNARE complexes formed between purified secretory vesicles can be disassembled before fusion (Tahara *et al*, 1998), though purposeful cycles of disassembly and reassembly have not been reported in this system. Reconstituted proteoliposome fusion systems offer advantages for mechanistic studies. In addition to vacuolar SNAREs, neuronal (Weber *et al*, 1998) and endosomal (Ohya *et al*, 2009) SNAREs have been successfully reconstituted into proteoliposomes. The neuronal *trans*-SNARE complex is functionally resistant to α SNAP and NSF (Weber *et al*, 2000). When a self-inhibitory domain of syntaxin was removed, fusion was accelerated by the addition of α SNAP and NSF (Weber *et al*, 2000). This is in contrast to proteoliposomes bearing vacuolar SNAREs, which do not fuse at all in the presence of Sec17p and Sec18p without HOPS (Mima *et al*, 2008). The difference may be attributable to the different lipid composition in the two proteoliposome

Figure 8 HOPS prevents Sec18p-mediated inhibition of vacuole fusion. **(A)** Fusion reactions containing BJ3505 and DKY6281 vacuoles were incubated at 27°C for 60 min with increasing amounts of Sec18p and compensatory amounts of Sec18p buffer. At the end of the reaction, Pho8p activity was assayed. **(B)** BJ3505 and DKY6281 vacuoles were mixed with either 0.57 μM Sec18p or control buffer, along with various amount of HOPS and compensatory amounts of HOPS buffer. Pho8p activity was assayed after 60 min incubation at 27°C (except for the ice control), Pho8p activity was assayed. Fusion signals, after subtracting the ice background signal, were averaged from three independent experiments. Error bars represent s.e.m.

Figure 9 Model of HOPS action. SNAREs on apposed biological membranes can form a *trans*-SNARE complex that is accessible to Sec17p and Sec18p in the absence of HOPS. On ATP hydrolysis, Sec17p and Sec18p can disassemble the *trans*-SNARE complex and thus prevent the merger of the two membranes. HOPS, by binding simultaneously to two apposed membranes, protects *trans*-SNARE complexes from Sec17p and Sec18p. The block is relieved on fusion as HOPS no longer binds two membranes, allowing post-fusion *cis*-SNARE complex to be primed for a second round of fusion. This model does not exclude other HOPS functions, such as tethering and proofreading.

fusion systems or to differences in the neuronal and vacuolar SNARE complexes, for example, neuronal *trans*-SNARE complexes may execute fusion at a rate faster than the rate of turnover by α SNAP and NSF. Reconstituted early endosome fusion requires a set of 17 recombinant proteins including SNAREs, α SNAP and NSF, Rab5 and their partners (Ohya *et al*, 2009). The first round of fusion takes place without α SNAP and NSF, because VAMP4 and syntaxin13/VTI1A/syntaxin6 are reconstituted separately on two different proteoliposomes. When added individually, α SNAP or NSF blocked this fusion, presumably either by preventing SNAREs from entering into *trans*-SNARE complexes or by inhibiting *trans*-SNARE complex function. A *trans*-SNARE complex assay similar to the one used in our study might distinguish these possibilities. Like reconstituted vacuolar fusion, the α SNAP/NSF-dependent endosomal fusion requires a protein complex that contains a Vps33p isoform, human VPS45. Biochemical analysis will be needed to address whether this complex preserves the endosomal *trans*-SNARE complex as HOPS does for the vacuole.

Recent studies of intra-Golgi traffic have shown that the COG tethering complex, which is required for the stability of the intro-Golgi SNARE complex (Shestakova *et al*, 2007), must interact with Sly1p, a Vps33 isoform, for Golgi SNARE-pairing (Laufman *et al*, 2009). If COG, like HOPS, is indeed preserving the *trans*-SNARE complex, then this new activity may be a common feature of oligomeric tethering factors.

Materials and methods

Yeast strains, vacuole isolation, and fusion reactions

Vacuoles were purified (Haas *et al*, 1994) from *Saccharomyces cerevisiae* strains BJ3505, DKY6281, BJ3505 *nyv1Δ*, DKY6281 *vam3Δ*, and DKY6281 *vam3Δ nyv1-CCIIIM* (Jun *et al*, 2007) for fusion and *trans*-SNARE complex assays. Fusion reactions (30 μl) contained 20 mM PIPES-KOH, pH 6.8, 200 mM sorbitol, 125 mM KCl, 6 mM MgCl₂, 1 mM ATP, an ATP regenerating system (1 mg/ml creatine kinase and 29 mM creatine phosphate), 10 mM coenzyme A, 3.3 μg/ml Pbi2p (I₂^B), 3 μg of *pep4Δ* vacuoles (from BJ3505 derivatives), and 3 μg of *pho8Δ* vacuoles (from DKY6281 derivatives). After incubation at 27°C, Pho8p phosphatase activity was assayed at 30°C for 5 min (Haas, 1995). Fusion units are micromoles of *p*-nitrophenol phosphate hydrolyzed per min per microgram *pep4Δ* vacuole.

Proteins, antibodies, and cross-linking

Vam7p and Vam7p-3Δ were purified according to Schwartz and Merz (2009) with the following modifications: (1) *Escherichia coli* Rosetta 2 (DE3) (Novagen) was used for protein expression; (2) induced bacteria were lysed by French Press (3 passes, 900 pounds per square inch); and (3) after affinity purification on Ni-NTA agarose (Qiagen), his₆-Vam7 proteins were exchanged into RB500 (20 mM HEPES-NaOH, pH 7.4, 500 mM NaCl, 10% (v/v) glycerol) for storage. Recombinant Vam3p, Vti1p, and Nyv1p were purified from Rosetta 2 (Novagen) (Mima *et al*, 2008). HOPS complex containing Vps33-GST was purified from yeast CHY31 (Starai *et al*, 2008). Sec17p was purified from Rosetta 2 (DE3) as described (Schwartz and Merz, 2009), except that the tagless Sec17p was exchanged into RB150 (20 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 10% (v/v) glycerol) for storage. His₆-Sec18 (Haas and Wickner, 1996), I₂^B (Slusarewicz *et al*, 1997), GST-2xFYVE domain (Gillooly *et al*, 2000), his₆-Gyp1-46 (Wang *et al*, 2003), Gdi1p (Starai *et al*, 2007), Y42A-PX domain (Boeddinghaus *et al*, 2002), α Ypt7, and its antigenic peptides (Eitzen *et al*, 2001), α VPS33 (Seals *et al*, 2000), and α Sec17 (Haas and Wickner, 1996) were prepared as described. α Porin I was a kind gift from Naomi Thorngren. MARCKS effector domain (Wang *et al*, 2001) was synthesized by the WM Keck Biotechnology Resource Center (New Haven, CT).

α Vam3 and α Vam3-N (specific for N-terminal domain) were affinity purified with immobilized GST-Vam3(ATM) and GST-Vam3-N(ATM), respectively. Proteins (10 mg) were cross-linked to 2.5 ml of Sulfolink coupling gel (Thermo Scientific, Rockford, IL) following the manufacturer's instructions. Serum (10 ml) from rabbits immunized with his₆-Vam3(ATM) was used for antibody purification (Jun *et al*, 2007).

For immunoprecipitation assays, antibodies (α Vam3, α Vam3-N, α Sec17, or α Porin I) were cross-linked to protein A Sepharose CL-4B (GE) as described (Harlow and Lane, 1998), except that 2 mg of IgG (1 OD₂₈₀ = 0.8 mg/ml) were used per 4 ml of protein A resin. Protein A resin cross-linked to antibodies was stored at 4°C in PBS containing 0.02% Na₃N. Immediately before use, resins were exchanged into elution buffer (0.2 M glycine, pH 2.2, 0.2 M NaCl), then equilibrated with solubilization buffer (see below).

Proteoliposome reconstitution and lipid mixing assay

SNARE proteoliposomes with POPC (42 or 44% for donor or acceptor), POPE (18%), soy PI (18%), POPS (4.4%), POPA (2.0%), CL (1.6%), ERG (8.0%), DAG (1.0%), PI(3)P (1.0%), PI(4,5)P₂ (1.0%), and fluorescent lipids (1.5% NBD-PE and 1.5% Rh-PE for donor; 1.0% dansyl-PE for acceptor) were prepared as described (Mima *et al*, 2008).

Fusion reactions (20 μ l) contained RB150 (20 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 10% (v/v) glycerol), 1 mM MgCl₂, 1 mM ATP, an ATP regenerating system, donor proteoliposomes (50 μ M lipids), and acceptor proteoliposomes (400 μ M lipids). Vam7p or Vam7-3A (0.2–1.8 μ M), HOPS (61 nM), Sec17p (0.17–2.7 μ M), and Sec18p (0.06–0.96 μ M) were added where indicated. To monitor lipid mixing, reaction mixtures (prepared on ice) were transferred to a 396-well plate (pre-incubated on ice) and the NBD fluorescent signal was measured (λ_{ex} = 460 nm, λ_{em} = 538 nm, λ_{ex} = 515 nm) in a SpectraMAX Gemini XPS plate reader (Molecular Devices) at 27°C for 30 min (Mima *et al*, 2008). Fusion is presented as the ratio of the fluorescence at any time to that at 1 min.

Trans-SNARE complex assay

Proteoliposome samples (450 μ M lipids; 20 μ l) were chilled (ice, 5 min). Each received 1.4 μ g of GST-Nyv1(ATM) and, after an additional 10 min, 800 μ l of RIPA buffer (25 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% NP40-Alternative, 1% deoxycholate, 0.1% SDS) containing 10 mM EDTA, protease inhibitor cocktail (0.46 μ g/ml leupeptin, 3.5 μ g/ml pepstatin A, 24 μ g/ml pefablock-SC), and 1 mM PMSF. GST-Nyv1(ATM) (70 μ g/ml) was added to block potential binding of native Nyv1p to Vam3p in detergent. Samples were nutated at 4°C for 20 min, then centrifuged (16 000 g, 4°C, 5 min). Supernatant (700 μ l) was incubated with 15 μ l of immobilized α Vam3 at 4°C for 1 h with nutation. After centrifugation (3000 g, 4°C, 2 min), the α Vam3 resin was resuspended in 600 μ l of RIPA buffer (with 10 mM EDTA and protease inhibitor cocktail), centrifuged (3000 g, 4°C, 2 min), and the supernatant was removed by centrifugation. After repeating this process three times, proteins were eluted with 2 $\times$ sample buffer (Sambrook *et al*, 1989) and subjected to SDS-PAGE (12%) and western blot analysis. The blots were scanned and analysed using software UN-SCAN-IT gel 5.3 (Silk Scientific).

To assay for vacuolar *trans*-SNARE complex, vacuoles from a 300- μ l reaction were centrifuged (11 750 g, 10 min, 4°C) and resuspended in 400 μ l of solubilization buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM MgCl₂, 10% glycerol, 0.5% NP40-Alternative) containing protease inhibitor cocktail and 1 mM PMSF. After 20 min at 4°C, samples were centrifuged (16 000 g, 20 min, 4°C) and supernatant (350 μ l) was incubated with 30 μ l α Vam3-N resin at 4°C overnight. Resin was resuspended in 600 μ l of solubilization buffer with protease inhibitor cocktail, centrifuged (3000 g, 4°C, 2 min), and the supernatant was removed. This process was repeated three times before proteins were eluted from the resin and assayed as above. For immunoadsorption experiments, vacuolar detergent extracts were incubated with resin bearing either α Sec17 or α Porin I (30 μ l) at 4°C for 1 h. Supernatant was collected after centrifugation (3000 g, 4°C, 2 min) and incubated (4°C, 1 h) with fresh resin and again centrifuged. Supernatant was then used for overnight incubation with α Vam3-N resin as described above. For experiments in Figure 1, a larger fusion reaction (4.83 ml) containing BJ3505 *nyv1* Δ vacuoles (483 μ g), DKY6281 *vam3*(Δ N) vacuoles (483 μ g), and 40 nM Vam7p was incubated at 27°C for 45 min with either no inhibitors, Gyp1-46

(2.5 μ M)/Gdi1p (0.6 μ M) or MED (10 μ M). Aliquots (30 μ l) were set aside for Pho8p activity assay. The remainder was resuspended in 6.4 ml solubilization buffer (described above). Detergent extracts were incubated with 480 μ l of immobilized α Vam3(N) at 4°C overnight. Proteins were eluted with 6 ml of 0.2 M glycine (pH 2.2), 0.2 M NaCl and divided into two equal aliquots. Ice-cold TCA (100%) was added to each to 12.5%. After vigorous mixing (5 s), samples were incubated on ice for 30 min and centrifuged (16 000 g, 4°C, 20 min). The pellet in each aliquot was resuspended in 3 ml of acetone (–20°C) and centrifuged (16 000 g, 4°C, 10 min). Supernatants were removed and pellets were dried in air for 5 min. One aliquot was subjected to SDS-PAGE and Sypro Ruby staining and the other to mass spectrometry.

Multidimensional protein identification technology

The protein digest was pressure loaded onto a fused silica capillary desalting column containing 5 cm of 5 μ m Polaris C18-A material (Metachem, Ventura, CA) packed into a 250- μ m internal diameter (i.d.) capillary with a 2- μ m filtered union (UpChurch Scientific, Oak Harbor, WA). The desalting column was washed with buffer containing 95% water, 5% acetonitrile, and 0.1% formic acid. After desalting, a 100- μ m i.d. capillary with a 5- μ m pulled tip packed with 10 cm 3- μ m Aqua C18 material (Phenomenex, Ventura, CA) followed by 3 cm 5- μ m Partisphere strong cation exchanger (Whatman, Clifton, NJ) was attached to the filter union and the entire split column (desalting column–filter union–analytical column) was placed inline with an Agilent 1100 quaternary HPLC (Palo Alto, CA) and analysed using a modified five-step separation described earlier (Washburn *et al*, 2001). The buffer solutions used were 5% acetonitrile/0.1% formic acid (buffer A), 80% acetonitrile/0.1% formic acid (buffer B), and 500 mM ammonium acetate/5% acetonitrile/0.1% formic acid (buffer C). Step 1 consisted of a 90-min gradient from 0 to 100% buffer B. Steps 2–4 had the following profile: 3 min of 100% buffer A, 2 min of X% buffer C, a 10-min gradient from 0 to 15% buffer B, and a 97-min gradient from 15 to 45% buffer B. The 2-min buffer C percentages (X) were 20, 50, 80%, respectively, for the five-step analysis. The final step, the gradient contained: 3 min of 100% buffer A, 20 min of 100% buffer C, a 10-min gradient from 0 to 15% buffer B, and a 107-min gradient from 15 to 70% buffer B.

As peptides eluted from the microcapillary column, they were electrosprayed directly into an LTQ two-dimensional ion trap mass spectrometer (ThermoFinnigan, Palo Alto, CA) with the application of a distal 2.4 kV spray voltage. A cycle of one full-scan mass spectrum (400–1400 *m/z*) followed by eight data-dependent MS/MS spectra at a 35% normalized collision energy was repeated continuously throughout each step of the multidimensional separation. Application of mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcalibur data system.

Analysis of tandem mass spectra

MS/MS spectra were analysed using the following software analysis protocol. Poor quality spectra were removed from the data set using an automated spectral quality assessment algorithm (Bern *et al*, 2004). MS/MS spectra remaining after filtering were searched with the SEQUESTTM algorithm (Eng *et al*, 1994) against the *Saccharomyces* Genome Database (December 2005) concatenated to a decoy database in which the sequence for each entry in the original database was reversed (Peng *et al*, 2003). All searches were parallelized and performed on a Beowulf computer cluster consisting of 100 1.2 GHz Athlon CPUs (Sadygov *et al*, 2002). No enzyme specificity was considered for any search. SEQUEST results were assembled and filtered using the DTASelect (version 2.0) program (Tabb *et al*, 2002; Cociorva *et al*, 2007). DTASelect 2.0 uses a linear discriminant analysis to dynamically set XCorr and DeltaCN thresholds for the entire data set to achieve a user-specified false-positive rate (5% in this analysis). The false-positive rates are estimated by the program from the number and quality of spectral matches to the decoy database. All the data sets can be found under VAM3 in the Yeast Resource Center Public Data Repository (Riffle *et al*, 2005).

Cis-SNARE complex disassembly assay

Proteoliposomes (100 or 3.3 μ M lipids) bearing Vam3p, Vti1p, Vam7p (either wild-type or truncated), and Nyv1p were incubated in 20 μ l with RB150, 1 mM MgCl₂, 1 mM ATP, an ATP regenerating

system, and (where indicated) HOPS, Sec17p, and Sec18p. After 30 min at 27°C, samples were chilled on ice for 5 min before receiving 2.8 µg of GST-Nyv1p (ΔTM) and 400 µl of RIPA buffer containing 10 mM EDTA, protease inhibitor cocktail, and 1 mM PMSF. After 20 min at 4°C, samples were centrifuged (16000g, 5 min, 4°C), and the supernatant (350 µl) was collected for immunoprecipitation as described above.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank Alex Merz (U Washington) for sharing Vam7p and Vam7p-3Δ constructs before publication and Amy Orr and Holly

Jakubowski for excellent technical support. This work was supported by NIH grant GM23377. Mass spectrometry analysis was supported by NIH grant P41 RR11823. Hao Xu and William Wickner designed the experiments and wrote the manuscript. Youngsoo Jun conceived the *trans*-SNARE complex assay and engineered the required yeast strains. James Thompson and John Yates performed mass spectrometry analysis and wrote the corresponding methods. William Wickner isolated SNARE proteins and the lyticase used for vacuole isolation. Hao Xu performed the rest of the experiments.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Barszczewski M, Chua JJ, Stein A, Winter U, Heintzmann R, Zilly FE, Fasshauer D, Lang T, Jahn R (2008) A novel site of action for alpha-SNAP in the SNARE conformational cycle controlling membrane fusion. *Mol Biol Cell* **19**: 776–784
- Bern M, Goldberg D, McDonald WH, Yates III JR (2004) Automatic quality assessment of peptide tandem mass spectra. *Bioinformatics* **20** (Suppl 1): i49–i54
- Boeddinghaus C, Merz AJ, Laage R, Ungermann C (2002) A cycle of Vam7p release from and PtdIns 3-P-dependent rebinding to the yeast vacuole is required for homotypic vacuole fusion. *J Cell Biol* **157**: 79–89
- Burd CG, Emr SD (1998) Phosphatidylinositol(3)-phosphate signaling mediated by specific binding to RING FYVE domains. *Mol Cell* **2**: 157–162
- Cheever ML, Sato TK, de Beer T, Kutateladze TG, Emr SD, Overduin M (2001) Phox domain interaction with PtdIns(3)P targets the Vam7 t-SNARE to vacuole membranes. *Nat Cell Biol* **3**: 613–618
- Cociorva D, L Tabb D, Yates JR (2007) Validation of tandem mass spectrometry database search results using DTASelect. *Curr Protoc Bioinformatics* **Chapter 13**: Unit 13.4
- Collins KM, Thorngren NL, Fratti RA, Wickner WT (2005) Sec17p and HOPS, in distinct SNARE complexes, mediate SNARE complex disruption or assembly for fusion. *EMBO J* **24**: 1775–1786
- Collins KM, Wickner WT (2007) Trans-SNARE complex assembly and yeast vacuole membrane fusion. *Proc Natl Acad Sci USA* **104**: 8755–8760
- Deak F, Xu Y, Chang WP, Dulubova I, Khvotchev M, Liu X, Sudhof TC, Rizo J (2009) Munc18-1 binding to the neuronal SNARE complex controls synaptic vesicle priming. *J Cell Biol* **184**: 751–764
- Eitzen G, Thorngren N, Wickner W (2001) Rho1p and Cdc42p act after Ypt7p to regulate vacuole docking. *EMBO J* **20**: 5650–5656
- Eng J, McCormack A, Yates J (1994) An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J Am Soc Mass Spectrom* **5**: 976–989
- Fratti RA, Jun Y, Merz AJ, Margolis N, Wickner W (2004) Interdependent assembly of specific regulatory lipids and membrane fusion proteins into the vertex ring domain of docked vacuoles. *J Cell Biol* **167**: 1087–1098
- Gillooly DJ, Morrow IC, Lindsay M, Gould R, Bryant NJ, Gaullier JM, Parton RG, Stenmark H (2000) Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells. *EMBO J* **19**: 4577–4588
- Gissen P, Johnson CA, Morgan NV, Stapelbroek JM, Forshew T, Cooper WN, McKiernan PJ, Klomp LW, Morris AA, Wraith JE, McClean P, Lynch SA, Thompson RJ, Lo B, Quarrell OW, Di Rocco M, Trembath RC, Mandel H, Wali S, Karet FE *et al* (2004) Mutations in VPS33B, encoding a regulator of SNARE-dependent membrane fusion, cause arthrogyrosis-renal dysfunction-cholestasis (ARC) syndrome. *Nat Genet* **36**: 400–404
- Grosshans BL, Ortiz D, Novick P (2006) Rabs and their effectors: achieving specificity in membrane traffic. *Proc Natl Acad Sci USA* **103**: 11821–11827
- Guo W, Roth D, Walch-Solimena C, Novick P (1999) The exocyst is an effector for Sec4p, targeting secretory vesicles to sites of exocytosis. *EMBO J* **18**: 1071–1080
- Haas A (1995) A quantitative assay to measure homotypic vacuole fusion *in vitro*. *Methods Cell Sci* **17**: 283–294
- Haas A, Conradt B, Wickner W (1994) G-protein ligands inhibit *in vitro* reactions of vacuole inheritance. *J Cell Biol* **126**: 87–97
- Haas A, Wickner W (1996) Homotypic vacuole fusion requires Sec17p (yeast alpha-SNAP) and Sec18p (yeast NSF). *EMBO J* **15**: 3296–3305
- Harlow E, Lane D (1998) *Using Antibodies: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press
- Hickey CM, Wickner W (2010) HOPS initiates vacuole docking by tethering membranes prior to trans-SNARE complex assembly. *Mol Biol Cell* (in press)
- Jun Y, Xu H, Thorngren N, Wickner W (2007) Sec18p and Vam7p remodel trans-SNARE complexes to permit a lipid-anchored R-SNARE to support yeast vacuole fusion. *EMBO J* **26**: 4935–4945
- Kutateladze TG, Ogburn KD, Watson WT, de Beer T, Emr SD, Burd CG, Overduin M (1999) Phosphatidylinositol 3-phosphate recognition by the FYVE domain. *Mol Cell* **3**: 805–811
- Laufman O, Kedan A, Hong W, Lev S (2009) Direct interaction between the COG complex and the SM protein, Sly1, is required for Golgi SNARE pairing. *EMBO J* **28**: 2006–2017
- Malsam J, Kreye S, Sollner TH (2008) Membrane fusion: SNAREs and regulation. *Cell Mol Life Sci* **65**: 2814–2832
- Markgraf DF, Peplowska K, Ungermann C (2007) Rab cascades and tethering factors in the endomembrane system. *FEBS Lett* **581**: 2125–2130
- Mayer A, Wickner W, Haas A (1996) Sec18p (NSF)-driven release of Sec17p (alpha-SNAP) can precede docking and fusion of yeast vacuoles. *Cell* **85**: 83–94
- McNew JA (2008) Regulation of SNARE-mediated membrane fusion during exocytosis. *Chem Rev* **108**: 1669–1686
- Mima J, Hickey CM, Xu H, Jun Y, Wickner W (2008) Reconstituted membrane fusion requires regulatory lipids, SNAREs and synergistic SNARE chaperones. *EMBO J* **27**: 2031–2042
- Ohya T, Miaczynska M, Coskun U, Lommer B, Runge A, Drechsel D, Kalaidzidis Y, Zerial M (2009) Reconstitution of Rab- and SNARE-dependent membrane fusion by synthetic endosomes. *Nature* **459**: 1091–1097
- Peng J, Elias JE, Thoreen CC, Licklider LJ, Gygi SP (2003) Evaluation of multidimensional chromatography coupled with tandem mass spectrometry (LC/LC-MS/MS) for large-scale protein analysis: the yeast proteome. *J Proteome Res* **2**: 43–50
- Peplowska K, Markgraf DF, Ostrowicz CW, Bange G, Ungermann C (2007) The CORVET tethering complex interacts with the yeast Rab5 homolog Vps21 and is involved in endo-lysosomal biogenesis. *Dev Cell* **12**: 739–750
- Price A, Wickner W, Ungermann C (2000) Proteins needed for vesicle budding from the Golgi complex are also required for the docking step of homotypic vacuole fusion. *J Cell Biol* **148**: 1223–1229
- Pulipparacharuvil S, Akbar MA, Ray S, Sevrioukov EA, Haberman AS, Rohrer J, Kramer H (2005) Drosophila Vps16A is required for trafficking to lysosomes and biogenesis of pigment granules. *J Cell Sci* **118** (Part 16): 3663–3673
- Riffle M, Malmstrom L, Davis TN (2005) The yeast resource center public data repository. *Nucleic Acids Res* **33** (Database issue): D378–D382

- Robinson M, Poon PP, Schindler C, Murray LE, Kama R, Gabriely G, Singer RA, Spang A, Johnston GC, Gerst JE (2006) The Gcs1 Arf-GAP mediates Snc1,2 v-SNARE retrieval to the Golgi in yeast. *Mol Biol Cell* **17**: 1845–1858
- Rojo E, Zouhar J, Kovaleva V, Hong S, Raikhel NV (2003) The AtC-VPS protein complex is localized to the tonoplast and the prevacuolar compartment in arabidopsis. *Mol Biol Cell* **14**: 361–369
- Sacher M, Jiang Y, Barrowman J, Scarpa A, Burston J, Zhang L, Schieltz D, Yates III JR, Abeliovich H, Ferro-Novick S (1998) TRAPP, a highly conserved novel complex on the cis-Golgi that mediates vesicle docking and fusion. *EMBO J* **17**: 2494–2503
- Sadygov RG, Eng J, Durr E, Saraf A, McDonald H, MacCoss MJ, Yates III JR (2002) Code developments to improve the efficiency of automated MS/MS spectra interpretation. *J Proteome Res* **1**: 211–215
- Sambrook J, Fritsch E, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Press
- Schwartz ML, Merz AJ (2009) Capture and release of partially zipped trans-SNARE complexes on intact organelles. *J Cell Biol* **185**: 535–549
- Seals DF, Eitzen G, Margolis N, Wickner WT, Price A (2000) A Ypt/Rab effector complex containing the Sec1 homolog Vps33p is required for homotypic vacuole fusion. *Proc Natl Acad Sci USA* **97**: 9402–9407
- Shen J, Tareste DC, Paumet F, Rothman JE, Melia TJ (2007) Selective activation of cognate SNAREpins by Sec1/Munc18 proteins. *Cell* **128**: 183–195
- Shestakova A, Suvorova E, Pavliv O, Khaidakova G, Lupashin V (2007) Interaction of the conserved oligomeric Golgi complex with t-SNARE Syntaxin5a/Sed5 enhances intra-Golgi SNARE complex stability. *J Cell Biol* **179**: 1179–1192
- Sinioglou S, Pelham HR (2002) Vps51p links the VFT complex to the SNARE Tlg1p. *J Biol Chem* **277**: 48318–48324
- Slusarewicz P, Xu Z, Seefeld K, Haas A, Wickner WT (1997) I2B is a small cytosolic protein that participates in vacuole fusion. *Proc Natl Acad Sci USA* **94**: 5582–5587
- Starai VJ, Hickey CM, Wickner W (2008) HOPS proofreads the trans-SNARE complex for yeast vacuole fusion. *Mol Biol Cell* **19**: 2500–2508
- Starai VJ, Jun Y, Wickner W (2007) Excess vacuolar SNAREs drive lysis and Rab bypass fusion. *Proc Natl Acad Sci USA* **104**: 13551–13558
- Stroupe C, Collins KM, Fratti RA, Wickner W (2006) Purification of active HOPS complex reveals its affinities for phosphoinositides and the SNARE Vam7p. *EMBO J* **25**: 1579–1589
- Stroupe C, Hickey CM, Mima J, Burfeind AS, Wickner W (2009) Minimal membrane docking requirements revealed by reconstitution of Rab GTPase-dependent membrane fusion from purified components. *Proc Natl Acad Sci USA* **106**: 17626–17633
- Sudhof TC, Rothman JE (2009) Membrane fusion: grappling with SNARE and SM proteins. *Science* **323**: 474–477
- Suvorova ES, Duden R, Lupashin VV (2002) The Sec34/Sec35p complex, a Ypt1p effector required for retrograde intra-Golgi trafficking, interacts with Golgi SNAREs and COPI vesicle coat proteins. *J Cell Biol* **157**: 631–643
- Suzuki T, Oiso N, Gautam R, Novak EK, Panthier JJ, Suprabha PG, Vida T, Swank RT, Spritz RA (2003) The mouse organellar biogenesis mutant buff results from a mutation in Vps33a, a homologue of yeast vps33 and Drosophila carnation. *Proc Natl Acad Sci USA* **100**: 1146–1150
- Tabb DL, McDonald WH, Yates III JR (2002) DTASelect and Contrast: tools for assembling and comparing protein identifications from shotgun proteomics. *J Proteome Res* **1**: 21–26
- Tahara M, Coorsen JR, Timmers K, Blank PS, Whalley T, Scheller R, Zimmerberg J (1998) Calcium can disrupt the SNARE protein complex on sea urchin egg secretory vesicles without irreversibly blocking fusion. *J Biol Chem* **273**: 33667–33673
- Thorngren N, Collins KM, Fratti RA, Wickner W, Merz AJ (2004) A soluble SNARE drives rapid docking, bypassing ATP and Sec17/18p for vacuole fusion. *EMBO J* **23**: 2765–2776
- Ungermann C, Nichols BJ, Pelham HR, Wickner W (1998a) A vacuolar v-t-SNARE complex, the predominant form *in vivo* and on isolated vacuoles, is disassembled and activated for docking and fusion. *J Cell Biol* **140**: 61–69
- Ungermann C, Sato K, Wickner W (1998b) Defining the functions of trans-SNARE pairs. *Nature* **396**: 543–548
- Wada Y, Ohsumi Y, Anraku Y (1992) Genes for directing vacuolar morphogenesis in *Saccharomyces cerevisiae*. I. Isolation and characterization of two classes of vam mutants. *J Biol Chem* **267**: 18665–18670
- Wang J, Arbuzova A, Hangyas-Mihalyne G, McLaughlin S (2001) The effector domain of myristoylated alanine-rich C kinase substrate binds strongly to phosphatidylinositol 4,5-bisphosphate. *J Biol Chem* **276**: 5012–5019
- Wang L, Merz AJ, Collins KM, Wickner W (2003) Hierarchy of protein assembly at the vertex ring domain for yeast vacuole docking and fusion. *J Cell Biol* **160**: 365–374
- Washburn MP, Wolters D, Yates III JR (2001) Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat Biotechnol* **19**: 242–247
- Weber T, Parlati F, McNew JA, Johnston RJ, Westermann B, Sollner TH, Rothman JE (2000) SNAREpins are functionally resistant to disruption by NSF and alphaSNAP. *J Cell Biol* **149**: 1063–1072
- Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, Parlati F, Sollner TH, Rothman JE (1998) SNAREpins: minimal machinery for membrane fusion. *Cell* **92**: 759–772
- Wickner W (2002) Yeast vacuoles and membrane fusion pathways. *EMBO J* **21**: 1241–1247
- Wickner W, Schekman R (2008) Membrane fusion. *Nat Struct Mol Biol* **15**: 658–664
- Wurmser AE, Sato TK, Emr SD (2000) New component of the vacuolar class C-Vps complex couples nucleotide exchange on the Ypt7 GTPase to SNARE-dependent docking and fusion. *J Cell Biol* **151**: 551–562