

Purification of active HOPS complex reveals its affinities for phosphoinositides and the SNARE Vam7p

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Coupling of Rab GTPase activation and SNARE complex assembly during membrane fusion is poorly understood. The homotypic fusion and vacuole protein sorting (HOPS) complex links these two processes: it is an effector for the vacuolar Rab GTPase Ypt7p and is required for vacuolar SNARE complex assembly. We now report that pure, active HOPS complex binds phosphoinositides and the PX domain of the vacuolar SNARE protein Vam7p. These binding interactions support HOPS complex association with the vacuole and explain its enrichment at the same microdomains on docked vacuoles as phosphoinositides, Ypt7p, Vam7p, and the other SNARE proteins. Concentration of the HOPS complex at these microdomains may be a key factor for coupling Rab GTPase activation to SNARE complex assembly.

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Introduction

Many of the proteins and lipids involved in membrane traffic are well-conserved (Jahn *et al.*, 2003). Rab GTPases are ras-like proteins required for membrane tethering (Allan *et al.*, 2000; Pfeffer, 2001). Diverse Rab effectors, which interact with GTP-bound Rab proteins, have been identified (Segev, 2001). Diverse multiprotein complexes also act in intracellular transport pathways (Peterson and Emr, 2001; Hsu *et al.*, 2004; Babst, 2005; Oka *et al.*, 2005); two, the conserved oligomeric Golgi complex and transport particle I complex, have been purified in active form (Walter *et al.*, 1998; Sacher *et al.*, 2001). SNARE proteins are integral or peripheral membrane proteins that bind each other to form four-helical SNARE complexes (Poirier *et al.*, 1998) that act at a late stage of membrane fusion (Weber *et al.*, 1998). Sec1/munc18 proteins bind SNAREs and SNARE complexes, promoting SNARE complex assembly (Pevsner *et al.*, 1994;

Carr *et al.*, 1999; Peng and Gallwitz, 2002). Lipids also play a role in membrane trafficking. Ergosterol is required for yeast vacuole fusion (Kato and Wickner, 2001), and cholesterol is involved in localization of SNAREs (Lang *et al.*, 2001). Phosphoinositides are required for vacuole fusion (Mayer *et al.*, 2000) and regulate the localization of both Rabs and SNAREs (Boeddinghaus *et al.*, 2002; Fratti *et al.*, 2004).

Vacuoles from *Saccharomyces cerevisiae* are used to study membrane tethering and fusion. Fusion of purified vacuoles can be assayed *in vitro* (Haas *et al.*, 1994). This reaction requires many of the same lipids and protein families as other fusion reactions, and thus uses the same mechanisms (Wickner and Haas, 2000). Vacuole fusion occurs in three stages: priming, docking, and mixing of lipid bilayers and luminal contents. During priming, SNARE complexes are disassembled by Sec18p and its co-chaperone Sec17p (Mayer *et al.*, 1996; Ungermann *et al.*, 1998a). In the first phase of docking, vacuoles tether; this requires the Rab GTPase Ypt7p (Mayer and Wickner, 1997). Proteins and lipids important for fusion then become enriched in a ring encircling the apposed patches of membrane on each tethered vacuole. This microdomain is termed the vertex ring (Wang *et al.*, 2002, 2003; Fratti *et al.*, 2004). After the vertex ring has formed, SNAREs from the apposed membranes bind each other to form *trans*-SNARE complexes (Ungermann *et al.*, 1998b; Wang *et al.*, 2003; Dietrich *et al.*, 2005). Finally, the membranes fuse around the vertex ring, internalizing the apposed membrane from each vacuole as a luminal vesicle (Wang *et al.*, 2002).

The homotypic fusion and vacuole protein sorting (HOPS) complex is a six-subunit complex that acts during the docking stage of yeast vacuole fusion (Price *et al.*, 2000b). HOPS complex subunits are also involved in transport between endocytic compartments, as well as transport to the vacuole (Cowles *et al.*, 1997; Rehling *et al.*, 1999; Srivastava *et al.*, 2000; Peterson and Emr, 2001). The HOPS complex is also termed the Class C vacuolar protein sorting (Vps) complex because it is composed of Vps proteins, which are encoded by genes required for proper protein sorting to the yeast vacuole (Raymond *et al.*, 1992). The first proteins shown to be in the HOPS complex were Vps11p/Pep5p, Vps16p, Vps18p/Pep3p, and Vps33p (Rieder and Emr, 1997). Later, Vps39p/Vam6p and Vps41p/Vam2p were found in a complex with these four proteins (Seals *et al.*, 2000; Wurmser *et al.*, 2000). Vps11p, Vps16p, Vps18p, and Vps33p also form a complex, lacking Vps39p and Vps41p, with Vps8p (Subramanian *et al.*, 2004). We will refer here to the complex of Vps11p, Vps16p, Vps18p, Vps33p, Vps39p, and Vps41p as the HOPS complex.

The HOPS complex interacts functionally and physically with Ypt7p and SNAREs. Vps39p is a nucleotide exchange factor for Ypt7p (Wurmser *et al.*, 2000). HOPS binds to GTP γ S-bound Ypt7p, and thus is also a Ypt7p effector (Seals *et al.*, 2000). The HOPS complex is required for SNARE

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complex assembly and stably associates with SNARE complexes on vacuoles (Price *et al*, 2000a; Sato *et al*, 2000; Collins *et al*, 2005). This interaction may involve Vps33p, a member of the Sec1/munc18 family (Rieder and Emr, 1997; Dulubova *et al*, 2001).

The specific functions of the HOPS complex and the mechanisms by which it couples activated Ypt7p to SNARE complex assembly are unknown. To directly investigate HOPS complex functions, we have developed a method to purify HOPS from yeast, and an assay to show that pure HOPS complex is active. We now report that Rab GTPase-dependent tethering of *vps11-1^{ts}* vacuoles requires pure HOPS complex. Rab GTPase, Rho GTPase, and SNARE catalysts of vacuole docking cannot complete their functions on *vps11-1^{ts}* vacuoles without pure HOPS complex. Pure HOPS complex binds directly to phosphoinositides and to the phox homology (PX) domain of the soluble SNARE Vam7p. Thus, HOPS, phosphoinositides, and Vam7p each have mutual affinities, explaining their interdependent assembly into the vertex ring with Ypt7p. This high local concentration of HOPS, SNAREs, and Ypt7p may be a key element in coupling active Ypt7p to SNARE complex assembly during vacuole docking.

Results

We purified the HOPS complex using a streptavidin-binding peptide (SBP) fused to Vps33p (Keefe *et al*, 2001). Purified vacuoles (Seals *et al*, 2000) bearing Vps33-SBP were lysed in detergent and subjected to streptavidin affinity chromatography. Only the other five known HOPS complex subunits co-purified at high levels with SBP-tagged Vps33 (Figure 1). Ypt7p interacts with the HOPS complex in the presence of GTP γ S (Seals *et al*, 2000), but no Ypt7p was detected in our HOPS preparations, even when GTP γ S was added during Vps33p-SBP purification (not shown). Membrane association of Ypt7p may be required for HOPS–Ypt7p interactions at native Ypt7p levels.

In addition to the other HOPS complex subunits, two proteins co-purify at low levels with Vps33-SBP in detergent solution (Figure 1A). Mass spectrometry analysis shows that the band below Vps33-SBP contains two peptides from Pho8p, the vacuolar alkaline phosphatase, and one peptide from the heat-shock protein Ssb1/2p. Because of the apparent molecular mass, we believe that this band is largely Pho8p. However, our final HOPS complex preparation is eluted in buffer lacking detergent and containing BSA, and contains neither the integral-membrane Pho8p protein (Supplementary Figure S1A) nor Pho8p activity (Supplementary Figure S1B). The faint band just above Vps33-SBP contains 56 peptides from three HOPS subunits and one peptide from the ER-luminal chaperone Kar2p, and thus likely represents a breakdown product of HOPS complex subunits.

To test whether pure HOPS complex was functional, we used an *in vitro* assay of homotypic vacuole fusion. This assay (Haas, 1995) uses vacuoles from two yeast strains. One strain has normal vacuolar proteases but is deleted in *PHO8*. The other is deleted in *PEP4*, which encodes vacuolar proteinase A, and thus contains inactive pro-Pho8p. *In vitro* fusion of these vacuoles allows proteolytic activation of pro-Pho8p. We introduced a temperature-sensitive *VPS11* allele, *vps11-1^{ts}* (Peterson and Emr, 2001), into these strains. Fusion

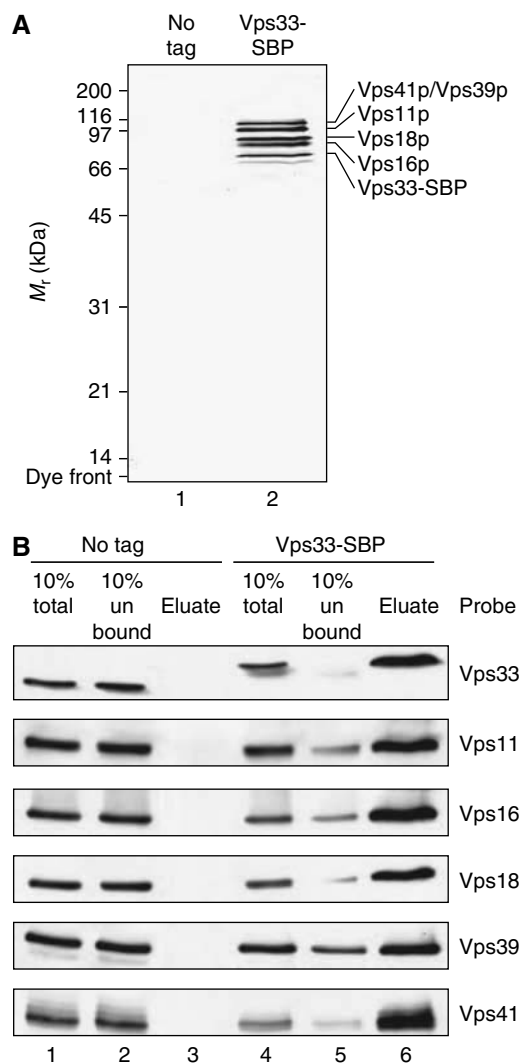


Figure 1 HOPS complex purification. (A) Lysates from BJ2168 (no tag) and CSY12 (Vps33-SBP) vacuoles were incubated with streptavidin Sepharose. Bound material was eluted in buffer containing Triton X-100 as described in Materials and methods and subjected to SDS-PAGE and silver staining. Lane 1, material purified from BJ2168 vacuoles; lane 2, material purified from CSY12 vacuoles. (B) Lysates from BJ3505 (lanes 1–3) and CSY12 (lanes 4–6) vacuoles were incubated with streptavidin Sepharose. Bound material was eluted in the presence of BSA and subjected to SDS-PAGE and immunoblotting. Lanes 1 and 4, vacuole lysate (representative of 10% of sample); lanes 2 and 5, material not bound to streptavidin Sepharose (representative of 10% of sample); lanes 3 and 6, material eluted from streptavidin Sepharose (representative of 100% of sample).

of vacuoles from *vps11-1^{ts}* strains was stimulated by pure HOPS (Figure 2A, squares). Fusion was further stimulated by a low concentration (3.7 nM) of recombinant Vam7p (rVam7p; Figure 2A, triangles). This low level of rVam7p stimulated fusion of *vps11-1^{ts}* vacuoles only slightly in the absence of pure HOPS complex (Figure 2A and C). High levels (2.7 μ M) of rVam7p only partially restored fusion without pure HOPS complex (Figure 2A, open arrow; Figure 2C, squares). Pure HOPS complex did not stimulate fusion of vacuoles from strains bearing the wild-type *VPS11* allele (Figure 2B and D). The modest (but statistically significant) inhibition of the fusion of *VPS11* vacuoles by

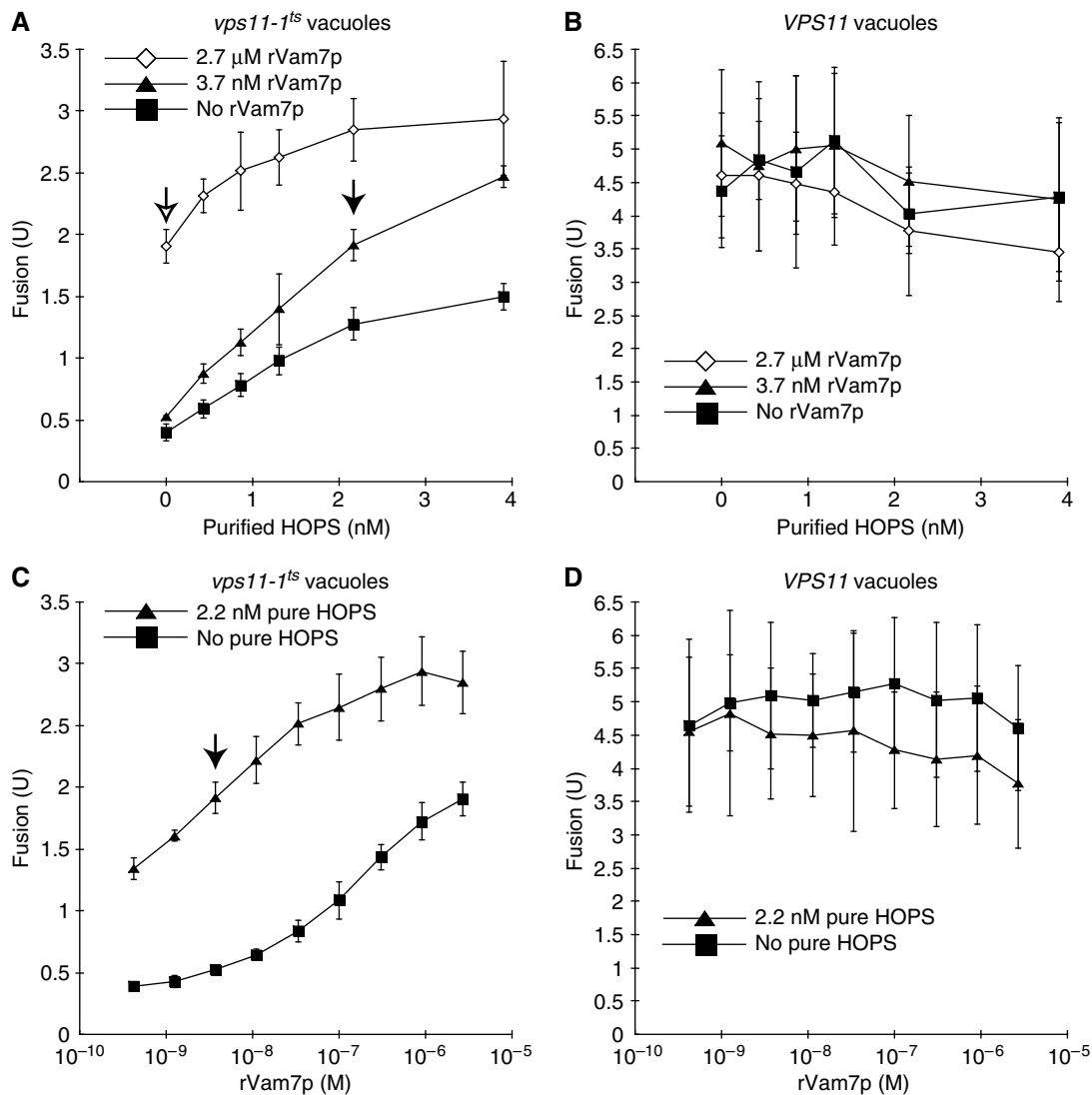


Figure 2 HOPS complex activity assay. *Vps11-1^{ts}* (CSY9 and CSY10) and *VPS11* (BY4742 *pep4Δ::kanMX6* and BY4742 *pho8Δ::kanMX6*) vacuoles were assayed for fusion in the presence of pure HOPS complex and rVam7p. HOPS buffer was added, so the total volume of pure HOPS and HOPS buffer was 9 μ l. Filled arrows indicate the fusion signal observed with the concentrations of pure HOPS complex (2.2 nM) and rVam7p was (3.7 nM) used in the fusion assays in the remainder of this paper. Means and standard deviations for fusion signals observed in three independent experiments are shown. (A) Pure HOPS added to *vps11-1^{ts}* vacuoles, with the indicated concentrations of rVam7p. (B) Pure HOPS added to *VPS11* vacuoles, with the indicated concentrations of rVam7p. (C) rVam7p added to *vps11-1^{ts}* vacuoles, with or without pure HOPS. (D) rVam7p added to *VPS11* vacuoles, with or without pure HOPS.

\sim 4 nM HOPS complex in the presence of 2.7 μ M rVam7p (Figure 2B, diamonds) may be due to their binding and sequestering other fusion factors. Thus, our pure HOPS complex is functionally active.

To determine whether the HOPS complex-dependent fusion of *vps11-1^{ts}* vacuoles uses the normal fusion pathway, we tested the effects of well-characterized inhibitors of vacuole fusion (Figure 3). Antibodies to Sec17p and Sec18p blocked fusion in the absence of rVam7p and partially inhibited fusion in the presence of rVam7p, consistent with the finding that rVam7p can bypass the priming stage of vacuole fusion (Thorngren *et al*, 2004). Fusion was inhibited by excess recombinant Sec17p (rSec17p). Antibodies directed against Ypt7p, Vps33p, or the SNARE Vam3p also inhibited fusion. Thus, HOPS complex-dependent fusion of *vps11-1^{ts}* vacuoles uses the same catalysts as fusion of *VPS11* vacuoles.

Vacuole tethering and docking require active HOPS complex

Which fusion catalysts can fulfil their function before HOPS complex action? To address this question, we incubated *vps11-1^{ts}* vacuoles with rVam7p but without pure HOPS complex for 30 min, and then added inhibitors followed by pure HOPS complex and continued the incubations for 55 min. Reactions achieved resistance to anti-Sec17p antibodies prior to HOPS addition, and thus had completed their requirement for priming (Figure 4). Without pure HOPS complex, however, reactions did not become resistant to excess rSec17p, which inhibits vacuole fusion during docking and reduces HOPS association with SNARE complexes (Wang *et al*, 2000; Collins *et al*, 2005), or to anti-Ypt7p antibodies, anti-Vps33p antibodies, Rho GDP dissociation inhibitor (RDI), or anti-Vam3p antibodies (Figure 4). The reduced fusion signal after incubation without pure HOPS complex

may be due to proteolytic sensitivity of *vps11-1^{ts}* vacuoles (Supplementary Figure S1B, sets 14 and 16); also, reactions in the third set contained HOPS for only 55 min, whereas reactions in the first two sets contained HOPS for 90 min. Reactions with pure HOPS complex added at the start of the incubation became resistant to each of these inhibitors within

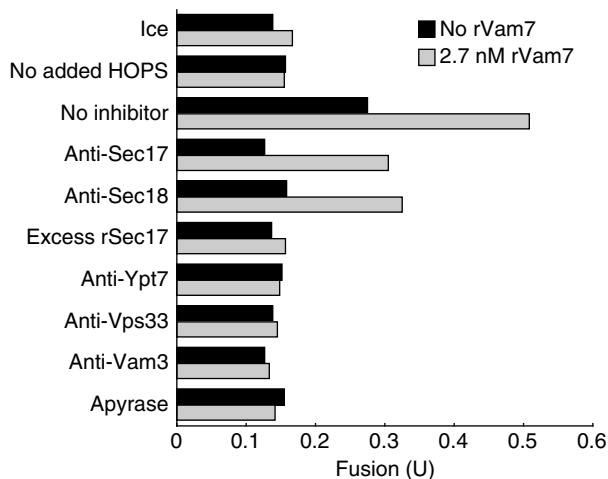


Figure 3 Authentic fusion of *vps11-1^{ts}* vacuoles. *Vps11-1^{ts}* vacuoles supplemented with pure HOPS complex were assayed for fusion in the absence (black bars) or presence (gray bars) of rVam7p and with the indicated inhibitors. All reactions received HOPS except for the reactions labeled 'no added HOPS', which received an equal volume of HOPS buffer.

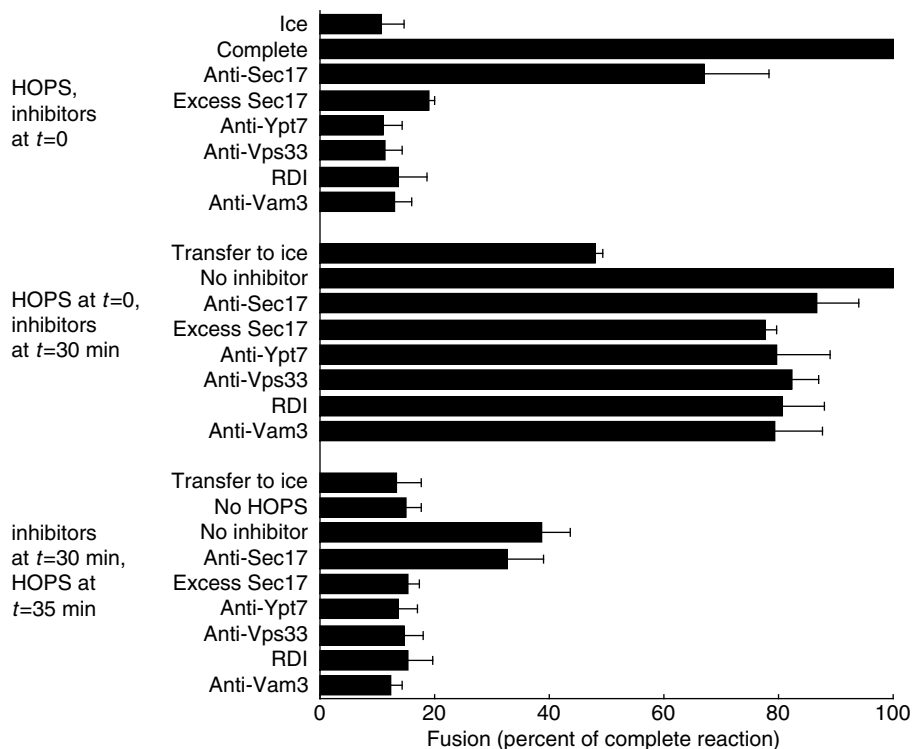


Figure 4 Staging of HOPS complex function. Reactions with *vps11-1^{ts}* vacuoles and 3.7 nM rVam7p were divided into three sets. One set received inhibitors and pure HOPS at the beginning of the assay and HOPS buffer at 35 min. A second set received pure HOPS at the beginning of the assay, inhibitors at 30 min, and HOPS buffer at 35 min. The third set received HOPS buffer at the beginning of the assay, inhibitors at 30 min, and pure HOPS at 35 min. All reactions were assayed for fusion at 90 min. Means and standard deviations of the fusion in three independent experiments, expressed as a percentage of the fusion signal of each 'complete' reaction (no inhibitor) before averaging, are shown.

30 min (Figure 4). Thus, Ypt7p, Vps33p (which is present on *vps11-1^{ts}* vacuoles; data not shown), Vam3p, and Rho GTPases cannot fulfil their function in the absence of HOPS complex activity. All of these fusion catalysts are needed for vacuole docking (Mayer and Wickner, 1997; Nichols *et al*, 1997; Seals *et al*, 2000; Eitzen *et al*, 2001). The HOPS complex is therefore needed for catalysts of docking, but not of priming, to complete their functions.

It has been proposed that HOPS may support membrane tethering (Price *et al*, 2000a; Srivastava *et al*, 2000; Richardson *et al*, 2004; LaGrassa and Ungermann, 2005), but the lack of pure, active HOPS complex has precluded a direct test of this model. We therefore incubated *vps11-1^{ts}* vacuoles, in the presence or absence of pure HOPS complex, under conditions that permit assay of membrane tethering (Mayer and Wickner, 1997; Wang *et al*, 2002). Pure HOPS complex increased the median number of vacuoles in each cluster. This is shown in representative fields (Figure 5A), in a cumulative distribution plot of all scored fields (Figure 5B), and in determination of median cluster size (Figure 5C). To test whether this increase required a Rab GTPase, we also incubated tethering reactions with Rab GDP GTPase, we also incubated tethering reactions with Rab GDP dissociation inhibitor (GDI) and Gyp1-46, a catalytic fragment of a Rab GTPase activating protein (Garrett *et al*, 1994; Will *et al*, 2001). Gyp1-46/GDI treatment abolished the increase in median cluster size due to pure HOPS complex. Tethering did not occur in the absence of pure HOPS complex, as the median cluster size without added HOPS was not significantly decreased by Gyp1-46/GDI (Figure 5C). Thus, the HOPS complex is required for Rab GTPase-dependent vacuole tethering.

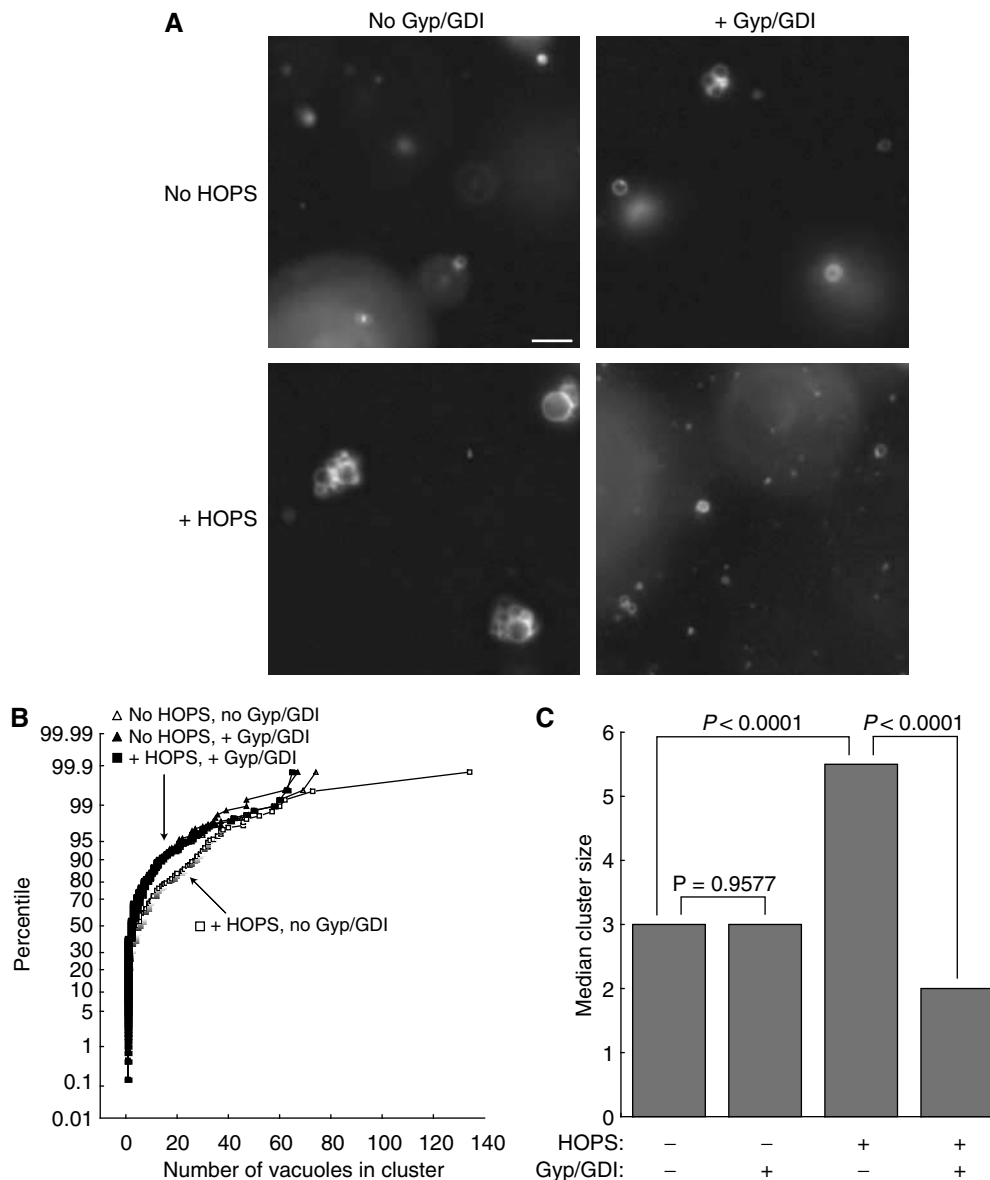


Figure 5 The HOPS complex is required for vacuole tethering. *Vps11-1^{ts}* vacuoles (CSY10) were incubated in the presence or absence of pure HOPS and Gyp1-46 and GDI where indicated; no rVam7p was added. Samples were randomized and mounted on slides for fluorescence microscopy. Random fields were imaged. For each sample, the number of vacuoles in ~100 vacuole clusters was counted. Data from three independent experiments were pooled for analysis. Bar, 5 μ m. (A) Representative images of vacuoles from each sample in one experiment. The enlarged vacuoles in samples with HOPS but without Gyp1-46/GDI reflect fusion, and cause an underestimation of the number of vacuoles that had initially tethered in these clusters. (B) Cumulative distribution plot of the pooled data. (C) Median cluster sizes for the four treatments.

The HOPS complex binds phosphoinositides

Phosphoinositides are required for enrichment of the HOPS complex at the vertex ring during docking (Fratti *et al*, 2004). To determine whether a direct interaction between the HOPS complex and phosphoinositides might contribute to this localization, we performed protein–lipid overlay assays using pure HOPS. Pure HOPS complex was incubated with nitrocellulose strips bearing adsorbed phospholipid spots. Bound HOPS complex was detected using antibodies to each HOPS subunit (Figure 6A). Each HOPS complex subunit associated with the same phosphoinositides; since pure HOPS complex, not individual subunits, was used in these assays, this suggests that the intact complex interacts with phosphoinositides. The HOPS complex interacted most strongly with singly phosphorylated phosphoinositides. The

HOPS complex did not associate strongly with phosphatidylinositol (PI) or other acidic phospholipids such as phosphatidylserine (PS) or phosphatidic acid (PA) (Figure 6A). Removal of trace amounts of Vam7p, a HOPS-binding protein (see below) that interacts with PI-3-phosphate (PI(3)P; Cheever *et al*, 2001), did not reduce the amount of HOPS complex bound to PI(3)P (Supplementary Figure S3).

To determine whether the HOPS complex can interact with phosphoinositides in a phospholipid bilayer, we performed binding assays using liposomes that contained each phosphoinositide, as well as liposomes bearing PS, PA, or unphosphorylated PI. Liposomes were mixed with pure HOPS complex and floated in a sucrose gradient, then harvested; bound HOPS was quantified by immunoblotting. HOPS complex bound to liposomes containing PI-4-phosphate (PI(4)P),

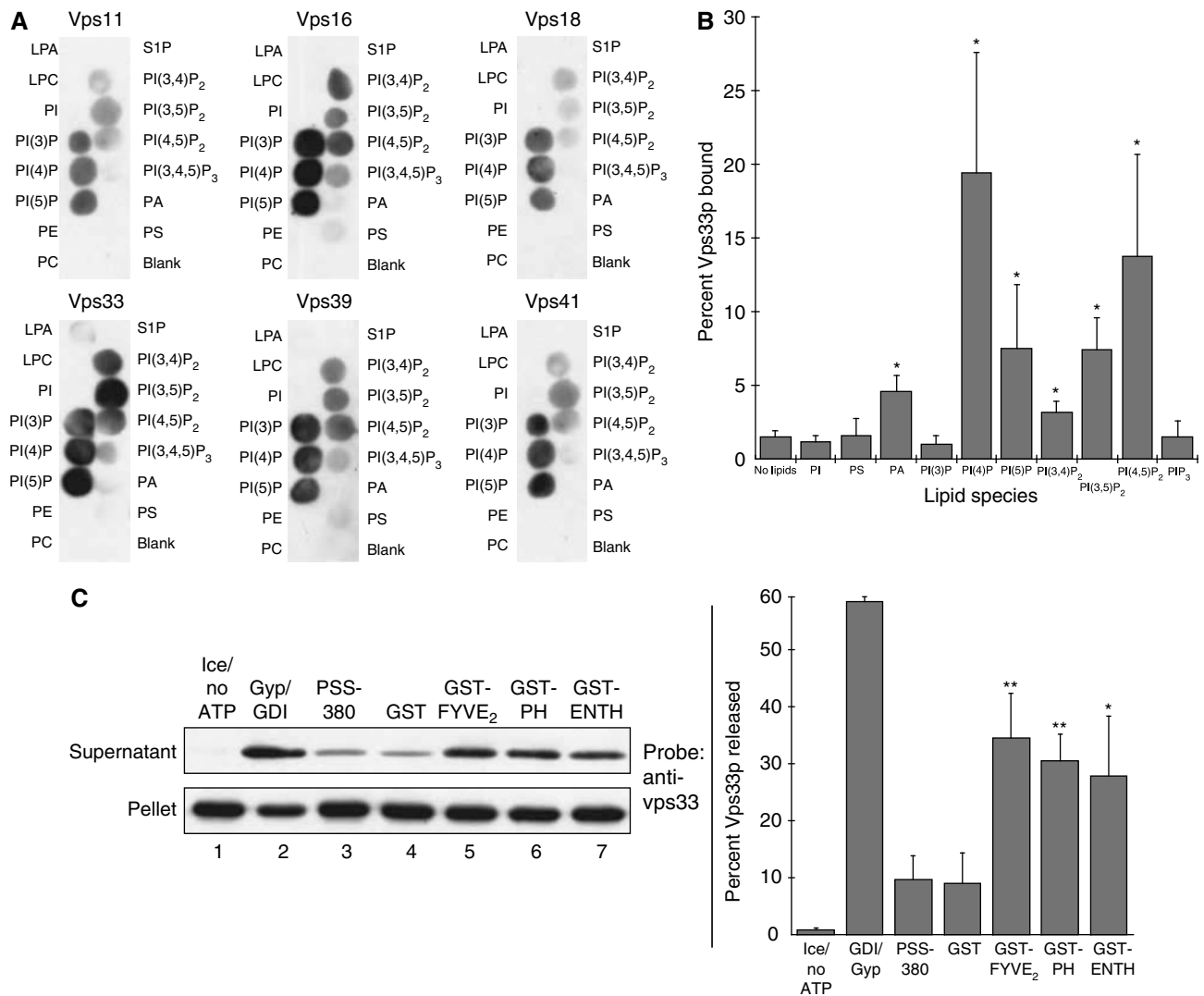


Figure 6 The HOPS complex binds phosphoinositides. **(A)** PIP MicroStrips (Echelon) were blocked with HBSTG (20 mM NaHEPES, pH 7.8, 3 mg/ml defatted BSA, 200 mM NaCl, 0.1% Tween-20, 5% glycerol) and incubated with pure HOPS (0.5 ml of 0.33 nM HOPS in HBSTG) at 4°C. Strips were washed four times with HBSTG and probed with antibodies in HBSTG, then washed four times with HBSTG; secondary detection was carried out with HRP-conjugated donkey anti-rabbit antibodies (GE). Strips were washed four times with HBSTG and bound antibody was detected by enhanced chemiluminescence (GE). **(B)** HOPS binding to liposomes was assayed by a modification of a described method (Matsuoka *et al*, 1998). Liposomes with PI were made by mixing CHCl₃ solutions of soy PC, soy PE, soy PI (Avanti), and rhodamine-DHPE (Invitrogen) at molar ratios of 50:25:25:0.025 and drying with N₂ gas and vacuum. Lipids were resuspended in 250 μl RB (20 mM NaHEPES, pH 7.8, 100 mM NaCl, 5% glycerol, 250 mM sorbitol; 8 mM total lipids), and subjected to freeze-thaw cycles (liquid N₂/bath sonication) until the suspension was clear. Liposomes with DOPA, brain PS (Avanti), or phosphoinositides (Echelon; in 1:2:0.8 CHCl₃:MeOH:H₂O) were prepared in the same way, with 20–25% of the PI replaced by the appropriate phospholipid. Liposomes (20 μl) or RB were mixed in 7 × 20 mm tubes (Beckman) with 10 μl pure HOPS (2.6 nM final) and 10 μl of HBSG (20 mM NaHEPES, pH 7.8, 200 mM NaCl, 5% glycerol, 250 mM sorbitol) with 1.8 M sucrose and 2 mg/ml defatted BSA, then incubated on ice for 30 min. This mixture was overlaid with 75 μl of HBSG with 0.5 M sucrose, 75 μl of HBSG with 0.3 M sucrose, and 50 μl of HBSG, then centrifuged (50 000 r.p.m., 1 h, 4°C, TLS-55 rotor). Liposomes (40 μl) were taken from the border between the top layers, and bound HOPS was quantified with a ChemiDoc system (UVP) after SDS-PAGE and immunoblotting for Vps33p, with a standard curve of pure HOPS. Liposome recovery was estimated using rhodamine-DHPE fluorescence (λ_{ex}/λ_{em} 510/580 nm); reported bound HOPS was corrected for liposome recovery. Mean percentages and standard deviations for at least three independent experiments are shown. **P* < 0.05 for differences relative to binding to PI liposomes, by one-way ANOVA. **(C)** HOPS release assay: fusion assays were supplemented as indicated and incubated on ice or at 27°C for 90 min, then sedimented (16 000 g, 15 min, 4°C). Supernatants and pellets were subjected to SDS-PAGE and immunoblotting for Vps33p. Blots were quantified by densitometry. Left, representative immunoblot. Right, mean percentages and standard deviations of Vps33p released in three independent experiments. ***P* < 0.01, **P* < 0.05, for differences relative to release in the presence of GST, by one-way ANOVA.

PI-3,5-bisphosphate (PI(3,5)P₂), or PI-4,5-bisphosphate (PI(4,5)P₂), but interacted only weakly with liposomes bearing PS, PA, or PI (Figure 6B). (*P* < 0.05 by one-way ANOVA for PI(4)P, PI(3,5)P₂, or PI(4,5)P₂-containing liposomes relative to PI or PA liposomes.) One difference between the results of the liposome assay and the overlay assay is that HOPS did not

bind liposomes bearing PI(3)P. A PI(3)P-binding FYVE domain construct (Gillooly *et al*, 2000), however, did bind these liposomes (Supplementary Figure S4). This difference may be due to an interaction between the HOPS complex and the hydrophobic portion of the bilayer. This phenomenon, seen in the FYVE domain of Early Endosomal Antigen 1

(EEA1; Kutateladze and Overduin, 2001), may force HOPS into an orientation that prevents specific interactions with PI(3)P.

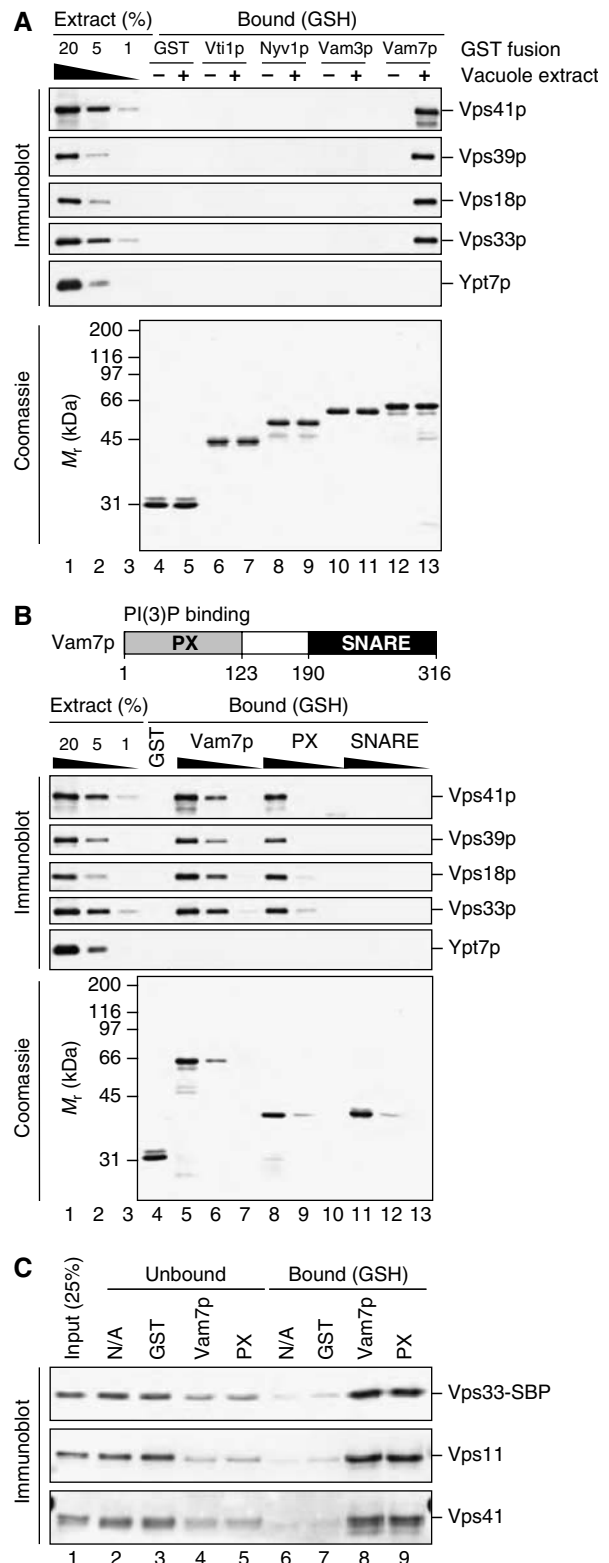
To determine whether the affinity of the HOPS complex for phosphoinositides contributes to its association with vacuoles, we incubated vacuole fusion reactions with ligands to specific phospholipids, then sedimented the vacuoles and assayed for HOPS complex release by SDS-PAGE and immunoblotting. Little HOPS complex was released when fusion reactions were incubated on ice, with PSS-380 (a PS ligand) or with GST (Figure 6B). However, HOPS complex was released when fusion reactions were incubated with ligands specific for phosphoinositides. GST fusions to a tandem FYVE domain specific for PI(3)P (Gillooly *et al*, 2000), a pleckstrin homology (PH) domain specific for PI(4)P (Weixel *et al*, 2005), or an ENTH domain specific for PI(4,5)P₂ (Rosenthal *et al*, 1999) caused HOPS complex release from vacuoles (Figure 6C). HOPS complex release may be caused by competition of these lipid ligands with HOPS for phosphoinositide binding. However, HOPS complex release may be an indirect effect due to release of HOPS-binding proteins, such as Vam7p (see below), which also bind phosphoinositides (Cheever *et al*, 2001). Release of Vam7p correlated almost perfectly with release of HOPS complex in these experiments (not shown), even when a PI(4)P or PI(4,5)P₂ ligand was present. Release of HOPS by these ligands may cause Vam7p release, as the Vam7p PX domain has no affinity for PI(4)P or PI(4,5)P₂ (Cheever *et al*, 2001).

The HOPS complex binds the PX domain of the soluble SNARE Vam7p

The HOPS complex associates with SNARE complexes (Collins *et al*, 2005), and GST fusions to Vam3p and its

SNARE domain interact with HOPS (Sato *et al*, 2000; Dulubova *et al*, 2001). However, it is not clear whether other individual SNAREs can also associate with the HOPS complex. We therefore incubated vacuole lysates with purified GST-tagged vacuolar SNAREs and examined bound proteins by immunoblotting. HOPS complex from vacuole lysates showed the strongest interaction with GST-Vam7p

Figure 7 The HOPS complex binds Vam7p and the Vam7p PX domain. (A) HOPS binds recombinant Vam7p. Vacuoles (1.2 mg) from BJ3505 yeast were sedimented (16 000 g, 10 min, 4°C), resuspended in 2 ml SB (20 mM HEPES-KOH, pH 7.4, 100 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 20% glycerol, 0.46 µg/ml leupeptin, 3.5 µg/ml pepstatin, 2.4 µg/ml pefabloc-SC, 1 mM DTT) and incubated on ice for 20 min. Insoluble material was removed by centrifugation (TLA 120.2, 52 000 r.p.m., 11 min, 4°C). A sample of lysate was removed, and 200 µl of SB (lanes 4, 6, 8, 10, and 12) or vacuole lysate (lanes 5, 7, 9, 11, and 13) was incubated with 5 µg each of pure GST (lanes 4 and 5) or GST fused to the cytoplasmic residues of indicated SNAREs (lanes 6 and 7: Vti1p; lanes 8 and 9: Nyv1p; lanes 10 and 11: Vam3p; lanes 12 and 13: Vam7p). Lanes 1–3 represent 20, 5, and 1% of each lysate added. Fusion proteins were retrieved with glutathione Sepharose and bound material was analyzed by SDS-PAGE and immunoblotting (top) or staining with Coomassie blue (40% of eluted material, bottom). (B) The HOPS complex interacts with the Vam7p PX domain. GST (lane 4) or GST fusions to full-length Vam7p (lanes 5–7), the PX domain (lanes 8–10), or the SNARE domain (lanes 11–13) were added (5 µg for GST alone, and 5, 1, and 0.2 µg for Vam7p, PX, and SNARE domains) to vacuole lysates prepared in parallel to those in (A). Lanes 1–3 represent 20, 5, and 1% of each lysate added. Fusion proteins were retrieved with glutathione Sepharose (GE) and bound material was analyzed by SDS-PAGE. (C) The HOPS complex binds directly to the Vam7p PX domain. Pure HOPS (0.65 nM; lane 1) was incubated with glutathione Sepharose and buffer (lanes 2 and 6) or 5 µg GST (lanes 3 and 7), GST-Vam7 (lanes 4 and 8), or GST-Vam7 PX domain (lanes 5 and 9), in a total volume of 200 µl of SB; bound material was eluted by heating with SDS. Unbound (lanes 2–5) and bound (lanes 6–9) material was analyzed by SDS-PAGE and immunoblotting.



(Figure 7A, lane 13). Only a weak association was seen between the HOPS complex and GST-Vam3p or GST-Nyv1p, and no association between HOPS and GST-Vti1p was seen (Figure 7A and longer exposures of the same immunoblots, not shown).

To determine which domain of Vam7p mediates the interaction between GST-Vam7p and the HOPS complex, we incubated vacuole lysates with GST fusions to full-length Vam7p, the amino-terminal PX domain of Vam7p, or the carboxy-terminal SNARE domain of Vam7p. HOPS complex from the lysates bound fusions of GST to full-length Vam7p or the PX domain of Vam7p but not a GST fusion to the Vam7p SNARE domain (Figure 7B).

Does the HOPS complex interact directly with the PX domain of Vam7p, or does this interaction require other proteins? To address this question, we incubated pure HOPS complex with purified fusions of GST to Vam7p or to the PX domain from Vam7p. HOPS complex, containing only the six known subunits (Figure 1A), bound efficiently to GST fusions to either full-length Vam7p or the Vam7p PX domain on glutathione beads (Figure 7C, lanes 8 and 9).

Discussion

The HOPS complex is at the center of vacuole docking mechanisms. It is an exchange factor and an effector for Ypt7p (Seals *et al*, 2000; Wurmser *et al*, 2000). It is required for SNARE complex assembly and remains bound to SNARE complexes after vacuole fusion (Price *et al*, 2000b; Sato *et al*, 2000; Collins *et al*, 2005). Purification of functional HOPS complex (Figures 1–3) has allowed us to show that HOPS is required for vacuole tethering (Figure 5). We have also shown that the HOPS complex directly binds phosphoinositides (Figure 6) and the PX domain of Vam7p (Figure 7), crucial components of the vertex docking ring that are required for vertex ring assembly (Wang *et al*, 2003; Fratti *et al*, 2004). The HOPS complex is required for other catalysts of docking, including SNAREs, Ypt7p, and Rho GTPases, to complete their functions (Figure 4).

The physical and functional interactions of the HOPS complex suggest a model of how it links Ypt7p activation to SNARE complex assembly. After Vps39p activates Ypt7p by catalyzing the exchange of bound GDP for GTP, HOPS may remain associated with GTP-bound Ypt7p. The HOPS complex and Ypt7p then act together to promote tethering. The isoprenyl anchors of Ypt7p and the affinity of the HOPS complex for phosphoinositides and the PX domain of Vam7p may promote enrichment of HOPS, Ypt7p, and Vam7p at the vertex ring. Both the HOPS complex and PI(3)P bind Vam7p through its PX domain, which may initiate the assembly of a SNARE complex associated with HOPS. HOPS remains associated with newly assembled SNARE complexes, where it may regulate or contribute to their roles in hemifusion and fusion. Finally, Sec17p displaces the HOPS complex from the SNARE complex, allowing HOPS to engage in another cycle of SNARE complex assembly and preparing the SNARE complex for disassembly by Sec17p and Sec18p.

Our finding that the HOPS complex binds phosphoinositides and the Vam7p PX domain suggests that HOPS is enriched at the vertex ring through direct interactions with Ypt7p, Vam7p, and PI(4,5)P₂. All of these HOPS-binding partners are enriched at the vertex ring, and inhibitors

directed at Ypt7p, Vam7p, or PI(4,5)P₂ block HOPS enrichment at the vertex ring (Wang *et al*, 2002, 2003; Fratti *et al*, 2004; data not shown). Some of the effect of phosphoinositide ligands on HOPS vertex enrichment may be indirect: both the PI(4,5)P₂-binding ENTH domain and the PX domain of Vam7p inhibit Vam3p vertex enrichment (Wang *et al*, 2003; Fratti *et al*, 2004), and anti-Vam3p F_{ab} fragments block enrichment of HOPS at the vertex ring (Wang *et al*, 2003).

The interaction between the HOPS complex and the Vam7p PX domain may initiate the assembly of SNARE complexes, possibly in combination with an interaction between HOPS and Vam3p. We have shown that the HOPS complex binds GST-tagged Vam7p preferentially over GST fusions to other SNAREs (Figure 7A). However, HOPS can also bind GST fusions to both full-length Vam3p and its SNARE domain (Sato *et al*, 2000; Dulubova *et al*, 2001). These results are not mutually exclusive: monomeric Vam7p and Vam3p may bind simultaneously or sequentially to HOPS before their assembly into a SNARE complex (Ungermann *et al*, 1999; Sato *et al*, 2000; Collins *et al*, 2005). This hypothesis is supported by the finding that a SNARE complex can assemble on a Sed5p–Sly1p complex (Peng and Gallwitz, 2002). Sed5p is a Vam3p homolog, and Sly1p is a Vps33p homolog, suggesting that Vps33p may be involved directly in SNARE complex assembly.

The multiple interactions of the HOPS complex are reminiscent of tethering factors that interact with proteins and lipids that can reside on different membranes. EEA1 tethers endosomal membranes by binding both Rab5 and PI(3)P (Lawe *et al*, 2002). p115 binds GM130, a Golgi matrix protein, and giantin, a protein from COPI vesicles (Nakamura *et al*, 1997; Sonnichsen *et al*, 1998). The exocyst complex binds the vesicular Rab Sec4p and the plasma membrane GTPase Rho1p (Boyd *et al*, 2004). This common feature of the HOPS complex and tethering factors, the ability to engage in multiple interactions, suggests that HOPS tethers membranes directly by binding to proteins and lipids in the apposed lipid bilayers.

The interaction between the HOPS complex and Pho8p is consistent with the requirement for Vps41p for formation of AP-3-dependent vesicles, which transport Pho8p from the Golgi apparatus to the vacuole (Rehling *et al*, 1999). Vps41p has been proposed to homo-oligomerize and act as a coat, perhaps stimulating vesicle budding (Darsow *et al*, 2001). If the HOPS complex is part of a coat for AP-3-dependent vesicles, then a HOPS–Pho8p interaction may contribute to sorting of Pho8p into these vesicles. Such an activity would be analogous to the COPII-dependent packaging of cargo into ER-derived vesicles, which requires interaction between the coat and the cargo protein Bet1p (Miller *et al*, 2003).

How does the HOPS complex bind phosphoinositides? The six HOPS complex subunits do not have any known lipid-binding motifs, although Vps11p and Vps18p have ‘really interesting new gene’ (RING) motifs (Schultz *et al*, 1998). RING domains are involved in protein–protein interactions by ubiquitin-protein ligases (Joazeiro and Weissman, 2000). However, RING domains resemble plant homeodomain domains (Aravind *et al*, 2003), one of which binds PI-5-phosphate (Gozani *et al*, 2003). It is also possible that the phosphoinositide-binding site in the HOPS complex contains residues from more than one subunit; it has been shown that a lipid-binding PH domain is formed by partial PH domains from PLC- γ 1 and TRPC3 (van Rossum *et al*, 2005).

It is likewise not known how the HOPS complex binds the Vam7p PX domain. The RING domain of Vps11p or Vps18p may mediate this interaction by interacting with the proline-rich loop between the $\alpha 1$ and $\alpha 2$ helices of the Vam7p PX domain (Cheever *et al*, 2001). The RING domain of the c-Cbl ubiquitin-protein ligase recognizes two proline-rich loops in the UbcH7 ubiquitin-conjugating enzyme (Zheng *et al*, 2000). However, the $\alpha 1$ - $\alpha 2$ loop of the Vam7p PX domain may be involved in phosphoinositide binding (Cheever *et al*, 2001). Also, Vps18p and Vps41p contain clathrin heavy chain repeats (Schultz *et al*, 1998), which mediate clathrin self-association (Ybe *et al*, 1999). These motifs may be involved in HOPS binding to Vam7p. Another possible Vam7p ligand is a WD40 repeat near the Vps41 amino terminus (Schultz *et al*, 1998). This motif is required for Vps41p self-association and binding of Vps41p to Apl5p (Darsow *et al*, 2001), and may also interact with Vam7p.

The availability of pure, active HOPS complex will be essential for uncovering the mechanisms by which HOPS carries out its membrane tethering activity and couples Ypt7p activation to SNARE complex assembly. New *in vitro* assays for intermediate steps of membrane fusion that require pure HOPS may reveal currently unknown activities for this complex. Pure, active HOPS complex will be invaluable for reconstitution of the docking reactions in yeast vacuole fusion.

Materials and methods

Reagents

Anti-Sec18p (Haas and Wickner, 1996), anti-Sec17p (Haas and Wickner, 1996), anti-Ypt7p (Eitzen *et al*, 2001), anti-Vps33p (Seals *et al*, 2000), anti-Vam3p (Nichols *et al*, 1997), GST-RDI (Eitzen *et al*, 2001), Gyp1-46 (Will *et al*, 2001), GDI (Haas *et al*, 1995), PSS-380 (Fratti *et al*, 2004), GST-ENTH (Rosenthal *et al*, 1999), GST-FYVE₂ (Gillooly *et al*, 2000), GST-PH (Weixel *et al*, 2005), GST-Vam7p and rVam7p (Merz and Wickner, 2004), GST-PX (Merz and Wickner, 2004), I₂^B (Slusarewicz *et al*, 1997), his₆-Sec17p (Haas and Wickner, 1996), and his₆-Sec18p (Thorngren *et al*, 2004) were described previously. Apyrase was from Sigma-Aldrich. A plasmid encoding a GST fusion to the Vam7p SNARE domain (residues 190–316) was the generous gift of Dr Alexey Merz.

Vacuole fusion assay

Vacuoles were purified and assayed for fusion as described (Haas, 1995). Vacuoles lacking Pep4p or Pho8p (3 μ g each) were in a volume of 30 μ l of 20 mM PIPES-KOH, pH 6.8, 200 mM sorbitol, 125 mM KCl, 5 mM MgCl₂, 15 μ M coenzyme A, 1 μ g/ml his₆-Sec18p, 7 μ g/ml I₂^B, 3.3 ng/ml leupeptin, 3.3 ng/ml 1,10 phenanthroline, 16.7 ng/ml pepstatin A, 0.8 ng/ml pefabloc-SC, 1 mg/ml creatine kinase, 1 mM MgATP, and 40 mM creatine phosphate. Reactions were incubated on ice or at 27°C for 90 min and Pho8p activity was measured (Haas, 1995). Fusion inhibitors were at the following concentrations: anti-Sec18p, 39 μ g/ml; anti-Sec17p, 113 μ g/ml; excess rSec17p, 4 μ g/ml; anti-Ypt7p, 100 μ g/ml; anti-Vps33p, 28 μ g/ml; anti-Vam3p, 133 μ g/ml; GST-RDI, 833 μ g/ml; apyrase, 1 U per reaction; GDI, 500 μ g/ml; his₆-Gyp1-46, 467 μ g/ml.

HOPS complex purification

A plasmid based on pFA6-GFP(S65T)-TRP1 (Longtine *et al*, 1998), with the streptavidin-binding peptide coding sequence (Keefe *et al*, 2001) in place of the GFP(S65T) coding sequence, was the generous gift of Dr John Flanagan. The yeast strain CSY12 was prepared by transforming BJ2168 cells (Zubenko *et al*, 1980; Gietz and Schiestl, 1995) with a PCR product amplified from this plasmid using primers (Wang *et al*, 2002) for carboxy-terminal tagging of Vps33p.

For purification of HOPS complex, 12 mg CSY12 vacuoles (Seals *et al*, 2000) were thawed, diluted in 25 ml of 20 mM NaHEPES, pH 7.8, 200 mM sorbitol, and reisolated by centrifugation (13 000 r.p.m., JA-20 rotor, 20 min, 4°C). Vacuoles were resuspended to 0.6 mg/ml in lysis buffer (20 mM NaHEPES, pH 7.8, 400 mM

NaCl, 5% glycerol, 0.5% Triton X-100, 5 mM β -mercaptoethanol, 0.46 μ g/ml leupeptin, 3.5 μ g/ml pepstatin, 2.4 μ g/ml pefabloc-SC, 1 mM PMSF) and incubated on ice for 20 min. Lysate was clarified (50 000 r.p.m., Type 60 Ti rotor, 30 min, 4°C) and incubated with streptavidin Sepharose High Performance (2 ml; GE Healthcare) for 3 h at 4°C. The resin was drained and washed four times with 20 ml lysis buffer, four times with 20 ml BSA buffer (20 mM NaHEPES, pH 7.8, 400 mM NaCl, 5% glycerol, 5 mM β -mercaptoethanol, 2 mg/ml BSA), and eluted with 0.5 ml fractions of HOPS buffer (BSA buffer with 2 mg/ml biotin). Fractions were tested for HOPS complex activity (see below). Fractions with activity were pooled and frozen in 52 μ l aliquots in liquid N₂. HOPS was quantified as described in Supplementary Figure S2. For SDS-PAGE/silver stain analysis, bound proteins were eluted with 2 mg/ml biotin in lysis buffer after washing with lysis buffer.

HOPS complex activity assay

VPS11 was deleted from strains BY4742 *pep4 Δ ::kanMX6* and BY4742 *pho8 Δ ::kanMX6* (ATCC) by transformation with a PCR product amplified from pFA6-His3MX6 (Longtine *et al*, 1998) to make strains that were then transformed with pMP160, which bears the *vps11-1^{ts}* allele (Peterson and Emr, 2001), to make CSY9 and CSY10, respectively.

Vacuoles were purified from CSY9 and CSY10 cells as described (Haas, 1995). Cultures were grown overnight in CSM-ura at 30°C. Cells (CSY9, 7 ml at OD₆₀₀ = 1; CSY10, 5 ml at OD₆₀₀ = 1) from these cultures were used to start cultures in 2% YPD, grown for ~24 h at 25°C, and used for vacuole purification.

To assay pure HOPS, vacuole fusion reactions with *vps11-1^{ts}* vacuoles were performed with pure HOPS and/or HOPS buffer. Sorbitol was added at 0.2 M to aliquots of pure HOPS complex or HOPS buffer before being added to fusion reactions. The [KCl] in the reactions was adjusted, so the sum of [NaCl] (from the HOPS buffer) and [KCl] was 125 mM. MgGTP was added at 10 μ M; this stimulates fusion of *vps11-1^{ts}* vacuoles by 10–20%, but does not stimulate fusion of VPS11 vacuoles (not shown). Where indicated, rVam7p was added.

Vacuole tethering assay

Tethering reactions were as described (Mayer and Wickner, 1997), except that they contained CSY10 vacuoles, pure HOPS (2.2 nM) or HOPS buffer, and 33 mM KCl (to keep the sum of [KCl] and [NaCl] at 100 mM). Reactions contained 6 μ g CSY10 vacuoles, 20 mM PIPES-KOH, pH 6.8, 200 mM sorbitol, 33 mM KCl, 0.5 mM MgCl₂, 20 μ M coenzyme A, 1 μ g/ml his₆-Sec18p, 2.7 μ M FM4-64 (Invitrogen), 0.7 mg/ml creatine kinase, 0.3 mM MgATP, 6 mM creatine phosphate, and 5 μ l HOPS buffer. Reactions were randomized and incubated at 27°C for 30 min. Low melting point agarose in 20 mM PIPES-KOH, 200 mM sorbitol (50 μ l of 0.6% solution) at 42°C was added and reactions were vortexed (3 s at medium setting). Aliquots (15 μ l) were placed on glass slides, overlaid with coverslips and immersion oil (nd 1.516; Olympus), and imaged.

Imaging used an Olympus BX51 microscope with a 100 W mercury arc lamp, a Plan Apochromat $\times 60$ objective (1.4 NA; Olympus), and a Sencam QE CCD camera (Cooke). A U-25ND25 neutral density filter and a U-RSL6 UV/IR filter (Olympus) were used to reduce incident light. A TRITC/Dil filter set (Chroma Technologies) was used to collect FM4-64 images. Images were recorded using IPLab (Scanalytics) and vacuole clusters were counted manually using ImageJ (NIH). Wilcoxon-Mann-Whitney tests on data pooled from three independent experiments were carried out using KaleidaGraph (Synergy Software).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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