

# Molecular architecture of the multisubunit homotypic fusion and vacuole protein sorting (HOPS) tethering complex

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**Membrane fusion within the eukaryotic endomembrane system depends on the initial recognition of Rab GTPase on transport vesicles by multisubunit tethering complexes and subsequent coupling to SNARE-mediated fusion. The conserved vacuolar/lysosomal homotypic fusion and vacuole protein sorting (HOPS) tethering complex combines both activities. Here we present the overall structure of the fusion-active HOPS complex. Our data reveal a flexible  $\approx 30$ -nm elongated seahorse-like structure, which can adopt contracted and elongated shapes. Surprisingly, both ends of the HOPS complex contain a Rab-binding subunit: Vps41 and Vps39. The large head contains in addition to Vps41 the SNARE-interacting Vps33, whereas Vps39 is found in the bulky tip of its tail. Vps11 and Vps18 connect head and tail. Our data suggest that HOPS bridges Ypt7-positive membranes and chaperones SNAREs at fusion sites.**

Eukaryotic cells rely on an elaborate and dynamic vesicular transport system to direct proteins and lipids to their destinations in the cell. Membrane fusion within the endomembrane system of eukaryotic cells is a multistage reaction, which includes an initial recognition, termed tethering, followed by mixing of the membrane bilayers (1, 2).

Tethering in most cases is controlled by Rab GTPases, which exist in an inactive membrane-bound or soluble GDP form and an active membrane-bound GTP form. Once activated by their respective guanine nucleotide exchange factor, such as the well-characterized transport protein particle (TRAPP) complex that acts on Rab1, GTP-Rabs residing on vesicles or organelles can bind to tethering factors, including long coiled-coil proteins like the early endosome antigen (EEA1), which captures vesicles at a distance and promotes their subsequent fusion (1, 3, 4). Multisubunit tethering complexes (MTCs), which are found on all endomembranes, have additional functions such as SNARE binding (1, 2). Of all known complexes, only the Dsl1 complex at the endoplasmic reticulum (ER) does not depend on a Rab GTPase for its apparent activity but rather is anchored to the ER via SNAREs (5, 6). All other MTCs, such as the exocyst, Golgi-associated retrograde protein (GARP) complex, or COG, bind their respective Rab-GTP and require this interaction for function (1). Some MTCs, like COG, the exocyst, depend on SLY1-20 (Dsl) complex, and homotypic fusion and vacuole protein sorting (HOPS), also bind SNAREs (1, 2) and support their assembly during membrane fusion (7). Structural data on coiled-coil and Rab-independent tethers (1) and the Rab-binding domain of the exocyst subunit Sec15 exist (8). However, molecular insight into the overall structure of a Rab-binding MTC has been lacking to date.

Within the endolysosomal system, two similar heterohexameric MTCs, the endosomal class C core vacuole/endosome tethering (CORVET) complex and the vacuolar HOPS, have been identified (9–11). They share four of their six subunits, namely the class C subunits Vps11, Vps16, Vps18, and the Sec1/Munc18-like Vps33, which interacts with SNAREs (12). The endosomal CORVET contains in addition the two subunits Vps3 and Vps8, which both bind the Rab5 homolog Vps21 in its GTP form (11, 13, 14). HOPS

binds the Rab7 GTPase Ypt7 via its subunits Vps41 and Vps39 (15, 16) and promotes fusion of late endosomes, AP-3 vesicles, and autophagosomes with vacuoles, as well as homotypic fusion (2, 17). All HOPS and CORVET subunits—except Vps33—likely consist of an N-terminal  $\beta$ -propeller and a C-terminal  $\alpha$ -solenoid region (13, 16). They thus differ structurally from the CATCHR (complex associated with tethering containing helical rods) complexes such as Dsl1, COG, GARP, and exocyst (1). HOPS is also the only complex for which an in vitro fusion assay has been established (7, 18). Moreover, HOPS is limiting for Ypt7-dependent fusion of proteoliposomes carrying vacuolar SNAREs (19, 20). HOPS—and likely CORVET—are conserved across species (21–23). Thus, HOPS is a prime candidate to unravel the mechanism of Rab-mediated tethering and the promotion of SNARE-mediated fusion.

Here we analyzed the HOPS complex by electron microscopy (EM) combined with single-particle analysis and tomography. HOPS forms an elongated particle with two Rab-binding sites formed by Vps39 and Vps41 at opposite ends, connected by a flexible linker between the two terminal lobes. The SNARE-binding site is proximal to one Rab-binding site, suggesting that HOPS coordinates Rab-mediated tethering with SNARE-driven fusion.

## Results

**HOPS Is an Elongated and Flexible Complex.** HOPS activity in in vitro fusion assays has been extensively documented (7, 16, 18), although its precise function has remained unclear. We thus reasoned that a functional understanding of tethering requires the elucidation of its architecture. To select its structure, we analyzed the HOPS complex by single-particle EM of negatively stained particles (Fig. 1 *A* and *B*). Overproduced HOPS was purified via a tandem affinity purification (TAP) tag on Vps41 from yeast and further resolved on glycerol gradients (Fig. 1*C*). Using a modified in vitro vacuole fusion assay that depends on the addition of purified HOPS to obtain fusion (18), we confirmed robust fusion activity of the complex in fractions 9–11 of the gradient (Fig. 1*D*; details in *Experimental Procedures*). When the complex was analyzed by EM, it partially disintegrated (Fig. S1 *A* and *B*). To obtain a sufficient number of intact HOPS particles, we further stabilized HOPS by applying the GraFix method, in which the complexes are centrifuged over

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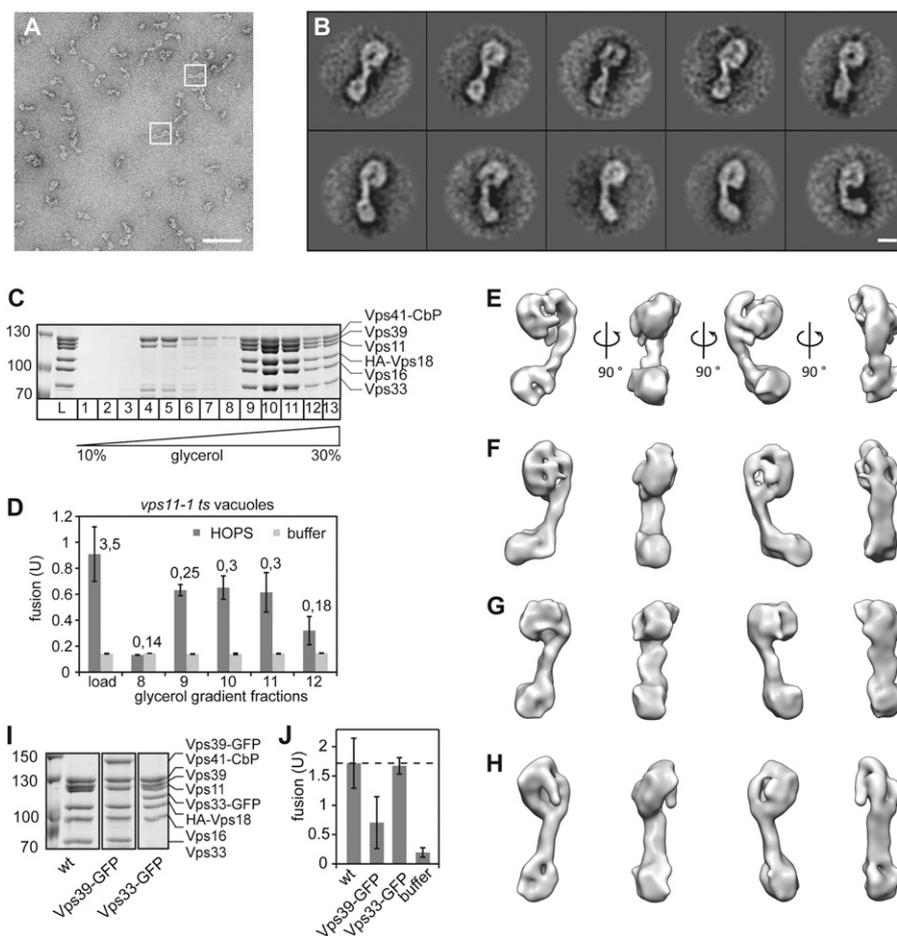
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**Fig. 1.** Isolation and structure of the HOPS complex. (A) Typical micrograph area of the negatively stained HOPS (Vps39-GFP) tethering complex. Representative particles are boxed in white. (Scale bar, 100 nm.) (B) Representative class averages, each containing between 12 and 27 particles. Fig. S2 shows the complete set of class averages. (Scale bar, 10 nm.) (C) Purification of HOPS via a glycerol gradient. Overproduced HOPS with TAP-tagged Vps41 (16) was purified from yeast via IgG Sepharose as described in *Experimental Procedures*, then separated on 10–30% glycerol gradients. (D) Fusion activity of HOPS. Indicated fractions of C were applied to reporter vacuoles from *vps11-1* strains. Six microliters of each fraction were analyzed per fusion reaction. Numbers indicate the protein concentration in the sample. (E–H) 3D reconstructions of the yeast HOPS complex using the random conical tilt approach and 3D multireference alignment. Four different conformations (E to H) were chosen. Shown are four different side views, after horizontal rotation of 90° around their longest axis. Table S1 shows the volumes of the structures. (Scale bar, 10 nm.) (I) Purification of wild-type and GFP-tagged HOPS complexes. Coomassie-stained SDS/PAGE gels of HOPS, HOPS (Vps33-GFP), and HOPS (Vps39-GFP) are shown. (J) Fusion activity. The different GFP variants were analyzed in fusion as in D. Fusion was compared to wild-type HOPS (wt) was set to 100%. SDs are given,  $n = 3$ .

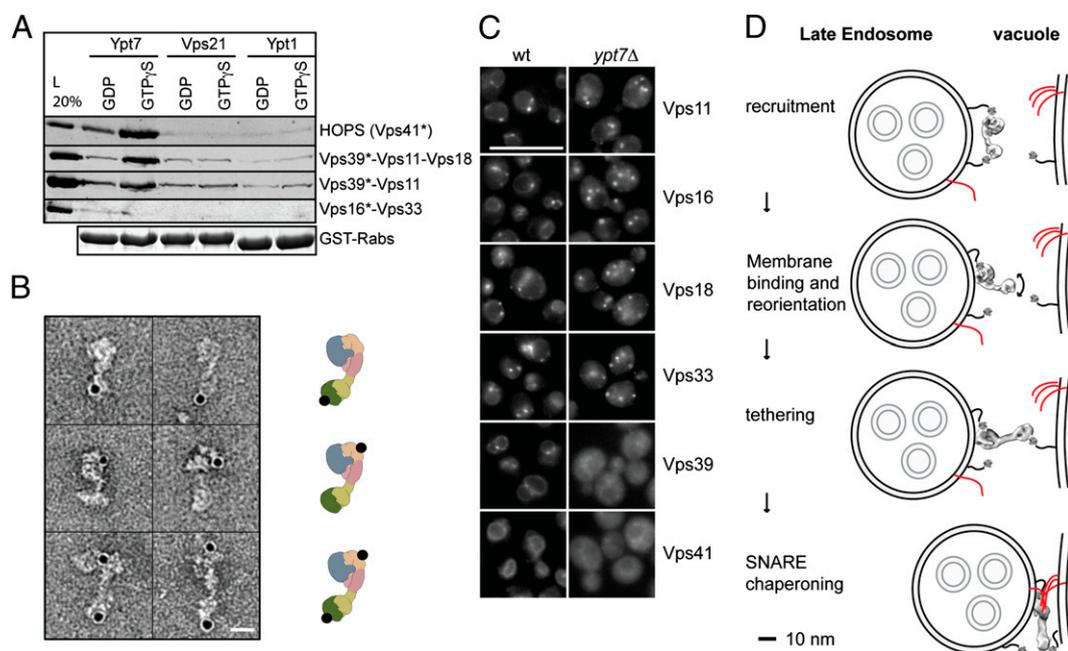
a glycerol gradient into an increasing concentration of glutaraldehyde (24). Importantly, mild intramolecular cross-linking occurs slowly while HOPS migrates into the gradient, and intermolecular cross-linking is prevented. As expected, HOPS stability was increased and its integrity conserved (Fig. S1 C and D). In comparison with the non-cross-linked HOPS, we did not notice any overall structural changes (Fig. S1 A–D). We therefore believe that the observed HOPS conformations (see below) reflect its overall flexibility and not cross-linking artifacts. In addition to that, we tested HOPS with GFP tags at different subunits (Fig. 1 A and B and Fig. S1 E and F) and found that HOPS with Vps39-GFP (Fig. 1 J) was more stable than wild-type HOPS (Fig. S1 C and D) and HOPS with Vps33-GFP (Fig. 1 I and Fig. S1 E and F). Interestingly, it was also only half as active as wild-type HOPS (Fig. 1 J). This is not due to GFP tagging per se as HOPS with Vps33-GFP showed robust activity (Fig. 1 I and J). Because of its reproducible remaining activity and higher stability, all subsequent structural analyses were performed with HOPS (Vps39-GFP). The class averages (Fig. 1 A and Fig. S2) revealed that HOPS complexes had a seahorse-like shape, with a deeper cavity in its head region and a bulky, highly flexible lobe at the end of its tail (Fig. 1 B and Movie S1).

Because of the flexible nature of the complex, we were not able to use cryo-electron microscopy to determine the 3D structure of HOPS. Therefore, we recorded image pairs of negatively stained complexes at tilt angles of 50° and 0° to obtain different views needed to calculate four different 3D density maps of HOPS with the random conical tilt approach (25) (Fig. 1 E–H). Tomography of cryo-negatively stained particles revealed the same overall structures as our reconstructions (Fig. S3). This confirmed that HOPS had not been flattened because of dehydration in our negative stain studies.

Our density maps showed that HOPS is an  $\approx 30$ -nm-long complex that can adopt several conformations without changing its overall composition (Fig. 1 E–H, Table S1, and Movies S2 and S3). Whereas it was obvious from the 2D projections (Fig. 1 B) that the end of the tail is very flexible, the 3D reconstructions showed that the head is also intrinsically flexible. The backbone proximal to the large head appears rather stiff and extends into the head. This results in contracted (Fig. 1 E) and elongated (Fig. 1 H) as well as intermediate shapes (Fig. 1 F and G), in which the distance between the head and tail is almost doubled (from 7 nm in the contracted to 14 nm in the elongated state).







**Fig. 4.** Two Rab-binding sites on a single HOPS complex. (A) Interaction of Ypt7 with Vps39. Rab pull-down with GTP $\gamma$ S- or GDP-loaded Rabs Ypt7, Vps21, and Ypt7 was done with purified HOPS or the HOPS subcomplexes. Bound proteins were eluted and analyzed on SDS/PAGE, followed by Western blotting against the CbP-tag (indicated with \*). A representative Coomassie gel shows GST-Rabs present in the pull-down. (B) HOPS and Ypt7-His<sub>6</sub> were incubated, cross-linked, and separated on a glycerol gradient. The complexes were then labeled with Ni-NTA-nano-gold and imaged in the electron microscope. *Left*: Raw particle images. *Right*: Localization of the gold-label graphically mapped to the model of HOPS as shown in Fig. 3. (Scale bar, 10 nm.) (C) Localization of GFP-tagged HOPS subunits. All HOPS subunits were genomically tagged at their C terminus with GFP and localized by fluorescence microscopy in wild-type (wt) and *ypt7* $\Delta$  cells. (Scale bar, 10  $\mu$ m.) (D) Model of HOPS function in fusion. SNAREs are in red. Details in text.

prevent efficient Rab inactivation (29, 30). Such a reorientation of HOPS would require that Vps39 let go of endosomal Ypt7, to then be free for binding Ypt7 on the vacuole surface. In agreement with this model, HOPS requires Ypt7 to tether proteoliposomes *in vitro* (31) and promote their Ypt7-dependent fusion (19, 20). At present we cannot explain the observed interaction between Vps41 and Vps39 (13), although it is possible that HOPS undergoes a closed conformation whereby the two subunits get in touch. Tethering of the membranes may be facilitated by the ability of HOPS to stretch outward (Fig. 1 *E–H* and *Movies S4* and *S5*). Elongated structures between endosomes and lysosomes have indeed been observed in ultrastructural studies, which could represent HOPS (32, 33). Closer apposition of the membranes would then allow Vps33 to facilitate SNARE assembly before membrane mixing. It is likely that the flexibility of HOPS also supports this process.

In addition to homotypic fusion, HOPS also acts in the fusion of AP-3 vesicles with vacuoles by binding the  $\delta$ -ear domain of the AP-3 subunit Apl5 (28, 34). For this interaction, the HOPS subunit Vps41 has to be phosphorylated by the vacuolar kinase Yck3. Phosphorylation at its membrane-interacting ALPS motif releases Vps41 from its close membrane apposition and thus exposes the Apl5 binding site (28). On the basis of our structural insights, we can now start to unravel the precise function of Vps41/HOPS in this process.

How does HOPS compare with known EM structures of tethering complexes? To date, EM structures are available of the Dsl1 complex, which operates without Rab binding, and the Golgi-localized COG complex. Both belong to the CATCHR family of helical tethering complexes. The Dsl1 complex also forms an elongated particle, which requires ER-localized SNAREs for its membrane localization (5), and may capture COPI vesicles via coat binding, whereas COG forms a three-legged structure that is reminiscent of the clathrin legs (35).

HOPS shows some similarity to the Dsl complex but also differs strongly. The Dsl complex seems to be stiffer, owing to its rod-like subunits, whereas HOPS has six, likely interconnected subunits. Five of the six HOPS subunits are related to known vesicle coat components and nuclear pore complexes (36–39), which form polymeric structures unlike HOPS. It is thus likely that additional hinge regions exist between these subunits, which would explain its flexibility. COG with its three flexible legs seems more like a coat but could also span membranes with its similar length of some 40 nm (35). It will thus be important to determine in the future how each complex behaves on membranes. To understand HOPS in detail, high-resolution structures of the subunits and mapping of their interfaces will be critical to determine its precise organization and test the proposed tethering model.

## Experimental Procedures

Details are provided in *SI Experimental Procedures*.

**Biochemical Procedures.** HOPS was purified from 2 L of YP medium with 2% (vol/vol) D-galactose that were grown for 48 h. Purification of HOPS and HOPS subcomplexes was done via the TAP protocol, as previously described (16). For preparation of HOPS for EM analyses, the buffer was as follows: 1 M NaCl, 50 mM Hepes/NaOH (pH 7.5), and 10% (vol/vol) glycerol. Glycerol gradient centrifugation with simultaneous cross-linking (GraFix) with glutaraldehyde was performed according to Kastner et al. (24). Vacuole fusion was done as described with vacuoles carrying the *vps11-1* allele (18).

**EM and Image Processing.** Samples were either directly prepared for EM as previously described (40) or first cross-linked on a glycerol gradient as described above. All images were taken with a JEOL JEM-1400 electron microscope equipped with a LaB<sub>6</sub> filament at an operation voltage of 120 kV. Particles were manually selected, aligned, and classified using reference-free alignment and K-means classification procedures implemented in SPARX and EMAN2 (41).

For the 3D reconstruction of HOPS (Vps39-GFP), image pairs were collected at tilt angles of 50° and 0°, and 4,800 particle pairs were selected from 200 image pairs using WEB, the display program associated with the SPIDER software package (42), which was used for further image processing steps. Random conical tilt reconstructions of the tilted particles were calculated from the seven best class averages by back-projection, followed by back-projection refinement. The resulting reconstructions were then submitted to several rounds of 3D multireference projection matching against the dataset of 24,900 single particles using SPARX. Four reconstructions remained stable during the refinement process with a resolution of  $\approx 29$  Å according to the 0.5 criterion (Fig. S6).

For visualization, analysis, and preparation of the figures, we used Chimera (43). Initial segmentation of the HOPS complex was performed automatically using the module Segment Map of the Chimera software package (44).

To locate subunits in the HOPS complex, we labeled HOPS (Vps33-GFP), HOPS (Vps39-GFP), HOPS-Vps41-CbP (wild type), and HOPS subcomplex

Vps39-CbP-Vps11-Vps18 with anti-GFP and anti-CbP antibodies, respectively. The data sets were submitted to reference-free alignment and *k*-means classification (40–50 particles per class) using SPARX. To determine the position of HOPS Ypt7 binding, TAP-purified HOPS complexes and 150  $\mu$ g purified His<sub>6</sub>-tagged Ypt7, preloaded with GTP $\gamma$ S according to Ostrowicz et al. (16), was incubated at 1:1 ratio for 1 h at 4 °C. After subsequent cross-linking by glutaraldehyde and separation on a glycerol gradient, the sample was labeled with Ni-NTA-nano-gold (Nanoprobes) for 1 min.

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