Structure of the HOPS tethering complex, a lysosomal membrane fusion machinery

Dmitry Shvarev1†, Jannis Schoppe2†, Caroline König2†, Angela Perz2, Nadia Füllbrunn2, Stephan Kiontke3, Lars Langemeyer2, Dovile Januliene1, Kilian Schnelle1, Daniel Kümmel4, Florian Fröhlich5, Arne Moeller1,6*, Christian Ungermann2,6*.

1 Osnabrück University, Department of Biology/Chemistry, Structural Biology section; 49076 Osnabrück, Germany.
2 Osnabrück University, Department of Biology/Chemistry, Biochemistry section; 49076 Osnabrück, Germany.
3 University of Marburg, Department of Plant Physiology and Photo Biology; 35043 Marburg, Germany.
4 University of Münster, Department of Chemistry and Pharmacy, Institute of Biochemistry; 48149 Münster, Germany.
5 Osnabrück University, Department of Biology/Chemistry, Molecular Membrane Biology group; 49076 Osnabrück, Germany.
6 Osnabrück University, Center of Cellular Nanoanalytic Osnabrück (CellNanOs); 49076 Osnabrück, Germany.

*Corresponding authors. Email: arne.moeller@uos.de (A.M.), cu@uos.de (C.U.).
† These authors contributed equally to this work.
Abstract

Lysosomes are essential for cellular recycling, nutrient signaling, autophagy, and pathogenic bacteria and viruses invasion. Lysosomal fusion is fundamental to cell survival and requires HOPS, a conserved heterohexameric tethering complex. On the membranes to be fused, HOPS binds small membrane-associated GTPases and assembles SNAREs for fusion, but how the complex fulfills its function remained speculative. Here, we used cryo-electron microscopy to reveal the structure of HOPS. Unlike previously reported, significant flexibility of HOPS is confined to its extremities, where GTPase binding occurs. The SNARE-binding module is firmly attached to the core, therefore, ideally positioned between the membranes to catalyze fusion. Our data suggest a model for how HOPS fulfills its dual functionality of tethering and fusion and indicate why it is an essential part of the membrane fusion machinery.

One-Sentence Summary

Using cryo-EM, we solved the structure of HOPS tethering complex and suggest its mechanistic role in lysosomal membrane fusion.

Key words:

HOPS, membrane fusion, vacuole, lysosome, Rab GTPase, tethering, cryo-EM
**Introduction**

Lysosomal fusion underlies a plethora of cellular processes. It is essential in the maintenance and upkeep of eukaryotic membranes and fundamental to secretion, endocytosis and autophagy (Saftig and Puertollano, 2020). Macromolecules from different trafficking pathways end up in lysosomes where they are degraded (Klionsky et al., 2021; Saftig and Puertollano, 2020). This process relies on multiple fusion events within the endomembrane system. In general, fusion depends on SNAREs, which are present on opposite membranes and zipper into four-helix bundles with the help of Sec1/Munc1 (SM) proteins (Jahn and Fasshauer, 2012; Südhof and Rothman, 2009; Wickner and Rizo, 2017). Each assembled SNARE complex contains three Q-SNAREs ($Q_a$, $Q_b$, and $Q_c$) and one R-SNARE, which are categorized according to the interaction of the glutamine and arginine residues in the central hydrophilic layer of the otherwise hydrophobic interfaces within the SNARE complex (Jahn and Fasshauer, 2012; Südhof and Rothman, 2009; Wickner and Rizo, 2017). Prior to fusion, specialized tethering complexes establish tight links between organelles and interact with SM proteins to promote fusion (Baker and Hughson, 2016; Kuhlee et al., 2015; Ungermann and Kümmel, 2019). Despite their central position in trafficking, the underlying mechanics of tethering complexes and how they catalyze membrane fusion remain unresolved.

The heterohexameric HOPS complex mediates fusion of late endosomes, autophagosomes and AP-3 vesicles with mammalian lysosomes or vacuoles in yeast (Beek et al., 2019; Spang, 2016; Wickner and Rizo, 2017), and is probably the best studied tethering complex. Fusion assays using yeast vacuoles or reconstituted SNARE-bearing proteoliposomes showed that HOPS is essential for membrane fusion at physiological SNARE concentrations (D’Agostino et al., 2017; Mima et al., 2008; Zick and Wickner, 2016). HOPS is the target of viruses such as SARS-CoV2 (Miao et al., 2021), and its inactivation blocks Ebola infections (Carette et al., 2011). Furthermore, multiple HOPS mutations can cause severe diseases ranging from Parkinson’s to lysosomal disorders (Beek et al., 2019; Sanderson et al., 2021; Welle et al., 2021).
Five out of six HOPS subunits (Vps11, 16, 18, 39, 41) are predicted to share a similar architecture, comprising an N-terminal β-propeller and a C-terminal α-solenoid domain (Figure 1A). Vps11 and Vps18 form the core and carry conserved C-terminal RING finger domains (Rieder and Emr, 1997), which are essential for HOPS formation (Hunter et al., 2017), but also show E3 ligase activity on their own (Segala et al., 2019). At the opposite sites, Vps41 and Vps39 bind to membrane anchored small GTPases (the Rab7-like Ypt7 in yeast) (Bröcker et al., 2012; Lürick et al., 2017; Ostrowicz et al., 2010), while Vps16 and the SM protein Vps33 establish the SNARE-binding module (Baker et al., 2015; Graham et al., 2013). Low resolution negative-stain electron microscopy analyses revealed the overall arrangement of HOPS (Bröcker et al., 2012; Chou et al., 2016), yet were insufficient to localize the exact position of its subunits and suggested significant flexibility within the particle.

The way HOPS fulfills its function remains speculative, and multiple mechanisms have been proposed, including a role as a bulky membrane stressor (D’Agostino et al., 2017) or, conversely, as a highly flexible membrane tether (Bröcker et al., 2012; Chou et al., 2016). In the absence of detailed structural data, it remains obscure how HOPS facilitates lysosomal fusion.

**Results**

**Structure of the HOPS complex**

Previously, structural studies were hampered by the low stability and flexibility of the complex, which required fixation through mild crosslinking for sample preparation and confined structural studies to negative stain analyses (Bröcker et al., 2012; Chou et al., 2016). To enable high-resolution cryo-electron microscopy (cryo-EM) of non-crosslinked HOPS, we vastly improved and accelerated our purification protocols and removed any delays during the sample preparation procedure (Figure 1B-D). Single-particle analysis including extensive classifications followed by
local refinements led to a composite structure with resolutions between 3.6 Å and 5 Å (Figure 1E and Figure 1 – Figure supplement 1-3).

HOPS forms a largely extended, slender structure extending approximately 430 Å in height and 130 Å in width, resembling a triangular shape (Figure 1E-G, Video 1). In the center of the modular complex, Vps11 and Vps18 align antiparallel through their elongated α-solenoids, establishing a large interface area of 1972 Å\(^2\) (Figure 1E-G, 2, Figure 2 – Figure supplement 4), which resulted in the highest resolution obtained within HOPS (Figure 1 – Figure supplement 3) and is comparable with protein interfaces in other complexes with similar structural elements as in HOPS (e.g. Kschonsak et al., 2022). Interestingly, AlphaFold predicts a long unstructured region within Vps11 (Q760 to D784), resulting in an upper and lower part of the subunit. However, this region is clearly resolved and organized in our density. In our model, the two core subunits create a central assembly hub for the four other subunits (Vps39, Vps41, Vps16, Vps33) that fulfil specific functions and localize to the periphery of the complex. The N-termini of Vps11 and Vps18 are located distally from the core and each carry a β-propeller, which can be deleted without affecting the complex formation (Figure 3A,D, Figure 1 – Figure supplement 4). At the C-termini, both Vps11 and Vps18 have long α-helices which are followed by RING finger domains (Figure 1E-G, 2A,B, Figure 2 – Figure supplement 1). Both features are key elements for the stability of the modular architecture and serve as anchor points for the additional subunits. In agreement, HOPS, carrying mutations in the RING finger domains of Vps11 (vps11-1) (Peterson and Emr, 2001), selectively lost Vps39, whereas only Vps41 was obtained from a similar Vps18 mutant (Figure 2 – Figure supplement 2).

Vps41 and Vps39 provide the Ypt7-interaction sites at their peripheral N-terminal regions. Their extended C-terminal helices, similar to those of Vps11 and Vps18, are tightly interlocked through coiled-coil motifs with the long C-terminal α-helices and RING finger domains from the
respective core subunits (Vps41 with Vps18, and Vps39 with Vps11) (Figure 2A,B). Additional
stability of Vps39 within the complex is provided by the interaction of the long α-helix at the C-
terminus of Vps39 with the β-propeller of Vps18 (Figure 2D). In our density, peripheral portions of 
both Vps41 and Vps39 are least well resolved indicating their considerable flexibility. Multi-
modular classification analyses revealed angular re-orientations of about 9° for Vps41 and 20° for 
Vps39 (Figure 2 – Figure supplement 3A-D) relative to the core, resulting in variable positions of 
the N-terminal β-propellers. At the top, Vps41 reaches out by approximately 100 Å in length and, 
similarly, Vps39 forms an elongated arch at the bottom (Figure 1E-G), positioning both Ypt7-
interacting units at the farthest ends of the complex.

The SNARE-binding element, composed of Vps16 and the SM protein Vps33, branches out 
to the lateral side of the complex approximately at the center of the structure (Figure 1E-G, 2). 
Vps16 shares a large interface with the coiled-coil motif formed by Vps18 and Vps41 and the N-
terminus of Vps18, which is stabilized through interactions between hydrophobic and charged 
residues (Figure 2C). Vps33 is in immediate contact with the structured loop of Vps18 (residues 
824-831) that connects the elongated helix with the RING finger domain (Figure 2C). This, as well 
as the role of RING finger domains in the interlocking of other subunits, explains, why mutations at 
RING domains result in devastating human diseases (Beek et al., 2019; Edvardson et al., 2015; 
Welle et al., 2021; Zhang et al., 2016) and cause failure of correct HOPS assembly (Figure 2 – 
Figure supplement 2). Overall, the SNARE binding module appears to be stably connected to the 
central core, while only the short C-terminal section of Vps16 α-solenoid (residues 739-798) 
displays high variability and is not resolved in our structure.

**HOPS couples tethering to fusion activity**

Tethering complexes bridge membranes by binding small GTPases, but also harbor or bind 
SM proteins (Ungermann and Kümmel, 2019). Reconstituted vacuole fusion is strictly HOPS and
Ypt7-dependent at physiological SNARE concentrations (Langemeyer et al., 2018; Zick and Wickner, 2016), suggesting that HOPS is not just a tether, but part of the fusion machinery (Baker et al., 2015; Wickner and Rizo, 2017). However, so far it was unknown how tethering and fusion activities of HOPS may be linked mechanistically. To address this, we first analyzed the N-terminal β-propellers of Vps41 and Vps39, the likely binding sites with Ypt7 (Lürick et al., 2017; Ostrowicz et al., 2010; Plemel et al., 2011). The intrinsic low affinity between HOPS and Ypt7 (Lürick et al., 2017) prevented reconstitution of the complex for structural studies, therefore, we instead relied on AlphaFold predictions. Additionally, we solved the structure of the β-propeller of *Chaetomium thermophilum* Vps39 by X-ray crystallography, which largely confirmed the predicted model (Figure 3 – Figure supplement 1A). Surprisingly, in the AlphaFold model, Ypt7 binding occurs at the α-solenoid of Vps39 where it does not directly interact with the β-propeller (Figure 3 – Figure supplement 1C), as originally expected. Furthermore, the binding site on Vps39 is placed approximately 5-6 nm above the membrane if Ypt7-anchored HOPS is in an upright position on supported lipid bilayers (Füllbrunn et al., 2021). Membrane-bound Ypt7 can still reach this site due to its 10 nm long hypervariable domain (not shown in the prediction).

In the predicted complex of Vps41 (residues 1-919) with Ypt7 (residues 1-185), the GTPase binds directly to the Vps41 β-propeller, as anticipated. However, it interacts on the opposite side from the membrane-interacting amphipathic lipid-packing sensor (ALPS) motif (Cabrera et al., 2010) (Figure 3 – Figure supplement 1B), suggesting that the hypervariable region of Ypt7 is required for binding, in analogy to Vps39. Curiously, in the predicted model, the ALPS motif faces away from the membrane which would hamper membrane binding. We noted, however, that this distal region of Vps41 (Figure 3 – Figure supplement 1B) displays substantial flexibility. The β-propeller of Vps41 might, therefore, be oriented differently in the structure than predicted by AlphaFold, which may bring the ALPS motif in contact with the bilayer if Vps41 is bound to Ypt7.
Nevertheless, future experimental data will need to confirm the predicted AlphaFold model of Vps41 and Vps39 interaction with Ypt7.

Vps39 binds Ypt7 far stronger than Vps41 (Auffarth et al., 2014; Lürick et al., 2017), and may be assisted by Vps18 to sandwich Ypt7, whereas Vps41 binds to Ypt7 apparently alone (Figure 3 – Figure supplement 1D). Such a dual interaction could explain both tighter binding and a preferred orientation of HOPS on membranes (Füllbrunn et al., 2021). To test the functional importance, we generated HOPS complexes lacking the β-propeller of Vps11, Vps18 or Vps41 (Figure 3A). All complexes were purified in equimolar stoichiometry (Figure 1 – Figure supplement 4A), and interacted with Ypt7, but not the Golgi Rab Ypt1 in GST-pull down assay, suggesting that at least one Rab-binding site is maintained in all truncated complexes (Bröcker et al., 2012; Lürick et al., 2017; Ostrowicz et al., 2010; Zick and Wickner, 2016, 2012) (Figure 1 – Figure supplement 4B).

To determine the activity of HOPS mutants, we compared tethering and fusion. For tethering, we incubated liposomes bearing Ypt7-GTP with each complex and quantified clustering after centrifugation (Füllbrunn et al., 2021; Lürick et al., 2017). HOPS lacking the Vps41 β-propeller was inactive as shown (Lürick et al., 2017), whereas HOPS with truncated Vps11 or Vps18 was fully functional and as efficient as wild-type HOPS (Figure 3B). In contrast, when added to SNARE and Ypt7-GTP bearing liposomes, only wild-type HOPS, but none of the mutant complexes promoted fusion (Figure 3C). This was particularly puzzling for HOPS lacking either the Vps18 or Vps11 β-propeller as they had full tethering activity (Figure 3B). Therefore, we compared the structural features of HOPS mutants with the wild type using negative stain EM. Deletions of the β-propellers of Vps41, Vps11 or Vps18 indeed resulted in a loss of protein density at the expected positions, while preserving the densities of all other subunits (Figure 3D). Interestingly, HOPS complexes lacking β-propellers in Vps11 or Vps18 showed an alteration in the relative orientation of Vps39 within the complex in some 2D class averages. This observed structural
variation might be a result of increased flexibility of mutant HOPS due to the lack of structural support by β-propellers of Vps18 and Vps11. We conclude that the β-propellers of Vps18 and Vps11 contribute to the overall structure of the HOPS complex or may play a stabilizing role during the fusion process, which would explain, why they are essential for the full activity of HOPS.

**Discussion**

Our data suggest a working model of how HOPS catalyzes fusion at lysosomes and vacuoles (Figure 4). According to our structure, the three major ligand binding sites of HOPS are arranged in a triangular fashion. While the two Ypt7-binding sites show significant conformational variability, the SNARE-interacting unit is firmly connected to the stable backbone formed by Vps11 and Vps18. For tethering, HOPS Vps39 and Vps41 bind Ypt7 on target membranes. During this process, HOPS remains upright on membranes (Füllbrunn et al., 2021). Then, the SM protein Vps33 and possibly other sites on HOPS (Krämer and Ungermann, 2011; Baker et al., 2015; Lürick et al., 2017, 2015; Song et al., 2020) bind SNAREs from the opposing membranes, and zipper them up toward their membrane anchor (Figure 4). Note, that this process can be blocked by Orf3a in the COVID-19 SARS-CoV-2 virus (Miao et al., 2021). In our model, the backbone of HOPS dampens the movement of the vesicles and acts as a lever arm holding on to SNAREs during zippering (D’Agostino et al., 2017). This three-point arrangement would cause membrane stress and could explain how HOPS catalyzes membrane fusion (Figure 4). The physiological function of HOPS can be bypassed if large complexes are redirected to SNAREs at the fusion site (D’Agostino et al., 2017; Song et al., 2017), which can even promote fusion of deficient SNARE complexes (Orr et al., 2022; Song et al., 2021). Zippered SNAREs may then dissociate from HOPS and allow access for α-SNAP and NSF to recycle SNAREs (Zhang and Hughson, 2021).

How tethering complexes contribute to fusion poses a long-standing question in the field. The process necessitates binding of two opposing membranes and exact coordination of the
zippering procedure of membrane-bound SNAREs. Previous analyses suggested strong flexibility along the entire HOPS structure, which was interpreted as a hallmark of tethering complexes and an essential prerequisite to their function (Bröcker et al., 2012; Chou et al., 2016; Füllbrunn et al., 2021; Ha et al., 2016). Structural models of a largely open HOPS, based on negative stain EM (Chou et al., 2016), supported the importance of HOPS structural flexibility. Flexibility was also observed in our previous negative stain EM structure (Bröcker et al., 2012). Instead, our cryo-EM data, collected on a highly pure and homogeneous sample that was not modified by any crosslinker or other fixative agents like negative stain, shows that HOPS flexibility is limited. Taking the obtained resolution as a measure of sample flexibility (Rawson et al., 2016), we conclude that the backbone and the SNARE-binding module are the least flexible parts of HOPS and appear to be stably associated with each other. In contrast, the membrane interacting units of Vps41 and Vps39 show some flexibility with 10-20° movements between particles, which substantially reduced their resolution (Figure 2 – Figure supplement 3). We relate this flexibility to their function within HOPS, where it may dampens the motion of HOPS between membranes and stabilizes the SNARE interaction, which are necessary for fusion (Laage and Ungermann, 2001; Krämer and Ungermann, 2011; Lürick et al., 2015; Baker et al., 2015; Song et al., 2020). Mammalian HOPS contains the same six subunits as yeast HOPS, which are generally highly conserved and will therefore function similarly (Beek et al., 2019; Kant et al., 2015). Our structure can hence be used to map disease prone mutations (Figure 4 – Figure supplement 1) and may thus explain the consequences on HOPS function.

Due to low binding affinities, no structures of tethering complexes bound to Rabs have been solved. Here, we used AlphaFold to predict the interfaces of HOPS with its bound Rab7-like Ypt7 protein to understand the positioning of HOPS during tethering (Figure 3 – Figure supplement 1). We suspect that this low binding affinity helps tethering complexes to also let go of the Rab when fusion progresses (Figure 4). Even though long coiled-coil tethers such as EEA1 can promote
SNARE-mediated membrane fusion (Murray et al., 2016), their cooperation with Rab GTPases in fusion is likely quite different from the mechanism proposed here (Ungermann and Kümmel, 2019). Some tethering complexes such as CORVET, CHEVI or FERARI have attached SM proteins (Beek et al., 2019; Solinger et al., 2020), others such as COG, GARP, Exocyst and Dsl cooperate with SM proteins (Ungermann and Kümmel, 2019), which may catalyze fusion similar to HOPS. For each of these complexes, in vivo models for their function exist, yet proteoliposome fusion assays in the presence of the required small GTPases are either not available or not yet completely developed (Balderhaar et al., 2013; Ha et al., 2016; Lee et al., 2022; Maib and Murray, 2022; Ren et al., 2009; Rossi et al., 2020; Solinger et al., 2020). We expect that similar approaches as established for HOPS will further support for the key role of tethering complexes and reveal their cooperation with SM proteins in SNARE-mediated membrane fusion.

The overarching principle suggested here for HOPS is not limited to lysosomal fusion but may extend to synaptic transmission, where the tether Munc13 and the SM protein Munc18 cooperatively catalyze the N- to C-terminal zippering of SNAREs during fusion (Lai et al., 2017; Rizo, 2022; Stepien et al., 2022; Stepien and Rizo, 2021). Both Munc18 and Vps33 interact similarly with the N-terminal part of the SNARE domains of the R- and Qa-SNARE and may promote assembly until the central zero-layer of the SNARE domain (Baker et al., 2015; Stepien et al., 2022).

During HOPS-mediated fusion, SNARE zippering beyond the zero-layer could then proceed, while the Vps33 lets go of the forming four helix bundle (Stepien et al., 2022). However, HOPS binds both the N-terminal extensions of the Qa-SNARE Vam3 and other SNAREs, possibly via different binding sites along the HOPS complex (Laage and Ungermann, 2001; Lürick et al., 2015; Song et al., 2020). This association may thus maintain the force on membranes to catalyze full fusion, even if the SM protein lets go of the assembling SNARE complex (Figure 4). In agreement, HOPS complexes with deficient Vps33 can catalyze fusion of proteoliposomes only if
the SNARE density is high (Baker et al., 2015). In turn, vacuoles expressing a Qa-SNARE Vam3 variant lacking the N-terminal extension, which is needed to bind HOPS, show diminished fusion (Laage and Ungermann, 2001). This suggests that HOPS supports SNAREs by templating the association of R- and Qa-SNAREs and by binding the N-terminal regions of SNAREs.

We believe that the deletion of the β-propeller in Vps11 or Vps18 result in a similarly deficient HOPS due to a less stable backbone (Figure 3). In either case, coupling between a stable backbone of HOPS and SNARE binding may be impaired and could result in less specific activity as fusion catalysts. Future experiments are required to determine the precise reason for their fusion deficiency.

Overall, our insights provide a novel blueprint to understand HOPS function, dynamics and regulation both in fusion and in other functions of its subunits (Beek et al., 2019; Elbaz-Alon et al., 2014; Hönscher et al., 2014; Montoro et al., 2021, 2018; Wong et al., 2020), and imply a general role of tethering complexes as a catalytic part of the fusion machinery.

References


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Competing interests: Authors declare that they have no competing interests.

Data and materials availability

The electron density maps of the yeast HOPS complex and the models and the C. thermophilum Vps39 β-propeller and the yeast HOPS complex have been deposited in the Electron Microscopy Data Bank with ID EMD-14964, EMD-14965, EMD-14966, EMD-14967, EMD-14968, EMD-14969, EMD-14970 and in the PDB with 7ZTY and 7ZU0.
Methods

**Yeast strains.** Yeast strains used in this study are listed in Supplemental File 1. In general, HOPS subunits were expressed under the control of the GAL1 promoter according to the standard protocol (Janke et al., 2004). For HOPS subunit truncation (Vps41, Vps11 and Vps18) of the N-terminal part, the GAL1 promoter was inserted into the genome at the respective position. The 3x-FLAG Tag was attached to the HOPS subunit Vps41, except for the 18 ΔN mutant.

**Protein expression and purification from *Escherichia coli.*** Rab GTPases for pulldown or tethering and fusion assays were expressed in *E. coli* BL21 (DE3) Rosetta cells. Cells were grown in Luria broth (LB) medium complemented with 35 µg/ml kanamycin and 30 µg/ml chloramphenicol. Cultures were induced at OD<sub>600</sub> = 0.6 with 0.5 mM isopropyl-β-d-thiogalactoside (IPTG) overnight at 16°C before harvesting by centrifugation (4,800 g, 10 min, 4 °C). Cells resuspended in buffer (150 mM NaCl, 50 mM HEPES/NaOH, pH 7.4, 10 % glycerol, 1 mM PMSF and 0.5-fold protease inhibitor mixture (PIC)) were lysed by a Microfluidizer (Microfluidics Inc.) and centrifugated at 25,000 g, 30 min, 4 °C. Supernatants were incubated with glutathione Sepharose (GSH) fast flow beads (GE-Healthcare) for GST-tagged proteins or nickel–nitriloacetic acid (Ni-NTA) agarose (Qiagen) for His-tagged proteins for 2 h at 4°C. The proteins were eluted with buffer (150 mM NaCl, 50 mM HEPES/NaOH, pH 7.4, 10 % glycerol) containing 25 mM glutathione or 300 mM imidazole. Buffer was exchanged via a PD10 column (GE Healthcare). For tag cleavage, TEV protease was added after washing and incubated over night. All proteins were stored at -80 °C.

**Purification of the 3xFLAG-tagged HOPS complex variants from yeast.** Two liters of yeast peptone (YP) medium containing 2 % galactose (v/v) were inoculated with 6 ml of an overnight
culture. Cells were grown for 24 h and harvested by centrifugation (4,800 g, 10 min, 4 °C). Pellets were washed with cold HOPS purification buffer (HPB, 1 M NaCl, 20 mM HEPES/NaOH, pH 7.4, 1.5 mM MgCl₂, 5% (v/v) glycerol). The pellet was resuspended in a 1:1 ratio (w/v) in HPB supplemented with 1 mM phenylmethylsulfonylfluoride (PMSF), 1× FY protease inhibitor mix (Serva) and 1 mM dithiothreitol (AppliChem GmbH) and afterwards dropwise frozen in liquid nitrogen before being lysed in a freezer mill cooled with liquid nitrogen (SPEX SamplePrep LLC).

The powder was thawed on ice and resuspended in HPB supplemented with 1 mM PMSF, 1× FY and 1 mM DTT using a glass pipette, followed by two centrifugation steps at 5,000 and 15,000 g at 4 °C for 10 and 20 min, respectively. After centrifugation, the supernatant was added to 2 ml of anti-FLAG M2 affinity gel (Sigma-Aldrich) and gently agitated for 45 min at 4 °C on a nutator.

Beads were briefly centrifuged (500 g, 1 min, 4 °C) and the supernatant was removed. Beads were transferred to a 2.5 ml MoBiCol column (MoBiTec) and washed with 25 ml of HOPS washing buffer (HWB, 1 M NaCl, 20 mM HEPES/NaOH, pH 7.4, 1.5 mM MgCl₂, 20% (v/v) glycerol). FLAG-peptide was added and incubated on a turning wheel for 40 min at 4 °C. The eluate was collected by centrifugation (150 g, 30 s, 4 °C) and concentrated in a Vivaspin 100 kDa MWCO concentrator (Satorius), which was previously incubated for 45 min with HWB containing 1% TX-100. The concentrated eluate was applied to a Superose 6 Increase 15/150 column (Cytiva) for size exclusion chromatography (SEC) and eluted in 50 μl fractions using ÄKTA go purification system (Cytiva). Peak fractions were used for further analysis.

Mass photometry. Mass photometry experiments were done using a Refeyn TwoMP (Refeyn Ltd.). Data was obtained using AcquireMP software and analysed using DiscoverMP (both Refeyn Ltd.). Glass coverslips were used for sample analysis. Perforated silicone gaskets were placed on the coverslips to form wells for every sample to be measured. Samples were evaluated at a final concentration of 10 nM in a total volume of 20 μl in the buffer used for SEC.
Cryo-EM sample preparation and data acquisition. Prior to cryo-EM analysis, HOPS samples were tested by negative-stain electron microscopy, using 2% (w/v) uranyl formate solution as previously described (Januliene and Moeller, 2021). Negative-stain micrographs were recorded manually on a JEM-2100Plus transmission electron microscope (JEOL), operating at 200 kV and equipped with a XAROSA CMOS 20 Megapixel Camera (EMSIS) at a nominal magnification of 30,000 (3.12 Å per pixel). For cryo-EM, 3 μl of 0.6–0.9 mg/ml of freshly purified wt HOPS complex were applied onto glow-discharged CF grids (R1.2/1.3) (EMS) and immediately plunge-frozen in liquid ethane using a Vitrobot Mark IV (Thermo Fisher Scientific) with the environmental chamber set to 100% humidity and 4°C. Micrographs were recorded automatically with EPU (Thermo Fisher Scientific), using a Glacios microscope (Thermo Fisher Scientific) operated at 200 kV and equipped with a Selectris energy filter and a Falcon 4 detector (both Thermo Fisher Scientific). Images were recorded in Electron Event Representation (EER) mode at a nominal magnification of 130,000 (0.924 Å per pixel) in the defocus range from −0.8 to −2.8 µm with an exposure time of 8.30 s resulting in a total electron dose of approximately 50 e− Å−2.

Cryo-EM image processing. All cryo-EM data processing (Figure 1 – Figure supplement 1) was performed in cryoSPARC v3.3.1 (Punjani et al., 2017). For all collected movies, patch motion correction (EER upsampling factor 1, EER number of fractions 40) and patch contrast transfer function (CTF) estimation were performed using cryoSPARC implementations. To solve the structure of the core part of HOPS (Figure 1 – Supplemental Figure 1E,F, 2), reference-free blob particle picking on 2186 pre-processed movies of the first dataset and particle extraction using a box size of 672 pixels (px, 0.924 Å per pixel) binned to 128 px was performed. Extracted particles were subjected to 2D classification to eliminate bad picks. Selected good 2D classes (representative good 2D classes are shown in Figure S3A) were used for template particle picking on 2186 movies.
from the first dataset combined with additional 6580 movies from the second dataset, preprocessed alike. After removal of duplicates, picked particles were extracted with the same box size and subjected to rounds of 2D classification, ab-initio reconstruction with multiple classes and 3D heterogeneous refinement to remove false positive particle picks. From the best class, particles were extracted using a box size of 672 px (0.924 Å per pixel) binned to 320 px and subjected to 2D classification. Particles from this 2D classification were also used for flexibility analysis (see below). 407,996 selected particles from good 2D classes were used for ab-initio reconstruction with six classes and followed by 3D heterogeneous refinement. This heterogeneous refinement resulted in two best classes containing 130,009 and 116,151 particles, respectively, which were further refined individually. For each of both classes, a Non-Uniform (NU)-refinement was performed, followed by particle extraction with a box size of 672 px (0.924 Å per pixel, without binning) with homogeneous and NU-refinement afterwards. Resulting consensus maps were used for local refinements of different parts of the structure (Figure 1 – Supplemenal Figure 1A, 2, 3B-E). First consensus NU-refinement that reached global resolution of 4.2 Å (Fourier shell correlation = 0.143) was used for local refinements of the upper “core” part of the complex (4 Å) and the SNARE-binding module (3.6 Å), the second consensus NU-refinement, resolved to 4.4 Å (FSC = 0.143), was used for local refinements resolving bottom parts of the complex (4.4 and 5 Å).

To better resolve distal parts of the complex, flexible Vps39 and Vps41 N-terminal fragments, the following approach was used (Figure 1 – Figure supplement 1B). Here, micrographs from first two datasets were combined with micrographs from two additional datasets of 2841 and 8338 movies. After template picking, about 3,896 million particles were extracted using a box size of 882 pixels (0.924 Å per pixel) and used for rounds of heterogeneous, homogeneous and NU-refinements to obtain 3D reconstructions, which best resolve Vps39 and Vps41. At these steps, binning was applied for particle extractions. Then, particles belonging to one of such best classes were subjected to a round of NU-refinement followed by 3D variability analyses using masks.
covering either Vps39 or Vps41. The further 3D variability display procedures were used to better sort particles. Finally, the best particles were extracted with the same box size (882 pixels, 0.924 Å per pixel) without binning and used for local refinements of Vps39 or Vps41 (Figure 1 – Figure supplement 1B, 3F,G).

For all local refinements, masks were generated in UCSF Chimera (Pettersen et al., 2004). During processing, no symmetry was applied. The quality of final maps is demonstrated in Extended Data Figure 3. All FSC curves were generated in cryoSPARC. Local resolutions of locally-refined maps (Figure S3B-G) were estimated in cryoSPARC and analyzed in UCSF ChimeraX (Pettersen et al., 2020). Dataset statistics can be found in Supplementary File 2.

To analyze the flexibility of the complex, a different processing scheme was applied (Figure 1 – Figure supplement 1A, dashed arrows). For this, a set of 383,881 good particles was selected after 2D classification of 320 px-binned particles (initial box size of 672 px with 0.924 Å per pixel). These particles were subjected to either ab-initio reconstruction and heterogeneous refinement with ten classes (Figure 2 – Figure supplement 3A,B) or several refinement rounds followed by 3D variability analysis (Figure 2 – Figure supplement 3D). Prior to 3D variability analysis, ab-initio reconstruction with one class, homogeneous and NU-refinement were performed.

**Model building and refinement.** Models of HOPS subunits were initially generated using AlphaFold (Jumper et al., 2021; Varadi et al., 2021) and docked into locally-refined maps using “Fit in Map” tool in UCSF ChimeraX (Pettersen et al., 2020). The N-terminal parts of Vps41 (residues 1-863) and Vps39 (residues 1-700) and the C-terminal part of Vps16 (from residue 739) with no well-resolved densities assigned, were removed. The AlphaFold model of Vps11 was initially split in two parts (“Vps11top”, residues 1-760, and “Vps11bottom”, residues 784-1025), which were first refined separately; the region predicted by AlphaFold as unfolded (residues 761-783) was deleted. Fitting of the C-terminal parts of Vps11 (residues 784-1025) and Vps39 (residues 701-
1045) was improved using Namdinator (Kidmose et al., 2019). Afterwards, models of single proteins were manually adjusted and refined in COOT (Emsley et al., 2010), followed by iterative rounds of refinements against corresponding locally-refined and their composite maps in Phenix (Liebschner et al., 2019) and COOT. In Phenix Graphical User Interface, real space refine tool (Afonine et al., 2018) with or without option “rigid body” was used. After several refinement rounds, two separate models were created by joining models of the upper (Vps33, Vps16, Vps41, Vps18, Vps11top) and lower (Vps11bottom, Vps39) parts of the complex. These partly-combined models were subjected to further several iterations of refinements in Phenix and COOT.

Afterwards, the two refined models were fused into a single model, which was again refined in Phenix and COOT. Sequences of the model in the bottom part of the complex were changed to polyalanines (residues 784-1025 in Vps11, residues 1-493 in Vps18, and residues 701-1045 in Vps39), since no assignment of side chains was possible at the resolution obtained there. In other parts of the complex, where blurred densities did not allow unambiguous model building, respective short fragments of the model were also replaced by polyalanine chains or deleted. Afterwards, the initially deleted part of Vps11 (residues 761-783) was built de novo according to the cryo-EM density (residues 769-783 were replaced by alanines). Finally, the complete model was subjected to another round of refinement in Phenix followed by manual refining in COOT. Model validation was performed using MolProbity (Williams et al., 2018). Figures were prepared using UCSF ChimeraX.

Model refinement and validation statistics can be found in Supplemental File 2.

**ALFA Pulldowns for mass spectrometry.** One liter of YP medium containing 2 % glucose (v/v) was inoculated with an overnight preculture. Cells were grown to OD$_{600}$ 1 at 26 °C, followed by 1 h incubation at 38 °C. Cultures were harvested by centrifugation at 4,800 g for 10 min at 4 °C). Pellets were washed with cold Pulldown buffer (PB), 150 mM KAc, 20 mM HEPES/NaOH, pH 7.4, 5% (v/v) glycerol, 25 mM CHAPS). The pellet was resuspended in a 1:1 ratio (w/v) in PB.
supplemented with Complete Protease Inhibitor Cocktail (Roche) and afterwards dropwise frozen in liquid nitrogen before lysed in 6875D LARGE FREEZER/MILL® (SPEX SamplePrep LLC).

Powder was thawed on ice and resuspended in PB by using a glass pipette followed by two centrifugation steps at 5,000 and 15,000 g at 4 °C for 10 and 20 min. The supernatant was added to 12.5 µl prewashed ALFA Selector ST beads (2,500 g, 2 min, 4 °C) (NanoTag Biotechnologies) and incubated for 15 min at 4 °C while rotating on a turning wheel. After incubation, beads were washed twice in PB and four times in PB without CHAPS. Samples were digested using PreOmics sample kit (iST Kit, preomics) and analysed in Q ExativePlus mass spectrometer (Thermo).

**GST Pulldowns.** Nucleotide specific interaction of the Rab-GTPase Ypt7 with purified HOPS variants was analysed in GST pulldowns using GST-Ypt1 or GST-Ypt1 as a negative control. 125 µg purified Rab-GTPases were preloaded with 1 mM GDP or GTP in the presence of 20 mM EDTA and Wash buffer (150 mM NaCl, 50 mM HEPES-NaOH, pH 7.4, 2 mM MgCl₂, 0.1 % Triton X-100) in a water bath for 30 min at 30 °C. For nucleotide stabilization, 25 mM MgCl₂ was added. Prewashed GSH Sepharose 4B GE Healthcare) were added to loaded Rab-GTPases and incubated for 1 h at 4 °C on a turning wheel. Beads were centrifuged for 1 min at 300 g before adding 25 µg of respective HOPS variants, followed by 1.5 h incubation at 4 °C on a turning wheel. Beads were washed three times with Wash buffer, followed by two elution steps with 600 µl Wash buffer containing 20 mM EDTA. After an incubation at room temperature for 20 min while rotating, the supernatant was TCA precipitated. 10 % of the final sample was loaded on a 7.5 % SDS gel next to 5 % of protein input. Samples were analysed via western blot using antibodies against the FLAG-Tag. Beads were boiled in 50 µl Laemmli buffer. 2 % of the sample was loaded on a 11 % SDS gel for Coomassie staining as Rab-GTPase loading control. Bands were quantified relative to the Rab-GTPase content.
Tethering assay. HOPS mediated tethering assays were performed as described (Füllbrunn et al., 2021). For this, ATTO488 labelled liposomes were prepared and loaded with prenylated Ypt7 (Langemeyer et al., 2018). 50 nmole liposomes were incubated with 50 pmole pYpt7:GDI complex together with GTP for 30 min at 27 °C. For reactions, 0.17 mM Ypt7-loaded liposomes were incubated with different concentrations of HOPS complex or buffer (300 mM NaCl, 20 mM HEPES-NaOH, pH 7.4, 1.5 mM MgCl2, 10 % (v/v) glycerol) for 10 min at 27 °C, followed by sedimentation for 5 min at 1,000 g. The overall tethered liposomes in the pellet fraction were determined in a SpectraMax M3 fluorescence plate reader (Molecular devices) comparing the ATTO488 fluorescent signal of the supernatant before and after sedimentation.

Fusion assays. Fusion assays and the purification of all proteins was performed as described (Langemeyer et al., 2018) with a protein to lipid ratio of 1:8,000. Reconstituted proteoliposomes (RPLs) were composed of the vacuole mimicking lipid mix (VML) (Zick and Wickner, 2014). One population of RPLs carried the SNARE Nyv1 and the other set contained Vti1 and Vam3. RPLs were preloaded with prenylated Ypt7 with the help of 100 mM Mon1-Ccz1 and 0.5 mM GTP (Langemeyer et al., 2018). Then, 25 nM HOPS complex, 50 nM Sec18, 600 nM Sec17, and finally 100 nM Vam7 were added. Fusion of liposomes was followed by content mixing of the RPLs and subsequent increase in fluorescence which was monitored in a SpectraMax M3 fluorescence plate reader (Molecular devices).

Cloning and protein purification of CtVPS391-500. Codon-optimized synthetic DNA (GenScript) encoding amino acids 1-500 of Vps39 from Chaetomium thermophilum (CtVp391-500; NCBI XP_006691033) was subcloned into a modified pET28a expression vector yielding an N-terminally His6-SUMO-tagged fusion protein (His6-SUMO-CtVps391-500). In short, His6-SUMO-CtVps39 was purified by Ni-NTA affinity chromatography followed by proteolytic cleavage with
SUMO protease at 4 °C overnight. SEC was performed to separate CtVps391-500 from the expression tag and SUMO protease and yielded >95% pure protein. To obtain phase information for structure determination, selenomethionine-substituted CtVps391-500 was prepared according to well-established methods (Doublié, 1997).

**Limited proteolysis and protein crystallization.** Since initial crystallization approaches did not yield protein crystals suitable for X-ray structure determination, flexible parts of CtVps391-500 were removed by limited proteolysis with α-Chymotrypsin (Merck) at 37° C for 10 minutes. Limited proteolysis was stopped by addition of protease inhibitors (Pierce™ Protease Inhibitor Tablets, EDTA-free, Thermo Fisher Scientific), and an additional SEC in buffer containing 20 mM BIS-TRIS pH 6.5, 200 mM NaCl and protease inhibitors (1:1000) was performed as a final polishing step. Best crystals were obtained by seeding at 12° C and a protein concentration of 7.5 mg/ml in a crystallization condition containing 0.1 M MES pH 7.25 and 20% PEG 2000 MME. Selenium-derivative crystals were flash-cooled in liquid nitrogen in latter condition with 25% glycerol as cryoprotectant.

**Crystal structure determination.** Anomalous X-ray data was collected from a single crystal at 100 K at beamline P13, EMBL Hamburg, Germany and diffraction data was processed using XDSAPP3 (Krug et al., 2012). Phase determination using single-wavelength anomalous dispersion at the selenium peak was not successful. A manually trimmed AlphaFold (Jumper et al., 2021) model of CtVps391-500 was used as a search template in molecular replacement and yielded a single solution with a TFZ score of 24.8 in phenix.phaser (Adams et al., 2010; Liebschner et al., 2019; McCoy et al., 2007), which was then used for MR-SAD phasing in phenix.autosol (Adams et al., 2010; Liebschner et al., 2019; Terwilliger et al., 2009) and subsequent density modification and automated model building using phenix.autobuild (Adams et al., 2010; Liebschner et al., 2019;
Terwilliger et al., 2009). Iterative cycles of model building in COOT (Emsley et al., 2010) and refinement using phenix.refine (Afonine et al., 2012; Liebschner et al., 2019; Terwilliger et al., 2009) led to a final model of CtVps391-500 with R-factors of Rwork 26.9% and Rfree 29.8%. The model contained no Ramachandran outliers with 96.74% residues within favoured regions. Crystallographic statistics are summarized in Supplemental File 3. PYMOL and UCSF ChimeraX was used for visualization and graphical analysis.
Figure legends

**Figure 1 with four supplements.** Composition and architecture of the yeast HOPS complex. (A) Domain architecture and size of HOPS subunits. Predicted domains and structural features are indicated. (B) Size exclusion chromatography (SEC) of the affinity-purified HOPS. Purification was done as described in Materials and Methods. (C) Mass photometry analysis. Peak fractions from SEC were analyzed for size. (D) Purified HOPS. Proteins from affinity purification (eluate) and SEC (red dashes in B) were analyzed by SDS-PAGE. (E) Overall architecture of the HOPS complex. Composite map from local refinement maps (Figure S1-3) was coloured by assigned subunits. One of consensus maps used for local refinements was low-pass-filtered and is shown as a transparent envelope. (F) Atomic model of the HOPS complex. For the N-terminal fragments of Vps41 and Vps39, which were not resolved to high resolution by local refinements, AlphaFold models are used and manually fitted into the densities of consensus maps (Figure 1 – Figure supplement 1,2). The triangular shape of the complex is highlighted with the approximate distance between the β-propellers of Vps41 and Vps39. **G,** Schematic representation of the HOPS complex indicates central features.

**Figure 1 - Figure supplement 1.** Cryo-EM data processing workflow. (A) Processing of the core part of the complex. 2,186 and 6,580 movies from two different datasets were preprocessed in parallel and used for initial particle sorting by 2D and 3D classifications using the box size of 128 pixels with 4.85 Å per pixel. After initial classifications, approximately 625,000 particles were selected and combined for further processing. A round of ab-initio reconstruction followed by hetero-refinement with five classes was performed to separate the best particles for further refinements. About 407,000 particles from three classes, which reached 9.9 Å resolution (Nyquist for the binned data), were extracted using the box size of 320 pixels with 1.94 Å per pixel, and another round of 2D classification was performed. Afterwards, a new round of ab-initio reconstruction and hetero-refinement with six classes was conducted. Particles from two classes that reached the best resolutions (7 and 7.1 Å) were further processed separately. The first of the selected two classes was refined by NU-refinement to 4.3 Å resolution, while the second class was refined to 4.9 Å. Subsequently, particles were extracted in full box size (672 pixels with 0.924 Å per pixel) and used for further homogeneous and NU-refinement, which resulted in two consensus maps, better resolving either upper or lower part of HOPS (reached 4.2 Å and 4.4 Å resolution, respectively). FSC curves generated in cryoSPARC are shown for both consensus maps. Afterwards, local refinements were applied to improve each of the consensus maps. Local refinements of the first map resulted in two maps covering the SNARE-binding module (3.6 Å resolution) and the upper part for the core of HOPS (4 Å resolution). Local refinements of the second consensus map provided maps of the bottom of HOPS core better covering either Vps18 (4.4 Å resolution) or Vps39 (5 Å resolution). The subset of particles used for generation of both consensus maps was in parallel probed for flexibility using 3D classification (Figure 2 – Figure supplement 3A,B) and 3D variability analyses (Figure 2 – Figure supplement 3D) (dashed arrows; see Materials and Methods for details). (B) Processing of the distal parts of HOPS. Movies used for core reconstruction (see A) were combined with movies from two additional datasets (2841 and 8338 movies, respectively) and preprocessed. After template picking, about 3.9 million particles...
were selected and subjected to several rounds of heterogeneous, homogeneous and NU-refinements to select classes best resolving upper (Vps41) and lower (Vps39) distal parts of HOPS. Afterwards, selected particles were used for a round of NU-refinement with a box size of 448 px with 1.82 Å per pixel. The particles from this NU-refinement were then subjected to 3D variability analysis with masks covering either the Vps41 or Vps39 volume. This was followed by 3D variability display in the cluster mode to further select particles. Finally, particles from best clusters were subjected to local refinements at a full box size of 882 pixels with 0.924 Å per pixel resulting in the maps resolving N-terminal regions of Vps39 (7.5 Å resolution) or Vps41 (6.9 Å resolution).

**Figure 1 – Figure supplement 2.** Consensus maps and corresponding local refinement maps. (A) Consensus cryo-EM map of the upper part of HOPS, shown as a transparent envelope, with two corresponding local refinement maps fitted (coloured). (B) the same consensus map, as in A, shown from different sides. (C) the low-pass-filtered consensus map from A, used in Figure 1E and viewed at a threshold, which allows to demonstrate the densities of N-terminal regions of Vps39 and Vps41. (D) The consensus map of the lower part of HOPS, shown as a transparent envelope, with two corresponding local refinement maps fitted (coloured). (E) The consensus map from D, shown from different sides. (F) The map from D, shown as in C.

**Figure 1 – Figure supplement 3.** Cryo-EM analysis of HOPS. (A) representative 2D class averages. Scale bar, 100 Å. (B-G) Local-resolution estimation, FSC curves and angular distribution plots generated in cryoSPARC for each of six local refinement maps (see Figure 1 – Figure supplements 1, 2 and Materials and Methods). Note, that in F and G, local resolution was not calculated for the peripheral areas of the maps due to mask limitations and is displayed in dark blue.

**Figure 1 – Figure supplement 4.** Biochemical analysis of HOPS mutants lacking N-terminal β-propellers. (A) SDS-PAGE of purified wild-type and mutant complexes. (B) Ypt7-interaction. HOPS wild-type and mutant complexes were added to immobilized GST-Ypt7 or Ypt1 loaded with GTP or GDP. Eluted proteins were analysed by SDS-PAGE and Western blotting. Coomassie gels of corresponding Rab GTPases are shown below.

**Figure 1 – source data.** Gels and graphs for Figure 1b, c and d.

**Figure 1 – Figure supplement 4 – source data.** Blots and gels corresponding the experiments.

**Figure 2 with four supplements.** Vps11 and Vps18 C-termini as central interaction hubs for all other subunits. Atomic model of HOPS with highlighted interaction sites between subunits. (A, B) Coiled-coil motifs followed by the RING finger domains (violet) are the key structural features of HOPS. (A) The Vps18 C-terminal hub. Vps18 and Vps41 interact via the coiled coil and the Vps18 RING finger domain (displayed as non-transparent cartoons). (B) The Vps11 C-terminal hub. Vps11 interacts via its RING finger domain and the coiled coil with Vps39 (displayed as non-transparent cartoons). (C) Connection of the SNARE binding module (Vps33 and Vps16) to the backbone of HOPS via interactions with the structured loop at the RING finger domain and the C-
terminus of Vps18 (displayed as non-transparent cartoons). (D) Vps39 connects by its C-terminal helix the β-propeller of Vps18, which provides additional stability in this part of HOPS.

Figure 2 – Figure supplement 1. Comparison of Vps11 and Vps18 RING finger domains. (A) Fragments of Vps11 and Vps18 depicting RING finger domains (yellow) following the long helices. Amino acids (orange) participating in folding of the RING finger domains are labeled. (B) Multiple sequence alignment of Vps11 and Vps18 RING finger domains from different model organisms (Drosophila melanogaster, Homo sapiens, Mus musculus, Saccharomyces cerevisiae, Danio rerio), made using MUSCLE (Edgar, 2004) and visualized in Jalview (Waterhouse et al., 2009), is shown; colouring by conservation. Conserved amino acids from a are labeled by red asterisks.

Figure 2 – Figure supplement 2. Key role of the RING finger domains of Vps11 and Vps18 in HOPS stability. (A) cartoon of cell growth, lysis, ALFA pull down and mass spectrometry analysis. See Materials and Methods for details. (B-E). Mass spectrometry analysis of Vps41 purified via the ALFA tag from the indicated strains and enriched proteins (green dots). Results of purification from wt (B), vps11-1 (C), vps11-3 (D) and vps18 (E) cells. (F), schematic representation of the results based on the structural model (as in Figure 1G).

Figure 2 – Figure supplement 3. Flexibility analysis of the HOPS complex. (A) Upper row, maps generated by a hetero-refinement with multiple classes from particles used for local refinements (see Figure 1 – Figure supplement 1A). All classes demonstrate different positions of densities at the distal ends relative to the rigid core (highlighted by red lines) of the volumes. Lower row, rotation angles calculated between the points indicated on the maps from above. (B) Angular difference calculated from angles in A, demonstrating limited movements of Vps41 and Vps39, as depicted in the cartoon to the right. C, representative 2D class averages show fuzzy densities of Vps41 and Vps39 owing to their flexibility. D, 3D variability analysis of particles used for local refinements (see Figure 1 – Figure supplement 1A). 3D density maps at negative (red) and positive (blue) positions along each variability component are shown.

Figure 2 – Figure supplement 4. Interactions between core subunits Vps11 and Vps18. Open-book view at the interface between Vps11 and Vps18 observed from the SNARE-binding module side of the complex. Surfaces colored by electrostatic potential and hydrophobicity are shown demonstrating a high degree of hydrophobic and electrostatic complementarity at the interface between the subunits.

Figure 2 – Figure supplement 2 – source data. Mass spectrometry raw data.
Figure 3 with one supplement: HOPS couples tethering and fusion activities. (A) Schematic representations of HOPS wild-type (as in Figure 1G) and mutants lacking N-terminal β-propeller domains (indicated by pink asterisks). (B) Tethering assay. Fluorescently labeled liposomes loaded with prenylated Ypt7-GTP or none were incubated with HOPS and mutant complexes. Tethering was determined as described in Materials and Methods. (C) Fusion assay. Fusion of proteoliposomes carrying vacuolar SNAREs were preincubated with Ypt7-GDI, GTP and Mon1-Ccz1. For fusion, HOPS wild-type or mutant and the soluble Vam7 SNARE were added (Langemeyer et al., 2020, 2018). Analysis was done as described (Zick and Wickner, 2016). See Materials and Methods. (D) Representative 2D class averages obtained from negative-stain analyses of wild-type HOPS and mutants. Pink asterisks indicate missing densities in the mutants.

Figure 3 – Figure supplement 1. Ypt7 interaction with Vps41 and Vps39. (A) Structure of the β-propeller of C. thermophilum Vps39 confirms the structure prediction. Crystal structure of C. thermophilum Vps39 (1-500) construct (cyan) is shown and superimposed on the model of S. cerevisiae Vps39 (dark green) generated by AlphaFold. Below, sequence alignment of Vps39 N-terminal fragments from C. thermophilum and S. cerevisiae is displayed. Alignment is made using MUSCLE (Edgar, 2004) and visualized in Jalview (Waterhouse et al., 2009); colouring by conservation. (B) Model of Vps41 (light green, residues 1-919) with Ypt7 (pink, residues 1-185) generated by AlphaFold (multimer mode). According to the model, Ypt7 binds to the β-propeller of Vps41 at the opposite side than the identified membrane-interacting ALPS motif (Cabrera et al., 2010). (C) Model of Vps39 (dark green, residues 1-926) with Ypt7 (pink, residues 1-185) was obtained as in A. Ypt7 binds to the inside of the α-solenoid of Vps39 according to the model. (D) The model from B, fitted to the atomic model of HOPS generated in this study (gray, see Figure 2). According to the AlphaFold model, Ypt7 has a contact site not only with Vps39 but also with Vps18 β-propeller.

Figure 3 – source data. Raw data for tethering (Figure 3B) and fusion assay (Figure 3C).
**Figure 4 with one supplement:** Working model for HOPS-mediated membrane tethering and fusion. The HOPS complex binds to Ypt7 on the vacuole and vesicles via Vps39 (dark green) and Vps41 (light green). SNARE proteins are recruited to HOPS by their N-terminal domains and the SNARE-binding module (dark and light brown). The stable central core of HOPS keeps the membranes in place, while Vps41 and Vps39 may function as dampers due to their limited flexibility. Consequentially, zippering of SNAREs, which is initiated by binding to Vps33 (dark brown), begins. As the N-terminal domains of SNAREs bind to HOPS, further SNARE zippering may occur with the HOPS backbone acting as a lever (not shown here). This may cause membrane stress and thus catalyzes fusion. HOPS may let go of Ypt7 and SNAREs thereafter. For details see text.

**Figure 4 – Figure supplement 1.** Point mutations affecting HOPS function. (A) Multiple sequence alignment of HOPS subunit fragments from different model organisms (*Drosophila melanogaster*, *Homo sapiens*, *Mus musculus*, *Saccharomyces cerevisiae*, *Danio rerio*) displaying known point mutations (red frames) (Beek et al., 2019; Peterson and Emr, 2001). (B) Mutations from a are labeled on the structure of HOPS (see Figure 2) as red spheres. In cases where mutations were described for a different organism, respective homologous residues in yeast were mapped according to the alignment in A.

**Supplementary File 1.** Yeast strains used in the study.

**Supplementary File 2.** Cryo-EM data collection, refinement and validation statistics

**Supplementary File 3.** Crystallographic data collection and refinement statistics (molecular replacement)

**Video 1.** Overall architecture of HOPS tethering complex: transition between ribbon and molecular surface representation.
**HOPS subunits**

<table>
<thead>
<tr>
<th>HOPS subunit</th>
<th>Vps11</th>
<th>Vps16</th>
<th>Vps18</th>
<th>Vps33</th>
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<td>918</td>
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**Predicted domains (UniProt)**

- RING finger domain
- CHCR domain
- disordered

**Structural features**

- β-propeller
- α-solenoid
- SM protein

**Retention volume [ml]**

- 0.5
- 1.0
- 1.5
- 2.0
- 2.5

**Absorbance at 280 nm [mAU]**

- 0
- 25
- 50
- 75
- 100
- 125
- 150
- 175

- 0.5
- 1.0
- 1.5
- 2.0
- 2.5

**Mass [kDa]**

- 0
- 40
- 80
- 120

**Counts**

- 10
- 13
- 17

**Figures**

- (a) Bar chart showing HOPS subunits with predicted domains and structural features.
- (b) Chromatogram graph showing retention volume and absorbance at 280 nm.
- (c) Graph showing retention volume and mass.
- (d) Image of HOPS purification.
- (e) Three-dimensional models of HOPS showing domains and structural features.
- (f) Detailed structural model highlighting SNARE-binding module and β-propellers.
- (g) Schematic view of protein interactions with Ypt7-binding and rigid core.
Vps18 and Vps41 coiled coil

Vps18 RING finger domain

Vps11 and Vps39 coiled coil

Vps11 RING finger domain

Vps18 structured loop and C-terminus

Vps39 N-terminal α-helix

Vps18 β-propeller

Vps18 α-solenoid domain
Patch motion correction, CTF estimation
Blob and template picking, duplicates removal - 816K particles
Ab-initio multiple-class reconstructions and hetero-refinements, duplicates removal - 145K particles
Ab-initio multiple-class reconstruction and hetero-refinement

2D classification, selection
383k particles
Ab-initio multiple-class reconstruction and hetero-refinement
407k particles
Ab-initio single-class reconstruction, homogeneous and NU-refinement

NU-refinement - 432k particles
Analysis of flexibility between classes (Extended Data Fig. 6a,b)
3D variability analysis (Extended Data Fig. 6d)

Rounds of hetero-, homo-, NU-refinements for the best classes resolving Vps41 or Vps39

2D classifications, ab-initio multiple-class reconstruction and hetero-refinement, duplicates removal - 480k particles

Homogeneous refinement
NU-refinement - 432k particles
3D variability and 3D variability display in cluster mode

Local refinement
Local refinement
Local refinement
Local refinement
3D variability and 3D variability display in cluster mode