Materials and Methods

Protein expression and purification

Full-length *S. cerevisiae* Vps4p E233Q, ΔN-Vps4p (residues 104–437) E233Q, and Vta1p were expressed and purified as previously described.[28] Briefly, proteins were expressed from a pET151 vector (Invitrogen) and auto-induced in BL21 codon + *Escherichia coli* cells in ZY media (37 °C for 6 h and 21 °C overnight). All three proteins were purified by Ni^{2+} Sepharose, Q-Sepharose, and Superdex-200 column chromatography. The histidine tag was removed by incubation with TEV protease (~1 mg/100 mg protein, 20 h, at 4 °C), and the mixture of cleaved and uncleaved Vps4p or Vta1p proteins was separated by Ni^{2+} Sepharose chromatography. The cleaved protein was collected in the flow-through, dialyzed into 25 mM Tris, pH7.4, 100 mM NaCl, and 1 mM DTT, concentrated, and used directly for cross-linking.

Analysis of the Vta1p-Vps4p complex

Vps4p and Vta1p were mixed at molar ratios of 2:1, 1:1, and 1:3 in the presence of ATP, and the resulting complexes were isolated by size-exclusion chromatography (as described above). Fractions corresponding to the Vta1p–Vps4p and free Vta1p complexes were subjected to SDS-PAGE; the component proteins were visualized by Coomassie Blue staining, and their band intensities were quantified and analyzed by densitometry on an Odyssey fluorescence scanner (Li-Cor, Lincoln, NE) using the Odyssey scanning software. Absolute protein concentrations were determined by comparing band intensities with standard curves made from known concentrations of pure Vps4p and Vta1p.

Cross-linking

Oligomeric Vps4p and Vta1p–Vps4p complexes were isolated by size-exclusion chromatography (Superdex-200HR) in buffer A (100 mM NaCl, 25 mM Tris–HCl, pH 7.5, 2 mM $MgCl_2$, 1 mM ATP, and 1 mM DTT) and cross-linked with 0.02% glutaraldehyde at a protein concentration of 20 μg/ml in 0.1 M potassium acetate, 2 mM magnesium acetate, 20 mM Hepes, pH 7.4, and 1 mM ATP following the protocol reported for ClpB.[45,47] After 20 min of incubation at room temperature, the reaction was quenched by addition of an equal volume of 1 M glycine. The cross-linked complexes were concentrated and re-analyzed by size-exclusion chromatography in buffer A without ATP and $MgCl_2$, and peak fractions were collected for EM analysis.

Cryo-EM data collection

Vps4p solutions (4 μl) at a concentration of 0.3 mg/ml were applied to holey carbon film-covered copper grids, and excess solution was blotted away with filter paper in a 100% relative humidity chamber. Grids were plunged into liquid ethane using a Vitrobot and transferred to 300 kV of an FEI G2 Polara transmission electron microscope equipped with a field emission gun and an energy filter. Projection images were recorded on a charge-coupled device (2 K × 2 K) under low-dose conditions at a nominal magnification of 50,000×, which resulted in a pixel size of 4.6 Å on the charge-coupled device. The images were taken with a range of defocuses from 2.5 to 5.5 μm of underfocus.

Image processing and three-dimensional reconstruction

Particle selection, contrast transfer function correction, and three-dimensional reconstruction were done using the EMAN software suite.[69] First, the power spectrum of each digital image was calculated, and only those images without drift were used for further analysis. Particles were picked manually using *boxer*: 9593, 4190, and 4445 particles were picked for the ΔN-Vps4p, full-length Vps4p, and Vta1–Vps4p complexes, respectively. The defocus of each image and the corresponding contrast transfer function were determined using *ctfit*. Phase corrections were applied to all particles. Symmetry analyses were performed as described in the main text.

The first three-dimensional reconstruction was of the full-length Vps4p complex. A reference-free initial model was generated using *startnrclasses* and *startAny*. Specifically, all the projection images were divided into 50 classes based on their similarity, and particles within each class were aligned and averaged to generate class averages. Eight representative class averages were chosen to build an initial three-dimensional model using the cross-common line method. Projections of this initial model were calculated to reclassify the particles and generate new class averages and a new three-dimensional model. This process was repeated iteratively until the model converged. Six-fold symmetry was imposed throughout the reconstruction process. A second reference-free initial model was produced using a different set of class averages and converged to essentially the same result. The first refined full-length Vps4p model was then used as the initial model to reconstruct the ΔN-Vps4p and Vta1p–Vps4p complexes. As controls, multiple reference-free models of the ΔN-Vps4p and Vta1p–Vps4p complexes were also calculated as described above for full-length Vps4p, and an additional model of the full-length Vps4p structure was produced using one of these ab initio models of ΔN-Vps4p as an initial reference. All the various models of ΔN-Vps4p, full-length Vps4p, and Vta1p–Vps4p confirmed that the complexes assembled as two stacked hexameric rings with a large central cavity in the top ring. In the final reconstructions, 4775, 1506, and 3497 particles were included for the ΔN-Vps4p, full-length Vps4p, and Vta1p–Vps4p complexes, respectively. The contour levels (thresholds) used for surface representations were estimated by assuming 0.81 Da/$Å^3$ (1.35 g/ml). The isosurface maps in Figs. 6, 8 and 9 were produced using the UCSF Chimera package.[84] The final ΔN-Vps4p, full-length Vps4p, and Vta1p–Vps4p structures will be deposited in the Electron Microscopy Database.

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