Vps4 disassembles an ESCRT-III filament by global unfolding and processive translocation

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The endosomal sorting complex required for transport (ESCRT) machinery is essential for budding and release of HIV-1 (ref. 1), multivesicular-body biogenesis2, cytokinesis3,4, exosome biogenesis5 and membrane wound repair6. The ESCRTs consist of ALIX, ESCRT-0, ESCRT-I and ESCRT-II, which bind to ubiquitin and viral and cargo proteins7; ESCRT-III, which severs membrane necks8; and Vps4, a member of the ATPases associated with diverse cellular activities (AAA+)9,10. Vps4 is required for the recycling of ESCRT-III from membrane-bound filaments back to the cytosol11 and probably has additional roles in the ESCRT pathway. As such, the absence or inhibition of Vps4 leads to a complete shutdown of HIV-1 budding and multivesicular-body formation.

There are seven ESCRT-III proteins in yeast and 12 in humans. These proteins are monomers or dimers in solution, but they carry out their functions as membrane-bound oligomeric assemblies. All ESCRT-III proteins share a common fold consisting of a long α1-α2 helical hairpin and three shorter helices, α3–α5 (refs. 12–14). The C termini of ESCRT-III proteins include autoinhibitory elements that prevent premature assembly15–17. ESCRT-III proteins form a variety of assemblies: filaments18, spirals19–22 and helical tubes9,13. These assemblies do not spontaneously dissociate. Rather, the enzymatic action of Vps4 is required to disassemble them.

Some ESCRT-III subunits contain C-terminal microtubule interacting (MIT)-interacting motifs (MIMs) that bind to Vps4 (refs. 23,24). These include the α-helical MIM1 motif of Vps2 (human CHMP2), Diid2 (CHMP1) and Ist1, and the extended MIM2 motif of Vps20 (CHMP6)25,26. Other ESCRT subunits bind to Vps4 with low affinity, if at all. Vps4 binds to the MIM1 and MIM2 motifs of ESCRT-III subunits via two sites on its N-terminal MIT domain23,24,26. In the presence of ATP, wild-type Vps4 exists predominantly as hexamers27 (although dodecamers predominate when ATP hydrolysis is blocked) and is therefore believed to function as a ring-shaped hexamer. In the absence of ATP, Vps4 exists as monomers and dimers, and available crystal structures of Vps4 reflect these inactive states28–31. EM reconstructions have been obtained for yeast Vps4 oligomers stabilized by the active site glutamate-residue mutant E233Q or AMP-PNP30,32,33 and have been interpreted in terms of dodecamers or tetradecamers consisting of two rings. None of these reconstructions have been at a resolution high enough to place the crystallographic Vps4 monomers. However, the lower ring in one of the reconstructions contains a central pore and is consistent with a hexameric model of Vps4 (ref. 34).

This model predicts that conserved tryptophan and leucine residues line the central pore. These residues are important for HIV-1 budding in vivo28. Mixed Vps4 hexamers containing a single catalytically active ATPase site and a single MIT domain can disassemble ESCRT-III as long as the putative central pore is intact35. These data suggest that portions of ESCRT-III subunits enter the pore during disassembly. In one model, ESCRT-III unfolds completely and translocates through the pore, similarly to substrates processed by the AAA+ unfoldase ClpX36,37. ClpX degrades proteins in conjunction with the peptidase ClpP, but it also has disassembly functions independent of ClpP38. In a second model, Vps4 breaks up filaments by locally disrupting contacts in the assembly. This would be similar to the mechanism of SNARE disassembly by NSF, which appears to unwind SNAREs but does not translocate them through the pore39. In the second model, the region entering the Vps4 pore would probably be limited to parts of the C-terminal region. We set out to address whether Vps4 denatures its substrate globally or whether a more localized perturbation is involved.

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We selected the chimeric ESCRT-III subunit Vps24-2 (Fig. 1a) as a model substrate because its core is a stably folded unit\(^{12}\), it forms homo-oligomeric filaments (Supplementary Fig. 1a) and it binds to Vps4 via the MIM1 of Vps2 (ref. 18). The Vps24-2 filament is the only homo-oligomeric ESCRT-III assembly that has been shown to be completely disassembled by Vps4 in vitro. We measured the baseline conformational dynamics of Vps24-2 filaments and its changes upon exposure to Vps4, using hydrogen/deuterium exchange and MS (HDX-MS). These experiments showed that Vps24-2 is completely unfolded concurrently with its removal from the filament. We went on to show that the unfolding process has close parallels with that of the well-characterized unfoldase ClpX. We then showed that unfolding is coupled to translocation through a central pore in the Vps4 hexamer. Pore-loop residues were required for disassembly, and they presumably act by directly engaging the substrate and mechanically pulling on it in response to ATP hydrolysis–driven conformational changes of the Vps4 ring. Finally, cross-linking between an engineered cysteine residue in the Vps4 pore and cysteines in Vps24-2 revealed that the core region of ESCRT-III comes into direct contact with the pore, indicating complete threading through the Vps4 hexamer pore.

**RESULTS**

**Dynamics of Vps24-2 monomers and filaments**

In order to establish the baseline dynamics of Vps24-2 monomers in solution and as assembled in filaments, we performed an HDX characterization of these states in the absence of Vps4. We obtained excellent peptide coverage (Supplementary Fig. 2) and examined peptides representing helices α1–α4. We collected Vps24-2 from the gel-filtration peak fraction corresponding to the monomer and immediately subjected it to HDX for various times. By 60 s, the majority of the monomeric protein had exchanged at least 50% of its protons (Fig. 1b and Supplementary Fig. 3). The core α1–α2 helical hairpin was the region least susceptible to exchange (Fig. 1b). Because Vps24-2 spontaneously forms filaments at high concentration, we also performed HDX studies of the monomer at higher concentration by using a construct containing maltose-binding protein (MBP) fused to the N terminus in order to inhibit polymerization (Fig. 1a and Supplementary Fig. 1b). We incorporated a C-terminal ssrA tag into this construct to facilitate control experiments with ClpX (described below). We observed similar HDX-exchange patterns for MBP–Vps24-2–ssrA and for the monomeric Vps24-2 construct (Fig. 1b and Supplementary Fig. 3b).

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**Fig. 1** Dynamics of a model ESCRT-III substate of Vps4. (a) Schematic diagram of MBP–Vps24-2–ssrA and Vps24-2 constructs used in the study. Residue numbers from Vps24 and Vps2 are shown for the construct boundaries. (b,c) Deuterion incorporation over time for the Vps24-2 monomer (b) and Vps24-2 filament assembly (c), mapped onto a Vps24-2 structural model based on the crystal structure of the human Vps24 ortholog, CHMP3 (ref. 12). The inset shows the color coding for different percentages of deuterion incorporation. The HDX experiments were repeated twice. The start and end amino acids of each α-helix are labeled at the far left of b. (d) HDX changes for Vps24-2 in filament assembly as compared to soluble monomers, color coded as shown (red and orange for increased HDX in the filament relative to the monomer; blue and green for decreased HDX). We induced polymerization of Vps24-2 by increasing its concentration to above 350 μM and incubating at 4 °C overnight, according to methods described in ref. 18 (Supplementary Fig. 1a). We carried out HDX with the resulting filamentous material (Fig. 1c) and found sharply reduced exchange for the N terminus of helix α1 and the entire α3 and α4 helices (Fig. 1c,d) as compared to the soluble monomer. In contrast, HDX of α5 increased in the filament as compared to the soluble monomer (Fig. 1c,d). This is consistent with a role for α5 as an autoinhibitory element in the soluble ESCRT-III monomer\(^{15,16}\).

We analyzed EX1 and EX2 exchange kinetics to probe the mechanism of exchange of the Vps24-2 monomer and filament (Fig. 2 and Supplementary Fig. 4). EX1 kinetics are diagnostic for the persistence of global unfolding for a time long enough for the majority of labile protons to be exchanged. In contrast, EX2 kinetics are indicative of a folded protein that samples an exchange-competent conformation for a time shorter than the intrinsic exchange rate of the labile protons\(^{40}\). We selected six peptides that fall entirely within the folded core of Vps24-2 for analysis (Fig. 2a). In the monomer, four of these peptides, from helix α1, α2 and α4 (Fig. 2a), exchanged gradually by an EX2 mechanism. EX2 behavior is evident from inspection of the spectra, which gradually shift to higher mass over time while remaining unimodal (Fig. 2b–d and Supplementary Fig. 4c).

It has been proposed that a peak-width change that remains within 2 Da of its initial value throughout the course of exchange is diagnostic for the EX2 mechanism\(^{41}\). To provide a quantitative assessment of the exchange mechanism, we evaluated peak widths as a function of time. The width of the peak, as defined at a threshold of 20% of the maximum value, remained within or near 2 Da of its initial value for the four peptides from helices α1, α2 and α4 at all time points measured (Fig. 2e–g and Supplementary Fig. 4d). Even though the peak-width change for peptides from the distal part of α1 (Supplementary Fig. 4b) and α3 (Supplementary Fig. 4f) remained within 2 Da at all time points measured, the deuteron incorporation for these two peptides (Supplementary Fig. 4a,e) was so rapid (~ 80% deuteron incorporation at 5 s and >90% deuteron incorporation by 30 s) that neither EX1 nor EX2 kinetics could be unambiguously assigned to these two peptides for the Vps24-2 monomer.

For the Vps24-2 filament, exchange was more gradual for all six peptides examined (Fig. 2b–d and Supplementary Fig. 4a,e).
Vps4 completely unfolds ESCRT-III substrate in the course of disassembly. (a) Folded (F)-to-unfolded (U) conversion for the indicated peptides in the presence of Vps4, color coded as in the Vps24 structural model in the inset. (b–d) Mass spectra of selected peptides from helices α1-α2 (b), α2 (c) and α4 (d) of Vps24-2 monomers (mono), filaments (fila) or filament +Vps4. Guanidine HCl (GdnHCl) controls and time points are indicated. Regions representing EX2 and EX1 behavior are indicated with F and U above the graph. (e–g) Peak-width analysis of the selected peptides at 5, 10, 20, 30, 40 and 60 s for selected peptides from helices α1-α2 (e), α2 (f) and α4 (g). Gray bars, 2-Da peak-width change allowance for peptides undergoing EX2 kinetics. (h) SDS-PAGE gel (uncropped image in Supplementary Data Set 1) and quantification bar chart of Vps4-mediated disassembly of Vps24-2 filaments (shown as percentage of filaments solubilized), analyzed by sedimentation. P, pellet; S, supernatant. Error bars, s.d. of three experiments.

Vps4 disassembles ESCRT-III by unfolding entire subunits

We quantified the disassembly of Vps24-2 filaments by Vps4 by monitoring the fraction of solubilized Vps24-2 in the supernatant after ultracentrifugation (Fig. 2h). Vps4 solubilized about 30% of the Vps24-2 within 10 s and 80% by 60 s (Fig. 2h). We therefore monitored HDX of Vps24-2 over the interval of 5–60 s (Fig. 2b–g and Supplementary Fig. 4). All six peptides from helices α1 to α4 manifested clearly visible bimodal distributions during exchange (Fig. 2b–d and Supplementary Fig. 4a,c,e). We carried out peak-width analysis for all the six peptides in the presence of Vps4 (Fig. 2e–g and Supplementary Fig. 4b,d,f). Maximum peak widths increased dramatically, to as much as 10 Da higher than the initial value for the peptide of amino acids (aa) 3–24 (Supplementary Fig. 4b). We interpret this as compelling evidence for an EX1 exchange mechanism that is dependent on the presence of Vps4. This provides strong evidence that Vps4 unfolds Vps24-2 in the process of solubilizing it. We obtained the rate of Vps24-2 conversion from the folded (F) to the unfolded (U) state by fitting two Gaussian peaks to the bimodal isotope cluster and dividing the areas of the bimodal isotope cluster by the isotope cluster area of the unfolded state (U/(F + U)). We observed similar conversion rates for all six peptides (Fig. 2a), a result consistent with their dependence on a single global unfolding process.

Vps4 and ClpX unfold MBP–Vps24-2–ssrA by a common mechanism

The ClpX unfoldase is among the most extensively characterized members of the AAA+ ATPase superfamily. ClpX engages soluble folded protein substrates and denatures them by processively translocating them through its central pore. In the context of the ClpXP protease, ClpX threads these denatured substrates directly into the internal degradation chamber of the associated peptidase ClpP for proteolytic cleavage, whereas in isolation ClpX releases translocated polypeptides on the other side of the ring. We included an 11-residue ssrA tag at the C terminus of MBP–Vps24-2–ssrA to allow its specific recognition by ClpX. We carried out parallel HDX studies of the processing of soluble MBP–Vps24-2–ssrA monomers by Vps4 and ClpX in order to make a side-by-side comparison of the unfolding mechanisms.

We incubated either Vps4 or ClpX, at similar molar concentrations, with soluble MBP–Vps24-2–ssrA and triggered substrate processing and proton exchange simultaneously by dilution of the solution to 5% of its previous concentration into ATP and 100% D2O. Under these conditions, we observed robust EX1-dominated exchange for peptides from the Vps24-2 portion of MBP–Vps24-2–ssrA (Fig. 3) at 10–60 s. Vps4 and ClpX unfolded the Vps24-2 portion of the construct to a similar extent, as judged by the bimodal spectra (Fig. 3b–d) and peak-width analysis (Fig. 3e–g). The total extent of unfolding was similar for ClpX and Vps4 when compared for all three peptides (Fig. 3h).

To address the relative potency of Vps4 and ClpX as generic unfoldases, we examined their ability to unfold regions of the MBP
portion of the fusion construct. When we treated substrate with ClpX, a peptide from the C-terminal portion of MBP (aa 347–360), encompassing helix XIII and helix XIV [Fig. 4a] showed around 7%, 16% and 30% unfolding at 10, 30 and 60 s (Fig. 4b,d). However, the same peptide showed around 5%, 8% and 8% unfolding at 10, 30 and 60 s when Vps4 was present. (Fig. 4b,d). An N-terminal peptide of MBP (aa 77–104), which maps to the center of the MBP helix [Fig. 4c], showed no visually detectable unfolding at 10 s by either Vps4 or ClpX. ClpX robustly unfolded the same peptide by 60 s (30%), with trace unfolding by Vps4 over the same time period (Fig. 4c). We also examined another four peptides in between these two peptides and found that all showed detectable bimodal spectra by 30–60 s in the presence of either ClpX or Vps4 (Supplementary Fig. 5). At the earliest time examined, 10 s, ClpX unfolded a two- to three-times-greater fraction of the C-terminal peptide of MBP as compared to the N terminus (Fig. 4d). By 60 s, the difference disappeared for ClpX, consistently with the known processive C-to-N unfolding mechanism. In the case of Vps4, the difference persisted, thus suggesting that Vps4 might be stalling after failing to disassemble the central region of MBP. As compared to Vps4, ClpX unfolds MBP to a 200%-greater extent in the C-terminal region and a 500%-greater extent in the central and N-terminal regions by 60 s. This seems in line with expectation, given that ClpX is a relatively nonspecific unfoldase (as long as the substrate contains an ssrA motif), whereas Vps4 is specialized for ESCRT-III disassembly. Although ClpX is, unsurprisingly, the more robust unfoldase, the main inference drawn from the MBP experiments is that Vps4 and ClpX are qualitatively similar in their processive unfolding of the model substrate.

**Substrates enter the central pore of Vps4**

The similarities in the kinetics of unfolding behavior seemed consistent with the previous suggestion that Vps4 might, by analogy to ClpX and other AAA+ unfoldases, translocate unfolded ESCRT-III subunits through the central pore (Fig. 5a) of the hexamer. In order to directly test the role of pore-loop residues in substrate disassembly, we generated the pore-loop double mutant Vps4W206A M207A (Fig. 5a). The basal ATPase activity of Vps4W206A M207A was slightly greater than half that of wild-type Vps4 (Vps4WT) (Supplementary Fig. 6b). However, Vps4W206A M207A was completely unable to solubilize Vps24-2 filaments (Fig. 5b,c). These data confirm that mutation of the pore loop directly blocks ESCRT-III disassembly, explaining the previously reported loss of function of human VPS4 pore-loop mutants in HIV-1 budding.

In order to determine whether the ESCRT core becomes exposed and directly interacts with the Vps4 pore during processive translocation, we applied site-directed cross-linking between an engineered cysteine in the Vps4 pore loop and cysteines introduced at positions 92 or 136 of Vps24-2. These two cysteines in Vps24-2 are located in α2 and α4 of the core domain, respectively (Supplementary Fig. 6c). We generated a cysteine-free Vps4 variant (Vps4CF, herein referring to Vps4C317A C376A) and used it to introduce a single cysteine at position 206 in the central pore (Vps4CF W206C). Vps4CF, as compared to wild type, had greater than half the ATPase activity and essentially identical Vps24-2 solubilization activity (Supplementary Fig. 6a,b). The ATPase activity of wild-type...
Vps4 and variants increased upon exposure to the Vps24-2 substrate, as has previously been found for other Vps4-substrate combinations.\(^49\)

We carried out cross-linking in the presence and absence of ATP or the nonhydrolyzable ATP analog AMP-PNP. In initial experiments, some cross-linking occurred between Vps4 CF 206C and Vps24-2 136C in the absence of nucleotide or in the presence of AMP-PNP (data not shown). We hypothesized that this behavior was due to the equilibration of Vps4 between the hexameric state and the monomeric state, in which the pore-loop Cys206 is solvent exposed and accessible for low-efficiency cross-linking even with folded Vps24-2. In order to protect monomeric Vps4 CF 206C from reaction, we truncated its N-terminal Vps2-binding MIT domain. The presence of the MIT domain is required for recruitment of ESCRT-III; therefore, the monomeric MIT-deleted construct (Vps4 CF 206C MIT) cannot, by design, bind to ESCRT-III. We combined Vps4 CF 206C MIT at a 1:8 molar ratio with full-length Vps4 CF to generate mixed hexamers that contain on average less than one cross-linkable Vps4 CF 206C MIT subunit (Fig. 6a). The hexamers used in the cross-linking studies described below contain, on average, five MIT domains contributed by the five full-length subunits. These mixed hexamers had basal and substrate-stimulated ATPase activity identical to that of homohexameric Vps4 CF (Supplementary Fig. 6b).

Vps24-2 136C cross-linked efficiently to the Vps4 CF 206C MIT subunits in mixed hexamers but did so only in the presence of ATP (Fig. 6b,c). No reaction was evident in the absence of nucleotide or in the presence of AMP-PNP. We mutated the catalytic amino acid Glu233 in the Walker B motif to glutamine to produce an ATP hydrolysis–deficient mutant in the cysteine-free background (Vps4 EQ CF). As a further control, we presented Vps4 EQ CF 206C MIT in mixed hexamers with full-length Vps4 EQ CF, which lacks ATPase activity\(^50\) (Supplementary Fig. 6b). We observed no cross-linking with this ATPase-inactivated Vps4 even in the presence of ATP. Finally, to explore whether the cross-linking behavior was general to other regions of the Vps24-2 structure, we replaced Ile92 of the Vps4 CF 206C MIT subunit with alanine to produce a Vps4W206A M207A mutant which lacks ATPase activity (Supplementary Fig. 6b). We observed no cross-linking with this ATPase-inactivated Vps4 even in the presence of ATP. Finally, to explore whether the cross-linking behavior was general to other regions of the Vps24-2 structure, we replaced Ile92 of the Vps4 CF 206C MIT subunit with alanine to produce a Vps4W206A M207A mutant which lacks ATPase activity (Supplementary Fig. 6b). We observed no cross-linking with this ATPase-inactivated Vps4 even in the presence of ATP.

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solvent. Cys92 reacted with about 60% of the efficiency of Cys136 (Fig. 6b,c), a result consistent with its more N-terminal position. The control reactions with no nucleotide, in the presence of AMP-PNP, or the mixture with ATP and the Walker B mutant Vps4EQ CF yielded no reaction with Vps24-292C (Fig. 6b,c). Finally, when we presented Vps4CF 206C MIT in mixed hexamers with Vps4W206A M207A CF, we observed no cross-linking to either Vps24-292C or Vps24-2136C in the presence of ATP (Fig. 6b,c). These data showed that only two parts of the ESCRT-III structural core come into direct contact with the hexamer pore in a manner that depends on ATP hydrolysis and intact pore loops.

**DISCUSSION**

The HDX and cross-linking data provide strong evidence for unfolding and processive translocation of a model ESCRT-III subunit by Vps4. These findings provide direct confirmation for a model that has been favored in the field for a decade yet has eluded direct verification. The global denaturation mechanism might seem excessive at first glance. In principle, less ATP might be consumed if only local regions of assembly contacts in ESCRT-III were destabilized. However, Vps4 functions in the disassembly of many different ESCRT-III subunits. It seems likely that the early-acting subunit Vps20 might form one type of contact with Snf7, which composing the bulk of the complex. Late-acting and less abundant subunits, such as Vps2, Did2 and Ist1, probably have yet other types of contacts. ESCRT-III forms a range of filaments and tubes of varying diameters, with the ones observed in vivo ranging from 1.5-µm-diameter midbodies in cytokinesis to 25-nm intralumenal bud necks in yeast multivesicular bodies. Although it has not yet been established that all ESCRT-III assemblies are functional or competent for disassembly, it is unlikely that their structural nature is identical. Global denaturation has a clear advantage in providing a single mechanism to deal with all of these situations.

In the context of ESCRT-III disassembly at sites of vesicle budding on membranes, the translocation mechanism offers some apparent benefits. The ESCRT complexes are likely to be densely packed at sites of vesicle budding and scission. Available EM images are consistent with the concept that the vicinity of these sites is sterically crowded. The translocation mechanism allows the newly liberated ESCRT-III subunit to emerge on the side of Vps4 that is distal to the membrane. This relocalization has the virtue of preventing the extracted ESCRT-III subunit from simply reassembling into the newly vacant site created by its own departure from the assembly.

Vps4 belongs to the meiotic clade of AAA+ ATPases. The other members of this clade include the microtubule-severing enzymes spastin and katanin, which are thought to function by removing individual tubulin subunits from microtubules. A small-angle X-ray scattering reconstruction of spastin showed that it is a hexamer with a central pore, similar to models proposed for Vps4 (ref. 52). On the basis of the observation that spastin possesses a central pore, it has been proposed that at least a portion of the tubulin monomer is unfolded and threaded through this pore in the course of disassembly. Given the close parallels between Vps4 and spastin, together with the results presented here, it seems reasonable to speculate that spastin could completely denature tubulin subunits and extrude them all the way through the central pore.

Some ESCRT-III subunits, such as Vps24, do not bind directly to Vps4, and the question remains as to how these subunits are removed. Once the Vps4-binding subunits are denatured, any noncovalent interactions with the remaining Vps24 subunits would be lost. One possibility is that Vps4 recruitment to directly-binding subunits also brings nonbinding ESCRT-III subunits such as Vps24 into sufficient proximity of the central processing pore for them to be engaged and translocated. A similar mechanism has been described for the degradation of heterodimeric substrates by the ClpAP protease, in which only one of the substrate monomers contains a degradation signal but delivers the second monomer by tethering it to the protease. A simple alternative explanation is that not all of the ESCRT-III subunits actually need to be processed through the central pore. Once a critical number of subunits are released from the lattice, remaining subunits might spontaneously or cooperatively dissociate. Such a mechanism would be consistent with our observation that over the same 1-min time frame, in which ~75% of Vps24-2 is disassembled, only ~35–40% of the Vps24-2 subunits undergo unfolding. This suggests that Vps4 may have to process as few as only one-half of the subunits in the assembly in order to completely break it apart.
Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

B.Y. conceived the project, created reagents, acquired data, analyzed data and wrote the manuscript; G.S. analyzed data; Q.S. acquired data; A.M. conceived the project and wrote the manuscript; J.H.H. conceived the project, analyzed data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Protein expression and purification. We generated a sequence encoding S. cerevisiae Vps24 (1–179 aa) and Vps2 (181–232 aa) through overlapping PCR and cloned it into the pMBP-His vector. We expressed the resulting Vps2-2 construct as an N-terminal TEV-cleavable His6-MBP fusion in E. coli Rosetta2 (DE3) at 20 °C overnight in LB medium. We purified the protein with His-Talon resin (Clontech). We mixed the eluted fractions from the His-Talon column with tobacco etch virus (TEV) protease (1:200 (w/w)) and dialyzed the pooled fractions against buffer QA (20 mM Tris-HCl, pH 8.0, 50 mM NaCl and 5 mM β-mercaptoethanol) overnight. We then loaded the cleaved samples onto a 5-ml HiTrap Q-Sepharose FF column (GE Healthcare) and eluted the material with a gradient of 0–100% buffer QB (20 mM Tris-HCl, pH 8.0, and 500 mM NaCl) in 12 column volumes. We pooled and gel-filtered the peak fractions containing untagged Vps24-2 in buffer A (20 mM Tris, pH 8.0, and 100 mM NaCl) on a HiLoad 16/60 Superdex 75 (GE Healthcare) column. We expressed full-length S. cerevisiae Vps4 (1–437) as a fusion protein with an N-terminal TEV-cleavable His6 tag. We carried out the expression and purification of Vps4 in the same way as for Vps2-2. We generated the cysteine-free Vps4 (Vps4CF) Vps4W206A M207A mutant and single-cysteine mutants of Vps4 and Vps2-2 through site-directed mutagenesis and purified them as for Vps2-2 except that the N-terminal histidine tag of Vps4 mutants was not cleaved off. K. Nyquist (Martin laboratory, University of California, Berkeley) gave us the ClpX and ClpP proteins.

We generated the MBP-Vps24-2–ssrA construct by adding an ssrA tag (AANDENYALAA) to the C terminus of Vps24-2 through overlapping PCR. We induced expression of the resulting fusion protein with 1 mM IPTG at 30 °C for 90 min. We purified MBP-Vps24-2–ssrA protein by His-Talon resin affinity chromatography (Clontech), gel filtration on a HiLoad 16/60 Superdex 200 (GE Healthcare) column and ion exchange on a HiTrap Q-Sepharose FF column (GE Healthcare).

Sedimentation analysis of filament disassembly. We assembled Vps24-2 filaments by concentrating the protein to at least 350 µM and incubating it at 4 °C overnight. We confirmed filament formation by ultracentrifugation of protein samples in a TLA100 rotor at 50,000 rpm for 30 min at 10 °C. After ultracentrifugation, the pellet (P) and supernatant (S) were diluted with equal volumes of SDS-PAGE loading buffer, and equal volumes of S and P fractions were analyzed on a 4–12% Bis-Tris gel run with MES (2-(N-morpholino)ethanesulfonic acid) buffer. Gels were visualized by Coomassie blue staining, and the intensity of bands was quantified with ImageJ.

For sedimentation analysis of Vps24-2 filament disassembly by Vps4, Vps4CF and the Vps4W206A M207A mutant, we mixed 3.6 µM Vps24-2 filament assembly with 10.2 µM Vps4 or mutant in buffer B (50 mM HEPES, pH 7.5, 100 mM KCl, and 5 mM MgCl2). We initiated reactions by adding ATP to a final concentration of 1 mM. After incubation at 30 °C for 10, 30, or 60 s, we quenched the reactions at 0 °C by adding EDTA to a final concentration of 50 mM. We subjected the samples to ultracentrifugation and analyzed them as described above. We obtained data from three independent repeats.

Hydrogen/deuterium-exchange mass-spectrometry measurements. For HDX-MS experiments with MBP–Vps24-2–ssrA, Vps24-2 monomer, and Vps24-2 filament assembly, we initiated amide hydrogen exchange by diluting 5-µl protein samples (67 µM for MBP–Vps24-2–ssrA, 25 µM for Vps24-2 monomer and 72 µM for filaments) into 95 µl D2O buffer (50 mM HEPES, pH 7.5, 100 mM KCl and 5 mM MgCl2) containing 1 mM EDTA at 30 °C. At various time points, we quenched the exchange reaction by cooling to 0 °C and adjusting pH to 2.2 through adding an equal volume of ice-cold quench buffer 1 (400 mM KH2PO4/K2HPO4, pH 2.2, and 6 M guanidine HCl). After incubation on ice for 20 s, we further diluted the quenched samples to 20% of their previous concentration through addition of ice-cold quench buffer 2 (400 mM K2HPO4/KH2PO4, pH 2.2) and injected samples into an HPLC-MS (Agilent 1100) system with in-line peptic digestion and desalting, immersed in ice bath. We eluted the desalted peptides and directly analyzed them with an Orbitrap Discovery mass spectrometer (Thermo). We extensively cleaned the HPLC system between samples. We prepared fully deuterated control samples by three cycles of drying and resolubilization in D2O buffer and 6 M guanidine HCl. We analyzed both nondeuterated and fully deuterated Vps24-2 samples (or MBP–Vps24-2–ssrA) to serve as 0% and 100% controls.

For MBP–Vps24-2–ssrA and Vps24-2 filament assembly with Vps4, we preincubated 67 µM MBP–Vps24-2–ssrA or 72 µM Vps24-2 filament assembly with 200 µM Vps4 on ice for 5 min. We initiated amide hydrogen exchange by dilution of the preincubated sample to 5% of its previous concentration with D2O buffer containing 1 mM ATP at 30 °C. The subsequent experimental procedure was as described above. After dilution to 5% of its previous concentration, we kept the protein concentration the same as in the sedimentation assays. For experiments on MBP–Vps24-2–ssrA with ClpX, we preincubated 52.5 µM MBP–Vps24-2–ssrA with 150 µM ClpX on ice for 5 min, and the subsequent experimental procedure was as described above. For deuteron-incorporation measurements of undigested protein at 11 different time points ranging from 10 s to 15 min, we performed experiments as above except that the in-line peptic digestion was omitted, and the elution was performed without gradient.

HDX data analysis. We performed initial peptide identification by running tandem MS/MS experiments. We identified peptides with PEAKS Studio 7 (http://www.bioinfor.com/). We carried out the initial analysis of the peptide centroids with HD-Examiner v1.3 (Sierra Analytics) and then manually verified every peptide to check retention time, charge state, m/z range and the presence of overlapping peptides. We calculated relative deuteration levels (%D) of the peptic peptides covering 95% of Vps24-2 by comparing the centroids of the molecular ion isotope envelope to that of the nondeuterated and fully deuterated samples with HD-Examiner v1.3. We adjusted the deuteron content for deuteron gain/loss during digestion and HPLC. For analysis of the EX2- to EX1 transition, we calculated the abundance of the two deuterated mass species by fitting two Gaussian peaks to the bimodal isotope cluster in Prism (GraphPad). The isotope cluster area of the unfolded state (U) was divided by the sum of the areas of the bimodal isotope cluster (U/(F + U)) and plotted versus the exchange time.

For deuteron incorporation measurements of undigested protein samples, we calculated the molecular weight (MW) of undigested sample with GPMAW 8.2 (Lighthouse Data), and we calculated the relative deuteron incorporation at each time point by dividing the deuteron incorporation at each time point (MW−DWundetermined) by the maximum deuteron incorporation (MWfully deuterated–MWundetermined).

Cross-linking. We mixed Vps4CF (or Vps4W206A M207A CF) with Vps4EQ CF 206C ΔMIT, and we mixed Vps4EQ CF 206C ΔMIT with Vps4EQ CF 206C ΔMIT, both at a molar ratio of 8:1. We then reduced the resultant mixture at 22 °C for 1 h with 5 mM DTT; concentrated it to 400 µM, and assembled it into hexamers by adding 2 mM AMP-PNP and 5 mM EDTA; this was followed by buffer exchange with Micro Bio-Spin columns (Bio-Rad) to remove excessive DTT.

We activated the introduced cysteine in the I92C or M136C mutants of Vps24-2 by formation of a mixed disulfide with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, Sigma). After buffer exchange with Micro Bio-Spin columns (Bio-Rad) to remove excessive DTNB, we mixed 1 µM DTNB-activated Vps24-2 single cysteine mutant with 5 µM preassembled Vps4 hexamer mixture in the presence of 10 mM ATP/MgCl2 or 10 mM AMP-PNP/EDTA or no nucleotide in cross-linking buffer (50 mM HEPES, pH 7.5, and 200 mM KCl). We allowed the cross-linking reaction to proceed in the dark for 1 min at 30 °C before we stopped it by adding 200 mM iodoacetic acid (IAA) in 400 mM Tris-HCl, pH 8.5, 6.2 M urea and 2 mM EDTA. We allowed alkylation with IAA to proceed in the dark at 22 °C for 30 min. We then loaded the samples onto nonreducing SDS-PAGE and detected disulfide-cross-linked Vps24-2–Vps4 complexes by western blot with an anti-polyhistidine antibody (R&D systems, monoclonal mouse IgG1, clone AD1.1.10, 1:5,000; validation at http://www.mdsystems.com/Products/MAB050/).

Electron microscopy. We concentrated Vps24 to 350 µM for overnight self-assembly at 4 °C (ref. 18), and we then diluted the resulting filaments to 3.5 µM for negative-stain EM. We placed 4-µl droplets of the sample on glow-discharged carbon-coated copper grids, and we negatively stained them with 2% (w/v) uranyl acetate solution. We examined the negatively stained samples under a transmission electron microscope (Tecnai-12; FEI) operated at 120 keV and recorded images with a charge-coupled device camera (4k TemCam-F416; TVIPS) at a magnification of 49,000×.
ATPase activity assay. We determined the basal ATPase activities of Vps4 wild type and mutants with the ADP-Glo Kinase assay kit (Promega) at room temperature. Briefly, we incubated 2 μM Vps4 in 25 μl reaction buffer (50 mM HEPES, pH 7.5, 200 mM KCl, 1 mM ATP and 10 mM MgCl2) with or without 4 μM Vps24-2. At different time points (ranging from 10 s to 1 min), we stopped the reactions by addition of 25 μL ADP-Glo reagent. After a 40-min incubation to allow for the complete depletion of unconsumed ATP, we added 50 μL kinase-detection reagent to simultaneously convert ADP to ATP and allow the newly synthesized ATP to be measured with a luciferase/luciferin reaction. After another incubation of 60 min, we measured luminescence with a plate-reading luminometer (GloMax, Promega) and correlated data to ADP concentrations with a standard ATP-to-ADP conversion curve generated in parallel. We interpreted the data in Prism (GraphPad).