Cryo-EM structures of the ATP-bound Vps4\textsuperscript{E233Q} hexamer and its complex with Vta1 at near-atomic resolution

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The cellular ESCRT-III (endosomal sorting complex required for transport-III) and Vps4 (vacuolar protein sorting 4) comprise a common machinery that mediates a variety of membrane remodelling events. Vps4 is essential for the machinery function by using the energy from ATP hydrolysis to disassemble the ESCRT-III polymer into individual proteins. Here, we report the structures of the ATP-bound Vps4\textsuperscript{E233Q} hexamer and its complex with the cofactor Vta1 (vps twenty associated 1) at resolutions of 3.9 and 4.2 Å, respectively, determined by electron cryo-microscopy. Six Vps4\textsuperscript{E233Q} subunits in both assemblies exhibit a spiral-shaped ring-like arrangement. Locating at the periphery of the hexameric ring, Vta1 dimer bridges two adjacent Vps4 subunits by two different interaction modes to promote the formation of the active Vps4 hexamer during ESCRT-III filament disassembly. The structural findings, together with the structure-guided biochemical and single-molecule analyses, provide important insights into the process of the ESCRT-III polymer disassembly by Vps4.
The ESCRT-III (endosomal sorting complex required for transport -III) machinery and the Vps4 (vacular protein sorting 4) contribute to the events of membrane budding away from the cytosol. They play essential roles in diverse cellular processes, including the multivesicular body biogenesis, the extracellular vesicle formation, enveloped virus budding, midbody abscission during cytokinesis, wound plasma membrane repair, neural pruning, nuclear pore complex assembly and nuclear envelope reformation. The dynamic assembly and disassembly cycle of ESCRT-III proteins between monomeric states in the cytosol and heteropolymers states on the membrane is critical for the efficient ESCRT-III function.

The disassembly of ESCRT-III is mainly mediated by the Vps4 protein that is a member of the type I AAA + ATPase (ATPase associated with various cellular activities) family and oligomerizes into a functional hexamer with elevated ATPase activity upon binding to ATP. Powered by ATP hydrolysis, the Vps4 oligomer, promoted by the cofactor protein Vta1 (Vps twenty associated 1), disassembles ESCRT-III polymers into individual ESCRT-III subunits available for recycling. Vps4 contains an MIT (microtubule interacting and trafficking) domain at the N terminus, a single ATPase cassette comprising a large ATPase domain and a small ATPase domain with a β-domain inserted within it, and a C-terminal helix. The structure of the ATPase cassette resembles those from other AAA + ATPase members, except for the β-domain that is responsible for the binding to Vta1 (refs 8,14). Vta1 promotion of Vps4 function in vivo and ATPase activity in vitro is largely determined by Vta1 structural features. The N-terminal domain (NTD) of Vta1 consists of two MIT motifs, important for the binding to ESCRT-III subunits Vps60 and Did2 (ref. 19). A flexible linker connects the NTD with the C-terminal domain (CTD) composed of a pair of antiparallel α-helices mediating Vta1 dimerization and Vps4 binding (Fig. 1a, lower panel).

During the past decade, numerous crystal structures of Vps4 have been reported. The overall structure is composed of a disordered MIT domain, an AAA ATPase cassette, an independent antiparallel β-strand-containing domain inserted within the small domain of the AAA ATPase cassette and a C-terminal helix packing against the large ATPase domain. The structure of the ATPase cassette of Vps4 is similar to those from other AAA + ATPase members, except for the β-domain. As expected, the nucleotide binds in a site between the large and small ATPase domains. Crystal structural studies reveal significant structural flexibility within the Vps4 molecule, displayed as en bloc rotations of up to 20° between the large and small ATPase domains, whereas little conformational changes within the individual domains, and that nucleotides binding may stabilize a relatively closed conformation.

Notably, only nucleotide-bound forms of SKD1 can be well superimposed onto the hexameric ring structures of nucleotide-bound p97 without any major steric clashes, whereas apo form of SKD1 showed less compatible interfaces between protomers, and this may explain why the apo form of SKD1 is mainly a monomer in solution.

Besides the reported crystal structures of Vps4, the electron microscopy structures of Vps4 in dodecamer or tetradecamer were also obtained claiming that the Vps4 oligomer is a two stacked ring structure with 12 or 14 subunits, but these structures appeared to contradict a biochemical analysis that showed that the functional form of Vps4 is hexameric. Although a recent study provided a crystal structure of the Vps4 hexameric ring, the used protein is a Vps4 homologue from Metallosphaera sedula, a prokaryotic organism that lacks endomembrane structures.

Moreover, this Vps4 hexamer was obtained in the absence of ATP that is completely different from the eukaryotic Vps4 hexamer whose assembly is ATP dependent. Thus, the structure of this MsVps4 hexamer cannot represent the structure of the active form of the eukaryotic Vps4 hexamer.

In this study, using Vps4 from yeast, we solved the structures of the ATP-bound Vps4E233Q hexamer at 3.9 Å resolution and its complex with Vta1 at 4.2 Å resolution, both determined by single-particle electron cryo-microscopy (cryo-EM) without any symmetry imposed. The near-atomic resolution structures reveal rich details for the arrangement of the Vps4E233Q subunits in the hexamer, the interactions between Vps4 and Vta1, and between Vps4 and Vta1. The structural findings, together with structure-guided biochemical and single-molecule fluorescence analyses, not only explain the assemblies of the Vta1-free Vps4 hexamer and the Vta1-bound Vps4 hexamer but also reveal the mechanisms of the ATP-dependent oligomerization of Vps4 and the Vta1-promoting activity on Vps4. Our results provide important insights into the disassembly process of the ESCRT-III polymer by the Vps4–Vta1 complex.

Results

Structures of ATP-bound Vps4 hexamer and Vps4–Vta1 complex.

We purified the full-length yeast Vps4 containing an E233Q mutation, which allows ATP binding but blocks ATP hydrolysis, and assembled Vps4E233Q oligomer in the presence of 1 mM ATP in vitro (Methods). The assembled Vps4E233Q appeared as a homogenous oligomer with a molecular weight of ~300 kDa, corresponding to a hexamer, as determined by size exclusion chromatography (SEC) with multiangle light scattering (MALSSupplementary Fig. 1a). We used single-particle cryo-EM method to obtain the three-dimensional (3D) reconstruction of the 300 kDa oligomer without imposing any symmetry at a resolution of 3.9 Å (Fig. 1b and Supplementary Fig. 1b–g). The near-atomic resolution of the EM map revealed clear major secondary structural elements with some bulky side chains identifiable (Supplementary Fig. 1e). The structure appeared as a hexameric ring, consistent with the MALSSupplementary Fig. 1a). The ATPase members, except for

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To investigate the structure detail of the ATP-bound Vps4 hexamer, we built atomic models within the cryo-EM map using the available crystal structure of the ATP-bound Vps4E233Q (PDB ID: 3EIH) as the starting model to refine (Fig. 1c and Supplementary Fig. 1h). Just as shown in the EM map, the hexameric ring structure exhibits a striking asymmetry in subunit juxtaposition (Fig. 1c,f,g). First, the ring deviates from a sixfold symmetric conformation. The subunit A shifts slightly away from the ring centre (Fig. 1c,f,g), and the interaction interfaces between it and its two adjacent subunits are only about half of those between the other subunits.
Figure 1 | Overall structures of the ATP-bound Vps4E233Q hexamer and its complex with Vta1. (a) Domain diagrams of the yeast Vps4 (upper) and Vta1 (lower). (b) Different views of the EM density map of the ATP-bound Vps4E233Q hexamer at 5.8 σ contour level. (c) The atomic model of the Vps4E233Q hexamer. Vps4E233Q subunits are colour coded. (d) Different views of the EM density map of the ATP-bound Vps4E233Q–Vta1 complex at the 3.9 σ contour level showing the positions of the Vta1. (e) The atomic model of the ATP-bound Vps4E233Q–Vta1 complex. Vps4E233Q subunits and Vta1 subunits are colour coded. (f) Different views of the helix α3 and C-terminal helix in the Vps4E233Q hexamer to show the spiral arrangement of Vps4 subunits. (g) A schematic diagram showing the topology of the Vps4E233Q subunits in the Vps4E233Q hexamer. The colouring scheme is the same as in (c).
(Supplementary Fig. 2a). This relatively weak interaction of subunit A with its neighbouring subunits may account for its flexibility. Moreover, the ring is not planar, in which subunits arrange in an upward spiral from subunit A to subunit F as manifested by the relative positions of the α3-helix and C-terminal helix in each subunit, resembling a split right-handed spiral ring (Fig. 1f,g). Notably, when superimposing the structures of each subunit, the large ATPase domain of the subunit A exhibits a slightly different position relative to the small ATPase domain as compared with the other five subunits that all perfectly align with each other (Supplementary Fig. 3a). Agreeing with this, densities for nucleotides are clearly visible in the nucleotide-binding pockets of subunit B through F, whereas there is no clear density in the nucleotide-binding pocket of subunit A, suggesting that subunit A is likely free of the nucleotide (Supplementary Fig. 4a). The current resolution limit and the quality of EM maps did not allow conclusive differentiation between ATP and ADP. To verify the nucleotide state in the hexamer, we measured the ATP and ADP levels in the Vta1-bound Vps4E233Q hexamer eluted from the SEC, and a major peak shifted to a lower elution volume suggesting that the bound nucleotide in the hexamer is almost impossible. Therefore, all of the above information suggests that the ADP molecule is almost nonexistent in the hexamer, resulting in one CTD, referred to as inner CTD, closer to the ring centre than the other one, referred to as outer CTD (Fig. 1e and Supplementary Figs 2b,3b). Notably, in our map, the helix z8 of the inner CTD is much longer than that of the outer CTD (Fig. 1e and Supplementary Figs 2b,3b), suggesting that the helix z8 is partially flexible, consistent with the crystal structure observation that the N-terminal part of helix z8 displays great conformational variability in the CTD dimer19.

Characterization of protein interactions in the structures. Our cryo-EM work represents the experimentally determined atomic model of the ATP-bound Vps4E233Q hexamer with or without the cofactor Vta1, allowing visualization of specific interactions governing Vps4 oligomerization in its active state. Since the structure of the Vps4E233Q hexamer present in the Vps4E233Q–Vta1 complex is very similar with that in the Vta1-free Vps4E233Q hexamer, we next only investigated the interaction in the Vta1-free Vps4E233Q hexamer in detail. When one Vps4E233Q molecule from each pair of the adjacent subunits aligned with each other, the one object is also well aligned, except for the pairs involving subunit A (Fig. 2a). This is expected as the interface areas between subunit A and its neighbouring subunits are much smaller as described above (Supplementary Fig. 2a). Therefore, we mainly focused on the analysis of the four similar interfaces and discussed interface between subunits C and D as a representative.

The inter-subunit interactions occur both between the large ATPase domains and between the large ATPase and the small ATPase domains including the β-domain (Fig. 2b). The large ATPase domain interaction contributed by helix z3 and z4 from one subunit, and the loops between strand β2 and helix z3 and between strand β3 and helix z4 from neighbouring subunit, largely composed of polar residues, indicating that electrostatic or hydrogen-bonding interactions dominate this interface (Fig. 2b,c). The interaction between the large and small ATPase domains including the β-domain involves the E147–R163 region comprising the C-terminal half of helix z1 and the following loop from one subunit, and the C-terminal half of helix z8 and the Trp388–Glu398 region of β-domain from the neighbouring...
subunit (Fig. 2b,d). Atomic interactions at this interface are versatile, involving polar, hydrophobic and aromatic residues, suggesting a combination of hydrogen bonds, salt bridges and van der Waals contacts (Fig. 2b,d). Additional contact occurs between residues in the C-terminal helix of one subunit and the corresponding amino acids in the linker region between helix 29 and C-terminal helix of the neighbouring subunit, exemplified by potential hydrogen bonds or salt bridges between Asp431 and...
Arg414 (Fig. 2b,e). To validate the above interfaces and residues involved in oligomerization of Vps4, we constructed Vps4 mutants based on the E233Q mutant: D201A, K215A, R241A and E247 for the large ATPase domain interactions (Fig. 2c); E147A, L151A, K154AF155A, L158AF159A, R163A, I351AR352A, W388A, L396A and E398A for interaction between the large and small ATPase domains including the β-domain (Fig. 2d); and R414A and D431A for the C-terminal helix interactions (Fig. 2e). All of these mutants can be purified but eluted on SEC with eliminated or substantially reduced amount of hexamers in the presence of ATP (Fig. 2f–h). Importantly, all of these residues are highly conserved in eukaryotes, suggesting a conserved mechanism of Vps4 oligomerization (Supplementary Fig. 7a). Previous studies based on the Vps4 hexamer models derived from the p97 hexameric structure and the crystal packing interactions in Vps4 crystal forms have discovered some residues, L151 and R352, essential for the oligomerization of Vps4 (Fig. 2i, j).12,21 These interactions are also observed in our structure and are at all the interface between the large and small ATPase domains (Fig. 2b, dg). Moreover, our structure discovered that the subunit interface between the large ATPase domains and between the C-terminal helices are also important for the hexamer assembly (Fig. 2b, c, e, f, b).

To further evaluate the functional relevance of the ATP-bound Vps4E233Q hexamer structure, we mutated subunit interface residues that substantially affect the Vps4E233Q assembly and compared the ATPase activities of these mutants with the wild-type Vps4. As shown in Supplementary Fig. 8, all of the mutants showed severely decreased ATPase activities to 20% of the wild-type activity and most of them reduced to 4% of wild-type Vps4. As shown in Supplementary Fig. 8, all of the mutants showed severely decreased ATPase activities to 20% of the wild-type activity and most of them reduced to 4% of wild-type Vps4. These results confirmed the functional relevance of the subunit interface discovered by the ATP-bound Vps4E233Q hexamer structure.

We next investigated the structure of the Vta1-bound Vps4E233Q complex to understand the mechanism of Vta1-promoting activity on Vps4. Vta1 CTD dimer bridges between two adjacent Vps4E233Q subunits in different modes (Fig. 2i). One mode is guided by the interactions of the β-domain and helices α7 and α9 of the small ATPase domain from one Vps4E233Q subunit with the regions around the tip of α8/α9 helix hairpin from the outer CTD of one CTD dimer and the N-terminal half of the helix α8 from the inner CTD of the same dimer, respectively (Fig. 2j). This interaction is consistent with the crystal structures of the Vta1 VSL dimer in complex with a Vps4 fragment14 and the Vta1 CTD dimer19, and a model generated by aligning these two crystal structures26 as reported previously. The other interaction mode, completely different from that observed before, occurs between the helix α7 of the neighbouring Vps4E233Q subunit and the α8/α9 helix hairpin tip of only the inner CTD, exemplified by potential polar contacts between Arg325 of Vps4, and Glu311 and Asp312 of Vta1 (Fig. 2i, k). The inner CTD binds to both of the adjacent Vps4E233Q subunits simultaneously and hence is more stable than the outer CTD that binds to only one Vps4E233Q subunit (Figs 1e and 2i). Also, the tip of α8/α9 helix hairpin from each CTD in one CTD dimer makes contribution to both of the binding modes by interacting with different regions of the adjacent Vps4E233Q subunits (Fig. 2i–k).

To corroborate the structural observations, we generated Vps4 mutant E233QR325A and examined its ability to associate with Vta1. Similar to the E233Q mutant, the E233QR325A mutant ran as a dimer without ATP and a hexamer in the presence of ATP on SEC, indicating this mutation does not affect the oligomerization ability of Vps4 (Supplementary Fig. 9). In the absence of ATP, both Vps4 mutant constructs can bind to Vta1 via the β-domain only at high concentration due to the weak interaction between Vps4 β-domain and Vta1 VSL as manifested by the relatively high Kd value of 85 μM (Fig. 2l–m). When ATP is present, Vps4E233Q assembles into hexamer forming an additional site for Vta1 to bind as revealed by our structure, and therefore should enhance the binding of Vta1. Indeed, in the presence of ATP, Vta1 can form complex with Vps4E233Q hexamer at a concentration as low as 5 μM of Vps4 (Fig. 2m). In contrast, when R325A is introduced to the additional binding site, Vta1 high-binding affinity to Vps4E233Q hexamer is lost (Fig. 2m). Together, these results demonstrated that the interaction between the helix α7 from Vps4E233Q and the tip of α8/α9 helix hairpin from Vta1 is dispensable for efficient binding of Vta1 to the ATP-bound Vps4E233Q hexamer.

We next examined the Vta1 effect on the stability of ATP-induced Vps4E233Q hexamer. We collected the SEC fractions of Vta1-free and Vta1-bound Vps4E233Q hexamers in the presence of ATP and reapplied them to SEC in the absence of ATP. Interestingly, we found that Vta1-free hexamers nearly all disassembled into dimers, whereas the Vta1-bound hexamers remained intact (Fig. 2n). As a control, treatment with apyrase to deplete ATP from the samples before loading to SEC led to full disassembly of both Vta1-free and Vta1-bound hexamers, indicating that ATP still binds to the Vta1-bound hexamer even in a solution lacking ATP once the complex forms (Fig. 2n). These experimental findings strongly argue that Vta1 binding may stabilize the conformation of the ATP-bound Vps4 hexamer, thus facilitating the bound ATP for subsequent hydrolysis. Accordingly, mutation construct E233QR325A of Vps4 hexamer at high concentration incubated with Vta1 disassembled

Figure 2 | Characterizations of protein interactions in the Vta1-free and the Vta1-bound Vps4E233Q hexamers. (a) Comparison of six pairs of the adjacent Vps4E233Q subunits. The colouring scheme is the same as in Fig. 1c, e. When one Vps4E233Q molecule (shown in ribbon representation) from each pair of the adjacent subunits aligned with each other, the other one (shown in cartoon representation) is also well aligned, except for the pairs involving subunit A (red arrow). (b) A focused view of the domains that mediate the interactions between the Vps4E233Q monomers (subunits C and D). Three boxed intermonomer interfaces are detailed in c–e. (c–e) The close-up views of the interfaces between the large ATPase domains (c), the large and small ATPase domains including the β-domain (d) and the C-terminal helices (e). Potential residues participated in the interactions are represented by spheres. Hydrophobic, aromatic, positively charged and negatively charged residues are coloured grey, pink, blue and red, respectively. The orientations of all close-up views are optimized to show the interactions. (f–h) SEC analyses of the oligomeric states of the Vps4E233Q mutants targeting the interface between the large ATPase domains (f), the large and small ATPase domains including the β-domain (g) and the C-terminal helices (h). (i) A focused view of the interactions between the Vta1 CTD dimer (subunits I and J) and the adjacent Vps4E233Q subunit pair (subunits C and D). Two different contact modes are highlighted by purple and blue dashed circles, respectively, and detailed in j and k. (j, k) The close-up views of the interactions between the Vps4E233Q subunit D and the CTD dimer (j), and the Vps4E233Q subunit C and the CTD dimer (k). (l) Assessments of the interactions by SEC between Vta1 and Vps4 mutants, E233Q and E233QR325A, at high concentration (100 μM) in the absence of ATP (l), and at low concentration (5 μM) in the presence of ATP (m). (n) Assessment of the stabilities of the Vta1-free Vps4E233Q hexamer and the Vta1-bound Vps4E233Q hexamer formed by different Vps4 mutants with or without apyrase treatment by SEC (See Methods for details)
immediately in a solution that lacked ATP (Fig. 2n). Notably, the residue 325 of Vps4 is highly conserved as a positively charged amino acid in eukaryotes, but diverse in crenarchaeota in which the Vta1 is absent, further indicating the importance of this residue for the function of Vta1 (Supplementary Fig. 7b).

The finding of our structure of the Vps4E233Q-Vta1 complex is the identification of the second site, R325 of Vps4 (Fig. 2n), which is highly conserved in eukaryotes, but diverse in crenarchaeota in which the Vta1 is absent, further indicating the importance of this residue for the function of Vta1 (Supplementary Fig. 7b).

Conformational changes and ATPase active centres. In our structure, we can clearly visualize ATP at the nucleotide-binding pocket of Vps4E233Q protein (Supplementary Fig. 4). This ATP-bound structure of Vps4E233Q provides us the opportunity to compare with the other nucleotide-bound structures to understand the mechanism of the ATP-dependent oligomerization of Vps4. In comparison with the ATP-S-bound Vps4E233Q (PDB ID: 3EIH)12, the ATP-bound Vps4E233Q in the hexamer (taking subunit D as representative) has conserved regions contributing to ATP binding (Fig. 3a–d). On the other hand, significant differences are observed in the position of the large ATPase domain, especially the surface-exposed helices including helices x3, x4 and x5 that are far away from the ATP-binding pocket of the subunit itself, exhibiting a significant movement between these two structures (Fig. 3a,b). Consequently, this change does not affect the conformation of the ATP-binding pocket of the subunit D (Fig. 3c), but instead leads to a relatively compact ATP centre of the neighboring subunit E by positioning Arg289, Asn261 and Asn265 residues into close proximity to the ATP of subunit E (Fig. 3d), enabling comprehensive close contacts with the ATP to not only allow efficient assembly of the hexamer but also result in elevated ATPase activity. Indeed, mutation of Arg289 to alanine or mutation of both Asn261 and Asn265 to alanine led to reduced ATP-dependent oligomerization activity and the ATPase activity (Fig. 3e,f). Specially, the mutation of Arg289, the highly conserved arginine finger, nearly failed assembly into the hexamer and retained only 7% of the wild-type ATPase activity (Fig. 3e,f). This observation is in agreement with the previous studies of the functions of the arginine finger. The arginine finger residues have been studied extensively and implicated in ATP hydrolysis and oligomer assembly. As for the Vps4 protein, mutations of the arginine finger residues have been studied extensively and implicated in ATP hydrolysis and oligomer assembly. As for the Vps4 protein, mutations of the arginine finger residues have been studied extensively and implicated in ATP hydrolysis and oligomer assembly. As for the Vps4 protein, mutations of the arginine finger residues have been studied extensively and implicated in ATP hydrolysis and oligomer assembly. As for the Vps4 protein, mutations of the arginine finger residues have been studied extensively and implicated in ATP hydrolysis and oligomer assembly. As for the Vps4 protein, mutations of the arginine finger residues have been studied extensively and implicated in ATP hydrolysis and oligomer assembly. 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Figure 3 | ATPase active centres. (a) Comparison of subunit D with the crystal structure of the ATPγS-bound Vps4E233Q (PDB ID: 3EIH)\textsuperscript{12}. The crystal structure is coloured grey, and the subunit D is coloured based on the distances between aligned Cα atom pairs with colouring scheme showing on the right. Helixes α3, α4 and α5 are labelled and marked by black arrows. (b) A focused view of the adjacent Vps4E233Q subunits D and E with each subunit superimposed with the crystal structure of the ATPγS-bound Vps4E233Q. Two ATPase active centres are boxed and detailed in (c,d). ATP molecules are shown in stick representation, and the map density shown in mesh. (c,d) Close-up views of the ATPase active centre in subunit D (c) and subunit E (d). Residues contributing to ATP binding are shown in sphere representation. Large shifts of R289, N261 and N265 in subunit D compared with those in the crystal structure are shown by red dashed boxes in (d). The orientations of all close-up views are optimized to show the positions of the residues contributing to ATP binding. All alignments were done using the residues in Walker A loop (Phe174-Tyr181), Walker B (Asp232-Gln233), sensor 1 (Asn277) and M307 as a reference. (e) SEC analyses of the oligomeric states of the Vps4 mutants. (f) The ATPase activity assays. Data are represented as the average of three independent experiments. Error bars represent s.d. (g) Comparison of the ATPase centres of Vta1-free and Vta1-bound Vps4E233Q hexamers. The colouring scheme is the same as in Fig. 1c.e. The ATP molecules in the Vta1-bound Vps4E233Q hexamers are displayed in stick model. The R289 residues (red arrows) in Vta1-free and Vta1-bound Vps4E233Q hexamers are coloured by pink and red, respectively. All alignments were performed using the subunits that the arginine fingers act on in trans.
their disappearances were 139 ± 12 and 90 ± 7 s, respectively, indicating that Vps4 stably associated with the filament for at least 90 s during the process of the filament disassembly. Otherwise, transient bound Cy5-Vps4 on the filaments would be quickly replaced by 100 nM unlabelled Vps4 presence in the solution. Furthermore, we found that the percentage of the active disassembly events significantly increases from 4.5% (n = 243) for Vps4 alone to 19.2% (n = 276) when additional Vta1 was present, suggesting more active Vps4 recruited to the filaments in the presence of Vta1. Since both our structures of the ATP-bound Vps4 and its complex with Vta1 and the previous studies of the oligomeric state of the Vps4 (ref. 21) indicate that the active form of Vps4 is hexameric, this observation in the single-molecule filament disassembly assays further suggests that more Vps4 hexamers recruited to the filaments in the presence of Vta1; that is, Vta1 serves as an assembly factor to help Vps4 oligomerize into active hexamers associated with filaments.

In this study, we present the cryo-EM structure of the ATP-bound Vps4E233Q hexamer that clearly demonstrates that the higher-order oligomer formed by Vps4E233Q upon binding to ATP is a hexamer. Previous studies reported that Vps4E233Q mutant assembled into higher-order oligomer containing 8–12 subunits in the presence of ATP by the gel filtration analysis.6,8,12,21,25. However, molecular mass determination by gel filtration may not be accurate due to the poor resolution of the
gel filtration retention times at this high-molecular-weight range and the effects of the molecule shapes on the measurement. Two EM structural studies also showed that Vps4\textsuperscript{E233Q} mutant assembled into double-ring structures with 12 subunits\textsuperscript{24,25}, and one EM study using wild-type Vps4 lacking the MIT domain and non-hydrolysable ATP analogues (AMPPNP) reported the Vps4 oligomer with 14 subunits, but these structures are all at a low-resolution range between 18 and 34 Å, and therefore the interpretation of these structures may not be reliable. Although we also used the E233Q mutant for the oligomer assembly, our near-atomic structures clearly reveal the assembled oligomer is a hexamer (Fig. 1), and this is also consistent with our MALS result showing a molecular weight of ~300 kD of the assembled oligomer (Supplementary Fig. 1a), corresponding to six Vps4 molecules in the oligomer.

In most published Vps4 crystal forms, Vps4 subunits assembled into a continuous helix with a sixfold screw axis, and if viewed along the screw axis, the projection of these molecules forms a hexameric ring\textsuperscript{8,11–13,20–22}. It should be noted that the arrangement of Vps4 subunits in the crystal forms is different from that in our ATP-bound Vps4\textsuperscript{E233Q} hexamer. The former is an opened helical assembly\textsuperscript{8,11–13,20–22}, while the latter is a closed spiral-shaped ring-like arrangement (Fig. 1b–g). Accordingly, the interface of the adjacent Vps4 subunits is also different. The helix in the crystal forms is stabilized by extensive interactions between the large and small AAA ATPase domains of the adjacent Vps4 subunits\textsuperscript{12}. These interactions are well preserved in our ATP-bound Vps4\textsuperscript{E233Q} hexamer (Fig. 2b,d,g). Moreover, our structure discovered interactions between the large ATPase domains and between the C-terminal helices essential for the hexamer assembly (Fig. 1b,c,e,f,h). Contrasting the large and small ATPase domain interactions, which are predominated by van der Waals contacts (Fig. 2d,g), the large ATPase domain interactions comprise mostly polar contacts (Fig. 2c,f). Compared with hydrophobic interactions, polar interactions usually exhibit faster kinetics and thus are more amenable to the biological function—in this case, ESCRTIII filament disassembly. Additionally, the interface between adjacent large ATPase domains is close to the centre pore of the hexameric ring that is essential for the function of Vps4. Therefore, the atomic interactions at this interface appear not only to be important for hexamer assembly, but also to have evolved to facilitate the underlying biological function.

One finding revealed by our structures is the split spiral arrangement of Vps4\textsuperscript{E233Q} subunits in both the Vps4\textsuperscript{E233Q} hexamer (Fig. 1f,g) and its complex with Vta1 (Supplementary Fig. 6c), and the biological relevance of the subunit interface in the hexamer was confirmed by the ATPase activity assays of the site-directed mutant proteins (Supplementary Fig. 8). The structural observation is reminiscent of that in other two AAA + ATPase members: the transcriptional activator NtrC1 (nitrogen regulatory protein C1) and the NSF (N-ethylmaleimide-sensitive factor)\textsuperscript{30,31}. Besides these AAA + ATPase members, the split spiral arrangement has been observed for some helicases, including the E1 helicase of bovine papilloma virus\textsuperscript{13}, the DnaB helicase from \textit{Geobacillus stearothermophilus}\textsuperscript{53} and the eukaryotic MCM (minichromosome maintenance protein complex) helicases\textsuperscript{34,35}. These ATPases have very different biological functions: NtrC1 is suggested to remodel RNA protein by directly interacting with region I that is predicted to form an α-helix\textsuperscript{30,36,37}. NSF disassembles SNARE complex that is a four-helix bundle\textsuperscript{38–40}; helicases unwind DNA that has a helical nature. Thus, the unifying structural feature of the split spiral-like ring likely plays a key role for these ATPases to exert forces derived from ATP hydrolysis on their target macromolecules.

**Methods**

**Protein expression and purification.** \textit{Saccharomyces cerevisiae} Vps4, Vta1, Snf7, Vps24 and Vps2 genes were amplified by PCR from yeast genomic DNA and directionally cloned into the pET28a vector (Novagen) with an N-terminal His\textsubscript{6} tag. Constructs expressing mutant proteins were generated with Quick Change Mutation Kit (Transgen) and verified by DNA sequencing. All proteins were expressed in \textit{Escherichia coli} BL21 (DE3) strains and induced with 0.5 mM isopropyl-1-β-D-thiogalactopyranoside (IPTG) at 18–21°C overnight. The proteins were further purified by SEC using a Superdex 200 10/300 GL column (GE Healthcare) in the fast performance liquid chromatography (FPLC) buffer containing 20 mM HEPS at pH 7.5, 500 mM NaCl, 10 mM imidazole, 1 mM phenylmethylsulfonyl fluoride and 2 mM β-mercaptoethanol (β-ME). The purified lysates were incubated on a Ni+-nitrilotriacetic acid column (GE Healthcare). The eluates were incubated with 2 U ml\textsuperscript{-1} apyrase (Sigma-Aldrich) at 4°C overnight. All proteins were further purified by SEC using a Superdex 200 10/300 GL column (GE Healthcare) in the fast performance liquid chromatography (FPLC) buffer containing 20 mM HEPS at pH 7.5, 100 mM NaCl and 1 mM dithiothreitol. The purity and quality of all the proteins were tested by SDS–PAGE.

**SEC with MALS.** SEC–MALS experiments were performed using an Agilent 1200 high-performance liquid chromatography system (Agilent Technologies, Santa Clara, CA, USA) coupled to a Wyatt DAWN HELEOS-II MALS instrument and a Wyatt Optilab REX differential refractometer (Wyatt, Santa Barbara, CA, USA). For chromatographic separation, 200 μl of 5 mM mg\textsuperscript{-1} (50 μM) Vps4\textsuperscript{E233Q} protein preincubated with 1 mM ATP at 4°C for 1 h was injected onto a Superdex 200 10/300 GL column (GE Healthcare) at a flow rate of 0.3 ml min\textsuperscript{-1} in the assembly buffer. The outputs were analysed by the ASTRA 6.0 software (Wyatt). MALS signals, combined with the protein concentration determined by the refractive index, were used to calculate the molecular mass.

**SEC analysis of oligomeric states and protein interactions.** To examine the oligomeric states of Vps4 mutants, each mutant protein at 100 μM was incubated with 1 mM ATP at 4°C for 1 h, and then loaded onto a Superdex 200 10/300 GL column (GE Healthcare) preequilibrated with the assembly buffer. To examine the interactions between Vps4 and Vta1 in the absence of ATP, Vps4 mutants at 100 μM were incubated with Vta1 at a molar ratio of 1:1.5 in the absence of ATP at 4°C for 1 h before being loaded onto the Superdex 200 10/300 GL column (GE Healthcare) preequilibrated with the FPLC buffer. To examine the interactions between Vps4 and Vta1 in the presence of ATP, Vps4 mutants at 5 μM were incubated with Vta1 at a molar ratio of 1:2 in the presence of 1 mM ATP at 4°C for 1 h before being loaded onto the Superdex 6 10/300GL column (GE Healthcare) preequilibrated with the assembly buffer.

**Stability assay.** The formed Vta1-free Vps4\textsuperscript{E233Q} hexamers and the Vta1-bound Vps4\textsuperscript{E233Q} hexamers eluted from SEC in the assembly buffer were individually reloaded onto the Superose 6 10/300 GL column (GE Healthcare) in the FPLC buffer. For the apyrase treatment, the 10 U ml\textsuperscript{-1} apyrase (Sigma-Aldrich) was added to the samples and incubated at 4°C overnight before SEC.

**ATPase activity assay.** ATPase activity assays measure released phosphate based on the formation of coloured complex between malachite green reagent and free phosphate according to the manufacturer’s instructions using the QuantiChrom ATPase/ATPase-Glo/ATPase-Glo Assay Kit (BioAssay Systems). Briefly, 1 μl of wild-type or mutant Vps4 proteins in the absence or presence of Vta1 in the ATPase assay buffer containing 20 mM HEPS at pH 7.5, 100 mM NaCl, 2 mM MgCl\textsubscript{2} and 5 mM ATP were incubated at 37°C for various times (0–4 min), and stopped by adding reagent from the kit. The solution was incubated at room temperature for 30 min, and immediately the absorbance at 620 nm was detected using a plate reader (Espire, Perkin Elmer). The released phosphate was calculated based on the absorbance standard curve established using KH\textsubscript{2}PO\textsubscript{4} standards.

**ATP/ADP quantification assay.** The 50 μM Vps4\textsuperscript{E233Q} and 100 μM Vta1 protein were incubated for 1 h on ice in the assembly buffer, and then loaded onto the Superose 6 10/300 GL column (GE Healthcare) in the FPLC buffer. The eluted Vps4\textsuperscript{E233Q}–Vta1 complex was used to examine the ATP and ADP levels by the bioluminescent detection-based method (Bioassay System EnzyLight ADP Assay Kit, EADP-100) according to the manufacturer’s instructions. The luminescence values were detected using a plate reader (Espire Multimode Plate Reader, Perkin
Elmer). The nucleotide levels were calculated based on the luminescence standard curves established using ATP and ADP standards. Three independent experiments were carried out on at least two different batches of purified Vps4E233Q and Vta1 proteins and each experiment was carried out in at least triplicate.

**Stoichiometry determination.** We used the fluorescence assay-based method to evaluate the stoichiometry of the ATP-bound Vps4E233Q–Vta1 complex. We constructed the single-cysteine mutants (containing only one cysteine residue) of Vps4 (Vps4E233Q(C70)) and Vta1 (Vta1E1230K45) by site-directed mutagenesis and purified them as other proteins. The concentrations of all proteins were measured by Pierce BCA Protein Assay Kit (Thermo). Vps4 and Vta1 proteins were concentrated to 4 and 10 mg ml\(^{-1}\) in the labelling buffer containing 50 mM HEPES, 100 mM NaCl and 1 mM TCEP (tris(2-carboxyethyl)phosphine), pH 7.4, respectively. Cy5 maleimide and Cy3 maleimide (GE Healthcare) were added into the Vps4 and Vta1 protein solutions, respectively, at the protein/nucleotide ratio of 1:4. Labelling reactions were performed in ice overnight, and terminated by 3 mM β-ME. The free dyes were removed by using the NAP-5 columns. Then, we generated the ATP-bound Vps4E233Q–Vta1 complex using these labelled proteins by incubating them at the molar ratio of 1:2 in the presence of ATP on ice for 1 h and then applying the mixtures to SEC in the assembly buffer to separate the complex. The fluorescence intensities of Cy5 and Cy3 of the complex were measured using a plate reader (Enspire Multimode Plate Reader, Perkin Elmer). Then, the protein concentrations of Vps4 and Vta1 in the complex were calculated according to the fluorescence intensities and the standard curves of the protein concentration versus the fluorescence intensity. Three independent experiments were carried out and each experiment was carried out in at least triplicate.

**Cryo-EM sample preparation and data collection.** The assembled ATP-bound Vps4E233Q hexamer eluted from SEC was concentrated to ~1 mg ml\(^{-1}\) and incubated with 0.02% glutaraldehyde for ~5 min on ice. Then, aliquots of 4 μl of this sample were applied to the glow-discharged Quantifoil R1/2/1.3 holey carbon grids (Quantifoil Micro Tools GmbH), blotted for 2 s and plunged frozen using 90% ethylene glycol (Beckman Coulter) as cryoprotectant. Pellet was resuspended in the buffer containing 50 mM HEPES at pH 7.4, 100 mM NaCl and 1 mM TCEP. All the particles were verified by SD–PAGE and negative staining.

**Model building.** For building the model of the ATP-bound Vps4E233Q hexamer, the crystal structure of the ATP-S-bound Vps4 (PDB ID: 3EHJ, chain A)\(^{12}\) was docked into the density of purified Vps4E233Q and Vta1 proteins (PDB ID: 3EHJ)\(^{12}\) and the Vta1 CTD dimers (PDB ID: 2RKL)\(^{29}\) as the starting models to refine. The atomic models were cross-validated according to previously described procedures\(^{25}\). Briefly, the coordinates of the final model were randomly displaced by 0.5 Å using the Phenix (PDB Tools) to remove potential model bias. The displaced model was then refined against one of the half maps (produced from a half set of all particles during refinement by RELION). FSC curves were calculated between the resulting model and half1 map (model versus half1 map, FSC\(_{free}\), that is, not used for refinement), the resulting model and half2 map (model versus half2 map, FSC\(_{new}\), that is, not used for refinement) and the protocol model and the final density map (model versus summap map) from all particles. The lack of significant separation between work and free FSC curves suggested that the models were not overfitted.

**ESCR-III filament reconstitution.** The ESCR-III filament reconstruction was validated by incubating Snf7\(^{52,54}\), Vps24 and Vps2 in the concentrations of 30, 25 and 25 μM, respectively, at 30 °C for 30 min (ref. 5). Then, the formed filaments were concentrated to 50,000 p.p.m. in a TLA-100 rotor (Beckman Coulter). Pellet was resuspended in the buffer containing 50 mM HEPES at pH 7.4, 100 mM NaCl and 1 mM TCEP. All the particles were filtered by 0.1 μm filter and used in the cryo-EM preparation.

**ESCR-III filament disassembly assay.** The reconstructed ESCR-III filaments were incubated with 1 μM Vps4 in the buffer containing 20 mM HEPES at pH 7.4, 2 mM ATP, 2 mM MgCl\(_2\), 1 mM TCEP at 25 °C for 30 min (Supplementary Fig. 10a) or indicated time points (Supplementary Fig. 10b) and then 50 mM EDTA were added to stop the reactions. The samples were centrifuged at 50,000 r.p.m. for 30 min in a TLA-100 rotor, and the supernatant and pellet were analysed by SDS–PAGE. The intensities of bands of Snf7 were quantified by ImageJ. Each experiment was carried out independently for three times.

**Vps4 protein and ESCR-III filament labelling.** Vps4 proteins were concentrated to 2 mg ml\(^{-1}\) in labelling buffer containing 50 mM HEPES, 100 mM NaCl and 1 mM TCEP, pH 7.4. Cy5 maleimide (GE Healthcare) was added into the protein solution at protein/fluorophore ratio of 14. Labelling reactions were performed on ice overnight, and terminated by 3 mM β-ME. The free dyes were removed by using a NAP-5 column. The labelling efficiency was 88% by EM. The labelled filaments were reconstituted from labelled with the Cy3 dye and biotin.Filaments, Cyaniem HS (Lumiprobe) and NHS-biotin (Thermo Scientific) were incubated at a molar ratio of 10:1:1:1 (Snf7\(^{52,54}\), monomer-Cyaniem HS-NHS-biotin) in the labelling buffer on ice for 2 h. The free dyes and biotins were removed by centrifugation at 50,000 r.p.m. for 30 min. The labelled filaments were precipitated and resuspended in 50 mM HEPES at pH 7.4, 100 mM NaCl. Because NHS esters (N-hydroxysuccinimide esters) target and crosslink with primary amines on the proteins and reconstituted filaments are formed by tens of or hundreds of monomers, each filament was expected to be labelled with tens of Cy3 and several biotin. Although it is hard to estimate the labelling efficiency of filaments, they can be specifically attached to our cover slip via streptavidin coating and exhibit an order of magnitude stronger fluorescence intensity than single Cy3 fluorescence. Therefore, in our single-molecule assay, decreasing of Cy3 fluorescence signals was used to quantify disassembling of the reconstituted filaments.

**Acquisition and analysis of single-molecule data.** Filaments were specifically attached to microscope flow cells formed by polyethylene glycol-oxidized slides, decorated with biotin-polylethylene glycol and streptavidin\(^{27}\). Single-molecule experiments were performed at 23 °C in the buffer containing 50 mM HEPES at pH 7.4, 100 mM NaCl with an oxygen scavenging system, containing 3 mg ml\(^{-1}\) glucose, 100 μg ml\(^{-1}\) glucose oxidase (Sigma-Aldrich), 40 μg ml\(^{-1}\) catalase (RBI Catalyst, 1,5-mitrobenzenoic acid (Sigma-Aldrich), 1.5 mM 6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid (Troxol, Sigma-Aldrich) added from a concentrated DSMO stock solution and
1% bovine serum albumin (Amresco) to reduce nonspecific binding. After filament immobilization, 100 nM of Vps4 labelled with Cy5 alone or together with 500 nM Vta1 was injected into a cell expressing ESCRT-II mediates the assembly and architecture of ESCRT-III: an endosome-associated heterooligomeric protein complex required for the AAA-ATPase Vps4p. Cryo-EM density maps were deposited in Electron Microscopy Data Bank with access code EMD-6733 (without mask), EMD-6735 and 5XMK for the ATP-bound Vps4p complex and bandpass filters, ET585/65m (Chroma, Cy3) and ET700/75m (Chroma, Cy5), respectively. The data that support the findings of this study are available from the corresponding authors on request. References

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Author contributions

S.S., C.C. and S.-F.S. designed the experiments. S.S. and L.L. purified all proteins and carried out all biochemical characterizations. L.L. and F.Y. conducted the cryo-EM analysis of the Vps4E233Q hexamer. S.S. and X.W. conducted the cryo-EM analysis of the Vps4E233Q–Vta1 complex. F.F. built the atomic models and analysed the interactions. M.Y. performed the single-molecule experiments. C.C. analysed the single-molecule data. C.C., X.L. and H.-W.W. participated in the manuscript editing and discussion. S.S. analysed the structures and biochemical data, and prepared the figures. S.S. and S.-F.S. wrote the manuscript with help from all authors.

Additional information

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