D1 ring in a cryo-EM reconstruction[44] and the N-terminal domain of the P97 hexamer was found both above the D1 ring[36] and around the D1 ring[35,37] in cryo-EM and crystal structures. Similarly, cryo-EM reconstructions of ClpB found that its N-terminal domain was flexible, but, nevertheless, six small protrusions around the central pore above the D1 ring were suggested to be the beginning of the flexible linker connecting the N-terminal domain to the D1 ring.[47] Thus, our assignment of the nipple-like density as the N-terminal domain over the top ring in the full-length Vps4p is consistent with these earlier reports. It is possible, however, that the flexible N-domains diffusing around the symmetry axis were cross-linked together, giving them an unnaturally well-ordered appearance. At 3.5σ, the molecular mass of the nipple-like density in Fig. 8a was estimated to be 45 kDa, corresponding to ~68% of the mass of six N-terminal domains (each N-domain is ~11 kDa).

Vta1p

The Vta1p co-factor interacts directly with Vps60p and with Vps4p, promoting Vps4p oligomerization and stimulating ATP hydrolysis.[10,30–32,75] The amino acid sequence of Vta1p and recent NMR experiments indicate that purified Vta1p is highly extended, with ordered N- and C-terminal regions joined by a long flexible linker (W.I.S., unpublished data). The C-terminal VSL domain (40 residues) forms a dimer of helical hairpins[30] (W.I.S., unpublished data), and this domain alone is necessary and sufficient for Vps4p binding, although it does not stimulate ATP hydrolysis as well as full-length Vta1p,[30] indicating that other Vta1p regions likely also contact Vps4p. Mutations in Vps4p β domain inhibit Vta1p binding,[28] suggesting that the VSL dimer may contact the Vps4p β domain.

Unfortunately, the resolution of our Vta1p–Vps4p reconstruction was not sufficient to locate or identify specific domains of Vta1p, but it did show that as when pure, in complex with Vps4p, the six Vta1p's are not highly ordered globular proteins because new globular densities large enough to account for the entire mass of Vta1p were not observed. Instead, addition of Vta1p increased the height and width of the nipple-like density above the bowl and added strong new density below the bottom ring ~85 Å away. At 3.5σ, the additional molecular mass of the nipple-like density in Fig. 7b was estimated to be 40 kDa, which accounts for only 32% of the mass of six Vta1ps (each Vta1p is ~41 kDa). One possibility is that the C-terminal VSL domains account for some of the extra density seen below and around the bottom ring of the Vta1p–Vps4p complex, near where the β domains of the bottom ring are expected (Fig. 7), where they might help stabilize the complex and stimulate ATP hydrolysis (see discussion of the role of the two rings below). The N-termini of Vta1p, which are known to interact with the ESCRT-III-like protein Vps60p, might then account for the additional density above the bowl, in a position to help the Vps4p MIT domains recruit and direct substrate into the central cavity. In any case, our finding that there are only six Vta1p subunits in the Vta1p–Vps4p complex is consistent with the notions that the two Vps4p rings are in different conformations and that only half the Vps4p molecules present the correct interface to bind Vta1p. The three symmetric Vta1p dimers expected could not, however, conform strictly to the 6-fold symmetry seen at this resolution, which might have obscured the relatively small contribution of their ordered domains. Higher-resolution structures will clearly be needed to resolve these ambiguities.

Implications for mechanism

The two rings in type II AAA ATPases may play different functional roles. While the D1 ring (top ring in Fig. 9) of NSF is the main site of ATP hydrolysis, the D2 ring (bottom ring in Fig. 9) exhibits little hydrolysis activity.[76,77] It has therefore been suggested that the D2 ring plays a mainly structural role by holding the complex together and that the D1 ring disassembles SNARE complexes through ATP hydrolysis-driven conformational changes (for a review, see Whiteheart *et al.*[78]). Interestingly, the roles of the two rings in p97 appear to be reversed with respect to NSF.[37] In the various cryo-EM reconstructions of ClpB throughout the ATP hydrolysis cycle, the top (D1) ring undergoes significant conformational changes, while the bottom (D2) ring stays relatively uniform.[47] The roles of the two rings in Vps4p are still unknown, but the asymmetric nature of the two rings shared by Vps4p and the type II AAA proteins suggests that their mechanisms may be similar. The ClpA, ClpB, and ClpX rings unfold protein substrates by translocating them through the central pore of the hexamer.[46,51–53,55,79] Similarly, key residues around the core of the p97 D2 hexameric ring block activity,[40] and alanine substitution mutations in the pore I residues predicted to reside in the central channel of Vps4 impair HIV budding *in vivo*.[28] It is therefore likely that Vps4 also disassembles ESCRT-III assemblies by feeding them through the central cavity of the lower ring.

As noted above, a plausible model for the bottom ring of Vps4 has been proposed based on comparisons with the structurally characterized D1 ring of p97.[28] In contrast, the conformation of the upper ring must be quite different. Comparison of upper and lower rings gives the impression of large-scale domain/subunit reorientations, as have been seen previously for the protein rings in the group I chaperonin, GroEL, where the apical domains of each identical subunit twist by 90° from the open substrate-binding state to the closed substrate-folding state.[80–83] Although the details remain to be determined, the open conformation of the upper ring of Vps4 suggests that this ring may function to create a large central chamber that can envelope ESCRT-III subunits in the course of removing them from their membrane-bound lattice.