molecular assemblies to be remodeled. This activity has been adapted for numerous diverse biological functions, including vesicle formation; membrane fusion and transport; protein unfolding, disaggregation and refolding; DNA replication, recombination, and repair; transcription; apoptosis; cytoskeletal regulation; and organelle biogenesis. The hallmark of AAA proteins is a 200- to 250-amino-acid ATP-binding domain named the AAA "domain" or "cassette."

The two human Vps4 proteins (VPS4A and VPS4B) and the unique Vps4 protein of yeast (Vps4p) all share a common domain organization. The proteins start with a three-helical bundle termed the microtubule interacting and transport (MIT) domain that binds the C-termini of a subset of the ESCRT-III/CHMP (changed multivesicular body protein) proteins[23–27] and is connected to the AAA cassette by what appears to be a semiflexible linker.[28] Crystal structures have revealed that the monomeric VPS4B and Vps4p AAA cassettes are composed of two domains, a large mixed-α/β domain and a smaller four-helix bundle.[28,29] These domains resemble other AAA cassettes, except that the four-helix bundle of Vps4 is split by the insertion of a small domain composed of a three-stranded β-sheet (termed the "β domain") that forms the binding site for the Vta1p/LIP5 co-factor.[28]

Vps4 proteins contain a single AAA cassette and are therefore classified as type I AAA proteins, whereas family members that contain two AAA cassettes in a single polypeptide, such as p97, VAT, NSF, ClpA, and ClpB, are classified as type II AAA proteins. Most type I AAA proteins, including ClpX, HslU, and katanin, form a single ring of six protomers.[22] Vps4 is unusual, however, in that it assembles into even larger complexes in the presence of ATP. Chemical cross-linking data suggested that the fully assembled Vps4 complex contained 10 subunits,[18] whereas gel filtration chromatography and equilibrium analytical ultracentrifugation experiments suggested a complex of 10–12 subunits, with 12 being the best fit.[28] It has therefore been proposed that Vps4 may form pentameric or hexameric double-ring structures, but structural data have been lacking. In the presence of ATP, Vta1p/LIP5 binds directly to the fully assembled Vps4 complex to form an even larger complex of unknown stoichiometry, with an estimated molecular mass of 1 MDa.[28] Vta1p/LIP5 enhances the stability and ATPase activity of assembled Vps4[30] and can also interact directly with the Vps60p/CHMP5 subset of ESCRT-III-like proteins.[10,31,32]

Over the last decade, major progress has been made in structural and functional studies of other AAA proteins that act on protein substrates,[33] including p97,[34–40] VAT,[41,42] NSF,[43,44] ClpB,[45–47] HslU,[48–50] ClpX, and ClpA.[51–57] These studies have revealed that AAA ATPase rings are typically hexameric but that other arrangements are also possible. For example, crystal structures have revealed that the clamp loader AAA ATPases form open pentameric rings.[58] Electron cryomicroscopy (Cryo-EM) studies of ClpB indicated 6-fold symmetry in all nucleotide states,[47] but there are also reports based on sedimentation equilibrium experiments that ClpB is a heptamer in the apo state.[59] EM studies of HslU have reported both hexameric and heptameric rings,[60] while all available crystal studies suggest hexameric rings.[48–50] The Lon protease was reported to be a seven-membered ring[61] by EM but to be hexameric in subsequent biochemical, biophysical, and crystallographic studies.[62–64] EM reconstructions of the apoptosome protein Apaf-1 indicated both hexameric[65] and heptameric[66] configurations. RuvB is reported to be a heptamer in the absence of substrate DNA duplex but is converted to a hexameric ring when it binds short strands of duplex DNA, implying that substrate binding changes its oligomeric state.[67] Finally, dynein forms asymmetric single-polypeptide rings with seven lobes.[68] Thus, the architecture of different AAA ATPases can vary considerably and must be determined experimentally.

To determine the architecture of Vps4 and to provide a basis for understanding how this complex enzyme functions, we imaged three Vps4p complexes by cryo-EM. The reconstruction of the smallest complex, formed by an N-terminally truncated Vps4p (ΔN-Vps4p) comprising just the AAA ATPase domain, reveals a core structure of two stacked hexameric rings that adopt strikingly different conformations and together form a bowl. While this core is preserved in the larger structures of full-length Vps4p and a Vta1p–Vps4p complex, additional densities reveal the locations of the substrate-binding N-terminal domains and shed light on the interactions of Vps4p with Vta1p.

## Results and Discussion

### Sample preparation

The *Saccharomyces cerevisiae* Vps4p E233Q point mutant was used for all cryo-EM reconstructions because this mutant protein binds but does not hydrolyze ATP, and it therefore forms stable higher-order Vps4p and Vta1p–Vps4p complexes in the presence of ATP.[18] Purified ΔN-Vps4p, full-length Vps4p, and mixtures of Vta1p and Vps4p formed oligomeric complexes in the presence of ATP and $MgCl_2$, as analyzed by size-exclusion chromatography (Fig. 1). Vta1p and Vps4p were previously shown to form a large complex of undetermined stoichiometry.[28] To investigate the stoichiometry, we mixed purified Vta1p and Vps4p in different ratios, purified the resulting complexes by gel filtration, and analyzed the molar ratios of the two proteins in each fraction using SDS-PAGE and standard curves of known protein concentrations (Fig. 2). As shown in Fig. 2a, Vta1p and Vps4p formed a single discrete complex when mixed in a 1:2 molar ratio (black curve). The expected 1:2 Vta1p/Vps4p ratio in this purified complex was confirmed by SDS-PAGE and