The C-terminal helix is a critical determinant for Vps4 assembly and ATPase activity and has elements conserved in other members of the meiotic clade of AAA ATPases

Parimala R. Vajjhala[1,2], Chau H. Nguyen[1,2], Michael J. Landsberg[1], Carol Kistler[1,2], Ai-Lin Gan[1,2], Glenn F. King[1], Ben Hankamer[1], and Alan L. Munn[1,2,3,4*]

1 Institute for Molecular Bioscience, The University of Queensland, St Lucia, QLD 4072, Australia

2 ARC Special Research Centre for Functional and Applied Genomics, The University of Queensland, St Lucia, QLD 4072, Australia

3 School of Biomedical Sciences, The University of Queensland, St Lucia, QLD 4072, Australia

4 School of Medical Science, Griffith University, Gold Coast, QLD 4222, Australia

Running Title: Role of the Vps4 C-terminal helix

Subdivision: Molecular cell biology

*Correspondence should be addressed to:

Dr. Alan L. Munn

Institute for Molecular Bioscience,

The University of Queensland, St Lucia, QLD 4072,

Australia

Ph: +61 7 3346 2017

Fax: +61 7 3346 2101

E-mail: A.Munn@imb.uq.edu.au

Key words

Macromolecular complex, membrane traffic, endocytosis, lysosome, vacuole

Abstract

Sorting of membrane proteins into intralumenal endosomal vesicles, multivesicular body (MVB) sorting, is critical for receptor downregulation, antigen presentation, and enveloped virus budding. Vps4 is an AAA-ATPase that functions in MVB sorting. **Although AAA ATPases are oligomeric**, mechanisms that govern **Vps4** oligomerisation **and activity** remain elusive. Vps4 has an N-terminal MIT domain required for endosome recruitment, an **AAA domain containing the ATPase catalytic site and** a β domain, and a C-terminal α-helix positioned close to the **catalytic site** in the 3D structure. Previous attempts to identify the role of the C-terminal helix **were unsuccessful**. **Here, we show that the C-terminal helix is important for Vps4 assembly and ATPase activity in vitro** and function in vivo, but not **endosome recruitment** or interactions with Vta1 or ESCRT III. Unlike the β domain, which is also important for Vps4 **assembly**, the C-terminal helix is not **required in vivo** for Vps4 homotypic interaction or dominant negative effects **of Vps4-E233Q, carrying a mutation in the ATP hydrolysis site. Vta1 promotes assembly of hybrid complexes comprising Vps4-E233Q and Vps4 lacking an intact C-terminal helix in vitro. Formation of catalytically active hybrid complexes demonstrates an intersubunit catalytic mechanism for Vps4p. One end of the C-terminal helix lies in close proximity to the second region of homology (SRH), which is important for assembly and intersubunit catalysis in AAA ATPases. We propose that SRH function requires an intact C-terminal helix. Co-evolution of a distinct SRH and C-terminal helix in meiotic clade AAA ATPases supports this possibility.**

Introduction

The exchange of material between the cell surface and interior is critical for many aspects of cell physiology including nutrient uptake, signal transduction and intercellular communication (reviewed in [1, 2]). Endosomes are dynamic organelles that receive internalised material and biosynthetic traffic en route to the lysosome/vacuole [3, 4]. They are active in multiple sorting processes including the sorting of certain membrane proteins into internal vesicles that form by invagination of the limiting membrane of the endosome. The internal vesicles give the endosome the appearance of a multivesicular body (MVB) and this sorting process is referred to as MVB sorting (reviewed in [5]). The MVB can either fuse with the lysosome leading to degradation of its contents or with the plasma membrane leading to release of the internal vesicles (exosomes), which are important for immune regulation and other biological functions [6]. MVB sorting of signalling receptors such as growth factor receptors is critical for their efficient silencing and subsequent degradation [7]. **The MVB sorting machinery also mediates other topologically similar membrane budding processes, including cytokinesis** [8] **and enveloped virus budding** [9], **and functions in autophagy** [10]. **In addition, the MVB compartment is important for loading of antigens on to MHCII complexes for antigen presentation** [11]. Intensive research efforts are currently aimed at achieving a detailed understanding of the roles of the numerous components of the MVB sorting machinery.

Vps4 is an ATPase of the AAA (ATPase associated with a variety of cellular activities) family [12, 13] that plays critical roles in multiple processes during endocytic trafficking. Vps4 is required for trafficking through endosomes and for MVB sorting within endosomes. In the absence of Vps4, the endosome forms an aberrant multilamellar compartment that accumulates endocytosed material, including receptors that normally recycle back to the plasma membrane, newly synthesised lysosomal proteins and recycling late Golgi proteins [13-16]. There are two mammalian isoforms of

VPS4, VPS4A and VPS4B, which both function in endocytic trafficking [17] and virus budding [18, 19] (reviewed in [9, 20]).

Members of the **AAA** superfamily contain one or two ATPase domains that assemble into one or two stacked hexameric rings. The ATPase catalytic site is located at the interface between adjacent **ATPase domains of** a ring and consists of three highly conserved motifs. One **ATPase domain** contributes the Walker A and B motifs that mediate nucleotide binding and hydrolysis respectively, while the **adjacent ATPase domain** contributes a **conserved** motif referred to as the second region of homology (SRH). **The SRH distinguishes the AAA family ATPases from other Walker-type ATPases [21]. A pair of conserved Arg residues within this motif activate ATPase activity in an adjacent ATPase domain** [22, 23] and have also been shown to be important for oligomerisation [22]. **These conserved Arg residues are normally separated by two residues. However, in the meiotic clade of AAA ATPases, which Vps4 belongs to, the conserved Arg residues are not separated [24].**

Conformational changes upon ATP binding and hydrolysis are proposed to mediate remodelling of a protein substrate as it feeds through the core **of an oligomeric ring formed by these AAA ATPases. Thus many** AAA ATPases function as protein disassembly machines (reviewed in [25]). ATPase activity of Vps4 is critical for disassembling the MVB sorting machinery, including the ESCRT complexes (endosomal sorting complexes required for transport) **0**-III and non-ESCRT components that assemble at the endosome membrane, thus allowing their reuse in subsequent rounds of MVB sorting [13, 26, 27]. However, several aspects of Vps4 function and assembly into an active oligomeric ATPase are poorly understood. Structural analysis of Vps4 revealed that it contains a single ATPase domain incorporating a structure rich in β strands (β domain), an N-terminal microtubule interacting and trafficking (MIT) domain [28-30], and a final C-terminal α-

helix [31]. In previous studies, we characterised the role of motifs in the different domains that are highly conserved between yeast and mammalian Vps4. These studies indicated that the N-terminal MIT domain has a dual role in recruitment to endosomes [32, 33] and substrate binding [32] while the β domain is required for a Vps4-Vps4 (i.e. homotypic) interaction and for interaction with another component of the MVB sorting machinery, Vta1p/ SBP1, both of which are important for Vps4 oligomerisation [31, 34, 35].

Here, we address the role of the **yeast** Vps4p C-terminal helix. In the 3D structure of mammalian and yeast Vps4, this helix lies close to the catalytic domain [31]. The close proximity of the C-terminal helix to catalytically important residues is strongly suggestive of a role in Vps4 **ATPase activity**. In addition, other members of the meiotic clade of AAA ATPases that Vps4 belongs to are also predicted to contain a C-terminal helix **with elements conserved with the C-terminal helix of** Vps4. Attempts to identify the role of the Vps4 C-terminal helix have been complicated by insolubility of a Vps4p mutant protein lacking the C-terminal helix [31, 36]. **Our approach has been to study the function of sequences conserved between yeast and human Vps4 that are present at the start and end of the C-terminal helix.** We show that the C-terminal helix, like the β domain, is not important for targeting to endosomes or for interaction with ESCRT III components, Vps2p, Vps20p, Snf7p or with non-ESCRT components, but is essential for Vps4p oligomerisation into an active ATPase **in vitro** and function in vivo. **However,** unlike the β domain, the C-terminal helix is not required for interaction with Vta1p, or for the Vps4p homotypic interaction in vivo. In addition, unlike the β domain, the C-terminal helix is not essential for Vps4p-E233Q, **which has a mutation in the ATP hydrolysis site**, to confer dominant negative effects. These indicate that the C-terminal helix and β domain contribute to Vps4p oligomerisation into a functionally active ATPase via independent mechanisms. **We also show that Vta1p can promote the assembly of a catalytically active hybrid complex comprising a Vps4p mutant protein**

lacking the conserved sequence at the end of the C-terminal helix and Vps4p-E233Q (which has a mutation in the ATP hydrolysis site). Therefore, although the sequence at the end of the C-terminal helix is essential for ATPase activity and assembly in vitro this requirement can be bypassed by the addition of Vta1p and a Vps4p protein containing an intact C-terminal helix. Based on our experimental data and bioinformatic analysis we propose a model for the role of the C-terminal helix in Vps4 assembly and ATPase activity.

Results

The C-terminal helix is essential for Vps4p function in vivo

Our approach to characterise the role of the C-terminal helix (Fig. 1A) was to perform a sequence alignment of yeast Vps4p and **human** VPS4A and 4B (Fig. 1B) to identify amino acids in the C-terminal helix that are highly conserved and predicted to be functionally important. **We identified sequences containing conserved amino acids at the start and end of the C-terminal helix** (Fig. 1B). To test their importance, we deleted the DNA sequence encoding these amino acids in a plasmid-borne copy of the *VPS4* gene. **We refer to the amino acid sequences deleted by their first three amino acids. The TRP sequence (TRPTVNEDDLLK) is at the start of the helix while the RDF sequence (RDFGQEGN) is at the end of the helix (Fig. 1B, C).** To determine whether the β domain has any functions in addition to those that we have previously identified [34], we also deleted a **conserved sequence (DELKEP)**, located at the end of the β domain (Fig. 1B, C). **We refer to this sequence as the DEL sequence.**

Plasmids encoding the Vps4p mutant proteins were introduced into *vps4Δ* cells and expression of the Vps4p mutant proteins was tested by immunoblotting of cell extracts (Fig. 1D). The expression level of each of the Vps4p mutant proteins was comparable to that of wild-type Vps4p. **Thus any loss of function of the mutant proteins in vivo cannot be attributed to lowered expression levels.** We subsequently tested the ability of the mutant Vps4p proteins to functionally substitute for Vps4p.

To assess the contributions of the Vps4p C-terminal helix and the previously uncharacterised β domain DEL sequence to Vps4p function in MVB sorting, we used a GFP-tagged marker known to undergo MVB sorting into the vacuole lumen [37]. This marker comprises Fth1p, an iron transporter that normally resides on the vacuole limiting membrane, conjugated to ubiquitin to

confer ubiquitin-dependent MVB sorting and to GFP for visualisation (Fth1p-GFP-Ub). The *vps4Δ* cells containing the above plasmids and expressing Fth1p-GFP-Ub were visualised by fluorescence microscopy to determine whether Fth1p-GFP-Ub was correctly MVB sorted and delivered to the vacuole lumen. In cells expressing wild-type Vps4p, Fth1p-GFP-Ub was observed in the vacuole lumen (Fig. 2A). However, in *vps4Δ* yeast expressing the mutant proteins or carrying empty vector, Fth1p-GFP-Ub appeared to be trapped in a compartment adjacent to the vacuole. Moreover, the small amount that reached the vacuole was present on the vacuole limiting membrane (Fig. 2A). We conclude that the C-terminal helix and the β domain DEL **sequence** are critical for Vps4p function in MVB sorting.

To investigate whether the Vps4p C-terminal helix and the β domain DEL **sequence** play major roles in vacuolar protein sorting, we tested the ability of the mutant proteins to correct vacuolar protein sorting defects of *vps4Δ*. Newly synthesised **vacuolar** proteins are delivered from the late secretory pathway to the vacuole via the MVB compartment. **In the late Golgi**, sorting of **soluble resident vacuolar proteins** from other cargo destined for the cell surface is mediated by a receptor, Vps10p, which continuously recycles between the late Golgi and the MVB [38]. **Transport of Vps10p from the MVB to the late Golgi is independent of the process of MVB sorting.** In *vps4Δ* cells, Vps10p along with several other late Golgi proteins becomes trapped in the MVB and is proteolytically degraded. The loss of Vps10p, results in **missorting and secretion of vacuolar proteins** into the extracellular medium [13, 39, 40]. **To test for vacuolar protein sorting**, we made use of the marker protein carboxypeptidase Y (CPY), which is a soluble resident protein of the vacuole. *vps4Δ* cells expressing wild-type Vps4p or the Vps4p mutant proteins or carrying vector alone were grown in contact with a filter and secreted proteins bound to the filter were detected by immunoblotting. Cells expressing wild-type Vps4p retained CPY intracellularly (Fig. 2B). In contrast, cells expressing Vps4p mutant proteins or carrying empty vector released CPY into the

medium allowing its detection on the filter (Fig. 2B). We conclude that the C-terminal helix and the β domain DEL sequence play an essential role in Vps4p function in vacuolar protein sorting.

vps4Δ cells exhibit a kinetic delay in transport of endocytosed material, including alpha factor and both water- and membrane-soluble dyes, to the vacuole [41, 42]. To assess the importance of the C-terminal helix and β domain DEL sequence in this Vps4p-dependent process, we compared the ability of the mutant and wild-type Vps4p proteins to restore efficient vacuolar accumulation of a fluid-phase marker, lucifer yellow (LY), in *vps4Δ* cells (Fig. 2C). **While there was some low level accumulation of LY in the vacuoles of cells expressing Vps4p mutant proteins (this varied from cell to cell)** expression of wild-type Vps4p restored efficient LY accumulation in the vacuoles **of all cells** (Fig. 2C). Therefore the Vps4p C-terminal helix and the β domain DEL sequence are important for efficient transport of fluid-phase markers to the vacuole.

The endocytic defects of *vps4Δ* cells are accompanied by a temperature-sensitive growth defect, which permits growth at 24 °C but not at 40 °C **[41, 42]**. Consistent with the restoration of endocytic functions, wild-type Vps4p but not the mutant Vps4p proteins rescued the temperature-sensitive growth defect of *vps4Δ* cells on solid medium (Fig. 2D). We conclude that the C-terminal helix and the β domain DEL sequence are important for Vps4p function in growth at elevated temperature.

Vps4p recruitment to endosomes is independent of the C-terminal helix

In work described above, we have shown that the C-terminal helix and the β domain DEL sequence are important for all Vps4p in vivo functions tested. One possible reason for this is a role for the conserved sequences in Vps4p recruitment to endosomes, as we **and others** have previously shown that recruitment of Vps4p to endosomes is essential for all Vps4p in vivo functions [32, 33]. To

assess a potential role for the C-terminal helix and the β domain DEL sequence in recruitment to endosomes, we compared the subcellular localisation of GFP-tagged wild-type and mutant Vps4p proteins expressed in *vps4Δ* yeast (Fig. 3). GFP-tagged wild-type and mutant Vps4p proteins localised to punctate cytoplasmic structures consistent with recruitment to endosomes. In contrast, a GFP-tagged Vps4p mutant protein that lacks the N-terminal MIT domain (Vps4p-CC) exhibited diffuse fluorescence throughout the cytoplasm consistent with a defect in endosomal recruitment as described previously [33, 34]. We conclude that the C-terminal helix and the β domain DEL sequence are not essential for Vps4p recruitment to endosomes.

The C-terminal helix is essential for Vps4p ATPase activity in vitro

Since the C-terminal helix was critical for in vivo function but not for recruitment to endosomes, we reasoned that it might be important for Vps4p ATPase activity. This is because the 3D structure of Vps4p shows that the C-terminal helix is positioned in close proximity to the ATPase catalytic site [31, 36] To assess the importance of the C-terminal helix as well as the β domain DEL sequence for Vps4p ATPase activity, wild-type and mutant Vps4p proteins **were purified** (Fig. 4A) **and the ATPase activity of each Vps4p protein was assayed** (Fig. 4B). Mutant Vps4p proteins lacking an intact C-terminal helix exhibited greatly diminished ATPase activity compared to wild-type Vps4p. Furthermore, consistent with our previous findings with a different Vps4p β domain mutant protein, Vps4p-GAI [34] **that was included for comparison**, loss of the DEL sequence also diminished Vps4p ATPase activity. We conclude that the C-terminal helix and the β domain DEL sequence are critical for Vps4p ATPase activity in vitro.

The Vps4p C-terminal helix is dispensable for all known Vps4p interactions

To determine whether the C-terminal helix and the β domain DEL sequence are important for interaction of Vps4p with other proteins, we tested the ability of the Vps4p mutant proteins to

interact with a set of known Vps4p-interacting proteins. Using a yeast two-hybrid assay (Fig. 5A), **we found** no evidence that any of the Vps4p mutations **diminished** interactions with Did2p or the ESCRT III components Vps2p, Snf7p and Vps20p, which we **and others** have previously shown interact with the Vps4p N-terminal MIT domain [32, 43, 44]. **Instead, the interaction with Vps20p appeared to be strengthened by the mutations.** Deletion of the TRP and RDF sequences also did not perturb interaction with Vta1p, which interacts with Vps4p via the C-terminal β domain. In contrast, deletion of the DEL sequence abolished interaction with Vta1p.

As an independent test of the importance of the C-terminal helix and β domain DEL sequence for known Vps4p protein interactions, we employed an in vitro protein-binding assay (Fig. 5B). This assay also allowed us to test Vps4p interaction with Bro1p, which binds Vps4p in vitro but does not exhibit yeast two-hybrid interaction with Vps4p [32, 45]. We also included the β domain mutant, Vps4p-GAI, for comparison in these experiments. Consistent with the yeast two-hybrid results described above, the C-terminal helix was dispensable for interaction with Vta1p, Did2p and the ESCRT III components, Vps2p and Vps20p. In addition, these experiments also showed that the C-terminal helix is dispensable for binding **to** Bro1p. Also consistent with the yeast two-hybrid data, the β domain DEL sequence, like the GAI sequence, was critical for binding to Vta1p but not for **any** other interaction including **that with** Bro1p. We conclude that the C-terminal helix is dispensable for all Vps4p interactions tested while the β domain DEL sequence is essential for binding to Vta1p.

Interactions between the Vps4p MIT domain and a subset of ESCRT III components are regulated by Vps4p ATPase activity [32, 46]. Our finding that the C-terminal helix is important for Vps4p ATPase activity suggests that loss of the C-terminal helix may abrogate ATPase-dependent dissociation **from these** ESCRT III components. We therefore compared binding of an ESCRT III

component, Vps20p, to wild-type and mutant Vps4p proteins in the presence and absence of ATP (Fig. 5C). Binding of Vps20p to the Vps4p mutant proteins lacking the DEL, TRP and RDF sequences showed at most a marginal decrease (≤14%) in the presence of ATP. In contrast, binding of Vps20p to wild-type Vps4p in the presence of ATP was considerably decreased (~60%). These data are consistent with our in vitro data showing that the C-terminal helix and β domain DEL sequence are critical for Vps4p ATPase activity. Furthermore, the data offer a possible explanation for the strengthened interaction of Vps20p with the Vps4p mutant proteins **that we observed** in vivo using the yeast two-hybrid assay.

Mutations in the C-terminal helix confer phenotypes that are either recessive or only partially dominant

Many *vps4* mutations confer dominant-negative phenotypes [33, 41, 47]. Therefore, we tested whether the Vps4p mutant proteins lacking the C-terminal helix TRP or RDF sequences or the β domain DEL sequence also confer dominant-negative phenotypes. Each mutant protein was expressed in wild-type cells and the effect on Vps4p-dependent functions was tested. MVB sorting (Fig. 6A) of the Fth1p-GFP-Ub marker to the vacuole lumen was partially inhibited in wild-type cells expressing the Vps4p-TRP mutant protein, although not as strongly as observed in cells expressing the dominant-negative Vps4p mutant protein, Vps4p-E233Q. In contrast, MVB sorting of Fth1p-GFP-Ub was normal in wild-type cells expressing wild-type Vps4p, Vps4p-DEL, or Vps4p-RDF mutant proteins or carrying vector only.

The partial dominant-negative effect of Vps4p-TRP was also observed in the assay for vacuolar protein sorting (Fig. 6B). Again this defect was not as strong as in cells expressing dominant-negative Vps4p-E233Q. In contrast, expression of the Vps4p- DEL or RDF mutant proteins in wild-type cells did not confer a dominant-negative effect on CPY sorting. None of the Vps4p mutant

proteins conferred any detectable dominant-negative effects on either fluid-phase endocytosis or growth at elevated temperature (Fig. 6C, D), although the Vps4p-E233Q mutant protein also conferred dominant-negative effects on both of these processes.

We conclude that the Vps4p-TRP mutant protein can confer a partial dominant-negative effect while the Vps4p-RDF and Vps4p-DEL mutant proteins cannot.

The C-terminal helix and β domain DEL sequence are essential for Vps4p oligomerisation in vitro

It has previously been proposed that wild-type Vps4p, like other AAA ATPases, functions as an oligomer in vivo although such an oligomer has been difficult to detect in vitro perhaps due to its transient nature. However, the Vps4p-E233Q mutant protein, which **has a mutation in the ATP hydrolysis site,** is known to form a stable oligomer in the presence of ATP in vitro [33]. To address the role of the C-terminal helix in ATP-dependent Vps4p oligomerisation in vitro, we introduced the C-terminal helix RDF mutation into a Vps4p-E233Q mutant protein and examined its effect on oligomer formation in vitro. Gel filtration analysis to resolve Vps4p complexes of different sizes showed that in the absence of ATP, Vps4p-E233Q has a molecular weight of ~92 kDa, which is consistent with the size of a dimer. However, in the presence of ATP, the shift in the elution profile is consistent with formation of a higher order oligomer with a molecular weight of ~350 kDa (Fig. **7A**).

In contrast, the elution profile of the Vps4p-E233Q-RDF double mutant protein indicated that the mutant protein has a predicted molecular weight of ~65kDa in the presence or absence of ATP **(Fig. 7B)**. This value is intermediate between that predicted for the monomer and dimer, and so we analysed the Vps4p-E233Q-RDF mutant protein using multi-angle laser light scattering (MALLS)

analysis, which unlike gel filtration is able to determine molecular weight independent of protein shape [48]. MALLS analysis of the predominant peak from gel filtration indicated that the Vps4p-E233Q-RDF mutant protein is a stable monomer (M_r 52 kDa). We conclude that the C-terminal helix is critical for the ability of Vps4p-E233Q to form a stable higher order oligomer in vitro in the presence of ATP.

Similarly, the elution profile of a Vps4p-E233Q mutant protein lacking the conserved β domain sequence, GAI, was consistent with a molecular weight of ~70 kDa in the presence or absence of ATP (**Fig. 7C**). Subsequent MALLS analysis showed that this mutant protein is also a monomer (M_r 44 kDa). These data are consistent with our previous yeast two-hybrid in vivo data [34].

We conclude that the Vps4p C-terminal helix and β domain both play essential roles in **dimerisation and** ATP-dependent formation of Vps4p higher order oligomers in vitro.

Mutations in the C-terminal helix of a dominant negative Vps4p mutant protein do not prevent it from conferring a dominant-negative phenotype

As an independent in vivo test of the role of the Vps4p C-terminal helix in oligomerisation, we employed the same strategy that we have previously used to assess the role of the β domain in Vps4p oligomerisation in vivo. In this strategy, we assess the ability of additional mutations to reduce the ability of Vps4p-E233Q to engage with and interfere with the function of wild-type Vps4p. We therefore deleted the C-terminal helix RDF and TRP sequences as well as the β domain DEL sequence in the dominant negative Vps4p-E233Q mutant protein and tested the ability of the double mutant proteins to elicit Vps4p mutant phenotypes in otherwise wild-type cells (Fig. 8). Deletion of the C-terminal helix RDF sequence did not appear to reduce the dominant negative effects of the Vps4p-E233Q mutant protein at 24 °C (**Fig. 8A, B**) but alleviated the effect

somewhat at elevated temperature (**Fig. 8C**). In contrast, deletion of the C-terminal helix TRP sequence **partially** reduced the dominant-negative effect of Vps4p-E233Q at each temperature tested (**Fig. 8A, B, C**). Consistent with our previous finding with the Vps4p β domain GAI sequence, deletion of the DEL sequence abrogated the dominant-negative effect of the E233Q mutation (**Fig. 8A, B, C**).

These data suggest that the RDF and TRP sequences in the C-terminal helix are not essential for interaction of Vps4p-E233Q with wild-type Vps4p in vivo, although loss of the TRP sequence weakens the interaction. In contrast, the DEL sequence is essential for interaction of Vps4p-E233Q with wild-type Vps4p. In summary, mutations in the C-terminal helix differ in their ability to abolish interaction of Vps4p-E233Q with wild-type Vps4p while both β domain mutations tested abolish this interaction.

The Vps4p C-terminal helix is not essential for homotypic interaction in vivo

In previous work we have shown that wild-type Vps4p exhibits a homotypic interaction (Vps4p-Vps4p) in the yeast two-hybrid system [34], which is consistent with biochemical data showing that wild-type Vps4p forms a dimer [33]. Our in vitro gel filtration data showing the role of the C-terminal helix in oligomerisation of the Vps4p mutant proteins described above suggest that the C-terminal helix, like the β domain, **may** play a critical role in homotypic interaction **in vivo**. To test whether the Vps4p C-terminal helix and the β domain DEL sequence are important for **Vps4p** homotypic interaction in vivo, we tested the ability of the mutant proteins to self-associate and to interact with wild-type Vps4p using the yeast two-hybrid system (Fig. 9). Consistent with our previous observation with the β domain GAI sequence [34], deletion of the DEL sequence in the β domain abolished the homotypic interaction with either wild-type or mutant Vps4p. Unexpectedly,

however, deletion of the C-terminal helix TRP and RDF sequences did not affect the homotypic interaction with wild-type or mutant Vps4p.

Despite the importance of the Vps4p C-terminal helix conserved sequences for oligomerisation in vitro, we surmise that these sequences are not essential for the Vps4p homotypic interaction in vivo. However, the β domain DEL sequence, like the GAI sequence, is essential for **Vps4p homotypic** interaction in vivo.

Vta1p promotes the assembly of Vps4p-RDF and Vps4p-E233Q mutant proteins into hybrid complexes that are catalytically active in vitro

Although the Vps4p-E233Q-RDF double mutant protein could not assemble into dimers in vitro, Vps4p-RDF retained the Vps4p homotypic interaction in vivo. Furthermore, the Vps4p-RDF-E233Q double mutant protein retained the ability to induce dominant-negative effects like Vps4p-E233Q. This suggests that loss of the RDF sequence does not abolish the ability of Vps4p-E233Q to engage wild-type Vps4p and inhibit its function in vivo. One possible explanation for the difference between our in vivo and in vitro findings is that in vivo Vta1p may allow assembly of otherwise assembly-incompetent Vps4p-E233Q-RDF with wild-type Vps4p. Vta1p is known to promote Vps4p assembly and ATPase activity in vitro [49, 50] and is expressed in the yeast-two hybrid strain used to test the homotypic interaction and in the strain used for phenotypic assays. Vta1p-dependent assembly would not occur in vitro since our in vitro experiments were performed using purified proteins and Vta1p was not included.

To test the ability of Vta1p to promote assembly of Vps4p-RDF, we examined whether addition of Vta1p could promote assembly of Vps4p-RDF into a catalytically active ATPase in vitro. We assessed assembly by monitoring ATPase activity because ATPase activity reflects

assembly of physiologically relevant complexes. Consistent with our hypothesis, the addition of Vta1p to Vps4p-RDF did stimulate the ATPase activity of Vps4p-RDF, however the activity was still significantly lower than that of wild-type Vps4p (Fig. 10A).

The RDF sequence lies in close proximity to the Arg residues within the SRH motif, which are important for both assembly and inter-subunit catalysis in AAA ATPases [23]. Therefore, we next considered the possibility that deletion of the RDF sequence may disrupt the function of the SRH motif and thereby affect assembly and ATPase activity. If this were true, then addition of a Vps4p protein with a functional SRH to the Vps4p-RDF mutant protein in *trans* might enable the formation of a catalytically active hybrid oligomer. We could not test the ability of wild-type Vps4p to promote ATPase activity of the Vps4p-RDF mutant protein since wild-type Vps4p already has ATPase activity and this could mask the activity stimulated in Vps4p-RDF. Instead, we tested the ability of Vps4p-E233Q, which has a mutation in the ATP hydrolysis site, to stimulate ATPase activity of Vps4p-RDF. Despite being defective in ATP hydrolysis, Vps4p-E233Q has both an intact SRH motif and C-terminal helix. In the presence of Vta1p, Vps4p-E233Q stimulated the ATPase activity of Vps4p-RDF to a considerably greater extent than Vta1p alone (Fig. 10A). Vps4p-E233Q alone stimulated the ATPase activity of Vps4p-RDF, although much more weakly than when Vta1p was also present (Fig. 10A). The stimulation observed with Vps4p-RDF was greater than that observed with wild-type Vps4p in the presence of Vps4p-E233Q and/or Vta1p (Fig. 10A). However, in the presence of higher concentrations of ATP, a greater stimulation of wild-type Vps4p activity may be obtained.

To test whether these effects are specific to Vps4p-RDF we examined the effect of Vps4p-E233Q and/or Vta1p addition on the ATPase activity of the other Vps4p mutant proteins

referred to in this study. Addition of Vps4p-E233Q and/or Vta1p did not have any apparent effect on the ATPase activity of the other Vps4p C-terminal helix mutant protein (Vps4p-TRP). Thus, the roles of the conserved sequences at the start and end of the C-terminal helix are distinct. Addition of Vps4p-E233Q and/or Vta1p did not stimulate the ATPase activity of the β domain mutant proteins (Vps4p-GAI and Vps4p-DEL), which cannot bind Vta1p.

We conclude that Vps4p-RDF and Vps4p-E233Q can assemble into a catalytically active hybrid complex and this assembly is promoted by Vta1p (Fig. 10B). Clearly, the RDF sequence at the end of the C-terminal helix is essential for ATPase activity, however, this requirement can be bypassed by the formation of a hybrid complex with Vps4p-E233Q, which has a mutation in the ATP hydrolysis site but which has a functional SRH and C-terminal helix.

Discussion

Several recent structural studies of the AAA ATPase, Vps4, have revealed features that are highly conserved between yeast and human Vps4 [30, 31, 36, 43, 44]. The challenge now is to determine how these structural features contribute to Vps4 function. Here, we focus our attention on the role of the C-terminal helix of Vps4p, which has been elusive. In the secondary structures of yeast and mammalian Vps4, the C-terminal helix is an independently folded structure that is separated from the ATPase domain by **a structured loop (Fig. 1C), [31, 36]**. However, in the **tertiary** structures, the C-terminal helix is in **close proximity** to the catalytic domain suggesting a **possible function in catalysis**. This C-terminal helix **appears to be** a common feature of the meiotic clade of AAA ATPases (Fig. 11), [31]. **The meiotic clade** includes katanin and fidgetin, which are important for cell division [51, 52], **and spastin, which is mutated in hereditary spastic paraplegia** [53].

Our **functional** characterisation of the Vps4 C-terminal helix is based on our analysis of two **sequences that contain amino acids highly conserved in Vps4 orthologues (Fig. S1), [31, 54]** and thus predicted to be functionally important. One of these, the TRP sequence, includes the start of the C-terminal helix as well as the **structured loop** between the ATPase domain and the C-terminal helix and in the 3D structure is positioned close to the ATP binding site (**Fig. 12A**). The second sequence, RDF, is at the end of the **C-terminal** helix, and in the 3D structure (**Fig. 12A**) is positioned close to the SRH motif. **The SRH motif contains Arg residues, which interact with the catalytic site of the neighbouring subunit and have been shown to be important for inter-subunit catalysis in other AAA ATPases** [22, 23]. We show that the C-terminal helix is essential for a range of Vps4p in vivo functions, including MVB sorting, fluid-phase endocytosis, vacuolar protein sorting, and growth at high temperature, based on analysis of the phenotypes conferred by mutations in **the** conserved **TRP and RDF** sequences. The C-terminal helix, like the β domain, is dispensable for recruitment to endosomes but is essential for Vps4p oligomerisation and ATPase

activity in vitro. In contrast to the β domain, however, the C-terminal helix is dispensable for the homotypic interaction in vivo and mutations in the C-terminal helix do not reverse the dominant-negative effects of Vps4p-E233Q. These **data** indicate that the contributions of the C-terminal helix and β domain to Vps4p oligomerisation are distinct. **We also show that Vta1p promotes formation of a catalytically active hybrid oligomer comprising a Vps4p mutant protein lacking the conserved RDF sequence and Vps4p-E233Q, which has a mutation in the ATP hydrolysis site.**

The C-terminal helix is not critical for Vps4p expression or its ability to adopt an overall folded structure. This conclusion is supported by several lines of evidence. First, the steady state expression level of each Vps4p mutant protein tested is equivalent to that of wild-type Vps4p. Second, each of the mutant proteins tested retained the ability to interact with a panel of known Vps4p interactors indicating that the N-terminal domain is correctly folded in each of the mutant proteins and that the β domain is correctly folded in the Vps4p proteins harbouring mutations in the C-terminal helix. This was demonstrated using both the yeast two-hybrid assay and in vitro protein binding assays except for the Bro1p interaction, which is not detectable using the yeast two-hybrid assay [32, 45]. Third, the ability of each mutant protein to be recruited to endosomes is consistent with the **N-terminal endosome targeting domain retaining its native structure**. **Fourth, we show that Vps4p-RDF retains the capacity for ATP hydrolysis since Vps4p-RDF/Vps4p-E233Q hybrid oligomers are catalytically active. This suggests that the folding of the ATPase domain is not grossly affected by loss of the RDF sequence.**

In a previous study we found that specific substitution of the charged amino acids R, D and E in the RDF sequence (RDFGQEG) at the end of the C-terminal helix (**to generate the Vps4p-RDE mutant protein**) had minimal effects on Vps4p function [34]. The only apparent phenotype of cells

expressing the Vps4p-RDE mutant **protein**, was a mild temperature-sensitive phenotype. In contrast, here we show that complete deletion of the RDF sequence abolishes all Vps4p functions. This suggests that other amino acids are more important than the charged amino acids, R, D, and E. Indeed, within the predicted C-terminal helices of the meiotic clade of AAA ATPases, the FG residues in the RDF sequence are the most highly conserved (Fig. 11, Fig. S1) and may be more critical for function.

The importance of the C-terminal helix for Vps4p ATPase activity is evident from both ATPase activity assays and our finding that mutation of the C-terminal helix abrogates ATP-dependent dissociation of Vps20p-Vps4p complexes in vitro. In addition, yeast two-hybrid assays suggest that the Vps20p-Vps4p interaction is stabilised in vivo by mutation of the C-terminal helix. This is consistent with an essential role of the C-terminal helix in Vps4p ATPase activity in vivo.

Loss of ATPase activity caused by mutations **in** the C-terminal helix may be due to defects in oligomerisation. Previous studies have shown that Vps4p-E233Q undergoes ATP-dependent assembly into a higher order oligomer in vitro [33]. However, mutation of the C-terminal helix prevents this oligomerisation in vitro so that Vps4p-E233Q-RDF exists as a monomer in the presence or absence of ATP. This is consistent with an essential role for the C-terminal helix in oligomerisation in vitro. Furthermore, phenotypic analysis of the Vps4p-E233Q-TRP double mutant suggests an important role for the C-terminal helix in Vps4p oligomerisation in vivo. Vps4p-E233Q, which is locked in the ATP-bound state, acts as a powerful inhibitor of Vps4p function in vivo. This dominant-negative inhibition is rationalised on the basis of interaction of Vps4p-E233Q with wild-type Vps4p and assembly of catalytically inactive hybrid oligomers. Mutations that affect Vps4p oligomerisation would be predicted to reverse the dominant-negative inhibition caused by the E233Q mutation. Indeed in a previous study, we showed that mutations in the β domain reverse

the **dominant-negative** inhibition caused by the E233Q mutation [34]. Interestingly, Vps4p-E233Q-TRP retained only partial dominance consistent with a role for the C-terminal helix in oligomerisation **in vivo**. However, mutation of the RDF sequence **at the end** of the C-terminal helix did not reverse the dominant-negative inhibition caused by Vps4p-E233Q in vivo. This suggests that despite the defect in oligomerisation caused by mutation of the C-terminal RDF sequence in vitro, this mutation does not prevent association of Vps4p-E233Q with wild-type Vps4p and inhibition of its activity in vivo.

We have previously described a homotypic interaction **involving** Vps4p using the yeast two-hybrid system [34]. Mutations in the β domain abolish this homotypic interaction in vivo. However, consistent with our phenotypic analysis of Vps4p-E233Q-RDF and Vps4p-E233Q-TRP double mutants, mutations in the C-terminal helix do not abolish the Vps4p homotypic interaction in vivo. Two main factors may explain the differences between the in vitro and the in vivo data in regard to Vps4p assembly. First, Vps4p-E233Q-RDF retains interaction with Vta1p, which has been proposed to play an important role in Vps4p oligomerisation [49]. Thus, in vivo, the presence of Vta1p may enable oligomerisation of Vps4p monomers, which due to mutation of the C-terminal helix, are unable to oligomerise in vitro and may promote the homotypic interaction and the dominant-negative effects of Vps4p-E233Q-RDF in vivo. **In contrast, loss** of the β domain, which abolishes Vta1p binding, would abolish Vps4p oligomerisation in vivo and in vitro **in the presence or absence of Vta1p**. Second, the Vps4p-E233Q-RDF mutant protein, while unable to interact with other Vps4p-E233Q-RDF mutant proteins, may still retain the ability to interact with wild-type Vps4p. This would explain why mutations in the C-terminal helix do not abolish the dominant-negative effect of Vps4p-E233Q on wild-type Vps4p in vivo.

Since Vps4p-E233Q and Vps4p-RDF are individually defective in ATP hydrolysis, the ATPase activity that is stimulated upon mixing the two proteins suggests assembly of a hybrid oligomer in vitro. This is consistent with previous studies showing that AAA ATPases assemble into oligomeric rings and that ATPase activity is dependent on stimulation of ATP hydrolysis in one subunit by conserved Arg residues in the SRH motif of an adjacent subunit within a ring [22, 23, 25]. Our finding that Vta1p promotes formation of a catalytically active Vps4p-RDF/Vps4p-E233Q hybrid complex in vitro supports our proposal that Vta1p may promote assembly of Vps4p-E233Q-RDF with wild-type Vps4p in vivo. This may explain why Vps4p-E233Q-RDF confers a dominant negative phenotype in vivo despite its inability to oligomerise in vitro. One might expect therefore that Vps4p-RDF may also assemble with wild-type Vps4p in vivo. However this would not **be predicted to** lead to dominant-negative effects since **the wild-type Vps4p would stimulate ATPase activity of Vps4p-RDF and thus the mixed oligomer would retain catalytic activity and function in vivo.**

By analogy to other AAA ATPases, which form hexameric rings [25], Vps4p-E233Q has been proposed to assemble into a dodecamer comprising two stacked hexameric rings [31]. While the 3D structure of this Vps4p-E233Q oligomer has yet to be elucidated, modelling of the **human** Vps4B ATPase domain and C-terminal helix into a hexameric ring, based on the oligomeric structure of the AAA ATPase p97, predicts that the C-terminal helix is well positioned to mediate intersubunit interactions between the two stacked rings [31]. Intriguingly, the RDF sequence in the crystal structure of the **human** Vps4B **(Fig. 12B)** is positioned in close proximity to Arg residues in the SRH that are important for intersubunit interactions in other AAA ATPases [22, 23]. Furthermore the TRP sequence is positioned very close to the ATP binding pocket in the adjacent subunit. Thus the TRP sequence at the beginning of the **C-terminal** helix and **the** RDF **sequence** at the end of the **C-terminal** helix may both have additional roles in mediating intersubunit interactions within a

ring. **Alternatively, the RDF and TRP sequences may modulate the functions of the SRH and the ATP binding pocket, respectively. This may in turn contribute to assembly into an active ATPase. The fact that Vps4p-RDF activity is stimulated by catalytically inactive Vps4p-E233Q, which has an intact C-terminal helix and SRH motif, is consistent with a role for the RDF sequence in modulating the function of the SRH.**

The C-terminal region of human Vps4B contains the β domain (β strands 7 and 8), the final helix of the AAA domain (α helix 10) and the C-terminal helix (α helix 11). This C-terminal region of Vps4 has been defined in the PFAM database as the "Vps4 oligomerisation domain"(PF09336) based on our previous study showing that the β domain is required for Vps4 oligomerisation [34]. According to the PFAM database there are 259 known proteins with elements of this Vps4 oligomerisation domain (a full list is available at http://pfam.sanger.ac.uk/family?acc=PF09336). With a few possible exceptions, these proteins are meiotic clade AAA ATPases (see below) (Fig. 11). Some of these proteins are likely to be Vps4 orthologues and contain all three structural elements of the Vps4 oligomerisation domain (i.e. β sheets 7 and 8, the AAA domain helix, and the C-terminal helix). However, the majority of these proteins are likely to be other meiotic clade AAA ATPases and have the AAA domain helix and the C-terminal helix but not the β domain.

The distinguishing feature of members of the meiotic clade of AAA ATPases is the SRH motif, which differs from that of other AAA ATPases [24]. The pair of Arg residues in the SRH motif, which mediate inter-subunit interactions important for catalysis, is not separated by two residues as in **non-meiotic clade** AAA ATPases (Fig. 11). In addition, a third Arg residue (**also within the SRH motif**) **frequently** precedes the conserved pair of Arg residues. Another distinguishing feature appears to be the presence of the C-terminal helix (**Fig. 11**). Moreover, **we find that** the residues

FG within the RDF sequence at the end of the Vps4p C-terminal helix are highly conserved in members of the meiotic clade of AAA ATPases (Fig. 11, Fig. S1). A striking observation in the 3D structure of human Vps4B is that the highly conserved Phe **440** residue in the C-terminal helix is positioned close to Arg **289** that is present in the SRH (Fig. 12B). **The corresponding Phe 432 and Arg 287 are also in close proximity in the yeast Vps4p structure (not shown).** It is **possible** that one outcome of deleting the RDF sequence in **yeast Vps4p** is to interfere with an interaction between **Phe 432** and **Arg 287** that in turn interferes with the function of the SRH and that this affects Vps4p assembly and ATPase activity.

In this study we also characterised a second conserved sequence, DEL, within the β domain. **Interestingly, this sequence has an insertion in the two plant Vps4 orthologues included in our alignment (Fig. S1).** We find that the DEL sequence is important for full ATPase activity in vitro, for Vps4p homotypic interaction and for the ability of the Vps4p-E233Q dominant negative mutant to engage with and inhibit wild-type Vps4p. This is consistent with the results of our previous study in which we analysed the phenotypes **that arise when** the GAI sequence (also within the β domain) is deleted. Also, like the GAI sequence, the DEL sequence is critical for Vps4p interaction with Vta1p, a protein proposed to stimulate Vps4p oligomerisation. Furthermore, gel filtration and multi-angle laser light scattering analysis shown here reveal that Vps4p-E233Q-GAI, unlike Vps4p-E233Q, is a monomer in vitro and does not assemble into a **higher** order oligomer upon addition of ATP. These findings further support the role we previously proposed for the β domain in mediating Vps4p dimerisation.

In summary, we have shown here that the Vps4p C-terminal helix is critical for Vps4p oligomerisation **and** ATPase activity **in vitro,** and for endosomal function in vivo, but is dispensable for interaction with ESCRT III and recruitment to endosomes. **We also show that**

Vta1p promotes the assembly of a catalytically active hybrid complex comprising a Vps4p mutant protein lacking the conserved RDF sequence at the end of the C-terminal helix and Vps4p-E233Q, which has a mutation in the ATP hydrolysis site. This demonstrates that the requirement for the conserved RDF sequence for assembly and activity can be overcome by addition of Vta1p and a second Vps4p molecule with an intact C-terminal helix. We also find evidence for the co-evolution of the C-terminal helix (in particular an FG motif at the end of the C-terminal helix) with the distinct SRH in the meiotic clade of AAA ATPases. Since the conserved FG motif at the end of the C-terminal helix lies in close proximity to the SRH in the 3D structure, we propose that the C-terminal helix may be important for the function of the SRH motif in Vps4p assembly and inter-subunit catalysis. It will be interesting in future work to investigate whether the functions of the C-terminal helix described here for Vps4p are conserved in other meiotic clade AAA ATPases **such as spastin, which is implicated in human neurological disorders.**

Experimental procedures

Media, reagents, strains and plasmids

YPUAD rich media and SD minimal media were prepared as described previously [46]. Lucifer Yellow (LY) carbohydrazide dilithium salt was obtained from Fluka AG (Buchs, Switzerland). Bathophenanthroline disulfonic acid (BPS) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG were from Sigma-Aldrich (St. Louis, MO, USA). HRP-conjugated rabbit anti-goat IgG was from Zymed (San Francisco, CA, USA). HRP-conjugated **goat** anti-mouse IgG and gel filtration standards were from Bio-Rad Laboratories (Hercules, CA, USA). Monoclonal anti-penta His antibody and Ni-NTA agarose were from Qiagen (Hilden, Germany). Immobilised glutathione on agarose was from Scientifix (Melbourne, Australia). Prestained protein molecular weight marker was from Fermentas (Hanover, MD, USA). PVDF membrane was from Millipore (Bedford, MA, USA). **Goat** polyclonal anti-**yeast** Vps4p antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and **rabbit** polyclonal anti-carboxypeptidase Y and anti-calmodulin antisera were gifts from H. Riezman (University of Geneva, Switzerland).

PCR primers used for plasmid constructions were from GeneWorks (Thebarton, Australia) and are listed in Table 1. *S. cerevisiae* strains and plasmids used in this study are listed in Tables 2 and 3, **respectively**. The sequence of all constructs was confirmed by automated DNA sequencing (Australian Genome Research Facility, Brisbane, Australia). Transformation of yeast with plasmid DNA was **performed** as described previously [32].

Construction of plasmids

Genomic DNA was prepared from *S. cerevisiae* as described previously [32] and PCR was carried out using the proof-reading DNA polymerase Pfu (Fermentas, Hanover, MD, USA). C-terminal DEL, TRP and RDF mutants were generated by site-directed mutagenesis using the same strategy

that we employed previously [34]. Oligonucleotides used are listed in Table 1. To generate pLexA or pB42 constructs, mutant *VPS4* **genes** were amplified without any upstream sequence and with suitable restriction sites for cloning **in-frame into these vectors**. To express mutants with a C-terminal GFP tag, **genes** were **PCR-**amplified without a stop codon and cloned in-frame into a YCplac111-based plasmid encoding **yeast codon optimised** yEGFP. **The yEGFP sequence was sub-**cloned into YCplac111 from pYM12 [55]. To express mutant **proteins** with a C-terminal hexa-His tag in *E. coli*, **coding sequences** were amplified **using** a **primer** that encodes C-terminal hexa-His tag and **cloned** downstream of the T7 promoter of pET11a or pET11d (Novagen, Madison, WI, USA).

Phenotypic assays

Assays for fluid-phase endocytosis, MVB sorting, carboxypeptidase Y sorting and temperature-sensitive growth were performed as described previously [34].

Western blot analysis of total yeast cell lysates

For western blot analysis of total cell lysates, AMY245 (*vps4Δ*) yeast carrying expression plasmids were grown at 24 °C overnight. Lysates were prepared as described previously [34] and were subjected to 10% SDS-PAGE. The proteins were transferred to a PVDF filter **and this** was then probed with a goat anti-yeast Vps4p polyclonal antibody and enhanced chemiluminescence.

ATPase activity assay

The 6His-tagged WT Vps4p or Vps4p mutant proteins were expressed in *E. coli* and purified on Ni-NTA agarose. The 6His-tagged proteins were eluted from the resin using 250 mM imidazole and analysed using SDS-PAGE. To assay for ATPase activity, wild-type or Vps4p mutant proteins (3 μg) in ATPase assay buffer [13] (0.1 M potassium acetate, 5 mM

magnesium acetate, 20 mM HEPES, pH 7.4) were incubated in 0.1 mM ATP in a 100 μl assay volume for 1 h at 30°C. Released inorganic phosphate was quantified using a phosphate detection kit (R&D Systems, Minneapolis, MN, USA). To test the effect of adding Vps4p-E233Q and GST-Vta1p, 6His-tagged Vps4p-E233Q was purified as above and GST-Vta1p was purified on glutathione agarose and eluted in assay buffer containing 5 mM glutathione. The wild-type and C-terminal mutant Vps4p proteins (1.3 μg) were incubated alone or with Vps4p-E233Q (1.3 μg) and GST-Vta1p (2 μg), either alone or together, in the presence of 0.1 mM ATP in a 100 μl assay volume for 1h at 30 °C. Inorganic phosphate released was quantified using the phosphate detection kit (as above).

In vitro protein binding assay

In vitro binding assays to compare the binding of 6His-tagged wild-type Vps4p or Vps4p mutant proteins to Vps4p-interacting proteins fused to GST or to test for ATPase-sensitive interaction with Vps20p were performed as previously described [32].

Yeast two-hybrid protein interaction analysis

Protein interactions were assayed using the Matchmaker LexA yeast two-hybrid system from Clontech (Palo Alto, CA, USA) as described previously [46]. Briefly, bait plasmids containing LexA fusion proteins were co-transformed into the yeast strain EGY48 along with prey plasmids encoding proteins fused to a **B42** activation domain and the reporter plasmid p8op-LacZ. To test for interaction, transformants were spotted onto synthetic galactose/raffinose complete medium lacking Ura, Trp, and His and containing X-gal. The strength of protein interactions was assessed by blue coloration on this medium.

Microscopy

Microscopy was performed using an Olympus BX51 (Olympus Australia Pty, Ltd., Mount Waverly, Australia) with a Nomarski filter for visualising vacuoles and the appropriate filters for viewing LY or GFP fluorescence.

Gel filtration chromatography

Gel filtration chromatography was performed on a Superdex 200 10/300 GL column (GE Healthcare). An aliquot containing ~250 μg of purified recombinant protein was loaded and the column was run at 0.5 ml/min using **either 0.1 M potassium acetate, 5 mM magnesium acetate, 20 mM HEPES, pH 7.4, ±1 mM ATP or 20 mM HEPES, 200 mM potassium chloride, 10 mM magnesium chloride, pH 7.5, ±1 mM ATP**.

Gel filtration chromatography-multiangle laser light scattering

Light scattering of eluates from gel filtration chromatography run in 0.1 M potassium acetate, 5 mM magnesium acetate, 20 mM HEPES, pH 7.4, 1 mM ATP, was monitored using a DAWN-HELEOS MALLS photometer (50mW solid-state laser operating at λ=658 nm), which provides scattering measurements at up to 16 angles (Wyatt Technology), combined with an Optilab rEX refractive index detector (Wyatt Technology). MALLS data was processed using ASTRA software **(Wyatt Technology)**. For molecular weight determination the differential refractive index response was used to determine protein concentration, assuming a specific refractive index increment (dN/dC) value of 0.190 mL/g. Data were fitted to a first order Debye model, assuming a second viral coefficient of zero. Photodiode arrays within the scattering detector were normalised using a 4 mg/mL solution of bovine serum albumin (Sigma-Aldrich).

Acknowledgement

This work was made possible by funding from the National Health and Medical Research Council of Australia (Project Grant 252750) to ALM **and core funding from the Queensland state government**.

References

1. Giebel B & Wodarz A (2006) Tumor suppressors: control of signaling by endocytosis. *Curr Biol* **16**, R91-92.
2. Polo S & Di Fiore PP (2006) Endocytosis conducts the cell signaling orchestra. *Cell* **124**, 897-900.
3. Prescianotto-Baschong C & Riezman H (2002) Ordering of compartments in the yeast endocytic pathway. *Traffic* **3**, 37-49.
4. Vida TA, Huyer G & Emr SD (1993) Yeast vacuolar proenzymes are sorted in the late Golgi complex and transported to the vacuole via a prevacuolar endosome-like compartment. *J Cell Biol* **121**, 1245-1256.
5. Gruenberg J & Stenmark H (2004) The biogenesis of multivesicular endosomes. *Nat Rev Mol Cell Biol* **5**, 317-323.
6. Keller S, Sanderson MP, Stoeck A & Altevogt P (2006) Exosomes: from biogenesis and secretion to biological function. *Immunol Lett* **107**, 102-108.
7. Katzmann DJ, Odorizzi G & Emr SD (2002) Receptor downregulation and multivesicular-body sorting. *Nat Rev Mol Cell Biol* **3**, 893-905.
8. Morita E, Sandrin V, Chung HY, Morham SG, Gygi SP, Rodesch CK & Sundquist WI (2007) Human ESCRT and ALIX proteins interact with proteins of the midbody and function in cytokinesis. *Embo J* **26**, 4215-4227.
9. Bieniasz PD (2006) Late budding domains and host proteins in enveloped virus release. *Virology* **344**, 55-63.
10. Rusten TE, Vaccari T, Lindmo K, Rodahl LM, Nezis IP, Sem-Jacobsen C, Wendler F, Vincent JP, Brech A, Bilder D & Stenmark H (2007) ESCRTs and Fab1 Regulate Distinct Steps of Autophagy. *Curr Biol* **17**, 1817-1825.
11. Trombetta ES & Mellman I (2005) Cell biology of antigen processing in vitro and in vivo. *Annu Rev Immunol* **23**, 975-1028.
12. Riezman H, Munn A, Geli MI & Hicke L (1996) Actin-, myosin- and ubiquitin-dependent endocytosis. *Experientia* **52**, 1033-1041.
13. Babst M, Sato TK, Banta LM & Emr SD (1997) Endosomal transport function in yeast requires a novel AAA-type ATPase, Vps4p. *Embo J* **16**, 1820-1831.
14. Yoshimori T, Yamagata F, Yamamoto A, Mizushima N, Kabeya Y, Nara A, Miwako I, Ohashi M, Ohsumi M & Ohsumi Y (2000) The mouse SKD1, a homologue of yeast Vps4p, is required for normal endosomal trafficking and morphology in mammalian cells. *Mol Biol Cell* **11**, 747-763.
15. Bishop N & Woodman P (2000) ATPase-defective mammalian VPS4 localizes to aberrant endosomes and impairs cholesterol trafficking. *Mol Biol Cell* **11**, 227-239.
16. Fujita H, Yamanaka M, Imamura K, Tanaka Y, Nara A, Yoshimori T, Yokota S & Himeno M (2003) A dominant negative form of the AAA ATPase SKD1/VPS4 impairs membrane trafficking out of endosomal/lysosomal compartments: class E vps phenotype in mammalian cells. *J Cell Sci* **116**, 401-414.
17. Scheuring S, Rohricht RA, Schoning-Burkhardt B, Beyer A, Muller S, Abts HF & Kohrer K (2001) Mammalian cells express two VPS4 proteins both of which are involved in intracellular protein trafficking. *J Mol Biol* **312**, 469-480.
18. Crump CM, Yates C & Minson T (2007) Herpes simplex virus type 1 cytoplasmic envelopment requires functional Vps4. *J Virol* **81**, 7380-7387.
19. Urata S, Noda T, Kawaoka Y, Morikawa S, Yokosawa H & Yasuda J (2007) Interaction of Tsg101 with Marburg virus VP40 depends on the PPPY motif, but not the PT/SAP motif as in the case of Ebola virus, and Tsg101 plays a critical role in the budding of Marburg virus-like particles induced by VP40, NP, and GP. *J Virol* **81**, 4895-4899.

20. Morita E & Sundquist WI (2004) Retrovirus budding. *Annu Rev Cell Dev Biol* **20**, 395-425.
21. Lupas AN & Martin J (2002) AAA proteins. *Curr Opin Struct Biol* **12**, 746-753.
22. Wang Q, Song C, Irizarry L, Dai R, Zhang X & Li CC (2005) Multifunctional roles of the conserved Arg residues in the second region of homology of p97/valosin-containing protein. *J Biol Chem* **280**, 40515-40523.
23. Yakushiji Y, Nishikori S, Yamanaka K & Ogura T (2006) Mutational analysis of the functional motifs in the ATPase domain of Caenorhabditis elegans fidgetin homologue FIGL-1: firm evidence for an intersubunit catalysis mechanism of ATP hydrolysis by AAA ATPases. *J Struct Biol* **156**, 93-100.
24. Frickey T & Lupas AN (2004) Phylogenetic analysis of AAA proteins. *J Struct Biol* **146**, 2-10.
25. Hanson PI & Whiteheart SW (2005) AAA+ proteins: have engine, will work. *Nat Rev Mol Cell Biol* **6**, 519-529.
26. Sachse M, Strous GJ & Klumperman J (2004) ATPase-deficient hVPS4 impairs formation of internal endosomal vesicles and stabilizes bilayered clathrin coats on endosomal vacuoles. *J Cell Sci* **117**, 1699-1708.
27. Lin Y, Kimpler LA, Naismith TV, Lauer JM & Hanson PI (2005) Interaction of the mammalian endosomal sorting complex required for transport (ESCRT) III protein hSnf7-1 with itself, membranes, and the AAA+ ATPase SKD1. *J Biol Chem* **280**, 12799-12809.
28. Ciccarelli FD, Proukakis C, Patel H, Cross H, Azam S, Patton MA, Bork P & Crosby AH (2003) The identification of a conserved domain in both spartin and spastin, mutated in hereditary spastic paraplegia. *Genomics* **81**, 437-441.
29. Takasu H, Jee JG, Ohno A, Goda N, Fujiwara K, Tochio H, Shirakawa M & Hiroaki H (2005) Structural characterization of the MIT domain from human Vps4b. *Biochem Biophys Res Commun* **334**, 460-465.
30. Scott A, Gaspar J, Stuchell-Brereton MD, Alam SL, Skalicky JJ & Sundquist WI (2005) Structure and ESCRT-III protein interactions of the MIT domain of human VPS4A. *Proc Natl Acad Sci U S A* **102**, 13813-13818.
31. Scott A, Chung HY, Gonciarz-Swiatek M, Hill GC, Whitby FG, Gaspar J, Holton JM, Viswanathan R, Ghaffarian S, Hill CP & Sundquist WI (2005) Structural and mechanistic studies of VPS4 proteins. *Embo J* **24**, 3658-3669.
32. Vajjhala PR, Catchpoole E, Nguyen CH, Kistler C & Munn AL (2007) Vps4 regulates a subset of protein interactions at the multivesicular endosome. *Febs J* **274**, 1894-1907.
33. Babst M, Wendland B, Estepa EJ & Emr SD (1998) The Vps4p AAA ATPase regulates membrane association of a Vps protein complex required for normal endosome function. *Embo J* **17**, 2982-2993.
34. Vajjhala PR, Wong JS, To HY & Munn AL (2006) The beta domain is required for Vps4p oligomerization into a functionally active ATPase. *Febs J* **273**, 2357-2373.
35. Fujita H, Umezuki Y, Imamura K, Ishikawa D, Uchimura S, Nara A, Yoshimori T, Hayashizaki Y, Kawai J, Ishidoh K, Tanaka Y & Himeno M (2004) Mammalian class E Vps proteins, SBP1 and mVps2/CHMP2A, interact with and regulate the function of an AAA-ATPase SKD1/Vps4B. *J Cell Sci* **117**, 2997-3009.
36. Xiao J, Xia H, Yoshino-Koh K, Zhou J & Xu Z (2007) Structural characterization of the ATPase reaction cycle of endosomal AAA protein Vps4. *J Mol Biol* **374**, 655-670.
37. Urbanowski JL & Piper RC (2001) Ubiquitin sorts proteins into the intralumenal degradative compartment of the late-endosome/vacuole. *Traffic* **2**, 622-630.
38. Piper RC, Cooper AA, Yang H & Stevens TH (1995) VPS27 controls vacuolar and endocytic traffic through a prevacuolar compartment in Saccharomyces cerevisiae. *J Cell Biol* **131**, 603-617.

39. Cereghino JL, Marcusson EG & Emr SD (1995) The cytoplasmic tail domain of the vacuolar protein sorting receptor Vps10p and a subset of VPS gene products regulate receptor stability, function, and localization. *Mol Biol Cell* **6**, 1089-1102.

40. Cooper AA & Stevens TH (1996) Vps10p cycles between the late-Golgi and prevacuolar compartments in its function as the sorting receptor for multiple yeast vacuolar hydrolases. *J Cell Biol* **133**, 529-541.

41. Zahn R, Stevenson BJ, Schroder-Kohne S, Zanolari B, Riezman H & Munn AL (2001) End13p/Vps4p is required for efficient transport from early to late endosomes in Saccharomyces cerevisiae. *J Cell Sci* **114**, 1935-1947.

42. Munn AL & Riezman H (1994) Endocytosis is required for the growth of vacuolar H(+)-ATPase-defective yeast: identification of six new END genes. *J Cell Biol* **127**, 373-386.

43. Obita T, Saksena S, Ghazi-Tabatabai S, Gill DJ, Perisic O, Emr SD & Williams RL (2007) Structural basis for selective recognition of ESCRT-III by the AAA ATPase Vps4. *Nature* **449**, 735-739.

44. Stuchell-Brereton MD, Skalicky JJ, Kieffer C, Karren MA, Ghaffarian S & Sundquist WI (2007) ESCRT-III recognition by VPS4 ATPases. *Nature* **449**, 740-744.

45. Bowers K, Lottridge J, Helliwell SB, Goldthwaite LM, Luzio JP & Stevens TH (2004) Protein-protein interactions of ESCRT complexes in the yeast Saccharomyces cerevisiae. *Traffic* **5**, 194-210.

46. Yeo SC, Xu L, Ren J, Boulton VJ, Wagle MD, Liu C, Ren G, Wong P, Zahn R, Sasajala P, Yang H, Piper RC & Munn AL (2003) Vps20p and Vta1p interact with Vps4p and function in multivesicular body sorting and endosomal transport in Saccharomyces cerevisiae. *J Cell Sci* **116**, 3957-3970.

47. Finken-Eigen M, Rohricht RA & Kohrer K (1997) The VPS4 gene is involved in protein transport out of a yeast pre-vacuolar endosome-like compartment. *Curr Genet* **31**, 469-480.

48. Folta-Stogniew E & Williams KR (1999) Determination of Molecular Masses of Proteins in Solution: Implementation of an HPLC Size Exclusion Chromatography and Laser Light Scattering Service in a Core Laboratory. *J Biomol Tech* **10**, 51-63.

49. Azmi I, Davies B, Dimaano C, Payne J, Eckert D, Babst M & Katzmann DJ (2006) Recycling of ESCRTs by the AAA-ATPase Vps4 is regulated by a conserved VSL region in Vta1. *J Cell Biol* **172**, 705-717.

50. Lottridge JM, Flannery AR, Vincelli JL & Stevens TH (2006) Vta1p and Vps46p regulate the membrane association and ATPase activity of Vps4p at the yeast multivesicular body. *Proc Natl Acad Sci U S A* **103**, 6202-6207.

51. Zhang D, Rogers GC, Buster DW & Sharp DJ (2007) Three microtubule severing enzymes contribute to the "Pacman-flux" machinery that moves chromosomes. *J Cell Biol* **177**, 231-242.

52. James PA & Talbot K (2006) The molecular genetics of non-ALS motor neuron diseases. *Biochim Biophys Acta* **1762**, 986-1000.

53. Salinas S, Carazo-Salas RE, Proukakis C, Schiavo G & Warner TT (2007) Spastin and microtubules: Functions in health and disease. *J Neurosci Res* **85**, 2778-2782.

54. Winter V & Hauser MT (2006) Exploring the ESCRTing machinery in eukaryotes. *Trends Plant Sci* **11**, 115-123.

55. Knop M, Siegers K, Pereira G, Zachariae W, Winsor B, Nasmyth K & Schiebel E (1999) Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. *Yeast* **15**, 963-972.

56. Gietz RD & Sugino A (1988) New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74**, 527-534.

57. Thompson JD, Higgins DG & Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673-4680.

58. Bennett-Lovsey RM, Herbert AD, Sternberg MJ & Kelley LA (2007) Exploring the extremes of sequence/structure space with ensemble fold recognition in the program Phyre. *Proteins* **70**, 611-625.

Supplementary material

Fig. S1. Sequence alignment of the C-terminal regions of Vps4, spastin, katanin and fidgetin from a range of species.

Table 1 Primers used for mutagenesis

Primer	Sequence
Vps4-DEL F	5’-TGGACGGATATTGAAGCTGATCTCACCATAAAGGAT-3’
Vps4-DEL R	5’-ATCCTTTATGGTGAGATCAGCTTCAATATCCGTCCA-3’
Vps4-TRP F	5’-TTAAAGGCTATCAAATCGCAAGAACAGTTCACTAGA-3’
Vps4-TRP R	5’-TCTAGTGAACTGTTCTTGCGATTTGATAGCCTTTAA-3’
Vps4-RDF F	5’-GAAGCAAGAACAGTTCACTTAGTCAATTGATTAACGTG-3’
Vps4-RDF R	5’-CACGTTAATCAATTGACTAAGTGAACTGTTCTTGCTTC-3’

Table 2 Yeast Strains used in this study

Strain	Genotype	Source
EGY48	*MATα his3 trp1 ura3 LexAop(×6)-LEU2*	Clontech
AMY245	*MAT***a** *vps4-Δ::KanMx leu2 ura3 his4 lys2 bar1*	[34]
RH1800	*MAT***a** *his4 leu2 ura3 bar1*	Riezman lab strain

Table 3 Plasmids used in this study

Plasmid	Description	Source
YCplac111	*CEN4 ARS1 LEU2 E. coli*/ yeast shuttle vector	[56]
pGEX5X-1	GST fusion expression vector	GE Healthcare
pET11d	T7 RNA polymerase-based gene expression vector	Novagen
p8op-lacZ	Two-hybrid reporter plasmid	Clontech
pLexA	Two-hybrid bait vector	Clontech
pB42AD	Two-hybrid prey vector	Clontech
pPL1640	*URA3 CEN* plasmid expressing Fth1p-GFP-Ub	[37]
pAM 349	Original library clone of *VPS20* in pB42AD (encoding Vps20p 3-221/end)	[46]
pAM 377	pGEX5X-1 expressing Vps20p with an N-terminal GST tag	[46]
pAM 378	pGEX5X-1 expressing Vta1p with an N-terminal GST tag	[46]
pAM 398	Original library clone of *VTA1* in pB42AD (encoding Vta1p 108-330/end)	[46]
pAM 451	pLexA expressing LexA fused to Vps4p	[46]
pAM 482	pET11a *E. coli* expression vector expressing Vps4p with a C-terminal 6His tag	[46]
pAM 496	Original library clone of *DID2*/*CHM1* in pB42AD (encoding Did2p/Chm1p 41-204/end)	[34]
pAM 813	YCplac111 expressing Vps4p	[34]
pAM 863	YCplac111 expressing Vps4p with a C-terminal yEGFP tag	[34]
pAM 870	pB42AD expressing the activation domain fused to Vps4p	[34]
pAM 916	YCplac111 expressing Vps4p Δ394-399 (Vps4p-DEL)	This study
pAM 917	pLexA expressing LexA fused to Vps4p-DEL	This study
pAM 920	YCplac111 expressing Vps4p Δ413-424 (Vps4p-TRP)	This study
pAM 921	pLexA expressing LexA fused to Vps4p-TRP	This study
pAM 922	YCplac111 expressing Vps4p-E233Q	[34]
pAM 932	YCplac111 expressing Vps4p Δ31-87 (Vps4p-CC) with a C-terminal yEGFP tag	[32]
pAM 934	pB42AD expressing the activation domain fused to Snf7p	[32]
pAM 961	YCplac111 expressing Vps4p Δ430-437/end (**Vps4p-**RDF)	This study
pAM 962	pLexA expressing LexA fused to Vps4p-RDF	This study
pAM 963	pB42AD expressing the activation domain fused to Vps4p-DEL	This study
pAM 964	pB42AD expressing the activation domain fused to Vps4p-TRP	This study
pAM 965	pB42AD expressing the activation domain fused to Vps4p-RDF	This study
pAM 966	pET11a *E. coli* expression vector expressing Vps4p-DEL with a C-terminal 6His tag	This study
pAM 967	pET11a *E. coli* expression vector expressing Vps4p-TRP with a C-terminal 6His tag	This study
pAM 969	pB42AD expressing the activation domain fused to Vps2p	[32]
pAM 974	pET11a expressing Vps4p-E233Q, Δ382-390 (Vps4p-E233Q-GAI)	This study
pAM 975	pET11a expressing Vps4p-GAI with a C-terminal 6HIS tag	This study
pAM 977	pGEX-4T expressing Snf7p with an N-terminal GST tag	[32]
pAM 982	pB42AD expressing the activation domain fused to Bro1p	[32]
pAM 987	pGEX-4T expressing Vps2p with an N-terminal GST tag	[32]
pAM 988	pGEX-4T expressing Did2p/Chm1p with an N-terminal GST tag	[32]
pAM 989	pGEX-4T expressing Bro1p with an N-terminal GST tag	[32]
pAM 998	YCplac111 expressing Vps4p-DEL with a C-terminal yEGFP tag	This study
pAM 999	YCplac111 expressing Vps4p-TRP with a C-terminal yEGFP tag	This study
pAM 1000	YCplac111 expressing Vps4p-RDF with a C-terminal yEGFP tag	This study
pAM 1006	YCplac111 expressing Vps4p-E233Q, Δ394-399 (Vps4p-E233Q-DEL)	This study
pAM 1007	YCplac111 expressing Vps4p-E233Q, Δ413-424 (Vps4p-E233Q-TRP)	This study
pAM 1008	YCplac111 expressing Vps4p-E233Q, Δ430-437 (Vps4p-E233Q-RDF)	This study
pAM 1009	pET11a *E. coli* expression vector expressing Vps4p-RDF with a C-terminal 6His tag	This study
pAM 1011	pET11a expressing Vps4p-E233Q, Δ430-437 (Vps4p-E233Q-RDF) with a 6His tag	This study

Figure legends

Fig. 1. Construction of Vps4p C-terminal mutants. *A.* Schematic representation of wild-type Vps4p. *B.* Alignment of C-terminal sequences of *S. cerevisiae* (*S.c.*) Vps4p and human (*H.s.*) VPS4A and VPS4B using ClustalW [57]. Conserved blocks deleted in individual mutant proteins are shown in bold. The secondary structure of the corresponding region of **yeast** Vps4p is also shown. *C.* Crystal structure of the **yeast** Vps4p ATPase domain and C-terminal helix [36] showing the location of residues that were mutated. The TRP, RDF and DEL sequences are shown in green, dark blue and red, respectively. The β domain and C-terminal helix are circled and labelled. The colour code for the non-mutated residues in the different domains is: **large** AAA subdomain, pink; **small** AAA subdomain, beige; non-mutated region of C-terminal α-helix, cyan; β domain, yellow. **Note: residues 387-396 containing part of the DEL sequence which is depicted as a ribbon is part of a structured loop.** *D.* Total cell lysates from AMY245 (*vps4Δ*) yeast cells carrying **centromeric** plasmids expressing wild-type Vps4p (*WT*), Vps4p-DEL (*D*), Vps4p-TRP (*T*), or Vps4p-RDF (*R*) mutant proteins or **carrying empty vector (*V*)** were subjected to western blotting using an anti-Vps4p polyclonal antibody. The Vps4-specific band and a non-specific (*NS*) band are indicated.

Fig. 2. Conserved sequences in the C-terminal helix and in the β domain are critical for Vps4p functions in vivo. *A.* Ubiquitin-dependent MVB sorting of Fth1p-GFP-Ub in AMY245 (*vps4Δ*) yeast cells carrying plasmids expressing wild-type (WT) Vps4p or Vps4p mutant proteins or carrying empty vector (YCplac111). Cells were incubated **at 24 °C** in YPUAD medium containing 100 μM bathophenanthroline disulphonic acid (BPS) for 6 h to chelate iron and induce Fth1p-GFP-Ub expression. Cells were then washed with buffer containing 1% sodium azide, 1% sodium fluoride, 100 mM phosphate, pH 8.0 to stop further transport. The same fields of cells are shown visualised by Nomarski (*left*) and fluorescence (*right*) optics. Scale bar, 5 μm. *B.* Vacuolar protein sorting in AMY245 (*vps4Δ*) yeast cells carrying plasmids expressing wild-type Vps4p or Vps4p

mutant proteins or carrying empty vector (YCplac111). Cells were grown on YPUAD solid medium for 2 days at 24 °C in contact with a nitrocellulose filter. Cells were eluted from the filter and CPY on the filter was detected by immunoblotting with anti-CPY antiserum. To test for cell lysis the blot was stripped and re-probed with an antibody to a cytoplasmic protein (calmodulin). *C*. LY uptake and vacuolar accumulation in AMY245 (*vps4Δ*) yeast cells carrying plasmids expressing wild-type Vps4p or Vps4p mutant proteins or carrying empty vector (YCplac111). The same fields of cells are shown visualised by Nomarski (*left*) and fluorescence (*right*) optics. Scale bar, 5 μm. *D*. Temperature-sensitive growth assay of AMY245 (*vps4Δ*) yeast cells carrying plasmids expressing wild-type Vps4p or Vps4p mutant proteins or carrying empty vector (YCplac111). Cells were serially diluted ten-fold and 7 μl aliquots were spotted onto YPUAD solid media and incubated at 24 °C (*left*) or 40 °C (*right*). Plates were photographed after 3 or 7 days, respectively.

Fig. 3. The conserved sequences in the C-terminal helix and β domain are not essential for recruitment of Vps4p to endosomes. AMY245 (*vps4Δ*) yeast cells **carrying centromeric plasmids** expressing GFP-tagged wild-type Vps4p, Vps4p-CC, Vps4p-DEL, Vps4p-TRP or Vps4p-RDF were grown in SD medium and the GFP-tagged proteins were visualised by fluorescence microscopy. Scale bar, 5 μm.

Fig. 4. Conserved sequences in the C-terminal helix and in the β domain are important for Vps4p-ATPase activity. *A*. Affinity-purified 6His-tagged wild-type Vps4p (*W*), **Vps4p-E233Q (*E*)**, Vps4p-GAI (*G*), Vps4p-DEL (*D*), Vps4p-TRP (*T*), and Vps4p-RDF (*R*) **were subjected to 10% SDS-PAGE and stained with Coomassie blue. *B.* The purified 6His-tagged wild-type Vps4p and Vps4p mutant proteins were assayed in vitro for ATPase activity at 30 °C. ATPase activity is expressed as nmol inorganic phosphate released/ h/ μg protein and shown graphically. The**

negative values in samples containing Vps4p-E233Q may be because ATP bound to this inactive protein inhibits autolysis.

Fig. 5. The TRP and RDF sequences in the C-terminal helix are not required for Vps4p protein interactions while the DEL sequence in the β domain is required for interaction with Vta1p. *A.* Yeast two-hybrid interaction analysis of wild-type Vps4p and Vps4p C-terminal mutants with Did2p, Vta1p, Vps2p, Vps20p, and Snf7p. EGY48 carrying pLexA-based bait plasmids and pB42AD-based prey plasmids as well as p8op-LacZ reporter plasmid were spotted onto medium containing X-gal. Plates were photographed after overnight incubation and two-hybrid interaction was assessed by blue colouration. Four independent transformants are shown **for each plasmid combination**. *B. In vitro* binding of 6His-tagged wild-type Vps4p and Vps4p mutant proteins to GST-tagged Did2p, Vta1p, Vps2p, Vps20p, and Bro1p or GST only. Bound protein was released **from the beads** with Laemmli sample buffer and subjected to SDS-PAGE and immunoblotting with a polyclonal anti-**yeast** Vps4p antibody. **An amount representing 5% of the input used for the vitro binding assay is also shown.** *C.* The 6His-tagged wild-type and mutant Vps4p proteins were incubated with glutathione agarose bearing GST-Vps20p in the presence or absence of ATP. Bound protein was detected as in *B.*

Fig. 6. The phenotypes conferred by mutation of the TRP sequence are partially dominant-negative while those conferred by mutation of the RDF and DEL sequences are recessive. RH1800 (wild-type) yeast cells **carrying centromeric plasmids** expressing wild-type Vps4p or a Vps4p mutant protein or carrying empty vector (YCplac111) were assayed for MVB sorting **of Fth1p-GFP-Ub** (*A*), CPY missorting **into the medium** (*B*), fluid phase endocytosis of LY (*C*) or **temperature-sensitive** growth (*D*) as in Fig. 2 except that cells in *A, C* and *D* were grown on SD **minimal** media **to maintain selection of the plasmids**. Scale bar, 5 μm.

Fig. 7. The RDF sequence in the C-terminal helix and the GAI sequence in the β domain are critical for Vps4p oligomerisation in vitro. The ability of the different **affinity-purified recombinant 6His-tagged** Vps4p mutant proteins to form oligomers was assessed by gel filtration chromatography. The elution positions of molecular weight standards are indicated on the chromatograms. The Vps4p-E233Q-RDF and Vps4p-E233Q mutant **proteins** were run using 0.1 M potassium acetate, 5 mM magnesium acetate, 20 mM HEPES, pH 7.4, ±1 mM ATP. The Vps4p-E233Q-GAI mutant was run using 20 mM HEPES, 200 mM potassium chloride, 10 mM magnesium chloride, pH 7.5, ±1 mM ATP. Retention times of the Vps4p-E233Q dimer and high order oligomer in both buffers were identical.

Fig. 8. **Mutations of the C-terminal helix in dominant-negative Vps4p-E233Q do not abrogate its dominant-negative effects.** RH1800 (wild-type) yeast cells **carrying centromeric plasmids** expressing wild-type Vps4p, Vps4p-E233Q, or the double mutants, Vps4p-E233Q-DEL, Vps4p-E233Q-TRP, Vps4p-E233Q-RDF, or carrying empty vector were assayed for MVB sorting of Fth1-GFP-Ub (*A*), CPY missorting (*B*), or **temperature-sensitive** growth (*C*) as in Fig. 6. Scale bar, 5 μm.

Fig. 9. The C-terminal helix TRP and RDF sequences are not required for the Vps4p homotypic interaction but the β domain DEL sequence is required. The interaction between various combinations of wild-type Vps4p and Vps4p-mutant proteins was assessed using the yeast two-hybrid technique. EGY48 carrying a p8op-LacZ reporter plasmid and pLexA-based bait plasmids or pLexA vector only, and pB42AD-based prey plasmids, or pB42 vector only, were spotted onto synthetic galactose-**raffinose** medium containing X-gal. Plates were photographed after incubation **for 2 days at 30 °C** and two-hybrid interaction was assessed by blue colouration. Four independent transformants are shown **for each plasmid combination**.

Fig. 10. Vta1p induces the assembly of catalytically active hybrid complexes comprising Vps4p-RDF and Vps4p-E233Q. *A.* Purified 6His-tagged wild-type Vps4p (*W*) and Vps4p mutant proteins, Vps4p-GAI (*G*), Vps4p-DEL (*D*), Vps4p-TRP (*T*), Vps4p-RDF (*R*), were mixed with 6His-tagged Vps4p-E233Q (*E*) or GST-Vta1p (*V*) or both and assayed in vitro for ATPase activity at 30 °C. ATPase activity is expressed as nmol inorganic phosphate released/ h/ mL assay mix as defined in Materials and Methods. The phosphate released upon incubation of ATP only in the buffer was subtracted from each sample. The negative values in samples containing Vps4p-E233Q may be because ATP bound to this inactive protein inhibits autolysis. *B.* Schematic depicting a possible model to explain how ATPase activity of Vps4p-RDF may be stimulated by Vps4p-E233Q and Vta1p in vitro. For simplicity, only a single oligomeric ring is shown, although Vps4p is proposed to form a double ring structure. Vps4p-RDF is only very weakly active on its own due to defects in both assembly and function of the SRH. Vps4p-E233Q can weakly assemble with Vps4p-RDF to form an active ATPase complex in which the SRH of Vps4p-E233Q stimulates the activity of Vps4p-RDF, however, assembly is inefficient. Vta1p promotes assembly of this Vps4p-RDF/Vps4p-E233Q hybrid oligomer such that there is efficient formation of a catalytically active Vps4p complex. Note that not all molecules in the hybrid oligomer will be oriented with the functional SRH and catalytic sites adjacent as depicted above, however, by chance some will assemble in this orientation and these will possess ATPase activity.

Fig. 11. A C-terminal helix is a characteristic feature of meiotic clade AAA ATPases. Sequence alignments of some of the proteins listed in the PFAM database that contain the Vps4 C-terminal oligomerisation domain (PF09336). The sequence of the SRH of each protein is also shown. The sequence of the SRH of a non-meiotic clade AAA ATPase, FtsH, is shown

for comparison. We also included the sequence of spastin, a well-known member of the meiotic clade, although it is not in the PFAM database. The secondary structure of the C-terminal sequences of these proteins as predicted using Phyre is also shown [58](H=helix, C=coil). (*S.c.* = *Saccharomyces cerevisiae* (yeast), *H.s.* = *Homo sapiens* (primate), *P.a.* = *Podospora anserina* (fungus), *A.g.* = *Ashbya gossypii* (fungus), *E.h*. = *Entamoeba histolytica* (protozoan), and *E.c.* = *Escherichia coli* (prokaryote))

Fig. 12. **In the Vps4 3D structure,** the C-terminal helix RDF sequence is located close to the SRH motif while the TRP sequence is located close to the Walker A and B motifs. *A.* Space filling model of the **human** Vps4B ATPase domain and C-terminal helix. Surface exposed regions of the TRP (green) and RDF (dark blue) sequences as well as the Arg residues in the SRH (red) and Walker A and B motifs (black) **are shown**. **The colour code for the remainder of the protein is: large AAA subdomain, pink; small AAA subdomain, beige; β domain, orange; non-mutated region of C-terminal α-helix, cyan.** *B.* Close up of **part of** the C-terminal helix **(residues 426-444)** and SRH **(residues 284-291)** of Vps4B **as they appear in the 3D structure to illustrate their close proximity**. Colour scheme is as in *A.* The side chains of the conserved F440 residue in the C-terminal helix and the three Arg residues (R289, R290, R291) in the SRH are shown. **Images were generated using MacPymol (http://www.pymol.org).**

Fig. S1. Sequence alignment of the C-terminal regions of Vps4, spastin, katanin and fidgetin from a range of species. Alignment was performed using ClustalW [57]. The secondary structure of the corresponding region is shown above the Vps4p sequence. Conserved blocks deleted in Vps4p in this study are underlined. (*S.c.* = *Saccharomyces cerevisiae* (yeast), *H.s.* = *Homo sapiens* (primate), *M.m.*= *Mus musculus* (rodent), *R.n.*= *Ratus norvegicus* (rodent), *X.l.*=

Xenopus laevis **(amphibian),** ***D.m.*****=** ***Drosophila melanogaster*** **(fruit fly),** ***C.e.*****=** ***Caenorhabditis elegans*** **(nematode),** ***A.t.*****=** ***Arabidopsis thaliana*** **(plant),** ***O.s.*****=** ***Oryza sativa*** **(rice i.e. plant))**