Vps51p links the VFT complex to the SNARE Tlg1p

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running title: assembly of the VFT complex
Summary

Intracellular membrane fusion requires the complex coordination of SNARE, rab/ypt and rab effector function. In the yeast *Saccharomyces cerevisiae*, fusion of endosome-derived vesicles with the late Golgi depends on a cascade of protein-protein interactions that results in the recruitment to Golgi membranes of a conserved docking complex, VFT. This complex binds to Ypt6-GTP, which is necessary for its localization to the Golgi, and also to the SNARE Tlg1p. We show here that the VFT complex contains a fourth, previously uncharacterized, subunit, Vps51p (Ykr020w). Yeast cells lacking *VPS51* have defects in vacuole morphology and recycling of the SNARE Snc1p to the plasma membrane, but still assemble a core VFT complex consisting of Vps52p, Vps53p and Vps54p that localizes properly to the Golgi. Binding to Ypt6-GTP is a property of Vps52p. In contrast, binding to Tlg1p is mediated by a short sequence at the N terminus of Vps51p. Recent evidence suggests that components of a number of rab/ypt effector complexes share a common, distantly related helical coiled-coil motif. We show that each VFT subunit requires this coiled-coil motif for assembly into the complex.
Introduction

Membrane traffic within eucaryotic cells requires an elaborate machinery to create transport carriers and to dock and fuse them to the correct membrane. Key components are the SNAREs, integral membrane proteins that, by the formation of helix bundles, draw membranes into close apposition (1-4). Earlier stages in the targeting process are regulated by peripheral membrane proteins, including the rab/ypt GTPases and their effectors, amongst which are a set of large protein complexes implicated in vesicle docking (5, 6).

Three such complexes, acting at different stages in the secretory pathway, have recently been shown to contain subunits with regions of weak but detectable similarity (for a review, see (6)). These were first characterized in yeast, though each has an animal cell homologue. The exocyst, with eight subunits, is involved in the fusion of vesicles with localized regions of the plasma membrane (7). The COG complex (Conserved Oligomeric Complex) also with eight subunits, is found on the Golgi apparatus and is thought to mediate return traffic from late to early Golgi compartments, and also perhaps from endosomes to Golgi (8, 9). In yeast, only four of the COG subunits are essential for growth, suggesting that the complex may consist of two tetrameric subcomplexes with distinct functions.

The third member of this family is the VFT complex (Vps Fifty three, also named GARP), which is found on late Golgi membranes and is required for the recycling of proteins from endosomes to the late Golgi (10, 11). It was originally described as a trimeric complex of Vps52p/Sac2p, Vps53p and Vps54p/Luv1p. Like many subunits of the COG and exocyst complexes, each of these proteins contains a conserved short potential amphipathic helix. In addition, Vps52p shows homology to a domain of the exocyst component Sec3p (8).
The extent to which these complexes share structural and functional features remains to be determined. VFT is recruited to membranes by Ypt6p (11), and COG is an effector of Ypt1p (9). However, while some exocyst components are bound to vesicles via the GTPase Sec4p others, notably Sec3p, are targeted to the plasma membrane by a separate mechanism that involves Rho1p and Cdc42p (7, 12, 13). Both VFT and COG complexes have been reported to bind SNAREs (9, 11), but this does not seem to be a feature of the exocyst. Despite some differences, the complexes may well share common features. It has been suggested that each may be based on a tetrameric organization, with the exocyst and COG complexes containing two tetramers (6).

We have previously shown that the VFT complex purified from yeast can bind independently to the GTP form of Ypt6p and to the N-terminal domain of the SNARE Tlg1p (11). These interactions suggest that VFT functions either to link endosome-derived vesicles containing Tlg1p with Golgi membranes marked by Ypt6p, or to activate Tlg1p and associated SNAREs on the Golgi for subsequent complex formation and membrane fusion. To analyze the interactions in more detail, we attempted to map the binding site for Tlg1p and were surprised to find that none of the known VFT subunits, expressed alone, could bind this SNARE. Here we present further characterization of the VFT complex, and show that although originally thought to be a trimer, it normally contains a fourth subunit, Vps51p. This subunit bears a short peptide sequence through which it interacts with Tlg1p. We also show that short amphipathic helices found in each of the four VFT components are necessary, though apparently not sufficient, for the assembly of the complex.

**Experimental Procedures**

**Yeast strains, Microbiological Techniques and Plasmids**
The yeast strains used in this work are listed in Table I. Recombinant GST- and His6-fusions were expressed using pGEX6P2 (Pharmacia-Amersham) in MC1061
and pET30a (Novagen) in BL21DE3 cells, respectively. The following plasmids were used for yeast expression: YCplac111 and Ycplac33, ARS1/CEN4 vectors with the LEU2 and the URA3 marker respectively (14); YEplac181, 2μ vector with the LEU2 marker (14); YCplac111-VPS52-PtA, YCplac111-VPS54-PtA, YCplac33-VPS54-myc, YCplac111-VPS52-GFP and YCplac111-VPS54-GFP (11).

**Gene deletion of the VPS51 gene**
The complete VPS51 ORF was deleted by generating two unique BamHI sites via PCR-mediated mutagenesis, one just after the start codon and the other just before the stop codon, removing the DNA between start and stop codon and inserting a BamHI fragment containing the HIS3 gene. The *vps51::HIS3*, alleles, carrying 5’ and 3’ flanking sequences, was excised and transformed into the SEY6210 strain.

**Construction of VPS51/52/53/54 fusion genes and truncation mutants**
To construct a VPS52 truncation mutant lacking its N-terminal coiled-coil domain, a PCR fragment coding for its promoter and 4 N-terminal residues was ligated with a second PCR fragment coding for residues 123 to 641. Similarly, the VPS53 truncation mutant was constructed by ligation of a PCR fragment coding for its promoter and 4 N-terminal residues with a second fragment coding for residues 104 to 801. The VPS54 mutant was constructed by ligating a fragment coding the promoter and the 267 N-terminal residues with a second fragment coding for residues 312 to 889. In all three cases, the last C-terminal residue was followed by the Tobacco Etch Virus (TEV) protease cleavage site and the Protein A tag. To delete the C-terminal coiled-coil domain of VPS51, a PCR fragment the 120 N-terminal residues was ligated with a second fragment encoding the Protein A tag. All constructs were cloned into the LEU2-based YCplac111 vector.

To construct VPS51-PtA or GFP C-terminal fusions, a BamHI site was introduced just before the VPS51 stop codon by PCR and a BamHI fragment coding for the TEV protease cleavage site followed by two IgG binding domains from *Staphylococcus aureus* protein A or GFP was inserted. In all experiments, VPS51-
PtA or GFP fusions were expressed from the \( VPS51 \) promoter on centromere vectors.

To express recombinant \( VPS51, VPS52 \) or \( VPS53 \), the full length genes were cloned in frame as PCR fragments into the pGEX6P2 vector. To construct the various \( VPS51 \) truncation mutants as GST fusions, PCR fragments encoding residues 1 to 47, 40, 34, 30 or 27, or 121 to 164, were cloned into pGEX6P2. To express the \( VPS52/53/54 \) coiled coil domains as recombinant His\(_6\)-fusions, the corresponding residues (1-139 for \( VPS52 \), 1-110 for \( VPS53 \) and 216-382 for \( VPS54 \)) were cloned as PCR fragments into the pET10a vector.

**Affinity purification of PtA-fusions and in vitro binding experiments**

Affinity purification of Vps52p-, Vps54p-, and Vps51p-PtA from yeast extracts was as previously described (11). TEV protease dependent elution of PtA fusions was as in (15). All E. coli strains expressing recombinant proteins were grown in 2XTY medium and induced at 30°C with 0.2 mM (GST fusions) or 0.5 mM (His\(_6\) fusions) IPTG. In vitro binding experiments using GST-Ypt6p, full-length GST-VPS51/52/53 or the various GST-VPS51 fragments were done as in (11).

To test the assembly of the coiled-coil domains of the various VFT subunits, 10 ml cultures of bacterial cells expressing His\(_6\)-Vps52(1-139), His\(_6\)-Vps53(1-110), and His\(_6\)-Vps54(216-382), were lysed in 1 ml of lysis buffer (20 mM Heps-KOH pH 7.4, 150 mM KCl, 0.2% Triton X-100). Aliquots (0.3 ml) of the three lysates were mixed together with a 0.3 ml aliquot of a lysate from a 10 ml culture of either GST-Vps51 full-length, GST-Vps51(121-164) or GST-expressing bacteria. Binding reactions were incubated for 2 hours. After that 30 \( \mu \)l of glutathione-sepharose beads were added and the reactions were incubated for a further 30 minutes. Beads were then washed four times with 0.5 ml lysis buffer and bound proteins were eluted with SDS sample buffer.

**Antibodies**

The antibody used to detect Protein A fusions was from DAKO (Cat. No Z0113).
The anti-myc polyclonal antibody was from Santa Cruz (Cat. No sc-789), the penta-His HRP conjugate from Qiagen (Cat. No 34460) and the anti- Tlg1p as described earlier (16).

**Mass spectrometric analysis**
Protein identification by mass spectrometry of tryptic fragments was performed as previously described (11). 8 tryptic peptides were obtained from the Vps51p band in the Vps54p-PtA pull-down (sequence coverage 50%, mean mass error 9.8 ppm). 26 peptides were obtained from the Vps53p band (sequence coverage 36%, mean error 37 ppm) and 15 peptides from Vps54p (sequence coverage 19%, mean error 12 ppm).

**Results**

**Identification and characterization of a fourth VFT subunit**

To search for novel VFT-interacting proteins we expressed either Vps52p or Vps54p tagged with protein A and purified the complex from yeast cells on IgG-Sepharose. Figure 1 shows a Coomassie stained gel of the eluted proteins. Analysis by mass spectrometry indicated that many of the minor bands were either irrelevant contaminants or breakdown products (data not shown), but one small protein (Ykr020w) was consistently present. As seen in Figure 1, tagging of Ykr020w with protein A resulted in co-purification of the other VFT components, confirming the interaction (see also below).

We recently learnt that Ykr020w is identical to the product of *VPS51*, a gene identified in the same genetic screen as *VPS52, VPS53* and *VPS54* (E. Conibear, personal communication cited in (6); (10)). For simplicity, we shall therefore refer to this protein as Vps51p. The gene was also identified in a screen for mutants that affect apical growth, and named *API3* (17). Furthermore, a very recent systematic survey of deletion mutants with vacuolar protein sorting defects has
identified the same gene, which was named \textit{VPS67} since at that time its identity with \textit{VPS51} was unknown (18). These genetic studies indicate that Vps51p, like the other components of the VFT complex, has a role in the sorting of soluble vacuolar proteins, a process that requires recycling of the sorting receptor Vps10p between Golgi and endosomes.

Vps52p, Vps53p and Vps54p, together with Ypt6p and its heterodimeric exchange factor Ric1p-Rgp1p, are also required for efficient recycling of the exocytic SNARE Snc1p from the plasma membrane back via the Golgi, and for proper vacuolar morphology (10, 11, 15). We therefore tested these phenotypes for \textit{vps51}. Figure 2A shows that the mutant has fragmented vacuoles, and accumulates GFP-Snc1p within cells. Thus, Vps51p has functions similar to those of the other VFT subunits.

We next examined the localization of a functional GFP-tagged version of Vps51p, expressed from its own promoter in the absence of endogenous Vps51p. Figure 2B shows that it had a punctate distribution characteristic of Golgi compartments in yeast, consistent with its association with the VFT complex on Golgi membranes.

**Formation of a VFT complex without Vps51p**

Despite its apparent role as an integral part of the VFT complex, loss of Vps51p had a milder effect on growth than did deletion of any of the other components. Figure 2C shows that although deletions of \textit{vps51}, \textit{vps52} and \textit{vps54} were all temperature-sensitive for growth at 37°C, at lower temperatures \textit{vps51} cells grew much more strongly than \textit{vps52} or \textit{vps54} cells. This suggests either that a form of the VFT complex lacking a fourth subunit retains some function, or that some other protein can substitute for Vps51p (though no obvious homologues exist in the \textit{S. cerevisiae} genome).
That some of the properties of the VFT complex do not depend on Vps51p was borne out by examination of the intracellular distribution of GFP-tagged proteins. We have previously shown that the complex has a punctate Golgi distribution, and that this is dependent on Ypt6p, without which the complex is dispersed throughout the cell (11). Significantly, GFP-tagged Vps52p, Vps53p and Vps54p each retain their punctate distribution in a vps51 null strain (Figure 3A). In contrast, deletion of VPS52 resulted in complete dispersal of Vps54-GFP (Figure 3A), and deletion of VPS54 had a similar effect on Vps53-GFP (data not shown).

To examine whether a VFT complex could form in the absence of Vps51p, we purified Vps52-protein A from a vps51 deletion strain. Figure 3B (left lane) shows that Vps53p and Vps54p could still be found associated with Vps52p, but the level of the complex was substantially reduced relative to cells that contain also Vps51p (right lane). We could detect no new protein associated with the VFT complex in the vps51 null strain, though because of the low yield of the complex it is difficult to exclude the possibility of a minor band. We conclude that a trimeric core VFT complex consisting of Vps52p, Vps53p and Vps54p can form, and that this is sufficient for correct targeting to the Golgi, but that for full stability and function of the complex the presence of Vps51p is essential.

**Vps51p mediates the binding of VFT to the SNARE Tlg1p via a short N-terminal peptide**

We have previously shown that the VFT complex, purified from yeast cells, can directly bind to the N-terminal domain of the SNARE Tlg1p, and that this interaction can also be detected in cells by cross-linking (11). In order to identify the Tlg1p-binding subunit within the VFT complex, we expressed full-length Vps51p, Vps52p and Vps53p as GST fusions in *E. coli*, and examined the ability of Tlg1p from cell lysates to bind to these fusions immobilized on glutathione-Sepharose beads. Vps54p was not included in this analysis because we were not able to stably express GST-Vps54p. Figure 4A shows that Tlg1p binds specifically only to Vps51p.
Comparison of the sequences of Vps51p homologues from related Saccharomyces species (paradoxus, mikatae and bayanus) available from the Saccharomyces Genome Database reveals regions at the N and C terminus in which 90% of the residues are identical in all these species, in contrast to the central portion of the proteins which is much more divergent. In addition, the last 40 residues have the potential to form an amphipathic coiled-coil (Figure 5A). Like a number of other SNAREs, the N-terminal domain of Tlg1p is predicted to form a three-helix bundle (19), and thus one possible mode of binding would be for a helix from Vps51p to pack against this bundle. To map the domain responsible for binding, we expressed various portions of Vps51p as GST fusions, and checked binding as for the full-length protein. As shown in Figure 4B, the Tlg1p binding site mapped not to the C-terminal coiled-coil, but to the N-terminus of Vps51p. More detailed mapping showed that residues 1-30, which are highly conserved, bound to Tlg1p as efficiently as did the full-length protein. In contrast, residues 1-27 were not sufficient for binding. Residues 15-30 are predicted to be helical, and if helical the whole region would have an amphipathic character (Figure 4B). Binding of such a structure to Tlg1p might be sufficient to alter the conformation, and hence activity, of this SNARE (see Discussion).

The coiled-coil domains of the VFT subunits are required for complex assembly

One intriguing recent finding is that a number of tethering complexes, including VFT, may be structurally related to each other (8). All VFT subunits contain short regions predicted to form amphipathic helices or coiled coils (see Figure 5A and B). The Vps53p and Vps54p N-terminal helices exhibit distant homology with similar domains found in components of the Sec34/35 complex and the exocyst. Furthermore, the Vps52p coiled coil is related to that of the exocyst component Sec3p. The function of these short helices is not understood. Since coiled-coils tend to assemble with each other, and each VFT subunit contains such a domain,
one possibility is that they form a four helix bundle that is required for complex assembly. Alternatively, these coiled-coil domains might interact with other proteins involved in membrane docking and fusion, such as Rabs/Ypts or SNAREs. To address these questions we performed a systematic deletion of the coiled coil domains found in the four VFT subunits (Figure 5A and C). We did not include in this analysis the second predicted coiled-coil found in the middle of Vps53p, because the amphipathic nature of this region is not conserved in related yeast species and also because, unlike the other helical domains, we were unable to express it in bacteria.

The short coiled-coil domains within the different VFT subunits are evidently important for function because their removal abolished the ability of Vps51p, Vps52p, Vps53p and Vps54p to complement the growth of the respective null mutants at 37°C (data not shown). The truncated proteins did, however, accumulate to the same levels in cells as the full-length proteins, and thus were unlikely to be grossly misfolded since this usually leads to rapid degradation (Figure 5C and unpublished data). We next tested the ability of the protein A-tagged full-length and truncated Vps51p, Vps52p, and Vps53p to co-precipitate myc-tagged Vps54p, or of protein A-tagged full-length and truncated Vps54p to co-precipitate myc-tagged Vps53p. As shown in Figure 5C, in each case removal of the coiled-coil domain from a subunit prevented its association with the VFT complex. In the case of Vps52p, the deletion also removed a short region on the N terminal side of the coiled-coil, but deletion of this alone (residues 2-50) did not affect complex formation (data not shown). We conclude that the coiled-coil motifs are essential for assembly into the VFT complex.

Since coiled-coils tend to assemble with each other, we then examined whether the isolated VFT coiled-coils would bind to each other and form a four helix bundle, similar to the one formed by SNARE helices. For this purpose, the predicted coiled-coil regions from Vps52p (residues 1-139), Vps53p (residues 1-110), and Vps54p (residues 216-382), expressed as His<sub>6</sub>-fusions were incubated with either the Vps51p coiled coil domain (residues 121-164), expressed as a GST fusion, or GST alone. No association of the helical domains could be detected.
when the GST proteins were pulled down on glutathione beads, indicating that at least under these conditions spontaneous assembly of the four helices did not occur (Figure 5D).

Overall, these data show that the coiled coil domains mediate interactions which are necessary for the assembly and function of the VFT complex \textit{in vivo}. However, it seems that interactions outside the helical regions are also required for the formation of a tetrameric structure.

**Vps52p binds to Ypt6-GTP**

An important interaction of the VFT complex is with Ypt6p in its GTP-bound form on Golgi membranes (11). Since Golgi targeting of the complex occurs in the absence of Vps51p, the Ypt6p interaction must involve one of the other subunits. To identify which one, we expressed protein-A tagged versions of Vps52p in a \textit{vps53} mutant, and Vps53p and Vps54p in a \textit{vps52} mutant. The absence of an essential VFT subunit in each case ensured that assembly of the complex could not occur, thus allowing the binding properties of the single proteins to be assessed. Cell extracts were then incubated with beads containing GST-Ypt6p. As shown in Figure 6, Vps52p had the ability to bind to Ypt6p and retained a preference for the GTP form, whereas the other subunits showed only background binding. This confirms our previous conclusion that binding to Tlg1p and Ypt6p are independent properties of the complex, now revealed to be mediated by different subunits, Vps51p and Vps52p.

**Discussion**

We have shown that the VFT complex is normally not trimeric but tetrameric. The fourth subunit previously escaped attention, probably because of its small size and the fact that it lacks internal methionine or cysteine residues, and thus
cannot be labeled with $^{35}$S. This subunit, Vps51p, is unusual in that it is not absolutely required for assembly, localization or even partial function of the VFT complex. Also, unlike the other subunits it is poorly conserved. Homologues can be found in closely related yeasts (20), but divergence is considerable and we have been unable to identify any homologue in higher eucaryotes.

Like the other subunits, Vps51p contains a coiled-coil motif. We have shown that each subunit requires its coiled-coil domain for assembly into the VFT complex, but that a Vps52/53/54p complex can form, albeit inefficiently, in the absence of Vps51p. Though indirect effects on the structures of the proteins cannot be ruled out, the simplest explanation is that the complex is stabilized by the formation of a four-helix bundle, or in the absence of Vps51p a three-helix bundle. Since similar coiled-coil motifs are a feature of the COG and exocyst components, this may be a general feature of these complexes.

The coiled-coil motifs invite comparison with the SNAREs, which also form four-helix bundles. However, the isolated helical domains of the VFT components did not form bundles when tested under conditions in which SNAREs readily do so (21). There are also other significant differences. Whereas the coiled-coil “SNARE motif” is the most strongly conserved part of these molecules and easily recognizable, reflecting formation of stable bundles recognized by a universal dissociation machinery, the coiled-coils in the VFT complex are no more conserved than the rest of the proteins, and seem to vary in length and exact position in different species. Furthermore, though some weak similarity between the subunits can be seen (Figure 5B), its significance is unclear and alignment of these yeast proteins with the mammalian COG and exocyst coiled-coil consensus is difficult. Thus, there may be additional interactions that drive assembly of these complexes, the coils adding a relatively non-specific stabilizing influence. Indeed, we noticed that whilst complete removal of one subunit results in reduced yield of the others (10), removal of the coiled-coil from any one protein did not significantly destabilize either it or other components of the complex (Figure 5C), perhaps because weak interactions between the proteins are still possible.
SNAREs undergo reversible assembly, and there is also evidence that the exocyst is assembled during the process of vesicle docking (7). Similarly, one could imagine that Vps51p binds to Tlg1p on vesicles, while the remaining VFT subunits are recruited to the Golgi membrane via the Ypt6p binding site of Vps52p, and that docking is mediated by VFT assembly. We have not, however, been able to find circumstances under which the complex dissociates in vivo, or under physiological conditions in vitro. Furthermore, the fact that vps51 mutants have a less severe growth phenotype than other VFT mutants implies that the complex has at least some activity that does not involve Vps51p.

Perhaps the most striking feature of Vps51p is its ability to bind, via a short N-terminal peptide, to the N terminal domain of Tlg1p. Loss of this interaction may account for the observation that vps51 cells have severe trafficking defects even though they can still assemble and localize a core VFT complex. The N terminal domain of Tlg1p is predicted to have a three-helix structure like that of syntaxin, and thus might, as with the neuronal syntaxin 1a and yeast Sso1p, bind to and sequester the C-terminal SNARE motif (22, 23). In such a scenario the N terminus of Vps51p, which also has the potential to form an amphipathic helix, could compete for the same binding site as the SNARE motif and hence release this motif for interaction with other SNAREs. It has also been shown that interaction of a pre-formed complex of Tlg1p, Tlg2p and Vti1p with Snc1p is markedly stimulated by peptides corresponding to the C terminal portion of Snc1p (24), an effect that has been ascribed to subtle conformational changes in the SNARE helices (25). Further studies may reveal whether the N terminal peptide of Vps51p has any effect on the assembly of Tlg1p-containing SNARE complexes, and thus whether the VFT complex can directly modulate SNARE activity.

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References


Footnotes

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Figure legends

**Figure 1** Identification of Vps51p as the fourth subunit of the VFT complex. Affinity purification of Vps52p-PtA (left lane), Vps54p-PtA (middle lane) and Vps51p-PtA (right lane) from *vps52Δ, vps54Δ* and *vps51Δ* strains respectively. The purified proteins eluted from the IgG-Sepharose column with low pH were analyzed by SDS-PAGE and Coomassie staining. The position of the PtA fusions and the co-purifying proteins are indicated by symbols and labeled in the right hand panel (omitting the Vps for clarity), with the positions of molecular weight markers (10kD ladder) also indicated. Vps51p was identified by mass-
spectrometry as described in Materials and Methods. Note that like many helical proteins Vps51p migrates more slowly than expected from its molecular mass (18.9 kD), and that the Vps51p-PtA fusion is expressed at very low levels. The prominent band often seen between Vps52 and Vps53 is the coat protein of the yeast virus-like particles, a common contaminant (11).

Figure 2 Characterization of VPS51. (A) Defects in vacuolar morphology and GFP-Snc1p re-cycling in a \textit{vps51} deletion mutant. Mutant and wild-type strains were grown at 30°C to exponential phase, labeled with the endocytic tracer dye FM4-64 for 15 min and chased for 1 hr. Note that only vacuoles are visible under these conditions. The strains were also transformed with a plasmid expressing GFP-Snc1p and analysed by confocal microscopy. Note the presence of plasma membrane fluorescence in wild type but not \textit{vps51Δ} cells. (B) Distribution of GFP-Vps51p, expressed from its own promoter in a \textit{vps51Δ} strain. (C) Comparison of the growth properties of the \textit{vps51Δ}, \textit{vps52Δ}, \textit{vps54Δ} and wild-type cells. Cells were spotted onto selective YEPD plates and grown at 30°C and 37°C for 2 days.

Figure 3 Localization of the VFT complex to the Golgi is independent of Vps51p (A) \textit{VPS52-GFP}, \textit{VPS53-GFP} and \textit{VPS54-GFP} were expressed from centromeric plasmids under the control of their own promoters, in the indicated strains (wild-type, \textit{vps51Δ}, \textit{vps52Δ}). (B) Assembly of the VFT complex in the \textit{vps51Δ} deletion mutant. Affinity purification of Vps52p-PtA from a \textit{vps51Δ vps52D} double deletion strain (left lane) or a \textit{vps52D} strain (right lane). Equal amount of spheroplasts from the two strains were used to purify the Vps52p-PtA fusion.

Figure 4 Vps51p mediates binding to the SNARE Tlg1p via a short N-terminal fragment (A) Vps51p binds to Tlg1p. Glutathione-Sepharose beads with bound GST or GST-Vps52p, GST-Vps53p or GST-Vps51p were incubated with detergent-solubilized cytosol from a wild-type yeast strain. Bound proteins were eluted and analyzed by western blot using anti-Tlg1p antibodies. (B) Mapping of the Tlg1-binding site to the N-terminus of Vps51p. Upper panel: Schematic representation of the various Vps51p fragments used to map its binding with Tlg1p. The black box indicates the position of the predicted coiled coil domain in
Vps51p; the numbers indicate the % of residues in each of three regions that are completely identical in four *Saccharomyces* species (see text). The sequence of the highly-conserved minimal binding region is shown, with stars indicating hydrophobic residues in a heptad repeat pattern corresponding to a predicted amphipathic helix. Lower panel: Binding of Tlg1p from cytosol to glutathione-Sepharose beads containing the indicated GST-Vps51p fragments. Data from three different experiments are outlined.

**Figure 5** The coiled coil domains within the VFT subunits are necessary but not sufficient for complex assembly. (A) Prediction of the probability of the four subunits of the VFT complex to form coiled coils. The length of the x-axis corresponds to the length of the proteins, with residue numbers indicated at the bottom of the figure. On the y-axis is plotted the probability of a coiled-coil being at each residue of the protein (26). (B) Sequence alignment of predicted coiled coil regions from the four subunits of the VFT complex. The heptad repeats are indicated by the black dots. Residues that are identical or similar in at least half the sequences are shown on a black or grey background respectively. (C) The coiled coil domains of the VFT subunits are necessary for complex assembly. All yeast strains used in this experiment were co-expressing two proteins: Vps54p-myc (lanes 1-6) or Vps53p-myc (lanes 7-8) together with full-length (FL) protein-A fusions of Vps51p (lane 1), Vps52p (lane 3), Vps53p (lane 5) and Vps54p (lane 7) or the respective truncation mutants lacking the predicted coiled coil domains Vps51ΔCC (lacking residues 121-164 – lane 2), Vps52CC (lacking residues 5-122 – lane 4), Vps53CC (lacking residues 5-103 – lane 6) and Vps54CC (lacking residues 268-311 – lane 8). Protein-A fusions were purified and eluted with low pH (lanes 1-2) or TEV-protease digestion (lanes 3-8) as described previously (15). Eluates were analyzed by western blot. Stars denote the position of the protein A fusions in the solubilized extracts (upper panel). The position of Vps54-myc and Vps53-myc in the extracts or eluates (lower panel) is indicated. Note that in lanes 5, 7 and 8 the PtA- and myc- fusions co-migrate. All fusion genes were expressed under the control of their own promoters from centromeric plasmids. (D) The coiled coil domains of the VFT complex do not assemble in a four helix bundle. Solubilized supernatants from bacteria expressing the predicted coiled coil
domains of Vps52p (residues 1-139; His$_6$-52CC), Vps53p (1-110; His$_6$-53CC), and Vps54p (216-382; His$_6$-54CC) as His$_6$-fusions were mixed with a bacterial supernatant containing either the Vps51p-predicted coiled coil domain (residues 121-164; GST-51CC) as a GST fusion or GST alone. GST-fusions were purified as described in Experimental Procedures. Samples of the supernatants of the His$_6$-fusions (left panel) and the bound fractions (middle panel) were analysed by western blot using an anti-His$_6$ antibody. Right panel: samples of the bound fractions stained with Coomassie blue.

**Figure 6.** Vps52p binds to Ypt6-GTP. Western blot analysis of fractions eluted from GST-Ypt6:GTP$_\gamma$S or GST-Ypt6:GDP columns that had been incubated with cytosol from strains expressing Vps52p-PtA, Vps53p-PtA or Vps54p-PtA from high-copy (2 micron) vectors in vps53Δ, vps52Δ, or vps52Δ deletion mutants, respectively. Blots were probed with anti-PtA antibodies. Bands corresponding to the bound proteins were quantitated by densitometry and normalized amounts are shown above the lanes.
### Table I. Yeast strains used in this study

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<th>Strain</th>
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<td>S. Emr</td>
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Figure 1
Figure 2
Figure 3

A

Wild-type  vps51  vps52

VPS54-GFP

VPS53-GFP

VPS52-GFP

B

Vps54p  Vps53p  Vps52p-PtA  Vps51p
Figure 4
Figure 5
Figure 6
Vps51p links the VFT complex to the SNARE Tlg1p
Symeon Siniossoglou and Hugh R. B. Pelham

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