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***TVP23* interacts genetically with the yeast SNARE *VTI1* and functions in retrograde transport from the early endosome to the late Golgi**

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Synopsis

SNARE proteins contribute to specific recognition between transport vesicles and target membranes and are required for fusion of membranes. The SNARE Vti1p is required for several transport steps between late Golgi, endosomes and the vacuole in the yeast *Saccharomyces cerevisiae*. Here we identified the late Golgi membrane protein *TVP23* as a multicopy suppressor of the growth defect in *vti1-2* cells. By contrast, the growth defect in *vti1-11* cells was not suppressed by *TVP23* overexpression. Deletion of *TVP23* aggravated the growth defect in *vti1-2* cells. Genetic interactions between *TVP23* and *vti1-2* were not found in transport from the late Golgi via the late endosome to the vacuole or in transport from the Golgi directly to the vacuole. These data suggest that Tvp23p is not involved in forward transport from the late Golgi. Therefore retrograde traffic to the late Golgi was analyzed. *vti1-2* cells accumulated GFP-Snc1p within the cell indicating that retrograde transport from the early endosome to the late Golgi was defective in these cells. Deletion of *TVP23* in *vti1-2* cells resulted in a synthetic defect in GFP-Snc1p recycling while *tv23Δ* cells had a slight defect. These data indicate that Tvp23p performs a partially redundant function in retrograde transport from the early endosome to the late Golgi. This transport step was unaffected in *vti1-11* cells providing an explanation for the allele specific multicopy suppression by *TVP23*.

Short title:

TVP23 in transport from the early endosome to the late Golgi

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membrane traffic

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soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)

Introduction

Membrane traffic in eukaryotic cells is mediated by transport vesicles, which bud from a donor organelle and fuse with a target membrane. Several protein-protein interactions are required for recognition between membranes. Members of the Rab/Ypt family of small GTPases have to be recruited to the vesicle membrane and activated by GEFs (guanine nucleotide exchange factor), which exchange GDP for GTP [1]. Tether proteins are associated with target membranes. They are heterooligomeric complexes or large coiled coil proteins [2]. Some tether proteins act as GEFs for Rabs. On the other hand some tether proteins function as Rab effectors, which bind only to the active GTP form. These interactions are required for the tethering of transport vesicles to target membranes. In addition, tether proteins form complexes with SNARE proteins. Specific SNAREs are found on the transport vesicle and target membrane [3]. SNAREs contain a C-terminal transmembrane domain or a posttranslational hydrophobic modification. They form SNARE complexes via the conserved SNARE motifs next to the membrane anchor. These SNARE complexes consist of a bundle of four different parallel α -helices. Side chains from all four helices interact in 16 stacked layers in the core of the bundle. SNAREs can be classified as R-, Qa, Qb and Qc-SNAREs according to sequence similarities [4]. Functional SNARE complexes are composed of one member of each class. SNARE complex formation bridges the gap between both membranes and is required for fusion of both membranes.

The baker's yeast *Saccharomyces cerevisiae* is a powerful model to study the molecular mechanism of membrane traffic. Several trafficking pathways leave from the late Golgi in addition to the secretory route to the plasma membrane [5]. Most vacuolar proteins are transported from the late Golgi to the late endosome, also called multivesicular body or prevacuolar compartment and in a second transport step to the vacuole, the equivalent of the mammalian lysosome. The vacuolar carboxypeptidase Y (CPY) serves as a marker for this pathway. By contrast, the vacuolar alkaline phosphatase (ALP) traffics from the late Golgi to the vacuole without a passage through the late endosome. In addition, there is traffic from the Golgi to the early endosome. Endocytosed proteins destined for degradation are transported from early endosomes via late endosomes to the vacuole. On the other hand, there is retrograde transport from the early endosome to the late Golgi to recycle proteins from the plasma membrane. Escaped late Golgi proteins are retrieved from the early endosome to maintain the identity of the late Golgi. Retrograde traffic is also observed from the late endosome to the late Golgi. This recycling route is taken by the CPY receptor Vps10p. The SNARE protein Vti1p is essential for these transport steps as part of three different SNARE complexes [6, 7]. The SNARE complex consisting of Ykt6p, Pep12p, Vti1p and Syn8p is required for transport to the late endosome in addition to the Rab/Ypt GTPase Vps21p and the CORVET tethering complex [8-10]. Ypt7p, the HOPS tethering complex and a SNARE complex consisting of Ykt6p, Vam3p, Vti1p and Vam7p are required for fusion with the vacuole [11]. The heterooligomeric GARP/VFT tethering complex interacts with Ypt6p and the SNARE Tlg1p in retrograde traffic from endosomes to the late Golgi [12, 13]. The SNARE complex in retrograde traffic to the late Golgi consists of Snc1p or Snc2p, Tlg2p, Vti1p and Tlg1p [14, 15]. Snc1p/Snc2p is also required on secretory vesicles for secretion together with the plasma membrane SNAREs Sso1p/Sso2p and Sec9p, which contributes two helices to the SNARE complex [16]. After incorporation into the plasma membrane Snc1p/Snc2p is

endocytosed and recycled via early endosomes to the late Golgi for another round of secretory vesicle formation [17].

In addition, Vti1p is involved in retrograde transport to the cis-Golgi, probably in a complex with Ykt6p, Sed5p and Sft1p [6, 18]. We have been studying the function of Vti1p with the help of different temperature-sensitive mutants. *vti1-11* cells do not grow at the restrictive temperature and are blocked in transport to the late endosome, to the vacuole and in retrograde traffic to the cis-Golgi. *vti1-2* cells are defective in transport to the late endosome and in fusion with the vacuole as well as in growth at the restrictive temperature [6, 7]. The growth defect of *vti1-2* cells was used for a multicopy suppressor screen to identify additional components of these transport pathways.

Here we identified the late Golgi protein *TVP23* as an allele specific genetic interaction partner of *VTI1*. We found that Tvp23p is involved in retrograde transport from the early endosome to late Golgi. We describe that this transport step is also defective in *vti1-2* cells.

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Experimental

Materials

Reagents were used from the following sources: enzymes for DNA manipulation from New England Biolabs (Beverly, MA), [³⁵S]methionine [³⁵S]cysteine (EXPRES35S35S protein labeling mix) from Perkin Elmer (Waltham, MA), fixed *Staphylococcus aureus* cells (Pansorbin) from Calbiochem (San Diego, CA), zymolyase from Seikagaku (Tokyo, Japan), FM4-64 from Invitrogen (Carlsbad, CA).

Plasmid manipulations were performed in the *E. coli* strain DH5α using standard media. Yeast strains were grown in rich media (1% yeast extract, 1% peptone, 2% dextrose, YEPD) or standard minimal medium (SD) with appropriate supplements.

Strains and plasmids

Yeast strains were constructed using standard genetic techniques. The strains FvMY7, FvMY24 and FvMY21 carrying the *vti1-1*, *vti1-2*, *vti1-11* mutation, respectively, in the SEY6210 strain background have been described before [6, 7, 19]. *tvp23Δ* and *tlg2Δ* strains in BY4742 background were obtained from the Euroscarf strain collection [20]. The *tvp23Δ::kanMX4* mutant locus was PCR-amplified from genomic DNA with about 200 bp of flanking regions and integrated into SEY6210 to yield ISY5, into FvMY24 resulting in ISY6 and into SNY18 (*pho8Δ::ADE2* [21]) to obtain AGY4. A 2 μ plasmid encoding TVP23 was generated by subcloning a 1.7 kb HindIII fragment containing nucleotides 612.924 to 614.653 from chromosome IV from a suppressor plasmid into Yep352 yielding pLS2. pSN55 encoded the A-ALP fusion protein [22], pGS416 encoded the GFP-Snc1p fusion protein [17] and Dsred-Sec7 was expressed from pTPQ128 [23]. The yeast strain AHY41 carries the mutant alleles *vps5Δ::HIS3* *pho8Δ::ADE2* [27].

Suppressor Screen

The growth defect of *vti1-2* cells at 37°C was used to identify multicopy suppressors after transformation with a YE24 2 μ genomic library [24, 25].

Transport assays

Yeast cells were grown in log-phase at 24°C and 0.5 OD preincubated at the indicated temperature for 15 minutes or grown and labeled at 30°C for A-ALP assays. Cells were labeled with [³⁵S]methionine [³⁵S]cysteine (100 μCi/0.5 OD) for 10 minutes, chased for 30 minutes and CPY was immunoprecipitated with a rabbit antiserum from cellular extracts (I) and medium (E), ALP only from cellular extracts. For A-ALP stability assays, the cells were labeled for 25 minutes, chased for indicated time points and A-ALP was immunoprecipitated with antiserum directed against ALP from cellular extracts (I) as described [7, 22, 26]. Cells lacking the retromer subunit Vps5p were used as a positive control (AHY41, *vps5Δ::HIS3* *pho8Δ::ADE2* [27]). ALP antiserum was kindly provided by T.H. Stevens (University of Oregon, USA). Immunoprecipitates were analyzed by SDS-PAGE and autoradiography. A BAS-1800 II (Fuji) was used for quantification.

Fluorescence microscopy

Yeast cells were grown to an early logarithmic stage in appropriate SD-minimal medium at 24°C for FM4-64 staining, Ds-Red-Sec7p and GFP-Snc1p localization. For FM4-64 endocytosis about 0.7 OD of cells were pelleted, resuspended in 120 μl YEPD and 2 μl 16mM FM4-64 were added. After 5 min incubation at 24°C cells were washed once with YEPD and immediately viewed under a fluorescence microscope

(Leica DM-5000 B) with the CCD camera Leica DFC350 FX. *tlg2Δ* and *typ23Δ* cells were shifted for 1h to 30°C and *vti1-2* cells to 37°C for the GFP-Snc1p – Ds-Red-Sec7p colocalization experiment. For GFP-Snc1p and DsRed-Sec7p localization cells were washed once with PBS and immediately viewed under a fluorescence microscope.

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Results

Identification of *TVP23* as a suppressor of the growth defect in *vti1-2* cells

vti1-2 cells are defective in transport from the late Golgi to the late endosome, in direct transport from the late Golgi to the vacuole and in homotypic vacuolar fusion. These cells grow very slowly at the restrictive temperature of 37°C. In order to identify genes cooperating with *VTI1* a multicopy suppressor screen was performed utilizing the growth defect of *vti1-2* cells at 37°C. A 2µ plasmid containing nucleotides 609,360 to 617,576 of chromosome IV improved growth of *vti1-2* cells at 37°C (Fig. 1A). Five full length genes are encoded in this segment: the telomer binding protein *Stn1p*, the nucleolar protein *Rrp8p*, *Tvp23p*, a regulator of pheromone receptor signaling (*Afr1p*) and the *Sss1p* subunit of the *Sec61p* translocation complex. *Tvp23p* was identified as a membrane protein in the late Golgi or early endosome [28]. As *Tvp23p* is the only protein encoded on this suppressor plasmid with a possible connection to post Golgi membrane trafficking a 2µ plasmid was generated, which encoded only *Tvp23p*. Overexpression of *TVP23* alone was sufficient to improve growth of *vti1-2* cells (Fig. 1A). Suppression was not observed with fragments of the suppressor plasmid without intact *TVP23* (data not shown). This indicates that *TVP23* is a multicopy suppressor of the growth defect in *vti1-2* cells. *tpv23Δ* cells grew as well as wild-type cells at all temperatures (Fig. 1B). Deletion of *TVP23* in *vti1-2* cells reduced the slow growth of *vti1-2* cells at 37°C after prolonged incubation even further (Fig. 1B). These data show that combination of *vti1-2* and *tpv23Δ* mutations resulted in a synthetic growth defect at high temperatures as additional genetic interaction. Suppression may be due to direct or more general effects. Therefore, *vti1-11* cells were tested, which are defective in an additional transport step, in retrograde transport to the cis-Golgi. The growth defect of *vti1-11* cells at 37°C was not suppressed by overexpression of *TVP23* (Fig. 1C). This allele specific effect suggests that *Tvp23p* functions in one of the trafficking steps defective in *vti1-2* cells.

Tvp23p was not involved in anterograde traffic to the vacuole

Effects of the *tpv23Δ* mutation on trafficking were tested. Transport from the late Golgi via the late endosome to the vacuole was investigated first. The vacuolar hydrolase carboxypeptidase Y (CPY) is modified to the pro form p1CPY in the ER and receives further glycosylation in the Golgi to a slightly larger p2CPY. p2CPY travels via the late endosome to the vacuole, there it is proteolytically cleaved to mature mCPY with lower molecular weight [5]. p2CPY is secreted into the medium upon block in transport from the late Golgi to the vacuole. Newly synthesized proteins were labeled with [³⁵S]cysteine and [³⁵S]methionine, chased with unlabeled cysteine and methionine and CPY immunoprecipitated from cellular extracts (I) and the medium (E). Samples were analyzed by SDS-PAGE and autoradiography to follow transport of newly synthesized CPY. CPY reached the vacuole as mCPY in wild-type cells (Fig. 2A). By contrast, nearly all CPY was secreted into the medium (E) in *vti1-2* cells at 33°C. Overexpression of *TVP23* from either the 8.2 kb 2µ suppressor plasmid or as the only gene on a 2µ plasmid did not improve sorting of CPY (Fig. 2A). Deletion of *TVP23* did not result in a CPY transport defect (Fig. 2B). To investigate whether lack of *Tvp23p* caused a synthetic defect CPY transport was analyzed at a semi-permissive temperature of 28°C. At this temperature only a partial CPY sorting defect was observed in *vti1-2* cells (Fig. 2B). This defect would be aggravated in case of a synthetic genetic interaction. However, sorting of CPY was comparable in

vti1-2 cells and in *vti1-2 tvp23Δ* cells. These data indicate that Tvp23p does not function in CPY transport from the late Golgi via the late endosome to the vacuole. The vacuolar membrane protein alkaline phosphatase (ALP) and CPY reach the vacuole via different transport routes. ALP is transported from the late Golgi to the vacuole without passage through the late endosome [5]. Arrival in the vacuole results in cleavage of the pre form pALP to two different forms of mature mALP with lower molecular weights. Newly synthesized protein were labeled with [³⁵S]cysteine and [³⁵S]methionine and ALP immunoprecipitated. Overexpression of *TVP23* from either 2 μ plasmid did not increase the amount of vacuolar mALP in *vti1-2* cells at 36°C (Fig. 3A). Only mALP and no pALP was detected in *tvp23Δ* cells indicating ALP transport was not affected by the absence of Tvp23p (Fig. 3B). The ratio between pALP and mALP was not different in *vti1-2* cells and in *vti1-2 tvp23Δ* cells at a semi-permissive temperature of 30°C (Fig. 3B). The lack of a multicopy suppression of as well as a synthetic defect with the *vti1-2* mutation indicate that Tvp23p is not involved in ALP transport from the late Golgi directly to the vacuole.

***tvp23Δ* and *vti1-2* cells were defective in transport from the early endosome to the late Golgi**

As we did not find any indication for a role of Tvp23p in anterograde transport from the late Golgi to the vacuole we analyzed retrograde traffic from the early endosome to the late Golgi. GFP-Snc1p serves as a marker for this transport pathway [17]. Snc1p is an R-SNARE on secretory vesicles targeted from the late Golgi to the plasma membrane [29]. After fusion it is incorporated into the plasma membrane. Snc1p is endocytosed and recycled from early endosomes to the late Golgi for further sorting into secretory vesicles. GFP-Snc1p is found in the plasma membrane in most wild type cells but there is also an intracellular pool (Fig. 4A). GFP-Snc1p accumulates in intracellular structures in cells defective in transport from the early endosome to the late Golgi such as *tlg2Δ* cells [17] which lack the SNARE Tlg2p required for this transport step (Fig. 4B). Slightly less *tvp23Δ* cells than wild-type cells accumulated GFP-Snc1p at the plasma membrane (Fig. 4C). GFP-Snc1p localized to the plasma membrane in 68.4 % of 790 *tvp23Δ* cells grown at 24°C analyzed in 4 independent experiments (SD 3.4 between experiments) compared to 74.1 % of wild-type cells (SD 1.3, 730 cells, three experiments). The difference was small but reproducible and statistically significant ($p = 0.043$, student's t-test). These data suggest that recycling of GFP-Snc1p maybe slightly affected in the absence of Tvp23p.

Transport from early endosomes to the late Golgi has not been investigated in *vti1* mutant cells. *vti1-1* cells display the mildest phenotype and are not temperature sensitive for growth. *vti1-1* cells are defective in transport from the late Golgi to the late endosome at 37°C. *vti1-1*, *vti1-2* and *vti1-11* cells were grown at 24°C and shifted to 30°C or 37°C for 1 hour. GFP-Snc1p was concentrated at the plasma membrane in *vti1-1* cells at all temperatures indicating that recycling of GFP-Snc1p was not defective (Fig. 5). *vti1-11* cells displayed a similar distribution of GFP-Snc1p as wild-type cells. Intracellular GFP-Snc1p was observed in some *vti1-11* cells at 37°C. However, increased temperature also resulted in intracellular GFP-Snc1p in wild-type cells. This indicates that GFP-Snc1p recycling was normal or only slightly affected in *vti1-11* cells. By contrast, GFP-Snc1p distribution was abnormal in *vti1-2* cells at all temperatures. Even at 24°C GFP-Snc1p was shifted from the plasma membrane to internal stores in 50% of the *vti1-2* cells. The severity of the phenotype was increased at elevated temperatures (Fig. 5). At 30°C only 27% of the *vti1-2* cells displayed GFP-Snc1p at the plasma membrane, 25 % of over 200 cells counted in

this set of experiments at 37°C. These data indicate that transport from the early endosome to the late Golgi was somewhat affected even at 24°C in *vti1-2* cells and that the transport block was stronger at 30°C and 37°C.

vti1-2 cells and *vti1-2 tvp23Δ* cells were examined for synthetic defects in GFP-Snc1p recycling at 24°C. GFP-Snc1p was localized to the plasma membrane of less *vti1-2 tvp23Δ* cells compared to *vti1-2* cells (Fig. 6B, 6C). 35 % of over 900 *vti1-2 tvp23Δ* cells analyzed in 3 independent experiments (SD 1.2 between experiments) displayed a GFP-Snc1p localization at the plasma membrane compared to 46 % of the *vti1-2* cells (SD 2.0, 720 cells in three experiments) at 24°C (Fig. 6D). These differences were statistically significant ($p = 0.0012$, student's t-test). The synthetic defect in GFP-Snc1p recycling in *vti1-2 tvp23Δ* cells provides further evidence for a role of Tvp23p in retrograde traffic from the early endosome to the late Golgi. It has been reported that GFP-Snc1p accumulates in early endosomes and transport vesicles mediating traffic between early endosomes and late Golgi upon block of recycling to the late Golgi [17]. These structures also contain late Golgi proteins because late Golgi proteins can escape to early endosomes and recycle from there to the late Golgi. In order to investigate whether GFP-Snc1p was localized to early endosomes the cells were allowed to endocytose the membrane dye FM4-64 for 5 min. As expected GFP-Snc1p and FM4-64 colocalized in *tlg2Δ* cells (Fig. 7A). A similar colocalization was observed in *vti1-2* as well as in *tvp23Δ* cells indicating that GFP-Snc1p accumulated in endocytic structures. DsRed-Sec7p was expressed as a late Golgi enzyme [23]. Considerable colocalization was seen between DsRed-Sec7p and GFP-Snc1p in *tlg2Δ* cells (Fig. 7B). This could be due to a mislocalization of DsRed-Sec7p to early endosomes because recycling from early endosome to late Golgi is blocked. On the other hand, GFP-Snc1p containing transport vesicles could be tethered to the late Golgi. DsRed-Sec7p and GFP-Snc1p showed comparable colocalization in *tlg2Δ*, *vti1-2* and *tvp23Δ* cells selected for internal GFP-Snc1p accumulation. Taken together these data demonstrate that GFP-Snc1p accumulated in similar internal structures in *tvp23Δ*, *vti1-2* and *tlg2Δ* cells. We conclude that *tvp23Δ* as well as *vti1-2* cells were defective in transport from early endosomes to the late Golgi.

Certain late Golgi proteins are retrieved from the late endosome back to the late Golgi. An example is dipeptidyl aminopeptidase A (DPAP A), which binds via the cytosolic domain to the retromer complex and to sorting nexins [27, 30]. This transport pathway can be investigated using the fusion protein A-ALP, which consists of the cytosolic tail of DPAP A required for trafficking fused to the transmembrane and luminal part of ALP used for immunoprecipitation with antiserum directed against ALP [22]. A-ALP recycles between late Golgi, early endosomes and late endosomes. If retrograde traffic from the late endosome is blocked unprocessed A-ALP (pA-ALP) is transported to the vacuole and cleaved to a smaller form mA-ALP. Therefore, loss of pA-ALP and generation of mA-ALP can serve as an assay for block in retrograde transport from the late endosome to the late Golgi. Wild type, *tvp23Δ* and *vps5Δ* cells lacking the retromer subunit Vps5p [27] were incubated with [³⁵S]cysteine and [³⁵S]methionine, chased with unlabeled cysteine and methionine for different time points and ALP immunoprecipitated. In *vps5Δ* cells about 50% of A-ALP was found as vacuolar mA-ALP after 60 min chase and processing was more prominent after 120 min chase (Fig. 8) as described [27]. The amounts of pA-ALP remaining were similar in wild type and *tvp23Δ* cells chased for 60 min and for 120 min while a band with a mobility similar to that of mA-ALP did not increase with time (Fig. 8). This

indicates that retrograde transport from the late endosome to the late Golgi was not significantly affected in *tvp23Δ* cells.

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Discussion

Here we studied different *vti1* mutant cells for defects in retrograde transport from the early endosome to the late Golgi. A marker protein recycling between endosomes and late Golgi was not useful because anterograde transport from the Golgi to endosomes is blocked in *vti1* mutant cells. Therefore we had to use a marker protein, which is endocytosed from the plasma membrane and recycled to the late Golgi such as GFP-Snc1p [17]. GFP-Snc1p trafficking was not affected in *vti1-1* cells and *vti1-11* cells. By contrast, *vti1-2* cells were defective in GFP-Snc1p recycling. Each of the three mutant alleles contains two amino acid exchanges in different positions in the N-terminal half of the SNARE motif or just before the SNARE motif [19]. The amino acid exchanges in the three *vti1* mutant proteins have to affect different SNARE complex partners in different ways in order to cause defects in various numbers and combinations of transport pathways (Fig. 9). The crystal structure of the mammalian late endosomal SNARE complex provides some hints due to the conserved structure [31]. One of the mammalian homologs of the Qb-SNARE Vti1p, vti1b is part of this complex together with syntaxin 7, syntaxin 8 and VAMP-8. Aspartic acid D157 of vti1b forms a salt bridge with arginine R164 of syntaxin 8. The equivalent amino acid residue in Vti1p is E145, which is changed to E145K in *vti1-1p* and to E145G in *vti1-11p*. The position of the Qc-SNARE syntaxin 8 in the SNARE complex is taken by Syn8p in transport to the late endosome, by Vam7p in transport to the vacuole and by Tlg1p in retrograde transport to the late Golgi. The salt bridge is only conserved in Syn8p with arginine R183 in the position equivalent to R164 of syntaxin 8, but filled by N151 in Tlg1p and A269 in Vam7p. Therefore, it is not surprising that *vti1-1* and *vti1-11* cells are defective in the Syn8p but not in the Tlg1p depended transport step. The second amino acid exchange in both alleles is found in amino acid residues, which form layers of interacting side chains in the hydrophobic core of the helical bundle. *vti1-1* cells with the G148R exchange in the -3 layer in *vti1-1p* are only defective in transport to the late endosome. By contrast, the L155F exchange in the -1 layer of *vti1-11p* has more severe consequences, because *vti1-11* cells are defective in transport to the vacuole and in retrograde transport to the cis Golgi in addition to a block in transport to the late endosome [6, 7]. *vti1-2p* contains the amino acid exchanges S130P just four amino acid residues N-terminal of the SNARE motif (-7 layer L134) and I151T in the -2 layer. *vti1-2* cells accumulated some intracellular GFP-Snc1p already at 24°C and a clear defect was observed at 30°C. Interestingly, transport of CPY to the late endosome was normal at 24°C and defective at 31°C in *vti1-2* cells. The restrictive temperature for ALP transport to the vacuole was 37°C. These data indicate that the SNARE partners of Vti1p in these three complexes react slightly differently to the same amino acid exchange.

The growth defect of *vti1-2* cells at elevated temperatures was aggravated in the absence of Tvp23p and suppressed upon overproduction of Tvp23p. By contrast, overexpression of *TVP23* did not suppress the growth defect of *vti1-11* cells at elevated temperatures. These data demonstrate an allele specific genetic interaction of *TVP23* with *VTI1* and suggest that Tvp23p functions in one or more of the pathways, which are defective in *vti1-2* cells. Absence of Tvp23p did not result in defects of transport of CPY from the Golgi via the late endosome to the vacuole or in transport of ALP directly to the vacuole as observed in this study and by Inadome et al. [32]. This does not exclude a role of Tvp23p in these transport pathways because a redundant protein could substitute for a lack of Tvp23p. As we observed a synthetic growth defect in *vti1-2 tvp23Δ* cells we should be able to identify a synthetic defect in

one of the transport pathways, which require Vti1p. However, we did not observe synthetic defects in CPY or ALP transport. The problem of redundant proteins can be circumvented if overexpression results in the suppression of a phenotype. However, overexpression of *TVP23* did not result in a suppression of the CPY or ALP sorting defects in *vti1-2* cells even though the growth defect was suppressed. Therefore we conclude that Tvp23p does not function in transport from the late Golgi to the late endosome or from the late Golgi directly to the vacuole. The lack of Tvp23p did not result in increased vacuolar degradation of the late Golgi protein A-ALP indicating that retrograde transport from the late endosome was not defective. Slightly less cells accumulated GFP-Snc1p at the plasma membrane in the absence of Tvp23p compared to wild-type cells. In addition, the absence of Tvp23p increased the GFP-Snc1p recycling defect in *vti1-2* cells. These data indicate that Tvp23p has a role in retrograde transport from the early endosome to the late Golgi. However, the block in transport was weak suggesting redundancy in the pathway or a modifying role. Our results show the first transport defects due to the lack of Tvp23p and are in agreement with earlier observations. Tvp23p was identified as a membrane protein of vesicles, which were immunoisolated with antibodies directed against the late Golgi and early endosome SNARE Tlg2p [28]. Tagged Tvp23p colocalizes with proteins of the late Golgi and early endosome [28]. Therefore, Tvp23p is found in the right subcellular localization to function in retrograde transport from the early endosome to the late Golgi. A synthetic growth defect was observed in *tv23Δ ypt6Δ* cells [28]. As the Rab/Ypt GTPase Ypt6p functions in retrograde transport from the early endosome to the late Golgi [33] this is consistent with a function of Tvp23p in the same pathway. However, a synthetic defect between two deletion mutants could also indicate a function in two parallel pathways. Coimmunoprecipitation revealed that Tvp23p binds to Yip4p and Yip5p [32]. Yip4p and Yip5p are Rab GTPase interacting factors [34]. Yip4p is able to bind all Rab GTPases found in yeast. Yip5p interacts with all yeast Rab GTPases except for Ypt6p. As Yip4p and Yip5p can bind each other the complex may function on Ypt6p. Yip4p and Yip5p belong to the YIP1 family of proteins. Yip3, a mammalian member of this protein family, recruits rab9 to the membrane by dissociating the soluble GDI (GDP dissociation inhibitor) -rab complex [35] and is therefore called a GDI displacement factor (GDF). It is possible that Yip4p and Yip5p serve as a GDF for Ypt6p. Tvp23p may play a role in this process. Recruitment of Ypt6p-GDP to the membrane increases the amount of substrate available for activation by the Ric1p-Rgp1p complex, which acts as GEF for Ypt6p [36]. Overproduction of Tvp23p may increase the efficiency of the tethering between Ypt6p and the GARP/VFT tethering complex. As GARP/VFT binds directly to the SNARE Tlg1p [13] this may allow for an efficient use of the residual activity of the Ykt6p, Tlg2p, Vti1p, Tlg1p SNARE complex in *vti1-2* cells. An alternative explanation would be a direct interaction between Tvp23p and Vti1p.

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Figure legends

Figure 1: Overexpression of *TVP* suppressed and absence of *Tvp23p* aggravated the growth defect in *vti1-2* cells at 37°C. Dilutions of cells were incubated at 24°C or 37°C on plates with rich medium. (A) *vti1-2* cells (FvMY24) overexpressing *TVP23* either from a 8.2 kb library 2μ plasmid or as the only open reading frame on a 2μ plasmid grew better at 37°C than *vti1-2* cells. WT: SEY6210 wild-type cells (B) *vti1-2 tvp23Δ* double mutant cells (ISY6) grew slower at 37°C than either *vti1-2* or *tvp23Δ* (ISY5) single mutant cells after prolonged incubation. (C) The growth defect in *vti1-11* cells (FvMY21) was not suppressed by overexpression of *TVP23* indicating that the suppression is allele specific. All mutations were introduced into the SEY6210 background.

Figure 2: Lack of genetic interaction between *vti1-2* and *TVP23* in transport of the vacuolar hydrolase CPY from the late Golgi via the late endosome to the vacuole. (A) *vti1-2* cells were defective in CPY transport via the late endosome to the vacuole at 33°C as indicated by the lack of mCPY in cellular extracts (I). Overexpression of *TVP23* either from a 8.2 kb library 2μ plasmid or as the only open reading frame on a 2μ plasmid did not suppress the CPY transport defect. (B) CPY transport was analyzed at the semi-permissive temperature of 28°C. CPY transport was unaffected in *tvp23Δ* cells. The partial CPY sorting defect in *vti1-2* cells was not aggravated in *vti1-2 tvp23Δ* double mutant cells. CPY traffic was analyzed by pulse-chase labelling, immunoprecipitation from cellular extracts (I) and the medium (E), SDS-PAGE, and autoradiography. All strains were derived from a SEY6210 background. WT: wild-type cells; p2CPY: Golgi proCPY; mCPY: mature vacuolar CPY

Figure 3: Lack of genetic interaction between *vti1-2* and *TVP23* in transport of the vacuolar phosphatase ALP from the late Golgi directly to the vacuole. (A) Similar amounts of pALP indicated that overexpression of *TVP23* either from a 8.2 kb library 2μ plasmid or as the only open reading frame on a 2μ plasmid did not suppress the ALP transport defect in *vti1-2* cells at 36°C. (B) ALP transport was analyzed at the semi-permissive temperature of 30°C. ALP reached the vacuole in *tvp23Δ* cells. The partial ALP transport defect in *vti1-2* cells was not aggravated in *vti1-2 tvp23Δ* double mutant cells. ALP traffic was analyzed by pulse-chase labelling, immunoprecipitation, SDS-PAGE, and autoradiography. All strains were derived from a SEY6210 background. WT: wild-type cells; pALP: proALP; mALP: mature vacuolar ALP (two bands)

Figure 4: Slightly impaired recycling of GFP-Snc1p to the late Golgi in *tvp23Δ* cells. Cells expressing GFP-Snc1p were analyzed by fluorescence microscopy. (A) GFP-Snc1p was localized predominantly to the plasma membrane in SEY6210 wild-type cells (WT). GFP-Snc1p was found in internal structures in *tlg2Δ* cells (BY4742 background) known for their block in transport from the early endosome to the late Golgi (B) GFP-Snc1p was found on the plasma membrane and in internal structures in *tvp23Δ* cells (ISY5, 6210 background, C). (D) Quantification of cells with a GFP-Snc1p plasma membrane (PM) localization. 68.4% of *tvp23Δ* cells had plasma membrane staining (795 cells in 4 independent experiments, SD 3.4 between experiments) compared to 74.1% of wild type cells (738 cells in 3 experiments, SD 1.3). The differences are statistically significant ($p = 0.043$, student's t-test).

Figure 5: Defective recycling of GFP-Snc1p to the late Golgi in *vti1-2* cells. Cells expressing GFP-Snc1p were grown at 24°C, shifted to the indicated temperature for 1 hour and analyzed by fluorescence microscopy. Most GFP-Snc1p was localized to the plasma membrane at all temperatures in *vti1-1* (FvMY7) and *vti1-11* (FvMY21) cells. By contrast some GFP-Snc1p was localized to internal structures at 24°C in *vti1-2* cells (FvMY24). The mislocalization was more pronounced at 30°C and at 37°C indicating that recycling to the late Golgi was blocked in *vti1-2* cells.

Figure 6: Synthetic defect in recycling of GFP-Snc1p to the late Golgi in *vti1-2 tvp23Δ* cells. *tvp23Δ* (ISY5, **A**), *vti1-2* (FvMY24, **B**) and *vti1-2 tvp23Δ* cells (ISY6, **C**) expressing GFP-Snc1p were grown at 24°C and analyzed by fluorescence microscopy. (**D**) Quantification of cells with a GFP-Snc1p plasma membrane (PM) localization. 35 % of *vti1-2 tvp23Δ* cells had plasma membrane staining (909 cells in 3 independent experiments, SD 1.2 between experiments) compared to 46 % of *vti1-2* cells (726 cells in 3 experiments, SD 2.0). The differences are statistically significant ($p = 0.0012$, student's t-test).

Figure 7: GFP-Snc1p colocalized with endosomal and late Golgi markers in *tvp23Δ* and *vti1-2* cells. (**A**) *tlg2Δ* (BY4742 background), *tvp23Δ* (ISY5), and *vti1-2* cells (FvMY24) expressing GFP-Snc1p were analyzed by fluorescence microscopy after incubation with the endocytosis marker FM4-64 for 5 min. Substantial overlap between intracellular GFP-Snc1p and FM4-64 was observed in these strains. (**B**) *tlg2Δ*, *tvp23Δ*, and *vti1-2* cells expressing GFP-Snc1p and Ds-Red-Sec7p were analyzed by fluorescence microscopy. Sec7p localizes to the late Golgi and to early endosomes upon block of recycling to the late Golgi. Many GFP-Snc1p positive intracellular structures contained Ds-Red-Sec7p in *tlg2Δ*, *tvp23Δ*, and *vti1-2* cells. A slight gamma-correction was applied for clarity of the merged images.

Figure 8: Transport from the late endosome to the late Golgi was not affected in *tvp23Δ* cells. Retrograde transport from the late endosome to the late Golgi was monitored using A-ALP, which contained a retrieval signal for recycling from the late endosome to the late Golgi. Block in retrograde traffic from the late endosome for example in *vps5Δ* cells results in a destabilization of pA-ALP and processing to the slightly smaller mA-ALP because it is transported to the vacuole. A-ALP consists of the cytosolic domain of dipeptidyl aminopeptidase A (DPAP A), which localizes to the late Golgi, fused to the transmembrane and luminal domain of alkaline phosphatase (ALP) for detection. Cells were pulse labeled at 30°C and chased for indicated time periods before immunoprecipitation. A-ALP was immunoprecipitated with antisera directed against ALP in cells deleted for *PHO8*, the gene encoding ALP to avoid immunoprecipitation of ALP. The stability of pA-ALP was comparable in wild-type (WT, SNY18) and *tvp23Δ* cells (AGY4) while a band with a mobility similar to mA-ALP did not increase with time. By contrast, the amounts pA-ALP and mA-ALP were comparable after 60 min chase and mA-ALP the predominant form after 120 min in *vps5Δ* cells (AHY41). pA-ALP, Golgi pro A-ALP; mA-ALP, vacuolar mature A-ALP

Figure 9: Amino acid exchanges in the different *vti1* mutant alleles result in different effects in membrane transport. (**A**) Vti1p consists of an N-terminal domain, which forms a three helix bundle (grey rods), the SNARE motif (amino acid residues 134 – 186) and a C-terminal transmembrane domain (black rod). Boxes indicate the amino acid residues, which participate in layers of interacting side chains within the centre of the SNARE complex. Amino acid exchanges are indicated: *vti1-1*

E145K, G148R (-3 layer); *vti1-2* S130P, I151T (-2 layer); *vti1-11* E145G, L155F (-1 layer). **(B)** Names of *vti1* mutant next to arrows indicate transport steps defective in this allele. Defect in *vti1-1* Golgi to LE; *vti1-2* Golgi to LE, fusion with the vacuole, EE to Golgi; *vti1-11* Golgi to LE, fusion with the vacuole, retrograde to cis Golgi. Four different SNARE complexes are required for these trafficking steps (boxes). Tvp23p is involved in EE to Golgi transport. LE: late endosome, EE: early endosome

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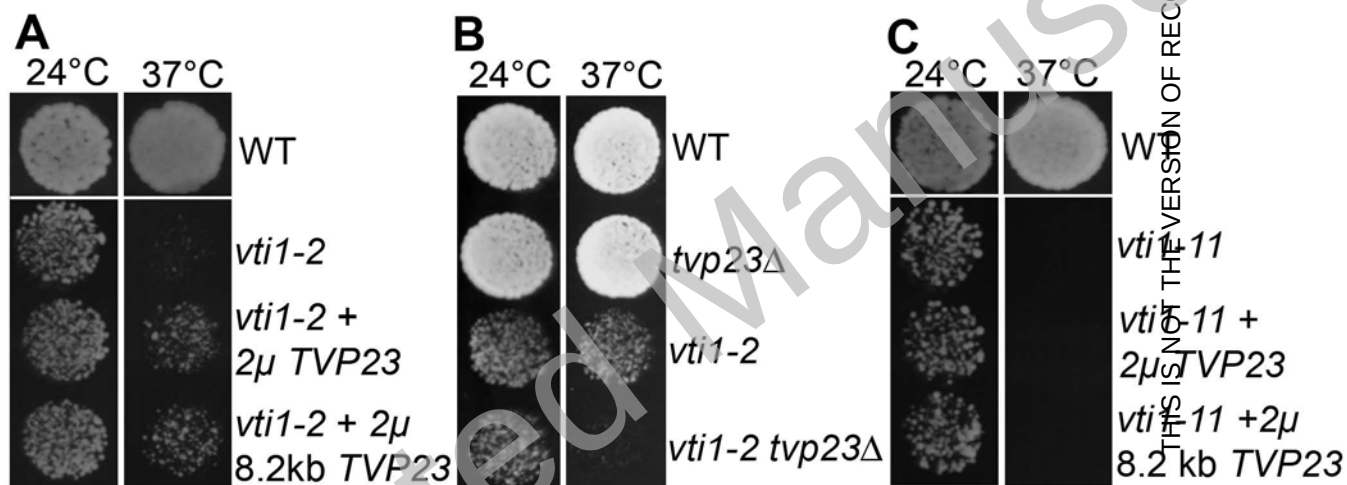


Figure 1

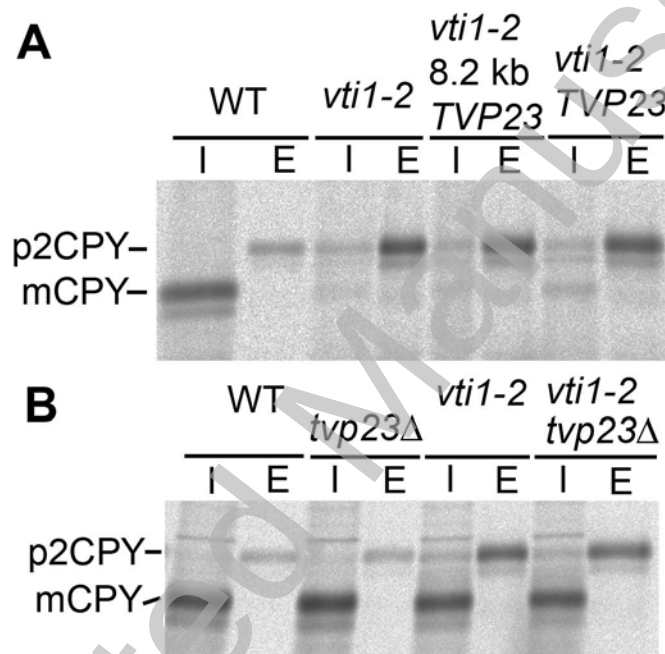


Figure 2

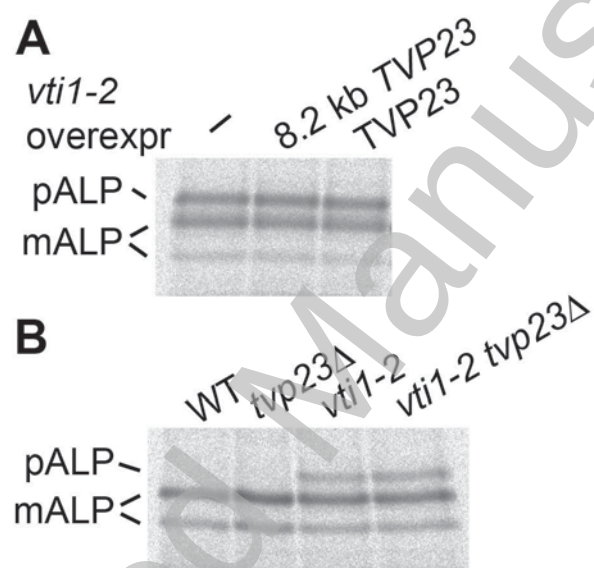


Figure 3

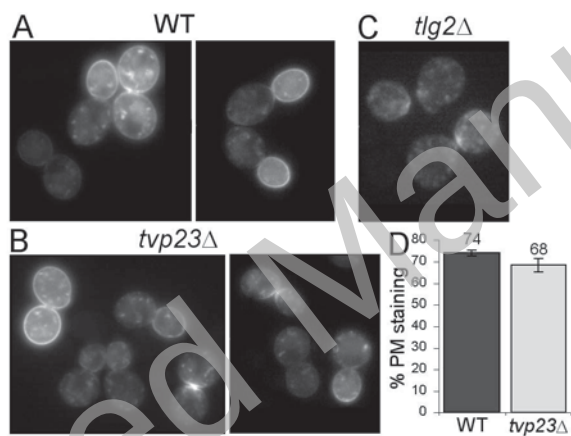


Figure 4

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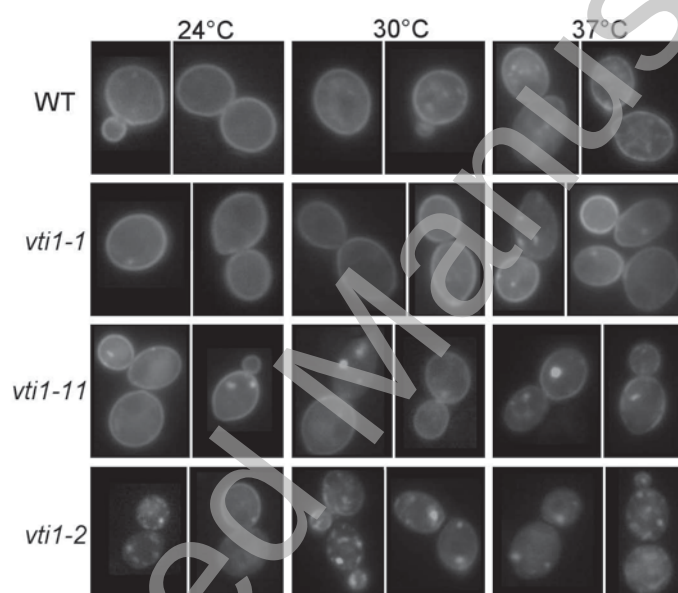


Figure 5

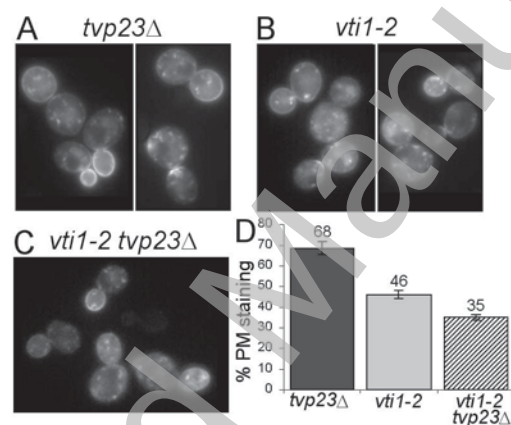


Figure 6

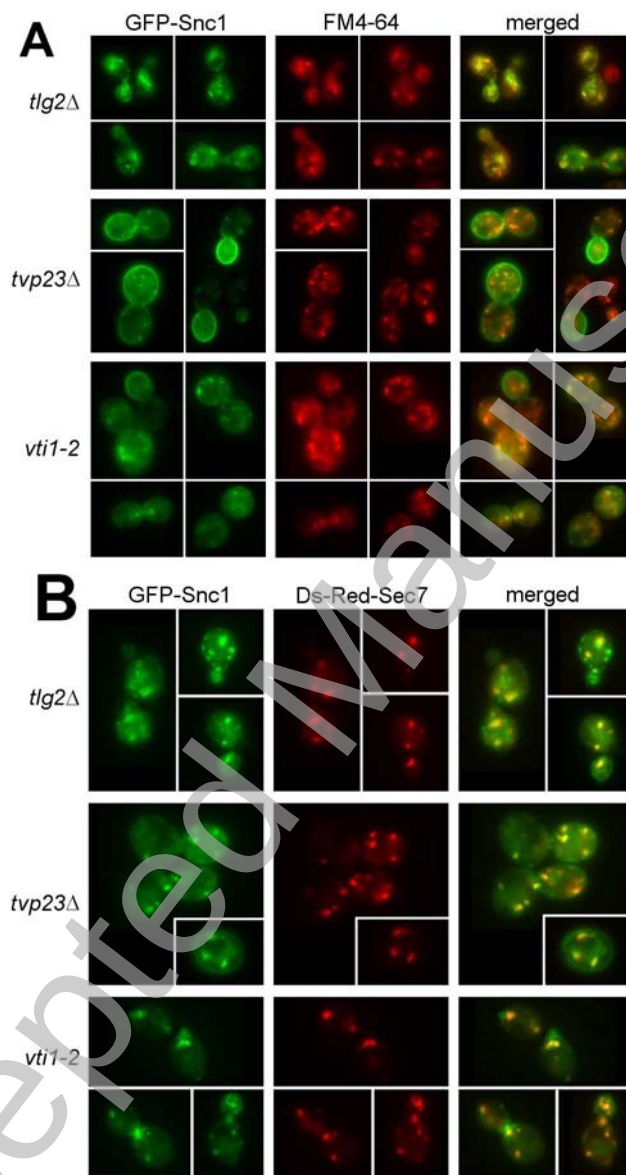


Figure 7

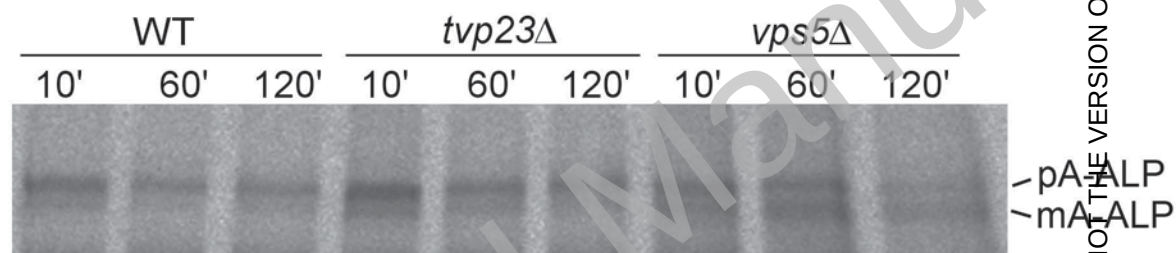


Figure 8

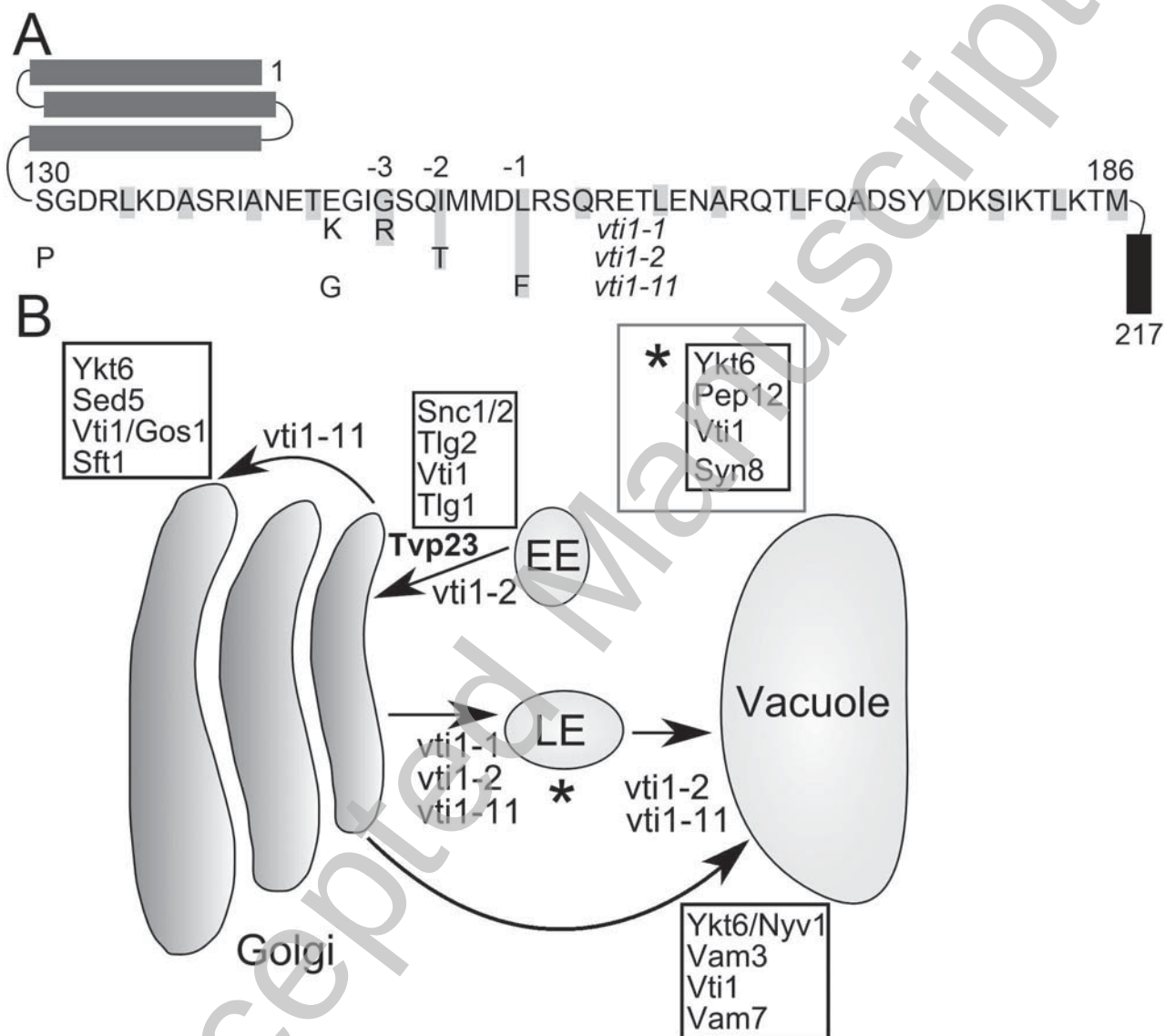


Figure 9