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Establishing a Role for the GTPase Ypt1p at the Late Golgi

Anthony Sclafani¹, Shuliang Chen⁴, Felix Rivera-Molina², Karin Reinisch², Peter Novick^{2,3}, and Susan Ferro-Novick^{1,4,++}

¹Department of Cell Biology, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT 06519, USA

²Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06520, USA

³Department of Cellular and Molecular Medicine, University of California at San Diego, La Jolla, CA 92093-0651

⁴Department of Cellular and Molecular Medicine, Howard Hughes Medical Institute, University of California at San Diego, La Jolla, CA 92093-0668

Abstract

GTPases of the Rab family cycle between an inactive (GDP-bound) and active (GTP-bound) conformation. The active form of the Rab regulates a variety of cellular functions via multiple effectors. Guanine nucleotide exchange factors (GEFs) activate Rabs by accelerating the exchange of GDP for GTP, while GTPase activating proteins (GAPs) inactivate Rabs by stimulating the hydrolysis of GTP. The GTPase Ypt1p is required for ER-Golgi and intra-Golgi traffic in the yeast *Saccharomyces cerevisiae*. Recent findings, however, have shown that a Ypt1p GEF, GAP and effector are all required for traffic from the early endosome to the Golgi. Here we describe a screen for *ypt1* mutants that block traffic from the early endosome to the late Golgi, but not general secretion. This screen has led to the identification of a collection of recessive and dominant mutants that block traffic from the early endosome. While it has long been known that Ypt1p regulates the flow of biosynthetic traffic into the cis side of the Golgi, these findings have established a role for Ypt1p in the regulation of early endosome-Golgi traffic. We propose that Ypt1p regulates the flow of traffic into the cis and trans side of the Golgi via multiple effectors.

Keywords

Rab; early endosome; Golgi; membrane traffic

Introduction

GTPases of the Rab family are key regulators of membrane traffic. Rabs have been shown to function at multiple stages of vesicle traffic, including cargo selection, vesicle tethering, and membrane fusion (1-3). More than 60 Rabs have been identified in mammalian cells, while 11 Rabs (called Ypt or Sec4) have been found in the yeast *Saccharomyces cerevisiae* (4). Rabs are molecular switches that cycle between their inactive (GDP bound) and active (GTP bound) forms. Because of their slow intrinsic rate of nucleotide exchange and hydrolysis, guanine nucleotide exchange factors (GEFs) are needed to accelerate the exchange of GDP

++Correspondence should be addressed to: Dr. Susan Ferro-Novick, Department of Cellular and Molecular Medicine, Howard Hughes Medical Institute, University of California at San Diego, GPL room 315, 9500 Gilman Drive, La Jolla CA 92093-0668, Phone: (858) 246-0466, Fax: (858) 534-7688, sfnovick@ucsd.edu.

for GTP, while GTPase activating proteins (GAPs) are needed to inactivate the Rab by stimulating GTP hydrolysis.

Rabs contain several conserved regions that include the P-loop, and the switch I and switch II effector domains. The P-loop is important for binding to the β and γ phosphate of the guanine nucleotide. Switch I and switch II domains adopt different conformations depending on the nucleotide binding state of the Rab. Effectors, which are crucial downstream targets, differentiate between the active and inactive forms of the Rab by interacting with the switch regions. They interact with one or both of the switch regions as well as other surfaces near the nucleotide binding pocket (2,5-7). Each Rab can recruit multiple effectors to perform its diverse regulatory functions (3).

Mutational analysis has revealed that Ypt1p functions in ER to Golgi and early Golgi traffic (8,9). Several recent observations, however, suggest that Ypt1p may also function at the late Golgi. First, a GEF for Ypt1p, TRAPP2, acts at the late Golgi and largely resides on this compartment (10,11). Additionally, an *in vivo* GAP for Ypt1p (Gyp1p) is required for traffic from the early endosome to the late Golgi (12). Finally, a known Ypt1p effector, COG, also mediates traffic at the late Golgi (13). Mutants with an early secretory block may have masked a requirement for Ypt1p in trafficking events at the late Golgi. To reveal a role for Ypt1p in traffic from the early endosome to the late Golgi, we screened for *ypt1* mutants that specifically block this stage of membrane traffic. Here we describe the isolation and characterization of a collection of recessive and dominant mutations in *ypt1* that block traffic through the early endosome, but not general secretion. These mutants do not accumulate ER or Golgi, but instead accumulate clusters of 60-70nm vesicles that may be endosomal-derived membranes that fail to fuse with the late Golgi. Thus, in addition to regulating the flow of traffic into the cis face of the Golgi, Ypt1p also appears to modulate vesicle traffic into the trans side of the Golgi.

Results

Screening for new *ypt1* mutants that block traffic at the late Golgi

To demonstrate a direct role for Ypt1p at the late Golgi, we screened for *ypt1* mutants that specifically block traffic from the early endosome to the late Golgi. Mutants were constructed by PCR mutagenesis using the plasmid shuffle method and *in vivo* homologous recombination (14). Briefly, a strain in which the chromosomal copy of *ypt1* was disrupted was balanced with *YPT1* on a *URA3* centromeric plasmid (pNB166), and transformed with a gapped *LEU2* plasmid and the mutagenized PCR product. To select against the *URA3* balancing plasmid, the transformants were counterselected on minimal plates containing uracil and 5-fluoroorotic acid (5-FOA). 5-FOA is metabolized into a toxic substance (5-fluorouracil) in cells containing a wild type *URA3* gene. Of the 406 transformants selected on 5-FOA, 103 survived (Figure 1A). Since secretion, but not early endocytic traffic is essential for viability in yeast, we screened for non-temperature-sensitive (ts) mutants that grow at 37°C. All but two of the strains that survived on 5-FOA plates were found to grow at 37°C and analyzed further (Figure 1A).

Since trafficking defects at the late Golgi can lead to the aberrant sorting of vacuolar hydrolases, we examined the trafficking of carboxypeptidase Y (CPY). CPY, a resident vacuolar protein which normally transits the secretory pathway enroute to the vacuole, is missorted in a mutant (*gyp1 Δ*) that lacks an *in vivo* GAP for Ypt1p (12,15). Using a plate assay that detects the secretion of CPY, we found that thirty of the mutants secreted CPY at a level greater than wild type. A representative plate assay is shown in Figure 1B. The release of CPY into the medium was truly a consequence of secretion and not cell lysis, as the cytosolic protein Adh1p was not released from the cells (Figure 1B, right).

To further analyze the new *ypt1* mutants, we examined the trafficking of GFP-Snc1p. Snc1p and its redundant homolog, Snc2p, are vesicle SNAREs that are required for the fusion of post-Golgi secretory vesicles with the plasma membrane (16). Snc1p is constitutively endocytosed and traffics from the early endosome to the late Golgi on its way back to the plasma membrane (17). In wild type cells, Snc1p is largely found on the plasma membrane and the bud neck (Figure 2). To analyze the *ypt1* mutants for trafficking defects, strains were transformed with a *GFP-SNC1* construct and examined by fluorescence microscopy. In all mutants GFP-Snc1p accumulated in internal puncta and in some mutants, GFP-Snc1p also appeared diffuse (Figure 2). The GFP-Snc1p recycling defect was quantified as the percent of cells displaying a mutant phenotype. We considered a defect significant when greater than 50% of the cells were defective. Nine of the 30 strains tested met our criteria (Figure 1A and Table S4). The growth of these mutants at 25°C, 35°C and 37°C is shown in Figure S1. The *ypt1* mutants defective in GFP-Snc1p recycling showed an accumulation of GFP-Snc1p in one or more puncta (Figure 2). While GFP-Snc1p puncta were also observed in the *ypt1-138* and *ypt1-151* mutants, some GFP-Snc1p appeared to be diffuse (Figure 2). The diffuse pool may represent GFP-Snc1p in small vesicles, while the pool that is present in puncta could be clusters of small vesicles. Of the 406 transformants examined in this study, nine *ypt1* mutants were not ts for growth and displayed GFP-Snc1p trafficking defects (Figure 1A). The localization of the late Golgi marker Sec7p, fused to GFP, was found to be normal (Figure S2). A GFP fusion to the FYVE domain of the early endosomal antigen 1 protein, which marks endosomal membranes that contain PI-3P (phosphatidylinositol-3-phosphate), was also analyzed (Figure S3) in these mutants and found to be normal.

To determine if GFP-Snc1p accumulates in early endosomes in the *ypt1* mutants, cells were labeled with FM4-64. FM4-64 is a lipophilic styryl dye that binds to the plasma membrane and labels endocytic compartments when it is internalized (18). The dye was either internalized for 5 min or 1 hr to mark early endosomes and the vacuole, respectively. In wild type, internalized FM4-64 was found in small puncta within the cytoplasm after 5 min (Figure 3, left). In the *ypt1-53* mutant, however, FM4-64 localized to large puncta that colocalized with GFP-Snc1p (Figure 3, left see arrows). After a 1 hr incubation, FM4-64 labeled the vacuole and no longer colocalized with GFP-Snc1p (Figure 3, right). The vacuole in *ypt1-53* and all other *ypt1* mutants isolated in this study appeared to be fragmented (Figure S4). Thus in the *ypt1-53* mutant, GFP-Snc1p which is unable to recycle efficiently, accumulates in an early endosomal compartment.

The *ypt1* mutants, defective in GFP-Snc1p recycling, were then assayed for invertase secretion defects. The enzyme invertase traffics from the ER through the Golgi and to the early endosome before it is secreted into the periplasm (19). To assay for secretion defects, cells were shifted to 37°C for 20 min, resuspended in low glucose media to derepress the synthesis of invertase, and incubated for 1 hr at 37°C. Invertase activity was measured and used to calculate the percent of invertase secreted. As a control for this experiment, *ypt1-3*, a ts strain that displays a defect in ER-Golgi traffic was analyzed. The *ypt1-3* mutant secreted approximately 25% of the total cellular invertase, while wild type secreted 75%. In the 9 mutants examined, the percent of invertase secretion was within one standard deviation of wild type (Figure S5). The mutants were also tested for secretion defects at 25°C and no defect was observed at either temperature (Figure S5; data not shown). This data implies that the new *ypt1* mutants we isolated are not defective in secretion.

CPY processing and general secretion is normal in the *ypt1* mutants

In order to analyze the new *ypt1* mutants for biosynthetic transport defects in more detail, we followed the intracellular trafficking of CPY. CPY is translocated into the ER where it becomes glycosylated (p1 CPY, 67 kDa) before it travels to the Golgi and undergoes further

carbohydrate modifications (p2 CPY, 69 kDa). Finally, it transits from the late Golgi to the vacuole where it is proteolytically cleaved to generate mature CPY (m CPY, 61 kDa) (20).

We first assayed CPY processing in cells grown at 25°C, or shifted to 37°C for 2 hr. Lysates from wild type, *sec18-1*, and the *ypt1* mutants that displayed Snc1p recycling defects were prepared and assayed by western blot analysis. In wild type cells, the mature form of CPY was the predominant form and little p1 CPY was observed (Figure 4A). To mark p1 CPY, the *sec18-1* mutant, which blocks ER-Golgi traffic, was shifted to 37°C for 2 hr (Figure 4A, top and bottom panels). At 25°C, only *ypt1-132* and *ypt1-138* accumulated significantly more p1 CPY than wild type (Figure 4A, top), however, *ypt1-132* did not accumulate p1 CPY at 37°C (Figure 4A, bottom). Two other mutants, *ypt1-11* and *ypt1-51*, accumulated p1 CPY at 37°C, but not 25°C (Figure 4A, bottom and top panels). The *ypt1-3* mutant, which blocks ER-Golgi traffic, only accumulated p1 CPY at 37°C (Figure 4A, bottom).

We also monitored CPY trafficking using pulse chase analysis. For this analysis, strains were shifted to 37°C for 20 min, pulse labeled for 4 min, and chased for 30 min. At the zero time point, only p1 CPY was present in all strains (Figure 4B). Following the 30 min chase, p1 CPY was converted into the mature form in wild type and the mutants, except for *ypt1-138*, which displayed a partial block in processing (Figure 4B). This analysis indicates that the kinetics of ER-Golgi traffic appears to be unaffected in most of the *ypt1* mutants we isolated.

To assay for defects in general secretion, cells were shifted to 37°C for 20 min, labeled for 5 min, and chased for 20 min. Proteins secreted into the medium were precipitated with TCA and resolved on an SDS polyacrylamide gel. A block in general secretion was found for *sec18-1*, but no obvious defect was observed for *ypt1-53* and *ypt1-151* (Figure 4C). No defect in general secretion was also observed for the *ypt1-166* and *ypt1-52* mutants (data not shown). Together these findings indicate that the new *ypt1* mutants we isolated, disrupt GFP-Snc1p recycling through the early endosome, but not general secretion.

The *ypt1* mutants are defective in the recycling of FM4-64

The recycling of FM4-64 into the medium was analyzed as a second endocytic marker in the *ypt1* mutants (21). FM4-64 follows one of two routes after it is internalized, a portion is recycled into the medium while the remainder traffics through the endocytic pathway before reaching the vacuole. FM4-64 is only fluorescent when bound to a membrane, but its fluorescence decreases as the dye is released into the medium (21). For this analysis, cells were allowed to internalize FM4-64 for 12 min, washed to remove the cell surface associated dye, resuspended in fresh medium and incubated at 25°C. The *gyp1Δ* mutant, which was previously shown to have a recycling defect, was used as a control in these studies (12). In wild type, FM4-64 fluorescence decreased from 100 % to ~45%, while fluorescence only decreased to ~60% in the *gyp1Δ* mutant (Figure 5A). Six of the *ypt1* mutants were tested and all of them showed a recycling defect with fluorescence decreasing from 100% to 60-70 % (Figure 5B, C, D). Thus, all *ypt1* mutants defective in GFP-Snc1p recycling also displayed a defect in the recycling of FM4-64.

Ypt1p colocalizes with the late Golgi marker Sec7p

Our ability to isolate *ypt1* mutants that specifically block recycling from the early endosome to the late Golgi suggests a possible role for Ypt1p at the late Golgi. This observation prompted us to determine if Ypt1p also partially resides on the late Golgi. To do this, we determined if Ypt1p colocalizes with Sec7p, a late Golgi marker (22,23). For this analysis, we fused mCherry (mCh) to the N-terminus of Ypt1p and assessed colocalization with Sec7p-GFP using a spinning disk confocal microscope. The tagged Ypt1p construct that we

made was functional, as it did not impair growth. Quantification of 200 cells (Figure 6) revealed that roughly 60% of the mCh-Ypt1p puncta (red) colocalized with the Sec7p-GFP puncta (green). Thus, Ypt1p functions at and partially localizes to a late Golgi compartment that is marked by Sec7p. The Ypt1p GEF, TRAPP^{II}, also resides on this compartment (10).

Clusters of vesicles accumulate in the *ypt1-53* mutant

The morphology of the *ypt1* mutants was examined by thin section electron microscopy and compared to wild type and the *ypt1-3* mutant that blocks ER-Golgi traffic (24). A mutant that harbors a mutation in the Trs130p subunit of the TRAPP^{II} complex (*trs130-2*), which blocks Golgi and early endosomal traffic, was also examined (10). For this analysis, wild type, *ypt1-3*, and *trs130-2* cells were shifted to 37°C for 2 hr. At 37°C, the *trs130-2* mutant accumulated aberrant Golgi structures (compare Figure 7A to 7B) called Berkeley bodies (10,25). In contrast, the *ypt1-3* mutant accumulated dilated ER (Figure 7C). Unlike *ypt1-3*, the *ypt1-53* mutant did not accumulate ER, but instead accumulated clusters (>5 vesicles) of 60-70 nm vesicles in either the bud tip, bud, or mother cell (Figure 7D, E). Approximately, 25% of the *ypt1-53* cells contained vesicle clusters. The vesicles within these clusters were larger than ER to Golgi vesicles (40-50 nm) and smaller than post-Golgi secretory vesicles (100 nm) (26,27). We speculate these membranes are endosomal vesicles that fail to fuse with the late Golgi. Consistent with this hypothesis is the observation that GFP-Snc1p accumulates in large puncta in the *ypt1-53* mutant. These puncta may represent the clusters of vesicles we see by EM. All other *ypt1* mutants (*ypt1-19*, *ypt1-51*, *ypt1-151*, and *ypt1-166*) examined by electron microscopy displayed a similar morphology (data not shown).

The identification of amino acids within the switch I domain of Ypt1p that are required for endocytic recycling

In order to identify the amino acid changes that result in the observed endocytic recycling defects, we sequenced all nine *ypt1* mutants. Since the mutations we identified were randomly spread throughout the entire *YPT1* coding sequence and the number of mutations in each allele varied from 3 to 10 (Figure 8), it was not obvious which residue or regions may be critical for endocytic recycling (Figure 8). For this reason, we decided to examine mutations within switch I and switch II, as well as severe amino acid substitutions outside of these regions (Figure 8). To test whether any of these individual mutations resulted in the same phenotype as the original allele, mutations were individually introduced into the *YPT1* gene by site-directed mutagenesis. Strains harboring these mutations were then analyzed for GFP-Snc1p and FM4-64 recycling defects (Table S5; Figure S6). Initially, three single mutations (I41M, K46E, and Q176L), identified in the *ypt1-53* mutant, were analyzed. The K46E (in switch I) and Q176L mutations did not have any effect on GFP-Snc1p recycling (Figure 9). However, the single I41M (in switch I) mutation resulted in a GFP-Snc1p trafficking defect similar in magnitude to *ypt1-53* (Figure 9A, Tables S4 and S5). The same result was obtained when FM4-64 recycling was examined (Figure S6A). Another mutation located in the switch I domain, T40A, was identified in the *ypt1-132* allele. This mutation alone could account for the GFP-Snc1p and FM4-64 recycling defects observed in *ypt1-132* (Figure 9B; Figure S6; Table S5).

In the switch II domain we focused on residue Q67, which was previously shown to be important for GTP hydrolysis (28). The Q67 residue was mutated in the *ypt1-138* and *ypt1-166* mutants (Figure 8). An earlier study demonstrated that mutating this residue reduces the intrinsic GTPase activity of Ypt1p, but not its ability to be stimulated by its GAP (28). When we constructed the *ypt1^{Q67H}* mutant by site-directed mutagenesis, the percent of cells with a recycling defect was significantly less than the *ypt1-138* mutant (Tables S4 and S5). The same result was obtained for the *ypt1^{Q67L}* mutation, which was previously shown to affect GFP-Snc1p trafficking (12). Surprisingly, the *ypt1^{Q67L}* and *ypt1-166* mutants

displayed similar FM4-64 recycling defects with fluorescence decreasing to ~60% (Figure S6D). Together, these findings imply that the phenotype observed in the *ypt1-138* and *ypt1-166* mutants likely results from multiple mutations. Consistent with this hypothesis, we found that neither the I38F (in switch I) nor the N179Y (outside of effector regions) mutation in *ypt1-166* blocked GFP-Snc1p trafficking (Table S5).

The *ypt1-19* mutant is dominant

We next wanted to determine if any of the *ypt1* mutant alleles we isolated are dominant. A dominant allele expresses its mutant phenotype in the presence of a wild type copy of *YPT1*. To identify dominant *ypt1* mutant alleles, a wild type strain was transformed with a *CEN* plasmid containing a mutant *ypt1* allele and then GFP-Snc1p trafficking was analyzed. Of the nine alleles we isolated, only *ypt1-19* was found to be dominant (Figure 10). This mutant harbored three mutations, D6V, F162V, and N177Y (Figure 8). In order to determine which of these mutations resulted in a gain of function phenotype, we introduced each of them into the *YPT1* gene and then tested their ability to disrupt GFP-Snc1p trafficking (Figure 10). Interestingly, the D6V and N177Y mutations did not perturb GFP-Snc1p recycling, but the F162V mutation led to a defect in ~30% of cells. This defect, however, was not as strong as the *ypt1-19* mutant (Figure 10). We then constructed *ypt1* plasmids containing combinations of these mutations and found that combining the D6V and F162V mutations resulted in dominant Snc1p and FM4-64 recycling defects that were comparable to *ypt1-19* (Figure 10, Figure S7). The single F162V mutation had an intermediate recycling defect (Figure 10, Figure S7). Thus, the dominant negative phenotype of the *ypt1-19* mutant requires both the D6V and F162V mutations.

Discussion

Several indirect observations have suggested that Ypt1p functions at the late Golgi/early endosome. First, the *in vivo* GAP for Ypt1p, Gyp1p, is required for traffic from the early endosome to the late Golgi (12,15). Second, two Ypt1p GEFs have been identified (11). These GEFs are multimeric exchange factors that share several subunits (11). One GEF (TRAPPI) is required for ER to Golgi traffic, while the other (TRAPPII) acts at a later stage of membrane traffic (10,25). TRAPPII largely resides on the late Golgi, and mutations in the Trs120p subunit of this complex disrupt traffic from the early endosome to the late Golgi (10). Third, a known Ypt1p effector, COG, is a multimeric complex that is required for traffic from the late Golgi/early endosome to the early Golgi (13). Interestingly, mutations in several of the genes that encode the subunits of this complex, also disrupt ER-Golgi and intra-Golgi traffic (29-31).

Here we describe the identification of new *ypt1* mutants that specifically block traffic from the early endosome to the late Golgi. The rationale for our study was based on the observation that a GAP, GEF, and an effector of Ypt1p are all known to function in retrograde traffic from the early endosome to the Golgi. Unlike previous genetic screens, we focused on the isolation of mutants that grow at 37°C (non-ts) and display endocytic trafficking defects, but not defects in secretion. The mutants we isolated do not accumulate ER or Golgi, but instead accumulate clusters of vesicles that are 60-70nm in size. These vesicles may be endosomal-derived membranes that fail to fuse with the late Golgi. Consistent with the observation that Ypt1p functions at the late Golgi, we also show this GTPase largely colocalizes with the late Golgi marker Sec7p.

Based on the findings we present here and previous studies, we propose that Ypt1p regulates the flow of traffic into both the cis and trans sides of the Golgi. Interestingly, three different GAPs (Gyp1, Gyp5, and Gyp8) have been shown to stimulate the hydrolysis of GTP on Ypt1p *in vitro* and they also functionally interact with Ypt1p *in vivo* (12,15,28). These three

GAPs have distinct localizations. Gyp5 is largely cytosolic, while Gyp8 and Gyp1 are found on punctate structures (28). Although Gyp8 and Gyp1 both reside on punctate structures, they do not colocalize with each other by fluorescence microscopy (28). Gyp1p regulates endosomal recycling and is likely to be the Ypt1p GAP that functions at the late Golgi (12,15,28). Together, these observations suggest that Gyp5, Gyp8 and Gyp1 act at three distinct trafficking steps.

Rabs can interact with multiple effectors that act at different stages of membrane traffic (3). For example, the T40A mutation we describe here specifically blocks traffic from the early endosome to the late Golgi. But when T40 is mutated to lysine (T40K), this mutation blocks ER-Golgi traffic (9). Thus, T40 interacts with effectors that function in ER-Golgi traffic and effectors that act at the late Golgi, and disrupting the interaction of Ypt1p with an effector on one pathway does not affect its interaction with an effector on another pathway.

Interestingly, the *ypt1-19* mutant contains two mutations, F162V and D6V, that lead to a dominant negative defect in late Golgi trafficking. Other dominant negative *ypt1* alleles have been identified (*ypt1^{D124N}* and *ypt1^{N121I}*), but both of these mutations inhibit growth and ER-Golgi traffic when overexpressed (32). Because the dominant negative *ypt1-19* mutant specifically blocks traffic at the late Golgi and does not display a growth defect, this mutant may sequester a Rab regulatory protein or effector that only functions at the late Golgi. The new *ypt1* mutants we describe here will be useful tools for studying the role of Ypt1p in late Golgi trafficking events.

Materials and Methods

Strains, Plasmids and Media

All strains were grown in YPD (2% peptone, 1% yeast extract 2% glucose) or synthetic complete media (0.67% yeast nitrogen base without amino acids (Difco), 2% glucose, with appropriate amino acids). Yeast transformation was performed using the lithium acetate method (33). All strains and plasmids used in this study are listed in Table S1 and Table S2.

Construction of *ypt1* mutants

New *ypt1* mutant alleles were isolated by random PCR mutagenesis (34) and *in vivo* homologous recombination (14). A diploid strain with one copy of *ypt1* disrupted by *HIS3* was transformed with a balancing *YPT1* plasmid (pNB166 *CEN URA3*). This strain was sporulated and dissected to generate a haploid strain that harbored a balancing plasmid and the chromosomal copy of disrupted *ypt1*.

A 1.7Kb fragment, containing the *YPT1* coding sequence and its 5' and 3' UTR, was amplified from yeast genomic DNA using two primers (YPT1XHOI and YPT1SACI) and inserted into the XhoI and SacI sites of pRS315 (*CEN LEU2*). This construct can rescue a *ypt1Δ* mutant. A gapped plasmid was created by digesting the plasmid with AvrII (cuts 200bp upstream of ATG) and SgrAI (cuts 20bp upstream of TGA) which released a 0.8Kb fragment. A template for PCR mutagenesis was generated by PCR using primers YPT1MF and YPT1MR. Random PCR mutagenesis was performed with the same primers and the following conditions: 7mM MgCl₂, 50mM KCl, 0.5mM MnCl₂, 10mM Tris pH 8.3, 0.1% Triton-X-100, dNTPs 1mM TTP, 1mM CTP, 200μM ATP, 200μM GTP, and 2.5units of Taq polymerase (New England Biolabs).

The haploid strain containing the balancing plasmid was transformed with 100 ng of the gapped *YPT1* plasmid and the PCR product. The PCR product had 100 to 200 bp homology at its 5' and 3' ends with the gapped plasmid, allowing for homologous recombination. The

cells were plated on minimal media, and then selected on 5-FOA (5-fluoroorotic acid) plates to counter select against the balancing plasmid.

Mutations were created in the *YPT1 CEN LEU2* plasmid by using the primers listed (Table S3) and the plasmid was transformed into the haploid strain containing the balancing plasmid. Transformants were selected on 5-FOA plates.

CPY plate assay

Stationary cultures of wild type, *gyp1Δ*, and *ypt1* strains grown in YPD were diluted to an OD₆₀₀=2.0 and 3ul of the cell suspension was pipetted onto YPD plates in duplicate and incubated for 3hr at 30°C. A nitrocellulose filter, soaked in distilled water, was applied to each plate before it was incubated for 18hr at 30°C. The filter was then washed with water to remove the cells, and blotted with α-CPY antibody or α-Adh1p antibody. Adh1p was analyzed to assess cell lysis.

In Vivo Labeling and Western Blot Analysis

CPY pulse chase experiments were performed as described before (10,35). Briefly, wild type, *sec18-1* and the *ypt1* mutants were grown overnight at 25°C in minimal media with the appropriate amino acids. Seven OD₆₀₀ units of cells were pelleted, resuspended in 1.575 ml of fresh media, pre-shifted to 37°C for 20 min, and pulse labeled for 4 min with 175uCi Easy Tag S³⁵ methionine (Perkin Elmer). Samples were chased with 10mM cysteine and 10mM methionine. Aliquots were taken at 0 and 30 min, and CPY was immunoprecipitated and analyzed on an 8% SDS polyacrylamide gel.

The general secretion assay was performed as described before (29) with a few modifications. Briefly, wild type, *sec18-1* and the *ypt1* mutants were grown overnight at 25°C in minimal media with the appropriate amino acids. Two OD₆₀₀ units of cells were pelleted and resuspended in 800ul of fresh media with 50μg BSA. Cells were pulsed with 100uCi S³⁵ methionine for 5 min and chased with 10mM cysteine and 10mM methionine for 20 min. Cells were pelleted and proteins secreted into the medium were precipitated with trichloroacetic acid (TCA). The precipitate was washed with ice-cold acetone, solubilized in sample buffer, and resolved on an 8% SDS polyacrylamide gel.

To analyze CPY processing at steady state, cells were grown overnight in YPD media at 25°C. The next day, the cells were shifted to 37°C for 2hr or incubated for an additional 2 hr at 25°C. Aliquots of cells were lysed with 5% BME, 2M NaOH and incubated on ice for 10 min. TCA was added (final concentration 10%) and samples were incubated for an additional 10 min. Samples were centrifuged, washed with 1M Tris, resuspended in 1× sample buffer and analyzed by western blot analysis (36). Bos1p was used as a loading control.

Invertase assays

Invertase assays were performed as described before (10). Briefly, cells were grown overnight at 25°C in YPD media. Two OD₆₀₀ units of cells were shifted to 37°C for 20 min. One OD₆₀₀ unit of cells was washed and then resuspended in 1ml of ice-cold 10mM NaN₃ and incubated on ice (0 min time point). The other OD₆₀₀ cell unit was washed and incubated in YP + 0.1% glucose for 1 hr to derepress the synthesis of invertase. Following derepression, cells were washed and resuspended in ice-cold 10mM NaN₃ (60 min time point). One half of each cell sample was spheroplasted and lysed in 0.5% Triton-X-100 to measure internal invertase. The cell suspension was used to assay for external invertase. Invertase activity was measured as described before (37).

Fluorescence microscopy

Strains expressing GFP-Snc1p were grown overnight at 25°C to early log phase in synthetic complete media lacking uracil. Cells were spun at room temperature and resuspended in fresh media and examined. Images were taken with an Axioimager Z1 microscope (Zeiss) under a 100× objective with a Zeiss AxioCamMRm Camera using AxioVision 4.5 SP1 software. All image processing was done using Photoshop 7.0 and Illustrator 10.0 (Adobe).

For the data shown in Figure 6, images were acquired on a Yokagawa spinning disc confocal microscope (PerkinElmer). The system was mounted onto an inverted microscope (IX71; Olympus) equipped with a 1 Kb × 1 Kb electron multiplying charge-coupled device camera (Hamamatsu Photonics), which was controlled by Ultraview ERS software (PerkinElmer). Cells were imaged with a 100 × 1.4 NA oil phase objective, yielding a pixel size of 87 nm. Excitation of GFP or mCherry was achieved using 488-nm argon and 568-nm argon/krypton lasers respectively (Melles Griot). Cells were grown in SD media to an $Abs_{600nm}=0.3-0.6$, washed and concentrated with fresh SD media, and incubated on ice. To avoid cell movement during microscopy, the cells were mounted on microscope slides containing a dried 1% low melt agarose pad dissolved in SD. For each sample a z-stack of 14-16 slices was generated, with optical sections spaced 400 nm apart. Alternated acquisition of the red and green fluorescent signals was used to acquire the images. Exposure time for mCherry and GFP images were 75 msec and 100 msec, respectively. Before the colocalization analysis, each optical section was digitally enhanced. Colocalization analysis was performed on the maximum intensity projection of 4-middle slices for each stack. The percentage of colocalization between the signals was calculated on a pixel by pixel basis using the colocalization threshold plug-in from Macbiophotonics Image J (38). The scatter plot of the pixel intensities for each channel was then used to calculate the number of colocalized pixels. To calculate the percentage of colocalization, we used the sum of intensities of the pixels above threshold that showed colocalization divided by the sum of intensities above threshold of the respective channel that did not colocalize.

For the data in Figure S2 and Figure S3, DsRed-FYVE was expressed from a 2µm plasmid. To induce expression, *ypf1* mutants containing the plasmid were grown in synthetic media lacking methionine. Because the *gyp1Δ* mutant required methionine for growth, 1.5 µM methionine was added to the growth medium. This concentration was low enough to allow induction of DsRed-FYVE. To view Sec7p-GFP, cells were grown in YPD media at 25°C. At an early log phase, the cells were pelleted at 4°C, resuspended in ice-cold media and examined by fluorescence microscopy as described above.

FM4-64 labeling and recycling assay

Strains expressing GFP-Snc1p were grown to early log phase in synthetic complete media lacking uracil. Cells were spun at room temperature, resuspended in fresh media, and FM4-64 (Molecular Probes, Invitrogen) was added to a final concentration of 40µM. To label early endosomes, cells were incubated for 5 min at 25°C, pelleted at 4°C and washed twice in ice-cold media. To label the vacuole, cells were incubated with FM4-64 for 10 min, centrifuged at room temperature and washed one time with fresh media. Cells were then incubated for 1 hr at 25°C to allow FM4-64 to reach the vacuole before they were centrifuged at 4°C and resuspended in cold media.

The FM4-64 recycling assay was performed as described before (21). Strains were grown in SC complete media overnight at 25°C to early log phase, pelleted, resuspended in fresh media containing 40 µM FM4-64, and incubated for 12 min. Following the incubation, cells were pelleted at 4°C, washed three times with ice cold synthetic complete media, resuspended in 20ul of ice cold media and kept on ice. For the assay, cells were diluted to an

OD₆₀₀ of .25 units per ml with warm media (25°C) and assayed immediately. Fluorescence measurements were obtained for 600 sec using a Fluorolog-3 spectrofluorimeter (Horiba Jobin Yvon) at a 90° angle. The excitation wavelength was 515 nm with a 10nm bandwidth and the emission wavelength was 680 nm with a 10nm bandwidth. Data was plotted as percent fluorescence with time zero equal to 100%.

Electron microscopy

Wild type, *trs130-2*, and *ypt1* mutant strains were grown at 25°C overnight. The next day, strains were either fixed or shifted to 37°C for 2 hr before fixation. Ten OD₆₀₀ units of cells were harvested on a Nalgene filter, resuspended in fixative (3% glutaraldehyde, 0.1M sodium cacodylate buffer pH 7.2, .5M sorbitol, 5 mM CaCl₂), incubated for 1hr at room temperature and stored overnight at 4°C. Fixed cells were washed twice with an ice-cold solution containing 0.1M sodium cacodylate buffer (pH 7.2), 0.5M sorbitol, and 5 mM CaCl₂. After washing, the fixed cells were incubated for 25 min at 37°C in 2 ml of the same buffer containing 0.25 mg/ml Zymolyase 100T. The zymolyase treatment was stopped by centrifugation at 4°C. After washing the samples 3 times with 0.1 M cacodylate buffer, cells were treated with 1% osmium tetroxide for 1hr. The cells were then washed with 0.1 M cacodylate buffer, water and 10% ethanol before a 1 hr incubation with 1% uranyl acetate in 10% ethanol. Samples were then dehydrated using a series of ethanol washes, embedded in LX112 resin, and baked at 80°C for two days. Images were obtained using a JEOL 1200 EX II transmission electron microscope equipped with a Gatan Orius 600 CCD camera. Vesicle diameter was measured using Image J software (National Institute of Health).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A

Summary of *ypt1* mutant screen

-Transformants	406	
-Transformants able to grow on 5-FOA plates	103	
	non ts*	ts*
-Mutants	101	2
-Mutants that secrete CPY	30	2
-Mutants displaying Snc1p trafficking defects	9	0
-Mutants with invertase secretion defects	0	2

*ts = temperature-sensitive for growth at 37°C

B

Figure 1.

Summary of *ypt1* mutant screen. (A) The Table shows the results from the mutant screen. (B) Wild type, *ypt1*, and *gyp1Δ* strains were spotted on YPD plates, incubated with a nitrocellulose filter and blotted for secreted CPY (left panel) and the cytosolic protein Adh1p (right panel), which was used as a lysis control.

Figure 2.

The *ypt1* mutant alleles display GFP-Snc1p recycling defects.

GFP-Snc1p was examined in wild type, the *ypt1* mutants and the *gyp1Δ* deletion strain. All strains were grown in selective media at 25°C.

Figure 3.

GFP-Snc1p accumulates in early endosomes in the *ypt1-53* mutant.

Wild type and the *ypt1-53* mutant, expressing GFP-Snc1p, were incubated with FM4-64 for 5 min or 60 min to label early endosomes and the vacuole, respectively. Arrows point to GFP-Snc1p structures that label with FM4-64 (bottom left).

Figure 4.

Analysis of CPY trafficking and general secretion in the *ypt1* mutants.

(A) Lysates were prepared from wild type and the *ypt1* mutants grown at 25°C (top) or shifted to 37°C for 2 hr (bottom) and then blotted with anti-CPY and anti-Bos1p antibodies. Bos1p was used as a loading control. The *sec18-1* mutant, shifted to 37°C for 2 hr, was used to identify p1CPY. (B) CPY was processed normally in the *ypt1* mutants at 37°C. Wild type, *ypt1*, and *sec18-1* strains grown at 25°C were shifted to 37°C for 20 min, pulse labeled for 4 min, and then chased. Samples were taken before (0 min) and after the chase (30 min). CPY was immunoprecipitated and analyzed for processing defects on an 8% SDS polyacrylamide gel. (C) General secretion is normal in the *ypt1-53* and *ypt1-151* mutants. Wild type, *ypt1*, and *sec18-1* strains were pulse labeled for 5 min and then chased for 20 min. Proteins secreted into the medium were TCA precipitated and resolved on an 8% SDS polyacrylamide gel.

Figure 5.

The *ypt1* mutants exhibit FM4-64 recycling defects.

Cells were allowed to internalize FM4-64 for 12 min at 25°C and then washed thoroughly with ice cold media. Fluorescence was measured every second for 10 min (600 sec total) immediately following resuspension in 25°C media. Data is plotted as % fluorescence over time. FM4-64 recycling in the *ypt1*Δ (A), *ypt1-52*, *ypt1-151* (B), *ypt1-53*, *ypt1-132* (C), *ypt1-138*, and *ypt1-166* (D) mutants was compared to wild type (A,B,C, D). All strains were tested a minimum of three times and the data from one representative experiment is shown.

Figure 6.

Ypt1p colocalizes with the late Golgi marker Sec7p.

Cells containing mCherry-Ypt1p (mCh-Ypt1p) and Sec7-GFP (NY2804) were analyzed with a spinning disc confocal microscope (Top panel). Arrows (Top panel, right) point to compartments that display colocalization. The graph (bottom panel) shows the percent of mCh-Ypt1p puncta that colocalize with Sec7-GFP puncta. Scale bar = 5 μ m, error bars = s.d.

Figure 7.

The *ypt1-53* mutant accumulates clusters of vesicles.

The *trs130-2* (B) and *ypt1-3* (C) mutants were grown in YPD medium at 25°C and shifted to 37°C for 2 hr prior to preparation for EM analysis. The EM phenotype of wild type was the same with cells grown at 25°C (A) or shifted to 37°C (not shown). The vacuole is fragmented in the *ypt1-53* mutant and roughly 25% of the cells contained a cluster of 60-70 nm vesicles (D, E, see arrow). The inset in panel D is magnified and shown in E. All size bars are 1 μm. Abbreviations are as follows: vacuole (Vac), Endoplasmic Reticulum (ER), vesicles (V), Berkeley bodies (B).

Figure 8.

A summary of mutations in the new *ypt1* mutants

All mutated amino acid changes are shown in (A) and a sequence alignment is shown in (B).

The switch I and switch II effector regions (11) are highlighted or underlined (A, B).

Mutations in bold were introduced individually or in combination by site-directed mutagenesis (A, B), dots indicate no mutation (B). (C) A ribbon diagram of Ypt1p in its active form (PDB 1D 1YZN). GPPNP (GTP analog) and Mg^{2+} in the active site are lavender. Residues that were mutated are shown in red (Q176, N177 and N179 at the very C-terminus are not in the structure and are not shown).

Figure 9. Single mutations in *ypt1* can recapitulate the GFP-Snc1p recycling defect seen in the *ypt1-53* and *ypt1-132* mutants. (A) The recycling defect in the *ypt1-53* mutant appears to be the consequence of the I41M mutation. Images of wild type, *ypt1-53*, *ypt1^{I41M}*, *ypt1^{K46E}*, and *ypt1^{Q176L}* expressing GFP-Snc1p cells are shown. (B) The recycling defect in the *ypt1-132* mutant appears to be the consequence of the T40A mutation. Images of the *ypt1-132* and *ypt1^{T40A}* mutants expressing GFP-Snc1p are shown.

Figure 10.

The *ypt1-19* mutant is dominant. The mutations in the *ypt1-19* mutant were analyzed for recycling defects. Only the *ypt1^{F162V}* and *ypt1^{D6V, F162V}* mutations were defective in GFP-Snc1p recycling. The dominant recycling defect in the *ypt1-19* mutant appears to be the consequence of the *ypt1^{D6V, F162V}* mutations.