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Analysis of subcellular localization and function of the yeast Rab6 homologue, Ypt6p, using a novel amino-terminal tagging strategy

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ABSTRACT

Ypt6p, the yeast homologue of mammalian Rab6, is involved in the multiple processes regulated by membrane trafficking such as vacuole maturation and membrane protein recycling. Although several lines of evidence suggest that Ypt6p is possibly localized to multiple membrane compartments, the precise localization of endogenous Ypt6p remains to be elucidated. In this study, we developed a novel method for N-terminal tagging of endogenous protein based on homologous recombination and investigated the subcellular localization and function of Ypt6p. Ypt6p and its GTP-bound form were predominantly localized to the *cis*- to *medial*-Golgi compartments whereas the GDP-bound form of Ypt6p was localized to the cytosol. Ric1p, a component of the specific GEF complex for Ypt6p, largely colocalized with Ypt6p in the early Golgi, and localization of Ypt6p changed to the cytosol in *ric1*Δ cells. On the other hand, Gyp6p, a putative GAP for Ypt6p, was localized to the *trans*-Golgi compartment and deletion of *GYP6* increased the localization of Ypt6p at the *trans*-Golgi, suggesting that Gyp6p promotes the dissociation of Ypt6p from the Golgi when arriving at the *trans*-Golgi compartment. Additionally, we demonstrated that overexpression of the GDP-bound form of Ypt6p caused defective vacuole formation and recycling of Snc1p to the plasma membrane. These results suggest that the GTP-binding activity of Ypt6p is necessary for *intra*-Golgi trafficking and protein recycling in the early Golgi compartment.

1. Introduction

Rab/Ypt GTPases are well-characterized regulators of intracellular membrane trafficking, functioning as molecular switches that alternate between GTP- and GDP-bound forms. The biological activity and localization of Rab/Ypt proteins are controlled by multiple factors, including guanine nucleotide dissociation inhibitor (GDI), guanine nucleotide exchange factor (GEF), and GTPase-activating protein (GAP) [1]. Covalent isoprenylation of the C-terminal cysteine residues is also required for proper localization and activity of Rab/Ypt proteins [2]. GDP-bound prenylated Rab/Ypt proteins bind to GDI, which masks their isoprenyl anchor and thereby maintains them in soluble cytosolic form [1]. Conversion of the GDP-bound Rab/Ypt into the GTP-bound form is catalyzed by a specific GEF, which promotes the exchange of GDP with GTP, resulting in binding of Rab/Ypt proteins to their effector proteins [1]. GTP-bound Rab/Ypt no longer associates with GDI and therefore can stably reside on membrane compartments via the prenylated C terminus. After arriving at the target membrane, Rab/Ypt proteins are inactivated by hydrolysis of the bound GTP to GDP, extracted from the membrane by GDI, and recycled back to the cytosol [1].

The mechanisms of trafficking along the secretory pathway are well conserved from yeast to mammalian cells [3]. In yeast, the secretory pathway is known to be regulated by sequential activation and inactivation of three types of Rab protein – Ypt1p, Ypt31p/32p and Sec4p – by their GEFs and GAPs [4,5]. A recent study has demonstrated a novel Rab-GAP cascade in the secretory pathway that regulates the conversion of Ypt6p to Ypt32p during *intra*-Golgi trafficking; Ypt6p was shown to reside at the *medial*-Golgi and to dissociate from the membrane upon arrival of Ypt31p/32p that recruit Gyp6p, a putative GAP for Ypt6p, to the Golgi [6]. Previous studies have reported that Ypt6p, together with

Abbreviations: GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; VPS, vacuolar protein sorting; GFP, green fluorescent protein.

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its GEF Ric1p/Rgp1p complex, mediates the fusion of vesicles from endosomes to the *trans*-Golgi by recruiting the Golgi-associated retrograde protein (GARP) complex [7,8]. These observations suggest roles of Ypt6p at the *medial*- or *trans*-Golgi, but the precise localization of endogenous Ypt6p has yet to be determined, and therefore the precise step(s) controlled by Ypt6p remains unclear.

Chromosomal GFP tagging of endogenous protein at the C terminus has become a standard technique for analyzing the localization of protein in yeast [9]. However, C-terminal tagging of Ypt6p, which contains conserved C-terminal cysteine residues that are required for prenylation, renders Ypt6p non-functional because it interferes with the latter's membrane anchoring. Thus, a novel strategy for tagging GFP at the N terminus is necessary for analyzing the localization and function of endogenous Ypt6p. We recently developed a novel method for N-terminal tagging of endogenous protein based on homologous recombination [10]. In the present study, using this novel method, we generated cells chromosomally expressing GFP-Ypt6p and determined the localization of Ypt6p. Additionally, based on the principles of this method, we developed new techniques for one-step introduction of specific point mutations into a gene and replacement of the promoter region of the gene at the chromosomal locus. Using these techniques, we demonstrate that Ypt6p and its GTP-bound form were predominantly localized to the *cis*- to *medial*-Golgi compartments whereas the GDP-bound form of Ypt6p was localized to the cytosol. We also demonstrate that overexpression of the GDP-bound form of Ypt6p caused defective vacuole formation and recycling of Snc1p to the plasma membrane.

2. Material and methods

2.1. Yeast strains, growth conditions, and plasmids

The yeast strains used in this study are listed in Supplementary Table 1. All strains were grown in standard rich medium (YPD) or synthetic medium (SM) supplemented with 2% glucose and appropriate amino acids. The N-terminal GFP tag was integrated at the endogenous locus of the *YPT6* gene as follows: 333-bp 5′ UTR of *YPT6* gene and the N-terminal fragment of the *YPT6* ORF (nt 1–210) were generated by PCR and cloned into the BamHI or BglII site of pBS-GFP-HIS3 vector [10] (pBS-P_{YPT6}-GFP-*YPT6*(1–210)-HIS3). To integrate GFP at the N terminus of the *YPT6* gene, the integration plasmid was linearized by XcmI and transformed into yeast. The extra region generated by insertion of the integration plasmid was removed by PCR-based homologous recombination as shown in Fig. 1A. GFP-tagged Ypt6(Q69L)p and Ypt6(T24N)p were generated as follows: The N-terminal fragment of the *YPT6* ORF (nt 1–636) was subcloned into BamHI-digested pBS and mutagenized using a PCR-based mutagenesis protocol. To create integration plasmids, these mutagenized fragments were replaced with the N-terminal fragment of the *YPT6* of pBS-P_{YPT6}-GFP-*YPT6*(1–210)-HIS3, and the integration plasmid was linearized by HindIII and transformed into yeast. The integration plasmid for Ypt6p overexpression was generated by replacing the *YPT6* promoter of pBS-P_{YPT6}-GFP-*YPT6*(1–210)-HIS3 with the *TPI1* promoter (418-bp 5′ UTR of *TPI1* gene). The C-terminal GFP or mCherry tagging of proteins was performed by PCR-based homologous recombination using pFA6a-GFP(S65T) or pFA6a-mCherry, respectively, as a template [9].

2.2. Fluorescence microscopy

Fluorescence microscopy was performed using an Olympus IX81 microscope equipped with a x100/NA 1.40 (Olympus) objective and Orca-AG cooled CCD camera (Hamamatsu), using Metamorph software (Universal Imaging). Simultaneous imaging of red and green fluorescence was performed using an Olympus IX81 microscope and an image splitter (Dual-View; Optical Insights) that divided the red and green components of the images with a 565-nm dichroic mirror and passed the red component through a 630/50 nm filter and the green component through a 530/30 nm filter. Fluorescence labeling of α-factor was performed as described previously [11].

2.3. Cell extract preparation and immunoblotting

Yeast cells were grown in YPD medium at 25 °C for 16–20 h. The cells collected from 50 mL of the cultures were washed twice with distilled water, frozen in liquid nitrogen, and powdered using mortar and pestle. After suspended the cell powder in lysis buffer (20 mM Tris–HCl, pH 8.0, 300 mM NaCl, 2 mM EDTA, 10 mM EGTA, 1% Triton X-100, 15% glycerol, 1 mM PMSF and protease inhibitor cocktail), cell lysates were prepared using ultrasonication. After being centrifuged, the supernatants were separated on SDS–PAGE and transferred onto PVDF membranes (Bio-Rad). The membrane was blocked overnight with 5% nonfat dry milk in phosphate buffer saline containing 0.05% Tween 20 (PBS-T) and incubated for 2 h at room temperature with anti-GFP antibody (Life Technologies) diluted in PBS-T containing 1% nonfat dry milk. After washing in PBS-T, the membrane was incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (GE Healthcare). Immunoreactive protein bands were visualized by exposing the membrane for 1 min to an ECL Plus chemiluminescence reagent (GE Healthcare).

3. Results and discussion

3.1. Endogenous Ypt6p is predominantly localized to the cis- to medial-Golgi compartments

In a recent study, we developed a novel method for N-terminal GFP tagging of endogenous protein [10]. Using this method, a GFP-tag was added to the N terminus of Ypt6p (Fig. 1A) (see Methods for details). To test the functionality of GFP-tagged Ypt6p, we examined its ability to complement the phenotype of *ypt6*Δ cells. A previous study had shown that *ypt6*Δ cells exhibit a temperature-sensitive growth defect [12]. As shown in Fig. 1B, we found that expression of GFP-Ypt6p was able to restore the growth defect of *ypt6*Δ cells at 39 °C. GFP-Ypt6p was clearly detected as numerous small puncta throughout the cytoplasm (Fig. 1C). The puncta of GFP-Ypt6p were highly colocalized with those of Vrg4p (~80.0%), a *cis*-Golgi marker, and Mnn5p (~70.6%), a *medial*-Golgi marker (Fig. 1D and E), but only partly colocalized with Sec7p (~28.8%), a *trans*-Golgi marker, or Vps10p (~27.5%), a marker for the *trans*-Golgi and late endosomal compartments [13,14] (Fig. 1D and E). Interestingly, Ypt6p was highly localized to COPI-coated vesicles labeled by Sec21p (~65.5%), whereas it was rarely localized in COPII-coated vesicles labeled by Sec13p (~8.0%) (Fig. 1D and E), suggesting the localization of Ypt6p at the early Golgi compartments. Such localization differs somewhat from previous observations showing that Ypt6p resides at the boundary between the *cis*- and *trans*-Golgi, 54% and 58% of GFP-Ypt6p being colocalized with *cis*- and *trans*-Golgi markers, respectively [6]. We also examined the localization of Ypt6p in endocytic compartments by colabeling GFP-Ypt6p with Alexa Fluor 594-α-factor (A594-α-factor) [11], and found that Ypt6p little colocalizes with A594-α-factor at any of the time points examined. From these results we concluded that endogenous Ypt6p is localized to the Golgi apparatus, particularly the *cis*- to *medial*-Golgi compartments.

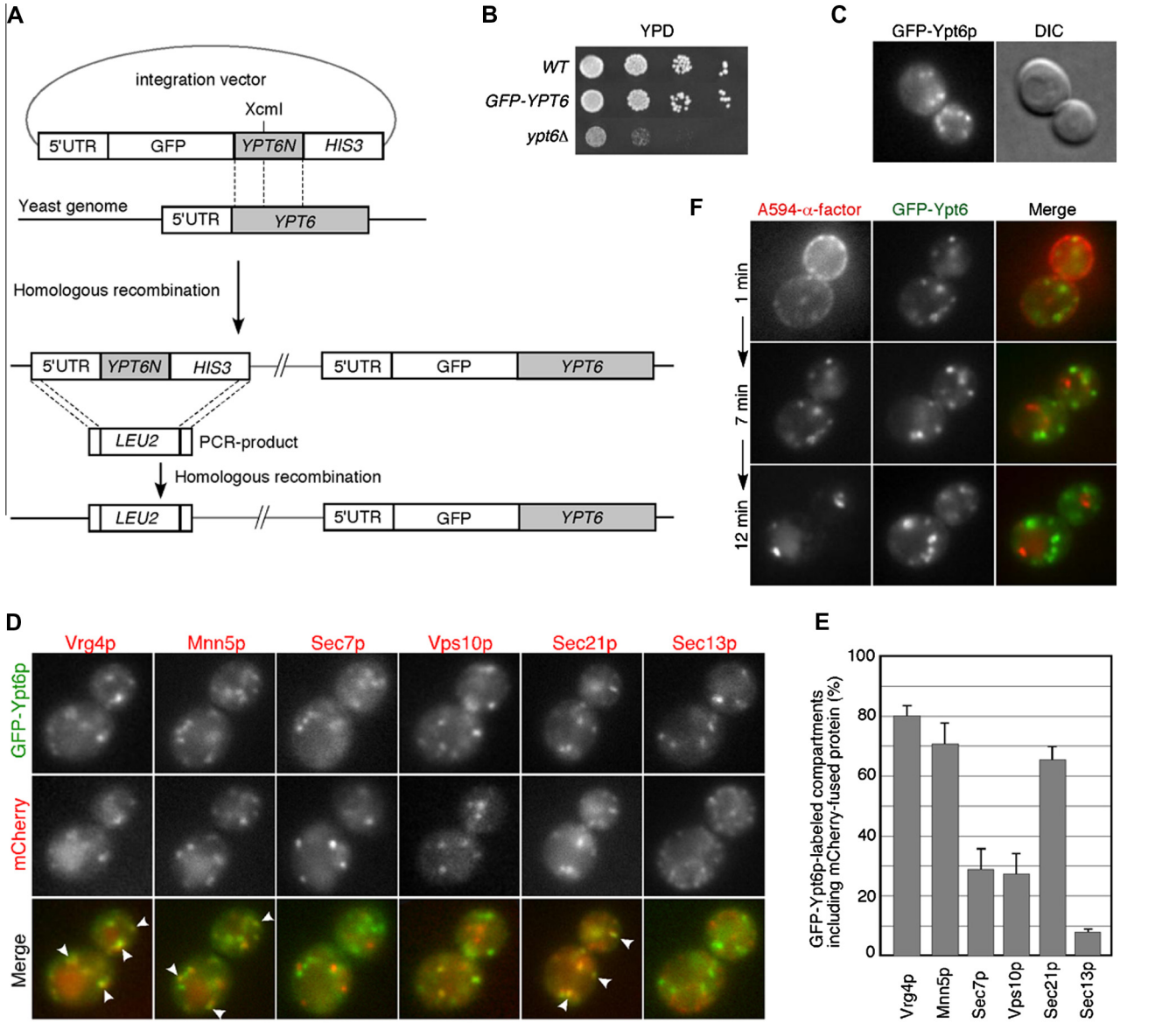

Fig. 1. Schematic representation of the method for GFP tagging of the chromosomal *YPT6* gene, and localization of GFP-Ypt6p. (A) The integration plasmid was digested with XcmI, transformed and integrated at the N terminus of the ORF of the chromosomal *YPT6* gene through homologous recombination. The extra region generated by the insertion of the integration plasmid was removed by PCR-based homologous recombination. (B) GFP-Ypt6p complemented the growth phenotype of *ypt6* Δ cells. Dilution series of cells was plated on YPD plates and incubated at 39 °C for 3 days. (C) The endogenous localization of GFP-Ypt6p in living cells. (D) Localization of GFP-Ypt6p and mCherry-tagged organelle markers in wild-type cells. Each image pair was acquired simultaneously using dual-channel two-dimensional (2D) imaging system. Arrowheads indicate examples of colocalization. (E) Quantification of colocalization of GFP-Ypt6p and mCherry-tagged organelle markers. The percentages of colocalization were calculated as the ratio of GFP-Ypt6p labeled endosomes ($n = 100$) colocalizing with each marker in each experiment. Error bars indicate the standard deviation (SD) from at least three independent experiments. (F) Localization of GFP-Ypt6p in endocytic compartments. The images were acquired simultaneously at 1, 7, and 12 min after washing out unbound A594-α-factor and warming the cells to 25 °C.

3.2. Ypt6p dissociates from and associates with the cis-Golgi compartment by cycling between the GTP-bound and GDP-bound states

To examine the localization of the GTP- or GDP-bound form of endogenous Ypt6p, we again utilized N-terminal tagging methods. It has been reported that substitution of a highly conserved threonine by glutamine (T24N) or an asparagine residue to leucine (Q69L) fixes Rab proteins to the GDP or GTP-bound form (Fig. 2A) [15]. To generate *YPT6*(*T24N*) or *YPT6*(*Q69L*) mutants, we subcloned the N-terminal fragment of Ypt6p containing each point mutation into the integration plasmid, and then linearized the plasmid with HindIII and used it for transformation of wild-type cells (Fig. 2B) (see Methods for details). These GFP-fused Ypt6p mutant proteins were expressed at similar levels to the wild-type proteins, although Ypt6(T24N)p migrated slightly more slowly on SDS–PAGE (Fig. 2C). As expected, the GDP-bound form of Ypt6p, Ypt6(T24N)p, exhibited cytosolic – and not punctate – localization (Fig. 2D, left panel). In contrast, the GTP-bound form of Ypt6p, Ypt6(Q69L)p, showed a punctate distribution in the cytosol (Fig. 2D, right panel), similar to wild-type Ypt6p. To further investigate the localization of the GTP- or GDP-bound form of Ypt6p, we next examined the effect of deletion of the putative Ypt6p GEF or GAP on Ypt6p localization. As a previous study has identified the Ric1p-Rgp1p protein complex as the GEF for Ypt6p, and shown that Ric1p is predominantly localized to the *trans*-Golgi compartment

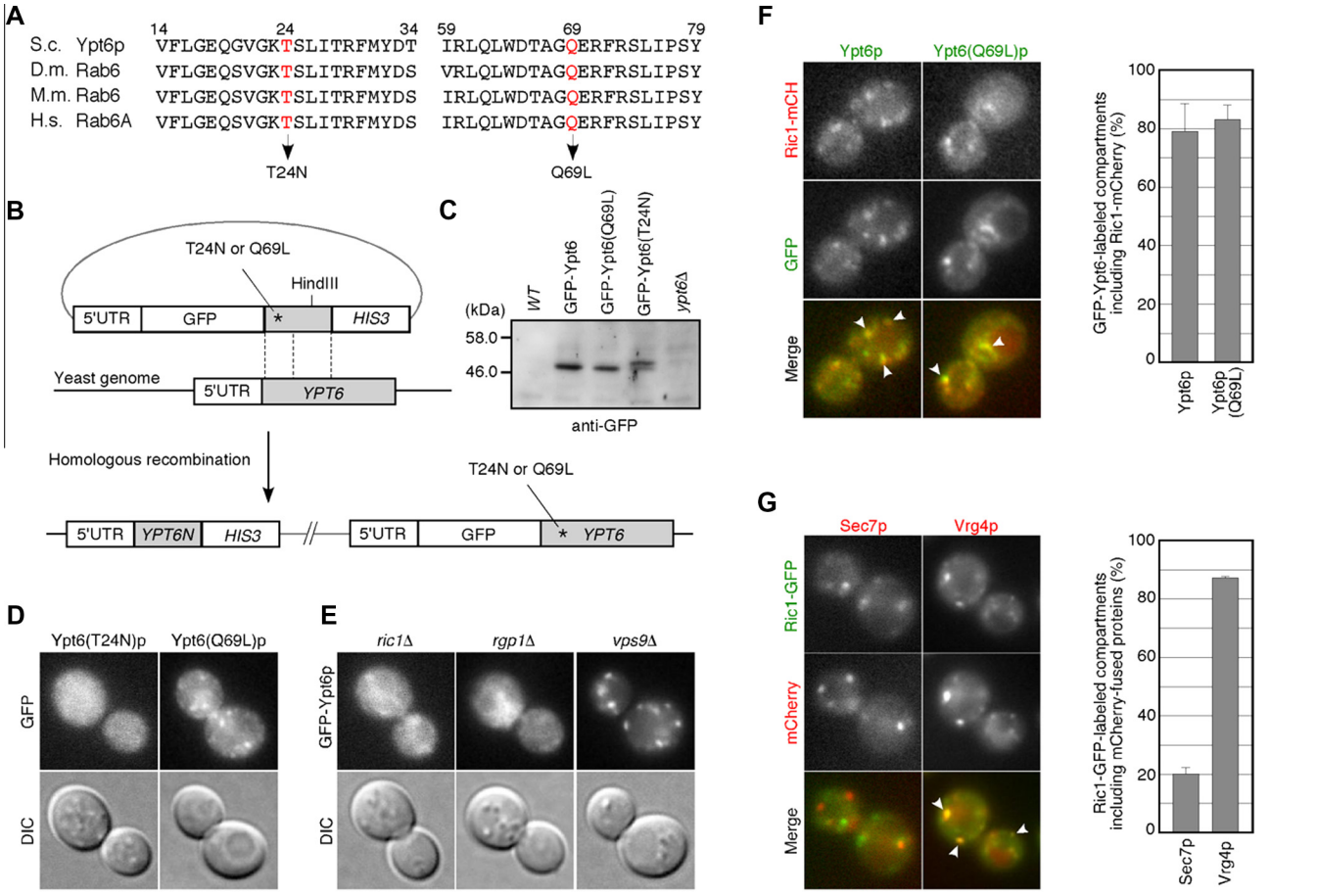

Fig. 2. Localization of putative GTP- or GDP-locked mutant of Ypt6p and Ric1p at the *cis*-Golgi compartment. (A) Sequence alignments of the GTP/GDP-binding region of Rab6 proteins, including sequences from *Saccharomyces cerevisiae* (S.c.), *Drosophila melanogaster* (D.m.), *Mus musculus* (M.m.), and *Homo sapiens* (H.s.). (B) Schematic representation of the method for introducing mutation in the chromosomal *YPT6* gene. (C) Immunoblots showing expression of GFP-fused Ypt6p, -Ypt6(Q69L)p, and -Ypt6(T24N)p. 10 μg of whole cell extracts from each strain was loaded per lane and immunoblotted with an anti-GFP antibody. (D) Localization of GFP-Ypt6(T24N)p, and -Ypt6(Q69L)p in living cells. (E) The endogenous localization of GFP-Ypt6p in *ric1*Δ or *rgp1*Δ cells. (F) Colocalization of mCherry (mCH)-fused Ric1p with GFP-Ypt6p or -Ypt6(Q69L)p in wild-type cells. Each image pair was acquired simultaneously using dual-channel imaging system. Quantification of colocalization is shown on the right. Data show mean ± SD from at least three experiments, with 100 GFP-Ypt6p or -Ypt6(Q69L)p labeled compartments counted per experiment. (G) Colocalization of GFP-fused Ric1p with Sec7p or Vrg4p in wild-type cells. Quantification of colocalization is shown on the right. Data show mean ± SD from at least three experiments, with 100 GFP-Ric1 labeled compertments counted per experiment.

[7], we examined the effect of *RIC1* or *RGP1* gene deletion on Ypt6p localization. Similarly to GFP-Ypt6(T24N)p, in *ric1*Δ or *rgp1*Δ cells, we observed that the localization of GFP-Ypt6p changed to the cytosol (Fig. 2E, left and center panels). In contrast, GFP-Ypt6p still showed a punctate localization in cells with deletion of the *VPS9* gene, encoding a GEF for Vps21p [16] (Fig. 2E, right panel). mCherry-tagged Ric1p was highly colocalized with both wild-type and GTP-bound Ypt6p (~79.0% or ~83.0%, respectively) (Fig. 2F) and the *cis*-Golgi labeled by Vrg4-GFP (~87.4%) (Fig. 2G), indicating that Ric1p-Rgp1p and the GTP-bound form of the Ypt6p complex reside predominantly in the *cis*-Golgi compartment. Taken together, these results suggest that Ypt6p changes its localization from the cytosol to the *cis*-Golgi by switching its state from the GDP-bound to the GTP-bound form.

3.3. Gyp6p inactivates Ypt6p in the trans-Golgi compartment

Yeast contains 11 GAPs that possess the TBC (Tre-2/Bub2/Cdc16) domain [17]. Among them, Gyp2p, Gyp8p and Gyp6p have been reported to be possible GAPs for Ypt6p [18–20], but it is still controversial which GAP(s) actually function as Ypt6p-GAP. A previous study has shown that deletion of the *GYP3* gene, encoding Vps21p-GAP, leads to migration of Vps21p from endosomes to the vacuole because the GTP-bound form of Vps21p cannot be extracted from the organelle membrane with which it associates [21,22]. Therefore we speculated that the localization of endogenous Ypt6p might migrate from the *cis*-Golgi in the absence of its specific GAP. By comparing Ypt6p localization in cells lacking each of the individual GAP genes, we found that deletion of the *GYP6* gene led to slight mis-localization of Ypt6p to the plasma membrane (Fig. 3A). We further examined the effect of *GYP6* deletion on the localization of Ypt6p in the Golgi. As shown in Fig. 3B, we found that the localization of Ypt6p partly migrated from the *cis*-Golgi to the *trans*-Golgi compartment (Fig. 3B). Quantitative analyses revealed that localization of GFP-Ypt6p at the *trans*-Golgi compartment was markedly increased in *gyp6*Δ cells (~54%, compared with ~23.1% in wild-type cells) (Fig. 3B). We also found that Gyp6-GFP was highly localized to the *trans*-Golgi compartment labeled by Sec7-mCherry, but little localized to the Vrg4p-residing Golgi compartment (Fig. 3C). These observations suggest that Ypt6p is hydrolyzed by Gyp6p and extracted from the membrane after arrival at the *trans*-Golgi compartment. To gain further information on the function of Ypt6p in the intracellular trafficking pathway, we deleted each of the eleven known yeast Rab GAPs in a *gyp6*Δ genetic background and tested the resulting double mutants for growth at 25 °C and 39 °C (Fig. 3D). Interestingly, loss

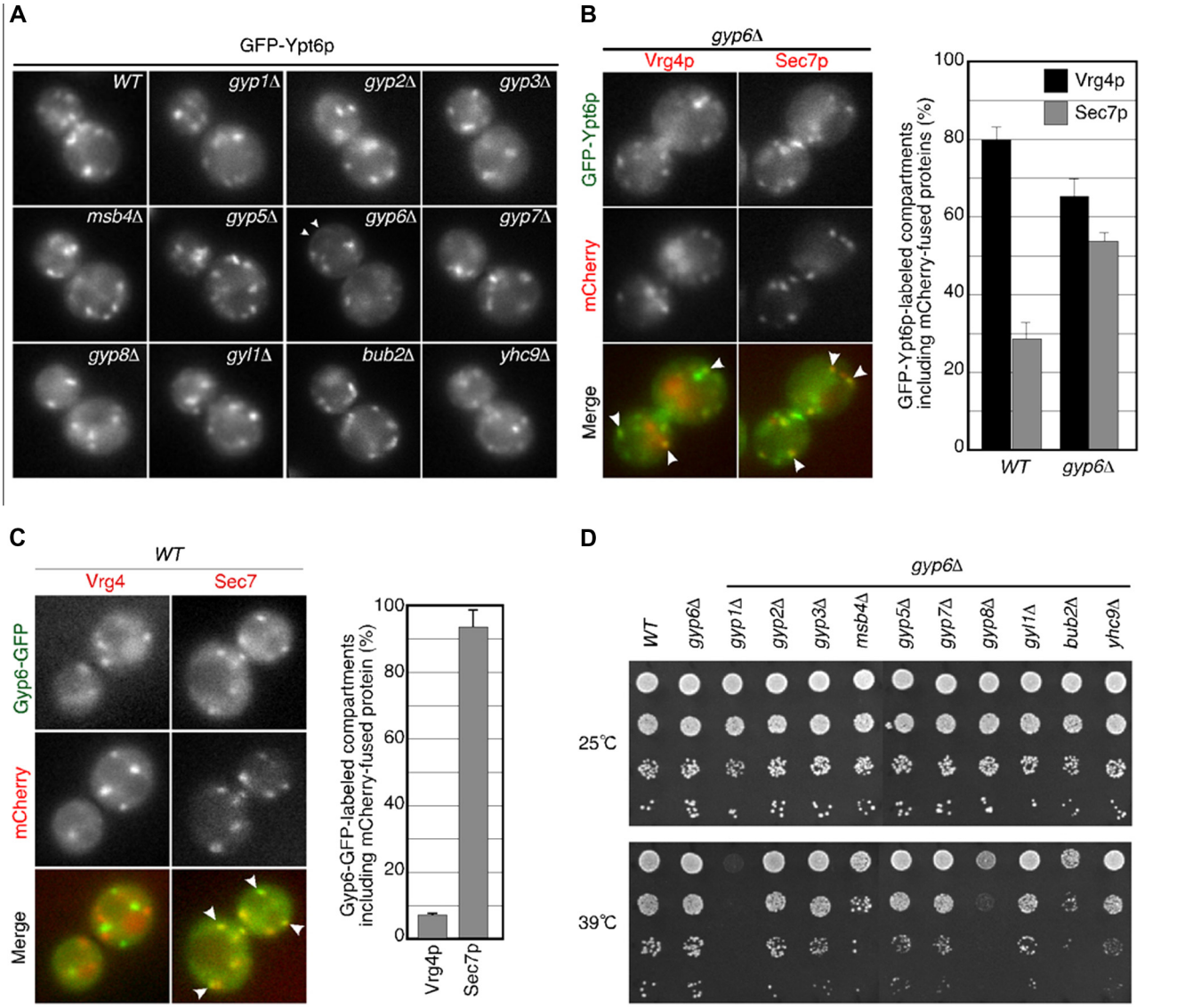

Fig. 3. Effect of deleting Rab-GAP genes on Ypt6p localization and localization of Gyp6p. (A) Localization of GFP-Ypt6p in cells depleted Rab-GAPs. Arrowheads indicate localization of GFP-Ypt6p at the plasma membrane. (B) Colocalization of GFP-Ypt6p with mCherry-fused Vrg4p or Sec7p in *gyp6*Δ cells. Each image pair was acquired simultaneously using dual-channel imaging system. Quantification of colocalization is shown on the right. Data show mean ± SD from at least three experiments, with 100 GFP-Ypt6p labeled compartments counted per experiment. (C) Colocalization of Gyp6-GFP with mCherry-fused Vrg4p or Sec7p in wild-type cells. The percentages of colocalization were calculated as the ratio of mCherry-fused protein localized in Gyp6-GFP positive compartments (n = 100) in each experiment. Error bars represent the SD from at least three experiments. Arrowheads indicate examples of colocalization. (D) Plates showing growth phenotypes of double mutants deleting *YPT6* and other GAP genes. Dilution series of cells was plated on YPD plates and incubated for 2–3 days at 25 or 39 °C, respectively.

of two GAP genes, *GYP1* and *GYP8*, resulted in synthetic growth defects when combined with the *gyp6*Δ mutation. It has been reported that both Gyp1p and Gyp8p are possible GAPs for Ypt1p [19,23]. Additionally, it is also shown that overexpression of Ypt1p suppresses the growth and secretion defects of the *ypt6* mutant [24] and the defective transport of Snc1p to the plasma membrane via the Golgi observed in the *rgd1*Δ mutant [25]. These observations suggest the function of Ypt6p in *intra*-Golgi trafficking from the *cis*- to *trans*-Golgi compartments.

3.4. Overexpression of the GDP-bound form of Ypt6p causes defective vacuole formation and recycling of Snc1p

We next utilized N-terminal tagging for overexpression of Ypt6p and analyzed the resulting effects. The promoter of the yeast triose phosphate isomerase (*TPI1*) gene is a constitutive and powerful promoter, and used frequently for overexpression of protein [26]. According to the yeast GFP fusion localization database (http://yeastgfp.yeastgenome.org), Tpi1p is present at about 207,000 molecules per cell, being nearly 30 times more abundant than Ypt6p (7720 molecules per cell). To overexpress Ypt6p and its GTP/GDP-bound forms at a similar level, we replaced the promoter region of the endogenous *YPT6* gene with the *TPI1* promoter, as shown in Fig. 4A. Western blotting using anti-GFP antibody revealed that the *TPI1* promoter markedly increased the levels of expression of wild-type and mutated Ypt6p (Fig. 4B). Under the *TPI1* promoter, the localization of Ypt6p and Ypt6(Q69L)p was similar to that under the *YPT6* promoter, except for ER localization (Fig. 4C, left and center panels). Ypt6(T24N)p was localized almost entirely to the cytosol, similarly to when it was expressed via its own promoter (Fig. 2D), although the fluorescence intensity was much higher (Fig. 4C, right panel). Comparison of the localization of mCherry-fused Vrg4p and Sec7p showed that overexpressed Ypt6p had a localization relatively similar to that of endogenous

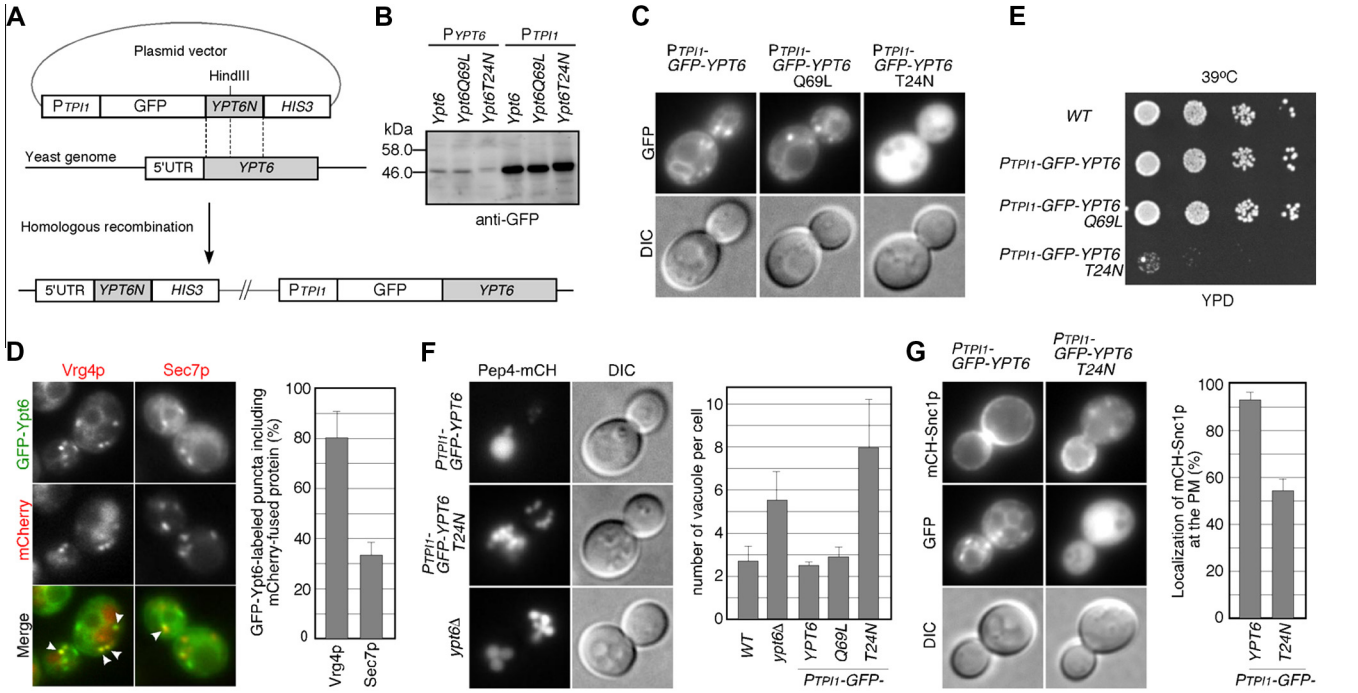

Fig. 4. Overexpression of Ypt6p GDP-locked mutant leads to defects in growth, vacuolar morphology, and intracellular trafficking. (A) Schematic representation of the method for replacing the promoter region of the chromosomal *YPT6* gene. (B) Immunoblots showing expression of GFP-fused Ypt6p, Ypt6(Q69L)p, and Ypt6(T24N)p from the *YPT6* or *TPI1* promoter. 10 μg of whole cell extracts from each strain was loaded per lane and immunoblotted with an anti-GFP antibody. (C) Localization of wild-type Ypt6p and GTP- or GDP-locked mutants of Ypt6p overexpressed by the *TPI1* promoter. (D) Localization of overexpressed GFP-Ypt6p in wild-type cells. Quantification of colocalization is shown on the right. Data show mean ± SD from at least three experiments, with 100 GFP-Ypt6p labeled compartments counted per experiment. (E) Plates showing growth phenotypes of cells overexpressing Ypt6p or its GTP/GDP-locked mutant. Dilution series of cells was plated on YPD plates and incubated for 3 days at 39 °C. (F) The integrity of the vacuole in wild-type and mutant cells. Cells expressing Pep4-mCherry(mCH) were grown early to mid-logarithmic phase in YPD medium at 25 °C and observed by fluorescence microscopy and DIC. Right histogram shows the number of vacuole observed in wild-type and mutant cells. Data show the mean ± SD of three experiments, with 50 cells counted at each time point per experiment. (G) Localization of mCherry(mCH)-Snc1p in cells overexpressing wild-type or GDP-locked Ypt6p. The bar graphs represent the percentages of cells exhibiting mCH-Snc1p localized at the membrane (PM) of mother cells. Data show mean ± SD from at least three experiments, with >100 cells counted for each strain per experiment.

Ypt6p: ~79.9% in the *cis*-Golgi and ~33.3% in the *trans*-Golgi (Fig. 4D). To examine the effect of overexpression of these proteins, we first analyzed the growth sensitivity of these cells to high temperature. Cells overexpressing Ypt6p or Ypt6(Q69L)p showed growth activity comparable to that of the parental wild-type cells (Fig. 4E). On the other hand, cells overexpressing Ypt6(T24N)p were inviable on YPD medium at 39 °C (Fig. 4E). Since it has been reported that Ypt6p-disrupted cells show characteristic fragmentation of vacuoles [7], we next analyzed the vacuolar morphology of cells overexpressing these proteins. As shown in the previous study [7], *ypt6*Δ cells contained fragmented vacuoles (Fig. 4F). Cells overexpressing Ypt6(T24N)p also exhibited severely fragmented vacuoles, whereas cells overexpressing Ypt6p or Ypt6(Q69L)p contained vacuoles that were morphologically similar to those of the parental wild-type cells (Fig. 4F). Quantitative analysis of the number of vacuoles demonstrated that overexpression of Ypt6(T24N)p led to more severe vacuolar fragmentation, in comparison to *ypt6*Δ cells (Fig. 4F), suggesting that Ypt6(T24N)p might exert a dominant-negative effect by inhibiting the function of endogenous Ypt6p effectors. Although the functions of Ypt6p in intracellular vesicle trafficking remain obscure, it has been shown that Ypt6p has a role in the recycling of exocytic SNARE Snc1p between the endosome and the Golgi, and that deletion of the *YPT6* gene blocks the recycling of Snc1p [7]. Similarly, we found that localization of GFP-Snc1 was partly shifted to the intracellular compartments in cells overexpressing Ypt6(T24N)p, but was unchanged in cells overexpressing wild-type Ypt6p (Fig. 4G). Thus, Ypt6p activity is necessary for recycling of protein from the Golgi to the plasma membrane. These results suggest that overexpression of protein using N-terminal tagging is useful for analysis of protein function. Using this method, it could also be possible to decrease or regulate protein expression by replacing the promoter with one showing low activity or one that can be regulated, such as the *GAL1* promoter and the *MET25* promoter [27].

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.002.

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