

## Trs20, Trs23, Trs31 and Bet5 participate in autophagy through GTPase Ypt1 in *Saccharomyces cerevisiae*

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**Abstract:** TRAPP (transport protein particle) is a large, highly conserved, multi-subunit complex. Four types of TRAPP complexes (I, II, III and most recently IV) have been identified in *Saccharomyces cerevisiae*. Studies on the roles of TRAPP II, TRAPP III and TRAPP IV specific subunits (Trs130, Trs85 and Trs33) have demonstrated that TRAPP II, TRAPP III and TRAPP IV activate the small GTPases that regulate autophagy. Up to now, the roles of the common TRAPP subunits have been well studied in vesicle transport. However, the roles of the common TRAPP subunits and their relationship to Ypt/Rab GTPases in autophagy are not clear. In this paper, we examined Trs20, Trs23, Trs31, and Bet5 (the common TRAPP subunits), which are required for starvation-induced autophagy and the cytoplasm-to-vacuole targeting (Cvt) pathway. During autophagy, GFP-Atg8 accumulates as single or multiple dots and is not recruited into the pre-autophagosomal structures (PAS) in *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells. Furthermore, these dots are linked to the endoplasmic reticulum in mutant cells. Additionally, overexpression of Ypt1, but not Ypt31, suppresses the autophagy defect in *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells. Based on these results, we concluded that Trs20, Trs23, Trs31, and Bet5 are required for autophagy, and that these common TRAPP subunits regulate autophagy partially through GTPase Ypt1, but not Ypt31.

**Key words:** autophagy; TRAPP; common TRAPP subunits; Ypt1; Ypt31

**Abbreviations:** Atg – autophagy-related gene; Cvt – cytoplasm-to-vacuole targeting; ER – endoplasmic reticulum; GEF – guanine nucleotide exchange factor; GFP – green fluorescent protein; PAS – phagophore assembly site/pre-autophagosomal structure; RFP, red fluorescent protein; TRAPP – transport protein particle; Ypt – yeast protein transport

### INTRODUCTION

Vesicle transport among different organelles in yeast is regulated by molecular switches known as Ypt/Rab GTPases, which are activated by guanine-nucleotide exchange factors (GEFs) [1,2]. The TRAPP (transport protein particle) complex is a large, highly conserved, multi-subunit complex [3]. Four forms of TRAPP complexes have been identified in yeast with GEF activities toward GTPases Ypt1 and Ypt31/32 [4-12]. Five common subunits (Bet3, Bet5, Trs20, Trs23 and Trs31) form the TRAPP I complex, which regulates endoplasmic reticulum (ER)-to-Golgi trafficking by activating Ypt1 [13,14]. TRAPP II, which contains components of TRAPP I plus five specific subunits (Trs120, Trs130, Trs65, Trs33 and Tca17), functions in trans-Golgi trafficking [10,15-17]. However, TRAPP

II has also been shown to play important roles in autophagy by activating Ypt31/32 [18]. TRAPP I and a specific subunit, Trs85, form the TRAPP III complex, is involved in autophagy and vesicle trafficking by activating Ypt1 [6,7]. According to a recent study, TRAPP I and a specific subunit, Trs33, form the TRAPP IV complex, which is also required for autophagy by activating Ypt1 [12].

Autophagy is a highly conserved process in eukaryotes that depends on the lysosome (vacuole in yeast) material degradation pathway, and is responsible for the removal of aging proteins and damaged/aging organelles and for developmental remodeling [19-21]. Autophagy is generally divided into selective and nonselective forms. In selective autophagy, substrates or organelles are degraded by specific forms

of autophagy. Vacuolar proteases, the precursor forms of Ape1 (hydrolase aminopeptidase I; prApe1) are synthesized in the cytoplasm and transported to the vacuole for further processing through a selective autophagy pathway named the cytoplasm-to-vacuole targeting (Cvt) pathway, which is a model for selective autophagy [20]. In contrast, during nonselective autophagy, portions of the cytoplasm, such as proteins and organelles, are recycled when the cell is under stress [22,23]. Autophagy usually refers to nonselective autophagy.

Findings from studies of specific subunits of Trs130, Trs85 and Trs33 show that TRAPP II, TRAPP III and TRAPP IV are required for autophagy [7,12,18]. However, the role of the common TRAPP subunits in autophagy is unclear. The common subunits, Bet3 and Trs20, were recently shown to maintain the stability of the TRAPP III complex and are involved in autophagy [24,25]. In addition, according to the results from a previous study, Bet3 affects both the TRAPP III complex and the function of the TRAPP I complex during autophagy through the GTPase Ypt1 [26]. However, researchers have not clearly identified the roles of the other common TRAPP subunits (Bet5, Trs20, Trs23 and Trs31) in autophagy or whether Ypt1 or Ypt31 suppress the autophagy defects in strains with mutations in these subunits.

As will be shown in this paper, Bet5, Trs20, Trs23 and Trs31, the common subunits of the TRAPP complex, are required for nonselective and selective autophagy. The *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells grow at the permissive temperature of 26°C but do not grow at 37°C. However, a defect in the autophagy phenotype is observed in these strains at the permissive temperature of 26°C. Furthermore, the Atg8 protein, a protein required to form the autophagosome, is not transported to the pre-autophagosomal structures (PAS) in the *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells. Finally, Ypt1, but not Ypt31, suppresses the autophagy defect in *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells under starvation conditions. Therefore, in this work, the common subunits of the TRAPP complex, Bet5, Trs20, Trs23 and Trs31, partially regulate autophagy through the GTPase Ypt1.

## MATERIALS AND METHODS

### Strains, plasmids and reagents

The yeast strains used in this study are listed in Table S1. The common TRAPP subunit mutants used here included *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* [27]. The desired genes were amplified from the background of the common TRAPP subunit mutants using polymerase chain reaction (PCR) and transferred to the strain SEY6210 for this study. For gene deletion, the entire gene coding region was replaced with a drug-resistance cassette using PCR amplification. Plasmids expressing *GFP-ATG8 (URA3)*, *RFP-APE1 (LEU2)* and *ATG9-3XGFP (URA3)* under the control of the endogenous promoter were described in a previous study [18]. For the genetic interaction experiments, pRS425 plasmids (2 µg, LEU2) expressing *YPT1* and *YPT31* were used [18]. The open reading frames (ORFs) of *TRS20*, *TRS23*, *TRS31* and *BET5*, along with 1000 bp of the 5'-promoter regions were cloned into the pRS425 vector.

The antibodies used in this study are as follows: mouse anti-GFP (Santa Cruz), rabbit anti-Ape1 (a gift from Y. Ohsumi), rabbit anti-pGK1 (Acris, Herford, Germany), and rabbit anti-glucose-6-phosphate dehydrogenase (G6PDH, Sigma-Aldrich, MO, United States). A horseradish peroxidase-conjugated goat anti-rabbit IgG antibody and an ECL kit were obtained from Millipore Corporation (MA, United States).

All chemical reagents were purchased from AMRESCO (OH, United States), unless indicated otherwise. SynaptoRed, also known as FM4-64, was purchased from Molecular Probes (OR, United States). Geneticin was obtained from Gibco Laboratories (NY, United States). Restriction enzymes and buffers were from Takara Biotechnology (Dalian, China).

### Yeast culture conditions and the induction of autophagy

Yeast strains were grown in rich yeast extract peptone dextrose (YPD) or selective (synthetic dropout, SD) media. Experiments in which autophagy was induced by nitrogen starvation were performed in SD medium lacking nitrogen (SD-N).

For live-cell fluorescence microscopy, yeast cultures were grown at 26°C in YPD or SD media to log phase. If the cells were subjected to nitrogen starvation, they were washed and transferred to SD-N for 2 h at 26°C. If necessary, FM4-64 was added to the liquid medium to a concentration of 1.6  $\mu$ M to stain the vacuoles during the last hour of incubation. Slides were visualized using a Nikon Eclipse Ti inverted research microscope (Tokyo, Japan). The results were quantified using Photoshop CS.

For the genetic interaction analysis, cells were grown in SD media overnight at 26°C, normalized to the same density and spotted onto agar plates in 10-fold serial dilutions. Plates were incubated at different temperatures for the genetic interaction assays.

### GFP-Atg8 and Ape1 processing assays

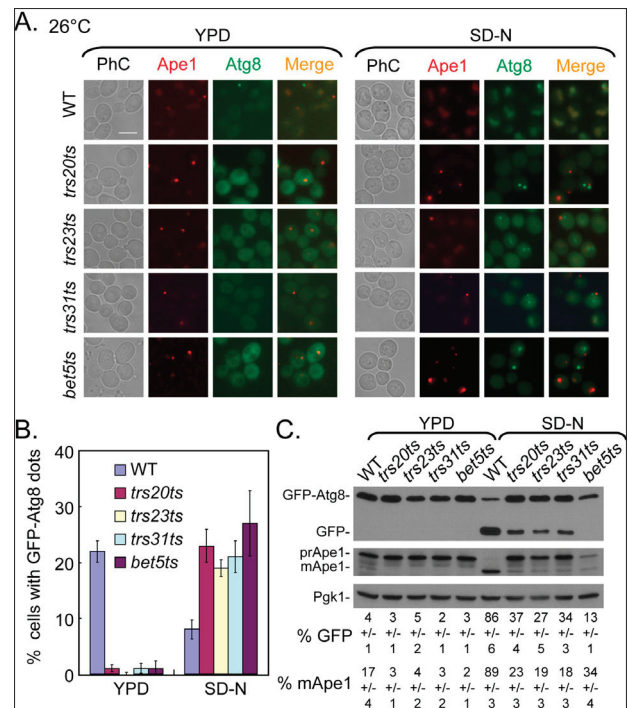
The GFP-Atg8 and Ape1 processing assays were used to detect the level of autophagy in different strains. The concrete steps were as follows: strains were grown at 26°C to an early log phase in YPD media. Autophagy was then induced by SD media and cells were kept at 26°C. Culture aliquots were collected at intervals of 2 h and GFP-Atg8 cleavage or pr-Ape1 maturation were determined by Western blot analysis of the cell extracts. Western blot analysis was performed as previously described [17] and repeated at least twice.

## RESULTS

### Common TRAPP subunits are required for both the Cvt pathway and starvation-induced autophagy

In yeast cells, four types of TRAPP complexes have been identified [7,12,14,28], and by studying the specific TRAPP subunits (Trs130 and Trs85), TRAPP II, TRAPP III and TRAPP IV complexes were shown to regulate autophagy by activating Ypt31/32 and Ypt1, respectively [7,12,18]. According to the results of our recent study, Bet3, a common TRAPP subunit, regulates autophagy by activating Ypt1 [26]. However, as it is not clear whether the other common subunits of TRAPP (Trs20, Trs23, Trs31 and Bet5) are involved in autophagy, we examined their involvement in this process.

We visualized the localization of Atg8 in wild-type (WT), *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* strains under non-starvation and starvation conditions to directly monitor the autophagy phenotype. The Cvt pathway, a model of selective autophagy, was analyzed using a nutrient enriched medium [20]. Thus, we treated all strains with YPD liquid medium to detect the Cvt pathway. About 22% of WT cells displayed a single GFP-Atg8 dot that colocalized with the PAS marker RFP-Ape1 (Fig. 1A and B), indicating a normal Cvt



**Fig. 1.** The Cvt pathway and starvation-induced autophagy are impaired in *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells. **A.** Atg8 and Ape1 were mislocalized in common TRAPP subunit mutant cells, *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts*. WT and mutant cells were transformed with GFP-Atg8 and RFP-Ape1 integration plasmids. The cells were grown in YPD medium to mid-log phase at 26°C; if the cells were subjected to nitrogen starvation, they were washed and transferred to SD-N at 26°C for 2 h. The experiments were repeated twice and representative results are presented. Bar – 5  $\mu$ m. **B.** Percentage of cells that had at least one GFP-Atg8 dot that was quantified. At least 300 cells from each strain were counted in at least three fields. Error bars represent the standard deviation. **C.** Ape1 maturation was blocked and GFP-Atg8 degradation was reduced in common TRAPP subunit mutant cells. The cells were grown and treated as described in A and were then collected for immunoblotting with an anti-GFP antibody to examine GFP-Atg8 degradation, an anti-Ape1 antibody to examine Ape1 maturation and an anti-Pgk1 antibody serving as a loading control. Quantification of the immunoblot assay was performed using IMAGEJ software. The graphs present data from an average of three experiments.

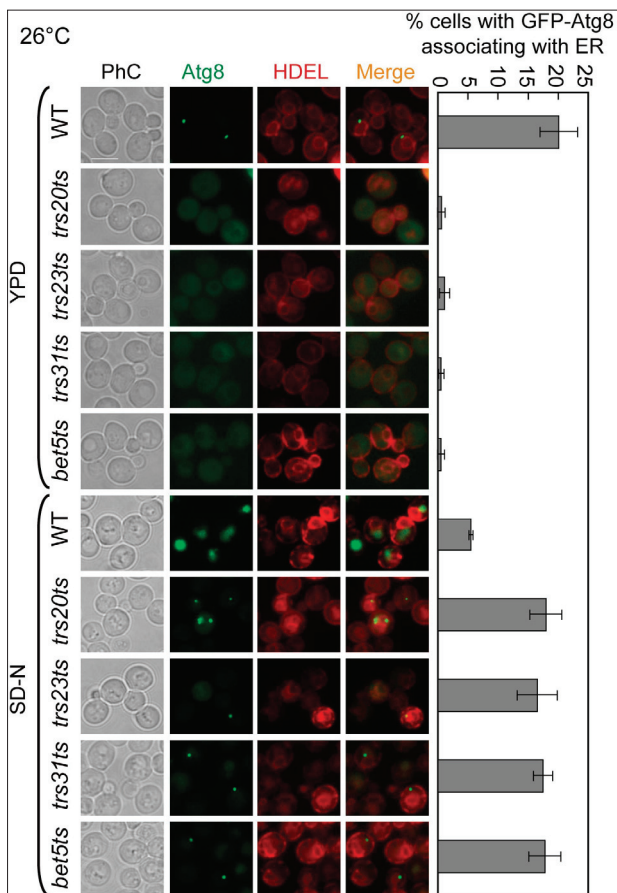
pathway. GFP-Atg8 was dispersed in the cytoplasm in four common TRAPP subunit mutant cells (Fig. 1A and B), suggesting a defective Cvt pathway. We also used the Ape1 processing assay to detect mature Ape1 in YPD medium. As expected, WT cells contained the mature form of Ape1 (mApe1) and the precursor form of Ape1 (prApe1) (Fig. 1C), suggesting a normal Cvt pathway. In contrast, *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells accumulated prApe1, but mApe1 was not detected, suggesting that the Cvt pathway was blocked (Fig. 1C), which was consistent with the findings in Fig. 1A. Autophagy was induced when cells were grown under starvation conditions (in SD-N medium), and GFP-Atg8 and RFP-Ape1 were normally delivered to the vacuole in WT cells (Fig. 1A). In contrast, GFP-Atg8 and RFP-Ape1 both accumulated as dots in the cytoplasm and were not delivered to the vacuole in four common TRAPP subunit mutant cells (Fig 1A and B), suggesting an autophagy defect. Meanwhile, the GFP-Atg8 dots were not colocalized with RFP-Ape1 (Fig. 1A). We used the GFP-Atg8 processing assay and Ape1 processing assay to monitor autophagy and extend our analysis. These results were consistent with the fluorescence phenotype, showing that most of the Atg8 was not degraded and most of the Ape1 had not matured in the mutant cells (Fig. 1C). Thus, Trs20, Trs23, Trs31 and Bet5 are required for the Cvt pathway and autophagy.

Trs20, Trs23, Trs31 and Bet5 are common subunits of the TRAPP complex, and their loss of function will affect the functions of the TRAPP I, II, III and IV complexes. Thus, we wanted to determine the functions of these common subunits of the TRAPP complex in autophagy. We compared the GFP-Atg8 phenotype in *trs85Δ* (TRAPP III specific subunit) mutant, *trs130ts* (TRAPP II specific subunit) mutant and *trs130ts trs85Δ* double-mutant cells (Fig. S1A). Because *trs130ts* is a temperature-sensitive mutant, normal autophagy is observed in this mutant at permissive temperatures (26°C) (Fig. S1A) [18]. When cultured at a high temperature (37°C), GFP-Atg8 formed multiple dots in more than 82% of *trs130ts* mutant cells through both the Cvt pathway and autophagy (Fig. S1A). Therefore, we focused on the different phenotypes observed in different TRAPP-specific subunit mutants under high-temperature conditions. In *trs85Δ* mutant cells, GFP-Atg8 was dispersed in the cytoplasm through the Cvt pathway (~97% of cells)

and was present in vacuoles and the cytoplasm as a result of autophagy (~92% of cells) (Fig. S1A). In contrast, GFP-Atg8 distribution in *trs130ts trs85Δ* double-mutant cells was similar to *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells through the Cvt pathway (~97% of cells); upon the induction of autophagy, GFP-Atg8 dots were formed in the cells, and one of the multiple GFP-Atg8 dots was colocalized with the PAS marker RFP-Ape1 in *trs130ts trs85Δ* cells (~72% of cells) (Fig. S1B). Because GFP-Atg8 is not transported to the PAS in *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells, the GFP-Atg8 phenotype in mutants of specific subunits of the TRAPP complex is different from that in *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells (Figs. 1A, S1A and B). In addition, Trs33 (TRAPP IV-specific subunit) is also involved in autophagy, and the GFP-Atg8 phenotype in the *trs33Δ* mutant is similar to *trs130ts*, as GFP-Atg8 formed multiple dots [18]. Thus, Trs20, Trs23, Bet5 and Trs31 are involved in autophagy, but these subunits may participate in autophagy but not through the TRAPP II, III and IV complexes.

#### **GFP-Atg8 accumulates in a region in contact with the ER in *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells under starvation-induced autophagy**

The endoplasmic reticulum (ER) has been shown to play an essential role in the formation of autophagosomes [29-31]. The autophagosomal structures formed during the processes used to form autophagosomes, PAS, phagophores, and autophagosomes are linked to the ER, which provides a double-lipid membrane for autophagosomes [29]. Atg8, a protein related to autophagy, is involved in the elongation and establishment of the autophagosome [32,33]. Since we showed that in *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutants Atg8 did not colocalize with the PAS marker RFP-Ape1 (Fig. 1A), we wanted to test whether Atg8 is located in a region in contact with the ER in these common TRAPP subunit mutants. We created strains expressing GFP-Atg8 together with the ER marker DsRed-HDEL. As shown in Fig. 2, a GFP-Atg8 dot was linked to the ER in WT cells in YPD liquid culture medium, but no GFP-Atg8 dots were observed in the *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells, suggesting the defective processing of GFP-Atg8. Upon the induction of autophagy, GFP-Atg8 was transported to the vacuole in WT cells normally, and no GFP-Atg8 dots were linked



**Fig. 2.** The GFP-Atg8 dots are linked to the ER in *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells. The Atg8 dots are connected to the ER in *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* cells. WT and all mutant cells were transformed with GFP-Atg8 and HDEL-DsRed integration plasmids. The cells were grown and treated as described in Fig. 1A. The experiments were repeated twice and representative results are presented. Bar – 5  $\mu$ m. Quantitation of the percentage of cells in which a GFP-Atg8 dot was linked to the ER. At least 300 cells from each strain were counted in at least three fields. Error bars represent the standard deviation.

to the ER. In contrast, in all mutant cells, the transport of GFP-Atg8 to the vacuole was blocked, and green dots linked to the ER accumulated. Thus, based on these data, GFP-Atg8 remained connected to the ER and was not transported to the vacuole in the *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells.

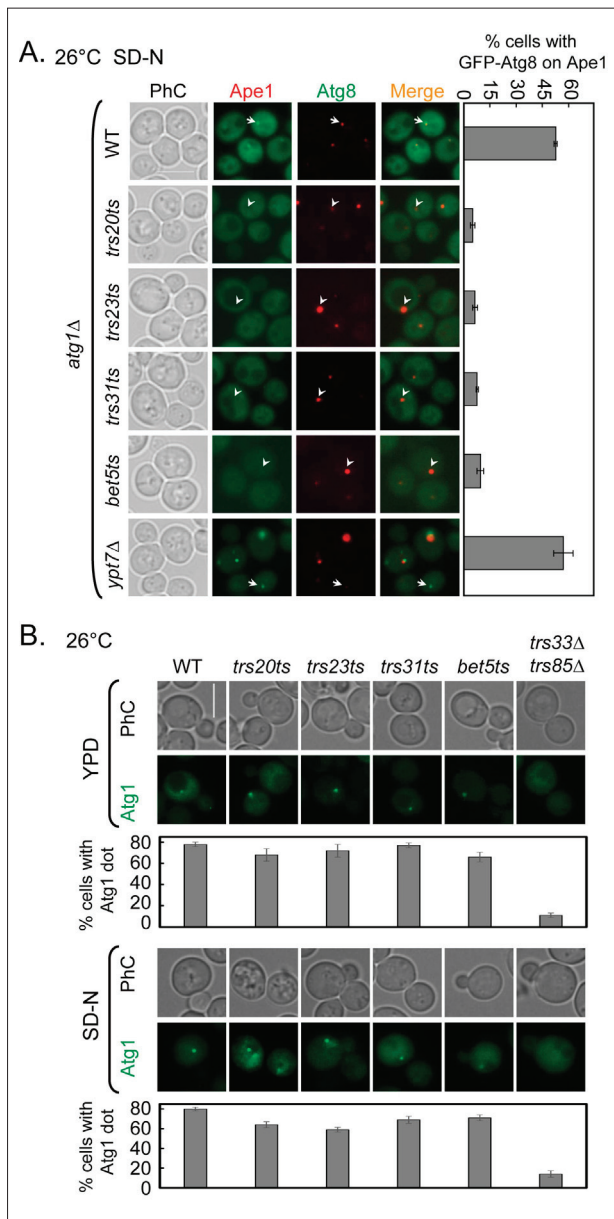
#### GFP-Atg8 dot formation depends on Atg1 in *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells

Atg1 is required for autophagy. The PAS is formed in cells lacking the ATG1 gene, but the autophago-

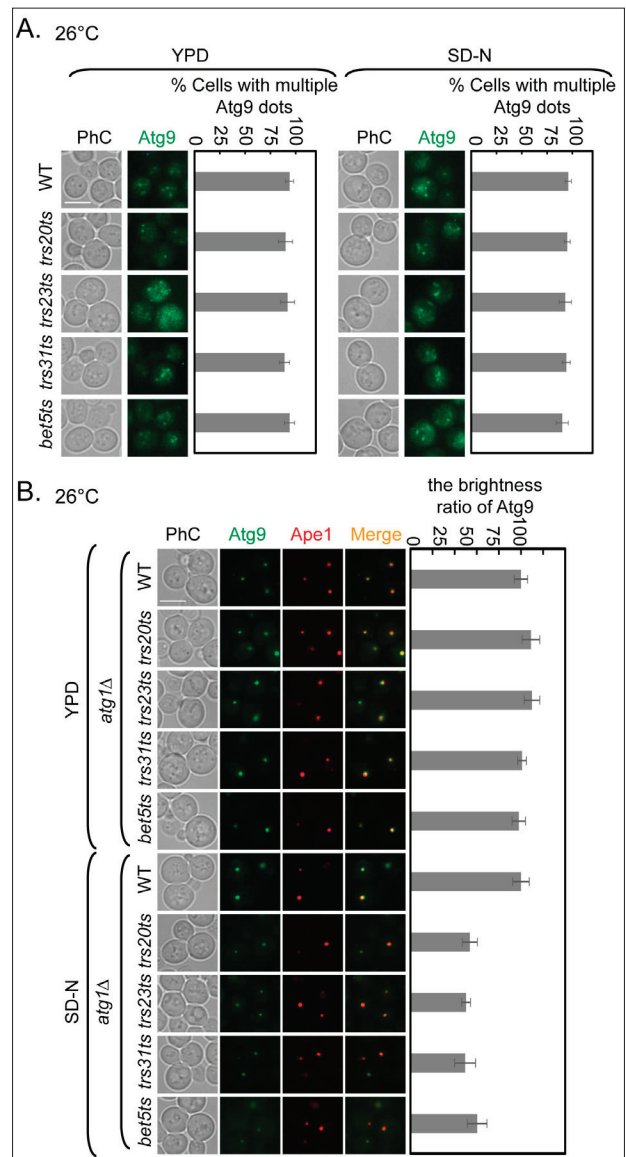
somal structures stop at this step; phagophores and autophagosomes are not formed in *atg1* $\Delta$  mutant cells [34,35]. In *atg1* $\Delta$  mutant cells, GFP-Atg8 dots accumulate and are believed to be the PAS [18,35]. According to the result shown in Fig. 1A, GFP-Atg8 was not delivered to the PAS. Thus, we wanted to determine whether the functions of Trs20, Trs23, Trs31 and Bet5 are upstream of Atg1. We deleted the ATG1 gene in WT, *trs20ts*, *trs23ts*, *trs31ts*, *bet5ts* and *ypt7* $\Delta$  strains to examine the localization of Atg8. If the common TRAPP subunits are upstream of Atg1, the deletion of ATG1 would not change the GFP-Atg8 phenotype in *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells. As shown in Fig. 3A, GFP-Atg8 dots accumulated and colocalized with RFP-Ape1, the PAS marker, in *atg1* $\Delta$  cells. The Rab GTPase Ypt7 functions downstream of Atg1 and Ypt7 participates in the fusion of the autophagosomes with the vacuole [36,37]. Multiple GFP-Atg8 dots accumulate in cells lacking YPT7. In control *ypt7* $\Delta$ *atg1* $\Delta$  mutant cells, GFP-Atg8 accumulated at a single dot that colocalized with RFP-Ape1. However, in *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells in which ATG1 had been deleted, GFP-Atg8 was dispersed in the cytoplasm and the dot did not form. This phenotype is not similar to *atg1* $\Delta$  mutant cells or common TRAPP subunit mutant cells. To extend the analysis of the relationship between common TRAPP subunits and Atg1, we examined the location of Atg1 in *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells under both non-starvation and starvation conditions. Specifically, in >78% of WT cells Atg1 localized to a single dot per cell and TRAPP III and IV regulated the localization of Atg1, which is diffuse in *trs33* $\Delta$ *trs85* $\Delta$  mutant cells (Fig. 3B) [12]. When compared to WT cells, Atg1 also localized to a single dot per cell in *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells (Fig. 3B). Based on this result, the relationship between the four common TRAPP subunits and Atg1 is not a strict upstream or downstream relationship, but is most likely parallel, regulating GFP-Atg8 transport.

#### Trs20, Trs23, Trs31 and Bet5 are required for the localization of Atg9 to the PAS

Atg9 is a transmembrane protein in yeast that is involved in autophagosome formation [38]. Atg9 is located in several intracellular organelles, including the mitochondria, and Golgi apparatus, and it nor-



**Fig. 3.** The recruitment of Atg8 to the PAS is affected in *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells. **A.** No GFP-Atg8 dots accumulated in *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells lacking *ATG1* under starvation conditions. Arrow – Ape1-RFP dot overlapping with GFP-Atg8. Arrowheads – Ape1-RFP dots that do not overlap with GFP-Atg8. The cells were grown and treated as described in Fig. 1A. Bar – 5 μm. The percentage of cells that exhibited colocalization of Atg8 and Ape1. At least 300 cells were counted in at least three fields for each strain. Error bars represent the standard deviation. **B.** Atg1 was compared in WT, *trs20ts*, *trs23ts*, *trs31ts*, *bet5ts* and *trs33Δ trs85Δ* mutant cells. Cells were transformed with a plasmid expressing pRS415-GFP-Atg1 grown under non-starvation and starvation conditions and analyzed using live-cell microscopy. Bar – 5 μm. The percentage of cells that exhibited the Atg1 dot. Results shown in this figure are representative of at least two independent experiments. Error bars represent the standard deviation.

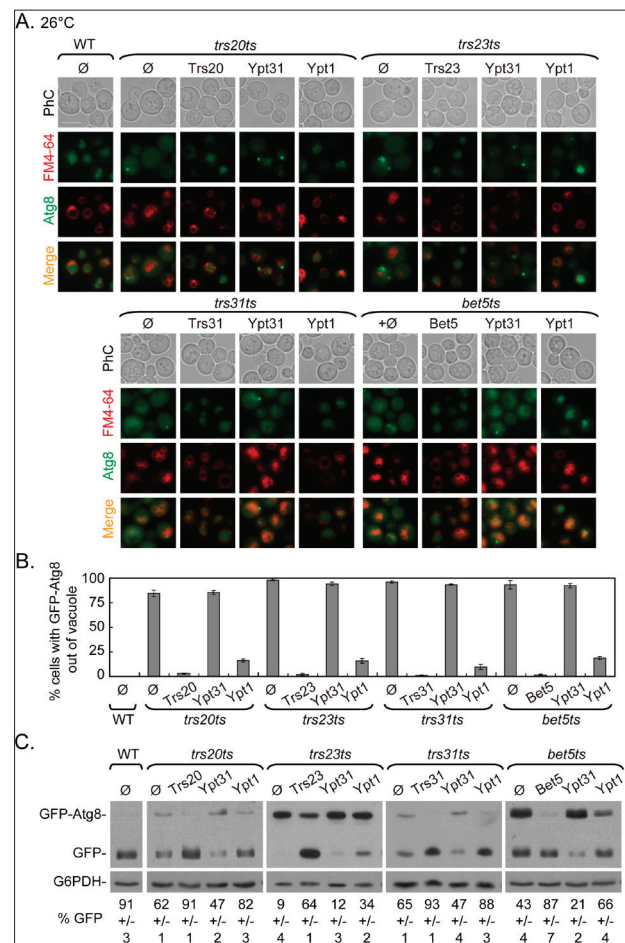


**Fig. 4.** Atg9 anterograde movement is less efficient in the *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells. **A.** WT and *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells were transformed with Atg9-3XGFP integration plasmids. The cells were grown in YPD medium to mid-log phase at 26°C; if the cells were subjected to nitrogen starvation, they were washed and transferred to SD-N medium for about 2 h to induce starvation. The experiments were repeated twice and representative results are presented. Bar – 5 μm. **B.** WT and *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells tagged with Atg9-3XGFP and RFP-Ape1 in the *atg1Δ* background. The cells were grown and treated as described in A. The results shown in this figure are representative of at least two independent experiments. Bar – 5 μm. Quantitative analysis of the brightness of the Atg9-3XGFP dot in each of the cells in (B) using ZEN 2012 software (Zeiss); the brightness of Atg9 in the WT is taken as 1. At least 300 cells from each strain were counted in at least three fields. Error bars represent the standard deviation.

mally cycles between the PAS and these organelles [39,40]. This process also requires several other Atg proteins; for example, the recycling of Atg9 from the PAS depends on the Atg1-Atg13 complex [34]. Thus, if cells lack *ATG1*, the recycling of Atg9 from the PAS is blocked and Atg9 remains trapped in the PAS [34]. Because Atg8 transport to the PAS depends on Atg9 and Atg8 is mislocalized in all mutant cells, we screened for the recycling of Atg9 in WT cells and the four common TRAPP subunits in mutant cells. Multiple Atg9-3XGFP dots were formed in all strains under non-starvation and starvation conditions (Fig. 4A), indicating that normal retrograde trafficking of Atg9 from the PAS occurred. When the *ATG1* deletion was present in background strains, under non-starvation and starvation conditions Atg9-3XGFP was concentrated as a single dot that colocalized with RFP-Ape1, the PAS marker, in WT cells. In addition, the localization of Atg9-3XGFP in all mutant cells was similar to WT cells. Atg9-3XGFP accumulated at a single dot and colocalized with RFP-Ape1 (Fig. 4B). However, the brightness of the Atg9-3XGFP dot in WT cells was greater than in the mutant cells under starvation conditions. The quantitative analysis of the results revealed that the brightness of Atg9-3XGFP in the mutant was only 50~60% of that in the WT (Fig. 4B), suggesting that anterograde trafficking of Atg9 to the PAS is not completely blocked.

### Ypt1 suppresses the autophagy defect in common subunits of TRAPP mutants

Four TRAPP complexes in yeast have been reported to exert GEF activity toward the Rab GTPases Ypt1 and Ypt31/32 [4-12]. In addition, the overexpression of *YPT1*, but not *YPT31*, suppressed the temperature sensitivity of *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells (Fig. S2), indicating that all plasmids used in this study were functional. Thus, we tested whether Ypt1 and Ypt31 rescued the autophagy defects observed in all mutant cells. First, we visualized the transport of GFP-Atg8 in all mutant cells overexpressing Ypt1 or Ypt31 under starvation conditions. When WT cells were transformed with the vector, GFP-Atg8 was transported to the vacuole normally (Fig. 5A and B). Additionally, overexpression of the gene missing in the mutant reversed the defect in GFP-Atg8 transport, whereas the overexpression of Ypt1, but not Ypt31,



**Fig. 5.** Ypt1, but not Ypt31, suppresses the GFP-Atg8 transport defect in the *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells. Empty plasmids (Ø) and plasmids expressing the gene lost in the mutant, Ypt1 and Ypt31 (all genes in pRS425 vectors) were transformed into WT and *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells. **A.** Ypt1, not Ypt31, suppressed the autophagy defect in *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells. The cells were grown as described in Fig. 4A and stained with FM4-64 during the last hour to label the vacuoles. The cells were washed twice and observed under a fluorescence microscope. Bar – 5 µm. **B.** Quantitation of the percentage of cells in which GFP-Atg8 was not observed in vacuoles. At least 300 cells from each strain were counted in at least three fields. Error bars represent the standard deviation. **C.** Ypt1, not Ypt31, suppressed GFP-Atg8 degradation in *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells. The cells were grown and treated as described in Fig. 1A and were then collected for immunoblotting with an anti-GFP antibody to examine GFP-Atg8 degradation; anti-Pgk1 antibody served as a loading control. Band densities in the blots from two independent experiments were quantified using IMAGEJ software (National Institutes of Health).

specifically suppressed the intracellular accumulation of GFP-Atg8 in all mutant cells. Second, we performed the GFP-Atg8 processing assay and the results were

consistent with the fluorescence phenotype, showing that Ypt1, but not Ypt31, can suppress the autophagy defect in all mutant cells (Fig. 5C). Thus, Trs20, Trs23, Trs31 and Bet5 regulate autophagy through Ypt1.

## DISCUSSION

Trs20, Trs23, Trs31 and Bet5 are the common subunits of the TRAPP complex [13,14]. In this study, we show that these four subunits were also involved in autophagy. We took advantage of the transport of GFP-Atg8 in *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells to directly monitor the autophagy phenotype. GFP-Atg8 was not transported to the vacuole and accumulated into one or more dots in the cytoplasm in all mutant cells; these Atg8 dots were not located in the PAS. However, the common subunits belong to four TRAPP complexes, I, II, III and IV, and we compared the defects in autophagy among strains expressing different TRAPP complexes to further define the functions of the common subunits. When the strain lacked the TRAPP III complex (*trs85Δ*), TRAPP II complex (*trs130ts*) or both the TRAPP III and TRAPP II complex (*trs85Δtrs130ts*), GFP-Atg8 was located in the PAS. Thus, Trs20, Trs23, Trs31, and Bet5 do not affect the function of the TRAPP II or TRAPP III complex in autophagy. In addition, we observed a slight impairment in the anterograde trafficking of Atg9 in these mutant cells, but the Atg9-3XGFP phenotype differed among these common subunit mutants and the specific subunit mutants. In *trs85Δ* and *trs130ts* mutant cells in the *atg1Δ* background, Atg9-3XGFP accumulated in multiple dots [18,41], and the phenotypes observed in *trs85Δ* and *trs130ts* mutants were different from those in *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells, which is consistent with the GFP-Atg8 phenotype. The TRAPP IV complex was recently shown to be required for autophagy, but multiple GFP-Atg8 dots accumulated in *trs33Δ* (specific subunit of TRAPP IV) mutant cells in our previous study [18]. This GFP-Atg8 phenotype in *trs33Δ* mutant cells differs from the phenotypes observed in *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells, suggesting that the defect in autophagy was not mediated by the TRAPP IV complex in these common subunit mutants. These phenotypic differences between common subunit and specific subunit mutants suggested that autophagy may be regulated by the

TRAPP I complex, but not the TRAPP II, TRAPP III and TRAPP IV complexes, in *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutant cells.

In yeast, autophagosome biogenesis depends on Atg8 located in the PAS [32]. In this study, Atg8 was not located in the PAS in *trs20ts*, *trs23ts*, *trs31ts* or *bet5ts* mutant cells, suggesting that the functions of Trs20, Trs23, Trs31 and Bet5 are to regulate the formation of autophagosomes. This result is consistent with the findings obtained with the *bet3ts* mutant (a common subunit of TRAPP complex mutant) [26]. The transport of Atg8 to the PAS depends on Atg9. When we examined the localization of Atg9, we found that Atg9 was located in the PAS, as the fluorescence signal was detected in the PAS in *trs20ts*, *trs23ts*, *trs31ts* and *bet5ts* mutants, although the intensity was obviously decreased. This decrease in the localization of Atg9 to the PAS may partially block the transport of Atg8 to the PAS. Previous studies have shown that COPII vesicles are not only involved in vesicle transport from the ER to the Golgi but also play roles in the transport from the ER to the PAS during autophagy [5]. Thus, Trs20, Trs23, Trs31 and Bet5 function as coregulators of COPII vesicle and Atg9-mediated transport pathways and are required for the transport of Atg8 to the PAS.

Four TRAPP complexes function as GEFs to activate the GTPases Ypt1 and Ypt31, thus regulating vesicle trafficking and autophagy [7,10-12,14,18]. The functions of all TRAPP complexes require the common subunits, Trs20, Trs23, Trs31 and Bet5. Overexpression of Ypt1, but not Ypt31, suppressed the autophagy defects in *trs23ts*, *trs20ts*, *trs31ts* and *bet5ts* mutant cells. Based on these results, we conclude that Trs20, Trs23, Trs31 and Bet5 are required for Ypt1-mediated autophagy, but not Ypt31-mediated autophagy. However, Ypt1 is involved in multiple steps in autophagy, and deletion of the common subunits did not lead to the complete loss of Ypt1 function. Thus, in the future, we will focus on determining which step of the autophagy process is regulated by the common subunits through Ypt1.

In conclusion, our work has shown a role for the common subunits Trs20, Trs23, Trs31 and Bet5 in the Cvt pathway and starvation-induced autophagy, in particular in the transport of Atg8 to the PAS. Our



genetic analysis suggested that autophagy defects in these common subunit mutants were partially caused by the reduced activity of Ypt1. These results contribute towards a comprehensive understanding of the roles of TRAPP and their regulation mechanisms in autophagy.

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