Trs33-Containing TRAPP IV: A Novel Autophagy-Specific Ypt1 GEF

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ABSTRACT Ypt/Rab GTPases, key regulators of intracellular trafficking pathways, are activated by guanine-nucleotide exchange factors (GEFs). Here, we identify a novel GEF complex, TRAPP IV, which regulates Ypt1-mediated autophagy. In the yeast *Saccharomyces cerevisiae*, Ypt1 GTPase is required for the initiation of secretion and autophagy, suggesting that it regulates these two distinct pathways. However, whether these pathways are coordinated by Ypt1 and by what mechanism is still unknown. TRAPP is a conserved modular complex that acts as a Ypt/Rab GEF. Two different TRAPP complexes, TRAPP I and the Trs85-containing TRAPP III, activate Ypt1 in the secretory and autophagic pathways, respectively. Importantly, whereas TRAPP I depletion copies Ypt1 deficiency in secretion, depletion of TRAPP III does not fully copy the autophagy phenotypes of autophagy-specific *ypt1* mutations. If GEFs are required for Ypt/Rab function, this discrepancy implies the existence of an additional GEF that activates Ypt1 in autophagy. Trs33, a nonessential TRAPP subunit, was assigned to TRAPP I without functional evidence. We show that in the absence of Trs85, Trs33 is required for Ypt1-mediated autophagy and for the recruitment of core-TRAPP and Ypt1 to the preautophagosomal structure, which marks the onset of autophagy. In addition, Trs33 and Trs85 assemble into distinct TRAPP complexes, and we term the Trs33-containing autophagy-specific complex TRAPP IV. Because TRAPP I is required for Ypt1-mediated secretion, and either TRAPP III or TRAPP IV is required for Ypt1-mediated autophagy, we propose that pathway-specific GEFs activate Ypt1 in secretion and autophagy.

KEYWORDS macro-autophagy; Ypt1; Ypt/Rabs; TRAPP complex; TRAPP III; GEF; Trs33; TrappC6A; TrappC6B

IN autophagy, cargo destined for degradation is engulfed by the double-membrane autophagosomes (APs), and is shuttled to the lysosome. Depending on the cargo and the growth conditions, autophagy can be generic or selective (Nair and Klionsky 2005; Nakatogawa *et al.* 2009). All autophagy pathways start with the formation of the preautophagosomal structure (PAS), which is comprised of the autophagy-specific proteins Atgs and membrane (Weidberg *et al.* 2011). Like all other intracellular trafficking pathways, autophagy is regulated by the conserved Ypt/Rab GTPases (Ao *et al.* 2014). When stimulated by guanine-nucleotide exchange factors (GEFs), Ypt/Rabs bind their downstream effectors, which include intracellular trafficking machinery components, like motors and tethers (Segev 2001). Recently, a role for Ypt/Rabs in coordination of intracellular trafficking steps and pathways has been proposed (Segev 2011; Lipatova *et al.* 2015).

In yeast, three Ypts regulate the different steps of autophagy: Ypt1 is required for the beginning of autophagy, PAS formation (Lipatova *et al.* 2012), while Vps21 and Ypt7 play a role in later steps that lead to the fusion of APs with the vacuole (the yeast lysosome) (Wang *et al.* 2002; Chen *et al.* 2014). Ypt31/32 were also implicated in autophagy, but the step is not clear (Zou *et al.* 2013). The established role of Ypt1 is the regulation of ER-to-Golgi transport (Segev 1991), and that of Vps21 and Ypt7 is in endocytosis (Schimmoller and Riezman 1993; Singer-Kruger *et al.* 1994). Interestingly, whereas both Vps21 and Ypt7 function in autophagy and endocytosis in the context of the same GEF-GTPase-effector modules (Wang *et al.* 2002; Chen *et al.* 2014), Ypt1 does not. Instead, two different TRAPP complexes, TRAPP I and TRAPP III, stimulate Ypt1 in the secretory and autophagy pathways, respectively (Lipatova *et al.* 2015). Likewise, in secretion and autophagy Ypt1 interacts with different effectors; *e.g.*, Atg11 is an autophagy-specific effector of Ypt1 (Lipatova *et al.* 2012).

Currently, three TRAPP complexes are known: I, II, and III, and their multiple subunits are conserved from yeast to human

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cells (Kim *et al.* 2016). TRAPP I contains four core essential subunits, and TRAPP III contains the nonessential Trs85 in addition to core-TRAPP and the Trs20 adaptor. Both TRAPP I and TRAPP III act as Ypt1 GEFs (Morozova *et al.* 2006; Cai *et al.* 2008; Lynch-Day *et al.* 2010). TRAPP II contains two essential large subunits, Trs120 and Trs130, the nonessential subunits Trs65 and Trs33, the Trs20 adaptor, and core-TRAPP. TRAPP II localizes to *trans*-Golgi, and, while still controversial, acts as a GEF for Ypt31/32 (Kim *et al.* 2016). A role for a third nonessential subunit, Trs33, was shown in the assembly of TRAPP II only in the absence of Trs65 (Tokarev *et al.* 2009) (Figure 1A).

Ypt1 is essential for autophagy based on the fact that the autophagy phenotypes of autophagy-specific *ypt1* mutations are as severe as those of core-*atg* deletions (Lynch-Day *et al.* 2010; Lipatova *et al.* 2012). In contrast, whereas Trs85 plays a role in autophagy, it is not essential for this process (Lipatova *et al.* 2012). The question that drove this research project is why Ypt1 is essential to autophagy whereas its autophagy-specific GEF is not. Here, we show that Trs33 plays a role in autophagy, and together with Trs85 is required for Ypt1-mediated PAS formation. Based on results presented here, we propose the existence of a new TRAPP complex, the Trs33-containing TRAPP IV, which together with TRAPP III activates Ypt1 in the onset of autophagy. Because all players are conserved from yeast to human cells, we propose that the human homologs of Trs33, TrappC6A and B, regulate Rab1-mediated autophagy.

Materials and Methods

Strains, plasmids, and reagents

Strains used in this paper are summarized in Supplemental Material, Table S1. Plasmids used in this study are summarized in Table S2. All chemical reagents were purchased from Fisher Scientific (Hampton, NH), except for the following: Nitrogen bases were purchased from US Biological (Swampscott, MA); ProtoGel for Western blots from National Diagnostics (Atlanta, GA); Bacto peptone and Bacto agar from BD Difco (Franklin Lakes, NJ); salmon testes DNA, amino acids, *p*-nitrophenyl phosphate, and protease inhibitors from Sigma (St. Louis, MO); glutathione Sepharose 4B beads from Amersham Biosciences (Little Chalfont, UK); glass beads from BioSpec Products (Bartlesville, OK); EDTA-free protease inhibitor mixture from Roche Diagnostics (Indianapolis, IN); restriction enzymes and buffers from New England Biolabs (Ipswich, MA).

Antibodies used in this study included mouse monoclonal anti-GFP (Roche Diagnostics), rabbit anti-GST (Molecular Probes, Eugene, OR), rabbit anti-Ape1 (a kind gift from Dr. Ohsumi), affinity-purified rabbit anti-Ypt1 (Segev *et al.* 1988), rabbit anti-G6PDH (Sigma), goat anti-rabbit HRP and goat anti-mouse HRP (GE Healthcare), and TexasRed dye-conjugated goat anti-rabbit (Jackson ImmunoResearch).

Yeast culture conditions and viability analysis

Medium preparation and yeast culture growth for nitrogen starvation shift experiments were done as described (Segev and Botstein 1987).

Protein level analyses

To determine levels of GST- or GFP-tagged proteins in yeast lysates, exponentially growing cell cultures ($7 \times OD_{600}$) were spun down, resuspended in 100 μl of Laemmli buffer, boiled, vortexed with glass beads, and subjected to Western blot analysis using appropriate antibodies. Preparation of protein lysates for Ape1 and GFP-Atg8 processing analyses was done as described (Cheong and Klionsky 2008). ImageJ was used for quantification of protein bands.

Autophagy assays

Cell survival, Atg8-GFP processing, and Ape1 processing assays were done as previously described (Lipatova *et al.* 2012). Alkaline phosphatase activity assay of Pho8Δ60 was done as previously described (Abeliovich *et al.* 2003).

GST pull-downs from yeast extracts

Yeast culture growth for pull-down experiments and purification of GST-tagged proteins was done as previously described (Morozova *et al.* 2006).

Microscopy

Live-cell microscopy was done as follows: Wild-type and mutant cells carrying constructs for expression of GFP-, YFP-, yEGFP-, RFP-, or mCherry-tagged protein(s) were grown to midlog phase in appropriate selective media. Fluorescent microscopy was performed using a deconvolution Axioscope microscope (Carl Zeiss) with FITC and TexasRed filter sets. Immuno-fluorescence microscopy using affinity-purified anti-Ypt1 antibodies was done as previously described (Segev *et al.* 1988). Colocalization was quantified by counting puncta that do or do not overlap on a single plane. For statistical analyses we used Student's *t*-test.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

Results

We have noticed that the autophagy phenotypes of the autophagy-specific *ypt1-1* mutation are more severe than those of *trs85*Δ mutant cells (Lipatova *et al.* 2012). While it was possible that Ypt1 can function in autophagy without being activated by an autophagy-specific GEF, we hypothesized that there is an additional GEF that can activate Ypt1 in autophagy. One candidate was Trs33, which was originally identified as a TRAPP I/II subunit using pull-down experiments (Sacher *et al.* 2001). However, unlike other TRAPP I subunits, Trs33 is not essential for viability (Sacher *et al.* 2000) or for the Ypt1-GEF activity of TRAPP I (Kim *et al.* 2006). Interestingly, a negative genetic interaction was reported in high-throughput screens between *TRS33* and *TRS85*. Specifically, *trs33*Δ *trs85*Δ double deletion cells exhibit slower growth than each of the single deletions (Schuldiner *et al.*

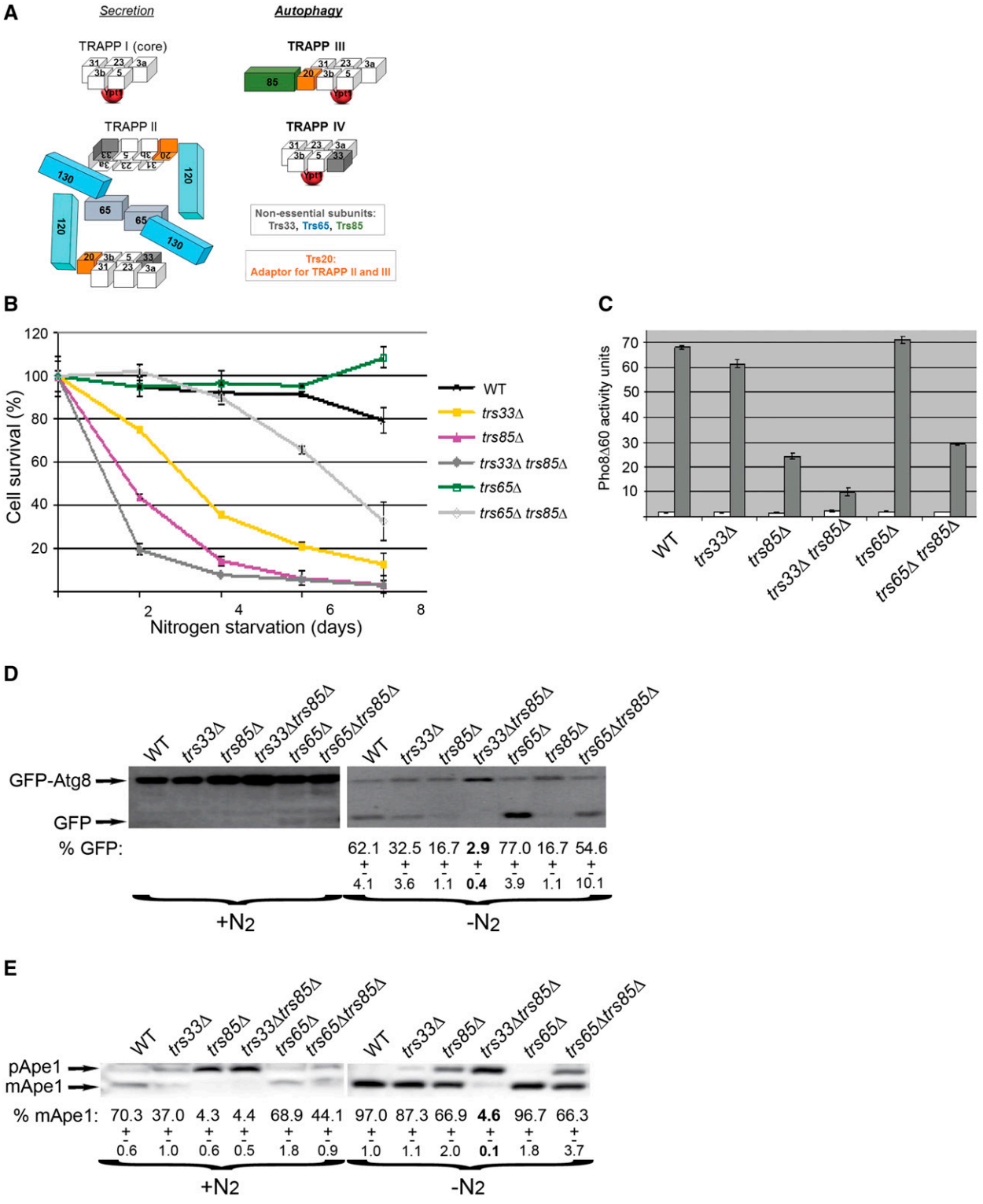

Figure 1 Deletion of *TRS33*, but not *TRS65*, exacerbates the autophagy phenotypes of *trs85*Δ mutant cells. (A) Diagram showing TRAPP complex composition. Three TRAPP complexes act as GEFs for Ypt1: I, III, and IV. We propose that TRAPP I is composed of core subunits, TRAPP III contains core TRAPP, the Trs20 adaptor, and the nonessential subunit Trs85 (Kim *et al.* 2016), and based on results presented here, TRAPP IV contains core TRAPP and the nonessential subunit Trs33. TRAPP II contains core TRAPP, Trs20, Trs120, Trs130, and the nonessential subunits Trs65 and Trs33 (in the absence of Trs65). Diagram and positioning of Ypt1 in TRAPP I and III are based on published structures (Cai *et al.* 2008; Tan *et al.* 2013); numbers stand for TrsN, except for Bet3 and Bet5. (B–E) Generic (B–D) and selective (E) autophagy phenotypes were determined in the following strains: wild type (WT), *trs33*Δ, *trs85*Δ, *trs33*Δ *trs85*Δ, *trs65*Δ, and *trs65*Δ *trs85*Δ mutant cells. (B) Cell survival during nitrogen starvation. Cells were shifted from rich (YPD) medium to medium without nitrogen and the percent of viable cells at indicated time points (*x*-axis) is shown as percent of viable cells at time 0 (*y*-axis). Right: strain

2005; Costanzo *et al.* 2010). Because *ypt1-1* mutant cells also exhibit slower growth than wild-type cells (Segev and Botstein 1987), we decided to explore a possible role for Trs33 in autophagy.

Trs33 is required for autophagy in trs85Δ mutant cells

The effect of *TRS33* deletion in wild-type and *trs85*Δ mutant cells on autophagy was determined. Generic autophagy was determined by survival, delivery of the cytosolic protein Pho8Δ60 to the vacuole, and processing of GFP-Atg8 and Ape1 under nitrogen starvation. The selective autophagy cytosol-to-vacuole (CVT) pathway was determined by processing of Ape1 under normal growth conditions (Lipatova *et al.* 2012). The *trs33*Δ mutation resulted in mild generic autophagy phenotypes under nitrogen starvation, *i.e.*, viability, alkaline phosphatase assay, processing of GFP-Atg8, and processing of Ape1. This single deletion also caused a defect in selective autophagy, based on Ape1 processing under normal growth conditions. The observed autophagy phenotypes of *trs33*Δ are less severe than those of *trs85*Δ. However, deletion of *TRS33* in *trs85*Δ mutant cells resulted in severe generic and selective autophagy phenotypes (Figure 1, B–E), more severe than those of either single deletion and similar to those reported for the *ypt1-1* mutation (Lipatova *et al.* 2012).

We have previously reported that Trs33 is important for TRAPP II assembly in the absence of the TRAPP II nonessential subunit Trs65 (Tokarev *et al.* 2009), and that mutation in the TRAPP II-specific subunit Trs130, *trs130ts*, can cause defects in autophagy at the nonpermissive temperature (Zou *et al.* 2013). Therefore, we wished to determine whether the effect of the *trs33*Δ mutation on autophagy is due to a defect in TRAPP II function. First, deletion of *TRS65* in wild-type cells does not cause generic or selective autophagy defects in WT cells. Second, deletion of *TRS65* in *trs85*Δ mutant cells does not cause more severe phenotypes than those of *trs85*Δ mutant cells (Figure 1, B–E). Moreover, we have previously shown that while the *trs130ts atg11*Δ double mutation results in a more severe autophagy phenotype than that of *atg11*Δ (Zou *et al.* 2013), the *trs85*Δ *atg11*Δ double mutation does not (Lipatova *et al.* 2012). Similar to *trs85*Δ, the *trs33*Δ *atg11*Δ double mutation also does not cause a more severe autophagy phenotype than that of the *atg11*Δ deletion (Figure S1), suggesting that Trs33 functions in autophagy in the context of the Ypt1-Atg11 module while TRAPP II does not. Together, these results indicate that the effects of *trs33*Δ on autophagy are not due to its role in TRAPP II assembly. In agreement with this conclusion we show below that Trs33 and Trs85, but not Trs65, are required for bringing TRAPP to PAS, and that Ypt31 does not suppress the autophagy defect of *trs33*Δ, while it can suppress that of *trs130ts* mutant cells (Zou *et al.* 2013).

Trs33 functions in autophagy through Ypt1

Ypt1 regulates autophagy (Lynch-Day *et al.* 2010; Lipatova *et al.* 2012) and a Trs33-containing TRAPP complex can act as its GEF *in vitro* (Kim *et al.* 2006). Therefore, we tested whether the role of Trs33 in autophagy is through Ypt1. First, we have previously shown that Ypt1 plays a role in the onset of autophagy: the formation of PAS (Lipatova *et al.* 2012). Specifically, whereas in >80% of wild-type cells, the core-Atg components – Atg11, Atg8, and Atg1 – localize to a single dot per cell that represents PAS or AP, PAS is not formed in >70% of *ypt1-1* mutant cells; *i.e.*, Atg11 and Atg8 show multiple dots and Atg1 is diffuse (even though their levels do not change) (Lipatova *et al.* 2012). Here, the effect of deletion of *TRS33* in wild-type and *trs85*Δ mutant cells on PAS formation was determined by following the cellular distribution of Atg11, Atg8, and Atg1. While the proportion of cells with multiple Atg11 or Atg8 dots is not significantly higher in *trs33*Δ and *trs85*Δ single mutant cells when compared to wild-type cells, ~75% of *trs85*Δ, but not *trs33*Δ, mutant cells exhibit diffuse Atg1 staining (Figure 2). This is in agreement with the more severe autophagy phenotypes of *trs85*Δ than those of *trs33*Δ mutant cells. Importantly, the *trs33*Δ *trs85*Δ double mutant cells exhibit more severe defects in Atg11, Atg8, and Atg1 distribution than the single deletions (Figure 2), similar to those of *ypt1-1* (Lipatova *et al.* 2012). As in the *ypt1-1* mutant cells, the changes in the distribution of PAS components were not caused by changes in GFP-Atg protein levels (Figure S2). These results support the idea that, like Ypt1, either a Trs33- or Trs85-containing TRAPP complex is required for PAS formation.

Second, we expect that overexpression of the Ypt substrate of a GEF might suppress the phenotypes of partial depletion of its GEF. For example, we have previously shown that overexpression of Ypt31, but not Ypt1, can suppress the autophagy phenotype of *trs130ts* (Zou *et al.* 2013). This result is in agreement with our view that TRAPP II acts as a

legend. (C) Pho8Δ60 alkaline phosphatase assay. Lysates were prepared from cells grown in YPD medium (open bars) and after 4 hr of nitrogen starvation (shaded bars), and ALP activity in the lysates was determined. Pho8Δ60 activity units represent nmol nitrophenol/mg protein. (D) GFP-Atg8 processing. Strains transformed with a plasmid expressing GFP-Atg8 were grown to midlog phase in rich medium and shifted to medium without nitrogen for 4 hr. Processing of GFP-Atg8 was determined by immuno-blot analysis using anti-GFP antibodies (percent GFP is shown at the bottom). All strains exhibit similar Atg8 processing under nonstarvation conditions (left). Under nitrogen starvation (right), >60% of the GFP-Atg8 protein in wild-type and *trs65*Δ mutant cells is processed to the GFP size, whereas this processing is defective in cells deleted for *TRS33* and/or *TRS85*. (E) Ape1 processing during normal growth and under nitrogen starvation. Cells were grown to midlog phase in rich medium and shifted to medium without nitrogen for 4 hr. Processing of Ape1 was determined by immuno-blot analysis using anti-Ape1 antibodies (percent mApe1 is shown at the bottom). In wild-type and *trs65*Δ mutant cells, ~70% of the Ape1 protein (pApe1) is processed into the mature form (mApe1) under nonstarvation conditions, whereas >95% is processed under starvation. Cells deleted for *TRS33* and/or *TRS85* exhibit an Ape1 processing defect under starvation and nonstarvation conditions. Error bars and ± represent SD; results shown in this figure are representative of at least two independent experiments.

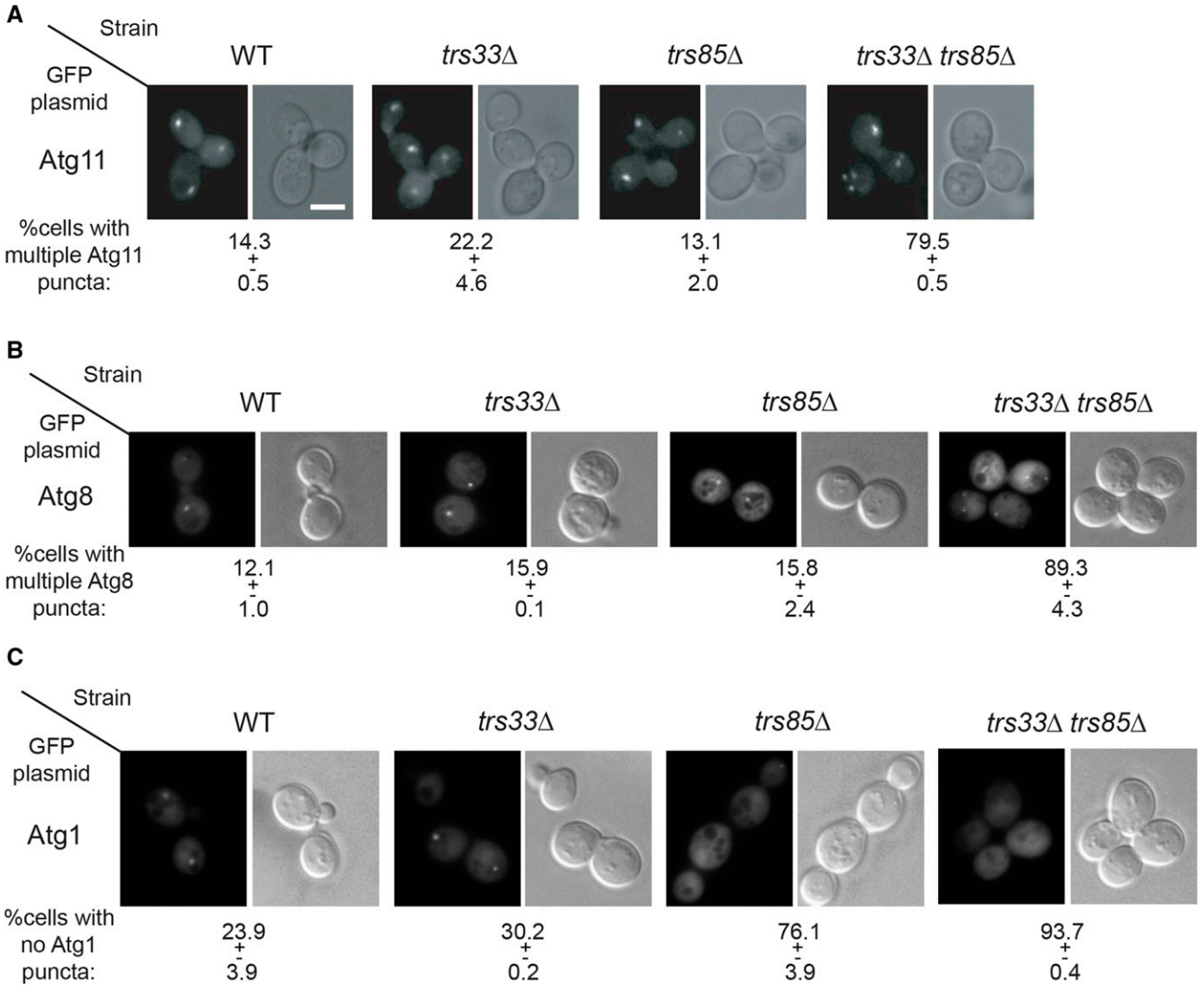

Figure 2 Deletion of *TRS33* in *trs85*Δ mutant cells causes a severe defect in PAS formation. The distribution patterns of PAS components, Atg11, Atg8, and Atg1, were compared in WT, *trs33*Δ, *trs85*Δ, and *trs33*Δ *trs85*Δ mutant strains. Cells were transformed with a plasmid expressing from the alcohol dehydrogenase *(ADH1)* promoter GFP-Atg11 (A), GFP-Atg8 (B), or GFP-Atg1 (C), grown under normal conditions, and analyzed using live-cell microscopy. Whereas in WT cells these PAS markers localize to a single dot per cell, in mutant cells Atg11 and Atg8 show multiple puncta, and Atg1 is diffuse. Shown for each strain, GFP (left) and DIC (right). Bottom: Percent cells with multiple Atg11 and Atg8 puncta (A and B) or with no Atg1 puncta (C). The differences between *trs33*Δ and WT are not statistically significant (*P*-value = 0.1 for GFP-Atg11, GFP-Atg8, and GFP-Atg1); for GFP-Atg1 the differences between *trs85*Δ and WT and between *trs85*Δ and *trs33*Δ *trs85*Δ are statistically significant (*P*-values are 0.0005 and 0.015, respectively). At least 45 cells visualized for each strain. ± represents SD. Results shown in this figure are representative of at least two independent experiments. ± represents SD. Bar, 5 μm.

Ypt31/32 GEF (Morozova *et al.* 2006). We have also shown that overexpression of Ypt1, but not Ypt31, can suppress the Ape1 processing defect of *trs85*Δ mutant cells (Lipatova *et al.* 2012), which is in agreement with the role of Trs85-containing TRAPP III as a GEF for Ypt1 (Lynch-Day *et al.* 2010). Here, we show that overexpression of Ypt1, but not Ypt31, can suppress the mild Ape1 processing defect of *trs33*Δ mutant cells (Figure 3A and Figure S3). In addition, overexpression of Ypt1 can partially suppress the growth defects of *trs33*Δ and *trs85*Δ mutant cells under nitrogen starvation (Figure 3B). In contrast, overexpression of Ypt1 cannot suppress the severe Ape1 and growth phenotypes of *trs33*Δ *trs85*Δ double mutant cells (Figure 3 and Figure S3). These results indicate that, like Trs85, Trs33 functions in autophagy through activation of Ypt1, and that Ypt1 activation by an autophagy-specific GEF is required for its function in autophagy.

Trs33 and Trs85 bring core-TRAPP and Ypt1 to PAS

If Trs33 and Trs85 act in the context of TRAPP to activate Ypt1, which in turn mediates PAS formation, we expect that in their absence, TRAPP and Ypt1 do not colocalize with PAS markers. The colocalization of the tagged core-TRAPP subunit Trs23 with the PAS marker mCherry-Ape1 was

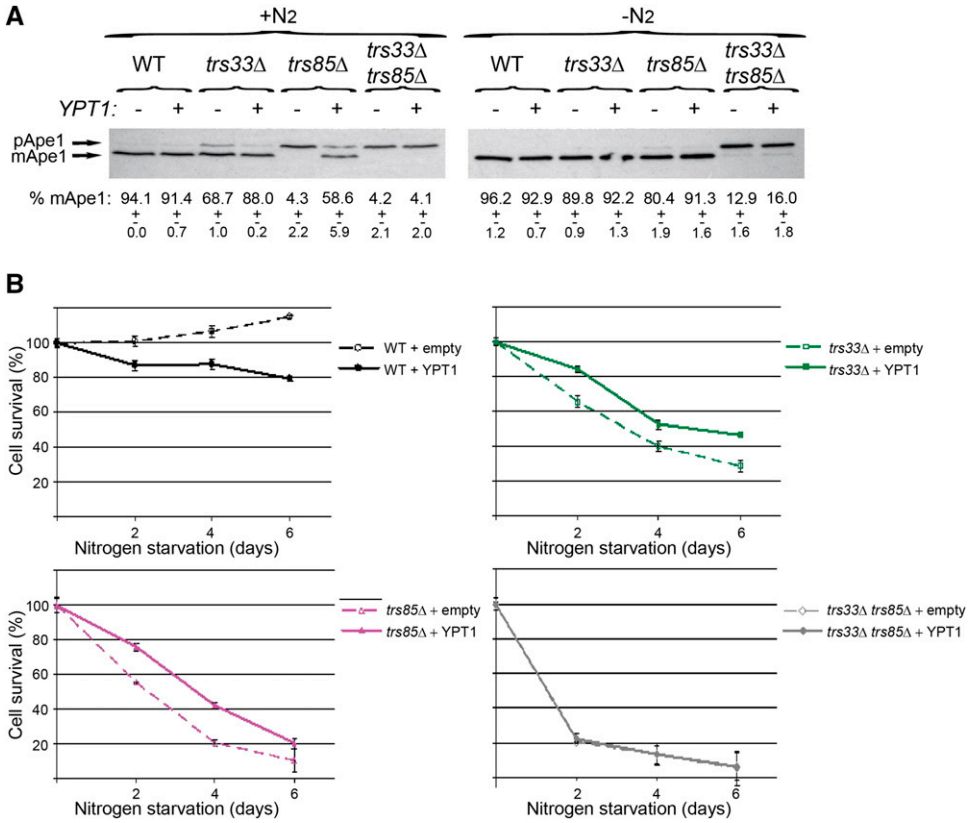

Figure 3 Overexpression of Ypt1 can suppress the autophagy phenotypes of *trs33*Δ and *trs85*Δ, but *trs33*Δ *trs85*Δ, mutant cells. WT, *trs33*Δ, *trs85*Δ, and *trs33*Δ *trs85*Δ mutant cells were transformed with a 2 μ plasmid for overexpression of Ypt1 (+), or empty plasmid as a negative control (−). Cells were tested for: (A) Ape1 processing under normal growth (left) and nitrogen starvation (right), and (B) viability under nitrogen starvation, as described for Figure 1. Results shown in this figure are representative of at least two independent experiments. Error bars and ± represent SD.

determined by live-cell microscopy in wild-type, *trs33*Δ, *trs85*Δ, and *trs33*Δ *trs85*Δ mutant cells. Whereas Trs23 colocalizes with Ape1 in ~80% of wild-type cells, colocalization was observed in only ~15% of the *trs33*Δ *trs85*Δ double mutant cells. The single deletions, *trs33*Δ and *trs85*Δ, result in a partial defect (~55%, Figure 4A). In contrast, deletion of the TRAPP II subunit Trs65 does not affect Trs23 localization to PAS, and does not exacerbate the *trs85*Δ phenotype (Figure 4A). These results suggest that together Trs33 and Trs85 are required for recruitment of core-TRAPP to PAS, and support the idea that Trs33 does not function through TRAPP II.

The colocalization of Ypt1 with the PAS marker yEGFP-Atg8 was determined in wild-type, *trs33*Δ, *trs85*Δ, and *trs33*Δ *trs85*Δ mutant cells, using immuno-fluorescence microscopy. Whereas colocalization of Ypt1 and Atg8 was observed in ~60% of wild-type cells, no colocalization was observed in the *trs33*Δ *trs85*Δ double mutant cells. Deletion of *TRS33* did not have an effect, while localization of Ypt1 to PAS was partially impaired in *trs85*Δ single mutant cells (Figure 4B). These results are in agreement with the severity of the autophagy phenotypes of the single and double mutant cells shown above.

Trs33 and Trs85 localize to PAS independently

The aforementioned evidence suggests that either Trs33 or Trs85 is required for bringing core-TRAPP and Ypt1 to PAS in the onset of autophagy. Therefore, we expect that Trs33 and Trs85 can be recruited to PAS independently of each other. To test this idea, the colocalization of fluorescently tagged Trs33 or Trs85 with a PAS marker was compared in wild type and in cells deleted for the other subunit. Trs33 tagged on the chromosome with yEGFP is functional based on the observation that, unlike *trs33*Δ, it does not cause a growth defect under nitrogen starvation (Figure 5A). Live-cell microscopy shows about four Trs33-yEGFP puncta/cell slice, of which 70% colocalizes with the core-TRAPP subunit Trs23, 55% colocalizes with a *trans*-Golgi marker (Chc1), and only 15% colocalizes with a *cis*-Golgi marker (Cop1) (Figure 5B). These results indicate that Trs33-yEGFP can incorporate into the TRAPP complex, and agree with our previous observation that Trs33 can function in the context of TRAPP II in the late Golgi (Tokarev *et al.* 2009). While Trs85-GFP was reported to colocalize with the Golgi marker Sec7 (especially under starvation) and not with a PAS marker (Shirahama-Noda *et al.* 2013), we have previously shown using bimolecular fluorescence complementation analysis that Trs85-Ypt1 interaction puncta do not colocalize with any exocytic compartment, including the Golgi (Lipatova *et al.* 2012). Thus, it is still unclear whether Trs33 and Trs85 colocalize with each other on secretory compartments.

In addition to its localization to the Golgi, in about 50% of wild-type cells that contain PAS marked with mCherry-Atg8, Trs33-yEGFP localizes to PAS (Figure 6A), and deletion of *TRS85* does not affect this localization. Functional Trs85-yEGFP (Lipatova *et al.* 2012) localizes to PAS in >80% of cells that contain PAS, and deletion of TRS33 does not affect this localization either (Figure 6B). Thus, the

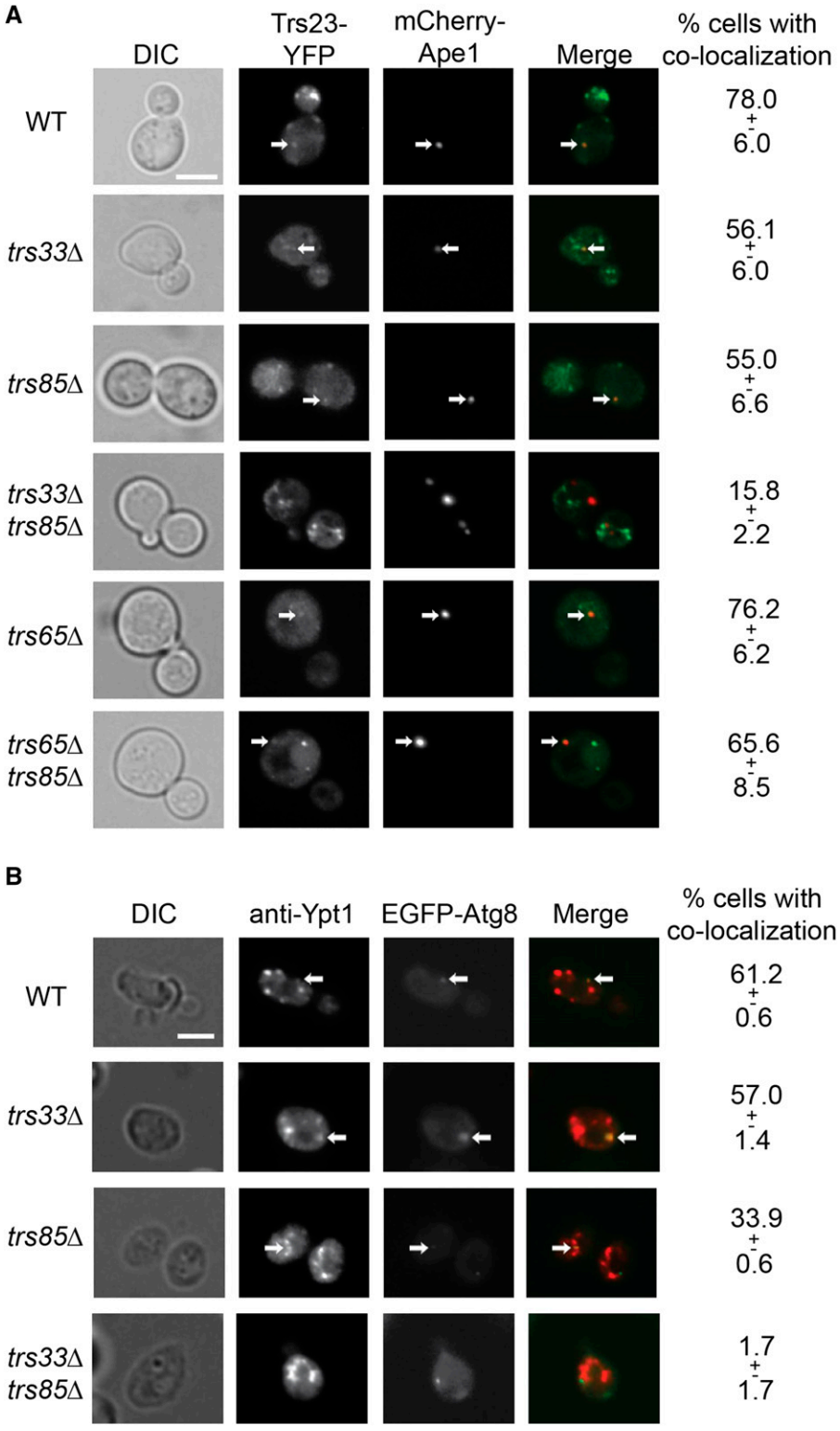

Figure 4 Trs33 and Trs85 are required for the localization of core-TRAPP and Ypt1 to PAS. (A) The core-TRAPP subunit Trs23 was tagged on the chromosome with YFP in the indicated strains (left column). Cells were transformed with a plasmid expressing the PAS marker mCherry-Ape1, and the colocalization of Trs23 and Ape1 was determined using live-cell microscopy. From top to bottom: WT, *trs33*Δ, *trs85*Δ, *trs33*Δ *trs85*Δ, *trs65*Δ, and *trs65*Δ *trs85*Δ. Shown from left to right: DIC, green, red, merge, and % cells with colocalization. The differences between WT and the single deletions *trs33*Δ and *trs85*Δ are statistically significant (*P*-values = 0.05). (B) Cells were transformed with a plasmid expressing the PAS marker EGFP-Atg8, and the localization of Ypt1 was determined by immuno-fluorescence microscopy. From top to bottom: WT, *trs33*Δ, *trs85*Δ, and *trs33*Δ *trs85*Δ. Shown from left to right: DIC, Ypt1, Atg8, merge, and % cells with colocalization. While the difference between WT and *trs33*Δ is not statistically significant (*P*-value = 0.1), the difference between WT and *trs85*Δ is (*P*-value = 0.0005). Arrows point to colocalizing puncta. At least 35 cells visualized for each strain. Results shown in this figure are representative of at least two independent experiments. ± represents SD. Bar, 5 μm.

localization of Trs33 and Trs85 to PAS is not dependent on each other.

The ability of Trs33 and Trs85 to localize to PAS independently suggests that they can assemble into separate TRAPP complexes. To test this idea, we used a GST pull-down assay. Trs33, Trs85, or the core-TRAPP subunit Bet3 were tagged on the chromosome with GFP in wild-type cells. The cells were transformed with a plasmid that expresses GST, GST-Bet5 (core-TRAPP), GST-Trs33, or GST-Trs85. The coprecipitation of GFP-tagged proteins with the GST-tagged protein (or GST as a negative control) was determined using immuno-blot analysis. The core-TRAPP

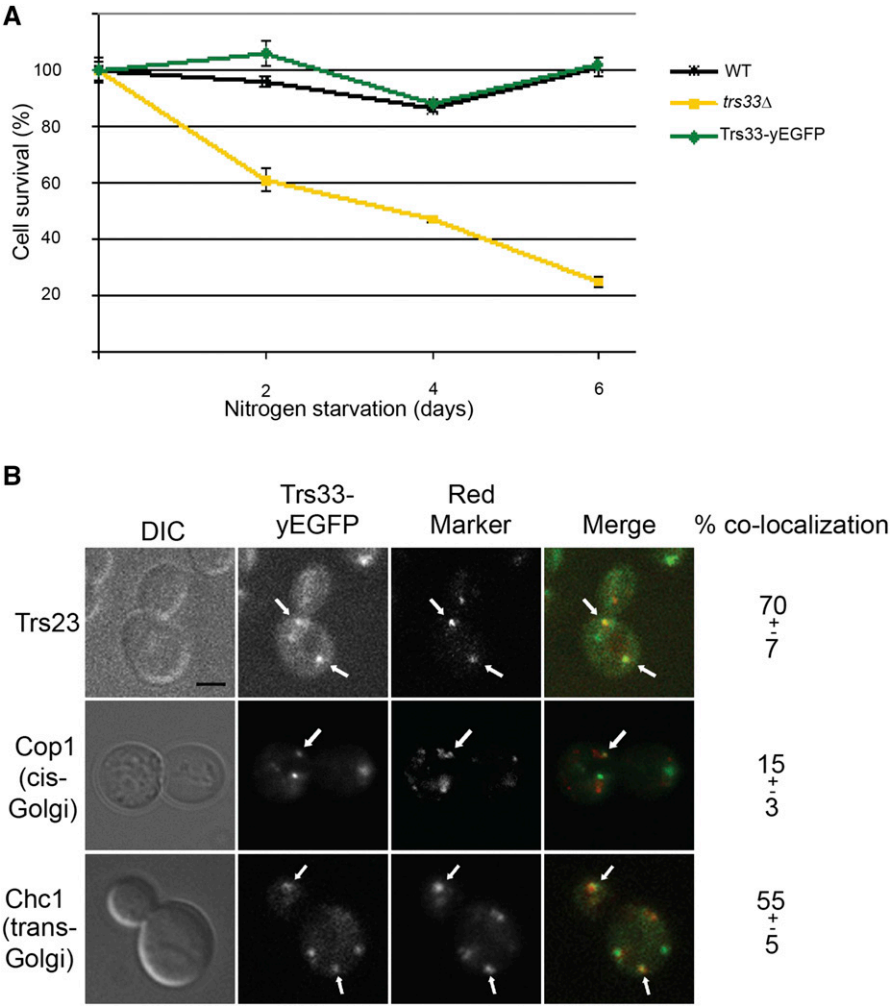

Figure 5 Trs33-yEGFP is functional and localizes to the Golgi. Trs33 was tagged with yEGFP at its C-terminus on the chromosome. (A) Trs33-yEGFP is functional autophagy. The survival of these cells under nitrogen starvation was compared with those of WT and *trs33*Δ mutant cells. Whereas *trs33*Δ mutant cells exhibit an autophagy phenotype, cells expressing Trs33-yEGFP behave like WT. Error bars represent SD. (B) Trs33-yEGFP colocalizes with a core-TRAPP subunit and Golgi markers. Trs33-yEGFP was tagged in cells also expressing RFP-tagged the core-TRAPP subunit Trs23 (top), the *cis*-Golgi marker Cop1, or the *trans*-Golgi marker Chc1. The colocalization of Trs33 and the red markers was determined using live-cell microscopy. Sown from left to right: DIC, green, red, merge, and % dots of Trs33-GFP that colocalize with the red marker. Arrows point to colocalizing puncta. At least 43 cells visualized for each strain. Results shown in this figure are representative of two independent experiments. Bar 2 μm.

subunit Bet3-GFP coprecipitated with GST-tagged Bet5, Trs33, and Trs85, but not GST. This result shows that all the GST-tagged proteins, including Trs33 and Trs85, can pull down a core-TRAPP subunit. In contrast, Trs33-GFP coprecipitated with GST-Bet5, while very little coprecipitates with GST-Trs85 or GST. Likewise, Trs85-GFP coprecipitated with GST-Bet5, but not with GST-Trs33 or GST (Figure 6, C–E). These results show that both Tss33-GFP and Trs85-GFP can precipitate with TRAPP (GST-Bet5). However, while GST-tagged Trs33 and Trs85 can pull down a core-TRAPP subunit (Bet3-GFP), they cannot pull down each other. These results support the idea that Trs33 and Trs85 form separate TRAPP complexes.

Discussion

Two autophagy-specific TRAPPs

Although Trs85 plays a role in autophagy, it is not essential for this process. Results presented here show that in addition to Trs85, another TRAPP subunit, Trs33, is important for activation of Ypt1 in autophagy. While *trs33*Δ mutant cells exhibit mild autophagy phenotypes, *trs33*Δ *trs85*Δ double mutant cells exhibit more severe autophagy defects than either deletion alone, similar to defects observed in *ypt1-1* mutant cells. In addition, like Ypt1 and Trs85, Trs33 plays a role in the onset of autophagy, namely PAS assembly. Moreover, like Trs85, Trs33 functions in the context of TRAPP to activate Ypt1, because either Trs33 or Trs85 is required for efficient recruitment of core-TRAPP and Ypt1 to PAS. The ability of overexpressed Ypt1 to suppress the autophagy defects of *trs33*Δ and *trs85*Δ mutant cells supports this idea. Finally, Trs33 and Trs85 can localize to PAS independently of each other, and form biochemically distinct TRAPP complexes. Therefore, we conclude that two TRAPP complexes can activate Ypt1 in autophagy: Trs85-containing TRAPP III and Trs33-containing TRAPP IV.

Trs33 is currently considered a TRAPP I/II subunit based on coprecipitation with core-TRAPP (Sacher *et al.* 2001). However, there is no evidence for a role for Trs33 with TRAPP I in secretion, and allocation to a specific TRAPP complex solely based on coprecipitation can be misleading because coprecipitation of TRAPP subunits is dependent on the purification conditions (Choi *et al.* 2011; Brunet *et al.* 2012). For example, based on coprecipitation with core-TRAPP, Trs85

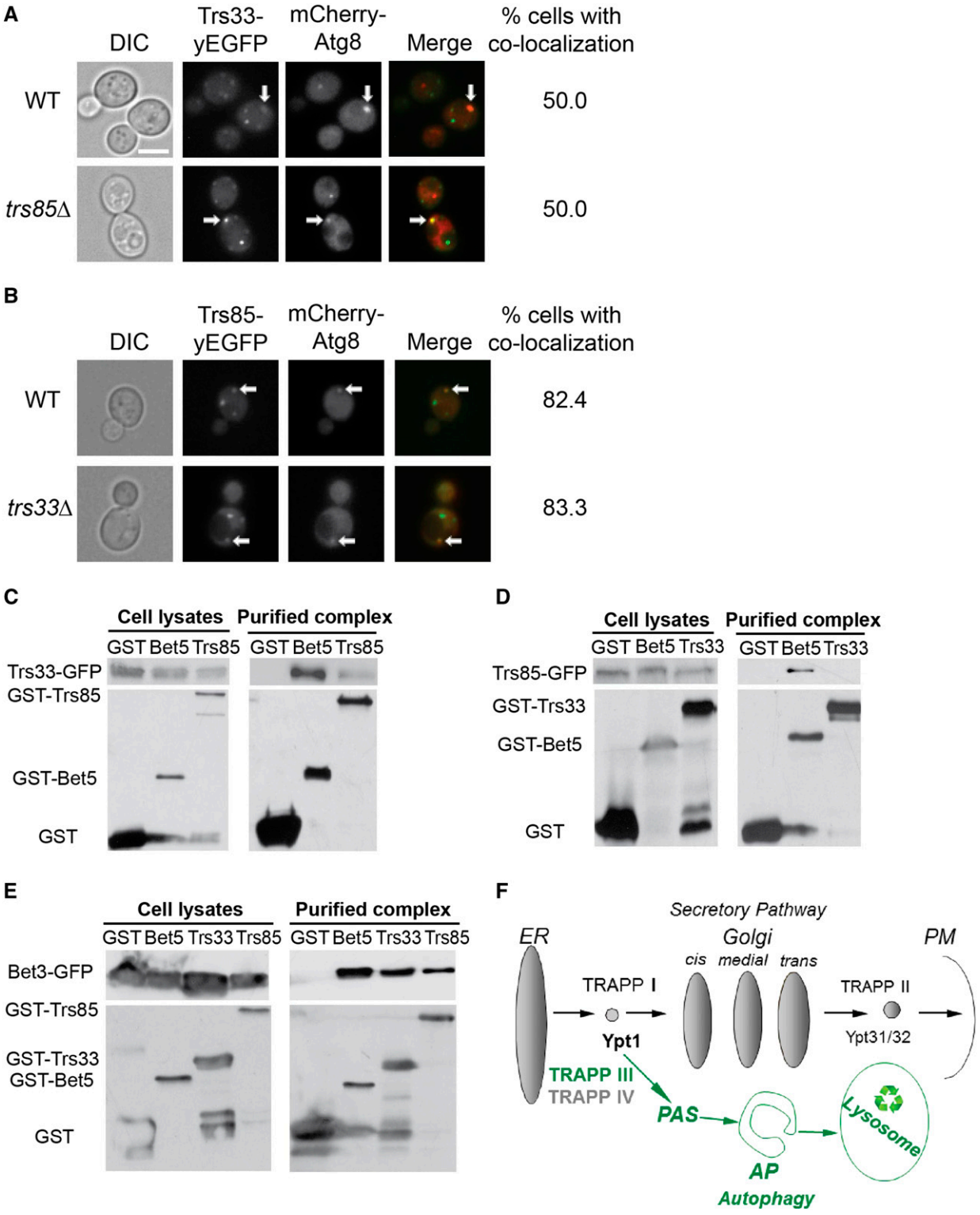

Figure 6 Trs33 and Trs85 localize to PAS independently from each other. (A) The localization of Trs33 to PAS is not affected by deletion of *TRS85*. Trs33 was tagged on the chromosome with yEGFP in WT (top) and *trs85*Δ mutant cells (bottom). The cells were transformed with a plasmid for expression of mCherry-Atg8. Shown from left to right: DIC, green, red, merge, and % cells with colocalization. (B) The localization of Trs85 to PAS is not affected by deletion of *TRS33*. Trs85 was tagged on the chromosome with yEGFP in WT and *trs85*Δ mutant cells. The cells were transformed with a plasmid for expression of mCherry-Atg8. Shown from left to right: DIC, green, red, merge, and % cells with colocalization. (A and B) Arrows point to colocalizing puncta; at least 40 cells visualized for each strain. Bar, 5 μm. (C–E) Trs85 and Trs33 are present on two separate TRAPP complexes. Cells expressing GFP-tagged Trs33 (C), Trs85 (D), or Bet3 (E) were transformed with a plasmid for expression of GST, GST-Bet5, GST-Trs85, or GST-Trs33. The coprecipitation of the GFP-tagged proteins (right) with the GST-tagged proteins from

was also considered to be in TRAPP I/II (Sacher *et al.* 2001). However, when a role for this subunit was shown in autophagy (Meiling-Wesse *et al.* 2005; Nazarko *et al.* 2005), the TRAPP complex containing Trs85 was termed TRAPP III (Lynch-Day *et al.* 2010). Likewise, based on a role for Trs33 in autophagy, distinct from that of Trs85, we term the Trs33-containing complex TRAPP IV (Figure 1A). The EM structure of TRAPP III was reported to contain core-TRAPP and Trs20 (Tan *et al.* 2013), and the latter is required for the assembly of Trs85 with TRAPP (Taussig *et al.* 2014). We suggest that TRAPP IV also contains core-TRAPP subunits based on coprecipitation with Bet3 and Bet5 (Figure 6, C and E) and colocalization with Trs23 (Figure 5B). However, we propose that Trs20 is not required for the assembly and the function of TRAPP IV in autophagy. First, Trs33 associates with TRAPP in the absence of Trs20 (Kim *et al.* 2006). Second, the autophagy phenotypes of *trs20* mutations are similar (not more severe) to those of the *trs85*Δ mutation (Brunet *et al.* 2013; Taussig *et al.* 2014), supporting the idea that Trs20 does not function with TRAPP IV in autophagy.

TRAPP III and IV could colocalize on PAS or APs based on the observation that Trs85 and Trs33 colocalize with Atg8 in ~80 and 50% of the cells, respectively (Figure 6, A and B). However, whereas the other Trs85 puncta colocalize with the membrane protein Atg9 (Lipatova *et al.* 2012), Trs33 colocalizes with *cis*- and *trans*-Golgi markers (Figure 5B). This different cellular distribution might reflect a distinct mechanism of recruitment of the two complexes to the autophagy pathway.

We have previously shown that Trs33 is required for formation of the Ypt31/32 GEF TRAPP II in the absence of another nonessential TRAPP subunit, Trs65 (Tokarev *et al.* 2009). The colocalization of Trs33-yEGFP mostly with a *trans*-Golgi marker agrees with this idea (Figure 5B). In addition, a role for TRAPP II in autophagy has been suggested based on autophagy defects of *trs130ts* mutant cells at their nonpermissive temperature (Zou *et al.* 2013). We show that the role of Trs33 in autophagy is not connected to TRAPP II or Ypt31/32 based on the following evidence: First, deletion of the TRAPP II-specific Trs65 subunit in wild-type or *trs85*Δ mutant cells does not exacerbate their autophagy phenotypes. Second, while the autophagy defects of *trs130ts* mutant cells can be suppressed by Ypt31 and not Ypt1 (Zou *et al.* 2013), those of *trs33*Δ mutant cells can be suppressed by overexpression of Ypt1 and not Ypt31 (shown here). Finally, the autophagy defects of the double mutant *atg11*Δ *trs130ts* are more severe than those of either single mutation, suggesting that Atg11 and the Trs130-containing TRAPP II function in parallel pathways (Zou *et al.* 2013). In contrast, deletion of *TRS33* does not exacerbate the autophagy phenotypes of the *atg11*Δ mutation (shown here), indicating that like Trs85 (Lipatova *et al.* 2012), Trs33 functions in the context of the same Ypt1 GTPase module as Atg11.

The existence of TRAPP III and IV raises the question of why two GEFs exist for the activation of a single Ypt, Ypt1, in a single process — PAS formation. One possibility is that in the absence of TRAPP III, which is the major Ypt1 GEF in generic autophagy as judged by the severity of the autophagy phenotypes, TRAPP IV compensates for its absence. The alternative is based on the breadth of the autophagy pathways, which are all dependent on PAS formation. Thus, while TRAPP III and IV compensate for each other in autophagy pathways that were tested, they might be specific for yet untested ones.

Pathway-specific Ypt/Rab GEFs

Based on previous data and results presented here, we conclude that pathway-specific GEFs enable Ypt/Rab-dependent regulation of two distinct pathways. Thus, Ypt1-dependent initiation of the secretory and autophagy pathways is regulated by TRAPP complexes: TRAPP I is required for Ypt1-mediated ER-to-Golgi transport, whereas TRAPP III or IV is required for Ypt1-mediated PAS assembly (Figure 6F). While the requirement of TRAPP I for Ypt1-dependent ER-to-Golgi transport is established (Sacher *et al.* 2001), the requirement of a GEF for Ypt1-mediated autophagy was not clear, because depletion of TRAPP III resulted in less severe autophagy defects than depletion of Ypt1. Here we show that TRAPP III or IV is required for Ypt1-mediated PAS assembly. Moreover, we show that overexpression of Ypt1 can suppress the autophagy defects of *trs33*Δ and *trs85*Δ single mutant cells, but not those of the double mutant. This finding indicates that Ypt1 needs a GEF to mediate autophagy. Thus, in cells deleted for one GEF, this function is provided by the remaining GEF, while in cells depleted for both GEFs, autophagy is completely blocked. One possible explanation for this dependency of Ypt1-mediated autophagy on a GEF is that at least one autophagy-specific GEF, TRAPP III or TRAPP IV, is essential for the recruitment of Ypt1 from the secretory pathway to autophagy.

We have proposed that Ypt/Rab GTPases coordinate shuttling of cargo from a single compartment to different destinations (Lipatova *et al.* 2015). For example, Ypt1, which regulates delivery of cargo from the ER to the secretory pathway and autophagy (Lipatova *et al.* 2013), is a candidate for coordination of these two pathways, and pathway-specific GEFs are candidates for enabling such coordination.

yeast cell lysates (left) was determined. Trs33-GFP coprecipitates with GST-Bet5, but not with GST or GST-Trs85 (C); Trs85-GFP coprecipitates with Bet5, but not with GST or Trs33 (D); Bet3-GFP coprecipitates with GST-Bet5, GST-Trs85, and GST-Trs33, but not with GST (E). Results shown in A–E are representative of at least two independent experiments. (F) Model for TRAPP complexes function in the secretory pathway and autophagy. Whereas TRAPP I and II regulate secretion (Sacher *et al.* 2001), TRAPP III and IV regulate autophagy [(Lynch-Day *et al.* 2010), and results presented here]. See text for discussion.

Conservation

Trs33 has two mammalian homologs, TrappC6A and TrappC6B (Kim *et al.* 2016). While hTrappC6A was implicated in neurodegenerative disease (Hamilton *et al.* 2011; Chang *et al.* 2015), nothing is currently known about its physiological role. The role of Ypt1 in autophagy is conserved and was shown for Rab1 (Zoppino *et al.* 2010). We propose that the role of Trs33 in autophagy is also conserved.

Acknowledgments

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Figure S1

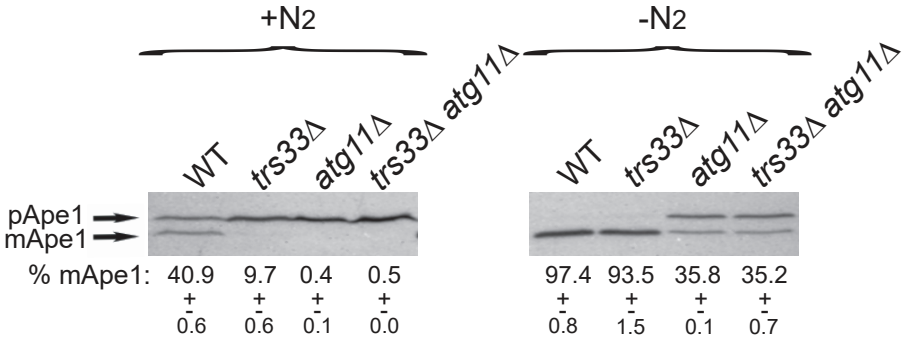
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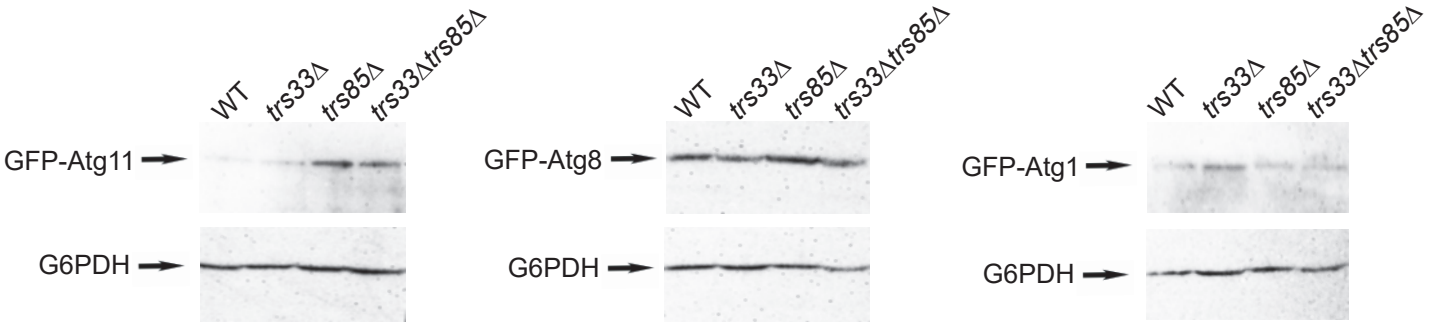
Figure S2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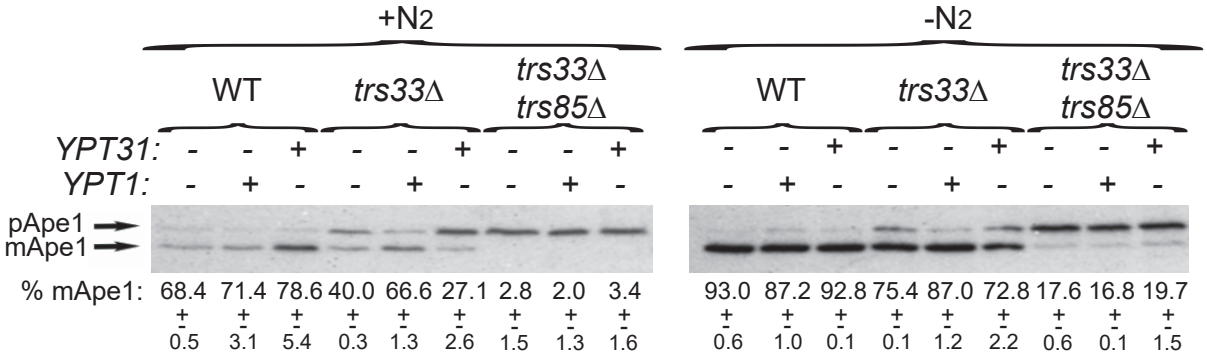
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Table S1. Yeast strains used in this study.

Strain	Alias	Genotype	Source
NSY825	BY4741	*Mat**a** leu2Δ0 ura3Δ0 his3Δ1 met15Δ0*	(Brachmann et al., 1998)
NSY1440	*trs85Δ*	NSY825 *trs85Δ::HYGRO*	(Lipatova et al., 2012)
NSY1834	*trs33Δ*	NSY825 *trs33Δ::KAN*	This study
NSY1111	*trs65Δ*	NSY825 *trs65Δ::KAN*	(Liang et al., 2007)
NSY1811	*trs33Δ trs85*Δ	NSY1440 *trs33*Δ*::KAN*	This study
NSY1835	*trs65Δ trs85*Δ	NSY1111 *trs85*Δ*::HYGRO*	This study
NSY1841	BY4741 *TRS23-YFP*	NSY825 *TRS23-YFP::SpHIS5*	This study
NSY1842	*trs33Δ TRS23-YFP*	NSY1834 *TRS23-YFP::SpHIS5*	This study
NSY1843	*trs85Δ TRS23-YFP*	NSY1440 *TRS23-YFP::SpHIS5*	This study
NSY1844	*trs33Δ trs85*Δ *TRS23-YFP*	NSY1811 *TRS23-YFP::SpHIS5*	This study
NSY1845	*trs65Δ TRS23-YFP*	NSY1111 *TRS23-YFP::SpHIS5*	This study
NSY1846	*trs65Δ trs85*Δ *TRS23-YFP*	NSY1835 *TRS23-YFP::SpHIS5*	This study
NSY128	DBY4975	*Matα ade2 his3-Δ200 leu2-3,112 lys2-801 ura3-52*	(Jedd et al., 1997)
NSY55	*ypt1-1*	*MATα his3-Δ200 leu2-3,112 ura3-52 ypt1-T40K*	(Lipatova et al., 2013)
NSY1528	TN124	*Mat**a** leu2-3,112 trp1 ura3-52 pho8::pho8*Δ*60 pho13*Δ*::LEU2*	(Noda et al., 1995)
NSY1530	TN124 *trs85*Δ	NSY1528 *trs85*Δ*::HYGRO*	(Lipatova et al., 2012)
NSY1836	TN124 *trs33*Δ	NSY1528 *trs33*Δ*::KAN*	This study
NSY1837	TN124 *trs33*Δ *trs85*Δ	NSY1530 *trs33Δ::KAN*	This study
NSY1838	TN124 *trs65*Δ	NSY1528 *trs65Δ::HYGRO*	This study
NSY1839	TN124 *trs65*Δ *trs85*Δ	NSY1530 *trs65Δ::NAT*	This study
NSY1847	BY4741 *TRS33-yEGFP*	NSY825 *TRS33-yEGFP::KAN*	This study
NSY1848	*trs85Δ TRS33-yEGFP*	NSY1440 *TRS33-yEGFP::KAN*	This study
NSY1524	NSY825 *TRS85-yEGFP*	NSY825 *TRS85-yEGFP::KAN*	(Lipatova et al., 2012)
NSY1849	*trs33Δ TRS85-yEGFP*	NSY1834 *TRS85-yEGFP::HYGRO*	This study
NSY1850	*COPI-RFP TRS33-yEGFP*	NSY862 *TRS33-yEGFP::HYGRO*	This study
NSY1851	*CHC1-RFP TRS33-yEGFP*	NSY863 *TRS33-yEGFP::HYGRO*	This study
NSY1499	*atg11*Δ	NSY825 *atg11*Δ*::KAN*	(Lipatova et al., 2012)
NSY1840	*atg11*Δ *trs33*Δ	NSY1499 *trs33*Δ*::HYGRO*	This study
NSY915	EJ758	*Mat**a** his3-*Δ*200 leu2-3,112 ura3-52 pep4*Δ*::HIS3*	(Martzen et al., 1999)
NSY1853	NSY915 *TRS33-yEGFP*	NSY915 *TRS33-yEGFP::KAN*	This study
NSY1854	NSY915 *TRS85-yEGFP*	NSY915 *TRS85-yEGFP::HYGRO*	This study
NSY1852	NSY915 *BET3-yEGFP*	NSY915 *BET3-yEGFP::KAN*	This study

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Table S2. Plasmids used in this study.

Plasmid	Alias	Genotype	Source
pNS274	YEp24	2μ, *URA3*, Amp^r	New England Biolabs, MA
pNS489	YEp24-*YPT1*	2μ, *URA3, YPT1*	(Morozova et al, 2006)
pNS229	YEp24-*YPT31*	2μ, *URA3*, *YPT31*	(Jones et al., 1999)
pNS581	pDH5	YFP-*SpHIS5*, Amp^r	Yeast Resource Center
pNS1191	pFA6a-3xHA-*KanMX6*	*KanMX6*, Amp^r	(Longtine et al, 1998)
pNS584	pAG32	*hphMX,* Amp^r	(Goldstein and McCusker, 1999)
pNS583	pAG25	*natMX4,* Amp^r	(Goldstein and McCusker, 1999)
pNS955	pKT127	yEGFP-*KanMX4*, Amp^r	(Sheff and Thorn, 2004)
pNS1532	pKT-*hphMX*	yEGFP-*hphMX*, Amp^r	This study
pNS1360	p416-yEGFP-*ATG8*	*CEN*, *URA3*, *ADH1* promoter-yEGFP-*ATG8*-*CYC1* terminator	(Lipatova et al, 2012)
pNS1359	p416-yEGFP-*ATG11*	*CEN*, *URA3*, *ADH1* promoter-yEGFP-*ATG11*-*CYC1* terminator	(Lipatova et al, 2012)
pNS1361	p416-yEGFP-*ATG1*	*CEN*, *URA3*, *ADH1* promoter-yEGFP-*ATG1*-*CYC1* terminator	(Lipatova et al, 2012)
pNS1362	p416-mCherry-*ATG8*	*CEN, URA3, ADH1* promoter-mCherry-*ATG8*-*CYC1* terminator	(Lipatova et al., 2012)
pNS1321	p416-mCherry-*APE1*	*CEN, URA3, ADH1* promoter-mCherry-Ape1-*CYC1* terminator	This study
pNS1408	pRS416-GFP-*ATG8*	*CEN, URA3,* GFP-*ATG8*	(Shintani and Klionsky, 2004)
pNS422	pYEX-4T-1	2μ, *URA3, leu2-d,* Amp^r, GST expressed under *CUP1* promoter	(Morozova et al., 2006)
pNS424	pYEX-4T-1-*BET5*	2μ, *URA3, leu2-d,* Amp^r, GST-Bet5 expressed under *CUP1* promoter	(Morozova et al., 2006)
pNS1448	pYEX-4T-1-*TRS85*	2μ, *URA3, leu2-d,* Amp^r, GST-Trs85 expressed under *CUP1* promoter	(Taussig et al., 2014)
pNS1570	pYEX-4T-1-*TRS33*	2μ, *URA3, leu2-d,* Amp^r, GST-Trs33 expressed under *CUP1* promoter	This study

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