Modular TRAPP Complexes Regulate Intracellular Protein Trafficking Through Multiple Ypt/Rab GTPases in *Saccharomyces cerevisiae*

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ABSTRACT Ypt/Rab are key regulators of intracellular trafficking in all eukaryotic cells. In yeast, Ypt1 is essential for endoplasmic reticulum (ER)-to-Golgi transport, whereas Ypt31/32 regulate Golgi-to-plasma membrane and endosome-to-Golgi transport. TRAPP is a multisubunit complex that acts as an activator of Ypt/Rab GTPases. Trs85 and Trs130 are two subunits specific for TRAPP III and TRAPP II, respectively. Whereas TRAPP III was shown to acts as a Ypt1 activator, it is still controversial whether TRAPP II acts as a Ypt1 or Ypt31/32 activator. Here, we use GFP-Snc1 as a tool to study transport in Ypt and TRAPP mutant cells. First, we show that expression of GFP-Snc1 in *trs85*Δ mutant cells results in temperature sensitivity. Second, we suggest that in *ypt1ts* and *trs85*Δ, but not in *ypt31*Δ*/32ts* and *trs130ts* mutant cells, GFP-Snc1 accumulates in the ER. Third, we show that overexpression of Ypt1, but not Ypt31/32, can suppress both the growth and GFP-Snc1 accumulation phenotypes of *trs85*Δ mutant cells. In contrast, overexpression of Ypt31, but not Ypt1, suppresses the growth and GFP-Snc1 transport phenotypes of *trs130ts* mutant cells. These results provide genetic support for functional grouping of Ypt1 with Trs85-containing TRAPP III and Ypt31/32 with Trs130-containing TRAPP II.

TRANSPORT of membranes and proteins through the endocytic and exocytic pathways connects membrane-bound intracellular compartments with the plasma membrane (PM) and the cell milieu. The endoplasmic reticulum (ER) is the compartment into which membrane and cargo proteins are translocated en route to all the other cellular compartments. From the ER, membrane and proteins are transported through the Golgi apparatus to the PM.

Ypt/Rab GTPases regulate trafficking between cellular compartments (Segev 2001a,b; Stenmark 2009). In yeast, Ypt1 is required for ER-to-Golgi transport (Segev *et al.* 1988; Jedd *et al.* 1995), whereas the Ypt31/32 functional pair plays a role at the *trans*-Golgi in both Golgi-to-PM and endosome-to-Golgi transport (Jedd *et al.* 1997; Chen *et al.* 2005). In addition, Ypt1 and Ypt31/32 play a role in autophagy (Segev and Botstein 1987; Geng *et al.* 2010; Lynch-Day *et al.* 2010). Autophagy is a cellular process induced by stress in which cytosolic and membrane proteins are engulfed by a double-membrane organelle termed autophagosome to be delivered to the lysosome for degradation (Nakatogawa *et al.* 2009; Yang and Klionsky 2009).

Ypt/Rabs are activated by guanine-nucleotide exchange factors (GEFs) and the GEF for Ypt1 and Ypt31/32 is the TRAPP complex. TRAPP is a multisubunit modular complex (Sacher *et al.* 2008), which exists in at least three forms. TRAPP I, which contains five essential subunits, acts as a GEF for Ypt1 (Jones *et al.* 2000; Wang *et al.* 2000), and is required for ER-to-Golgi transport (Sacher *et al.* 1998). Trs85, a TRAPP subunit, nonessential for cell viability, plays a role in autophagy (Meiling-Wesse *et al.* 2005; Nazarko *et al.* 2005). A Trs85-containing TRAPP complex, termed TRAPP III, also acts as a Ypt1 GEF (Lynch-Day *et al.* 2010). TRAPP II, which contains Trs120 and Trs130 in addition to TRAPP I subunits, functions at the *trans*-Golgi

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(Sacher *et al.* 2001). However, currently the Ypt GEF specificity of the TRAPP II complex is under dispute.

On the basis of biochemical and genetic evidence, we have proposed that TRAPP I activates Ypt1 in ER-to-Golgi transport, whereas Ypt31/32 act at the *trans*-Golgi in both Gogli-to-PM and endosome-to-Golgi transport, and TRAPP II acts as their GEF at least in the former step (Jones *et al.* 2000; Chen *et al.* 2005; Morozova *et al.* 2006). This model is currently being challenged claiming that Ypt1 functions in endosome-to-Golgi transport in addition to its role in ER-to-Golgi transport (Sclafani *et al.* 2010), and that TRAPP II acts as a GEF for Ypt1 in both steps (Cai *et al.* 2008; Barrowman *et al.* 2010; Yip *et al.* 2010). Here we study the physiological relationship between Ypt1 and Ypt31/32 and two TRAPP complexes, TRAPP III and TRAPP II. Data presented in this article provide support to the idea that TRAPP II acts with Ypt31/32, and not with Ypt1, in endosome-to-Golgi transport.

GFP-Snc1 has been extensively used as a PM recycling marker in yeast (Lewis *et al.* 2000; Chen *et al.* 2005; Montpetit and Conibear 2009; Sclafani *et al.* 2010). Snc1 is a v-SNARE that plays a role in fusion of *trans*-Golgi vesicles with the PM. For multiple rounds of vesicle fusion, Snc1 recycles back from the PM to the Golgi via endosomes (Lewis *et al.* 2000). We have previously used GFP-Snc1 to reveal a role for Ypt31/32 in endosome-to-Golgi transport (Chen *et al.* 2005). Recently, a role for Ypt1, together with TRAPP II, has been proposed in the recycling of GFP-Snc1 from the PM using *YPT1* alleles defective specifically in Snc1-GFP trafficking (Sclafani *et al.* 2010). In addition, a role for Trs85 in endosome-to-Golgi transport was also suggested on the basis of intracellular accumulation of Snc1-GFP in *trs85*Δ mutant cells (Montpetit and Conibear 2009). In contrast, our results suggest that in *ypt1ts* and *trs85*Δ*ts* mutant cells, GFP-Snc1 accumulates in the ER, supporting a role for Ypt1 and Trs85 in exit of membrane proteins from the ER. Because accumulation of GFP-Snc1 in the ER of *trs85*Δ*ts* mutant cells is coupled with a cell-growth defect, we propose that accumulation of proteins in the ER can cause cell toxicity. Moreover, overexpression analyses support functional grouping of Ypt31/32 with the Trs130-containing TRAPP II complex in endosome-to-Golgi transport and of Ypt1 with the Trs85-containing TRAPP complex in ER exit of overexpressed membrane proteins. It is not clear whether this role of Trs85 is related to its established function in autophagy.

Results

Biochemical analysis has shown that TRAPP I acts as a Ypt1 GEF (Jones *et al.* 2000; Wang *et al.* 2000). However, the substrate for TRAPP II complex is currently controversial (Morozova *et al.* 2006; Cai *et al.* 2008). Recently, the Trs85-containing TRAPP III complex was suggested to act as a GEF for Ypt1 and not Ypt32 (Lynch-Day *et al.* 2010). However, in the published experiment there was no positive control for a Ypt32 GEF and does not include Ypt31. Here, we add biochemical support to the idea that TRAPP III acts as a GEF for Ypt1, but not Ypt31/32, by including the missing positive control. The Ypt GEF activity of the Trs85-containing TRAPP complex purified from yeast lysates was compared to that of the Bet5-containing TRAPP complexes, using a GDP-release assay. Because the Bet5 subunit is common to the three known TRAPP complexes, purified GST-Bet5 TRAPP contains all the TRAPP complexes including TRAPP II. Under our reaction conditions, in addition to acting as a Ypt1 GEF, GST-Bet5 purified TRAPP acts as a GEF for Ypt31 and Ypt32. In contrast, the GST-Trs85 purified complex acts as a GEF only for Ypt1 (Supporting Information, Figure S1). Thus, the Trs85-containing TRAPP, like TRAPP I, acts as a GEF for Ypt1, but not for Ypt31/32.

To study the physiological relationship between Ypt1 and Ypt31/32 and two TRAPP complexes, TRAPP II and TRAPP III, we followed the transport of GFP-Snc1 in mutants defective in Ypt and TRAPP function.

*GFP-Snc1 accumulates in the ER of ypt1ts and trs85*Δ*ts mutant cells*

The functional pair of Snc1 and Snc2 acts as a v-SNARE that cycles between the Golgi, PM, and endosomes (Protopopov *et al.* 1993). GFP-Snc1 has been used commonly as a PM recycling marker in yeast (Lewis *et al.* 2000). In wild-type (wt) cells, GFP-Snc1 localizes mostly to the PM, whereas in mutant cells defective in PM recycling, it accumulates in endosomes. Using this marker, we have previously shown that Ypt31/32 play a role in endosome-to-Golgi transport (Chen *et al.* 2005). A role for several TRAPP subunits in this step, including the two TRAPP II-specific subunits, Trs120 and Trs130 (Cai *et al.* 2005), and the three nonessential TRAPP subunit, Trs33, Trs65, and Trs85 (Montpetit and Conibear 2009), was also suggested on the basis of accumulation of intracellular GFP-Snc1. Recently, a role for Ypt1 in endosome-to-Golgi transport was suggested (Sclafani *et al.* 2010), and *trs85*Δ was identified in a screen for genes required for endosome-to-Golgi transport (Montpetit and Conibear 2009). Even though a role for Trs85 and Ypt1 in autophagy has been established (Segev and Botstein 1987; Lynch-Day *et al.* 2010), they could also play a role in endosome-to-Golgi transport, individually or together. For example, Ypt31/32 and Trs130 function both in exit from the *trans*-Golgi and in endosome-to-Golgi transport (Jedd *et al.* 1997; Sacher *et al.* 2001; Cai *et al.* 2005; Chen *et al.* 2005). Therefore, we reexamined whether Ypt1 and Trs85 function in the recycling of GFP-Snc1 from the PM.

GFP-Snc1 was integrated into the *URA3* locus in wt, *ypt1ts*, and *trs85*Δ mutant cells (these cells express both Snc1 and Snc2) (Lewis *et al.* 2000). GFP-Snc1–expressing *trs85*Δ, but not *trs65*Δ, mutant cells exhibit a temperature-sensitive growth phenotype (Figure 1A). The linkage of the temperature-sensitive growth phenotype with overexpression of GFP-Snc1 in *trs85*Δ mutant cells was verified by cosegregation of the temperature sensitivity with integrated GFP-Snc1 and *trs85*Δ, and by overexpression of GFP-Snc1

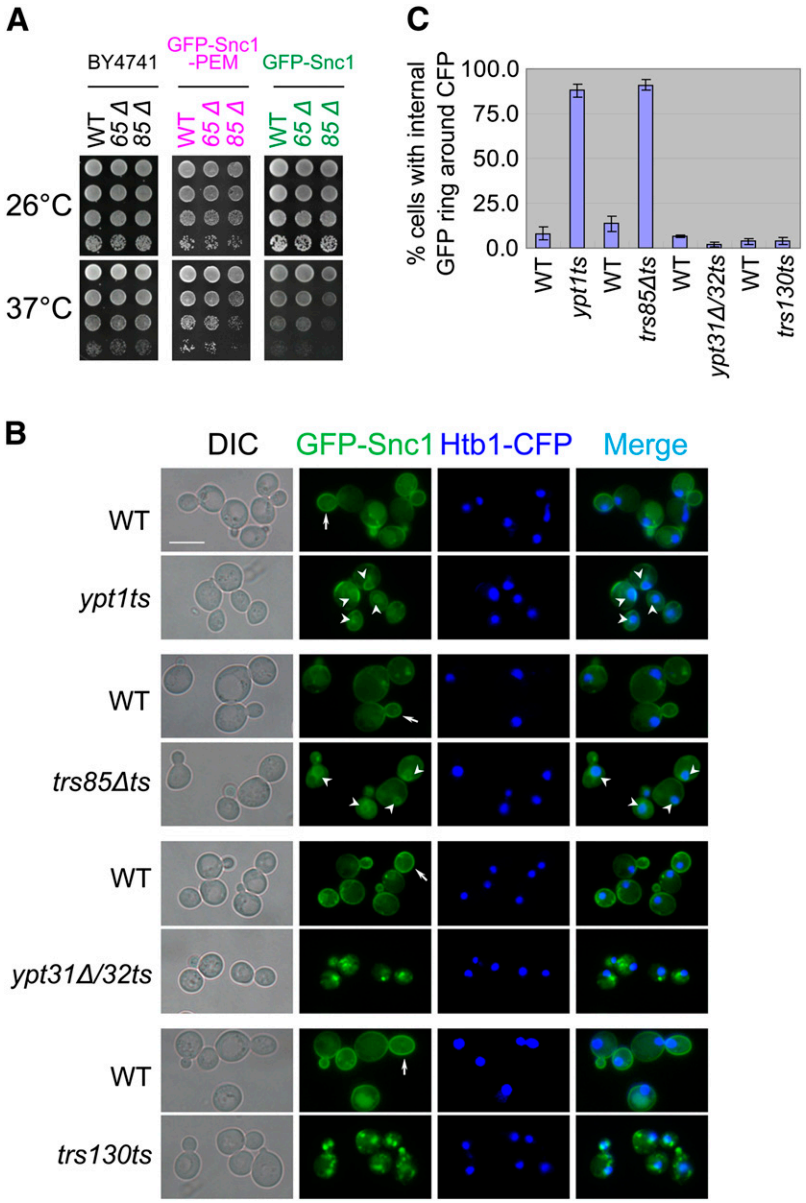

Figure 1 GFP-Snc1 accumulates in the rings around the nuclei of *ypt1ts* and *trs85Δts* mutant cells. (A) *trs85Δ*, but not *trs65Δ* mutant cells expressing GFP-Snc1 or GFP-Snc1-PEM exhibit a temperature-sensitive growth phenotype. The *TRS85* or *TRS65* gene was deleted in the following three strains: wild type (BY4741, left), chromosomally tagged GFP-Snc1-PEM (YLY1582, middle), and chromosomally tagged GFP-Snc1 (YLY130, right). Cells were grown on YPD plates at 26° (top) and 37° (bottom). Four 10-fold serial dilutions are shown from top to bottom. (B) GFP-Snc1 accumulates in the ER of *ypt1ts* and *trs85Δts*, but not of *ypt31Δ/32ts* and *trs130ts* mutant cells. Wild-type and mutant cells expressing chromosomally tagged GFP-Snc1 and Htb1-CFP, as a nuclear marker, were grown in YPD medium at 26° to midlog phase and then shifted to 37° for 1.5 hr. GFP-Snc1 and Htb1-CFP were visualized using live-cell fluorescence microscopy. In *ypt1ts* and *trs85Δts*, but not in *ypt31Δ/32ts* or *trs130ts*, GFP-Snc1 accumulates around nuclei, which is indicative of ER localization. Bar, 7 μm. Arrows point to GFP-Snc1 on the PM and arrowheads to perinuclear GFP-Snc1 circle. (C) Quantification of data presented in B: shown is percentage of cells with an internal GFP-Snc1 ring around the nucleus, which is marked with Htb1-CFP. At least 200 cells were counted in at least two fields for each strain. Error bars represent SD.

from a 2μ plasmid (Figure S2). Thus, overexpression of GFP-Snc1 in *trs85Δ* mutant cells confers a temperature-sensitive phenotype. We termed the mutant strain in which GFP-Snc1 is integrated and *TRS85* is deleted *trs85Δts*. This mutant was used as a tool in microscopic analysis to study GFP-Snc1 transport phenotype, and in overexpression analysis, to determine genetic interactions between the Ypts and TRAPP subunits.

The accumulation of GFP-Snc1 in *ypt1ts* and *trs85Δts* mutant cells at their permissive (26°) and nonpermissive (37°) temperatures was determined using live-cell microscopy (Figure S3). In both *ypt1ts* and *trs85Δts* mutant cells, GFP-Snc1 accumulates internally at 37°. This internal GFP-Snc1 accumulation was compared to the accumulation in *ypt31Δ/32ts* and *trs130ts* mutant cells, in which GFP-Snc1 accumulates in endosomes already at the permissive temperature (under conditions that allow Golgi-to-PM transport) (Chen *et al.* 2005). However, whereas in *ypt31Δ/32ts* and *trs130ts* mutant cells, GFP-Snc1 accumulates in puncta, the internal accumulation in *ypt1ts* and *trs85Δts* mutant cells appears as rings. Because Ypt1 functions in ER-to-Golgi transport and in yeast ER appears as rings around the nucleus (Hampton *et al.* 1996; Huh *et al.* 2003), we wished to determine whether the GFP-Snc1 rings in *ypt1ts* and *trs85Δts* mutant cells are around the nucleus.

To determine the cellular compartment in which GFP-Snc1 accumulates in *ypt1ts* and *trs85Δts* mutant cells, it was colocalized with nuclear and endosomal markers. The nucleus was visualized by Htb1-CFP (histone H2B) (Michelsen *et al.* 2006). In both *ypt1ts* and *trs85Δts* mutant cells, GFP-Snc1 accumulates in rings around the nucleus labeled with Htb1-CFP. In contrast, in *ypt31Δ/32ts* and *trs130ts* mutant cells, the puncta are not associated with the nucleus (Figure 1, B and C).

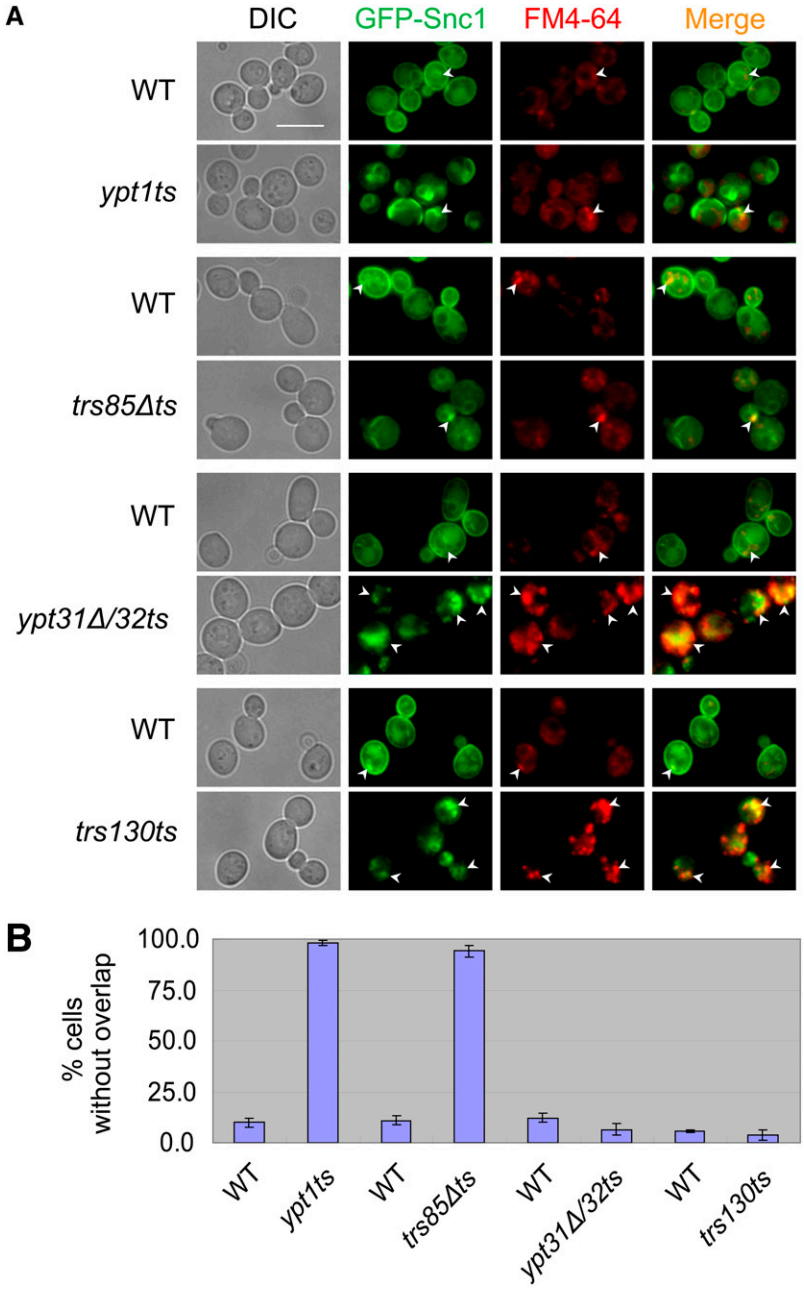

Figure 2 GFP-Snc1 accumulates in endosomes of *ypt31Δ/32ts* and *trs130ts*, but not of *ypt1ts* and *trs85Δts* mutant cells. (A) Wild-type and mutant cells expressing chromosomally tagged GFP-Snc1 were stained with FM4-64 for 5 min to label early endosomes and were visualized using live-cell fluorescence microscopy. Cells grown in YPD medium at 26° to midlog phase were shifted to 37° for 85 min, stained with FM4-64 for 5 min, and kept on ice until visualization. Bar, 7 μm. Arrowheads point to GFP-Snc1 that localizes to early endosomes marked with FM4-64. (B) Quantification of data presented in A shown is percentage of cells with internal GFP-Snc1 that does not overlap with FM4-64. At least 200 cells were counted in at least three fields for each strain. Error bars represent SD.

Localization of GFP-Snc1 to endosomes was determined by staining cells with the fluorescent dye FM4-64 for 5 min. As expected for a protein that recycles through endosomes, there is some localization of GFP-Snc1 to endosomes in wild-type cells. In *ypt1ts* and *trs85Δts* mutant cells the limited colocalization of GFP-Snc1 with the endosomal staining is similar to that seen in wild-type cells. In contrast, under the same conditions (as previously reported, Cai *et al.* 2005; Chen *et al.* 2005), in *ypt31Δ/32ts* and *trs130ts* mutant cells, intracellular GFP-Snc1 localizes mostly to endosomes (Figure 2).

Together, the colocalization analysis of GFP-Snc1 with nuclear and endosomal markers suggest that in *ypt1ts* and *trs85Δts*, but not in *ypt31Δ/32ts* and *trs130ts*, mutant cell Snc1 accumulates in the ER.

The nonrecycling marker, GFP-Snc1-PEM, accumulates in the ER of ypt1ts and trs85Δ mutant cells

To further characterize the GFP-Snc1 localization defect in *ypt1ts* and *trs85Δts* mutant cells, we used the GFP-Snc1-PEM protein. This hybrid protein contains the Sso1 *trans*-membrane domain and two point mutations, V40A and M43A, which interfere with endocytosis and cause accumulation on the PM (Lewis *et al.* 2000). Therefore, GFP-Snc1-PEM does not accumulate in endosomes in mutant cells defective in PM recycling (Galan *et al.* 2001; Lafourcade

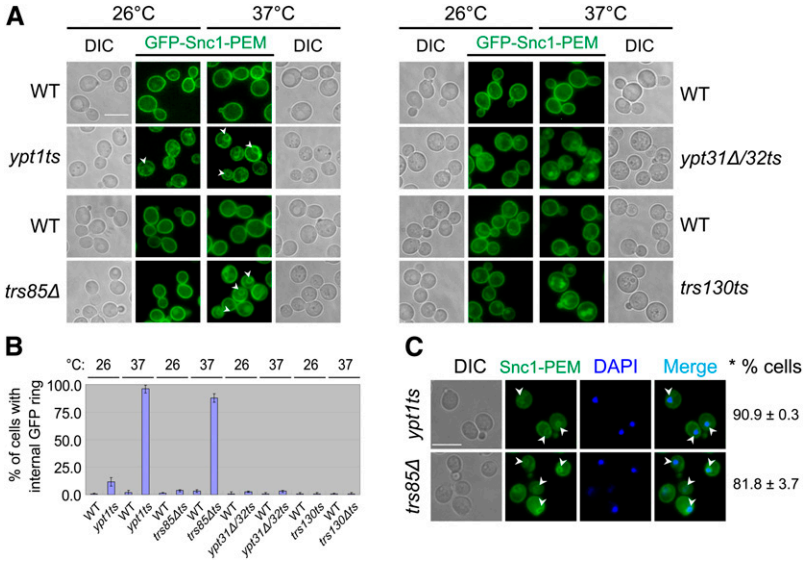

Figure 3 GFP-Snc1-PEM accumulates in the rings around the nuclei of *ypt1ts* and *trs85Δts* mutant cells. (A) Like GFP-Snc1, GFP-Snc1-PEM accumulates in ring-like structures in *ypt1ts* and *trs85Δ*, but not in *ypt31Δ/32ts* or *trs130ts* mutant cells. Wild-type and mutant yeast cells expressing chromosomally tagged GFP-Snc1-PEM were used for live-cell fluorescence microscopy as described in Figure S3 legend. GFP-Snc1-PEM accumulates inside *ypt1ts* and *trs85Δ* mutant cells under the same conditions that cause intracellular accumulation of GFP-Snc1; namely 26° and 37° for *ypt1ts* and 37° for *trs85Δ*. In contrast, GFP-Snc1-PEM does not accumulate inside *ypt31Δ/32ts* or *trs130ts* mutant cells at 26°, under conditions that GFP-Snc1 does accumulate (see Figure S3). DIC images (on each side) show the contour of cells. Bar, 7 μm. Arrowheads point to internal GFP-Snc1-PEM. (B) Quantification of data presented in A: shown is percentage of cells with an internal GFP-Snc1-PEM ring. At least 200 cells were counted in at least three fields for each strain; error bars represent SD. (C) The GFP-Snc1-PEM rings in *ypt1ts* and *trs85Δ* mutant cells are around the nuclei. *Ypt1ts* and *trs85Δ* mutant cells were grown at 26° to midlog phase and shifted to 37° for 1.5 hr. The cells were fixed in ethanol, stained with DAPI to mark nuclei, and visualized by fluorescence microscopy. Arrowheads point to internalized GFP-Snc1-PEM. The GFP-Snc1-PEM ring-like structures accumulating in these mutant cells are around nuclei indicating ER staining. Bars, 7 μm. Right: *% cells indicates percentage of cells with an internal GFP-Snc1-PEM ring around the nucleus, which is stained with DAPI. ± represents SD.

et al. 2004; Chen *et al.* 2005). Importantly, this protein can be used as a probe for defects in the biosynthetic transport of GFP-Snc1 because it is not endocytosed.

In wild-type cells, GFP-Snc1-PEM accumulates on the PM both at 26° and 37°. As expected, in *ypt31Δ/32ts* and *trs130ts* mutant cells at 26°, GFP-Snc1-PEM localizes mostly to the PM (Figure 3A). This localization supports the idea that in these mutant cells, intracellular accumulation of GFP-Snc1 is caused by a PM recycling defect. At 37°, GFP-Snc1-PEM accumulates in both *ypt31Δ/32ts* and *trs130ts* mutant cells (Figure 3A). This intracellular accumulation of GFP-Snc1-PEM indicates a block in transport of this protein to the PM. Because at 37°, *ypt31Δ/32ts* and *trs130ts* mutant cells are defective in Golgi-to-PM transport (Jedd *et al.* 1997; Sacher *et al.* 2000), GFP-Snc1-PEM probably accumulates in the Golgi.

In *ypt1ts* and *trs85Δ* mutant cells at 26°, GFP-Snc1-PEM localizes to the PM and, especially in *ypt1ts* mutant cells, it also accumulates inside the cells. At 37°, while there is GFP-Snc1-PEM on the PM, it also localizes to internal rings (Figure 3, A and B). Moreover, like the GFP-Snc1 rings that accumulate in these mutant cells at 37°, the GFP-Snc1-PEM rings also surround the nucleus (Figure 3C). Finally, like overexpression of GFP-Snc1, overexpression of GFP-Snc1-PEM in *trs85Δ*, but not *trs65Δ* mutant cells confers a cell growth defect (Figure 1A). Thus, we suggest that GFP-Snc1-PEM, like GFP-Snc1, accumulates in the ER of *ypt1ts* and *trs85Δ* mutant cells.

These results suggest that, whereas in *ypt31Δ/32ts* and *trs130ts* mutant cells GFP-Snc1 and GFP-Snc1-PEM accumulate in endosomes and Golgi, respectively, in *ypt1ts* and *trs85Δ* mutant cells both GFP-Snc1 and GFP-Snc1-PEM accumulate in the ER en route to the PM.

Overexpression of Ypts in TRAPP mutants

Recently, the observation that *ypt1* mutant cells, like TRAPP II-specific subunit mutants, accumulate internalized GFP-Snc1 was used to support the claim that TRAPP II acts as a GEF for Ypt1 to regulate endosome-to-Golgi transport (Sclafani *et al.* 2010). To test this idea, we performed an overexpression analysis. The effect of overexpression of Ypt1 or Ypt31/32 on the following two phenotypes of *trs85Δts* and *trs130ts* mutant cells was determined: internal accumulation of GFP-Snc1 and cell growth.

Ypt1 and Ypt31 were overexpressed from 2μ plasmids. Overexpression of Ypt1 specifically suppresses the growth defect of *ypt1ts*, but not *ypt31Δ/32ts* mutant cells. Conversely, overexpression of Ypt31 suppresses the growth defect of *ypt31Δ/32ts*, but not *ypt1ts* mutant cells (Figure S4).

If Ypt1 plays a role in GFP-Snc1 recycling from the PM together with the Trs130-containing TRAPP II complex, it was expected that overexpression of Ypt1 would suppress the GFP-Snc1 recycling defect of *trs130ts* mutant cells. However, we found that Ypt31/32, but not Ypt1, can suppress this defect in *ypt31Δ/32ts* and *trs130ts* mutant cells (Figure 4, C–E and Figure S5, B and C). In contrast, Ypt1, but not Ypt31, can suppress the intracellular accumulation of GFP-Snc1 in *ypt1ts* and *trs85Δts* mutant cells (Figure 4, A, B, and E). Ypt32 also cannot suppress the intracellular accumulation of GFP-Snc1 in *ypt1ts* and *trs85Δts* mutant cells (data not shown). These results argue against the idea that Ypt1 plays a role with Trs130-containing TRAPP II in endosome-to-Golgi transport and further support the physiological relationship between Ypt31/32 and the Trs130-containing TRAPP II complex.

If the growth defect of the *trs85Δts* mutant cells is caused by ER accumulation of GFP-Snc1, we expected that suppression of

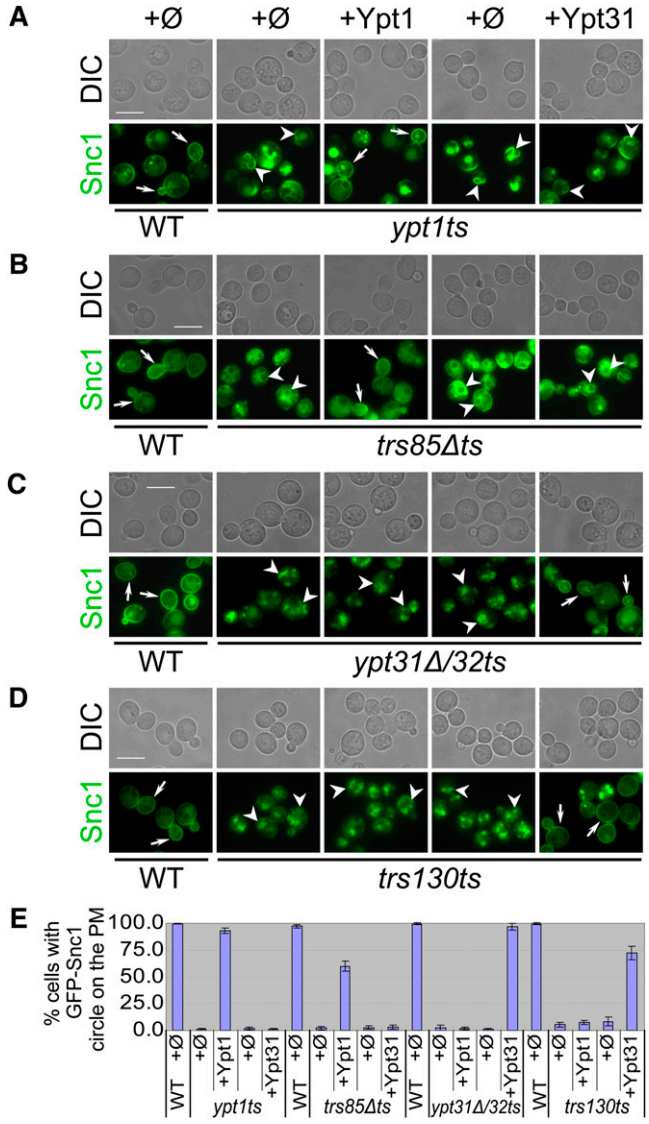

Figure 4 Ypt1 suppresses the GFP-Snc1 transport phenotype of *trs85Δts*, whereas Ypt31 suppresses the phenotype of *trs130ts*. The localization of chromosomally tagged GFP-Snc1 was determined by live-cell fluorescence microscopy in wild-type and mutant cells transformed with 2μ plasmids: Ypt1, and Ypt31, and their corresponding empty plasmids (φ, pRB684 and pRS425, respectively). Cells were grown in selective medium (to keep the plasmids) to midlog phase at 26° and then shifted to 37° for 1.5 hr. Plasmids are shown at the top of A. Bar, 7 μm. Wild-type cells transformed with the different plasmids show the same GFP-Snc1 PM localization; shown on the left are wild-type cells transformed with empty vector (φ, pRB684). All mutants accumulate internalized GFP-Snc1 (compare the left panels for wild type and mutant with empty plasmid (φ). Overexpression of Ypt1, but not Ypt31, suppresses the GFP-Snc1 phenotype of *ypt1ts* (A) and *trs85Δts* (B) mutant cells. In contrast, overexpression of Ypt31, but not Ypt1, suppresses the GFP-Snc1 phenotype of *ypt31Δ/32ts* (C) and *trs130ts* (D). In the GFP-Snc1 panels, arrows point to GFP-Snc1 on the PM in wild-type and suppressed mutant cells; arrowheads point to intracellular GFP-Snc1 seen in mutant cells. (E) Quantification of data presented in A–D. Shown is percentage of cells with GFP-Snc1 on the PM. At least 200 cells were counted in at least four fields for each strain. Error bars represent SD.

the GFP-Snc1 ER accumulation by overexpression of Ypt1 should also suppress the temperature growth defect of these cells. Moreover, if Ypt31/32, and not Ypt1, function together with TRAPP II, we expect that overexpression of Ypt31/32, but not Ypt1, would suppress the growth defect of *trs130ts* mutant cell. Indeed, overexpression of Ypt1, but not Ypt31, can suppress the temperature-sensitive growth phenotype of *ypt1ts* and *trs85Δts* mutant cells (Figure 5A and Figure S4A). Overexpression of Ypt32 also cannot suppress the temperature-sensitive growth phenotype of *ypt1ts* and *trs85Δts* mutant cells (data not shown). In contrast, the temperature-sensitive growth phenotype of *ypt31Δ/32ts* and *trs130ts* mutant cells can be suppressed by overexpression of Ypt31 or Ypt32, but not Ypt1 (Figure 5B and Figure S4B; Figure S5A).

In summary, genetic analysis presented here reveals that while overexpression of Ypt1 can suppress the two GFP-Snc1–induced phenotypes of *trs85Δts* mutant cells, GFP-Snc1 accumulation in the ER and temperature sensitivity, it does not suppress those of *trs130ts* mutant cells. Importantly, the two GFP-Snc1–induced phenotypes of *trs130ts* mutant cells, internal accumulation of GFP-Snc1 and temperature sensitivity, can be suppressed by overexpression of Ypt31/32. Therefore, these results provide genetic support to grouping Ypt1 with Trs85-containing TRAPP III and Ypt31/32 with Trs130-containing TRAPP II (Figure 5C).

Discussion

Here, the v-SNARE GFP-Snc1 is used as a transport marker to support two ideas. First, GFP-Snc1 and GFP-Snc1-PEM accumulate in the ER of *ypt1ts* and *trs85Δ* mutant cells en route to the PM. Second, overexpression analysis supports physiological grouping of Ypt1 with the Trs85-containing TRAPP III complex and of Ypt31/32 with the Trs130-containing TRAPP II complex (Figure 5C). The importance of each of these conclusions is discussed below.

Using GFP-Snc1 as a cargo marker

Using traditional exocytic markers, like invertase and CPY, it has been established that *ypt1ts* mutant cells exhibit an ER transport block (Jedd *et al.* 1995), whereas *ypt31Δ/32ts* and *trs130ts* exhibit a Golgi block (Jedd *et al.* 1997; Sacher *et al.* 2001; Cai *et al.* 2005). Using CPY (Prc1) as a marker, it was recently concluded that *trs85Δ* mutant cells do not exhibit an ER block for this luminal vacuolar protease (even though a weak kinetic block is observed; Figure S3A) (Lynch-Day *et al.* 2010).

Here, we show that the membrane-associated GFP-Snc1 can also be used as an exocytic cargo marker. In wild-type cells, GFP-Snc1 is inserted into the ER membrane like all other proteins that contain a *trans*-membrane domain. From the ER, the v-SNARE Snc1 is transported to the Golgi where it plays a role in Golgi-to-PM transport. Snc1 can then be recycled back to the Golgi through endosomes for multiple rounds of vesicular transport (Lewis *et al.* 2000). GFP-Snc1 has been used as a PM-recycling marker to show that

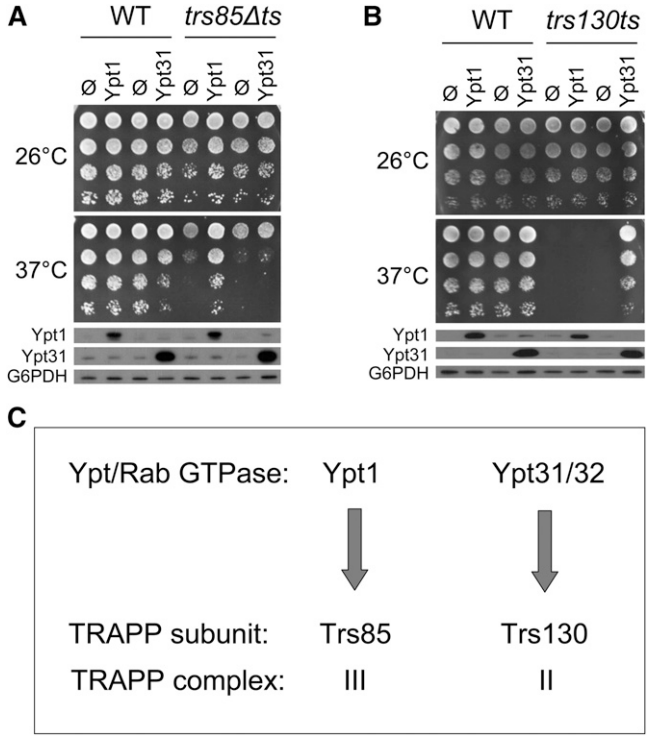

Figure 5 Ypt1 suppresses the growth defect of *trs85Δts*, whereas Ypt31 suppresses the growth defect of *trs130ts*. Mutant cells, *trs85Δts* (A), and *trs130ts* (B), and their corresponding wild-type cells, all expressing chromosomally tagged GFP-Snc1, were transformed with the following 2μ plasmids: Ypt1 and Ypt31, and their corresponding empty plasmids (φ, pRB684 and pRS425, respectively). Cell growth on plates was tested at 26° and 37°; four 10-fold serial dilutions are shown from top to bottom. Overexpression of Ypt1, but not Ypt31, suppresses the growth defect of *trs85Δts* (A). In contrast, overexpression of Ypt31, but not Ypt1, suppresses the growth defect of *trs130ts* (B). Overexpression of Ypt1 and Ypt31 in the transformants was verified by immunoblot analysis using anti-Ypt1 and anti-Ypt31 antibodies, respectively; G6PDH indicates equal loading (shown at the bottom of each panel). (C) Summary of the overexpression analysis described here: Specific suppression of *trs85Δts* and *trs130ts* mutant cells by Ypt1 and Ypt31/32, respectively, supports physiological grouping of Ypt1 with Trs85-containing TRAPP III and Ypt31/32 with Trs130-containing TRAPP II complex.

ypt31Δ/32ts and *trs130ts* mutant cells are defective in endosome-to-Golgi transport (Cai *et al.* 2005; Chen *et al.* 2005). Here, we show that GFP-Snc1 can also be used as an exocytic cargo marker in mutants defective in exit from the ER. Whereas in *ypt31Δ/32ts* and *trs130ts* it accumulates in endosomes or the Golgi, in *ypt1ts* and *trs85ts* it accumulates in the ER. The apparent discrepancy between transport of CPY (Lynch-Day *et al.* 2010) and GFP-Snc1 (here) in *trs85Δ* mutant cells might be due to the difference between the two markers: CPY is an endogenous luminal protein, whereas GFP-Snc1 is a tagged overexpressed membrane protein.

Ypt1 and Trs85 act in ER exit

Accumulation of internalized GFP-Snc1 in *trs85Δ* and *ypt1* mutant strains has been previously reported. It was taken as an indication that Ypt1 and Trs85 play a role in endosome-to-Golgi transport (Montpetit and Conibear 2009; Sclafani *et al.* 2010). Here we show that in *ypt1ts* and *trs85Δts* mutant cells, GFP-Snc1 accumulates in the ER and not during PM recycling. This idea is supported by three observations: First, GFP-Snc1 accumulates in rings around the nucleus, which do not overlap with internalized FM4-64, suggesting ER localization. Second, similar accumulation of the non-recycling GFP-Snc1-PEM protein, indicates that this accumulation is not caused by a PM recycling defect, but, rather by a transport defect. Third, the observation that overexpression of Ypt1 cannot suppress the endosomal accumulation of GFP-Snc1 in *trs130ts* mutant cells, but can suppress the ER accumulation of GFP-Snc1 in *trs85Δts* mutant cells, supports the idea that Ypt1 does not have a role in TRAPP II-mediated endosome-to-Golgi transport. Therefore, we can conclude that Trs85 does not play a role in endosome-to-Golgi transport. As for Ypt1, while *ypt1ts* mutant cells do not exhibit a block in this transport step, the idea that specific *YPT1* alleles are defective in endosome-to-Golgi transport (Sclafani *et al.* 2010) is not addressed here, because these alleles were not used in this study. However, data presented here indicate that even if Ypt1 plays a role in endosome-to-Golgi transport, it does not act together with TRAPP II in that step (see below).

Why does GFP-Snc1 accumulate in the ER of *ypt1ts* and *trs85Δts* mutant cells? There are two possible explanations for this phenotype. Because Ypt1 is required for ER-to-Golgi transport and *ypt1ts* mutant cells exhibit a block in this step at their restrictive temperature (Jedd *et al.* 1995), it is possible that GFP-Snc1 is blocked in the ER of *ypt1ts* and *trs85Δts* mutant cells en route to the Golgi. In this scenario, at high temperatures, Trs85 plays a role in ER-to-Golgi transport. Alternatively, because both Ypt1 and Trs85 play a role in autophagy (Segev and Botstein 1987; Lynch-Day *et al.* 2010), it is possible that in *ypt1ts* and *trs85Δts* mutant cells excess GFP-Snc1 fails to be shuttled through the autophagy pathway for degradation in the lysosome/vacuole. Future studies should address this question.

Deletion of *TRS85* results in temperature sensitivity only if GFP-Snc1 protein is overexpressed in this mutant strain. The correlation shown here between ER accumulation of GFP-Snc1 and GFP-Snc1-PEM at 37° and a temperature-sensitive growth phenotype of *trs85Δ* mutant cells expressing these tagged proteins, suggests that accumulation of proteins in the ER can cause cell toxicity. Such toxicity can be related to the role of Trs85 and Ypt1 in autophagy. Alternatively, it can be caused by an ER-to-Golgi transport block due to depletion of exocytic machinery by the ER-accumulated GFP-Snc1.

Grouping Ypt1 with Trs85 and Ypt31/32 with Trs130

We have previously shown that Ypt1 and Ypt31/32 are essential for Golgi entry and exit, respectively (Segev *et al.* 1988; Jedd *et al.* 1995, 1997). We have also shown that TRAPP I and TRAPP II act as GEF for Ypt1 and Ypt31/32, respectively (Jones *et al.* 2000; Morozova *et al.* 2006). This idea is supported by biochemical and genetic evidence

(Morozova *et al.* 2006; Liang *et al.* 2007; Tokarev *et al.* 2009) and agrees with the assignment of TRAPP I and TRAPP II to the *cis*- and *trans*-Golgi, respectively, in the exocytic pathway (Sacher *et al.* 1998, 2001). However, this view has been challenged by negative biochemical results (Wang and Ferro-Novick 2002; Cai *et al.* 2008; Yip *et al.* 2010), and a role for Ypt1 with TRAPP II in endosome-to-Golgi transport was suggested (Sclafani *et al.* 2010). Data presented here further support a physiological role of Ypt31/32, and not Ypt1, with TRAPP II also in endosome-to-Golgi transport. While it is possible that GFP-Snc1 accumulates in endosomes in addition to its accumulation in the ER of *ypt1* mutant cells not used in the current study, overexpression analysis presented here argues against the possibility that it acts with TRAPP II in this transport step. Thus, our genetic analysis further supports grouping of Ypt31/32, but not Ypt1, with TRAPP II in endosome-to-Golgi transport.

We propose that the modular TRAPP complexes act as GEFs for multiple Ypts, and not just of Ypt1. Currently, there is a dispute regarding the Ypt specificity of the different TRAPP complexes. Results presented here agree with previously published data that Trs85-containing TRAPP III acts as a GEF for Ypt1 (Lynch-Day *et al.* 2010). Importantly, we show here that TRAPP III does not act as a Ypt31/32 GEF under conditions in which GST-Bet5-purified TRAPP has such an activity (Figure S1). Our overexpression analysis provides genetic support to the idea that Ypt1 acts together with Trs85, whereas Ypt31/32 act together with Trs130 (Figure 5C).

In summary, studies presented here further support our view of a role for TRAPP I and Trs85-containing TRAPP III with Ypt1 in exit from the ER and a role for TRAPP II with Ypt31/32 in exit from the *trans*-Golgi and in the recycling of PM proteins to the Golgi.

Materials and Methods

Strains, plasmids, and reagents

Strains and plasmids used in this study are summarized in Table S1. For genetic interaction experiments, Ypt1, Ypt31, and Ypt32 in 2μ plasmids and their corresponding empty plasmids (φ, pRB684, pRS425, and yep351, respectively) were used. All yeast transformations were done using the lithium acetate method (Gietz *et al.* 1992). *Escherichia coli* transformation was done using electroporation.

For live-cell microscopy observation, GFP-Snc1 was integrated into NSY340 (*ypt31Δ/32ts*) and NSY128 (wild type) with pRS406 GS GFP-Snc1 (pNS568) (Lewis *et al.* 2000) to create YLY132 and YLY130, respectively. *TRS85* was deleted from the wild-type strain (YLY130) with KAN^R cassette to create *trs85Δts* (YLY1347). YLY130 was used to mate with NSY1082 (*ypt1ts*), NSY991 (*TRS130-HA*), and NSY992 (*trs130ts*). The diploids were sporulated and tetrads were dissected to obtain the following GFP-Snc1–tagged mutants and wild-type strains: YLY1665 (*ypt1ts*) and YLY1664 (YPT1), YLY1771 (*trs130ts*) and YLY1770 (TRS130). GFP-Snc1-PEM was integrated into *ypt3Δ/32ts*, *ypt1ts*, *trs130ts*, and their corresponding wild-type strains with pRS406 GSSOM GFP-Snc1-PEM (pNS571) (Lewis *et al.* 2000) to create YLY1613 (*ypt1ts-GFP-Snc1-PEM*), YLY1583 (*ypt31Δ/32ts-GFP-Snc1-PEM*), YLY1585 (*trs130ts-GFP-Snc-PEM*), and their corresponding wild-type strains. *TRS85* was deleted from YLY1582 to obtain YLY1651 (*trs85Δ-GFP-Snc-PEM*). Htb1-CFP was integrated into the above GFP-Snc1–tagged strains using pYL227 (a gift from B. Schwappach, Georg-August-Universität, Göttingen, Germany) for marking the nucleus.

Antibodies used in this study are: rabbit antiglucose-6-phosphate dehydrogenase (G6PDH; Sigma-Aldrich), affinity-purified rabbit anti-Ypt31 (Jedd *et al.* 1997), affinity-purified rabbit anti-Ypt1 (Segev *et al.* 1988), and horseradish peroxidase linked antirabbit and antimouse IgG (Amersham Biosciences, Little Chalfont, UK).

All chemical reagents were purchased from Amersco (Fair Lawn, NJ), unless otherwise noted. Other reagents used in this study: SynaptoRed, also known as FM4-64 (Molecular Probes, Eugene, OR), DAPI (Roche Diagnostics, Indianapolis, IN), Geneticin (Gibco Laboratories, Grand Island, NY), and restriction enzymes and buffers (Takara Biotechnology, Dalian, China).

Yeast culture conditions

For genetic interaction, cells were grown overnight at 26° in YPD or minimal (SC) media, normalized to the same density and spotted onto agar plates in 10-fold serial dilutions. Plates were incubated at different temperatures for genetic interaction assays. For live-cell fluorescence microscopy, yeast cultures were grown at permissive temperature (26°) in rich (YPD) or selective (when plasmid is used) media to log phase and switched to a restrictive temperature (37°) for 1.5 hr. Cells were either directly observed or subjected to DAPI staining or FM4-64 staining as described below.

Fluorescence microscopy

Direct fluorescence microscopy of temperature-sensitive yeast cells was performed as described in Chen *et al.* (2005) to localize Snc1, Snc1-PEM, and Htb1. DAPI staining was performed according to protocol suggested by the manufacturer. FM4-64 staining for endosomes (5 min) was done as previously described (Sclafani *et al.* 2010). Slides were visualized using Nikon inverted research microscope Eclipse Ti (Tokyo, Japan). More than five fields were collected for each sample. Each experiment was repeated at least twice.

Cell lysates and immunoblot analysis

For checking the expression level of Ypt1 or Ypt31 in cells used for the genetic interaction assay, five OD_{600} of overnight cell cultures were lysed in 100 μl of Laemmli buffer supplemented with equal volume of glass beads (BioSpec Products, Bartlesville, OK) and vortexed for 2 min. The supernatant of a 13,000 rpm spin (2 min) was subjected to

immunoblot analysis with anti-Ypt1 or anti-Ypt31 antibodies, together with anti-G6PDH for serving as a loading control.

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Literature Cited

Barrowman, J., D. Bhandari, K. Reinisch, and S. Ferro-Novick, 2010 TRAPP complexes in membrane traffic: convergence through a common Rab. Nat. Rev. Mol. Cell Biol. 11: 759–763.

Cai, H., Y. Zhang, M. Pypaert, L. Walker, and S. Ferro-Novick, 2005 Mutants in trs120 disrupt traffic from the early endosome to the late Golgi. J. Cell Biol. 171: 823–833.

Cai, Y., H. F. Chin, D. Lazarova, S. Menon, C. Fu *et al.*, 2008 The structural basis for activation of the Rab Ypt1p by the TRAPP membrane-tethering complexes. Cell 133: 1202–1213.

Chen, S. H., S. Chen, A. A. Tokarev, F. Liu, G. Jedd *et al.*, 2005 Ypt31/32 GTPases and their novel F-box effector protein Rcy1 regulate protein recycling. Mol. Biol. Cell 16: 178–192.

Galan, J. M., A. Wiederkehr, J. H. Seol, R. Haguenauer-Tsapis, R. J. Deshaies *et al.*, 2001 Skp1p and the F-box protein Rcy1p form a non-SCF complex involved in recycling of the SNARE Snc1p in yeast. Mol. Cell. Biol. 21: 3105–3117.

Geng, J., U. Nair, K. Yasumura-Yorimitsu, and D. J. Klionsky, 2010 Post-Golgi Sec proteins are required for autophagy in Saccharomyces cerevisiae. Mol. Biol. Cell 21: 2257–2269.

Gietz, D., A. St Jean, R. A. Woods, and R. H. Schiestl, 1992 Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. 20: 1425.

Hampton, R. Y., A. Koning, R. Wright, and J. Rine, 1996 In vivo examination of membrane protein localization and degradation with green fluorescent protein. Proc. Natl. Acad. Sci. USA 93: 828–833.

Huh, W. K., J. V. Falvo, L. C. Gerke, A. S. Carroll, R. W. Howson *et al.*, 2003 Global analysis of protein localization in budding yeast. Nature 425: 686–691.

Jedd, G., C. Richardson, R. Litt, and N. Segev, 1995 The Ypt1 GTPase is essential for the first two steps of the yeast secretory pathway. J. Cell Biol. 131: 583–590.

Jedd, G., J. Mulholland, and N. Segev, 1997 Two new Ypt GTPases are required for exit from the yeast trans-Golgi compartment. J. Cell Biol. 137: 563–580.

Jones, S., C. Newman, F. Liu, and N. Segev, 2000 The TRAPP complex is a nucleotide exchanger for Ypt1 and Ypt31/32. Mol. Biol. Cell 11: 4403–4411.

Lafourcade, C., J. M. Galan, Y. Gloor, R. Haguenauer-Tsapis, and M. Peter, 2004 The GTPase-activating enzyme Gyp1p is required for recycling of internalized membrane material by inactivation of the Rab/Ypt GTPase Ypt1p. Mol. Cell. Biol. 24: 3815–3826.

Lewis, M. J., B. J. Nichols, C. Prescianotto-Baschong, H. Riezman, and H. R. Pelham, 2000 Specific retrieval of the exocytic SNARE Snc1p from early yeast endosomes. Mol. Biol. Cell 11: 23–38.

Liang, Y., N. Morozova, A. A. Tokarev, J. W. Mulholland, and N. Segev, 2007 The role of Trs65 in the Ypt/Rab guanine nucleotide exchange factor function of the TRAPP II complex. Mol. Biol. Cell 18: 2533–2541.

Lynch-Day, M. A., D. Bhandari, S. Menon, J. Huang, H. Cai *et al.*, 2010 Trs85 directs a Ypt1 GEF, TRAPPIII, to the phagophore to promote autophagy. Proc. Natl. Acad. Sci. USA 107: 7811–7816.

Meiling-Wesse, K., U. D. Epple, R. Krick, H. Barth, A. Appelles *et al.*, 2005 Trs85 (Gsg1), a component of the TRAPP complexes, is required for the organization of the preautophagosomal structure during selective autophagy via the Cvt pathway. J. Biol. Chem. 280: 33669–33678.

Michelsen, K., T. Mrowiec, K. E. Duderstadt, S. Frey, D. L. Minor *et al.*, 2006 A multimeric membrane protein reveals 14–3-3 isoform specificity in forward transport in yeast. Traffic 7: 903–916.

Montpetit, B., and E. Conibear, 2009 Identification of the novel TRAPP associated protein Tca17. Traffic 10: 713–723.

Morozova, N., Y. Liang, A. A. Tokarev, S. H. Chen, R. Cox *et al.*, 2006 TRAPPII subunits are required for the specificity switch of a Ypt-Rab GEF. Nat. Cell Biol. 8: 1263–1269.

Nakatogawa, H., K. Suzuki, Y. Kamada, and Y. Ohsumi, 2009 Dynamics and diversity in autophagy mechanisms: lessons from yeast. Nat. Rev. Mol. Cell Biol. 10: 458–467.

Nazarko, T. Y., J. Huang, J. M. Nicaud, D. J. Klionsky, and A. A. Sibirny, 2005 Trs85 is required for macroautophagy, pexophagy and cytoplasm to vacuole targeting in Yarrowia lipolytica and Saccharomyces cerevisiae. Autophagy 1: 37–45.

Protopopov, V., B. Govindan, P. Novick, and J. E. Gerst, 1993 Homologs of the synaptobrevin/VAMP family of synaptic vesicle proteins function on the late secretory pathway in S. cerevisiae. Cell 74: 855–861.

Sacher, M., Y. Jiang, J. Barrowman, A. Scarpa, and J. Burston *et al.*, 1998 TRAPP, a highly conserved novel complex on the cis-Golgi that mediates vesicle docking and fusion. EMBO J. 17: 2494–2503.

Sacher, M., J Barrowman, D Schieltz, J. R Yates 3rd. and S. Ferro-Novick, 2000 Identification and characterization of five new subunits of TRAPP. Eur. J. Cell Biol. 79: 71–80.

Sacher, M., J. Barrowman, W. Wang, J. Horecka, Y. Zhang *et al.*, 2001 TRAPP I implicated in the specificity of tethering in ER-to-Golgi transport. Mol. Cell 7: 433–442.

Sacher, M., Y. G. Kim, A. Lavie, B. H. Oh, and N. Segev, 2008 The TRAPP complex: insights into its architecture and function. Traffic 9: 2032–2042.

Sclafani, A., S. Chen, F. Rivera-Molina, K. Reinisch, P. Novick *et al.*, 2010 Establishing a role for the GTPase Ypt1p at the late Golgi. Traffic 11: 520–532.

Segev, N., 2001a Ypt and Rab GTPases: insight into functions through novel interactions. Curr. Opin. Cell Biol. 13: 500–511.

Segev, N., 2001b Ypt/rab gtpases: regulators of protein trafficking. Sci. STKE 2001: re11.

Segev, N., and D. Botstein, 1987 The ras-like yeast YPT1 gene is itself essential for growth, sporulation, and starvation response. Mol. Cell. Biol. 7: 2367–2377.

Segev, N., J. Mulholland, and D. Botstein, 1988 The yeast GTP-binding YPT1 protein and a mammalian counterpart are associated with the secretion machinery. Cell 52: 915–924.

Stenmark, H., 2009 Rab GTPases as coordinators of vesicle traffic. Nat. Rev. Mol. Cell Biol. 10: 513–525.

Tokarev, A. A., D. Taussig, G. Sundaram, Z. Lipatova, Y. Liang *et al.*, 2009 TRAPP II complex assembly requires Trs33 or Trs65. Traffic 10: 1831–1844.

Wang, W., and S. Ferro-Novick, 2002 A Ypt32p exchange factor is a putative effector of Ypt1p. Mol. Biol. Cell 13: 3336–3343.

Wang, W., M. Sacher, and S. Ferro-Novick, 2000 TRAPP stimulates guanine nucleotide exchange on Ypt1p. J. Cell Biol. 151: 289–296.

Yang, Z., and D. J. Klionsky, 2009 An overview of the molecular mechanism of autophagy. Curr. Top. Microbiol. Immunol. 335: 1–32.

Yip, C. K., J. Berscheminski, and T. Walz, 2010 Molecular architecture of the TRAPPII complex and implications for vesicle tethering. Nat. Struct. Mol. Biol. 17: 1298–1304.

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GENETICS

Supporting Information
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Modular TRAPP Complexes Regulate Intracellular Protein Trafficking Through Multiple Ypt/Rab GTPases in *Saccharomyces cerevisiae*

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DOI: 10.1534/genetics.112.139378

File S1

Supporting Materials and Methods

Cell Lysates and Protein Complexes Purification

Ypt1, Ypt31 and Ypt32 proteins expressed in bacteria were purified as described previously (Jones *et al.*, 2000); protein concentration was ~0.5 mg/ml. Yeast cell extracts and purified protein complexes were prepared and analyzed as described previously (Morozova *et al.*, 2006). For purifying TRAPP complexes, cells were grown in SD-Ura medium overnight, and then diluted into SD-Ura-Leu media at OD_{600} of 0.2 to grow overnight. Cells were re-inoculated into 1L medium at OD_{600} of 0.2 to cell OD_{600} of 0.8, and then yeast cells expressing GST-Bet5, GST-Trs85, and GST under the CUP1 promoter were induced with 0.25 mM $CuSO_4$ for 6 h at 26°C. Cell breakage buffers were supplemented with an EDTA-free protease-inhibitors cocktail (Roche Diagnostics). Protein concentrations were determined by a Bio-Rad protein assay (Bio-Rad, Hercules, CA). Ten micrograms of yeast whole cell lysates or 0.2 µg GST-associated complexes were loaded on 10% SDS-polyacrylamide gel electrophoresis (PAGE). Gels were run, and proteins were transferred to polyvinylidene difluoride membranes and subjected to immuno-blot analysis.

GDP Release Assays

GDP release assays were performed as described previously (Morozova *et al.*, 2006). GST-, GST-Bet5-, or GST-Trs85-associated complexes were purified from yeast cell lysates and added as a source of GEF to GDP-release assays with bacterially purified Ypt1, Ypt31 or Ypt32 loaded with ^{3}H-GDP.

References

Brachmann, C.B., Davies, A., Cost, G.J., Caputo, E., Li, J., Hieter, P., and Boeke, J.D. (1998). Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast *14*, 115-132.

Jones, S., Jedd, G., Kahn, R.A., Franzusoff, A., Bartolini, F., and Segev, N. (1999). Genetic interactions in yeast between Ypt GTPases and Arf guanine nucleotide exchangers. Genetics *152*, 1543-1556.

Jones, S., Newman, C., Liu, F., and Segev, N. (2000). The TRAPP complex is a nucleotide exchanger for Ypt1 and Ypt31/32. Mol Biol Cell *11*, 4403-4411.

Lewis, M.J., Nichols, B.J., Prescianotto-Baschong, C., Riezman, H., and Pelham, H.R.B. (2000). Specific retrieval of the exocytic SNARE Snc1p from early yeast endosomes. Mol Biol Cell *11*, 23-38.

Liang, Y., Morozova, N., Tokarev, A.A., Mulholland, J.W., and Segev, N. (2007). The role of Trs65 in the Ypt/Rab guanine nucleotide exchange factor function of the TRAPP II complex. Mol Biol Cell *18*, 2533-2541.

Ma, H., Kunes, S., Schatz, P.J., and Botstein, D. (1987). Plasmid construction by homologous recombination in yeast. Gene *58*, 201-216.

Michelsen, K., Mrowiec, T., Duderstadt, K.E., Frey, S., Minor, D.L., Mayer, M.P., and Schwappach, B. (2006). A multimeric membrane protein reveals 14-3-3 isoform specificity in forward transport in yeast. Traffic *7*, 903-916.

Morozova, N., Liang, Y., Tokarev, A.A., Chen, S.H., Cox, R., Andrejic, J., Lipatova, Z., Sciorra, V.A., Emr, S.D., and Segev, N. (2006). TRAPPII subunits are required for the specificity switch of a Ypt-Rab GEF. Nat Cell Biol *8*, 1263-1269.

Sciorra, V.A., Audhya, A., Parsons, A.B., Segev, N., Boone, C., and Emr, S.D. (2005). Synthetic genetic array analysis of the PtdIns 4-kinase Pik1p identifies components in a Golgi-specific Ypt31/rab-GTPase signaling pathway. Mol Biol Cell *16*, 776-793.

Sikorski, R.S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics *122*, 19-27.

Yamamoto, K., and Jigami, Y. (2002). Mutation of TRS130, which encodes a component of the TRAPP II complex, activates transcription of OCH1 in Saccharomyces cerevisiae. Curr Genet *42*, 85-93.

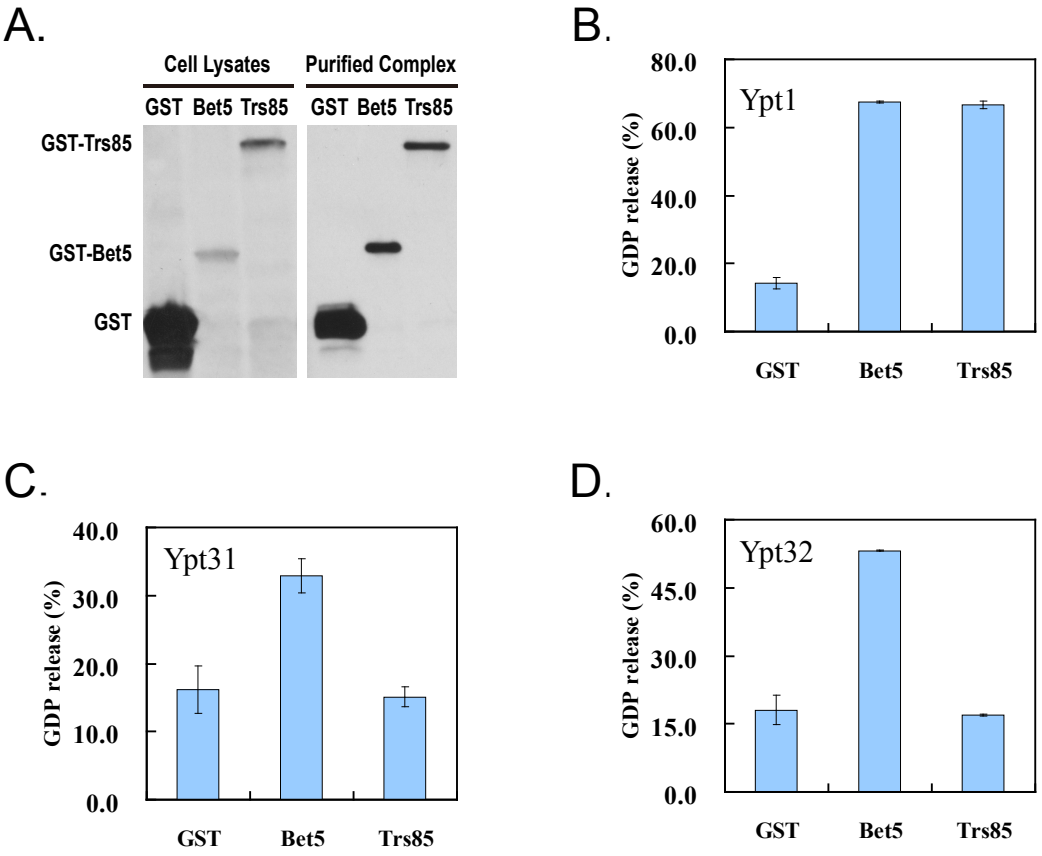
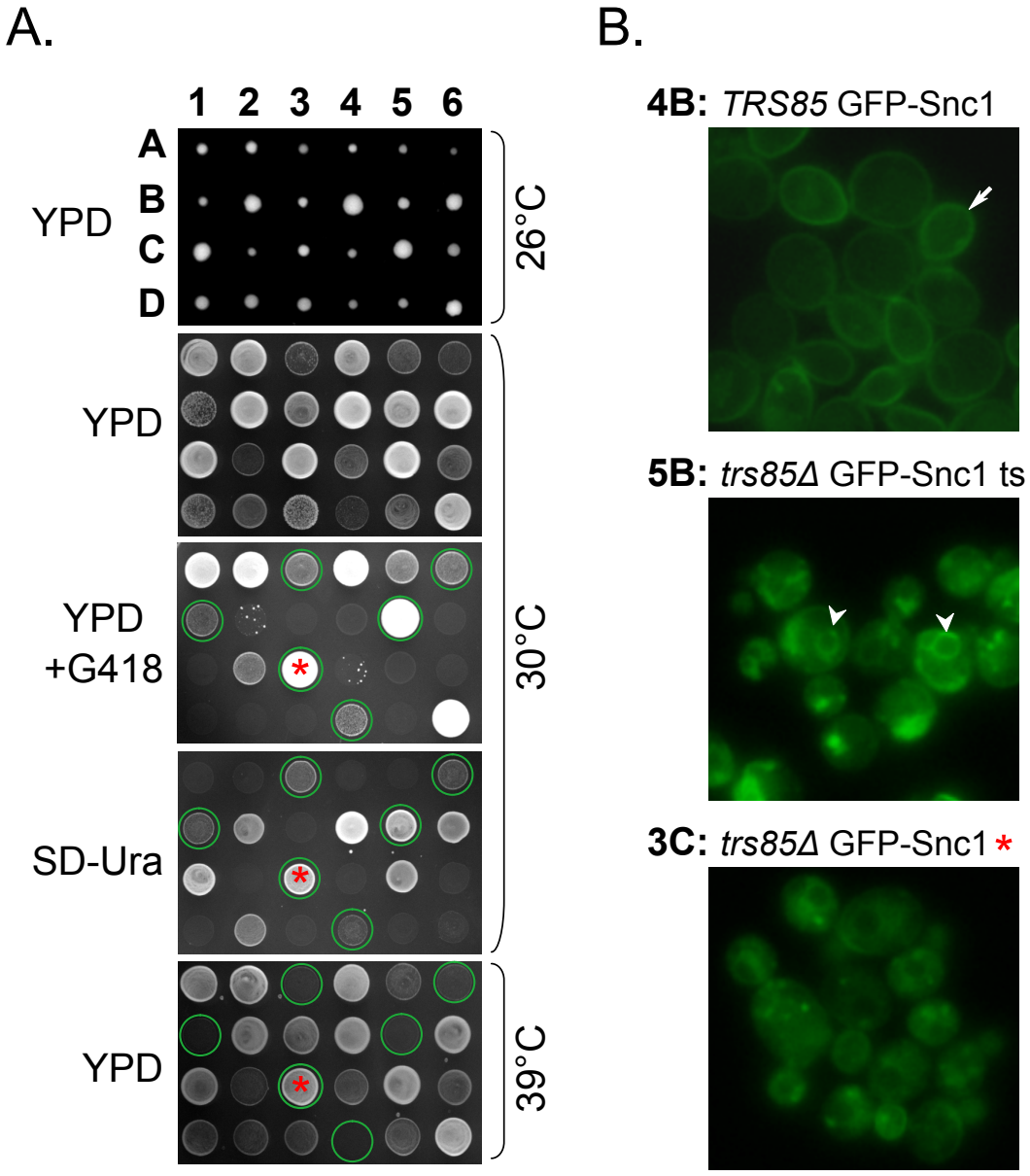


Figure S1 GST-Bet5, but not GST-Trs85, complex purified from yeast lysates can act as a GEF for Ypt31 and Ypt32. A. GST-Bet5 and GST-Trs85 complexes were purified from yeast cell lysates expressing the different GST-tagged proteins from a plasmid; GST was used as a negative control. B. Both GST-Bet5 and GST-Trs85 purified complexes can act as Ypt1 GEFs. Equal amount of GST-purified complexes were used as a GEF source in a GDP-release assay with bacterially expressed Ypt1 protein. Percent GDP release at 36 minutes is shown. C. Purified GST-Bet5, but not GST-Trs85 complexes can act as a GEF for Ypt31. The experiment was done as described in panel B, except that Ypt31 protein was used for the GDP-release assay and percent GDP release at 50 minutes is shown. D. Purified GST-Bet5, but not GST-Trs85 complexes can act as a GEF for Ypt32. The experiment was done as described in panel B, except that Ypt32 protein was used for the GDP-release assays. Percent GDP release at 30 minutes is shown. Results shown in panels B, C and D are the average of duplicate reactions and are representative of at least three independent experiments. Error bars represent standard deviation.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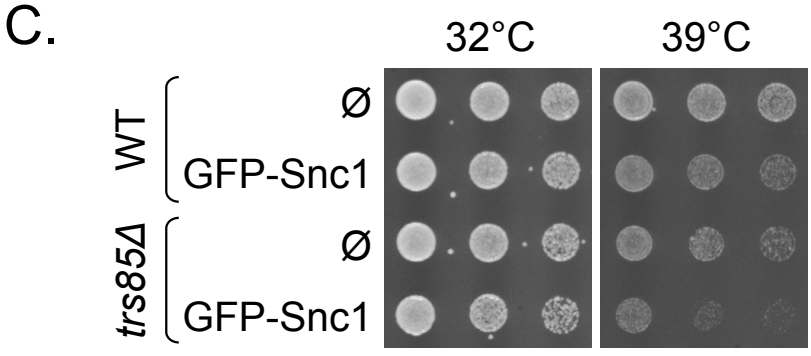
3C: trs85Δ GFP-Snc1 *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GFP-Snc1

Figure S2 A temperature-sensitive growth phenotype is linked to over-expression of GFP-Snc1 in *trs85Δ* mutant cells. A. Segregation of a temperature-sensitive growth phenotype with *trs85Δ* GFP-Snc1. The following two haploid strains were mated: *MATα* GFP-Snc1::*URA3* (YLY130) and *MATa trs85Δ*::*KAN*^{R} (YLY919) strain on YPD at 26°C. Diploids were selected, sporulated, dissected and grown on YPD at 26°C (top). Spores (A-D) from six consecutive tetrads (1-6) were inoculated in YPD medium and spotted on various plates, which were incubated at the indicated temperatures. Shown from top to bottom: Growth on YPD at 30°C, YPD+G418 (30°C) for *trs85Δ*::*KAN*^{R}, SD-Ura (30°C) for GFP-Snc1::*URA3*, and YPD at 39°C for temperature sensitivity. Green circles show *KAN*^{R} *URA3* spores. All *KAN*^{R} *URA3* spores are temperature sensitive for growth except for spore 3C (*). B. GFP-Snc1 localization in wild type and temperature-sensitive (ts) spores. Spores expressing GFP-Snc1 from panel A were examined using live-cell microscopy for their GFP-Snc1 phenotype at 37°C, as described for Figure 1B. The GFP-Snc1 phenotype is shown for three spores from top to bottom: 4B, *TRS85* GFP-Snc1 (arrows point to GFP-Snc1 on the PM in wild type cells); 5B, *trs85Δ* GFP-Snc1 ts (arrowheads point to the intracellular GFP-Snc1 rings); 3C, *trs85Δ* GFP-Snc1 not ts (*), GFP-Snc1 accumulates inside cells at a lower level than in ts cells. C. Temperature sensitive growth phenotype by expression of GFP-Snc1 from a plasmid in *trs85Δ* mutant cells. Wild type and *trs85Δ* mutant cells were transformed with a high-copy 2μ *URA3* empty plasmid, as a negative control, or the same plasmid expressing GFP-Snc1 from the *TPI* promoter. Shown is growth on SD-Ura plates at 32° and 39°C (10-fold serial dilutions from left to right). Over-expression of GFP-Snc1 in *trs85*Δ mutant cells results in a temperature-sensitive growth phenotype at 39°C. Results shown in this panel are representative of four independent transformants.

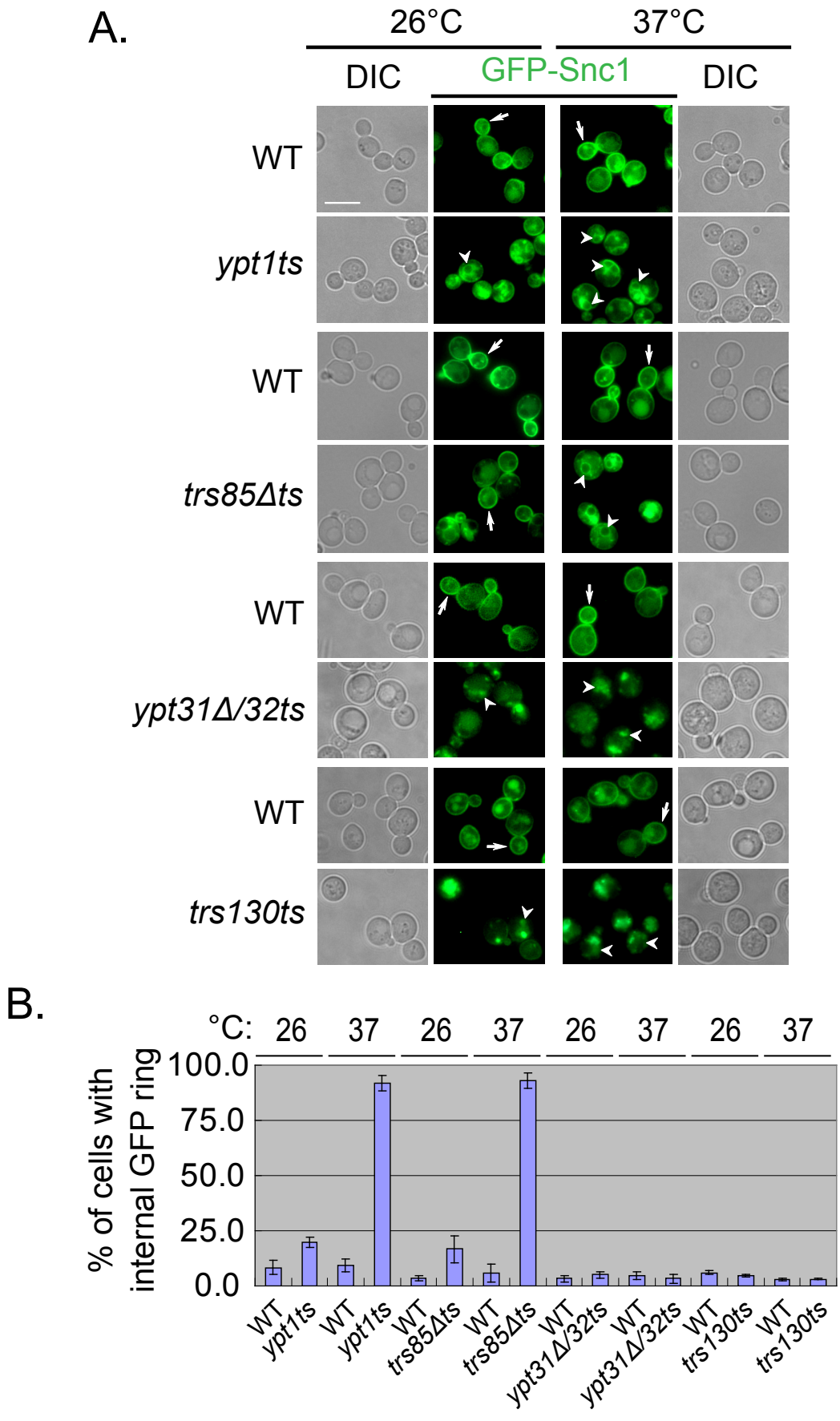
A.
26°C
37°C
DIC
GFP-Snc1
DIC
WT
ypt1ts
WT
trs85Δts
WT
ypt31Δ/32ts
WT
trs130ts
B.
°C: 26 37 26 37 26 37 26 37
% of cells with internal GFP ring
100.0
75.0
50.0
25.0
0.0
WT
ypt1ts
WT
ypt1ts
WT
trs85Δts
WT
trs85Δts
WT
ypt31Δ/32ts
WT
ypt31Δ/32ts
WT
trs130ts
WT
trs130ts

Figure S3 Accumulation of intra-cellular GFP-Snc1 in *ypt1ts* and *trs85Δts* mutant cells is different from its accumulation in *ypt31Δ/32ts* and *trs130ts*. A. Accumulation of internal GFP-Snc1 in *ypt1ts* and *trs85Δts* mutant cells. Wild type and *ypt1ts*, *trs85Δts*, *ypt31Δ/32ts* and *trs130ts* mutant cells expressing chromosomally-tagged GFP-Snc1 were grown to mid-log phase in YPD medium at 26°C (left) or shifted to 37°C for 1.5 hours (right). GFP-Snc1 localization was visualized by live-cell fluorescence microscopy. In wild type cells, GFP-Snc1 localizes mainly to the PM. In all four mutant strains GFP-Snc1 accumulates inside cells. Whereas the intracellular GFP-Snc1 accumulation is evident as puncta in *ypt31Δ/32ts* and *trs130ts* mutant cells already at the permissive temperature, in *ypt1ts* and *trs85Δts* it is apparent as rings mostly at the restrictive temperature. Arrows point to GFP-Snc1 on PM, and arrowheads point to internalized GFP-Snc1. DIC images (on each side) show the contour of cells; Bar, 7 mm.
B. Quantification of data presented in panel A. Shown is percent of cells containing an internal GFP-Snc1 ring. At least 100 cells were counted in at least three fields for each strain; error bars represent STDEV.

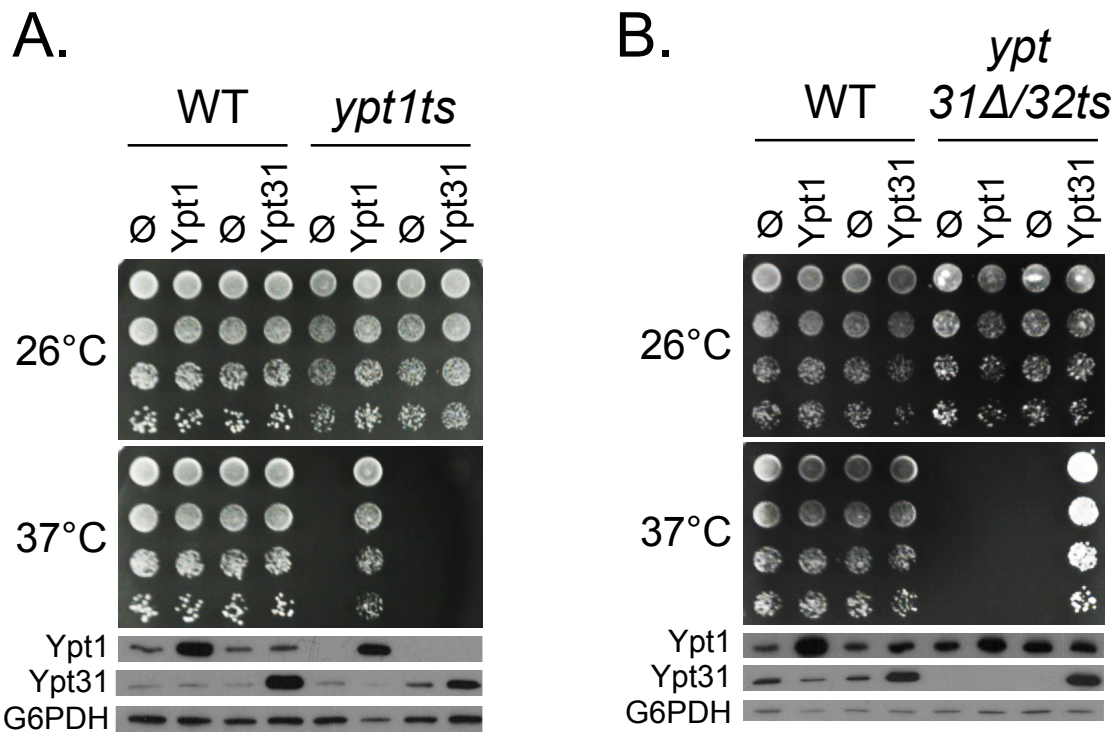

Figure S4 Ypt1 suppresses the growth defect of *ypt1ts*, whereas Ypt31 suppresses the growth defect of *ypt31Δ/32ts*. Mutant cells, *ypt1ts* (A) and *ypt31Δ/32ts* (B), and their corresponding wild-type cells, all expressing chromosomally tagged GFP-Snc1, were transformed with the following 2m plasmids: Ypt1, and Ypt31, and their corresponding empty plasmids (*φ* : pRB684 and pRS425, respectively). Cell growth on plates was tested at 26°C and 37°C; four ten-fold serial dilutions are shown from top to bottom. Over-expression of Ypt1, but not Ypt31, suppresses the growth defect of *ypt1ts* (A). In contrast, over-expression of Ypt31, but not Ypt1, suppresses the growth defect of *ypt31Δ/32ts* (B). Over-expression of Ypt1 and Ypt31 in the transformants was verified as in Figure 5.

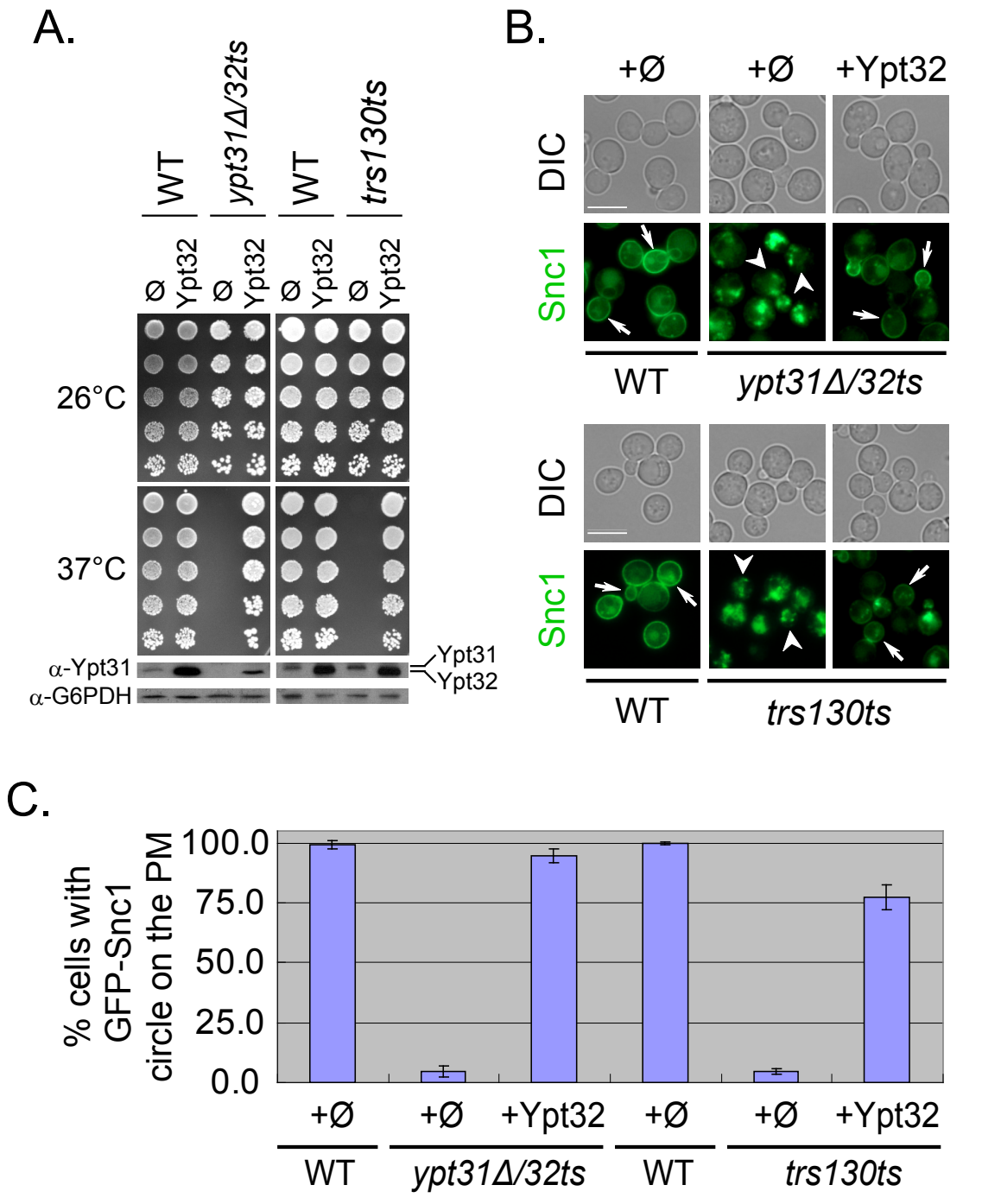

Figure S5 Ypt32 suppresses the growth defect and GFP-Snc1 phenotype of *ypt31Δ/32ts* and *trs130ts*. A. Over-expression of Ypt32 suppresses the growth defect of *ypt31Δ/32ts* and *trs130ts*. Mutant cells, *ypt31Δ/32ts* and *trs130ts*, and their corresponding wild-type cells, all expressing chromosomally tagged GFP-Snc1, were transformed with Ypt32, and its corresponding empty 2m plasmids (*φ*: yep351). Cell growth on plates was tested as in Figure S4. Over-expression of Ypt32 in the transformants was verified by immunoblot analysis using anti-Ypt31 antibody which

also recognize Ypt32 as smaller size; G6PDH indicates equal loading (shown at the bottom of each panel). B. Ypt32 suppresses the GFP-Snc1 phenotype of *ypt31Δ/32ts* and *trs130ts*. Cells used in panel A were grown to mid-log phase in SD-Leu medium at 26°C and incubated at 37°C for 1.5 hours before GFP-Snc1 localization was visualized by live-cell fluorescence microscopy. Overexpression of Ypt32 in WT has the same GFP-Snc1 phenotype as overexpression of empty plasmids in it. Only overexpression of empty plasmids in WT is shown. Arrows point to GFP-Snc1 on the PM in wild type and suppressed mutant cells; arrowheads point to intracellular GFP-Snc1 seen in mutant cells.
C. Quantification of data presented in panel B. Shown is percent of cells with GFP-Snc1 on the PM. At least 290 cells were counted in at least four fields for each strain; error bars represent STDEV.

Table S1 Yeast strains and plasmids used in this study

A. Strains

Strain	Alias	Genotype	Source*
NSY825	BY4741	*MATa his3-1 leu2Δ met15Δ ura3Δ*	(Brachmann *et al.*, 1998)
YLY1342		BY4741, *TRS65::Hyg*	
YLY919		BY4741, *TRS85::Kan*	
NSY128	YPT31	*MATα ade2 his3-Δ200 leu leu2-3,112 lys 2-801 ura 3-52 gal*	(Jones *et al.*, 1999)
NSY340	*ypt31Δ/32ts*	*MATα ura 3-52 lys2 leu2 ypt31Δ/32ts*	(Jones *et al.*, 1999)
NSY991	VSY459, TRS130	*MATa leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ura3-52 TRS130-HA:HIS3MX6*	(Sciorra *et al.*, 2005)
NSY992	*trs130ts*	*MATa leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ura3-52 trs130-(33 aa truncation)-HA^{ts}:HIS3MX6*	(Sciorra *et al.*, 2005)
NSY1081	YPT1	*MATa ura3-52 Leu2 his3*	(Liang *et al.*, 2007)
NSY1082	*ypt1ts*	*MATa ura3-52 Leu2 his3 ypt1A136D*	(Liang *et al.*, 2007)
YLY130	GFP-Snc1	NSY128, *GFP-Snc1::URA3*	
YLY132	GFP-Snc1 *ypt31Δ/32ts*	NSY340, *GFP-Snc1::URA3*	
YLY1770	GFP-Snc1 TRS130	*MATa his3 lys2 leu2 TRS130-HA:HIS3MX6 GFP-Snc1::URA3*	
YLY1771	GFP-Snc1 *trs130ts*	*MATa his3 lys2 leu2 trs130-(33 aa truncation)-HA^{ts}:HIS3MX6 GFP-Snc1::URA3*	
YLY1664	GFP-Snc1 YPT1	*MATa ade2 his3 leu2 lys2 GFP-Snc1::URA3*	
YLY1665	GFP-Snc1 *ypt1ts*	*MATa ade2 his3 leu2 lys2 ypt1A136D GFP-Snc1::URA3*	
YLY1347	GFP-Snc1 *trs85Δts*	YLY130, *TRS85::Kan*	
YLY1340	GFP-Snc1 *trs65Δ*	YLY130, *TRS65::Hyg*	
YLY1582	GFP-Snc1-PEM	NSY128, *GFP-Snc1-PEM::URA3*	
YLY1583	GFP-Snc1-PEM *ypt31Δ/32ts*	NSY340, *GFP-Snc1-PEM::URA3*	
YLY1584	GFP-Snc1-PEM TRS130	NSY991, *GFP-Snc1-PEM::URA3*	
YLY1585	GFP-Snc1-PEM *trs130ts*	NSY992, *GFP-Snc1-PEM::URA3*	
YLY1612	GFP-Snc1-PEM YPT1	NSY1081, *GFP-Snc1-PEM::URA3*	
YLY1613	GFP-Snc1-PEM *ypt1ts*	NSY1082, *GFP-Snc1-PEM::URA3*	

YLY1651	GFP-Snc1-PEM *trs85Δ*	YLY1582, *TRS85::Kan*
NSY915	EJ758	*MATa his 3 delta leu200-3, 112 ura3-52 pep4::his3*

B. Plasmids

Plasmid	Alias	Genotype	Source*
pNS568		pRS406 GS GFP-Snc1	(Lewis *et al.*, 2000)
pNS571		pRS406 GSSOM GFP-Snc1-PEM	(Lewis *et al.*, 2000)
pYL227		Htb1-CFP NUF-3' UTR in pRS303	(Michelsen *et al.*, 2006)
pNS114	pRB684	2μ, *LEU2*	(Ma *et al.*, 1987)
pNS993		pRS11-*YPT1*	(Liang *et al.*, 2007)
pNS180	pRS425	2μ, *LEU2*	(Sikorski and Hieter, 1989)
pNS781		pRS425-Ypt31	(Sciorra *et al.*, 2005)
pYL151	Yep351	2μ, *LEU2*	(Yamamoto and Jigami, 2002)
pYL283		Yep351-Ypt32	
pNS563	pRS426	2 μ, *URA3*	(Sikorski and Hieter, 1989)
pNS1404	2μ GFP-Snc1	pNS563 with TPIp GFP-Snc1 (BamHI-PspXI from pNS568)	

*All strains and plasmids, if not marked otherwise, were constructed in this study.