

# Mutants in *trs120* disrupt traffic from the early endosome to the late Golgi

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**T**ransport protein particle (TRAPP), a large complex that mediates membrane traffic, is found in two forms (TRAPPI and -II). Both complexes share seven subunits, whereas three subunits (Trs130p, -120p, and -65p) are specific to TRAPP II. Previous studies have shown that mutations in the TRAPP II-specific gene *trs130* block traffic through or from the Golgi. Surprisingly, we report that mutations in *trs120* do not block general secretion. Instead, *trs120* mutants accumulate aberrant membrane structures that resemble Berkeley bodies and

disrupt the traffic of proteins that recycle through the early endosome. Mutants defective in recycling also display a defect in the localization of coat protein I (COPI) subunits, implying that Trs120p may participate in a COPI-dependent trafficking step on the early endosomal pathway. Furthermore, we demonstrate that Trs120p largely colocalizes with the late Golgi marker Sec7p. Our findings imply that Trs120p is required for vesicle traffic from the early endosome to the late Golgi.

## Introduction

The yeast *Saccharomyces cerevisiae* internalizes extracellular fluid, plasma membrane proteins, and ligands bound to their receptors (Riezman, 1993). The endocytosis of membrane proteins from the plasma membrane to the vacuole can be divided into several steps that involve at least two classes of endosomes, early and late endosomes (Singer-Kruger et al., 1993; Hicke et al., 1997; Prescianotto-Baschong and Riezman, 1998). The late endosome, or prevacuolar compartment, is where the endocytic pathway converges with the *vacuolar protein sorting* (*vps*) pathway (Davis et al., 1993; Babst et al., 1997).

Traffic from endosomes to the trans-Golgi is required for the transport of at least three different types of cargo proteins. Soluble vacuolar proteins, such as carboxypeptidase Y (CPY), are examples of the first type of cargo. Vps10p, a sorting receptor for CPY, forms a complex with this vacuolar protease at the trans-Golgi and then enters vesicles targeted to a prevacuolar compartment where Vps10p and CPY are dissociated. CPY is transported to the vacuole, whereas Vps10p is recycled back to the trans-Golgi via a retrograde pathway (Marcusson et al., 1994; Cooper and Stevens, 1996). Resident proteins of the late Golgi, which recycle between the endosome and Golgi, are examples of a second type of cargo. Well-characterized examples

of Golgi resident proteins are Kex2p and DPAP-A (dipeptidyl aminopeptidase A), which act in the late Golgi to modify the mating pheromone  $\alpha$  factor as it traverses the secretory pathway (Fuller et al., 1988). Specific signals in the cytoplasmic portion of these proteins are required for their retrieval from endosomes (Wilcox et al., 1992; Nothwehr et al., 1993). The third class of proteins are integral membrane plasma membrane proteins, such as the exocytic SNARE Snc1p. These proteins are endocytosed from the plasma membrane and travel through the early endosome before they reach the late Golgi, where they are recycled back to the plasma membrane (Lewis et al., 2000). Several proteins have been identified that regulate traffic between endosomes and the late Golgi, including proteins that are required for vesicle budding, targeting, and fusion.

In this paper, we show that Trs120p, a component of the multiprotein complex called transport protein particle (TRAPP) II (Sacher et al., 2001), is required for the trafficking of proteins from the early endosome. TRAPP II is one of six large complexes that have been implicated in the tethering of transport vesicles to an acceptor compartment in *S. cerevisiae* (for reviews see Guo et al., 2000; Whyte and Munro, 2002). Unlike other essential subunits of the TRAPP complex, mutants in *trs120* do not appear to block general secretion. However, the *trs120-2*, *-4*, and *-8* mutants block the recycling of GFP-Snc1p, which traffics from the early endosome to the late Golgi. The trafficking of a second marker protein that follows a similar recycling pathway, chitin synthase III (Chs3p), is also defective in these mutants. Interestingly, the same

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Abbreviations used in this paper: Chs3p, chitin synthase III; COP, coat protein; CPY, carboxypeptidase Y; PI[3]P, phosphatidylinositol-3-phosphate; TAP, tandem affinity purification; TRAPP, transport protein particle; *vps*, *vacuolar protein sorting*.

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*trs120* mutants mislocalize subunits of the coat protein I (COPI) complex (also called coatomer). Our findings support a role for Trs120p in the tethering of vesicles that recycle through the early endosome.

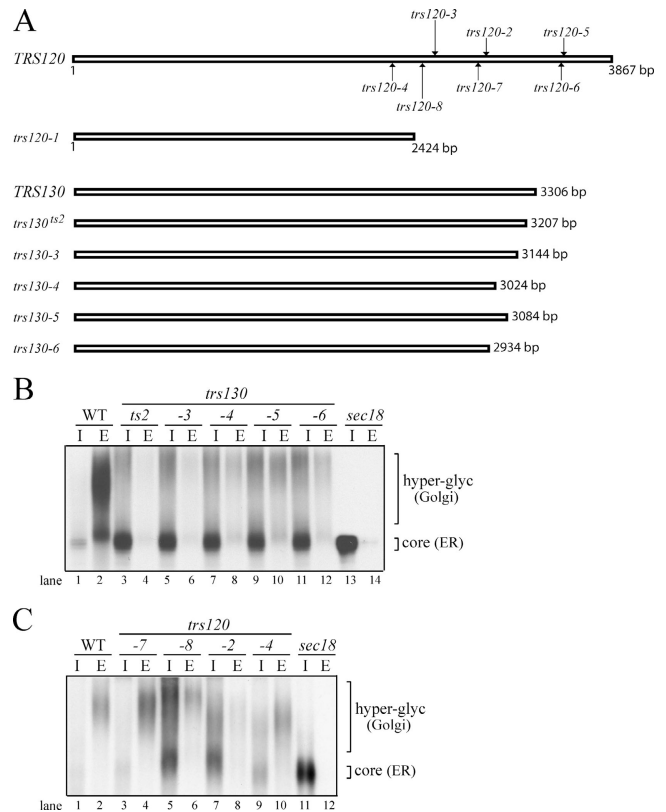
## Results

### Invertase secretion is not blocked in all *trs120* temperature-sensitive mutants

Experiments have shown that there are two forms of the TRAPP complex, TRAPPI and -II. TRAPPI and -II share seven subunits, whereas three subunits (Trs130p, -120p, and -65p) are unique to TRAPP II (Sacher et al., 2001). Mutational analysis and in vitro transport studies have revealed that TRAPPI acts in ER–Golgi traffic to mediate the tethering of COPII vesicles to the Golgi, whereas mutants in the TRAPP II-specific subunit Trs130p disrupt Golgi traffic (Sacher et al., 2001).

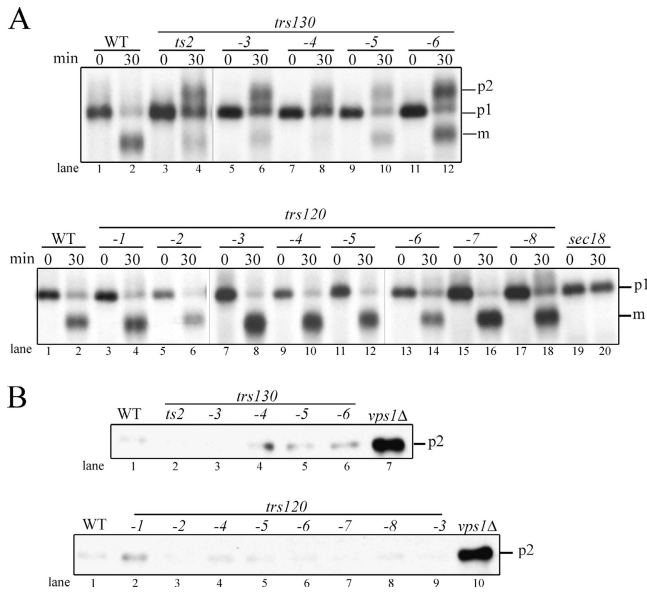
To further characterize the function of TRAPP II in membrane traffic, we constructed a collection of temperature-sensitive mutations in genes that encode essential TRAPP II-specific subunits. Initially, PCR mutagenesis was used to isolate mutants in *trs120*. However, after this approach failed to yield mutants, transposon mutagenesis was performed. The location of the transposon insertion in each of the seven mutants we isolated is shown in Fig. 1 A. An eighth mutant, *trs120-1*, was constructed by truncating the last 481 amino acids of Trs120p (Fig. 1 A). Further deletion of the COOH terminus of Trs120p led to cell death. Five truncation mutants in *trs130* were also constructed. These mutants deleted the region of *TRS130* that encodes the last 33–124 amino acids (Fig. 1 A). Deletion of *TRS65*, which encodes the third TRAPP II-specific subunit, did not affect the growth of yeast or membrane traffic (Sacher et al., 2001).

Initially, these temperature-sensitive mutants were examined for defects in the trafficking of invertase. Invertase is translocated into the lumen of the ER, where it is core glycosylated. The ER form of invertase, which is ~80 kD, accumulates in mutants that block traffic from the ER to the Golgi complex (*sec18* mutant in Fig. 1, B [lane 13] and C [lane 11]). The 80-kD form of invertase is further glycosylated in the Golgi and then transits through the early endosome before it is secreted into the periplasm (Harsay and Schekman, 2002). External invertase migrates on SDS–polyacrylamide gels as a heterogeneous species with an apparent molecular mass of 100–140 kD (Fig. 1 B, lane 2). To determine whether mutants in *trs130* and *-120* block invertase secretion, we shifted wild-type and mutant cells to the restrictive temperature (37°C) for 20 min to impose a block in secretion. Cells were resuspended in low-glucose medium to derepress the synthesis of invertase and radiolabeled for 60 min with [<sup>35</sup>S] ProMix. As shown in Fig. 1 B, all *trs130* mutants exhibited a near complete or partial defect in secretion at the nonpermissive temperature, accumulating the heterogeneous high molecular mass form of invertase and partially glycosylated forms. Invertase assays revealed that this defect was clearly temperature sensitive (Table S1, available at <http://www.jcb.org/cgi/content/full/jcb.200505145/DC1>). An analysis of the carbohydrate modification indicated that the accumulated



**Figure 1. The analysis of invertase secretion in *trs130* and *-120* mutants.** (A) A schematic diagram of *trs120* and *-130* mutants used in this study. The *trs120-1* mutant was made by truncating 481 amino acids from the COOH terminus of Trs120p. The other *trs120* mutants were made by transposon mutagenesis as described in Materials and methods. The arrows point to the position where the transposon is inserted in each mutant. Five *trs130* mutants were made by truncating 33, 54, 74, 94, and 124 amino acids from the COOH terminus of Trs130p. Invertase secretion is blocked in all *trs130* mutants (B) but not all *trs120* mutants (C). Wild-type (WT) and mutant cells were grown at 25°C and preincubated at 37°C for 20 min. Cells were resuspended in low-glucose medium to induce the synthesis of invertase and were radiolabeled for 60 min. Samples were then processed for the immunoprecipitation of internal (I) and periplasmic (E) invertase. To facilitate the detection of the high molecular mass form of invertase in *trs130* mutants, cells were transformed with the *SUC2* gene on a 2- $\mu$ m plasmid.

invertase contained  $\alpha$ 1-3 mannose (later Golgi modification; Sacher et al., 2001; unpublished data), implying that it reached the late Golgi (Graham and Emr, 1991; Brigance et al., 2000). These results indicate that mutations in *trs130* confer a defect in membrane traffic at multiple steps in the Golgi, including the late Golgi, where invertase is fully glycosylated. In contrast, invertase assays revealed partial secretion defects in only two *trs120* mutants (*trs120-2* and *-8*). Radiolabeling experiments demonstrated that these mutants showed a partial block in secretion and accumulated both partially and fully glycosylated forms of invertase. Both mutants also secreted some external invertase (Fig. 1 C and see Table S1 for quantitative assays). The forms of invertase that accumulated in *trs120-2* and *-8*, as well as two mutants that did not accumulate significant amounts of invertase, are shown in Fig. 1 C. The defect observed in the *trs120-2* and *-8* mutants was reproducibly seen in four separate experiments.

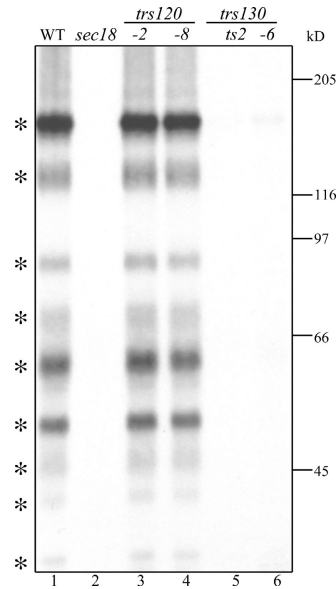


**Figure 2. The analysis of CPY trafficking in *trs130* and *-120* mutants.** (A) Mutants in *trs130*, but not *-120*, display a defect in CPY trafficking. Wild-type (WT) and mutant cells were grown at 25°C, preincubated at 37°C for 20 min, labeled for 4 min, and chased for 30 min. Aliquots of samples were removed at the indicated time points and immunoprecipitated with anti-CPY antibody. (B) Mutants in *trs130* and *-120* do not secrete CPY into the growth medium. The growth medium was collected from wild-type and mutant cells that were shifted to 37°C for 2 h, processed for TCA precipitation, and blotted with anti-CPY antibody. The secretion of CPY from the *vps1Δ* mutant is shown as a control.

### The loss of *trs120* function does not block the trafficking of CPY

To monitor the trafficking of a second marker protein, we analyzed the vacuolar protease CPY. CPY is synthesized, translocated, and glycosylated in the lumen of the ER (p1 form) before it is transported to the Golgi (p2), where it receives additional carbohydrate modifications. On transport to the vacuole, CPY is proteolytically activated to the mature form (Stevens et al., 1982). To examine CPY trafficking, wild-type and mutant cells were preincubated at 37°C for 20 min, pulse labeled for 4 min, and chased for 30 min (Fig. 2 A). At the end of the chase, all *trs130* mutants accumulated small amounts of the p1 form of CPY as well as a heterogeneous smear of CPY that accumulated between the p1 and p2 forms of CPY. Small amounts of mature CPY were also observed at the end of the chase (Fig. 2 A, top), suggesting that Trs130p is not completely inactivated at 37°C. These results are consistent with our previous proposal that TRAPP2 may be required for intra-Golgi traffic (Sacher et al., 2001). This finding is also consistent with the observation that fully glycosylated invertase accumulates in the late Golgi in all *trs130* mutants. In contrast, the kinetics of anterograde CPY trafficking in all *trs120* mutants was the same as wild type (Fig. 2 A, bottom).

CPY is sorted away from secreted proteins in the trans-Golgi by the sorting receptor Vps10p (Vida et al., 1993; Marcussen et al., 1994). Failure to sort CPY from the TGN or to retrieve Vps10p from a late endosomal compartment leads to a *vps* phenotype and the secretion of CPY into the growth medium (Bankaitis et al., 1986; Rothman and Stevens, 1986).



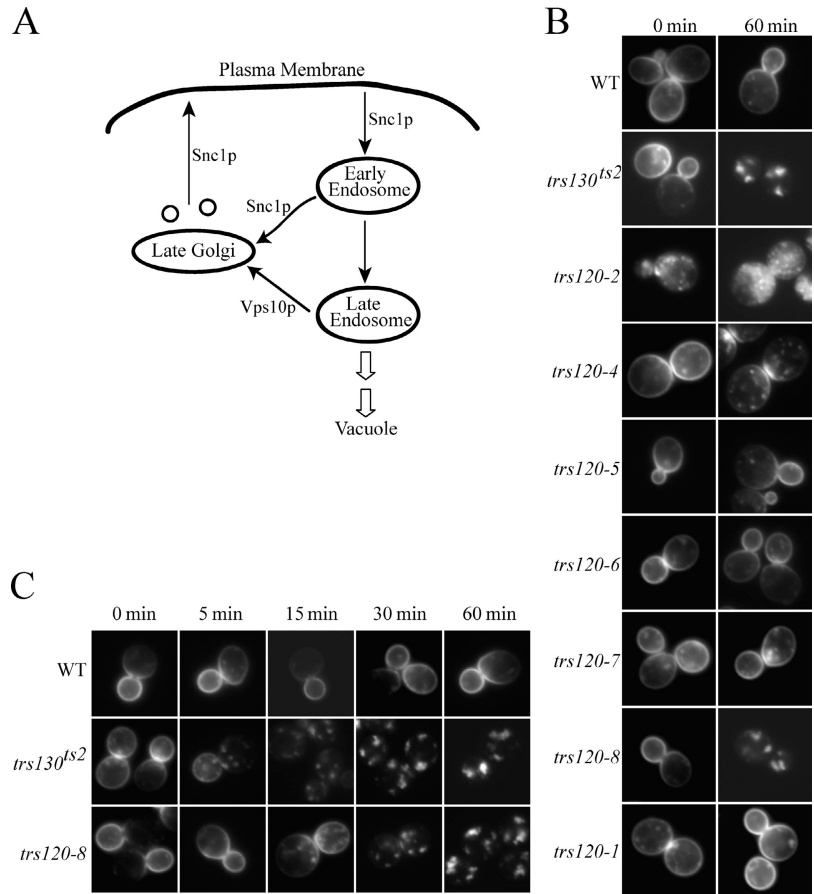
**Figure 3. Mutants in *trs120* do not block general secretion.** Cells were grown to early log phase at 25°C, preincubated to 37°C for 20 min, radio-labeled for 15 min, and then chased for 30 min. The growth medium was separated from the cells by centrifugation, processed for TCA precipitation, and analyzed by SDS-PAGE on a 10% gel as described previously (Kim et al., 2001). The asterisks mark proteins secreted into the medium in wild-type (WT) cells.

To determine whether the loss of *trs120* or *-130* function leads to a *vps* phenotype, wild-type and mutant cells were grown to early log phase, pelleted, resuspended in fresh growth medium, and shifted to 37°C for 2 h. Proteins secreted into the growth medium were precipitated with TCA and assayed for the presence of CPY using Western blot analysis. Although the secretion of CPY was detected in the *vps* mutant, *vps1Δ* (Fig. 2 B, top [lane 7] and bottom [lane 10]), significant amounts of CPY were not detected in the *trs120* and *-130* mutants (Fig. 2 B).

### Trs120p is not required for traffic on the exocytic pathway

The finding that *trs120-2* and *-8* mutants do not block the trafficking of CPY and only partially block invertase secretion prompted us to determine whether there is a general block in secretion in these mutants. To assay for a general secretion defect, we shifted wild-type and mutant cells to 37°C for 20 min, pulse labeled them for 15 min, and chased them for 30 min. Cells were pelleted, and the proteins secreted into the medium were precipitated with TCA and resolved on an SDS-polyacrylamide gel (Fig. 3). Surprisingly, although the *sec18*, *trs130<sup>ts2</sup>*, and *trs130-6* mutants blocked secretion (Fig. 3, compare lane 1 with lanes 2, 5, and 6), no obvious defect was observed in the *trs120-2* and *-8* mutants (lanes 3 and 4). The block in secretion was complete in *sec18* but not in *trs130* mutants (see Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200505145/DC1>, for darker exposure of the autorad). Therefore, although mutations in the *trs130* gene lead to a defect in the trafficking of proteins within the Golgi, the loss of *trs120* function does not result in a significant block in secretion.

Figure 4. **Mutants in *trs120* and *-130* are defective in GFP-Snc1p recycling.** (A) The recycling pathway of GFP-Snc1p. (B) GFP-Snc1p was examined in wild-type (WT) and mutant cells that were grown at 25°C (0 min) or shifted to 37°C for 60 min. (C) GFP-Snc1p was examined in wild type, *trs130<sup>ts2</sup>*, and *trs120-8* mutants at 0, 5, 15, 30, and 60 min after a shift to 37°C.



### The recycling of Snc1p and Chs3p is defective in *trs120* mutants

Our findings indicate that Trs120p may not be required for general secretion. However, the analysis of invertase trafficking revealed a defect in invertase secretion in the *trs120-2* and *-8* mutants (Fig. 1 C and Table S1). Invertase has been shown to transit through the early endosome before reaching the cell surface (Harsay and Schekman, 2002). Therefore, one explanation for the defect in invertase secretion in the *trs120-2* and *-8* mutants is that Trs120p may be required for the trafficking of proteins through the early endosome. To begin to address this question, we examined the recycling of GFP-Snc1p in the *trs120* mutants we constructed, as well as in all *trs130* mutants.

Snc1p is the yeast homologue of synaptobrevin. With its homologous partner, Snc2p, it mediates the fusion of post-Golgi vesicles with the plasma membrane (Protopopov et al., 1993). Snc1p largely resides on the plasma membrane but is rapidly endocytosed and traffics through the early endosome before it reaches the late Golgi, where it is incorporated into another round of secretory vesicles (Fig. 4 A; Lewis et al., 2000). To monitor the recycling of GFP-Snc1p, we grew wild-type and mutant cells at the permissive temperature (25°C) or shifted them to 37°C for 60 min. In wild-type cells, GFP-Snc1p was generally found at regions of polarized growth and the plasma membrane. Interestingly, after a 60-min shift to 37°C, little GFP-Snc1p was found at the cell surface in the *trs120-2* and *-8* mutants (Fig. 4 B). In both mutants, GFP-Snc1p was

present on intracellular membranes in >80% of the cells. In *trs120-2*, GFP-Snc1p was found in small punctate structures at 25 and 37°C. The recycling defect in *trs120-2* resulted in a growth defect at 25°C, and when this defect was more pronounced at 37°C, the cells died. An increased cytoplasmic haze was also observed (Fig. 4 B). This cytosolic haze may correspond to the presence of GFP-Snc1p in transport vesicles. In support of this hypothesis, we found that the *trs120-2* mutant accumulated three to five times more vesicles than wild type (see Fig. 9). Although no significant defect in invertase secretion was found in the *trs120-4* mutant, GFP-Snc1p was found in punctate structures in ~40% of the cells (Fig. 4 B). Thus, *trs120* mutants that exhibited defects in invertase secretion also displayed severe defects in GFP-Snc1p recycling. In contrast, all *trs130* mutants were defective in GFP-Snc1p recycling and, like *trs120-8*, GFP-Snc1p accumulated in larger structures in these mutants (Fig. 4 B, *trs130<sup>ts2</sup>*).

As shown in Fig. 4 C, GFP-Snc1p-containing structures that accumulate in *trs120* and *-130* mutants became larger during a 60-min incubation at 37°C. To address whether the enlarged structures are early endosomes or Golgi, we labeled *trs130<sup>ts2</sup>* and *-120-8* mutant cells that were shifted to 37°C for 30 min with the styryl dye FM4-64. The early endosome is labeled after a brief incubation (5 min) with this dye (Vida and Emr, 1995). At the 5-min time point, the GFP-Snc1p-containing structures were labeled with FM4-64 (Fig. 5), implying that at least some of the Snc1p accumulates in the early endosome at

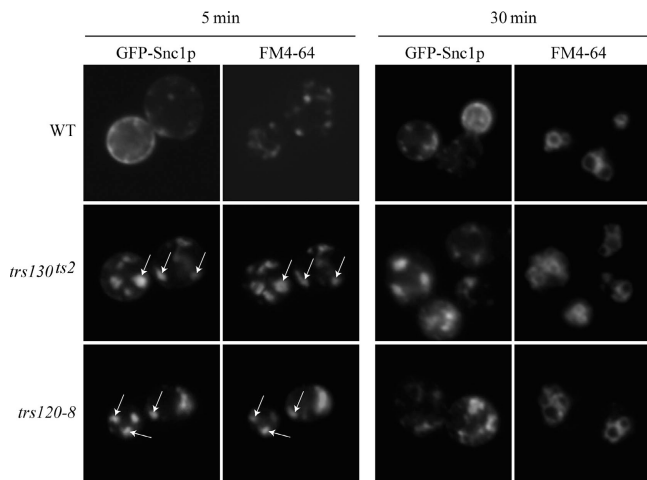


Figure 5. **GFP-Snc1p localizes to early endosomes in the *trs130<sup>ts2</sup>* and *-120-8* mutants.** Cells were labeled with FM4-64 as described in Materials and methods. FM4-64 was internalized for 5 or 30 min.

37°C in the *trs130<sup>ts2</sup>* and *-120-8* mutants. At the later time point (30 min), FM4-64 was no longer detected in the GFP-Snc1p-containing structures and instead stained the vacuolar membrane, which was fragmented in both mutants (Fig. 5).

To determine whether another marker protein that travels through the early endosome is also defective in these *trs120* and *-130* mutants, we analyzed the cell surface enzyme Chs3p. Chs3p follows a recycling pathway that is similar to Snc1p. It localizes to the mother-bud junction, the cell surface, and punctate intracellular structures called chitosomes (Chuang and Schekman, 1996; Valdivia et al., 2002). To monitor the recycling of Chs3p, we grew wild-type and mutant cells at the permissive temperature or shifted them to 37°C for 30 min. Quantitation of ~100–150 cells revealed that at 37°C Chs3p-GFP localized to the mother–bud neck region in ~51% of wild-type cells. A dramatic decrease in the targeting of Chs3p-GFP to the bud neck was observed in the *trs130<sup>ts2</sup>* (8%), *-120-2* (10.5%), and *-120-8* (9%) mutants at this temperature, whereas a modest decrease was seen in the *trs120-4* (29%) mutant (Fig. 6). The *trs120-2* mutant also displayed a partial defect (30%) at 25°C, whereas no defect was observed in the *trs130<sup>ts2</sup>*, *-120-4*, and *-120-8* mutants at this temperature (Fig. 6). These findings indicate that mutants in *trs120* and *-130* display defects in the targeting of Chs3p to the bud neck. Furthermore, the temperature dependence and the severity of the defect for each of the affected mutants was the same as observed for GFP-Snc1p.

#### TRAPP<sub>II</sub> localizes to a late Golgi/early endosomal compartment

Previous fractionation studies indicated that TRAPP<sub>I</sub> and <sub>II</sub> cofractionate with each other and early Golgi markers on a sucrose density gradient (Sacher et al., 2001). Our finding that mutations in *trs130* and *-120* block the trafficking of Snc1p and Chs3p warranted a reexamination of the localization of TRAPP<sub>II</sub>.

In an attempt to better resolve the different compartments of the Golgi by subcellular fractionation, we modified the sucrose density gradient we had previously used. In earlier studies, a

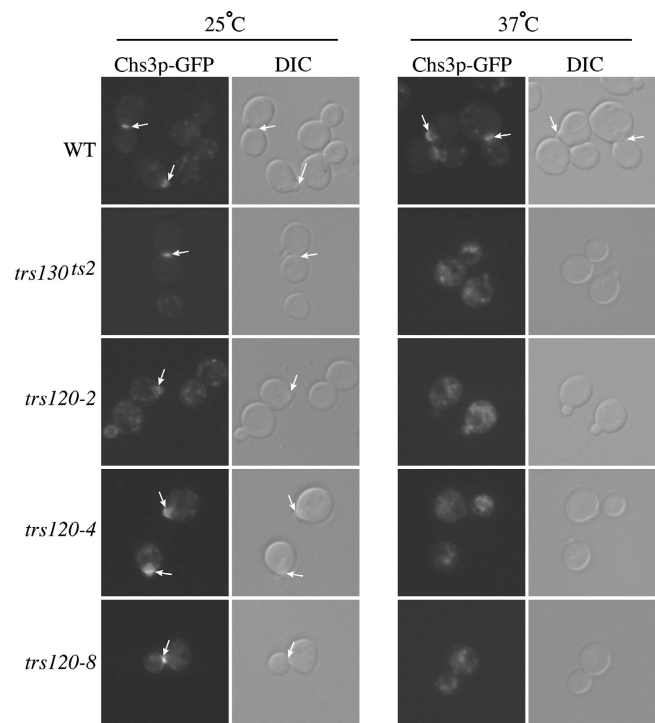
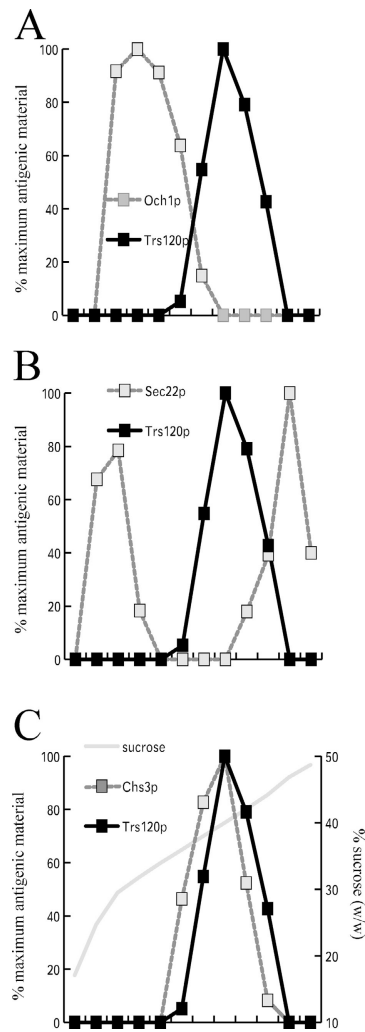


Figure 6. **Mutants in *trs120* and *-130* are defective in the trafficking of Chs3p-GFP.** Chs3p-GFP was examined in wild-type and mutant cells that were grown at 25°C or shifted to 37°C for 30 min. Arrows point to bud necks and incipient bud sites, which are visible in the differential interference contrast (DIC) image.

step gradient containing 11 different concentrations of sucrose, ranging from 18 to 60%, was used. The TRAPP<sub>II</sub> complex fractionated between 26 and 48% sucrose on this gradient (Sacher et al., 2001). For this paper, we designed a new step gradient containing 11 1-ml steps that ranged from 26 to 50% sucrose (see details in Materials and methods). Och1p, which marks the earliest known carbohydrate-modifying compartment in the yeast Golgi, was used as an early Golgi marker (Nakayama et al., 1992; Brigance et al., 2000), whereas Chs3p marked both the late Golgi and early endosomes (Valdivia et al., 2002). Late Golgi and early endosomes are difficult to resolve by sucrose density analysis (Holthuis et al., 1998; Valdivia et al., 2002). Thus, it is unclear whether late Golgi markers solely mark this compartment or if they also mark the early endosome. With our gradient conditions, Och1p peaked in fraction 4 (Fig. 7 A), whereas Chs3p peaked in fraction 8 (Fig. 7 C). The SNARE Sec22p peaked in fractions 3 and 11 (Fig. 7 B). The later peak (at 47% sucrose) represents the fraction of Sec22p that resides on the ER, whereas the earlier peak (at 29% sucrose) is Golgi-bound Sec22p (Barrowman et al., 2000). This result confirms that our new gradient conditions clearly resolve early and late Golgi subcompartments. To monitor the localization of TRAPP<sub>II</sub> on this gradient, we added a 13-x-myc tag to the genomic copy of *Trs120p* as described previously (Longtine et al., 1998). Tagged *Trs120p* is functional, as the presence of the tag did not impede growth or the assembly of the TRAPP<sub>II</sub> complex. *Trs120p* largely cofractionated with Chs3p (Fig. 7 C) but not with Och1p and Sec22p (Fig. 7,



**Figure 7. Trs120p resides on a late Golgi/early endosomal compartment.** Lysates were prepared and fractionated on a sucrose density gradient as described in Materials and methods. Trs120p does not colocalize with the early Golgi marker Och1p (A) or the ER-Golgi SNARE Sec22p (B). Trs120p largely cofractionates with the late Golgi/early endosomal marker Chs3p (C).

A and B). These findings imply that Trs120p resides on a late Golgi/early endosomal compartment that is marked by Chs3p.

Using a monoclonal anti-myc antibody, we performed indirect immunofluorescence to localize Trs120p-myc in cells containing the late Golgi marker Sec7p-GFP. Quantitation of 86 cells revealed that 314 of the 331 Trs120p-myc-containing puncta colocalized with Sec7p-GFP (Fig. 8 A, top). A total of 448 Sec7p-GFP-containing puncta were identified in the 86 cells that were quantitated, and 314 of these puncta colocalized with Trs120p-myc. Similar results were obtained when we examined the localization of Trs130p fused to 3-x-GFP with Sec7p-DsRed (Fig. 8 A, bottom). Furthermore, quantitation of >280 puncta revealed that ~50% of the Sec7p-DsRed puncta colocalized with Chs3p-GFP and ~60% of the Chs3p-GFP puncta colocalized with Sec7p-DsRed (Fig. 8 B). Thus, Chs3p and Sec7p largely colocalize with each by direct fluorescence. The localization of TRAPP II was also monitored with respect to the FYVE domain of early endosomal antigen 1, which

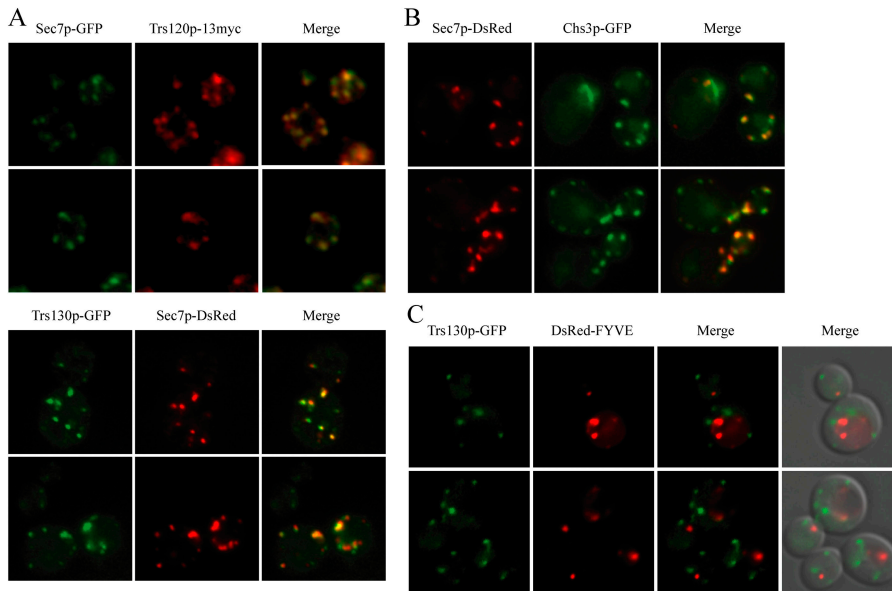
marks phosphatidylinositol-3-phosphate (PI[3]P)-containing endosomal membranes (Burd and Emr, 1998; Gaullier et al., 1998; Katzmann et al., 2003). Trs130p-3-x-GFP did not colocalize with DsRed-FYVE, which was found on punctate structures adjacent to the vacuole (Fig. 8 C). Together, our findings indicate that TRAPP II resides on a late Golgi/early endosomal compartment that contains Sec7p and Chs3p. Furthermore, this compartment appears to be distinct from PI(3)P-containing endosomal membranes.

### Aberrant membrane structures accumulate in mutants defective in recycling

To determine whether *trs120* mutants defective in early endosomal traffic accumulate membrane structures, the phenotype of wild-type, *trs120-2*, *-120-4*, *-120-8*, and *-130<sup>ts2</sup>* mutant cells was examined morphologically by EM after a 2-h shift to 37°C (Fig. 9). Previous experiments demonstrated that mutants in *trs130* accumulate Berkeley bodies (Sacher et al., 2001). Berkeley bodies are believed to be aberrant Golgi. They are somewhat varied in shape and either form a toroid or contain two curved membranes with an enclosed lumen (Novick et al., 1980). As previously reported (Sacher et al., 2001), the *trs130<sup>ts2</sup>* mutant accumulated Berkeley bodies (Fig. 9, compare B with A). Interestingly, although the *trs130<sup>ts2</sup>* mutant blocks secretion and *trs120* mutants do not, the *trs120* mutants accumulated aberrant membrane structures that resemble Berkeley bodies. Quantitation of 50 cells revealed that the *trs120-2* (Fig. 9 D) and *-8* mutants accumulated a comparable number of aberrant membrane structures that resemble Berkeley bodies. Furthermore, the number of aberrant membrane structures in these mutants was comparable with the number of Berkeley bodies in the *trs130<sup>ts2</sup>* mutant. In contrast, the *trs120-4* mutant accumulated 5–10 times fewer aberrant membranes than the *trs120-2* and *-8* mutants (Fig. 9 C). Therefore, the accumulation of aberrant membrane structures in the *trs120* mutants appears to correlate with the severity of the defect in early endosomal traffic. The *trs120* and *-130* mutants also accumulated vesicles (<100 and >100 nm in size; Fig. 9, B and D). However, the origin of these vesicles is currently unknown.

### Mutants in *trs120*, which display a recycling defect, also mislocalize coatamer subunits

Mutants bearing mutations in genes that code for TRAPP II-specific subunits were previously reported to display synthetic lethal interactions with mutants containing mutations in genes encoding COPI subunits (Sacher et al., 2001). COPI is a heptameric complex that was originally identified for its role in the budding of vesicles from the Golgi (Rothman and Wieland, 1996). Interestingly, COPI has been shown to interact with other tethers (Suvorova et al., 2002). It also has been implicated in recruiting Snc1p into vesicles bound for the late Golgi (Lewis et al., 2000). These findings suggested a possible link between TRAPP II and COPI and prompted us to analyze the localization of coatamer in the *trs120* and *-130* mutants we constructed.



**Figure 8. TRAPP II largely colocalizes with Sec7p-GFP but not with PI(3)P-containing endosomal membranes.** (A, top) Cells containing Sec7p-GFP and Trs120p-13myc were fixed and processed for immunofluorescence. (bottom) Cells containing Trs130p-GFP and Sec7p-DsRed were viewed by direct fluorescence microscopy. (B) Chs3p-GFP-containing puncta also largely colocalized with Sec7p-DsRed. Cells containing Sec7p-DsRed and Chs3p-GFP were viewed by direct fluorescence microscopy. (C) Cells containing Trs130p-GFP and DsRed-FYVE were viewed by direct fluorescence microscopy. Trs130p-GFP did not colocalize with DsRed-FYVE, a marker for PI(3)P-containing endosomal membranes.

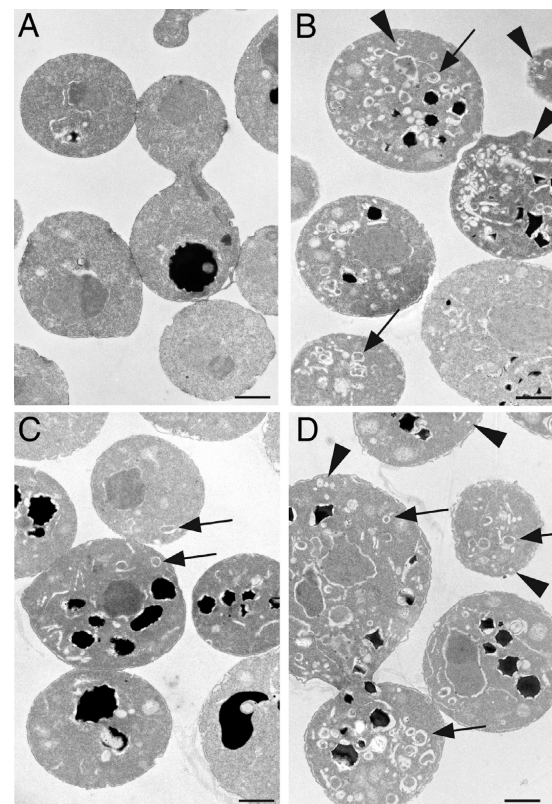
To monitor the localization of COPI, we used Sec21p-GFP, the  $\gamma$  subunit of COPI fused to GFP (Hosobuchi et al., 1992; Rossanese et al., 2001). In wild-type cells shifted to 37°C for 15 min, Sec21p-GFP localized to punctate structures in the cytoplasm (Fig. 10 A). Interestingly, three *trs120* mutants (*trs120-2*, *-4*, and *-8*) displayed defects in the localization of Sec21p-GFP. Besides puncta, a uniform haze throughout the cytosol was also observed in these mutants (Fig. 10 A). The same result was obtained with permissively grown cells. In contrast, as shown in Fig. 10 A, *trs130* mutants showed wild-type staining. The localization of a second coat subunit, Ret2p-GFP, was also defective in the same *trs120* mutants (Fig. 10 B). The cytosolic haze observed with COPI subunits is not attributable to cleaved GFP, as none was detected by immunoblotting whole cell lysates with an anti-GFP antibody (unpublished data).

The increased cytosolic haze of Sec21p-GFP (or Ret2p-GFP) observed in *trs120* mutants may be soluble coatomer, COPI vesicles that accumulate in the cytosol, or dispersed membrane structures. To rule out the possibility that the cytosolic fluorescence resulted from the dispersal of Golgi membranes, the integrity of the Golgi was monitored by fluorescence microscopy using Och1p-HA as an early Golgi marker (Nakayama et al., 1992; Brigance et al., 2000) and Sec7p-GFP as a late Golgi marker (Franzusoff et al., 1991; Rossanese et al., 2001). Sec7p-GFP is a particularly good marker to analyze because the majority of the Sec7p-containing puncta colocalize with Trs120p (Fig. 8 A). In the *trs120* mutants, the localization, distribution, and morphology of Och1p and Sec7p were indistinguishable from wild type (Fig. 10 A), indicating that the integrity of the early and late Golgi was not perturbed in these mutants. Our data indicates that mutants in *trs120*, but not *-130*, disrupt the localization of COPI in vivo.

#### TRAPP interacts with coatomer

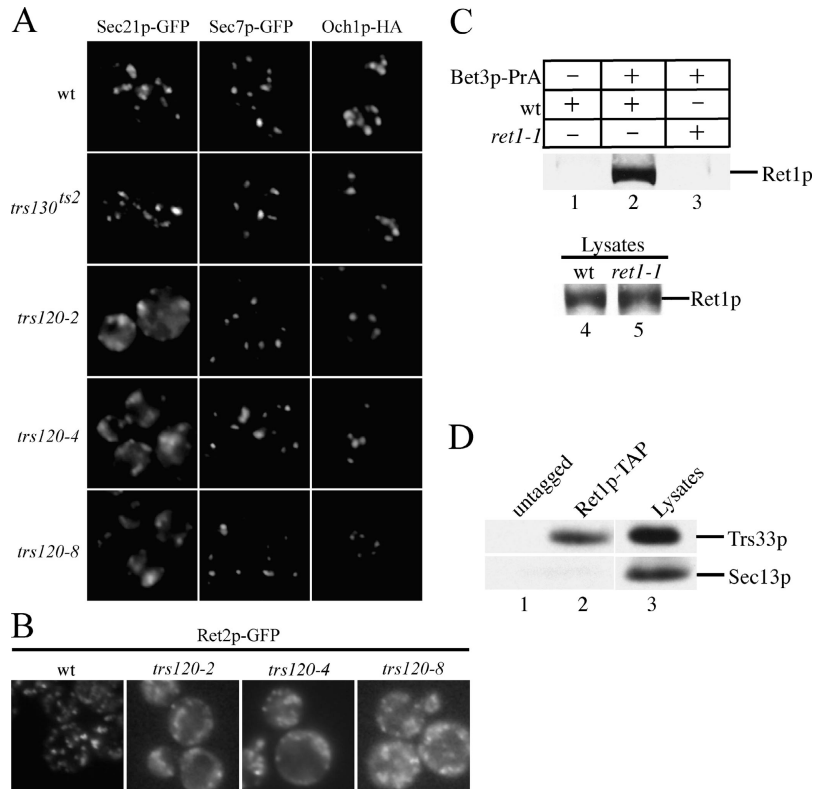
The observation that coatomer (Sec21p-GFP) is mislocalized in certain *trs120* mutants prompted us to determine whether TRAPP II physically interacts with coatomer. To address this

possibility, protein A-tagged Bet3p (Bet3p-PrA) was immobilized on IgG-Sepharose beads, and the washed TRAPP-containing beads were incubated with either wild-type or *ret1-1* mutant lysates (Fig. 10 C, lanes 2 and 3). As a control, beads



**Figure 9. Mutants in *trs120* and *-130* accumulate aberrant membrane structures.** Wild-type and mutant cells were shifted to 37°C for 2 h in YPD (yeast extract/peptone/dextrose) medium and processed for EM as described previously (Newman and Ferro-Novick, 1987). (A) Wild type, (B) *trs130<sup>s2</sup>*, (C) *trs120-4*, and (D) *trs120-2*. The arrows point to Berkeley bodies or structures that resemble Berkeley bodies. Arrowheads point to vesicles. Bars, 1  $\mu$ m.

**Figure 10. The *trs120-2*, *-4*, and *-8* mutants display a defect in the localization of coatomer subunits.** (A) Wild-type (wt) and mutant cells containing Sec21p-GFP, Sec7p-GFP, or Och1p-HA were grown to early log phase at 25°C and then shifted to 37°C for 15 min. For the analysis of Och1p-HA, cells were processed for immunofluorescence using a monoclonal anti-HA antibody. (B) Wild-type and mutant cells containing Ret2p-GFP were grown at 25°C and then shifted to 37°C for 15 min. (C) To immobilize TRAPP on beads, 50 mg of lysate prepared from a Bet3p PrA-tagged strain (lanes 2 and 3) was incubated with 30  $\mu$ l of IgG-Sepharose beads for 2 h at 4°C. As a control, an untagged lysate (lane 1) was incubated with beads in the same way. The beads were washed three times with 1 ml of wash buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM DTT, 2.5 mM MgCl<sub>2</sub>, 1% Triton X-100, and 1 $\times$  Protease inhibitor cocktail) and then incubated with 50 mg of wild type (lanes 1 and 2) or *ret1-1* lysate (lane 3) for 3 h. After the incubation, the beads were washed four times with 1 ml of wash buffer, eluted with 0.2 M glycine, pH 2.8, and neutralized before loading onto an SDS-polyacrylamide gel. To detect Ret1p in lysates, equal amounts of wild-type (lane 4) and *ret1-1* (lane 5) lysates were subjected to SDS gel electrophoresis and blotted with anti-coatomer antibody. (D) Lysates prepared from a Ret1p TAP-tagged (lane 2) or untagged (lane 1) strain were incubated with 30  $\mu$ l of IgG-Sepharose beads for 2–3 h at 4°C. The beads were washed and treated as described in C. The lysate containing TAP-tagged Ret1p (lane 3) was subjected to SDS-PAGE and blotted with anti-Trs33p and anti-Sec13p antibodies.



that lacked TRAPP were also treated with a wild-type lysate (Fig. 10 C, lane 1). Bound proteins were eluted, subjected to SDS gel electrophoresis, and immunoblotted to detect the coatomer subunit Ret1p. We found that Ret1p bound to the TRAPP-containing beads but not control beads (Fig. 10 C, compare lanes 1 and 2). The average of several binding experiments revealed that  $\sim 0.1\%$  of the total Ret1p bound to TRAPP. Furthermore, a decrease in binding was observed when the TRAPP-containing beads were incubated with a *ret1-1* mutant lysate (Fig. 10 C, compare lanes 2 and 3). This decrease in binding appeared to be the consequence of the *ret1-1* mutation, as no reduction in Ret1p was observed in the mutant lysate (Fig. 10 C, compare lanes 4 and 5).

A reciprocal binding experiment was performed as depicted in Fig. 10 C, using tandem affinity purification (TAP)-tagged Ret1p and wild-type lysate. Proteins bound to the beads were eluted and probed with antibodies directed against the TRAPP subunit Trs33p and the COPII subunit Sec13p (COPII subunit required for budding from the ER; Barlowe et al., 1994), which was used as a specificity control. Coatomer bound to TRAPP, but no binding was observed to Sec13p (Fig. 10 D, lanes 1–3). On average,  $\sim 0.1\%$  of the TRAPP bound to TAP-tagged Ret1p. Therefore, although the interaction between TRAPP and coatomer appears to be weak, it is specific.

## Discussion

### Trs120p is required for traffic from the early endosome

To address the role of Trs120p in the TRAPP complex, we constructed a collection of eight mutations in *trs120*. Surpris-

ingly, none of these mutants block the trafficking of CPY and only two (*trs120-2* and *-8*) disrupt the trafficking of invertase, a secreted protein that transits through the early endosome before reaching the yeast periplasm (Harsay and Schekman, 2002). Interestingly, both of these mutants severely disrupt the trafficking of two marker proteins (GFP-Snc1p and Chs3p-GFP) that recycle from the plasma membrane through the early endosome. Another mutant, *trs120-4*, partially blocks the trafficking of Snc1p and Chs3p but does not significantly block invertase secretion. Because general secretion is not blocked in the *trs120-2* and *-8* mutants, we attribute the defect in invertase secretion to the severe block in traffic on the endosomal pathway. No defect in general secretion was detected in the *trs120-8* mutant even after a prolonged incubation at the restrictive temperature, conditions that clearly disrupt endocytic traffic (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200505145/DC1>). Thus, the block in endocytic traffic is the primary defect in *trs120* mutants and not the consequence of disrupting exit from the late Golgi.

The recruitment of proteins into vesicles that recycle from the early endosome to the Golgi is likely to be mediated by a cytoplasmic coat. In mammalian cells, the COPI complex has been implicated in the sorting and recycling function of early endosomes (Aniento et al., 1996; Daro et al., 1997), and studies in yeast have also suggested a role for COPI on this pathway. Specifically, GFP-Snc1p recycling was found to be defective in mutants harboring mutations in COPI subunits (Lewis et al., 2000). Interestingly, we find here that COPI subunits are mislocalized in *trs120* mutants defective in GFP-Snc1p recycling. An increased cytosolic haze of coatomer was observed in *trs120-2*, a mutant that accumulates GFP-Snc1p in



small vesicular structures. We speculate that these structures may be COPI vesicles that are unable to tether. Our finding that TRAPP interacts with COPI in yeast lysates supports this hypothesis. An intriguing possibility is that TRAPP II may tether vesicles via COPI. Further studies will be needed to determine whether TRAPP directly interacts with coatamer or if this interaction is mediated by another component. A cytosolic haze of coatamer was also observed in the *trs120-4* and *-8* mutants. However, the mislocalization defect was not as severe as in *trs120-2*, and GFP-Snc1p accumulated in large punctate structures in these mutants. COPI may be more soluble in the *trs120-4* and *-8* mutants. However, because coatamer readily dissociates from membranes upon cell lysis, we were unable to use subcellular fractionation to directly address this possibility.

In contrast to *trs120*, mutants in *trs130* display general defects in secretion. All mutants in *trs130* examined here accumulate some of the ER form of invertase and CPY and a heterogeneous collection of Golgi forms of both marker proteins. The trafficking of GFP-Snc1p and Chs3p-GFP is also defective in *trs130* mutants. These findings are consistent with a role for Trs130p in traffic to and through the Golgi complex, as well as traffic between the Golgi and early endosomes. Therefore, Trs130p may be required at multiple steps on the secretory and endocytic pathways, whereas Trs120p appears to be required on only the endocytic pathway. Given the different phenotypes of *trs120* and *-130* mutants reported here, one possibility is that Trs120p acts independently of the TRAPP II complex. However, we have shown that the quantitative depletion of Bet3p from lysates also results in the complete depletion of Trs120p and *-130p* (Fig. S3, available at <http://www.jcb.org/cgi/content/full/jcb.200505145/DC1>). Thus, Trs120p is exclusively found in a TRAPP II complex.

#### TRAPP II and a possible Pik1p-Ypt31p signaling pathway

Recently, a synthetic genetic array analysis with *pik1*, a mutant strain defective in the phosphatidylinositol 4-kinase, was performed to identify components of a Pik1p signaling pathway (Sciorra et al., 2005). This screen identified *drs2Δ* and *ypt31Δ* as possible candidates, as well as mutants in genes that encode TRAPP subunits (*trs33* and *trs65*). Drs2p is an integral Golgi membrane protein required for the formation of clathrin-coated vesicles (Chen et al., 1999; Gall et al., 2002), whereas Ypt31p is a GTPase required for Golgi traffic. The exact role of Ypt31p in Golgi traffic, however, has remained controversial (Benli et al., 1996; Jedd et al., 1997). Interestingly, both Pik1p and Ypt31p were shown to localize to the Sec7p compartment in this study and Drs2p was reported to reside in the late Golgi in an earlier study (Chen et al., 1999). With our finding that TRAPP II largely localizes to a compartment containing Sec7p, it is now clear that all of the components identified in this screen appear to localize to the same compartment of the Golgi.

Genetic studies have also shown that the overexpression of *YPT31* suppresses the temperature-sensitive growth phenotype of a *trs130* mutant (Wang and Ferro-Novick, 2002; Yamamoto and Jigami, 2002; Zhang et al., 2002), and *YPT31* locked in its GTP form suppresses the lethality of a *trs33Δtrs65Δ* dou-

ble mutant (Sciorra et al., 2005). These findings have led to the speculation that TRAPP II may be a guanine nucleotide exchange factor for Ypt31p (Jones et al., 2000; Sciorra et al., 2005). Biochemical studies, however, have failed to support this hypothesis. Specifically, the quantitative depletion of TRAPP II from cytosol did not reduce Ypt31p exchange activity (Wang and Ferro-Novick, 2002). Although the exact relationship of TRAPP II to Ypt31p is unclear, these genetic studies clearly demonstrate that Ypt31p acts downstream of TRAPP II.

#### The relationship of TRAPP II to other tethers that act on endosomal-Golgi pathways

Together, our findings support the hypothesis that Trs120p is required for the recycling of proteins from the early endosome. The endocytic pathway is known to intersect with the *vps* pathway at the prevacuolar compartment (Piper et al., 1995). The observation that mutants in *trs120* do not secrete CPY implies that Trs120p is required for the retrieval of proteins on the endocytic, but not *vps*, pathway. Subcellular fractionation and immunofluorescence studies have shown that TRAPP II localizes to a late Golgi/early endosomal compartment that is marked by Sec7p and Chs3p. The localization of Trs120p and the phenotype of mutants in *trs120* are consistent with a role for Trs120p in the tethering of vesicles that recycle from the early endosome to a late Golgi compartment. Thus, while TRAPP I tethers COPII vesicles at the cis face of the Golgi, TRAPP II may play an analogous role with COPI vesicles at the most distal compartment of the Golgi.

TRAPP II is one of six large putative tethering complexes that have been identified. Each of the known tethering complexes appears to act at distinct trafficking steps (for review see Whyte and Munro, 2002). Two other tethering complexes, COG (conserved oligomeric Golgi) and GARP (Golgi-associated retrograde protein; VFT [*vps* fifty-three]), have also been proposed to act on endosomal-Golgi pathways. COG largely localizes to the cis-Golgi (Kim et al., 2001) and may tether retrograde trans-Golgi/endosomal vesicles en route to the cis-Golgi (Whyte and Munro, 2001; Zolov and Lupashin, 2005). GARP largely colocalizes with Kex2p and has been implicated in retrograde traffic from both the early and late endosome to the Kex2p compartment (Conibear et al., 2003). Unlike *trs120* and *-130* mutants, defects in COG or GARP (VFT) subunits also lead to defects on the *vps* pathway and result in the secretion of CPY (Spelbrink and Nothwehr, 1999; Suvorova et al., 2002; Conibear et al., 2003). Thus, TRAPP II, COG, and GARP appear to be mediating different tethering events on endosomal-Golgi pathways. Additional studies will be needed to precisely determine the molecular mechanism by which TRAPP II mediates traffic through the early endosome.

## Materials and methods

#### Plasmid and strain construction

The *trs120-1* mutant and all *trs130* mutants were constructed by a PCR-mediated method developed by Longtine et al. (1998). The remaining *trs120* mutants were made by transposon mutagenesis. One copy of *TRS120* was disrupted in a diploid strain that was transformed with a

balancing plasmid containing *TRS120*. Diploid cells were sporulated and dissected, and haploid cells containing the balancing plasmid were selected. These cells were then transformed with a plasmid containing transposon-mutagenized *trs120* (plasmid construction was based on the Transposon Construction Vector from Epicentre Technologies). Transformants were stamped on fluoroarotic acid plates to select against the balancing plasmid and screened for temperature sensitivity. DNA from the mutant cells was extracted and sequenced. *Ret2p-GFP-*, *Chs3p-GFP-*, *Trs120p-13myc-*, and *Trs130p-13myc-*-containing strains were constructed using a PCR-based method developed by Longtine et al. (1998).

#### In vivo labeling and immunoprecipitation

For the analysis of CPY secretion, ~7 ml ( $OD_{600} = 1.0$ ) of wild-type and mutant cells grown at 25°C were preshifted to 37°C for 20 min in 1.6 ml of synthetic minimal medium that was supplemented with the appropriate amino acids. Cells were pulse labeled for 4 min with 250  $\mu$ Ci of [<sup>35</sup>S] Pro-Mix (GE Healthcare) and chased for the indicated time points in the presence of 10 mM methionine and 10 mM cysteine. Samples were then processed for immunoprecipitation as described in Rossi et al. (1995).

For the analysis of invertase secretion, 3 ml ( $OD_{600} = 1.0$ ) of wild-type and mutant cells grown at 25°C were preshifted to 37°C for 20 min in synthetic minimal medium (supplemented with amino acids) with 2% glucose. The cells were then transferred into medium containing 0.1% glucose to derepress the synthesis of invertase and labeled with 250  $\mu$ Ci of [<sup>35</sup>S] ProMix for 60 min. Internal and periplasmic invertase was immunoprecipitated as described in Rossi et al. (1995).

#### Fluorescence microscopy

Cells expressing GFP-Snc1p, Sec21p-GFP, Ret2p-GFP, Sec7p-GFP, Chs3p-GFP, Sec7p-DsRed, DsRed-FYVE, and Trs130p-GFP were grown overnight at 25°C to early log phase ( $OD_{600} = 0.3-0.6$ ). Approximately 5  $OD_{600}$  units of cells were pelleted, resuspended in 250  $\mu$ l of ice-cold growth medium, and observed as described below. For temperature shift experiments, cells were pelleted and resuspended in prewarmed medium. The incubation was performed at 37°C. Cells were subsequently harvested at 4°C, resuspended in ice-cold growth medium, and observed as described below. To visualize Och1p-HA and Trs120p-myc, indirect immunofluorescence was performed as described previously (Estrada et al., 2003). Cells were observed with a fluorescence microscope (Axiophot; Carl Zeiss MicroImaging, Inc.) using a 100 $\times$  oil-immersion objective. Images were captured with a charge-coupled device camera (Orca ER; model C4742-95; Hamamatsu Photonics) and the OpenLab 3.08 imaging software (OpenLab, Inc.) and processed using Photoshop 7.0 and Illustrator 10.0 (Adobe).

#### Subcellular fractionation

Density gradient analysis was performed as described in Barrowman et al. (2000), with modifications. Cells were grown overnight in synthetic minimal medium (with amino acids) to early log phase ( $OD_{600} = 0.5-1.0$ ). Approximately 200  $OD_{600}$  units of cells were harvested, converted to spheroplasts, and lysed in 3 ml of cold lysis buffer as described in Barrowman et al. (2000). 1 ml of lysate was loaded onto the top of a sucrose gradient that contained 11 1-ml steps (26, 30, 32, 34, 36, 38, 40, 42, 44, 46, and 50% sucrose wt/wt in 10 mM Hepes, pH 7.4, and 1 mM MgCl<sub>2</sub>). Gradients were centrifuged at 38,000 rpm in a SW40.1 rotor (Beckman Instruments) at 4°C for 18 h. 1-ml fractions were collected from the top of the gradient. Fractions were boiled in SDS sample buffer. Subsequently, 25  $\mu$ l of each fraction was analyzed by SDS-PAGE using the ECL method for immunodetection. For the detection of Chs3p, fractions were first subjected to TCA precipitation as described previously (Estrada et al., 2003). Samples were quantitated using NIH image 1.61/68K software. The data plotted represents a quantification of the number of pixels in a band, a measurement that is proportional to the band intensity.

#### FM4-64 uptake

To label cells with FM4-64, 4  $OD_{600}$  units of cells were grown to early log phase at 25°C and then shifted to 37°C for 30 min. Cells were harvested, resuspended in 200  $\mu$ l of medium prewarmed to 37°C, and incubated with 40  $\mu$ M FM4-64 for 5 min. Cells were washed once with fresh medium. Images were taken immediately or 25 min after the addition of fresh medium.

#### Online supplemental material

Fig. S1 shows that general secretion is partially blocked in *trs130* mutants. Fig. S2 shows that *trs120-8* mutant cells specifically block the endocytic pathway. Fig. S3 shows that Trs120p and -130p are exclusively present in

the TRAPP complex. Table S1 shows the quantitative analysis of invertase secretion in *trs120* and -130 mutants. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200505145/DC1>.

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