Ypt1p is essential for retrograde Golgi-ER transport and for Golgi maintenance in *S. cerevisiae*

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Summary

The small GTPase Ypt1p of the Rab family is required for docking of ER-derived transport vesicles with the Golgi prior to fusion. However, the identity of the Rab protein that mediates docking of Golgi-derived COPI vesicles with the ER in retrograde transport remains elusive. Here, we show that in yeast Ypt1p is essential for retrograde transport from the Golgi to the ER. Retrieval of gpαF-HDEL (glycolylated pro-α-factor with an HDEL tag at the C-terminus) was blocked in *Δypt1/SLY1-20* membranes at the restrictive temperature in vitro. Moreover, Ypt1p and the ER-resident t-SNARE Ufe1p interact genetically and biochemically, indicating a role for Ypt1p in consumption of COPI vesicles at the ER. Ypt1p is also essential for the maintenance of the morphology and the protein composition of the Golgi. Interestingly, the concentrations of the Golgi enzymes Anp1p and Mnn1p, the cargo protein Emp47p and the v-SNARE Sec22p were all substantially reduced in Golgi from a *Δypt1/SLY1-20* strain as compared with wild-type Golgi, while the concentration of Arf1p and of coatomer were mildly affected. Finally, COPI vesicles generated from *Δypt1/SLY1-20* Golgi membranes in vitro were depleted of Emp47p and Sec22p. These data demonstrate that Ypt1p plays an essential role in retrograde transport from the Golgi to the ER.

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Introduction

Proteins destined for secretion are first translocated into the lumen of the ER where they are core glycosylated. Subsequently, they are incorporated into COPII vesicles, which are en route to the Golgi complex. Upon arrival in the Golgi complex, proteins are further modified and sorted at the *trans*-Golgi network to reach their final destinations. Protein and membrane traffic at the ER-Golgi interface is bi-directional. Transport factors such as SNARE proteins, cargo receptors and ER-resident proteins that have escaped the ER retention system, are packaged into COPI vesicles and retrieved back to the ER in order to preserve organelle integrity of both the ER and the Golgi complex.

Fusion of both COPII and COPI vesicles with their respective target membranes is tightly regulated. In principle, the general fusion process can be subdivided into tethering, docking and fusion (Bonifacino and Glick, 2004). Tethering factors at the Golgi are Uso1p, TRAPPI and the COG complex, whereas at the ER membrane this function seems to be fulfilled by Dsl1p, Tip20p and probably other yet unspecified proteins (Andag et al., 2001; Kamena and Spang, 2004; Ram et al., 2002; Reilly et al., 2001). Docking of vesicles employs Rab/Ypt proteins. Ypt1p is the Rab protein required for anterograde ER-Golgi transport, whereas the Rab essential for retrograde transport remains elusive (Segev, 2001b). Finally, a recognition step mediated by the SNARE proteins leads to close opposition of the membranes of the vesicle and the target compartment and subsequently to fusion of the lipid bilayers. The precise mechanism by which fusion occurs is still a matter of debate (Peters et al., 2001; Weber et al., 1998).

Rab/Ypt proteins are small GTPases of the ras superfamily. They cycle between a GTP-bound (active) and a GDP-bound (inactive) form. The yeast genome encodes 11 *YPT*s. Ypt51/52/53p, Ypt10p and Ypt7p act in endocytosis en route to the vacuole, whereas Ypt1p, Ypt31p and Ypt32p function in exocytosis (Buvelot Frei et al., 2006; Segev, 2001a). Ypt6p is thought to be involved in both endocytosis and exocytosis (Li and Warner, 1996). Ypt11p seems to be involved in ER inheritance (Buvelot Frei et al., 2006). Ypt1p is crucial for the ER to Golgi transport and is also important for intra Golgi transport (Bacon et al., 1989; Baker et al., 1990; Jedd et al., 1995; Segev et al., 1988). Ypt31p and Ypt32p are partially functionally redundant and are both involved in protein exit from the trans-Golgi (Benli et al., 1996; Jedd et al., 1997). The switch between the GDP-bound and the GTP-bound state is mediated by specific guanine nucleotide exchange factors (GEFs) whereas GTPase activating proteins (GAPs) regulate the hydrolysis of GTP. GDI, the GDP dissociation inhibitor, is an additional regulator of Rab/Ypt proteins that sequesters the GDP-bound form of the GTPase and prevents the exchange of GDP for GTP, thereby inhibiting recruitment to the membrane (Araki et al., 1990; Garrett et al., 1994; Sasaki et al., 1990). A single GDP dissociation inhibitor, Gdi1p, has been identified in *Saccharomyces cerevisiae*. *GDI1* is essential for cell viability and can probably act on all Ypt proteins (Dirac-Svejstrup et al., 1994; Haas et al., 1995; Peter et al., 1994; Ullrich et al., 1993).

Despite the importance of Rab/Ypt proteins and although vesicle fusion at the ER-Golgi interface has been subject of intensive research, it is still not known which Rab/Ypt protein functions at

the fusion step of COPI vesicles with the ER. In this study, we identify Ypt1p as the Rab involved in the retrograde transport from the Golgi to the ER. In addition, we provide evidence for an additional function of Ypt1p in maintaining Golgi identity and integrity.

Results

Gdi1p inhibits retrieval of [^{35}S]gpαF-HDEL from the Golgi to ER in vitro

To identify the Rab protein involved in fusion of retrograde transport vesicles with the ER, we used a cell-free transport system called the round-trip assay (Spang and Schekman, 1998). The round-trip assay has been used successfully to characterize several proteins involved in retrograde transport from the Golgi to the ER (Kamena and Spang, 2004; Poon et al., 1999; Spang et al., 2001). Essentially, the round-trip assay recapitulates the transport of a reporter protein from the ER to the Golgi complex and back to an acceptor ER (supplementary material Fig. S1). As a reporter we use radioactively labeled prepro-α-factor with an HDEL tag at the C-terminus ([^{35}S]ppαF-HDEL) (Dean and Pelham, 1990). A glucan trimming reaction provides the means to determine successful retrograde transport to the acceptor ER. Trimmed and untrimmed forms can be distinguished by their differential mobilities on SDS-PAGE, and the trimmed form of [^{35}S]gpαF-HDEL is produced only upon successful return to the ER. The untrimmed band represents [^{35}S]gpαF-HDEL present in the Golgi or in vesicles that have docked but not fused and cannot be correlated to the amount of trimmed [^{35}S]gpαF-HDEL.

We first asked, whether retrograde transport actually requires the action of a Rab protein. The GDP dissociation inhibitor Gdi1p binds the GDP-bound form of Rab proteins and can inhibit the exchange of GDP for GTP (Sasaki et al., 1990). We took advantage of the presence of only one Rab-specific GDI in yeast, which most probably acts on all yeast Rabs (Ypts). We performed a round-trip assay and compared the retrieval efficiency of [^{35}S]gpαF-HDEL to the ER in the presence and in the absence of exogenously added purified Gdi1p. The amount of [^{35}S]gpαF-HDEL that reached the ER in the presence of Gdi1p was significantly reduced compared with the control (Fig. 1A, compare lanes 1 and 2). This reduction of the signal in the presence of Gdi1p was independent of its ability to inhibit COPII vesicle fusion with the Golgi because purified Gdi1p was only added to the reaction after the COPII vesicles had fused with the Golgi. Furthermore, after the fusion of the COPII vesicles with the Golgi, the membranes were washed extensively prior to the addition of cytosol, in order to avoid any residual fusion activity of COPII vesicles with the Golgi complex. Therefore, Gdi1p blocks retrieval of [^{35}S]gpαF-HDEL to the ER in vitro, and therefore establishes a requirement for a Ypt protein in retrograde transport from the Golgi to the ER.

Retrieval of [^{35}S]gpαF-HDEL is defective in *Δypt1/SLY1-20* in vitro

The result above indicates that retrograde transport depends on the action of at least one Ypt. We sought to determine which Ypt is involved in retrograde transport from the Golgi to the ER and decided to follow those Rabs involved in exocytosis, namely Ypt1p, Ypt31/32p and Ypt6p. *YPT1* is essential, but the loss of *YPT1* can be rescued by the expression of a mutation in *SLY1*, *SLY1-20*, which renders the strain temperature sensitive (ts) (Dascher et al., 1991; Ossig et al., 1991). Sly1p was isolated as suppressor of *YPT1* loss of function. Sly1p is a member of the Sec1p family and may promote target SNARE complex formation at the Golgi (Dascher et al., 1991; Kosodo et al., 2002; Peng and Gallwitz, 2002). We also used a second ts mutant, *ypt1-3*, which has been used extensively for in

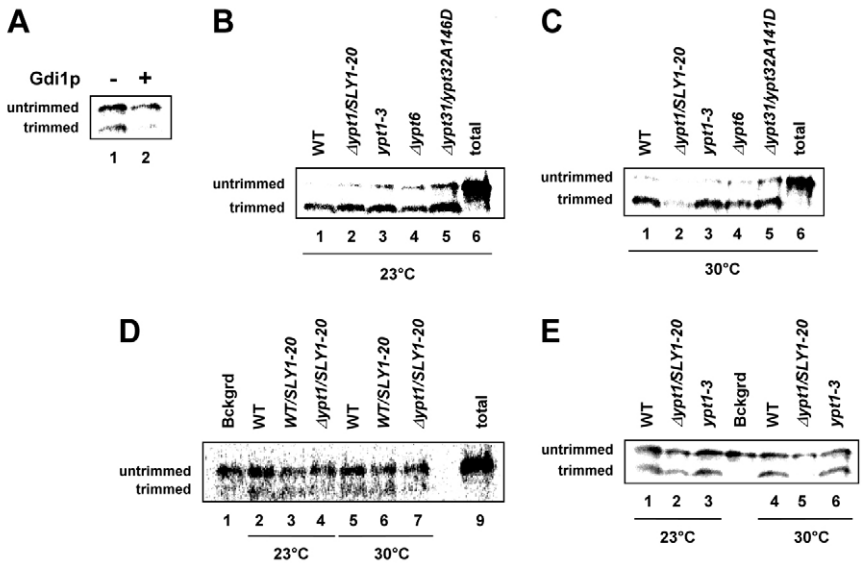


Fig. 1. Ypt1p is required for retrograde transport from the Golgi to the ER. (A) Addition of GDI reduced the retrieval efficiency of [^{35}S]gpαF-HDEL to the ER. A round-trip assay was performed with wild-type semi-intact cells. GDI was added to the round-trip assay after the fusion of COPII vesicles with the ER. The amount of trimmed [^{35}S]gpαF-HDEL in lane 2 was reduced to 63% compared with that in the buffer control (100%). In the buffer control 1.14% and after addition of GDI 0.73% of the [^{35}S]gpαF-HDEL incorporated into COPII vesicles at the donor ER reached the acceptor ER and was trimmed. (B) *YPT* mutants are not defective in retrograde transport at the permissive temperature. Semi-intact cells from different ts-mutants and wild type were used as acceptor membranes in a round-trip assay. The last step, the retrieval from the Golgi to the ER, was performed at the permissive temperature, 23°C. All semi-intact cells gave a comparable signal of trimmed [^{35}S]gpαF-HDEL. (C) Deletion of *YPT1* results in a reduction of retrograde transport from the Golgi to the ER. In parallel to the assay in B an assay was performed, in which the temperature was raised in the last step to 30°C. This temperature should represent at least a semi-restrictive temperature for most ts-mutants. The signal did not alter significantly for most mutants when compared with wild type, but the signal of retrieved and trimmed [^{35}S]gpαF-HDEL was strongly diminished in *Δypt1/SLY1-20* acceptor membranes. (D) *SLY1-20* expression does not contribute to the defect in retrograde transport. A retrieval assay was performed as described above. In *Δypt1/SLY1-20* membranes the signal in the retrograde transport assay was drastically reduced at 30°C, but the transport in WT/*SLY1-20* was as efficient as in WT. (E) *ypt1-3* is not defective in retrograde transport in vitro. A retrieval assay was performed to compare two different *YPT1* mutants side by side. *Δypt1/SLY1-20* membranes were unable to allow retrograde transport of [^{35}S]gpαF-HDEL at the restrictive temperature, *ypt1-3* membranes behaved like wild-type semi-intact cells. 'Bckgrd' is an assay using wild-type membranes, but cytosol was omitted in the last incubation step (transport from the Golgi to the ER). The retrieval efficiency was determined as percentage of trimmed [^{35}S]gpαF-HDEL of the reporter that was incorporated into COPII-coated vesicles at the ER. At 20°C: WT 2.9 %, *Δypt1/SLY1-20* 2.34 %, *ypt1-3* 3.15 %; at 30°C: WT 2.92 %, *Δypt1/SLY1-20* 1.28%, *ypt1-3* 3.05%.

vivo studies (Cao et al., 1998; Morsomme and Riezman, 2002). The *Δypt31/ypt32A141D* is a temperature-sensitive strain bearing a point mutation in *YPT32* in a *Δypt31* background (Jedd et al., 1997). *YPT6* is a non-essential gene but deletion of the gene results in growth defects at 37°C (Li and Warner, 1996). The use of semi-intact cells derived from these strains in the round-trip assay combined with a temperature shift in the last step of the assay should enable us to determine, which Ypt is required for retrograde transport from the Golgi to the ER.

When the round-trip assay was performed at the permissive temperature, the retrieval of [^{35}S]gpαF-HDEL in the *ypt* mutant membranes was indistinguishable from that in the wild-type membranes (Fig. 1B). By contrast, when we shifted the incubation temperature in the last stage (retrieval from the Golgi to the ER) of the round-trip assay to 30°C, which should be at least a semi-restrictive temperature for these mutants, the amount of retrieved [^{35}S]gpαF-HDEL to the ER in *Δypt1/SLY1-20* membranes was strongly reduced (Fig. 1C, lane 2). The presence of the *SLY1-20* plasmid itself did not exert any negative effect on the retrograde transport, because the retrieval efficiency of [^{35}S]gpαF-HDEL was not altered in a wild-type strain expressing *SLY1-20* (Fig. 1D). All other mutants, including *ypt1-3*, showed retrieval efficiencies similar to that of the wild type (Fig. 1C,E). The temperature shift to 30°C in the last step of the assay might still be permissive for *ypt1-3* in vitro. However, raising the temperature further is not possible in this in vitro system, because the membranes become leaky and make the interpretation of the assay impossible. In addition, Ballew et al. (Ballew et al., 2005) showed that Ypt6p becomes essential in *Δypt1/SLY1-20*, indicating that it can take over part of the Ypt1p functions. Therefore, it is possible that Ypt6p compensates for the transport defect in *ypt1-3* in vitro. Nonetheless, the results indicate that the defect in retrograde transport might be due to the loss of Ypt1p function in *Δypt1/SLY1-20*.

Ypt1-3 semi-intact cells are permissive for anterograde transport to the Golgi in vitro

To determine whether *ypt1-3* might be functional at 30°C in vitro, we performed an anterograde ER-Golgi transport assay (Fig. 2). The role of Ypt1p in fusion of COPII vesicles with the Golgi is well established (Cao et al., 1998). Furthermore, a constitutive defect at the permissive temperature in the fusion of COPII vesicles with the Golgi could potentially abolish the round-trip and lead to a reduction of the amount of trimmed [^{35}S]gpαF-HDEL as was seen with the *Δypt1/SLY1-20* strain. To test this possibility, we measured the amount of anti-α-1,6-linked mannose-precipitable [^{35}S]gpαF-HDEL, which is an indicator of the arrival of the reporter in the Golgi. At the permissive temperature, the amount of precipitable [^{35}S]gpαF-HDEL was similar in all membranes tested (Fig. 2A). Additionally, as expected, at the restrictive temperature, the *Δypt1/SLY1-20* mutant strain displayed a drastic reduction in the transport of [^{35}S]gpαF-HDEL to the Golgi (Fig. 2B). This result confirms that there is no constitutive defect in anterograde transport in *Δypt1/SLY1-20* under the conditions of our assay. However, the *ypt1-3* semi-intact cells were permissive for anterograde transport from the ER to the Golgi *in vitro* even at the restrictive temperature (Fig. 2B). We conclude, therefore, that the decrease in function of the *ypt1-3* mutant is insufficient to be detectable in our assay. Taken together, these results suggest that Ypt1p is essential for retrograde transport form the Golgi to the ER.

To substantiate our finding that Ypt1p is involved in retrograde transport, we scored the secretion of Kar2p. The chaperone Kar2p is an ER-resident protein, which can escape to the Golgi and is retrieved back to the ER by the HDEL-receptor Erd2p (Semenza et al., 1990). However, if retrograde transport is defective, Kar2p is secreted. We assessed secretion of Kar2p by colony blot after incubation at 30°C (Fig. 2C). We chose 30°C as semi-permissive temperature for the secretion assay, because all strains still grew at about the same rate at this temperature, so that unspecific effects caused by differences in cell number and growth behavior could be excluded. Although no Kar2p was secreted in the wild-type strain, both *ypt1-3* and *ypt1D136A* secreted Kar2p efficiently, similar to that of *tip20-5* and *dsl1-22*, two retrograde transport mutants with established Kar2p secretion phenotypes (Andag et al., 2001; Cosson et al., 1997). Hence *ypt1-3* and *ypt1D136A* are defective in retrograde transport in vivo. This phenotype can probably be compensated for in our in vitro assays. The *Δypt1/SLY1-20* strain also secreted Kar2p, though to a lesser degree. This result was not unexpected, since *Δypt1/SLY1-20* is a much stronger mutant than the other *ypt1* alleles in the assay and has defects at lower temperatures. Anterograde transport is likely to be more strongly affected in *Δypt1/SLY1-20* than in *ypt1-3* and *ypt1D136A*. The *ypt6* and *ypt31/32* mutants also showed some Kar2p secretion, which is most likely due to an interference with Golgi function. However, a more direct effect is also possible at least for Ypt6 (Ballew et al., 2005). The Kar2p signal was not due to cell lysis, because the cytoplasmic protein Pgk1p was never detected for any of the strains grown at 30°C. Taken together, we find that multiple mutants in *YPT1* secrete Kar2p, supporting a role for Ypt1p in retrograde transport from the Golgi to the ER.

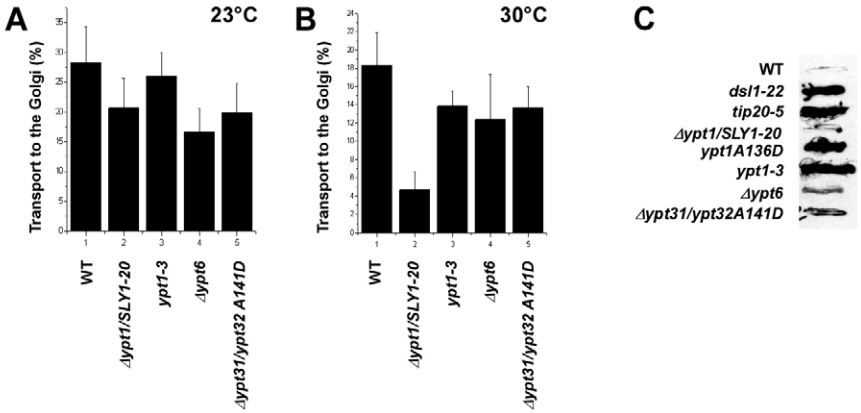


Fig. 2. Behavior of *YPT* mutants in anterograde transport and in a Kar2p secretion assay. (A,B) An ER-Golgi transport assay. The arrival of [^{35}S]gpαF-HDEL in the Golgi was monitored by precipitation with α-1,6-mannose antibodies and protein A-Sepharose. The amount of gpαF-HDEL precipitated by α-1,6-mannose antibodies was normalized to the amount of budded gpαF-HDEL from the ER. The average and standard deviation from at least three independent experiments are shown. The ER-Golgi transport assay was performed at (A) 23°C and (B) 30°C. (C) Mutants in *YPT1* secrete Kar2p. Colony blot of different *YPT* mutants grown overnight at 30°C. Secreted Kar2p was detected by immunoblotting.

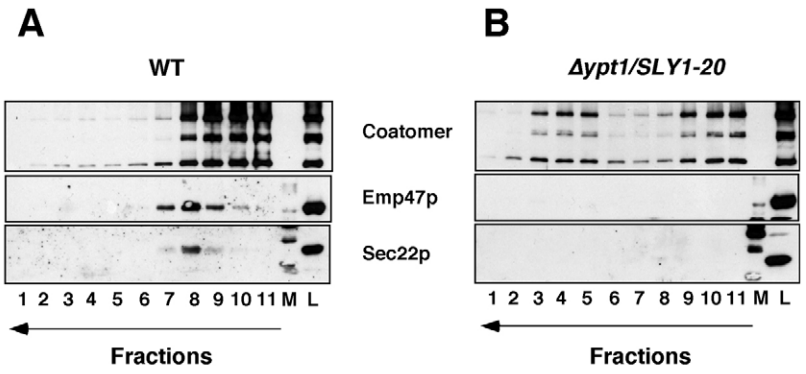


Fig. 3. COPI-coated vesicles from *Δypt1/SLY1-20* Golgi are depleted from cargo and SNAREs. Golgi membranes enriched from either wild-type (A) or *Δypt1/SLY1-20* (B) cells were incubated with Arf1p, coatomer and GTP-γ-S, to generate COPI vesicles. The vesicles were separated by velocity sedimentation centrifugation. The vesicle peak was collected and floated on a buoyant density gradient. Fractions were collected from the top, precipitated, and analyzed by immunoblotting. Sec22p is a v-SNARE in the ER-Golgi shuttle and Emp47p is a vesicle cargo. The arrows indicate the movement of the lipid particles in the gradient.

Enriched *Δypt1/SLY1-20* Golgi membranes are unable to bud functional COPI vesicles in vitro

The best-characterized function of Ypt1p is in the docking of ER-derived vesicles to the Golgi membrane (Cao et al., 1998). Yet, recent investigations have shown that Ypt1p is also required for the sorting of GPI-anchored cargo molecules into ER-derived vesicles (Morsomme and Riezman, 2002). Furthermore, another recent study reports a role for Rab1, the mammalian orthologue of Ypt1p, in cargo uptake into COPI vesicles from the Golgi (Alvarez et al., 2003). If Ypt1p plays a similar role in yeast, then the defect observed in our round-trip assay might arise either from a failure in COPI vesicle generation at the Golgi or, alternatively, from defective consumption of COPI vesicles at the ER. Therefore, we investigated a possible role for Ypt1p in COPI vesicle formation in an in vitro Golgi budding assay (Spang and Schekman, 1998). Enriched Golgi membranes from wild-type or *Δypt1/SLY1-20* cells were incubated with coatomer, Arf1p and guanine nucleotide. The resulting COPI vesicles were purified, first over a sedimentation gradient and then by buoyant density centrifugation. As expected, the wild-type membranes formed normal COPI-coated vesicles as judged by the presence of the cargo Emp47p and the v-SNARE Sec22p in the vesicle fraction (Fig. 3A). By contrast, Golgi membranes from the *Δypt1/SLY1-20* mutant formed abnormal COPI-coated vesicles (Fig. 3B). Although, a coatomer signal was obtained in higher migrating fractions, which indicates a lower buoyant density, these fractions contained much less Emp47p and Sec22p (Fig. 3B). Thus, these vesicles might represent at least partially defective COPI vesicles because they did not contain normal amounts of Sec22p (Spang and Schekman, 1998). Together, these data support a requirement of Ypt1p in the formation of COPI-coated vesicles at the Golgi.

The Golgi is altered in *Δypt1/SLY1-20* mutant cells

Does the defect in COPI vesicle generation arise form a partially dysfunctional Golgi complex? To investigate this possibility, we first compared, by immunoblotting, the content of different Golgi proteins and Golgi-associated proteins in wild-type and *Δypt1/SLY1-20* Golgi membranes. As for the experiments above, the Golgi used for this analysis was enriched from cells that had been grown at the permissive temperature for *Δypt1/SLY1-20*, 23°C. Surprisingly, the concentrations of the Golgi enzymes Anp1p and Mnn1p, the cargo Emp47p and the v-SNARE Sec22p were all dramatically

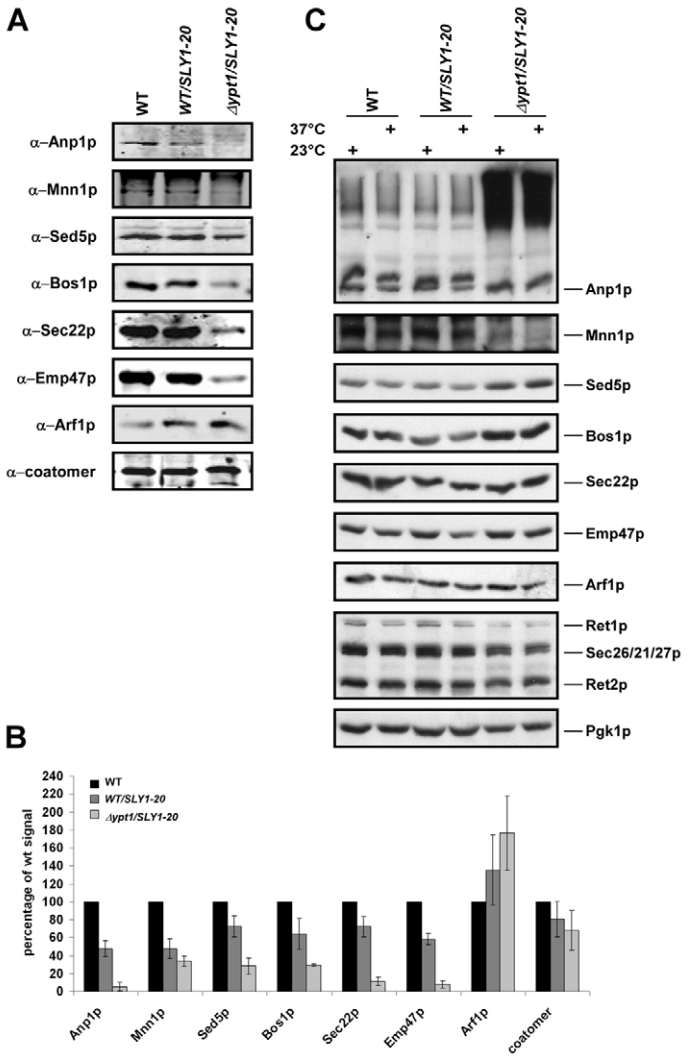


Fig. 4. Wild-type and *Δypt1/SLY1-20* Golgi differ in their protein content. Immunoblots of equal amounts of Golgi membranes enriched from wild-type, wild-type expressing *SLY1-20* and *Δypt1/SLY1-20* cells that were grown at 23°C. The blots were developed with antibodies directed against the Golgi enzymes Anp1p and Mnn1p, the SNAREs Sed5p, Bos1p and Sec22p, the cargo Emp47p, and the COPI components Arf1p and coatomer. (A) A representative collection of immunoblots. (B) The immunoblots were quantified using a Licor Odyssey system. The average and standard deviation from at least three independent experiments are shown. (C) Proteins that are lost from *Δypt1/SLY1-20* Golgi are not degraded. Total cell lysates were prepared from cells that were grown at 23°C or shifted to 37°C. Equal amounts of protein were loaded for SDS-PAGE and analyzed by immunoblotting with antibodies as described above and with anti-Pgk1 as a loading control.

reduced, whereas the concentration of coatomer remained constant and the level of Arf1p was increased (Fig. 4A,B). About half of the t-SNARE Sed5p and the v-SNARE Bos1p were lost in the *Δypt1/SLY1-20* Golgi. Expression of *SLY1-20* in the wild-type background also caused a reduction of Anp1p, cargo and SNAREs, although this reduction was less dramatic. This loss of proteins from the *SLY1-20* Golgi did not cause any obvious growth phenotype (data not shown) nor did it interfere with COPI vesicle formation in vitro.

Sed5p-GFP and Emp47p-myc accumulate in the ER and Anp1p-GFP is dispersed in *Δypt1/SLY1-20* mutants

Next, we wanted to determine the fate of the proteins lost from *Δypt1/SLY1-20* Golgi membranes. They could either be degraded or accumulate in other organelles or structures in the cell. To distinguish between these two possibilities, we compared the protein levels in total yeast lysates derived from strains grown at 23°C or shifted for 1 hour to 37°C, which allows the induction of a comparable acute response in the different strains. Surprisingly, no big changes in the protein level of Golgi or Golgi-associated proteins were detected irrespective of the strain background or the temperature (Fig. 4C). Therefore, we next determined the subcellular localization of the Sed5p-GFP, Emp47p-myc, Anp1p-GFP and Sec7p-dsRed in wild-type, WT/*SLY1-20* and *Δypt1/SLY1-20* at 23°C or after shifting to 37°C (Fig. 5). Consistent with the immunoblot data, all proteins were detected also at 37°C. Although no significant difference in protein localization was observed upon shift to 37°C in wild-type and WT/*SLY1-20* cells, Sed5pGFP and Emp47p-myc accumulated in the ER and the Anp1p-GFP and the Sec7p-dsRed signal became diffuse in *Δypt1/SLY1-20*. Moreover, a partial defect had been observed already for Emp47p, Anp1p and Sec7p at 23°C, indicating that this mutant has defects even at 23°C and these defects gradually increase with a rise in temperature. The effects observed could be due to a strong decrease in anterograde traffic, a defect in retrograde transport or a combination of both, because Ypt1p is required for the fusion of vesicles at the Golgi and the ER. Taken together our data suggest that Ypt1p plays a role in the maintenance of the Golgi complex.

The *Δypt1/SLY1-20* Golgi is at least partially functional at 23°C

Since, the localization of Anp1p and Sec7p was already disturbed at 23°C in *Δypt1/SLY1-20* cells, we wondered whether efficient glycosylation could still take place. Surprisingly, the extent of glycosylation observed in the *Δypt1/SLY1-20* mutant strain was comparable to or even greater than that in the wild type, independent of the incubation temperature (Fig. 6A). This result confirms that, despite the loss of Anp1p and other proteins, the Golgi was still functional. A similar result was observed when we analyzed a mutant in the ARF-GEF *SEC7*, which possesses an abnormal Golgi morphology at the restrictive temperature (Achstetter et al., 1988). In addition, *Δypt1/SLY1-20* cells were not osmo-sensitive, which indicates that the extracellular matrix was functional. Taken together, our results suggest that despite the lack of at least one glycosylation enzyme, glycosylation in the Golgi occurred very efficiently.

The result above was somewhat unexpected, because Ypt1p is essential for the fusion of COPII vesicles with the Golgi and hence a reduction of extensively glycosylated proteins could have been expected. One explanation for the observed glycosylation pattern might be that, most of the glycosylated proteins were not turned over during the 1-hour shift to 37°C. To further investigate this possibility, we performed a pulse-chase experiment and followed the maturation of the vacuolar carboxypeptidase C (CPY). Upon arrival in the ER, proCPY is core-glycosylated (p1). Further glycosylation occurs in the Golgi complex (p2), and in the vacuole the mature form (m) is present. In wild-type and WT/*SLY1-20* cells all CPY was converted into the mature form after 30 minutes of chase at 23°C and at 37°C (Fig. 6B). By contrast, maturation of CPY was strongly delayed in *Δypt1/SLY1-20* at 23°C. However, the processing of CPY seemed to occur normally, indicating that

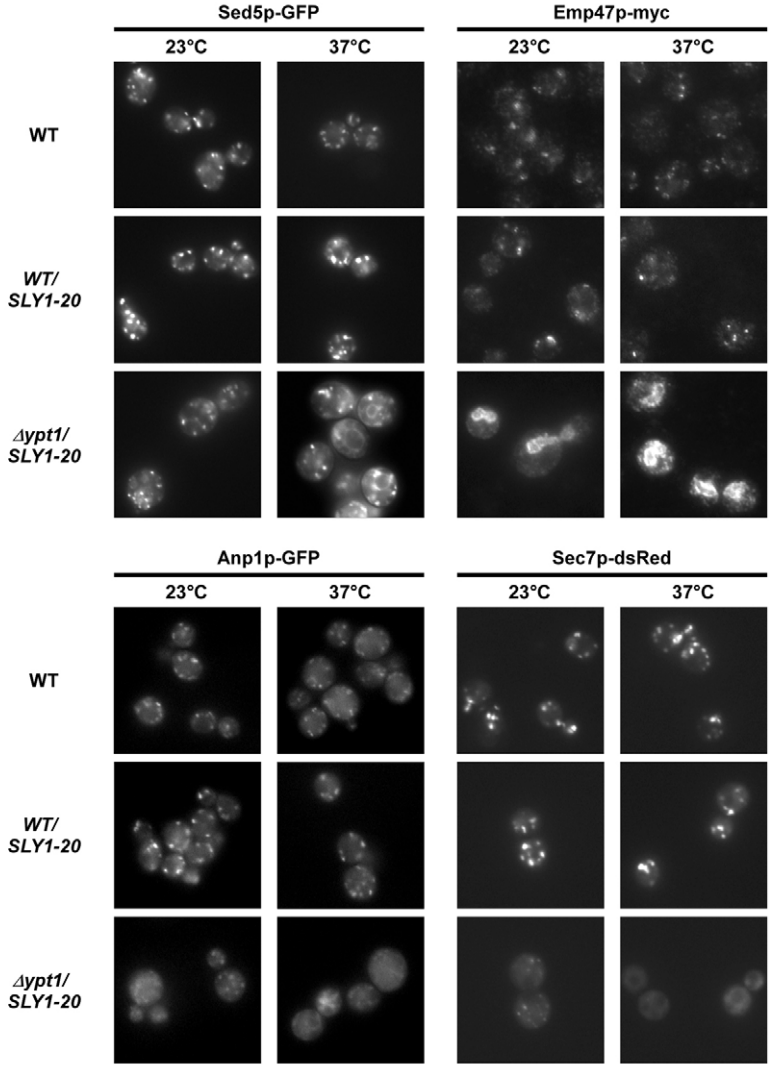


Fig. 5. The localization of various Golgi proteins is altered in *Δypt1/SLY1-20* cells. Cells were grown to early log phase at 23°C. One half of the culture was shifted to 37°C for 1 hour. Cells were either examined directly (Anp1p-GFP, Sed5p-GFP, Sec7p-dsRed) or treated for immunofluorescence (Emp47p-myc) with mouse anti-myc and goat-anti mouse IgGs coupled to CY3.

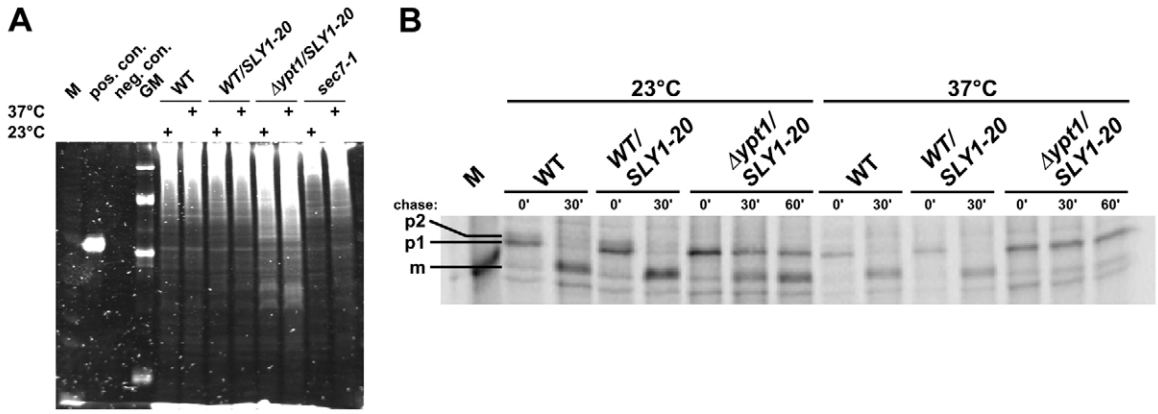
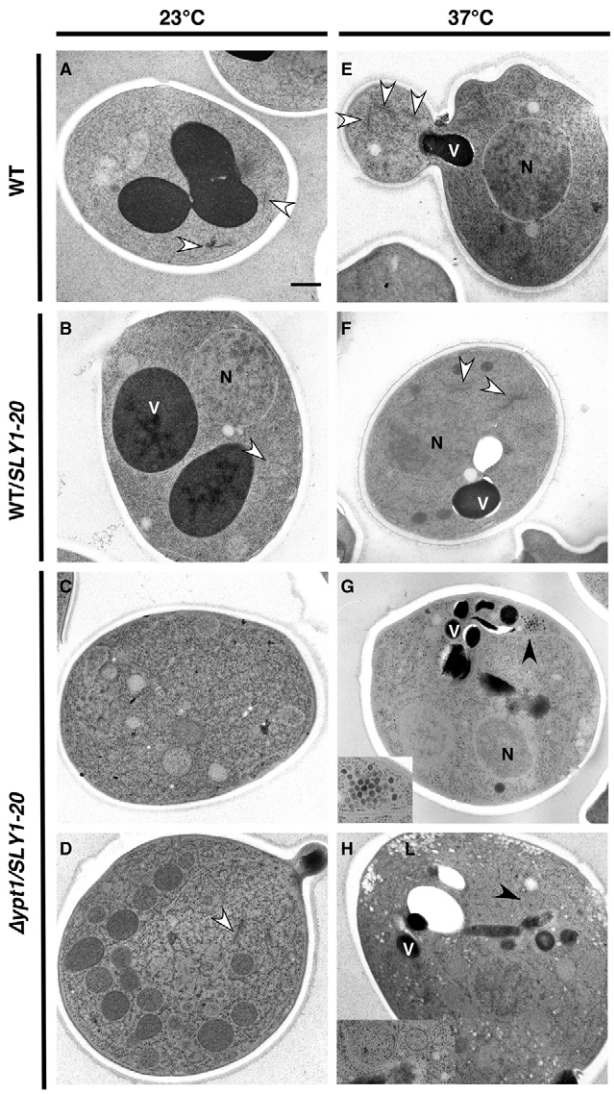


Fig. 6. Golgi function is impaired in *Δypt1/SLY1-20* cells. (A) Glycosylation is enhanced in *Δypt1/SLY1-20* cells. Strains were grown overnight at 23°C to early to mid log phase and shifted for 1 hour to 37°C where indicated. Total yeast lysates were prepared and equal amounts of protein were separated by SDS-PAGE. Glycosylated proteins were visualized with a Pro-Q Emerald 300 Kit. More glycoproteins were detected in *Δypt1/SLY1-20* cells irrespective of the temperature. M, marker; pos. con., positive control (HRP); neg. con., negative control, Sec24p; GM, CandyCane glycoprotein molecular mass standards: (from top to bottom) 180 kDa; 82 kDa; 42 kDa; 18 kDa. (B) CPY outer chain glycosylation still occurs at 23°C and is abolished 37°C in *Δypt1/SLY1-20* cells. A pulse-chase experiment was performed at either 23°C or 37°C. Transport of CPY to the vacuole was delayed, yet the glycosylation occurred normally at 23°C in *Δypt1/SLY1-20* cells. At 37°C transport between the ER and the Golgi is blocked. Hence no CYP maturation could occur. Note that a wild-type strain expressing *SLY1-20* behaved like the wild type in the assay.



the Golgi must be at least partially functional. As expected, the p1 of CPY accumulated in the *Δypt1/SLY1-20* cells at 37°C as the transport to the Golgi was blocked.

Therefore, we decided to investigate the Golgi morphology further by electron microscopy (Fig. 7). Although expression of *SLY1-20* in wild-type yeast had no effect on the morphology of the cells and its organelles, Golgi cisternae were less frequently observed in *Δypt1/SLY1-20* cells even at 23°C. In addition the vacuole seemed fragmented (compare Fig. 7A,B with C,D). Upon shift of *Δypt1/SLY1-20* cells for 1 hour to 37°C, no normal Golgi cisternae could be identified, the ER was dilated and vesicle accumulations were frequently observed, consistent with a block in the ER-Golgi transport shuttle (Fig. 7G). Furthermore, lipid-rich structures were present (Fig. 7G,H). Therefore, *Δypt1/SLY1-20* cells have a vesicle transport and a Golgi morphology defect.

Ypt1p interact physically and genetically with Ufe1p

Our results suggest a role for Ypt1p in Golgi maintenance and in the generation of functional COPI vesicles. Is Ypt1p also required for COPI vesicle consumption? If so, we should be able to establish a direct interaction between Ypt1p and an ER t-SNARE. Ypt1p binds to the t-SNARE Sed5p at the Golgi (Lupashin and Waters, 1997). Similarly, Ypt1p should bind the t-SNARE on the ER membrane protein Ufe1p. We performed GST-pull down assays using GST-SNARE fusion proteins and purified His_6-Ypt1p. GST-Ufe1p as well as GST-Sed5p could specifically recruit His_6-Ypt1p (Fig. 8, lanes 2 and 6). By contrast, the GST fusion protein of Snc1p, which

Fig. 7. Ultrastructural analysis of *Δypt1/SLY1-20* cells. Wild-type, wild-type expressing *SLY1-20* and *Δypt1/SLY1-20* strains were grown to early to mid-log phase and half of the culture was shifted to 37°C for 1 hour. The cultures were processed for ultrastructural analysis. Thin sections were stained with lead citrate and uranyl acetate. (A-D) Cells grown at 23°C; (E-H) cells shifted for 1 hour to 37°C. (A,E) wild-type; (B,F) wild-type expressing *SLY1-20*; (C,D,G,H) *Δypt1/SLY1-20*. White arrowheads point to individual Golgi cisternae. The black arrowhead in G points to an accumulation of vesicles [an enlargement (×2) is shown in the inset]. The black arrowhead in H is directed towards strange membraneous profiles. An enlargement (×2) is shown in the inset. N, nucleus; V, vacuole. Bar (in A), 500 nm.

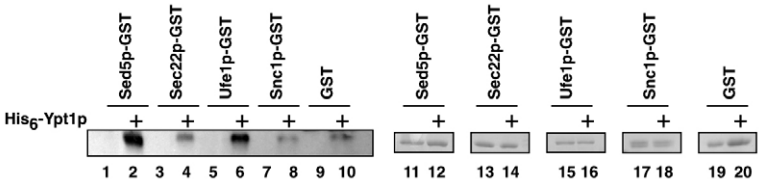


Fig. 8. Ypt1p interacts directly with Ufe1p-GST. The transmembrane domain of the SNAREs was replaced by GST. Equal amounts of GST-tagged SNAREs were immobilized on GSH-agarose and incubated with purified His_6-Ypt1p. His_6-Ypt1p bound to the SNAREs was detected by immunoblotting (lanes 1-10). The amount of immobilized SNARE proteins is shown in lanes 11-20.

functions in post Golgi transport, bound only background amounts of His_6-Ypt1p (Fig. 8, compare lanes 8 and 10). GST-Sec22p binding was slightly over the background (Fig. 8, lane 4). Binding of His_6-Ypt1p to GST-Ufe1p was specific because when we used His_6-Ypt7p instead of His_6-Ypt1p no association with GST-Ufe1p was detected (data not shown). Ypt7p is the Rab protein required for all fusion steps with the vacuole. These results demonstrate a direct physical interaction between the Rab Ypt1p and the ER t-SNARE Ufe1p. To corroborate these findings, we aimed to establish a genetic interaction between *YPT1* and *UFE1*. For that we crossed the temperature-sensitive *ypt1-3* strain with a strain in which *UFE1* was chromosomally deleted and the temperature-sensitive *ufe1-1* mutation was present on a CEN plasmid (Fig. 9A). Sixty-eight tetrads were dissected, 21 of which had only two viable spores at 23°C and 37°C, indicating that they carried the wild-type alleles of *YPT1* and *UFE1*. Because *ufe1-1* was only present on a plasmid, we determined the transmission rate during sporulation (Fig. 9B). The plasmid was transmitted in the wild-type spores with 36% efficiency. Given this transmission rate, we should have been able to recover tetrads with three or four spores growing at 23°C and only two spores growing at 37°C, reflecting the temperature-sensitive phenotype of p*ufe1-1 Δufe1* and *ypt1-3* (Fig. 9A). However, this combination was never obtained, demonstrating that *UFE1* and *YPT1* interact genetically. By contrast crossing *ypt1-3* with *tip20-8,* a mutant that was recently shown to allow back fusion of COPII vesicles with the ER (Kamena and Spang, 2004), we could isolate viable double mutant haploids, indicating that the genetic interaction between *YPT1* and *UFE1* is indeed specific. These results demonstrate that Ypt1p interacts with Ufe1p both physically and genetically, consistent with an involvement of Ypt1p in the fusion process of COPI vesicles with the ER.

Discussion

Ypt/Rab proteins are essential for the fusion of membranes in the cell by displacing inhibitory factors from the SNAREs. Here, we demonstrate that Ypt1p is the Rab required for retrograde transport of COPI vesicles from the Golgi to the ER. Remarkably, Ypt1p might be required at each organelle-vesicle transition step in the ER-Golgi shuttle, namely (1) budding of COPII vesicles from the ER; (2) fusion of COPII vesicles with the Golgi; (3) generation of COPI-coated vesicles at the Golgi; and (4) consumption of Golgi-derived vesicles at the ER.

Ypt1p is required for sorting of GPI-anchored proteins into COPII vesicles at the ER (Morsomme and Riezman, 2002) and the role of Ypt1p in fusion of COPII vesicles with the Golgi is well established (Cao et al., 1998; Cao and Barlowe, 2000). However, no requirement of Ypt1p in retrograde transport from the Golgi to the ER had been demonstrated. Using an in vitro assay that recapitulates retrograde transport from the Golgi to the ER, we were able to show that Ypt1p is involved in this pathway. Consistent with data by Morsomme and Riezman (Morsomme and Riezman, 2002) on the role of Ypt1p in cargo inclusion we found that, in the absence of Ypt1p, COPI vesicles were still formed at the Golgi, yet these vesicles contained less of the cargo Emp47p and of the v-SNARE Sec22p than did

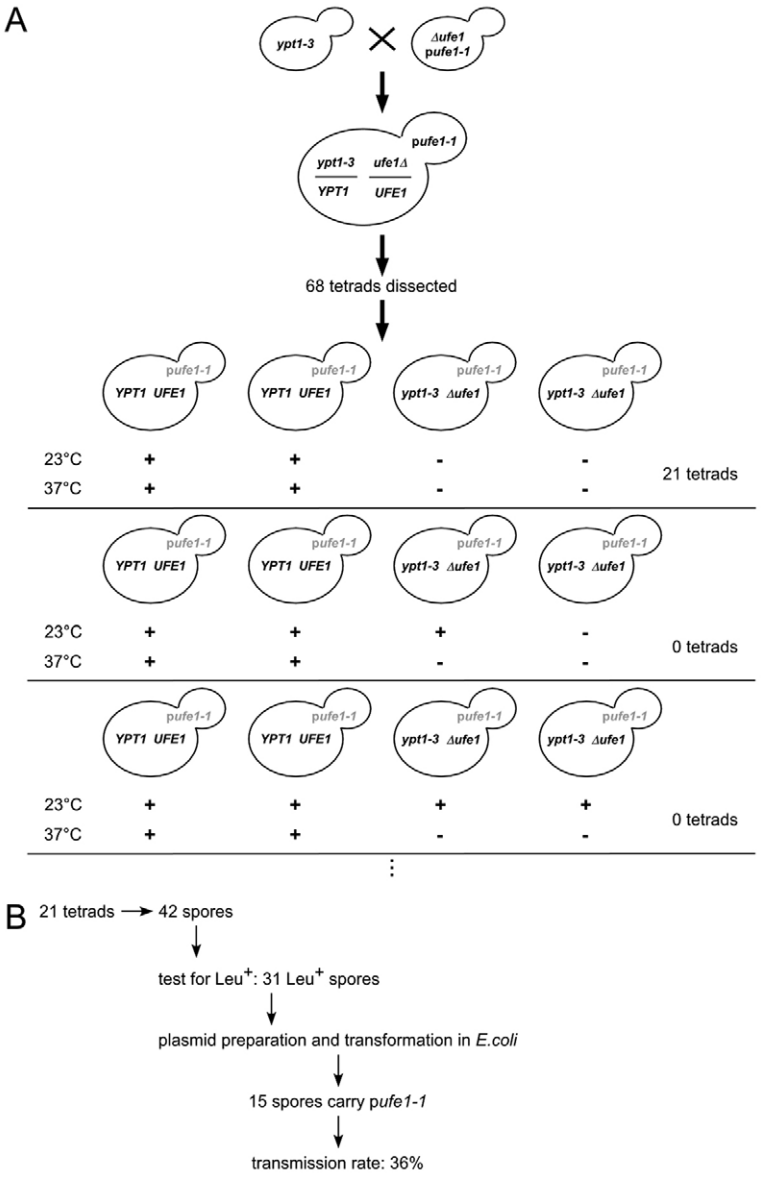


Fig. 9. *UFE1* and *YPT1* interact genetically. (A) Schematic outline of the cross between *ypt1-3* and *Δufe1* p*ufe1-1*. A subset of the meiotic outcomes is shown. (B) Determination of the transmission rate of p*ufe1-1* in wild-type yeast spores.

vesicles from wild-type cells. These COPI vesicles might only contain minor amounts of cargo, because the protein to lipid ratio was significantly decreased compared with COPI vesicles derived from wild-type Golgi membranes. In mammalian cells, the overexpression of a dominant-negative form of Rab1 causes displacement of coatomer from Golgi membranes and a BFA phenotype, indicating that Rab1 is required in a Arf1-dependent recruitment step of coatomer at the Golgi (Alvarez et al., 2003). In yeast, we could not detect a loss of coatomer from Golgi membranes in the *Δypt1/SLY1-20* mutant. However, the expression of *SLY1-20* might prevent the dissociation of coatomer from the Golgi. Yet, the protein composition of the mutant Golgi was altered. Surprisingly, the cells still performed glycosylation efficiently, and the cells were still inert against osmotic stress. In addition, we observed a change in morphology of the Golgi or a loss of Golgi cisternae by electron microscopy, which indicates that neither the absolute concentration of some Golgi proteins or peripheral Golgi proteins nor the Golgi morphology are important for survival in yeast.

Finally, to complete the cycle, we propose that Ypt1p is required for fusion of COPI vesicles with the ER. This hypothesis is based on our demonstration of a direct interaction between Ypt1p and the ER t-SNARE as well as on genetic analysis. Collectively, these data suggest that Ypt1p is not only involved in vesicle consumption but also vesicle generation at each step in the ER-Golgi shuttle. This rather surprising mechanism might be re-used at different places in the cell by different Rabs/Ypts. Recently, Vonderheit and Helenius (Vonderheit and Helenius, 2005) suggested a role for Rab7 in cargo sorting at the early endosome and in the formation of late-endosome-targeted transport vesicles. Rab7 has been implicated previously in fusion of transport intermediates with the late endosome and the lysosome (Bucci et al., 2000; Meresse et al., 1995) and the conversion from early to late endosomes (Poteryaev et al., 2007; Rink et al., 2005). Whether Rab7 is necessary for the generation of vesicles from the late endosomes that might be targeted to the TGN to recycle SNAREs and other transport factors remains to be determined. Yet, a common picture seems to emerge where the role of Ypts/Rabs is not limited to docking and fusion of vesicles and organelles, but also extends to cargo recruitment and transport carrier formation. However, this also implies that the regulation of the Ypts/Rabs must be more complicated than thus far anticipated. A focus of research in the near future must be to identify new upstream regulators of Rab proteins, in order to understand their regulation, which can no longer be limited to cycles of activation and inactivation.

Materials and Methods

Yeast methods, strains and antibodies

Standard yeast genetic techniques and media were used throughout (Sherman, 1991). Yeast strains used in this study are listed in Table 1.

Polyclonal rabbit antibodies directed against coatomer, Arf1p, Sec22p, Bos1p, Sed5p, Emp47p, Kar2p, α-1,6-linked mannose residues, Anp1p, Mnn1p, and Ypt1p and mouse monoclonal anti-myc antibodies were used in this study. His_6-Ypt7p was detected using the Super Signal West HisProbe Kit (Pierce Biotechnology Inc., Bonn, Germany)

Preparation of perforated yeast spheroplasts and cytosol

Perforated yeast spheroplasts (semi-intact cells) were prepared as described previously (Spang and Schekman, 1998).

To obtain cytosol, yeast cells were grown to early- to mid-log phase in YPD medium at either 23°C or 30°C. Cells were harvested by centrifugation and washed twice with water. The cell pellet was resuspended in a minimal volume of buffer B88 (20 mM Hepes pH 6.8, 250 mM sorbitol, 150 mM potassium acetate, 5 mM magnesium acetate) and pipetted into liquid nitrogen. The cell beads were ground up under liquid nitrogen in a blender (Worthington Biochemical, Lakewood, NJ) for large-scale preparations or in a mortar for small-scale preparations. The cell powder was thawed in an ice-water bath, and was then diluted 1:1 with B88, and 1 mM DTT (dithiothreitol) and protease inhibitors were added. The lysate was centrifuged (5 minutes at 3000 ***g***, 15 minutes at 20,000 ***g***, 1 hour at 100,000 ***g***). The 100,000 ***g*** supernatant was collected, carefully avoiding the pellet and the lipids that floated to the top.

Table 1. Yeast strains used in this study

Strain	Genotype	Source
RSY1169	*MATa leu2-3,112 ura2-53 pep4::URA3 gls1-1*	R. Schekman
SEY6210	*MATa ura3 leu2 his4 trp1 lys2 suc2-Δ9*	P. Cosson
tip20-8	*MATα ura3 leu2 his4 trp1 lys2 suc2-Δ9 tip20-8*	P. Cosson
CBY900	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63*	C. Barlowe
CBY901	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 SLY1-20*	C. Barlowe
CBY903	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 SLY1-20 ypt1::HIS3*	C. Barlowe
Δypt6	*MATα ade2-101oc his3-200 leu2-1 lys2-801am trp1-63 ura3-52 ypt6::kan*	A. Spang
Ypt31/32	*MATta ypt32 A141 D-his ypt31::kan*	D. Gallwitz
PC137	*MATa ura3-1 leu2–1 his4-619 trp1–9 lys2-801am suc2–9 tip20-5*	P. Cosson
YUA1-9c	*MATα ade2 ura3 leu2 his3 lys2 dsl1-22*	H. D. Schmitt
RSY1163	*MATa ura3-52 leu2,3,-112 ade2-101 kar2-133*	R. Schekman
RSY976	*MATa ura3-52 ypt1-3*	R. Schekman
YAS134	*MATα his4 ura3 YPT1::ypt1-A136 D-LEU*	N. Segev
YAS959	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 pRS316*	This study
YAS976	*MATa ura3 leu2 his4 lys2 bar1 sec7-1 pRS316*	This study
YAS2058	*MATα ura3-52 ufe1::TRP1 pRS315-ufe1-1*	This study
YAS1751	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 pRS315-SED5-GFP*	This study
YAS1752	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 SLY1-20 pRS315-SED5-GFP*	This study
YAS1753	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 SLY1-20 ypt1::HIS3 pRS315-SED5-GFP*	This study
YAS1742	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 LEU2::EMP47-myc*	This study
YAS1743	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 SLY1-20 LEU2::EMP47-myc*	This study
YAS1744	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 SLY1-20 ypt1::HIS3 Yiplac128*$^{+}$	This study
YAS1745	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 ANP1::ANP1-xyEGFP-kanMX4*	This study
YAS1746	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 SLY1-20 ANP1::ANP1-xyEGFP-kanMX4*	This study
YAS1747	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 SLY1-20 ypt1::HIS3 ANP1::ANP1-xyEGFP-kanMX4*	This study
YAS1748	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 pTPQ128**	This study
YAS1749	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 SLY1-20 pTPQ128**	This study
YAS1750	*MATa his3Δ200 ura3-52 leu2-1 trp1Δ63 SLY1-20 ypt1::HIS3 pTPQ128**	This study

*pTPQ128: pRS415-ADH-SEC7-dsRed.

Purification of coatomer, Sec23/24p, Sec13/31p, Sar1p, Lma1p, Uso1p, Sec18p, Ypt1p, Ypt7p and *N*-myristoylated Arf1p

The purifications of Sar1p, Sec23/24p, Sec13/31p, coatomer, myc-tagged Uso1p, Sec18-His_6, *N-myr*-yArf1p and the Lma1p complex were performed as described previously (Barlowe, 1997; Barlowe et al., 1994; Hosobuchi et al., 1992; Kahn et al., 1995; Salama et al., 1993; Xu et al., 1997). His_6-Ypt1p and His_6-Ypt7p were prepared following the ΔN17-Arf1p-His_6 purification protocol (Rein et al., 2002).

In vitro round-trip assay

Stage I

Translocation. The translocation reaction using [^{35}S]ppαF-HDEL and *gls1-1* as donor membranes was performed as described previously (Spang and Schekman, 1998).

Stage II

Budding. To the membranes of the stage I reaction, we added 25 μg/ml Sar1p, 25 μg/ml Sec23/24p complex, 75 μg/ml Sec13/31p complex, 50 μM GTP and an ATP regeneration system (Baker et al., 1988). The reaction mixture was incubated for 30 minutes at 20°C, chilled for 5 minutes on ice, and subjected to a medium speed centrifugation (12,000 ***g***, 30 seconds), which retained COPII vesicles in the supernatant fraction (MSS). An aliquot of the MSS was saved to determine the efficiency of the retrograde transport.

Stage III

Fusion. The MSS from stage II was supplemented with an ATP regeneration system, 50 μM GTP, 1 μM Lma1p, 1 μg/ml Sec18p, 1.5 μg/ml Uso1p and 600 μg/ml perforated spheroplast membranes from the *GLS1* strain. Fusion was allowed to take place for 20 minutes at 20°C.

Stage IV

Retrieval. Cytosol was added to a final concentration of 2 mg/ml or the same volume of B88 was added to the non-cytosol control. Reactions were incubated for 30 minutes at either 23°C (permissive temperature for ts strains) or 30°C (semi-permissive temperature for ts strains). The reaction mixture was chilled on ice for 5 minutes and the acceptor ER sedimented by centrifugation at 12,000 ***g*** for 30 seconds. The pellet was washed once with 2.5 M urea in B88 for 10 minutes on ice and twice with B88. Fusion with the acceptor ER was measured by precipitation of protease-protected [^{35}S]gpαF-HDEL with concanavalin A-Sepharose followed by separation of untrimmed [^{35}S]gpαF-HDEL from trimmed [^{35}S]gpαF-HDEL by SDS-PAGE. The read out takes advantage of the different glycosylation patterns of the reporter in the donor ER and the acceptor ER. The donor ER is defective in glucosidase I, the enzyme, which together with glucosidase II, is responsible for trimming the *N*-glycans of glucosylated proteins prior to their ER exit. The acceptor membranes always contained a functional glucosidase I, so that successful retrieval of the reporter to the acceptor ER is monitored by trimming of the *N*-glycans. Trimmed and untrimmed forms of [^{35}S]gpαF-HDEL have different electrophoretic mobilities and can be discriminated by SDS-PAGE. In the experiments using Gdi1p, the membranes were washed twice with B88 after stage III and retrieval was performed either in presence of purified Gdi1p (1.72 μg/ml) or complemented with the same volume of B88.

In vitro forward-transport assay

Stage I

Translocation. The translocation reaction using either [^{35}S]ppαF-HDEL or [^{35}S]ppαF was performed as described above.

Stage II

Transport. An aliquot (10 μl) of the stage I membranes was incubated with an ATP regeneration system, 50 μM GTP, and 2.5 mg/ml cytosol for 1 hour at 30°C. Each reaction was performed in quadruplicate, and the final volume of each reaction was 50 μl. After the incubation, the samples were chilled for 5 minutes at 4°C and centrifuged for 30 seconds at 12,000 ***g***. A 30 μl aliquot of the supernatant collected from the meniscus was treated with trypsin followed by trypsin inhibitor as described previously (Rexach and Schekman, 1991). The membranes were solubilized with 1% SDS and heated for 5 minutes at 95°C. For each set of experiments, two reactions were precipitated with concanavalin A-Sepharose or antibodies directed against α-1,6-linked mannose modifications and protein A-Sepharose. Washed immunoprecipitates were quantified in a liquid scintillation counter (Beckman Instruments, Krefeld, Germany). The budding efficiency was determined by comparing the amount of protease-protected [^{35}S]gpαF-HDEL in the supernatant to the total amount of [^{35}S]gpαF-HDEL translocated into the ER in the stage I reaction precipitated with concanavalin A-Sepharose. The efficiency of the fusion of COPII vesicles with the Golgi is given as the ratio of anti-α-1,6-linked mannose precipitated counts over concanavalin A-Sepharose precipitated counts.

Golgi-budding assay

The in vitro Golgi-budding assay was performed as described by Spang and Schekman (Spang and Schekman, 1998) with modifications. Enriched Golgi membranes were incubated with 0.1 mM GTP, coatomer (100 μg/ml) and Arf1p (80 μg/ml) for 30 minutes at 30°C in a volume of 200 μl. After chilling on ice, samples were loaded on top of a Ficoll-sucrose gradient consisting of 0.4 ml 60% (wt/wt) sucrose, 0.8 ml 7.5% (wt/wt) Ficoll, 1 ml 5%, 4%, 3% and 0.8 ml 2% (wt/wt) Ficoll in 15% sucrose, 20 mM Hepes pH 6.8, and 5 mM magnesium acetate. Vesicles were separated from the Golgi by centrifugation for 2 hours at 35,000 rpm (SW55 rotor; Beckman Instruments). Fractions (400 μl) were collected from the top of the gradient. Fractions 5-7 were pooled, mixed with an equal volume of 80% Nycodenz in 20 mM Hepes pH 6.8, 150 mM potassium acetate, 5 mM magnesium acetate (B150), and overlaid with 600 μl 30%, 25%, 20% and 15%, and 400 μl 10% Nycodenz in B150. The gradient was centrifuged for 16 hours at 40,000 rpm (SW55 rotor). Fractions (300 μl) were collected from the top, TCA-precipitated, and analyzed by immunoblotting.

CPY pulse-chase analysis

Cells were grown to an OD_{600} of 1, and the cell equivalent of an OD_{600} of 2.5 was resuspended in 200 μl HC-MET-D. Cells were preincubated for 15 minutes, incubated at 23°C or 37°C for 30 minutes and then labeled for 10 minutes with 100 μCi/ml [^{35}S]methionine (GE Healthcare, Freiburg, Germany). The radioactive product was chased with an excess of methionine and cysteine for the indicated time points. Cells were lysed and carboxypeptidase C (CPY) was immunoprecipitated using anti-CPY antibodies and protein A-Sepharose (GE Healthcare). The immunoprecipitates were resolved by SDS-PAGE, and the bands were visualized with a PhosphoImager (GE Healthcare).

GST pull-down assay

SNARE-GST fusion proteins (5 μg) were immobilized onto 25 μl 50% glutathione-agarose slurry (GE Healthcare) for 30 minutes at 4°C. Unbound proteins were removed by three washes with BBP (25 mM Hepes, pH 6.8, 1 mM DTT, 0.5 mM $MgCl_2$, 300 mM potassium acetate, 0.2% Triton X-100). Beads were incubated with His_6-Ypt1p (100 pmol) or His_6-Ypt7p (100 pmol) in BBP for 1 hour at 4°C. The total reaction volume was 500 μl. After binding, beads were washed three times with BBP, transferred to a fresh tube, washed once with 20 mM Hepes, pH 6.8, and then heated to 65°C for 10 minutes in sample buffer. Eluted proteins were analyzed by SDS-PAGE followed by Coomassie Blue staining or immunoblot.

Kar2p secretion assay and glycostain

Secretion of the ER-resident Kar2p was assessed as described by Andag et al. (Andag et al., 2001). The detection was performed with antibodies directed against Kar2p or Pgk1p.

For the detection of glycosylated proteins, equal amounts of total yeast lysates were separated by SDS-PAGE and visualized using a Pro-Q Emerald 300 Kit from Molecular Probes (Mobitec, Göttingen, Germany) according to the manufacturer's recommendations. The yeast lysates were prepared according to Spang et al. (Spang et al., 2001).

Fluorescence microscopy and electron microscopy

Yeast cells were grown to early log phase and where indicated shifted for 1 hour to 37°C. The cells were immobilized on ConA-coated slides, mounted with Citifluor and GFP fluorescence was detected using an Axioplan microscope equipped with an Axiocam (Zeiss, Oberkochen, Germany). The detection of Emp47-myc by immunofluorescence was performed as described previously (Spang et al., 2001). For electron microscopy analysis, the cells were frozen under high pressure and treated as described in Sandmann et al. (Sandmann et al., 2003).

Genetic interaction analysis between *YPT1* and *UFE1*

To test for genetic interaction between *YPT1* and *UFE1*, *ypt1-3* was crossed to a deletion of *UFE1*, which is kept alive by the ts-sensitive mutation *ufe1-1* on a CEN plasmid. For sporulations, diploids were grown overnight at 37°C as a patch on GNA pre-sporulation plates (5% glucose, 1% yeast extract, 3% nutrient broth, 2% agar). Cells from the patch were incubated for 5 days at 30°C in liquid sporulation medium (1% potassium acetate, 0.005% zinc acetate) on a roller wheel. Tetrads were dissected on YPD plates and incubated at 23°C. Tetrads with two living and two dead spores were analyzed further. The two living spores always carried the wild-type copy of both *YPT6* and *UFE1*. Deletion of *UFE1* is lethal by itself. Therefore, we had to determine the inheritance rate of the *ufe1-1* ts plasmid in the surviving wild-type spores. Plasmid DNA was isolated and retransformed into *E. coli*. Thirty-six percent of the wild-type spores contained the *ufe1-1* plasmid. However, we never recovered a spore with the *Δypt6 Δufe1* p*ufe1-1* genotype in our tetrad analysis.

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