

The yeast p24 complex is required for the formation of COPI retrograde transport vesicles from the Golgi apparatus

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The p24 family members are transmembrane proteins assembled into heteromeric complexes that continuously cycle between the ER and the Golgi apparatus. These cargo proteins were assumed to play a structural role in COPI budding because of their major presence in mammalian COPI vesicles. However, this putative function has not been proved conclusively so far. Furthermore, deletion of all eight yeast p24 family members does not produce severe transport phenotypes, suggesting that the p24 complex is not essential for COPI

function. In this paper we provide direct evidence that the yeast p24 complex plays an active role in retrograde transport from Golgi to ER by facilitating the formation of COPI-coated vesicles. Therefore, our results demonstrate that p24 proteins are important for vesicle formation instead of simply being a passive traveler, supporting the model in which cargo together with a small GTPase of the ARF superfamily and coat subunits act as primer for vesicle formation.

Introduction

COPI vesicle-mediated retrograde transport from the Golgi apparatus to the endoplasmic reticulum (ER) is crucial for eukaryotic cell physiology (Semenza et al., 1990). The molecular mechanisms that drive COPI vesicle generation are not completely understood (Spang, 2002). The current model, known as priming complex model (Springer et al., 1999), proposes that Arf1p, a small GTPase, is attracted to the Golgi membrane, where it is activated by a guanine exchange factor (ArfGEF). Membrane-bound, activated Arf1p then recruits coatamer, a preassembled complex of seven subunits, which forms a priming complex with a GTPase-activating protein (ArfGAP) and a transmembrane protein (cargo or SNARE). Once enough COPI priming complexes are established, they associate laterally to form a larger, polymeric coat on the Golgi membrane. This coat polymerization leads to the deformation of the membrane and, subsequently, to the COPI vesicle generation.

In addition to the coat components, transmembrane proteins might also be important for the budding process by acting

as a primer to form priming complexes (Springer et al., 1999). Specific COPI vesicle passengers, such as the members of the p24 family, have been suggested to play a role in COPI vesicle-mediated flow (Stamnes et al., 1995; Bremser et al., 1999). The p24 proteins are assembled into heteromeric complexes that continuously cycle between ER and Golgi compartments (Sohn et al., 1996; Rojo et al., 1997; Fullekrug et al., 1999; Belden and Barlowe, 2001b). In yeast, at least four members of the p24 family (Emp24p, Erv25p, Erp1p, and Erp2p) function in the p24 complex (Marzioch et al., 1999). The yeast p24 complex plays a specialized role in selective cargo recruitment into specific ER-derived vesicles (Muñiz et al., 2000, 2001). Nevertheless, this is not likely to be the only function of the p24 complex in the early secretory pathway (Elrod-Erickson and Kaiser, 1996; Bremser et al., 1999; Lavoie et al., 1999; Emery et al., 2003). The p24 proteins have been assumed to play a structural role in COPI vesicle formation because of their ability to bind COPI proteins and their major presence in mammalian COPI vesicles (Stamnes et al., 1995; Sohn et al., 1996). Indeed, mammalian p24 cytosolic tails displayed on liposomes can stimulate the formation of COPI vesicles (Bremser et al., 1999). Consistent with these findings and based on further *in vitro* experiments, it has been proposed that mammalian p24 proteins could inhibit

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Abbreviations used in this paper: CPY, carboxypeptidase Y; UPR, unfolded protein response.

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GTP hydrolysis by slowing down ArfGAP activity, thereby ensuring that coatomer can remain on the membrane in order to form COPI priming complexes required for vesicle formation (Goldberg, 2000; Lanoix et al., 2001). This model predicts a critical role for p24 proteins in COPI vesicle biogenesis. However, deletion of all eight yeast p24 family members does not produce severe transport phenotypes or morphological alterations in the endomembrane system, suggesting that p24 complex is not essential for COPI function (Springer et al., 2000). Therefore, whether p24 proteins are just COPI coat-binding cargo proteins or are required for COPI coated vesicle formation itself still remains a matter of debate. Here, we provide evidence that yeast p24 complex plays an active role in COPI vesicle budding from Golgi membranes.

Results and discussion

The *emp24Δ* and *erv25Δ* mutations interact genetically with mutant alleles of genes encoding for COPI vesicle coat components

To gain more insights into the functions of the yeast p24 complex in the early secretory pathway, we performed a screen for mutations that induce synthetic lethality upon disruption of the *EMP24* gene. We identified a genetic interaction between *EMP24* and *GLO3*, a gene encoding for an ArfGAP involved in COPI vesicle formation (Lewis et al., 2004). Confirming this result, *GLO3* could rescue the viability of the *emp24Δ glo3Δ* double mutant (Fig. 1 A). This genetic interaction is also true for other p24 members. As expected, the *erv25Δ glo3Δ* double mutant was inviable but could be complemented by *GLO3* (Fig. 1 A). Deletion of *EMP24* or *ERV25* destabilizes the other three proteins of the complex, leading to a complete loss of p24 function (Marzioch et al., 1999). Thus, it is rather likely that other members of the p24 family play a similar role as Emp24p and Erv25p with respect to interaction with Glo3p.

Two partially redundant ArfGAPs operate in retrograde traffic from Golgi to ER: Glo3p and Gcs1p (Poon et al., 1999). However, neither *emp24Δ* nor *erv25Δ* showed genetic interaction with *gcs1Δ* (Fig. 1 B). We also found that overexpression of *GCS1* was not able to restore growth of the *emp24Δ glo3Δ* double mutant (unpublished data). These results show that the genetic interaction between p24 coding genes and *GLO3* is specific and are consistent with previous findings that Glo3p and Gcs1p have only partially overlapping functions (Poon et al., 1999; Lewis et al., 2004; Yahara et al., 2006).

Because the ArfGAP Glo3p is a component of the COPI vesicle coat through interactions with the coatomer subunits Sec21p and Sec27p (Eugster et al., 2000; Lewis et al., 2004), we wanted to investigate the genetic interaction of p24 mutations with mutations in *SEC21* and *SEC27*. *emp24Δ* and *erv25Δ* showed a synthetic growth defect when combined with *sec21-1* (Fig. 1 C) and *sec27-1* (unpublished data) mutations.

Altogether, these genetic interactions between *emp24Δ* or *erv25Δ* and genes encoding COPI vesicle coat proteins involved in Golgi to ER transport strongly suggest that p24 proteins could participate in the formation of COPI vesicles.

The p24 complex is necessary for efficient trafficking from the Golgi to the ER when Glo3p GAP activity is reduced

The data presented in the previous paragraph suggest that the p24 family members play a more active role in the generation of COPI-coated vesicles than previously anticipated. In the wild-type situation, this role may not be essential. However, when the ArfGAP activity provided by Glo3p is missing or compromised, p24 function in COPI vesicle generation could become vital. To investigate this possibility, we assessed the lack of p24 function in the *ret4-1* mutant, a temperature-sensitive mutant allele of *GLO3* (Dogic et al., 1999). The T66I mutation in *ret4-1*, which is localized to the GTPase-activating domain of Glo3p, reduces the GAP activity toward ARF1 in vitro. We found that *emp24Δ ret4-1* mutant cells were viable at 24°C, but they grew slower than respective single mutant cells (Fig. 2 A). Furthermore, deleting *EMP24* increased the temperature sensitivity of the *ret4-1* allele, which is consistent with the observed genetic interaction between *emp24Δ* and *glo3Δ* (Fig. 2 A). Next, we investigated whether the *emp24Δ* deletion aggravates the slight defect of the *ret4-1* mutant in retrograde trafficking. Because anterograde and retrograde traffic between the ER and the Golgi are interdependent, a block in retrograde transport usually has an indirect effect on anterograde transport. Thus, we analyzed the post-translational processing of several early secretory pathway cargoes such as the vacuolar hydrolase, carboxypeptidase Y (CPY) (Stevens et al., 1982), and the secreted enzyme invertase (Esmon et al., 1987) in the *emp24Δ ret4-1* mutant, as an indirect way to measure defects in retrograde trafficking. Mutations in several COPI genes result in the accumulation of the immature CPY form (p1) at the ER and a general decrease in the glycosylation state of the secreted invertase (Gaynor and Emr, 1997). Likewise, the lack of p24 protein function in a *ret4-1* mutant led to an accumulation of the p1 form of CPY (Fig. 2 B) and exacerbated the impairment of the invertase glycosylation and secretion in the single mutants (Fig. 2 C), which is symptomatic of a severe defect in retrograde transport.

To assess more directly the defect of *emp24Δ ret4-1* double mutant cells in retrograde trafficking, we determined the secretion of the ER molecular chaperone Kar2p. Most mutants that block retrograde transport secrete elevated levels of ER resident proteins with an HDEL signal, including Kar2p, into the media because the HDEL receptor Erd2p fails to retrieve the ER escaped proteins back from the Golgi (Semenza et al., 1990). Previous studies have shown that overproduction of Erd2p mitigates Kar2p secretion (Semenza et al., 1990; Belden and Barlowe, 2001a). Nevertheless, in some trafficking mutants, such as p24 mutants, the activation of the unfolded protein response (UPR) also contributes to Kar2p secretion by exceeding the capacity of the HDEL-retrieval pathway (Belden and Barlowe, 2001a). Wild-type cells did not secrete Kar2p into the media, but *emp24Δ*, *ret4-1*, and *emp24Δ ret4-1* cells secreted significant amounts of Kar2p (Fig. 2 D). Interestingly, when *ERD2* was overexpressed in *emp24Δ* and *ret4-1* mutants Kar2p is not secreted into the growth media, whereas overexpression of *ERD2* in *emp24Δ ret4-1* cells did not reduce Kar2p secretion (Fig. 2 D). This defect in Kar2p retention might be due to an exacerbation of the

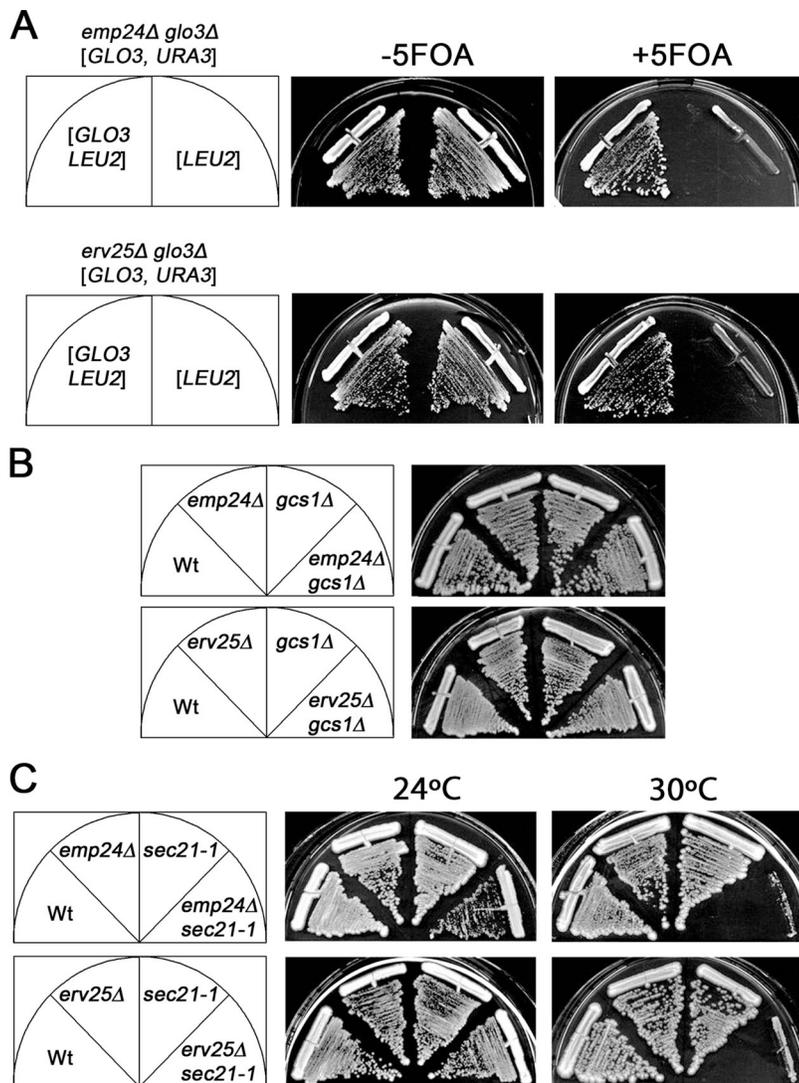


Figure 1. The *emp24Δ* and *erv25Δ* mutations interact genetically with mutant alleles of genes encoding for COPI vesicle coat components. (A) The *emp24Δ glo3Δ* strain with the *URA3* based plasmid bearing *GLO3* pMMY63 was transformed with *LEU2*-based empty vector pRS315 or *GLO3* encoding plasmid pMMY67. Double transformants were replica plated at 30°C on SD-leu-ura or on 5-FOA medium to induce loss of pMMY63. (B) The *emp24Δ* or *erv25Δ* strains were crossed with a *gcs1Δ* strain and the resulting haploid spores were tested for growth at 30°C on YPUAD. (C) *emp24Δ* or *erv25Δ* strains were crossed with a *sec21-1* strain and the resulting haploid spores were tested for growth at 24 and 30°C and grown on YPUAD.

UPR activation in *emp24Δ ret4-1* compared with *emp24Δ*. To address this possibility, we measured the UPR induction from a reporter construct (pJC31) that contains the 22-bp UPRE (unfolded protein responses element) of *KAR2* fused to *LacZ* (Cox and Walter, 1996). As shown in Fig. 2 E, β -galactosidase activity was not increased in the *emp24Δ ret4-1* cells compared with the *emp24Δ* strain. Therefore, we assumed that Kar2p secretion in these cells is mainly caused by a severe defect in retrieval of the Erd2p receptor rather than an overloading of the HDEL-retrieval system. The observed Kar2p secretion was not due to the cell lysis, as hexokinase, a cytosolic protein, was not found in the medium (Fig. 2 D).

Because COPI mutations also cause accumulation of ER membranes (Duden et al., 1994), we examined the morphology of the ER in *emp24Δ ret4-1*. We visualized the ER marker GFP-HDEL by fluorescence, which highlights the nuclear envelope as well as peripheral ER elements (Rossanese et al., 2001). As previously observed (Prinz et al., 2000), GFP-HDEL showed a massive proliferation of ER membranes in a *sec21-1* temperature-sensitive strain under conditions when retrograde transport is blocked (Fig. 2 F). Likewise, striking elaborations of the ER were apparent

in *emp24Δ ret4-1* cells, whereas the ER pattern observed in *emp24Δ* and *ret4-1* cells was identical to that in wild-type cells. Together, these data show that *emp24Δ ret4-1* double mutant cells exhibit COPI mutant-like phenotypes, supporting the idea of the involvement of the p24 complex in COPI function.

The p24 complex is required for COPI vesicle budding from Golgi membranes

Our genetic and biochemical findings suggest that the p24 complex might participate in retrograde transport from Golgi to ER by promoting the formation of COPI vesicles. We assessed directly this hypothesis by using an in vitro Golgi vesicle budding assay (Spang and Schekman, 1998). Enriched Golgi membranes devoid of ER from wild-type and *emp24Δ* strains were subjected to a vesicle budding assay by incubating them in the presence of GTP. Vesicles containing the COPI cargo Emp47p were produced by wild-type Golgi-enriched membranes (Fig. 3 A). In contrast, Golgi membranes from the *emp24Δ* mutant strain failed to form COPI vesicles (Fig. 3 A). This result indicates that the p24 complex plays an important role in COPI vesicle generation from Golgi membranes.

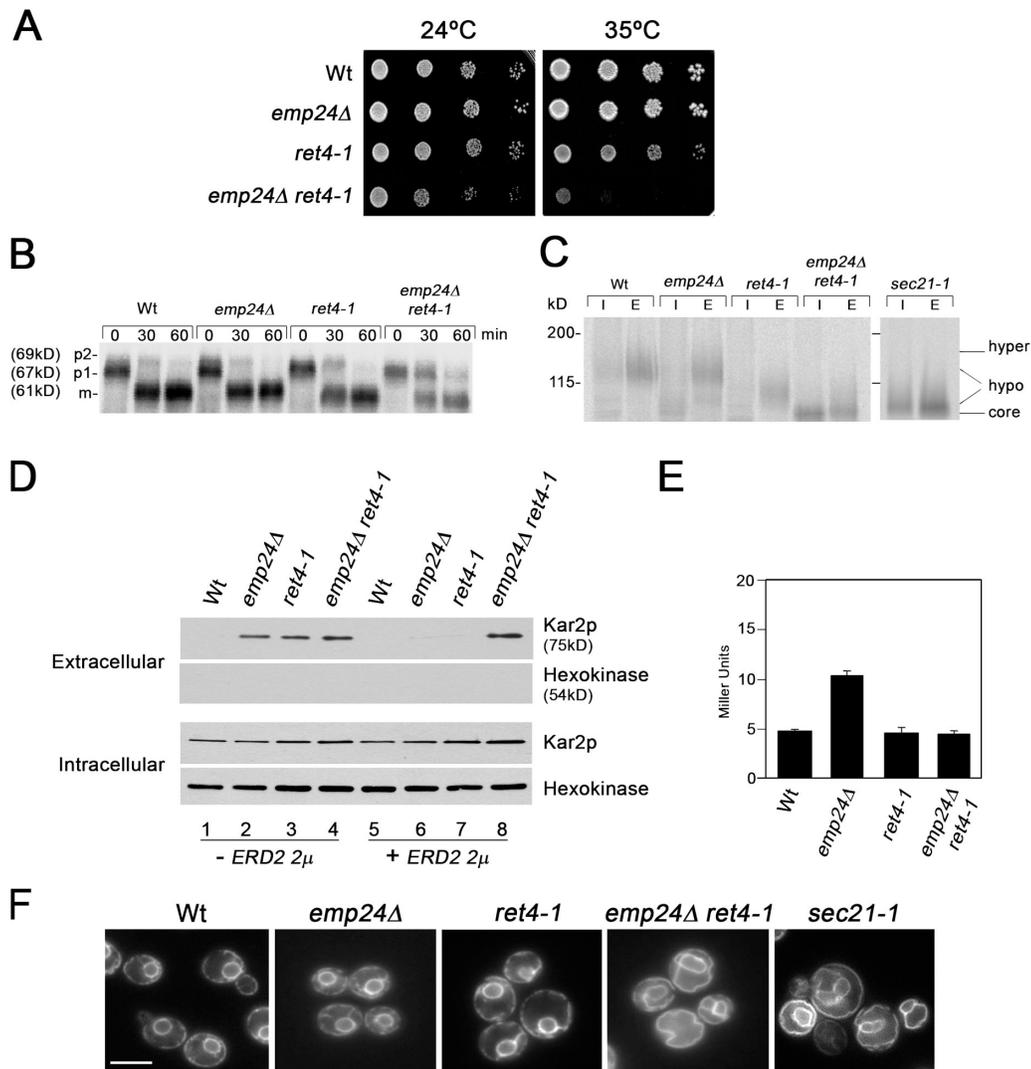


Figure 2. The *emp24Δ ret4-1* double mutant cells exhibit COPI mutant-like phenotypes. (A) *emp24Δ* strain was crossed with a *ret4-1* strain and the resulting haploid spores were tested for growth at 24 and 35°C on YPUAD. (B) Proliferating cells were radiolabeled for 5 min, chased for 30 min at 24°C, and lysed. CPY was immunoprecipitated, resolved by SDS-PAGE, and analyzed by Phospholmager. ER (p1), Golgi (p2), and vacuole (m) CPY forms are indicated. (C) Low glucose-induced cells were pulse-labeled for 5 min and chased for 30 min at 24°C. Cells were converted to spheroplasts, and separated into intracellular (I) and extracellular (E) fractions from which invertase was immunoprecipitated, resolved by SDS-PAGE, and analyzed by Phospholmager. Migration positions of core glycosylated, hypo-, and hyperglycosylated invertase are indicated. (D) Cells without (1, 2, 3, and 4) or with (5, 6, 7, and 8) *ERD2 2μ* plasmid (pJS209) were transferred to fresh media for 1 h at 24°C. Proteins contained in the cell culture supernatant were concentrated by TCA precipitation, resolved by SDS-PAGE, and Kar2p and Hexokinase were detected by immunoblotting. (E) β -Galactosidase assays were performed on strains harboring the reporter construct (pJC31). (F) Fluorescence images of cells expressing the ER-localized protein *GFP-HDEL*. Cells were grown at 24°C, except *sec21-1* cells, which were grown at 24°C and then shifted 40 min at 37°C. Bar, 5 μ m.

The p24 complex requirement for budding was observed only under coat-limiting conditions. Increasing the amount of coatomer and Arf1p in the budding assays restored COPI vesicle generation from *emp24Δ* Golgi membranes (Fig. 3 B). This COPI dosage dependence of budding strongly suggests that p24 proteins might facilitate the budding process by stabilizing coatomer on the Golgi membrane. This possibility is supported by a previous report showing that overexpression of *EMP24* suppresses *sec21-3* temperature sensitivity (Sandmann et al., 2003). Thus, the phenotype enhancement in the *emp24Δ sec21-1* mutant might be due to the failure of cargo proteins to create enough priming complexes that would support budding in the absence of p24 proteins. Increasing the stability of coatomer on

Golgi membranes might rescue the *emp24Δ sec21-1* mutant phenotype. Because ArfGAPs have been shown to potentiate the binding of coatomer to cargo proteins and consequently promote the stabilization of COPI priming complexes (Rein et al., 2002; Lee et al., 2005), we tested whether the temperature sensitivity of *emp24Δ sec21-1* and *erv25Δ sec21-1* is suppressed by overexpression of *Glo3p* and *Gcs1p*. Indeed, overexpression of *GLO3* was able to restore growth of *emp24Δ sec21-1* and *erv25Δ sec21-1* at the restrictive temperature, whereas overexpression of *GCSI* did not rescue the lethal phenotypes (Fig. 4 A). This result is consistent with the role of *Glo3p* as a specific component of the COPI vesicle coat through association with the coatomer subunit *Sec21p* (Lewis et al., 2004), and provides

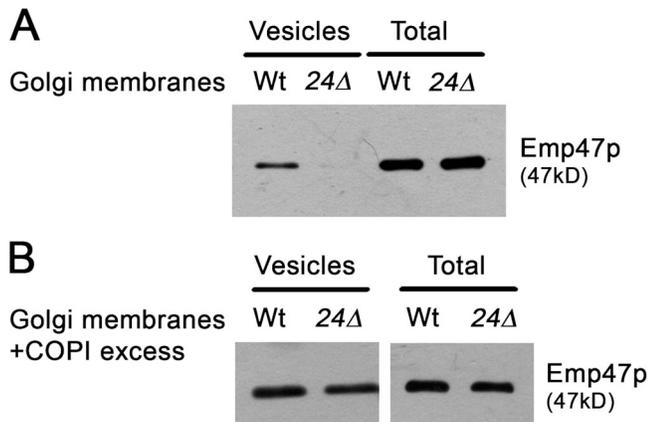


Figure 3. Golgi membranes derived from *emp24Δ* mutant cells are defective for the generation of COPI vesicles *in vitro*. Vesicles were generated from wild-type and *emp24Δ* (RH4443) Golgi membranes, which were incubated with GTP in the absence (A) or the presence of an excess of COPI components (B). The vesicles were purified over a velocity gradient, and subsequently floated on a Nycodenz gradient. Vesicle-containing fractions were collected and pooled, TCA precipitated, resolved on SDS-PAGE, and analyzed by immunoblot using antibody against Emp47p.

further evidence that p24 family proteins are important to stabilize priming complexes on Golgi membranes, which allows efficient polymerization of the COPI coat.

If our hypothesis is correct that p24 proteins are relevant for COPI priming complex formation, they should efficiently recruit COPI coat components such as coatamer and Glo3p. It has already been demonstrated that Emp24p and Erv25p bind coatamer (Belden and Barlowe, 2001b). Next, we tested whether Glo3p is also able to interact specifically with p24 proteins. Peptides corresponding to the C-terminal 10 amino acids of Emp24p or Erv25p were coupled to Sepharose beads and incubated with cytosol. We detected binding of Glo3p to both tail sequences (Fig. 4 B). In contrast, Gcs1p was not recruited to either the Emp24p or the Erv25p tail, supporting the idea that Gcs1p is not part of the COPI coat complex. Furthermore, recombinant Glo3p bound directly to the p24 tails (Fig. 4 C). Our data demonstrate that p24 proteins recruit COPI coat components. Together, our results show that p24 proteins can form COPI priming complexes and that these complexes are required to form retrograde COPI-coated vesicles efficiently from Golgi membranes.

UPR compensates the loss of retrograde transport function in the absence of p24 proteins

We have established in the previous paragraph that p24 complex form priming complexes. However, p24 genes are not essential in yeast. Indeed, deletion of all eight p24 family members does not produce severe transport phenotypes (Springer et al., 2000). Yet, subclasses of secretory proteins accumulate in the ER. How can this apparent discrepancy between the importance of p24 proteins for vesicle generation and the mild phenotypes of their deletion be reconciled? Perhaps a compensatory mechanism exists that helps the cell to cope with the loss of p24 proteins. Indeed, deletion of *ERV25* leads to UPR activation (Belden and Barlowe, 2001a). Moreover, the concomitant loss of *ERV25* (or *EMP24*)

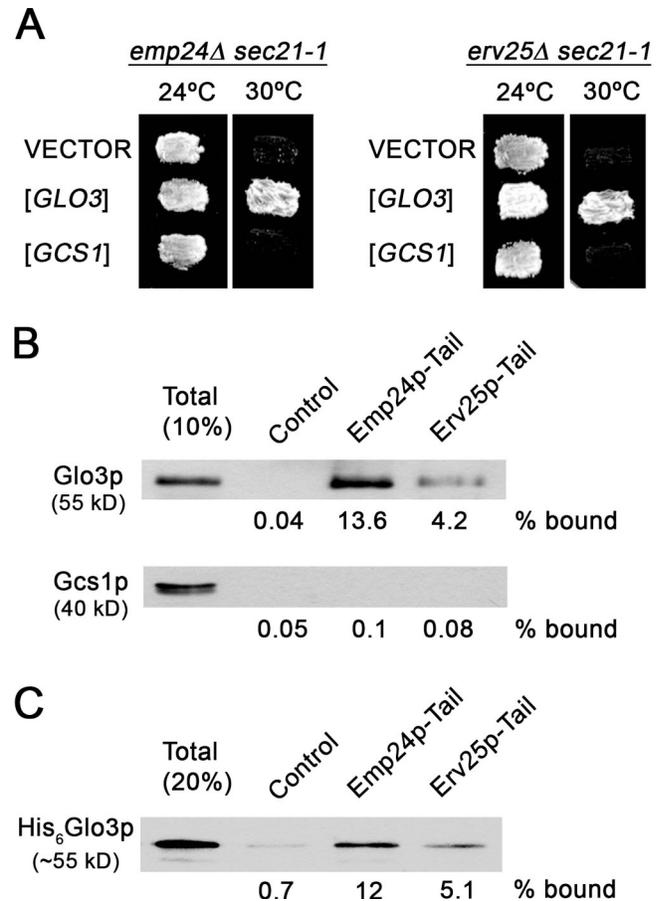
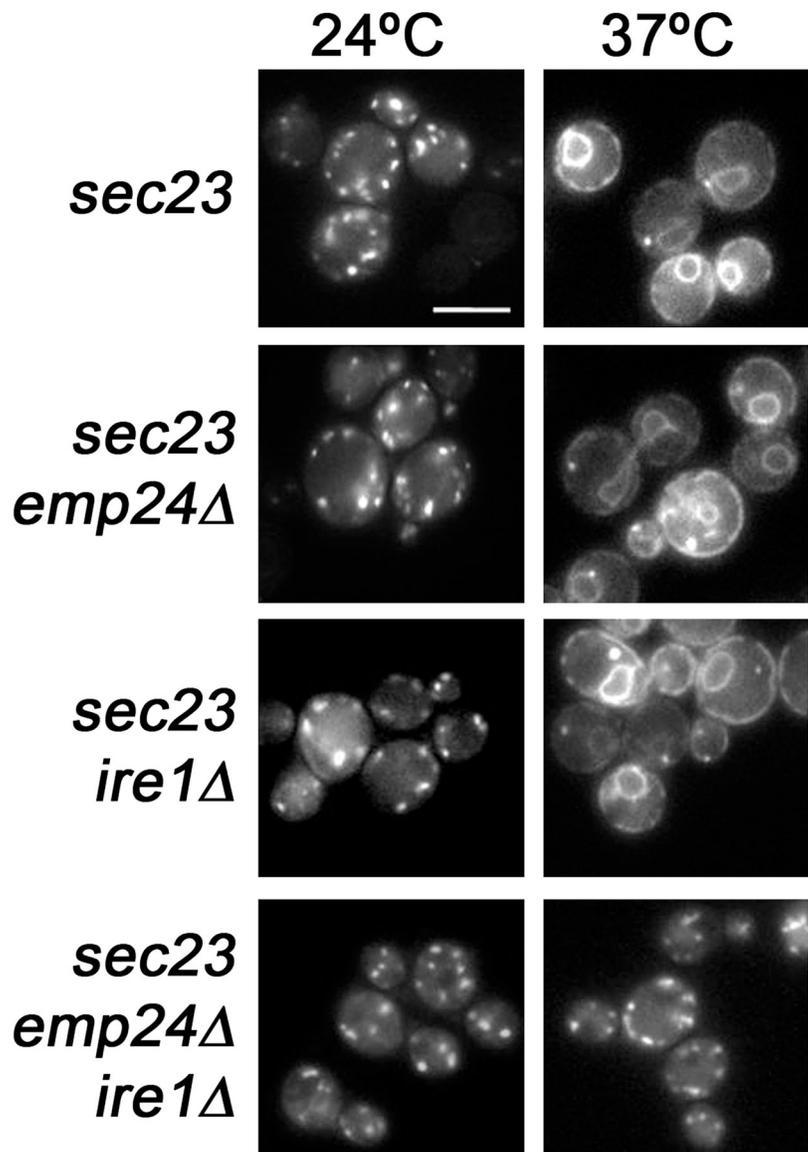


Figure 4. Specific genetic and physical interactions between p24 members and COPI coat components. (A) The *emp24Δ sec21-1* and *erv25Δ sec21-1* cells were transformed with empty plasmid (YEp352) or a high-copy plasmid carrying either the *GLO3* (pPPL43) or the *GCS1* gene (pPP421). Transformed cells were tested for growth at 24 and 30°C. (B and C) Synthetic peptides corresponding to cytoplasmic domains of Emp24p (RRFFEVTSLV) and Erv25p (KNYFKTKHII) were coupled to thiopropyl-Sepharose beads and incubated with cytosol (B) or recombinant Glo3p (C). Bound material was resolved by SDS-PAGE and analyzed by immunoblot using antibodies against Glo3p and Gcs1p.

and *IRE1*, an ER-localized kinase that activates the UPR, results in slow growth and more severe transport defects (Belden and Barlowe, 2001a). Accordingly, a genetic interaction between *emp24Δ* and *hac1Δ*, a transcriptional activator for a set of UPR-regulated genes, was observed during the genetic screen (unpublished data). We wanted to test directly whether UPR activation is responsible for the lack of an obvious retrograde trafficking defect in the *emp24Δ* strain. We assessed this possibility by determining whether the Golgi protein Rer1p, which cycles through the ER (Sato et al., 1997), is efficiently recycled back to the ER in an *emp24Δ ire1Δ* strain. We blocked ER exit with the temperature-sensitive *sec23-1* (COPII) allele to trap cycling proteins in the ER (Sato et al., 1997). If retrograde transport of Rer1p-GFP depends on both the p24 complex and on UPR, an *emp24Δ ire1Δ sec23-1* triple mutant strain should not accumulate Rer1p in the ER upon shift to the restrictive temperature (37°C). As shown in Fig. 5, in *sec23-1*, *emp24Δ sec23-1*, and *ire1Δ sec23-1* mutant cells at 24°C, Rer1p-GFP showed punctuate staining typical for Golgi in yeast, whereas at 37°C, the ER-characteristic nuclear

Figure 5. **UPR compensates the loss of retrograde transport function in the absence of p24 proteins.** Cells expressing Rer1p-GFP were observed by fluorescence microscopy at 24°C, or after the shift to 37°C for 20 min. Bar, 5 μ m.



ring staining was observed. In contrast, when Rer1p-GFP was expressed in *emp24Δ ire1Δ sec23-1* triple mutant cells at 37°C, no ER staining was observed and Rer1-GFP remained in the Golgi (Fig. 5). This result demonstrates that retrograde traffic is affected in the *emp24Δ ire1Δ sec23-1* mutant cells at 37°C, indicating that UPR can compensate for the loss of p24 function in retrograde transport from Golgi to the ER. This compensatory effect could rely on the control that the UPR exerts over many aspects of secretory function, including retrograde transport. Indeed, genes involved in Golgi retrieval, such as genes encoding for several coatamer subunits (i.e., SEC27) are up-regulated by the UPR (Travers et al., 2000). Because the p24 complex requirement for budding is overcome by increasing the amount of coatamer, it remains possible that the up-regulation of the COPI genes helps the cell to cope with the loss of the p24 proteins. In addition, it is also possible that activity of COPI is up-regulated post-translationally by the UPR, for instance by phosphorylation. Indeed, the phosphorylation of several coatamer subunits has been reported (Sheff et al., 1996).

This study further supports the notion that certain transmembrane cargoes, such as the p24 protein family members, might be essential components in the regulation of vesicle formation instead of simply being a passive traveler. p24 proteins could recruit initially the deactivated form of Arf1p, (Gommel et al., 2001) facilitating the formation of a COPI priming complex and subsequently enabling efficient budding. In addition, the ability of p24 proteins to oligomerize and to present multiple coatamer-binding motifs might promote COPI budding by docking coatamer more firmly to the membrane (Bethune et al., 2006). Overexpression of coatamer-binding tails in oligomeric complexes has been shown to overcome the lethality of *sec21-3* at the nonpermissive temperature presumably by stabilizing coatamer on the Golgi (Sandmann et al., 2003). Moreover, p24 complex might play a key role in the stabilization of priming complexes by modulating the ArfGAP activity (Goldberg, 2000; Lanoix et al., 2001). Thus, other cargo proteins with lower coatamer affinity, like the single tail exposing Emp47p, could be captured by stabilized priming complexes and then be sorted into COPI vesicles.

Materials and methods

Yeast strains and growth conditions

Strains of *Saccharomyces cerevisiae* used for this work are listed in Table S1 (available at <http://www.jcb.org/cgi/content/full/jcb.200710025/DC1>). Standard genetic manipulations were used throughout (Sherman, 1991). Strain RH6148 was made by replacement of *EMP24* ORF by natMX (Goldstein and McCusker, 1999) disruption cassette in the Y2922 background. Strain MMY104 was obtained by replacing the entire *IRE1* coding sequence of RH1433 with a *HphMX* disruption cassette (Goldstein and McCusker, 1999). Strains MMY103 and MMY91 were constructed by crossing MMY104 with RH4443. Strain MMY369 was constructed by crossing RH441 with PC238. Strains MMY409, MMY410, MMY419, and MMY518 were constructed by crossing MMY369 and RH1469 with BGY418. Cells were grown in either rich medium YPUAD (1% yeast extract, 2% peptone, 2% glucose, and 40 mg/ml each of adenine and uracil) or minimal medium SD (0.67% yeast nitrogen base without amino acids, 2% glucose, and the required nutrients).

Synthetic lethal screen

Synthetic lethal screen was performed as described by Tong et al. (2001), using an *emp24Δ* mutant (RH6148) as a bait strain. The yeast knockout collection (YKO) and wild-type strain Y2922 were provided by C. Boone (University of Toronto, Ontario, Canada; Tong et al., 2001). In total, 72 double-deletion combinations were found that resulted in a potential synthetic growth defect.

Plasmids

To generate pMM63 and pMM67, the *SacI*-*SmaI* fragment from pNY24-3HA-GLO3 (Yahara et al., 2006) containing the *GLO3* gene was subcloned in the vectors pRS416 and pRS315, respectively. Other plasmids used in this study are pSKY5/RER1-0 (a CEN-based *URA3* plasmid carrying Rer1-GFP; Sato et al., 1997), pPPL43 and pPP421 (a 2 μ -based *URA3* plasmid with *GLO3* and *GCS1*, respectively; Poon et al., 1999), pJS209 (a 2 μ -based *URA3* plasmid with *ERD2*; Semenza et al., 1990), and pJC31 (a CEN-based *TRP1* plasmid with *UPRE1,2-CYC-lacZ*; (Cox and Walter, 1996).

Pulse-chase analysis of CPY and invertase

Radiolabeling and immunoprecipitations were performed as described by Sutterlin et al. (1998). For invertase secretion, cells were induced as described by Kübler et al. (1994) and internal and external invertase was assayed as described by Gaynor and Emr (1997).

Analysis of Kar2 secretion

Extracellular Kar2p secretion was analyzed as described by Belden and Barlowe (2001a).

β -Galactosidase assay

Assays of β -galactosidase activity in extracts of yeast cells containing the *UPRE-lacZ* fusion construct, pJC31, were performed as described by Cox and Walter (1996).

In vitro Golgi budding assay

Golgi membranes and COPI components were purified as described by Spang and Schekman (1998). The Golgi budding assay was performed as described by Lewis et al. (2004) with several modifications. For the Golgi budding reactions, membranes were incubated in the presence of 0.1 mM GTP without (coat-limited conditions) or with an excess of coatomer (250 μ g/ml), and Arf1 protein (80 μ g/ml) at 30°C for 30 min in a total volume of 180 μ l. Samples were loaded on a FicolI-sucrose gradient consisting of 135 μ l of 60% (wt/vol) sucrose, 360 μ l of 7.5%, 450 μ l of 5, 4, and 3%, and 360 μ l of 2% (wt/wt) FicolI in 15% (wt/vol) sucrose in 20 mM Hepes, pH 6.8, 5 mM Mg(OAc)₂, and 150 mM KOAc (B88*). The vesicles were separated from the Golgi apparatus by centrifugation for 90 min at 42,000 rpm (TLS55 rotor; Beckman Coulter). 540 μ l from the top was discarded and the next 540 μ l were mixed with an equal volume of 80% Nycodenz in B88*, and overlaid with 276 μ l of 35, 25, 20, and 15% and 184 μ l of 10% Nycodenz in B88*. The gradients were centrifuged for 16 h at 50,000 rpm (TLS55 rotor; Beckman Coulter). Fractions (150 μ l) were collected from the top. Vesicle-containing fractions (5, 6, 7) were collected and pooled, TCA precipitated, resolved on SDS-PAGE, and analyzed by immunoblot.

Pull-down assay

Synthetic peptides corresponding to the 10 C-terminal amino acids of Emp24p (RRFFEVTSLV) and Erv25p (KNYFKTKHII) with an N-terminal

cysteine residue were generated (JPT Peptide Technologies) and linked to thiopropyl-Sepharose 6B (GE Healthcare) as described by Belden and Barlowe (2001b). Cytosol from a *pep4Δ* strain (RH732) was obtained as described by Muñiz et al. (2000). Recombinant Glo3p was purified as described by Rein et al., (2002). In vitro binding reactions were performed as described by Belden and Barlowe (2001b). The immunoblot was quantified by densitometry.

Light microscopy

For fluorescence microscopy of GFP-tagged strains, log-phase cells grown in minimal media were observed directly. Acquisition was performed at 24°C using a Leica DMR microscope equipped with an objective lens (HCX PL APO 63x/1.32 OIL Ph3), a DC 350F camera, and Image Manager 50 v1.20 following the instructions of the manufacturer.

Online supplemental material

Table S1 provides information about the yeast strains used during this study. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200710025/DC1>.

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