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Article

# COPII Coat Composition Is Actively Regulated by Luminal Cargo Maturation

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## Summary

**Background:** Export from the ER is an essential process driven by the COPII coat, which forms vesicles at ER exit sites (ERESs) to transport mature secretory proteins to the Golgi. Although the basic mechanism of COPII assembly is known, how COPII machinery is regulated to meet varying cellular secretory demands is unclear.

**Results:** Here, we report a specialized COPII system that is actively recruited by luminal cargo maturation. Glycosylphosphatidylinositol-anchored proteins (GPI-APs) are luminal secretory proteins anchored to the membrane by the glycolipid GPI. After protein attachment in the ER lumen, lipid and glycan parts of the GPI anchor are remodeled. In yeast, GPI-lipid remodeling concentrates GPI-APs into specific ERESs. We found that GPI-glycan remodeling induces subsequent recruitment of the specialized ER export machinery that enables vesicle formation from these specific ERESs. First, the transmembrane cargo receptor p24 complex binds GPI-APs as a lectin by recognizing the remodeled GPI-glycan. Binding of remodeled cargo induces the p24 complex to recruit the COPII subtype Lst1p, specifically required for GPI-AP ER export.

**Conclusions:** Our results show that COPII coat recruitment by cargo receptors is not constitutive but instead is actively regulated by binding of mature ligands. Therefore, we reveal a novel functional link between luminal cargo maturation and COPII vesicle budding, providing a mechanism to adjust specialized COPII vesicle production to the amount and quality of their luminal cargos that are ready for ER exit. This helps to understand how the ER export machinery adapts to different needs for luminal cargo secretion.

## Introduction

Eukaryotic secretory proteins are synthesized and inserted into the ER, where they undergo folding, assembly, and posttranslational modifications. Matured secretory proteins are then transported in lipid vesicles to the Golgi apparatus. These vesicles are generated by polymerization of cytosolic coat protein complex COPII, which locally bends the ER membrane at specific domains called ER exit sites (ERESs). For efficient ER export, most secretory proteins are concentrated at ERESs and packaged into nascent COPII-coated vesicles through interaction with the COPII cargo-binding subunit Sec24p. Active concentration into vesicles of those cargo molecules that cannot directly interact with Sec24p, like soluble secretory proteins, requires specialized transmembrane proteins, which act as adaptors or cargo receptors by linking cargo with the COPII coat. However, neither the precise mechanism by which cargo receptors select mature from immature secretory proteins nor how this selection is coupled to COPII vesicle biogenesis are well understood [1–3].

We have addressed these fundamental issues by further investigating how mature glycosylphosphatidylinositol-anchored proteins (GPI-APs) are efficiently exported from the ER. GPI-APs constitute a particular category of luminal secretory cargos, which contain a soluble protein attached by a conserved posttranslational glycolipid modification, the GPI anchor, to the external leaflet of the plasma membrane, where they perform diverse but important physiological functions [4]. The core structure of the GPI anchor precursor consists of a phospholipid moiety (acyl-phosphatidylinositol) with a glycan backbone (Man4-(EtNP)Man3-(EtNP)Man2-(EtNP)Man1-GlcN, where EtNP is ethanolamine-phosphate, Man is mannose, the numbers represent the positions of the Man in the anchor, and GlcN is glucosamine) and is made by a series of sequential reactions at the ER membrane. It is then added in the ER lumen by a GPI-transamidase to newly synthesized proteins containing a GPI attachment signal sequence at their C terminus. GPI-APs leave the ER in COPII vesicles and travel via the Golgi to the plasma membrane [5, 6].

The presence of the GPI anchor confers to the anchored proteins special trafficking features along the secretory pathway [7]. Indeed, in yeast, GPI-APs are segregated from other secretory proteins and concentrated into specific ERESs, from where they are subsequently incorporated into distinct COPII vesicles [5, 8]. Interestingly, this concentrative sorting of GPI-APs at ERESs is independent of the COPII machinery but requires a lipid-based mechanism implying the structural remodeling of the GPI anchor [8]. This process begins immediately after protein attachment to the GPI anchor and, in yeast, consists of the inositol deacylation by Bst1p followed by lipid remodeling, which involves the replacement of the original lipid moiety by another lipid, containing a very long saturated fatty acid, usually ceramide [9]. The acquisition by the GPI anchor of such long and saturated lipids has been proposed to lead to the association of GPI-APs with ceramide-enriched membrane domains that could finally sort them into specific ERESs [8].

Subsequent packaging of lipid-remodeled GPI-APs into COPII vesicles requires the p24 proteins [10–12], a class of abundant type I transmembrane proteins with a luminal domain and a short cytoplasmic tail that harbors both COPII and COPI coat-binding signals [13–17]. The p24 proteins assemble in a heteromeric complex that cycles between the ER and Golgi compartments [16, 18–21]. Experimental

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evidence from yeast and mammalian cell systems suggests a specific role of the p24 complex as a transmembrane adaptor or cargo receptor that could link remodeled GPI-APs to the cytosolic COPII coat, ensuring their selective incorporation into nascent COPII vesicles [10–12]. Nevertheless, the exact motif on GPI-APs recognized by the p24 complex and how this recognition is regulated and coupled to COPII coat assembly is still unknown. Previous studies have shown that GPI-APs depend on Lst1p, an isoform of the COPII cargo-binding subunit Sec24p, for specific capture into vesicles that are slightly larger than conventional COPII vesicles [22–24]. Because the p24 proteins can also bind Lst1p in addition to Sec24p, it has been suggested that the p24 complex might connect GPI-APs to Lst1p, although this hypothesis remains to be experimentally addressed [25].

Previous studies in mammalian cells have revealed additional specific requirements for ER export of GPI-APs. In addition to the inositol deacylation, the glycan portion of the GPI anchor undergoes another remodeling process prior to ER exit [26]. The initial side-chain EtNP on the second mannose of the glycan portion is removed by a specific phosphodiesterase (PGAP5). Here, we have identified Ted1p as the functional ortholog of mammalian PGAP5 in yeast and provide for the first time direct evidence suggesting that, once remodeled by Ted1p, the glycan structure of the GPI anchor is physically recognized by the p24 complex, which subsequently connects remodeled GPI-APs with Lst1p, but not with Sec24p. Moreover, we found that recognition of remodeled GPI-APs stimulates the p24 complex to preferentially bind Lst1p, suggesting an active role of GPI anchor remodeling in the formation of the specialized COPII coat. Therefore, our findings reveal a novel functional link between cargo maturation and COPII vesicle budding, providing a mechanism by which COPII vesicle production is adjusted to the amount of transport-competent proteins generated.

## Results

### Ted1p Is the Yeast Functional Ortholog of the Mammalian GPI-Glycan Remodelase PGAP5

In mammalian cells, it has been recently reported that, after protein attachment, the glycan structure of the GPI anchor undergoes a remodeling process prior to ER exit, consisting of the removal of the side-chain EtNP placed at the second mannose of the GPI-glycan, carried out by an ER phosphoesterase, PGAP5 [26]. Importantly, ER-to-Golgi transport of mammalian GPI-APs in *pgap5* mutant cells is impaired, indicating that the presence of this specific side-chain EtNP on the GPI-glycan prevents the efficient ER export of GPI-APs. In yeast, there are two potential functional homologs of PGAP5, Ted1p and Cdc1p, which contain a conserved phosphoesterase domain and are also localized at the ER [26]. A previous study has shown that Ted1p is required for the efficient ER-to-Golgi transport of GPI-APs [27]. Furthermore, we observed that, like in the case of PGAP5, this transport requirement is specific for GPI-APs (Figure S1 available online) and also depends on the phosphoesterase activity of Ted1p because expression of Ted1p with mutation in the conserved phosphoesterase active site caused accumulation of the ER form of the GPI-AP Gas1p (Figure 1A). Moreover, Ted1p was partially localized at ERESs (Figure S2) similarly to PGAP5 in the mammalian system [26].

We next tested whether Ted1p performs the same GPI-glycan-remodeling reaction as PGAP5 by specifically removing the side-chain EtNP from the second mannose on the glycan backbone of the GPI anchor. In yeast, this specific EtNP is added to the GPI-glycan by the enzyme Gpi7p during GPI anchor precursor biosynthesis before GPI attachment to the protein [28]. Therefore, if the EtNP added by Gpi7p is specifically removed by Ted1p, the *gpi7Δ* mutation should then be epistatic to the *ted1Δ* mutation. Consistent with this, we observed that the *gpi7Δ* mutation suppresses the GPI-AP ER export defect caused by the *ted1Δ* mutation. The ER form of Gas1p detected in *ted1Δ* mutant cells was no longer accumulated in *gpi7Δ ted1Δ* double mutant cells (Figure 1B). Similarly, the GPI-AP Cwp2p was retained in the ER in *ted1Δ* mutant cells as judged by the ER-characteristic nuclear ring staining, whereas no ER staining was observed in *gpi7Δ ted1Δ* double mutant cells (Figures 1C and 1D). Moreover, accumulation of the ER form of Gas1p in *ted1Δ* mutant cells was significantly diminished by expression of human PGAP5 cDNA (Figure 1E). Altogether these results strongly indicate that Ted1p functions as the yeast ortholog of the mammalian PGAP5 by removing a specific EtNP on GPI-glycan (Figure 1F). By contrast, the other yeast PGAP5 homolog *CDC1* seems not to be involved in GPI-AP trafficking, because the *cdc1* decreased abundance by mRNA perturbation mutant cells didn't show any obvious Gas1p transport defect and the *gpi7Δ* mutation does not restore their growth defect (Figure S3). Consistent with our data, it has been shown recently that Cdc1p is a phosphoesterase that removes specifically the side-chain EtNP placed at the first mannose of the GPI-glycan, which is not important for trafficking but facilitates the integration of GPI-APs into the yeast cell wall [29].

### Ted1p Activity Is Not Required for GPI-AP Concentration at ERES

Once established that Ted1p acts as a GPI-glycan remodelase, we next investigated why GPI-glycan remodeling is required for efficient ER export of GPI-APs. GPI-lipid remodeling has been shown to be necessary for ER exit of GPI-APs because it drives their concentration at ERESs [8]. Therefore, we then addressed whether the export function of Ted1p is also to facilitate this concentrative process (Figures 2A and 2B). Cargo concentration at ERESs can be observed in yeast under fluorescence microscope by blocking the COPII vesicle budding with the temperature-sensitive *sec31-1* (COPII) allele, as previously established [8]. In *sec31-1* mutant cells at 37°C, Cwp2-Venus showed punctate staining that corresponds to its accumulation into ERESs [8]. As a negative control, we used the disruption of *BST1*, which inactivates the lipid-remodeling pathway and thus blocks GPI-AP concentration at ERESs. As previously shown, in *bst1Δ sec31-1* double mutant cells, Cwp2-Venus is not accumulated in ERESs at 37°C, displaying a typical ER nuclear ring staining [8]. However, the same punctuate pattern observed in *sec31-1* mutant cells was also reproduced in the *ted1Δ sec31-1* strain incubated at 37°C. We confirmed that these dot-like structures containing Cwp2p are ERESs by colocalization with the ERES marker Sec13p (Figures S4A and S4B). These data show that GPI-AP concentration at ERESs does not require GPI-glycan remodeling by Ted1p. Consistently, Ted1p activity is also not required for GPI-lipid remodeling because the GPI anchors of the *ted1Δ* strain contained the same remodeled GPI-lipid species as in wild-type strain (Figure 2C). Therefore, we conclude that GPI-lipid remodeling, but not GPI-glycan remodeling, drives GPI-AP concentration at ERESs (Figure 2D), suggesting that Ted1p function in ER export must take place at a step after ERES formation. But what could be then this later function?

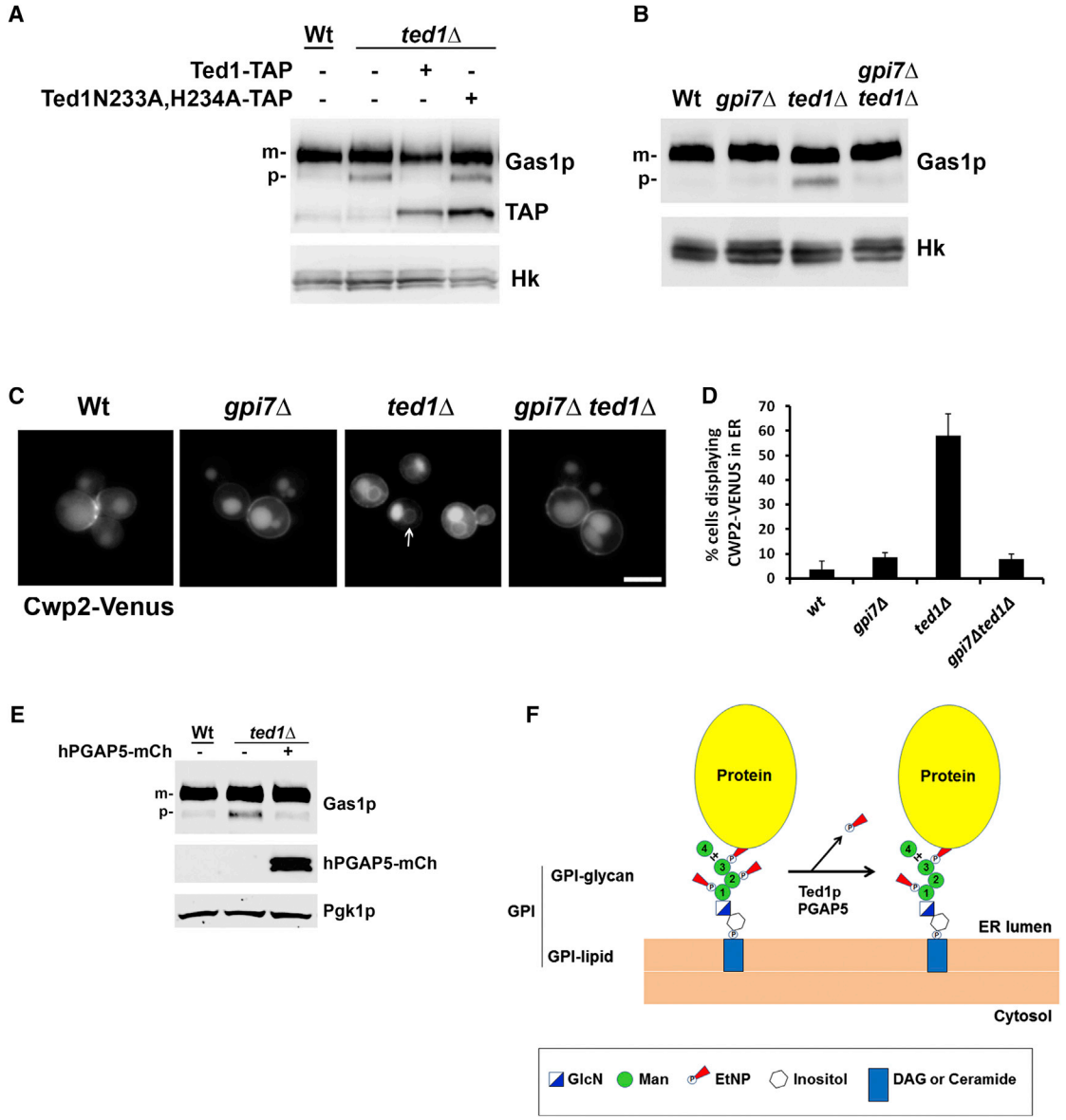


Figure 1. Ted1p Is the Yeast Functional Ortholog of the Mammalian GPI-Glycan Remodelase PGAP5

(A) Efficient ER-to-Golgi transport of GPI-APs requires Ted1p phosphoesterase activity. Expression of catalytic dead mutant Ted1p causes accumulation of the ER form of the GPI-AP Gas1p. Extracts prepared from wild-type cells and *ted1Δ* mutant cells transformed with an empty vector, a TAP-tagged *TED1*, or TAP-tagged *ted1*N233A,H234A mutant allele were analyzed by western blot for Gas1p and hexokinase (loading control). ER (p) and Golgi (m) Gas1p forms are indicated.

(B–D) *gpi7Δ* mutation suppresses the GPI-AP ER export defect caused by the *ted1Δ* mutation. (B) The ER form of Gas1p detected in *ted1Δ* mutant cells is no longer accumulated in *gpi7Δ ted1Δ* double mutant cells. Western blot analysis for Gas1p and hexokinase (loading control) of cell extracts prepared from wild-type and deletion strains. (C) The ER-characteristic nuclear ring staining (white arrow) showed by the GPI-AP Cwp2-Venus in *ted1Δ* mutant cells indicating ER retention is not observed in *gpi7Δ ted1Δ* double mutant cells. Live images of wild-type and deletion strains expressing Cwp2-Venus at 24°C. The scale bar represents 5 μm. (D) Quantification of several micrographs described in (C). The graph plots the average percentage of cells displaying Cwp2-Venus in the ER. $n \geq 100$. Error bars indicate the SD.

(E) Expression of human PGAP5-mCherry cDNA complements the GPI-AP transport-defective phenotype of the *ted1Δ* mutant strain. Western blot analysis for Gas1p and Pgk1p (loading control) of cell extracts prepared from wild-type and *ted1Δ* mutant strains.

(F) Schematic representation of the GPI-glycan remodeling by the ER phosphoesterase PGAP5/Ted1p.

See also Figures S1–S3.

## The Cargo Receptor p24 Complex Acts as a Lectin for the Remodeled GPI-Glycan

We have previously established that the ER export of GPI-APs comprises at least two consecutive steps [10, 11]. First, GPI-APs are sorted and concentrated at their specific ERESs upon GPI-lipid remodeling. In a second step, the p24 complex recognizes lipid-remodeled GPI-APs to package them into COPII vesicles. Because Ted1p, like the p24 complex [10], is not involved in the first step of GPI-AP concentration at ERESs, it could be then required for the subsequent step of GPI-AP recognition by the p24 complex. Indeed, Ted1p was functionally related with the p24 proteins in a previous genetic screen

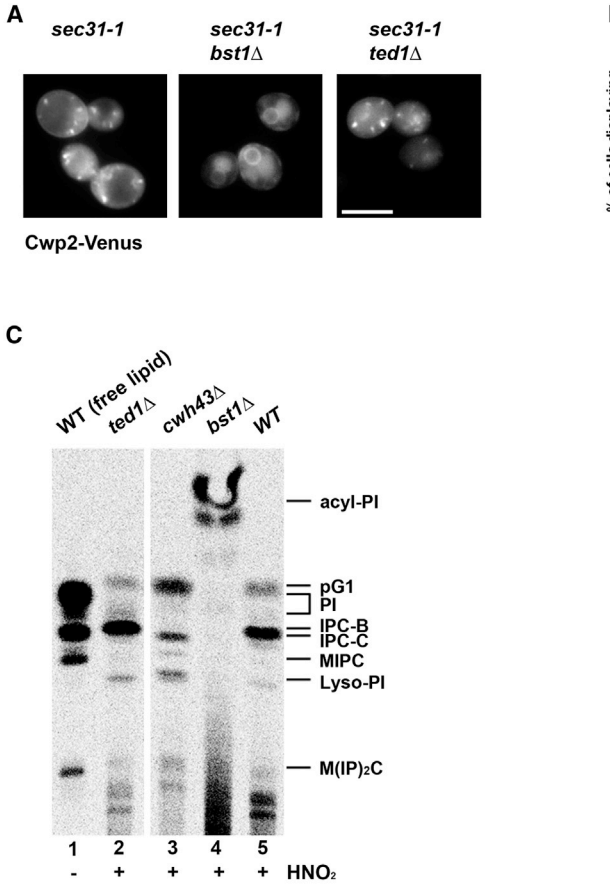


Figure 2. Ted1p Is Not Required for Concentration of GPI-AP Cargo into ERESs

(A) Fluorescent micrographs of live *sec31-1*, *sec31-1 bst1Δ*, and *sec31-1 ted1Δ* cells expressing Cwp2-Venus at 37°C. The scale bar represents 5 μm. Raw images.

(B) Quantification of several micrographs described in (A). The graph plots the average percentage of the cells, for which Cwp2-Venus is found in dot-like structures. n, number of cells plotted; $72 \leq n \leq 86$. Error bars indicate the SD.

(C) Lipid remodeling of the GPI anchor is normal in *ted1Δ* cells. Wild-type, *ted1Δ*, *bst1Δ*, and *cwh43Δ* strains were labeled with [3H] myo-inositol for 2 hr at 25°C. The labeled PI moieties were prepared from GPI-APs and analyzed by thin-layer chromatography using the solvent system (55:45:10 chloroform-methanol-0.25% KCl). Lipids extracted from wild-type cells (lane 1) were used as a standard. IPC-B, inositolphosphorylceramide consisting of 4-hydroxysphinganine and a C26:0 fatty acid; IPC-C, inositolphosphorylceramide consisting of 4-hydroxysphinganine and a hydroxylated C26:0 fatty acid; lyso-PI, lyso-phosphatidylinositol; MIPC, mannosylinositolphosphorylceramide; M(IP)2C, mannosyldi(inositolphosphoryl) ceramide; pG1, phosphatidylinositol with a C26:0 fatty acid in sn-2 position; PI, phosphatidylinositol [30, 31].

(D) Export functions of GPI-anchor-remodeling pathways in yeast.

See also Figure S4.

[27]. Therefore, we examined a possible correlation between Ted1p activity and the cargo receptor role of the p24 complex. For this purpose, we addressed whether the phosphoesterase activity of Ted1p is required for the GPI-AP recognition by the p24 complex. As seen in Figure 3A, the p24 protein Emp24p can be efficiently coimmunoprecipitated with Cwp2-Venus in wild-type cells. This physical interaction was specific because Erv29p, an unrelated cargo receptor for soluble secretory proteins, was not recovered after Cwp2-Venus immunoprecipitation (IP). Nevertheless, we found that, in *ted1Δ* mutant cells expressing the phosphoesterase-inactive Ted1p, Emp24p could not be coprecipitated with the GPI-AP Cwp2-Venus, whereas expression of wild-type Ted1p allowed the association of Emp24p with Cwp2-Venus (Figure 3B). These findings indicate that removal of the EtNP by Ted1p regulates the recognition of GPI-APs by the p24 complex, which is consistent with the previous observation that in mammalian cells the *pgap5* mutation also reduces the interaction of p24 proteins with GPI-APs [12]. However, these observations cannot rule out an indirect effect of the GPI-glycan remodelase inactivation on p24 binding. A direct effect would imply that the presence of the EtNP directly prevents the binding of the p24 complex to GPI-APs. If this hypothesis is correct, the loss of function of Gpi7p should lead to the production of GPI anchors without the EtNP on the second mannose, which would allow the binding of the p24 complex to the GPI-AP even in the absence of Ted1p. We tested this hypothesis using our coimmunoprecipitation (coIP) assay. As seen in Figure 3C, in the *gpi7Δ* mutant strain, Emp24p was coprecipitated with Cwp2-Venus, although slightly less efficiently than in the wild-type (WT) strain. By contrast, in the *ted1Δ* mutant strain, it was not coprecipitated at all. This binding defect was rescued by the *gpi7Δ* mutation because Emp24p and Cwp2-Venus coprecipitated in the *gpi7Δ ted1Δ* double mutant strain with the same efficiency as in the *gpi7Δ* mutant strain. This result shows that the EtNP physically hinders the efficient recognition of GPI-APs by the p24 complex, which therefore only occurs after Ted1p removes that EtNP.

Taken together, these data open the possibility that the p24 proteins interact physically with the glycan portion of the GPI anchor after removal of the side-chain EtNP. Indeed, previous homology modeling of the luminal domains of p24 proteins has shown that they assume a structure often observed in sugar-binding/processing proteins [12, 32]. We directly addressed whether the p24 proteins physically recognize the GPI-glycan structure by using synthetic GPI-glycans in an in vitro binding assay. The glycan portion of the GPI anchor without side-chain EtNPs ($Man_4$-$Man_3$-$Man_2$-$Man_1$-GlcN-InoP) can be chemically synthesized and coupled to a matrix, which allows pull-down experiments [33]. Here, we directly tested the ability of several

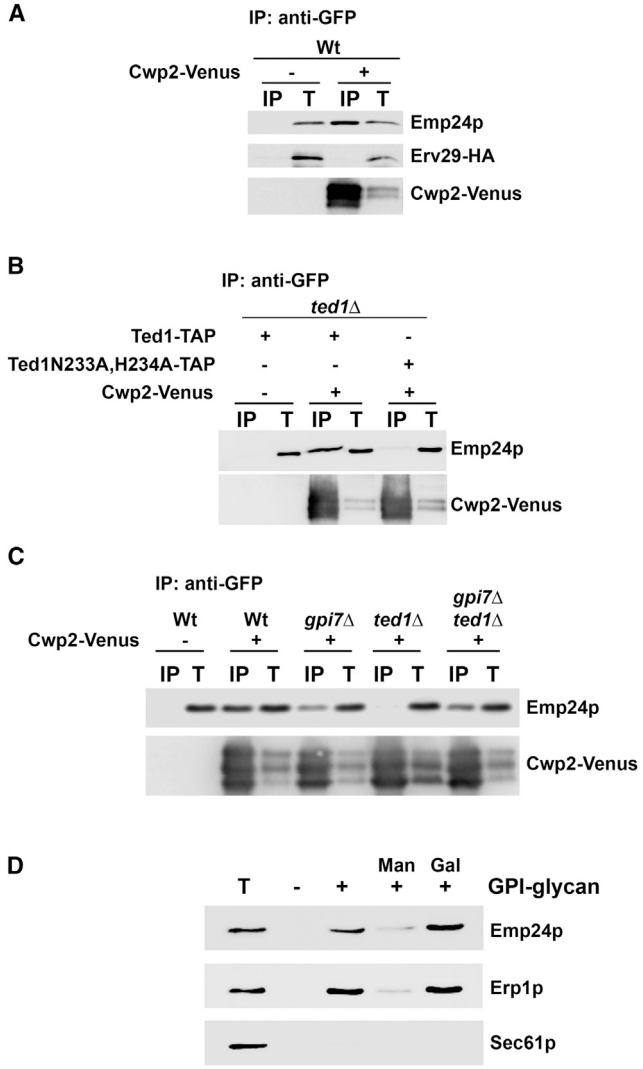


Figure 3. The Cargo Receptor p24 Complex Acts as a Lectin for Remodeled GPI-Glycans

(A–C) CoIP assay between Cwp2-Venus and the p24 complex subunit Emp24p or Erv29-HA. (A) Emp24p specifically binds Cwp2-Venus. (B) Ted1p activity regulates GPI-AP recognition by p24 proteins. (C) The EtNP on the second mannose of the GPI-glycan prevents the p24 binding to GPI-APs. Enriched ER fractions of wild-type and deletion strains expressing indicated tagged proteins were processed for IP as in Figure 2.

(D) The p24 proteins specifically recognize remodeled GPI-glycans in vitro. Synthetic glycans corresponding to mature GPI-glycans were coupled to agarose beads and incubated with wild-type cell extracts in the absence or presence of 300 mM of mannose and galactose. Bound material was resolved by SDS-PAGE and analyzed by immunoblotting using antibodies against Emp24p, Erp1p, and Sec61p. Total (T) represents a fraction of the solubilized input material.

p24 proteins, including Emp24p and Erp1p, to specifically interact with the remodeled form of GPI glycans without side-chain EtNPs by performing a pull-down assay after incubating the GPI glycan matrix with a solubilized cell extract. Both p24 proteins, Emp24p and Erp1p, were efficiently coprecipitated with the mature GPI glycan (Figure 3D). This association was specific because an unrelated protein such as the translocon subunit Sec61p was not recovered after the pull-down. A characteristic of lectin interaction with complex glycans is that they can be competed specifically with sugars that comprise part of the recognition site. Therefore, we performed competition experiments to determine whether the p24 proteins display affinity for the mannoses of the GPI-glycan. As shown in Figure 3D, the p24 protein binding to the GPI glycan was strongly reduced by the presence of mannose, but not by other sugars like galactose, which is not a component of the GPI-glycan. Therefore, these results provide direct evidence that the p24 complex functions as a lectin by specifically recognizing the mature GPI glycan.

## Cargo Binding Stimulates the p24 Complex to Specifically Recruit the COPII Subtype Lst1p

We have shown in the previous paragraph that the remodeled glycan structure of the GPI anchor is specifically recognized by the p24 complex. Next, we investigated how this recognition is coupled to the COPII coat assembly for ER export of GPI-APs. COPII coat assembly is initiated by the formation of a prebudding complex that comprises the small guanosine triphosphatase (GTPase) Sar1p, the dimer Sec24p/Sec23p, and a transmembrane cargo molecule specifically recognized by Sec24p [22, 34]. Subsequent recognition and polymerization of prebudding complexes by the outer layer of the COPII coat Sec31p/Sec13p leads to COPII coat assembly and vesicle budding [30, 35]. Sec24p presents alternative isoforms or paralogs such as Lst1p, which has been shown to be required in vitro for the efficient capture of GPI-APs into COPII vesicles [22]. Interestingly, the cytosolic tail of the p24 proteins can interact with both Sec24p and Lst1p to form different types of prebudding complexes [23]. Furthermore, we have previously shown that disruption of the specific p24-binding sites on Lst1p impairs the ER-to-Golgi transport of GPI-APs [10]. Taken together, these data suggest the idea that the p24 complex might connect Lst1p in the cytosol with GPI-APs in the ER lumen to form specific prebudding complexes that ensure GPI-AP packaging into COPII vesicles. We directly addressed this hypothesis in vivo by quantifying, using crosslinking analysis, the extent of association between Lst1p and GPI-APs in wild-type and *emp24Δ* cells. As seen in Figure 4A, Lst1-mCherry could be efficiently crosslinked to Cwp2-Venus in wild-type, but not in the *emp24Δ* mutant strain. This association was specific because Sec24p was not crosslinked to Cwp2-Venus. These results provide direct evidence that the role of the p24 complex in the ER export of GPI-APs is to specifically link them with Lst1p, but not with Sec24p, the major COPII cargo-binding subunit. Moreover, because GPI-glycan remodeling regulates p24-complex-binding to GPI-APs, this specific interconnection with Lst1p must also be dependent on Ted1p. Indeed, we found that the p24 complex is unable to connect Lst1p with GPI-APs in the *ted1Δ* mutant strain (Figure 4A). As expected, this defect can be rescued by the *gpi7Δ* mutation (Figure S5). These results suggest a mechanism to ensure that only correctly remodeled GPI-APs can be connected by the p24 complex to Lst1p for ER export in specialized COPII vesicles. In agreement with this model, we found that Lst1p is not involved in concentration of GPI-APs at ERESs (Figures 4B and 4C), suggesting that specialized prebudding complexes containing Lst1p are only formed at a late stage of COPII vesicle budding.

Next, we investigated the sequence of interactions among GPI-APs, the p24 complex, and Lst1p that leads to the formation of the prebudding complex. For this purpose, we analyzed whether the p24 complex is still able to recognize GPI-APs in the absence of Lst1p. As shown in Figures 4D and 4E,

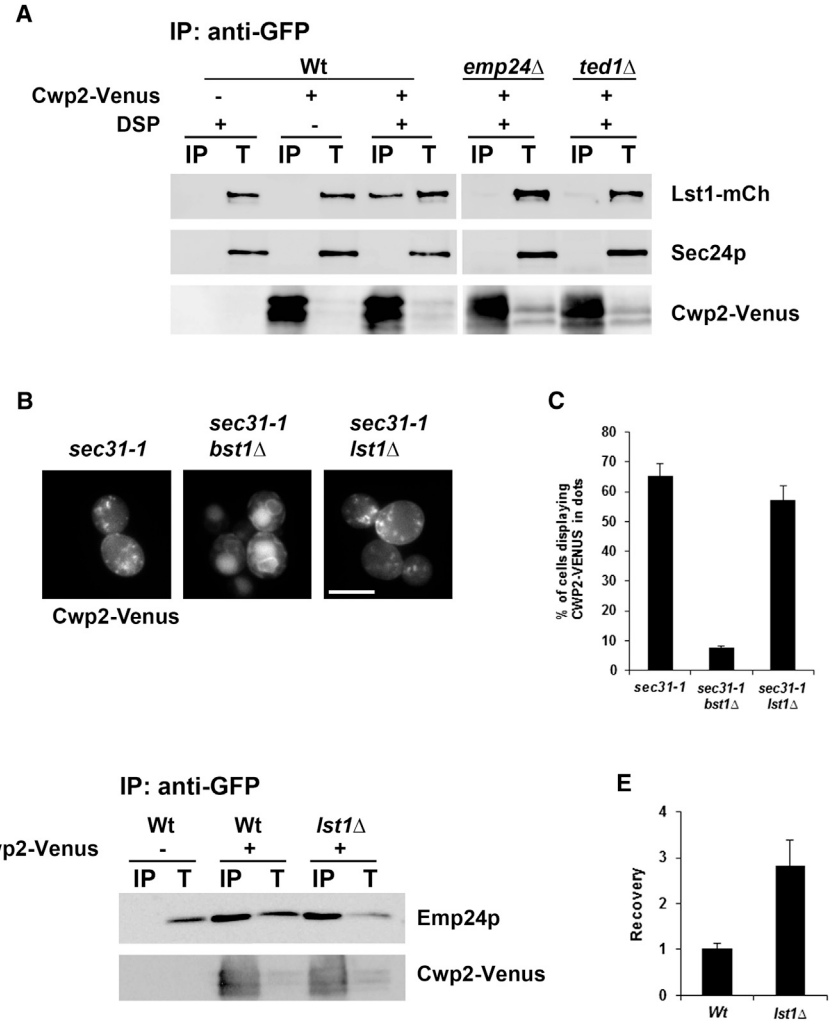


Figure 4. The p24 Complex Forms Specialized Prebudding Complexes by Binding First to Remodeled GPI-APs and Then to the Specific COPII Subunit Lst1p

(A) The p24 complex connects remodeled GPI-APs preferentially with the specific COPII subunit Lst1p, but not with Sec24p. Crosslinking assay between Cwp2-Venus and Lst1-mCherry or Sec24p. Extracts of wild-type and deletion strains expressing Cwp2-Venus and Lst1m-Cherry were incubated with (+) and without (−) DSP, solubilized, and immunoprecipitated with anti-GFP antibody, followed by IB with anti-mCherry or anti-Sec24p antibodies. T represent a fraction of the solubilized input material.

(B) Lst1p is not required for GPI-AP cargo concentration into ERESs. Fluorescent micrographs of live *sec31-1*, *sec31-1 bst1Δ*, and *sec31-1 lst1Δ* cells expressing Cwp2-Venus at 37°C. The scale bar represents 5 μm. Raw images.

(C) Quantification of several micrographs described in (B). The graph plots the average percentage of the cells, for which Cwp2-Venus is found in dot-like structures. $72 \leq n \leq 86$. Error bars indicate the SD.

(D) Lst1p is not required for GPI-AP recognition by the p24 complex. CoIP assay between Cwp2-Venus and Emp24p. Enriched ER fractions of wild-type and *lst1Δ* strains expressing Cwp2-Venus were processed for IP as in Figure 2.

(E) Quantification of three experiments described in (D). The graph plots the average percentage of the recovery of Emp24p in *lst1Δ* mutant strain normalized to the recovery in the wild-type strain. Error bars indicate the SD.

See also Figure S5.

Emp24p was even more efficiently coprecipitated with Cwp2-Venus in the null mutant *lst1Δ* than in the wild-type strain. Therefore, GPI-AP recognition by the p24 complex does not require the COPII coat binding, suggesting that p24 interaction with GPI-APs constitutes an intermediate stage in their ER export. Thus, the p24 complex interacts first with GPI-APs before recruiting Lst1p to form the prebudding complex for COPII budding.

This finding prompted us to study whether cargo binding influences the ability of the p24 complex to interact with Lst1p. We addressed this possibility by assessing the amount of Lst1p bound by the p24 complex in the absence or the presence of its cargo, the remodeled GPI-APs. We observed that in the *ted1Δ* mutant, in which the p24 receptor is not loaded with GPI-APs, Lst1p was significantly less crosslinked to Emp24p than in the wild-type strain (Figures 5A and 5B). Importantly, this defect is specific for Lst1p because the extent of Sec24p crosslinked to the p24 complex was not reduced in the *ted1Δ* mutant compared to the wild-type. We confirmed that the lack of GPI-AP cargo recognition directly caused the crosslinking defect between the p24 complex and Lst1p in *ted1Δ* mutant by showing that this defect can be rescued by the *gpi7Δ* mutation (Figures 5A and 5B). In addition, it is not the consequence of an altered p24 distribution because Emp24-CFP shows the same typical ER-Golgi localization in *ted1Δ* mutant cells as in wild-type cells (Figure 5C). Therefore, these results collectively show that binding of remodeled GPI-AP cargo stimulates the p24 complex to specifically interact with Lst1p and thus form prebudding complexes containing this Sec24p homolog.

Considering the great abundance of GPI-APs in yeast, the conclusion that GPI-AP cargo binding induces the formation of p24-Lst1p complexes leads to the possibility that it also might contribute to the overall recruitment of Lst1p to the ER membrane. To test this possibility, we analyzed by differential fractionation the subcellular distribution of Lst1p and Sec24p in wild-type and *ted1Δ* mutant strains. We found that Lst1p, but not Sec24p, was less associated to the ER membrane fraction in the *ted1Δ* mutant compared to the wild-type strain (Figures 5D and 5E). This result indicates that the p24 complex specifically recruits Lst1p to the ER membrane in response to cargo recognition. Because this recognition takes place in specific GPI-AP-containing ERESs, cargo-loaded p24 receptors should then recruit Lst1p to these specialized ER membrane domains. We addressed this issue by colocalization analysis. As mentioned above, to visualize GPI-AP cargo accumulated in ERESs, we have to block COPII vesicle budding by incubating the *sec31-1* mutant strain at restrictive temperature. Using this method, we could observe colocalization of Lst1p with concentrated Cwp2-Venus in GPI-AP containing ERESs (Figures 6A and 6B). However, this colocalization was dramatically diminished in *sec31-1 ted1Δ* mutant strain, showing that GPI-AP glycan remodeling is required for Lst1p

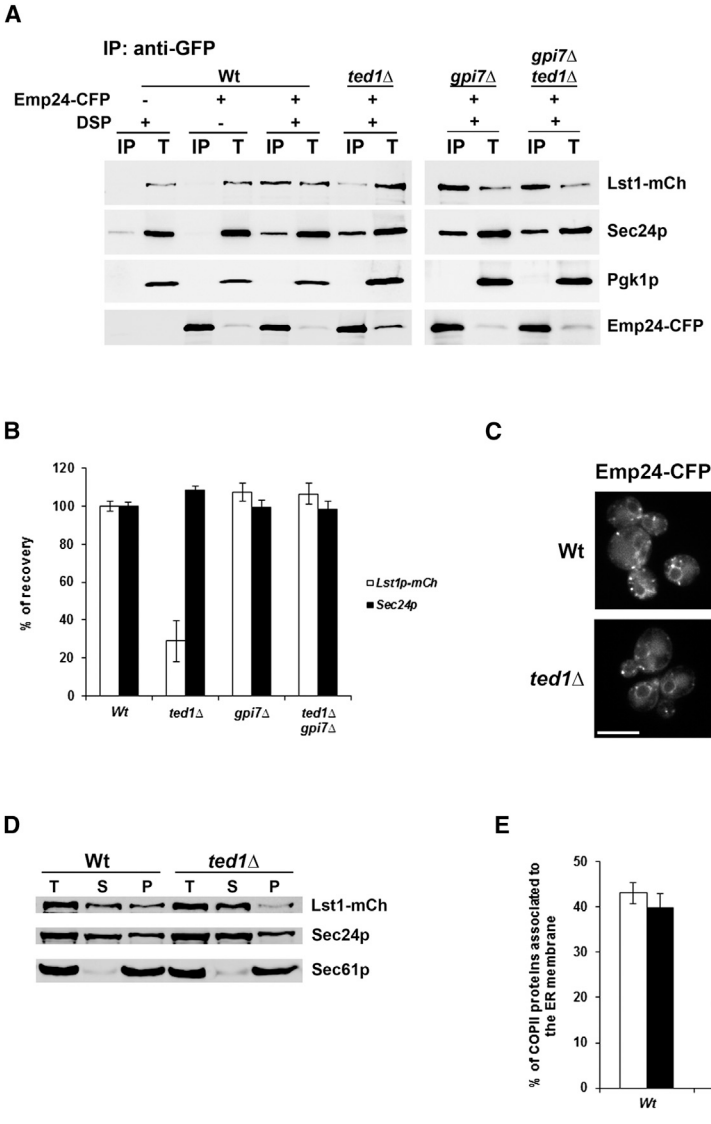


Figure 5. Cargo Binding to the p24 Complex Specifically Stimulates Lst1p Recruitment

(A) Crosslinking assay between Emp24-CFP and Lst1-mCherry or Sec24p. Extracts of wild-type and deletion strains expressing Emp24-GFP and Lst1-mCherry were processed for crosslinking and IP as in Figure 4A.

(B) Quantification of three experiments described in (A). The graph plots the average percentage of the recovery of Lst1p and Sec24p in different mutant strains normalized to the recovery in the wild-type strain. Error bars indicate the SD.

(C) Live images of wild-type and *ted1Δ* strains expressing Emp24-CFP at 24°C. The scale bar represents 5 μm.

(D) *ted1Δ* mutation reduces Lst1p association to the ER membranes. T lysates prepared from wild-type and *ted1Δ* cells were centrifuged at 13,000 × *g*, and the supernatant (S) and the ER membrane pellet (P) fractions were analyzed by western blot for Lst1p, Sec24p, and Sec61p.

(E) Quantification of three experiments described in (D). The graph plots the average percentage of the ER membrane association of Lst1p and Sec24p. Error bars indicate the SD.

targeting to the specialized GPI-AP containing ERESs. Altogether, our findings strongly support the idea that GPI anchor remodeling plays an active role in the recruitment of the specialized COPII coat specifically required for the efficient ER export of GPI-APs.

## Discussion

The COPII coat forms vesicles at ERESs to transport secretory proteins to the Golgi apparatus. However, it is unclear how the COPII coat works together with cargo receptors to select fully assembled proteins into forming vesicles [1–3]. In this study, we provide direct new mechanistic insight into the previously unknown mode of action of ER cargo receptors. First, we show that the cargo receptor for GPI-APs, the p24 complex, acts as a lectin by selectively recognizing the mature glycan structure of the GPI anchor once remodeled by Ted1p. Moreover, we found that binding of the remodeled cargo changes the COPII specificity of the p24 complex to nucleate the recruitment of the specific COPII subunit Lst1p to the GPI-AP containing ERESs. These results strongly suggest that the recruitment of the COPII coat by cargo receptors is not constitutive but instead is actively regulated by binding of mature ligands. Therefore, we reveal here a specialized COPII vesicle budding system that responds to the presence of a subset of mature luminal cargos. This system requires GPI anchor remodeling, which plays a role in sorting and concentrating GPI-APs at their specific ERESs (GPI-lipid remodeling) and subsequently promotes the recruitment of the specialized ER export machinery that enables vesicle formation from these ERESs (GPI-glycan remodeling). In this model (Figure 7), the p24 complex is first recruited to specific ERESs, where it binds to concentrated, remodeled GPI-APs. Next, this interaction stimulates the p24 complex to selectively recruit and stabilize Lst1p-Sec23p prebudding complexes, which afterward favor the assembly of the outer COPII layer to generate specialized COPII vesicles enriched in GPI-APs.

Vesicle formation and packaging of GPI-APs from their specific ERESs exhibit special COPII coat requirements such as an increased dependence on Sec13p and the presence of Lst1p [25]. Sec13p has been proposed to confer sufficient rigidity to the coat in order to overcome the potential membrane-bending force associated with GPI-AP-enriched membranes [36]. In addition, Lst1p could be required for adjusting this coat rigidity by creating a larger-diameter vesicle bud. Consistent with this possibility, it has been shown that COPII vesicles formed in vitro with mixed Sec24p-Lst1p coats are slightly larger than conventional Sec24p vesicles [22]. Thus, Lst1p could help to accommodate potentially large cargos such as clusters of GPI-APs or oligomers of the plasma membrane ATPase Pma1p, whose export from ER is also facilitated by Lst1p [24]. In this context, our results indicate that the p24 complex specifically binds remodeled GPI-APs to recruit and stabilize Lst1p at specific ERESs and thus enable the formation of the COPII vesicles for GPI-AP ER

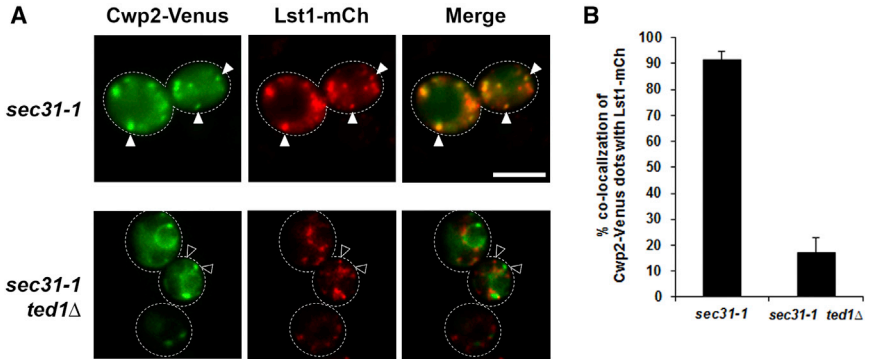


Figure 6. GPI-Glycan Remodeling Induces Lst1p Targeting to the Specialized GPI-AP Containing ERESs

(A) Fluorescence micrographs of live *sec31-1* and *sec31-1 ted1Δ* cells expressing Cwp2-Venus (green) and Lst1-mCherry (red) at 37°C. Fine dashed line, cell shape. White arrowheads: co-localizing dots. Open arrowheads: noncolocalizing dots. The scale bar represents 5 μm.

(B) Quantification of several micrographs described in (A). The graph displays the means of the percentage of colocalization per cell of Cwp2-Venus dots with Lst1-mCherry dots in *sec31-1* (n = 40) and in *sec31-1 ted1Δ* (n = 42). Error bars indicate the SD.

export. Indeed, Lst1p, but not Sec24p, is connected with GPI-APs, despite the fact that both subunits can be bound in vivo by the p24 proteins. This dual specificity of p24 complex could explain its incorporation into both GPI-AP- and non-GPI-AP-containing COPII vesicles for delivery to the Golgi [5, 11]. Therefore, it is conceivable that the p24 complex binds Lst1p and Sec24p in ERESs for GPI-APs and non-GPI-APs, respectively. Nevertheless, the fact that the p24 complex interacts more efficiently with Lst1p when it is bound to GPI-APs raises the question of the underlying selective mechanism. One possibility is that the specific binding of the remodeled GPI-AP triggers a conformational change of the p24 proteins that selectively increases their affinity for Lst1p, but not for Sec24p. Alternatively, a special lipid environment could contribute to the initial selective recruitment of Lst1p to the specific ERESs for GPI-APs, which could be later differentially captured by the p24 proteins and thus stabilized on the membrane in GPI-AP-containing prebudding complexes. Consistent with this possibility, it has been proposed that GPI-APs are clustered in ceramide-enriched microdomains at their specific ERESs, which are generated upon GPI-lipid remodeling [8].

This study further supports the notion that luminal cargo plays a key regulatory role in the formation of COPII vesicles instead of simply being a passive traveler. In the particular case of GPI-APs, we show in vivo that the GPI anchor remodeling plays a critical role in the activity of the p24 complex as selective COPII coat nucleator. The p24 complex could facilitate specialized coat assembly on specific ERESs for GPI-APs in several ways. First, the ability of p24 proteins to oligomerize and to present multiple COPII-binding signals could contribute to firmly dock the Lst1p/Sec23p inner coat subunits to the membrane even after GTP hydrolysis by Sar1p. The formed stable prebudding complexes might act as structural scaffolds for the recruitment of the Sec31/13p outer coat and thus subsequently lead to productive vesicle budding, as suggested by previous in vitro studies [37, 38]. In retrograde traffic, the p24 complex is also thought to promote COPI vesicle budding by acting as a primer to induce COPI coat polymerization [39, 40]. Furthermore, the fact that the cytosolic tail of the p24 protein Emp24p is able to directly bind Sar1p in vitro independent of its guanine nucleotide status raises the possibility that the p24 complex might also regulate the Sar1p GTPase cycle [16]. Initial binding to Sar1p could help to bring Lst1p/Sec23p to the inner coat. Next, the Sar1p released after GTP hydrolysis could be rapidly captured again by the p24 complexes to be activated by Sec12p and initiate a new cycle [37]. Finally, Sar1p bound to the p24 complex could also be relevant for the fission and release of GPI-AP-containing vesicles [41, 42]. In any cases, further investigation will be required to test this model.

The transitory presence of the side-chain EtNP added by Gpi7p just before GPI attachment to the protein suggests that GPI glycan remodeling by Ted1p could operate as a quality control system licensing the ER export of GPI anchors only after they have been correctly added to proteins. Because the p24 complex recognizes the GPI anchor and not the protein part, a requirement of protein attachment for Ted1p activation would ensure that GPI anchors do not interact with the p24 complex until they are attached to proteins. Thus, this system could prevent the premature ER export of free GPI molecules, increasing the efficiency of the GPI-anchoring process. Additionally, free GPI export might interfere with integration of GPI-AP into the cell wall. The quality control by Ted1p might also monitor the GPI-lipid remodeling, which occurs immediately after GPI attachment, in order to couple the processes of GPI-AP concentration at specific ERESs with the recruitment of the specialized ER export machinery to form vesicles from these ERESs. In this sense, the fact that Gpi7p facilitates the last step of the GPI-lipid-remodeling process [43], in which the primary GPI-lipid is replaced by a very-long-chain ceramide [44], suggests that Ted1p may function preferentially after ceramide remodeling and thus favor the p24 interaction with ceramide-based GPI-APs. Indeed, ceramide could be required for optimal GPI-glycan recognition by the p24 complex as we observed a slight reduction of p24 binding with GPI-APs in *gpi7Δ* mutant cells compared to wild-type cells. In agreement with this possibility, it has been recently shown that the mammalian homolog of Emp24p interacts with a specific sphingomyelin through its transmembrane domain [45]. In conclusion, GPI-glycan remodeling by Ted1p could act as a quality control system to enable p24 recognition and efficient ER export of completely matured GPI-APs. Further testing of this possible quality control and to determine the regulatory mechanism that activates Ted1p to trigger the p24-mediated ER export will be subjects of future research.

Finally, our findings reveal a novel functional link between luminal cargo maturation and COPII vesicle budding. We show that GPI anchor remodeling actively promotes the formation of specialized COPII vesicles for the efficient ER export of GPI-APs. Because GPI anchor remodeling takes place after protein attachment, this also suggests that COPII vesicle production is fine-tuned by the number of proteins that are correctly anchored and ready for ER export. The existence of this type of regulatory mechanism could help to better understand how the ER export can be adapted to different needs for luminal cargo secretion, which occurs in many specialized secretory cells.

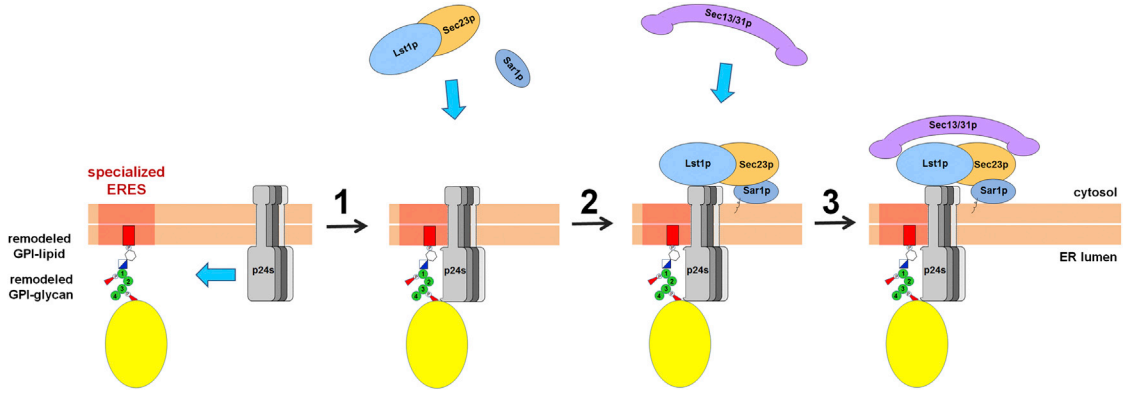


Figure 7. Model for an Actively Regulated ER Export of GPI-APs in Yeast

Graphical representation of the actively regulated vesicle budding system used by GPI-APs for efficient ER export. After protein attachment, lipid and glycan parts of the GPI anchor are remodeled. The GPI-lipid remodeling sorts and concentrates GPI-APs at their specific ERESs, and the GPI-glycan remodeling allows the subsequent recruitment to these ERESs of the p24 complex, which acts as a lectin, by recognizing the remodeled GPI-glycan moiety of GPI-APs (1). Binding of the remodeled cargo prompts the p24 complex to nucleate the selective recruitment of the specific inner COPII coat Lst1p/Sec23p (2). The formed, stable prebudding complexes act as structural scaffolds for the recruitment of the outer COPII coat Sec13/31p and thus subsequently lead to productive vesicle budding (3).

## Experimental Procedures

### Yeast Strains and Plasmids

Strains of *Saccharomyces cerevisiae* used for this work are listed in Table S1. The plasmid expressing Cwp2-Venus (pRS416ADH-CWP2-VENUS) has been made in a previous study [8]. In order to construct the plasmid expressing Ted1-TAP, we first C-terminal TAP tagged the wild-type strain on the genomic locus of *TED1*, using PCR-based homologous recombination. From the resultant strain, we amplified by PCR the coding region of the TAP-tagged *TED1* including its endogenous promoter, using PCR with high-fidelity polymerase and cloning into pRS315 (LEU CEN), making plasmid VGp152. The plasmid expressing Ted1N233A,H234A-TAP (VGp249) was derived from VGp152 by PCR-based site-directed mutagenesis, changing N233A and H234A simultaneously. The human *PGAP5* (*MPPE1*) coding sequence [46] was tagged with BamHI- and SacI-cleavable sequences by PCR amplification of a human cDNA and subcloned into p2UGPD [47] by standard procedures resulting in plasmids pRWE101 and pRWE102, respectively.

### Native Coimmunoprecipitation

The native coimmunoprecipitation experiments were performed on enriched ER fractions as described [48]. In brief, 100 optical density 600 units of yeast cells were washed twice with TNE buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonylfluoride, and protease inhibitor cocktail; Roche Diagnostics) and disrupted with glass beads, after which cell debris and glass beads were removed by centrifugation. The supernatant was then centrifuged at 13,000 × *g* for 15 min at 4°C. The pellet was resuspended in TNE, and digitonin was added to a final concentration of 1%. The suspension was incubated for 1 hr at 4°C with rotation, after which insoluble components were removed by centrifugation at 13,000 × *g* for 60 min at 4°C. For immunoprecipitation of Cwp2-Venus, the sample was first preincubated with bab agarose beads (ChromoTek) at 4°C for 1 hr and subsequently incubated with GFP-Trap_A (ChromoTek) at 4°C for 3 hr. The immunoprecipitated beads were washed five times with TNE containing 0.2% digitonin, eluted with SDS sample buffer, resolved on SDS-PAGE, and analyzed by immunoblot.

### Crosslinking Assay

The crosslinking assay was performed on cell extracts as described [10].

### GPI-Glycan Pull-Down Assay

The GPI-glycan was synthesized and coupled to agarose beads as described [33]. An enriched ER fraction from wild-type cell extracts, obtained as above, was solubilized with 0.5% Triton X-100 in coupling buffer (50 mM Tris and 5 mM EDTA [pH 8.5]), after which insoluble components were removed by centrifugation at 13,000 × *g* for 30 min at 4°C. The suspension was incubated with the GPI-glycan matrix in the absence or presence of 300 mM of mannose (Sigma) or galactose (Sigma) for 1 hr at 4°C. The beads were washed five times with coupling buffer containing 0.5% Triton X-100, eluted with SDS-sample buffer, resolved on SDS-PAGE, and analyzed by immunoblot.

### Differential Fractionation

Differential fractionation was performed as described by Chen et al. [49] with some modifications. A total of 100 ml of cells was harvested at 5 × $10^6$ cell/ml, washed with 10 mM sodium azide, spheroplasted, lysed, layered on a 1 ml sorbitol cushion (1.7 M sorbitol and 50 mM potassium phosphate [pH 7.5]), and centrifuged at 6,500 rpm for 3 min. The pellet was lysed in 1 ml of lysis buffer (20 mM HEPES [pH 7.4] with protease inhibitors) and centrifuged at 2,500 rpm for 2 min in a microfuge. The supernatant (600 μl) was removed (T) and spun at 13,000 × *g* for 30 min. The supernatant (S) was saved, and the pellet (P) was resuspended in 600 μl of lysis buffer.

### GPI Anchor Remodeling

Phosphatidylinositol (PI) moieties of GPI anchor were isolated from GPI-APs labeled with [3H] myo-inositol as described previously [31, 50]. The lipids were separated by thin-layer chromatography using solvent system (55:45:10 chloroform-methanol-0.25% KCl) and visualized using FLA-7000 (Fujifilm).

### Light Microscopy

For fluorescence microscopy of Cwp2-Venus and Emp24-CFP, log-phase cells grown in minimal media were observed directly. For accumulation of Cwp2-Venus at ERESs, log-phase cells grown in minimal media were incubated 45 min at 37°C [8]. Acquisition was performed using an Olympus BX61 microscope equipped with an objective lens (PlanApo 100×/1.40 OIL), a DP60 camera, and Image Manager 50 v1.20 following the instructions of the manufacturer. Colocalization experiments were performed using a Leica DM6000B microscope equipped with an objective lens (HCX PL APO 100×/1.40 OIL PH3 CS), L5 (GFP y VENUS), TX2 (mCherry) filters, a DFC350FX camera, and LAS AF software following the instructions of the manufacturer.

### Supplemental Information

Supplemental Information includes five figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.11.039.

### Author Contributions

M.M. and J.M.-L. provided conception and design. J.M.-L., A.M.P.-L., A.A.-R., M.E.M., R.E.W., T.O., and K.F. acquired the data. M.M., J.M.-L., H.R., and K.F. analyzed and interpreted the data. M.M., J.M.-L., R.E.W., H.R., and A.M.P.-L. drafted or revised the article. D.V.S., P.H.S., H.R., and V.G. contributed essential reagents.

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**References**

1. Dancourt, J., and Barlowe, C. (2010). Protein sorting receptors in the early secretory pathway. Annu. Rev. Biochem. *79*, 777–802.
2. Jensen, D., and Schekman, R. (2011). COPII-mediated vesicle formation at a glance. J. Cell Sci. *124*, 1–4.
3. Geva, Y., and Schuldiner, M. (2014). The back and forth of cargo exit from the endoplasmic reticulum. Curr. Biol. *24*, R130–R136.
4. Ferguson, M.A.J., Kinoshita, T., and Hart, G.W. (2009). Glycosylphosphatidylinositol anchors. In Essentials of Glycobiology, Second Edition, A. Varki, R.D. Cummings, J.D. Esko, H.H. Freeze, P. Stanley, C.R. Bertozzi, G.W. Hart, and M.E. Etzler, eds. (Cold Spring Harbor: Cold Spring Harbor Laboratory Press).
5. Muñiz, M., Morsomme, P., and Riezman, H. (2001). Protein sorting upon exit from the endoplasmic reticulum. Cell *104*, 313–320.
6. Doering, T.L., and Schekman, R. (1996). GPI anchor attachment is required for Gas1p transport from the endoplasmic reticulum in COP II vesicles. EMBO J. *15*, 182–191.
7. Mayor, S., and Riezman, H. (2004). Sorting GPI-anchored proteins. Nat. Rev. Mol. Cell Biol. *5*, 110–120.
8. Castillon, G.A., Watanabe, R., Taylor, M., Schwabe, T.M., and Riezman, H. (2009). Concentration of GPI-anchored proteins upon ER exit in yeast. Traffic *10*, 186–200.
9. Fujita, M., and Kinoshita, T. (2012). GPI-anchor remodeling: potential functions of GPI-anchors in intracellular trafficking and membrane dynamics. Biochim. Biophys. Acta *1821*, 1050–1058.
10. Castillon, G.A., Aguilera-Romero, A., Manzano-Lopez, J., Epstein, S., Kajiwara, K., Funato, K., Watanabe, R., Riezman, H., and Muñiz, M. (2011). The yeast p24 complex regulates GPI-anchored protein transport and quality control by monitoring anchor remodeling. Mol. Biol. Cell *22*, 2924–2936.
11. Muñiz, M., Nuoffer, C., Hauri, H.P., and Riezman, H. (2000). The Emp24 complex recruits a specific cargo molecule into endoplasmic reticulum-derived vesicles. J. Cell Biol. *148*, 925–930.
12. Fujita, M., Watanabe, R., Jaensch, N., Romanova-Michaelides, M., Satoh, T., Kato, M., Riezman, H., Yamaguchi, Y., Maeda, Y., and Kinoshita, T. (2011). Sorting of GPI-anchored proteins into ER exit sites by p24 proteins is dependent on remodeled GPI. J. Cell Biol. *194*, 61–75.
13. Stamnes, M.A., Craighead, M.W., Hoe, M.H., Lampen, N., Geromanos, S., Tempst, P., and Rothman, J.E. (1995). An integral membrane component of coatomer-coated transport vesicles defines a family of proteins involved in budding. Proc. Natl. Acad. Sci. USA *92*, 8011–8015.
14. Sohn, K., Orci, L., Ravazzola, M., Amherdt, M., Bremser, M., Lottspeich, F., Fiedler, K., Helms, J.B., and Wieland, F.T. (1996). A major transmembrane protein of Golgi-derived COPI-coated vesicles involved in coatomer binding. J. Cell Biol. *135*, 1239–1248.
15. Schimmöller, F., Singer-Krüger, B., Schröder, S., Krüger, U., Barlowe, C., and Riezman, H. (1995). The absence of Emp24p, a component of ER-derived COPII-coated vesicles, causes a defect in transport of selected proteins to the Golgi. EMBO J. *14*, 1329–1339.
16. Belden, W.J., and Barlowe, C. (2001). Distinct roles for the cytoplasmic tail sequences of Emp24p and Erv25p in transport between the endoplasmic reticulum and Golgi complex. J. Biol. Chem. *276*, 43040–43048.
17. Dominguez, M., Dejgaard, K., Füllekrug, J., Dahan, S., Fazel, A., Paccaud, J.P., Thomas, D.Y., Bergeron, J.J., and Nilsson, T. (1998). gp25L/emp24/p24 protein family members of the cis-Golgi network bind both COP I and II coatomer. J. Cell Biol. *140*, 751–765.
18. Marzioch, M., Henthorn, D.C., Herrmann, J.M., Wilson, R., Thomas, D.Y., Bergeron, J.J., Solari, R.C., and Rowley, A. (1999). Erp1p and Erp2p, partners for Emp24p and Erv25p in a yeast p24 complex. Mol. Biol. Cell *10*, 1923–1938.
19. Rojo, M., Pepperkok, R., Emery, G., Kellner, R., Stang, E., Parton, R.G., and Gruenberg, J. (1997). Involvement of the transmembrane protein p23 in biosynthetic protein transport. J. Cell Biol. *139*, 1119–1135.
20. Füllekrug, J., Suganuma, T., Tang, B.L., Hong, W., Storrie, B., and Nilsson, T. (1999). Localization and recycling of gp27 (hp24gamma3): complex formation with other p24 family members. Mol. Biol. Cell *10*, 1939–1955.
21. Hirata, R., Nihei, C., and Nakano, A. (2013). Isoform-selective oligomer formation of Saccharomyces cerevisiae p24 family proteins. J. Biol. Chem. *288*, 37057–37070.
22. Miller, E., Antonny, B., Hamamoto, S., and Schekman, R. (2002). Cargo selection into COPII vesicles is driven by the Sec24p subunit. EMBO J. *21*, 6105–6113.
23. Miller, E.A., Beilharz, T.H., Malkus, P.N., Lee, M.C., Hamamoto, S., Orci, L., and Schekman, R. (2003). Multiple cargo binding sites on the COPII subunit Sec24p ensure capture of diverse membrane proteins into transport vesicles. Cell *114*, 497–509.
24. Shimoni, Y., Kurihara, T., Ravazzola, M., Amherdt, M., Orci, L., and Schekman, R. (2000). Lst1p and Sec24p cooperate in sorting of the plasma membrane ATPase into COPII vesicles in Saccharomyces cerevisiae. J. Cell Biol. *151*, 973–984.
25. D'Arcangelo, J.G., Stahmer, K.R., and Miller, E.A. (2013). Vesicle-mediated export from the ER: COPII coat function and regulation. Biochim. Biophys. Acta *1833*, 2464–2472.
26. Fujita, M., Maeda, Y., Ra, M., Yamaguchi, Y., Taguchi, R., and Kinoshita, T. (2009). GPI glycan remodeling by PGAP5 regulates transport of GPI-anchored proteins from the ER to the Golgi. Cell *139*, 352–365.
27. Haass, F.A., Jonikas, M., Walter, P., Weissman, J.S., Jan, Y.N., Jan, L.Y., and Schuldiner, M. (2007). Identification of yeast proteins necessary for cell-surface function of a potassium channel. Proc. Natl. Acad. Sci. USA *104*, 18079–18084.
28. Flury, I., Benachour, A., and Conzelmann, A. (2000). YLL031c belongs to a novel family of membrane proteins involved in the transfer of ethanolaminephosphate onto the core structure of glycosylphosphatidylinositol anchors in yeast. J. Biol. Chem. *275*, 24458–24465.
29. Vazquez, H.M., Vionnet, C., Roubaty, C., and Conzelmann, A. (2014). Cdc1 removes the ethanolamine phosphate of the first mannose of GPI anchors and thereby facilitates the integration of GPI proteins into the yeast cell wall. Mol. Biol. Cell *25*, 3375–3388.
30. Antonny, B., and Schekman, R. (2001). ER export: public transportation by the COPII coach. Curr. Opin. Cell Biol. *13*, 438–443.
31. Fujita, M., and Jigami, Y. (2008). Lipid remodeling of GPI-anchored proteins and its function. Biochim. Biophys. Acta *1780*, 410–420.
32. Theiler, R., Fujita, M., Nagae, M., Yamaguchi, Y., Maeda, Y., and Kinoshita, T. (2014). The $\alpha$-helical region in p24$\gamma$2 subunit of p24 protein cargo receptor is pivotal for the recognition and transport of glycosylphosphatidylinositol-anchored proteins. J. Biol. Chem. *289*, 16835–16843.
33. Azzouz, N., Kamena, F., and Seeberger, P.H. (2010). Synthetic glycosylphosphatidylinositol as tools for glycoparasitology research. OMICS *14*, 445–454.
34. Kuehn, M.J., Herrmann, J.M., and Schekman, R. (1998). COPII-cargo interactions direct protein sorting into ER-derived transport vesicles. Nature *391*, 187–190.
35. Bi, X., Mancias, J.D., and Goldberg, J. (2007). Insights into COPII coat nucleation from the structure of Sec23.Sar1 complexed with the active fragment of Sec31. Dev. Cell *13*, 635–645.
36. Copic, A., Latham, C.F., Horlbeck, M.A., D'Arcangelo, J.G., and Miller, E.A. (2012). ER cargo properties specify a requirement for COPII coat rigidity mediated by Sec13p. Science *335*, 1359–1362.
37. Sato, K., and Nakano, A. (2005). Dissection of COPII subunit-cargo assembly and disassembly kinetics during Sar1p-GTP hydrolysis. Nat. Struct. Mol. Biol. *12*, 167–174.
38. Tabata, K.V., Sato, K., Ide, T., Nishizaka, T., Nakano, A., and Noji, H. (2009). Visualization of cargo concentration by COPII minimal machinery in a planar lipid membrane. EMBO J. *28*, 3279–3289.
39. Aguilera-Romero, A., Kaminska, J., Spang, A., Riezman, H., and Muñiz, M. (2008). The yeast p24 complex is required for the formation of COPI

retrograde transport vesicles from the Golgi apparatus. J. Cell Biol. *180*, 713–720.

40. Bremser, M., Nickel, W., Schweikert, M., Ravazzola, M., Amherdt, M., Hughes, C.A., Söllner, T.H., Rothman, J.E., and Wieland, F.T. (1999). Coupling of coat assembly and vesicle budding to packaging of putative cargo receptors. Cell *96*, 495–506.
41. Bielli, A., Haney, C.J., Gabreski, G., Watkins, S.C., Bannykh, S.I., and Aridor, M. (2005). Regulation of Sar1 NH2 terminus by GTP binding and hydrolysis promotes membrane deformation to control COPII vesicle fission. J. Cell Biol. *171*, 919–924.
42. Lee, M.C., Orci, L., Hamamoto, S., Futai, E., Ravazzola, M., and Schekman, R. (2005). Sar1p N-terminal helix initiates membrane curvature and completes the fission of a COPII vesicle. Cell *122*, 605–617.
43. Benachour, A., Sipos, G., Flury, I., Reggiori, F., Canivenc-Gansel, E., Vionnet, C., Conzelmann, A., and Benghezal, M. (1999). Deletion of GPI7, a yeast gene required for addition of a side chain to the glycosylphosphatidylinositol (GPI) core structure, affects GPI protein transport, remodeling, and cell wall integrity. J. Biol. Chem. *274*, 15251–15261.
44. Yoko-O, T., Ichikawa, D., Miyagishi, Y., Kato, A., Umemura, M., Takase, K., Ra, M., Ikeda, K., Taguchi, R., and Jigami, Y. (2013). Determination and physiological roles of the glycosylphosphatidylinositol lipid remodelling pathway in yeast. Mol. Microbiol. *88*, 140–155.
45. Contreras, F.X., Ernst, A.M., Haberkant, P., Björkholm, P., Lindahl, E., Gönen, B., Tischer, C., Elofsson, A., von Heijne, G., Thiele, C., et al. (2012). Molecular recognition of a single sphingolipid species by a protein's transmembrane domain. Nature *481*, 525–529.
46. Vuoristo, J.T., and Ala-Kokko, L. (2001). cDNA cloning, genomic organization and expression of the novel human metallophosphoesterase gene MPPE1 on chromosome 18p11.2. Cytogenet. Cell Genet. *95*, 60–63.
47. Pittman, J.K., Cheng, N.H., Shigaki, T., Kunta, M., and Hirschi, K.D. (2004). Functional dependence on calcineurin by variants of the Saccharomyces cerevisiae vacuolar Ca2+/H+ exchanger Vcx1p. Mol. Microbiol. *54*, 1104–1116.
48. Fujita, M., Yoko-O, T., and Jigami, Y. (2006). Inositol deacylation by Bst1p is required for the quality control of glycosylphosphatidylinositol-anchored proteins. Mol. Biol. Cell *17*, 834–850.
49. Chen, S., Cai, H., Park, S.K., Menon, S., Jackson, C.L., and Ferro-Novick, S. (2011). Trs65p, a subunit of the Ypt1p GEF TRAPPII, interacts with the Arf1p exchange factor Gea2p to facilitate COPI-mediated vesicle traffic. Mol. Biol. Cell *22*, 3634–3644.
50. Guillas, I., Pfefferli, M., and Conzelmann, A. (2000). Analysis of ceramides present in glycosylphosphatidylinositol anchored proteins of Saccharomyces cerevisiae. Methods Enzymol. *312*, 506–515.