

Remodeling of secretory compartments creates CUPS during nutrient starvation

David Cruz-Garcia,^{1,2} Amy J. Curwin,^{1,2} Jean-François Popoff,^{1,2} Caroline Bruns,^{1,2} Juan M. Duran,^{1,2} and Vivek Malhotra^{1,2,3}

¹Cell and Developmental Biology Programme, Centre for Genomic Regulation (CRG), 08003 Barcelona, Spain

²Universitat Pompeu Fabra (UPF), 08002 Barcelona, Spain

³Institució Catalana de Recerca i Estudis Avançats (ICREA), 08010 Barcelona, Spain

Upon starvation, Grh1, a peripheral membrane protein located at endoplasmic reticulum (ER) exit sites and early Golgi in *Saccharomyces cerevisiae* under growth conditions, relocates to a compartment called compartment for unconventional protein secretion (CUPS). Here we report that CUPS lack Golgi enzymes, but contain the coat protein complex II (COPII) vesicle tethering protein Uso1 and the Golgi t-SNARE Sed5. Interestingly, CUPS biogenesis is independent of COPII- and COPI-mediated membrane transport. Pik1- and Sec7-mediated membrane export from the late Golgi is required

for complete assembly of CUPS, and Vps34 is needed for their maintenance. CUPS formation is triggered by glucose, but not nitrogen starvation. Moreover, upon return to growth conditions, CUPS are absorbed into the ER, and not the vacuole. Altogether our findings indicate that CUPS are not specialized autophagosomes as suggested previously. We suggest that starvation triggers relocation of secretory and endosomal membranes, but not their enzymes, to generate CUPS to sort and secrete proteins that do not enter, or are not processed by enzymes of the ER–Golgi pathway of secretion.

Introduction

Secretory cargoes containing a signal sequence enter the ER, where they are processed, folded, and then packaged into coat protein complex II (COPII)-coated vesicles for trafficking to the Golgi. Within the Golgi, cargoes are further processed, sorted, and transported to different cellular destinations, including the extracellular space. However, there are many examples of secreted proteins that do not contain a signal sequence for translocation into the ER (Rabouille et al., 2012). This class of proteins lack any known, common structural feature or posttranslational modification. In *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Dictyostelium discoideum*, the 87-aa signal sequence-lacking protein Acb1 (AcbA in *D. discoideum*) is secreted under conditions of nutrient deprivation (Kinseth et al., 2007; Duran et al., 2010; Manjithaya et al., 2010). In *D. discoideum*, secreted AcbA is proteolytically cleaved to generate a 34-aa peptide, SDF2, which triggers rapid encapsulation of pre-spore cells (Anjard and Loomis, 2005). How Acb1 is secreted, how the enzyme required for the production of SDF2 is expressed on the exoplasmic face of the plasma membrane, and whether Acb1 undergoes

any modification before its release from the cells is not known (Malhotra, 2013). GrpA and Grh1, the respective *D. discoideum* and yeast homologues of the mammalian Golgi-associated proteins GRASP55 and GRASP65, respectively, are required for AcbA/Acb1 secretion and production of functionally active SDF2, but their exact role in this overall pathway is not known (Kinseth et al., 2007; Duran et al., 2010; Manjithaya et al., 2010). Interestingly, in *S. cerevisiae*, Grh1 localizes to ER exit sites and early Golgi membranes under growth conditions, and upon starvation, Grh1 redistributes to a large compartment called compartment for unconventional protein secretion (CUPS; Bruns et al., 2011). CUPS are present at 1–3 copies/cell, localized in the vicinity of the Sec13-containing ER exit sites; they lack Golgi processing enzymes and endocytic markers (Bruns et al., 2011). The function of CUPS in Acb1 secretion is not known, but it has been suggested that CUPS collect Acb1 from the cytoplasm and then generate small Acb1-enriched vesicles for its further processing and secretion (Malhotra, 2013). What is the

Correspondence to Vivek Malhotra: vivek.malhotra@crgeu

Abbreviations used in this paper: COPI, coat protein complex I; COPII, coat protein complex II; CUPS, compartment for unconventional protein secretion; PI3P, phosphatidylinositol 3-phosphate; PI4P, phosphatidylinositol 4-phosphate.

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molecular composition of CUPS, how do they form, and what is their fate when starved cells return to nutrient-rich conditions?

We now report that formation of CUPS depends on COPII- and COPI-independent membrane recruitment from the Golgi. The formation and maintenance of CUPS also requires membrane export from late Golgi/endosomes. Upon return of cells to growth condition, CUPS are reabsorbed into the ER in a COPI-dependent process. The description of this hybrid membrane compartment and its dynamics follows.

Results and discussion

CUPS contain the vesicle tethering protein *Uso1* and the Golgi t-SNARE *Sed5*

Grh1 is a peripheral membrane protein that has been reported to be associated with early Golgi membranes (Behnia et al., 2007) and ER exit sites in growing yeast cells (Levi et al., 2010). Our previous data showed that incubation of cells in 2% potassium acetate (starvation medium) caused relocation of Grh1 from ER exit sites/early Golgi to CUPS (Bruns et al., 2011). Our present experiments confirm the previous data and reveal that Grh1 redistribution to CUPS is much faster and can be detected as early as 30 min after incubation in starvation medium (Fig. 1, A and B).

The attachment of Grh1 to Golgi membranes is mediated in part by a protein called Bug1 (Behnia et al., 2007); we have shown previously that binding of Grh1 to CUPS is also Bug1 dependent (Bruns et al., 2011). Bug1 and Grh1 interact genetically with *Uso1* (Behnia et al., 2007), which is a long peripheral protein that tethers COPII vesicles with Golgi membranes before their fusion (Nakajima et al., 1991; Cao et al., 1998). We first tested whether *Uso1* is associated with CUPS. Cells coexpressing endogenously tagged Grh1-2×mCherry and *Uso1*-2×GFP were visualized by fluorescence microscopy. In cells grown in complete synthetic medium to mid-log phase, *Uso1* was located in a small number of elements that were scattered in the cytoplasm and frequently colocalized with (or were adjacent to) Grh1-positive structures (Fig. 1 A). Within 30 min of starvation, *Uso1* and Grh1 colocalized in CUPS, and this colocalization was maintained for up to 4 h of starvation (Fig. 1 A).

We then asked whether *Uso1* is required for the recruitment of Grh1 to CUPS by using a strain harboring the temperature-sensitive allele, *uso1-1*. First, we confirmed that the assembly of CUPS in wild-type cells expressing Grh1-2×GFP was not affected by incubation at the nonpermissive temperature of 37°C (Fig. 1 B). In *uso1-1* cells starved at 25°C, Grh1 was located in CUPS-like structures, although at a lower frequency than in starved wild-type cells (Fig. 1, B and C). Moreover, the number of *uso1-1* cells showing a diffuse distribution and/or multiple, small Grh1-2×GFP puncta was higher than that observed for the wild-type cells (Fig. 1, B and C). Inactivation of *Uso1* function by incubation at 37°C prevented the association of Grh1-2×GFP with CUPS-like structures in starved *uso1-1* cells, as revealed by an increase in the proportion of cells displaying a diffuse/small punctate distribution of Grh1, compared with wild-type starved cells (Fig. 1, B and C). These data reveal that *Uso1* is required for Grh1 recruitment to CUPS.

To investigate whether Grh1 is necessary for the association of *Uso1* with CUPS membranes, we examined the localization of *Uso1* in a *grh1Δ* strain. When *GRH1* was deleted, the distribution of *Uso1*-GFP in growing cells was more diffuse than that observed in wild-type cells (Fig. 1 D). This effect on the *Uso1* localization was more pronounced in starved cells, where the localization of *Uso1*-GFP to CUPS was barely detectable (Fig. 1 D), indicating that Grh1 is required for *Uso1* recruitment to CUPS.

Because *Uso1* is required for the fusion of ER-derived COPII vesicles with Golgi membranes (Cao et al., 1998), we tested whether the Golgi-specific t-SNARE *Sed5* (Hardwick and Pelham, 1992) was localized to CUPS. Fluorescence microscopy analysis of cells coexpressing Grh1-2×mCherry and GFP-*Sed5* revealed that in growing cells the Grh1-labeled structures colocalized with or were in close proximity to *Sed5* (Fig. 1 E). Under starvation conditions, Grh1 and *Sed5* were found to colocalize in CUPS (Fig. 1 E). *Sed5* was also detected in puncta devoid of Grh1 in starved cells (Fig. 1 E). These results reveal the presence of a pool of membrane-anchored *Sed5* protein in CUPS, which suggests that Golgi membranes are used for the formation of CUPS.

To further test the localization of *Uso1* and *Sed5* in CUPS, we performed confocal microscopy followed by a quantitative colocalization analysis between Grh1-2×mCherry and *Uso1*-2×GFP or GFP-*Sed5* in cells starved for 2 h. As controls, we used two early Golgi enzymes, *Mnn2*-GFP and *Mnn5*-GFP. This analysis revealed that the Manders' colocalization coefficients between Grh1 and *Uso1* or *Sed5* were significantly higher than those obtained for the pairs Grh1/*Mnn2* or Grh1/*Mnn5* (Fig. S1), further indicating that CUPS contain Golgi proteins, such as *Sed5*, but are largely devoid of Golgi enzymes.

CUPS form independent of COPII- and COPI-mediated membrane transport

Are COPII components involved in the biogenesis of CUPS? In our previous work, we addressed this question by analyzing the redistribution of Grh1 to CUPS after 4 h of starvation in conditions where COPII vesicle production was blocked in a thermosensitive *sec12* strain (Bruns et al., 2011). We decided to explore this finding further by testing the involvement of the COPII coat proteins *Sec13* and *Sec31*, and to gauge the role of COPII-mediated transport at earlier time points of starvation. Mid-log phase cells expressing Grh1-2×GFP were preincubated at 37°C for 45 min prior to starvation to ensure that the tested *Sec* protein was inactivated at the beginning of starvation. The analysis by fluorescence microscopy revealed that the distribution of Grh1-2×GFP in growing *sec13-1* and *sec31-1* cells was similar to that observed in the control wild-type cells (Fig. 2, A and B). In wild-type cells starved at 37°C, Grh1-2×GFP was localized to one to three large dots in the majority of cells. Similarly, Grh1 was found to be distributed in CUPS in most of the *sec13-1* and *sec31-1* cells starved at 37°C (Fig. 2, A and B). Thus, conventional COPII vesicle-mediated transport of membranes is not required to form CUPS.

Sed5 is required for COPI-mediated intra-Golgi transport (Banfield et al., 1995; Nichols and Pelham, 1998). We therefore

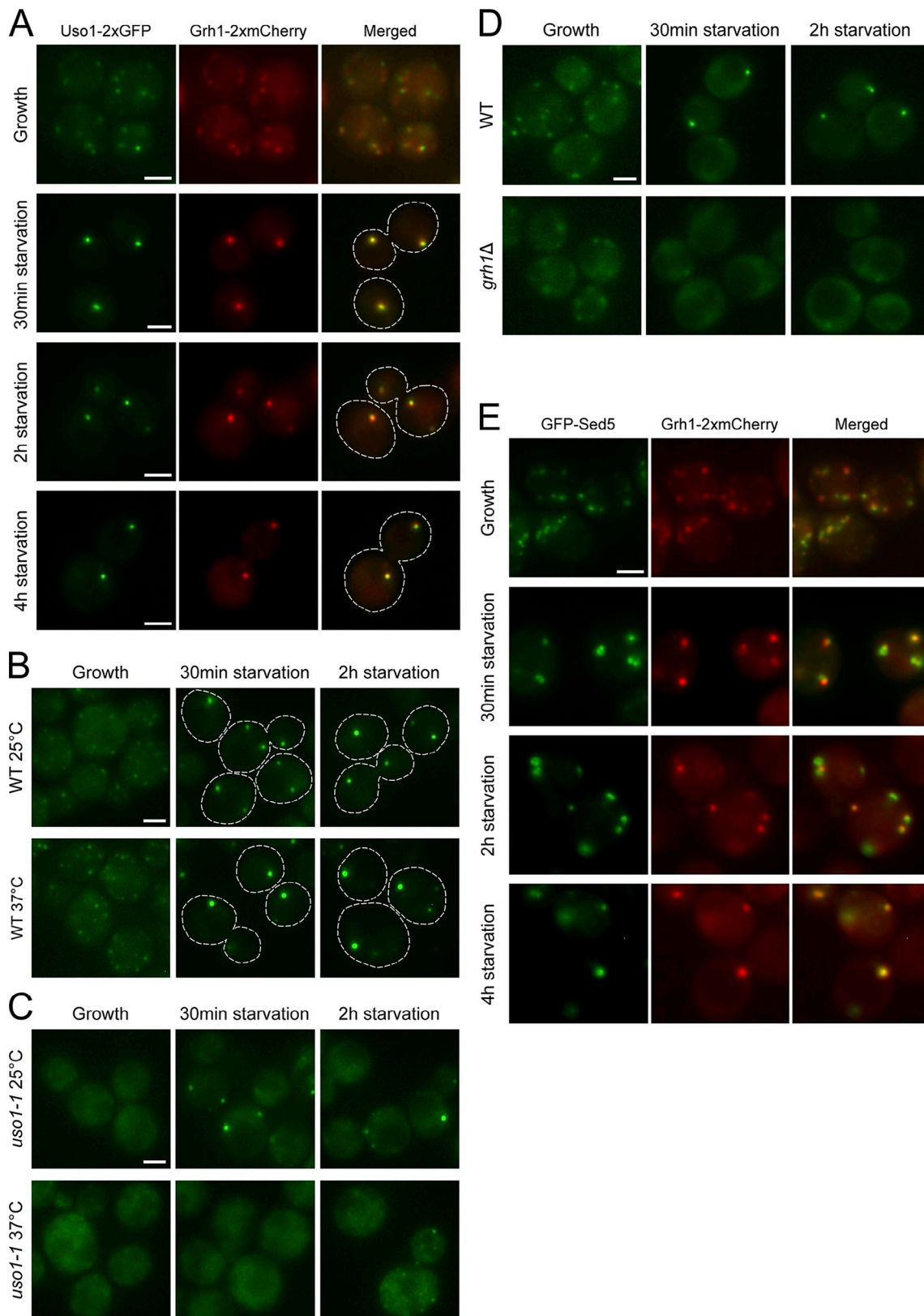


Figure 1. **Uso1 and Sed5 colocalize with Grh1 in CUPS.** (A) Cells coexpressing Uso1-2xGFP and Grh1-2xmCherry were grown in complete medium and imaged by fluorescence microscopy (Growth), or centrifuged and resuspended in 2% potassium acetate to induce starvation and visualized by fluorescence microscopy at 0.5, 2, and 4 h after incubation in starvation medium. (B and C) Wild-type (B) and *uso1-1* (C) cultures expressing Grh1-2xGFP were grown at 25°C, then either held at 25°C or shifted for 45 min to 37°C, followed by incubation in starvation medium at 25°C or 37°C, respectively, for 2 h. Cells were observed by fluorescence microscopy just before starvation (Growth) and after 0.5 and 2 h of starvation. (D) Wild-type or *grh1Δ* cells expressing Uso1-GFP were cultured in growth medium or starved for 2 h, and the distribution of Uso1-GFP was monitored by fluorescence microscopy. (E) Cells expressing GFP-Sed5 and Grh1-2xmCherry were processed and analyzed as described in A. Broken lines indicate cell boundaries. Bars, 2 μ m.

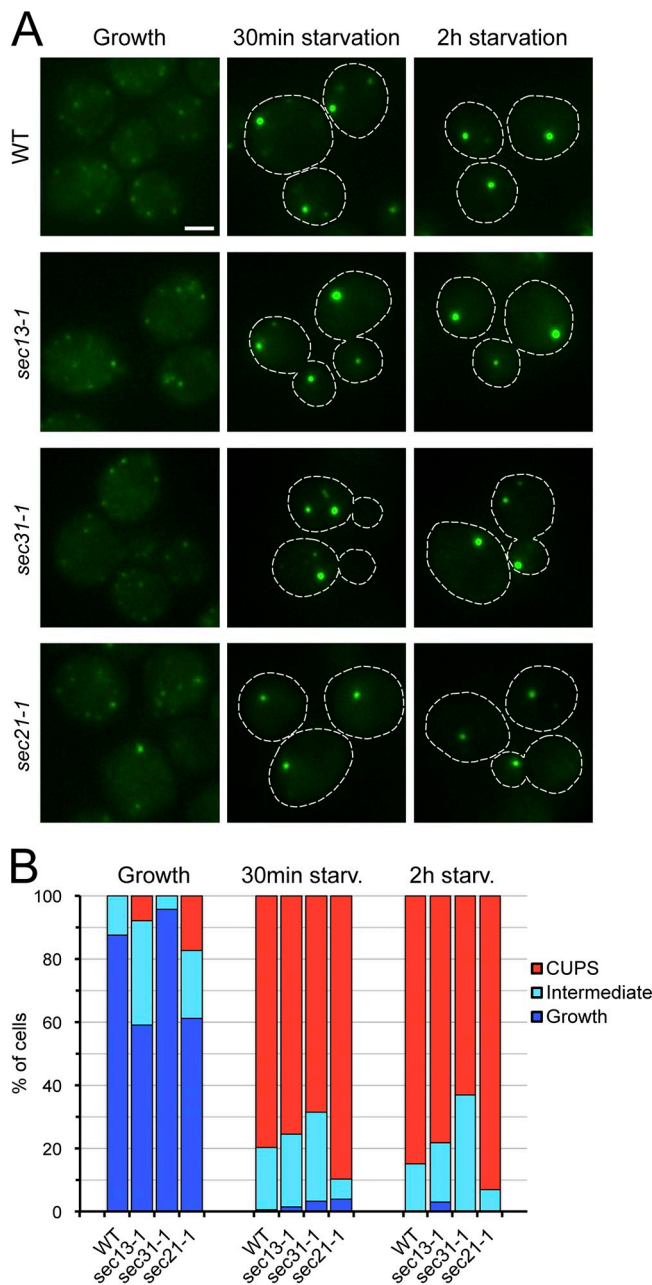


Figure 2. COPII- and COPI-mediated membrane transport is not necessary for CUPS formation. (A) Wild-type, *sec13-1*, *sec31-1*, and *sec21-1* cells expressing Grh1-2xGFP were grown to mid-log phase at 25°C, then preincubated at 37°C for 45 min followed by washing of the cells and incubation in 2% potassium acetate at 37°C for 2 h. Cells were imaged by fluorescence microscopy just before starvation (Growth) and within 0.5 and 2 h of starvation. Broken lines indicate cell boundaries. Bar, 2 μm. (B) Quantification of the different Grh1-2xGFP distributions observed in the conditions tested in A. The data shown are from a single representative experiment out of two repeats. For each condition in the experiment shown, $n \geq 80$ cells.

tested the involvement of COPI-coated vesicles in CUPS formation using the conditional mutant strain, *sec21-1* (Hosobuchi et al., 1992; Letourneur et al., 1994). When growing *sec21-1* cells were incubated at 37°C, Grh1-2xGFP retained a wild-type distribution in most of the population, with a small percentage of cells displaying a localization of Grh1 in CUPS-like structures

(Fig. 2, A and B). Nutrient starvation at 37°C did not affect the relocation of Grh1-2xGFP to CUPS in the *sec21-1* strain (Fig. 2, A and B). This result indicates that COPI-dependent membrane transport is not necessary for CUPS biogenesis. Taking into account that COPI vesicles have been proposed to play a role in the localization of Golgi processing enzymes (Tu et al., 2008), the lack of involvement of COPI vesicle-mediated transport in CUPS biogenesis is consistent with the absence of Golgi enzymes in CUPS (Fig. S1; Bruns et al., 2011).

We then asked whether CUPS formed in wild-type cells were the same as in COPII or COPI mutants. We tested this hypothesis by monitoring the distribution of Anp1, an early Golgi marker. Anp1-RFP did not colocalize with Grh1-2xGFP-containing CUPS in wild-type cells under growth or starvation condition (Fig. S2). Similarly, in the COPII (*sec31-1*) or COPI (*sec21-1*) mutant cells under starvation, CUPS did not contain Anp1-RFP (Fig. S2). Therefore, a defect in COPII- or COPI-dependent transport does not create CUPS that are biochemically different from those that form in wild-type cells.

The biogenesis of CUPS requires membrane export from the late Golgi

To investigate whether membrane transport from the late Golgi is involved in the formation of CUPS, we used strains bearing thermo-sensitive mutations in the ARF-GEF Sec7 and in the phosphatidylinositol 4-kinase Pik1, two essential proteins localized primarily at the late Golgi. In these mutant strains (*sec7-1* and *pik1-104*), the export of cargo from the late Golgi is blocked or delayed (Achstetter et al., 1988; Franzusoff et al., 1991; Walch-Solimena and Novick, 1999). The analysis by fluorescence microscopy of growing *sec7-1* and *pik1-104* cells incubated at 37°C revealed that the distribution of Grh1-2xGFP in these conditions was similar to that observed in the control wild-type strain (Fig. 3, A and B). In contrast, when both *sec7-1* and *pik1-104* cells were starved at 37°C, Grh1 failed to relocate completely to CUPS, and it was found in few larger CUPS-like structures and multiple small puncta dispersed throughout the cytoplasm (Fig. 3, A and B). This defect in Grh1 redistribution to CUPS was observed as early as 30 min after starvation and was also visible in cells starved for 2 h (Fig. 3, A and B). These results indicate the requirement of Sec7 and Pik1 for the complete assembly of CUPS and suggest that transport from the late Golgi is involved in CUPS biogenesis.

We have shown previously that Vps34, a phosphatidylinositol 3-kinase, is required for CUPS formation (Bruns et al., 2011). Based on the new observation that the phosphatidylinositol 4-kinase Pik1 is required for early events in CUPS formation, we retested the dependence on Vps34 for this process. A *VPS34* deletion strain was incubated in starvation medium, and the location of Grh1-2xGFP was examined at various time points after starvation. The loss of Vps34 did not strongly alter the formation of CUPS within 30 min of starvation (Fig. 3, C and D). However, at later time points and especially within 4 h of starvation, Grh1 distribution was affected in the *vps34Δ* cells as revealed by the significant percentage of cells showing numerous small Grh1-2xGFP-containing structures (Fig. 3, C and D; Bruns et al., 2011). Together, these results suggest

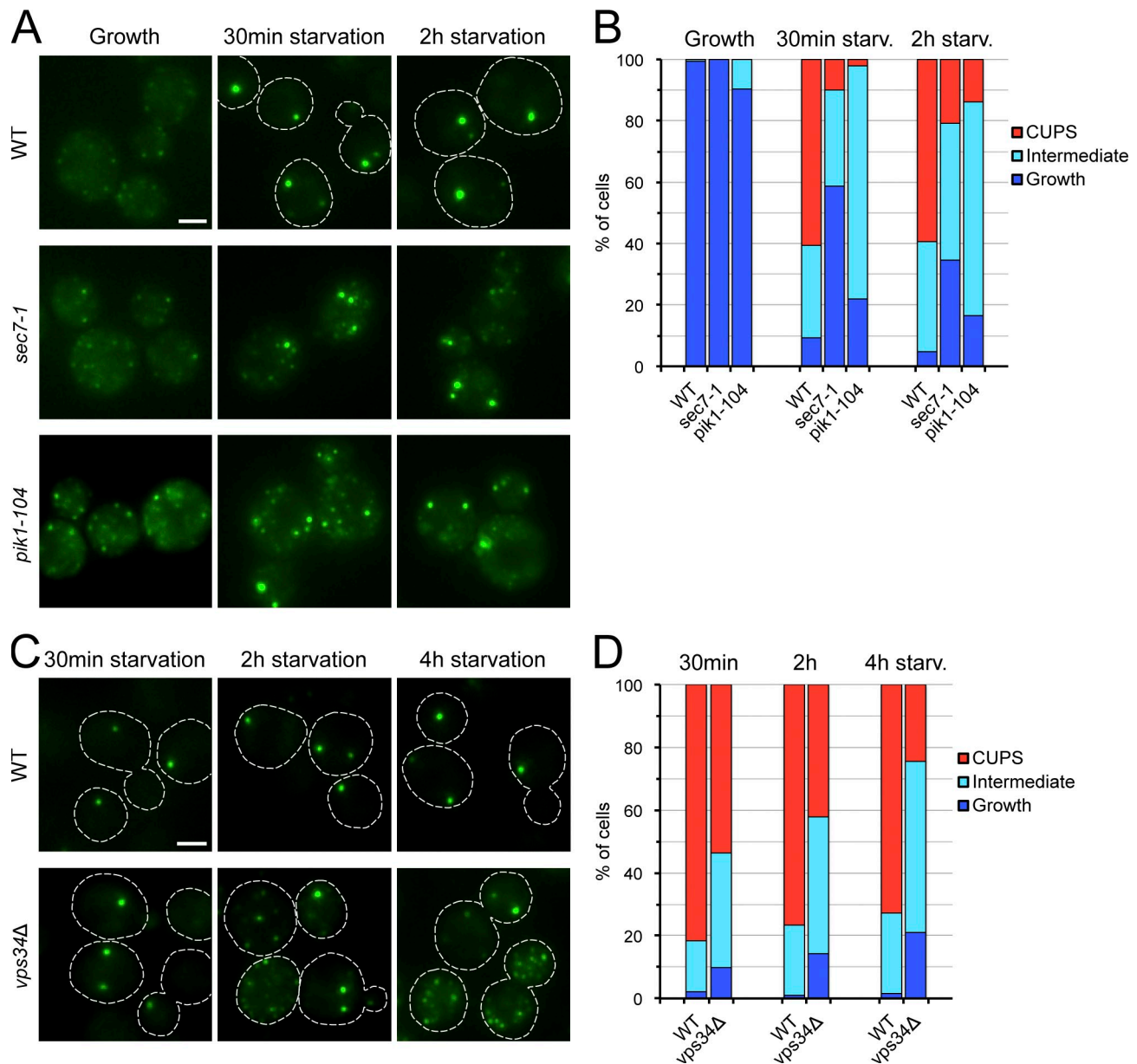


Figure 3. Sec7, Pik1, and Vps34 are required for the formation and/or maintenance of CUPS. (A) Wild-type, *sec7-1*, and *pik1-104* cells expressing Grh1-2xGFP were processed and analyzed as described in Fig. 2. (B) Quantification of the phenotypes observed for the localization of Grh1-2xGFP in A. The data shown are from a single representative experiment out of three repeats. For each condition in the experiment shown, $n \geq 50$ cells. (C) Wild-type and *vps34Δ* cells expressing Grh1-2xGFP were grown to mid-log phase, then starved for 4 h and observed by fluorescence microscopy within 0.5, 2, and 4 h of starvation. (D) Quantification of the different Grh1-2xGFP distributions observed in the conditions tested in C. The data shown are from a single representative experiment out of three repeats. For each condition in the experiment shown, $n \geq 180$ cells. Broken lines indicate cell boundaries. Bars, 2 μm .

that PI3P production by Vps34 is not necessary for CUPS biogenesis but is instead required for the maintenance of Grh1-containing CUPS during starvation.

Next, we tested whether CUPS contain PI4P and PI3P, the enzymatic products of Pik1 and Vps34, respectively. Cells coexpressing Grh1-2xGFP and the phosphoinositide-specific probes mCherry-Osh2-2xPH or mRFP-EEA1-FYVE were visualized by fluorescence microscopy at different time points after starvation. Our results revealed that CUPS do not contain the amount of PI4P and PI3P measured by this approach (Fig. S3, A and B). These findings suggest that Pik1 and Vps34 are required for the export of specific cargoes from Golgi

and endosomes, respectively, and not for their activities at the CUPS.

The biogenesis of CUPS is induced by glucose starvation

Incubation of cells in 2% potassium acetate triggers the formation of CUPS. However, this treatment could potentially be signaling via glucose- and/or nitrogen-sensing pathways. We therefore tested specifically whether CUPS form upon nitrogen or glucose starvation. Mid-log phase cells expressing Grh1-2xGFP were washed, placed in medium lacking either glucose or nitrogen, and visualized by fluorescence microscopy after

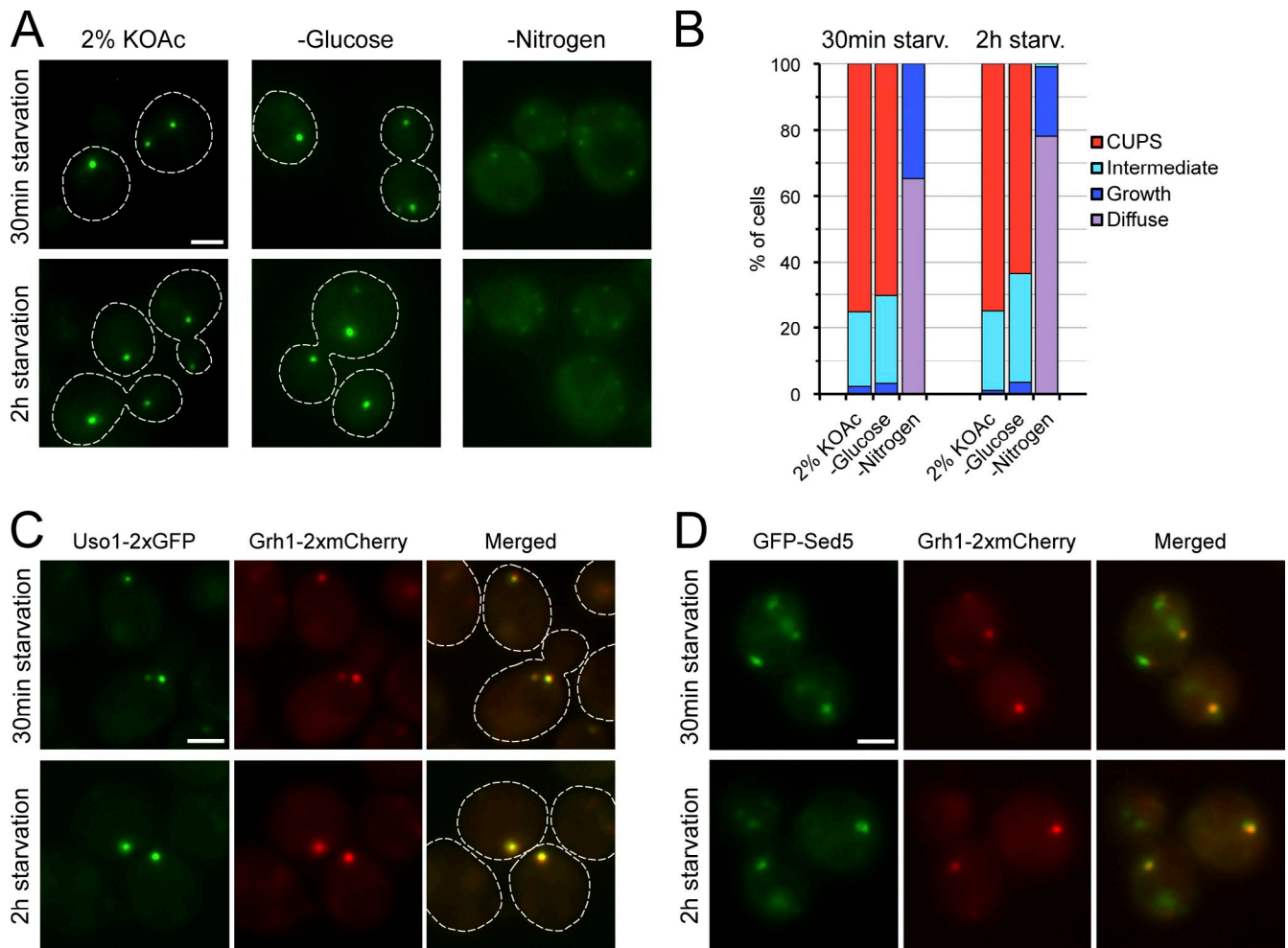


Figure 4. CUPS formation is specifically triggered by glucose deprivation. (A) Grh1-2xGFP-expressing cells were grown in rich medium, washed, and cultured in 2% potassium acetate, SC-D (–glucose), or SD-N (–nitrogen) media for 0.5 or 2 h and visualized by fluorescence microscopy. (B) Quantification of the phenotypes observed for the distribution of Grh1-2xGFP in A. The data shown are from a single representative experiment out of two repeats. For each condition in the experiment shown, $n \geq 120$ cells. (C and D) Cells coexpressing Uso1-2xGFP and Grh1-2xmCherry (C) or GFP-Sed5 and Grh1-2xmCherry (D) were grown in complete medium, then centrifuged and resuspended in SC-D medium to induce glucose deprivation and visualized by fluorescence microscopy at 0.5 and 2 h after incubation in the starvation medium. Broken lines indicate cell boundaries. Bars, 2 μ m.

30 min and 2 h of incubation. Grh1 relocated to CUPS upon glucose deprivation, whereas it displayed a diffuse distribution with some faint cytoplasmic puncta in nitrogen-starved cells (Fig. 4, A and B). Colocalization analysis in cells coexpressing Grh1-2xmCherry and either Uso1-2xGFP or GFP-Sed5 revealed that both Uso1 and Sed5 are found in CUPS at 30 min and 2 h after glucose deprivation (Fig. 4, C and D). However, Anp1-RFP and Sec7-2xmCherry, two Golgi markers excluded from CUPS in potassium acetate-starved cells (Bruns et al., 2011), did not colocalize with Grh1-2xGFP-containing CUPS in cells starved for glucose for 2 h (Fig. S3, C and D). These results highlight the involvement of signals that are triggered upon glucose starvation in the events leading to CUPS biogenesis. In addition, our data reveal that CUPS and the enlarged Sec7-containing Golgi membranes observed in glucose-deprived cells (Aoh et al., 2011) are different compartments.

We previously reported the location of the pre-autophagosome markers Atg8 and Atg9 to CUPS (Bruns et al., 2011). However, our data indicate that COPII-mediated transport

is not required for CUPS biogenesis, and that their formation is not activated by nitrogen starvation or rapamycin treatment but is rather induced by glucose deprivation. This contrasts with the assembly of pre-autophagosomal structures that requires COPII-dependent transport (Graef et al., 2013), and it is activated by rapamycin or nitrogen starvation. Thus, we reevaluated the localization of Atg8 and Atg9 in potassium acetate-starved cells. Our results revealed a lack of colocalization of mCherry-Atg8 and mRFP-Atg9 with Grh1-2xGFP-containing CUPS in a significant number of cells (Fig. S3 E). We cannot rule out the possibility that these components are recruited transiently and thus escape our detection, but they are clearly not a stable feature of CUPS.

CUPS are reabsorbed into the ER upon return to growing conditions

Grh1 relocates from CUPS to ER exit sites/early Golgi when starved cells are incubated in nutrient-rich medium (Fig. 5 A; Bruns et al., 2011). Grh1 is a peripheral membrane protein, and

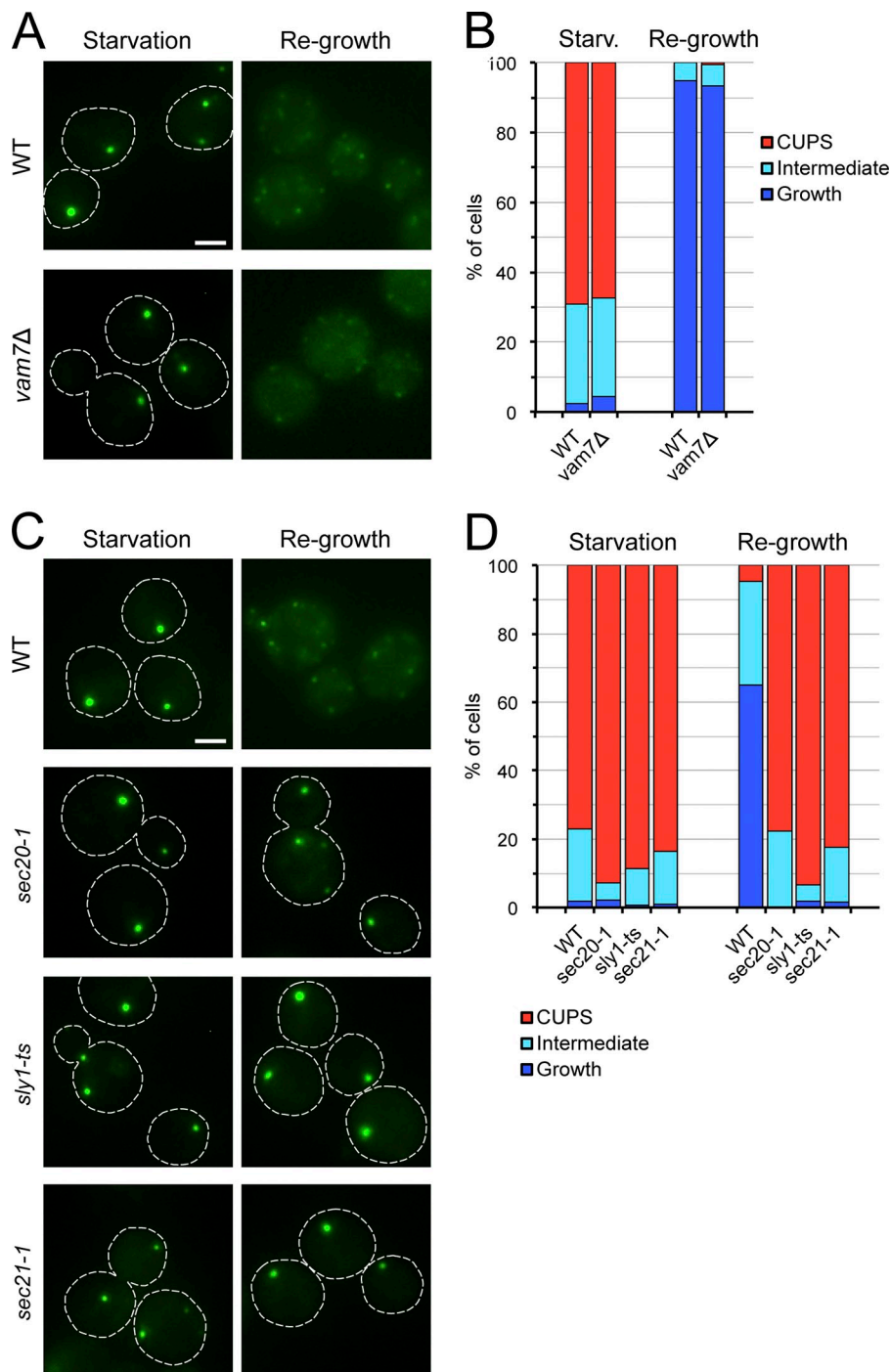


Figure 5. Retrograde transport to the ER is required for Grh1 relocation upon return to growth conditions. (A) Wild-type and *vam7Δ* strains expressing Grh1-2×GFP were starved in 2% potassium acetate for 3 h at 25°C and observed by fluorescence microscopy, or washed to remove the starvation medium and cultured in complete medium for 1 h before examination by fluorescence microscopy. (B) Quantification of the Grh1-2×GFP distribution observed in A. The data shown are from a single representative experiment out of two repeats. For each condition in the experiment shown, $n \geq 130$ cells. (C) Wild-type, *sec20-1*, *sly1-ts*, and *sec21-1* cells expressing Grh1-2×GFP were starved for 2 h at 25°C, then shifted to 37°C for 1 h followed by incubation in nutrient-rich medium for 1 h at 37°C. Cells were observed by fluorescence microscopy just before incubation in rich medium and within 1 h of incubation in rich medium. (D) Quantification of the phenotypes observed for the distribution of Grh1 in C. The data shown are from a single representative experiment out of two repeats. For each condition in the experiment shown, $n \geq 90$ cells. Broken lines indicate cell boundaries. Bars, 2 μm .

this finding therefore does not reveal whether CUPS are reabsorbed into the ER, the Golgi, or any other membrane compartment such as the vacuole. We first asked if Grh1 relocation was affected in a *vam7Δ* strain, which is defective in membrane fusion with the vacuole (Sato et al., 1998). Deletion of *VAM7* did not alter the distribution of Grh1-2×GFP in starved cells or in cells returned to nutrient-rich conditions as compared with the wild-type strain (Fig. 5, A and B), which indicates that CUPS are not reabsorbed into the vacuole upon return to growth conditions.

Next, we investigated whether fusion with ER membranes is required for the relocation of Grh1 after return to

growth medium. To test this, we used a strain harboring a temperature-sensitive mutation in Sec20, an ER-localized SNARE-like protein involved in ER membrane fusion (Cosson et al., 1997; Lewis et al., 1997). Wild-type and *sec20-1* cells expressing Grh1-2×GFP were starved for 2 h at 25°C to allow CUPS formation, then shifted to 37°C and further incubated for 1 h followed by washing to remove the starvation medium and incubation in nutrient-rich medium for 1 h at 37°C. Upon return to growth conditions at 37°C, Grh1-2×GFP relocated from CUPS to multiple small puncta in wild-type cells, whereas it remained in a CUPS-like distribution in *sec20-1* cells (Fig. 5, C and D). To ascertain further the role of ER membrane

fusion by a Sec20-mediated reaction, we tested the requirement for Sly1, a protein of the Sec1/Munc-18 family that is needed for this membrane fusion event (Li et al., 2005). Inactivation of Sly1 by incubation of starved *sly1-ts* cells at 37°C prevented the redistribution of Grh1-2xGFP after returning to nutrient-replete medium (Fig. 5, C and D). These results indicate that upon return to normal growth conditions, CUPS are absorbed into the ER by a Sec20- and Sly1-dependent membrane fusion process.

Does ER reabsorption of CUPS membranes require COPI vesicle-mediated membrane transport? To address this question, Grh1-2xGFP distribution was analyzed in *sec21-1* cells, which exhibit defects in membrane transport to the ER at nonpermissive temperatures (Letourneur et al., 1994). *Sec21-1* cells failed to relocate Grh1 from CUPS when starved cells were incubated in nutrient-rich medium at 37°C (Fig. 5, C and D). Thus, CUPS membranes are consumed by COPI vesicle-dependent traffic.

In summation, our data suggest that CUPS form by the fusion of at least two classes of membranes. One is derived from early Golgi that contain Grh1, Bug1, the membrane tethering factor Uso1, and the t-SNARE Sed5. It is important to note that not all Sed5 relocated to CUPS in starved cells, which displayed some Sed5-containing elements lacking Grh1. The second are vesicles produced from the late Golgi in a Sec7- and Pik1-dependent manner. The fusion of these two sets of membranes is likely mediated by Sed5 and Uso1, and this structure is the fundamental unit of CUPS. CUPS then mature by addition of membranes from the endosomes by a Vps34-dependent process. We expect that many other components are added to CUPS to obtain a fully functional compartment. Upon returning cells to growth conditions, CUPS fuse by a COPI-, and Sly1-dependent process with Sec20-containing membranes of the ER.

CUPS are devoid of the glycosylating enzymes contained in the Golgi. This is reasonable given that these modifications are not required for the sorting, trafficking, and function of signal sequence-lacking proteins such as Acb1 that cannot enter the ER. This also explains the lack of COPI involvement in CUPS biogenesis. However, proteins such as Acb1 still have to be sorted from the total pool of cytosolic proteins and released in a signal-dependent manner. CUPS, a hybrid of the secretory and endosomal compartments, likely provide this sorting function by recruiting Acb1, or a processing activity, for its export from cells without involving the functions of ER-Golgi-endosomes. In other words, cells use portions of compartments of the conventional secretory pathway and endosomes to create a new compartment, CUPS, for the export of signal sequence-lacking proteins that are functional in the extracellular space. In higher eukaryotes, many cytokines, galectins, and insulin-degrading enzymes are secreted without the involvement of ER-Golgi (Malhotra, 2013). Do higher eukaryotes also make CUPS in a signal-dependent manner? The organization of yeast and mammalian Golgi is very different. Does the morphology and composition of CUPS vary accordingly? The identification of CUPS in other eukaryotes will help us understand this fundamentally important process.

Materials and methods

Strains

All yeast strains used in this study are listed in Table S1. The parental thermosensitive strains were provided by C. Boone (University of Toronto, Ontario, Canada; Li et al., 2011). Strains expressing C-terminally 2xyeGFP- and/or 2xyomCherry-tagged proteins were constructed by a PCR-based targeted homologous recombination using the pYM-2xyeGFP-HIS3MX6 and pFA6a-link-2xyomCherry-CaURA3 plasmids (Janke et al., 2004). Deletion of *GRH1* or *VPS34* was performed by replacing the open reading frame of interest with a KanMX4 cassette using PCR-based targeted homologous recombination. Strains coexpressing Grh1-2xmCherry and Mnn2-GFP or Mnn5-GFP, and those coexpressing Grh1-2xGFP and Anp1-RFP were made by mating the pertinent haploids, followed by sporulation and selection of the appropriate spore-derived clones.

Plasmids

pYM-2xyeGFP-HIS3MX6 was constructed by inserting a linker-yeGFP fragment (linker sequence: GAGAIL) in frame with the C terminus of the yeGFP in the pYM44 plasmid (Janke et al., 2004) by Gibson assembly (Gibson et al., 2009) with two PCRs using the following primers: 5'-TGGTATGGATGAATTGTACAAAGGTGCTGGAGCAATTCTGTCT-3' and 5'-AGTGGC-GCGCAGTTATTGTACAATTC-3' to amplify the linker-yeGFP fragment from the pYM44 plasmid, and 5'-TAACCTGCGGCCACTTCTAAATAAG-3' and 5'-TTTGTACAATTCATCCATACCATGGGTAATACCA-3' to amplify the linearized pYM44 plasmid. To generate the pFA6a-link-2xyomCherry-CaURA3 plasmid, a linker-yomCherry fragment (linker sequence: GSG-AGAGAGAGA) was amplified by PCR using the pFA6a-link-yomCherry-CaURA3 plasmid (Sheff and Thorn, 2004) as a template and the primers 5'-ATAGTCGACGGATCCGGAGCAGGTGCTGGTGTGCTGGAG-CAGTTAGCAAAGGCGAGGAAGATAACATG-3' and 5'-ACATAATTAA-TGCTCCAGCACCAGCACCTTGTACAGTTCATCCATACCACCTG-3', and cloned in frame with the N terminus of the yomCherry in the pFA6a-link-yomCherry-CaURA3 plasmid using the Sall and PacI restriction sites. SnapGene software (GSL Biotech; available at www.snapgene.com) was used for molecular cloning procedures. The centromeric plasmid for expression of GFP-Sed5 under the control of the ADH promoter (pRS315-prADH-GFP-Sed5) was provided by J. Gerst (Weizmann Institute of Science, Rehovot, Israel; Weinberger et al., 2005). The episomal plasmid coding for two tandem copies of the Osh2 PH domain fused to mCherry (pRS425-prGDP-mCherry-2xPH^{Osh2}) was provided by S. Emr (Cornell University, Ithaca, NY). The plasmids for the expression of mRFP-EEA1-FYVE (pRS416-prADH-mRFP-FYVE^{EEA1}), mCherry-Atg8 (pRS316-prATG8-mCherry-Atg8), and mRFP-Atg9 (pRS416-prCUP1-mRFP-Atg9) have been described previously (Bruns et al., 2011).

Media and medium shift procedures

Yeast cells were grown in synthetic complete (SC) media (0.67% yeast nitrogen base without amino acids, 2% glucose supplemented with amino acid drop-out mix; Sigma-Aldrich). Glucose starvation medium (SC-D) consisted of SC medium that lacked glucose. The nitrogen starvation medium (SD-N) consisted of 0.17% yeast nitrogen base lacking amino acids and ammonium sulfate, and 2% glucose. To induce nutrient starvation, mid-log phase cells were harvested by centrifugation at 3,000 g, washed twice in 2% potassium acetate, SC-D, or SD-N, and subsequently incubated in 2% potassium acetate, SC-D, or SD-N at 1 OD_{600nm}/ml for the indicated time intervals. For re-feeding experiments, starved cells were collected by centrifugation, resuspended in SC medium at 0.3 OD_{600nm}/ml, and incubated for 1 h. Unless otherwise indicated, cells were grown at 25°C.

Epifluorescence microscopy

After incubation in the appropriate medium, ~1.5 OD_{600nm} of cells were harvested by centrifugation at 3,000 g for 3 min, resuspended in a small volume of the corresponding medium, spotted on a microscopy slide, and subsequently live-imaged at 25°C with a DM16000 B epifluorescence microscope (Leica) equipped with a DFC 360FX camera (Leica) using an HCX Plan Apochromat 100x 1.4 NA objective lens. Images were acquired using LAS AF software (Leica) with 0.5–2 s exposure times. For the quantification of the localization phenotypes of Grh1-2xGFP, whole cell z stacks with a step size of 0.4 μm were captured as described above. The corresponding maximum-projection images were created with ImageJ 1.45r and the number of cells showing the following phenotypes for the distribution of Grh1-2xGFP was manually counted: diffuse, growth (>5 small punctate structures), CUPS (1–3 large dots), or intermediate (1–3 large dots and several small punctate structures). At least three microscopic fields (≥50 cells) were examined for each condition. Results represent the percentage of cells with each distribution phenotype and are representative of at least two experiments.

Confocal fluorescence microscopy

After incubation in starvation medium for 1.5 h, ~ 0.05 OD_{600nm} of cells were plated in starvation medium on Concanavalin A-coated (Sigma-Aldrich) Lab-Tek chambers (Thermo Fisher Scientific) and were allowed to settle for 30 min at 25°C. Then, whole cell z stacks with a step size of 0.3 μ m were acquired using a spinning-disk confocal microscope (Revolution XD; Andor Technology) with a Plan Achromat 100 \times 1.45 NA objective lens equipped with a dual-mode electron-modifying charge-coupled device camera (iXon 897 E; Andor Technology) and controlled by the iQ Live Cell Imaging software (Andor Technology). Colocalization coefficients were calculated with ImageJ 1.45r and the JACoP plugin (Bolte and Cordelières, 2006).

Online supplemental material

Fig. S1 shows the high degree of colocalization between Grh1 and Uso1 or Sed5 and the low colocalization between Grh1 and the early Golgi enzymes Mnn2 or Mnn5 in starved cells. Fig. S2 shows that the early Golgi marker Anp1-RFP does not accumulate in CUPS formed in the COPII or COPI mutant cells. Fig. S3 shows the lack of localization of mCherry-Osh2-2 \times PH, mRFP-EEA1-FYVE, Anp1-RFP, Sec7-2 \times mCherry, mCherry-Atg8, and mRFP-Atg9 in Grh1-2 \times GFP-labeled CUPS. Table S1 lists the yeast strains used in this study. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201407119/DC1>.

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References

- Achstetter, T., A. Franzusoff, C. Field, and R. Schekman. 1988. SEC7 encodes an unusual, high molecular weight protein required for membrane traffic from the yeast Golgi apparatus. *J. Biol. Chem.* 263:11711–11717.
- Anjard, C., and W.F. Loomis. 2005. Peptide signaling during terminal differentiation of *Dicystostelium*. *Proc. Natl. Acad. Sci. USA.* 102:7607–7611. <http://dx.doi.org/10.1073/pnas.0501820102>
- Aoh, Q.L., L.M. Graves, and M.C. Duncan. 2011. Glucose regulates clathrin adaptors at the trans-Golgi network and endosomes. *Mol. Biol. Cell.* 22:3671–3683. <http://dx.doi.org/10.1091/mbc.E11-04-0309>
- Banfield, D.K., M.J. Lewis, and H.R. Pelham. 1995. A SNARE-like protein required for traffic through the Golgi complex. *Nature.* 375:806–809. <http://dx.doi.org/10.1038/375806a0>
- Behnia, R., F.A. Barr, J.J. Flanagan, C. Barlowe, and S. Munro. 2007. The yeast orthologue of GRASP65 forms a complex with a coiled-coil protein that contributes to ER to Golgi traffic. *J. Cell Biol.* 176:255–261. <http://dx.doi.org/10.1083/jcb.200607151>
- Bolte, S., and F.P. Cordelières. 2006. A guided tour into subcellular colocalization analysis in light microscopy. *J. Microsc.* 224:213–232. <http://dx.doi.org/10.1111/j.1365-2818.2006.01706.x>
- Bruns, C., J.M. McCaffery, A.J. Curwin, J.M. Duran, and V. Malhotra. 2011. Biogenesis of a novel compartment for autophagosome-mediated unconventional protein secretion. *J. Cell Biol.* 195:979–992. <http://dx.doi.org/10.1083/jcb.201106098>
- Cao, X., N. Ballew, and C. Barlowe. 1998. Initial docking of ER-derived vesicles requires Uso1p and Ypt1p but is independent of SNARE proteins. *EMBO J.* 17:2156–2165. <http://dx.doi.org/10.1093/emboj/17.8.2156>
- Cosson, P., S. Schröder-Köhne, D.S. Sweet, C. Démollière, S. Hennecke, G. Frigerio, and F. Letourneur. 1997. The Sec20/Tip20p complex is involved in ER retrieval of dilysine-tagged proteins. *Eur. J. Cell Biol.* 73:93–97.
- Duran, J.M., C. Anjard, C. Stefan, W.F. Loomis, and V. Malhotra. 2010. Unconventional secretion of Acb1 is mediated by autophagosomes. *J. Cell Biol.* 188:527–536. <http://dx.doi.org/10.1083/jcb.200911154>
- Franzusoff, A., K. Redding, J. Crosby, R.S. Fuller, and R. Schekman. 1991. Localization of components involved in protein transport and processing through the yeast Golgi apparatus. *J. Cell Biol.* 112:27–37. <http://dx.doi.org/10.1083/jcb.112.1.27>
- Gibson, D.G., L. Young, R.Y. Chuang, J.C. Venter, C.A. Hutchison III, and H.O. Smith. 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods.* 6:343–345. <http://dx.doi.org/10.1038/nmeth.1318>
- Graef, M., J.R. Friedman, C. Graham, M. Babu, and J. Nunnari. 2013. ER exit sites are physical and functional core autophagosome biogenesis components. *Mol. Biol. Cell.* 24:2918–2931. <http://dx.doi.org/10.1091/mbc.E13-07-0381>
- Hardwick, K.G., and H.R. Pelham. 1992. SED5 encodes a 39-kD integral membrane protein required for vesicular transport between the ER and the Golgi complex. *J. Cell Biol.* 119:513–521. <http://dx.doi.org/10.1083/jcb.119.3.513>
- Hosobuchi, M., T. Kreis, and R. Schekman. 1992. SEC21 is a gene required for ER to Golgi protein transport that encodes a subunit of a yeast coatomer. *Nature.* 360:603–605. <http://dx.doi.org/10.1038/360603a0>
- Janke, C., M.M. Magiera, N. Rathfelder, C. Taxis, S. Reber, H. Maekawa, A. Moreno-Borchart, G. Doenges, E. Schwob, E. Schiebel, and M. Knop. 2004. A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. *Yeast.* 21:947–962. <http://dx.doi.org/10.1002/yea.1142>
- Kineth, M.A., C. Anjard, D. Fuller, G. Guizzanti, W.F. Loomis, and V. Malhotra. 2007. The Golgi-associated protein GRASP is required for unconventional protein secretion during development. *Cell.* 130:524–534. <http://dx.doi.org/10.1016/j.cell.2007.06.029>
- Letourneur, F., E.C. Gaynor, S. Hennecke, C. Démollière, R. Duden, S.D. Emr, H. Riezman, and P. Cosson. 1994. Coatomer is essential for retrieval of dilysine-tagged proteins to the endoplasmic reticulum. *Cell.* 79:1199–1207. [http://dx.doi.org/10.1016/0092-8674\(94\)90011-6](http://dx.doi.org/10.1016/0092-8674(94)90011-6)
- Levi, S.K., D. Bhattacharyya, R.L. Strack, J.R. Austin II, and B.S. Glick. 2010. The yeast GRASP Grh1 colocalizes with COPII and is dispensable for organizing the secretory pathway. *Traffic.* 11:1168–1179. <http://dx.doi.org/10.1111/j.1600-0854.2010.01089.x>
- Lewis, M.J., J.C. Rayner, and H.R. Pelham. 1997. A novel SNARE complex implicated in vesicle fusion with the endoplasmic reticulum. *EMBO J.* 16:3017–3024. <http://dx.doi.org/10.1093/emboj/16.11.3017>
- Li, Y., D. Gallwitz, and R. Peng. 2005. Structure-based functional analysis reveals a role for the SM protein Sly1p in retrograde transport to the endoplasmic reticulum. *Mol. Biol. Cell.* 16:3951–3962. <http://dx.doi.org/10.1091/mbc.E05-02-0114>
- Li, Z., F.J. Vizeacoumar, S. Bahr, J. Li, J. Warringer, F.S. Vizeacoumar, R. Min, B. Vandersluis, J. Bellay, M. Devit, et al. 2011. Systematic exploration of essential yeast gene function with temperature-sensitive mutants. *Nat. Biotechnol.* 29:361–367.
- Malhotra, V. 2013. Unconventional protein secretion: an evolving mechanism. *EMBO J.* 32:1660–1664. <http://dx.doi.org/10.1038/emboj.2013.104>
- Manjithaya, R., C. Anjard, W.F. Loomis, and S. Subramani. 2010. Unconventional secretion of *Pichia pastoris* Acb1 is dependent on GRASP protein, peroxisomal functions, and autophagosome formation. *J. Cell Biol.* 188:537–546. <http://dx.doi.org/10.1083/jcb.200911149>
- Nakajima, H., A. Hirata, Y. Ogawa, T. Yonehara, K. Yoda, and M. Yamasaki. 1991. A cytoskeleton-related gene, uso1, is required for intracellular protein transport in *Saccharomyces cerevisiae*. *J. Cell Biol.* 113:245–260. <http://dx.doi.org/10.1083/jcb.113.2.245>
- Nichols, B.J., and H.R. Pelham. 1998. SNAREs and membrane fusion in the Golgi apparatus. *Biochim. Biophys. Acta.* 1404:9–31. [http://dx.doi.org/10.1016/S0167-4889\(98\)00044-5](http://dx.doi.org/10.1016/S0167-4889(98)00044-5)
- Rabouille, C., V. Malhotra, and W. Nickel. 2012. Diversity in unconventional protein secretion. *J. Cell Sci.* 125:5251–5255. <http://dx.doi.org/10.1242/jcs.103630>
- Sato, T.K., T. Darsow, and S.D. Emr. 1998. Vam7p, a SNAP-25-like molecule, and Vam3p, a syntaxin homolog, function together in yeast vacuolar protein trafficking. *Mol. Cell. Biol.* 18:5308–5319.
- Sheff, M.A., and K.S. Thorn. 2004. Optimized cassettes for fluorescent protein tagging in *Saccharomyces cerevisiae*. *Yeast.* 21:661–670. <http://dx.doi.org/10.1002/yea.1130>
- Tu, L., W.C. Tai, L. Chen, and D.K. Banfield. 2008. Signal-mediated dynamic retention of glycosyltransferases in the Golgi. *Science.* 321:404–407. <http://dx.doi.org/10.1126/science.1159411>
- Walch-Solimena, C., and P. Novick. 1999. The yeast phosphatidylinositol-4-OH kinase pik1 regulates secretion at the Golgi. *Nat. Cell Biol.* 1:523–525. <http://dx.doi.org/10.1038/70319>
- Weinberger, A., F. Kamena, R. Kama, A. Spang, and J.E. Gerst. 2005. Control of Golgi morphology and function by Sed5 t-SNARE phosphorylation. *Mol. Biol. Cell.* 16:4918–4930. <http://dx.doi.org/10.1091/mbc.E05-02-0101>