Unconventional secretion of Acb1 is mediated by autophagosomes

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Starving *Dictyostelium discoideum* cells secrete AcbA, an acyl coenzyme A–binding protein (ACBP) that lacks a conventional signal sequence for entering the endoplasmic reticulum (ER). Secretion of AcbA in *D. discoideum* requires the Golgi-associated protein GRASP. In this study, we report that starvation-induced secretion of Acb1, the *Saccharomyces cerevisiae* ACBP orthologue, also requires GRASP (Grh1). This highlights the conserved function of GRASP in unconventional secretion. Although genes required for ER to Golgi or Golgi to cell surface transport are not required for Acb1 secretion in yeast, this process involves autophagy genes and the plasma membrane t-SNARE, Sso1. Inhibiting transport to vacuoles does not affect Acb1 secretion. In sum, our experiments reveal a unique secretory pathway where autophagosomes containing Acb1 evade fusion with the vacuole to prevent cargo degradation. We propose that these autophagosome intermediates fuse with recycling endosomes instead to form multivesicular body carriers that then fuse with the plasma membrane to release cargo.

Introduction

An estimated 30% of human genes encode proteins carrying an N-terminal amino acid sequence that targets them to the ER. Most of these proteins are transported from the ER to the Golgi and are secreted from cells by an extensively studied and well-known conventional mode of protein secretion.

Rubartelli et al. (1990) previously reported that interleukin-1β, which lacks a signal sequence for targeting to the ER, was secreted from cells. This was a surprise at the time, but since then, a large number of proteins have been found that lack a signal sequence and are secreted by eukaryotic cells. This class of proteins includes FGF2, the β-galactoside–specific lectins galectin 1 and 3, blood coagulation factor XIIIa, some members of the interleukin family, macrophage migration inhibitory factor, the engrailed homeoprotein, and the acyl coenzyme A–binding protein (ACBP), AcbA (Grundmann et al., 1988; Lutomski et al., 1997; Joliot et al., 1998; Menon and Hughes, 1999; Flieger et al., 2003; Kinseth et al., 2007; Nickel and Seedorf, 2008).

The best-known example of an unconventionally secreted protein in the yeast *Saccharomyces cerevisiae* is the mating pheromone a-factor (Kuchler et al., 1989). After lipid modification, this protein is secreted directly across the cell surface by a dedicated ABC transporter, Ste6. However, this does not appear to be the mechanism for transport of AcbA because pharmacological inhibition of ABC transporters in *Dictyostelium discoideum* does not affect secretion of this protein (Kinseth et al., 2007). Moreover, secretion of AcbA in *D. discoideum* requires the Golgi membrane–associated protein GRASP, which is not required for secretion of a-factor from yeast (Kinseth et al., 2007). Noncanonical transport of α-integrin in *Drosophila melanogaster* embryos has also been shown to require dGRASP (Schotman et al., 2008). The phosphoinositide lipid PI(4,5)P$_2$ is necessary for the unconventional secretion mechanism of FGF2 in mammalian cells (Temmerman et al., 2008), but little else is known about its secretory pathway.

We have analyzed the *S. cerevisiae* homologue of AcbA, Acb1, which also lacks a signal sequence. Surprisingly, when
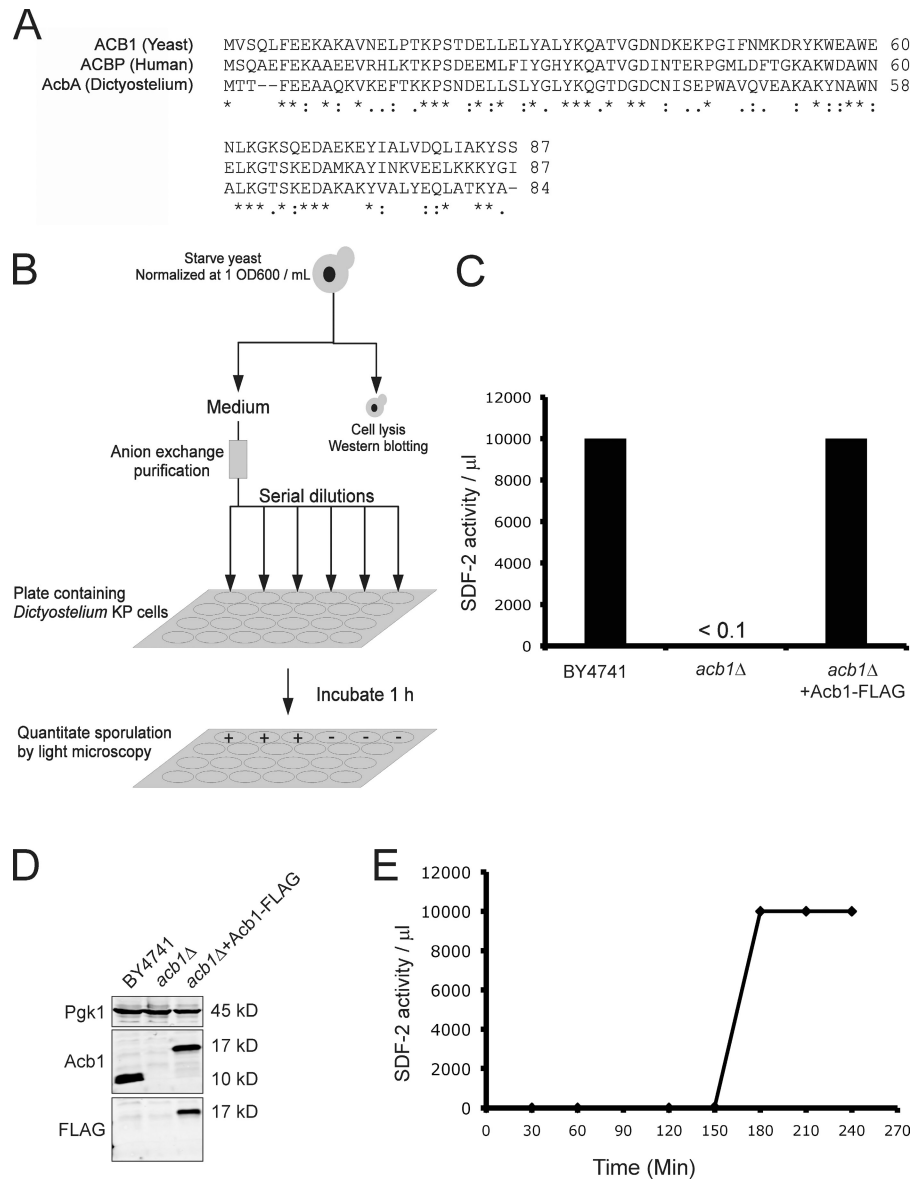
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Figure 1. **Yeast release SDF-2–like material.**
(A) Amino acid sequence of the AcbA homologues in *S. cerevisiae*, *D. discoideum*, and *Homo sapiens* are aligned. (B) The assay for the activity of Acb1. Yeast cells were starved for the time indicated. The cells were separated from the medium by low speed centrifugation and lysed. The lysates were used for detection of the intracellular levels of Acb1 and the control protein 3-phosphoglycerate kinase (Pgk1). The medium was processed to concentrate Acb1 activity, which was then tested for its ability to sporulate *D. discoideum* KP cells and quantified. (C) Developed KP cells were incubated with buffer in which yeast had been starved for 4 h and the number of spores counted 1 h later. (D) Protein levels were assessed by Western blotting the yeast cell lysates with an anti-Pgk1p (3-phosphoglycerate kinase) antibody, which is a cytosolic protein used as a control, and anti-Acb1. Anti-Flag antibody shows expression of the tagged version of Acb1-Flag. (E) Time course of secretion. Yeast cells were starved, and samples were taken every 30 min up to 4 h. The buffer was assayed for SDF-2–like activity. The data show an average of three experiments.

A

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ACB1 (Yeast)        MVSQLFEEKAKAVNELPTKPSTDELLELYALYKQATVGDNDKEKPGIFNMKDRYKWEAWE 60
ACBP (Human)        MSQAEFEKAAEEVRHLKTKPSDEEMLFIYGHYKQATVGDINTERPGMLDFTGKAKWDAWN 60
AcbA (Dictyostelium) MTT--FEEAAQKVKEFTKKPSNDELLSLYGLYKQGTDGDCNISEPWAVQVEAKAKYNAWN 58
                    *    **: *: *..: .*** :*:* :*. ***.* ** : ..*  .:.  : *::**:

                    NLKGKSQEDAEKEYIALVDQLIAKYSS 87
                    ELKGTSKEDAMKAYINKVEELKKKYGI 87
                    ALKGTSKEDAKAKYVALYEQLATKYA- 84
                    ***.*:***   *:   ::* **.
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B

C

D

E

yeast cells are starved for nitrogen, we found that they secrete Acb1 into the medium where it is processed into signals that can stimulate rapid encapsulation of *D. discoideum*. Secretion of Acb1 requires autophagy genes and membrane fusion with the plasma membrane in a process that cooperates with the Golgi GRASP protein Grh1, the early endosomal compartments, and the multivesicular body (MVB) sorting pathway. The discussion of this unique secretory pathway follows.

Results

Starvation induces secretion of Acb1 in *S. cerevisiae*

The genome of *S. cerevisiae* contains a single gene (*ACB1*) that encodes a protein similar to *D. discoideum* AcbA and human ACBP (Fig. 1 A). All three homologues are small (10 kD) proteins that lack signal sequences. Late in development of *D. discoideum*, AcbA is secreted and proteolytically processed into a 34–amino acid peptide, SDF-2, which triggers rapid

encapsulation of prespore cells (Anjard and Loomis, 2005). Likewise, human ACBP is secreted from glial cells and processed into the peptides TTN and ODN, which modulate the GABA$_A$ ionotrophic receptor (Costa and Guidotti, 1991). The similarity of the human peptides to SDF-2 is sufficient to stimulate rapid encapsulation of *D. discoideum* prespore cells, although the affinity is ~1,000-fold lower than for SDF-2, which is active at 1 pM. The yeast Acb1 protein is also highly similar to AcbA. Therefore, we used the *D. discoideum* bioassay to determine whether Acb1 was secreted and processed into active peptides (Fig. 1 B).

Because production of SDF-2 is a developmentally regulated process in *D. discoideum* initiated by removal of nutrients, we starved growing *S. cerevisiae* cells in acetate buffer for 4 h before collecting the medium and testing for SDF-2–like activity. The medium was passed over an anion exchange resin that concentrates and purifies SDF-2, and the eluate was serially diluted before adding to *D. discoideum* KP cells that had developed at low density for 18 h in buffer containing cAMP.
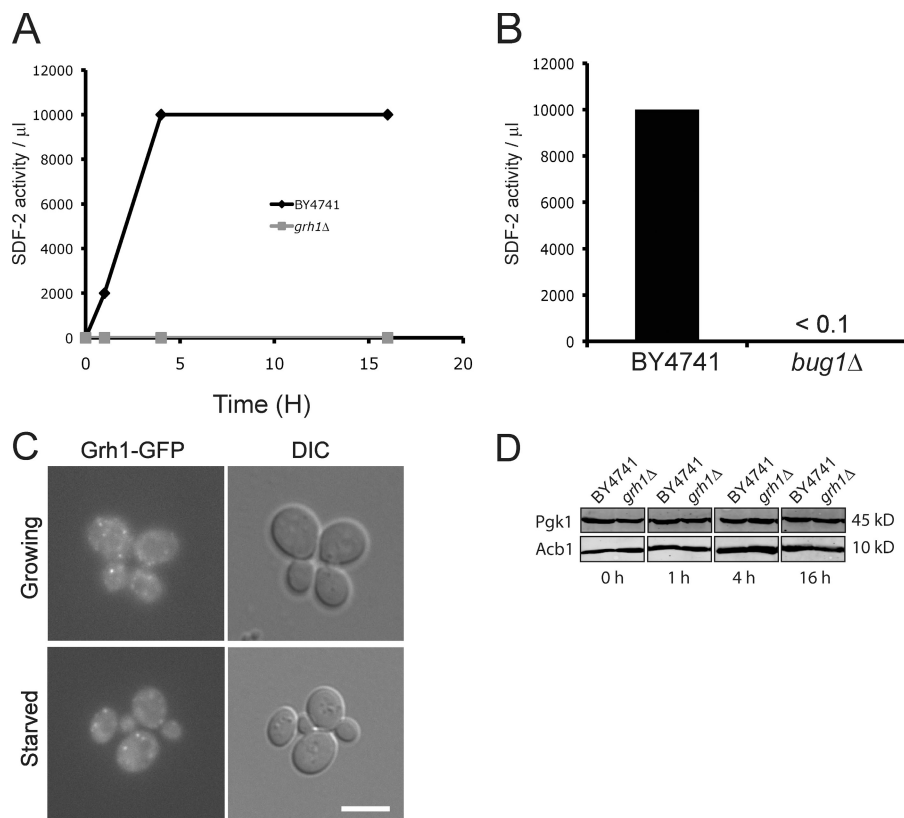
Figure 2. **GRASP is required for Acb1 secretion.** (A) Wild-type and grh1Δ yeast were incubated in starvation buffer for 0, 1, 4, and 16 h, and the supernatant was tested in the bioassay. (B) Wild-type and bug1Δ yeast were starved for 4 h and the supernatant analyzed in the bioassay. (C) A wild-type strain expressing a GFP-tagged version of Grh1 was starved for 4 h and visualized by fluorescence microscopy. Starvation does not change the punctate pattern of Grh1 localization. Bar, 5 µm. (D) Cells were treated as in A, and the cell lysates were analyzed to monitor the respective protein levels. Deletion of Grh1 does not affect the intracellular levels of 3-phosphoglycerate kinase or Acb1. The data show an average of three experiments.

We found that we could dilute the purified fraction released by yeast 10,000-fold and still induce rapid encapsulation of the tester KP cells (Fig. 1 C).

To directly show that the activity was dependent on Acb1, we tested medium from cultured mutant cells lacking the *ACB1* gene. After 4 h of nitrogen starvation, mutant *acb1*Δ yeast cells failed to secrete any material that was active in the bioassay (Fig. 1 C). However, *acb1*Δ cells that expressed a tagged version of Acb1 from a centromeric plasmid were found to secrete as much SDF-2–like activity as wild-type cells (Fig. 1 C). Thus, the defect in the *ACB1* deletion strain in the bioassay was solely the result of the lack of Acb1. We used rabbit polyclonal anti-Acb1 antisera to confirm that Acb1 was absent from *acb1*Δ cell extracts (Fig. 1 D). We also confirmed that a band of the expected size was recognized by both anti-Acb1 and anti-Flag antibodies from extracts of *acb1*Δ cells expressing Acb1-Flag from a plasmid (Fig. 1 D).

SDF-2 is recognized in *D. discoideum* KP cells by the receptor histidine kinase DhkA that controls the activity of an intracellular cAMP phosphodiesterase (Anjard and Loomis, 2005). *D. discoideum* KP cells lacking DhkA fail to respond to SDF-2. Likewise, mutant *D. discoideum* KP cells lacking DhkA fail to respond to the activity purified from the media of starved wild-type yeast cells, showing that the same receptor is responsible for response to SDF-2 and the yeast Acb1-derived signal (unpublished data).

We next determined whether release of the Acb1-derived signal from yeast cells was continuous or regulated at a particular stage during starvation. Media aliquots were collected for bioassay from a wild-type yeast culture every 30 min after the shift to starvation conditions. No activity was observed in response to media samples harvested during the first 150 min of starvation. Subsequently, bioassay activity increased rapidly and reached peak activity within 30 min (Fig. 1 E). There was no further increase in activity after 3 h of starvation, indicating that SDF-2–like material is produced in a concerted, regulated pulse. These results, indicating that no activity was detected before 150 min of starvation and that there was no increase in the activity for 2.5–3 h after nitrogen starvation, argue against the possibility that cells undergo random lysis and release of Acb1 during starvation. Moreover, yeast cells remain viable for >3 h in starvation medium and resume growth when shifted back to rich medium (unpublished data).

The Golgi-associated GRASP protein Grh1 is required for unconventional Acb1 secretion

The Golgi membrane–associated protein GRASP in *D. discoideum* is required for secretion of AcbA (Kinseth et al., 2007). Therefore, we asked whether this function of GRASP is conserved in *S. cerevisiae*. Yeast cells lacking the GRASP gene (*GRH1*) were tested for Acb1 secretion with the bioassay. The starvation media prepared from *grh1*Δ cell cultures lacked SDF-2–like activity even after 16 h of starvation (Fig. 2 A). The absence of activity in the *grh1*Δ culture medium was not caused by changes in the intracellular levels of Acb1 in *grh1*Δ cells as compared with wild-type cells (Fig. 2 D).

In yeast, the protein Bug1 mediates localization of Grh1 to Golgi membrane compartments (Behnia et al., 2007). Yeast cells lacking Bug1 were incubated under starvation conditions

and tested for Acb1-derived signal secretion. The *bug1Δ* mutant cells failed to produce any SDF-2–like activity (Fig. 2 B). We also tested whether starvation altered the normal location of Grh1. Yeast expressing Grh1-GFP were kept in rich medium (YPD) or starvation medium and visualized by fluorescence microscopy. Under both conditions, Grh1 shows a similar punctate, intracellular distribution (Fig. 2 C). Together, these findings indicate an essential role for membrane-associated Grh1 in secretion of Acb1 in yeast.

The conventional secretory pathway is not required for secretion of Acb1

Acb1 lacks a conventional signal sequence for targeting to the ER and is therefore most likely secreted without entering the ER–Golgi pathway. To provide formal proof that secretion of Acb1 is independent of the conventional early secretory pathway and to further explore how Acb1 is secreted, we tested several yeast *sec* mutants for Acb1 secretion. For this, we used temperature-conditional alleles of *SEC23* that encode a COPII component required for cargo export from the ER, *SEC7* that encodes an Arf guanine nucleotide exchange factor required for export from the Golgi, and *SEC1* that encodes a regulator of secretory vesicle fusion with the plasma membrane (Barlowe et al., 1994; Sata et al., 1998; Carr et al., 1999). As a control and to assess whether Acb1 secretion is mediated by membrane trafficking, we also included the *sec18-1* temperature-conditional mutant. Loss of Sec18/NSF function impairs several intracellular SNARE-dependent membrane fusion events (Graham and Emr, 1991). Mutant yeast cells were incubated at permissive (25°C) and nonpermissive (37°C) temperatures under starvation conditions, and the media from each condition were prepared and tested in the bioassay. Strikingly, the *sec23*, *sec7*, and *sec1* mutants produced SDF-2–like material at both permissive and nonpermissive temperatures, whereas the NSF mutant (*sec18-1*) only produced activity at the permissive temperature (Fig. 3 A). Intracellular levels of Acb1 and a control cytosolic protein (Pgk1) were monitored to ensure that *sec* mutants do not alter the expression of these proteins (Fig. 3 B). These findings demonstrate that the secretion of Acb1 in yeast is not via the conventional ER to Golgi to cell surface pathway. But the fact that Sec18/NSF is required for Acb1 secretion implies the involvement of a SNARE-dependent membrane fusion event.

As mentioned, the yeast mating pheromone a-factor lacks a signal sequence and is secreted without entering the ER–Golgi pathway. This peptide pheromone is translocated across the plasma membrane by the ABC transporter, Ste6 (Kuchler et al., 1989). We have shown previously that in *D. discoideum*, chemical inhibition of ABC transporters did not affect AcbA secretion (Kinseth et al., 2007). In this study, we have found that MAT-α haploid yeast cells that do not express the Ste6 ABC transporter fully secrete Acb1-derived signal activity (see Fig. 5), thus excluding a role for Ste6 function in this pathway. Our result that Sec18/NSF was required for Acb1 secretion suggests the involvement of a vesicular intermediate that fuses with the plasma membrane. Whether Acb1 secretion in yeast is further mediated by the function of
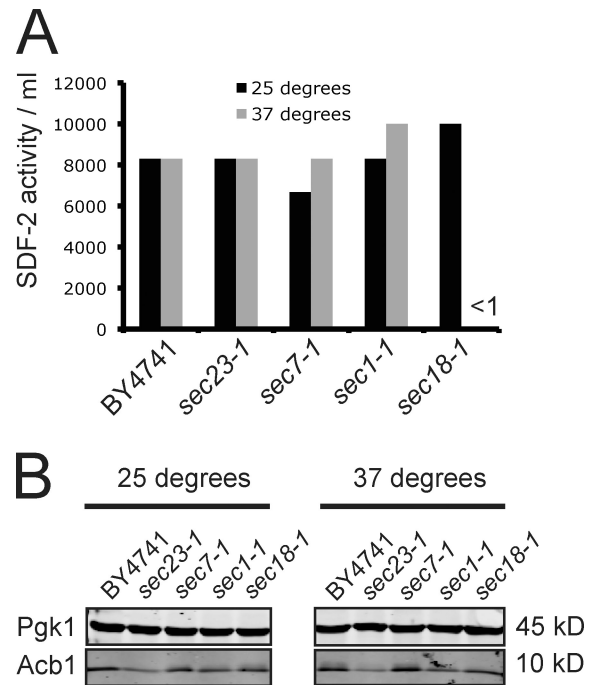

Figure 3. **Conventional secretory pathway is not required for Acb1 secretion.** (A) Yeast were grown in rich medium at 25°C, washed, and starved for 4 h in acetate buffer at the permissive (25°C) or restrictive temperature (37°C). SDF-2–like activity was determined in the bioassay. The data show an average of three experiments. (B) Cells from A were lysed and Western blotted with anti-Pgk1 and anti-Acb1 antibodies to monitor protein levels at the permissive and nonpermissive temperature.

plasma membrane ABC transporters, other than Ste6, remains to be tested.

Acb1 secretion requires autophagy genes

We have previously suggested the involvement of an exosome/autophagosome-like intermediate in AcbA secretion, and our new data prompted us to formally test this working hypothesis (Kinseth et al., 2007). Our previous data (Fig. 1 E) indicated that Acb1 secretion is significantly delayed from onset of starvation, suggesting that specialized membrane compartments may be generated for this process. Autophagosomes are vesicles with double membranes that assemble upon nutrient starvation in all eukaryotic cells. Once formed, the outer membrane of the autophagosome usually fuses with the vacuolar membrane, delivering its cargo into the vacuolar lumen (Klionsky and Emr, 2000; Lum et al., 2005). We reasoned that a similar process could be used to incorporate yeast Acb1 into a specialized autophagosome, which then fuses with the plasma membrane rather than the vacuole. To test this hypothesis, we examined *atg5*-, *atg7*-, *atg8*-, and *atg12*-null mutants that are impaired in various stages of autophagy (Mizushima et al., 1998; Kuma et al., 2002; Nakatogawa et al., 2007; Xie et al., 2008) for production of Acb1-derived activity in the bioassay. Each of the *ATG* gene deletion mutants failed to produce SDF-2–like material, suggesting that components of the autophagy pathway are necessary for secretion of Acb1 (Fig. 4 A). The lack of SDF-2–like activity cannot be attributed to a general failure in protein synthesis under amino acid deprivation in the *atg* mutants, as the
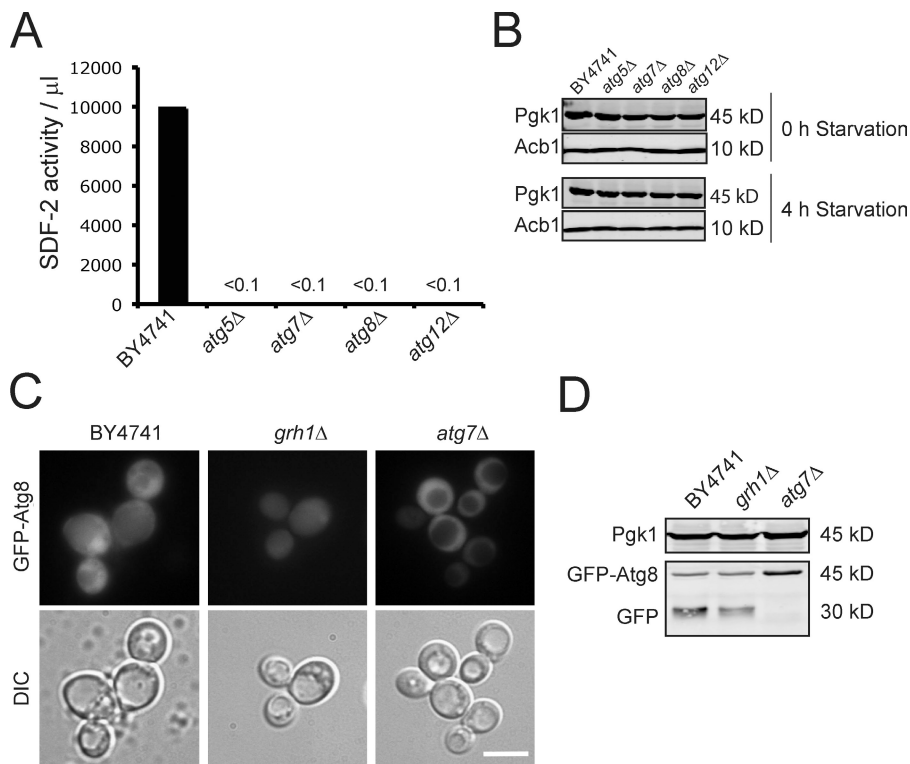
Figure 4. Autophagy genes are required for Acb1 secretion. (A) Yeast mutants deleted of atg genes 5, 7, 8, and 12 were incubated in starvation medium for 4 h. The buffer was tested in the bioassay. The data show an average of three experiments. (B) Cells described in A were Western blotted with anti-Acb1 antibody. Atg mutants do not show any change in the intracellular levels of Acb1. (C) Wild-type yeast, grh1Δ, and atg7Δ were transformed with a plasmid expressing GFP-Atg8 under the control of its endogenous promoter. The cells were starved as in A. Wild-type and grh1Δ strains show GFP fluorescence inside the vacuolar compartment. However, the atg7Δ strain lacked GFP fluorescence in the vacuole because of a defect in autophagy. Bar, 5 μm. (D) Wild-type, grh1Δ, and atg7Δ cells expressing GFP-Atg8 were starved for 4 h, and cell lysates were Western blotted with anti-GFP antibody to monitor the vacuolar proteolysis of GFP-Atg8. Wild type and grh1Δ, but not atg7Δ cells, showed a lower band with the apparent molecular weight of GFP alone caused by the proteolysis of GFP-Atg8 by vacuolar proteases (Shintani and Klionsky, 2004).

inhibition of protein synthesis in a wild-type strain (using cycloheximide) does not prevent the sporulation of KP cells (unpublished data). Moreover, we found that Acb1 was expressed at similar levels in wild-type and *atg* mutant cells before and after starvation (Fig. 4 B).

Is Grh1 (GRASP) therefore required for autophagosome formation and autophagy? Yeast lacking Grh1 (*grh1Δ* cells) were tested for import of the autophagosomal marker GFP-Atg8 into the lumen of the vacuole, which is an indicator of autophagy. Wild-type, *grh1Δ*, and *atg7Δ* cells expressing GFP-Atg8 were incubated under starvation conditions and visualized by fluorescence microscopy to monitor the localization of GFP-Atg8 (Fig. 4 C). As expected, GFP fluorescence was observed in the vacuole of wild type but not in *atg7Δ* cells. The *grh1Δ* cells show GFP inside the vacuolar lumen. Upon transport to the vacuole lumen, GFP-Atg8 undergoes proteolytic cleavage to release stable GFP. Control, *grh1Δ*, and *atg7Δ* cells were starved, and the cell lysates were monitored by Western blot analysis with anti-GFP antibody. The full-length GFP-Atg8 fusion protein as well as the proteolytically clipped GFP product was detected in both wild-type and *grh1Δ* mutant cell lysates (Fig. 4 D). In *atg7Δ* mutant cells, as expected, GFP-Atg8 was not cleaved in the vacuole as a result of a general defect in autophagosome formation and autophagy.

Acb1-containing autophagosomes bypass fusion with the vacuole

Once formed, autophagosomes usually fuse with the vacuole where their cargo is degraded by the vacuolar enzymes. The Vam7/Vam3 SNARE complex regulates the fusion of autophagosomes with the vacuolar membrane (Schimmöller and Riezman, 1993; Haas et al., 1995; Darsow et al., 1997; Sato

et al., 1998). The fusion of autophagosomes with the vacuole also requires the small GTPase Ypt7 (Haas et al., 1995). Yeast without Vam3 (*vam3Δ* cells) or Ypt7 (*ypt7Δ* cells) were incubated in starvation medium, and the conditioned media were tested for SDF-2–like activity. There was no obvious defect in Acb1 secretion by these mutants (Fig. 5 A). Therefore, fusion of autophagosomes containing Acb1 with the vacuole is not required for starvation-induced release of Acb1 in yeast.

Ypt6 is a Rab GTPase that has been implicated in recycling of endosomal components. In its absence, the organization and function of endosomes is perturbed as a result of defects in components between the Golgi endosomal compartments (Luo and Gallwitz, 2003). Therefore, we tested the requirement of Ypt6 in Acb1 secretion. Mutant *ypt6Δ* cells were incubated under starvation conditions, and the resulting medium was tested in the *D. discoideum* bioassay. Media preparations from cells lacking Ypt6 did not contain measurable amounts of Acb1 in the bioassay (Fig. 5 A). These data suggest that endosomal sorting, but not transport to vacuoles, is required for delivery of Acb1-containing carriers to the plasma membrane and subsequent secretion of Acb1.

The yeast vacuolar/endocytic compartments contain three syntaxin (t-SNARE) homologues: Vam3, Pep12, and Tlg2. Vam3 is located at the vacuole and is required for membrane docking and fusion events (Darsow et al., 1997). Pep12 is enriched on late endosomal membranes and is required for biosynthetic traffic from the Golgi to the vacuole (Becherer et al., 1996). Tlg2 regulates membrane traffic through the yeast endocytic system and is also required for the autophagosome-mediated traffic of specific vacuolar hydrolases to the vacuole (Nichols et al., 1998). This pathway, called the cvt pathway (cytoplasm to vacuole transport), is more selective than starvation-induced

Figure 5. **Early endosomal and MVB components are required for Acb1 secretion.** (A) Wild-type and deletion mutants for the genes shown were starved for 4 h, and the SDF-2–like activity in the buffer was determined in the bioassay. The data show an average of three experiments. (B) Yeast from A was lysed, and equal amounts were Western blotted with anti-Pgk1 and anti-Acb1 antibody. Deletion mutants are compared with their wild-type isogenic strain (BY4741 or SEY6210). The intracellular levels of Acb1 are not affected in yeast mutants tested in this experiment.
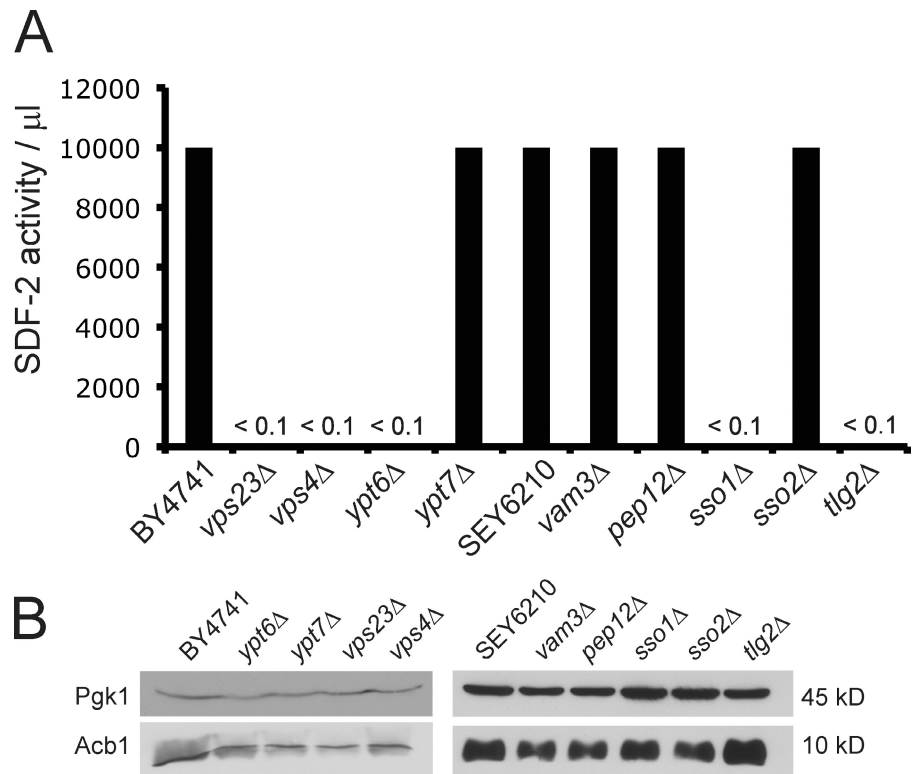
macroautophagy and is mediated by cargo-specific adaptors (Abeliovich et al., 1999). We asked if any of these t-SNAREs are involved in the trafficking steps that mediate Acb1 secretion. Interestingly, neither *VAM*3 nor *PEP12* deletion affected the secretion of Acb1 (Fig. 5 A). However, deletion of *TLG*2 impaired secretion of SDF-2–like material (Fig. 5 A). All of the mutants described in Fig. 5 A were also tested to monitor intracellular levels of Acb1. As shown in Fig. 5 B, none of the mutants tested affected expression of Acb1.

Therefore, autophagosomes containing Acb1 do not fuse with the vacuole, given the lack of effect of *pep12*Δ, *ypt7* Δ, and *vam3*Δ mutations. Nevertheless, the involvement of Ypt6 and Tlg2 indicates that an endosomal compartment is involved in Acb1 trafficking to the plasma membrane and that this is mediated by specific population of autophagosomes (Fig. 4 A).

Acb1 secretion requires components of MVB pathway

Next, we wanted to further dissect the pathway of Acb1 secretion after autophagosome formation along the endosomal pathway. We noted that in mammalian cells, autophagosomes fuse with MVB endosomal compartments to generate hybrid organelles called amphisomes (Fader and Colombo, 2006; Liang et al., 2008). After their formation, amphisomes fuse with lysosomes, and their content is degraded. Although the MVB pathway has not yet been clearly linked to autophagy in yeast, we asked whether this convergence between the autophagosomal pathway and the endosomal pathway was necessary for the secretion of Acb1. Therefore, we tested the ability of different class E *vps*/ESCRT mutants (impaired in various stages of the yeast MVB sorting pathway) to secrete Acb1. For this, we

used *vps23*Δ and *vps4*Δ mutant cells. Vps23 is a component of the ESCRT-I complex involved in ubiquitin-dependent sorting of proteins into the MVB sorting pathway. Vps4 is an ATPase responsible for ESCRT-III disassembly and is required for MVB protein sorting. Mutations of these genes (and other class E *VPS* genes) impair protein sorting at abnormal endosome structures (class E compartments) and thus delay and misroute trafficking through and out of these abnormal endosomal compartments (Babst et al., 1997; Katzmann et al., 2003). Strikingly, neither *vps23*Δ nor *vps4*Δ cells secrete active Acb1 as measured in the KP cell assay (Fig. 5 A). These results further suggest that the Acb1-containing autophagosomes may not fuse directly with the plasma membrane upon formation but that they fuse first with endosomes or MVBs en route to the plasma membrane. This fits well with our findings that *tlg2*Δ and *ypt6*Δ mutant yeast cells do not secrete Acb1.

Acb1 secretion requires the plasma membrane t-SNARE Sso1

There are two t-SNAREs at the yeast cell surface, Sso1 and Sso2 (Aalto et al. 1993). Cells lacking either Sso1 or Sso2 were grown in starvation medium, and their media were then tested for secretion of Acb1 in the *D. discoideum* KP cells sporulation assay. Interestingly, medium from yeast grown under starvation conditions secreted Acb1 in an Sso1-dependent manner (Fig. 5 A). Sso2 was not essential for Acb1 secretion (Fig. 5 A). Collectively, our results indicate that fusion of Acb1-containing autophagosomes is a regulated process that is precisely timed (Fig. 1 E) independent of the activity of Sec1 (Fig. 3 A) and mediated by a specific cell surface SNARE protein, Sso1 (Fig. 5 A).
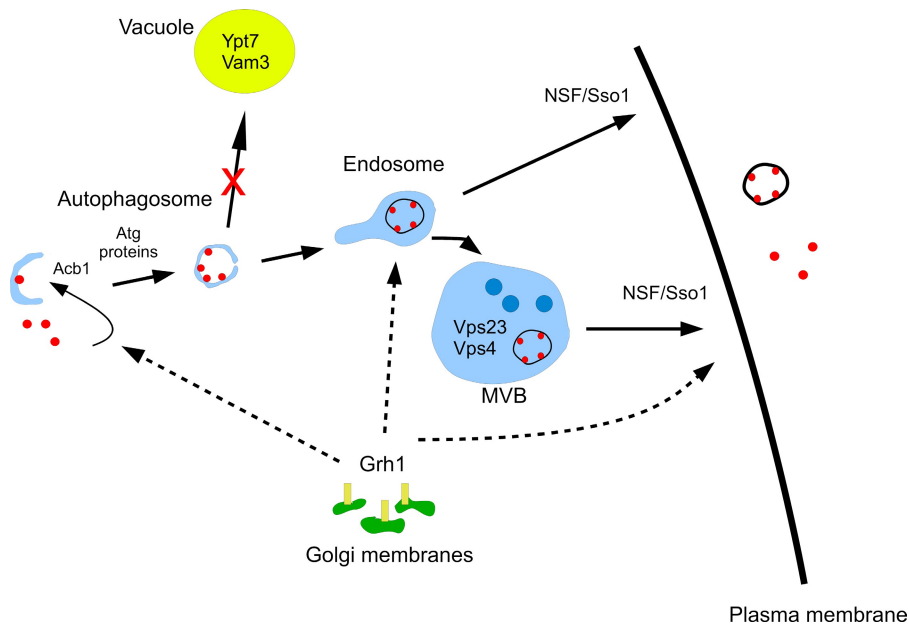
Figure 6. **The Acb1 secretion pathway.** Cytosolic Acb1 is packaged into autophagosomes, which fuse with early endosomes. The early endosomes containing Acb1 either fuse directly with the cell surface or more likely mature into an MVB. The MVB fuses with the cell surface to release exosomes containing Acb1. Grh1 is required for the secretion of Acb1 and has to be membrane associated for its role in this pathway. However, the exact site of action for Grh1 in unconventional secretion remains unknown.

Discussion

In both *S. cerevisiae* and *D. discoideum*, ACBPs Acb1 and ACBP, respectively, are secreted as a single burst when the cells are nitrogen starved. ACBP is also secreted from mammalian brain cells and processed to signaling peptides. This ancient conserved signaling mechanism uses an unconventional secretion mechanism for release of the precursor proteins. However, in each case, only a small amount of cytosolic ACBP is released, precluding the use of Western blotting to assess their external levels. Therefore, we used an exquisitely sensitive bioassay to monitor secretion of Acb1 in yeast (Anjard et al., 1998). Using this assay, we have determined the consequences of loss of specific yeast genes known to be involved in membrane trafficking with respect to Acb1 secretion, uncovering a unique pathway for secretion of proteins lacking signal sequences. We found that Acb1 is secreted from cells without entering the ER–Golgi pathway. Unlike a-factor, secretion of Acb1 in *S. cerevisiae* does not appear to be mediated by an ABC transporter. Instead, our data reveal a complicated alternative secretory mechanism, which rivals the complexity of the conventional secretory pathway. Based on the large number of genes involved in secretion of Acb1, we have delineated the following sequence of events (Fig. 6).

Acb1 is packed into an autophagosome
The fact that loss of the secretory pathway components Sec23, Sec7, and Sec1 did not inhibit release of Acb1 is strong evidence that the conventional ER–Golgi–cell surface pathway is not required for secretion of Acb1. However, Sec18/NSF was required for secretion of Acb1, which points to the involvement of a vesicular intermediate. The requirement for the autophagy genes *ATG5*, *7*, *8*, and *12* for Acb1 secretion imply the involvement of autophagosomes. Atg5 and Atg12, together with Atg16, form a complex required for the initial assembly of autophagosomes (Mizushima et al., 1998; Kuma et al., 2002). Atg8 then

binds to the membrane upon conjugation with the lipid phosphatidylethanolamine by the action of Atg7 and is also required for autophagosomal membrane expansion, tethering, and hemi fusion (Nakatogawa et al., 2007; Xie et al., 2008).

Acb1-containing autophagosomes escape fusion with the vacuole
The contents of autophagosomes are normally degraded when they fuse with and enter the vacuole. If this were to happen with Acb1-containing autophagosomes, there would be no secretion of Acb1. It is noteworthy that Ypt7, which is the GTPase involved in the fusion of autophagosomes with the vacuole (Haas et al., 1995), is not required for Acb1 secretion. Furthermore, neither Vam3, a t-SNARE needed for the fusion of autophagosomes with the vacuole (Reggiori et al., 2004), nor the t-SNARE Pep12, which functions at the prevacuolar late endosomal compartment (Becherer et al., 1996), have a role in Acb1 secretion. Thus, Acb1-containing autophagosomes do not fuse with the vacuole. But how are Acb1-containing autophagosomes diverted to the cell surface? Our findings that Tlg2 and Ypt6 are required for Acb1 secretion suggest the involvement of a population of early endosomes bound for the plasma membrane. In other words, the autophagosomes containing Acb1 may adopt or acquire early endosome features that allow them to recycle en route to secretion.

The involvement of components of the MVB pathway
Our findings reveal the involvement of Vps23 and Vps4 in Acb1 secretion. Vps23 is a component of the ESCRT-I complex, which recognizes ubiquitinated, transmembrane proteins at endosomes and promotes invagination of membrane and cargo into the lumen of an MVB. In the absence of Vps23, the initiation of membrane invagination is inhibited, thus preventing cargo sorting into a lumenal vesicle of an MVB (Babst et al., 1997; Katzmann et al., 2003). Vps4 recycles ESCRT-III

complex subunits for new rounds of MVB formation. Because both of these proteins are required for Acb1 secretion, we entertain the two following possibilities: fusion of autophagosomes containing Acb1 with an MVB before fusion with the plasma membrane or fusion of autophagosomes containing Acb1 directly with the plasma membranes (see following paragraph). Fusion of MVBs with the plasma membrane is known to occur in some mammalian lymphoid cell lines to produce exosomes or secreted vesicles that are released to the extracellular medium (Simons and Raposo, 2009; Théry et al., 2009). If autophagosomes containing Acb1 fuse directly with the plasma membrane, the requirement for Vps23 and Vps4 would have to be an indirect consequence of perturbation in endosomal dynamics that affects the fate of early endosomes. For example, some as yet unidentified factor necessary for Acb1 secretion may become mislocalized to the abnormal endosomal compartments that accumulate in cells lacking ESCRT function. Future studies will be required to distinguish between these two possible steps in the secretion of Acb1.

The final step in Acb1 secretion

The last step in this pathway is mediated by fusion with the cell surface through the SNARE Sso1. Sso1 and Sso2 are highly homologous t-SNAREs at the cell surface, and either is sufficient for growth of yeast. However, it has been reported that yeast lacking Sso2 sporulate, whereas yeast lacking Sso1 are sporulation defective (Jäntti, et al., 2002). Thus, it appears that these two proteins are only partially redundant and that the t-SNARE Sso1 is specifically required for the fusion of Acb1-containing autophagosomes with the plasma membrane during starvation.

The involvement of GRASP in unconventional secretion

Our new findings reveal that, as in *D. discoideum* secretion of AcbA, secretion of the yeast homologue Acb1 requires GRASP and is triggered by starvation. GRASP has also been found to play an essential role in secretion of Acb1 during starvation of the distantly related yeast *Pichia pastoris* (see Manjithaya et al. in this issue). But what exactly is the role of GRASP in this reaction? Schotman et al. (2008) have reported that GRASP is required for the docking/targeting/tethering of ER-derived vesicles directly to the cell surface in early *Drosophila* embryos, i.e., without trafficking through the Golgi. They also reported that some of the GRASP protein is localized to the plasma membrane in these cells (Schotman et al., 2008). It is noteworthy that preventing GRASP attachment to Golgi membranes, as in the case of yeast lacking the *BUG1* gene, blocks Acb1 secretion. Thus, although GRASP has to be membrane associated for its function in Acb1 secretion, its exact role in this process remains unclear.

A new secretory route

In sum, we have uncovered a new pathway for secretion of specific proteins that do not enter the conventional ER–Golgi pathway. These unconventionally secreted proteins are released under specific developmental conditions. Although the function of unconventionally secreted proteins is known and appreciated,

the advantage of this pathway of secretion for the overall cell physiology remains unknown. For future discussion, we classify unconventional secretion into two classes: type I and type II. Cell surface–specific ABC transporters, such as Ste6 in *S. cerevisiae*, mediate type I unconventional secretion. FGF2 and galectin-1 of the mammalian cells may be transported in a similar manner (Seelenmeyer et al., 2008). Type II unconventional secretion involves a vesicular carrier and requires genes of the autophagy pathway and the Golgi attachment of GRASP. An important question for the future is how unconventionally secreted type II cargoes are sorted for packing into autophagosomes for extracellular release rather than degradation in lysosomes/vacuoles.

Materials and methods

Antibodies
Mouse monoclonal antibody against yeast 3-phosphoglycerate kinase was obtained from Invitrogen (clone 22C5). Rabbit polyclonal antibody anti-Acb1 was a provided by J. Knudsen (University of Southern Denmark, Odense, Denmark). Anti-Flag M2 mouse monoclonal antibody was obtained from Sigma-Aldrich. Mouse monoclonal antibody anti-GFP was obtained from Roche.

Western blotting
A volume corresponding to 1 OD_{600} of cells was removed, and the cells were harvested and resuspended in 1 ml of cold water. The cell suspensions were mixed with 150 µl 1.85 M NaOH and 7.5% β-mercaptoethanol (freshly prepared) and placed on ice for 15 min. 150 µl 55% TCA (wt/vol; stored in the dark) was added, and the mixture was incubated for 10 min on ice. The cells were pelleted for 10 min at 14,000 rpm at 4°C or room temperature. The supernatant was removed, the cells centrifuged briefly for a second time, and all residual traces of TCA were aspirated off. The pellet was resuspended in 100 µl HU buffer (8 M urea, 5% SDS, 200 mM Tris, pH 6.8, and 1 mM EDTA with bromophenol blue as coloring and pH indicator, 1.5%) per OD_{600} of cells. Denaturation of the proteins was performed for 10 min at 65°C. Volume corresponding to 0.2 OD_{600} was loaded on 12% acrylamide gels.

Microscopy
Cells were harvested by centrifugation in a tabletop centrifuge (300 *g* for 2 min), resuspended in nonfluorescent medium or starvation medium, mounted and live imaged at 25° in a microscope (DMI 6000 B; Leica) equipped with a camera (IXON plus; Andor) using a Plan Apo 100× 1.4 NA objective. Images were taken using LAS-AF software (Leica) with the same exposure times.

Strains, media, and plasmids
Yeast strains used are summarized in Table I. All knockouts, unless otherwise stated, are BY4741 based (ORF substituted with KanMX cassette). Yeast cells were grown in rich YPD (1% yeast extract, 2% peptone, and 2% glucose) or synthetic minimal media (SC glucose; 0.67% yeast nitrogen base, 2% glucose, amino acids, and vitamins as needed). Starvation experiments were made using 2% potassium acetate. For construction of the JMD18 strain, a PCR-based modification was used to tag the genomic Grh1 protein. Primers F2 5′-ATCCTCGCCCCACCACCA-CAAAAACAGTCATCCTCTGATCGGATCCCGGGTTAATTAA-3′ and R1 5′-ATTTTGCTATGAAACAAAACCAAAAGACTAACCAGGAAAGGAATTC-GAGCTCGTTTAAAC-3′ were used to amplify a DNA fragment using the plasmid pFA6a (GFPS65T)-HIS3MX6 (Longtine et al., 1998) as a template. PCR product was transformed in a BY4742 strain using a lithium acetate method. ACB1 gene was amplified by PCR from *S. cerevisiae* genomic DNA and cloned in HindIII–EcoRI sites in pRS416. The protein was tagged in C terminus by introducing an oligonucleotide coding for the 3×Flag sequence between EcoRI and SacI restriction sites. GFP-Atg8 was cloned in a pRS316 plasmid under the control of its endogenous promoter (Suzuki et al., 2001) and was provided by Y. Ohsumi (National Institute for Basic Biology, Okazaki, Japan).

Sample collection for Acb1 secretion
Yeast was grown in YPD to early log phase ($OD_{0.6–0.8}$). Cells were pelleted by centrifugation and washed with cold water, repelleted, and suspended in

Name	Genotype	Source
BY4741	MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0	EUROSCARF
BY4742	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0	EUROSCARF
JMD018	BY4742, GRH1-GFP:: HIS3	This study
JMD021	BY4741, acb1Δ::KanMX [Acb1-3xFlag, CEN-URA]	This study
RSY11	MATα ura3-52 s18-1 leu2-3,2-112	Emr Laboratory[a]
SF309-2C	MATα mal gal2 Sec23-1	Emr Laboratory[a]
SF611-6A	MATa sec7-1 trp 1-289 his4-580am leu2-3,2-112 ura3-52	Emr Laboratory[a]
NY768	Matα sec1-1 leu2-3,2-112 ura3	Emr Laboratory[a]
SEY6210	MATα leu2–3, 112 ura3–52 hisΔ200 trp–Δ901 lys2–801 suc2-Δ9	Robinson et al., 1988
JMD030	SEY6210; vam3Δ::HIS3	Emr Laboratory[a]
CBY31	SEY6210; pep12Δ::HIS3	Emr Laboratory[a]
JMD031	SEY6210; sso1Δ::HIS3	Emr Laboratory[a]
JMD032	SEY6210; sso2Δ::HIS3	Emr Laboratory[a]
HAY73	SEY6210; tlg2Δ::URA3	Abeliovich et al., 1999

[a]Weill Institute for Cell and Molecular Biology, Cornell University, Ithaca, NY.

the appropriate volume of starvation medium to obtain a concentration of 1 OD/ml. Cells were subsequently incubated at 25°C during the indicated times. Medium was collected by pelleting the cells in a tabletop centrifuge at 500 g for 5 min. The supernatant was recovered and recentrifuged for 10 min at 3,000 g, filtered through a 0.45-μm mesh filter (Millipore) syringe, and frozen at −20°C until used.

KP cell sporulation assay

We used the assay for SDF-2 that has been previously described (Anjard et al., 1998, 2009). Samples were collected on exchange resin (A-25; Anion), washed with 100 mM NaCl, and eluted with 400 mM NaCl. The KP strain is a previously described derivative of strain Ax2 overexpressing PKA under the control of its endogenous promoter (Anjard et al., 1998). Vegetative cells were harvested during exponential growth and resuspended in 1 ml buffer (20 mM MES, pH 6.2, 20 mM NaCl, 20 mM KCl, 1 mM MgSO$_4$, and 1 mM CaCl$_2$). 4.5×10^4 KP cells were resuspended in 12.5 ml of buffer containing 5 mM cAMP. 500 μl aliquots were distributed in each well of a 24-well dish to a density of 2×10^3 cells/cm^2. The cells were incubated overnight at 23°C before addition of test samples or defined products. Counting spores and undifferentiated cells 1 h after addition of the samples scored induction of spore formation. SDF-2 activity was determined by serial dilution of the samples before addition to KP cells. A unit is defined as the reciprocal of the highest dilution that showed activity.

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