The yeast arrestin-related protein Bul1 is a novel actor of glucose-induced endocytosis

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ABSTRACT  Yeast cells have a remarkable ability to adapt to nutritional changes in their environment. During adaptation, nutrient-signaling pathways drive the selective endocytosis of nutrient transporters present at the cell surface. A current challenge is to understand the mechanistic basis of this regulation. Transporter endocytosis is triggered by their ubiquitylation, which involves the ubiquitin ligase Rsp5 and its adaptors of the arrestin-related family (ART). This step is highly regulated by nutrient availability. For instance, the monocarboxylate transporter Jen1 is ubiquitylated, endocytosed, and degraded upon exposure to glucose. The ART protein Rod1 is required for this overall process; yet Rod1 rather controls Jen1 trafficking later in the endocytic pathway and is almost dispensable for Jen1 internalization. Thus, how glucose triggers Jen1 internalization remains unclear. We report that another ART named Bul1, but not its parologue Bul2, contributes to Jen1 internalization. Bul1 responds to glucose availability, and preferentially acts at the plasma membrane for Jen1 internalization. Thus, multiple ARTs can act sequentially along the endocytic pathway to control transporter homeostasis. Moreover, Bul1 is in charge of Jen1 endocytosis after cycloheximide treatment, suggesting that the functional redundancy of ARTs may be explained by their ability to interact with multiple cargoes in various conditions.

INTRODUCTION

Endocytosis contributes to plasma membrane homeostasis and is critical for cellular adaptation. Whereas the molecular mechanisms driving endocytosis of ligand-bound receptors (e.g., EGF [epidermal growth factor] receptor) are relatively well characterized (Sigismund et al., 2012), less is known about the endocytosis of nutrient transporters. Transporter abundance regulates the uptake capacity of cells and their ability to proliferate, and altering the regulation of uptake systems in cancer cells is a strategy for therapeutic interventions (McCracken and Edinger, 2013). Thus, a deeper understanding of the physiological regulation of nutrient transporters is needed.

The yeast Saccharomyces cerevisiae is an ideal model system to study how eukaryotic cells cope with changes in extracellular nutrients (Broach, 2012). These changes modify transcriptional programs to allow a rapid metabolic rewiring; they also reconfigure nutrient transport systems present at the plasma membrane through selective endocytosis (Haguenauer-Tsapis and André, 2004). A current challenge is to understand how nutrient-signaling pathways drive the endocytosis of specific nutrient transporters.

Transporter ubiquitylation triggers their endocytosis (MacGurn et al., 2012). This reaction is performed by the Nedd4-like E3 ubiquitin ligase Rsp5 whose metazoan homologues also contribute to endocytosis (Hein et al., 1995; Galan et al., 1996; Rotin and Kumar, 2009). The discovery of Rsp5 adaptor proteins of the arrestin-related family (ARTs: arrestin-related trafficking adaptors) improved our understanding of the regulation of this step (reviewed in Becuwe et al., 2012a; Piper et al., 2014). ARTs promote Rsp5 association with transporters to facilitate their ubiquitylation, but also warrant the timeliness of this interaction with respect to environmental cues.
glucose-induced internalization of Jen1. Moreover, Jen1 internalization was also observed in a mutant lacking nine arrestin genes, suggesting the involvement of other adaptors (Becuwe and Leon, 2014).

Bul1 and Bul2 are regulators of Gap1 (general amino acid permease) trafficking (Helliwell et al., 2001; Soetens et al., 2001; Risinger and Kaiser, 2008). They act as Rsp5 adaptor proteins (Merhi and André, 2012), sometimes redundantly with ARTs (Nikko and Pelham, 2009; Crapeau et al., 2014) and may share structural features with them (Nikko and Pelham, 2009). We thus considered the possible contribution of Bul1 and Bul2 in Jen1 trafficking.

In WT cells, Jen1-GFP internalization in endosomes is observed as early as 5 min after glucose treatment as previously described (Becuwe and Leon, 2014), and is followed by the vacuolar delivery of Jen1-GFP within 60 min (Figure 1A). It is noteworthy that glucose also represses Jen1 transcription and promotes the degradation of the corresponding transcripts (Lodi et al., 2002; Chambers et al., 2004; Mota et al., 2014), allowing one to observe the trafficking of preexisting Jen1 in the absence of neosynthesis. As reported, Jen1-GFP internalization still occurred in rod1Δ cells, with the persistence of a signal at the plasma membrane and partial targeting to the vacuole at late time points (Figure 1A; Becuwe and Leon, 2014). Strikingly, the deletion of BUL1 in the rod1Δ background almost completely abolished Jen1-GFP internalization (Figure 1A). Bul1 and Bul2 share many functions (Helliwell et al., 2001; Soetens et al., 2001; Abe and Iida, 2003; Pizzirusso and Chang, 2004; Liu et al., 2007; Nikko and Pelham, 2009; Merhi and André, 2012; Crapeau et al., 2014; Villers et al., 2017), yet BUL2 deletion had no effect on Jen1 sorting (Figure 1, A and B), indicating that Bul1 alone is responsible for the "remnant internalization" reported earlier in the rod1Δ mutant (Becuwe and Leon, 2014). Jen1-GFP trafficking was not altered in the single bul1Δ mutant, confirming that Rod1 remains the major ART for the glucose-induced endocytosis of Jen1 (Figure 1C).

To precisely place the action of Bul1, we used the fact that Jen1 vacuolar targeting is abolished in a strain lacking Vps52, a subunit of the VFT tethering complex (Conibear and Stevens, 2000; Siniossoglou and Pelham, 2001) in which the endosome-to-TGN retrograde pathway is disrupted (Figure 1D). In vps52Δ, Jen1 accumulates in endosomal structures (Figure 1E; Becuwe and Leon, 2014) that did not colocalize with the TGN or the vacuole (Supplemental Figure S1). Of note, these structures originated from the plasma membrane, and not from intracellular compartments, as judged by the inhibitory effect of latrunculin A (an actin polymerization inhibitor and potent inhibitor of endocytosis) on their appearance (Figure 1, E and F). The additional deletion of ROD1 increased Jen1 localization to the plasma membrane, but did not fully prevent this intracellular accumulation, confirming that internalization still partially occurs in the absence of Rod1 (Figure 1, E and F). In the triple vps52Δ rod1Δ bul1Δ mutant, Jen1 internalization was more severely compromised as Jen1 further accumulated at the plasma membrane (Figure 1, E and F). Therefore, Bul1 promotes Jen1 internalization.

**RESULTS AND DISCUSSION**

**Bul1 functionally assists Rod1 in the glucose-induced endocytosis of the lactate transporter Jen1 and acts primarily at the plasma membrane**

In rod1Δ cells, Jen1 internalization was delayed but still partially occurred, suggesting the involvement of (an)other adaptor(s) for the glucose-induced internalization of Jen1. Moreover, Jen1 internalization was also observed in a mutant lacking nine arrestin genes, suggesting the involvement of other adaptors (Becuwe and Leon, 2014).

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**Bul1 is responsible for the residual ubiquitylation and degradation of Jen1 observed in the rod1Δ strain**

Jen1-GFP was degraded to comparable levels in WT and rod1Δ cells after 60 min glucose treatment, but it was partially protected from degradation in the single rod1Δ mutant and was almost not degraded in the rod1Δ bul1Δ mutant (Figure 2, A and B). Bul1 may thus act as an "alternative adaptor" when Rod1 is absent. Earlier studies showed that Jen1 ubiquitylation is readily detectable on total protein extracts, causing a decreased electrophoretic mobility of a fraction of Jen1 (Paiva et al., 2009; Becuwe et al., 2012b;
Becuwe and Leon, 2014). The absence of this shift in the rod1Δ bul1Δ mutant suggested a defective ubiquitylation in this strain (Figure 2, A–C). Jen1-GFP was immunopurified in denaturing conditions 10 min after glucose treatment, and Jen1-GFP ubiquitylation was compared in WT, rod1Δ, and rod1Δ bul1Δ cells (Figure 2D). Jen1 ubiquitylation was decreased but not fully abolished in rod1Δ cells, as reported earlier (Becuwe et al., 2012b). The additional deletion of BUL1 further impeded Jen1 ubiquitylation, showing that Bul1 is able to sustain a certain level of Jen1 ubiquitylation.

Expression of a TGN-localized version of Rod1 leads to an increased dependency toward the function of Bul1 for Jen1 degradation

We constructed a TGN-targeted version of Rod1 by fusing it to the PH (pleckstrin homology) domain of the human protein FAPP1 (four-phosphate-adaptor protein 1), which interacts with both the TGN-localized PtdIns(4)P and Arf1 (Dowler et al., 2000; He et al., 2011). A PHFAPP1-GFP fusion localizes to the TGN in yeast (Levine and Munro, 2002) and so did Rod1-PHFAPP1-GFP (as defined by the trans-Golgi marker Sec7-mCherry; Franzusoff et al., 1991), even in nutrient conditions that do not naturally drive Rod1-GFP localization to the TGN (Figure 3A; Glc O/N and Lac, 4 h). Of note, Rod1-PHFAPP1-GFP was more cytosolic in lactate medium than when glucose was present, probably because the TGN pool of PtdIns(4)P is strongly influenced by glucose availability (Faulhammer et al., 2005; Demmel et al., 2008). Regardless, Rod1-PHFAPP1-GFP was more strongly associated to the TGN than endogenous Rod1-GFP upon glucose addition to lactate-grown cells (Figure 3A).

We then used this tool to evaluate the function of Rod1 at the TGN using Jen1 degradation as a proxy. The expression of Rod1-PHFAPP1 restored Jen1 degradation in a rod1Δ background as efficiently as Rod1 (Figure 3, B and C). We hypothesized that this was only possible because endogenous Bul1 may compensate for the loss of Rod1 function at the plasma membrane. Although Rod1-PHFAPP1 expression in a rod1Δ bul1Δ background partially restored Jen1 degradation, in line with the importance of TGN-based sorting in this process, it was significantly less efficient than WT Rod1 to do so (Figure 3, B and C). Thus, a stronger association of Rod1 to the TGN leads to an increased dependency toward the function of Bul1 for Jen1 degradation. This supports the idea that multiple Rsp5 adaptors sequentially act at several subcellular locations during transporter endocytosis.

Bul1 phosphorylation is regulated by glucose availability

Several ARTs are targeted by nutrient-signaling kinases/phosphatases (O’Donnell et al., 2010, 2013, 2015; MacGurn et al., 2011; Becuwe et al., 2012b; Merhi and André, 2012; Llopis-Torregrosa et al., 2016; Hovsepian et al., 2017). In particular, nitrogen availability...
Bul1 was constitutively ubiquitylated in our conditions. However, we always used ammonium as a nitrogen source; this could explain why the appearance of the slower-migrating species of Bul1, revealing that it was due to Bul1 phosphorylation (Figure 4, C and D). Thus, Bul1 is phosphorylated in the absence of glucose and dephosphorylation upon glucose addition, establishing Bul1 as a new glucose-responsive adaptor.

We previously reported that the glucose-induced dephosphorylation of Rod1 coincides with a change in its subcellular localization (Becuwe and Leon, 2014). Unfortunately, we were not able to study Bul1 localization because GFP-tagged versions of Bul1 were not functional (unpublished data).

The glucose-induced dephosphorylation of Bul1 requires Sit4
The PP1 phosphatase Glc7_reg1 is required for Rod1 dephosphorylation in response to glucose (Becuwe et al., 2012b); however, in a reg1Δ mutant, Bul1 dephosphorylation still occurred (Figure 5A) suggesting the involvement of another phosphatase. Previous work indicated that Sit4 regulates Snf1 phosphorylation (Bozaquel-Morais et al., 2010) and acts redundantly with Glc7_reg1 for Snf1 dephosphorylation when glucose levels are restored (Ruiz et al., 2011). Because Sit4 is also required for Bul1 dephosphorylation in response to ammonium (Merhi and André, 2012), we tested its involvement in the glucose-induced dephosphorylation of Bul1. Indeed, Sit4 contributed to this regulation because Bul1 remained partially phosphorylated after glucose treatment in the sit4Δ mutant (Figure 5, B and C).

We then studied the potential involvement of Sit4 in Jen1 endocytosis. For this experiment, we used a galactose-inducible version of Jen1 (Paiva et al., 2009) because endogenous Jen1 was not expressed in the sit4Δ mutant (unpublished data). In sit4Δ cells, Jen1 was endocytosed in response to glucose similarly to the WT (Figure 5, D and E). However, the deletion of Sit4 in the rod1Δ mutant increased the endocytosis defect observed in the single rod1Δ mutant. Although this could be indirect, this reminds one of the situation observed with Bul1, which is not required per se for endocytosis, but becomes important when Rod1 is absent.

Bul1 is also required for the cycloheximide-induced endocytosis of Jen1
Bul1/Bul2, as targets of the TORC1-regulated kinase Npr1, are ideal players of nitrogen-regulated endocytosis (Helliwell et al., 2001; Soetens et al., 2001; Merhi and André, 2012). Interestingly, Jen1 is also endocytosed upon cycloheximide (CHX) treatment (Becuwe et al., 2012b), a condition used to trigger transporter endocytosis in a Npr1-dependent manner (MacGurn et al., 2011). Jen1 endocytosis after CHX treatment occurred independently of Rod1, but the deletion of Bul1 strongly interfered with this process, and with no additional effect of the deletion of Rod1 (Figure 5, F and G). Thus, Jen1 is an endocytic cargo for Bul1 after CHX treatment. If glucose indeed relieves Bul1 of its phosphoinhibition through its Sit4-dependent dephosphorylation, and because Bul1 is activated similarly in the presence of rich N sources, this may explain why Bul1 targets Jen1 both after glucose and CHX treatment. These results are in line with a recently published paper in which Bul1 was found to participate in Jen1 endocytosis in response to CHX, independently of Rod1 (Talaia et al., 2017).

In conclusion, whereas the most essential function of Rod1 in Jen1 trafficking in response to glucose occurs at the TGN, Bul1 acts primarily at the plasma membrane. This sequential action of ARTs may allow a fine-tuning of transporter trafficking with respect to the regulation of Bul1/2 phosphorylation through a balanced activity of the TORC1-regulated kinase Npr1 and Sit4, a type 2A–related protein phosphatase. This allows Gap1 endocytosis to occur only when a rich N source is present in the medium (Merhi and André, 2012). So, we investigated whether Bul1 posttranslational modifications also responded to glucose availability.

In protein extracts from glucose-grown cells, Bul1-Flag migrated as a doublet (Figure 4A) much like what is observed in ammonium-treated cells (Merhi and André, 2012), in which Bul1 is ubiquitylated. Mutation of the Rsp5-binding motifs on Bul1 (Bul1-Pym), which abrogates Bul1 ubiquitylation (Merhi and André, 2012), led to a disappearance of the slower-migrating species of Bul1, revealing that it corresponded to ubiquitylated species (Figure 4, A and B). Here, we always used ammonium as a nitrogen source; this could explain why Bul1 was constitutively ubiquitylated in our conditions. However, we noticed a decreased mobility of Bul1-Flag species (both ubiquitylated and nonubiquitylated forms) when cells were grown in lactate medium. Treatment of these samples with alkaline phosphatase demonstrated that this was due to Bul1 phosphorylation (Figure 4, C and D). Thus, Bul1 is phosphorylated in the absence of glucose and dephosphorylation upon glucose addition, establishing Bul1 as a new glucose-responsive adaptor.

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We show that the Sit4-dependent Bul1 dephosphorylation, a prerequisite for its activation during ammonium-induced endocytosis of Gap1 (Merhi and André, 2012), also occurs after glucose treatment. Sit4 also regulates phosphorylation of Snf1, suggesting that its activity may be stimulated by glucose (Bozaquel-Morais et al., 2010; Ruiz et al., 2012). Thus, independent signals (glucose or nitrogen) may converge onto Sit4 to promote Bul1 dephosphorylation and possibly, its activation. It was possible that Bul1 only substitutes for Rod1 during glucose-induced endocytosis because of the void created by the absence of Rod1. To test this hypothesis, we expressed an inactive mutant version of Rod1, Rod1-PYm (which no longer interacts with Rsp5) and tested whether this would prevent Bul1 function, that is, whether this would phenocopy the rod1∆ bul1∆ double deletion. The results indicate that this is not the case (Supplemental Figure S3), suggesting that Bul1 is still active despite the expression of Rod1-PYm. This is in line with our previous results using an inactive, nonubiquitylatable Rod1-KR mutant that failed to restore Jen1 degradation when expressed in a rod1∆ mutant but did not aggravate the trafficking defect (Becuwe et al., 2012b). Regardless of whether Bul1 acts only in the context of the rod1∆ mutant or also contributes to endocytosis in a WT context, Bul1 might be able to do so because 1) Jen1 is a target of Bul1 in other conditions (e.g., endocytosis in response to CHX, or other signals; Talaia et al., 2017) and 2) Bul1 can be activated by glucose. We propose that the multiplicity of actors involved in signaling pathways that are solicited upon a given nutrient change may translate into multiple ART activations and redundancy, and ultimately contribute to an increased robustness in the adaptive response.

**MATERIALS AND METHODS**

**Yeast strains, transformation, and growth conditions**

Strains are listed by occurrence in each figure in Supplemental Table 1. Supplemental Table 2 describes their full genotype and references. All strains are derivatives of the BY4741 strains. Yeast was transformed by standard lithium acetate/polyethylene glycol procedure. Cells were grown in synthetic complete (SC) medium containing 2% (wt/vol) glucose or 0.5% (vol/vol) Na-lactate (pH 5.0; Sigma). For lactate inductions, cells were grown overnight in SC-glucose medium, harvested in early exponential phase (A600 = 0.3), resuspended in the same volume of SC-lactate medium, and grown for 4 h (A600 = 0.5) before the addition of glucose (2% wt/vol, final concentration).
FIGURE 4: Glucose regulates Bul1 phosphorylation. (A) Western blot from total protein extracts prepared from cells expressing either Bul1-Flag or Bul1(PYm)-Flag and grown as indicated. (B) Line scan of the Western blot presented in A showing pixel intensity (relative to the max intensity for each lane) over 100 pixels. (C) Western blot on total protein extracts prepared from cells expressing either Bul1-Flag or Bul1(PYm)-Flag and grown as indicated, and treated or not with cold-tolerant phosphatase (CIP). (D) Line scan of the Western blot presented in C.

Plasmids

The plasmids used in this study are listed in Supplemental Table 3. Plasmids pMA121 (YCp- BUL1-FLAG; URA3) and pMA136 (YCp- BUL1(PYm)-FLAG; URA3) were a kind gift from Bruno André, Université Libre de Bruxelles, Gosselies, Belgium (Merhi and André, 2012). For the Rod1-PhFAPPI1-GFP plasmid (pSL164; rod1-PhFAPPI1- GFP), the FAPPI1 sequence (a kind gift from Chris Stefan, MRC Laboratory for Molecular Cell Biology, University College London, UK, and Catherine Jackson, Institut Jacques Monod, Paris, France) was amplified by PCR (using primers oSL349/oSL350, XmaI/XmaI) and cloned at the XmaI site into pSL93 (p416-based, URA3-URA3) and pMA136 (YCp-URA3-URA3) and pMA121 (YCp-URA3-URA3) and pMA136 (YCp-URA3-URA3), respectively (10 µl) were loaded on SDS–PAGE gel. Resuspended in 100 µl of TCA (10%, vol/vol), and lysed with glass beads for 10 min at 4°C. Beads were removed, the lysate was centrifuged for 1 min at room temperature at 16,000 x g, and the resulting pellet was resuspended in TCA-sample buffer (Tris-HCl 50 mM, pH 6.8, dithiothreitol 100 mM, SDS 2%, bromphenol blue 0.1%, glycerol 10%, containing 200 mM of unbuffered Tris solution) at a concentration of 50 µl/initial OD unit, before being denatured at 37°C for 10 min in a thermomixer (1400 rpm). Samples (10 µl) were loaded on SDS–PAGE gel. Phosphatase treatment was performed as previously described (Becuwe et al., 2012b).

Immunoprecipitation of Jen1-GFP in denaturing conditions

To immunoprecipitate Jen1-GFP, 50 OD equivalents of yeast cells grown for 4 h in lactate medium and treated with 2% glucose for 10 min were used. Yeast cells were mechanically disrupted with glass beads using a vortex at 4°C in 500 µl ice-cold RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 2 mM EDTA, 50 mM NaF, 1% NP-40, 0.5% Na-deoxycholate [Sigma-Aldrich], 1% glycerol) containing protease inhibitors (cOmplete Protease Inhibitor Cocktail [Roche Life Sciences], 2 mM phenylmethylsulfonyl fluoride [Sigma-Aldrich], and 20 mM NEM [N-ethylmaleimide; Sigma-Aldrich]) using four cycles of lysis (2 min), each separated by 2 min on ice. The lysate was then gently incubated on rotation on a wheel for 30 min at 4°C for solubilization. Then, 500 µl of wash buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 5% glycerol, containing 20 mM NEM) was added and the sample was vortexed to mix and further rotated for 1 h at 4°C for better solubilization. After vortexing for 3 min, the lysate was centrifuged at 10,000 x g for 10 min at 4°C. The cleared lysate (supernatant) was then transferred to another tube, to which were added 10 µl of GFP-Trap MA magnetic agarose beads (ChromoTek GmbH, Planegg-Martinsried, Germany) prewashed twice in 1 ml of wash buffer. The sample was rotated overnight at 4°C. The beads were then collected using a magnetic rack and washed twice by rotating for 15 min at 4°C with 1.5 ml ice-cold wash buffer. The beads were further washed for 30 min at room temperature with 1.5 ml Tris-buffered saline buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl), containing 0.1% Tween 20. The beads were then collected and resuspended in 50 µl of 2x urea sample buffer (150 mM Tris-HCl, pH 6.8, 6M urea, 6% SDS, 0.01% bromphenol blue) and then incubated for 30 min at 37°C in a thermomixer (1400 rpm). Then, 50 µl of boiling buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% SDS, 20% glycerol) was added and the sample was further incubated for 1 h at 37°C in a thermomixer (1400 rpm).

Antibodies, immunoblotting, and quantifications

We used monoclonal antibodies raised against GFP (clones 7.1 and 13.1; cat #11814460001; Roche Life Sciences, Meyland, France), Flag (clone M2, ref. F1804, Sigma), anti-ubiquitin (clone P4D1, ref. sc-8017; Santa Cruz Biotechnology, Dallas, TX), and rabbit polyclonal antibodies against 3-phosphoglycerate kinase (PGK, ref. NE130/7S; Nordic-MUBio BV, Susteren, Netherlands). Horseradish peroxidase–coupled secondary antibodies were from Sigma (anti-mouse, ref A5278; anti-rabbit, ref. A6154). Chemiluminescence signals were
For Figures 2B and 3C, the ratio of the free GFP signal over the total GFP signal was calculated for the 60 min time point ($n = 3$ biological replicates). An unpaired, two-sided $t$ test was performed using Prism 7.0 (GraphPad software, La Jolla, CA) and the $p$ values are indicated (NS: $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). For line scans (Figures 2C, 4, B and D, and 5C), a line was drawn in the center of the lane, along the area of interest, and the intensity profile was plotted. Lanes were aligned with each other to correct for potential heterogeneity in the migration using a reference band present in all lanes.

In all figures except Figure 2, total proteins were visualized in gels using a trihalo compound incorporated in SDS–PAGE gels (stain-free TGX gels, 4–20%; Bio-Rad) after 1 min UV-induced photoactivation and imaging using a Gel Doc EZ Imager (Bio-Rad). In Figure 2, samples were loaded on SDS–PAGE 4–12% gels (Bis-Tris) from Invitrogen that we found more suitable for ubiquitin visualization.

Fluorescence microscopy and quantifications

Cells were mounted in the appropriate culture medium and imaged at room temperature with a motorized Olympus BX-61 fluorescence microscope equipped with an Olympus PlanApo 100× oil-immersion objective (1.40 NA), a QIClick cooled monochrome camera (QImaging, Surrey, BC, Canada), and the MetaVue acquisition software (Molecular Devices, Sunnyvale, CA). GFP-tagged proteins were visualized using a GFP filter set (41020 from Chroma Technology, Bellows Falls, VT; excitation HQ480/20×, dichroic Q505LP, emission HQ535/50m). mCherry-tagged proteins were visualized using an HcRedI filter set (41043 from Chroma Technology, Bellows Falls, VT; excitation HQ575/50×, dichroic Q610lp, emission HQ640/50m). Vacuolar staining was obtained by incubating cells with 100 µM CMAC (7-amino-4-chloromethylcoumarin; ThermoFisher) for 10 min under agitation at 30°C; cells were then washed twice with the appropriate medium and observed with a DAPI (4′, 6-diamidino-2-phenylindole) filter set (31013v2; Chroma Technology Corp.; excitation D365/10×, dichroic Q610lp, emission D460/50m). Images were processed in ImageJ for levels and for contrast when indicated.

For quantification of intracellular/peripheral fluorescence signals, a first ellipse was drawn around each cell ($n > 50$ cells) using ImageJ, and a second ellipse was drawn inside the cell so as to exclude the plasma-membrane localized signal. The integrated density (IntDens) of both acquired with the LAS-4000 imaging system (Fuji, Tokyo, Japan). Quantifications were performed using ImageJ (National Institutes of Health) on nonsaturated blots from independent experiments ($n \geq 3$).
of these regions of interest were measured, to which the background noise for each ellipse was subtracted, giving a corrected value of IntDens (IntDensCorr). Background noise was calculated by multiplying the area of the ellipse of interest by the average gray value per pixel as measured in a control region. The difference (IntDensCorr[1st ellipse]−IntDensCorr[2nd ellipse]) was taken as a value of the peripheral (plasma membrane) signal. An unpaired, two-tailed t test was performed using Prism 7.0 (GraphPad Software; La Jolla, CA), and the p values are indicated (NS: p > 0.05; *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001).

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