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RESEARCH ARTICLE

Alpha-arrestins Aly1/Art6 and Aly2/Art3 regulate trafficking of the glycerophosphoinositol transporter Git1 and impact phospholipid homeostasis

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Abstract

Background information: Phosphatidylinositol (PI) is an essential phospholipid, critical to membrane bilayers. The complete deacylation of PI by B-type phospholipases produces intracellular and extracellular glycerophosphoinositol (GPI). Extracellular GPI is transported into the cell via Git1, a member of the Major Facilitator Superfamily of transporters at the yeast plasma membrane. Internalized GPI is degraded to produce inositol, phosphate and glycerol, thereby contributing to these pools. *GIT1* gene expression is controlled by nutrient balance, with phosphate or inositol starvation increasing *GIT1* expression to stimulate GPI uptake. However, less is known about control of Git1 protein levels or localization.

Results: We find that the α -arrestins, an important class of protein trafficking adaptor, regulate Git1 localization and this is dependent upon their interaction with the ubiquitin ligase Rsp5. Specifically, α -arrestin Aly2 stimulates Git1 trafficking to the vacuole under basal conditions, but in response to GPI-treatment, either Aly1 or Aly2 promote Git1 vacuole trafficking. Cell surface retention of Git1, as occurs in *aly1* Δ *aly2* Δ cells, is linked to impaired growth in the presence of exogenous GPI and results in increased uptake of radiolabeled GPI, suggesting that accumulation of GPI somehow causes cellular toxicity. Regulation of α -arrestin Aly1 by the protein phosphatase calcineurin improves steady-state and substrate-induced trafficking of Git1, however, calcineurin plays a larger role in Git1 trafficking beyond regulation of α -arrestins. Interestingly, loss of Aly1 and Aly2 increased phosphatidylinositol-3-phosphate on the limiting membrane of the vacuole, and this was further exacerbated by GPI addition, suggesting that the effect is partially linked to Git1. Loss of Aly1 and Aly2 leads to increased incorporation of inositol label from [³H]-inositol-labelled GPI into PI, confirming that internalized GPI influences PI balance and indicating a role for the α -arrestins in this regulation.

Conclusions: The α -arrestins Aly1 and Aly2 are novel regulators of Git1 trafficking with previously unanticipated roles in controlling phospholipid distribution and balance.

Abbreviations: a.u., arbitrary units; CDP, cytidine diphosphate; DMSO, dimethyl sulfoxide; GPI, glycerophosphoinositol; LatA, Latrunculin A; PI, phosphatidylinositol; PI(3)P, phosphatidylinositol-3-phosphate; PI(3,5)P₂, phosphatidylinositol-3,5-bisphosphate; PI(4)P, phosphatidylinositol-4-phosphate; PI(4,5)P₂, phosphatidylinositol-4,5-bisphosphate; PS, phosphatidylserine

Significance: To our knowledge, this is the first example of α -arrestin regulation of phosphatidylinositol-3-phosphate levels. In future studies it will be exciting to determine if other α -arrestins similarly alter PI and PIPs to change the cellular landscape.

KEYWORDS

calcineurin, E3 ubiquitin ligase, endocytosis, microscopy, protein trafficking adaptors

INTRODUCTION

Eukaryotic membrane-bound organelles have unique lipid compositions that alter their function and influence protein trafficking to and from these compartments (Behnia & Munro, 2005; Strahl & Thorner, 2007; Klug & Daum, 2014; Casares et al., 2019). Imbalances in lipids, nucleotides, or amino acids often result in cellular toxicity (Kim et al., 2000; Risinger et al., 2006; Ljungdahl & Daignan-Fornier, 2012; Shyu et al., 2019; Ruiz et al., 2020). Here we focus on regulation of the glycerophosphoinositol (GPI) transporter Git1 and consider how elevated cellular GPI, which increases phosphatidylinositol (PI), alters phospholipid homeostasis (Patton et al., 1995; Patton-Vogt & Henry, 1998).

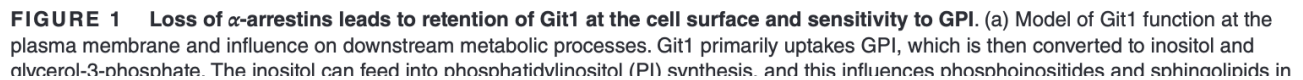
In normal metabolism, PI is deacylated by B-type phospholipases to produce GPI (Angus & Lester, 1972; Patton et al., 1995). GPI, along with the complex inositol-containing sphingolipids, are the major products of PI turnover (Angus & Lester, 1972). GPI, and to a lesser extent, glycerophosphocholine, enters the cell via the Git1 transporter (Patton-Vogt & Henry, 1998; Fisher et al., 2005). Internalized GPI is converted to inositol, phosphate, and glycerol, thus contributing to the cellular pools of these metabolites (Patton-Vogt & Henry, 1998). Git1 was first cloned for its ability to restore growth to the inositol auxotroph, *ino1*, (Patton-Vogt & Henry, 1998), demonstrating that GPI uptake via Git1 provides inositol to cells (Almaguer et al., 2003; Almaguer et al., 2004). Not surprisingly, transcription of *GIT1* is regulated by inositol, and more exquisitely by phosphate (Almaguer et al., 2003; Almaguer et al., 2004). The *GIT1* promoter contains binding sites for Pho2 and Pho4 (Almaguer et al., 2004), transcription factors that respond to phosphate limitation by cooperatively binding to genes needed for phosphate uptake and/or scavenging (Vogel et al., 1989; Magbanua et al., 1997; Shao et al., 1998). The *GIT1* promoter further contains inositol and choline responsive elements (ICREs) for the heteromeric Ino2/Ino4 transcription factors, which stimulate *GIT1* expression during inositol starvation.

Once GPI enters the cell through Git1, released free inositol fluxes into PI, which in turn is a precursor to two important classes of lipids: polyphosphoinositides and sphingolipids (Figure 1a) (Angus & Lester, 1972; Patton

minor class of membrane lipids produced by phosphorylation/dephosphorylation of carbons 3, 4, and 5 in the inositol ring of PI. Each organelle and/or subdomain is enriched for a specific class of phosphorylated PI (Di Paolo & De Camilli, 2006; Strahl & Thorner, 2007). Phospholipids are important binding sites on membranes that regulate trafficking and signaling events. In contrast, sphingolipids are a major lipid species with roles endocytosis, actin organization, response to heat stress, calcium sensitivity, and cell cycle regulation (Klemm et al., 2009; Epstein et al., 2012; Klug & Daum, 2014; Montefusco et al., 2014). PI is used in de novo sphingolipid synthesis at two steps in the complex sphingolipid base formation (see Figure 8c) (Dickson et al., 2006; Klug & Daum, 2014; Montefusco et al., 2014); perturbations in PI alter sphingolipid synthesis, with increasing PI favoring elevated sphingolipid biosynthesis (Henry et al., 2012).

GPI transport via Git1 impacts PI synthesis and downstream biosynthetic pathways, making it critical to define the factors that govern this protein's stability and/or trafficking. Git1 is a 12-pass transmembrane domain protein that functions at the PM and is a member of the Major Facilitator Superfamily (MFS) (Mariggio et al., 2006). It contains sugar transporter-like domains, suggestive of a shared structural relationship to the glucose transporters in *Saccharomyces cerevisiae*. While there is no clear mammalian ortholog of Git1, mammalian glucose transporter Glut2 can uptake GPI and is considered functionally analogous (Mariggio et al., 2006). The trafficking of the other transporters needed for inositol uptake, such as Itr1 and Itr2, and members of the related glucose transporter family, including Hxt1, Hxt3, and Hxt6, are controlled by the α -arrestins, a family of protein trafficking adaptors (Nikko & Pelham, 2009; Harada et al., 2010; O'Donnell et al., 2015; Hovsepian et al., 2017; Ivashov et al., 2020). We sought to determine if the α -arrestins might similarly regulate Git1 trafficking, whose removal from the cell surface was previously linked to clathrin-mediated endocytosis (Almaguer et al., 2006).

The α -arrestins, conserved from yeast to man, act as bridges between membrane proteins (referred to as cargos) and Rsp5, a ubiquitin ligase that regulates the endocytosis of several nutrient permeases (Rotin & Kumar, 2009). Internalization of many nutrient transporters is induced by excess substrate and



with the endocytic machinery (Blondel et al., 2004; Lin et al., 2008; Nikko & Pelham, 2009; Ivashov et al., 2020). Modification of cargos by even a single ubiquitin is often sufficient to induce internalization (Shih et al., 2000; Raiborg et al., 2002; Stringer & Piper, 2011). However, many cargos lack the motifs to directly recruit Rsp5. Instead, the α -arrestins selectively bind to membrane cargos and simultaneously interact with the WW-domains of Rsp5 via their PPxY motifs (Gupta et al., 2007; Lin et al., 2008; Nikko et al., 2008; Becuwe et al., 2012; O'Donnell et al., 2013; Alvaro et al., 2014). Thus, α -arrestins link membrane cargo to the machinery needed for selective endocytosis of an expanding array of membrane proteins (reviewed in this issue of *Biology of the Cell* [Kahlhofer et al., 2020]).

Relevant to our studies, α -arrestin Art5 controls inositol influx by regulating the inositol-stimulated endocytosis of inositol transporter Itr1, and perhaps Itr2 (Nikko & Pelham, 2009; Yamagami et al., 2015). Alterations in cellular inositol balance, as occurs in response to mis-regulation of Itr1, have broad reaching implications in the cell, including causing changes to mitochondrial cardiolipin production by altering CDP-diacylglycerol levels (Harada et al., 2010; Tamura et al., 2013) and restoring polarized trafficking to cells where phospholipid flippases are mutated (Yamagami et al., 2015). Thus, ties between inositol balance, phospholipids and α -arrestins are established.

Here we add new players in regulating GPI and inositol in cells; we show that the paralogous *arrestin-like yeast proteins* 1 and 2 (Aly1 and Aly2) act as adaptors for Git1. This is a novel membrane protein cargo for these α -arrestins, which regulate the trafficking three amino acid permeases, a G-protein coupled receptor, an arsenite transporter, and a P-type ATPase (Hatakeyama et al., 2010; O'Donnell et al., 2010, 2013; Crapeau et al., 2014; Hager et al., 2018; Wawrzycka et al., 2019; Nishimura et al., 2020; Sen et al., 2020). Only a handful of transporters are linked to the Alys to date, but the diversity of cargos regulated by this family is rapidly expanding (Savocco et al., 2019). In this study, we show that α -arrestins operate in basal, unstimulated conditions to control the steady-state turnover of Git1 and are induced to internal-

ize Git1 in the presence of GPI. Excessive uptake of GPI is toxic, though the mechanism of this toxicity remains unclear. Consistent with earlier studies, we find that Git1 is regulated in part by the clathrin-mediated endocytic pathway (Almaguer et al., 2006); however, an intracellular sorting pathway also controls Git1 since endocytic blocks do not fully recapitulate the defective trafficking observed with loss of the Alys.

While Aly1 and Aly2 share functional similarities, Aly2 plays a dominant role in trafficking Git1, which is especially evident in basal turnover of Git1. Interestingly, while α -arrestin Aly1 is regulated by the protein phosphatase calcineurin (CN) (O'Donnell et al., 2013), however we found only modest contributions for this pathway in Git1 steady-state trafficking. When we explored GPI substrate-induced phenotypes we found that a phosphomimetic allele of Aly1 failed to rescue *aly1 Δ aly2 Δ* sensitivity to GPI and the mutant allele that mimicked the dephosphorylated state of Aly1 was better able to control GPI-induced Git1 internalization. Thus, CN appears to play a role in Aly1-mediated, substrate-induced trafficking of Git1.

Finally, loss of the Alys results in a sharp shift in phosphatidyl-inositol-3-phosphate (PI(3)P) balance in the endomembrane system, with a dramatic increase in PI(3)P levels in the limiting membrane of the vacuole (Schu et al., 1993; Burd & Emr, 1998; Gillooly et al., 2000). This alteration in PI(3)P does not appear to reflect an overall alteration of PIPs as other phosphoinositides are largely unchanged in the absence of the Alys. While uptake of GPI exacerbates this defect, leading to more PI(3)P on the vacuolar membrane in both wild-type and *aly1 Δ aly2 Δ* cells, it is unlikely that this change in PI(3)P abundance in *aly1 Δ aly2 Δ* is entirely due to GPI uptake via Git1. However, we confirm that [³H]-inositol derived from exogenously supplied [³H]-inositol-GPI is incorporated into PI (Patton et al., 1995; Patton-Vogt & Henry, 1998), the precursor to both the inositol-containing complex sphingolipids and PIPs and that label incorporation into PI increases in the absence of Aly and Aly2. Overall, we find that the α -arrestins Aly1 and Aly2 are new regulators of GPI uptake via Git1 and, thereby, impact cellular PI levels and downstream lipid species.

PRS316-derived plasmids expressing either nothing (vector) or the indicated α -arrestins on SC medium lacking uracil and leucine and containing 50 μ M GPI. Growth is shown at 2 days after incubation at 30°C. (c) WT or 9Arr Δ cells containing Git1-GFP expressed from the *TEF1* promoter and either a pRS316 empty vector or plasmid expressing the indicated α -arrestins were imaged by confocal fluorescence microscopy. (d and e) The PM and vacuolar fluorescence intensities from the cells depicted in C were quantified using either our manual analysis (d) or automated quantification (e) pipelines and the distributions of PM/intracellular fluorescence ratios in arbitrary units (a.u.) were plotted as scatter plots. The horizontal midline in black represents the median and the 95% confidence interval is represented by the white error bars. A yellow dashed line is added as a reference for the median ratio for wild-type cells and the gray dashed line provides a reference for the median value for the 9Arr Δ cells. Kruskal–Wallis statistical analyses with Dunn's post hoc test were performed, and statistical significance compared to the 9Arr Δ strain containing vector control is presented. (f) Whole cell extracts from cells expressing Git1-GFP and α -arrestin-containing plasmids were analyzed by SDS–PAGE and immunoblotting. Git1-GFP and the GFP-breakdown product abundance is assessed with an anti-GFP antibody and the anti-Zwf1 (G6PDH) antibody is used as a loading control. The ratio of the signal intensities of the GFP breakdown product to the full-length Git-GFP is presented below each lane. Molecular weights of standards are shown at right measured in kilodalton

RESULTS

Trafficking of Git1 is impaired in the absence of α -arrestins Aly1 and Aly2

We sought to determine if the α -arrestins control Git1 localization. To avoid confounding issues caused by endogenous *GIT1* gene expression, we expressed Git1-GFP from the constitutive *TEF1* promoter and examined growth of cells lacking 9 of the 14 yeast α -arrestins (referred to as 9Arr Δ [Nikko & Pelham, 2009]) on medium with Git1's substrate, GPI. The 9Arr Δ cells were exquisitely sensitive to growth on medium containing GPI, while wild-type cells grew well at the concentrations of GPI employed (Figure 1b). Functional complementation assays with plasmids expressing each of the missing α -arrestins in the 9Arr Δ cells showed that the paralogous α -arrestins Aly1 or Aly2 restored growth of 9Arr Δ cells on GPI. The 9Arr Δ cells' sensitivity to GPI might arise due to excessive GPI uptake, which be caused by increased Git1 activity or abundance at the PM, and/or imbalance of downstream metabolites (Figure 1a). We assessed Git1-GFP steady-state localization using confocal microscopy. Consistent with potentially elevated GPI import in the 9Arr Δ cells, Git1 was retained at the PM while in wild-type cells Git1 localized faintly to the PM with substantial vacuole fluorescence (Figure 1c). Git1 at the PM in 9Arr Δ cells was reduced in cells expressing *ALY2* and the PM/vacuole fluorescence ratio in cells expressing either *ALY1* or *ALY2* was significantly reduced, demonstrating augmented Git1-GFP trafficking to the vacuole (Figures 1c,d and S1a). However, plasmids expressing the other α -arrestins failed to improve growth on GPI or reduce the retention of Git1 at the cell surface (Figures 1b,c and S1a). Manual quantification of the PM and vacuolar fluorescence intensities for Git1-GFP supported these conclusions, showing significantly reduced PM/vacuole fluorescence ratios in wild-type cells compared to the 9Arr Δ cells (Figure 1d). Addition of either Aly1 or Aly2 significantly reduced the PM/vacuole ratio of 9Arr Δ cells, with Aly2 having the more robust effect (Figure 1d), however only Aly2 significantly reduced the PM fluorescence of Git1-GFP (Figure S1a), suggesting that over this time-course of exposure to GPI (2 h treatment) Aly2 is more effective than Aly1 at reducing the surface pool of Git1-GFP. However, over the longer-time course of a growth assay on GPI (as in Figure 1b), Aly1 may also reduce the surface abundance Git1-GFP, thereby accounting for this altered sensitivity. The other α -arrestins did not significantly reduce the PM fluorescence or PM/vacuole fluorescence ratio (Figures 1d and S1a), yet some did increase the PM/vacuole ratio (Art10 and Art5) based on the manual quantification only. This difference between the manual and automated quantification may reflect

Our lab has made extensive use of manual fluorescence quantification (O'Donnell et al., 2015; Prosser et al., 2015; Chandrashekarappa et al., 2016; Hager et al., 2018; Soncini et al., 2020) and the process is a time consuming and limiting factor for analyses. For manual quantification, we typically quantify ~100 cells with at least two full imaging fields being measured (O'Donnell et al., 2015). To facilitate this kind of quantification, we developed an automated pipeline using an R-script program we wrote called "CellQuant" (see Materials and Methods; script available at www.odonelllab.com and on Github at <https://github.com/sah129/CellQuant>). We re-ran our analyses to measure the PM and vacuolar fluorescence intensities for the images in Figure 1c using this automated pipeline and had strong agreement with our manual data set. The time taken to obtain this quantification was a fraction of that needed for the manual quantification (~30 min compared to >20 h) (Figure 1e). The imaging data, quantification, and GPI growth assays were consistent with the results of Git1-GFP immunoblot analyses, where we found less full-length Git1-GFP in protein extracts from wild-type cells than in those from 9Arr Δ cells. In wild-type cells significant signal was observed for the free GFP-breakdown product, often associated with cleaved GFP in the vacuole after degradation of GFP-tagged transporters (Li et al., 1999; Nikko & Pelham, 2009), while in the 9Arr Δ cells there was very little GFP-breakdown product (Figure 1f). Addition of Aly1 or Aly2 to 9Arr Δ cells increased the free GFP-breakdown product abundance and reduced the Git1-GFP abundance, thereby elevating the ratio of free GFP/Git1-GFP (Figure 1f). Expression of the other α -arrestins in the 9Arr Δ cells had modest effects on total Git1-GFP levels and did very little to increase the abundance of the GFP-breakdown product, however Art1 and Csr2 elevated the GFP/Git1-GFP ratio above the vector control and in other replicates we saw elevated GFP/Git1-GFP ratios for Art10 (Figure 1f, data not shown). Taken together these data show that the α -arrestins Aly1 and Aly2 help traffic Git1 to the vacuole for degradation, either by stimulating its endocytosis or its intracellular sorting from the Golgi and/or endosomes to the vacuole. We do not exclude the possibility that other α -arrestins may play added roles in Git1 trafficking; indeed many α -arrestins often regulate a single transporter under disparate conditions (Nikko & Pelham, 2009) and the elevated free GFP breakdown product suggests that some of them are at least partially operational with Git1. Additionally, these findings show that our new automated quantification pipeline is effective for measuring PM and vacuole fluorescence abundance, and we will assess its utility throughout this paper.

To this point our analyses hinged on the 9Arr Δ cells, however this background is missing only 9 of the 14 yeast α -arrestins. Given the data indicating that addition

if the other α -arrestins that are intact in 9Arr Δ cells might contribute to Git1 trafficking. A recent paper demonstrated that the α -arrestins and other adaptors that bind the ubiquitin ligase Rsp5 may compete for a limited pool of Rsp5 and therefore restrict its function (MacDonald et al., 2020). We observed that when α -arrestins other than Aly1 or Aly2 were expressed in the 9Arr Δ background there may be elevated Git1 PM/vacuole ratios (Figure 1d), a result that might be obtained if the expression of an α -arrestin unable to traffic Git1 interfered with the activity of the remaining endogenous α -arrestin. The 9Arr Δ cells retain 5 other α -arrestins, including Bul1 and Bul2, which have overlapping functions in other Aly1/Aly2-mediated trafficking events (Novoselova et al., 2012; Crapeau et al., 2014). We next determined if the Buls influence Git1 trafficking. GPI growth assays showed only a slight change in sensitivity for *bul1* Δ *bul2* Δ cells relative to wild-type cells (Figure S2a). Deletions of *ALY1* *ALY2* and *BUL2* showed similar sensitivities to GPI as the *aly1* Δ *aly2* Δ cells, though the *aly1* Δ *aly2* Δ *bul1* Δ cells displayed a bit more resistance to GPI than *aly1* Δ *aly2* Δ (Figure S2a). These data suggest that the Buls may play a role in Git1 trafficking. Furthermore, imaging of Git1 in the absence of Buls showed greatly reduced Git1 at the PM in all cells lacking any combination of Buls (Figure S1b–d). Perhaps this is due to increased protein degradation/turnover in the absence of these α -arrestins, which has been reported previously (Savocco et al., 2019) and could be due to a lack of competition by remaining α -arrestins for the Rsp5 ubiquitin ligase. Alternatively, the Bul proteins may influence expression from the *TEF1* promoter used for *GIT1* in these experiments, though most effects of α -arrestins described to date have been post-transcriptional in nature. Interestingly, while the loss of *bul1* Δ *bul2* Δ caused mild sensitivity to GPI and *bul1* Δ or *bul2* Δ alone did not alter the GPI sensitivity, each of these mutants reduced the PM abundance of Git1-GFP fluorescence (Figure S2b–d), indicating an added unknown secondary cause for sensitivity to GPI in these *bul* Δ backgrounds that is not strictly linked to the abundance of Git1-GFP at the cell surface. Given the complex results with these mutants, the contributions of Buls to Git1 trafficking will be explored in more detail elsewhere but these findings highlight the fact that GPI sensitivity cannot simply be correlated to Git1 retention at the cell surface. Perhaps there are elements that alter the activity of the transporter at the PM; Git1 is a protein symporter and elevated pH has been shown to decrease Git1 activity (Almaguer et al., 2004) but beyond this nothing is known about Git1 activity regulation. From here on we focus our experimental attention on the roles of Aly1 and Aly2.

To further confirm that Aly1 and Aly2 are important for Git1 regulation, we assessed the sensitivity of *aly1* Δ

tized cells to GPI, with loss of *ALY2* causing the more severe inhibition (Figure 2a). Cells lacking both *ALY1* and *ALY2* were more sensitive to GPI than either single mutant and had the same degree of GPI sensitivity as the 9Arr Δ cells (Figure 2a). Fluorescent imaging of Git1-GFP in *aly1* Δ *aly2* Δ cells revealed Git1 retention at the PM (Figure S1b) and significantly increased PM/vacuole ratio in these cells that is comparable to that of 9Arr Δ , as judged by both our manual and automated quantification pipelines (Figure 2b–d). Loss of *ALY2* alone increased Git1-GFP at the PM, while deletion of *ALY1* had no significant impact, suggesting that under these steady-state conditions Aly1 has a modest impact on Git1 trafficking (Figures 2b–d and S1b). Protein extracts from *aly2* Δ or *aly1* Δ *aly2* Δ cells revealed little difference in Git1-GFP abundance between them but both strains had less free GFP than the *aly1* Δ or wild-type cells and an overall reduction in the GFP/Git1-GFP ratio that was comparable to that of 9Arr Δ cells (Figure 2e). However, in 9Arr Δ cells the Git1-GFP was more abundant than in *aly1* Δ *aly2* Δ cells (Figure 2b–e) suggesting some contribution by the other α -arrestins or Bsd2, an Rsp5 adaptor operating in the Golgi, to Git1 trafficking under these conditions (as also indicated by Figure 1).

To confirm that the plasmid-borne, GFP-tagged version of Git1 was functional and accurately reflected events that regulate endogenous Git1, we performed short-term radiolabeled GPI transport assays (5 min) in wild-type and *aly1* Δ *aly2* Δ cells with and without the Git1-GFP plasmid. These cells were grown in high phosphate medium, where expression of Git1 would be at basal levels and not induced as it would be under phosphate or inositol starvation conditions (Almaguer et al., 2003). Even under these high phosphate conditions, we found that loss of α -arrestins *ALY1* and *ALY2* increased the rate of GPI transport into cells by more than ~3-fold compared to wild-type cells (Figure 2f). To ensure that the Git1-GFP construct was functional, we also assessed radiolabeled GPI uptake in wild-type or *aly1* Δ *aly2* Δ cells harboring the Git1-GFP plasmid. In cells expressing Git1-GFP from a constitutive promoter, GPI uptake was increased by ~100-fold in wild-type cells grown in high phosphate medium (compare Figure 2f,g). Cells lacking *ALY1* and *ALY2* nearly doubled this rate of uptake (Figure 2g), again consistent with retention and/or elevated activity of Git1 at the cell surface in the absence of these α -arrestins. In addition to these uptake assays, we examined the ability of the Git1-GFP plasmid to rescue growth of *git1* Δ on medium lacking phosphate and containing GPI. Earlier studies found that cells can grow when supplemented with GPI as the sole phosphate source (Almaguer et al., 2004). Growth on media containing GPI the sole phosphate source was rescued to wild-type levels in *git1* Δ cells expressing the Git1-GFP plasmid (Figure S3a). These

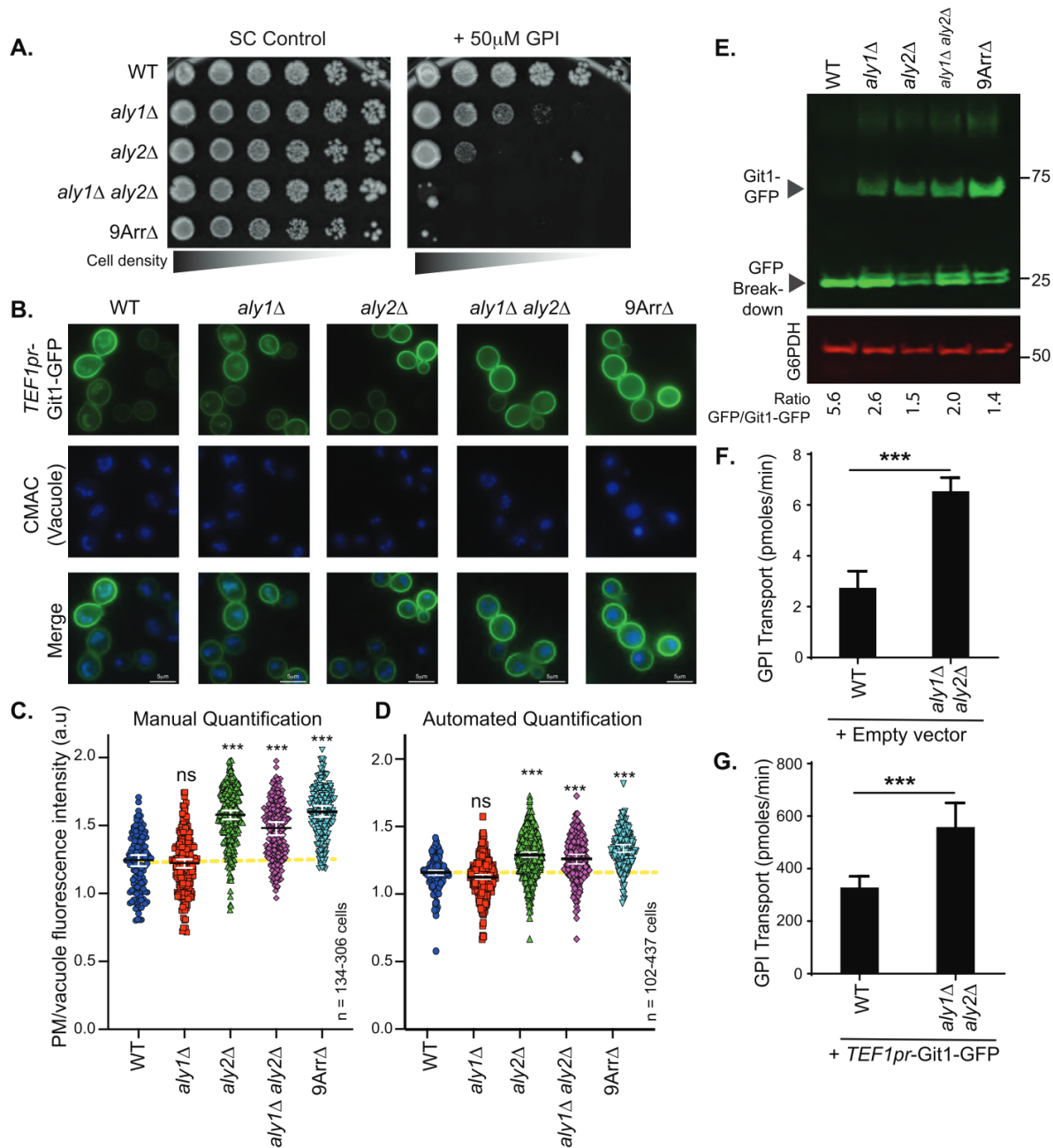


FIGURE 2 Aly1 and Aly2 regulate Git1 localization at the cell surface and loss of these α -arrestins causes GPI sensitivity. (a) Growth of serial dilutions of WT, *aly1* Δ , *aly2* Δ , *aly1* Δ *aly2* Δ , or 9Arr Δ cells containing the TEF1pr-GIT1-GFP plasmid on SC medium lacking leucine and containing 50 μ M GPI. Growth is shown at 2 days after incubation at 30°C. (b) The indicated cells containing Git1-GFP expressed from the TEF1 promoter were stained with CMAC and imaged by fluorescence microscopy. (c and d) The PM and vacuolar fluorescence intensities from the cells depicted in (b) were quantified using either our manual (c) or automated quantification (d) pipelines and the distributions of PM/vacuolar fluorescence ratios in arbitrary units (a.u.) were plotted as scatter plots. The horizontal midline in black represents the median and the 95% confidence interval is represented by the white error bars. A yellow dashed line is added as a reference for the median ratio for wild-type cells. Kruskal–Wallis statistical analyses with Dunn’s post hoc test were performed, and statistical significance compared to the wild-type cells is presented. (e) Whole cell extracts from the indicated cells containing Git1-GFP expressed from the TEF1 promoter were resolved by SDS-PAGE and immunoblotted with an anti-GFP antibody (to detect Git1-GFP and the GFP breakdown product) and anti-Zwf1 antibody (G6PDH) as a loading control. The ratio of the signal intensities of the GFP breakdown product to the full-length Git-GFP is presented below each lane. Molecular weights of standards are shown at right measured in kilodalton. (f and g) GPI radio-labeled uptake assays were performed as described in Surlow et al. (2014). WT and *aly1* Δ *aly2* Δ cells containing a LEU2-marked TEF1pr-GFP empty vector (f) or a LEU2-marked TEF1pr-GIT1-GFP plasmid (g) were grown at 30°C to logarithmic phase and shifted into synthetic media lacking inositol and choline and containing 10 mM inorganic phosphate. A total of 50 μ l of 25 μ M of [3 H]GPI (f) or 75 μ M of [3 H]GPI (g) was added to 200 μ l of

In limiting phosphate conditions Git1-GFP expression in *aly1Δ aly2Δ* cells is not toxic, perhaps because it is used as an added phosphate source here (Figure S3a).

To more carefully assess regulation of endogenous Git1, we made a chromosomally integrated, GFP-tagged version of this gene under its own promoter. We then examined Git1-GFP localization in cells grown in medium that contained high or low levels of phosphate, with or without inositol. Expression of Git1 is upregulated in response to phosphate and inositol limitation by the Pho2/Pho4 and Ino2/Ino4 transcription factors (Almaguer et al., 2003). As expected, regardless of the inositol content, in high phosphate medium there was little to no detectable Git1 by fluorescence microscopy (Figure 3a,b, note that 9ArrΔ cells in these conditions were sick and faint GFP fluorescence observed was due to a dead cell). When cells were incubated in low phosphate medium Git1-GFP fluorescence was observed (Figure 3c,d). Under low phosphate conditions, the wild-type cells had predominantly vacuolar GFP fluorescence, yet *aly1Δ aly2Δ* or the 9ArrΔ cells retained Git1-GFP at the PM. Of note, there was reduced vacuole fluorescence in 9ArrΔ cells compared to *aly1Δ aly2Δ* cells, indicating that other α -arrestins or Bsd2 are involved in the Git1 trafficking. The fluorescence intensity for each of these was slightly brighter when cells were additionally incubated in medium without inositol, consistent with the earlier studies of *Git1* expression (Almaguer et al., 2003). Manual quantification of this data found a substantially brighter PM/vacuole fluorescence ratio in the *aly1Δ aly2Δ* and 9ArrΔ cells in low phosphate medium with or without inositol (Figure 3e,f) and that 9ArrΔ is higher still than the *aly1Δ aly2Δ* cells. We performed automated quantification of these data but used a slightly different algorithm that used the DIC channel to define the cell surface since the PM fluorescence for the wild-type cells was too low and difficult to capture via our standard approach (see section "Methods"). These data agree with our manual quantification, although there was some compaction in the range of values identified in the automated quantification (Figure S3b,c). Taken together, these findings are consistent with our observations that the α -arrestins, in particular Aly1 and Aly2, contribute to Git1 trafficking and their absence results in increased levels of Git1 at the cell surface. However, there must also be other α -arrestins able to contribute to Git1 trafficking as the defect in the 9ArrΔ cells was more severe than that observed in *aly1Δ aly2Δ* alone.

We further explored the role of Aly1 and Aly2 in regulating Git1 function under shifting phosphate conditions using short-term GPI transport assays (Surlow et al., 2014). When wild-type cells were shifted from high to low phosphate medium to induce Git1 expression, GPI transport increased ~14-fold (Figure 3g). In *aly1Δ aly2Δ* cells grown in low phosphate medium, the GPI

phate medium (Figure 3g). Together, these data demonstrate that the loss of *ALY1* and *ALY2* increases GPI uptake by endogenous, untagged Git1 in response to phosphate starvation and are consistent with our findings that Git1-GFP is retained at the cell surface in *aly1Δ aly2Δ* cells (Figures 2b–e, 3c–f, and 3b,c).

Git1 internalization is induced by GPI and is dependent upon α -arrestins Aly1 and Aly2

Our experiments to this point indicate that Aly1 and Aly2 play a role in the steady-state internalization and/or trafficking to the vacuole of Git1, with Aly2 performing most of this trafficking and Aly1 only altering Git1 localization when added to the 9ArrΔ cells (Figure 1b–f). For many transporters, addition of their substrate triggers conformational changes that stimulate their vacuolar sorting and/or endocytic turnover (Gournas et al., 2010; Keener & Babst, 2013; Ghaddar et al., 2014; Gournas et al., 2017). We monitored Git1-GFP in response to GPI addition in wild-type cells treated with GPI and found that Git1 fluorescence at the PM was reduced and fluorescence shifted to the lumen of the vacuole in wild-type cells after GPI addition (Figure 4a,b). We examined Git1-GFP breakdown post GPI addition via immunoblotting and found that in WT cells the GFP/Git1-GFP ratio was much higher, but in *aly1Δ aly2Δ* or 9ArrΔ cells the ratio only increased slightly or not at all (Figure 4c). More detailed analysis of Git1 localization in response to GPI in WT or *aly1Δ aly2Δ* cells containing vector or Aly1 or Aly2 complementing plasmids showed that the GPI-induced shift in Git1 localization was largely lost in *aly1Δ aly2Δ* cells (Figure 4d,e and S4a). Though some modest reduction in PM/vacuole ratio is observed at 2 h post GPI addition, suggesting a GPI-induced and Aly1/Aly2-independent mechanism of reducing Git1 abundance during the 2-h GPI exposure (Figure 4a–c). This idea is confirmed in the immunoblots where a slight reduction in Git1-GFP is also seen post GPI addition in *aly1Δ aly2Δ* or 9ArrΔ extracts (Figure 4c), again consistent with an α -arrestin independent regulation of Git1. Plasmid-borne Aly1 in *aly1Δ aly2Δ* cells increased Git1-trafficking to the vacuole upon GPI addition (Figures 4d,e and S4a compare 60 min treatment between Aly1 vs. vector control). Interestingly, the ratio of PM/vacuole intensity in *aly1Δ aly2Δ* cells is initially still significantly higher than in wild-type cells when Aly1 was present, consistent with our earlier data showing that Aly1 plays a minor role in Git1 steady-state trafficking to the vacuole (Figures 2 and 4d,e). Addition of Aly2 to *aly1Δ aly2Δ* cells restored basal trafficking of Git1 to the vacuole, as indicated by the reduced PM/vacuole fluorescence ratios observed prior to GPI addition, which was not significantly differ-

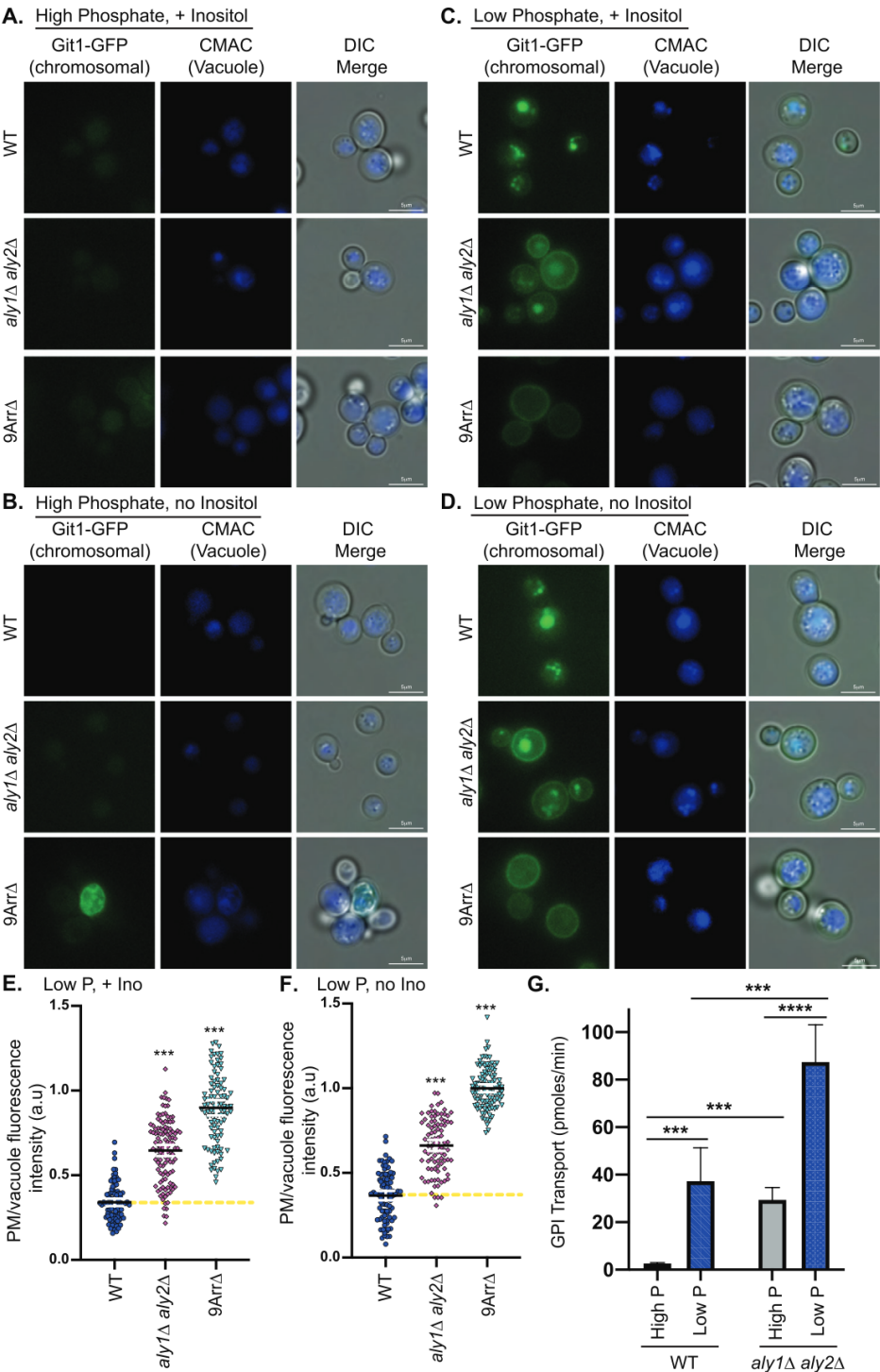
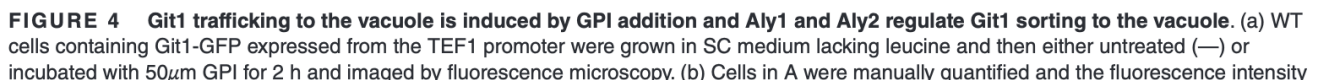


FIGURE 3 **Aly1 and Aly2 regulate endogenous Git1 transporter localization and function.** (a–d) The indicated cells containing a chromosomally integrated Git1-GFP at its endogenous locus were grown in medium containing high (10 mM) or low (100 μ M) phosphate with or without inositol for 12 h, stained with CMAC and imaged using a fluorescence microscope. (e and f) The images presented in panels (c) and (d) were quantified using our manual quantification methodology and the distributions of PM/vacuolar fluorescence ratios in arbitrary units (a.u.) were plotted as scatter plots. The horizontal midline in black represents the median and the 95% confidence interval is represented by the white error bars. A yellow dashed line is added as a reference for the median ratio for wild-type cells. Kruskal–Wallis statistical analyses with Dunn's post hoc test were performed and statistical significance compared to wild-type cells is presented. (g) GPI radio-labeled uptake assays were performed as described in Surlow et al. (2014). WT and *aly1Δ aly2Δ* cells were grown at 30°C to logarithmic phase and shifted into synthetic media lacking inositol and choline and containing 100 μ M (low) or 10 mM (high) inorganic phosphate. [³H]GPI was added to 200 μ l of processed



was not as impacted by Aly2, with only modest drops in the PM/vacuole ratios at 60- and 120-min post GPI addition, similar to what was observed for *aly1Δ aly2Δ* cells with just a vector control. Importantly, our manual and automated quantification of this imaging (Figures 4e and S4a, respectively) are consistent with one another. Taken together, these data support a model whereby Aly1 may play a more prominent role in the GPI-induced trafficking of Git1, while Aly2 is very effective at stimulating steady-state trafficking of Git1 to the vacuole. As borne out in earlier experiments, other factors also alter the localization of Git1 in response to GPI as even in the *aly1Δ aly2Δ* cells there was a significant reduction in the PM/vacuole fluorescence post GPI addition (Figure 4d,e, see 120 min timepoint for *aly1Δ aly2Δ* with vector). Perhaps this could be ascribed to Bul1/Bul2 function or another trafficking adaptor that can operate in this GPI-stimulated turnover pathway when the ALYs are deleted. Immunoblot analyses of Git1-GFP in *aly1Δ aly2Δ* cells containing vector, Aly1 or Aly2 plasmids and treated with GPI support the imaging data, demonstrating increased GFP/Git1-GFP ratios post GPI addition when Aly1 or Aly2 are present in these cells (Figure 4f).

To begin to define the relative contributions of endocytic trafficking versus intracellular sorting on Git1 accumulation in the vacuole under basal conditions and post GPI addition, we examined the Git1-GFP localization and GPI sensitivity of mutant cells lacking endocytic components. Consistent with our earlier findings, loss of *ALY1* and *ALY2* resulted in exquisite sensitivity to GPI and increased PM/vacuole ratios for Git1-GFP fluorescence (Figures 5a–c and 2a–c). Loss of the endocytic factor *END3*, which encodes an early arriving component of clathrin-mediated endocytic patches, similarly caused sensitivity to GPI and increased PM/vacuole fluorescence compared to wild-type (Figure 5a–c). Interestingly, deletion of *APL3* or *APL1*, which encode the α - and β -adaptin, respectively, of clathrin-associated protein complex 2 (AP-2), each gave rise to increased sen-

sitivity to GPI, with *apl1Δ* cells having a more severe phenotype than *apl3Δ* cells (Figure 5a). While the role of AP-2 cargo selection for clathrin-mediated endocytosis is well-documented in mammals, in yeast this complex is only associated with the internalization of a few membrane proteins, though it clearly associates with early endocytic patches (Carroll et al., 2009; Carroll et al., 2012). We examined AP-2 in these studies because Aly1 and Aly2 physically interact with these adaptins (O'Donnell et al., 2010). Loss of *APL1* led to increased PM/vacuole ratios, while *apl3Δ* significantly impaired vacuolar trafficking of Git1, but not as robustly as *apl1Δ* (Figure 5b,c). Interestingly, none of these endocytic factors (*end3Δ*, *apl1Δ*, or *apl3Δ*) increased the PM/vacuole ratio as much as loss of the ALYs (Figure 5b,c). Based on these data, we posit that some steady-state vacuolar trafficking of Git1 occurs via endocytosis, as vacuole-localized fluorescence diminishes in the endocytic mutants. Yet it appears that the *aly1Δ aly2Δ* cells additionally impair intracellular sorting of Git1. These data further suggest that AP-2 may play a role in regulating Git1 endocytosis.

To examine substrate-induced internalization and intracellular trafficking, we next examined the localization of Git1-GFP in cells treated with GPI that had either been preincubated in latrunculin A (LatA), which prevents actin polymerization to selectively block endocytosis while leaving intracellular sorting intact (Ayscough et al., 1997), or its vehicle control DMSO. Wild-type cells treated with LatA retained higher PM/vacuole fluorescence ratios after GPI addition (Figure 5d,e). However, the ratio was still significantly higher than the DMSO-treated cells after 2 h of GPI, indicating that some of the trafficking to the vacuole was likely due to endocytosis while a portion of the trafficking was independent of this LatA-blocked pathway. Interestingly, the LatA-treated cells were not statistically different from the DMSO-treated cells prior to GPI addition, suggesting an intracellular sorting route for Git1 (Figure 5d,e). In *aly1Δ aly2Δ* cells treated this way, GPI induced a

extracts from untreated cells or those incubated with GPI for 2 h and containing Git1-GFP expressed from the *TEF1* promoter were resolved by SDS-PAGE and immunoblotted with an anti-GFP antibody and revert total protein stain of the transfer membrane was used as a loading control. The ratio of the signal intensities of the GFP breakdown product to the full-length Git-GFP is presented below each lane. Molecular weights of standards are shown at left measured in kilodalton. (d) WT or *aly1Δ aly2Δ* cells containing Git1-GFP expressed from the *TEF1* promoter and either an empty pRS316 vector or one expressing the indicated α -arrestin were cultured in SC media lacking uracil and leucine and containing 50 μ M GPI for the times indicated before being imaged by fluorescence microscopy. (e) The PM and vacuolar fluorescence intensities from the cells depicted in (a) were quantified using our manual quantification pipeline and the distributions of PM/vacuolar fluorescence ratios in arbitrary units (a.u.) were plotted as scatter plots. The horizontal midline in black represents the median and the 95% confidence interval is represented by the gray error bars. Kruskal–Wallis statistical analyses with Dunn's post hoc test were performed, and statistical significance compared to the 0 min point for each graph is presented as asterisks (*) or indicated as not significant (ns). Additionally, the 0 min point for each of the strains is compared back to the wild-type with vector control and these are presented in blue text as daggers (†) or indicated as not significant (ns) where applicable. A yellow dashed line is presented to facilitate comparisons to the WT with vector control at the $t = 0$ min point, and a gray dashed line is presented to facilitate comparisons to the *aly1Δ aly2Δ* mutant with vector at the $t = 0$ min point. (f) Whole cell extracts from untreated cells or those incubated with GPI for 2 h and containing Git1-GFP expressed from the *TEF1* promoter were resolved by SDS-PAGE and immunoblotted with an anti-GFP antibody and Revert total protein stain of the transfer membrane was used as a loading control. The ratio of the signal

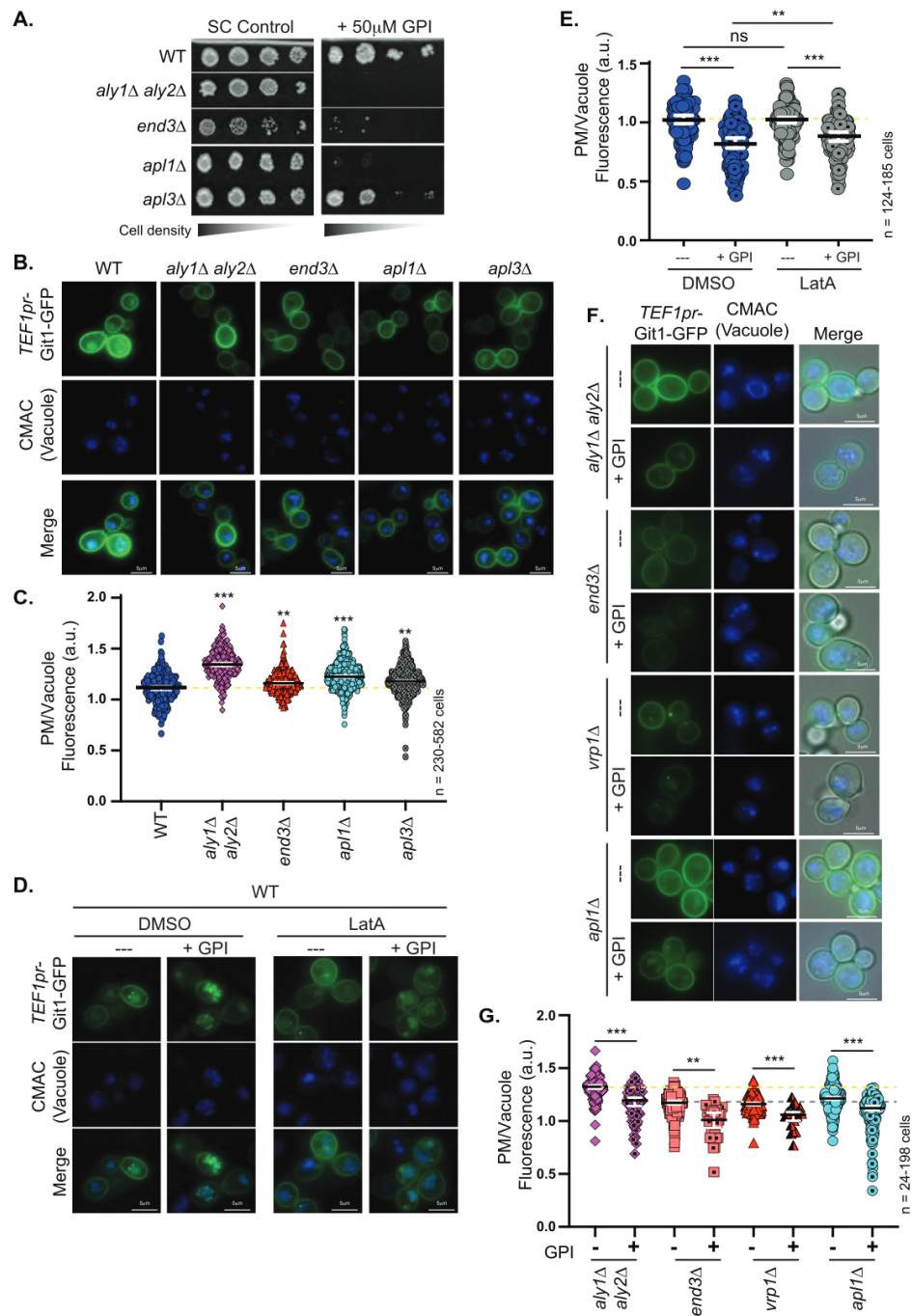


FIGURE 5 GPI induces clathrin-dependent endocytosis of Git1. (a) Growth on SC medium lacking leucine with or without 50 μ M GPI for serial dilutions of cells from the indicated yeast strains containing a *LEU2*-marked *TEF1pr-GIT1*-GFP plasmid is shown 2 days post-incubation at 30°C. (b) The indicated cells expressing Git1-GFP from the *TEF1* promoter were stained with CMAC and imaged by fluorescence microscopy. (c, e and g) The PM and vacuolar fluorescence intensities from the cells depicted in b, d or f, respectively, were quantified using our automated quantification pipeline and the distributions of PM/intracellular fluorescence ratios in arbitrary units (a.u.) were plotted as scatter plots. The horizontal midline in black represents the median and the 95% confidence interval is represented by the white error bars. A yellow dashed line represents the median ratio for untreated wild-type (in c and e) or *aly1 Δ aly2 Δ* (in g) cells to facilitate comparisons. Kruskal–Wallis statistical analyses with Dunn's post hoc test were performed and statistical significance is indicated with asterisks. In (c), all statistical comparisons indicated are relative to the WT control. (d) WT cells containing Git1-GFP expressed from the *TEF1* promoter were cultured in SC media lacking leucine and containing 200 μ M latrunculin A or an equal volume of DMSO for 120 min prior either addition of 50 μ M GPI or no treatment, and incubated for 120 min further before being stained with CMAC and imaged by fluorescence microscopy. (e) Cells containing Git1-GFP expressed from the *TEF1* promoter were cultured in SC media lacking leucine and containing 50 μ M GPI (+ GPI) or no treatment (—) and incubated for 120 min before staining with CMAC and fluorescence microscopy imaging.

small amount of Git1-GFP trafficking to the vacuole (as measured by decreased PM/vacuole fluorescence ratio) and Aly1 addition reduced this ratio further but the effect was muted in the LatA-treated cells (Figure S4b,c). In cells expressing Aly2 only, there was no impact of adding GPI to LatA-pretreated cells (Figure S4b,c). Thus, although GPI induces endocytosis of Git1 and this accounts for a portion of the decreased PM/vacuole ratio observed, the persistent decrease in the PM/vacuole ratio observed in LatA-treated cells after GPI addition suggests that there is a contribution of intracellular sorting to the vacuole for Git1-GFP in GPI-treated cells. Aly1 appeared better able to stimulate this intracellular sorting, as LatA-treated Aly1-expressing cells significantly dropped the PM/vacuole ratios after GPI treatment. In contrast, Aly2 did not seem to operate in this pathway as cells expressing Aly2 failed to reduce PM/vacuole ratios after LatA and GPI treatments.

To further assess this regulation, we next treated endocytic mutants with GPI and monitored PM/vacuole fluorescence. Cells lacking the ALYs have reduced PM/vacuole ratio post GPI addition and GPI significantly reduced the PM/vacuole ratio in *end3Δ*, *vrp1Δ* and *apl1Δ* cells (Figures 5f,g and S4b,c). Note that we included Vrp1, which encodes the yeast form of the actin nucleator verprolin, in these studies as an orthogonal approach to using the *end3Δ* cells as these were very sick upon GPI addition. This persistent reduction in PM/vacuole ratios post GPI addition in LatA-treated or endocytic mutant strains suggests that either a clathrin-independent endocytic (Prosser et al., 2015) and/or intracellular sorting route exists to shuttle Git1-GFP to the vacuole post GPI addition. Since LatA prevents both clathrin-mediated and -independent endocytosis the persistent internalization of Git1 observed in LatA-treated cells suggests that the intracellular sorting function is more likely.

α-Arrestins Aly1 and Aly2 must interact with Rsp5 to regulate Git1 trafficking

α-Arrestins contain L_P PxY motifs which allow for association with WW-domain-containing proteins. In yeast, each of the α-arrestins has been shown to use these L_P PxY motifs to engage the ubiquitin ligase Rsp5, which harbors three WW-domains (Figure 6a) (Gupta et al., 2007; Lin et al., 2008; O'Donnell et al., 2013; Becuwe & Leon, 2014). In addition to binding Rsp5, the α-arrestins bind to membrane proteins, thereby bringing the ubiquitin ligase near its membrane client proteins that lack their own L_P PxY motifs. Ubiquitination of membrane proteins serves as a signal for their endocytosis, with many of the early arriving endocytic proteins having ubiquitin-interaction motifs that aid in cargo clustering

but many of the α-arrestins themselves are monoubiquitinated by this ubiquitin ligase (Gupta et al., 2007; Lin et al., 2008; Becuwe et al., 2012; O'Donnell et al., 2013; Ho et al., 2017). This mono-ubiquitination is thought to be activating for α-arrestin function, and in some cases is required for α-arrestin recruitment to the membrane (Lin et al., 2008; MacGurn et al., 2011). More recently, a study by MacDonald et al. indicates that the monoubiquitination of the adaptor itself can act as an added anchor point for binding to the Rsp5 ubiquitin ligase (MacDonald et al., 2020). We sought to determine if Aly1 and Aly2 required interaction with Rsp5 and/or monoubiquitination to regulate Git1. We used two classes of Aly1 or Aly2 mutants. The first had each of the L_P PxY motifs mutated to L_P PxA and is called the “PY-less” mutant. We have shown that PY-less mutants of Alys fail to bind Rsp5, are no longer mono-ubiquitinated and fail to execute all the clathrin-mediated endocytic functions ascribed to date (O'Donnell et al., 2013; Prosser et al., 2015). The second mutant class has the single lysine needed for α-arrestin mono-ubiquitination mutated to arginine. We mapped this monoubiquitination site on Aly1 to lysine 379 and on Aly2 to lysine 392 using mass spectroscopy analyses and demonstrated previously that these K-to-R mutants are no longer ubiquitinated (Hager et al., 2018). We next introduced plasmid-borne versions of each of these mutants into *aly1Δ aly2Δ* cells and assessed their GPI sensitivity and Git1 trafficking. In keeping with all earlier observations, the “PY-less” mutant versions of either Aly1 or Aly2 were completely non-functional in these assays, and so *aly1Δ aly2Δ* cells containing Aly1^{PY-less} or Aly2^{PY-less} remained sensitive to GPI and the Git1-GFP PM/vacuole balance remained constant (Figure 6b–d). The Aly1^{K379R} mutant was as resistant to GPI as cells expressing Aly1 and slightly better at inducing steady state or substrate-induced trafficking of Git1 (Figure 6b–d). In contrast, cells with Aly2^{K392R} mutant were more sensitive to GPI than Aly2 cells. The steady-state trafficking of Git1-GFP to the vacuole appeared to be somewhat impaired in cells expressing Aly2^{K392R} but upon GPI addition this mutant reduced PM/vacuole ratios as effectively as Aly2 (Figure 6b–d).

These data demonstrate that while binding of Aly1 and Aly2 to Rsp5 is needed for their function with Git1, monoubiquitination of either of these α-arrestins has only a modest if any impact on their ability to traffic Git1.

Aly1-mediated trafficking of Git1 is modestly regulated by the protein phosphatase CN

Our earlier studies showed that α-arrestin Aly1 binds the protein phosphatase CN through a canonical 8-amino acid “PxIxIT” motif, in this case with the

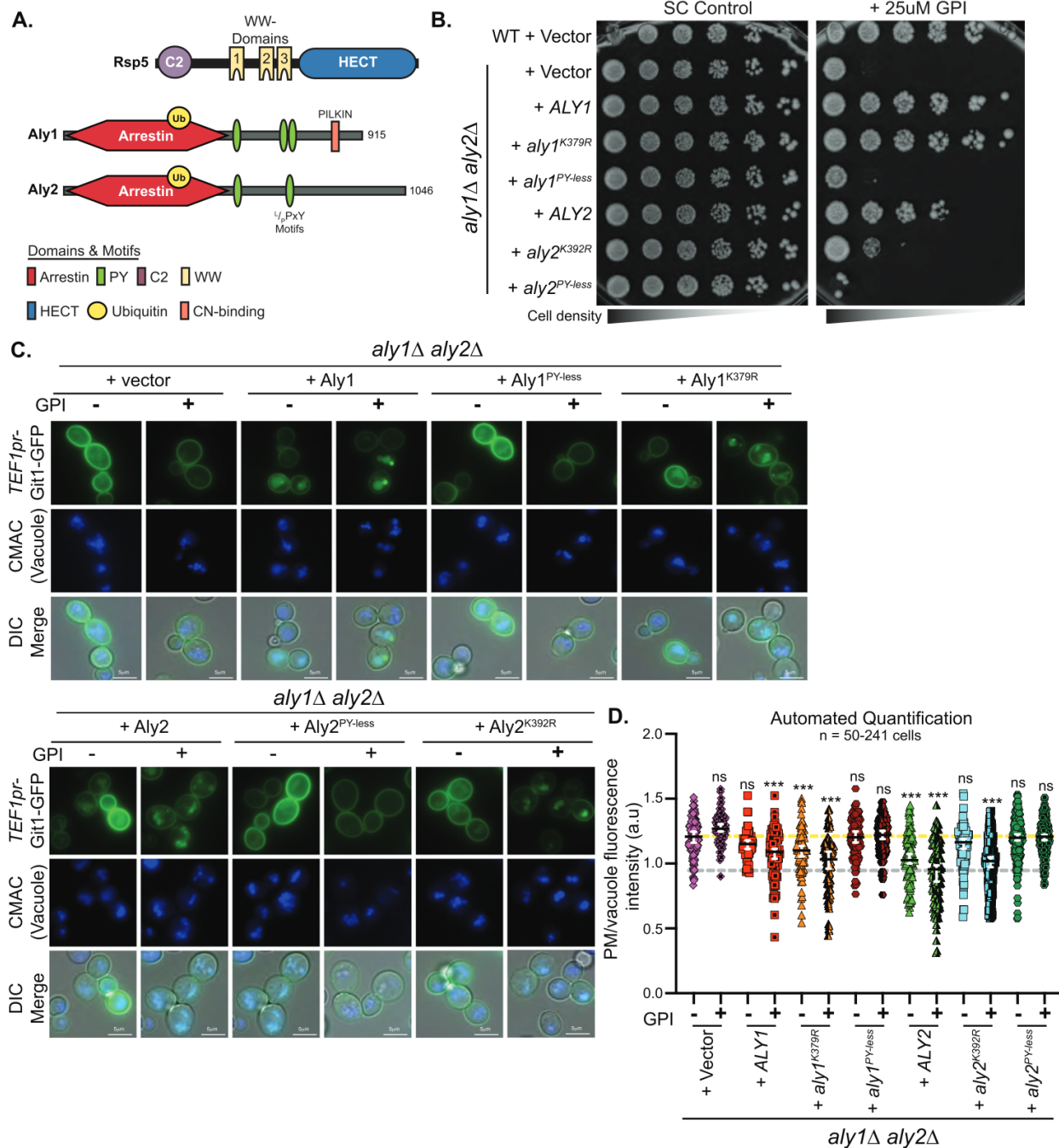


FIGURE 6 **Aly1- and Aly2-mediated trafficking of Git1 requires interaction with Rsp5.** (a) Schematic of Rsp5 and Aly1/Aly2 domain and motif architecture. The site of monoubiquitination on Aly1 and Aly2 is indicated as a yellow circle. (b) Growth of serial dilutions of WT or *aly1Δ aly2Δ* cells containing a *LEU2*-marked *TEF1pr-GIT1*-GFP plasmid and plasmids expressing the indicated α -arrestin variant on SC medium lacking uracil and leucine and containing 25 μ M GPI is presented. Growth is shown after 2 days of incubation at 30°C. (c) WT or *aly1Δ aly2Δ* cells containing Git1-GFP expressed from the *TEF1* promoter and either an empty pRS316 vector or expressing the indicated α -arrestin cultured in SC media lacking leucine and containing 50 μ M GPI (+ GPI) or no treatment (—), and incubated for 120 min before staining with CMAC and fluorescence microscopy imaging. (d) The PM and vacuolar fluorescence intensities from the cells depicted in (c) were quantified using our automated quantification pipeline and the distributions of PM/vacuolar fluorescence ratios in arbitrary units (a.u.) were plotted as scatter plots. The horizontal midline in black represents the median and the 95% confidence interval is represented by the white error bars. Kruskal-Wallis statistical analyses with Dunn's post hoc test were performed and statistical significance compared to untreated *aly1Δ aly2Δ* cells with vector is indicated with asterisks or as not significant (ns) where applicable. A yellow dashed line that represents the median of the untreated *aly1Δ aly2Δ* cells containing a vector control and a gray dashed line that represents the median of *aly1Δ aly2Δ* + Aly2 cells treated with GPI is provided to facilitate comparisons

calmodulin-dependent protein phosphatase conserved from yeast to man (Roy & Cyert, 2009, 2020). In humans, CN regulates T-cell differentiation, cardiac function, and impacts memory and learning (Klee & Haiech, 1980; Klee & Means, 2002). In yeast, CN helps cells adapt to stressors by regulating the nuclear translocation of the Crz1 transcription factor (Stathopoulos & Cyert, 1997; Cyert, 2003). However, CN also senses and regulates lipid homeostasis; specifically, it helps control sphingolipid balance through its downstream substrates (Bultynck et al., 2006; Tabuchi et al., 2006). Given that GPI transport through Git1 influences PI and sphingolipid levels in cells (Figure 1a), we sought to determine if CN-dependent regulation of Aly1 might influence its role in Git1 trafficking. To do this we made use of mutants that altered CN-regulation of Aly1 in two ways. First, we used Aly1 mutants that lack the CN binding site—either Aly1^{ΔPILKIN}, where the site was deleted, or Aly1^{AAAAAA}, where each residue in this motif was mutated to alanine. In each of these mutants, CN can no longer bind to or dephosphorylate Aly1, and therefore Aly1 is retained in its phosphorylated state (O'Donnell et al., 2013), providing that the kinases needed to phosphorylate Aly1, which remain undefined, are active. Second, from our earlier studies we identified five residues—T250, S252, S568, S569, and S573—whose modification was dependent on CN (O'Donnell et al., 2013). Here we use Aly1 mutants where each of these sites was converted to alanine (referred to as Aly1^{5A}), mimicking the dephosphorylated state, or a glutamic acid (referred to as Aly1^{5E}), mimicking the phosphorylated state (O'Donnell et al., 2013). These site mutations bypass the need for kinase activity and directly modulate the CN-regulated sites.

Cells expressing Aly1 or Aly1^{5A} (mimics constitutively dephosphorylated Aly1) gave rise to the strongest restoration of growth on GPI, while the Aly1^{ΔPILKIN} and Aly1^{AAAAAA} (which cannot be dephosphorylated by CN) gave a slightly more modest rescue of the growth on GPI than wild-type Aly1. Finally, Aly1^{5E} (mimics constitutively phosphorylated Aly1) only marginally improved growth on GPI (Figure 7a). When we examined the localization of Git1 under steady-state conditions, we again find that addition of Aly1 to *aly1Δ aly2Δ* cells gave only a modest reduction in the PM/vacuole ratio of Git1 that was not significantly different from the vector (Figure 5a,b). The mutant versions of Aly1 that lack the CN-binding site, Aly1^{ΔPILKIN} and Aly1^{AAAAAA}, and the CN-regulated phospho-mimetic Aly1^{5E} all failed to significantly alter the PM/vacuole Git1 fluorescence intensities when compared to the vector control (Figure 5a,b). It could be the case that under these conditions the kinase responsible for phosphorylating Aly1 is active and therefore retains these mutants as well as the wild-type Aly1 in an inactive state. However, cells containing Aly1^{5A}, which

ratios compared to *aly1Δ aly2Δ* cells with a vector control (Figure 5a,b). To better assess Aly1 function under conditions where it would be more active, we next explored GPI-induced trafficking of these same cells and found that when GPI was added Aly1, Aly1^{ΔPILKIN}, Aly1^{AAAAAA}, Aly1^{5A}, and Aly1^{5E} all significantly reduced the PM/vacuole fluorescence ratio in comparison to *aly1Δ aly2Δ* containing vector alone (Figure 7b,c). However, the Aly1^{5A} mutation that mimics the dephosphorylated form, stimulated vacuolar trafficking of Git1 best in these conditions (Figure 7b,c). Taken together, these data further support a model whereby Aly1 plays a modest role in the steady-state trafficking of Git1, however GPI addition stimulates Aly1's role in Git1 trafficking to the vacuole. Finally, in response to GPI the dephosphorylation mimetic allele, Aly1^{5A}, is best able to traffic Git1 to the vacuole while the phosphorylated mimetic, Aly1^{5E}, modestly rescued growth of *aly1Δ aly2Δ* on GPI-containing medium. These results are consistent with CN acting as a modest positive regulator of Aly1-mediated trafficking of Git1.

While these data suggest that CN—acting through Aly1—is a modest positive regulator of Git1 trafficking to the vacuole, we were surprised to see that when we deleted *Cnb1*, the regulatory subunit of CN, Git1 trafficking to the vacuole was greatly increased (Figure 8a,b). In contrast to the modest effects observed when exploring CN-regulation of Aly1, this dramatic change in steady-state localization of Git1 in the absence of CN function suggests that CN plays a largely inhibitory role on the vacuolar sorting of Git1. We sought to define the genetic relationship between the Alys and CN in controlling Git1 localization. We found that *cnb1Δ* cells were resistance to GPI and trafficked Git1 to the vacuole, which addition of GPI did not alter (Figure 8d,e). Importantly, loss of Aly1 and Aly2 completely blocked the *cnb1Δ*-induced resistance to GPI and prevented Git1 transit to the vacuole (Figure 8d,e), demonstrating that the Aly1/Aly2 regulation of Git1 is epistatic to that of *Cnb1*.

Aly-dependent alterations in sphingolipid and phosphoinositide metabolism

The GPI internalized via Git1 releases free inositol that is incorporated into PI. Since PI is a precursor to sphingolipid and phosphoinositide production (Figures 1a and 8c), we probed these pathways for alterations when either α -arrestin-mediated or general protein trafficking were perturbed and queried regulation by CN. First, we examined the sensitivity of cells lacking Aly1 and/or Aly2 to myriocin, a drug that inhibits the enzymes Lcb1 and Lcb2 which act in the first step of the de novo sphingolipid synthesis pathway (Figure 8c), and aureobasidin, a drug that blocks the phosphatidylinos-

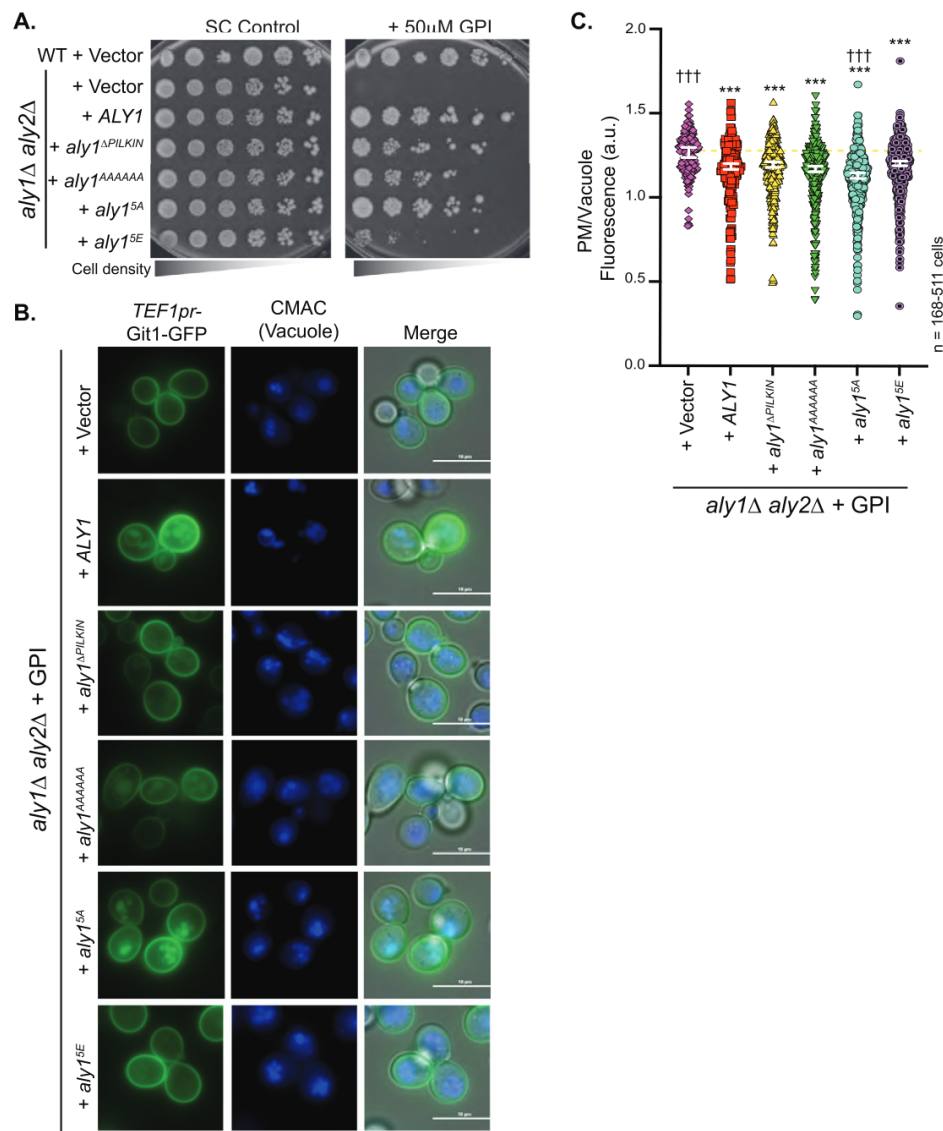


FIGURE 7 Calcineurin-mediated regulation of *Aly1* plays a modest role in *Git1* trafficking. (a) Growth of serial dilutions of WT or *aly1Δ aly2Δ* cells containing a *LEU2*-marked *TEF1pr-GIT1*-GFP plasmid and an empty pRS316 vector or one expressing the indicated α -arrestins on SC medium lacking uracil and leucine and containing 50 μ M GPI is shown after 2 days of incubation at 30°C. (b) Cells lacking *ALY1* and *ALY2* with a plasmid expressing Git1-GFP from the *TEF1* promoter and an empty pRS316 vector or one expressing the indicated α -arrestins were treated with 50 μ M GPI for 2 h, stained with CMAC and imaged by fluorescence microscopy. (c) The PM and vacuolar fluorescence intensities from the cells depicted in (b) were quantified using our automated quantification pipeline and the distributions of PM/intracellular fluorescence ratios in arbitrary units (a.u.) were plotted as scatter plots. The horizontal midline in black represents the median and the 95% confidence interval is represented by the white error bars. Kruskal-Wallis statistical analyses with Dunn's post hoc test were performed and statistical significance compared to the *aly1Δ aly2Δ* cells with vector is indicated with asterisks or as not significant (ns) where applicable. The ††† symbols represent p -values of <0.001 and is used to mark comparisons between *aly1Δ aly2Δ* expressing *ALY1* and each of the other conditions. In these conditions, most mutants are not statistically different from wild-type *Aly1* (no marker shown); *Aly1*^{5A} is the only mutant that has significantly lower PM/vacuole ratios than wild-type *Aly1* while the vector control has significantly higher PM/vacuole ratios. In each case, a yellow dashed line is provided that represents the median of the *aly1Δ aly2Δ* cells containing a vector control to facilitate comparisons

(IPC) in sphingolipid synthesis (Figure 8c) (Angus & Lester, 1972; Dickson et al., 2006; Epstein et al., 2012; Klug & Daum, 2014; Montefusco et al., 2014). Since elevated [³H]-GPI uptake leads to higher conversion of the inositol label into PI (Figure 10c) and PI is required

that *Aly* loss might make cells resistant sphingolipid synthesis inhibitors. However, this is not what we observed. Loss of *Aly1* and *Aly2* caused modest sensitivity to myriocin and aureobasidin (Figure 8d), that was rescued by addition of phytosphingosine (PHS) (Figure

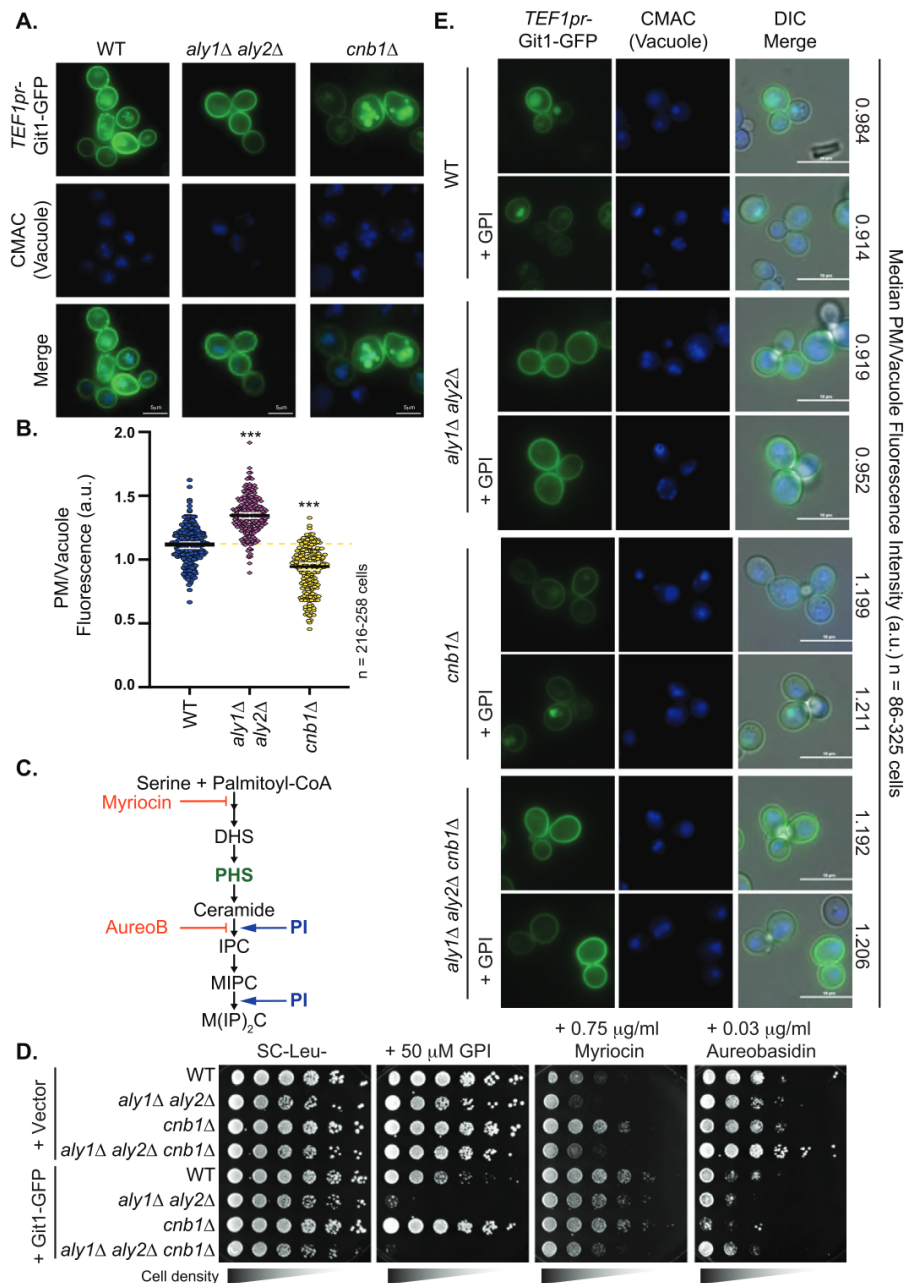


FIGURE 8 Loss of CN increases Git1 trafficking to the vacuole, but *aly1Δ aly2Δ* is epistatic to CN regulation. (a) WT, *aly1Δ aly2Δ*, or *cnb1Δ* cells containing Git1-GFP expressed from the *TEF1* promoter were stained with CMAC and imaged by fluorescence microscopy. (b) The PM and vacuolar fluorescence intensities from the cells depicted in (a) were quantified using our automated quantification pipeline and the distributions of PM/vacuolar fluorescence ratios in arbitrary units (a.u.) were plotted as scatter plots. The horizontal midline in black represents the median and the 95% confidence interval is represented by the white error bars. Kruskal–Wallis statistical analyses with Dunn's post hoc test were performed and statistical significance compared to the wild-type cells is indicated with asterisks. A yellow dashed line is provided that represents the median of wild-type cells to facilitate comparisons. (c) Schematic of the sphingolipid biosynthetic pathway demonstrating the points where myriocin and aureobasidin (AureoB) impair the pathway (red lines) and the sites where PI is incorporated into the complex base production in the lower half of the pathway (PI with blue arrows). The intermediates in the pathway are shown but the enzymes are not indicated. For simplicity, some intermediates are not shown and instead, multiple arrowheads are provided for those steps where two or more enzymatic steps have been condensed. (d) Growth of serial dilutions of the cells indicated containing either a vector or a *LEU2*-marked *TEF1pr-GIT1*-GFP plasmid on SC medium lacking leucine and containing the indicated concentrations of GPI, myriocin, or aureobasidin are shown after 2–4 days of incubation at 30°C. (e) WT, *aly1Δ aly2Δ*, *cnb1Δ*, or *aly1Δ aly2Δ cnb1Δ* cells containing Git1-GFP expressed from the *TEF1* promoter were treated with 50 μM GPI for 2 h, stained with CMAC and imaged by fluorescence microscopy. Automated quantification of the PM and vacuole fluorescence intensities was performed and the median PM/vacuole fluorescence intensity for each is presented in arbitrary units (a.u.) on the

(Figure 8c). Other endocytic mutants, including *vrp1Δ* and *end3Δ*, or ESCRT mutants *vps4Δ* and *vsp27Δ*, which each likely increase membrane protein abundance at the PM, similarly showed sensitivity to myriocin that can, in all but *end3Δ* cells, be partially rescued by PHS addition (Figure S6a). Given the importance of sphingolipids in the PM and other membranes, it is not surprising that defects in membrane trafficking may change sphingolipid balance. Many trafficking mutants have defects in sphingolipid balance, including the Golgi-associated retrograde protein (GARP) complex (Frohlich et al., 2015), regulators of Niemann-Pick type C disease (Newton et al., 2018), and Rab proteins (Choudhury et al., 2002). Based on these data it appears that the α -arrestins Aly1 and Aly2 influence sphingolipid homeostasis as well, however the linkage to Git1 regulation is unclear.

We next examined *cnb1Δ* cells and found they were resistant to myriocin and sensitive to aureobasidin (Figure 8d), consistent with a role for CN as an effector of sphingolipid biosynthesis (Dunn et al., 1998; Bultynck et al., 2006; Tabuchi et al., 2006; Muir et al., 2014). However, the *aly1Δ aly2Δ cnb1Δ* cells were wild-type like in their sensitivity to myriocin but completely resistant to aureobasidin, suggesting a more complex interplay between these α -arrestins and CN. Given that CN impinges upon the sphingolipid biosynthesis pathway at multiple points, including (i) dephosphorylation of Lac1/Lag1, (ii) regulation of Csg2, and (iii) dephosphorylation of the Slm proteins (Dunn et al., 1998; Bultynck et al., 2006; Tabuchi et al., 2006; Muir et al., 2014), it is unlikely that the genetic interaction would solely be explained via the α -arrestins. Considering these broad roles in sphingolipid regulation for CN, it is interesting that the *aly1Δ aly2Δ* reversed the aureobasidin sensitivity for *cnb1Δ* cells (Figure 8d). To begin to determine if any of these phenotypes were linked to Git1 trafficking, we over-expressed Git1-GFP in each of these mutants and found that this increased resistance to myriocin in all but the *cnb1Δ* cells, while increasing sensitivity to aureobasidin (Figure 8d). This result was not expected since we thought elevated Git1 would increase GPI uptake and PI levels, which should aid in sphingolipid biosynthesis. While these data further define a role for Git1, CN, and endocytic trafficking in influencing sphingolipids, the underlying mechanism for these altered sensitivities to sphingolipid inhibition remain to be fully elucidated.

In addition to feeding PI into the sphingolipid biosynthetic pathway, uptake of GPI through Git1 and its subsequent release of inositol could elevate PI to cause changes in membrane phospholipid production. To survey the phospholipid distributions in cells where Aly1 and Aly2 were lost, we examined the localization and abundance of probes for phosphatidylserine (PS, Lact-C2-GFP probe) as well as phosphatidyl-

GFP-P4Mx1 probes), phosphatidylinositol 3-phosphate (PI(3)P, GFP-FYVE-Eea1 probe), and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂, GFP-2xPH-Plc δ). We found no significant changes in PI(4)P or PI(4,5)P₂ in the absence of α -arrestins Aly1 or Aly2 (Figure S7a–c). However, there were dramatic changes in PI(3)P and PS in the *aly1Δ aly2Δ* mutant compared to wild-type cells (Figures 9a,b and S7d,e). Interestingly, the PI(3)P, which typically marks the endosomal compartment in wild-type cells, was significantly brighter and distributed to the limiting membrane of the vacuole in the absence of the Alys. Mutations that disrupt endocytosis and the trafficking of Git1, such as *end3Δ*, *vrp1Δ*, and *apl1Δ*, did not result in similar elevations of PI(3)P on the limiting membrane of the vacuole (Figure 9c), though for *end3Δ* there was an issue with the expression of the GFP-Eea1-FYVE probe (Figure 9d). The other mutants examined had equivalent expression of the GFP-Eea1-FYVE to that observed in WT cells (Figure 9d). Though it is somewhat unclear how these specific phospholipid species, and not others, would be influenced by α -arrestins, we sought to determine if alteration of PI(3)P could in any way be linked to GPI uptake through Git1. We treated cells with GPI and monitored GFP-FYVE-Eea1 localization and abundance changes. We found that even in wild-type cells treated with GPI, PI(3)P-associated fluorescence increased with brighter PI(3)P signal on the limiting membrane of the vacuole (Figure 10a,b). In the absence of ALY1 and ALY2, not only did cells start out with more PI(3)P on the vacuolar membrane, but the abundance further increased upon GPI addition (Figure 10a,b). To further assess the linkage between GPI uptake and PI conversion as possibly fueling these changes in phospholipid balance, we provided cells with [³H]-inositol-GPI and monitored the presence of the inositol label across extracellular, soluble intracellular, and membrane fractions (Table 1). These assays were performed using the endogenous Git1 transporter under growth conditions that would allow for *Git1* expression (see Section “Methods”). As shown in Table 1 and Figure 10c, *aly1Δ aly2Δ* cells incorporated a greater percentage of inositol labels from exogenous GPI into the cell as both a TCA-extractable (water-soluble) fraction and into a particulate membrane fraction as compared to wild-type cells during the labeling period. Roughly 2.5 times the amount of soluble intracellular inositol label and four times as much PI was found in *aly1Δ aly2Δ* as compared to wild-type (Table 1). This result is consistent with increased stability, and hence activity, of Git1 at the PM in *aly1Δ aly2Δ* cells. It is important to note that the labeled metabolites detected, including PI, do not represent total internal pools, but rather represent only those molecules that received inositol label from exogenous [³H]-GPI. In addition to this pool, de novo synthesis of inositol will be a major source of unlabeled inositol and that pool is not

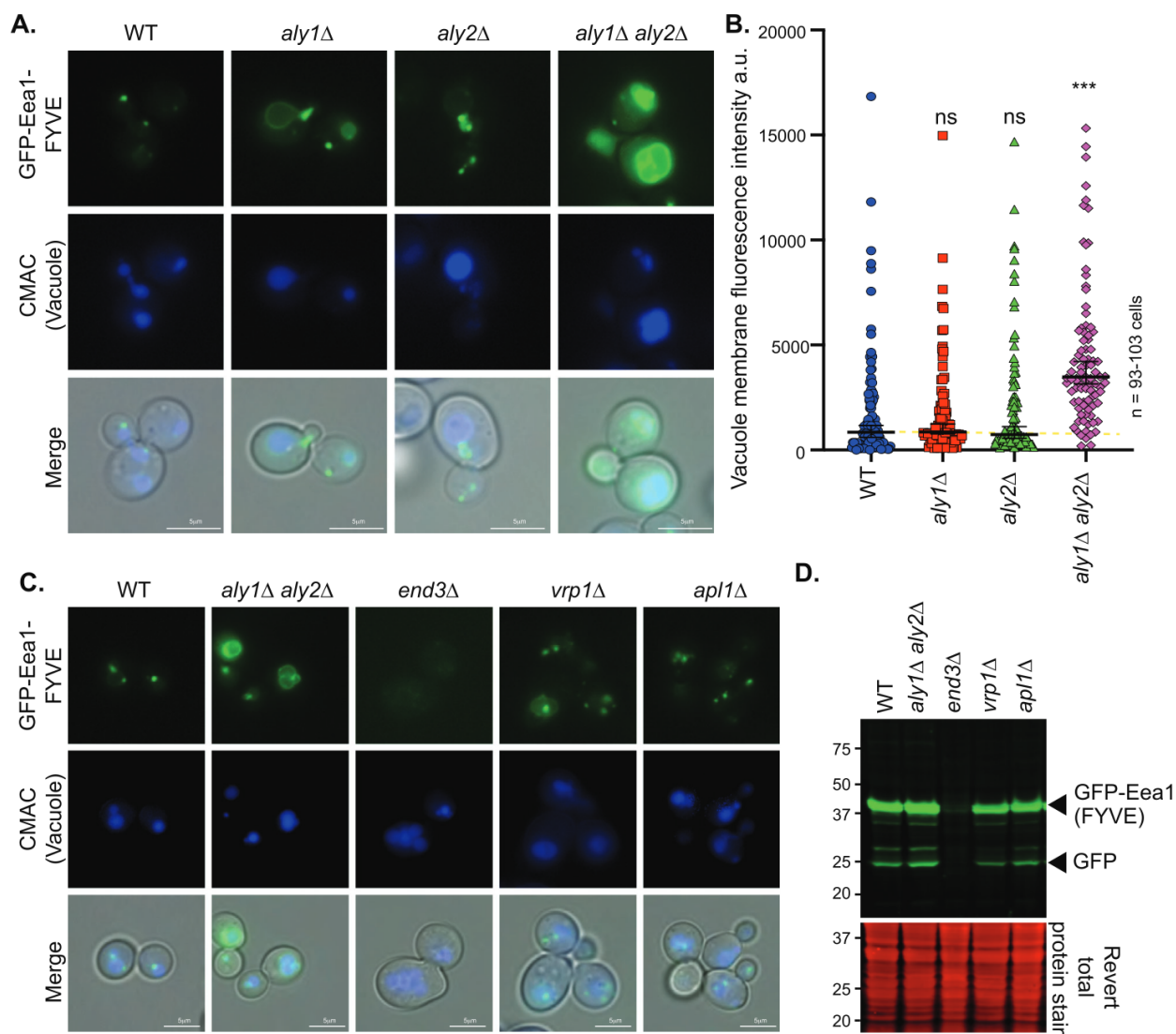


FIGURE 9 Loss of Aly1 and Aly2 disrupts phospholipid balance in the endomembrane system. (a) The indicated cells were transformed with the plasmid expressing a GFP-fused FYVE domain of Eea1, which marks PI(3)P. Cells were stained with CMAC and imaged by fluorescence microscopy. (b) The GFP fluorescence at the limiting membrane of the vacuole from the images in (a) was quantified by manually drawing a line around the outside of the CMAC stained vacuoles and overlaying this mask onto the GFP channel. The quantified fluorescence intensities (arbitrary units, a.u.) are presented as scatter plots. The horizontal midline in black represents the median and the 95% confidence interval is represented by the black error bars. Kruskal–Wallis statistical analyses with Dunn's post hoc test were performed and statistical significance compared to the wild-type cells is indicated with asterisks or as not significant (ns) where applicable. A yellow dashed line is provided that represents the median of wild-type cells to facilitate comparisons. (c) The indicated cells were transformed with the plasmid expressing a GFP-fused FYVE domain of Eea1, which marks PI(3)P. Cells were stained with CMAC and imaged by fluorescence microscopy. (d) Whole cell extracts from cells expressing the GFP-fused FYVE domain of Eea1 were resolved by SDS-PAGE and immunoblotted with an anti-GFP antibody and Revert total protein stain of the transfer membrane was used as a loading control. Molecular weights of standards are shown at left measured in kilodaltons

et al., 2014). These data confirm the known linkage between GPI uptake and incorporation of released inositol into PI and show that the α -arrestins Aly1 and Aly2 play an important role in this regulation (Table 1) (Patton et al., 1995; Patton-Vogt & Henry, 1998; Almaguer et al., 2003; Almaguer et al., 2004). This is not a completely unexpected functional link between GPI uptake,

pathways are well-documented. However, our findings do not demonstrate that all of the aberrant PI(3)P accumulation on *aly1* Δ *aly2* Δ vacuoles is due to GPI uptake via Git1. In fact, under the conditions used for the PI(3)P imaging assays we would expect very little Git1 to be at the PM and so there must be additional mechanisms by which *aly1* Δ *aly2* Δ cause elevated PI(3)P that remain to

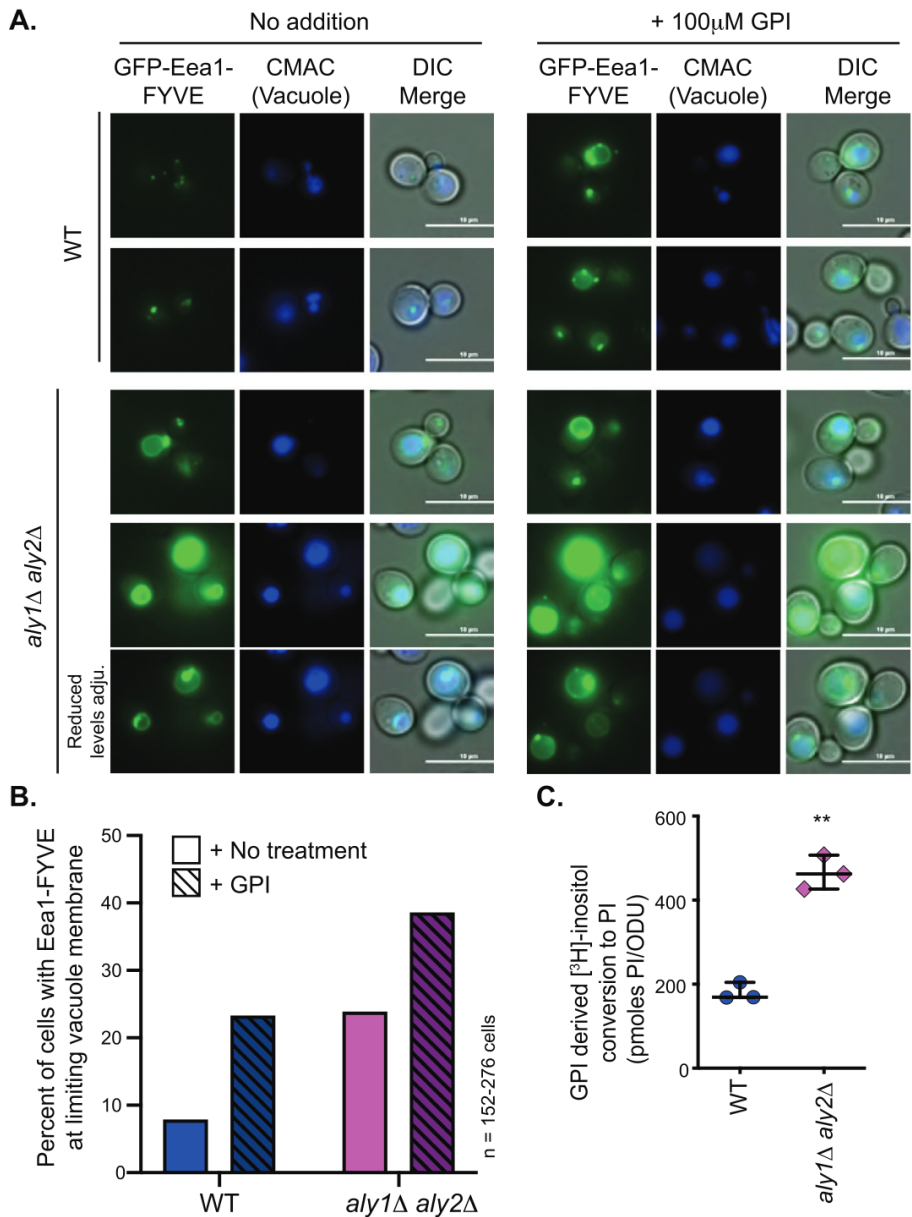


FIGURE 10 GPI uptake is converted into PI and increases PI3P at the limiting membrane of the vacuole. (a) The indicated cells were transformed with the plasmid expressing a GFP-fused FYVE domain of Eea1, which marks PI(3)P. Cells were grown to mid-log phase and either untreated (no addition) or treated with 100 μM GPI for 2 h, stained with CMAC and imaged by fluorescence microscopy. (b) Images of cells captured in (a) were evenly adjusted. Cells with evident GFP staining of vacuolar limiting membrane were counted in comparison to the total number of vacuoles in the image, as assessed using the CMAC stain. The percentage of cells with limiting membrane fluorescence is presented for each treatment. All cells in at least three images per condition were counted for an *n* = 150 at minimum per condition. (c) Radiolabeled [³H]-GPI uptake via the endogenous Git1 transporter and conversion to [³H]-PI was measured in wild-type or *aly1Δ aly2Δ* cells. Cells were grown under low phosphate conditions to induce *GIT1* expression in the presence of 5 μM [³H-inositol]-GPI, and intracellular and membrane fractions were isolated. The conversion of [³H]-GPI into [³H]-PI was monitored by using TLC and liquid scintillation counting. The graph presented represents three independent biological replicates that were measured and a Student's *t*-test was used to assess significance. A more detailed breakdown of these results is presented in Table 1 where both the water-soluble and membrane fractions are compared

DISCUSSION

Our findings demonstrate that α-arrestins Aly1 and Aly2 regulate trafficking of Git1 transporter to the vacuole by

to the presence of its substrate, GPI, and both basal and substrate-induced internalization are dependent upon the Alys. There is an interesting division of labor between the Alys, with Aly2 controlling Git1 trafficking

TABLE 1 Uptake and conversion of [³H]-GPI into [³H]-PI

Strain	Rep.	Extracellular			Intracellular			Membrane		
		%	Ave. (%)	St.Dev. (%)	%	Ave. (%)	St.Dev. (%)	%	Ave.	St.Dev. (%)
WT	1	74.73	76.34	1.40	18.53	17.48	1.28	6.74	6.61	0.12
	2	77.03			16.44			6.53		
	3	77.25			16.20			6.55		
aly1Δ aly2Δ	1	35.34	31.98	3.91	42.95	44.06	1.79	21.71	23.96	2.24
	2	27.69			46.12			26.19		
	3	32.90			43.11			23.98		

Strains were grown in the presence of 5 μM [³H]-inositol-GPI (see Section “Methods”) and then extracellular, intracellular and membrane fractions were isolated. Lipids were extracted from the membrane fraction and resolved by TLC as described in *Methods*. The average (Ave.) and standard deviation (St.Dev.) of three individual cultures (biological triplicates) are presented. It should be noted that this experiment was repeated with label of lower specific activity and similar results were obtained.

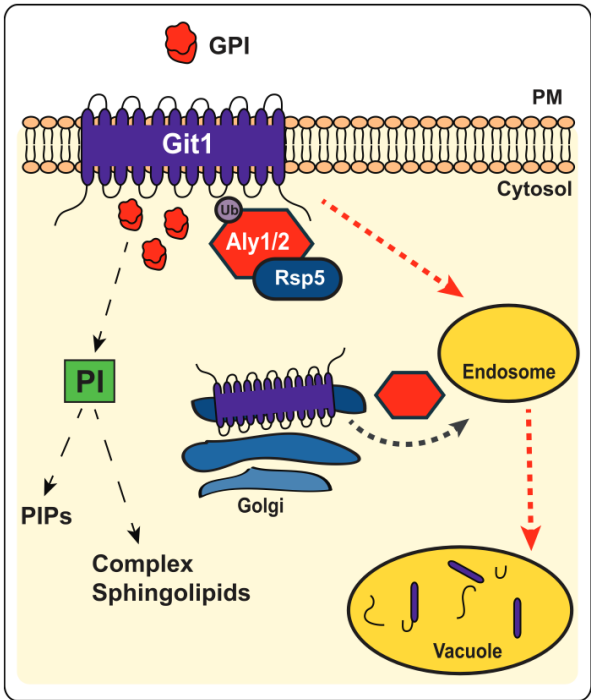


FIGURE 11 Model of α -arrestin regulation of Git1 trafficking. α -Arrestins are responsible for trafficking of Git1 to the vacuole during both steady-state conditions, where Aly2 is predominantly active, and in response to its substrate GPI, where Aly1 seems to play a more important role. Additionally, an intracellular sorting pathway that is independent of endocytosis contributes to Git1 trafficking to the vacuole and this pathway is also α -arrestin dependent (indicated by gray dashed arrow). Association with the ubiquitin ligase Rsp5 is required for α -arrestin-mediated trafficking of Git1. The endocytic pathway (indicated by red dashed lines) appears to be clathrin-dependent as disruption of clathrin-associated factors, like End3 and AP-2, leads to retention of Git1 at the cell surface. Uptake of GPI via Git1 may alter the sphingolipid balance in cells and seems to directly influence the distribution of membrane phospholipids, including elevating PI3P on the vacuolar membrane

depends upon clathrin, consistent with earlier studies (Almaguer et al., 2006), as we can block at least some Git1 endocytosis using mutations in clathrin-coated pit

AP-2. This latter finding is interesting since there are very few endocytic cargos associated with AP-2 in yeast and we have previously found that Alys can each interact directly with AP-2 (O'Donnell et al., 2010). Finally, both Aly1 and Aly2 must interact with the ubiquitin ligase Rsp5 to control Git1 trafficking, though their function is not dramatically hampered by loss of their respective mono-ubiquitination sites.

While endocytosis plays a clear role in Git1 trafficking, intracellular sorting also contributes to Git1 trafficking to the vacuole. Evidence in support of an intracellular sorting mechanism driving Git1 to the vacuole includes the fact that *end3Δ* or *LatA*-treated cells do not retain as much Git1 at the PM as *aly1Δ aly2Δ* cells, suggesting that these α -arrestins help sort Git1 to the vacuole in addition to regulating endocytosis. *LatA* is effective at blocking the impact of Aly2 on Git1 localization but does not seem as effective at blocking Aly1-mediated trafficking of Git1 to the vacuole. In addition, the block to vacuolar trafficking of Git1 in *aly1Δ aly2Δ* cells upon GPI addition is incomplete, suggesting that other adaptors or pathways are also important. Indeed, we find a role for the Buls in altering Git1 protein abundance and trafficking, and this is not the first example of a functional overlap between the Alys and the Buls (Crapeau et al., 2014). Additional α -arrestins may play minor roles in Git1 trafficking since (i) *aly1Δ aly2Δ* cells are not as effective as *9ArrΔ* cells (where the Buls are intact) in retaining endogenous Git1 at the cell surface and (ii) in the *9ArrΔ* cells, Aly1 is more effective at trafficking Git1 under basal conditions than it is in the *aly1Δ aly2Δ* cells, suggesting that it may still be competing with other α -arrestins for access to the cargo in this latter cell type.

α -Arrestin Aly1 is dephosphorylated by the protein phosphatase CN and this activity is needed for optimal Aly1-mediated endocytosis of the aspartic acid permease Dip5 in response to excess substrate (O'Donnell et al., 2013). However, the role of CN-regulation in Git1 trafficking is more complex than this, perhaps due to CN's broader functions in regulating sphingolipid homeostasis. Somewhat surprisingly, mutants of Aly1 that

Git1, perhaps indicating that the as-yet unknown kinase responsible for countering CN function is not fully active under these conditions. However, a mutant of Aly1 that side-steps the need for kinase function, like the one where the CN-regulated phosphorylation sites were all mutated to alanines to mimic a constitutively dephosphorylated state, significantly improved Git1 trafficking to the vacuole in both steady-state and substrate-induced conditions. This finding is in keeping with the idea that dephosphorylation improves an α -arrestin's ability to induce trafficking to the vacuole.

It was surprising then to see that loss of the regulatory subunit of CN resulted in robust steady-state trafficking of Git1 to the vacuole, indicating that CN has an inhibitory role in Git1 trafficking. Further, loss of the α -arrestins Aly1 and Aly2 in the *cnb1* Δ strains completely blocked this trafficking, demonstrating that the negative regulation CN exerts on this pathway occurs, either directly or indirectly, via the α -arrestins. Perhaps the altered sensitivities to myriocin and aureobasidin, two drugs that inhibit steps in the sphingolipid biosynthetic pathway, upon Git1 overexpression is linked to this element of CN regulation. Indeed, complex sphingolipid production is reduced under conditions where cells are starved for inositol and/or PI and elevated in conditions where there is excess inositol and/or PI (Henry et al., 2014). When Git1 is retained at the cell surface it should provide added GPI and, subsequent to its breakdown, inositol for PI synthesis. This should then augment sphingolipid production and provide resistance to drugs that impede the pathway (Henry et al., 2012; Henry et al., 2014). However, counter to this anticipated model we find increased sensitivity to aureobasidin when Git1 is overexpressed in cells. The reason for this phenotype is unclear, but it is important to note that these inhibitor studies were performed in the absence of exogenous GPI (Figure 8d) to avoid its toxicity phenotype. Without GPI supplementation, the extracellular levels of GPI available for transport are estimated to be 1–2 μ M per OD in a wild-type cell (Angus & Lester, 1972; Patton-Vogt & Henry, 1998) and so may not have a dramatic impact on PI levels. Instead, the observed sensitivity result may be related to a broader role for the Alys in altering trafficking of multiple membrane proteins and/or changing the signaling landscape. In the end, while our data suggest interesting connections between α -arrestins, Git1, CN, and sphingolipid balance, the mechanistic links remains to be resolved.

A striking finding from this work is the dramatic alteration in certain phospholipid species associated with loss of the Alys and exacerbated by addition of GPI as monitored through imaging. While PI(3)P is typically sequestered to endosomal membranes, we show a substantial increase in PI(3)P on the limiting membrane

phenotype can be linked at least in part to the activity of Git1, since addition of GPI to either wild-type or *aly1* Δ *aly2* Δ cells increased PI(3)P on vacuolar membranes, it seems unlikely that all of the PI(3)P accumulation in the absence of Alys is due solely to Git1. In yeast cells, PI conversion to PI(3)P occurs through the action of Vps34, the PI 3-kinase (Schu et al., 1993). Perhaps this enrichment in PI(3)P in *aly1* Δ *aly2* Δ cells, which is exacerbated upon GPI addition, is due to hyperactivity Vps34. Conversely, aberrant Fig4, the PI(3,5)P₂ phosphatase localized to vacuole and nuclear periphery (Gary et al., 2002), or Fab1, the vacuole-localized PI(3)P kinase that generates PI(3,5)P₂, activity may contribute to the elevated PI(3)P. However, given the relatively small pool of PI(3,5)P₂ in cells this seems somewhat less likely (Hasegawa et al., 2017).

Conversion of PI(3)P back into PI is regulated by the PI(3)P phosphatase, Ymr1, and the synaptojanin-like phosphatases, Sjl2 and Sjl3 (Parrish et al., 2004). In mutants lacking Ymr1 and Sjl3 there is aberrant accumulation of PI(3)P on the limiting membrane of the vacuole and a vacuole fragmentation phenotype (Parrish et al., 2004). While there is no observable vacuole fragmentation in *aly1* Δ *aly2* Δ , loss of Ymr1/Sjl activity could contribute to the elevated PI(3)P on vacuoles. It will be interesting to see the influence of GPI on the activity of these many PIP regulatory pathways, as it seems feasible that the mechanism by which Git1 retention at the cell surface leads to GPI-induced toxicity could be through alteration of PIP levels. Indeed, maintaining the balance of PIPs in cells is critical as defects in phospholipid metabolism and regulation can be toxic (Strahl and Thorner, 2007; Shyu et al., 2019).

Future studies may reveal a broader role for α -arrestins in contributing to phospholipid balance in cells and help uncover the underlying mechanism behind these phospholipid changes. While many components that disrupt protein trafficking alter phospholipid ratios in the cell, the link here between excess GPI uptake due to Git1 retention at the cell surface, conversion of internalized GPI to PI and specific enrichment of PI(3)P at the vacuolar membrane in the absence of α -arrestins Aly1 and Aly2 represent significant new findings and demonstrate that α -arrestins contribute to phospholipid balance in a previously unanticipated way.

MATERIALS AND METHODS

Yeast strains and growth conditions

All yeast strains used in this study are derived from the BY4741 genetic background of *S. cerevisiae* (S288C in origin) and are described in detail in Table 2. Yeast were grown in synthetic complete (SC) medium lack-

TABLE 2 Yeast strains

Strain	Genotype	Reference or source
BY4741	<i>MATa his3Δ1 leu2Δ0 LYS2 met15Δ0 ura3Δ0</i>	Open Biosystems (Winzeler et al., 1999)
EN60 (9ArrΔ)	<i>ecm21Δ::KanMX csr2Δ::KanMX bsd2Δ rog3Δ::NatMX rod1Δ ygr068cΔ aly1Δ aly2Δ ylr392cΔ::HIS3 his3Δ0 ura3Δ0 leu2Δ0</i>	(Nikko and Pelham, 2009)
<i>aly1Δ</i>	<i>MATa his3Δ1 leu2Δ0 LYS2 met15Δ0 ura3Δ0 aly1Δ::KanMX4</i>	Open Biosystems (Winzeler et al., 1999)
<i>aly2Δ</i>	<i>MATa his3Δ1 leu2Δ0 LYS2 met15Δ0 ura3Δ0 aly2Δ::KanMX4</i>	Open Biosystems (Winzeler et al., 1999)
<i>aly1Δ aly2Δ</i> (alias D2-6A)	<i>MATa his3Δ1 leu2Δ0 MET15 lys2Δ0 ura3Δ0 aly1Δ::KanMX4 aly2Δ::KanMX4</i>	(O'Donnell et al., 2010)
<i>end3Δ</i>	<i>MATa his3Δ1 leu2Δ0 LYS2 met15Δ0 ura3Δ0 end3Δ::KanMX4</i>	Gift from D. Prosser
<i>apl1Δ</i>	<i>MATa his3Δ1 leu2Δ0 LYS2 met15Δ0 ura3Δ0 apl1Δ::KanMX4</i>	Open Biosystems (Winzeler et al., 1999)
<i>apl3Δ</i>	<i>MATa his3Δ1 leu2Δ0 LYS2 met15Δ0 ura3Δ0 apl3Δ::KanMX4</i>	Open Biosystems (Winzeler et al., 1999)
<i>cnb1Δ</i>	<i>MATa his3Δ1 leu2Δ0 LYS2 met15Δ0 ura3Δ0 cnb1Δ::KanMX4</i>	Open Biosystems (Winzeler et al., 1999)
<i>bul1Δ bul2Δ</i>	<i>MATa his3Δ1 leu2Δ0 MET15 LYS2 ura3Δ0 bul1Δ::KanMX4 bul2Δ::KanMX4</i>	(O'Donnell et al., 2010)
<i>bul1Δ</i>	<i>MATa his3Δ1 leu2Δ0 LYS2 met15Δ0 ura3Δ0 bul1Δ::KanMX4</i>	Open Biosystems (Winzeler et al., 1999)
<i>bul2Δ</i>	<i>MATa his3Δ1 leu2Δ0 LYS2 met15Δ0 ura3Δ0 bul2Δ::KanMX4</i>	Open Biosystems (Winzeler et al., 1999)
<i>bul1Δ aly1Δ aly2Δ</i>	<i>MATα his3Δ1 leu2Δ0 LYS2 met15Δ0 ura3Δ0 bul1Δ::KanMX4 aly1Δ::NatMX4 aly2Δ::URA3</i>	(O'Donnell et al., 2010)
<i>bul2Δ aly1Δ aly2Δ</i>	<i>MATα his3Δ1 leu2Δ0 LYS2 met15Δ0 ura3Δ0 bul2Δ::KanMX4 aly1Δ::NatMX4 aly2Δ::URA3</i>	(O'Donnell et al., 2010)
<i>bul1Δ bul2Δ aly1Δ aly2Δ</i>	<i>MATα his3Δ1 leu2Δ0 LYS2 met15Δ0 ura3Δ0 bul1Δ::KanMX4 bul2Δ::KanMX4 aly1Δ::NatMX4 aly2Δ::URA3</i>	(O'Donnell et al., 2010)
<i>cnb1Δ</i>	<i>MATa his3Δ1 leu2Δ0 LYS2 met15Δ0 ura3Δ0 cnb1Δ::HphMX4</i>	This study
<i>cnb1Δ aly1Δ aly2Δ</i>	<i>MATa his3Δ1 leu2Δ0 MET15 lys2Δ0 ura3Δ0 aly1Δ::KanMX4 aly2Δ::KanMX4 cnb1Δ::HphMX4</i>	This study
<i>vrp1Δ</i>	<i>MATa his3Δ1 leu2Δ0 LYS2 met15Δ0 ura3Δ0 vrp1Δ::KanMX4</i>	Open Biosystems (Winzeler et al., 1999)
<i>vps4Δ</i>	<i>MATa his3Δ1 leu2Δ0 LYS2 met15Δ0 ura3Δ0 vps4Δ::KanMX4</i>	Open Biosystems (Winzeler et al., 1999)
<i>vps27Δ</i>	<i>MATa his3Δ1 leu2Δ0 LYS2 met15Δ0 ura3Δ0 vps27Δ::KanMX4</i>	Open Biosystems (Winzeler et al., 1999)
BY4741 Git1-GFP	<i>MATa GIT1-GFP::LEU2 his3Δ1 leu2Δ0 LYS2 met15Δ0 ura3Δ0</i>	This study
<i>aly1Δ aly2Δ</i> Git1-GFP	<i>MATa GIT1-GFP::LEU2 his3Δ1 leu2Δ0 MET15 lys2Δ0 ura3Δ0 aly1Δ::KanMX4 aly2Δ::KanMX4</i>	This study

as described in Johnston et al. (1977) or YPD medium where indicated. Liquid medium was filter sterilized and solid medium for agar plates had 2% agar w/v added before autoclaving. Yeast cells were grown at 30°C unless otherwise noted.

Plasmid and DNA manipulations

Plasmids used in this work are described in Table 3. PCR amplifications for generating plasmid constructs were performed using Phusion High Fidelity DNA Polymerase (ThermoFisher Scientific, Waltham, MA) and sequence insertions were verified using Sanger sequencing (Genewiz, South Plainfield, NJ). Plasmids were transformed into yeast cells using the lithium acetate method (Gietz & Schiestl, 2007) and selected for on the appropriate SC medium lacking the specific nutrient marker.

Serial dilution growth assays

Yeast cells were grown overnight in liquid culture at 30°C in either SC or YPD medium as appropriate. The saturated culture's absorbance at 600 nm (A_{600}) was measured and a starting concentration of $A_{600} = 1.0$ (which corresponds to approximately 1.0×10^7 cells/ml) was generated. This starting density was diluted in 5-fold series, plated onto the indicated medium and grown for 3–6 days as indicated at 30°C. Plate images were captured on an HP flatbed scanner or using a Chemidoc XRS+ imager (BioRad, Hercules, CA) and all were evenly modified in Photoshop (Adobe Systems Incorporated, San Jose, CA). For GPI containing plates, the GPI was obtained from the Patton-Vogt lab and the final concentration used is indicated in each figure. The myriocin (EMD Millipore, Burlington, MA) and aureobasidin (Takara Contech, Shiga, Japan) concentrations

TABLE 3 Plasmids

Name	Description	Reference or source
pRS415- <i>TEF1pr-GIT1</i> -GFP	The Git1 coding region, lacking its stop codon, was PCR amplified with primers containing restriction enzyme adaptor sites and cloned into <i>SpeI</i> and <i>HindIII</i> sites of pRS415- <i>TEF1pr</i> (described in Mumberg et al., 1995) and fused in frame at the C-terminus to eGFP. Between Git1 and eGFP there is a 5 amino acid linker (Ala-Gly-Ala-Gly-Ala-Gly). CEN <i>LEU2</i>	This study
pRS316	CEN <i>URA3</i>	(Sikorski & Hieter, 1989)
pRS316-Aly1	Genomic clone of <i>ALY1</i> expressed from its own promoter; CEN <i>URA3</i>	(O'Donnell et al., 2010)
pRS316-Aly2	Genomic clone of <i>ALY2</i> expressed from its own promoter; CEN <i>URA3</i>	(O'Donnell et al., 2010)
pRS316-Art5	Genomic clone of <i>ART5</i> expressed from its own promoter; CEN <i>URA3</i>	(O'Donnell et al., 2015)
pRS316-Ldb19	Genomic clone of <i>LDB19</i> expressed from its own promoter; CEN <i>URA3</i>	(O'Donnell et al., 2015)
pRS316-Art10	Genomic clone of <i>ART10</i> expressed from its own promoter; CEN <i>URA3</i>	(O'Donnell et al., 2015)
pRS316-Csr2	Genomic clone of <i>CSR2</i> expressed from its own promoter; CEN <i>URA3</i>	(O'Donnell et al., 2015)
pRS316-Ecm21	Genomic clone of <i>ECM21</i> expressed from its own promoter; CEN <i>URA3</i>	(O'Donnell et al., 2015)
pRS316-Rod1	Genomic clone of <i>ROD1</i> expressed from its own promoter; CEN <i>URA3</i>	(O'Donnell et al., 2015)
pRS316-Rog3	Genomic clone of <i>Rog3</i> expressed from its own promoter; CEN <i>URA3</i>	(O'Donnell et al., 2015)
pRS316-Aly1 ^{K379R}	This construct was subcloned by digesting the inserted <i>ALY1</i> promoter and coding sequence from pRS426-Aly1 ^{K379R} described in Hager et al. (2018) and ligating it into pRS316. CEN <i>URA3</i>	This study.
pRS316-Aly1 ^{PPxYless}	This construct was subcloned by digesting the inserted <i>ALY1</i> promoter and coding sequence from pRS426-Aly1 ^{PPxYless} described in Prosser et al. (2015) and ligating it into pRS316. CEN <i>URA3</i>	This study.
pRS316-Aly2 ^{K392R}	This construct was subcloned by digesting the inserted <i>ALY2</i> promoter and coding sequence from pRS426-Aly2 ^{K392R} described in Hager et al. (2018) and ligating it into pRS316. CEN <i>URA3</i>	This study.
pRS316-Aly2 ^{PPxYless}	This construct was subcloned by digesting the inserted <i>ALY2</i> promoter and coding sequence from pRS426-Aly2 ^{PPxYless} described in Prosser et al. (2015) and ligating it into pRS316. CEN <i>URA3</i>	This study.
pRS316-Aly1 ^{ΔPILKIN}	This construct was subcloned by digesting the inserted <i>ALY1</i> promoter and coding sequence from pRS426-Aly1 ^{ΔPILKIN} (O'Donnell et al., 2013) and ligating it into pRS316. CEN <i>URA3</i>	This study.
pRS316-Aly1 ^{AAAAAA}	This construct was subcloned by digesting the inserted <i>ALY1</i> promoter and coding sequence from pRS426-Aly1 ^{AAAAAA} (O'Donnell et al., 2013) and ligating it into pRS316. CEN <i>URA3</i>	This study.
pRS316-Aly1 ^{5A}	This construct was subcloned by digesting the inserted <i>ALY1</i> promoter and coding sequence from pRS426-Aly1 ^{5A} (O'Donnell et al., 2013) and ligating it into pRS316. CEN <i>URA3</i>	This study.
pRS316-Aly1 ^{5E}	This construct was subcloned by digesting the inserted <i>ALY1</i> promoter and coding sequence from pRS426-Aly1 ^{5E} (O'Donnell et al., 2013) and ligating it into pRS316. CEN <i>URA3</i>	This study.
p416-Lact-C2-GFP	Plasmid expresses C2 domain of Lact protein, which recognizes PS, fused to GFP.	Addgene plasmid #22853 (Yeung et al., 2008)
pRS426-GFP-2xPH(Plcδ)	Plasmid expresses 2 tandem copies of the PH domain of Plcδ protein, which recognizes PI(4,5)P ₂ , fused to GFP.	Addgene plasmid #36092 (Stefan et al., 2002)
pRS426-GFP-FYVE(Eea1)	Subcloned from pRS424-GFP-FYVE(Eea1) from Addgene plasmid #36096 (Burd & Emr, 1998) into pRS426. Plasmid expresses the FYVE domain from Eea1, which recognizes PI(3)P, fused to GFP.	This study
pRS426-GFP-2xPH(Osh2)	Subcloned from pRS424-GFP-2xPH(Osh2) from Addgene plasmid #36095 (Stefan et al., 2011) into pRS426. Plasmid expresses two tandem copies of the PH domain from Osh2, which bind to PI(4)P, fused to GFP.	This study
pRS416-GFP-P4Mx1	Subcloned from NES-EGFP-P4Mx1 (Addgene plasmid 108121) into pRS416 for expression in yeast. This plasmid uses the P4Mx1 to detect PI(4)P in cells	(Hammond et al., 2014)

Yeast protein extraction and immunoblot analyses

Whole cell extracts of yeast were made using the trichloroacetic acid (TCA) extraction method as previously described (Hager et al., 2018) and as modified from (Volland et al., 1994). Extracts were resolved by SDS-PAGE and proteins were identified using immunoblotting with mouse anti-GFP antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and a rabbit anti-Zwf1 (glucose-6-phosphate dehydrogenase, referred to as G6PDH) antibody (Sigma, St. Louis, MO) as a protein loading control. As an alternative protein loading control, immunoblot membranes were stained after transfer with Revert (Li-Cor Biosciences, Lincoln, NE) total protein stain and detected using the Odyssey FC infrared imaging system. Anti-mouse or anti-rabbit secondary antibodies conjugated to IRDye-800 or IRDye-680 (Li-Cor Biosciences, Lincoln, NE) were used to detect primary antibodies, visualized on an Odyssey FC infrared imaging systems (Li-Cor Biosciences). Quantification of band intensities on immunoblot scans was performed using Image J software (NIH, Bethesda, MD). In brief, each band was boxed, and the mean pixel intensity measured. The background fluorescence was subtracted from each measurement and then the ratio of the GFP-breakdown band over the full-length Git1-GFP band is presented below the immunoblot figures.

Fluorescence microscopy

To assess Git1-GFP or GFP-tagged phospholipid probe localization and/or abundance cells were grown overnight to saturation in SC medium, inoculated into fresh SC medium at an A_{600} of 0.2 and grown at 30°C until they reached mid-log phase ($A_{600} = 0.5-0.7$). Where indicated, cells were treated with 50 μ M GPI for the indicated time prior to imaging. Cells treated with latrunculin A (LatA) (Molecular Probes, Eugene, OR) were incubated with 200 μ M LatA or an equivalent volume of the dimethyl sulfoxide (DMSO) vehicle control. To visualize vacuoles, cells were stained with 250 μ M Cell Tracker Blue CMAC (7-amino-4-chloromethylcoumarin) dye (Life Technologies, Carlsbad, CA). Cells were then plated onto 35 mm glass bottom microwell dishes that were poly-D-lysine coated (MatTek Corporation, Ashland, MA) and imaged using a Nikon Ti inverted microscope (Nikon, Chiyoda, Tokyo, Japan) outfitted with an Orca Flash 4.0 cMOS camera (Hamamatsu, Bridgewater, NJ) and a 100 \times objective (numerical aperture, 1.49) or one outfitted with a swept-field confocal scan head, EMCCD camera (iXon3; Andor, Belfast, UK) and a 100 \times objective (numeri-

using NIS-Elements software (Nikon) and all images within an experiment were captured using identical settings. Images were cropped and adjusted equivalently using Photoshop software (Adobe Systems Incorporated).

Image quantification and statistical analyses

For all manual fluorescence quantification analyses, fluorescence intensity was measured using ImageJ software (National Institutes of Health, Bethesda, MD). For cell surface fluorescence quantification, a 2-pixel thick line was hand drawn over the plasma membrane (PM) signal and the mean pixel intensity measured with mean background pixel intensity subtracted (as described in O'Donnell et al. (2015)). For vacuolar fluorescence quantification, the vacuole profile was defined by using images captured of cells stained with CMAC dye (Life Technologies) which was then overlaid on the GFP images and the GFP signal within the vacuole mask was measured using ImageJ (NIH). The mean background fluorescence intensity was subtracted for the vacuole measures and each vacuole measure was paired to the corresponding plasma membrane quantification. Prism software (GraphPad Software, San Diego, CA) was then used to perform Kruskal-Wallis statistical analyses with Dunn's post hoc test. In all cases, significant p values from these tests are represented as: * p value <0.1; ** p value <0.01; *** p value <0.001; ns, p value >0.1 or in some instances where indicated ††† p value <0.001.

For our automated quantification pipeline, we developed an R program called *CellQuant*. The program detects the plasma membrane of cells using a Gaussian blur and simple threshold on the grayscale GFP image. An adaptive threshold based on the minimum plasma membrane radius is then used on the grayscale CMAC vacuole stain to help identify the vacuoles and constrain their size to features that should be smaller than the cell itself. Cells with no detectable vacuolar region are discarded and the fluorescent signal is measured in the GFP channel. The program further pairs the PM fluorescent output measures with the vacuoles and returns the PM/vacuole fluorescent ratios, which are used extensively in this paper. The source code for this pipeline is available at www.odonnelllab.com and <https://github.com/sah129/CellQuant>. In addition, the Docker image for the program is hosted on Docker Hub at <https://hub.docker.com/r/odonnelllab/cellquant>, with command-line installation instructions available on GitHub. There are tutorials that can assist individuals who would like to use this pipeline in setting it up on their own systems and we are happy to help users in applying this approach to their

Radiolabeled [³H]-GPI transport assays and conversion to [³H]-PI

For assays monitoring endogenous Git1 activity in the presence or absence of α -arrestins, BY4741, and *aly1*Δ *aly2*Δ cells (biological triplicates of each) were grown aerobically overnight at 30°C in yeast nitrogen base (YNB) medium altered to lack inositol and to contain a low level (200 μM) of KH₂PO₄ inorganic phosphate and 2% glucose. Amino acid concentrations were as described in Hanscho et al. (2012). These conditions were needed to allow for robust expression of the endogenous Git1 transporter. The next day, single cultures were used to inoculate two fresh cultures containing either 100 μM (low) or 10 mM (high) inorganic phosphate at an A₆₀₀ of 0.2. All cultures were allowed to reach an A₆₀₀ of 0.5, at which point they were harvested and assayed for transport activity as described below.

Transport assays to monitor endogenous Git1 transport or *TEF1pr*-Git1-GFP transport of GPI were performed as described in Surlow et al. (2014). For the Git1-GFP assays, cells were precultured in high phosphate medium only, as described above, as low phosphate medium was not required to induce expression of Git1. Cells were then harvested by filtration, washed with water and resuspended in 100 mM citrate buffer [pH 5] to an A₆₀₀ = 5.0. The assays were initiated by adding 50 μl of 75 μM [³H]-GPI to 200 μl of cell suspension. After a 4-min incubation at 30°C, the assays were stopped by the addition of ice cold distilled, deionized water. Samples were then filtered over Whatman GFC filters (Whatman number 1822-025), washed with 10 ml of ice cold distilled, deionized water, and resuspended in 10 ml of scintillation fluid for radioactivity counts. A student's *t*-test was used to assess significance and the data from three biological replicates are shown with each having two technical replicates performed (six replicates in total). GPI was purchased from Sigma Chemical Company, Tritium labeled GPI ([³H]-inositol-GPI) was produced through the deacylation of phosphatidyl[³H]-myo-inositol purchased from American Radiolabeled Chemicals, as described (Hama et al., 2000).

For assessments of the conversion of GPI to PI after uptake (as in Table 1 and Figure 10c), wild-type or *aly1*Δ *aly2*Δ cultures (biological triplicate) were grown aerobically overnight at 30°C in YNB medium altered to lack inositol and contain a low level of KH₂PO₄ (200 μM) and 2% glucose. Amino acid concentrations were as described (Hanscho et al., 2012). The medium lacked inositol (I-) and contained a low KH₂PO₄ level to induce expression of the *GIT1* transporter for GPI uptake. The overnight cultures were diluted to log phase in fresh media containing a high level of KH₂PO₄ (10 mM) and allowed to double. To begin labeling, 4 ml cultures normalized to OD₆₀₀ = 0.2 were spiked with 5 μM [³H]-

lowing aerobic growth at 30°C for 5 h. A membrane fraction, a TCA-extractable intracellular fraction and an extracellular fraction were isolated as described previously (Surlow et al., 2014). The percentages of radioactive counts in each fraction were determined using a liquid scintillation counter. Lipids were extracted from the membrane fraction and separated by thin layer chromatography as described previously (Surlow et al., 2014). Radioactive (American Radiolabeled Chemicals Inc.) and nonradioactive (Avanti Polar Lipid) PI standards were used to verify the migration of PI on the TLC plate. Tritium labeled GPI ([³H]-inositol-GPI) was produced through the deacylation of phosphatidyl[³H]-myo-inositol, as described in Hama et al. (2000).

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CONFLICTS OF INTEREST

The authors have no competing or conflicting interests to declare.

AUTHOR CONTRIBUTIONS

Benjamin P. Robinson collected the majority of the data and wrote and revised the manuscript. Sarah Hawbaker wrote the automated quantification pipeline, ensured it functioned correctly, and trained users, as well as edited the manuscript. Annette Chiang collected all data for the revision, the data on phospholipid localization and edited the manuscript. Eric M. Jordahl and Ceara K. McAtee performed data analysis and edited the manuscript. Saniket Anaokar, Alexiy Nikiforov, and Philip Ziegler collected data on lipid uptake. Ray W. Bowman II collected preliminary data for the manuscript, built several strains and reagents needed for the study and edited the manuscript. Jana Patton-Vogt conceptualized the study and edited the manuscript. Allyson F. O'Donnell conceptualized the studies, performed the experiments, wrote, and revised the manuscript.

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