

RESEARCH ARTICLE

Physical, genetic and functional interactions between the eisosome protein Pil1 and the MBOAT O-acyltransferase Gup1

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One sentence summary: Yeast MBOAT O-acyltransferase Gup1 interacts physically, genetically and functionally with the eisosomal Pil1, and has a role in eisosome formation and cell wall integrity, and during arsenite and acetate stress.

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ABSTRACT

The *Saccharomyces cerevisiae* MBOAT O-acyltransferase Gup1 is involved in many processes, including cell wall and membrane composition and integrity, and acetic acid-induced cell death. Gup1 was previously shown to interact physically with the mitochondrial membrane VDAC (Voltage-Dependent Anion Channel) protein Por1 and the ammonium transceptor Mep2. By co-immunoprecipitation, the eisosome core component Pil1 was identified as a novel physical interaction partner of Gup1. The expression of *PIL1* and Pil1 protein levels were found to be unaffected by *GUP1* deletion. In $\Delta gup1$ cells, Pil1 was distributed in dots (likely representing eisosomes) in the membrane, identically to *wt* cells. However, $\Delta gup1$ cells presented 50% less Pil1-GFP dots/eisosomes, suggesting that Gup1 is important for eisosome formation. The two proteins also interact genetically in the maintenance of cell wall integrity, and during arsenite and acetic acid exposure. We show that $\Delta gup1 \Delta pil1$ cells take up more arsenite than *wt* and are extremely sensitive to arsenite and to acetic acid treatments. The latter causes a severe apoptotic *wt*-like cell death phenotype, epistatically reverting the $\Delta gup1$ necrotic type of death. Gup1 and Pil1 are thus physically, genetically and functionally connected.

Keywords: *Saccharomyces cerevisiae*; co-immunoprecipitation; Gup1 Pil1; eisosomes; cell wall; arsenite

INTRODUCTION

The yeast Gup1 protein, which belongs to the MBOAT family of O-acyltransferases, affects many cellular processes in a yet unknown biochemical fashion (Lucas et al. 2016). The *Saccharomyces cerevisiae* *GUP1* deleted strains are best described in

association with cell wall composition and stability (Ferreira et al. 2006, 2010), with lipid metabolism and functionality (Oelkers et al. 2000), with implications in glycosylphosphatidylinositol (GPI)-anchor remodeling (Bosson, Jaquenoud and Conzelmann 2006) and with membrane structure, particularly with

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the assembly and integrity of lipid ordered domains/rafts (Ferreira and Lucas 2008). Other evidence supports the involvement of Gup1 in cytoskeleton polarization, intracellular trafficking and vacuole morphology (Lucas et al. 2016). *GUP* genes have been found in virtually all eukaryotic genomes sequenced so far. In higher eukaryotes, Gup1 (designated as HHATL) was described to operate as a negative regulator of the Hedgehog pathway (Abe, Kita and Niikura 2008), important in developmental processes regulating morphogenesis, differentiation and patterning, and in proliferation and cell fate during embryogenesis and wound healing (Varjosalo and Taipale 2007; Lee, Zhao and Ingham 2016). Morphogenesis phenotypes have been associated with *GUP1* deletion in the fungal pathogen *Candida albicans*, which upon deletion of the two *GUP1* copies loses virulence and the corresponding ability to differentiate, adhere, invade and form biofilms (Ferreira et al. 2010). Moreover, the deletion of *GUP1* in *S. cerevisiae* impairs acetic acid-induced regulated cell death (RCD) (Tulha et al. 2012). Thus, a better understanding of the Gup1, its interaction partners and cellular functions might be useful for future development of antifungal drugs.

Yeast Gup1 localizes to the plasma membrane, to the Endoplasmic reticulum (ER) (Holst et al. 2000; Bleve et al. 2005) and to the mitochondria (Holst et al. 2000; Tulha and Lucas 2018). These multiple localizations suggest multiple functions and multiple partners. Accordingly, Gup1 has been shown to physically interact with the yeast mitochondrial membrane VDAC protein Por1 (Tulha and Lucas 2018) and the plasma membrane-localized ammonium transceptor Mep2 (Van Zeebroeck et al. 2011). Whole-genome screenings (SGD database) suggest a larger physical interactome that, nevertheless, requires confirmation. The Gup1–Por1 partnership is involved in the processes of RCD in response to acetic acid, and cell wall integrity maintenance in response to injury-causing drugs (Tulha and Lucas 2018). On the other hand, the Gup1–Mep2 partnership affects *MEP2* expression, as well as proper membrane distribution and transport activity of Mep2 (van Zeebroeck et al. 2011). In view of the extensive phenotypes associated with *GUP1* deletion and the multiple intracellular localizations of Gup1 (Lucas et al. 2016), the present work aimed at identifying novel physical interaction partners using the co-immunoprecipitation (Co-IP) methodology previously proven successful (Tulha and Lucas 2018). Under non-stress physiological conditions, the protein Pil1, an eisosome core structural protein (Walther et al. 2006; Olivera-Couto et al. 2011), was identified as a physical partner of Gup1. The present work shows that the *GUP1* deleted mutant has ~50% less eisosomes than *wt* cells.

Eisosomes act as sensors of changes in membrane tension, with consequences in the response to stress caused by environment, nutrition, and plasma membrane and cell wall perturbing conditions and agents (reviewed by Lanze et al. 2020). These responses include changes in (i) cell wall composition and integrity, (ii) lipid homeostasis, (iii) plasma membrane architecture and related cell signaling, (iv) morphogenesis, (v) resistance to antifungal drugs and (vi) the manifestation of several virulence factors. All of these were previously described in association with *GUP1* deletion in *S. cerevisiae* and/or *C. albicans* (reviewed by Lucas et al. 2016). The results in this current study show that Gup1 and Pil1 interact genetically and functionally in cell wall integrity. The interaction of these proteins also has consequences in the sensitivity to arsenite, and in acetic acid-promoted RCD.

MATERIALS AND METHODS

Strains and growth conditions

The yeast strains used in this study are listed in Table 1. Yeasts were cultivated on YPD (1% yeast extract (Panreac AppliChem, Spain), 2% peptone (BD Difco, France), 2% glucose (LabChem, Portugal), or YNB without amino acids and nitrogen (BD Difco, France) (0.175%), with 0.5% $(\text{NH}_4)_2\text{SO}_4$ (Panreac AppliChem, Spain) and 2% glucose, supplemented according to auxotrophic requirements. Batch liquid cultures were grown at 30°C and 200 rpm orbital shaking in a 1:3 liquid-to-air ratio. Solid media contained 2% agar.

Constructions

The plasmids used in this work derived from pYES2 vector (Invitrogen/Thermo Scientific, USA) and were previously described: pYES-GFP (Tulha and Lucas 2018) and pYES2-GUP1-GFP (Bleve et al. 2011). The double mutant *S. cerevisiae* $\Delta gup1 \Delta pil1$ was constructed by replacing the *GUP1* gene in BY4741 $\Delta pil1$ (MATa *ura3* Δ 0 *leu2* Δ 0 *his3* Δ 1 *met15* Δ 0 YGR086c::kanMX4 from Euroscarf, Germany) with the *HIS3* disruption cassette amplified from the p416 GPD plasmid (ATCC® 87360) using the primers A and B in Table S1 (Supporting Information), by homologous recombination using standard protocols (Ito et al. 1983). Transformants were selected in YNB with 2% glucose and supplements without histidine (Panreac AppliChem, Spain). Positive clones were confirmed by colony PCR (polymerase chain reaction) using the primers C and D in Table S1 (Supporting Information). Strain BY4741 $\Delta gup1$ *PIL1*-GFP was constructed by deleting the *GUP1* gene from the BY4741 $\Delta pil1$ *PIL1*-GFP strain (Huh et al. 2003), using the KanMx disruption cassette amplified from plasmid pUG6 (Euroscarf), using the primers E and F in Table S1 (Supporting Information), by homologous recombination as above. Transformants were selected in YNB with 2% glucose and supplements with geneticin (Gibco, Thermo Fisher Scientific) (200 mg/L). Positive clones were confirmed by colony PCR using the primers C and G listed in Table S1 (Supporting Information).

Co-immunoprecipitation methodology

The Co-IP procedure and the protein identification were performed as described before (Tulha and Lucas 2018). Peptide mass fingerprinting and MALDI-TOF was purchased from the Unidad de Proteómica, Parque Científico de Madrid, UCM.

Microscopy

Fluorescence microscopy was done in a Leica Microsystems DM-5000B epifluorescence microscope with the GFP Filter (Ex Bp 470/40 and Em Bp 525/40), and a 100× oil-immersion objective. Images were acquired through a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems image software. The same system was used to observe and photograph cells by light microscopy. All the data in this work derive from observing at least 300 living cells per independent batch and at least three batches. Cells were cultivated and collected as mentioned in the text/legends.

qRT-PCR

Total RNA isolation and qRT-PCR procedures were done as described in Tulha and Lucas (2018) using the primers listed in

Table 1. Yeast strains used in this study. (ns: non specified)

Strain	Genotype	Source
<i>S. cerevisiae</i> W303-1A wt	MATa <i>leu2-3112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15</i>	ns
<i>S. cerevisiae</i> W303-1A Δ <i>gup1</i>	MATa <i>leu2-3112 trp1-1 can1-100 ura3-1 ade2-1 YGL084c::HIS5</i>	Holst et al. (2000)
<i>S. cerevisiae</i> W303-1A Δ <i>gup1</i> pYES2 \emptyset	MATa <i>leu2-3112 trp1-1 can1-100 ura3-1 ade2-1 YGL084c::HIS5 [pYES2(URA3)]</i>	Tulha and Lucas (2018)
<i>S. cerevisiae</i> W303-1A Δ <i>gup1</i> pYES2-GFP	MATa <i>leu2-3112 trp1-1 can1-100 ura3-1 ade2-1 YGL084c::HIS5 [pYES2(URA3)]</i>	Tulha and Lucas (2018)
<i>S. cerevisiae</i> W303-1A Δ <i>gup1</i> pYES2-GUP1-GFP	MATa <i>leu2-3112 trp1-1 can1-100 ura3-1 ade2-1 YGL084c::HIS5 [pYES2(URA3)]</i>	Tulha and Lucas (2018)
<i>S. cerevisiae</i> BY4741 wt	MATa <i>ura3Δ0 leu2Δ0 his3Δ1 met15Δ0</i>	Euroscarf
<i>S. cerevisiae</i> BY4741 Δ <i>gup1</i>	MATa <i>ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 YGL084c::kanMX4</i>	Euroscarf
<i>S. cerevisiae</i> BY4741 Δ <i>pil1</i>	MATa <i>ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 YGR086C::kanMX4</i>	Euroscarf
<i>S. cerevisiae</i> BY4741 Δ <i>gup1</i> Δ <i>pil1</i>	MATa <i>ura3Δ0 leu2Δ0 met15Δ0 YGR086C::kanMX4 YGL084c::HIS3</i>	This study
<i>S. cerevisiae</i> BY4741 Δ <i>pil1</i> PIL1-GFP	MATa <i>ura3Δ0 leu2Δ0 met15Δ0 YGR086C::PIL1-GFP-HIS3</i>	Huh et al. (2003)
<i>S. cerevisiae</i> BY4741 Δ <i>gup1</i> Δ <i>pil1</i> PIL1-GFP	MATa <i>ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 YGR086C::PIL1-GFP-HIS3 YGL084c::kanMX4</i>	This study

Table S2 (Supporting Information). The mRNAs quantification was normalized to the 18S gene. The comparative Ct method analysis ($2^{-\Delta\Delta Ct}$ method) (Schmittgen and Livak 2008) was used to analyze the results.

Western blotting

Yeasts were cultivated in 5 mL YPD at 30°C and 200 rpm orbital shaking up to OD_{600nm} 1.0, and were collected by centrifugation (5 min at 5000 rpm). The pellet was resuspended in 200 μ L of 0.2 M NaOH (Sigma-Aldrich, Germany) with 2% β -mercaptoethanol (Promega) and precipitated with 400 μ L 20% trichloroacetic acid (Panreac AppliChem, Spain), washed with 500 μ L acetone and solubilized in 100 μ L 2 \times loading buffer [5 \times loading buffer (10% w/v SDS, 10 mM β -mercaptoethanol, 20% v/v glycerol (Sharlau, Spain), 0.2 M Tris-HCl (Sigma-Aldrich, Germany) pH 6.8, 0.05% w/v bromophenol blue (Sigma-Aldrich, Germany)) diluted with 1 \times Tris-EDTA (TE) buffer]. Samples were run on a 10% SDS-PAGE, transferred to a PVDF membrane (Roche, Portugal) (54 mA 1.5 h), blocked with 5% non-fat milk in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (Sigma-Aldrich, Germany), and incubated overnight at 4°C with the primary antibody mouse monoclonal anti-GFP 1:2 500 (Roche, Portugal) and mouse monoclonal anti-Pgk1 (cytoplasmic phosphoglycerate kinase) 1:10 000 (Invitrogen/Thermo Scientific, USA). Dilutions of the antibodies were prepared using the above blocking mix. Membranes were incubated at room temperature for 1.5 h with secondary antibody against mouse IgGs (1:10 000) (Sigma-Aldrich, Germany), revealed by chemiluminescence (Amersham ECL, GE Healthcare, USA) according to the manufacturer's instructions, and visualized in a ChemiDoc equipment (XMS BioRad).

Phenotypic assessment

Drop tests

Drop tests were performed using cell suspensions at OD₆₀₀ = 1, collected from mid-exponential YPD grown cultures (OD₆₀₀ = 0.6–0.8). Four 10-fold serial dilutions in sterile water were made, and 5 μ L of each suspension was applied on solid YPD, YPG (YP with 2% w/v glycerol), YPE (YP with 2% v/v ethanol (Carlo Erba, France)) or YPD supplemented as described in the text. Results were scored after 3 days of incubation at 30°C, unless otherwise stated.

Sensitivity to high amounts of acetic acid

Yeast strains were grown until mid-exponential growth phase (OD₆₀₀ = 0.6–0.8) on YNB with 2% glucose and supplements and resuspended to a final OD₆₀₀ = 0.2 in identical fresh medium, adjusted to pH 3.0 with HCl and containing 150 mM of acetic acid (Panreac AppliChem, Spain). Incubation took place for 180 min at 30°C. At determined time points, 40 μ L from a 10⁻⁴ cell suspension were inoculated onto YPD agar plates and colony forming units (c.f.u.) were counted after 48 h incubation at 30°C. The percentage of viable cells was estimated considering 100% survival as the number of c.f.u. obtained at time 0 (T₀). The integrity of the membrane of cells exposed to acetic acid was assessed as previously described (Tulha et al. 2012), i.e. after incubation with acetic acid, cells were harvested, washed and resuspended in PBS containing 4 μ g/mL propidium iodide (PI) (Sigma-Aldrich, Germany), incubated for 10 min at room temperature in the dark and analyzed in an Epics[®] XL (Beckman Coulter) flow cytometer. A minimum of 20 000 cells from each sample were analyzed per experiment.

Sensitivity to arsenite

Cells growing exponentially in YNB with 2% glucose and supplements at an OD₆₀₀ = 1, were serially diluted 10-fold, and 5 μ L of each suspension was applied onto plates supplemented with 2% glucose in the absence or presence of sodium arsenite [As(III)] at the indicated concentrations. Growth was monitored after 2–3 days at 30°C.

Arsenic uptake

Exponentially growing cells in YNB with 2% glucose and supplements were either untreated or exposed to 1 mM As(III) for 1 h, collected and washed twice in ice-cold water. The cell pellet was resuspended in water, boiled for 10 min and centrifuged to collect the supernatant. The arsenic content of each sample was determined by ICP-MS. Prior to analysis, the samples were diluted 20 times using water from a GenPure water purification system (Thermo Scientific, Barnstead, resistivity 18.2 M Ω cm) and acidified to 1% volume HNO₃ (Sharlau, nitric acid 65% for trace analysis). The analysis was performed on an iCAP Q ICP-MS (Thermo Fisher Scientific, USA) with an SC-FAST automated sampling introduction system (Elemental Scientific, USA). The instrument was operated in the Kinetic Energy Discrimination (KED) mode with He as the collision gas to remove potential interference from argon chloride. Calibration was performed using a set of As standards with concentrations up to 500 μ g L⁻¹.

A 1 $\mu\text{g L}^{-1}$ indium solution was continuously injected for internal standardization. The detection limit estimated based on six blank analyses was 0.1 $\mu\text{g As L}^{-1}$.

RESULTS AND DISCUSSION

Pil1 is a physical partner of Gup1; implications in the number of eisosomes

Previously, two Gup1 physical partners were identified, the plasma membrane ammonium transceptor Mep2 (van Zeebroeck *et al.* 2011) and the mitochondrial VDAC Por1 (Tulha and Lucas 2018). Por1 was found by co-immunoprecipitating Gup1-GFP with an anti-GFP antibody, while Mep2 was found using a split-ubiquitin two-hybrid system. Using this last approach, specifically adapted for membrane proteins, Miller *et al.* (2005) presented a HTP assay in which $\pm 30\%$ of the *S. cerevisiae* annotated membrane proteins including Gup1, did not display any interaction with other proteins or themselves. Bearing in mind these discrepancies, the former Co-IP approach (Tulha and Lucas 2018) was chosen to search for further putative Gup1 partners. This was done using solubilized protein lysates of *S. cerevisiae* W303 cells expressing this Gup1-GFP chimeric protein under the control of the strong galactose-induced GAL1 promoter (Bleve *et al.* 2005; Tulha and Lucas 2018). Its induction for 16 h caused extensive membrane/ER proliferation (Bleve, di Sansebastiano and Grieco 2011). This was obviated by using a galactose-induction time of 6 h, which neither displayed membrane proliferation structures (Tulha and Lucas 2018), nor caused the release of fluorescence into the vacuole (not shown). Silver stained sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) of the results from one such assay (Fig. 1), shows several new protein bands. These were excised and analyzed by Peptide Mass Fingerprinting using MALDI-TOF. Only one band yielded an unequivocal answer: a protein of ~ 38 kDa that was identified as Pil1 (score 63, covering 39% of the amino acid sequence) (Fig. 1).

Pil stands for phosphorylation inhibited by long-chain bases. Pil1 is a member of the BAR domain protein family (Habermann 2004), which comprises proteins that contribute to shape membrane curvatures, participating in the early steps of endocytosis and sensing. Pil1 and Lsp1, another BAR domain protein, form the eisosome structural core (Walther *et al.* 2006; Olivera-Couto *et al.* 2011). Eisosomes configure furrow-like invaginations of the plasma membrane that aggregate thousands of Pil1 and Lsp1 molecules (Ziółkowska *et al.* 2011; Douglas and Konopka 2014; Olivera-Couto *et al.* 2015). These proteins directly bind to lipids and form a curved structure that locates in the cytoplasmic side of MCC domains (membrane compartment containing Can1) (Malínská *et al.* 2004; Strádalová *et al.* 2009; Malínský, Opekarová and Tanner 2010; Douglas *et al.* 2011; Douglas and Konopka 2014; Foderaro, Douglas and Konopka 2017). Pil1 interacts electrostatically with the negatively charged phosphoinositide phosphatidylinositol (4,5)-biphosphate (PI(4,5)P₂), concentrated in the cytoplasmic layer of MCC domains (Olivera-Couto *et al.* 2011; Ziółkowska *et al.* 2011), forcing the membrane to bend (Foderaro, Douglas and Konopka 2017). MCCs are also rich in complex lipids and ergosterol (Douglas *et al.* 2011; Foderaro, Douglas and Konopka 2017).

Gup1 is a membrane protein with 11–12 transmembrane domains (Holst *et al.* 2000). Many of the previously described phenotypes associated with the deletion of GUP1 are associated with the plasma membrane (Lucas *et al.* 2016). Besides the changes in lipid composition (Oelkers *et al.* 2000), the Δgup1

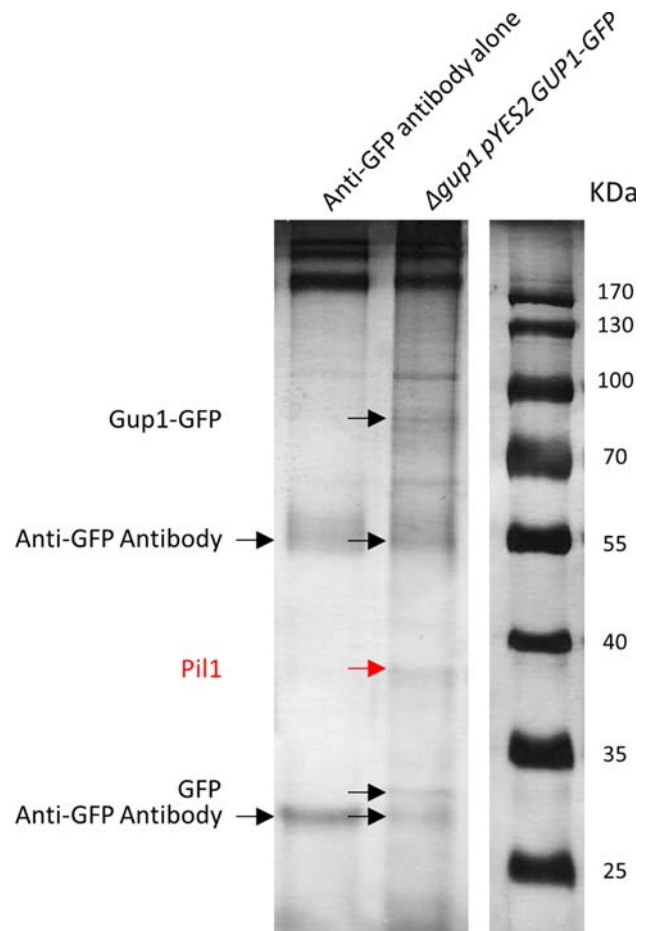


Figure 1. SDS-PAGE of the Co-IP assay. Co-IP of whole cell lysates of *S. cerevisiae* W303-1A Δgup1 expressing Gup1-GFP using an anti-GFP antibody (lane 2). Control SDS-PAGE of the antibody alone suspended in loading buffer (lane 1). Bands were excised and identified by LC-MS/MS.

mutant displays (i) an even distribution of ergosterol (Ferreira and Lucas 2008), (ii) the mislocalization of the Pma1 proton pump and (iii) inefficient Stl1-mediated glycerol/H⁺ symport (Ferreira *et al.* 2005). All of these, including the malfunction of active permeases, were previously associated with eisosome disassembly through PIL1 deletion (Malínská *et al.* 2003; 2004; Grossmann *et al.* 2007; 2008). Eisosomes harbor numerous sensors, signaling effectors and transporters, and play important roles in regulation and signaling-associated processes, as well as in membrane organization (Douglas *et al.* 2011; Foderaro, Douglas and Konopka 2017; Moharir *et al.* 2018). The Pil1 sub-cellular distribution/localization was therefore assessed in the Δgup1 mutant comparing to wt cells, using the strains expressing the chromosomal chimeric insertion Pil1-GFP, as a mean to assess the integrity, distribution and number of eisosomes.

In the wt strain, Pil1-GFP distributed as expected in punctate patches (Fig. 2A), likely corresponding to the eisosomes (Moreira *et al.* 2009; Strádalová *et al.* 2009). This type of distribution was preserved in the Δgup1 mutant (Fig. 2A). However, in the absence of Gup1, the number of these Pil1 associated-punctate structures, which are widely used to quantify the number of eisosomes per cell (e.g. Moreira *et al.* 2009; Strádalová *et al.* 2009), was found to be significantly reduced (Fig. 1B). More than 300 cells from each of ≥ 3 independent batches of Δgup1 and wt were

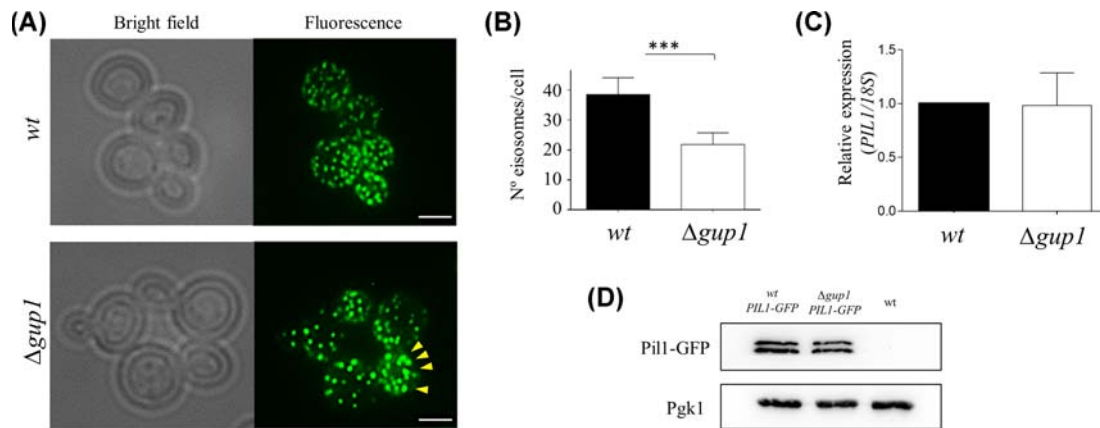


Figure 2. Effects of deleting *GUP1* from *S. cerevisiae* BY4741 on the subcellular localization and distribution of Pil1 (A), the number of eisosomes/cell (B) and the expression of *PIL1* (C). Cells were cultivated in YNB with 2% glucose and supplements until mid-exponential growth phase. (A) Pil1-GFP chromosomal insertion shows the location and distribution of Pil1 in live cells. Arrows indicate fluorescent dots bigger than average. Bars = 5 μ m. (B) The number of eisosome structures was counted in 300 cells per experiment. Results are representative of at least three independent experiments. *** $P < 0.001$ t-test. (C) Relative expression of *PIL1* by qRT-PCR. *PIL1* expression was normalized against 18S and represented relative to the levels in the wt strain as calculated by the comparative Ct method analysis ($2^{-\Delta\Delta CT}$ method). (D) Western blotting of anti-GFP antibody against whole protein extracts from $\Delta pil1$ *PIL1*-GFP (positive control), $\Delta gup1$ $\Delta pil1$ *PIL1*-GFP and wt (negative control). Loading control was made using anti-Pgk1 antibody.

quantified in this fashion. The wt and the $\Delta gup1$ mutant presented 38.6 ± 5.7 and 21.9 ± 3.9 eisosomes/cell, respectively, i.e. the mutant had $\sim 56\%$ less eisosomes than the wt strain. Noticeably, some of the Pil1-GFP punctate patches in $\Delta gup1$ cells appeared bigger than average (arrows in Fig. 2A). The cause of such a significantly reduced number of eisosomes was further investigated. A small number of eisosomes has been associated with reduced amounts of Pil1 protein (Moreira et al. 2009). Therefore, the expression of *PIL1* in the $\Delta gup1$ strain was quantified by qRT-PCR and found to be identical to that in wt cells (Fig. 2C). Additionally, the relative amounts of Pil1-GFP protein in $\Delta gup1$ mutant and wt cells were assessed by western blotting using an anti-GFP antibody confirming that the amounts of protein are similar in both strains (Fig. 2D). These results suggest that *PIL1* expression is not affected by the absence of Gup1, and that the reduced number of eisosomes per cell observed in the $\Delta gup1$ strain is not a direct consequence of a reduction in Pil1 protein levels.

The deletion of *GUP1* causes profound alteration in membrane lipid composition (Oelkers et al. 2000; Bosson, Jaquenoud and Conzelmann 2006) and organization/structure (Bosson, Jaquenoud and Conzelmann 2006; Ferreira and Lucas 2008). The mutant has less phospholipids, and more diacyl- and triacyl-glycerols (Oelkers et al. 2000) and lyso-PI (Bosson, Jaquenoud and Conzelmann 2006). Thus, it is possible that the absence of Gup1 influences the number of eisosomes by decreasing the available amounts of PI(4,5)P2. Eisosomes have been proposed to maintain phosphoinositide homeostasis, particularly that of PI(4,5)P2 (Fröhlich et al. 2014; Kabeche et al. 2014, Kabeche, Howard and Moseley 2015b). The other way round, membrane association of Pil1 with PI(4,5)P2 was proposed to be the limiting step in eisosome formation (Moreira et al. 2009; Olivera-Couto et al. 2011). Accordingly, the reduction of PI(4,5)P2 causes a decrease in the total number of eisosomes (Karotki et al. 2011; Kabeche et al. 2015a). This is therefore a possibility to be explored in the future.

Genetic interactions between Gup1 and Pil1

To evaluate the extent of the effect of the interaction between Gup1 and Pil1, the double mutant $\Delta gup1 \Delta pil1$ was created. Single and double mutants and wt were used to assay some of the

most prominent phenotypes previously associated with $\Delta gup1$ (Lucas et al. 2016). These phenotypes were assessed in the same culture conditions as before, many of which rely on cultivating the cells on glucose. Previous work using the $\Delta end3-1$ mutant suggested that Gup1 is internalized by endocytosis when the cells are shifted to glucose (Bleve et al. 2005). Yet, the expression of *GUP1* remains almost constant under glucose repression or derepression conditions (Oliveira and Lucas 2004; Lewis et al. 2014; Kunkel, Luo and Capaldi 2019), suggesting that if endocytosis occurs, the protein is probably swiftly replaced, supporting the numerous *GUP1* deletion phenotypes observed before.

Effect of cell wall-injuring drugs

Cells were grown in the presence of cell wall-damaging drugs such as Calcofluor White (CFW), caffeine and Congo Red (CR). CFW and CR bind cell wall chitin, thereby interfering with proper cell wall assembly (Roncero and Durán 1985; Ram and Klis 2006). These drugs operate differently, as inferred from distinct transcriptional profiles induced by either drug (Kuranda et al. 2006). CR induces the alteration of the expression of a small group of genes, almost completely restricted to cell wall remodeling functions (Kuranda et al. 2006), suggesting it specifically affects the Cell Wall Integrity (CWI/PKC) pathway. CFW on the other hand, triggers both HOG and CWI/PKC pathways (Alonso-Monge et al. 1999; García-Rodríguez, Durán and Roncero 2000). It alters the expression of a larger set of genes from RNA metabolism, transport, organelle biogenesis and the response to stress (Kuranda et al. 2006). Caffeine affects TORC1-mediated activation of the CWI pathway (Lum et al. 2004; Kuranda et al. 2006).

Results of growth on YPD at 30°C in the presence of these drugs (Fig. 3A, left) show that $\Delta gup1$ has a strong CFW sensitivity consistent with a primary defect in cell wall biogenesis and composition (Ferreira et al. 2006). CR, on the other hand, surprisingly affected more the wt than the $\Delta gup1$ mutant. This was unexpected since CR binds chitin in the cell wall and $\Delta gup1$ has 90% more chitin than wt cells (Ferreira et al. 2006). Possibly, the high amount of chitin in the mutant cell wall might cause a saturation-derived limit for the effect of CR. The $\Delta pil1$ mutant did not show any significant sensitivity to CFW or CR in comparison with the wt. However, the $\Delta gup1 \Delta pil1$ double mutant

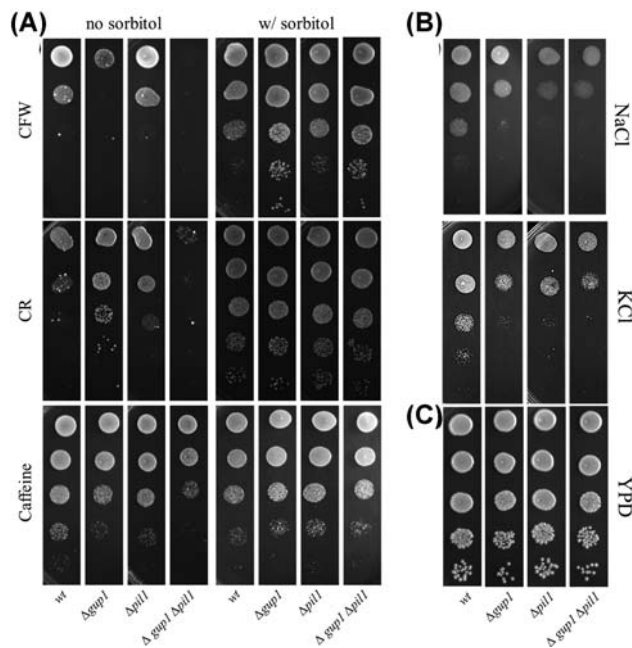


Figure 3. (A) Phenotypes of *S. cerevisiae* BY4741 wt, $\Delta gup1$, $\Delta pil1$ and $\Delta gup1 \Delta pil1$ strains in response to cell wall-injuring chemicals, CR—Congo Red (100 mg/L), CFW—Calcofluor White (50 μ g/mL) and caffeine (12 mM). Osmotic remediation of the phenotypes was tested with 1 M sorbitol. Results were scored after 3 days of incubation at 30°C. (B) Identical assays in YPD supplemented with 1 M NaCl or 1.5 M KCl. (C) Control assay in YPD. One representative experiment is shown.

was unable to grow in the presence of either drug, and was 10-fold more severely affected by caffeine than either single mutant (Fig. 3A, left). The osmotic stabilization of the cells by 1-mM sorbitol (Levin 2005) restored growth (Fig. 3A, right). Taken together, these results indicate that Gup1 and Pil1 affect related processes at the level of the cell wall.

A role (direct or indirect) of eisosomes in the regulation of the synthesis of cell wall components, as well as their spatial distribution, has been suggested (Foderaro, Douglas and Konopka 2017), possibly independently of the CWI/PKC pathway (Ferreira et al. 2006; Wang et al. 2011). The $\Delta gup1$ mutant appears to be responding regularly at the level of CWI/PKC pathway Slt2 phosphorylation (Ferreira et al. 2006) and, consistently (Munro et al. 2007; Lenardon et al. 2009), it has 90% more chitin than wt (Ferreira et al. 2006). Although there is no information available on the composition of cell wall in the $\Delta pil1$ strain, it is known that Pil1 complexes with β -1,3-glucan synthase Fks1 (Gavin et al. 2002), allowing to expect that the $\Delta pil1$ mutant is affected in glucan synthesis as reported to occur in other eisosome mutants (Wang et al. 2011; Foderaro, Douglas and Konopka 2017). The extreme effect of CFW and CR, and the exacerbated effect of caffeine on $\Delta gup1 \Delta pil1$ show that the cell wall is severely affected when the two proteins Gup1 and Pil1 are absent, to the point of impairing growth. This confirms that eisosomes are crucial for the maintenance of the wall integrity as previously suggested (Foderaro, Douglas and Konopka 2017). Particularly, the effect of caffeine suggests that the TORC1-mediated CWI/PKC activation is affected, possibly at the level of steps that are common to CFW and CR responses (Wanke et al. 2008).

Effect of hyperosmotic salt stress

There is solid evidence for cooperative cross-talk between HOG and PKC/CWI pathways in cell wall biogenesis (Rodríguez-Peña

et al. 2010). Namely, HOG controls the basal expression of several cell wall proteins as well as chitin synthetases (Lenardon et al. 2009). Transcription of the PKC/CWI effector SLT2 in response to high osmolarity seems to be dependent on both Hog1 and the PKC/CWI pathway-governed transcription factor Rlm1 (Rodríguez-Peña et al. 2010). To assess the ability of the mutants to withstand high osmotic stress, the single and double mutants were grown on YPD and YNB with 2% glucose and supplements with 1 M NaCl or 1.5 M KCl (Fig. 3B). The three mutants showed moderate and equal sensitivity to these salts in comparison to the wt strain. The $\Delta gup1$ mutant is moderately sensitive to salt stress, in association with defective intake of glycerol for osmoregulation purposes (Holst et al. 2000; Ferreira et al. 2005). Moreover, MCC eisosome domains have been associated with the response to stress injuries, including hyper and hypoosmotic stress (Dupont et al. 2010; Berchtold et al. 2012; Kabeche, Howard and Moseley 2015b). The observation that both single and double mutants have the same sensitivity to osmotic stress suggests that Gup1 and Pil1 might act in the same pathway during hyperosmotic stress response. A possible pathway might include the aquaglyceroporin Fps1 that closes in response to hyperosmotic stress to promote the retention of glycerol. A failure to close Fps1 renders cells sensitive to high osmolarity (Tamás et al. 1999).

Growth in the presence of arsenite and arsenite influx

In addition to its role in glycerol transport across the plasma membrane, Fps1 also mediates uptake of the metalloid arsenic in form of trivalent arsenite [As(III)] into cells (Wysocki et al. 2001). To test whether Gup1 and Pil1 might affect Fps1, we grew cells in the presence of sodium arsenite. Deletion of *GUP1* causes a strong sensitivity to As(III), which is aggravated in the $\Delta gup1 \Delta pil1$ mutant (Fig. 4A), while growth of the $\Delta pil1$ mutant was largely unaffected. The intracellular amounts of arsenic were measured after 1 h incubation with 1 mM As(III). The double mutant accumulated ~50% more arsenic than the wt and the single mutants (Fig. 4B). Thus, in the absence of both *GUP1* and *PIL1*, cells accumulate more arsenic and become highly sensitive to this metalloid. The aquaglyceroporin Fps1 is the main entry pathway of As(III) in cells growing on glucose (Wysocki et al. 2001). Previous studies have shown that Fps1 activity is regulated by the MAP kinases of the HOG (Hog1) and CWI/PKC (Slt2) pathways (Thorsen et al. 2006; Ahmadpour et al. 2016). Likewise, Hog1 and Slt2 are activated by As(III) (Thorsen et al. 2006; Ahmadpour et al. 2016; Lee and Levin 2018). The present results suggest that Fps1 might be deregulated (i.e. more of the Fps1 molecules are open) in the double mutant, allowing a higher influx of As(III). Fps1 also mediates the entry of acetic acid into cells (Mollapour and Piper 2007; Lindahl et al. 2017). Thus, if Fps1 does not close properly in the $\Delta gup1 \Delta pil1$ mutant, it should allow the entrance of more acetic acid, promoting increased sensitivity of the double mutant to this compound leading to faster/stronger death of the cells.

Acetic acid-induced cell death

Previously, we showed that the *GUP1* deleted mutant is greatly sensitive to acetic acid, although it dies with features that are more necrosis- than apoptosis-like, in opposition to what is observed for wt cells (Tulha et al. 2012). The previously reported physical association of Gup1 with the mitochondrial inner membrane VDAC Por1 (Tulha and Lucas 2018), was shown to be implicated in that process. Therefore, the strains used in this study were challenged with acetic acid apoptosis-inducing conditions.

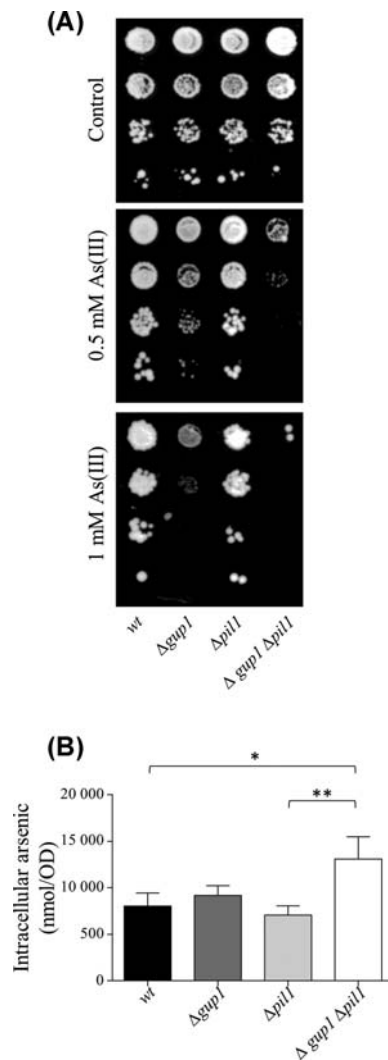


Figure 4. (A) Phenotypes of *S. cerevisiae* BY4741 wt, $\Delta gup1$, $\Delta pil1$ and $\Delta gup1 \Delta pil1$ strains in the presence of sodium arsenite [As(III)]. Results were scored in YNB with 2% glucose and supplements after 3 days of incubation at 30°C. (B) Intracellular arsenic. Cells were exposed to 1 mM As(III) during 1 h, washed and intracellular arsenic content measured in three independent biological replicates. Data represent mean \pm standard deviation of at least three independent experiments. ** $P < 0.01$; * $P < 0.1$ one-way ANOVA followed by Tukey Mean Difference test.

The $\Delta pil1$ mutant was more affected than $\Delta gup1$, which in turn was more affected than wt, while nearly all $\Delta gup1 \Delta pil1$ cells died upon treatment (Fig. 5A). Subsequently, the number of surviving cells presenting membrane disruption was determined by staining the cells with PI. The integrity of the plasma membrane and consequent impermeability to PI allows to discriminate between apoptotically or necrotically dying cells, although it does not discriminate between primary or secondary necrosis (Eisenberg et al. 2010; Carmona-Gutierrez et al. 2018). Results in Fig. 5B show the percentage of PI⁺ cells. The wt and $\Delta gup1$ strains presented results similar to previously reported (Tulha et al. 2012). The decrease in viability of wt cells (~45%) was not accompanied by a correspondent increase in loss of plasma membrane integrity (~5%), which points to a RCD event. The decrease in cell survival observed in $\Delta gup1$, on the other hand, was accompanied by loss of plasma membrane integrity as expected from cells mostly dying of a non-regulated death. The

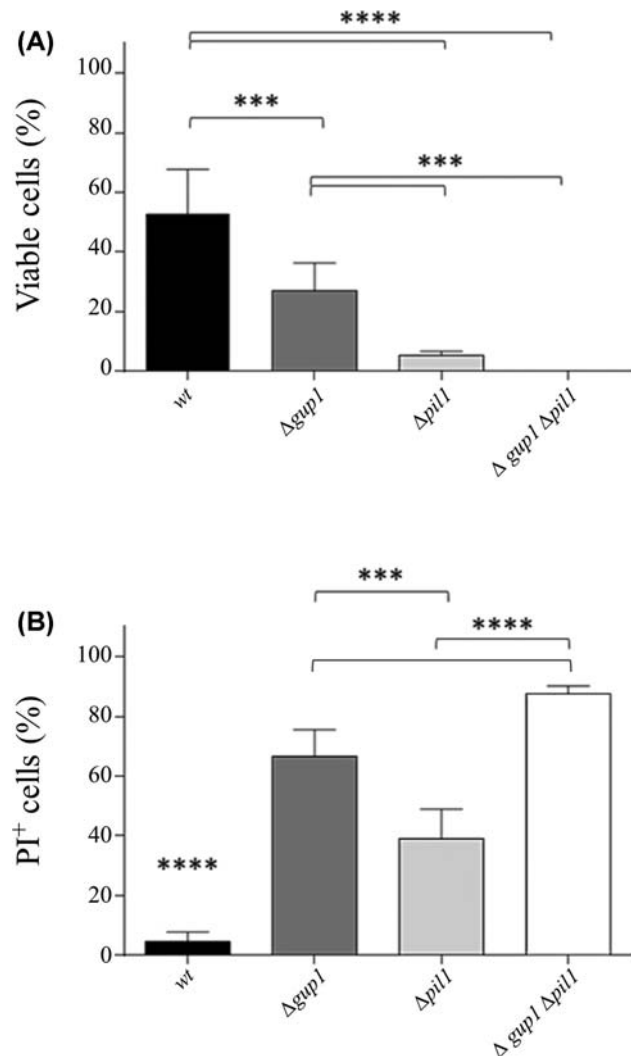


Figure 5. Response of *S. cerevisiae* BY4741 wt, $\Delta gup1$, $\Delta pil1$ and $\Delta gup1 \Delta pil1$ strains to acetic acid-induced cell death. Exponentially growing cells on YNB with 2% glucose and supplements were treated with 150 mM acetic acid for 3 h. (A) Viability was determined by c.f.u. assay (results were normalized with 100% survival corresponding to the total c.f.u. at T_0). (B) Graphic representation of the percentage of cells displaying positive PI staining, assessed by flow cytometry. Data represent mean \pm standard deviation of at least three independent experiments. **** $P < 0.0001$; *** $P < 0.001$ one-way ANOVA followed by Tukey Mean Difference test.

$\Delta pil1$ mutant presented a much smaller percentage of surviving c.f.u. (Fig. 5A), which displayed an intermediate percentage of cells that lost membrane integrity (Fig. 5B), suggesting that the fraction of the population dying necrotically could be dying of a secondary necrosis. Finally, the absence of both Gup1 and Pil1 almost killed the entire cell population, almost 100% of the cells being PI⁺. The integrity of the membrane rafts and the distribution of ergosterol, which are so severely disrupted in $\Delta pil1$ (Walther et al. 2006; Grossmann et al. 2007, 2008) and in $\Delta gup1$ (Ferreira and Lucas 2008), were suggested to affect cell survival to acetic acid and induced apoptosis (Mollinedo 2012), although there is yet no mechanistic understanding. The results obtained here are consistent with the hypothesis that the disruption of eisosomes derived from GUP1 and PIL1 deletion could cause Fps1 to be improperly regulated (unable to

close properly), promoting death-inducing influx of acetic acid into cells.

FINAL REMARKS

The present work revealed the plasma membrane eisosome structural protein Pil1 as a new physical interaction partner of the MBOAT *O*-acyltransferase Gup1. *GUP1* deletion resulted in a substantial reduction of the number of eisosomes per cell, estimated by measuring the number of Pil1-GFP punctate patches, while the expression of *PIL1* and the Pil1 protein levels remained unchanged. Thus, Gup1 may be implicated in eisosome formation/assembly. Pil1 preferentially binds to a minor phospholipid present in the membranes—PI(4,5)P2. The $\Delta gup1$ mutant is affected in membrane composition, presenting less phospholipids, and more diacylglycerol (Oelkers et al. 2000), which can be obtained by PI(4,5)P2 hydrolysis. Thus, such alterations at the level of PI homeostasis could be the cause for the reduction in the number of eisosomes.

Phenotypical analyses of the single and double *GUP1* and *PIL1* mutants revealed that Gup1 and Pil1 interact genetically. This interaction was found to be mostly negative. Some of the phenotypes were aggravated or only visible in the double mutant. The cell wall injuring drugs CFW and CR impaired growth of the double mutant. Thus, Gup1 and Pil1 are functionally implicated in cell wall biogenesis, possibly through the CWI/PKC pathway, which is the common denominator of these drugs.

One well-documented consequence of the disruption in *PIL1* is the mislocalization of several important signaling effectors, namely, the Sho1 and Sln1 activators of the HOG pathway (Tanigawa et al. 2012). Accordingly, $\Delta pil1$ was shown here to be affected by salt stress. Hyperosmotic stress induces a HOG-mediated response culminating, among others, in the production and intracellular accumulation of the osmolyte glycerol (Hohmann 2009). Hyperosmotic stress also results in closure of Fps1 so that the cell can retain glycerol (Tamás et al. 1999). Hog1, the MAP kinase of the HOG-pathway, has been shown to act as a negative regulator of Fps1 activity (Thorsen et al. 2006; Lee et al. 2013). A lower activity of Hog1, due to mislocalization of Sho1 and Sln1, was expected to result in an increased number of open Fps1 molecules. Besides glycerol, Fps1 is also permeated by the trivalent metalloid ions arsenite and antimonite (Wysocki et al. 2001), xylitol (Tani, Tagushi and Akamatsu 2017) and acetic acid (Mollapour and Piper 2007). In this work, it is shown that the $\Delta gup1 \Delta pil1$ mutant accumulated elevated levels of arsenic and were As(III) sensitive. Furthermore, when exposed to toxic acetic acid concentration, $\Delta pil1$ dies much more than $\Delta gup1$, and the double mutant is almost fully killed. Thus, acetic acid is most probably flooding the $\Delta gup1 \Delta pil1$ mutant, as observed to occur with arsenite. Whether the increased influx in the double mutant is indeed mediated by Fps1, as well as the molecular details of why Fps1 is deregulated in this strain, remains to be firmly established. We note that the proportion of acetic acid-induced cell death is not equivalent to the proportion of As(III) intake in either of the four yeast strains. The $\Delta pil1$ takes up approximately the same amount of arsenic as *wt*, but it dies almost 10× more of acetic acid, both strains dying of apoptosis. On the other hand, the deletion of *GUP1* causes a drastic change in this type of death, which becomes necrotic-like (Tulha et al. 2012). This is not maintained in the double mutant, in which the acetic acid-treated cells were almost all PI⁺, implying that the deletion of *PIL1*, apparently epistatically reverts the necrosis-like death

observed as the consequence of the deletion of *GUP1*. Thus, the mechanisms underlying the observed phenotypes are likely multifactorial.

Eisosomes delimit furrow-like membrane invaginations associated with MCC domains, which concentrate sphingolipids, sterols as well as signaling molecules involved in the regulation of (i) TORC2-Ypk1, by temporarily sequestering Sln1/2, therefore indirectly controlling sphingolipid synthesis (Berchtold et al. 2012); (ii) HOG pathway, interfering with the partitioning of the Sln1 and Sho1 sensors (Tanigawa et al. 2012); and (iii) CWI pathway, in which case eisosomes appear to be instrumental in sensing mechanical stress (Berchtold et al. 2012; Kabeche et al. 2014; Foderaro, Douglas and Konopka 2017). Previously, Gup1 protein was suggested to act at a hub between the CWI/PKC1, TORC2/YPK, TORC1/Pkh1/2 and HOG/Sho1 pathways in close association with PI(4,5)P2 (Lucas et al. 2016). This hypothesis is reinforced by the present results.

Summing up, this work presents evidence of physical, genetic and functional interactions between Gup1 and Pil1. Phenotypes associated with the deletion of *GUP1* are numerous and mostly common to those caused by *PIL1* deletion (compare Lucas et al. 2016 with Lanze et al. 2020). Phenotypes associated with the double deletion of these two proteins include defective acetic acid-promoted RCD, as well as arsenite uptake, suggesting that Fps1 might be deregulated in the absence of both proteins. Other phenotypes show that the interaction of these proteins affects cell wall integrity, the most prominent phenotype ever described in association with the deletion of *GUP1*. Most importantly, results show that the absence of Gup1 does not affect the expression of *PIL1* but strongly suggest it affects the number and size of eisosomes. *GUP1* deletion in the fungal pathogen *C. albicans* affects its virulence and ability to differentiate, adhere, invade and form biofilms (Ferreira et al. 2010). Thus, a better understanding of Gup1, its interaction partners and cellular functions might be useful for future development of anti-fungal drugs.

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SUPPLEMENTARY DATA

Supplementary data are available at [FEMSyr](https://www.femsyr.com) online.

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AUTHOR CONTRIBUTIONS

JT—acquisition and interpretation of the data; writing of the manuscript; MA-R, LAE and SR—acquisition of data; revising the manuscript; and CL and MJT—conception of the study; interpretation of the data; writing of the manuscript.

Conflict of Interest. None declared.

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